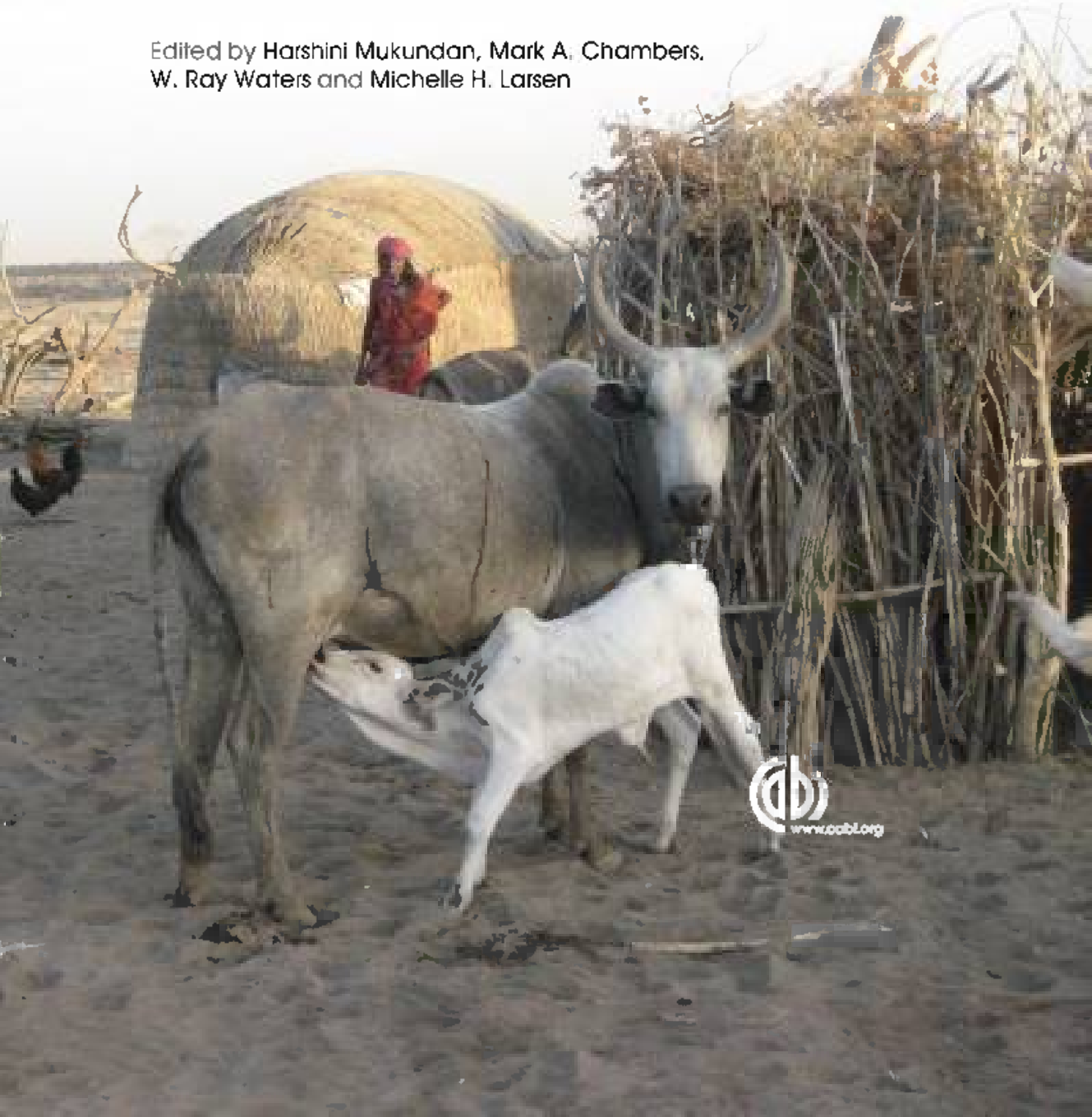
Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals

The Many Hosts of Mycobacteria

Edited by Harshini Mukundan, Mark A. Chambers, W. Ray Waters and Michelle H. Larsen



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Introduction – The Many Hosts of Mycobacteria: An Interdisciplinary Approach to Understanding Mycobacterial Diseases

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Mycobacteria have been associated with human and animal disease for millennia. In particular, tuberculosis (TB) continues to cause significant human morbidity and mortality worldwide. The discovery in 1882 of the tubercle Bacillus, *Mycobacterium tuberculosis*, by the German physician and microbiologist Robert Koch was met with great enthusiasm as it defined the infectious nature of the disease. By 1915, a collaboration between the physician Albert Calmette and the veterinarian Camille Guérin resulted in the development of an attenuated strain of the bovine tubercle Bacillus, *Mycobacterium bovis*, that later became the basis of the Bacille Calmette–Guérin (BCG) vaccine, which is one of the most widely used childhood vaccines in the world. This discovery is a wonderful example of how, for centuries, collaborations among multiple scientific disciplines have positively impacted the control of infectious disease.

Since 2007, scientists from the United States Department of Agriculture's Agricultural Research Service (USDA, ARS), the Albert Einstein College of Medicine (AECOM) and the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) have been convening the workshop *Many Hosts of Mycobacteria*. The workshop was founded on a principle of cross-disciplinary inclusion and the belief that by bringing together all members of the mycobacterial research community we could achieve a better understanding of mycobacteria and the diseases caused by them, and thus contribute to knowledge and the development of products to improve global health.

When researching a human infectious disease, experimental animal models are often employed to create or test hypotheses. However, the study of natural infections in animals, and the information that can be gained from them, is often overlooked. The knowledge that can be obtained by determining mechanisms that drive host specificity for related pathogens, as well as understanding disease transmission and differences in disease progression and presentation for the same pathogens in different hosts, can enhance our understanding of human disease. Focusing only on human disease also neglects the role of wildlife, livestock and other peri-domestic animals in transmission of infectious diseases. By bringing together a multidisciplinary team of leading scientists studying mycobacterial infections in humans and animals, *Many Hosts*

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of Mycobacteria created a venue for sharing knowledge about the spectrum of mycobacterial diseases, exploring host–pathogen variability, and understanding what the commonalities and differences in disease presentation and host specificity teach us.

Promoting discussion among experts in scientific disciplines that traditionally do not interact has resulted in the critical evaluation of mycobacterial infections in natural and artificial hosts and the identification of areas of mutual interest and collaboration. Participants in the workshop span from basic scientists to clinicians, animal modellers and product developers to individuals with zoological and wildlife expertise to cover the breadth of known species of mycobacteria and the various hosts that harbour them.

Many Hosts of Mycobacteria was initially driven by the TB research community's need to better understand and interpret the results of studies of candidate vaccines against human TB that were tested in a variety of animal species – cattle, non-human primates, guinea pigs and mice. Different animal models are useful for understanding particular aspects of human TB disease, but not all of these animals are practical models for many research needs because of size or cost. Initial discussions focused on differences in the host immune responses, but quickly evolved to include perspectives on how the molecular and pathophysiologic differences between various mycobacterial pathogens can manifest with a distinct host or tissue specificity and can be exploited in experimental models. As the workshop progressed, it became clear that these interdisciplinary discussions were providing unique insights that could not be gained from studying individual mycobacteria or hosts alone. By comparing the similarities and differences between each of the mycobacterial pathogens and hosts, the participants gained a better understanding of all mycobacteria. The concept of 'comparative mycobacteriology' was born as a framework to evaluate disease pathology and promises to contribute to different aspects of infectious disease research, which could ultimately improve vaccines, diagnostics and therapeutics for mycobacterial diseases such as TB and leprosy.

Although the greatest need for development of interventions for mycobacterial diseases is to improve human health, there are also many important applications for animals. Cattle are often infected from wildlife reservoirs such as deer, badgers or possums. Once an infection in livestock is discovered, the entire herd is typically culled, leading to considerable economic losses. In resource-limited settings where families are dependent on cattle for food and income, infected animals are frequently not culled and may lead to infection of humans through the consumption of meat or milk. A vaccine for cattle or the wildlife reservoirs of mycobacteria may minimize these infections. Zoo animals such as elephants can contract TB from their handlers. Colonies of non-human primates that are used in research are also affected by *M. tuberculosis* infection resulting in the removal and loss of valuable animals and research. A diagnostic that allows earlier detection and isolation to prevent transmission may be able to help protect these important animals.

The *Many Hosts of Mycobacteria* workshop has fostered an interdisciplinary approach and unique collaborations that benefit multiple scientific communities. The sharing of ideas and results across disciplines has allowed for enhancement of research in *M. tuberculosis*, *M. bovis*, *M. leprae* and other non-tuberculous mycobacteria. Most of all, it has increased the knowledge of researchers who are developing vaccines, diagnostics and drugs to prevent, diagnose and treat some of the oldest pathogens of man. This book serves to share the lessons learned from these workshops not only within the mycobacterial community, but also to a wider audience, as this approach may benefit other fields of research.

About the Cover

Mycobacterial diseases affect humans, livestock and wildlife in all regions of the world. Pathogenic mycobacteria can infect a wide variety of species, passing between them in complex webs of infection. We chose the cover photograph to illustrate vividly the intimate human–animal interface in a region of the

world (Sub-Saharan Africa) with a relatively high prevalence of bovine and human tuberculosis, leprosy and Buruli ulcer. The intent of the image is to capture the One Health essence of mycobacterial disease research. The suckling calf in the image demonstrates the role of ingestion of non-pasteurized milk in *Mycobacterium bovis* transmission. The indigenous Zebu cattle and Ethiopian tribesman portray genetic and socio-economic factors affecting control measures in diverse populations. In the background, chickens (likely to be harbouring *M. avium*) represent the confounding role of non-tuberculous mycobacteria for development of improved vaccines and diagnostic tests. In addition, iconic wildlife species (e.g. lions, elephants and rhinoceros) are native to Sub-Saharan Africa and survival of these species is hindered by chronic and debilitating infections with tuberculous mycobacteria species. (Photograph taken by Rea Tschopp (Wildlife Veterinarian/Epidemiologist at the Armauer Hansen Research Institute in Addis Ababa, Ethiopia).)

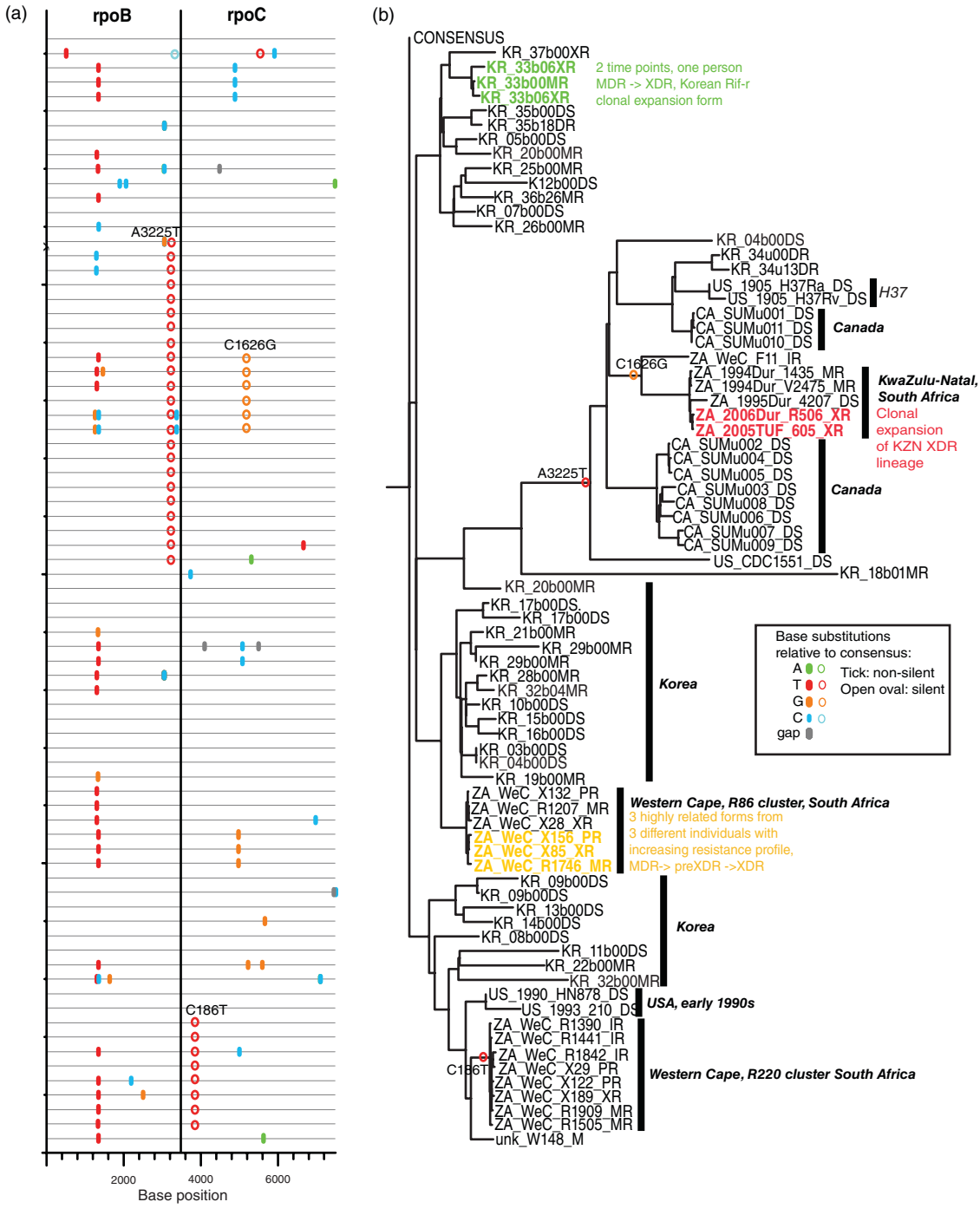

Plate 1. Mutational patterns associated with Rif resistance from sequences with known drug sensitivity, organized according to phylogenetic relationships.

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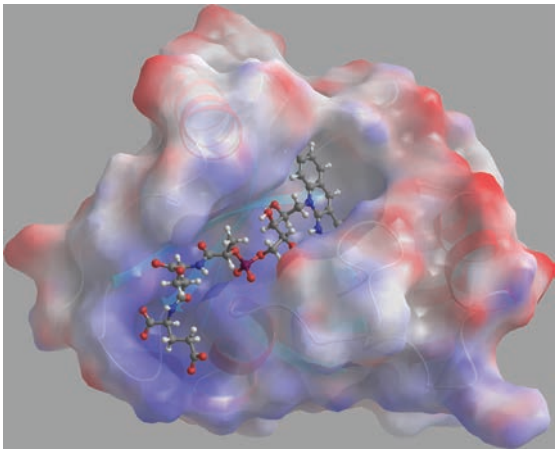

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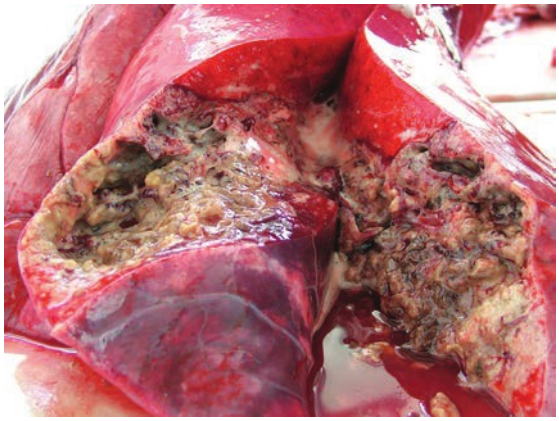

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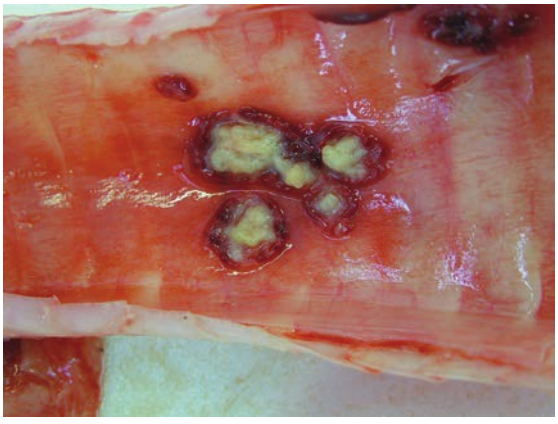

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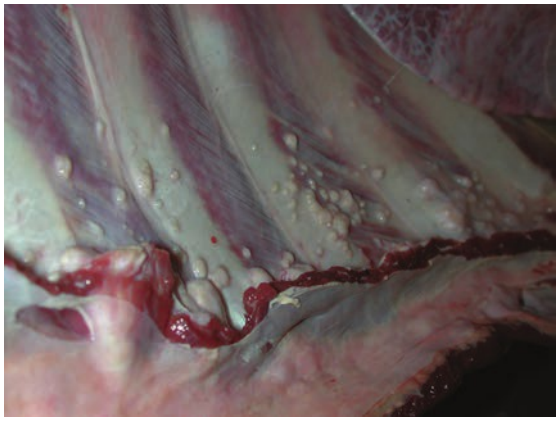

Plate 2. Crystal structure of the deazaflavin-dependent nitroreductase (Ddn) from Mtb with cofactor F420 (50). Despite a wealth of knowledge on Ddn as an activator of these important drug candidates little biological information on their physiological role has been obtained.

Plate 3. Large caseous lesion with cavitation in the lungs of a skin-test-negative alpaca with a cough and continuous hiccups. No other symptoms. The photograph shows a liquefactive flowing abscess, devastating the lung tissue, potentially resulting in highly infectious exhaled breath. (Photograph courtesy of James Barnett, APHA.)

Plate 4. Lesions of the tracheal mucosa in an apparently healthy, skin-test-negative alpaca euthanized as a direct contact of a herd mate with a cough. The pale purulent matter in the centre of the ulcerated lining contains *Mycobacterium bovis*. (Photograph courtesy of James Barnett, APHA.)

Plate 5. Parietal pleura of a llama with pearlescent lesions of tuberculous pleurisy. (Photograph courtesy of Tim Crawshaw, APHA.)

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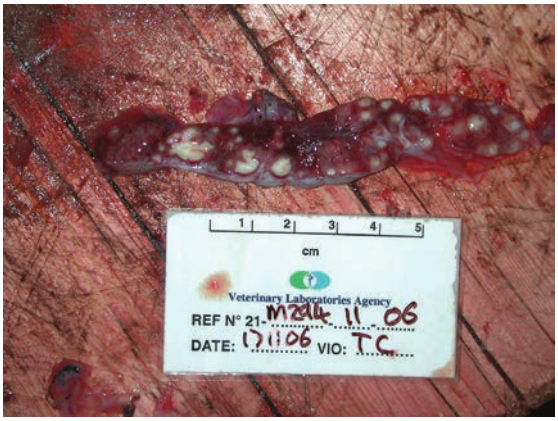

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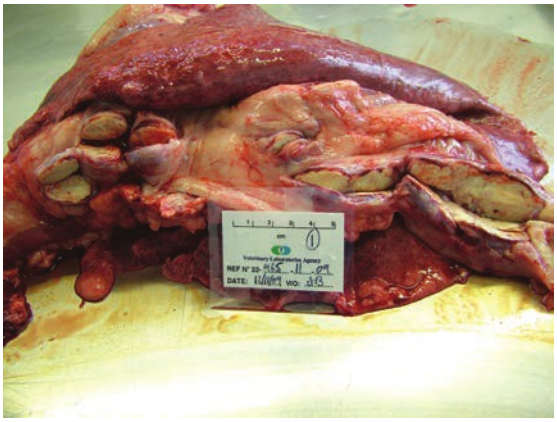

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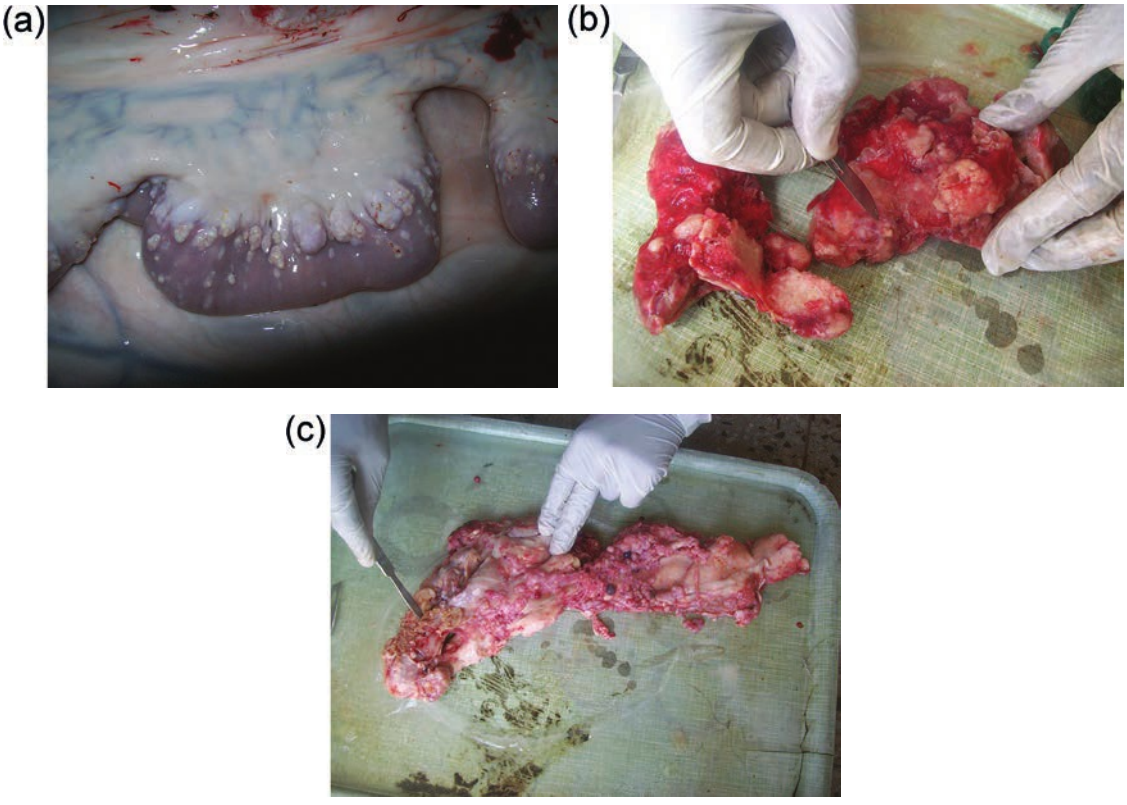

Plate 6. Multiple foci in llama mediastinal lymph node showing scattered caseous foci. (Photograph courtesy of Tim Crawshaw, APHA.)

Plate 7. Dorsal view of the thoracic viscera of an alpaca showing grossly enlarged (20×) bronchial and mediastinal lymph nodes almost completely with caseous material and containing *Mycobacterium bovis*. This alpaca was also skin-test-negative with no outward symptoms other than 'not his usual self'. (Photograph courtesy of James Barnett, APHA.)

Plate 8. Tuberculous lesions on the mesenteric lymph node and wall of intestine (a), hepatic (b) and cranial mediastinal (c) lymph nodes of a *Mycobacterium bovis*-infected dromedary in Ethiopia. (Photographs courtesy of BTB Research Group – ALIPB – Addis Ababa University, Ethiopia.)

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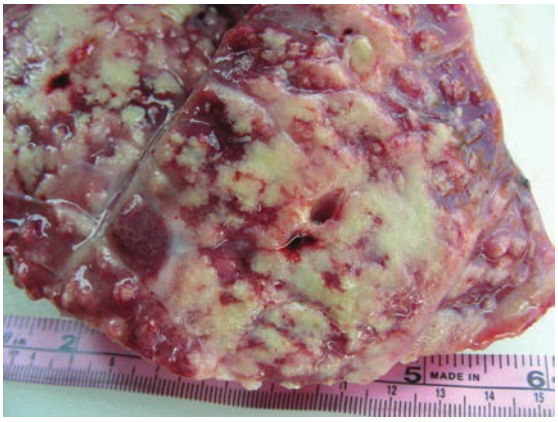

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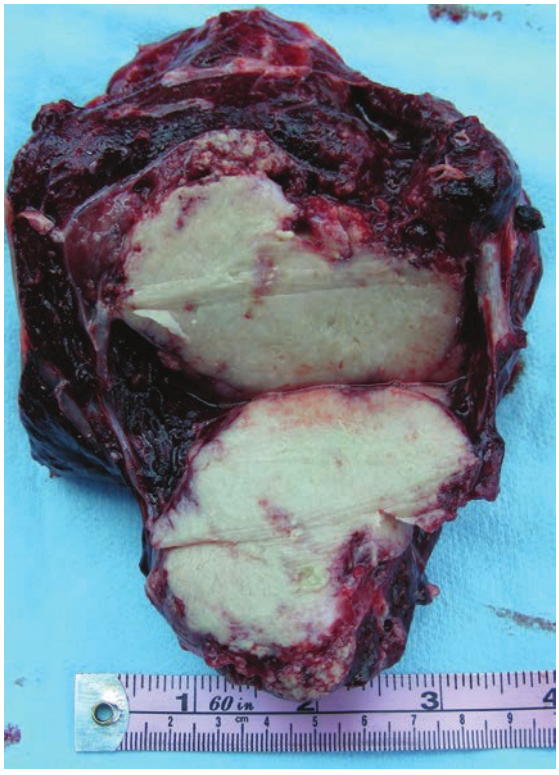

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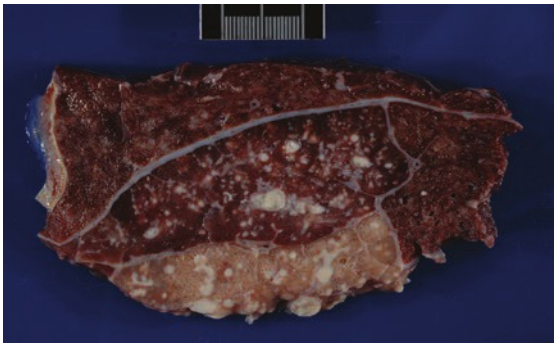

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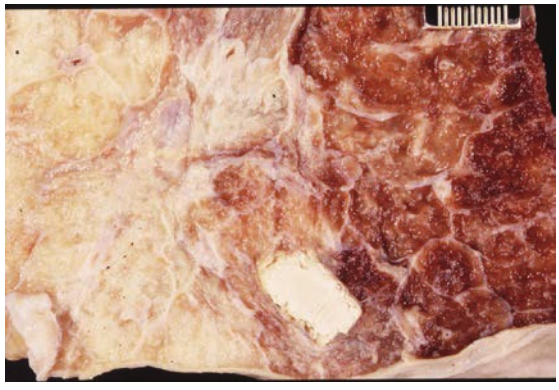

Plate 9. Lung from Elephant TB Stat-Pak®- and MAPIA-reactive African elephant. Trunk wash culture negative but culture positive for *Mycobacterium tuberculosis* at necropsy. Miliary to confluent granulomatous pneumonia. Note the variable appearance from granular to smooth and waxy or 'lardaceous'. (Image taken by Susan Mikota.)

Plate 10. Lung from Elephant TB Stat-Pak®- and MAPIA-reactive African elephant. Trunk wash culture negative but culture positive for *Mycobacterium tuberculosis* at necropsy. Large solid granuloma with 'lardaceous' texture and scattered chalky foci of mineralization. (Image taken by Susan Mikota.)

Plate 11. Asian elephant, Elephant TB Stat-Pak® and MAPIA reactive; trunk wash culture negative but culture positive for *Mycobacterium tuberculosis* at necropsy. Partially fixed lung with localized region with miliary granulomas, interlobular septal fibrosis and plugging of bronchioles with thick mucopurulent exudate. (Image taken by Dr Steven Kubiski, UC Davis Veterinary Medical Teaching Hospital.)

Plate 12. Asian elephant post-treatment for TB. Lung with regionally extensive consolidation and fibrosis (right side of image) and large white foci of mineralization (old granulomas). Culture and PCR were negative. (Image taken by L.J. Lowenstine, UC Davis Veterinary Medical Teaching Hospital.)

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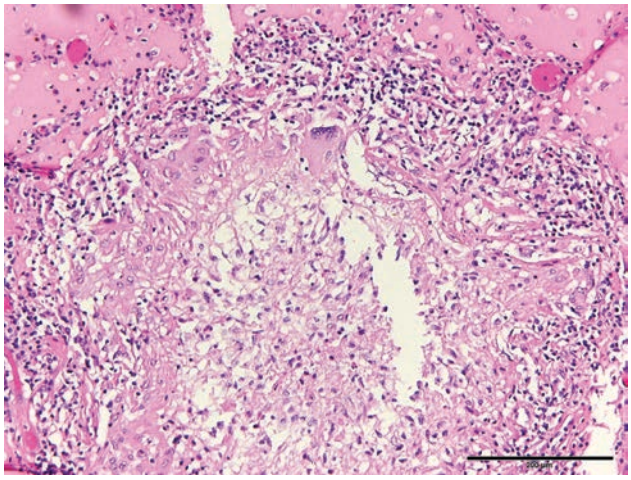

Plate 13. Asian elephant, Elephant TB Stat-Pak® and MAPIA reactive; trunk wash culture negative but culture positive for *Mycobacterium tuberculosis* at necropsy. Histiocytic granuloma with a single multinucleated giant cell and peripheral, infiltrating lymphocytes and neutrophils. Surrounding alveoli (at top of image) are flooded with oedema (haematoxylin and eosin, H&E). (Image taken by L.J. Lowenstine.)

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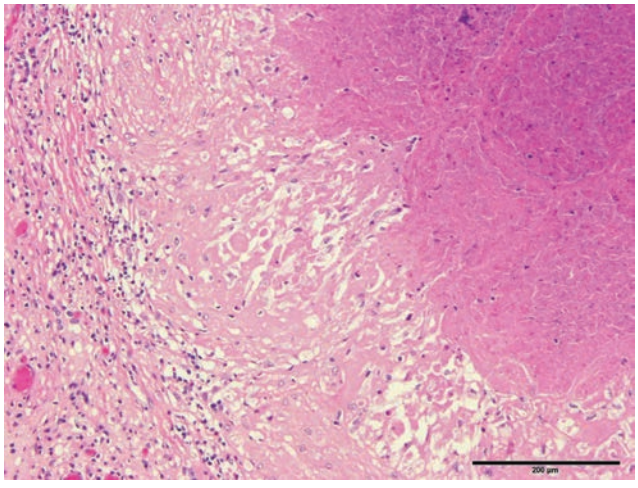

Plate 14. Asian elephant, Elephant TB Stat-Pak® and MAPIA reactive; trunk wash negative but culture positive for *Mycobacterium tuberculosis* at necropsy. Pulmonary granuloma with central caseous necrosis (upper right), histiocytes, neutrophils and lymphocytes and a thin rim of fibrosis (haematoxylin and eosin, H&E). Acid-fast stains were negative. (Image taken by L.J. Lowenstine.)

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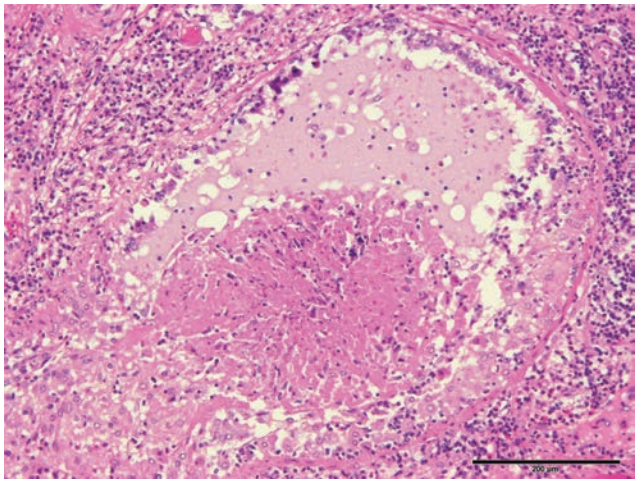

Plate 15. Asian elephant, Elephant TB Stat-Pak® and MAPIA reactive; trunk wash culture negative but culture positive for *Mycobacterium tuberculosis* at necropsy. Necrotizing and histiocytic bronchiolitis with peribronchiolar infiltration by lymphocytes and neutrophils (haematoxylin and eosin, H&E). (Image taken by L.J. Lowenstine.)

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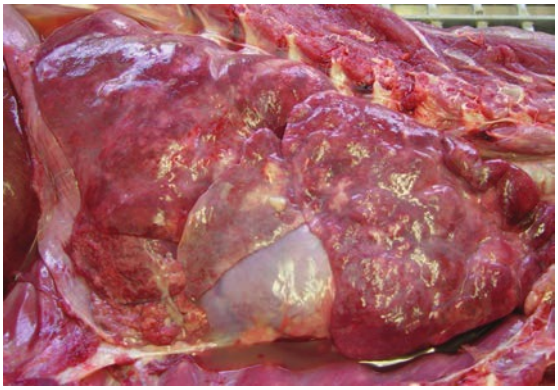

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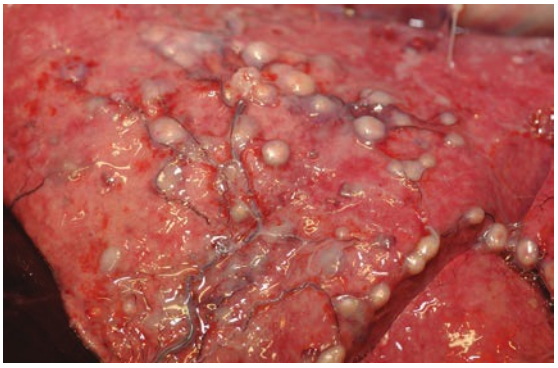

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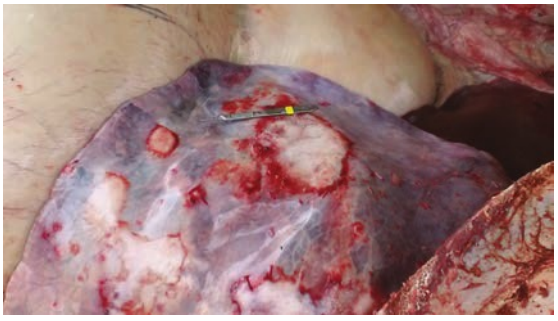

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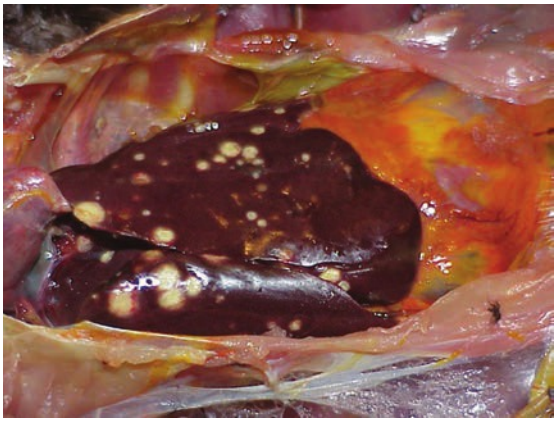

Plate 16. *Mycobacterium bovis* in lungs of a lion (*Panthera leo*).
Plate 17. Pulmonary lesions associated with *Mycobacterium kansasii* infection in a bontebok (*Damaliscus pygaragus dorcas*).
Plate 18. Pulmonary lesions associated with *Mycobacterium tuberculosis* infection in a black rhinoceros (*Diceros bicornis*).
Plate 19. *Mycobacterium avium* lesions in the liver of a white-winged wood duck (*Asarcornis scutulata*).

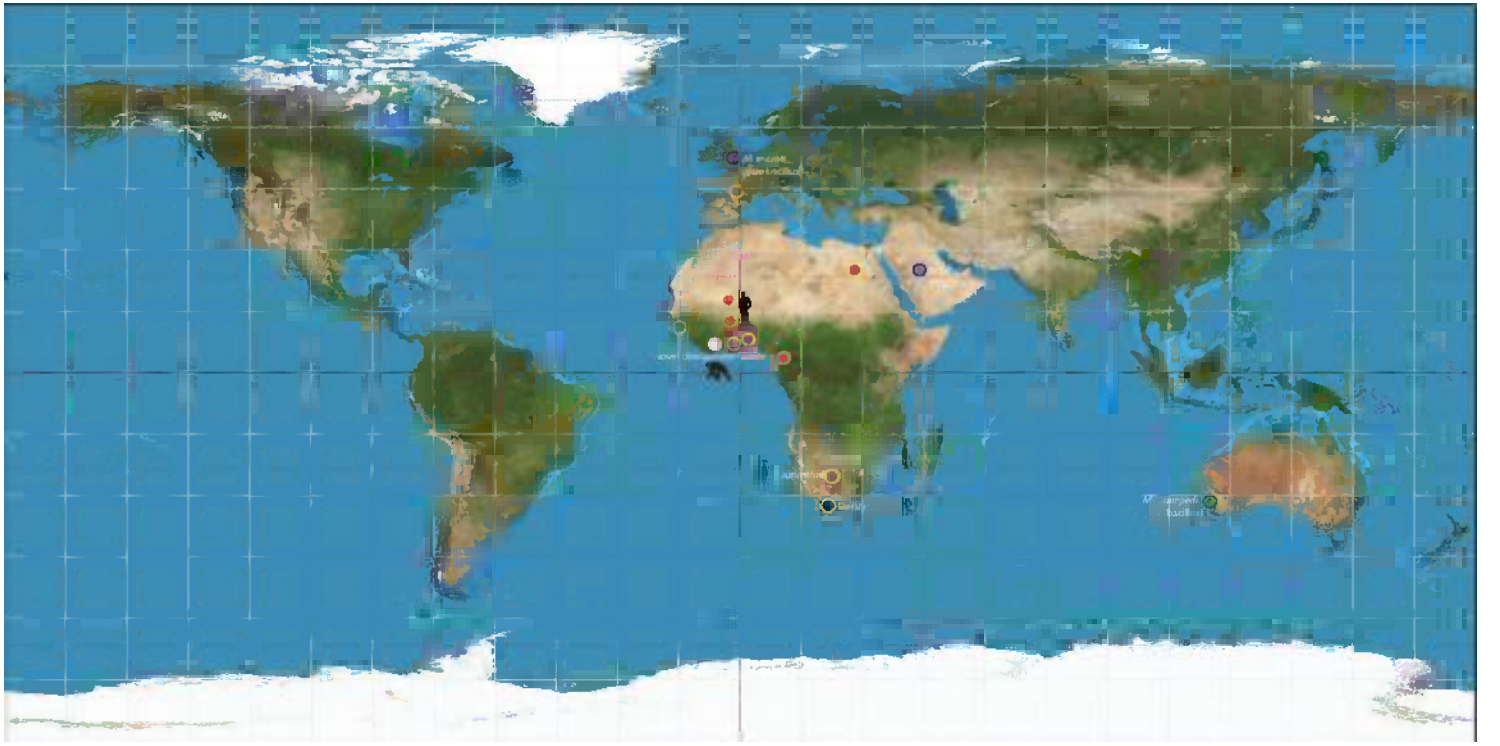

Plate 20. Origin of lineage six wildlife-associated TB strains in southern Africa (circles with yellow boundary) and human-associated *M. africanum* in northwest Africa (orange circles with yellow boundary). Other wildlife-adapted TB strains identified in the phylogenetic schematic (circles with black boundary) are included for comparison.

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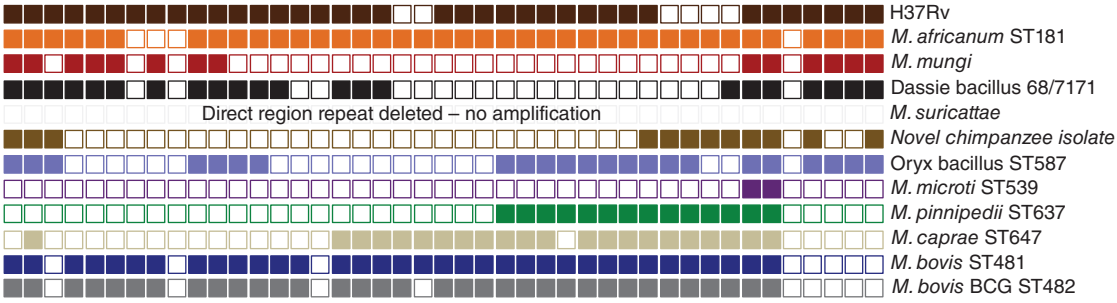

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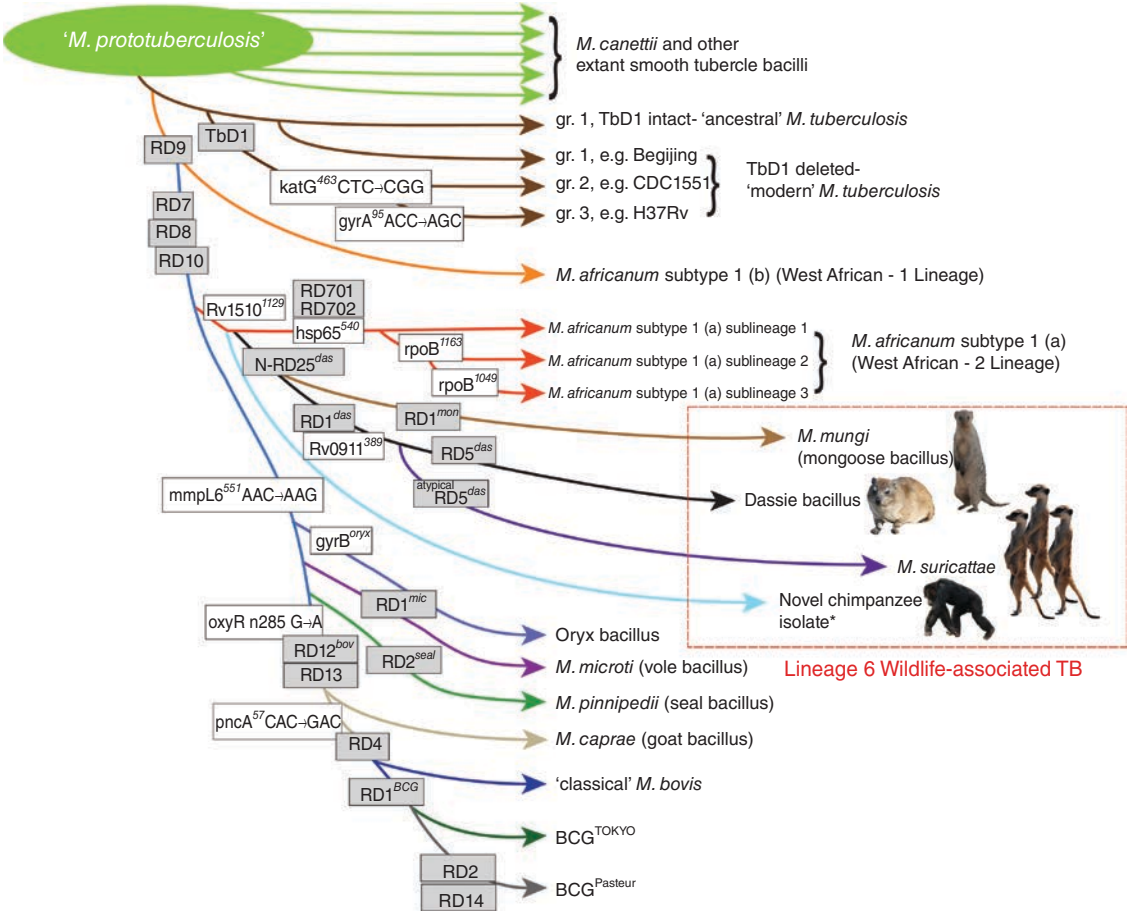

Plate 21. Spoligotype of lineage six wildlife-associated TB strains compared with representative spoligotypes from other *Mycobacterium tuberculosis* complex species, modified from Alexander *et al*. (2010).
Plate 22. Schematic of the phylogenetic relationships between *Mycobacterium tuberculosis* complex species, with the lineage of six wildlife-associated TB strains highlighted (red box). The phylogenetic relationships are based on single-nucleotide polymorphisms (white boxes) and the presence or absence of regions of difference (grey boxes), modified from Alexander *et al*., 2010.

1 Introduction and Epidemiology of *Mycobacterium tuberculosis* Complex in Humans

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History of Tuberculosis

Tuberculosis (TB) is arguably one of the most devastating diseases that have afflicted mankind from time immemorial. Known by many different names throughout history, such as phthisis, scrofula, consumption, King's Evil, lupus vulgaris, the white plague and 'captain of all these men of death', the scourge remains a significant public health concern. Perhaps the earliest evidence of TB comes from skeletal remains from burial sites from the latter part of the last Stone Age. Both macroscopic as well as microscopic evidence of TB, using modern scientific methods, has been found from excavations of mummified bodies from tombs from ancient Egypt dating as far back as 2400 BC (Allison *et al*., 1961; Nerlich *et al*., 1997; Zink *et al*., 2003). Drawings, pottery and statues of ancient Egypt that date up to 3000 BC have shown physical deformities that appear to show typical characteristics of TB of the spine (Vasiliadis *et al*., 2009; Dyer, 2010).

The first available writings about 'phthisis', meaning 'wasting away' in Greek, by Hippocrates (~460–370 BC) in his *Of Epidemics* dates as far back as 400 BC. Hippocrates, who is largely thought to be the father of modern medicine, believed phthisis was caused by growths in the lung, which he referred to as *tubercular*. He described phthisis as the most widespread disease of the era and provided detailed descriptions of the disease that included fevers, sweats, cough and wasting which closely resemble those of TB. The devastating nature of the disease even led Hippocrates to advise other physicians to avoid visiting 'consumption' patients with advanced disease because they would inevitably die and destroy the reputation of the attending physician. As pulmonary phthisis was commonly seen among close family members, Hippocrates and others widely considered the disease to be hereditary, a notion that persisted over a century. Aretæus, a Greek physician monk, described 'consumption' as 'a disease with a poor prognosis that was characterized by a chronic discharge of opaque, whitish yellow fluid from the lungs' (Dyer, 2010, p. 31). He associated people with a pale, slender and weak body type to be highly likely to develop TB. Another Greek physician, Clarissimus Galen (130–200 AD) downplayed the prevailing consideration of TB as a hereditary disease and instead came up with another theory that suggested transmission from person to person as

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

another way by which TB could be spread. This alternate proposition ushered in the possibility, even at this very early stage, of an infectious nature of the disease that would ultimately be proved to be right. Later Girolamo Franscatoro (1478–1553), an Italian physician, suggested that phthisis could be transmitted by invisible particles which he called *seminara*, and that the disease was a result of a lung ulcer. Franscatoro was also the proponent of the use of the term *phthisis* to be restricted to the description of only pulmonary consumption instead of its common use that referred to all cases of 'wasting'. The development of techniques for performing postmortems by Andreas Vesalius (1514–1564) and his colleagues in the 16th century further advanced knowledge of TB by introducing a way in which specific symptoms could be associated with the cause of death.

The precise pathological and anatomical descriptions of the disease only began to appear in the 17th century when in 1679 a Dutch physician, Franciscus de la Boë (Sylvius), identified the 'tubercle' as a consistent characteristic change in the lungs and other areas of 'consumptive' patients. One of Sylvius' students, Thomas Willis (1621–1675), related the localized lesions in the lungs and other organs to the general wasting away of the body. Another of his students, Richard Morton (1637–1698), described the three stages of phthisis: initial inflammation, formation of tubercles, and progression to ulcers and fully fledged consumption disease. Together, Willis and Morton described a form of TB that affected lymph nodes in the neck, which they called scrofula. In 1702, Mange went on to describe the pathological features of miliary TB.

In 1720, an English physician known as Benjamin Marten described the single-celled organisms (contagious microscopic animalcula) and speculated that TB might be caused by 'wonderfully minute living creatures' which could enter the body and generate lesions and symptoms of phthisis. However, it is thought that most of his work was not taken seriously because it was not published, only appearing among daily newsprints among other non-scientific material (Doetsch, 1978). The first experimental evidence that consumption could be transmitted from humans to cattle and from cattle to rabbits was demonstrated in 1865 by Jean-Antoine Villemin, a French military surgeon. The definitive cause of TB being the tubercle bacilli was only conclusively demonstrated by the German bacteriologist Hermann Heinrich Robert Koch in 1882 when he isolated and cultured bacilli from crushed tubercles. He made his findings public at the Physiological Society of Berlin on 24 March 1882, and later in an article entitled *Die Ätiologie der Tuberculose*. Three years later, Paul Erhlich discovered the acid-fastness of the TB bacillus (Burke, 1955; Allen and Hinkes, 1982). In 1890, Koch presented findings of a material he had isolated from the tubercle bacilli. He called this tuberculin and wrote that it could 'render harmless the pathogenic bacteria that are found in a living body and do this without disadvantage to the body' (Koch, 1890). Koch even inoculated himself with the tuberculin from which he developed what he termed an unusually violent attack and fever, and also made him wonder whether the test could be used as a diagnostic test for TB (Koch, 1891). The reaction to tuberculin observation was soon picked up and used to develop a skin test that begun to be used widely as a diagnostic tool in cattle. The tuberculin test was subsequently used to assess exposure of humans to the tubercle bacilli and has remained the main screening test for TB exposure to the present day. Koch's work in unravelling the causative agent of TB was recognized with the Nobel Prize in Medicine or Physiology in 1905.

Mycobacterium tuberculosis, the organism that causes the majority of TB cases in humans, belongs to a closely related cluster of species called the *M. tuberculosis* (Mtb) complex (MTBC). This complex includes *M. bovis* (Karlson and Lessel, 1970), which primarily causes bovine TB in cattle, deer and elk, but also causes TB in humans (albeit to a lesser extent), as do *M. africanum* (Castets *et al*., 1968) and *M. canettii* (van Soolingen *et al*., 1997). Other members of the complex such as *M. microti* (Wells and Oxon, 1937), host-adapted *M. caprae (*Aranaz *et al*., 1999), *M. pinnipedii* (Cousins *et al*., 2003) and the newly described member of the Mtb complex, *M. mungi* (Alexander *et al*., 2010), have been found infecting goats, seals and banded mongooses, respectively, suggesting that if one were to look hard

enough among other social mammals, other host-adapted members of the complex could be identified (Marcel Behr, 2014, personal communication to L.E. Via). Other MTBC species would most likely be found infecting social herbivores and omnivores, as the life history of the organism requires a reasonable density of hosts for successful transmission. Recent genomic analysis of *M. canettii* strains, which have a much larger genome and colony morphology distinct from most other MTBC, has suggested that the species may be more closely related to the ancestral tubercle bacilli than the MTBC (Supply *et al*., 2013). The natural reservoir for this species, if it is not humans, is currently unknown.

Consistent documentation of TB remained unavailable until around the 17th century when TB fatalities had reached high proportions in Europe and became the major cause of death by the 20th century. Tuberculosis, which was largely considered to be a disease of the poor, had by this time become established and even afflicted royalty. Over the years it had affected many famous personalities including St Francis of Assisi, Charlotte Brontë, John Keats, George Orwell, Eleanor Roosevelt and Vivian Leigh (Moorman, 1940; Zink *et al*., 2005; Ducati *et al*., 2006).

Pathogenesis of TB and Routes of Infection

The pathogenesis of Mtb was tragically illustrated when 250 infants were mistakenly 'vaccinated' with virulent bacilli rather than the intended *M. bovis* BCG vaccine stock in Lübeck, Germany, in 1930 (Luca and Mihaescu, 2013). Twenty-nine per cent of the infants died within the first year, but another 135 showed signs of infection yet recovered unaided by existing antibiotic therapy. In the early streptomycin clinical trials of adults with pulmonary TB, roughly 50% showed improvement when assigned to bed rest alone (Fox *et al*., 1999). Once exposed to Mtb, those who do not develop primary symptomatic disease are estimated to have a 10% lifetime risk of developing clinical disease (Corbett *et al*., 2003). Tuberculosis in humans is mainly transmitted via the inhalation of infectious droplet nuclei produced by an infectious host while coughing, sneezing or talking. The lungs are the most common site of infection although TB lesions can be found in any part of the body. Other methods of transmission include inoculation and ingestion (Walker, 1910). Transmission by infection was mainly noted among butchers when bovine tuberculous material gained access to the body via small cuts and wounds. Transmission by ingestion, also fairly common at one time for bovine TB, is thought to be fairly uncommon now because most of the milk that is consumed now is pasteurized. Though rare, there have also been cases of transplacental transmission of TB (Lee *et al*., 1998; Chen and Shih, 2004; Abramowsky *et al*., 2012).

Tuberculosis infection typically begins when tubercle bacilli aerosolized by someone with infectious TB are inhaled by a susceptible host. The droplet nuclei carrying the bacilli are often small enough to be inspired to the terminal alveoli where the bacteria are engulfed by professional macrophages and may be killed. If some bacilli survive this initial innate immune response, they start replicating in the macrophage and can migrate to nearby epithelial cells (Urdahl *et al*., 2011). The bacilli can also be disseminated by macrophages to the local lymph nodes using the lymphatic system, and to other parts of the body via the bloodstream, where they can infect other cells. The inflammatory response triggered by this process results in the migration and accumulation of additional immune cells such as neutrophils and lymphocytes to the primary infection site, eventually forming the initial granulomatous lesion or Ghon focus (Gonzalez-Juarrero *et al*., 2001; Doherty and Andersen, 2005). If the immune system fails to contain the infection, bacilli in the granuloma multiply and cause the granuloma to increase in size and cellularity, which leads to necrosis, local disease spread and in some cases cavity formation in the lungs. If the bacilli are spread though the blood or lymphatic system, miliary TB may ensue. The inflammatory processes that ensue produce the typical symptoms that are seen in active TB patients, such as weakness, fever, weight loss, night sweat, chest pain, dyspnoea, cough and haemoptysis.

If the immune system manages to contain the infection, the granulomas may shrink and calcify, trapping the bacilli inside, where they can persist in a dormant, non-replicative state for a long time constituting an asymptomatic or latent TB infection. Immune competent individuals latently infected by TB have a 10% lifetime risk of developing clinical disease (Corbett *et al*., 2003). The persisting bacilli contained in the Ghon focus and other initial lesions have been hypothesized to start multiplying again due to changing host conditions including advancing age, waning of the immune system, malnutrition, alcoholism, diabetes, immunization with BCG (Stead, 1967) and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), resulting in clinical TB disease. The premise that bacilli in granulomas are solely responsible for disease reactivation has been countered by necropsy studies that have found viable and infective bacilli in unaffected areas of the lung tissues (Feldman and Baggenstoss, 1938; Bishai, 2000) and in adipose tissues surrounding several organs (Neyrolles *et al*., 2006). More recent non-human primate studies have presented an even more complex picture, which suggests the presence of different types of lesions that vary from liquefied cavities to non-necrotic hypoxic lesions, with and without any viable bacilli with heterogeneous response to anti-TB treatment (Barry *et al*., 2009).

Early Intervention in TB Management

The milestone reached by the unequivocal demonstration by Robert Koch in 1882 that *M. tuberculosis* was the causative agent of TB did not immediately lead to significant improvement in its treatment. Early interventions were advanced by Leopold Auenbrugger (1722–1809), who associated the variety of sounds produced by tapping the chest with different symptoms of TB. Observations from this technique were later refined and used to develop a technique called percussion, which is still used today. Further breakthroughs were achieved via the discovery of X-rays by Wilhelm Conrad von Röntgen (1845–1923) in 1885, which was improved upon by Thomas Edison such that, by the 1920s, the technique proved helpful in the diagnosis and assessment of TB (Daniel, 2006).

One of the practices of treating TB that persisted for a long time was bleeding patients. Another related method was blood cupping that involved drawing blood from TB lesions with a premise that the bleeding would draw the infection from the lesions. When these practices declined, the use of various ointments, including administration of solutions such as iodine in the 1840s, was popularized until the use of cod-liver oil gained favour (Johnson, 1933).

The manner in which TB was treated changed fundamentally when a Siberian botany student, Hermann Brehmer, who had TB, was advised by his physician to seek out a healthier climate to help heal the disease. Following his physician's advice, Hermann travelled to the Himalayan mountains where he continued to work on his research and returned home free of the disease and studied medicine. After completing his medical studies in 1854, Hermann established an institution in Germany, where beds of TB patients were placed on balconies to expose them to continuous fresh air, and good nutrition was provided. This anecdotal observation led to the establishment of sanatoria throughout Europe and the USA with an emphasis on a regimen based on suitable climate, rest, good nutrition, fresh air and sunshine to treat chronic lung diseases, including TB (Kinghorn, 1921). However, sanatoria were subsequently closed down around the 1960s partly because of the slow healing process and because there was no major difference in case fatality rates among patients in sanatoria from those outside (Grzybowski and Enarson, 1978).

The next attempt at trying to cure TB was a process called collapse therapy by which TB could be cured by shrinking the lung, a technique initiated by an Italian physician Carlo Forlanini (1847–1918) in 1888. The procedure involved injecting air or nitrogen into the interpleural space, increasing the pressure until the lung collapsed. The premise was that the collapsed lung would be given a chance to rest while it repaired itself and that the process would cut off the oxygen supply to the TB bacteria, presumably killing them (Sakula, 1983).

Major advances in the management of TB were only realized following the isolation of streptomycin (the first antibiotic) by Selman Waksman in 1944; this was bactericidal against *M. tuberculosis* (Schatz *et al*., 1944). This was followed by the introduction of para-amino salicylic acid for treating TB, discovered by Jorgen Erik Lehmann (Lehmann, 1946). In 1952, Gerhard Domagk discovered yet another anti-tuberculosis drug, isoniazid (Lancaster, 1990) that would become one of the cornerstone drugs for TB treatment.

Susceptibility and Spread of TB Infection

Nearly everyone is susceptible to TB infection though the risk is higher among certain populations. The populations with a higher risk comprise individuals who have impaired immunity, and those who are constantly exposed to infectious TB patients. The latter group includes residents of high TB incidence settings, and people who either cohabit or are in close contact with infectious patients. For example, results from a recent household contact study found 6.9% of the 1206 TB contacts tested harboured *M. tuberculosis.* This study also found that most (89.2%) of the infected contacts were adults and that the majority (62.7%) of these contacts were close relatives, including 14.5% spouses (Singh *et al*., 2013). Another study detected TB infection in 64.6% of the contacts with a further 1.8% being TB culture positive. Close relatives, older age and cohabitation were also found to be associated with TB among contacts elsewhere (Sia *et al*., 2010). More recently, a review of data from studies that investigated the prevalence of latent and active TB infection and annual incidence of TB among contacts of patients with TB found that 51.5% of the contacts of TB patients in the studies from low- and middle-income settings were latently infected with TB, while 1.2% actually had active TB (Fox *et al*., 2013).

The contacts of TB patients who were found with active TB encompassed immunosuppressed individuals who lived or worked in institutionalized facilities such as hospitals, nursing homes, correctional facilities and homeless shelters. In addition, several factors have been shown to affect susceptibility to TB. Some of these factors include high bacillary load of infectious TB in the index case, proximity and length of exposure to an infectious case, co-infection with diseases or conditions that impair the immune system, malnutrition and young age (Narasimhan *et al*., 2013), abuse of alcohol and genetic factors (Davies and Grange, 2001) as well as diabetes (Kim *et al*., 1995; Alisjahbana *et al*., 2006; Jeon and Murray, 2008; Reed *et al*., 2013). Children under the age of five and those living with human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) are particularly prone to TB infection (Fox *et al*., 2013).

The role of genetics in susceptibility to TB has been debated for a long time (Davies *et al*., 1999). Evidence has even been demonstrated in a number of monozygotic and dizygotic twin studies (Simonds, 1957; Comstock, 1978) though it has been difficult to exclude the role of environmental factors (van der Eijk *et al*., 2007). Lately, the role of genetics in susceptibility to TB has received considerable attention, with current data suggesting an association between resistance to TB and host genetics. One reaffirmed the association of WT1 chr11 (rs2057178) genetic locus with TB susceptibility (Chimusa *et al*., 2013) and another found an association between a number of polymorphisms in the NRAMP1, VDR, HLA-DRB1 and HLA-DQB1 (Wu *et al*., 2013). Several other studies have implicated genetic polymorphisms such as the toll-like receptor 9 gene (Torres-García *et al*., 2013), HLA-A, B and DRB1 alleles (Mishra *et al*., 2013), P2X7 A1513C (rs3751143) gene polymorphism (Areeshi *et al*., 2013), genetic variations in the dicer 1, ribonuclease type III (DICER) mRNA (Song *et al*., 2013), polymorphisms in the Chr18q11.2 locus (Wang *et al*., 2013), ALOX5 (Pontillo *et al*., 2013; Shen *et al*., 2013) HSPEP1-MAFB genes (Mahasirimongkol *et al*., 2012), MRC1 polymorphism (Zhang *et al*., 2012), MCP-1 -2518 A/G polymorphism (Ben-Selma *et al*., 2011); SLC11A1 gene polymorphisms (Jin *et al*., 2009; Stagas *et al*., 2011); markers on chromosomes 15q and Xq (Bellamy *et al*., 2000), NRAMP1 and TNFA (Shaw *et al*., 1997). It has been hypothesized that resistance of a population to

TB may largely be based on the historic exposure of the population to the disease (Stead, 1992). Racial differences have been implicated in a study that administered a skin test among people in homeless shelters in which higher positive skin test results were observed among blacks compared to Caucasians. This difference has been thought to be due to the resistance to TB developed by Caucasians, particularly in Europe where TB has been endemic for a much longer period (Dubos and Dubos, 1952).

Epidemiology of TB

Today, TB constitutes one of the leading causes of morbidity and mortality worldwide, ranking only second to HIV/AIDS as the most causative agent of death. According to current data, a total of 6.1 million TB cases were reported to the World Health Organization (WHO) by national TB programmes worldwide with 5.7 million being new cases and 0.4 million cases being retreatment cases. India and China accounted for 39% of the cases, while the WHO African Region accounted for 23% of the cases in 2012. Thus, based on current estimates, between 11 and 13 million prevalent cases (equivalent to 169 cases per 100,000 population) occurred in 2012 of which about 8.6 million people (equivalent to 122 cases per 100,000 population) were incident cases, with an estimated 1.3 million fatalities. A group of 22 countries, collectively called high burden countries (HBCs) by the WHO, contributed a total of 81% of the 8.6 million global TB incident cases. The majority of the cases occurred in South-east Asia and the West Pacific Region (58%) while the African Region accounted for 27% of the total cases, and also recorded the highest rates of cases and deaths relative to population at 255 incident rates per 100,000 population (WHO, 2013). The highest contribution of cases to the global total was from India (26%) and China (12%), whereas 'South Africa and Swaziland had the highest incidence rates per capita (about one new case for every 100 people each year)' (WHO, 2013, p. 6). There was a wide variance in the TB incidence rates among countries, with the lowest being about ten cases per 100,000 population being mostly found in high-income countries and the highest rates being in low-income countries. The best estimate for the countries with the highest incidence rates was 1000 per 100,000 population per year for South Africa and Swaziland. There has been a gradual downward trend in the global incidence rates of TB from 2001, with a rate of 2% being recorded between 2011 and 2012 (WHO, 2013). Consequently, although the estimated global prevalence rate (169 cases per 100,000 population) above is still very high, it represents a 37% global decline since 1990 which, in addition to the mortality rate that has also fallen by nearly half (45%) since then, underscores the tremendous progress that has been made thus far.

With regard to the Millennium Development Goals (MDG) global 2015 targets, substantial progress has been made where a number of the set targets are within reach. These include the falling incidence rates of TB worldwide over the last decade, albeit slowly. The 45% recorded mortality rate of TB in 2015 is just 5% shy of the 50% target. In addition, the regions of the Americas and Western Pacific have already achieved the 2015 targets. Also, seven of the 22 HBCs have equally met the 2015 target for reduction of TB incidence, prevalence and mortality. However, there remain some challenges: for example, it is unlikely that the 50% reduction in TB prevalence in the community, which was at 37% in 2012, will be reached by 2015. Also, 11 of the 22 HBCs are unlikely to meet the targeted goals. The same is true with the target for MDR-TB (WHO, 2013).

Strategies of TB Control

The introduction of effective drug treatments in the mid-1940s led to tremendous declines in global TB rates until the early 1980s when the trend was reversed, in part due to the advent of HIV/AIDS. The gradual increase in the TB cases reached epidemic proportions resulting in the declaration of TB as a public health emergency by the WHO in 1993, with a call for governments worldwide to prioritize an increase in scale of TB control efforts (Raviglione, 2003). To back up this declaration,

several efforts were put in place by the WHO. The first was the launch of the recommended TB strategy control, which was later named Directly Observed Therapy Short-course (DOTS) that relied upon five elements: political commitment, case detection utilizing smear microscopy, standardized short-course chemotherapy, regular uninterrupted supply of all essential anti-TB drugs and programme supervision and evaluation. This was followed by the launch of the Stop TB Partnership in 1998 with the ambitious goal of eliminating TB as a public health problem by 2050. Other efforts to tame TB included the declaration of the MDGs by the United Nations in 2000, which committed nations to a new global partnership to reduce extreme poverty by setting out a series of time-bound targets for 2015, and advanced the cause for TB control (United Nations, 2013).

The DOTS strategy was initially developed as a public health approach to control TB in a cost-effective manner in resource-limited situations, with emphasis on prioritizing smear-positive patients. With time, however, a number of public health challenges arose, including the TB/HIV co-epidemic and the emergence of *M. tuberculosis* isolates that were resistant to at least rifampicin and isoniazid; these isolates were termed multidrug resistant (MDR). These challenges necessitated some changes in the global environment towards a more human approach to public health. This led to the redesign of disease control efforts that were more patient-centred, and directed towards universal access to care, culminating in the launch of the Stop TB Strategy in 2006 (WHO, 2006). The main goal of the Stop TB Strategy was to reduce substantially the global burden of TB by 2015 in line with the MDG and Stop TB Partnership targets and to achieve major progress in the research and development of the tools needed for TB elimination. The 2015 targets were to reduce the prevalence and mortality of TB by 50% compared to the prevailing rates in 1990. This strategy was updated to constitute the Global Plan to Stop TB 2011–2015, which provides clearer action items and guidance on what needs to be done to achieve the set goals by 2015 (WHO, 2011). The main difference between the DOTS strategy and the Stop TB Strategy was the enhancement of the concept of patient-centred care for all individuals with TB. The current Stop TB Strategy (WHO, 2013) comprises six components:

1. Pursue high-quality DOTS expansion and enhancement.
2. Address TB/HIV, MDR-TB and the needs of poor and vulnerable populations.
3. Contribute to health system strengthening based on primary health care.
4. Engage all care providers.
5. Empower people with TB and communities through partnership.
6. Enable and promote research.

TB and HIV Co-infection

Tuberculosis and HIV are responsible for the majority of the mortality observed worldwide as a result of communicable diseases. The reported TB incident cases in 2012 included about 1 million (13%) people who were co-infected with HIV. Altogether, 37% of the estimated TB/HIV co-infected cases resided in the WHO African Region countries, and collectively accounted for 75% of all TB/HIV co-infections worldwide. However, the estimated percentage of people living with HIV has remained steady over the recent years at 13% worldwide. About three-quarters of the deaths in 2012 occurred in the African and South-east Asian Regions, with India and South Africa accounting for nearly one-third of the global fatalities. About one-half of the TB patients in some African countries are additionally infected with HIV. With regard to gender, 34% of the estimated 8.6 million cases in 2012 were among women with the African and South-east Asian Regions accounting for 68% of the cases. Of the ~410,000 female deaths in 2012 nearly one-half occurred among HIV-positive cases (WHO, 2013).

The global notification of TB among children (≤15 years) was estimated to be 530,000 new cases, representing about 6% of the global incidence cases. An estimated 74,000 HIV-negative children with TB died in 2012, accounting for about 8% of the total estimated deaths. The case fatality rates among HIV-positive children were not available (WHO, 2013). The detrimental association between TB and HIV

appears to potentiate each condition in many aspects such as pathogenesis, epidemiologic profile, clinical presentation, treatment and prevention, not to mention the associated socio-economic issues. This is clearly evidenced by the fact that all areas with high TB cases are also high HIV-prevalent countries (Nunn *et al*., 2007). This is further substantiated by the observation that HIV infection is the major risk for progression of latent TB infection into active disease and that risk of developing active TB is significantly higher among TB/HIV co-infected patients (10% annual risk) compared to those solely infected with *M. tuberculosis* (8–10% lifetime risk) (Bloom and Murray, 1992). Although HIV-positive TB patients are generally shown to be less infectious than their HIV-negative counterparts (Cruciani *et al*., 2001), the mortality rate among this group is comparatively very high, i.e. 13.7% versus 0.5%, respectively (Murray *et al*., 1999).

Apart from the above, HIV infection alters the clinical picture of HIV-positive TB patients. For example, there is a high rate of false-positive skin tests among HIV patients (Barnes *et al*., 1991; Syed Ahamed Kabeer *et al*., 2009). In addition, atypical chest X-ray findings and/or TB patients with sputum smear negative results are not uncommon among HIV patients (Corbett *et al*., 2003; Mendelson, 2007; Hanekom *et al*., 2010). TB/HIV co-infected patients also frequently tend to suffer from extrapulmonary TB (Fätkenheuer *et al*., 1999) predominantly caused by opportunistic non-tuberculous mycobacteria such as the *M. avium* complex, *M. chelonae*, *M. fortuitum* and *M. kansasii* (Brennan and Nikaido, 1995). In a recent study, HIV infection has been shown to alter Cluster of Differentiation (CD)4 T cell memory phenotype among extrapulmonary TB patients (Matthews *et al*., 2012). The use of some TB drugs such as thiacetazone is contraindicated in HIV-infected patients due to some adverse side effects, thereby reducing the already limited options for proper management of TB (Kuaban *et al*., 1997) especially if one considers that the highest burden of TB is in settings that also have high prevalent rates of HIV. Because of these reasons, HIV has for some time been considered to be the most important single predictor of TB incidence in Africa (Corbett *et al*., 2003).

Treatment of TB

Tuberculosis is now a fairly curable disease for which effective treatment is available globally. The aims of TB treatment are to cure the patient and restore quality of life and productivity, prevent death from active TB or its late effects, prevent relapse, and prevent development and transmission of drug resistance (WHO, 2010).

The drugs are grouped mainly in two categories, namely first-line and second-line drugs, depending on their application. First-line drugs are administered for 6 or 8 months in what is commonly referred to as 'short-course chemotherapy' (SCC). This strategy targets treatment of drug-susceptible TB and is divided into two phases: the intensive phase and the continuation phase. The intensive phase covers the first 2 months of treatment and aims to kill actively growing and semi-dormant bacilli, thereby reducing the duration of infectiousness of an individual. The continuation phase lasts between 4 and 6 months depending on disease site and drug combination used and is intended to eliminate bacilli that are still multiplying and also reduce the risk of failure and relapses. In general, four categories of treatment can be distinguished according to the diagnostic status of the patient (smear positivity and treatment history). Within each category various options exist. The type of regimen used in a particular country depends on affordability, coverage by public health services and competence of the staff at a peripheral level (WHO, 2010).

First-line Drugs

The first-line drugs are mainly bactericidal and combine a high degree of efficacy with a relative toxicity to the patient during treatment and are mainly used in the treatment regimens of non-MDR-TB. They comprise isoniazid (H), rifampicin (R), streptomycin (S), ethambutol (E) and pyrazinamide (Z) (WHO, 2010).

H acts by inhibiting mycolic acid synthesis (Winder and Collins, 1970). Mutations in the *kat*G gene that encodes the catalase-peroxidase that activates the prodrug have been the most

frequently associated with H resistance (75–85%) (Garcia de Viedma, 2003). R, on the other hand, inhibits RNA synthesis by binding to the β-subunit of the RNA polymerase (Musser, 1995), and is the most potent sterilizing agent of all the first-line drugs. Mutations in the *rpo*B gene account for >98% of R-resistant isolates (Traore *et al*., 2000). Resistance to R is also commonly used as an indicator for MDR-TB (Cho *et al*., 2013). Together, H and R constitute the most powerful bactericidal TB drugs and are active against all populations of the TB bacilli.

S is bactericidal against rapidly multiplying TB bacilli. It acts by inhibiting protein synthesis and damaging cell membranes, which results in the death of the bacteria. Mutations in the *rrs* and *rps*L genes account for 65–75% resistance to S (Finken *et al*., 1993).

E is bacteriostatic and has a synergistic action with more powerful drugs to prevent the emergence of resistant bacilli. It inhibits the synthesis of the cell wall by interfering with the transfer of D-arabinose into cell wall arabinogalactans (Mikusova *et al*., 1995). Arabinogalactans are complex branched polysaccharides that connect mycolic acids to the inner peptidoglycan of the cell wall (Brennan and Nikaido, 1995). Mutations in the *emb*CAB operon coding for different arabinosyl transferases account for about 70% of resistant strains (Garcia de Viedma, 2003).

Z is bactericidal but is only active in an acid intracellular environment. It acts by inhibiting mycolic acid synthesis (Zimhony *et al*., 2000). Resistance to Z is mediated via mutations in the *pnc*A gene, encoding for pyrazinamidase (Scorpio and Zhang, 1996).

Fixed-dose TB Tablets

The WHO has developed and recommended formulations of a model list of essential anti-TB drugs and fixed-dose combinations (FDCs) of drugs (www.who.int/medicines/publications/essentialmedicines/en). FDC tablets contain different combinations of drugs, such as HR, HE, HRZ and HERZ. The efficacy of these FDCs has been shown to be comparable to the single tablet regimens, at least in smear-positive pulmonary TB patients (Bartacek *et al*., 2009). Some of the advantages of using FDC tablets include preventing the development of drug resistance, simplification of treatment and management, and reduction of misuse of the drugs for treatment of conditions other than TB. The main disadvantage with FDC tablets lies in the difficulty in handling side effects.

The WHO has also issued recommendations for treating TB in persons living with HIV. The recommendations state that TB patients with known positive-HIV status and all TB patients living in HIV-prevalent settings should receive daily TB treatment at least during the intensive phase and if possible for the continuation phase (Hopewell *et al*., 2006; WHO, 2010).

Second-line Drugs

Second-line drugs are reserve drugs that are only used in situations where the first-line drugs are failing. In general, they are less effective than first-line drugs but are more toxic, more expensive and require lengthy periods of administration of up to 2 years. They include fluoroquinolones such as ciprofloxacin and ofloxacin, which act by inhibiting type II topoisomerase (Wang, 1996) and aminoglycosides like kanamycin and amikacin (Edson and Terrell, 1999). Other drugs included in this category are viomycin, capreomycin (Herr and Redstone, 1966), ethionamide and para-aminosalicyclic acid. Other drugs include gatifloxacin, moxifloxacin and dyarilquinoline (Andries *et al*., 2005). Streptomycin is the oldest and perhaps the only drug that is used to treat drug-susceptible TB and extensively drug-resistant TB.

Acquisition of Drug Resistance

The increasing level of resistance to available TB drugs has necessitated the repurposing of old drugs and development of new anti-TB drugs. Drug resistance is a result of acquisition of spontaneous genetic mutations that occur naturally in individual mycobacteria.

Typically, the rates at which these mutations are acquired are so low that this mechanism would not lead to the clinical drug resistance of *M. tuberculosis* to TB drugs that is seen today. For example, the natural rate at which H and R acquire mutations is 3.5×10^{-6} and 3.1×10^{-8}, respectively. It is thus perceived that much of the resistance we normally encounter may be attributed to irregular drug intake due to either non-compliance or availability, poor quality of drugs in some instances and co-infection with non-tuberculous mycobacteria (NTM). The prolonged exposure to a single drug or suboptimal therapy may lead to the selection and expansion of resistant Mtb strains. In addition, the possibility of acquiring double spontaneous mutations to H and R, in the above example, is very low (9×10^{-14}). This suggests that resistance to more than one drug will most likely occur following sequential acquisition of mutations of different drugs as a result of sustained treatment failure (Traore *et al*., 2000).

Hope of New TB Agents

There has been an enhanced effort by many drug companies to invest in TB drug development. Presently, at least ten new or repurposed TB drugs are in the late phases of clinical development. In 2012, bedaquiline became the first new drug in a long time to be approved by the Federal Drug Administration (FDA) in the USA for treating MDR-TB patients. A number of other drugs including linezolid, sutezolid (PNU-100480), PA-824, SQ-109 and AZD-5847 are in Phase II clinical trials (WHO, 2013). In addition, other drugs are already in Phase III trials. For example, studies that evaluated the substitution of H by moxifloxacin in the intensive treatment phase and used rifampetine in the continuation phase reported favourable results (Jindani *et al*., 2014). Two other trials investigating the use of gatifloxacin instead of E or the substitution of moxifloxacin by either E or I are in progress. A third trial is currently evaluating the use of delamanid (OPC-67683) for treatment of MDR-TB (WHO, 2013).

Worldwide Prevalence of Drug Resistance

The bulk of the available data on global TB drug resistance has been systematically collected by the Global Project on Anti-Tuberculosis Drug Resistance Surveillance that was instituted by WHO in conjunction with the International Union against Tuberculosis and Lung Disease (IUATLD) from 1994 onwards. The data show that drug resistance among *M. tuberculosis* isolates is ubiquitous (WHO, 1997, 2000, 2004, 2008, 2010; Pablos-Méndez *et al*., 1998; Espinal *et al*., 2001; Aziz *et al*., 2006; Wright *et al*., 2009). Data exist from two-thirds of the WHO's 193 member states with at least two data sets being contributed by 71 countries. According to the 2008 global data on drug resistance among new cases, drug resistance to at least one anti-TB drug ranged from 0% to 56.3% Baku city (Azerbaijan), while the corresponding MDR rates ranged from 0% to 22.3% in Baku city. On the other hand, resistance to at least one anti-TB drug among previously treated TB patients ranged from 0% to 85.9% in Tashkent (Uzbekistan) with the highest MDR rates being recorded in Baku city, Azerbaijan (55.8%) and Tashkent (60%). According to global estimates, any drug resistance ranged from 0% in three European countries to 85.9% in Tashkent, Uzbekistan. Data on extensively drug-resistant TB (XDR-TB) available from 11 countries showed that, by and large, the XDR proportions among MDR-TB were lower in Central and Western Europe, the Americas and in the Asian countries (range 0–30%). The data from nine countries of the former Soviet Union indicated approximately 10% of MDR-TB cases were also XDR ranging from 4% in Armenia to 24% in Estonia (WHO, 2008). The proportion of XDR cases in MDR-TB cases in the latter countries was more worrisome because the percentages are based on high absolute numbers of cases compared to the former countries where the absolute number of MDR cases were few.

More recent data show new MDR-TB rates that ranged from 0% to 28.9%, with the highest rate being reported in Murmansk (Russian Federation), while the percentage among previously treated cases varied from 0% to 65.1%. Of the 38 countries and territories

that reported XDR cases, more than ten cases of XDR-TB were reported in only 6 (15%) instances (Zignol *et al.*, 2012). Overall data showed MDR rates of 3.4% (95% CI:1.9–5.0) and 19.8% (95% CI:14.4–25.1) among new and previously treated patients, respectively (WHO, 2013).

In general, the trends show that most low-burden TB countries exhibit stable drug resistance rates and absolute numbers of TB cases but there are increasing MDR rates in the Baltic States and in countries of the Russian Federation (WHO, 2008).

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2 Comparative Mycobacteriology of the *Mycobacterium tuberculosis* Complex

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Introduction

> I feel quite confident that the comparative study of tubercle bacilli will lead to some definite understanding on certain important questions, and eventually to more light on the whole subject of tuberculosis from the preventive as well as the therapeutic side.
> (Theobald Smith, 1898)

The *Mycobacterium tuberculosis* complex (MTBC) is a group of highly genetically related pathogens that cause tuberculosis (TB) in mammalian species. However, the very name of the complex underlines the fact that our knowledge of these pathogens is dominated by studies on the human pathogen, *M. tuberculosis*. Of course this is entirely justified; *M. tuberculosis* is a major global pathogen that exacts a horrendous burden in terms of mortality and morbidity so it is appropriate that it is the cornerstone of the complex. In the same way as *M. tuberculosis* is the best studied human tubercle bacillus, our knowledge of the animal-adapted strains has been dominated by studies with *M. bovis*. Again, given the economic importance of bovine TB and the potential for zoonotic transmission to humans, this is entirely expected. However, taking *M. tuberculosis* and *M. bovis* as the human- and animal-adapted 'poles' of the complex, our focus on these pathogens to the exclusion of other members has restricted, and potentially skewed, our understanding of diversity, virulence and host adaptation within the MTBC. Referring to Theobald Smith above, have we really exploited comparative studies of the tubercle bacilli to their full potential, or have we regarded the MTBC as merely *M. tuberculosis* plus some animal pathogens of lesser import? Herein we discuss our current understanding of the make-up of the MTBC, focusing on comparisons of *M. tuberculosis* and *M. bovis* as the exemplar human- and animal-adapted strains, and look to what studies of these pathogens can teach us about the evolution of the MTBC specifically and the emergence of host adaptation in pathogens in general. We also speculate on how our current focus on *M. tuberculosis* and *M. bovis* may have hindered our appreciation of fundamental concepts such as virulence, evolution and host adaptation of the tubercle bacilli.

The *M. tuberculosis* Complex (MTBC)

The constituent members of the MTBC can be broadly split into the human- and animal-adapted strains. The major human pathogens,

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

where no obvious animal reservoir has been identified, are *M. tuberculosis* and *M. africanum* subtypes 1 and 2. The animal-adapted strains have been isolated from a range of wild and domesticated animals and are named after their host of initial/most frequent isolation, including *M. bovis* (Smith, 1898; Karlson and Lessel, 1970), *M. microti* (Wells, 1946), *M. caprae* (Aranaz *et al*., 1999), *M. pinnipedii* (Cousins *et al*., 2003), *M. orygis* (van Ingen *et al*., 2012), *M. mungi* (Alexander *et al*., 2010) and the 'Dassie bacillus' (Smith, 1960; Cousins *et al*., 1994). However, it should be noted that these species names do not define exclusivity in host range. MTBC members can infect a range of mammals to greater or lesser degrees; the central feature of host adaptation is the ability to sustain within a host population. So *M. bovis* can infect and cause disease in humans; however, the capacity of *M. bovis* to transmit between immune-competent humans is severely limited compared to *M. tuberculosis* (Francis, 1950; Magnus, 1966). Conversely, the capacity of *M. tuberculosis* to sustain in human populations is remarkable, with an estimated one-third of the world's population latently infected with *M. tuberculosis* (WHO, 2014). Yet, while *M. tuberculosis* has been isolated from sporadic cases of TB in animals such as cattle, elephants, dogs and cats, these represent reverse zoonoses from infected humans rather than *M. tuberculosis* sustaining itself in these animal populations (Alexander *et al*., 2002).

The positioning of *M. canetti* (van Soolingen *et al*., 1997), and other smooth tubercle bacilli (STB), in the MTBC is an area of debate (Brisse *et al*., 2006; Smith, 2006; Becq *et al*., 2007). Obviously if we define the MTBC as a group of *genetically related* strains that cause TB in mammals the STB represent a problem, as they are an out-group, with substantial levels of diversity compared to the other members of the complex. Comparative whole-genome analysis of five STB strains isolated from patients from East Africa (Supply *et al*., 2013) showed that the STB have extensive genetic diversity, with multiple instances of horizontal gene transfer, genomes 10–115 kb larger than *M. tuberculosis*, and 25-fold more single nucleotide polymorphisms (SNPs) compared to members of the MTBC. Therefore the STB represent a distinct group of human TB pathogens; whether they also cause TB in animals is an open question. A central argument in favour of an animal reservoir is lack of evidence for human–human transmission of *M. canetti*, suggesting that the ability of *M. canetti* to sustain within the human population is limited. An alternative explanation would be an environmental reservoir (Koeck *et al*., 2011), such as soil or water, perhaps similar to that seen for *M. ulcerans* (Bratschi *et al*., 2014), but again no clear evidence is available.

There has been an increasing identification and subdivision of strains in the MTBC, with additions over the last decade including *M. orygis* and *M. mungii*. This expansion of species within the MTBC is a product of the increasing sophistication of molecular methods to differentiate strains based on spoligotyping, deletion typing and whole-genome sequencing. It is beyond question that genomics has had, and will continue to have, an immense impact on our understanding of the MTBC, and we will turn to this next.

MTBC Genomics

In the current age of high throughput genomics, with the capacity to sequence multiple *M. tuberculosis* genomes in a matter of weeks, it is worthwhile taking a brief historical perspective on genomics of the MTBC. One of the first attempts to perform genomic comparison across the complex was by Mahairas *et al.* who used subtractive hybridization techniques to identify three large-scale deletions, region of difference (RD) R1–RD3, that were absent from *M. bovis* BCG but present in the genome of virulent *M. bovis* (Mahairas *et al*., 1996). The publication of the *M. tuberculosis* H37Rv genome (Cole *et al*., 1998) heralded the start of the genomic age of the MTBC proper, with the *M. tuberculosis* genome providing for the first hybridization-based comparative approaches using microarrays or clone-arrays (Behr *et al*., 1999; Gordon *et al*., 1999). These analyses revealed a set of deletions from *M. bovis* and BCG and were the first to throw doubt on pre-existing ideas that *M. tuberculosis* had evolved from *M. bovis* when man domesticated cattle. The completion of the genome of *M. bovis* AF2122/97 provided further

clarification of the evolution of these strains, confirming the close genetic identity between the human and bovine strains but showing that the genome of *M. bovis* had no unique genes per se compared to *M. tuberculosis* (Brosch *et al*., 2002; Garnier *et al*., 2003). Given the lack of substantial genetic recombination in the MTBC (Namouchi *et al*., 2012), the loss of genetic regions from *M. bovis* served to underline the fact that *M. bovis* could not be the progenitor of *M. tuberculosis*. The identification of the TbD1 locus as the first region found to be absent from *M. tuberculosis* H37Rv relative to *M. bovis* AF2122/97 provided a useful marker (Brosch *et al*., 2002), with screening for presence/absence of the TbD1 locus across *M. tuberculosis* strains allowing strains to be grouped as TbD1-intact or 'ancient' (a genome configuration closer the common ancestor) or TbD1-deleted or 'modern' (more distant from the common ancestor such as *M. tuberculosis* H37Rv). The picture that emerged from these initial genome studies was an evolutionary scenario where all MTBC strains arose from a common progenitor, but with extant *M. tuberculosis* being closer to the common ancestor of the MTBC and hence rejecting the notion that *M. tuberculosis* in humans arose from *M. bovis* in cattle at the time of domestication (Brosch *et al*., 2002; Mostowy *et al*., 2002).

Using a limited number of complete genome sequences to identify deletions (or SNP panels), that are then screened across large numbers of isolates, can generate distorted phylogenies. Hershberg *et al*. sought to address these concerns by applying multi-locus sequence typing (MLST) across the MTBC, using 89 gene fragments across 108 MTBC strains, an approach that disclosed six major lineages in the MTBC (Hershberg *et al*., 2008). In this phylogeny, *M. tuberculosis* showed much greater genetic diversity, and hence possibly phenotypic diversity, than previously assumed and the animal-adapted strains grouped together with *M. africanum* subtype 2. The move from MLST to whole-genome sequencing provided even greater resolution to the MTBC phylogenies, with an analysis of 21 MTBC strain genomes generating a phylogeny entirely congruent with previous constructs (Comas *et al*., 2010). Expansion of these genome analyses to encompass 259 MTBC strains and overlaying them with data on human mitochondrial haplogroups showed a striking correlation between global MTBC and human population distributions (Comas *et al*., 2013). These analyses suggested that the MTBC may have been a pathogen of humans for at least 70,000 years, with increased population sizes during the Neolithic demographic transition being a key driver in the success of the MTBC (Comas *et al*., 2013).

Comparative analyses of the MTBC and non-tuberculous mycobacteria (NTM), such as *M. kansasii* and *M. marinum*, have delivered insight into the initial evolutionary steps of the tubercle bacilli (Veyrier *et al*., 2011; Behr, 2013). What is evident is that free-living saprophytic or opportunistic pathogens in the mycobacteria genus have larger genome sizes and show evidence for recombination (Stinear *et al*., 2008; Gordon *et al*., 2009; Doig *et al*., 2012). These features, taken in the context of the diversity of Actinobacteria as a whole, suggest that the progenitor of the MTBC was a free-living saprophyte that obtained novel functions through horizontal gene transfer that provided selective advantages in the face of competition from other environmental microbiota (e.g. allowing it to resist digestion by protozoa). These traits served well for infection and maintenance within higher eukaryotes and eventually allowed adaptation to mammals as opportunistic and then obligate pathogens. Once the MTBC common ancestor was then trapped within its mammalian niche, the initial accretion of genetic material was counterbalanced by deletion events, resulting in the RD loci that act as unique markers for clades (Smith *et al*., 2009). Whether loss of these loci provided any selective advantage to the emerging clones, or was merely the fixation of deleterious mutations in small populations, remains to be defined.

The narrative of MTBC evolution is therefore of an environmental bacteria that host adapted to preferred mammalian host niches; *M. tuberculosis* is the pinnacle of a globally successfully pathogen that has ridden the waves of human migration out of Africa and flourished among burgeoning human populations. *M. bovis* is the MTBC animal-adapted strain of choice as it infects cattle, a

cornerstone of modern agriculture and a clear risk for zoonotic transmission from contaminated dairy or meat products. The exquisite ability of MTBC pathogens to infect and sustain across a diverse array of mammalian species is striking; elucidating the molecular events behind these host adaptation events will shed light not only on what makes the MTBC such a successful group of pathogens, but also the mechanism behind the emergence of new host-adapted pathogens per se.

Host Preference

Observations of the distinct host preferences of tubercle bacilli date back to the work of Koch, Von Behring, Smith and others. In seminal work Theobald Smith examined eight human isolates (one isolated from a pet of a TB patient); six isolates from cattle; and single isolates from a pig, cat and horse for their microscopic appearance, cultural characteristics and pathogenesis in mice, guinea pigs, rabbits, pigeons and cattle (Smith, 1898). The bovine isolates 'grew less vigorously for a number of generations' in coagulated serum, while 'bovine bacilli tend to remain short; human bacilli are either more slender from the start or become so during cultivation'. In terms of pathogens, bovine bacilli had 'a much greater pathogenic activity towards rabbits, guinea pigs and cattle' than human isolates. This work defined the bovine tubercle bacilli as distinct from the human isolates, and furthermore defined the distinctive virulence of the isolates in animal models (Smith, 1898). Emil von Behring took Smith's observations an entrepreneurial step forwards by generating a live attenuated *M. tuberculosis* vaccine strain for use in cattle (Bovovaccine) but which was never widely used due to concerns with residual virulence (Linton, 2005).

These classic experiments of Smith on host preference have been revisited using well-characterized MTBC bacilli, namely the genome sequenced strains *M. tuberculosis* H37Rv and *M. bovis* AF2122 (Whelan *et al*., 2010). Cattle infected with *M. tuberculosis* H37Rv and *M. bovis* AF2122 became positive to skin-test and interferon gamma release assays; however, the *M. tuberculosis* H37Rv-infected cattle showed no pathological signs of disease (even though the same *M. tuberculosis* H37Rv seed lot used to infect cattle caused disease in guinea pigs). Although these experimentally infected cattle did not show obvious pathological signs, cattle naturally infected with *M. tuberculosis* that show typical TB granulomatous lesions and are culture positive for *M. tuberculosis* have been identified in multiple studies in regions such as Ethiopia, Nigeria or China (Cadmus *et al*., 2006; Berg *et al*., 2009; Chen *et al*., 2009). One can however argue that rather than *M. tuberculosis* sustaining in these cattle populations, they instead represent reverse zoonotic infections in countries where the burden of human TB disease is high and immune status of cattle is compromised (Ocepek *et al*., 2005; Ameni *et al*., 2011). It could be argued, therefore, that cattle infected with *M. tuberculosis* recapitulate key presentations of TB in humans, from latent infection to active disease. The bovine–*M. tuberculosis* infection model may therefore present a unique model in defining the *M. tuberculosis*–host dynamic.

Animal-adapted Strains

With the increasing resolution of genome-based phylogenies it has become apparent that the animal-adapted strains of the MTBC described to date are most closely related to MTBC lineage 5 and 6, also known as *M. africanum* (Comas *et al*., 2010, 2013). Coupled with the evidence for an African origin for the MTBC, it is likely that the diversity of animal-adapted MTBC strains will only be revealed in its full glory when we isolate and characterize tubercle bacilli infecting assorted wild animals in Africa. A case in point is the isolation of a novel MTBC strain from banded mongooses in Botswana (Alexander *et al*., 2010). This strain, tentatively designated *M. mungi*, was unusual in that it caused high mortality in banded mongooses and appeared to be transmitted via a non-respiratory route. These characteristics may suggest that this is a newly emergent pathogen for the mongoose, or perhaps point to a particular susceptibility in this population. A novel tubercle bacillus has also been isolated from a chimpanzee in the Côte d'Ivoire (Coscolla *et al*., 2013). Whole-genome

sequencing and comparative analyses revealed that while this strain was most similar to lineage 6, it was part of a distinct lineage that had not been sampled before. Whether this 'chimpanzee strain' represents the first isolate of a distinct species remains to be seen, but both of these cases suggest the true diversity of animal-adapted MTBC awaits full description.

As the identification and differentiation of animal isolates of the MTBC continue apace, we should be cautious in defining each novel animal isolate as a new species. An obvious first consideration is whether the animal represents a 'maintenance' host that can truly sustain the pathogen population through transmission cycles and to which the pathogen is adapted, or is a 'spillover' host that the pathogen has been introduced to from an exogenous source and where the pathogen cannot sustain as it is not host-adapted. The complexity of this situation can be seen with *M. bovis*, which can sustain in maintenance hosts as diverse as cattle, badgers, brush-tailed possums, wild boar, bison and white-tailed deer, and where a spillover host is humans. A more holistic approach is therefore to think of the host-adapted MTBC as 'ecotypes', a concept first explored for bacteria by Cohan (2002) and applied to the MTBC by Maynard Smith and colleagues (2006). The bacterial ecotype concept suggested by Cohan assumes that divergence within a species is constrained by periodic selective sweeps, with a new ecotype emerging when a species adapts to a new niche such that it is then immune to selection in the ancestral population. Applying this concept to the MTBC, one can define ecotypes as evolutionarily related groups (clades) that infect and sustain within distinct host populations (each host representing a new niche); the fixed molecular differences such as RD and SNPs that are currently used to define species within the MTBC show that selective sweeps have not homogenized diversity across the clades. So the MTBC can be seen as a set of host-adapted ecotypes; for example, the *M. tuberculosis* clade is a human-adapted ecotype. This therefore provides a framework to define the emergence of truly novel ecotypes in the MTBC and allows us to search for host-adaptive mutations. The inclination to designate each new animal isolate as a separate 'species' may complicate discourse around host adaptation; for example, the differentiation of *M. caprae* as a distinct species from *M. bovis*, with the suggestion of 'caprine' host adaptation, may be a step too far.

The application of next-generation genome sequencing to clinical isolates, with its power to reveal evolutionary relatedness across strains, looks set to expand the membership of the MTBC further over the coming years. The low cost of genome sequencing compared to its resolving power, and the promise that novel MTBC strains await discovery in wild mammals, suggest that sequencing of animal isolates should become as common an occurrence as is currently being suggested for human isolates of *M. tuberculosis* (Walker *et al*., 2013).

Pathoadaptation and Virulence Factors

The key to a pathogen's life cycle is the possession of virulence systems that enable it to sustain in a host population. These virulence factors run the gamut from battering rams that act across multiple hosts, to lock-picks that open a host-specific backdoor, to set up infection. Defining virulence factors involved in host adaptation is complex, not least because 'virulence' depends on context; the host's immune status and genetic background have a major effect on the outcome of infection (Casadevall and Pirofski, 2000). While great strides have been made in defining virulence factors in the MTBC, these have largely been defined on the interaction between *M. tuberculosis* mutants and murine infection models. Below we highlight some of the difficulties in this approach, and suggest that analysis of comparative virulence might provide new perspectives on MTBC virulence.

RD1

RD1 was originally identified by Mahairas and colleagues as a region deleted from *M. bovis* BCG relative to virulent *M. bovis* or *M. tuberculosis* (Mahairas *et al*., 1996). Deletion

of RD1 from BCG was the principal event in the attenuation of the vaccine strain due to loss of the encoded ESX-1 system (Pym *et al*., 2002; Bitter *et al*., 2009); inactivation of ESX-1 machinery or ESX-1-secreted effectors attenuate *M. tuberculosis* and *M. bovis* in animal models (Wards *et al*., 2000; Lewis *et al*., 2003). Synthesis and function of the ESX-1 type VII secretion system must demand a significant energy commitment from the bacterial cell; this presumably was a key reason for the *in vitro* selection of an RD1-deleted variant of *M. bovis* through repeated subculture during the derivation of BCG. Hence ESX-1 can fairly be described as a locus that is essential for virulence in these strains and models. However, ESX-1 systems are clearly not required for *M. mungi*, *M. microti* or the Dassie bacillus to sustain in their respective host populations as all of these strains have RD1-like regions deleted (Brodin *et al*., 2002; Mostowy *et al*., 2004; Alexander *et al*., 2010). This independent loss of a major virulence system in *M. mungi* and *M. microti* is intriguing, and could indicate a selective advantage to loss of ESX-1 in these MTBC strain-host combinations; however, what this advantage may be is unclear. Furthermore, the environmental NTM *M. kansasii* is highly attenuated in most experimental hosts relative to *M. tuberculosis* and *M. bovis*, yet *M. kansasii* contains the orthologous RD1 genes and can secrete ESAT-6 and CFP10 (Arend *et al*., 2002). Hence, the presence of an intact ESX-1 locus is not sufficient for virulence. Furthermore, loss of ESAT-6 and CFP10 secretion does not always attenuate *M. tuberculosis*; in work by Chen *et al*. (2013) site-directed mutagenesis of EspA, a protein that is cosecreted with ESAT-6 and CFP10 by ESX-1, produced a recombinant bacillus where ESAT-6::CFP10 secretion was blocked but virulence was unaffected. This result suggests that even in the well-studied mouse model there are significant gaps in our understanding of the role of ESX-1 in virulence.

MPT70 and MPT83

Two antigens that show differential expression across the MTBC are MPT70 and MPT83 (also known as MPB70 and MPB83 in *M. bovis*). MPT83 is a lipoprotein that is post-translationally glycosylated, while MPB70 is secreted with no post-translational modifications (Wiker *et al*., 1996). *M. bovis* shows constitutive high-level expression of these antigens, while *M. tuberculosis* has low-level expression but shows induction during intracellular growth (Schnappinger *et al*., 2003). Expression of MPB70 and MPB83 is under the control of the SigK regulon, with high-level expression in *M. bovis* resulting from a loss of negative regulation due to a mutation in the gene encoding the anti-sigma factor RskA (Charlet *et al*., 2005). Intriguingly, constitutive high expression of MPB70 and MPB83 is also seen in *M. orygis* by an independent missense mutation in *rskA* to that seen in *M. bovis*. The upregulation of the SigK regulon in *M. bovis* and *M. orygis* through independent mutations would suggest a selective advantage for increased expression of MPB83, MPB70 and other constituents of the regulon; however, the nature of this advantage is unclear.

Insights into the function of MPB70 and MPB83 have been accumulating through a range of studies. The solution structure of MPB70 revealed it to have a novel fold with similarity to fasciclin domain proteins that are involved in protein–protein interactions (Carr *et al*., 2003). Chambers and colleagues (2010) used the human monocyte THP-1 cell line to show that N-acylated MPB83 peptide was a TLR1/2 agonist driving expression of matrix metalloproteinase 9 and TNF-α. Interestingly, recombinant MPB83, devoid of any post-translational modification, also stimulated the production of MMP-9 from THP-1 cells, with this stimulation blocked by blocking antibodies against TLR1/2 (Chambers *et al*., 2010). This observation was extended by Chen *et al*. (2012), who again showed that recombinant MPT83 was a TLR2 agonist that drove expression of TNF-α, IL-6 and IL-12 p40 from the murine RAW267.4 cell line. Their results suggested that recombinant MPT83 was as potent a TLR-2 ligand as Pam_3CysSK_4, suggesting that high-level expression of MPB83 by *M. bovis* could be a powerful driver of innate immune responses. Hence testing the virulence of recombinant *M. bovis* mutants with a functional RskA anti-sigma factor may generate valuable data on the role of these

proteins during infection. However, for *M. tuberculosis*, it has been shown that inactivation of *sigK* does not attenuate in murine models (Schneider *et al*., 2014), suggesting either that the SigK regulon does not play a major role in *M. tuberculosis* virulence, or that the mouse model of primary progressive disease is suboptimal for understanding the role of the SigK regulon during a prolonged infectious cycle.

Lipids

Mycobacteria, whether pathogenic or harmless saprophytes, produce an esoteric array of cell wall lipids (Brennan and Nikaido, 1995). This no doubt reflects their common origin as environmental bacteria, with cell wall lipids providing the perfect coating to protect the bacterium from environmental vagaries such as dehydration or predator attack and with hydrophobic interactions promoting microcolony and biofilm formation. The effectiveness of lipids to provide a protective shield *in vivo* for MTBC is beyond question, yet these are not simply passive defences but biologically active structures that can drive and modulate immune responses in the host. Our knowledge of host interactions with mycobacterial lipids is largely drawn from cellular *in vitro* systems or mouse models; while these have provided immensely useful, they may not provide ideal systems to address possible host-adaptive roles for mycobacterial lipids.

Taking one mycobacterial lipid as an exemplar, the phenolic glycolipids (PGL) of the MTBC have known roles in virulence and immune modulation. These glycolipids are built on a core of phthiocerol dimycoserosate (DIM), with members of the MTBC having variation in the carbohydrate structures that are linked to DIM (Brennan, 2003); thus, *M. bovis* produces mycoside B, a monosaccharide variant with 2-*O*-methylrhamnose as the terminal sugar, a structure also seen in *M. microti* and *M. pinnipedii* (Malaga *et al*., 2008). A minority of *M. tuberculosis* strains produce a trisaccharide variant, with mutations in an assortment of glycosyltransferases, methyltransferases and polyketide synthases responsible for the variable production across *M. tuberculosis* lineages (Reed *et al*., 2004; Malaga *et al*., 2008; Simeone *et al*., 2010, 2013). Hence variation in PGL structures across the MTBC is apparent, but this variation occurs in the context of many other genetic differences, confounding simple linkages between lipid presence/absence to virulence. To address these problems Tabouret and colleagues have used elegant genetic approaches to reprogramme the synthesis of PGL across the MTBC, allowing the impact of lipid modifications to be assessed in isogenic backgrounds; for example, by switching the mycoside B variant of *M. bovis* BCG to the PGL variant expressed by *M. leprae* (Tabouret *et al*., 2010). Added to this, complete chemical synthesis of the PGL-tb has been recently reported, allowing the activity of this glycolipid to be studied in isolation (Barroso *et al*., 2012). Full chemical synthesis of the *para*-hydroxybenzoic acid derivatives (*p*HBADs), that contain an identical glycosylated phenolic moiety to PGLs, has been reported and have been used to show that *p*HBAD variants in isolation can suppress the production of IFNγ and IL-17 by stimulated murine splenocytes (Bourke *et al*., 2014). Teasing apart the roles of PGL and *p*HBADs variants on host interaction across the MTBC now appears feasible.

Sulfolipids (SL) are trehalose-containing glycolipids that are only expressed by *M. tuberculosis* in the MTBC (Brennan, 2003). The role of these lipids in virulence has been ambiguous, as while SL-negative mutants of *M. tuberculosis* do not show an attenuation phenotype in murine infection models (Rousseau *et al*., 2003), SL drives pro-inflammatory cytokine secretion by monocytes (Pabst *et al*., 1988). Clarity on these seemingly divergent phenotypes has been provided by a detailed analysis of SL mutants on backgrounds where the synthesis of other mycobacterial lipids (DIM, or di and poly-acyl trehalose) have also been blocked (Passemar *et al*., 2014). It was shown that DIM exerts a dominant effect in terms of mycobacterial virulence, with SL mutants showing no phenotype in a DIM+ background but a slight (but non-significant) decreased fitness in DIM− strains. Work by Gilmore and colleagues has also suggested that expression of sulfolipid negatively regulates growth of *M. tuberculosis* in human THP-1 cells, but that in murine RAW264.7 cells, or a murine infection model, lack of sulfolipid

does not attenuate (Gilmore *et al*., 2012). The attenuation defect in human cells was attributed to the production of antimicrobial peptides that are absent from murine cells. The potential of sulfolipid to act as a species-specific virulence factor warrants further attention.

Conclusions

The simplest way to assay whether a potential virulence factor has a role in experimental disease is to disrupt the responsible gene(s) and test for bacterial counts and/or pathology in a standardized infection model. While this approach has uncovered dozens of genes required for full virulence of *M. tuberculosis*, the presence of these same virulence genes in environmental bacteria and their absence in host-adapted members of the MTBC suggests that a more nuanced perspective of virulence is required. Furthermore, bacteria that are more virulent for humans can be less virulent for cattle (e.g. *M. tuberculosis*), and vice versa (in the case of *M. bovis*). Virulence is not linear, and by extension, bacteria of greater or lesser virulence are not forcibly expected to enjoy respectively more or less success.

In the case of the MTBC, to transmit between hosts, these organisms must survive host immunity and then exploit this response to cause 'just enough' pathology. An excessively virulent bacterium will either disseminate in the host, resulting in non-transmissible disease (e.g. TB meningitis) or simply kill the host through progressive pulmonary pathology. Conversely, insufficiently virulent bacteria may achieve a productive infection but will not be expelled in high enough numbers to cause transmissible disease. Both extremes select against propagation of the bacteria. *M. tuberculosis* inhabits a 'goldilocks zone', where median time from disease to death in the pre-antibiotic era was 2.5 years. Through millennia of co-evolution, MTBC organisms have been selected to generate the appropriate amount of pathology to generate a transmissible focus of pathology, in a host that is otherwise intact. The variants in the MTBC noted above may be the result of fine-tuned adjustments in response to hosts with gradations of natural resistance that would perturb transmission away from equilibrium. Finally, it has long been known that crowding is a risk factor for both human TB and bovine TB. It is possible that variants of the MTBC are not only adapted to cause disease in their respective hosts, but also adapted to transmit in the habitat where their hosts reside, be it burrows (*M. mungi*) or seashores (*M. pinnipedii*), groups (Dassie bacillus) or herds (*M. caprae*).

The MTBC represent the ideal group of pathogens to explore concepts in One Health, with collaboration across human, veterinary and environmental spheres offering new insights into pathogen evolution, virulence and disease transmission. In our quest for new drugs, diagnostics and vaccines to combat human TB we have underexploited the rich data available from comparative studies across the MTBC. With advances in genome-sequencing technologies, and cognisant of undiscovered animal-adapted strains lying in wait, we are now poised to look afresh at the diversity across the MTBC and the nature of host adaptation and virulence. The concepts uncovered in such an endeavour promise to illuminate our search for new disease control tools to fight TB, a fact not lost on Theobald Smith, as noted in our Introduction, nor on Emil von Behring in his studies on bovine TB (von Behring, 1901): 'I need hardly add that the fight against cattle tuberculosis only marks a stage on the road which leads finally to the effective protection of human beings against the disease'; it is time to pay renewed heed to these calls.

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3 Immunopathogenesis of Tuberculosis in Humans

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Global Burden of Tuberculosis

Mycobacterium tuberculosis (Mtb) is an ancient human scourge that is the etiological agent of tuberculosis (TB) and is the principal member of the *M. tuberculosis* complex (MTBC) of genetically related human mycobacterial pathogens (Wirth *et al*., 2008). The most common ancestor of the MTBC complex dates back nearly 40,000 years (Wirth *et al*., 2008) and Mtb DNA can be found in the mummified remains of individuals stricken with the disease thousands of years ago (Nerlich *et al*., 1997; Donoghue *et al*., 2010), demonstrating that Mtb evolution and human history are intertwined. In 2012, there were almost 9 million new TB cases and 1.3 million deaths associated with TB (WHO, 2013). Additionally, two thirds of the world population, or 2 billion people, are estimated to have latent Mtb infection (LTBI). While latently infected individuals control Mtb infection and are clinically asymptomatic, they retain a significant risk of progressing to TB by reactivation of latent Mtb when immune-compromised. This is due to the ability of Mtb to persist within the lungs of individuals and the inability of host immunity to completely eradicate mycobacteria from host tissue (WHO, 2013). TB remains a significant global public health burden despite the availability of the vaccine *M. bovis* Bacille Calmette Guérin (BCG), which is effective in preventing TB meningitis and disseminated TB in infants but does not confer reliable protection against pulmonary TB or reactivation from LTBI in children and adults (WHO, 2013). The high rates of TB transmission worldwide and the increasing cases of multi- and pan-drug resistant strains of Mtb and co-morbidities with other diseases, infectious or otherwise, underscore the importance of understanding how Mtb interacts with its human host (WHO, 2013).

Pathogenesis of Human Tuberculosis

Clinical presentation and diagnosis

Mtb is transmitted person-to-person by inhalation of aerosolized droplets containing bacteria, but multiple factors including proximity, the duration of contact with actively infected individuals, mycobacterial load and immunocompetency of the infected individual together determine the efficiency of transmission (Dye and Williams, 2010; Shenoi *et al*., 2010). Once lung deposition occurs, Mtb is taken up by alveolar macrophages and infection proceeds

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

to either symptomatic primary active TB disease (active TB; ATB), or is contained by host immunity, resulting in LTBI, which represents a spectrum of clinically asymptomatic Mtb infection (Barry *et al*., 2009).

Approximately 5–10% of individuals who are exposed to Mtb are estimated to develop active TB disease, characterized by persistent cough accompanied by sputum production, weight loss, weakness and night sweats (Zumla *et al*., 2013). A radiographic examination and detection of bacteria in patient's sputum are the primary diagnostic methods after physical examination and patient history. Primary TB is known to present as an infiltration in the lower lung. This can be followed by cavitation, the physical manifestation of lung tissue destruction and a sign of progressive disease (Hopewell, 1994). Secondary tuberculosis results from reactivation of latent infection and has distinct radiographic features, usually showing upper lung involvement and/or cavitation, which is more commonly seen in secondary TB compared to primary TB (Hopewell, 1994). Cavitation is less common in immunodeficient individuals where immune hyporesponsiveness and extrapulmonary infections are more common due to failure to contain the pathogen (Pitchenik and Rubinson, 1985; Chaisson *et al*., 1987). Infection with human immunodeficiency virus (HIV) represents one of the leading causes of secondary, acquired immunodeficiency. The high, and rising, numbers of HIV/TB co-infections have significantly changed the landscape for human TB diagnosis. Before the HIV epidemic, reports of pulmonary TB in the USA comprised approximately 85% of all cases, and disseminated, extrapulmonary TB made up the remnant 15% (Farer *et al*., 1979). However, in co-infected patients, extrapulmonary involvement is seen in the majority of all reported cases and clearly demonstrates that HIV infection severely compromises the ability to contain mycobacterial infection (Small *et al*., 1991).

Currently, diagnosis of TB relies on assessment of overt clinical symptoms, radiographic findings and sputum-based diagnostic tests that aim to detect the presence of Mtb in sputum, or in bronchoalveolar lavage (BAL) if the patient is not producing sputum. However, detection of acid-fast bacilli (AFB) by smear microscopy is insensitive, and microbiological culture of Mtb from sputum, which may take 6–8 weeks, is used when available to confirm Mtb. The introduction of a real-time nucleic acid amplification-based molecular method (Xpert Mtb/RIF) is now recommended by the World Health Organization (WHO) as the primary diagnostic tool for TB, and efforts are under way to increase access to Xpert in resource-constrained settings. An added advantage of Xpert is the ability to simultaneously determine resistance to the first line drug rifampicin, which is often a marker of a multidrug-resistant Mtb strain (Helb *et al*., 2010; WHO, 2013). However, despite these recent advances, sputum-based diagnostic tests fail to detect many TB cases and incidence rates for sputum-negative TB are high, particularly in HIV-infected individuals. Thus, better non-sputum based diagnostic tests are urgently needed.

The most commonly used non-sputum based tests, Interferon Gamma Release Assays (IGRAs), differentiate between Mtb infection and BCG vaccination and are an improvement over the Tuberculin Skin Test (TST) (Horsburgh and Rubin, 2011), though neither of these tests have the ability to distinguish between active TB and LTBI. Distinguishing between active and latent disease impacts treatment decisions since individuals with symptomatic active TB disease receive multidrug therapy for 6–9 months while individuals with LTBI are recommended to receive 9 months of Isoniazid Preventive Therapy (IPT) or the newly introduced, shorter, 3-month regimen of isoniazid and rifapentine. These issues underscore the need for improved diagnostics that accurately identify TB cases and rule out active TB in individuals with LTBI.

Granulomas

Mtb-infected individuals are able to contain bacteria within hallmark lesions in the lung, termed granulomas. Classically, granulomas were thought to be an immunologic walling off of bacteria to limit dissemination (Saunders and Cooper, 2000). The increased rates of reactivation TB observed with the advent of anti-TNF therapeutic antibodies implicated

this cytokine in the granuloma's ability to contain bacteria and supported the notion that constant immune pressure is needed to prevent reactivation (Keane *et al*., 2001). However, recent knowledge suggests that the granuloma is a dynamic environment shaped by both bacterial- and host-driven processes. The initiation of the granuloma starts with the infected macrophage and continues with the development of multinucleated giant cells (MGCs) and lipid-filled foamy macrophages surrounded by a ring of lymphocytes encapsulated in a fibrotic cuff (Saunders and Cooper, 2000; Russell *et al*., 2009). Early immunophenotyping of granulomas using immunofluorescence microscopy on frozen sections from active TB patients showed that lymphocytes comprised approximately 44% of the cellular infiltrate and that the majority were HLA-DR$^+$ (Randhawa, 1990). Depending on the oxygenation level and inflammatory milieu present within the granuloma microenvironment, Mtb is able to induce differential gene responses presumably in order to adapt to the changing immune milieu (Fenhalls *et al*., 2002; Timm *et al*., 2003).

A more recent study looking at the differences in the granulomas of humans and C57BL/6 mice indicates that similarities and significant differences exist especially concerning oxygenation levels during chronic infection. Specifically, both mouse and human granulomas have observable B cell aggregates and similar spatial organization showing a distinct pattern of cellular distribution, but human granulomas are far more hypoxic, and the type of cells that organize around the B cell aggregates are different between mice and humans (Tsai *et al*., 2006). Using a hypoxyprobe to biochemically assay oxygen tension in the tuberculous granuloma, a study found that, similar to human infection, tuberculous granulomas in guinea pigs, rabbits, and nonhuman primates are also hypoxic (Via *et al*., 2008).

In vitro human granuloma systems have been developed recently and have produced several insights into the formation and processes important in the Mtb granuloma. The first report demonstrated that purified protein derivative (PPD) from BCG and Mtb can induce a granuloma *in vitro* and that macrophages are sufficient to start the granuloma via MGC formation (Puissegur *et al*., 2004). The *in vitro* system showed that oxygenated mycolic acids on Mtb trigger monocyte-derived macrophages to differentiate into foamy macrophages and that these macrophages are characterized by high lipid content and serve as a reservoir for a dormant-like state of Mtb in lipid engulfed phagosomes (Peyron *et al*., 2008). Similarly, another study showed that Mtb lipids can induce granuloma formation, but that only pro-inflammatory glycolipids allow for the formation of MGCs from infected macrophages; this process was dependent on TLR2 and the host cell fusion machinery (Puissegur *et al*., 2007). Although permissive for Mtb growth, MGCs show an intact microbial defence system with functional NADPH oxidase activity, but also resemble mature dendritic cells in that they lose their phagocytic capacity while retaining antigen presentation (Lay *et al*., 2007). More recently, *in vitro* human granuloma systems implicate Mtb granuloma formation in contributing to Mtb dormancy and resuscitation (Kapoor *et al*., 2013). Together, recent advances in studying granuloma formation point to a complex and dynamic process that involves both host and pathogen processes which are yet to be fully understood.

Inflammation and cell death

A balance between pro- and anti-inflammatory responses against Mtb plays an important role in the outcome of disease. Too little inflammation leads to impaired bacterial control, but too much inflammation can lead to irreversible immunopathology and tissue damage. An example of this balance is demonstrated in studies that originated by studying *M. marinum* infection in zebrafish, which identified polymorphisms in the *lta4h* gene encoding for an enzyme that mediates production of pro-inflammatory and chemoattractant eicosanoids (Tobin *et al*., 2010). Zebrafish with mutations in this locus were hypersusceptible to *M. marinum* infection due to their inability to mount a productive innate inflammatory response via production of leukotrienes due to redirection of substrates into anti-inflammatory lipoxins (Tobin *et al*., 2010). This study was validated in a TB meningitis cohort in Vietnam

demonstrating a heterozygote advantage in balancing inflammatory and anti-inflammatory responses against this extreme form of TB (Tobin *et al*., 2010).

Other eicosanoids have been shown to regulate inflammatory responses by manipulating production of lipid mediators of cell death. Prostaglandin E2 (PGE2) is an eicosanoid that regulates synaptotagmin-7, a calcium sensor that helps reseal plasma membrane microdisruptions (Divangahi *et al*., 2009). Infection of human macrophages with virulent Mtb leads to the production of lipoxin A4 (LXA4), which blocks PGE2 biosynthesis and is able to induce macrophage necrosis instead of apoptosis (Divangahi *et al*., 2009). Another mechanism specifically employed by virulent Mtb to tip the balance in favour of macrophage necrosis includes the proteolysis of annexin-1, which prevents its crosslinking by tissue transglutaminase on the cell surface and formation of the apoptotic envelope (Gan *et al*., 2008). It has long been known that infection with virulent strains of Mtb modulates the type of cell death that occurs, preferentially inducing necrosis and pyroptosis over apoptosis (Keane *et al*., 2000; Duan *et al*., 2001; Chen *et al*., 2006). Apoptosis leads to fewer viable bacteria (Oddo *et al*., 1998) and promotes cross presentation (Brookes *et al*., 2003), without damaging inflammation caused by necrosis. However, inflammation remains a crucial factor in the recruitment and activation of innate and adaptive immune cells that combat infection. Finding the right balance between protection and pathology is key in shaping the outcome of infection in human TB.

In situ studies of human tuberculosis

Much knowledge has been gained from studies examining samples that have been derived from the lungs of patients with pulmonary TB. An older study shows that CD4 T cell reactivity to Mtb PPD is different when cells were recovered from pleural fluid and lung tissue of TB patients than from peripheral blood (Barnes *et al*., 1989). Cellular responses in TB patients with and without HIV co-infection were measured from BAL samples from TB-involved lung segments and compared to uninvolved segments and to peripheral blood responses (Law *et al*., 1996). The study found significant differences in the absolute cell counts and percentage of neutrophils and lymphocytes recovered between samples from TB-involved segments to uninvolved segments, and especially between BAL samples and peripheral blood (Law *et al*., 1996). Although much has been learned regarding human cellular responses to TB from analysing cellular responses in the periphery, it is important to keep in mind that physiologic infection occurs primarily in the lung and that the unique cell types and microenvironment found within the lung are likely to play a critical role in shaping the immune response against Mtb.

Human Immunity to Tuberculosis

Given the nature of Mtb pathogenesis, human immunity to TB necessarily involves the coordination of innate and adaptive immunity. Myeloid cells constitute the cellular niches for Mtb, and immune pressure in the lungs early after transmission comes from innate responses to infection. Adaptive immune responses, however, are critical in helping contain the pathogen. In the following sections, we will discuss the state of knowledge for the major components of innate and adaptive immunity as well as highlight the genetic studies that have proved invaluable in identifying critical pathways necessary for resistance to mycobacterial infection. Finally, we will delve briefly into TB and various comorbidities before giving an update on the current state of TB vaccine development.

Innate Immune Responses to Tuberculosis

Innate immunity is a crucial component of the immune response against Mtb. While myeloid cells serve as niches for bacterial replication, innate antimicrobial pathways activated early and throughout infection play a role in limiting disease. However, Mtb employs myriad potent mechanisms for evading antimicrobial responses and subverting the

innate immune crosstalk with adaptive immunity and elucidating these pathways provides novel targets for anti-TB therapeutics and vaccines.

Macrophages

Recognition and uptake of bacteria

Alveolar macrophages are one of the first host cell types to encounter Mtb in the lungs and much of the knowledge regarding the innate immune response in human TB stems from *in vitro* studies on macrophages. Receptor-mediated phagocytosis of the bacterium involve a wide variety of cell surface receptors that recognize a range of mycobacterial ligands. In studies across human and animal models of infection with Mtb, C-type lectins (e.g. mannose receptor, DC-SIGN, Dectin-1), complement receptors (e.g. complement receptor 3), collectins (e.g. surfactant proteins A and D, mannose-binding lectin), scavenger receptors (e.g. MARCO, SR-A1, CD36, SR-B1), Fc receptors (e.g. FcγR), glycophosphatidylinositol (GPI)-anchored membrane receptors (e.g. CD14) and toll-like receptors (TLRs) (e.g. TLR-2, TLR-4, TLR-9) have all been implicated in the recognition of mycobacterial glycolipids, lipoproteins and host-derived factors in order to mediate bacterial uptake (Philips and Ernst, 2012). While many of these receptors were identified using animal models, studies using human monocytic cell lines, blood monocyte-derived macrophages and primary alveolar macrophages validate the contributions of many of these receptors in human TB.

Mannose-binding lectin (MBL) belongs to a family of proteins called collectins, which fall into the family of soluble C-type lectins involved in the recognition of sugars. In the uninfected, steady state, MBL is involved in the recognition and clearance of apoptotic cells via calreticulin- and CD91-mediated phagocytosis (Ogden *et al*., 2001). In human Mtb infection, MBL recognizes mannosylated lipoarabinomannan (ManLAM) and phosphatidylinositol mannosides (PIMs) (Philips and Ernst, 2012). Similarly, the transmembrane C-type lectin, mannose receptor, ligates Mtb lipoarabinomannan and activates macrophage peroxisome proliferator activated receptor gamma (PPARγ) expression in a phospholipase A2-dependent manner (Rajaram *et al*., 2010). Activation of PPARγ leads to the induction of IL-8 and cyclooxygenase 2 expression, which regulate inflammatory responses via arachidonic acid metabolites. Interestingly, the PPARγ pathway is specifically utilized by virulent Mtb, as infection with BCG does not efficiently activate PPARγ (Rajaram *et al*., 2010).

Another receptor that mediates uptake in human cells is the dendritic cell specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN). Despite the name, DC-SIGN is a C-type lectin also present on the surface of human macrophages. Its function in macrophages is primarily in the recognition, and subsequent phagocytosis, of mannosylated carbohydrates on pathogen surfaces, such as Mtb ManLAM. In a study comparing patients with confirmed pulmonary TB with healthy controls or patients with other lung pathologies such as asthma and sarcoidosis, DC-SIGN was shown to be a marker specifically upregulated in alveolar macrophages in patients with TB (Tailleux *et al*., 2005).

While animal models of infection have been able to delineate the individual contributions of many more receptors, it is clear that coordinated ligation of a heterogeneous set of receptors is the physiological scenario. Furthermore, factors in the lung microenvironment itself are likely to play a role in bacterial uptake, adding to the complexity. For example, a study examining human alveolar lining fluid and lung factors demonstrated that the Mtb cell envelope is affected by lung hydrolases, resulting in decreased bacterial association with and intracellular growth within macrophages (Arcos *et al*., 2011). Overall, it is clear that Mtb is recognized by numerous receptors and induces a network of receptor-mediated signalling pathways in macrophages.

Macrophage effector functions

Despite the complicated nature of bacterial uptake, a large body of evidence demonstrates that Mtb is able to survive and replicate in the macrophage phagosome by inhibiting phagosomal maturation and acidification and

resisting macrophage antimicrobial functions. Bacterial pathogen-associated molecular patterns (PAMPs) are present on Mtb and recognized by a variety of pattern recognition receptors (PRRs), both on the cell surface and intracellularly, leading to downstream macrophage effector functions. TLR2 recognition of mycobacterial ManLAM leads to downstream NF-κB activation and iNOS gene transcription (Brightbill *et al*., 1999). Nitric oxide (NO) production in human macrophages is linked to NOS2 and NOS3 expression (Jung *et al*., 2013) and staining with monoclonal antibodies specific for NOS2 in the BAL of TB patients demonstrates clear TB-specific induction of macrophage NOS2 mRNA compared to healthy controls (Nicholson *et al*., 1996; Rockett *et al*., 1998). Studies in mouse models support the idea that NO production is tightly linked with resistance to Mtb (MacMicking *et al*., 1997; Scanga *et al*., 2004). However, the role of NO in human TB infection appears to be more complex, especially regarding its effects downstream of IFNγ. In contrast to murine studies, studies examining the role of NO during *in vitro* infection of human alveolar macrophages and primary monocytes post-IFNγ treatment demonstrate no bactericidal or bacteriostatic effects against Mtb (Thoma-Uszynski *et al*., 2001; Jung *et al*., 2013).

Another major area of discrepancy between human and murine TB studies seems to be the effectiveness of IFNγ in activating macrophages. Studies describing murine knockout strains and humans with polymorphisms in the IFNγ/IL-12 axis have definitively established IFNγ as critical in host resistance to Mtb (Cooper *et al*., 1993; Flynn *et al*., 1993; Jouanguy *et al*., 1996; Ottenhoff *et al*., 1998; Chackerian *et al*., 2001). Clinically, IFNγ in the pleural fluid and BAL of patients with confirmed pulmonary TB is increased when compared to healthy controls, providing evidence that IFNγ plays a prominent role in human TB infection (Barnes *et al*., 1990; Robinson *et al*., 1994; Newport *et al*., 1996; Jouanguy *et al*., 1999). However, while murine studies show that IFNγleads to efficient macrophage activation and restriction of bacterial growth, studies in human macrophages are not concordant with those shown in mice (Rook *et al*., 1986a). Early observations in human macrophages confirm that IFNγ leads to the production of antimicrobial molecules effective against toxoplasma (Nathan *et al*., 1983) and *Leishmania* parasites, but does not help control Mtb replication (Douvas *et al*., 1985). One mechanism proposed for the attenuated macrophage response to IFNγ is Mtb-mediated inhibition of critical STAT1 interactions augmenting antimicrobial gene responses (Ting *et al*., 1999). Specific downstream consequences of IFNγ signalling during infection include the induction of autophagy pathways (Gutierrez *et al*., 2004), maturation of phagosomes and upregulation of high affinity IgG Fc receptor (CD64) (Delneste *et al*., 2003), MHC class II (Schroder *et al*., 2004), interferon regulatory factors (IRFs), indoleamine 2,3-dioxygenase (IDO) (MacKenzie *et al*., 1999) and several pro-inflammatory cytokines and chemokines. Multiple reports suggest that Mtb is capable of regulating gene-specific expression downstream of IFNγ signalling in human macrophages. One of the most important genes downstream of IFNγ signalling that is modulated by Mtb in macrophages is MHC class II expression via downregulation of the class II transactivator (CIITA) necessary for MHC class II processing (Fenton *et al*., 1997; Kincaid and Ernst, 2003; Wang *et al*., 2005). This has important implications with regards to macrophage antigen presentation and is likely to play a significant role in reported defects in antigen recognition by Mtb-specific lymphocytes in the granuloma (Egen *et al*., 2011).

Vitamin D modulates human macrophage effector functions

The initial observation on the effect of vitamin D on human macrophages came from studies utilizing it as a synergistic treatment with IFNγ. Reactivity of vitamin D metabolites with human monocytes demonstrated additive synergy with IFNγ against Mtb (Rook *et al*., 1986b). Studies using the bioactive 1,25-dihydroxy-vitamin D3 clearly demonstrated that vitamin D treatment of human macrophages restricts Mtb replication (Crowle *et al*., 1987). The mechanism for the antimicrobial effects relies on phosphatidylinositol 3-kinase (Sly *et al*., 2001) as well as TLR ligation

(Liu *et al*., 2006). As with IFNγ in murine studies, vitamin D induces phagosomal maturation via downregulation of tryptophan-aspartate-containing coat protein (TACO; coronin-1), which associates with and retains immature phagosomes (Anand and Kaul, 2003). Importantly, vitamin D treatment results in macrophage induction of hCAP-18, a gene encoding the pro-form of the antimicrobial peptide LL-37 (cathelicidin), and trafficking of LL-37 to phagosomes leads to subsequent bacterial killing (Liu *et al*., 2007; Martineau *et al*., 2007b). Vitamin D has wide-ranging effects on human macrophages during Mtb infection and its importance was made clear in a study that suggests that the antimicrobial effects of autophagy, phagosomal maturation and the production of antimicrobial peptides in macrophages are vitamin D dependent (Rockett *et al*., 1998; Fabri *et al*., 2011).

However, the role of vitamin D *in vivo* during human TB infection remains unclear. Several epidemiological studies have shown a correlation between vitamin D insufficiency and pulmonary TB susceptibility among different ethnicities and lifestyles (Martineau *et al*., 2011), but no correlation could be found between vitamin D and LL-37 serum concentrations (Yamshchikov *et al*., 2010). Additionally, clinical trials using an intervention with vitamin D in pulmonary TB patients in Guinea-Bissau showed no significant protection compared to placebo (Wejse *et al*., 2009).

Dendritic Cells

Dendritic cells (DCs) are the major population of cells that take up and present antigen to lymphocytes and constitute a crucial link between innate and adaptive immunity. While alveolar macrophages and recruited tissue macrophages constitute a significant source of target cells for bacterial replication, Mtb can also infect DCs, although past reports have indicated that there are only low levels of bacterial replication within this niche (Tailleux *et al*., 2003a). In human monocyte-derived dendritic cells (moDC), mannose receptors, CD11b and CD11c, which are all expressed on DCs, recognize Mtb ligands. However, DC-SIGN serves as the major receptor for Mtb entry into moDCs via recognition of Man-LAM (Tailleux *et al*., 2003b). While the function of DC-SIGN on macrophages seems primarily to involve phagocytosis, its functionality in DCs is to bind ICAM-2 on endothelial surfaces to allow for tethering, rolling and extravasation in order to migrate to sites of inflammation (Geijtenbeek *et al*., 2000a,b). ICAM-3 on T cells also binds DC-SIGN and forms the initial structures that become the immunological synapse (van Kooyk and Geijtenbeek, 2002).

During Mtb infection, ligation of DC-SIGN by Mtb LAM leads to the induction of IL-10, an anti-inflammatory cytokine that has immunoregulatory roles during infection. DC-SIGN signalling during Mtb infection prevents LPS-induced DC maturation and suggests that the receptor might be co-opted by Mtb in order to interfere with proper maturation and induction of adaptive immunity (Geijtenbeek *et al*., 2003). Other studies suggest that mannosylated liporarabinomannans are capable of inducing a negative signal that inhibits IL-12 production through both mannose receptor and DC-SIGN (Nigou *et al*., 2001).

Given the importance of DCs in migrating to draining lymph nodes and activating naïve lymphocytes, the subversion of DC responses, which will impair the onset, magnitude and quality of the subsequent adaptive immune response, is likely to be an important Mtb immune evasion strategy. This is reflected in studies showing that the capacity of Mtb-infected moDCs to induce lymphoproliferation and activation of naïve and memory CD4 and CD8 T cells is impaired (Hanekom *et al*., 2003). Although impairment of phenotypic maturation is one potential mechanism for attenuated capacity to stimulate lymphocytes, it is possible that antigen availability might also play a significant role. DCs differ from macrophages in terms of the stability of peptide-MHC complexes at the cell surface after prolonged TLR signalling, with mature DCs downregulating a ubiquitin E3 ligase, MARCH1, in order to retain peptide-MHC complexes at the cell surface for up to 100 h for prolonged antigen presentation (Cella *et al*., 1997; De Gassart *et al*., 2008). A study looking at the timeline of Mtb antigen availability

suggests that MHCII cycling from the phagosome to the plasma membrane is induced by DC maturation but may precede the presence of loadable peptide antigens, thereby leading to Mtb immune evasion from CD4 T cells (Hava *et al*., 2008).

Neutrophils

Neutrophils are one of the primary effector cells discharging antimicrobial effectors throughout the course of bacterial infection. Their ability to generate large quantities of antimicrobial peptides (AMPs) and the nonspecific nature of AMPs released makes neutrophils a double-edged sword during immune reactions. During TB infection, neutrophils are the predominant cell type found in the BAL and sputum of active pulmonary TB patients and second only to lymphocytes within the lung cavity itself (Eum *et al*., 2010). A study examining the peripheral white blood cells in contacts of active TB patients shows an inverse correlation between development of pulmonary TB and neutrophil count. In that study, whole blood depletion of neutrophils led to poor induction of AMPs and failure to restrict BCG and Mtb growth (Martineau *et al*., 2007a). The environmental niche of Mtb is an important factor in determining the effectiveness of neutrophil degranulation. While extracellular bacteria are exposed to AMPs in a much more direct fashion, infected macrophages afford Mtb some measure of protection against direct AMP action. However, apoptotic neutrophils and purified neutrophil granules, both of which contain AMPs, can be taken up by infected macrophages. The AMPs traffic to the early endosomal compartment, which contains Mtb, and are able to impair bacterial replication (Tan *et al*., 2006).

A recently reported role for neutrophils beyond the induction of AMPs is the cell surface expression of programmed death ligand 1 (PD-L1), which has been shown in the context of numerous other infections to ligate programmed death receptor 1 (PD-1), leading to impaired lymphocyte responses (Sharpe *et al*., 2007). Whole blood transcriptional analysis from active TB patients show that PD-L1 is highly expressed and that its expression on neutrophils was primarily responsible (McNab *et al*., 2011). A similar whole blood transcriptional analysis designated a 393 transcript signature that differentiated active TB infection from healthy controls with LTBI, and an 86 gene signature that primarily involved a neutrophil-driven interferon inducible signature that uniquely identified TB infection from other inflammatory diseases (Berry *et al*., 2010). Such transcriptional assays have elucidated new and important pathways for neutrophils during Mtb infection in humans, but it must be noted that such assays done in whole blood are unlikely to be completely reflective of in situ conditions within the lung. Thus, application of these and other systematic methodologies to the physiologic site of infection will be an important next step in elucidating the role of specific innate immune cells in human TB.

Natural Killer Cells

Natural killer (NK) cells are granular innate lymphocytes that possess potent cytolytic activity without the need for prior antigen sensitization. Classically, IFNγ production and cytolytic activity are the two main effector functions of NK cells. Mtb can bind directly to the natural cytotoxicity receptor (NCR) NKp44 on NK cells via various cell wall components (Esin *et al*., 2013), and human NK cells can lyse Mtb-infected macrophages *in vitro* via interactions with C-type lectins and NCRs expressed on NK cells (Vankayalapati *et al*., 2002, 2005). IL-22 production by NK cells can inhibit intracellular growth of Mtb *in vitro* by enhancing phagolysosomal fusion (Dhiman *et al*., 2009). Moreover, NK cells can link innate and adaptive immune responses by promoting CD8 T cells to produce IFNγ and lyse Mtb-infected monocytes *in vitro* (Vankayalapati *et al*., 2004).

Although few studies have investigated the phenotypic and functional profiles of NK cells in Mtb-infected humans, mounting evidence indicates progressive functional impairment of NK cell activity with increasing mycobacterial load and TB disease progression. Decreased frequencies of NK cell subsets have been described in patients with newly

diagnosed pulmonary TB, coincident with decreased expression of the NCRs NKp30 and NKp46 and decreased NK cell IFNγ production capacity (Bozzano *et al*., 2009). The cytolytic capacity of NK cells is decreased in TB patients relative to healthy controls, with partial restoration of NK cell function following reduction in mycobacterial load by initiation of anti-TB treatment (Nirmala *et al*., 2001). Patients with tuberculous pleurisy exhibit NK cells with high levels of ICAM-1 and, under *ex vivo* conditions, are able to activate autologous lymphocytes (Schierloh *et al*., 2009) and have high levels of chemokine receptor and TLR expression (Pokkali *et al*., 2009).

In addition to direct killing, NK cells also modulate other cellular responses during TB pathogenesis in humans. Activated NK cells promote γδ T cell proliferation via CD54, TNFα, GM-CSF and IL-12 (Zhang *et al*., 2006). Activated NK cells are also able to restrict the expansion of regulatory T cells in an NKG2D/NKp46-dependent manner via recognition of an NK ligand, ULBP1 (Roy *et al*., 2008). Conversely, factors in the microenvironment during infection can also affect NK cell function. Monocyte-derived IL-10 is capable of downregulating NK cell expression of adhesion molecules, lytic activity and production of IFNγ (Schierloh *et al*., 2005).

Human Immunogenetics and Tuberculosis Susceptibility

Although much has been gleaned from *in vitro* and descriptive studies using samples comparing healthy individuals with LTBI and TB patients, much of the knowledge regarding human innate and adaptive immune pathways involved during Mtb infection relies upon clinical observations from patients with genetic polymorphisms. Mutations in five autosomal genes (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12Rβ*) and a mutation in an X-linked gene describe the spectrum of individuals affected by Mendelian susceptibility to mycobacterial disease (MSMD). Studies on MSMD involve patients infected with classically avirulent mycobacteria, including environmental mycobacteria and BCG (Ottenhoff *et al*., 1998). Deficiencies in the IFNγ receptor proteins, IFNGR1 and IFNGR2, have been documented in young patients and verifies that IFNγ signalling is important for controlling mycobacterial infections as these individuals are always infected early in life and disease is often fatal (Jouanguy *et al*., 1996; Newport *et al*., 1996; Dorman and Holland, 1998; Jouanguy *et al*., 1999). Additionally, molecules important for the intracellular signalling of IFNγ have also been shown to be important for mycobacterial resistance, as individuals with heterozygous germline STAT-1 mutations lose gamma-interferon activating factor (GAF) expression, an important transcription factor mediating IFNγ induced gene expression, and suffer from recurrent mycobacterial infections (Dupuis *et al*., 2001).

A mutation in NEMO, an intracellular protein involved in the activation of NF-κB, predisposes individuals to recurrent infections. Two specific mutations in the NEMO leucine zipper domain specifically impair CD40-mediated IL-12 production in monocytes and DCs (Filipe-Santos *et al*., 2006). Defects in IL-12p40 lead to low levels of IFNγ and susceptibility to mycobacterial infections (Altare *et al*., 1998a,b; Elloumi-Zghal *et al*., 2002; Picard *et al*., 2002). However, patients with IL-12 polymorphisms are less likely to suffer from fatal infections due to the ability to treat with exogenous IL-12 (Fieschi *et al*., 2003). Mutations in IL-12Rβ1, the most frequent genetic factor associated with MSMD, have provided clues towards host factors involved in mycobacterial immunity. These individuals are susceptible to primary mycobacterial infections, but BCG vaccination is capable of inducing resistance, suggesting that IL-12/IL-23 signalling in humans may not be completely required for secondary immunity (Altare *et al*., 1998a,b; de Jong *et al*., 1998; Altare *et al*., 2001; Caragol *et al*., 2003; Fieschi *et al*., 2004).

Mutations and polymorphisms in innate pathways also predispose individuals to recurrent infections in general and to mycobacteria in particular. Polymorphisms in TLR2 and TLR9 in Africans and Caucasians are strongly associated with pulmonary TB (Velez *et al*., 2010). Other TLR polymorphisms have also been linked to TB and include TLR1 (Ma *et al*., 2007), TLR8 (Davila *et al*., 2008) and

the intracellular signalling molecule TIRAP (Khor *et al*., 2007), though mechanisms for these associations are still unclear. A gain of function gene variant in caspase-1 coupled with a loss of function for inhibitory CARD8 leads to increased levels of IL-1β and inflammatory diseases such as rheumatoid arthritis, but isolated macrophages from individuals with such polymorphisms are more efficient at restricting Mtb growth *in vitro*, indicating that Mtb inhibits optimal inflammasome activation (Eklund *et al*., 2014). Relatedly, polymorphisms in the Il1 gene cluster and macrophage chemoattractant protein 1 (MCP-1) that affect IL-1 production and MCP-1, respectively, predispose individuals to TB due to insufficient innate pro-inflammatory responses against bacterial infection (Bellamy *et al*., 1998; Wilkinson *et al*., 1999; Flores-Villanueva *et al*., 2005).

Other innate immune pathways associated with TB infection include polymorphisms in genes encoding for surfactant proteins (Madan *et al*., 2002), cytokines such as TNF (Correa *et al*., 2005; Amirzargar *et al*., 2006), IL-6 (Amirzargar *et al*., 2006; Larcombe *et al*., 2008), IL-18 (Han *et al*., 2011), and IL-10 (Delgado *et al*., 2002; Shin *et al*., 2005; Zhang *et al*., 2011), chemokines such as RANTES (Chu *et al*., 2007; Ben-Selma *et al*., 2011), CXCL10 (Tang *et al*., 2009), and IL-8 (Ma *et al*., 2003) and effector proteins such as NOS2 (Jamieson *et al*., 2004; Gomez *et al*., 2007; Moller *et al*., 2009; Velez *et al*., 2009). The implications of these findings are unclear as the mechanism for many of these associations are still undefined. Finally, two major polymorphisms in the vitamin D receptor, uncovered in a population with low serum vitamin D3 metabolites, show a link between vitamin D responsiveness and susceptibility to TB, and validate *in vitro* studies on vitamin D-mediated induction of a variety of antimicrobial responses (Wilkinson *et al*., 2000).

Additionally, other association studies in humans linking TB susceptibility to candidate genes have uncovered genes that validate several previous studies in murine models. Human NRAMP1 (natural-resistance associated macrophage protein 1) is a human ortholog of a murine gene by the same name and encodes for a multipass membrane protein that shuttles divalent transition metals across the plasma membrane (Barton *et al*., 1999). Since the initial association between TB susceptibility and NRAMP1 polymorphisms found in a Gambian population (Bellamy *et al*., 2000), several other association studies have validated the role that NRAMP1 plays in resistance to intracellular pathogens and Mtb in particular (Greenwood *et al*., 2000; Malik *et al*., 2005; Li *et al*., 2006). Candidate gene approaches have also identified CD209 gene variants, encoding DC-SIGN, as associated with adult pulmonary TB in a South African population (Barreiro *et al*., 2006). Thus, human immunogenetic studies have validated several mechanisms of resistance against Mtb infection but the inherent complexity and multigenic nature of the immune response against pathogens leaves much to be discovered.

Adaptive Immune Response to Tuberculosis

The adaptive immune response helps control Mtb infection, though the precise mechanisms for this control are still under intense investigation. Although adaptive immunity represents an attractive target for the development of a vaccine against Mtb, the lack of good correlates of protection is a significant challenge that needs to be overcome through a more detailed understanding of the roles that T and B cells play in human TB.

Unconventional T cells in Mtb Infection

γδ T cells

γδ T cells represent a minority of peripheral $CD3^+$ T cells, and are enriched in epithelial surfaces including the liver and skin, as well as in mucosal surfaces including respiratory, digestive and reproductive organs (Bonneville *et al*., 2010). In contrast to conventional T cells bearing αβ T cell receptors (TCR), the γδ T cells utilize a restricted repertoire of TCR genes, and recognize non-peptide antigens such as microbial metabolites and phosphoantigens (Carding and Egan, 2002). γδ T cells possess a range of antimicrobial functions through the

production of pro-inflammatory cytokines, chemokines and cytotoxic granules, and are able to kill infected cells through expression of death-inducing receptors, including CD95 (Fas) and TNF-related apoptosis-inducing ligand receptors (TRAILR) (Qin *et al*., 2009).

γδ T cells have been identified that recognize isopentyl pyrophosphate and prenyl pyrophosphate derivatives from Mtb (Tanaka *et al*., 1994, 1995). The role of γδ T cells in Mtb infection was initially evaluated in mouse models of infection, where high numbers of activated γδ T cells were observed in the draining lymph nodes of Mtb-infected mice, and γδ T cells responded to Mtb antigen stimulation *in vitro* (Janis *et al*., 1989). In humans, γδ T cells represent a large proportion of Mtb-reactive T cells in peripheral blood (Haregewoin *et al*., 1989; Kabelitz *et al*., 1990, 1991; Boom *et al*., 1992). Further analysis of human γδ T cells indicates that the predominant subset of Mtb-reactive γδ T cells expresses the Vγ9 and Vδ2 gene segments (De Libero *et al*., 1991; Kabelitz *et al*., 1991; Panchamoorthy *et al*., 1991).

Vγ9Vδ2 T cells have been reported to reduce the viability of intracellular Mtb in infected macrophages (Dieli *et al*., 2000), and Mtb-infected monocytes have been shown to stimulate proliferation of Vγ9Vδ2 T cells (Havlir *et al*., 1991). Additional studies have determined that Vγ9Vδ2 T cells can reduce the viability of both intracellular and extracellular Mtb through a granulysin and perforin-dependent manner (Dieli *et al*., 2001). In addition to direct killing of Mtb-infected cells, Vγ9Vδ2 T cells have the capacity to modulate the immune response to Mtb indirectly, for example by mediating upregulation of costimulatory molecules CD40, CD80, CD86 and HLA-DR on dendritic cells (DCs) and promoting maturation of immature Mtb-infected DCs (Meraviglia *et al*., 2010). Novel antimicrobial defence mechanisms of γδ T cells have been described in humans, including production of granzyme A by Vγ9Vδ2 T cells, which triggers TNF-α production by monocytes, thus inhibiting intracellular growth of Mtb (Spencer *et al*., 2013).

Accumulating evidence indicates γδ T cells can play an important role in immunity to Mtb in patients with TB disease. One study indicated that the capacity of γδ T cells to expand upon stimulation with Mtb antigens is diminished in pulmonary TB patients (Barnes *et al*., 1992); another study reported that increases in the proliferative capacity of γδ T cells correlate with reduced IFNγ production capacity (Sanchez *et al*., 1994); and a further study has reported increased frequencies of γδ T cells producing the pro-inflammatory cytokine IL-17 in patients with active TB (Peng *et al*., 2008). Loss of Vγ9Vδ2 T cells in peripheral blood and BAL has been correlated with TB disease progression (Li *et al*., 1996). A potential mechanism resulting in selective loss of Vγ9Vδ2 T cell population is the induction of CD95 (Fas) and CD95L (FasL) on Vγ9Vδ2 T cell following stimulation with mycobacterial antigens, thus triggering apoptosis via the CD95/CD95L pathway (Li *et al*., 1998; Manfredi *et al*., 1998).

CD1-restricted T cells

CD1 molecules are a family of MHC class I-like molecules with limited polymorphisms that present glycolipid antigens to T cells. The five types of CD1 molecules in humans have been divided into two groups based on differences in protein sequences and transcriptional responses to pathogens; Group 1 includes CD1a, CD1b, CD1c and CD1e, whereas Group 2 includes CD1d only (Strominger, 2010).

Several studies have provided compelling evidence for an important role for CD1-restricted T cells in the human immune response to Mtb infection. Mycobacterial lipids presented by Group 1 CD1 molecules have been shown to stimulate proliferation and cytokine production by T cells (Moody *et al*., 2000; Ulrichs *et al*., 2003; Gilleron *et al*., 2004; Layre *et al*., 2009; Kasmar *et al*., 2011; Montamat-Sicotte *et al*., 2011; Ly *et al*., 2013; Seshadri *et al*., 2013). In addition to IFNγ production, mycobacterial lipid-specific CD1-restricted T cells can kill Mtb-infected cells *in vitro* (Porcelli *et al*., 1989; Sieling *et al*., 1995; Stenger *et al*., 1997).

The first description of microbial antigen presentation by CD1 molecules was Mtb lipid antigens presented by CD1b (Porcelli *et al*., 1992). CD1b-restricted T cells targeting glycerol monomycolate (Layre *et al*., 2009) and glucose

monomycolate (Ulrichs *et al*., 2003) have been detected in peripheral blood of individuals with LTBI, but not uninfected individuals, or patients with active TB disease. CD1b-restricted T cells targeting mycobacterial sulphoglycolipid have also been detected in individuals with LTBI and in patients with TB disease (Gilleron *et al*., 2004). Mycolic acid, a mycobacterial cell wall lipid and virulence factor, has been identified as a lipid antigen presented by CD1b (Beckman *et al*., 1994), and T cells specific for mycolic acid have been detected in individuals with LTBI and in patients with TB disease, but not in uninfected, BCG-vaccinated controls (Montamat-Sicotte *et al*., 2011). These mycolic acid-specific T cells were found to be CD1b-restricted, produced IFNγ and IL-2, and decreased in frequency in TB patients following treatment, thus suggesting a relationship between bacterial load and the frequency of mycolic acid-specific CD1b-restricted T cells (Montamat-Sicotte *et al*., 2011). More recently, CD1 tetramers have been constructed to detect CD1-restricted T cells in peripheral blood directly *ex vivo*. CD1b tetramers loaded with Mtb glucose monomycolate have identified populations of CD1b tetramer+ cells in patients with TB disease that are primarily CD4+ (Sieling *et al*., 2000; Kasmar *et al*., 2011), and were antigen-specific, as no cells were stained with empty CD1b tetramers (Kasmar *et al*., 2011).

CD1a has been shown to present dideoxymycobactin (Moody *et al*., 2004), a precursor of Mtb mycobactin (Madigan *et al*., 2012). CD1c-restricted T cell lines have been derived against lipid extracts from Mtb (Beckman *et al*., 1996), and recognize phospholipid antigens, including hexosyl-1-phosphoisoprenoids and mannosyl-beta1-phosphodolichols (Moody *et al*., 2000). Similar to CD1b-restricted T cells targeting Mtb lipid antigens, CD1c-restricted T cell responses have been described in Mtb-infected individuals, but not in Mtb-naïve individuals. Taken together, these data clearly demonstrate the presence of CD1-restricted T cells targeting Mtb glycolipid antigens in humans with latent infection and with active TB disease. However, their precise role in containment of Mtb infection *in vivo*, and their potential use as novel vaccine antigens, remain an area of active investigation.

Mucosal-associated invariant T cells (MAIT)

Mucosal-associated invariant T (MAIT) cells, a subset of innate effector cells, are enriched in tissues such as the intestinal mucosa, lung and liver, and represent 1–8% of T cells in blood (Martin *et al*., 2009; Gold and Lewinsohn, 2013; Le Bourhis *et al*., 2013). MAIT cells respond to bacterial and fungal infections and recognize antigens through a non-polymorphic MHC class I-related molecule 1 (MR1) (Treiner *et al*., 2003). Pterin-containing compounds that arise from riboflavin synthesis in bacteria and yeast have been shown to bind to MR1 and activate MAIT cells (Kjer-Nielsen *et al*., 2012). No viral antigens have yet been identified that can activate MAIT cells (Gold and Lewinsohn, 2013).

MAIT cells in humans were initially identified as cells that express an αβ TCR and utilize a semi-invariant α chain, Vα7.2-Jα33, which pairs with a limited number of Vβ segments (Porcelli *et al*., 1993). In humans, MAIT cells co-expressing Vα7.2 and CD161 have been found in the double negative (CD4−CD8−), and CD8-single positive T cell populations (Martin *et al*., 2009; Dusseaux *et al*., 2011). The capacity of MAIT cells to differentiate into memory T cell populations remains unknown; however, MAIT cells in cord blood express CD45RA and CCR7, whereas MAIT cells in the periphery express CD45RO and lack CCR7 expression, suggesting some capacity for antigen-driven differentiation (Gold *et al*., 2013).

CD8+ MAIT cells reactive to Mtb have been identified in humans, although these cells are considered to be Mtb-reactive, rather than Mtb-specific (Gold and Lewinsohn, 2013). In contrast with CD1-restricted T cells specific for Mtb glycolipid antigens, Mtb-reactive MAIT cells can be found in the blood of uninfected individuals. Mtb-reactive MAIT cells have been reported to be nearly undetectable in the blood of patients with active TB, compared with individuals with LTBI and healthy controls (Gold *et al*., 2010). The low level of MAIT cells observed in the blood of patients with active TB may be due to migration of these cells to the site of infection (Le Bourhis *et al*., 2010), consistent with studies reporting fivefold higher frequencies of Mtb-reactive

MAIT cells in the lung, compared with blood (Gold *et al*., 2010). MAIT cells isolated from thymus, lung and blood can rapidly respond to Mtb-infected cells by production of IFNγ and TNF-α (Gold *et al*., 2013), and also have the capacity to kill Mtb-infected cells *in vitro* (Gold and Lewinsohn, 2013). The enrichment of MAIT cells in the lung and rapid effector activity against Mtb suggests these cells may contribute to the early innate immune response to Mtb exposure.

The Adaptive Immune Response to Mtb: CD4 and CD8 T Cells

Infection of macrophages by the intracellular bacterium Mtb is thought to lead to a predominance of antigen presentation through the MHC class II antigen processing pathway, thereby activating CD4 T cells as the primary subset of T cells responding to Mtb. However, accumulating evidence indicates Mtb antigens are secreted into the cytosol of infected cells (Lewinsohn *et al*., 2006; van der Wel *et al*., 2007), thus enabling processing and presentation by the MHC class I pathway and subsequent activation of CD8 T cell responses. Cross-presentation of Mtb antigens mediated by uptake of apoptotic vesicles by DCs has also been described as a mechanism leading to presentation of Mtb antigens by MHC class I molecules (Winau *et al*., 2006).

CD4 T cells

The majority of Mtb-infected individuals mount robust CD4 T cell responses involving T helper 1 (Th1) cytokines such as IFNγ and TNF-α, which are critical for activating macrophages and containing bacteria within granulomas in the lung. The importance of CD4 T cells in contributing to protective immunity in Mtb infection has been demonstrated by previous studies that indicated increased susceptibility to mycobacterial diseases in IFNγ-deficient mice and in humans with IL-12 or IFNγ-receptor abnormalities (Cooper *et al*., 1993; Flynn *et al*., 1993; Jouanguy *et al*., 1996, 1999; Cooper *et al*., 1997), as well as studies documenting the significantly increased risk of TB disease in HIV-infected individuals, in whom CD4 T cells are depleted (Lawn *et al*., 2009). The importance of TNF-α in mediating successful immune control of Mtb is evidenced by observations of reactivation of LTBI (Keane *et al*., 2001) when patients with autoimmune diseases have received therapeutic neutralizing anti-TNF-α monoclonal antibodies.

Measurement of IFNγ has been the most commonly used method for detecting Mtb-specific CD4 T cell responses. However, measurement of IFNγ production alone is often not sufficient to differentiate individuals with latent infection (LTBI) from those with active TB disease, nor is it sufficient to identify latently infected individuals who are at most risk of progressing to active TB disease (Wallis *et al*., 2013). Studies from individuals with chronic viral infections indicate that T cells with the capacity to produce multiple cytokines simultaneously ('polyfunctional T cells') are associated with greater functional capacity and may be more effective at containing viral replication (Kannanganat *et al*., 2007a; Ciuffreda *et al*., 2008). Recent studies have assessed differences in polyfunctional cytokine production profiles of Mtb-specific CD4 T cells in individuals with LTBI and with pulmonary TB, with some studies indicating increased Mtb-specific polyfunctional CD4 responses in TB patients (Sutherland *et al*., 2009; Caccamo *et al*., 2010), and others indicating either decreased polyfunctional responses in TB patients (Day *et al*., 2011; Harari *et al*., 2011), or no difference (Streitz *et al*., 2011). Differences in experimental approaches and cohort characteristics may underlie the differential conclusions reported in these studies. Nevertheless, reports of reduced polyfunctional cytokine production in individuals with smear-positive pulmonary TB are consistent with progressive T cell dysfunction in the context of high mycobacterial loads, as has been well described in human chronic viral infections (Kannanganat *et al*., 2007a,b; Makedonas and Betts, 2011). Furthermore, the observation that Mtb-specific polyfunctional cytokine production capacity increases in TB patients after TB treatment-mediated resolution of disease provides further evidence of the association between bacterial load and Mtb-specific CD4 T cell functional capacity

(Day *et al*., 2011). A recent study showed that the phenotype of Mtb-specific CD4 T cells correlated with Mtb load. Frequencies of Mtb-specific, IFNγ-producing CD4 T cells expressing the immune activation markers CD38 and HLA-DR and the intracellular proliferation marker Ki-67 were significantly higher in patients with active pulmonary TB compared to LTBI and correlated with response to anti-TB treatment. These markers distinguished active and latent TB with 100% specificity and greater than 96% sensitivity and could serve as biomarkers of active TB and treatment response (Adekambi *et al*., 2015).

In addition to production of the Th1 cytokines IFNγ, TNF-α and IL-2, an important role has been recognized for Mtb-specific Th17 cells producing the pro-inflammatory cytokine IL-17. In mouse models of Mtb infection, Th17 cells produce chemokines that recruit Th1 CD4 T cells to the lungs (Khader *et al*., 2007). Mtb-specific Th17 cells have been identified in peripheral blood and BAL fluid of BCG-vaccinated individuals and individuals with LTBI (Scriba *et al*., 2008). However, other reports have indicated Mtb-specific Th17 cells are absent in patients with active TB disease (Perreau *et al*., 2013), suggesting these cells may be deficient in individuals who develop disease, or that Th17 cells have migrated from the peripheral blood to the lungs in persons with active disease.

In addition to cytokine production, Mtb-specific CD4 T cells have been described to have other important effector functions including cytotoxicity and proliferative capacity, a hallmark of memory T cells. Individuals with LTBI typically demonstrate robust CD4 T cell proliferative capacity to antigens such as CFP10, ESAT-6 and PPD; by contrast, patients with active TB disease are characterized by poor proliferative capacity to the same antigens (Govender *et al*., 2010; Day *et al*., 2011). CD4 T cells isolated from individuals with LTBI have been shown to express cytoxic molecules such as granzyme A and B, granulysin and perforin, and are able to lyse Mtb-infected monocytes by a perforin and Fas/Fas ligand independent mechanism (Canaday *et al*., 2001).

Efforts to systematically and comprehensively define CD4 T cell epitopes in Mtb have been hampered by the sheer size of the genome, comprising over 4000 open reading frames (Cole *et al*., 1998). The first genome-wide analysis of Mtb-specific CD4 T cell epitopes has been published recently, in which several novel T cell antigens were described that are contained within three broadly immunodominant antigenic islands (Lindestam Arlehamn *et al*., 2013). These genome-wide epitope studies were conducted only in healthy individuals with LTBI, and thus further studies in TB patients are needed to determine whether the spectrum of antigen recognition differs in individuals with latent versus active TB. These data demonstrate the broad spectrum of antigens simultaneously targeted in Mtb-infected individuals, and underscore the need to intensify efforts to define T cell epitopes across different states of Mtb infection. The online database Immune Epitope Database and Analysis Resource (www.iedb.org) maintains an up-to-date database of T cell epitopes identified in Mtb-infected humans.

Populations of memory T cell subsets with distinct functional programmes can be identified according to the combinatorial expression of surface molecules, their stage of differentiation and evaluation of their functional properties (Sallusto *et al*., 2010). Surface markers that have been used to define phenotypically distinct populations of antigen-specific memory T cells include CD45RO, CD45RA, CD62L, CD28, CD27, CD57, CD127 and CCR7 (Pantaleo and Harari, 2006). Phenotypic analysis of IFNγ+ Mtb-specific memory CD4 T cells in individuals with LTBI indicates these cells lacked expression of activation markers, did not express the proliferative molecule Ki67, did not express molecules associated with apoptosis and were largely CD45RA−CCR7− (Adekambi *et al*., 2012), indicative of a effector memory (TEM) phenotype (Sallusto *et al*., 1999). Studies in patients with active pulmonary TB also indicate a predominance of IFNγ+ Mtb-specific CD4 T cells with a TEM phenotype (defined as CD45RA−CD27−), with significantly lower proportions of Mtb-specific CD45RA−CD27+ central memory (TCM) CD4 T cells (Goletti *et al*., 2006). However, in another study, analysis of Mtb-specific CD4 T cells in individuals with LTBI using MHC class II tetramers revealed the majority of tetramer+ cells were CD45RA−CCR7+, indicating a TCM phenotype, and were contained in the

CXCR3+CCR6+ subset of memory T cells (Lindestam Arlehamn *et al*., 2013). These studies highlight the heterogeneity in Mtb-specific CD4 T cell phenotypes, which are likely to vary depending on antigen specificity and Mtb infection and disease status.

Regulatory T cells (Treg)

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are a specialized population of CD4 T cells that play a critical role in preventing autoimmunity and have the capacity to down-regulate effector CD4 and CD8 T cell responses. Increasing evidence indicates that the frequency of Tregs is increased both in peripheral blood and at the site of active disease in TB patients compared to healthy controls; furthermore, depletion of $CD4^+CD25^+$ T cells increases Mtb-specific T cell responses *in vitro* (Guyot-Revol *et al*., 2006; Ribeiro-Rodrigues *et al*., 2006; Chen *et al*., 2007; Hougardy *et al*., 2007; Garg *et al*., 2008). In a study of Cambodian TB patients who were anergic to PPD by delayed type hypersensitivity (DTH) skin test reaction, T cells from anergic patients produced the suppressive cytokine IL-10, but not IFNγ and failed to proliferate (Boussiotis *et al*., 2000). However, increased frequencies of Tregs at the time of disease may also serve a beneficial role by suppressing inflammatory Th1 responses and thus serving to temper the immune response and limiting tissue damage, as suggested from studies of Mtb-infected cynomologus macaques (Green *et al*., 2010). This notion is further supported by studies in mice that indicated Mtb-specific Treg cells expand early in infection, and are later selectively eliminated in response to IL-12 (Shafiani *et al*., 2013). Together these data suggest an important role for Tregs in mediating a balanced regulatory and pro-inflammatory response to ensure sufficient levels of immune activation, while also dampening potentially pathological immune responses.

CD8 T cells

In addition to CD4 T cells, increasing evidence indicates CD8 T cells play an important role in control of Mtb infection. The earliest evidence of the importance of CD8 T cells in control of Mtb infection was provided by experiments of Mtb infection of mice deficient in the β2-microglobulin (β2m) gene (Flynn *et al*., 1992), required for MHC class I assembly and trafficking, and mice deficient in CD1 and transporter associated with antigen processing (TAP)-1 genes (Behar *et al*., 1999; Sousa *et al*., 2000). Mice deficient in CD8 T cells and molecules in the MHC class I antigen processing pathway have higher bacterial loads in the lungs, and succumb sooner to TB than wild-type mice. Depletion of CD8 T cells in non-human primates, which mimic Mtb infection and disease in humans more closely than mouse models, results in loss of BCG vaccine-induced immunity and reduced immunity to Mtb challenge (Chen *et al*., 2009).

CD8 T cells produce effector functions, such as cytokine production and secretion of cytotoxic granules, which can suppress Mtb growth. Similar to CD4 T cells, two of the major cytokines produced by CD8 T cells are IFNγ and TNF-α, which directly activate macrophages and promote the production of nitrogen and oxygen radicals to suppress Mtb growth (Chan *et al*., 1992; MacMicking *et al*., 1997, 2003). CD8 require perforin to suppress Mtb growth (Stenger *et al*., 1997), and also contain the anti-mycobacterial protein granulysin in their cytotoxic granules, which can directly kill intracellular Mtb (Stenger *et al*., 1998; Ernst *et al*., 2000). In addition to mediating suppression of Mtb growth in blood-derived macrophages *in vitro* (Brookes *et al*., 2003), CD8 T cells have also been reported to suppress Mtb growth in autologous alveolar macrophages (Carranza *et al*., 2006).

In Mtb-infected humans, CD8 T cell responses to epitopes in CFP10 (Shams *et al*., 2004), ESAT-6 (Lalvani *et al*., 1998; Pathan *et al*., 2000) and the Ag85 complex (Klein *et al*., 2001; Caccamo *et al*., 2006) have been described. Testing of a large panel of CD8 T cell clones against a panel of synthetic peptide arrays of immunodominant antigens revealed that Mtb-specific CD8 T cell responses are directed at a limited number of epitopes, the majority of which are restricted by HLA-B alleles (Lewinsohn *et al*., 2007, 2013).

The frequency of cytokine-producing Mtb-specific CD8 T cells has been reported to be higher in patients with active TB, compared with individuals with LTBI (Day *et al*., 2011). In addition, previous studies have reported greater proportions of TB patients with detectable cytokine-producing Mtb-specific CD8 T cells in peripheral blood, compared with individuals with LTBI, with approximately 60% of TB patients displaying an IFNγ+ CD8 response to the immunodominant antigens CFP10 and ESAT-6, compared with only 15–20% of individuals with LTBI (Day *et al*., 2011; Harari *et al*., 2011; Rozot *et al*., 2013). One possible explanation for the increased detection of Mtb-specific CD8 T cells in patients with active TB is that these cells require a higher antigen stimulation threshold, compared with Mtb-specific CD4 T cells, and thus are driven to proliferate *in vivo* in the context of high bacterial load. This notion is supported by *in vitro* studies indicating CD8 T cells preferentially target macrophages with high bacillary burdens (Lewinsohn *et al*., 2003), and additional studies indicating significantly higher frequencies of Mtb-specific CD8 T cells in children with active TB disease, compared with children recently exposed to Mtb (Lancioni *et al*., 2012). Despite higher frequencies of cytokine-producing Mtb-specific CD8 T cells in peripheral blood of patients with TB, other functions of Mtb-specific CD8 T cells have been described to be progressively impaired in patients with active TB, including decreased cytolytic activity and production of cytotoxic molecules (Smith *et al*., 2000; Andersson *et al*., 2007), and significantly impaired proliferative capacity (Govender *et al*., 2010; Day *et al*., 2011; Rozot *et al*., 2013).

Studies of memory phenotypes of Mtb-specific CD8 T cells have indicated predominately terminally differentiated effector memory T cells (TEMRA) in individuals with LTBI, and of TEM cells in patients with active TB disease (Caccamo *et al*., 2009; Rozot *et al*., 2013). Immunoregulatory molecules that have been associated with CD8 T cell dysfunction and immune exhaustion in chronic viral infections, including CD160, PD-1 and 2B4 (Wherry, 2011), have been reported to be expressed at low levels on Mtb-specific CD8 T cells, both in the setting of latent infection and active TB disease (Rozot *et al*., 2013). By contrast to studies in chronic viral infections (Jones *et al*., 2008), expression of the immunoregulatory molecule Tim-3 by Mtb-specific CD4 and CD8 T cells has been reported to be associated with high effector functional capacity (Qiu *et al*., 2012). The molecular mechanisms underlying the expansion of cytokine-producing Mtb-specific CD8 T cells, which apparently lack the capacity to effectively control Mtb replication in patients with active TB, remain to be determined.

The Humoral Immune Response to Mtb: B Cells and Antibodies

As an intracellular pathogen of macrophages, studies of the immune response to Mtb have historically largely been focused on T cell immunity. However, mounting evidence indicates an important role for B cells and antibody (Ab) responses in shaping the immune response to numerous intracellular pathogens, including *Leishmania major*, *Salmonella enterica*, *Francisella tularensis*, *Ehrlichia chaffeensis* and species of *Plasmodium* (Culkin *et al*., 1997; Su *et al*., 1997; Langhorne *et al*., 1998; Yang and Brunham, 1998; Mastroeni *et al*., 2000; Li and Winslow, 2003; Woelbing *et al*., 2006). B cells play a central role in cellular immunity by stimulating proliferation, survival and differentiation of T cells through expression of costimulatory molecules and production of cytokines and antibodies. More recently, Abs have been shown to bind to pathogens for intracellular detection by the cytosolic Ab Fc receptor TRIM21, which triggers innate immune signalling, activation of antimicrobial functions, production of pro-inflammatory cytokines and induction of adaptive immunity (McEwan *et al*., 2013). These findings demonstrate a role for Abs in intracellular bacterial infections by binding to cytosolic TRIM21 and thus triggering activation of immune signalling.

B cells

Aggregates of B cells are present in high numbers in human granulomas (Ulrichs *et al*.,

2004; Tsai *et al*., 2006), and are generally localized in the lymphocytic cuff of the granuloma (Kozakiewicz *et al*., 2013b). Evidence for modulation of the B cell compartment in patients with TB disease is provided by whole blood gene expression profiling of TB patients prior to and after completion of TB treatment, which indicated significant changes in expression of B cell-associated genes, including IgM, IgD, CD79, CD19, CD22, BLK and Pax5, after initiation of TB treatment (Cliff *et al*., 2013). B cells have been shown to moderate inflammatory progression and susceptibility to pulmonary TB in challenge experiments in mice (Maglione *et al*., 2007). Moreover, B cells may play a role in modulating the immune response to Mtb by production of cytokine such as IL-10, and production of Ab that can engage Fcγ receptors and trigger signalling pathways (Maglione *et al*., 2008; Maglione and Chan, 2009). Further studies in mice have indicated B cells regulate infiltration of lung neutrophils by modulating the IL-17 response (Kozakiewicz *et al*., 2013a).

Antibodies

Antibodies to Mtb proteins have been reported in approximately 90% of TB patients (Lyashchenko *et al*., 1998). Numerous studies have investigated the presence of circulating Ab to Mtb proteins to evaluate their utility as biomarkers for TB disease. Serological tests have been developed commercially; however, the sensitivity and specificity of these tests are poor, with sensitivities ranging from 0.09% to 59.7%, and specificities ranging from 53% to 98.7% (Steingart *et al*., 2007a,b, 2009). However, a recent systems biology approach screening plasma samples against the entire Mtb proteome by microarray has identified specific sets of antigens that are recognized by Abs in patients with active TB disease, and further suggest that Ab levels correlate with bacterial burden (Kunnath-Velayudhan *et al*., 2010). This systems biology approach to identify Ab targets of Mtb indicates patients with active TB harbour Ab responses that target a very narrow spectrum of only 0.5% the entire Mtb proteome, the majority of which were extracellular proteins. Ab responses against 13 proteins were associated with active TB disease; these proteins are components of PPD and Mtb extracts, and most have been reported to be antigenic targets of B cells and/or T cells (Kunnath-Velayudhan *et al*., 2010).

Studies in mice have indicated administration of high-dose intravenous immunoglobulin (IVIG) may increase protection against TB (Roy *et al*., 2005). In humans, serum immunoglobulin G (IgG) Abs to lipoarabinomannan (LAM) have been implicated in conferring protection against disseminated TB disease (Costello *et al*., 1992; Pethe *et al*., 2001), and have been reported to promote mycobactericidal activity by innate cells and enhance stimulation of mycobacteria-specific CD4 and CD8 T cell responses (de Valliere *et al*., 2005). Antibodies to the mycobacterial Ag85 complex have been associated with favourable outcomes in patients with TB (Sanchez-Rodriguez *et al*., 2002).

Antibodies utilize multiple effector mechanisms to mediate control of pathogens, including neutralization, opsonization, antibody-dependent cytotoxicity (ADCC) and activation of the complement cascade, all of which play a significant role in the control of numerous viral, bacterial and fungal infections (Willcocks *et al*., 2009). Ab-dependent cellular phagocytosis (ADCP) has been shown to improve clearance of bacteria by functional monocytes, macrophages and neutrophils (Maeda *et al*., 1996). ADCP of mycobacteria by human macrophages has been linked to high intracellular Ca2+ concentrations that enhance phagosomal maturation and promote killing of intracellular bacteria and phagocytic clearance of Mtb (Malik *et al*., 2000). ADCC recruitment of NK cells results in high levels of cytokine secretion, including IFNγ, which is critical for protection against TB (Cooper *et al*., 1993; Flynn *et al*., 1993; Jouanguy *et al*., 1996, 1999). The importance of these antimicrobial mechanisms of bacterial control is clearly evidenced by the fact that many species of bacteria have evolved elaborate defence mechanisms against this activity, including a large family of enzymes that specifically cleave the Fc-portion of the Ab or the sugar moiety on the Fc-domain involved in altering Ab affinity for Fc receptors (Vincents *et al*., 2004; Ryan *et al*., 2008; Brezski *et al*., 2009). However, little is known about ADCC and ADCP humoral immune responses

in the context of Mtb infection in humans, which have largely been overlooked due to the fact that Abs targeting internal regions of Mtb are considered to have little impact on classical Ab-mediated neutralization. However, it is plausible that Abs targeting internal Mtb proteins, which may be transiently expressed on the surface of infected cells, may afford these humoral immune responses with the ability to recruit innate immune cells to rapidly destroy or clear infected cells, thereby promoting control of Mtb.

TB and Co-morbidities

Persons with chronic diseases such as TB are more likely to develop other co-morbidities with non-communicable diseases. Many high TB burden countries also have high co-morbidity with communicable and non-communicable diseases, and associations have been described between TB and diabetes mellitus, smoking, alcoholism, malnutrition, chronic lung diseases, immunosuppressive treatment and cancer (Fox and Menzies, 2013; Marais *et al*., 2013). Thus the host immune responses to infectious pathogens such as Mtb, as well as vaccine-induced immune responses, are likely to be modulated by co-infections and by non-communicable diseases.

TB and HIV

Co-infection with HIV is the single greatest risk factor for reactivation of LTBI and progression to TB disease: while HIV-uninfected individuals have a 5–10% lifetime risk of development of TB, HIV-infected individuals have a 10% annual risk of developing TB disease (Jones *et al*., 1993; Leroy *et al*., 1997; Havlir and Barnes, 1999; Toossi, 2003), with the risk of developing TB disease doubling in the first year of HIV infection (Sonnenberg *et al*., 2005).

HIV infection results in profound immune suppression and depletion of CD4 T helper cells, a critical component of the immune response to Mtb. HIV can also infect alveolar macrophages (Landay *et al*., 1993; Sierra-Madero *et al*., 1994; Nakata *et al*., 1995), the primary target of Mtb infection. Antiretroviral therapy reduces the incidence of TB by as much as 67% across all strata of CD4 counts in HIV-infected individuals (Lawn *et al*., 2011; Suthar *et al*., 2012). However, despite antiretroviral treatment, HIV-infected individuals remain at elevated risk for TB disease, compared with HIV-uninfected individuals (Girardi *et al*., 2005). Mtb/HIV co-infection can also be detrimental to HIV disease progression: HIV viral loads in co-infected individuals are increased in both plasma (Goletti *et al*., 1996) and at sites of Mtb infection (Nakata *et al*., 1997; Lawn *et al*., 2001), and TNF-α production in response to Mtb infection can further drive activation of HIV replication in macrophages (Kedzierska *et al*., 2003).

Increasing evidence indicates HIV infection compromises granuloma formation, with HIV-infected individuals often displaying systemic TB disease, characterized by lack of well-defined granulomas (de Noronha *et al*., 2008; Diedrich and Flynn, 2011). Histological studies have demonstrated decreased numbers of CD4 T cells in granulomas from TB patients with acquired immune deficiency syndrome (AIDS) (Shen *et al*., 1988). Furthermore, HIV infection impairs multiple functions of macrophages, including cytokine production, apoptosis, autophagy, oxidative burst activity and vacuole acidification (Spear *et al*., 1990; Mwandumba *et al*., 2004; Patel *et al*., 2007, 2009; Zhou and Spector, 2008; Kumawat *et al*., 2010), thus impairing the overall ability of macrophages to kill intracellular Mtb.

Cytokine-producing Mtb-specific T cells have been described in the blood of HIV-infected individuals (Zhang *et al*., 1994; Chapman *et al*., 2002; Lawn *et al*., 2007; Geldmacher *et al*., 2008, 2010), with generally lower frequencies of cytokine-producing Mtb-specific T cells when compared with HIV-uninfected individuals. Studies on the ability of antiretroviral treatment to restore Mtb-specific T cell functional capacity have yielded inconsistent results (Schluger *et al*., 2002; Sutherland *et al*., 2006, 2010; Wilkinson *et al*., 2009). Phenotypic analysis of Mtb-specific T cells in HIV-infected individuals with LTBI indicates an early to intermediate differentiated population of effector memory T cells, with the proportion of Mtb-specific IL-2-producing cells correlating inversely with HIV viral load (Day *et al*., 2008). Furthermore, HIV preferentially infects

Mtb-specific CD4 T cells in co-infected individuals, resulting in specific depletion of Mtb-specific CD4 T cell responses in peripheral blood (Geldmacher *et al*., 2008, 2010). Additional studies using BAL samples indicate depletion and impairment of mycobacteria-specific T cells in the lungs of HIV-infected individuals, compared with HIV-uninfected individuals (Kalsdorf *et al*., 2009; Jambo *et al*., 2011).

HIV-infected individuals have an increased risk of developing active TB disease and an increased risk of death due to TB (Whalen *et al*., 1997, 2000). However, the mechanisms whereby infection with HIV perturbs protective immune responses to Mtb, besides depletion of CD4 T cells, have not been fully elucidated. Recent studies comparing antigen-specific CD8+ T cells in HIV-infected and HIV-uninfected persons with LTBI report decreased degranulation and proliferative capacity of Mtb-specific CD8 T cells in the presence of HIV co-infection indicating that CD8 functions in LTBI are impaired by HIV (Kalokhe *et al*., 2015). Intensive characterization of the immune response to Mtb, in the setting of HIV co-infection, is necessary to fully understand why co-infection with HIV increases the risk of developing active TB by 20–40-fold, compared with HIV-uninfected persons (Selwyn *et al*., 1989; Getahun *et al*., 2010).

Helminths and TB

Helminths are parasitic worms that can reside in tissues, such as filarial or schistosomes, or in the intestinal lumen, including hookworms, *Nippostrongylus brasiliensis*, *Ascaris* species, and the whipworm *Trichuris trichiura*. Co-infections with Mtb and helminths are prominent in Sub-Saharan Africa (Jackson *et al*., 2009), and helminth co-infection has been shown to modulate the host immune response to Mtb in both human and animal studies.

Tissue injury induced by helminths promotes release of cytokine alarmins, which promote type 2 cytokine production, including IL-4, IL-5 and IL-13 by Th2 cells, basophils and eosinophils (Anthony *et al*., 2007). Exposure of macrophages to IL-4 and IL-13 suppresses classically activated macrophages (M1), and instead promotes generation of alternatively activated macrophages (M2) (Martinez *et al*., 2009). Studies in mice indicate engagement of the IL-4 receptor signalling pathway in helminth infections promotes differentiation of M2 macrophages that attenuate the innate immune defence against Mtb (Potian *et al*., 2011). While M1 macrophages are pro-inflammatory, and contribute to immunity against Mtb by using nitric oxide synthase (NOS2) to generate reactive nitrogen species (Nathan and Shiloh, 2000), M2 macrophages secrete anti-inflammatory IL-10 and TGF-β, and mediate resistance against helminth infections (Anthony *et al*., 2007). Proteins secreted and excreted by helminths can inhibit DC activation and the production of pro-inflammatory cytokines, and instead promote production of the anti-inflammatory cytokines IL-10 and TGF-β by DCs (White and Artavanis-Tsakonas, 2012). Moreover, helminth infections are associated with differentiation of CD103−CD11clo regulatory DCs, which promote the generation of Tregs from naïve precursor T cells and do not efficiently prime effector T cells (Grainger *et al*., 2010; Li *et al*., 2011; Smith *et al*., 2011; Aranzamendi *et al*., 2012). Thus the helminth-induced Th2 cytokine response, the generation of M2 macrophages and editing of DC effector functions together result in dampening of classical macrophage effector functions and Th1 immune responses necessary for immunity to Mtb.

In patients with TB disease, co-infection with helminths has been shown to be associated with more advanced TB disease (Resende Co *et al*., 2007). Decreased IFNγ production and increased IL-10 production in mycobacteria-stimulated whole blood cultures *in vitro* have been described for TB patients co-infected with helminths, compared with patients with TB alone (Resende Co *et al*., 2007). Mycobacteria-specific T cell proliferative response and Th1 and Th17 cytokine responses are suppressed in individuals with LTBI who reside in helminth endemic regions (Rougemont *et al*., 1977; Stewart *et al*., 1999; Babu *et al*., 2009b; George *et al*., 2013). PPD and Mtb-specific IL-23 and IL-17 production is reportedly decreased in TST positive individuals with filariasis, compared with TST-positive individuals without filariasis

(Babu *et al*., 2009b). Mycobacteria-specific T cell immunity in individuals with LTBI and helminth co-infection is markedly enhanced after de-worming (Elias *et al*., 2001; Babu *et al*., 2009a), thus providing further evidence of the ability of helminth infections to directly modulate the host immune response to Mtb.

Diabetes and TB

The global burden of diabetes indicates a prevalence of 9.8% worldwide in adults over 25 years of age, and approximately 200 million cases worldwide (Danaei *et al*., 2011). The link between TB and diabetes mellitus has long been recognized: there is a high incidence of TB in patients with diabetes mellitus, and the risk of developing TB disease has been correlated with the severity of diabetes mellitus (Dooley and Chaisson, 2009). Case–control studies indicate the relative odds of developing TB disease are between 2.4 and 8.3 in diabetic patients, compared with non-diabetic patients; similar associations between increased risk for TB disease in diabetic patients have been described in longitudinal cohort studies (Stevenson *et al*., 2007; Jeon and Murray, 2008). Moreover, the risk of treatment failure and death from TB are more than six-times higher in diabetic pulmonary TB patients, compared with non-diabetic patients with TB (Oursler *et al*., 2002; Dooley *et al*., 2009; Baker *et al*., 2011; Faurholt-Jepsen *et al*., 2012; Jimenez-Corona *et al*., 2013).

The immunological mechanisms contributing to increased risk of TB disease in patients with diabetes mellitus have not been fully elucidated. However, studies suggest several functions of macrophages that are important in Mtb infection, including chemotaxis, antigen presentation, phagocytosis and activation, are impaired in diabetic patients (Moutschen *et al*., 1992; Chang and Shaio, 1995a,b; Wang *et al*., 1999), thus potentially contributing to ineffectual control of Mtb replication by macrophages. Regarding T cell immunity, impaired proliferative capacity (MacCuish *et al*., 1974; Chang and Shaio, 1995a), altered PPD-specific cytokine production profiles (Restrepo *et al*., 2008), and increased frequencies of Th1 and Th17 cells (Kumar *et al*., 2013) have been described in TB patients with diabetes mellitus, compared with non-diabetic TB patients. The soaring rates of diabetes, due in part to global increases in obesity, combined with the growing rates of diabetes in high TB burden countries, necessitate intensification of efforts to diagnose, treat and optimize clinical management of diabetic patients with Mtb infection.

TB Vaccines

The only currently licensed vaccine against TB is Bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis* (Calmette, 1931; Behr *et al*., 1999). Although the degree of protection against TB is highly variable (Colditz *et al*., 1994), BCG has been shown to confer protection against severe forms of childhood TB, including miliary TB and TB meningitis (Rodrigues *et al*., 1993). The variable efficacy of TB has been attributed in part to geographical location, exposure to environmental mycobacteria and the memory T cell profile induced by the vaccine. BCG does not confer protection against pulmonary TB disease in adults (Colditz *et al*., 1994), the most common clinical manifestation of the disease and the largest source of disease transmission. However, some studies have suggested BCG may provide protection against acquisition of Mtb infection (Soysal *et al*., 2005; Eisenhut *et al*., 2009).

A major knowledge gap in TB vaccine development is the lack of validated clinical correlates of risk of TB disease, or clinical correlates of protection against TB. Although studies from human and animal models have demonstrated the importance of Th1 immune responses in Mtb infection, they do not correlate with BCG-mediated protection against TB (Kagina *et al*., 2010). Yet most TB vaccines currently in clinical trials have been designed to induce Th1 immunity, thus efforts to evaluate efficacy of novel TB vaccine candidates are hindered by the lack of known correlates of protection against TB.

There is some evidence that suggests natural infection with non-tuberculous mycobacteria (NTM), as well as long-term control of LTBI, provides some protection against development of TB disease following re-exposure

to Mtb (Fine, 1995; Weir *et al*., 2006; Andrews *et al*., 2012). The only TB vaccine to show some level of protection in phase III trials is the DAR-901 vaccine, consisting of inactivated whole cell *M. vaccae*, which was administered in five doses to HIV-infected individuals with CD4 counts ≥200 cells/μl. Vaccination with *M. vaccae* was associated with significant protection against TB in HIV-positive individuals, compared with the placebo group (von Reyn *et al*., 2010).

Multiple approaches are currently being employed to develop novel TB vaccine candidates. Protein subunit vaccines require the use of an adjuvant to induce Th1 immunity; current protein subunit TB vaccines in clinical trials include M72/AS01E, H1-IC31, H4-IC31, H1-CAF01, H56-IC31 and ID93/GLA-SE (Lalvani *et al*., 2013). Viral vectors do not require adjuvants, but previous exposure to the vector may attenuate vaccine-induced responses; current viral vector-based TB vaccine candidates currently in various stages of clinical trials include MVA85A/AERAS-485 and AERAS-402/Crucell Ad35 (Lalvani *et al*., 2013). Most TB vaccines in development now are designed for use as booster vaccines to be administered after priming with BCG; in some cases, novel TB vaccine candidates are being developed to replace BCG. An alternative approach to improving the BCG vaccine is to re-engineer strains of BCG, for example the construction of the recombinant BCG strain, rBCG30, that over-expresses Ag85B (Horwitz, 2005). VPM 1002 is another recombinant BCG strain that has been engineered to expresses lysteriolysin from *Listeria monocytogenes*, which allows the mycobacterium to escape from the vacuole to the cytosol and therefore enhance MHC class I antigen presentation to CD8 T cells, and also carries a urease deletion mutation to enhance acidification of the vacuole, improving release of the mycobacterium (Grode *et al*., 2013). Developed as a therapeutic vaccine, the TB vaccine candidate RUTI is generated by growing Mtb under stress, then fragmenting, detoxifying and delivering in antigens in liposomes (Cardona, 2006; Vilaplana *et al*., 2010).

As of 2013, there were 14 TB vaccine candidates in various phases of clinical trials (Lalvani *et al*., 2013). The first novel TB vaccine candidate to be tested in a phase IIb trial in infants was MVA85A, a modified vaccinia Ankara virus expressing Ag85A from Mtb. Vaccination with MVA85A did not provide protection against TB disease in infants, despite inducing high levels of cytokine-producing CD4 T cells. Moreover, there was no correlation between the magnitude of vaccine-induced immunity, as measured by the frequency of Th1 cytokine-producing T cells, and protection against TB (Tameris *et al*., 2013). Despite the disappointing lack of protective efficacy against TB disease, there is much that stands to be learned from the outcome of the phase IIb MVA85A trial that will hopefully guide the future development of TB vaccine candidates, including optimization of the route, dose, mode of antigen delivery and formulation of novel vaccine candidates, in addition to promoting research agendas that prioritize development of new tools for predicting and evaluating vaccine efficacy.

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4 Current Methods for Diagnosis of Human Tuberculosis and Considerations for Global Surveillance

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Introduction

Tuberculosis (TB) is a chronic infectious disease with an estimated 8.6 million cases worldwide; it was responsible for 1.3 million deaths globally in 2012, including 320,000 in individuals with HIV co-infection (World Health Organization, 2013b). Although the overall TB incidence is decreasing globally (45% decrease since 1990), this is not mirrored in large parts of Asia and Africa where the pathogen remains highly endemic (Zumla *et al*., 2013). Only 5.8 million (66%) of the global estimates of TB cases were notified to public health services. This difference has been attributed to patients not seeking and/or accessing healthcare, failure to diagnose TB infection and the underreporting of diagnosed TB cases (World Health Organization, 2013b), and improved diagnostic assays may be beneficial in decreasing this difference.

There are many considerations for the development of effective diagnostics for active TB infection. One major issue is the undetermined incubation period (latency) (Dye *et al*., 1999). The transition from latency to active disease is a pressing problem affecting diagnosis and global control of TB. Approximately one-third of the world's population is potentially infected with *Mycobacterium tuberculosis* (latent TB), but only 5–10% develop clinical symptoms (active TB) during their lifetime (Barry *et al*., 2009). However, the perception of the clinical manifestation of TB is changing from one of binary expression (i.e. latent and active) to a spectral expression of disease. The spectrum of disease progression in TB-infected people is determined by interplay between bacterial load and host immune response. At one extreme, the infection is eliminated without priming the antigen specific T cells with sterile tissue (innate immune response), resulting in fewer bacteria (pauci-bacillary) with less caseous cavitation (quiescent infection); while at the other, more bacteria (multi-bacillary) manifest with liquefied cavitation in the lungs (Barry *et al*., 2009). Understanding this spectrum of disease is critical to the development of diagnostics specifically targeting active TB.

Another critical issue influencing diagnostic success is the fact that the manifestation of

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

active TB is regulated by multiple parameters, all of which complicate design and application of clinical diagnostics for the disease. Prevailing co-morbidities (e.g. diabetes) and co-infections (HIV/AIDS) can also significantly affect disease manifestation (Porter and McAdam, 1994; Lonnroth *et al*., 2009), and influence diagnostic success.

Infection with *M. tuberculosis* most frequently manifests as pulmonary disease, but systemic infection can occur and may involve lymph nodes, pleura, urogenital tract, bones and joints, central nervous system, bowel, peritoneum, pericardium and skin (extra-pulmonary TB (EPTB)) (Carrol *et al*., 2001; Golden and Vikram, 2005). EPTB is more common in children, immune-compromised people with co-morbidities and the elderly.

Finally, the slow-replication rate (18–24 h) of *M. tuberculosis* is a significant barrier to rapid confirmatory diagnosis of active infection. As a result, laboratory diagnosis and conventional drug-susceptibility testing (which require culture) take at least 6–8 weeks. This poses a major challenge in the management of the individual patient as well as to laboratory-based surveillance and TB control programmes (see Detection of Immune Response Markers for the Diagnosis of TB, below).

The emergence of drug-resistant TB has created the additional need for tests to be able to provide resistance profiles. Currently, complete profiles can only be obtained by means of culture-based methods. Molecular tests can be used to determine the presence of mutations in genes coding for drug targets when such mutations have been linked with phenotypic resistance to that drug. So far, no other tests are able to provide information on drug resistance, and development of such capabilities is required especially considering the increase in incidence of drug-resistant TB.

All of these factors significantly influence effective diagnosis of active TB. The search for a reliable diagnostic test for active TB is ongoing, and remains an elusive target. Characteristics of an ideal diagnostic test include sensitivity, specificity, minimal sample processing, ease of execution, speed, cost and others. However, the relative significance of each of these parameters is different in different settings. For instance, a test that is performed for diagnosis of active TB in a resource-poor population in which TB is endemic should be simple, inexpensive and highly sensitive and should differentiate between active disease and previous exposure to the pathogen; for active cases, further testing should be available to provide information about drug-resistance profiles. However, diagnosis of TB in immigrants/travellers in a developed country need not essentially be associated with negligible cost, or be technically simple. This chapter attempts to comprehensively present all available strategies for the diagnosis of TB. The description of the various tests should be read with these criteria in mind. Indeed, each diagnostic paradigm described below has associated advantages and disadvantages. However, the choice of use in a given scenario is based uniquely on the needs of that population, the prevalent co-morbidities and the availability of resources.

Diagnostic tests for TB can be broadly categorized as tests that: (i) detect microbes/components of microbes; or (ii) detect components of the immune response to microbes. The former includes microscopy, culture, antigen detection and nucleic acid detection assays, while the latter includes tests that detect antibodies and activated T cells. In addition to the basic assay design, the sensitivity of these tests also depends on the type and quality of the specimen, the concentration of microbes or microbial components in the specimen, the stage of disease and the volume of specimen, as well as the skill of the technologist handling the processing and reading of results.

Direct Detection of *Mycobacterium tuberculosis*

Sputum smear microscopy

In many low- to middle-income countries, especially in Africa and Asia, direct detection of acid-fast bacilli in expectorated sputum is still the primary method for the diagnosis of pulmonary TB in humans (Tuberculosis Coalition for Technical Assistance, 2006). Indeed, the current guidelines of the World Health Organization (2011b) and the International

Union Against Tuberculosis and Lung Disease (Reider *et al*., 2007) both indicate that microscopic examination of sputum is an essential step in the identification of pulmonary TB in suspected patients. Further, Standard 2 of the International Standards for Tuberculosis Care categorically states that all patients suspected of having pulmonary TB should have at least two, and preferably three, sputum specimens made available for microscopic testing, of which two need to be positive before the diagnosis is confirmed (Tuberculosis Coalition for Technical Assistance, 2006). In practice, these guidelines can be difficult to implement. Difficulty in obtaining sputum from patients, as well as delay times in return of diagnostic information, can result in doctors electing to proceed with TB treatment without smear results (for example, see Tafuma *et al*., 2014). The sensitivity of microscopy for the diagnosis of TB can vary from as high as 80%, to as low as 20%, depending on existing co-morbidities (see discussion below) (Urbanczik, 1985; Steingart *et al*., 2006b) and health care-seeking behaviour in a population. Although sputum smear microscopy has lower sensitivity compared to many other tests, it identifies the most infectious individuals, and is therefore critical to prevent further transmission of disease. A key advantage of this technology is that it is cheap and affordable in various populations with different socio-economic levels (Steingart *et al*., 2006a).

The original acid-fast staining technique, which allows mycobacteria to be visualized by microscopy, was developed by the German researcher, Robert Koch, in the late 1870s to 1880s. The technique, which has changed little since its original discovery, is based on the observation that mycobacteria are acid-fast bacilli, which means that certain dyes will not be released from the bacterial cell by a solution of acid-alcohol, which will remove these dyes from most other bacteria. Today, two staining methods are predominantly used. The Ziehl–Neelsen (ZN) staining technique visualizes acid-fast bacilli (AFB) by bright field microscopy, while the Auramine-O staining technique requires fluorescent microscopy for visualization of mycobacteria.

The number of bacilli/ml of sputum varies in TB cases depending on the stage of disease and HIV sero-status. ZN-stained smears are likely to yield negative smear results if the number of AFB is less than 5000 bacilli/ml of sputum, while a consistently positive result requires at least 500,000 bacilli/ml sputum.

One of the major issues with AFB is the significant dependence on skill: the commitment and efficiency of the microscopist, as well as the duration of slide viewing, impacts detection of AFB on a smear. It is recommended that a ZN smear be viewed for at least 20 min, which will allow for visualization of 100 high power fields. In busy laboratories, however, staff may not be motivated to read a single slide for 20 min. Fatigue from reading too many slides can also lead to decreased concentration. Countries with high prevalence of TB process a large number of sputum smears. These countries typically have laboratory personnel exclusively trained in AFB-microscopy techniques. In South Africa, for example, training of such dedicated microscopists typically takes 2–5 days. This is likely to be inadequate and may contribute to the already poor sensitivity of smears.

Fluorescence microscopy is the preferred staining technique for diagnosis in high TB endemic countries. The field of vision during viewing is 17 times greater than conventional light microscopy, with a higher sensitivity. A recent meta-analysis comparing Auramine-O staining with ZN staining found that fluorescence microscopy of sputum smears offered a 10% greater sensitivity (Tuberculosis Coalition for Technical Assistance, 2006). This improved sensitivity was observed in low-grade smears as well. However, the need for a dark room for fluorescence microscopy makes this technique difficult to implement in peripheral and resource-poor settings. The introduction of new low-cost microscopes that use light-emitting diode (LED) technology enables viewing of Auramine-O stained slides independent of a dark room. This technique for reading smears has the potential for roll-out to remote areas. Automated digitized viewing of smears is currently employed only in larger diagnostic laboratories.

For all its advantages, acid-fast microscopy suffers from several critical disadvantages that limit its application. Its main use is

for the diagnosis of pulmonary TB. Although microscopy can be applied to specimens from patients with extra-pulmonary manifestations of the disease, because of the paucibacillary nature of these manifestations, the sensitivity is very low (Perkins, 2000; Luelmo, 2004; Desikan, 2013). Further, use of sputum microscopy is limited to adults, adolescents and children capable of producing sputum, thereby not covering the physically/mentally challenged and very young paediatric populations (Shingadia and Novelli, 2003; Perkins *et al*., 2006). Also, the sensitivity of the technology is severely limited with a bacterial load <5000–10,000 organisms/ml in sputum, thereby compromising the diagnosis of mild or early disease (Desikan, 2013).

Another limitation is the extreme variability with respect to the methodology used in different laboratories. To facilitate global standardization in sputum microscopy, the Global Drug Facility, a component of the Stop TB partnership established in 2001, is developing a smear microscopy kit to implement worldwide. Although not free, these kits are one method of improving the quality and availability of existing diagnostics for global use (www.Stoptb.org).

The biggest concern for the use of sputum microscopy is the significantly larger failure rate in individuals with HIV co-infection. The rapid upsurge in the incidence of TB since the mid-1980s is partially attributed to the wide spread of infection with HIV and associated AIDS (Timpe and Runyon, 1954; Colebunders *et al*., 1989; Elliott *et al*., 1990). Observations from countries with high HIV–TB co-infection rates indicate that the sensitivity of sputum microscopy is significantly lower in the HIV-infected TB patient, than otherwise (World Health Organization, 1988; Swai *et al*., 2011). In a study of 380 patients that compared HIV seronegative patients with pulmonary TB to seropositive patients in Dakar, Senegal, Samb *et al*. (1999) have shown that the latter are more likely to have negative sputum smears. Whereas some early studies contradict this (Smith *et al*., 1994), it is increasingly becoming accepted that HIV co-infection significantly reduces the likelihood of a positive sputum smear, making sputum microscopy an unreliable technique for the diagnosis of pulmonary TB in populations with high HIV incidence (Samb *et al*., 1999; Steingart *et al*., 2006b; Matee *et al*., 2008).

Culture

Conventional culture

For many decades, diagnosis of active TB has been made based on clinical presentation, X-rays, sputum microscopy and culture. Of these modalities, culture remains the gold standard for confirmatory diagnosis of active TB infection. However, the method is expensive, technically demanding, requires laboratory resources and is time consuming. As a result, culture is infrequently performed in under-resourced settings.

Jensen's modification of the Lowenstein medium, combined with trisodium phosphate as mucus digesting and decontaminating agent, has long been the most used methodology for culture of mycobacteria (Levin *et al*., 1950). Trisodium phosphate is ideal for preparation of sputum samples, as it has been shown to not kill *M. tuberculosis* even after 72 h of exposure (Spendlove *et al*., 1949). The methodology is more sensitive and specific than sputum microscopy (Singh and Kashyap, 2012), but the fact that it takes up to 8 weeks to provide a result is a major limitation in the use of this method.

The introduction of agar-based Middlebrook media instead of the egg-based Lowenstein–Jensen (L–J) media, as well as liquid media, has decreased the average time to result to 2–3 weeks, although incubation for 6 weeks is still required for optimal sensitivity. Indeed, the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, USA) recommends a turnaround time not exceeding 21 days for the isolation of *M. tuberculosis* (Styrt *et al*., 1997), which is clearly not achievable using conventional methodologies. Further, with the rise in prevalence of drug-resistant TB, biosafety Level 3 containment is becoming increasingly necessary for handling and culture of *M. tuberculosis*. Such laboratories are not always available. When available, they are expensive facilities requiring more extensive training and experience.

Microscopic observation of mycobacterial growth

Microscopic observation of broth- or agar-based cultures is a technologically simple and rapid testing method that has been employed for the diagnosis of TB (Shiferaw *et al*., 2007; Brady *et al*., 2008; Lazarus *et al*., 2012). The broth-based methodology, simply termed microscopic observation of drug sensitivity (MODS) (www.hardydiagnostics.com/tbmodskit.html) is well developed and does not require any new instrumentation other than an inverted light microscope. This diagnostic is based on the visualization of the characteristic cording growth pattern of *M. tuberculosis* by a trained microbiologist. The technology is largely simple, easy to adapt and costs <US$5.00 per specimen, making it affordable for use in many countries worldwide (Caviedes *et al*., 2000; Moore *et al*., 2004). However, it requires an inverted microscope which, unlike light/optical microscopes, is not routinely present in all laboratories. Therefore, some equipment installation/modification may be required for effective, more global use. Kidenya *et al*. (2013) performed a comparative study of MODS versus traditional culture in a cohort of 321 TB suspects in Tanzania, and found that liquid assay with MODS detected a significantly higher proportion of cases than L–J culture methodology: 89% versus 77%. The median time to diagnose TB was also much shorter by this method, 9 days (interquartile range 7–13) compared to 21 days (interquartile range 14–28) by L–J culture (p <0.0001). Further, the cost of the technology, assuming availability of the microscope, was $4.56 per sample, compared to $11.35 per specimen for L–J culture (Kidenya *et al*., 2013). Other studies have also produced similar results, especially in atypical populations such as paediatric patients (Ha *et al*., 2009) and patients with tubercular meningitis (Caws *et al*., 2007). Thus, MODS is an excellent rapid substitute to traditional culture for confirmatory diagnosis of TB. However, as with all culture techniques, the technology requires skilled technologists trained in observing cording and in handling highly contagious broth cultures.

Automated culture systems

The BACTEC 460TB™ (Becton Dickinson (BD), Sparks, MD, USA) was the first system for the automated culture and characterization of mycobacteria. The system promises detection within 4–8 days, differentiation in 3–6 days and a measure of drug-susceptibility within 4–12 days of initiation. These rapid result times are well within the CDC guidelines, and also provide information on the drug-resistance phenotypes (Siddiqi and Rusch-Gerdes, 2006). The system, however, is radiometric and uses Middlebrook 7H12 broth medium containing ^{14}C-labelled palmitic acid for the detection of bacterial growth, and is specifically suited for culture of sputum samples (Morgan *et al*., 1983; Kirihara *et al*., 1985; Sewell *et al*., 1993). Cutler *et al*. (1994) have shown that the use of BACTEC 460TB, in addition to L–J medium for culture of extra-pulmonary TB specimens, increased isolation rates by 20%, suggesting a value for this technology to improve yields in paucibacillary TB cases. Aggarwal *et al*. (2008) used the BACTEC 460TB system with L–J medium for diagnosis of patients with cutaneous TB, indicating that the rapid outcome is the biggest benefit.

To eliminate production of radiometric waste, BD developed the mycobacteria Growth Indicator Tube (MGIT) for fluorescence-based detection of mycobacterial growth. The BACTEC 960™ (Becton Dickinson, Sparks, MD, USA) is an automated system (https://www.bd.com/ds/productCenter/MT-BactecMgit960.asp) for the detection of fluorescence in these MGIT tubes, scanning every 60 min for increased fluorescence. Fluorescence intensity determines whether the test sample contains viable organisms, with a positive tube containing approximately 105–106 colony-forming units per millilitre (CFU/ml). Cultures without signs of growth for a minimum of 42 (maximum 56) days are considered negative. Other companies have followed BD in the development of automated systems, and like BACTEC, these have proved extremely valuable for the efficient and rapid detection of mycobacteria. However, the cost of culture and system set-up are still prohibitive in many populations (Tortoli *et al*., 1999, 2002; Kontos *et al*., 2004). Cruciani *et al*. (2004)

performed a systematic review and meta-analysis of BACTEC MGIT 960 for the diagnosis of mycobacteria, and found that the system demonstrated a specificity of 81.5% and a sensitivity of 85.8% in a study of 1381 strains of the pathogen from 14,745 clinical specimens. In the same study, the sensitivity and specificity of BACTEC 460 was found to be 99.6% and 99.9%, respectively. The lack of radioactive waste is seen as a clear advantage of the BACTEC MGIT 960, in addition to the shorter time to detection and convenience of use.

Detection of Immune Response Markers for the Diagnosis of TB

Tuberculin skin test

The earliest diagnostic assay for TB that does not use microscopy or culture was the tuberculin skin test (TST). TST is also referred to as the Mantoux test, Mendel–Mantoux test, Heaf test or Pirquet test, depending on the method by which the tuberculin, also known as purified protein derivative (PPD), is applied. The test was first described by Robert Koch in 1890, and further developed independently by the French physician Charles Mantoux in 1907 and the German physician Felix Mendel in 1908. The skin test antigen (PPD, tuberculin) is derived from sterilized culture filtrates of *M. tuberculosis*, and is a precipitate of several different non-specific molecules. Thus, PPD contains cross-reactive antigens with other mycobacteria, not specific to *M. tuberculosis*, and has limited scope in identifying LTBI in the individuals with prior BCG vaccination (Wang *et al*., 2002). Currently, the test is still used in the USA for the detection of possible exposure to *M. tuberculosis*. For the Mantoux test, the antigenic preparation is injected intradermally, and the response is quantitatively assessed as an indicator of the immune response to bacterial proteins. The test is positive if the diameter of the induration is greater than 10 mm in 2 days, and a positive result is indicative of TB exposure (not active disease).

There are several issues with the TST test: (i) it is non-specific, and does not discriminate between exposure to pathogenic and non-pathogenic mycobacteria; (ii) the results are subjective, in particular in differentiating between erythema and induration; (iii) measurement of induration can be difficult, especially in dark-skinned people; (iv) it does not differentiate between response due to BCG vaccination and exposure to *M. tuberculosis*; (v) once the test is performed, the individual cannot retake it for 6 months–1 year; (vi) false positives can occur simply by touching the injected area, making the results highly inconclusive; (vii) the result can be read only after 48 h, requiring a second visit to the physician's office; and (viii) other conditions such as infectious mononucleosis, sarcoidosis, malnutrition and HIV co-infection can result in false negative results. Because of all these reasons, the skin test is only minimally used, especially in endemic areas, for the diagnosis of active TB (Wang *et al*., 2002).

Biomarker-based assays

Biomarkers are loosely defined as discriminative molecular signatures that can, in this case, inform on disease. By this description, biomarkers can be pathogen or host molecules. The diagnosis of infection through the detection of molecules that are shed from or secreted by the microbial cells obviates the need to obtain a specimen from the site of infection. If these host or pathogen molecules are soluble, small enough and capable of passing through natural barriers, they may be present in blood, urine or other biological samples. This section, therefore, encompasses serological assays, interferon γ- (IFNγ) based assays and pathogen–biomarker-based assays for the diagnosis of TB, and outlines their relative advantages and disadvantages based on available literature.

Antibody-detection assays

Antibody-based tests, also known as serological tests since the specimen used is invariably serum, are tests that indicate the recognition of pathogens by the human immune system. Such immune response-based tests have historically been used for many

diseases, with varying degrees of success (Daniel, 1990; Bothamley, 1995; Chan *et al*., 2000; Laal, 2004). Currently, antibody detection tests for TB are adapted to one of two general formats: enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic assays.

ELISAs. ELISAs are typically designed for 96-well plate formats and, therefore, can be performed with high throughput. Most antibody-detection ELISA kits comprise microtiter plates with wells coated with mycobacterial antigens. The presence of antibodies that interact with these antigens in the infected host is subsequently probed (Janeway *et al*., 2005). To ensure specificity and sensitivity, and to facilitate antigen-antibody interactions at even low concentrations, ELISAs typically require several hours of incubation and thorough washing steps. The binding affinity of the antigen with the antibody, and associated non-specific interactions, are the limiting steps in the development of good ELISA assays. Recently, ELISAs have been completely automated, making them easy to perform and execute. This, of course, is of no advantage in resource-poor settings, where availability of laboratory and automation resources and the associated costs are both prohibitive.

IMMUNOCHROMATOGRAPHIC ASSAYS. These are simple, often inexpensive assays similar to home pregnancy tests and other lateral-flow strip tests in that the antigen is often pre-coated on a membrane (typically nitrocellulose). Subsequent application of the serum elicits an antigen–antibody binding which can be visualized colorimetrically. These tests are rapid and can be performed in the field without major technical expertize, making them ideal for resource-poor populations.

Serological tests overcome many disadvantages of sputum microscopy in that they are simple to perform, yet standardized in format, making them comparable in different global populations. Unfortunately, however, for the diagnosis of TB the performance of such diagnostic tests is extremely poor, especially in high-burden countries (Small and Perkins, 2000; Walsh and McNerney, 2004). The ease of performance and the relatively low costs have led to an uncontrolled use of diagnostic kits of different antigen composition, antigen source, chemical composition and output. Several studies reviewing one or more of these serological assays exist in the literature (Steingart *et al*., 2007, 2012). A systematic review and meta-analyses of serological tests addressed their accuracy in the diagnosis of both smear-positive and smear-negative TB, and also their specificity (healthy controls) (Steingart *et al*., 2007) and found that none of the commercial tests evaluated performed well enough to replace sputum smear microscopy. These studies criticized the lack of methodological rigour in the establishment of these tests, while strongly recommending the identification of novel biomarkers of immunodiagnostic potential. Yet, many countries across the world such as Afghanistan, Brazil, Cambodia, India, China, Kenya, Myanmar, Nigeria, Pakistan and Russia continue to use serological tests for TB diagnosis (Grenier *et al*., 2012). Over 1.5 million TB ELISA kits are sold in India alone, for example, every year (Specter, 2010). Yet, a recent study in India found that using serological tests instead of sputum microscopy would result in about a fourfold increase in costs for the Indian TB control programme, and more false-positive diagnosis (Steingart *et al*., 2012). To this end, the World Health Organization (WHO) has issued a strong recommendation against the use of currently available serological assays for the diagnosis of TB. Recently, the Indian government also banned the import or sale of TB serological tests in India.

The exact reasons for the failure of the TB serological tests remain poorly understood. For one, only 10% of those exposed to *M. tuberculosis* will actually develop active infection. The characterization of 'latency' in TB is also poorly understood, and it is possible that those exposed to the pathogen may develop an immune response that accounts for the high false positivity of serological assays. Vaccination with BCG at birth is another reason for cross-reactive immune responses and false positives. These and other technical reasons, such as the poor sensitivity of lateral-flow immunoassay platforms, can account for the failure rate. The wide acceptance of poorly characterized diagnostics in countries such as India was studied by Jaroslawski and

Pai and they suggest that in the absence of validated, point-of-care diagnostics for TB, serological assays address a 'perceived need' among providers and patients alike (Jaroslawski and Pai, 2012).

Interferon-γ release assays

The discovery that *M. tuberculosis*-specific antigens induce strong interferon γ (IFNγ) responses from sensitized T cells allowed for the development of several assays that exploit this phenomenon (Dockrell and Weir, 1998; Andersen *et al*., 2000; Lalvani, 2003; Barnes, 2004). The basic principle of the interferon-γ release assay (IGRA) is that T cells of individuals exposed to *M. tuberculosis* antigens will produce IFNγ. This IFNγ production can be observed *in vitro*, and quantitated. Early versions of the test using PPD as the stimulating antigen (Andersen *et al*., 2000) resulted in specificity limitations comparable with the TST. However, newer versions of the test have focused on the use of ESAT6 and CFP10, proteins encoded by genes within the Region of Difference 1 (RD 1) of the *M. tuberculosis* genome. Indeed, attenuated mycobacterial vaccine strains such as BCG, environmental and non-pathogenic mycobacteria such as *M. avium*, and common non-tuberculous mycobacteria (with the exception of *M. kansasii*, *M. marinum* and *M. szulgi*) lack these two antigens (Lalvani, 2003; Barnes, 2004), allowing for enhanced specificity and sensitivity of accurate diagnosis using IGRAs (Ariga and Harada, 2008).

This resulted in the development of commercially available diagnostic kits such as QuantiFERON®-TB Gold (QFT-G; Cellestis Ltd, Carnegie, VIC, Australia); QuantiFERON®-TB Gold in – Tube (QFT-G-IT; Cellestis Ltd, Carnegie, VIC, Australia) and the T-SPOT TB (T-SPOT®, Oxford Immunotec Ltd, Abingdon, UK), all of which have been extensively studied for the diagnosis of active TB infection in adults and children.

Like the TST, IGRAs are based on the T-cell response to *M. tuberculosis* antigens. However, IGRAs have been shown to have significant advantages over conventional TSTs: (i) IFNγ release assays are performed *in vitro*, and do not involve the measurement of a subjective parameter such as skin induration (Dockrell and Weir, 1998; Andersen *et al*., 2000; Lalvani, 2003; Barnes, 2004); (ii) the assays have been adapted for the diagnosis of both latent and active tuberculosis; (iii) IGRAs offer a simple way to distinguish infection from BCG vaccination, a significant issue with the TST test in endemic countries where children are vaccinated at birth; (iv) monitoring prognosis and treatment response is enhanced; and (v) there is improved diagnosis of tuberculosis in children and paediatric populations where conventional tests such as sputum microscopy have high failure rates.

The original QuantiFERON®-TB (Cellestis Ltd, Carnegie, VIC, Australia) assay is a whole blood assay that measures IFNγ responses to mycobacterial PPD in an ELISA format. This assay is US Food and Drug Administration (FDA) approved and is being used in many countries. The improved QuantiFERON®-TB Gold (Cellestis Ltd, Carnegie, VIC, Australia) assay uses ESAT6 and CFP10 as the primary antigens (Mazurek and Villarino, 2003). The T-SPOT TB assay detects the interaction of ESAT6 and CFP10 using peripheral blood mononuclear cells, in an ELISPOT (immunoblot) format (Lalvani, 2003, 2004). In addition, there are many laboratory-based tests designed on similar principles that have not yet transitioned into commercial use (Dockrell and Weir, 1998; Andersen *et al*., 2000; Barnes, 2004). All these tests, although based on the same design principles, vary extensively in assay format, incubation time, sensitivity, specificity and stability. A number of systematic reviews have examined the performance metrics of the commercially available platforms in different populations (Pai *et al*., 2004, 2008; Menzies *et al*., 2007). Pai *et al*. (2004) reviewed 75 articles that met their inclusion criteria and found that the use of RD-1 specific antigens such as ESAT6 and CFP10 results in higher specificity, greater correlation with exposure and less cross-reactivity due to BCG vaccination and non-TB mycobacteria (NTM) bacteria, compared to the use of TST antigens. Sollai *et al*. (2014) systematically reviewed the use of IGRAs for the diagnosis of tuberculosis in paediatric populations. They reviewed 31 studies in 6183

children for the QuantiFERON®-TB Gold assay, 14 studies (2518 children) for the T-SPOT TB assay and 34 studies (6439 children) for the TST assay in both high- and low-income countries across the world. They found that both IGRAs showed no better performance than TST in low-income countries (85–93% versus 90%), but higher IGRA specificity with respect to TST was found in high-income countries (97–98% versus 92%). Similarly, the success of IGRAs was found to be better in immuno-competent children, versus compromised ones in the same study. The influence of high HIV-prevalence in low-income countries should be considered in the interpretation of these results. Other significant yet subjective factors include costs, availability of clinicians and health care workers, patient acceptability, ease of distribution and storage, availability of freezers and laboratory resources should be taken into account.

The quantitative extrapolation of systematic reviews should be considered in the light of a recent report by Chang and Leung (2010). This study notes that many systematic reviews of IGRA performance have examined multiple assays with a focus on pooled estimates of sensitivity and specificity rather than the actual predictive value of a positive or negative test (Pai *et al*., 2004, 2008; Menzies *et al*., 2007), which suggests that predicative values cannot be effectively combined across different settings in meta-analysis studies, and that likelihood ratios should not be pooled in systematic review studies (Zwinderman and Bossuyt, 2008). By using the hierarchical SROC model and the bivariate random effects model and using a discrimination between latent and active tuberculosis disease, the authors found that at a 90% certainty threshold, QuantiFERON®-TB assay can diagnose latent tuberculosis but QuantiFERON®-TB-IT (Cellestis Ltd, Carnegie, VIC, Australia) and T-SPOT TB assays cannot. None of these platforms were able to diagnose tuberculosis disease, whereas T-SPOT TB can exclude this disease among middle-aged and older patients (Chang and Leung, 2010). These discrepant results raise questions regarding currently used parameters for the review and meta-analysis of diagnostic assays, and question the true efficacy of the assays in the field.

Detection of Pathogen Markers for the Diagnosis of TB

Antigen biomarker-based assays

Infection with *M. tuberculosis*, or any pathogen for that matter, results in the secretion of specific biomarkers and virulence factors. Some of the molecules, termed pathogen-associated molecular patterns (PAMPs), induce host immune response (Akira and Takeda, 2004), and are responsible for bacterial virulence, evasion of phagocytosis and propagation. Detection of these biomarkers, pathogen signatures, in the host allows for the specific, sensitive and selective detection of active disease. Bekmurzayeva *et al*. (2013) have tried to identify a panel of immunodominant tuberculosis-specific antigens, namely early secretory antigenic target 6 (ESAT6), antigen 85 complex (Ag85), culture filtrate protein 10 (CFP10) and MPT64 and collectively examine their value in diagnosing active TB. Each of these antigens has been previously explored for the diagnosis of TB. Kashyap *et al*. (2005) have developed indirect ELISA assays for the detection of Ag85 and demonstrated its value in the diagnosis of tuberculous meningitis by applying this ELISA on cerebrospinal fluid. More recently, the same group used this assay to measure Ag85 in sera from patients with pulmonary TB (Kashyap *et al*., 2007). In this study, they found that the Ag85 ELISA demonstrates 82% sensitivity (95% CI, 67–93%) and 86% specificity (95% CI, 57–98%). The assay indicated 96% positivity in clinically confirmed patients and demonstrated potential application to the diagnosis of active TB.

Although several mycobacterial biomarkers have been studied for their use in diagnostic tests, the test most researched and used is, arguably, LAM (lipoarabinomannan; Chatterjee and Khoo, 1998; Means *et al*., 1999; Pathak *et al*., 2005; Lawn, 2012) (Fig. 4.1). This is a 17–19 kDa heat stable lipoglycan that forms an integral component of the mycobacterial cell wall, accounting for 15–18% of total bacterial weight (Chatterjee and Khoo, 1998). LAM is an immunogenic virulence factor that is secreted by the metabolically active bacterium during infection and results in the activation

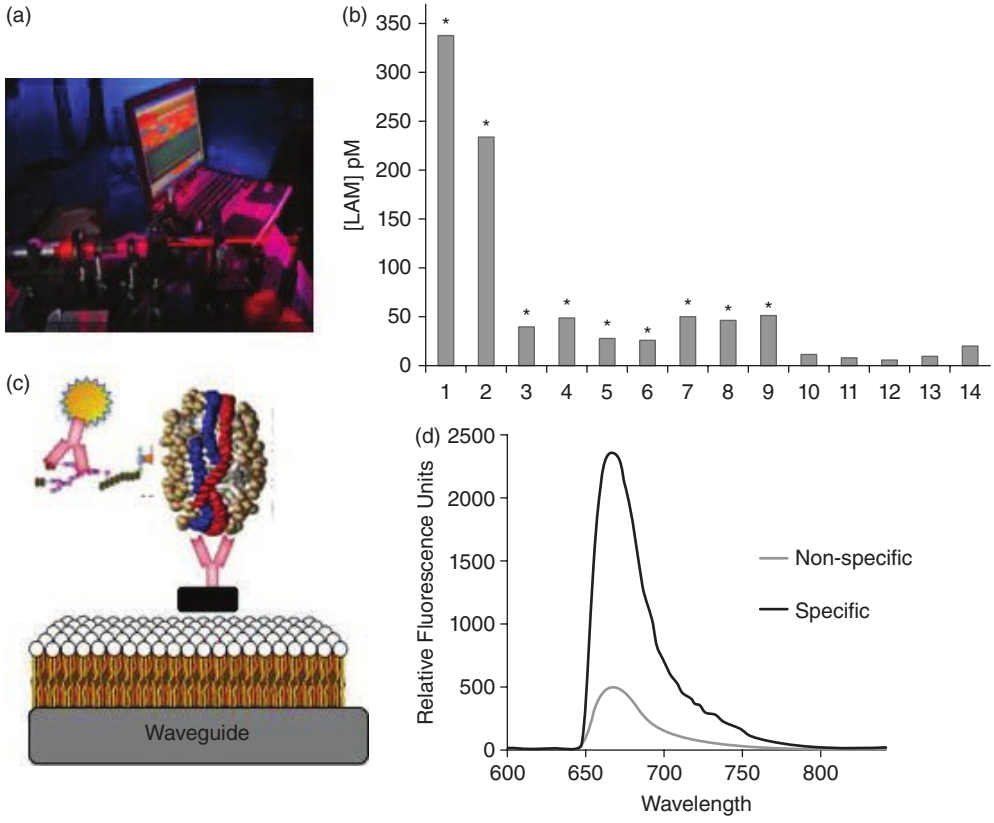

Fig. 4.1. Detection of lipoarabinomannan (LAM). (a) Using a waveguide-based optical biosensor platform developed at the Los Alamos National Laboratory (LANL). (b) Demonstrates the detection of lipoarabinomannan in blinded urine samples from South Korea using a sandwich immunoassay approach on the waveguide-based biosensor. *indicates confirmatory positive detection on diseased individuals, and the rest are controls. (c) Indicates the format for a lipoprotein capture method developed at LANL for the detection of the biomarker in host blood. Herein, the amphiphilic biomarker is captured on the waveguide surface by virtue of its association with host HDL, and a fluorescently labelled antibody targeting the biomarker is used as the reporter. (d) Represents the detection in a patient sample using this approach. This also represents the standard readout from the spectrometer interface associated with the waveguide instrument. y-axis is Relative Fluorescence Units from the instrument plotted as a function of wavelength (x-axis).

of the host innate immune response (toll-like receptor agonist) (Means *et al*., 1999).

Many investigators have shown that LAM is secreted in urine during active infection, forming the basis for many rapid diagnostic assays for this biomarker. Hamasur *et al*. (2001) were the first to demonstrate the use of mycobacterial lipoarabinomannan in urine as a diagnostic for TB. They showed two methods, a catch-up ELISA and a Dipstick test for the detection of the biomarker, and demonstrated excellent specificity in a preliminary evaluation. This was followed by a study in 200 TB patients and 800 non-TB patients in an Ethiopian centre (Tessema *et al*., 2001). This study demonstrated sensitivity and specificity of the LAM ELISA for diagnosing TB in patients versus controls as 74% and 86.9%, respectively, indicating significant promise for this method as a diagnostic test for active TB. These results were all the more significant because a urinary LAM-based TB diagnostic would be simple, devoid of major biosafety concerns (it can be performed without a BSL2/3

laboratory), extremely rapid (especially when compared to skin test and culture), non-invasive and specific to active disease, thereby revolutionizing the field. These possibilities resulted in the development of several commercial rapid detection assays for TB based on lipoarabinomannan detection. A prototype urinary LAM test kit was developed by Chemogen Inc. (Portland, ME, USA) and a commercial version is now marketed as the ClearView® TB ELISA (Alere Inc., Ballybrit, County Galway, Ireland; formerly, Inverness Medical Innovations, Waltham, MA, USA) (Inverness Medical Innovations Clearview TB ELISA).

However, recent studies have failed to demonstrate adequate sensitivity for TB diagnosis under routine conditions in unselected patients. Indeed, a recent systematic evaluation of urinary LAM-based assays determined that whereas the assay has several characteristics that make it attractive for the diagnosis of TB, it demonstrates sub-optimal sensitivity for clinical use in its present form. However, the authors did not exclude the usefulness of the diagnostic method and its practical advantages, especially in smear-negative patients (HIV-positive, paediatric populations and others). Indeed, the ClearView® TB ELISA is currently licensed for the evaluation of HIV-positive individuals because several studies have clearly shown a better predicative value of LAM for the diagnosis of active infection in this population (Shah *et al*., 2009; Dheda *et al*., 2010; Peter *et al*., 2010). Several theories have been proposed to explain this high predicative value of LAM measurement in HIV-positive individuals. Some investigators (Boehme *et al*., 2005; Shah *et al*., 2010) have suggested that the immune-compromised state of HIV-positive patients results in a greater multiplication rate of *M. tuberculosis*, thereby increasing bacillary burden. Thus, measurement of urinary LAM may be an indicator of bacillary burden at least in HIV-positive patients. They also suggest that the lack of cavity formation in this population could also account for greater shedding of LAM in blood and, hence, presence in urine. Chan *et al*. (2000), however, suggest that the larger degree of antigen–antibody complex formation in TB patients without immune suppression interferes with the excretion of LAM in urine. The reduction of nephrin in human podocytes by HIV-1 Tat, and the associated enhanced glomerular permeability, has also been suggested as a reason for the enhanced urinary concentration of LAM in this population (Doublier *et al*., 2007). Whatever the reason, the use of LAM as a biomarker for active infection in this population, and in others with high smear false negative rates, is becoming increasingly apparent. A recent study by Wood *et al*. (2012) echoed this conclusion. The authors found that whereas the overall success of LAM ELISA in diagnosing active TB was 16.1%, the sensitivity of the assay dramatically increased in HIV-positive patients. Indeed, they showed that in patients with CD4 counts of ≥200, 100–199, 50–99 and <50 cells/μl, the sensitivity of the LAM ELISA assay was 15.2%, 32%, 42.9% and 69.2%, respectively. Further, this study demonstrated the value of LAM as a prognostic indicator, as urinary LAM was found to decrease steadily after the initial 2 weeks of TB treatment (Wood *et al*., 2012).

If some of the known disadvantages of the measurement are overcome, the assay has significant promise in replacing smear microscopy as a routine diagnostic in endemic, high-burden populations. These disadvantages include cross-reactivity of the LAM assay with common oral flora such as Actinobacteria and *Candida* species, which can lower the predictive value of positive LAM assays (Dheda *et al*., 2010). Studies from our laboratory have shown that common sample processing methods such as filtration and dialysis result in the adsorption of LAM to the membranes, and hence a significant reduction in concentration in the elutant (Mukundan *et al*., 2012a) (Fig. 4.1). Hence, development of standardized sample processing methods is critical to enhance the success rate of LAM measurements in urine. Our study also found that urinary concentrations of LAM in HIV-negative individuals from South Korea were in the picomolar range, which is much lower than the limit of detection of current ELISA and immunochromatographic methods (Mukundan *et al*., 2012b). It is therefore important to note that in the absence of concentration methods, the use of more sensitive

sensor technology may be required to improve the reliability of LAM assays in HIV-negative populations. However, the relative disadvantage of increased costs with such technology, and the limitations of its widespread application, should be considered carefully. The biochemical nature of LAM should be taken into account in the design and development of assays for its detection. LAM is a lipoglycan and the use of conventional sampling and detection technologies designed for proteins, for the detection of this biomarker, is sub-optimal at best. Detection of LAM in blood has also proved difficult, although some researchers have shown that the biomarker is secreted and present in the host cardiovascular system (Sada *et al*., 1992). Recent studies have shown that LAM remains elusive in blood because the amphiphilic lipoglycan associates with other carrier molecules, and is not present in free form in blood plasma, as is biochemically predictable. Indeed, association of LAM with high-density lipoprotein (HDL) may play a significant role in its transport, recognition and clearance from blood and, as preliminary studies show, detection in blood (Mukundan *et al*., 2012a; Sakamuri *et al*., 2013) (Fig. 4.1).

Thus, several biomarkers such as Ag85, LAM and others (including ESAT6 and CFP10) have been explored for the diagnosis of TB. While the use of LAM has shown some promise, the use of other biomarkers has been limited. It is possible that the combined measurement of multiple biomarkers may offer additional reliability to the use of these molecules in the diagnosis of active TB.

Nucleic acid-based tests

With the advent of multi- and extremely drug resistance (MDR/XDR) TB (Mesfin *et al*., 2014), especially in combination with HIV co-infection, the need for rapid, specific and sensitive diagnostics that can also include characterization of emerging drug-resistance has become all the more critical. Currently highly specific and sensitive nucleic acid-based tests (NATs) are available for detection of gene sequences of the *M. tuberculosis* complex from clinical specimens and cultures (Niemz and Boyle, 2012; Boehme *et al*., 2013). These tests have the potential to overcome the limitations of conventional diagnostic procedures, and to simultaneously provide critical information regarding drug resistance. In this section, we present a range of NATs for the characterization of single and multiple targets, using either partially or fully automated platforms, for diagnosis of TB.

Detection of single target by cycling amplification (i.e. polymerase chain reaction (PCR))

After the discovery of PCR technology in 1983 many nucleic acid amplification tests (NAAT) were developed which have shown significant potential in the diagnosis of many diseases over the past decade (Niemz *et al*., 2011). PCR involves *in vitro* amplification of small segments of the genome, resulting in the generation of multiple copies, using specific primers (Sambrook *et al*., 1989). The first report on the use of the PCR method for detection of mycobacteria was based on the genus specific amplification of a 383 bp DNA fragment from the 65 kDa heat shock protein of mycobacteria, and its hybridization to specific oligonucleotide probes (Hance *et al*., 1989). This assay was then extended to detect mycobacteria in clinical specimens and 15 of 35 specimens studied were found to be positive (Brisson-Noel *et al*., 1989). This initial feasibility demonstration resulted in the development of several assays, some of which were based on the conserved and variable region of 16S rRNA gene, which is used for phylogenetic analysis at species level (Brisson-Noel *et al*., 1989). Later, insertion sequence elements *IS6110* and *IS986*; repetitive elements chromosomal direct repeats, TRC4, PGRS (polymorphic guanine–cytosine repetitive sequence); and a wide range of gene targets such as *dnaJ* (*Rv0352*), *devR* (*Rv3133c*), MPT-64 (*Rv1980c*), 38 kDa protein gene (*Rv0934*), *hupB* (*Rv2986c*), MTP-40 (*Rv2351c*), Antigen 85B (*Rv1886c*), *pncA* (*Rv2043c*) and *PPE8* (*Rv0355*) were used for PCR assays (Kolk *et al*., 1992; Soini *et al*., 1992; Groenen *et al*., 1993; Pierre *et al*., 1993; Verma *et al*., 1994). Although the specificity of these assays were high, the sensitivities were low, so in some assays a

second PCR or re-amplification of the first PCR product (nested PCR) was performed with another set of inner primers. This was shown to be effective for some of the gene targets such as 38 kDa protein gene (Sjobring *et al*., 1990; Miyazaki *et al*., 1993). A number of other PCR-based techniques such as PCR single-strand confirmation polymorphism, dideoxy fingerprinting, hemi-nested PCR, PCR heteroduplex analysis and PCR combined with restriction enzyme assay have been applied for the detection of *M. tuberculosis* using different gene targets (Rodrigo *et al*., 1992; Whelen *et al*., 1995; Kiepiela *et al*., 1998; Liu *et al*., 1998; Kim *et al*., 2001), but these technologies were only used in research laboratory settings.

The ligase chain reaction (LCR) (LCx™ *M. tuberculosis* assay (LCxMtb, Abbott Laboratories, Abbott Park, IL, USA) utilizes two pairs of primers hybridized to the complimentary DNA; the ligase enzyme anneals the two fragments only if they are exact match to the template DNA; the ligated pairs then serve as the template for new primers; and the reaction is continued as a regular PCR. The gene target for this assay is protein antigen B, which is involved in phosphate metabolism of *M. tuberculosis* and is specific to the *M. tuberculosis* complex (Tortoli *et al*., 1997). In a study from several centres in Australia, the overall sensitivity and specificity of the assay was found to be 69.7% and 99%, respectively, offering initial promise (Gilpin *et al*., 2007). However, sequence deletion in the probe binding sites of the *M. tuberculosis* protein antigen B was observed in some clinical isolates, resulting in false negative results (Gilpin *et al*., 2002).

The use of real-time PCR method in diagnosis is now rapidly expanding, since it combines the *two steps* of PCR amplification and detection into *one step*, and hence is more rapid (it also reduces hands-on time post-amplification) and sensitive. Real-time PCR uses fluorescein-labelled probes with two flanking primers in the reaction. The amplified product is detected by two methods either using the fluorescent dye (SYBR Green® (Life Technologies, Grand Island, NY, USA)) or the other which relies on the fluorescence energy resonance transfer (FRET) such as TaqMan® (Life Technologies, Grand Island, NY, USA) probes, molecular beacons, FRET hybridization probes or Scorpions™ (Sigma Aldrich, St Louis, MO, USA). All the above probes were used for the detection of mycobacteria using different gene targets such as 16S rRNA gene DNA, internal transcribed spacer (ITS) region, insertion element *IS6110*, senX3-regX3 intergenic region, MPT-64 and 65 kDa heat shock protein (Parashar *et al*., 2006; Sanjuan-Jimenez *et al*., 2013). The COBAS® (Roche Diagnostics, Laval, Quebec, Canada) TaqMan® Mtb (CTM-Mtb; Roche Diagnostics, Basel, Switzerland) uses real-time technology for the detection of the 16s rRNA gene DNA sequence (Kim *et al*., 2011; Yang *et al*., 2011). Recently, the WHO approved the fully automated Xpert® Mtb/RIF assay (Cepheid, Sunnyvale, California) for the diagnosis of TB in sputum samples (World Health Organization, 2011a). Xpert® Mtb/RIF assay is a hemi-nested PCR assay that utilizes five molecular beacons to detect 81 bp regions within the rifampicin resistance core of *M. tuberculosis* RNA polymerase B *(rpoB)*, of which one of the molecular beacons detects an *M. tuberculosis* complex-specific DNA sequence. This assay is discussed in detail in the later sections of this chapter. Truenat Mtb™ (MolBio Diagnostics Private Ltd, Goa, India) and Genedrive® Mtb iD® (Epistem, Manchester, UK) are two of several semi-automated platforms in development and undergoing field evaluation based on real-time PCR (Niemz and Boyle, 2012).

Detection by isothermal and other amplification methodologies

Isothermal nucleic acid amplification methods use a single reaction temperature, whereas PCR requires different temperatures for DNA denaturation, primer annealing and extension and hence, a thermocycler. In many cases, isothermal amplification can be performed in a simple inexpensive water bath, and therefore, is more suitable for low-resource settings (Niemz *et al*., 2011). Several isothermal amplification methods have been reported, and some of the methods used for TB diagnosis follow.

Strand displacement amplification achieves isothermal amplification of DNA in a two-step process: (i) the target sequence with restriction site is generated; and (ii) the actual target

sequence is amplified by repeated nicking strand displacement, and priming of the displaced strands (Walker *et al*., 1992). The amplified target is detected by hybridization with an oligonucleotide probe. The BD ProbeTec™ Mtb test developed by Becton-Dickinson (Sparks, Maryland, USA) utilizes this strand displacement amplification coupled with real-time detection. Fluorescent signal is generated as the target is amplified. The BD ProbeTec™ Mtb test is based on the *M. tuberculosis* complex-specific insertion sequence element IS*6110* as the gene target for TB diagnosis. In Taiwan the overall sensitivity and specificity of the BD ProbeTec™ Mtb test with 1066 clinical respiratory samples was found to be 63.2% and 98.4%, respectively (Mazzarelli *et al*., 2003).

Loop mediated amplification (LAMP) is another isothermal amplification method developed by Notomi *et al*. (2000) at Eiken Chemical Company (Tokyo, Japan). This method requires four to six primers that recognize the six distinct regions in the target sequence to form stem loop structure. A strand displacing DNA polymerase enzyme initiates the synthesis and two of the primers form the loop structure for further amplification process. LAMP reactions are performed at 60–64°C using simple incubator and associated reagents are very stable for 1 month at room temperature. Also, the amplification reaction results in the production of magnesium pyrophosphate; this makes the buffer turbid, and can be visually observed (Mori *et al*., 2013). Together, these factors make LAMP well suited for applications in the field in resource-poor regions of the world (Mori and Notomi, 2009). Loopamp® TB detection assay (Eiken Chemical Co. Ltd, Taito-ku, Tokyo, Japan) for *M. tuberculosis* detection was developed based on insertion sequence IS*6110* as the gene target (Boehme *et al*., 2007). This simple TB LAMP assay (Loopamp® TB detection assay) was evaluated in several countries and was found to have a higher sensitivity (81.4–92.7%) compared to microscopy with slightly less specificity (80.4–98.3%), and the feedback from these evaluations was found to be favourable (Neonakis *et al*., 2011; Boehme *et al*., 2013; Yuan *et al*., 2014). A WHO expert panel recently reviewed this assay, but did not recommend it due to insufficient data. If future studies are just as promising, this assay may soon be released for the diagnosis of TB worldwide (World Health Organization, 2013b). Later several other TB LAMP assays were developed with other gene targets, including 16S*rDNA*, *gyrB* and rimM (Neonakis *et al*., 2011). As with other technologies, LAMP TB detection assay is also less sensitive in smear-negative samples (48–56%) (Boehme *et al*., 2007; Boehme *et al*., 2013).

Helicase-dependent amplification and cross-priming amplification are two other isothermal amplification methods used for TB diagnosis. The *M. tuberculosis* helicase-dependent amplification-based assays were developed based on IS*6110* and *rpoB* gene targets and utilized the helicase enzyme for unwinding associated double-stranded DNA (Motre *et al*., 2011; Torres-Chavolla and Alocilja, 2011; Ao *et al*., 2012). Cross-priming amplification methods (NATeasy™ TB) were developed by Ustar Biotechnologies, Hangzhou, China (Fang *et al*., 2009). These isothermal amplification assays are dependent on DNA polymerases, and utilize multiple cross-linked primers for strand displacement. The DNA target is amplified under constant temperature, and the amplified products are detected in an inbuilt lateral-flow assay (Xu *et al*., 2012). NATeasy TB assay is currently in development, and aims to integrate the DNA extraction with nucleic acid detection. This will overcome the cumbersome nucleic acid extraction process, which is not performed manually for many technologies. Initial evaluation indicates that sensitivity for the smear-positive/culture positive and smear-negative/culture positive sputum samples was found to be 96.9% and 87.5%, respectively (Fang *et al*., 2009), which is promising.

Another approach utilized recently by researchers is the detection of pathogen-specific RNA, instead of DNA, allowing not only for effective diagnosis but also the discrimination of viable- from non-viable bacteria. This can, if successful, afford the specific diagnosis of active TB infection. The assay, known as *M. tuberculosis* detection test (MTD test), was developed based on transcription-mediated amplification (Abe *et al*., 1993). Herein, pathogen RNA is released from the cells by sonication, 16S rRNA is transcribed using reverse

transcriptase and amplified with RNA polymerase in an isothermal reaction. This assay has been commercialized by Gen-Probe Inc., San Diego, CA, USA, as Gen-Probe Amplified *M. tuberculosis* (MTD) test™. Guerra *et al*. (2007) reported that the Gen-Probe Amplified *M. tuberculosis* (MTD) test has 93.1% and 93.5% overall sensitivity and specificity, respectively. Gene-Trak Company designed another assay with RNA as a template, which is based on signal amplification method and is commonly known as Qβ assay (Gene-Trak Company, Framingham, MA, USA; Shah *et al*., 1995). In this format, two probes (one capture containing a tag and one detector containing the Qβ replicon) are used, which bind the target 23S rRNA. The target is immobilized on a solid support using the capture probe and the Qβ detector probe, and is recovered and further amplified isothermally (An *et al*., 1995). The advantage of using RNA as a target for diagnostics is that it is more abundant in the cell compared to DNA. The short lifespan, the abundance of RNAases and contamination with DNA all make the specific interrogation of RNA difficult.

Simultaneous detection of multiple targets by Multiplex PCR, microarray and other technologies

Multiplex PCR is used to amplify and detect two or more gene targets using more than one set of primers. This not only allows increased sensitivity, but also the detection of multiple bacterial pathogens or drug-resistance mutations in a single assay. Some of the gene targets used are more general, such as the 65 kDa heat shock protein which is present in most mycobacteria, whereas others are more specific and provide species differentiation, such as IS*6110* for *M. tuberculosis* complex and 33/34 spacer region for *M. bovis* (Yeboah-Manu *et al*., 2001). Multiplex PCR has been applied for the diagnosis and characterization of known drug-resistance phenotypes in both pulmonary and extra-pulmonary TB patients. Some gene targets, such as IS*6110*, are used for diagnosis; the others, containing mutations associated with resistance for rifampicin (*rpoB*), isoniazid (*inhA* and *katG*) and streptomycin (*strA* and *rrs*), are used for evaluating for drug susceptibility (Bifani *et al*., 1996). The amplified products are sequenced or hybridized to DNA strips for identifying the mutations in the gene targets associated with drug resistance. Multiplex PCR is also used for molecular typing methods such as spoligotyping (chromosomal direct repeats locus) and tandem-repeat typing of *M. tuberculosis* isolates (Sun *et al*., 2009; de Beer *et al*., 2014).

DNA microarrays are used to identify or quantify multiple targets in a single assay. Microarrays are currently not employed for routine use in clinical laboratories. However, their simplicity and high throughput outcome make them likely candidates for future use in such a setting. Affymetrix Inc. (Santa Clara, CA, USA), for instance, developed a mycobacteria probe array based on the sequence regions of 16s rDNA and rpoB loci of the pathogen, allowing for both detection and species identification in a microarray format (Troesch *et al*., 1999). Low-cost DNA microarrays for detection and evaluation of *M. tuberculosis* drug-resistance gene markers have been developed (Aragon *et al*., 2006; Kim *et al*., 2006; Park *et al*., 2006). These low-cost DNA microarrays were tested using drug resistance and susceptible *M. tuberculosis* isolates and the results were found to be comparable to DNA sequencing results (Kim *et al*., 2006).

Detection of TB by hybridization (FISH, RFLP, Line Probe)-based assays

Hybridization assays such as *in situ* hybridization and restriction fragment length polymorphism (RFLP) were commonly used for *M. tuberculosis* identification before the PCR assays were introduced for diagnosis (Cohn and O'Brien, 1998). Fluorescence *in situ* hybridization (FISH) assays employ fluorescently labelled probes specific to the gene target, and the binding is directly visualized in the clinical specimen using a fluorescence microscope. For differentiating *M. tuberculosis* complex from other NTM, peptide nucleic acid (PNA) probes for 16S rRNA were used in TB PNA FISH assay (Stender *et al*., 1999), which was tested directly in patient sputum, as well as in liquid and solid cultures from sputum, and sensitivity of the Mtb probe was found to

be 87%, 98% and 99% in direct sputum, solid and liquid cultures (Stender *et al*., 1999; Hongmanee *et al*., 2001), respectively.

Line probe assays (LPA) involve PCR amplification of target DNA from the clinical specimen using primers labelled with biotin or digoxigenin (Kolk *et al*., 1992). The PCR products are subsequently hybridized to the oligonucleotide probes, which are immobilized on a strip. LPA were endorsed by WHO in 2008 (World Health Organization, 2008). One format of LPA used for the diagnosis of TB, the Amplicor™ *M. tuberculosis* test (Roche Molecular Systems, Branchburg, New Jersey, USA), uses *M. tuberculosis* 16s rDNA as the gene target (D'Amato *et al*., 1995). This assay was evaluated in Kenya using the sputum samples from 1396 TB suspects (Kivihya-Ndugga *et al*., 2004). The sensitivity and specificity of the Amplicor assay is found to be 93% and 84%, respectively, and also the sensitivity is not affected by HIV status. However, this Amplicor assay was modified by the introduction of real-time PCR technology in the LightCycler® *Mycobacterium* Detection system (Roche Diagnostics GmbH, Mannheim, Germany). Later, Innogenetics (Zwijndrecht, Belgium) developed INNO-LiPA Rif TB® assay which is based on the rifampicin resistance-determining region of the *M. tuberculosis* RNA polymerase B (*rpoB*). This assay is not only capable of diagnosing *M. tuberculosis* but also determining the rifampicin drug resistance. In the INNO-LiPA Rif TB® assay the resistance determining region of the *M. tuberculosis rpoB* is initially PCR amplified, and then reverse hybridized to nine probes designed to detect the nucleotide changes in the *rpoB* sequence which confers the resistance (Rossau *et al*., 1997). However, the INNO-LiPA Rif TB assay is no longer commercially available since the other LPA product, which is commercially available, has the ability to detect gene mutation in not only rifampicin but also isoniazid. Currently GenoType® MTBDRPlus (Hain Lifescience, Nehren, Germany) is the only LPA used in diagnosing drug-resistant TB globally (www.hain-lifescience.de/en/products/microbiology/mycobacteria/genotype-mtbdrplus.html). The MTBDRPlus contains wild type and mutation probes for the rifampicin and isoniazid resistance determining regions of the *M. tuberculosis rpoB*, *katG* and *inhA* on the immobilized strip. The PCR amplified product from the DNA of the clinical specimen is hybridized to the probes on the immobilized strip, and the detection is colorimetric reaction on the strips. The sensitivity and specificities of this assay are discussed in detail later in this chapter.

Sample processing and handling for NAT-based detection

The results of NAT are greatly influenced by the purity of the nucleic acids and the extraction methodology, sample storage and other parameters. Sample collection, type of specimen used, nucleic acid extraction methods and inhibitors present after nucleic acid extraction can all influence the results of a NAT assay, making it technically difficult and laboratory intensive (Dineva *et al*., 2007).

The clinical samples from which the nucleic acids are extracted for TB diagnosis include sputum, cerebrospinal fluid, bronchiolar alveolar lavage, pleural fluid, abscess, lymph, biopsy, fine-needle aspiration and other body fluids (Mehta *et al*., 2012). To date, there is no single standardized protocol for the collection of, and the extraction of nucleic acids from, all categories of clinical samples of significance to TB diagnosis. This lack of standardization can have serious ramifications on the outcome of the several NAT assays currently being used or evaluated. For instance, early methods of nucleic acid extraction such as the phenol/chloroform method are no longer recommended due to the corrosive nature of the phenol, affecting assay reproducibility (Honore-Bouakline *et al*., 2003; Aldous *et al*., 2005; Santos *et al*., 2010). More recently column purification methods wherein the nucleic acids are bound to silica matrix membrane after the bacterial lyses, then washed extensively to remove macromolecules, inhibitors and other contaminants and then finally eluted, are being used. Comparative studies on nucleic acid extraction tests indicate that the commercially available kits based on column purification are better compared to manual, since they are rapid, eliminate inhibitors, reduce contamination and also provide a higher yield of nucleic acids (Santos *et al*., 2010). Several semi- and

fully automated NAT platforms have been designed for nucleic acid extraction from the clinical specimens, which allows for standardization of extraction. The GeneXpert Mtb/RIF assay, for instance, is a completely closed and fully automated system (cartridge-based) with a nucleic acid extraction, amplification and detection process, hence reduces hands-on time and detection of undesirable contaminants. This fully automated approach is the first of its kind in TB diagnosis (Lawn *et al*., 2013).

Another issue with diagnostics is the acquisition of an adequate volume of clinical specimen of suitable quality. Most molecular assays are based on detection in sputum, which is difficult to get from children, HIV infected and EPTB patients (Gilpin *et al*., 2007; Gonzalez-Angulo *et al*., 2012). Recent studies have therefore focused on the development of induction and concentration methods to extract this specimen from all categories of patients (Gonzalez-Angulo *et al*., 2012). Even after collection, digestion and decontamination of the sputum specimen becomes another critical component, and it is essential to ensure removal of all contaminants in the sample (Steingart *et al*., 2006b; Chatterjee *et al*., 2013). Acids, bases and detergents are used for this purpose, given mycobacteria are resistant to these agents, but most other pathogens are not. Still, some of the chemicals are too weak to completely remove all contaminants in the sputum, while others are too strong and destroy significant number of mycobacteria and relevant biomarkers. An ideal decontamination method for sputum is still an elusive target.

Commercially available NAT technology endorsed by WHO- MTBDR plus and GeneXpert

Although several TB NATs have been developed and evaluated, only two of them are currently endorsed by WHO as tests for TB diagnosis, and these are LPA and GeneXpert Mtb/RIF assays (World Health Organization, 2008, 2011a). The first NAT and only LPA assay to be endorsed is the Genotype MTBDR plus assay (World Health Organization, 2008). The pooled sensitivity and specificity on cultured bacteria from meta-analysis data for rifampicin was found to be 98% and 99%, respectively, and for isoniazid 84% and 99%, respectively (Ling *et al*., 2008; Bwanga *et al*., 2009). The cost of the assay, when performed directly from the sputum, was found be less compared to the TB drug-susceptibility testing (DST) culturing. Scott *et al*. (2011) performed a systematic analysis of 311 participants in South Africa (HIV prevalence, 70%, n = 215) and found that the sensitivity of the Xpert system was 6% greater than conventional assays and other molecular tests (84%). Most significantly, TB detection among smear-negative, culture positive patients was 61% (11/18) for this technology, compared to 28% (5/18) for GenoType MTBDR*plus*® (Hain Lifescience GmbH, Nehren, Germany) and 22% for the LightCycler® *Mycobacterium* Detection system (Roche Diagnostics GmbH, Mannheim, Germany) . However, laboratory infrastructure and well-trained personnel are required to conduct the experiments. Moreover, the sensitivity of this assay was found to be lower in smear-negative and HIV-positive individuals, and was not approved for use in specimens from these patients (Boehme *et al*., 2013).

GeneXpert Mtb/RIF assay is a fully integrated, automated, specimen processing to result platform (World Health Organization, 2011a) (Box 4.1). When *M. tuberculosis* was spiked into healthy clinical sputum samples in the preclinical assessment of the assay, the limit of detection was found to 131 CFU/ml of sputum (Helb *et al*., 2010), which is lower than smear microscopy (5000–10,000 CFU/ml), but higher than liquid culture (10–50 CFU/ml) (American Thoracic Society and Centers for Disease Control, 2000). The automatic specimen processing significantly reduces the biohazard associated with handling sputum. Several studies have explored the diagnostic accuracy of GeneXpert Mtb/RIF assay in both high-income and resource-limited settings (Lawn *et al*., 2013). A recent systematic review of 27 studies published in February 2014, containing 9557 pulmonary TB patients using the GeneXpert Mtb/RIF assay, showed that the pooled sensitivity and specificity for TB diagnosis was 89% and 99%, respectively,

Box 4.1. South Africa: A Case Study.

In South Africa, these NAAT tests did not feature in the diagnostic algorithm until drug-resistant TB became a public health concern. Currently, two commercially available tests, the Xpert® Mtb/RIF (Cepheid, Sunnyvale, CA, USA) and the lineprobe assay (GenoType MTBDR*plus*®) are used. PCR-based tests have two problems. One is contamination of negative specimens with microbes, unamplified nucleic acid or the amplification products from other specimens. The most significant issue is amplicon contamination, since these can contaminate clothing of laboratory workers, equipment, etc. GeneXpert® minimizes the contamination problem. The line probe assay, however, is contamination prone.

A concern with the GeneXpert® system is the occurrence of false positives in the detection of rifampicin resistance. The premise of the test is that a single mutation in the *rpo*B gene is rare, and therefore its occurrence is likely to be an indicator of multiple drug resistant phenotypes being expressed. The line probe assay uses a nitro-cellulose strip that contains a number of wild type and resistance-conferring mutation probes which bind short DNA sequences obtained by multiplex PCR from regions in the *rpo*B gene of the clinical isolate. It differs from GeneXpert® in that the line probe assay detects specific mutations. Mutations for which there is no complimentary probe included in the strip will be missed.

The issue of over-diagnosis and, hence, over-medication is a critical one to populations such as South Africa, especially considering the low number of novel therapeutic options in the pipeline for the treatment of drug-resistant TB.

and for rifampicin resistance, 95% and 98%, respectively (Steingart *et al*., 2014). However the sensitivity was found to be lower in smear-negative (67%), EPTB (80%), children (65.1–75.9%) and HIV-positive individuals (79%), possibly for the same reasons that sputum smear microscopy has lower success rate in these populations (Steingart *et al*., 2014). Despite these promising results, GeneXpert Mtb/RIF assay has some limitations, such as its inability to differentiate live from dead organisms (and hence, exposure from infection) and mixed infections, and as mentioned above, poor performance with many subpopulations of TB patients (Box 4.1). Further, the assay has some operational limitations, especially in resource-poor countries, such as high cost, need for trained operators, unlimited power supply and annual calibration requirements.

Other Diagnostics Paradigms for Tuberculosis

Chest X-rays are routinely used for screening and diagnosis of active TB, and to detect lung lesions in high-burden populations (Jeong and Lee, 2008). Sometimes chest X-rays are used as a confirmatory diagnostic, to discriminate between exposure (latency) and actual infection. However, chest X-rays sometimes may present as normal, even with active disease, and further, the images cannot reliably differentiate TB from pneumonia and other lung infections (Woodring *et al*., 1986). Computed (Axial) Tomography (CT-scan) is more sensitive than chest X-rays, and has also been evaluated for screening and diagnosis of smear-negative patients in developed countries, where the resource is readily available. CT-scan uses X-rays for imaging, but the image can be viewed in multiple planes, increasing the diagnostic reliability. The specificity of diagnosis using chest X-rays of primary TB (34%), reactivation (59%) (Woodring *et al*., 1986) is poor when compared to CT-scan (91%) (Lee *et al*., 1996). More recently PET (positron emission tomography)-scan technology has been evaluated for TB diagnosis. PET imaging is very sensitive, providing granularity to the cellular level, and can assess the disease in real time. These factors make the technology more sensitive and capable of early diagnosis of active TB infection. PET imaging uses radioactive tracers that emit positrons for three-dimensional imaging. The commonly used radioactive tracer in oncology 18-Flurodeoxyglucose (^{18}F-FDG) was also used for TB diagnosis (Kim *et al*., 2008; Treglia *et al*., 2011). However, it was later observed that the high uptake of ^{18}F-FDG and high retention rate of the inflammatory cells causes a significant spike in false positives in the diagnosis of

active TB (Treglia *et al*., 2011). To overcome this issue, Kim *et al*. (2008) used a dual phase FDG-PET method, where two PET-scan images, instead of one, were performed at 60 and 120 min after ^{18}F-FDG administrations, to identify and distinguish active and inactive lesions. The sensitivity and specificity of the method was found to be 71% and 100%, respectively. Yet another alternative tracer, ^{11}C-choline to ^{18}F-FDG, was found to differentiate TB lesions from lung cancer (Hara *et al*., 2003), adding specificity to the diagnosis. However the costs of CT-scan (US$1500–3000) and PET-scan (US$3000–6000) are very high compared to chest X-rays, making them prohibitive in developing countries. Also, more advanced and well-managed laboratory resources are required for their effective implementation.

Global Biosurveillance of Tuberculosis and Need for Effective Diagnostics

The WHO has been publishing global reports on the surveillance of TB worldwide since 1997, with the goal of providing a comprehensive and up-to-date assessment of the TB epidemic, and deciding on financing requirements of intervention and countermeasure strategy development to fight the disease. The report collates data from 197 countries across the world, representing 99% of the world's TB cases. The 2013 report (World Health Organization, 2013b) estimates that 8.6 million people developed TB in 2012, of which 1.1 million (13%) were estimated to be HIV-positive; 450,000 people worldwide were diagnosed with MDR TB, of whom 170,000 died of the disease. Twenty-nine per cent of all TB cases were reported from Southeast Asia and 27% from Africa. The Western Pacific accounted for 19% of the cases. India and China alone account for 26% and 12% of total cases, respectively.

Despite these alarming numbers, the WHO reports a positive trend in TB control globally. Nearly 20 years after the WHO declaration of TB as a global health emergency, the organization declared in its 2013 surveillance report that major progress has been made towards 2015 global health targets within the context of the Millennium Development Goals (MDGs) (World Health Organization, 2013a). Specifically, incidence of TB is reported to have been falling worldwide for about a decade, achieving the MDG target at the rate of 2% per year. Globally, TB mortality is reported to have reduced by 45% since 1990; and of the 22 high-burden countries accounting for 80% of the world's active TB cases, seven have already met their target for reduction in TB incidence, prevalence and mortality. Yet, several countries globally (especially 11 of the 22 high-burden countries) are not on target to achieve the expected reduction in TB associated mortality and incidence by 2015. Also, global progress towards targets for diagnosis and treatment of MDR/XDR TB is reported to be far off track, and less than 25% of individuals with MDR TB were detected in 2012.

Even though notifications of TB cases appear to have stabilized globally, 67% of the 8.6 million cases reported in 2012 were new infections, of which 3.6% were MDR cases. Globally, 20% of previously treated TB patients with relapse have drug-resistant forms.

Biosurveillance of TB relies on accurate diagnosis of the disease, and containing the growing MDR/XDR TB problem will depend on accurate rapid diagnosis of drug resistance to enable appropriate treatment. As outlined, the current state of TB diagnostics worldwide is far from ideal. Serological assays are still routinely used in high-burden countries such as India, resulting in significant skewing of reported data. East Africa suffers from a high burden of HIV infection, as do certain provinces in China. The significant failure of sputum microscopy in these populations compounds the surveillance efforts for the effective control of the disease, and biases the data towards lower numbers.

India, for instance, has more new TB cases annually than any other country worldwide, contributing to one-fifth of the global burden of TB in 2009. India uses smear microscopy as the primary diagnostic paradigm for biosurveillance of disease prevalence, and a study of the success of this technology with other determinants of TB epidemiology in India (e.g. HIV co-infection, diabetes) has not

been performed, although their relative risk for TB disease has been called out in a recent report from the Indian government (India Central TB Division, 2012).

TB surveillance in China is also dependent on a positive sputum smear, suggesting that individuals with extra-pulmonary TB, paediatric patients, HIV-positive patients and others may not be accurately represented in the case statistics from this region. TB is the leading cause of death from infectious diseases among adults in China. Some recent studies have attempted to tabulate the prevalence of drug-resistant TB in China. He *et al*. (2008) performed a systematic analysis of drug-resistant TB prevalence in ten Chinese provinces using smear microscopy for initial identification, followed by DST. They concluded that MDR TB levels varied significantly between provinces but were systematically higher than the global estimated average of 4.8%. Cheng *et al*. (2013) recently concluded that delayed diagnosis and treatment of tuberculosis is associated with a heightened transmission risk. To prevent further spread, these delays had to be significantly minimized, as can only be done by suitable and effective diagnostic paradigms.

Treatment of TB, required for effective control, requires an accurate understanding of the drug-resistant profile in the patient. Indeed, treating with combination chemotherapy without knowing the resistance profile of the individual is a critical issue both for the patient in question, and for the progression of the epidemic. Consequently, timely diagnosis of the resistance profile of the individual is an urgent need for the global control of drug-resistant TB. The recent understanding that drug-resistant strains of the pathogen can acquire compensatory mutations that allows them to survive, transmit and spread in a population is especially concerning, as it changes the requirements of an effective global TB surveillance architecture. This was recently demonstrated with respect to the role of Rif-resistance compensatory mutations in the spread of MDR/XDR TB. Rapid molecular diagnostics that provide an accurate assessment of drug resistance prior to treatment enable physicians to treat their patients with therapeutic drug combinations that are most likely to be effective (Box 4.2). As cost reduction, simplicity of operation and availability of such tests continue to improve, they also have the potential to help retard the spread of XDR/MDR TB at the population level. Combining what we learned from two prior TB studies provides a series of observations that highlights this second point (Ford *et al*., 2012; Song *et al*., 2014). Rif acts through binding to the RNA polymerase protein at the site of mRNA synthesis, and Rif-resistance mutations are focused in the section of the *rpoB* gene that encodes this region, the region targeted by the GeneXpert Mtb/RIF assay. Such mutations come with a fitness cost to the mycobacterium that carries them (Song *et al*., 2014); reductions in fitness may in turn reduce transmissibility. Compensatory mutations along the

Box 4.2. The Clinical Diagnostics Research Consortium (CDRC). Susan E. Dorman, MD; Professor, Johns Hopkins School of Medicine.

The Tuberculosis Clinical Diagnostics Research Consortium (TB-CDRC) is an interdisciplinary consortium of scientists, clinicians and support personnel whose mission is to provide data on the performance of investigational diagnostics and their potential impact on TB management algorithms in endemic countries. The TB-CDRC was established in 2009 through a contract from the National Institutes of Health/National Institute for Allergy and Infectious Diseases to Johns Hopkins University. The TB-CDRC conducts rigorously implemented, quality, monitored diagnostic accuracy studies according to Good Clinical Practice guidelines. These studies are conducted at one or more of six affiliated clinical study sites located in Uganda, South Africa, Brazil, South Korea, Kenya and China. In addition to conducting diagnostics accuracy studies, the TB-CDRC informs and advises scientists and manufacturers on further development and refinement of diagnostics that have promise to accelerate and/or improve the accuracy of TB diagnosis and the rapid detection of drug resistance. The TB-CDRC has also performed TB diagnostics-related economic analyses and mathematical modelling work with the aim of answering questions of relevance to policy makers.

RNA elongation tunnel, generally in the *rpoC* gene encoding a distinct subunit of the RNA polymerase, can restore function and are very commonly found in conjunction with the initial resistance mutations (Song *et al*., 2014). In a Korean population, Rif-resistant strains with compensatory mutations were observed to arise independently through convergent evolution, but also were identified in an extensive clonal transmission chain of linked cases (Song *et al*., 2014). Revisiting the genetic samples from the XDR outbreaks in South Africa (Ioerger *et al*., 2009), we found that a likely Rif-resistance compensatory mutation was present in the clonal XDR outbreak in KwaZulu-Natal (Song *et al*., 2014) (Plate 1). We also found evidence of recurrence of a transmitted Rif-resistance mutation coupled with a compensatory mutation in the non-clonal outbreak in the Western Cape (Ioerger *et al*., 2010). In the latter case, the accumulation of different patterns of resistance to other TB drugs was apparently emerging on the same Rif-resistant backbone. This scenario is consistent with inadequate combination therapy spurring increasing levels of drug resistance. These findings (Plate 2) suggest that our understanding of the fitness and genomics of drug-resistant TB is inadequate both with respect to the design of effective diagnostics, and consequently effective surveillance of the pathogen spread.

Summary and Future Directions

An ideal diagnostic for active TB in humans remains an elusive, but urgently needed target (Plate 1). The recent increase in multi- and extensively drug-resistant variants of *M. tuberculosis*, and the failure of conventional diagnostic paradigms in the context of co-infection with HIV, further add to the urgency. The failure to diagnose infected patients (false negatives), the mistaken diagnosis of uninfected individuals (false positives) and the cost and delayed diagnosis of drug-resistant variants (resulting in delayed treatment or in providing inappropriate drug combinations), all compound the problem of increasing TB incidence, and complicate implementation of surveillance and control programmes (Dorman, 2010) (Box 4.2). Although many new technologies are rapidly being developed, several of which show excellent promise, the race for the perfect diagnostic for this challenging disease is far from over. Indeed, the former director of the Wellcome Trust, Dr Mark Walport, has said that 'We are still using antiquated diagnostic and treatment regimes for control and management (of TB) and these are only effective in optimal conditions. With the emergence of TB strains resistant to all anti-TB drugs, we are greatly in need of a more effective tool-box' (Walport, 2009). There is thus a clear and imminent need for cost-effective, sensitive and specific diagnostic tools that can perform well, especially in the context of HIV co-infection, thereby facilitating a decrease in disease spread and associated mortality. This warrants an increase in funding for diagnostic research (US$41.9 million in 2007, compared to US$170 million for drugs). Better control of the disease and effective treatment can be achieved only with efficient diagnosis of active TB infection. Further, the success of any new tools in an endemic setting is predicated by the need for better understanding of host–pathogen biology, especially with multiple co-morbidities (HIV/AIDS, malnutrition, diabetes) and others. Understanding the systemic interaction of *M. tuberculosis* with the human host may pave the way to more successful technologies with greater translational value, as have the elucidation of the *M. tuberculosis* genome and the emergence of public–private global partnerships to effectively combat this challenge. TB is a chronic disease that has been ravaging humankind for centuries. A drastic, truly paradigm-shifting transformative change in our attitude towards the disease and its etiological agent may be required to resolve this complex problem permanently. To quote Dr Susan Dorman (Professor of Medicine, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, Maryland), 'What's new in TB diagnostics? A lot, but not enough'. An integration of commercial factors (cost effectiveness, laboratory requirements), valid development (translational science, sample preparation and characterization, reproducible results), new programmatic approaches and better algorithms, validation

studies in endemic populations with extensive co-morbidity issues and extensive basic research with systemic applications (beyond cell culture) is required to realize this dream. An integration of funding institutions (National Institutes of Health (NIH)), recommending bodies (e.g. WHO, Centers for Disease Control and Prevention, USA (CDC)), foundations (Gates Foundation, Foundation for Innovative New Diagnostics (FIND)), researchers (academics, national laboratories) and commercial entities will help in the execution of an effective path towards an ideal TB diagnostic.

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5 Development of Next-generation TB Vaccines: Comparative Approaches in Humans and Animals

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Although BCG vaccine remains useful for neonatal vaccination in human populations and for veterinary control of mycobacterial diseases in cattle and other animals, its inability to prevent global TB in adults (Kaufmann, 2006) has inspired a decades-long effort to discover new, more effective TB vaccines for human disease. There has been considerable progress in many aspects of TB vaccine development but many challenges remain. Mice, guinea pig and non-human primate TB challenge models are commonly used for testing TB vaccine candidates (McShane *et al*., 2012), but cattle and other animals have also been used to investigate the host immune responses and effectiveness of certain vaccine candidates (see Chapter 10, this volume; Waters *et al*., 2012; Buddle *et al*., 2013). The development and use of animal challenge models for TB is discussed elsewhere in this publication. In this chapter, we will summarize recent advances in TB vaccine development with an emphasis on what knowledge has been gained over the past decade and what key questions need to be addressed. Moreover, we will discuss how a collaborative approach among investigators developing vaccines for human and animal mycobacterial diseases has the potential to facilitate vaccine development.

New TB Vaccines in Human Studies

More than 16 candidate TB vaccines have entered human clinical trials over the past decade. A list of specific vaccine candidates in trials have been published in reviews (Hokey and Ginsberg, 2013; Velmurugran *et al*., 2013) and can be found in the 'TB Vaccine Pipeline document' (http://www.newtbvaccines.org/vaccine-candidates/) and on the Aeras (http://www.aeras.org/) and Tuberculosis Vaccine Initiative (www.tbvi.eu) websites. The investigational vaccines encompass a variety of approaches including viral-vectored vaccines, protein-adjuvanted subunit vaccines, live vaccines and whole cell extracts. In 2013, the results of a vaccinia-vectored vaccine expressing Mtb antigen 85A (McShane *et al*., 2004) were disappointing in that they demonstrated no efficacy in a very effectual trial setting (Tameris *et al*., 2013). This was the first clinical trial to evaluate the efficacy of a new, preventative TB vaccine candidate against clinical TB or *Mycobacterium tuberculosis* infection, and results were therefore of considerable interest to the vaccine research community. In this trial, conducted in South Africa from 2009 to 2012, the MVA85A vaccine was safe and well tolerated, confirming similar findings from previous Phase I and

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Phase IIa clinical trials using this vaccine. The vaccine was given months after all the infants had received BCG vaccine and it cannot be assumed that similar results would have been obtained in other populations. There is some evidence that it induces a stronger immune response in older age groups than in infants, therefore it is possible that adults, adolescents and older children could be better target populations for this vaccine. This study did demonstrate that a high-quality trial of a novel TB vaccine can be conducted and produce robust results in a high TB burden setting.

A few additional vaccines are in Phase II trials and outcomes are anticipated in the next few years. M72 +$AS01_E$ is a protein subunit vaccine containing a fusion protein of the *M. tuberculosis* antigens 32A and 39A in the adjuvant $AS01_E$ designed to prevent pulmonary TB (Leroux-Roels *et al.*, 2010). AERAS-402/Crucell Ad35 is an adenovirus-vectored vaccine candidate expressing three *M. tuberculosis* antigens: Ag85A, Ag85B and TB10.4, and is designed as a booster vaccine for infants, adolescents and adults (Abel *et al*., 2010). AERAS-402/Crucell Ad35 and MVA85A have also been combined to try to drive a balanced CD4+/CD8+ immune response. Three additional protein subunit adjuvanted vaccines, initially developed by the Statens Serum Institute in Copenhagen, Denmark: Hybrid 1 + IC31 containing Ag85B and ESAT-6 in an adjuvant (Christensen *et al*., 2010), IC31; Hybrid 56 + IC31 containing antigens 85B and ESAT6 as well as Ag Rv2660, which is expressed during latency (Lin *et al*., 2011); and Hybrid 4 + IC31, a fusion protein candidate that expresses Ag85B and TB10.4 now being developed with Sanofi Pasteur (Aagaard *et al*., 2009), have been in Phase IIa clinical trials in Africa.

Additional vaccines in early-phase clinical trials include ID93 + GLA-SE, a recombinant fusion protein formulated in the novel adjuvant, GLA-SE. It is being developed by the Infectious Disease Research Institute (IDRI) in collaboration with Aeras and expresses three *M. tuberculosis* virulence antigens (Rv2608, Rv3619 and Rv3620) and one *M. tuberculosis* latency antigen (Rv1813) (Bertholet *et al*., 2008). RUTI, a non-live vaccine based on fragmented *M. tuberculosis* bacteria, is being developed as an immunotherapeutic vaccine (Vilaplana *et al*., 2010). The Ad5 Ag85A vaccine is an adenovirus serotype 5 vector expressing Ag85A being developed by McMaster University with support from CanSino, a Chinese biotechnology company based in Tianjin (Xing *et al*., 2009). In addition, AnHui Longcom, a Chinese pharmaceutical company, is studying *M. vaccae*, a non-living lysate preparation from the non-pathogenic bacterium, as therapy prevention of disease in latently infected subjects with a PPD >15 mm.

Two live recombinant mycobacterial vaccines are under investigation in clinical trials as BCG replacement vaccines. VPM 1002 is a live recombinant vaccine derived from the Prague strain of BCG into which the listerolysin gene from *Listeria monocytogenes* has been cloned and the urease gene deleted to improve immunogenicity (Desel *et al*., 2011). Clinical studies have been completed in South Africa and the vaccine is now owned by the Serum Institute of India, which plans further development on the Indian subcontinent. MTBVAC is being developed by the University of Zaragosa, Institut Pasteur, BIOFABRI and the Tuberculosis Vaccine Initiative (TBVI). It is a live *M. tuberculosis* strain attenuated via deletions of the *pho*P and *fad*D26 genes (Martin, 2006). It is the first live attenuated *M. tuberculosis* vaccine to enter a Phase I clinical trial. There is also renewed interest in using BCG vaccine to revaccinate individuals previously vaccinated at birth with BCG, particularly in high transmission areas (Dye, 2013).

It is interesting to note that a number of these vaccines have been tested in large animals with positive results (Vordermeier *et al*., 2002). As most vaccines for wildlife species including white-tailed deer, possums, badgers, wild boar and African buffalo have concentrated on BCG vaccination (Buddle, 2013), for the purposes of this chapter we will concentrate on the advances in the development of cattle TB vaccines, since this is the large animal species in which most new TB vaccine candidates have been tested.

A large number of field trials of BCG in cattle were carried out in the early 20th century where, as for humans, the degree of BCG-induced protection varied considerably between studies (reviewed in Waters *et al*., 2012), reflecting the difficulty in controlling

the variables associated with such studies. Two recent field studies undertaken in Ethiopia and Mexico in situations where the prevalence and transmission rates of *M. bovis* infection were high have been encouraging with a protective efficacy of approximately 60% (Lopez-Valencia *et al*., 2009; Ameni *et al*., 2010). The most consistently successful approach to improving the efficacy of BCG in cattle has been the use of virally vectored vaccines that boost the immune response primed by BCG. In this context, both MVA85A and Ad5 Ag85A have improved the protection conferred by BCG to cattle under experimental conditions (Vordermeier *et al*., 2009; Dean *et al*., 2014). This vaccination strategy has also been shown to protect goats against infection *M. caprae* (Perez de Val *et al*., 2012).

Next-generation TB Vaccine Strategies

A number of next-generation TB vaccines are also in preclinical investigations and are illustrated in the 'TB Vaccine Pipeline document' (see above) and have been discussed elsewhere (Brennan *et al*., 2012b). There is an emphasis on vaccines that work through mechanisms different from those presently in human clinical trials and on new delivery approaches such as intranasal or oral (Xing *et al*., 2009; Jin *et al*., 2010). The most common strategy for targeting new TB vaccines for humans is to boost a primary BCG vaccination, which is commonly given to neonates in most countries endemic for TB (McShane and Hill, 2005). Many vaccines using this approach have been investigated in mice and guinea pig animal models for TB (Brennan, 2012c). As described above this strategy has also been investigated in large animals with promising results (Vordermeier *et al*., 2009; Dean *et al*., 2014) and recent results have demonstrated that intrapulmonary boosting of BCG-vaccinated cattle with Ad5 Ag85A boosted both lung mucosal and systemic (PBMC) responses (Whelan *et al*., 2012). Since in many endemic countries the increase in incidence of pulmonary TB begins during adolescence and often peaks between 20 and 30 years of age, a newer strategy is to test candidate TB vaccines in adolescent and adult populations. Vaccines to address this target population are at early stages of human clinical study.

Research on the pathogenesis of tuberculosis and on investigational vaccines in humans and animals over the past decade has increased our knowledge of the disease and host immune response but has also highlighted major gaps in our understanding of both. One rather understated message has been that the development of an effective and safe vaccine for tuberculosis will not be an easy task for a number of reasons:

1. The pathogenesis of TB is not completely understood. Recent evidence suggests that populations of infected individuals differ in their potential to progress towards active TB disease (Kagina *et al*., 2010; Kaufmann *et al*., 2010) and that TB disease itself is a complex process highlighted, for example, by the birth and death of granulomas in the lung (Lin *et al*., 2013).
2. No specific immune response to TB disease or to vaccination has been shown to correlate with protection. In fact, in populations in areas of high TB transmission, recurrence of TB in drug-treated individuals occurs at a very high rate (Verver *et al*., 2005), suggesting that TB disease itself may not elicit immunity that prevents reinfection or relapse. Vaccines, therefore, will need to induce an immunity different from disease (Young and Verreck, 2012).
3. The work of Comas *et al*. (2010) suggests that immunodominant Mtb antigens are actually conserved by the pathogen throughout evolution, suggesting that the response to these antigens (which includes Ag85 found in some new vaccines) is beneficial to the organism.
4. Mtb has evolved a number of mechanisms to evade the host's immune response which includes altering antigen presentation in cells harbouring the organism thereby allowing *M. tuberculosis* to exist within macrophages and other antigen-presenting cells (Flynn and Chan, 2003). It is clear that the challenges for vaccine production are many, but TB research has identified the major questions to be addressed and can guide new vaccine research strategies.

Strategies for facilitating TB vaccine development, clinical testing, manufacturing, and

for eventual access and introduction were highlighted more than a decade ago (Brennan and Fruth, 2001; Kaufmann, 2006). More recently, a list of key questions and recommendations for accelerating TB vaccine development has been provided in 'Tuberculosis Vaccines: a strategic blueprint for the next decade' (Brennan and Thole, 2012). Some of the key questions are found in Box 5.1. (Readers are also directed to other 'Opinion Pieces' on research and discovery, biomarkers, clinical trials, methods for selecting new vaccines and advocacy found in *Tuberculosis* 92(Suppl 1), March 2012.) Understanding the life cycle of *M. tuberculosis* in the human host highlighted by Kaufmann (2011), and the 'immunological life cycle of tuberculosis' as proposed by Ernst (2012), is critical for developing a mechanistic approach to TB vaccine development. Using this concept, a diverse portfolio of candidate TB vaccines should attempt to address one or more of the following:

- Prevent infection.
- Demonstrate sterilizing immunity.
- Prevent relapse or reinfection.
- Protect through novel mechanisms such as antibodies.
- Block transmission.
- Elicit effective immunity in the lung using novel vaccine delivery vectors or methods.

Related to these is a strategy to define correlates of immunity/protection and to use standardized assays in clinical trials that can evaluate a broad selection of immune responses to vaccines. Clinical studies should also include assessment of 'biomarkers' using a systems biology approach and to incorporate other new molecular and immunological approaches towards novel antigen identification (Rappuoli and Aderem, 2011; Evans *et al*., 2013; Lindestam Arlehamn *et al*., 2013).

The extensive array of immunological and molecular reagents available for use in the study of bovine responses to TB infection, along with the availability of experimental and natural transmission models of infection in cattle, mean that it is perhaps in the definition of correlates of immunity/protection that there may be the greatest synergies to be realized between bovine and human TB vaccine development. Indeed, there are already a number of areas that have benefitted from such an approach. Gamma interferon release assays were developed for the detection of TB infection in cattle in the form of the BOVIGAM assay many years before the development of QuantiFERON-TB and T-SPOT TB tests for humans. Humans and cattle share two of the major antigenic targets in ESAT-6 and CFP10 (Pollock and Andersen, 1997a,b; van Pinxteren *et al*., 2000) and antigen Rv3615c has also been identified as a useful diagnostic reagent for both cattle and humans (Sidders *et al*., 2008; Millington *et al*., 2011). Moreover, recent studies suggest *M. tuberculosis*-infected humans demonstrate a similar PE/PPE immune recognition hierarchy to *M. bovis*-infected cattle (Vordermeier *et al*., 2012). Taken together, these studies point to a similarity in antigen recognition between *M. bovis* infection in cattle and *M. tuberculosis* infection in humans.

One of the most powerful tools to address the issues of identifying predictors and correlates of vaccine efficacy is the bovine model of

Box 5.1. Key Questions for TB Vaccine Development. Adapted from Brennan *et al*., 2012a.

Why are certain *M. tuberculosis*-infected individuals resistant to TB disease, and what are the implications for vaccines?
What are the key cells and effector pathways that control host protective immunity to *M. tuberculosis*?
Can more relevant models of human TB disease be developed?
Are antibody responses to TB vaccines relevant to protection?
What are the best clinical strategies for showing that vaccines can prevent reactivation of latent TB disease and for studying therapeutic TB vaccines?
Will a standardized rational selection process be used by vaccine developers for selection of the best TB vaccines, and will they participate in comparative preclinical and clinical studies?
What are the best criteria for measuring the public health impact of TB vaccines?
What innovative approaches can be used to mobilize resources for TB vaccines?

infection itself. Unlike small animal models of TB infection where vaccine protection is measured in the reduction in disease burden as determined by a reduction in colony-forming units per organ, the outcome of vaccination experiments in cattle is normally threefold: vaccinated animals are either not protected, or animals are partially protected (defined as presenting significant reductions in pathology), or animals are 'fully protected in that they present at post-mortem without visible or histological signs of disease' (e.g. Vordermeier *et al*., 2002, 2009). Comparing animals from the same group that are not protected with those that are protected is a very powerful approach to biomarker discovery as it provides clear phenotypes with which to define biomarkers of protection (Vordermeier *et al*., 2009). Potential biomarkers identified using these experimental models can then be verified in natural transmission models and full-scale vaccine field trials. As the testing of cattle vaccines moves from experimental challenge models to natural transmission models and field trials as described below, parallel immunogenicity studies in cattle and humans, the sharing of clinical trial designs, the standardization of sample collection and assay selection, and harmonization of biomarker screening and immunological readouts for correlate work promises to accelerate the efforts towards a new animal and human TB vaccine.

Progress on Vaccines for Bovine TB

The development of cattle vaccines to prevent infection from other cattle or wildlife reservoirs of infection is an increasingly important goal for the global control of bovine tuberculosis and offers a number of opportunities for the human and bovine TB research communities to come together to take advantage of the opportunities that will be created by this increased endeavour. The focus for the development of a bovine TB vaccine will be on a vaccine that can prevent infection and block onward transmission. However, an additional requirement for the successful introduction of cattle vaccines is the development of complementary diagnostic reagents that allow the discrimination of infected from vaccinated animals (so-called DIVA tests) so that a test and slaughter approach to TB control can be continued in the face of vaccination. One further complication that will need to be overcome if cattle vaccination is to become a useful tool for the control of bovine tuberculosis is the EU-wide prohibition to vaccinate cattle against bovine TB. Thus, for cattle vaccines to form part of a bovine TB control strategy in EU countries, a number of national and EU laws and directives would need to be revised. Recently, in response to a request from the European Commission, the European Food Standards Authority published its opinion on the requirements of field trials for bovine tuberculosis vaccination. This opinion provides advice relating to the design of field trials to test the safety and performance of a vaccine for bovine TB along with a complementary DIVA test (EFSA, 2013) and will inform the design of future vaccine trials. The implementation of such trials offers the opportunity to test vaccine efficacy and safety in a robust way and provide important opportunities to identify correlates of immunity and protection to natural transmission of infection, which, as discussed above, may also help inform human TB vaccine development.

A Unified Strategy for Exploring TB Vaccines for Human and Animal Diseases

TB vaccine development in both humans and animals has been fraught with considerable challenges due to a lack of understanding of the underlying biological mechanisms and the absence of correlates of protection that can guide vaccine design, animal experiments or be used as a credible end point in clinical trials. Since researchers are in some cases using similar vaccines including BCG or new-generation TB vaccines to investigate the ability of vaccines to prevent disease in humans and animals, it would seem beneficial to synergize efforts across the veterinary and human fields, foster collaboration on key issues, share knowledge and coordinate the advance of TB vaccine research.

Key questions that could be addressed include:

- Can studies of vaccines in animals of agricultural importance inform the development of human vaccines and vice versa?
- Are there areas of synergy, such as defining correlates of immunity and performing comparative vaccine trials that can facilitate progress in both fields?
- What is the extent of the TB problem in cattle and other animals regionally and globally and can it be impacted by vaccination?
- What are the market dynamics of TB vaccines?
- What is the economic impact of TB in humans, livestock and wildlife?
- What international agencies may be interested in supporting joint activities that advance the goal of developing TB vaccines to benefit humans and animals?

A Working Group facilitated by the Animal Health and Veterinary Laboratories Agency (AHVLA, now known as the Animal and Plant Health Agency, APHA) and Aeras was formed in late 2013 to address these issues and includes participants from New Zealand, Mexico, the USA, the UK, Argentina, Chile and a number of other countries (see Aeras website, www.aeras.org, for relevant information). This group will initially focus on harmonizing TB vaccine efforts with researchers studying vaccines in cattle. Activities include an effort to map all past, present and upcoming studies in cattle by cataloging which vaccines are being tested, the specimens being collected in trials, immunological readouts being examined and a number of other key criteria used to study vaccines in field studies. Identifying gaps to better synergize efforts across human and animal TB vaccine research by sharing clinical trial designs, standardizing sample collection and assay selection, and harmonizing biomarker screening and immunological readouts for correlate work promises to accelerate the efforts towards a new animal and human TB vaccine. Since there are no perfect animal models that mimic human TB disease, cattle offer a fairly robust model with potential benefits for testing vaccines prior to human testing. This has been shown clearly in the use of cattle for the successful natural transmission studies of TB vaccines (Lopez-Valencia *et al*., 2009; Ameni *et al*., 2010). A number of immunological responses, as well as systems biology approaches and functional bacteriocidal assays, can be measured in the cattle model, which can help inform human vaccine development (see Chapter 10, this volume; Aranday-Cortes *et al*., 2012; Bhuju *et al*., 2012). There has been considerable effort to develop epidemiology and economics forecasting in human TB vaccine development to facilitate the clinical study and future introduction of TB vaccines in human populations. Since accurate and current data on bovine disease in cattle are not readily available for many countries and regions, understanding the global burden and economic impact of *M. bovis* should, similarly to human TB disease, facilitate the design of field trials and the licensure and marketing of vaccines for veterinary use. Working groups such as the one described here should help policy makers, funders and government officials to expand engagement with the broader vaccine community to gain support for joint TB vaccine projects that accelerate the development of TB vaccines.

Summary

In this chapter we discuss the current challenges that have slowed the development of novel vaccines for mycobacterial diseases and compare the major questions in vaccine development encountered by researchers investigating the potential of vaccines to control mycobacterial disease in both humans and cattle. The synergies, particularly the demanding challenges of identifying correlates of vaccine immunity and of constructing novel vaccines that prevent infection and transmission, are highlighted. We suggest that a combined effort, which includes a working group of investigators interested in advancing vaccines for human and bovine disease, may be an effective strategy for reaching our common goal of introducing safe and effective vaccines to control mycobacterial disease.

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6 The Continuing Co-evolution of *Mycobacterium tuberculosis* and *Homo sapiens*

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Introduction and Historical Context

Tuberculosis (TB) is a disease of antiquity, with evidence of human disease extending through most, if not all, of recorded human history. The oldest human remains to show evidence of disease were found in Turkey and dated to approximately 500,000 BP (Before Present) (Kappelman *et al*., 2008). This specimen of *Homo erectus* displayed pathology consistent with TB meningitis on the endocranial surface of a frontal bone; although molecular evidence of infection was not obtained, leading other authors to question this diagnosis (Roberts *et al*., 2009). Numerous studies have more conclusively identified TB DNA from somewhat more recent human remains from, for example: Egyptian mummies from 2050 BC to 500 BC (Zink *et al*., 2001, 2003); Peruvian mummies from 1000 BP (Salo *et al*., 1994); Lithuanian skeletal remains from the 15th to 17th centuries AD (Faerman and Jankauskas, 2000); and many more samples (nicely reviewed in Anastasiou and Mitchell, 2013). Even more definitively, both TB DNA and stable lipids completely unique to TB have been conclusively identified in remains from a woman and infant excavated from a Neolithic settlement in the eastern Mediterranean from about 9000 BP (Hershkovitz *et al*., 2008). *Mycobacterium tuberculosis* (Mtb) has thus been afflicting humankind for at least the last 10,000 years, possibly considerably longer, and has therefore both shaped and been shaped by human evolution.

The original source of TB infection in man is unknown but the coincidental timing of the earliest definitive palaeolobiological samples and the domestication of cattle has given rise to the hypothesis that humans acquired the disease from close contact with these animals. TB DNA and lipids have been identified from Pleistocene bison from Wyoming dated to 17,000 BP, predating rigorously confirmed human samples (Rothschild *et al*., 2001; Lee *et al*., 2012). However, more recent comparative genomics of bacilli of the TB complex show clear evidence of reductive evolution of *M. bovis* from Mtb, suggesting that the bovine ancestor evolved by deletion from a progenitor that was likely to be a human pathogen (Brosch *et al*., 2002). The genome sequences are thus more consistent with a scenario in which humans infected their domesticated animals than vice versa. There is no known environmental niche for Mtb, and human–human transmission is the only documented mode of propagation. Mtb is therefore an obligate human pathogen.

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

The Global Pool of Mtb Strain Lineages

The mycobacteria that form the Mtb complex have identical 16S rRNA sequences, look highly similar in morphology (Fig. 6.1) and have nearly identical genome sequences, yet they show very different pathogenicity across a wide spectrum of mammalian hosts. Mtb, *M. africanum* and *M. canettii* nearly exclusively infect humans (and are often referred to as the 'Mtb complex'); *M. microti* infects primarily rodents; and *M. bovis* infects a wide variety of animals, including cattle. The molecular basis for these varied host tropisms remains unknown and the extent to which, within human disease, such tropisms are geographically linked and possibly therefore linked to human adaptations are likewise unknown. The study of unique, relatively large deletions (ranging from 2 to 12.7 kb) among bacilli of this complex initially revealed the broad outline of the evolutionary scheme among currently circulating strains (Brosch *et al*., 2002). Virtually all of the oldest strains, those most closely resembling the progenitor organism in terms of lack of large deletions, are human pathogens bolstering the suggestion that the zoonotic diseases originated from humans. The apparently oldest of these deletions (TbD1) within the Mtb complex has been used to delineate 'modern' Mtb strains that represent many of the strains presently contributing to disease, while strains that lack that deletion are referred to as 'ancestral'.

One of these ancestral strains of Mtb is referred to as the 'Canettii' strain and appears to be a living relic. This strain is only rarely isolated from humans and is fundamentally different from prototypical Mtb strains in that it shows a smooth (as opposed to rough and waxy) morphology on agar, has a lower than 37°C optimal growth temperature, is less virulent in mice and appears to have retained the capacity for horizontal gene transfer – all attributes plausibly associated with a free-living progenitor of the Mtb complex of organisms (Supply *et al*., 2013). Genetically such strains show a total genome size slightly larger than other members of the Mtb complex with nucleotide identities of over 97% compared to modern Mtb strains, and appear to be recombinagenic, unlike other Mtb strains. Loss of the ability to perform horizontal gene

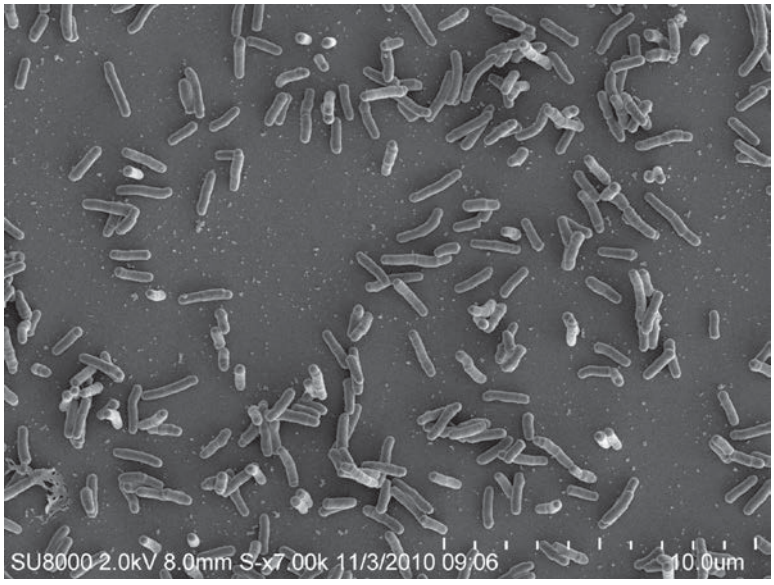

Fig. 6.1. Mtb typical morphology in scanning electron microscopy. Bacterial rods of varying length indicate actively growing cells; cell division results in a doubling of length before daughter bacilli separate to form individual rods 1 μm in length.

transfer is thought to have occurred in response to the lack of advantage to such transfer in strains that cause (largely) clonal infections in individual hosts. The Canettii strain therefore appears to represent a sort of missing link between the ubiquitous environmental mycobacteria and the obligate pathogens of the Mtb complex, and infection of early humans with a progenitor of this strain may have been the initiating organism in the origin of human TB.

Large-scale whole-genome sequencing of geographically diverse Mtb has more recently utilized single nucleotide polymorphisms (SNPs) to provide granularity to the deletion analysis previously described. Such analyses have revealed remarkable parallels between the phylogeny of Mtb complex organisms and that of human mitochondrial genomes and strongly support the hypothesis that expansion of Mtb from a fairly clonal population occurred simultaneously with Neolithic expansion of modern humans starting at about 70,000 BP (Comas *et al*., 2013). This expansion coincided with the first permanent human settlements that resulted in increased population density, providing more opportunity for an obligate pathogen to expand and adapt to specific human genetic variants. During expansion over the next 70,000 years the bacterial strains involved accumulated more than 30,000 SNPs. These SNPs cluster extant strains into seven major lineages of Mtb complex organisms, three of which are considered modern and all of which are relatively geographically distinct on a global scale (although some outliers to this will be discussed later).

Perhaps as a consequence of loss of the ability to perform horizontal gene transfer following adaptation as an obligate human pathogen, SNP accumulation and chromosomal deletions appear to have emerged as the twin hallmarks of Mtb evolution. Whether such changes could mediate host-adaptive changes was addressed in recent work using RNA-seq of representative lineages and, remarkably, showed that clustering of transcriptional profiles can recapitulate genome-(SNP) based phylogenetic groupings. Therefore many of these SNPs have phenotypic consequences, at least in terms of alterations in gene expression (Rose *et al*., 2013).

Reductive Evolution and Gene Decay Contribute to Host–Pathogen Specificity

The related leprosy bacillus, *M. leprae*, may be the extreme example of reductive evolution, having jettisoned fully half its genome as 'pseudogenes' – gene remnants so heavily mutated they no longer encode functional proteins. This genome downsizing perhaps explains why the bacillus appears incapable of growth outside the human host to which it is adapted (Eiglmeier *et al*., 2001). Although there are fewer examples of reductive adaptation to species tropism in the Mtb complex, one such lies in the regulatory system controlling the bacterial response to oxidative stress. Most bacteria induce a response to challenge with reactive oxygen species through a protein mediator called oxyR that senses these species and coordinates a cellular response at the transcriptional level. Presumably Mtb no longer found it beneficial to have such a system where the default state was off and as a consequence the oxyR pseudogene contains numerous deletions and frameshifts and is no longer functional, resulting in an oxidative stress response system that is constitutively induced (Sherman *et al*., 1995). Thus far the oxyR pseudogene appears to be a feature conserved in all Mtb complex lineages and therefore represents an adaptation to life as a human pathogen rather than to a specific human lineage.

There are, however, tantalizing hints that other regulatory functions may be adapting in a lineage-specific fashion. The main family of proteins differentially expressed in a lineage-specific fashion by RNA-seq were the toxin–antitoxin modules representing more than 10% of differentially expressed transcripts (Rose *et al*., 2013). These proteins, originally characterized as 'addiction modules' encoded by plasmids to ensure their maintenance in daughter cells, are typically co-expressed in pairs consisting of an unstable antitoxin and a stable toxin (Pandey and Gerdes, 2005). Failure to maintain expression of the antitoxin results in 'intoxication', usually manifested as growth arrest mediated by toxic endonuclease (or protease) activity. Mtb has an amazing 62 annotated pairs of such proteins, more than any

other bacterium, and these may represent transcription-independent regulatory systems that alter multiple cellular activities, adapting the bacillus to the lineage-specific host environment.

The East Asian 'Beijing' lineage of organisms offers another insight. This lineage is noteworthy for having spread to regions of the world where it did not evolve and causing TB disease of unusual severity with an increased rate of extrapulmonary complications (Reed *et al*., 2007). Organisms of the Beijing lineage have been reported from Europe, Africa and South America and there are innumerable, often conflicting, reports as to their association with a wide variety of severe clinical sequellae (Schurch *et al*., 2011). One virulence factor that has been shown to be unique to these strains is the immunosuppressive phenolic glycolipid (PGL-Tb). PGL-Tb is a glycosylated form of an abundant cell envelope lipid, phthiocerol dimycocerosate, and has potent immunomodulatory activity *in vitro*. In mice, Beijing strains proficient in PGL-Tb are significantly more lethal than isogenic strains in which production of this lipid has been specifically deleted (Reed *et al*., 2004). However, not all Beijing strains produce PGL-Tb. The Beijing lineage has been further subdivided into five subtypes based upon four additional deletions yet even within these subtypes there are some strains that produce PGL-Tb and others that do not (Reed *et al*., 2007). Even the genetic basis for loss of PGL-Tb expression varies among Beijing subtypes. Thus PGL-Tb would appear to be either a molecule currently undergoing negative selective pressure in the East Asian lineages, having been lost in most global Mtb lineages, or a molecule representing a rare form of the bacillus that is re-expanding due to the lack of host-specific adaptations in populations where it is emerging that have adapted to Mtb lineages that do not produce PGL-Tb.

Immunologic Selection of Global Mtb Lineages

Approximately 4 billion doses of the Bacillus Calmette–Guérin (BCG) vaccine have been administered worldwide and it is estimated by the World Health Organization that 100 million newborn human infants receive BCG every year (Kaufmann, 2011). The magnitude of the selective pressure this imposes on the bacillus to escape vaccine-induced immunologic pressure is hard to estimate. It is widely agreed that BCG is largely ineffective in reducing adult cases of TB (but probably does reduce paediatric cases of TB meningitis) and the results of controlled trials are highly divergent for reasons that remain poorly understood but may be associated with prior infection with other mycobacterial strains that do not cause disease- or host-specific immunological responses that vary according to geography (Mangtani *et al*., 2014). BCG use remains controversial since there are no validated correlates of protective immunity (Evans *et al*., 2013) and little understanding of the impact of current vaccination strategies. The recent failure of a large-scale Phase 2b trial with a BCG booster (a modified Vaccinia Ankara virus expressing the Mtb antigen 85A) to induce protection from development of TB disease highlights further how little is understood about the consequences of modulation of the human immune response to Mtb infection (Tameris *et al*., 2013).

Mtb relies on manipulating the human immune response to survive and transmit, and there is considerable evidence of both species- and lineage-specific adaptations directed at the human immune system. Innate immune responses to various Mtb strains, for example, vary dramatically according to strain lineage (Krishnan *et al*., 2011; Portevin *et al*., 2011; van Laarhoven *et al*., 2013). The argument has been made that T-cell epitopes show a higher degree of conservation in Mtb lineages than other genes and that this hyperconservation points to an important beneficial role for the bacillus in experiencing a potent immune response at a critical point in the disease process (Comas *et al*., 2010). These and similar findings have led some authors to conclude that recent phenomena, such as the global expansion of the Beijing lineage, are perhaps a direct consequence of the widespread vaccination programs with BCG (Parwati *et al*., 2010). Such assertions cannot, of course, be proved directly but should serve as a caution to carefully consider the wisdom of

proposed intervention strategies in light of the uncertainty of modulating the human immune response against a background of the highly diverse set of global phenotypes represented by various extant Mtb lineages. An effective vaccine against modern lineages may serve only to provide an opportunity for the re-emergence of cryptic 'ancestral' lineages.

Chemotherapeutic Pressures and the Evolution of New Mtb Variants

The widespread application of effective TB chemotherapy offers perhaps the clearest and most definitive area for the study of the evolutionary plasticity of Mtb. Quadruple drug short-course chemotherapy was only introduced in the 1970s as a means to control the bacterium, and the emergence of resistance to individual drugs began to appear even before. Selection of drug-resistant mutants of TB, after the loss of horizontal gene transfer during evolution from the Canetti-like progenitor, is exclusively due to SNPs and deletions making tracing of selective events relatively straightforward. The development of tools to genetically manipulate the bacillus made confirming causation of such genetic changes routine (Jacobs *et al*., 1987). However, identifying SNPs or deletions involved with resistance and confirming their phenotype does not always yield simple interpretable mechanisms of action. Mtb is notable for the number of 'prodrugs' used to eradicate it that require some form of metabolic activation, often quite complex oxidative or reductive transformation that have numerous cellular sequellae. Still, there are some clear examples of evolution to drug resistance, acquisition of fitness defects as a consequence and even generation of suppressor mutations that compensate for such defects that highlight the plasticity of the microbe in adaptation to the human host; several are presented in detail below.

KatG and isoniazid

The initial chemotherapy studies to treat TB involved single drug treatment with newly discovered agents. Resistance to streptomycin and isoniazid was therefore noted in about 85% of patients undergoing monotherapy within 3 and 4 months of starting therapy (Crofton and Mitchison, 1948; Dye *et al*., 1953). Gardiner Middlebrook (for whom the standard Mtb *in vitro* growth media is named) was among the first to notice that bacilli that had acquired resistance to isoniazid did so at a cost; when such organisms were re-injected into guinea pigs they were no longer able to cause disease or caused attenuated forms of the disease (Middlebrook and Cohn, 1953). It was another 40 years before it was determined that loss of KatG, the catalase–peroxidase responsible for isoniazid activation within Mtb, was the preferred route of resistance acquisition *in vitro* (Zhang *et al*., 1992). The situation proved more complex *in vivo* since loss of the protection to oxidative challenge afforded by KatG resulted in attenuation of the bacilli, an attenuation that could be compensated for by overproduction of the alkylhydroperoxidase AhpC (Sherman *et al*., 1996). This study was the first to show that suppressor mutations that ameliorate the impact of resistance-determining alleles were a viable evolutionary strategy for Mtb to adapt to the consequences of the selective pressure applied by chemotherapy. This study was limited by the fact that loss-of-function KatG mutations were a fairly rare occurrence in human infection with Mtb, where the preferred mutation was far more often an alteration in the residue Ser315, which allowed adventitious recognition of isoniazid by KatG.

RpoB/C and rifamycins

Rifampicin binds directly to the bacterial RNA polymerase, occluding the tunnel used by growing mRNA, thereby abolishing transcription (Campbell *et al*., 2001). Rifampicin resistance develops in Mtb by direct alteration of the drug-binding site resulting in bacteria with very slightly slower overall growth rates *in vitro*. In early studies it was noted that isolates selected for rifampicin resistance *in vitro* displayed this depressed growth rate, while isolates obtained from patients with identical mutations did not, arguing that these

bacteria had evolved second-site suppressor mutations that compensated for the growth defect of the resistance generating mutation (Gagneux *et al*., 2006). Subsequent epidemiological studies of rifampicin-resistant isolates confirmed and extended the spectrum of potential compensatory mutations (Comas *et al*., 2012; de Vos *et al*., 2013). These associative studies left open two important questions:

1. The magnitude of the fitness defect was sufficiently small that it could only be reliably measured in competitive culture assays, wherein wild type and mutant strains were co-cultured, and the growth advantage of the wild type strain quantified after multiple generations. It was hard to imagine how this small fitness defect translated into a sufficiently strong selective pressure in patients to elicit selection of compensatory mutations.
2. The putative compensatory mutations were located far in space, often on the adjacent β′ subunit encoded by rpoC, making it hard to imagine how these mutations were compensating for alterations near the active site where rifampicin bound.

The first of these questions was answered by carefully exploring the conditions under which the bacillus was cultivated. Under conditions of severe nutrient deprivation, sufficient to induce the bacterial starvation response, the magnitude of the fitness defect became highly magnified such that apparent growth rates could be directly compared and the rifampicin-resistant mutants grew two–threefold more slowly than wild type organisms (Song *et al*., 2014). The answer to the second question became clear with the appearance of the crystal structure of the RNA polymerase from *Escherichia coli* bound to the regulatory molecule ppGpp, a hyperphosphorylated nucleotide that signals cell starvation (Mechold *et al*., 2013). This molecule binds at the interface of the β′ and ω subunits of the polymerase, near the sites of the putative compensatory mutations, suggesting a critical role of this region of the polymerase in adapting to growth under nutrient-limited conditions. This work allowed the construction of a detailed structural hypothesis for the forced evolution of the bacillus to pressure in the form of rifampicin treatment. In addition it was shown that such evolution had occurred convergently in different strains resulting in optimal selection of resistance and compensated mutations that resulted in fully fit progeny strains (Song *et al*., 2014).

Cofactor F420 and nitroimidazoles (PA-824 and delaminid)

Cofactor F420 is a unique deazaflavin cofactor that structurally resembles a flavin but performs redox chemistry analogous to nicotinamide cofactors. The chemistry of this unique cofactor has been most well characterized in the methanogens where it plays a pivotal role in the production of methane (Deppenmeier, 2002). This cofactor is found in many mycobacterial species and across the Mtb complex of organisms but not, interestingly, in *M. leprae* (Manjunatha *et al*., 2006b). The role of the cofactor in the biochemistry of the Mtb complex organisms is unknown, although it has been proposed to play a role in detoxifying lethal species of reactive nitrogen species (Purwantini and Mukhopadhyay, 2009) and an enzyme utilizing reduced F420 has been shown to play a role in producing some species of mycolic acid subtypes in the cell envelope (Purwantini and Mukhopadhyay, 2013).

The interest in this cofactor derives largely from its role in activating two nitroimidazole prodrugs, PA-824 and OPC67683 (now known as Delamanid) that are currently in clinical trials for the treatment of TB (Diacon *et al*., 2012; Gler *et al*., 2012). Both *in vitro* and *in vivo* the major mechanism for the acquisition of resistance to these two drugs is loss of the ability to biosynthesize, or reduce, cofactor F420 (Stover *et al*., 2000). F420-mediated activation of either drug results in intracellular release of nitric oxide, which is highly lethal for the organism (Singh *et al*., 2008). The crystal structure of the enzyme that 'activates' these drugs has been solved in complex with F420 yet the physiological role for this enzyme and cofactor remain elusive (Plate 2) (Cellitti *et al*., 2012). Mutants selected for resistance to PA-824 that have lost the ability to synthesize F420 do not appear to be compromised for growth and arise at a high

frequency (Manjunatha *et al*., 2006a). The genes encoding the biosynthetic and redox coupling enzymes involved in producing reduced F420 appear polymorphic among existing Mtb complex members but thus far appear to encode functional proteins (Feuerriegel *et al*., 2011). The single exception to this is the Canetti strain that has mutations in the gene involved in mediating activation of the prodrugs. The lack of phenotype of the resistant bacilli, high frequency of emergence of resistance to these drugs and the fact that at least one member of this group of proteins is already dysfunctional in a contemporary lineage all suggest the possibility that resistance may emerge across Mtb lineages fairly quickly and with low cost to the organism, which may limit the clinical utility of this class of agents.

The Impact of TB on Modern Human Genetics

Unlike the application of chemotherapy, which has occurred largely within the observable time frame of the last 70 years, the impact of TB on the human host has been over a protracted time course making establishing cause and effect much more difficult to rigorously establish. None the less there are striking examples of modern day maladies that can be plausibly linked to selective pressures exerted by the bacillus on the human population that are worth considering. The full impact of human selection by Mtb may never be known with certainty, but when one considers that as recently as the 19th century fully one-quarter of all human deaths were from TB and that these deaths largely occurred in individuals of prime reproductive age, the potential for strong selective pressure becomes very clear. Particularly among diseases that occur late in life that would not have been historically important for human populations with far shorter median lifespans than enjoyed now, trade-offs that enhanced survival to disease at the expense of post-reproductive disease risk may have strongly shaped our genetic make-up. Such impacts may have had strong geographical biases that correlate with the existing strain clade-specific adaptations by the bacillus to survive within selected populations. This section will highlight some of the more compelling current examples.

TB and rheumatoid arthritis

Probably nothing has done more to elevate the awareness of adults in the USA and Western Europe that TB remains an important health issue than the advent of widespread use of anti-tumour necrosis factor (TNF) treatment for rheumatoid arthritis and Crohn's disease. In 2001 a clear link between anti-TNF treatment and the rapid progression of TB disease was first established, resulting in the US Food and Drug Administration (FDA) requiring the label of such products to carry a warning relating to risk of reactivation TB in patients who may have occult infection (Keane *et al*., 2001). TNF is a highly potent inflammatory cytokine regulating not only the body's defence to infectious agents but also a wide range of other vital systems including control of tumours, sleep regulation and bone resorption (Fiers, 1991). Inappropriate overproduction of TNF has been directly linked to rheumatoid arthritis although the genetics of this relationship is complex (Brennan *et al*., 1992).

TNF is also a critical element of the host response to Mtb boosting the intracellular killing of bacilli and maintaining the structure of the granuloma containing the infection (O'Garra *et al*., 2013). The importance of the latter function in human TB has been proved conclusively by the inadvertent reactivation of TB in patients treated with anti-TNF therapy for rheumatoid arthritis. This dual opposing role of TNF has led some authors to hypothesize that our immune system has been selected for enhanced survival to Mtb infection at the expense of increasing our sensitivity towards rheumatoid arthritis (Aguillon *et al*., 2006). Although impossible to prove the logic that TB tends to occur in people of prime reproductive age while arthritis tends to occur in people of post-reproductive age, it does suggest that selection by Mtb for the survival of people with endogenously high TNF levels is very plausible.

The relationship of TB and diabetes

The simple fact that malnutrition is consistently found as a risk factor for TB while obesity appears to be protective argues for a connection between TB and the metabolic disease experienced by modern humans (Leung *et al*., 2007). TNF and inflammatory status again provide a mechanistic link as obesity is strongly associated with multiple inflammatory processes (Ferrante, 2007). These associations have led some authors to speculate that TB may have been an important selective factor in the contemporary high rates of metabolic syndrome and diabetes (Roth, 2009).

Obesity may have been selected in human populations on the basis of longer-term survival to TB but today the confluence of two epidemics – diabetes and TB – is proving deadly. Diabetics are far more likely to die from TB than non-diabetics and the immune dysregulation experienced by diabetics may make them more susceptible to initial infection (Martinez and Kornfeld, 2014). As metabolic syndrome spreads in emerging economies in TB-endemic regions due to western-style diets, more reports on the linkage between diabetes and TB emerge. What was once protective during the industrial revolution in 19th-century Europe may now be magnifying the modern TB epidemic.

TB and cancer

In 1929 Raymond Pearl was the first to propose an association between lung cancer and TB (Goldman, 2002). Based on 7500 autopsies he concluded (incorrectly as it turns out) that TB and malignancies were antagonistic, a finding that motivated many therapeutic trials for various malignancies using the vaccine strain BCG. Ultimately these efforts were successful for bladder cancer, and intravesical instillation of BCG remains the standard of care for treating high-risk, non-muscle-invasive disease today (Redelman-Sidi *et al*., 2014).

The relationship of TB with cancer is complex, but in non-smokers, prior TB is a strong risk factor for subsequent development of adenocarcinoma (Pallis and Syrigos, 2013). This is thought to arise as a consequence of the destructive lung pathology that occurs with TB disease although precise molecular details have not been established. As argued previously for diabetes, selective pressure for pro-mutagenic host defence mechanisms (such as DNA-damaging reactive oxygen and nitrogen species) important for protection from TB early in life might plausibly be selected despite their higher oncogenic risk.

Conclusions

Clearly Mtb and modern humans have co-evolved to a very significant extent and we are only really beginning to appreciate the complexities of some aspects of this relationship. The emergence of drug resistance, and the epistatic compensatory mutations that restore fitness to resistant organisms, is becoming well documented but only for a small handful of drugs. Such considerations at this point do not impinge on any aspect of the TB drug development pipeline, but they most certainly should. Likewise the basic lack of understanding of why most people contain TB disease while others fail to do so makes prospects for augmenting protection in naïve people quite poor.

Given the complexity of the human relationship with TB it is worth wondering if eradicating the disease is a realistic prospect. The heightened virulence of the Beijing clade of Mtb is but one example of the consequences of large-scale human population mixing that is a fact of life in our increasingly global world. How other host-adapted but geographically isolated strains of Mtb will interact with new hosts is at this point unknown but represents a significant risk. The evolutionary bottleneck for the bacillus is transmission; its continued survival depends on being able to become airborne in an appropriate environment to infect a new host and yet very little is understood about most aspects of the transmission event. This process is probably one of the largest areas of opportunity for future TB research.

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7 The Global Distribution of *Mycobacterium bovis*

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Introduction

The *Mycobacterium tuberculosis* complex (Mtb complex) of bacteria includes an ever-growing number of named species of pathogenic bacteria all causing a very similar pathology (tuberculosis) in many different mammals (Smith *et al*., 2006a). The most important member of the Mtb complex is *M. tuberculosis* which is, both currently and historically, responsible for high morbidity and mortality in humans. *M. bovis*, however, is the commonest cause of tuberculosis (bovine tuberculosis, bTB) in bovids. The preferred host of *M. bovis* is domesticated cattle, although this pathogen can also be isolated from man and many other mammals (Smith *et al*., 2006a). The sequence divergence within the Mtb complex is minimal; around one change in 2000 bp (0.05%), and many different species have been 'shoehorned' into this complex based on differences in the host they were initially isolated from. Because the definition of 'species' is controversial in general (Mallet, 2010), and more so within the Bacteria and Archaea (Schleifer, 2009), it has been suggested that it would be more informative to refer to the host-adapted clades within the Mtb as 'ecotypes' of *M. tuberculosis* rather than as species (Smith *et al*., 2006b); and it might be better to refrain from naming new species within the Mtb complex that are identified merely because they have common genotypes and are isolated, occasionally, from the same host. For example, eight strains of the same rare genotype were reported in domestic cats from Newbury, Berkshire, UK, over a very short time period (2008–2013). The authors managed to resist the urge to name this genotype *M. catus*, mainly because the same genotype had frequently been isolated from cattle in the same area (Roberts *et al*., 2014).

There is no empirical evidence for the transfer of genes or genetic material between strains in the Mtb complex of bacteria; the population appears totally clonal. However, an analysis of the population structure of 24 genome sequences of *M. tuberculosis* using complex population analysis software identified signals of recombination within the Mtb complex, and suggested that since, the most recent common ancestor (*M. tuberculosis*) has evolved via a mixture of processes that include mutation, natural selection and also recombination. Recombination was identified on all branches of the *M. tuberculosis* lineage. In particular, Namouchi *et al*. (2012) identified very high rates of linkage disequilibrium for

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

sites that were very close on the chromosome (less than 500 bp apart) and this linkage disequilibrium decayed as the sites were further apart. When the identified recombinant sites were removed from the data set linkage disequilibrium became constant, and uniformly low, throughout the studied chromosomes. Although these data superficially seem to be evidence for recombination it can also be explained by other mechanisms such as compensatory mutations. For an initial mutation that is slightly deleterious, but which rises to reasonable frequency by either drift or background selection, it is expected that clones bearing compensatory mutations would subsequently rise in frequency. It is not unreasonable to suggest that these compensatory mutations would be more likely in the same gene or operon as the original deleterious mutation; that is, closely linked to the original deleterious mutation. This mechanism would generate linkage disequilibrium between closely linked sites in a clonal population. Furthermore, clusters of mutations and compensatory mutations would also be identified by Sawyer's runs test, as possible recombination fragments donated from outside the Mtb complex, an observation suggested by Namouchi *et al*. (2012) as further evidence of recombination within the Mtb complex.

In contrast, two very large-scale analyses of Mtb complex using whole-genome sequences suggest, from both empirical evidence and analysis, that recombination is rare or absent in the Mtb complex (Comas *et al*., 2013; Pepperell *et al*., 2013). The very low rates of homoplasies (identical mutations in different lineages) and the congruence between different molecular markers found in phylogenetic reconstructions of nucleotide sequence data of the Mtb complex are hallmarks of clonality (Maynard Smith and Smith, 1998; Comas and Gagneux, 2009). Furthermore, a more targeted analysis of 16 informative mutations among 501 strains of *M. bovis* from the British Isles concluded that the absence of homoplasies in the single nucleotide polymorphisms (SNP) phylogeny was consistent with *M. bovis* being a clonally reproducing organism, and that this conclusion was supported by the unambiguous assignment of genotypes to specific SNP lineages (Allen *et al*., 2013). This observation is important because the British isolates of *M. bovis* are closely related, both geographically and temporally. That is, they represent a sample of isolates from within a population where recombination is most likely to be observed because the strains have the opportunity to meet and recombine, which is a possible criticism of the two very large-scale analyses of Mtb complex using whole-genome sequences where the isolates were gathered from throughout the world and it could be suggested that local, small-scale recombination would be overlooked. The weight of empirical evidence therefore suggests the absence of recombination within the Mtb complex since the most recent common ancestor.

Bovine tuberculosis has been reported from every continent where domesticated cattle are found (Smith, 2011) and it is suggested that *M. bovis* evolved as a series of clonal complexes; groups of closely related isolates all descended from a most recent common ancestor with unique molecular markers and shared characteristics (Smith *et al*., 2003; Muller *et al*., 2009; Berg *et al*., 2011; Smith *et al*., 2011). To date, four clonal complexes of *M. bovis* have been defined by the presence of specific molecular markers, and each with distinct spoligotype signatures – two are geographically localized to Africa while the other two have a wider distribution.

The Identification of Clonal Complexes

A clonal complex of *M. bovis* strains is marked by any mutation that was present in the single cell that was the most recent common ancestor of the clonal complex and is therefore present in all members of a clonal complex by descent. The best molecular markers are deletions because they have unique start and end points and therefore are much less likely to occur independently in unrelated lineages; however, SNPs can also act as markers for specific clonal complexes. All members of the clonal complex will carry the molecular marker and the presence of the marker defines membership of the clonal complex. However, it is possible to make a preliminary identification

of a clonal complex using spoligotyping which is one of the most common molecular epidemiological techniques applied to the Mtb complex (Kamerbeek *et al*., 1997). Spoligotyping results in a 'barcode' for an isolate representing the presence or absence of 43 spacers that are unique to the Direct Variable Repeat region. Each barcode is given a name and it is assumed that spoligotype patterns evolve by the deletion of single, or groups of, spacer sequences. All members of a clonal complex will have a spoligotype pattern either the same as that found in the most recent common ancestor or a spoligotype pattern descended from the ancestral spoligotype pattern by deletion of specific spoligotype spacers. Often a spoligotype signature can be assigned for each clonal complex but because spoligotype spacers are frequently lost, a property that makes them useful for molecular epidemiology, it also means that a signature spoligotype pattern is not necessarily a marker for defining a strain as a member of a clonal complex; a spoligotype pattern that identifies a strain as a member of a clonal complex may have occurred in another lineage. Spoligotype patterns are very useful for initial screening of spoligotype data; a strain identified with a clonal complex specific spoligotype signature can then be tested by deletion assay (Smith *et al*., 2003, 2011; Muller *et al*., 2009; Berg *et al*., 2011).

A better use of large-scale spoligotype pattern data is to test for the presence of clonal complexes in a country. If the signature spacers of the clonal complex of interest for most strains from a country or region in a spoligotype survey are intact, then the assumption is that the clonal complex is not present, although it can be a good idea to assay a representative set of isolates for the specific molecular marker of the clonal complex.

The African 1 (Af1) Clonal Complex of *M. bovis*

Bovine tuberculosis has been shown to be present in most countries in Africa, but the true extent of the disease has not been evaluated and genotype surveys are often limited in scope (Ayele *et al*., 2004). The first major clonal complex of *M. bovis* was identified in large-scale spoligotype surveys of *M. bovis* from Cameroon, Nigeria, Mali and Chad. It was observed that spacer 30 was frequently absent in spoligotype patterns from these West–Central African countries (Njanpop-Lafourcade *et al*., 2001; Hilty *et al*., 2005; Cadmus *et al*., 2006; Muller *et al*., 2008). To confirm that the strains found at high frequency in West–Central Africa were all members of the same clonal complex we identified an informative deletion of chromosomal DNA by micro-array studies. We named this deletion RDAf1 and a simple PCR assay was developed to detect the RDAf1 deletion. When this deletion was tested in large surveys of isolates from these West–Central African countries, RDAf1 was found to be deleted in over 96% of the 300 strains assayed. All strains deleted for RDAf1 had lost spoligotype spacer 30, and the spoligotype signature of this clonal complex was determined to be the loss of spacer 30. We named this clonal complex of *M. bovis* African 1 (Af1) and concluded that *M. bovis* in these West–Central African countries were part of a single clonal complex of strains in which both RDAf1 and spacer 30 were deleted (Muller *et al*., 2009).

We also showed that strains of the Af1 clonal complex were not present in our samples of isolates from other African countries (Algeria, Burundi, Ethiopia, Madagascar, Mozambique, South Africa, Tanzania and Uganda). Although we identified isolates with spacer 30 missing they did not carry the defining deletion of the RDAf1 region and therefore were not members of the Af1 clonal complex. Furthermore, large population surveys of *M. bovis* strains from Europe, North and South America and Iran were examined for the spoligotype signature of the Af1 clonal complex (loss of spacer 30) and where possible were tested for the Af1-specific deletion. It was concluded that strains of the Af1 clonal complex were not at high frequency in any region outside of West–Central Africa. Recently strains of *M. bovis* with spacer 30 missing in their spoligotype pattern, and presumably members of the Af1 clonal complex, have also been identified in Niger and Ghana, reinforcing the geographical localization of this clonal complex to West–Central Africa (personal communication, 2011 – Dr A. Razac and Prof. S. Niemann).

African 2 – From East Africa

Many of the strains of *M. bovis* isolated from East Africa also showed a distinct spoligotype signature which was the loss of spacers 3–7. This spoligotype signature was found in strains in Uganda, Ethiopia Tanzania and Burundi. In representative strains from East Africa we identified a specific deletion of chromosomal DNA, called RDAf2, and strains with this deletion were found in over 65% of *M. bovis* isolates from these East African countries. The spoligotype signature of this clonal complex, which we named African 2 (Af2), was the absence of spacers 3–7 (Berg *et al*., 2011).

By reciprocal deletion analysis of isolates we showed that the RDAf2 region was intact in strains of the Af1 clonal complex and vice versa. Strains of Af1 and Af2 are, therefore, mutually exclusive and do not share any phylogenetic history since the most recent common ancestor of each clonal complex. In a similar manner to the analysis of the Af1 clonal complex (above) we found that strains with the spoligotype signature of Af2 were not present in cattle isolates from other African countries or in the New World or Europe.

A Globally Distributed Clonal Complex of *M. bovis* – European 1

Although strains of the Af1 and Af2 clonal complex of *M. bovis* are rarely, if ever, found in cattle outside of Africa, a third clonal complex of *M. bovis* has been identified with a global distribution. Strains of bTB found throughout the British Isles (England, Scotland, Wales, Northern Ireland and the Republic of Ireland), in contrast to strains from France, shared similar spoligotype patterns (Smith *et al*., 2006a). In further analyses it was found that over 99% of *M. bovis* strains isolated in the British Isles were deleted for a specific chromosomal region (RDEu1) and all lacked spacer 11 in their spoligotype patterns. This group of strains, defined by deletion RDEu1 and marked by the loss of spoligotype spacer 11, are members of an *M. bovis* clonal complex which was named European1 (Eu1) (Smith *et al*., 2011). We were able to show by reciprocal deletion analysis that isolates of the Eu1, Af1 and Af2 clonal complexes were all phylogenetically independent.

Deletion analysis of strain collections representing the current population structure of *M. bovis* in Italy, Belgium and France identified few strains of the Eu1 clonal complex. In contrast, Eu1 isolates were found at a frequency of about 6% of the strains from Portugal and Spain. Isolates from humans from Germany, the Netherlands and Sweden were surveyed for the RDEu1 deletion on the assumption that these people had been infected with bTB prior to its elimination from cattle (Smith *et al*., 2011). If these human strains do reflect the population of bTB circulating in cattle prior to its elimination, then in these three mainland European countries the Eu1 clonal complex was also rare in cattle. However, although the Eu1 clonal complex was at low frequency in most of mainland Europe, an analysis of spoligotype patterns from all over the world showed that some of the distinct Eu1 spoligotype patterns found in the British Isles were also found in many other countries outside of Europe.

To assay the global distribution of this clonal complex, representative strains from many countries were surveyed for the clonal complex specific RDEu1 deletion. Strains of the Eu1 clonal complex were found to be common in many former British colonies, such as New Zealand, South Africa, Australia, Canada and the USA. However, it was not found in African countries (except South Africa), some of which were dominated by Af1 and Af2 clonal complexes. Furthermore, the population of several North and South American countries were dominated by strains with the RDEu1 deletion. In general, the British Isles, some former British colonies, North America and South America (with the exception of Brazil) have the Eu1 clonal complex at high frequency. In contrast, most of mainland Europe, Iran and Africa (with the exception of South Africa), are dominated by other clonal complexes of *M. bovis*. Strains of the Eu1 clonal complex were also identified in both South Korea and Kazakhstan, presumably following import of cattle from regions where Eu1 was common. The Eu1 clonal complex of *M. bovis* is therefore a globally

important clonal complex of *M. bovis*. The spoligotype signature of this clonal complex is the loss of spacer 11; in all cases strains deleted for RDEu1 were also deleted for spacer 11 in their spoligotype pattern. It is assumed that the most recent common ancestral cell of the Eu1 clonal complex was deleted for both RDEu1 and spacer 11 in its spoligotype pattern.

It has been suggested that the reduced diversity of Eu1 in the British Isles compared to mainland European countries was the result of a population bottleneck caused by the test-and-slaughter procedure used to control bTB over the past 100 years (Smith *et al*., 2006a). However, this suggestion will have to be re-evaluated in the light of the global distribution of the Eu1 clonal complex strains with identical spoligotype patterns to those found in the British Isles (SB0130 and SB0140, www.Mbovis.org) (Smith and Upton, 2012). If the UK was the primary distribution point for the Eu1 clonal complex then it would seem that Eu1 strains with archetypal spoligotype patterns were present at reasonable frequency in the UK in the early 19th century. That is, the Eu1 clonal complex may have been dominant in the UK prior to any meaningful implementation of bTB control. A population bottleneck is still the best explanation for the reduced diversity of *M. bovis* in the British Isles. However, this bottleneck may have happened during the primary introduction of the disease to these islands after the last Ice Age (Smith *et al*., 2006a).

European 2 from the Iberian Peninsula

M. bovis isolates from the Iberian Peninsula are dominated by strains with spoligotype patterns deleted for spacer 21 (Aranaz *et al*., 1996, 2004; Gortazar *et al*., 2005; Duarte *et al*., 2008, 2009; Rodriguez *et al*., 2009). Whole-genome sequencing of three Spanish strains with spacer 21 missing in their spoligotype pattern revealed a series of SNPs and subsequent screening of a selection of these SNPs identified one in the gene *guaA* that is specific to these strains. This group of strains from Spain and Italy that are missing spoligotype spacer 21 represents a new clonal complex of *M. bovis*, defined by the SNP profile and with a distinct spoligotype signature. We named this clonal complex European 2 (Eu2) and found that it was present at low frequency in both France and Italy and absent from the British Isles.

Strains of *M. bovis* with spacer 21 deleted are also found in the USA, Mexico, Brazil, Taiwan and Kruger National Park in South Africa, although it will require sequencing of the informative SNP in *guaA* to prove that these strains are all members of the Eu2 clonal complex (Zumarraga *et al*., 1999; Zanini *et al*., 2005; Viana-Niero *et al*., 2006; Jou *et al*., 2008; Michel *et al*., 2008, 2009; Milian-Suazo *et al*., 2008; Rodwell *et al*., 2010).

Other Clonal Complexes of *M. bovis*

Although the Af1 clonal complex of *M. bovis* is common in Mali, a second group of strains with a distinct spoligotype signature makes up 40% of strains, suggesting that another clonal complex of *M. bovis* is present in this country. For the Af2 clonal complex things are more complicated and many minor clonal complexes can be found in the East African countries dominated by Af2. In Madagascar all strains of *M. bovis* lack spacers 4, 5, 8 and 10, suggesting the presence of another dominant clonal complex of *M. bovis* on this island; this clonal complex has provisionally been named African 3 (Af3) but has not been investigated further (Rasolofo Razanamparany *et al*., 2006). In most of mainland Europe, Algeria, Iran and Zambia many of the isolates of *M. bovis* have spoligotype patterns derived from that found in the vaccine strain BCG; this spoligotype pattern is the ancestral spoligotype pattern of *M. bovis* (Haddad *et al*., 2001; Kubica *et al*., 2003; Allix *et al*., 2006; Boniotti *et al*., 2009; Munyeme *et al*., 2009; Sahraoui *et al*., 2009). Without distinct spoligotype signatures it can be difficult to identify clonal complexes by spoligotype pattern alone. Whole genome sequencing (WGS) is likely to elucidate the phylogenetic relationships between isolates from these countries.

The Phylogeography of Af1, Af2, Eu1 and Eu2

The domination of the Eu1 clonal complex in the British Isles and its presence in many former British colonies raises the interesting possibility that the Eu1 clonal complex of strains may have been distributed from the British Isles in cattle. Domesticated cattle were introduced into former colonies from Europe, a region that has a long history of bTB, and it seems unlikely that the disease travelled from the former colonies to Europe. The similarity of the spoligotype patterns found in the British Isles and former trading partners and colonies also suggests that the global distribution of the Eu1 clonal complex may have happened only relatively recently (within 200–300 years). It has been suggested that the modern cattle types that were bred in the British Isles and subsequently distributed all over the world (Decker *et al*., 2009) would provide a perfect vehicle for the global dissemination of the dominant British clonal complex of *M. bovis* (Smith *et al*., 2011). For example, Hereford beef cattle that were bred in Herefordshire, UK, in the 18th century are the most widely distributed beef breed in the world and have been exported from the UK since the early 19th century (Porter, 1991). Hereford stock has since been used to improve many cattle breeds across the world and this may explain the presence of the Eu1 clonal complex in Kazakhstan where, in the early 20th century, Herefords were imported to improve the local breeds (Porter, 1991). Herefords were imported to Kazakhstan from England and Uruguay, both areas where Eu1 strains are common, and it is not unreasonable to suggest that this resulted in the introduction of the Eu1 clonal complex of *M. bovis* into Kazakhstan. If Hereford beef cattle from the UK initially distributed the Eu1 clonal complex it has not remained within that breed; Eu1 has also been isolated from dairy cattle throughout the world (Smith *et al*., 2011). Interestingly, not all former British colonies are dominated by the Eu1 clonal complex; strains of the Eu1 clonal complex are rare in the former British colonies of Nigeria, Uganda and Tanzania (Muller *et al*., 2009; Berg *et al*., 2011).

If the initial vehicle for the global distribution of Eu1 were Hereford cattle then the dispersal of this clonal complex to countries throughout the world is more complex than direct export from the UK. The movement of domesticated cattle to Australia, South Africa and New Zealand was not directly from the UK but involved either former British colonies or parts of South America where the Eu1 clonal complex is now common. Finding Eu1 strains in South Korea also shows a complex secondary dispersal of this clonal complex. South Korea repopulated its cattle herds with stock from Australia, Canada and the USA in the 1950s and 60s after the Korean war (Wee *et al*., 2009) and we have suggested that the Eu1 clonal complex of *M. bovis* was introduced from one of these sources (Smith *et al*., 2011). Although this clonal complex may have been initially distributed from the UK to former British colonies and trading partners in specialized cattle breeds such as Herefords, it has subsequently been dispersed throughout the world by international trade in cattle. If the British Isles were the originator of the global dispersal of the Eu1 clonal complex it should be pointed out that bTB must have been imported into these islands sometime in the last 10,000 years or so since the last Ice Age. It is interesting to note the high percentage of Eu1 strains in Spain and Portugal and that recolonization of the British Isles by fauna from the Iberian Peninsula after the last Ice Age is not unheard of (O'Meara *et al*., 2012). It is possible that bTB in the British Isles originated in the Iberian Peninsula and was introduced since the last Ice Age.

The Eu2 clonal complex of *M. bovis* is common in the Iberian Peninsula and it has been suggested that a group of *M. bovis* strains entered the Iberian Peninsula on the land route through the Pyrenees or on maritime routes along the Mediterranean, and that these founder strains subsequently underwent expansion, colonizing cattle naïve to bTB in the Iberian Peninsula (Rodriguez-Campos *et al*., 2013). The origin of Spanish and Portuguese *M. bovis* may well be revealed when whole-genome sequencing becomes available for French and Italian strains. It is interesting to note that strains with the Eu2 spoligotype spacer signature (absence of spacer 21) are common in Brazil as well as South Africa, suggesting that strains of the Eu2 clonal complex originating from the Iberian Peninsula may have a global distribution.

The widespread dispersal of Eu1 (and perhaps Eu2) can be contrasted with the Af1 and Af2 clonal complexes which are both localized to specific parts of Africa. It has been suggested that for both these African clonal complexes the simplest explanation for their distribution is that an ancestral strain, bearing both the deletion that defines each clonal complex and the spoligotype signature, spread throughout each of the regions where these clonal complexes are found. Subsequently, country-specific population structures could have arisen within each country. In this respect these African clonal complexes are like the Eu1 clonal complex in many countries such as Australia and New Zealand where the presence of the Eu1 clonal complex may reflect the unique introduction of the Eu1 clonal complex. Spoligotype surveys have shown that the Af1 clonal complex is virtually the only *M. bovis* clonal complex present in three of the West–Central African countries where this clonal complex is found, and this observation has led to the suggestion that the Af1 clonal complex spread between these countries in cattle naïve to bTB. However, in East Africa where the Af2 clonal complex is localized, non-Af2 strains of *M. bovis* are present at reasonably high frequency (between 5% and 33%). There is no obvious relationship between the spoligotype patterns of these non-Af2 strains identified in East Africa and whole-genome sequencing of selected isolates, suggesting that these non-Af2 strains are not phylogenetically related to the Af2 clonal complex (unpublished data). The non-Af2 strains may have been present before the introduction of the Af2 clonal complex or may have been introduced from neighbouring countries that have not yet been surveyed for bTB. However, the phylogeography of bTB in East Africa may be resolved as the genotypes surveys from neighbouring countries and whole-genome sequencing of the isolates becomes available.

Global Distribution of Other Animal-adapted Strains of the MTBC

It is reasonable to assume that the global distribution of *M. bovis* has resulted from international movement of its main host, domesticated cattle, and the same widespread distribution is also true for *M. tuberculosis*, the human-adapted member of the Mtb complex, which has been distributed worldwide by man. We can contrast the global distribution of *M. bovis* and *M. tuberculosis* with the other animal-adapted clades or ecotypes (Smith *et al*., 2006b) of the Mtb complex, such as *M. africanum*, *M. microti*, *M. pinnipedii* and *M. caprae* which, in general, are geographically localized to parts of one continent (Aranaz *et al*., 1999; Kubica *et al*., 2003; Duarte *et al*., 2008; Smith *et al*., 2009; de Jong *et al*., 2010). However, the distribution of these different animal-adapted clades does raise a number of questions.

It is generally well known that strains of *M. africanum* (Lineages 5 and 6, West Africa 1 and 2) are geographically localized to West Africa (de Jong *et al*., 2010; Comas *et al*., 2013) whereas the new Lineage 7 strains of *M. tuberculosis* are, at the moment, localized to the Horn of Africa (Firdessa *et al*., 2013). It has been noted that it is surprising that *M. africanum* is rarely isolated in the New World where it would be expected following the transport of millions of individuals from West Africa during the transatlantic slave trade (Gagneux, 2012). One possible explanation for the absence of *M. africanum* in America is that it was not at high frequency in West African humans during the period of the slave trade (mid-16th to early 19th centuries). In a similar manner it is surprising that strains of *M. caprae*, a very close relative of *M. bovis* and capable of infecting domesticated cattle, is rarely found outside of Spain and Central and Western European countries (Rodriguez *et al*., 2011), although I note the report of *M. caprae* from cattle in China (Zeng *et al*., 2013). Why is *M. caprae* not found in South America?

Finally, *M. microti* and *M. pinnipedii* shared a most recent common ancestor after their divergence from the main animal-adapted lineage (Brosch *et al*., 2002; Smith *et al*., 2006a,b), and yet the global distribution and host distribution of these clades seems to be at odds with their close phylogenetic relationship. *M. pinnipedii*, except for isolates recovered from zoos, is associated with marine mammals from the southern hemisphere (Cousins *et al*.,

2003; Loeffler *et al*., 2014), whereas *M. microti* is primarily associated with small mammals in Europe, mainly Britain but also France and Italy (Burthe *et al*., 2008; Smith *et al*., 2009; Panteix *et al*., 2010). Strains of *M. microti* have never been recovered in Ireland, which suggests that the distribution of this organism and its primary host in Western Europe is associated with the last Ice Age. But, overall, it is difficult to reconcile the geographical distribution and host affinity of these two clades with their close phylogenetic relationship.

The Future: Whole-genome Sequencing

Whole-genome sequencing has now become so cheap and simple, and the data so easy to produce, that it can revolutionize our understanding of the global distribution, phylogenetic relationships and host adaptations of the clades within the Mtb complex. It is easy to predict that the application of 'desktop' whole-genome sequencing machines will have the greatest impact on the molecular epidemiology of bTB (Roberts *et al*., 2014). However, to fully exploit this technology on a global scale will require a change in the way molecular epidemiologists do business. Whole-genome sequencing merely produces data for further analysis. It is not necessary – indeed it is probably counter-productive – that these data are produced on a local, regional or even national level. Expert and experienced international centres for whole-genome sequencing can rapidly, cheaply and at high quality produce standardized data that can be returned for local analysis. Whole-genome sequencing data should be considered as a 'resource' that is bought for further analysis locally.

I, for one, am looking forward to this 'brave new world' of rapid and cheap whole-genome sequencing which will, without doubt, shed light on some of the questions posed above.

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8 Immunopathogenesis of *Mycobacterium bovis* Infection of Cattle

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Introduction

Infection with the tubercle bacillus, and the ensuing immunopathogenesis, is a quintessential example of the complex and ancient interplay between host and pathogen (reviewed in Cooper and Torrado, 2012; Reece and Kaufmann, 2012). Upon entry into the host, *Mycobacterium tuberculosis* has a unique ability to delay onset of adaptive immune responses in mice or humans (reviewed in Ottenhoff, 2012). Likewise, adaptive responses are delayed with *M. bovis* infection of cattle (Vordermeier *et al*., 2009a; Waters *et al*., 2009). This delay in the adaptive response is likely to give the pathogen both a foothold for infection (a critical mass of tubercle bacilli) and a defined niche to dictate the ensuing response (Cooper, 2009). The tubercle bacillus is also armed with a multitude of immune evasion tactics enabling intracellular survival and persistence. Examples given by Ottenhoff (2012) include:

1. Inhibition of important host defence mechanisms such as phagosome maturation and phagolysosome fusion, autophagy, antigen processing and presentation, apoptosis and interferon (IFN)-γ receptor signalling.
2. Capacity to escape from the hostile phagosome to the cytoplasm.
3. Mechanisms to inhibit and scavenge toxic oxygen and nitrogen intermediates.

Once infection is established, primarily lymphocytes and macrophages are recruited to the site of infection in humans and cattle, forming granulomas. Granulomas are dynamic structures with continual movement of host cells into and out of the structure, orchestrated by the host as well as by the pathogen (Ramakrishnan, 2012). With humans, various stages of tuberculosis are recognized including active (either primary or post-primary), latent and reactivation or secondary disease (Barry *et al*., 2009; Young *et al*., 2009; Hunter, 2011). Primary disease occurs when immunocompetent individuals are first infected with *M. tuberculosis*. With this form, the bacillus typically spreads from the initial site of infection to regional lymph nodes (and potentially elsewhere) until immunity develops and the infection is controlled. Only 5–10% of individuals infected

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

with *M. tuberculosis* develop clinical manifestations of primary tuberculosis within 2 years after exposure. The majority of infected individuals develop latent tuberculosis, defined as having evidence of *M. tuberculosis* infection by tuberculin skin test or IFNγ release assay (IGRA) without clinical signs of disease (Lin and Flynn, 2010). Latent infection is a dynamic equilibrium in which the host controls but does not clear the bacillus (Behr and Waters, 2013). With *M. bovis* infection of cattle, the disease is generally considered slowly progressive, without clear delineation of the various stages associated with tuberculosis of humans (Pollock *et al*., 2006). As with human tuberculosis (originally described in Bayle, 1810; Laënnec, 1837), the disease in cattle may be defined microscopically according to the degree of fibrosis, necrosis, caseation and mineralization (Wangoo *et al*., 2005). Thus, there are many similarities in the immunopathogenesis of bovine and human tuberculosis, and a few distinct differences.

Aerosol and intratracheal inoculation are routinely used for experimental biology purposes to infect cattle with virulent *M. bovis*, each resulting primarily in a respiratory tract infection including lungs and lung-associated lymph nodes (reviewed in Vordermeier, 2010). Disease severity is dose- and time-dependent, closely mimicking natural infection of cattle (Buddle *et al*., 1994; Palmer *et al*., 2002). Recently, unique insights into *M. bovis* transmission have been gained through 'in contact' studies in which sentinel cattle are exposed to *M. bovis*-infected cattle in a model of natural infection (Khatri *et al*., 2012). With each of these routes of exposure, experimental approaches permit disease confirmation through post-mortem examination with laboratory analysis by histopathology and bacterial culture, defining the relationship between dose and route of infection, immune response and the pathogenesis of infection (Waters *et al*., 2012). The bovine infection model also provides significant opportunities to investigate the basis of genetic susceptibility and impacts of co-infection on pathogenesis and diagnostic techniques (Kao *et al*., 2007; Flynn *et al*., 2009; Claridge *et al*., 2012). Finally, access to naturally infected cattle provides a unique opportunity to evaluate vaccine and ante-mortem testing strategies, particularly as animals are often available for post-mortem inspection for infection confirmation as well as for gross and microscopic assessment of lesions. Thus, intervention strategies may be directly assessed, as opposed to being inferred based upon immunologic and clinical parameters.

Pathogenesis

The caseonecrotic granuloma is one of the hallmarks of tuberculosis. With the aim of gaining an understanding of the temporal evolution of the granuloma, researchers have performed sequential analysis of granulomas in experimentally infected cattle (Cassidy *et al*., 1998; Palmer *et al*., 1999). Microscopic granulomas can be seen as early as 7–15 days after experimental infection with 10^5 colony-forming units of *M. bovis* (Palmer *et al*., 2007). Early lesions, categorized as Stage I (initial, Fig. 8.1) granulomas (Wangoo *et al*., 2005; Palmer *et al*., 2007) are composed of accumulations of epithelioid macrophages with low numbers of lymphocytes, neutrophils and Langhan's multinucleated giant cells. Importantly, necrosis is absent in Stage I granulomas. Between 21 days and 60 days after experimental infection there is a steady progression through granuloma stages. Stage I granulomas are followed by Stage II (solid, Fig. 8.1) granulomas that are similar to Stage I granulomas but have central infiltrates of neutrophils and lymphocytes and a thin fibrous capsule. Central necrosis may be present. Stage III (necrotic, Fig. 8.1) granulomas exhibit complete fibrous encapsulation and significant central necrosis. Stage IV (necrotic and mineralized, Fig. 8.1) granulomas are characterized by multiple coalescing caseonecrotic granulomas with multicentric necrosis, dystrophic mineralization and thick fibrous encapsulation. By 60 days after experimental infection, granulomas of all four stages may be present on the same microscopic section of tissue. In fact, granulomas with morphology typical of Stage I may be seen in proximity to granulomas of more advanced stages, and are sometimes referred to as satellite granulomas. Acid-fast bacilli can be present in all stages

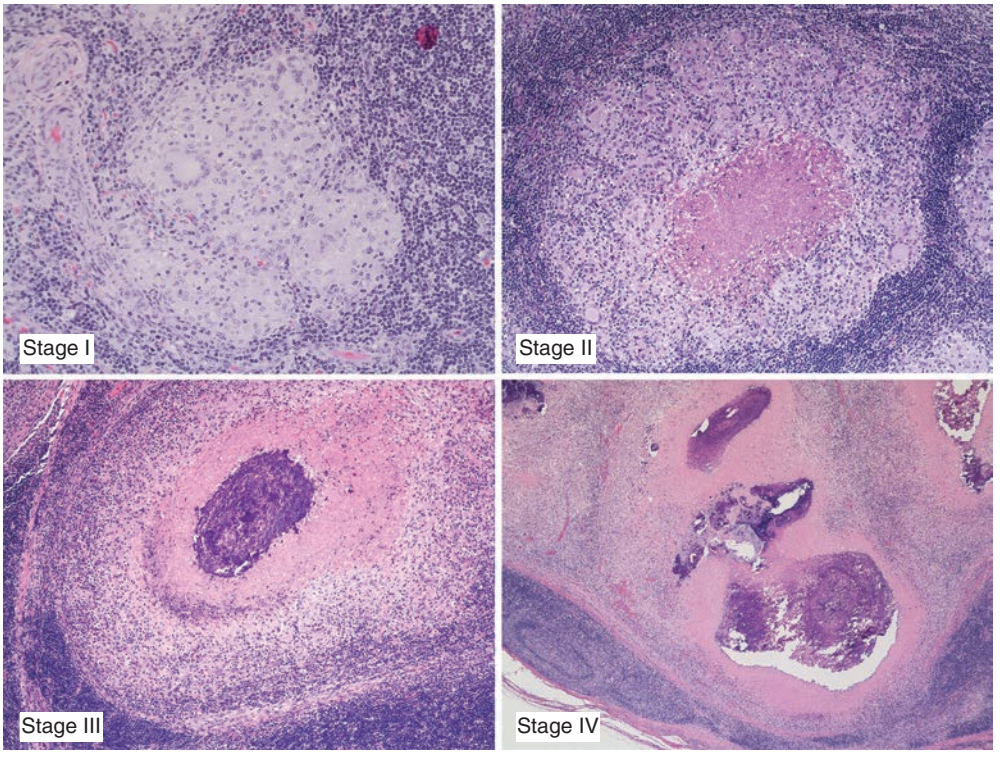

Fig. 8.1. Microscopic tuberculous lesions are staged (I–IV) based on adaptations of the criteria described by Wangoo *et al*. (2005). Stage I (initial) granulomas are characterized by accumulations of epithelioid macrophages with low numbers of lymphocytes and neutrophils. Multinucleated giant cells may be present but necrosis is absent. Acid-fast bacilli are often absent or present in low numbers within macrophages or multinucleated giant cells. Stage II (solid) granulomas are characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes may be present as well as multinucleated giant cells. Necrosis when present is minimal. Stage III (necrotic) granulomas are characterized by complete fibrous encapsulation. Necrotic cores are surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. Stage IV (necrotic and mineralized) granulomas are characterized by thick fibrous capsules, irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contain foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surround necrotic areas and there may be moderate to dense infiltratres of lymphocytes. Acid-fast bacilli are often present in moderate numbers primarily located within the caseum of the necrotic core. Magnifications: Stage I, 20×; Stage II, 10×; Stage III, 10× and Stage IV, 4×.

but have been shown to be most numerous in Stage IV granulomas (Palmer *et al*., 2007).

T-cell immunity is considered essential in clearance of mycobacterial infections. As such, $CD3^+$ $CD4^+$ T cells are the predominant lymphocyte subtype in granulomas of all stages. Lower numbers of $CD8^+$ T cells, γδ T cells, as well as B cells, can be found in Stages I–III. Stage IV granulomas may contain dense clusters of B cells in the periphery or outside the capsule. γδ T cells have been described in both early- (Palmer *et al*., 1999) and late-stage granulomas (Wangoo *et al*., 2005).

It has been shown that the dose of infection when administered within a relatively low dose range (i.e. 1–1000 colony-forming units, CFU) does not necessarily correlate to the level of pathology once infection takes place (Johnson *et al*., 2007). However, in this same study it was shown that the proportion of

cells expressing IFNγ was directly affected by the infection dose. Thus, the nature of the immune response observed at the site of infection may vary according to the initial bacterial load; the higher the infectious dose, the higher the proportion of IFNγ positive cells in the granuloma.

In another study, Aranday-Cortes *et al*. (2013) determined that the presence of certain cytokines and chemokines was related to the stage of progression of the granuloma. Thus, IL-17A and CXCL9 were found to be transcribed to a greater extent in type I as compared to type IV granulomas. CXCL10 was also found to be transcribed to a greater extent in type I as compared to type IV granulomas; however, this was not a linear relationship, as type II and type III granulomas expressed lower CXCL10 than type IV granulomas. These results indicate that there may be characteristic responses in the development of the granuloma that could inform whether a lesion is progressing or is being contained. However, the data also indicate that the immune response during tuberculosis is complex and may not necessarily follow a linear pattern of progression.

An intended goal of vaccination is to elicit a more rapid and targeted immune response upon subsequent exposure to the pathogen, thereby limiting granuloma development. Johnson *et al*. (2006) found that granulomas in cattle vaccinated with bacille Calmette-Guerin (BCG) subsequently challenged with *M. bovis* were reduced in number and size as compared to granulomas found in non-vaccinated cattle challenged with *M. bovis*. Importantly, the percentage area stained for lymphocytes (i.e. primarily $CD3^+$ and γδ TCR^+ cells) and macrophages (i.e. $CD68^+$ cells) was reduced in BCG-vaccinated as compared to non-vaccinated animals after challenge with virulent *M. bovis*. Additionally, staining for B cells (i.e. $CD79^+$ cells) and IFNγ clustered more intensely in the medullary regions of lymph nodes of vaccinated animals as compared to those of controls. Thus, the priming of cattle with BCG affects the subsequent immune response to the pathogen at the site of infection.

It is likely that the classification of the TB granuloma into stages of development as well as determining the immune response within and in the surrounding areas of lesions will inform future studies in vaccine and diagnostic developments. Dean *et al*. (2014) recently used the classification of the TB granuloma as a tool to determine the level of vaccine protection against *M. bovis*. Future studies aimed at finding correlates between peripheral and local immune responses will permit the development of tools to assist discrimination between vaccinated and infected animals, as well as defining responses favourable for controlling infection. This in turn will inform vaccine and diagnostic development.

Innate Immune Responses and Initiation of Adaptive Immune Responses

Dendritic cells and macrophages

Dendritic cells (DC) are a heterogeneous population of professional antigen-presenting cells (APC) which are essential mediators of immunity (Banchereau and Steinman, 1998) and tolerance (Steinman *et al*., 2003). They originate from stem cell precursors in the bone marrow which give rise to circulating myeloid or lymphoid precursors within the blood that enter tissues and reside as immature DC. These cells act as sentinels and respond to infection, inflammatory signals or tissue damage by migrating away from the periphery towards lymph nodes where immune responses may be induced. During migration DC undergo maturation characterized by an increased expression of costimulatory molecules, adhesion molecules, reduced endocytosis and redistribution of intracellular MHC II to the cell surface. Thus DC are primed to effectively stimulate naïve or memory T-cell responses within the lymph nodes.

In cattle there is evidence (as in other species) for considerable heterogeneity in DC populations: both functional and phenotypic divergence has been reported, and cells of both myeloid and lymphoid origin are present (Howard and Hope, 2000; Miyazawa *et al*., 2006; Reid *et al*., 2011). Subpopulations of DC draining the skin (McKeever *et al*., 1991; Howard *et al*., 1997; Brooke *et al*., 1998) and mucosal

surfaces have been described which have differential capacity not only to stimulate T-cell responses (Howard *et al*., 1997) but which also display divergent capacities to interact with mycobacteria. Monocyte-derived DC (MoDC) infected with *M. bovis* or BCG induced effective memory $CD4^+$ and $CD8^+$ T-cell responses (Hope *et al*., 2000). However, assessment of *ex vivo* populations of DC isolated from afferent lymphatic vessels revealed that only a subset could uptake and present antigens from BCG or *M. bovis* (Hope *et al*., 2012). This could impact vaccine efficacy or the induction of protective immunity to infection.

Macrophages are a diverse population of specialized phagocytic cells which are essential for host defence, homeostasis and wound repair. They are derived from bone marrow precursors and circulating blood monocytes which differentiate into resident macrophages or DC upon tissue entry (Verschoor *et al*., 2012). Macrophages are considered the major host cell for mycobacteria *in vivo* and, due to the respiratory route of entry, alveolar macrophages have a pivotal role during mycobacterial immune responses. Macrophages express an array of cell surface receptors that recognize mycobacteria including toll-like receptors (TLRs) (Means *et al*., 1999), the macrophage mannose receptor that identifies mannosylated glycoproteins, Fc receptors which can bind opsonized cells, and complement receptors (Ernst, 1998). The early interactions of mycobacteria with DC in the respiratory tract also involve TLRs including TLR2, 4 and 9 but, binding to dendritic cell specific intracellular adhesion molecule 3, grabbing non-integrin (DC-SIGN) is particularly important for uptake of *M. tuberculosis* (Means *et al*., 1999; Tsuji *et al*., 2000; Von Meyenn *et al*., 2006). The downstream events triggered by binding and uptake lead to divergent responses in macrophages when compared with DC with respect to bacterial killing/survival, cytokine and cell surface molecule expression, antigen presentation and trafficking, all of which can directly influence the outcome of infectious challenge.

In vitro bovine monocyte-derived macrophages and MoDC were equally phagocytic for *M. bovis* or BCG, yet higher numbers of bacilli were retained within MoDC compared with macrophages, reflecting preferential survival of mycobacteria within DC. In addition, DC infected with *M. bovis* or BCG up-regulated CD80, CD40 and MHC II, which are required for antigen presentation and stimulation of T-cell responses. Coupled with the ability of DC to migrate towards lymph nodes this could indicate that infection of DC would prime T-cell immune responses to infection. Different cytokine profiles have also been reported for macrophages and DC in response to mycobacterial exposure: typically, MoDC secrete high levels of IL-12 associated with induction of IFNγ by T cells and NK cells (Denis *et al*., 2007) whereas macrophage responses are dominated by TNFα, IL-1β and IL-10 (Hope *et al*., 2004). In addition, infected MoDC and macrophages have been shown to express inflammatory chemokines (Widdison *et al*., 2011; Siddiqui and Hope, 2013) to attract NK cells and granulocytes *in vitro*. The expression profiles of key chemokines were significantly different between MoDC and macrophages (Siddiqui and Hope, unpublished observations), suggesting the potential chemoattraction of different subsets of lymphocytes. *In vivo* chemokine gradients produced by *M. bovis*-infected APC would contribute to inflammatory influx into the tissues and granuloma formation.

Mycobacteria can persist within macrophages and DC by preventing phagosome maturation and evading lysosome fusion (Flynn and Chan, 2003; Nguyen and Pieters, 2005; Rohde *et al*., 2007) thereby evading intracellular destruction and reducing antigen presentation and subsequent T-cell responses. The production of nitric oxide (NO) is a key response contributing to the capacity of cells to control intracellular bacteria. In bovine studies, NO production was predominantly seen in macrophages (Denis and Buddle, 2008) and could be significantly enhanced by the addition of IFNγ (Piercy *et al*., 2007). The activation of macrophages with IFNγ promotes intracellular killing, at least in part through the induction of autophagy which has been shown to contribute not only to destruction of intracellular *M. tuberculosis* and BCG but also to increase antigen presentation to T cells (Gutierrez *et al*., 2004; Puleston and Simon, 2014).

Thus, early differential interactions of *M. bovis* with APC are central to the downstream events occurring post-infection. Further reciprocal stimulation of innate and adaptive cells

will contribute to the eventual outcome of exposure and determine whether disease or immunity ensues.

Natural killer cells

NK cells are large granular lymphocytes which are traditionally cells of the innate immune system but as essential drivers of the adaptive immune response, NK cells are found at the interface between innate and adaptive immunity. NK cells have two main effector functions: cytotoxicity of target cells through the release of preformed granules containing perforin and granulysin (Reefman *et al*., 2010) and cytokine production including IFNγ, TNFα, GM-CSF (Fehniger *et al*., 1999; Cooper *et al*., 2001; Boysen *et al*., 2006), IL-10 (Fehniger *et al*., 1999; Cooper *et al*., 2001) and IL-22 (Cella *et al*., 2009; Dhiman *et al*., 2009). NK cell function and maintenance of self-tolerance is determined by a complex interplay between activating and inhibitory NK cell receptors (Long *et al*., 2013). These germ line encoded receptors enable NK cells to distinguish cellular stress related ligands, MHC class I molecules and MHC class I-like molecules. In addition to key roles in innate defence, NK cells are located at the bridge between innate and adaptive immune responses through interactions with APC and have recently been shown to have features of adaptive immunity including 'memory'. Evidence for NK cell memory was first identified in mice (O'Leary *et al*., 2006; Sun *et al*., 2009) and human memory-like NK cells have been shown to migrate into tuberculous pleural fluid via a CXCR3 and CXCR4 axis (Fu *et al*., 2011, 2013).

Phenotypically distinct subsets of NK cells with divergent functions have been described across species. Human NK cells are defined as CD3- with variable expression of CD56 and CD16 (Cooper *et al*., 2001). Similarly, discrete subsets of NK cells also exist in mice which are differentiated based on their expression of CD27 and CD11b (Chiossone *et al*., 2009). NKp46 (CD335), a natural cytotoxicity receptor expressed exclusively by NK cells, is a pan species marker that identifies this heterogeneous cell population (Moretta *et al*., 2005; Walzer *et al*., 2007b). The development of a monoclonal antibody specific to this receptor has allowed for the characterization of NK cells in cattle (Storset *et al*., 2004). Bovine NK cells can be subdivided into two subsets based on their differential expression of the adhesion/costimulatory molecule, CD2 (Boysen *et al*., 2006). The NKp46+ CD2+ and NKp46+ $CD2^{-}$/low (referred to as $CD2^{-}$ herein) subsets are predominantly localized within the peripheral blood and lymph nodes/afferent lymph respectively (Boysen *et al*., 2006; Lund *et al*., 2013) and these subsets have divergent functions with the $CD2^{-}$ NK cells being more functionally active (Boysen *et al*., 2006, 2008). Within bovine peripheral blood, NK cells account for 0.5–10% of the total lymphocyte population with an increased prevalence in neonatal calves, particularly those aged between 8 and 120 days old (Kulberg *et al*., 2004; Graham *et al*., 2009).

NK cells are responsive during mycobacterial infection *in vivo*. Following infection of mice with *M. tuberculosis* or *M. bovis* BCG, increased numbers of activated NK cells are recruited to the lungs where they secrete IFNγ (Junqueira-Kipnis *et al*., 2003). During studies of active *M. tuberculosis* infection in humans, NK cells infiltrate the lungs where they are localized to granulomas (Portevin *et al*., 2012). Furthermore, the importance of NK cell- and γδ T cell-derived IFNγ during BCG immunization of infants has been recently defined, highlighting the significance of innate effector cells during anti-mycobacterial immune responses (Zufferey *et al*., 2013). The frequency, phenotype and function of NK cells during *M. bovis* or *M. bovis* BCG infection in cattle have not yet been fully defined.

Early interactions between populations of innate immune cells, particularly NK cells and DC, are fundamental in determining the nature of the adaptive immune response. Nevertheless, direct activation of NK cells has been reported, for example binding of *M. bovis* BCG to NK cells via NKp44 (Esin *et al*., 2008) or recognition of *M. bovis* via TLR2 expressed by human NK cells (Marcenaro *et al*., 2008). However, for the most part, NK cells require indirect activation through interplay with APC in order to become functionally mature (Lucas *et al*., 2007). In cattle, Hope *et al*. (2002) described interactions between NK cells and DC in the context of mycobacteria. An NK-like

population was shown to proliferate and produce IFNγ in response to *M. bovis* BCG infected DC (Hope *et al*., 2002). More recently it was shown that preferential stimulation of $CD2^-$ NK cells occurred following co-culture of bovine NK cells with *M. bovis*-infected DC (Siddiqui and Hope, 2013). This subset was effectively recruited in response to chemokines secreted by the DC resulting in significant expression of IFNγ which could potentiate Th1 bias and the development of protective immunity.

A central role for IL-12 in the cross-talk between DC and NK cells is evident from a number of studies. DC-derived IL-12 acting in synergy with IL-18 is essential to drive cytokine production by NK cells (Ferlazzo *et al*., 2004; Chaix *et al*., 2008). Bovine DC-secreted IL-12 in response to *M. bovis* or *M. bovis* BCG (Hope *et al*., 2004) and blockade of IL-12 secretion partially abrogated the capacity of NK cells to produce IFNγ in response to infected DC (Siddiqui and Hope, 2013). Importantly, the ability of DC to produce IL-12 depends on rapid IFNγ production by NK cells (Gerosa *et al*., 2002; Piccioli *et al*., 2002; Mailliard *et al*., 2003), resulting in a positive feedback loop involving secretion of IL-12 and IFNγ from mature DC and NK cells, respectively. This demonstrates the reciprocal nature of NK-DC interactions with effects on both cell populations being evident. DC that have been in contact with NK cells were shown to increase IL-12 (Van Elssen *et al*., 2010), CCR7 expression and, in cattle showed enhanced expression of MHC class II (Siddiqui and Hope, 2013). Thus NK cells may influence the adaptive immune response by inducing effective APC.

Bovine NK cells stimulated with IL-2, IL-15, IL-2/12 or IL-12/15 restrict the replication of *M. bovis* BCG within monocyte-derived and alveolar macrophages (Endsley *et al*., 2006). Additionally, NK cells proliferate and produce IFNγ in response to interactions with *M. bovis*-infected macrophages, which enhances the release of IL-12 and NO by activated macrophages (Denis *et al*., 2007). Thus exposure of macrophages to NK cells augments mycobacteria killing. Whether bovine NK cells exposed to *M. bovis*-infected DC are increased in their cytolytic potential for immature DC, as demonstrated for human NK cells (Ferlazzo *et al*., 2004), remains to be elucidated.

Protective immunity against *M. bovis* infection in cattle is driven by Th1 polarized immune responses which are characterized by IFNγ production (Buddle *et al*., 2005). Interactions between NK cells and DC *in vivo* were shown in murine models to mediate the induction of Th1 responses through the secretion of NK cell-derived IFNγ (Martín-Fontecha *et al*., 2004). Co-localization between NK cells, DC and T cells has been demonstrated in lymph nodes indicating a role for innate immune cell interactions in shaping T-cell responses *in vivo* (Bajenoff *et al*., 2006; Lucas *et al*., 2007; Walzer *et al*., 2007a). Further studies are essential to determine if bovine NK cells, DC and T cells co-localize *in vivo* and whether the reciprocal interactions between NK cells and DC can influence downstream responses of $CD4^+$ and $CD8^+$ T cells such that protective immunity may be induced.

Polar and apolar lipid fractions

The first point of contact between *M. bovis* and its host is likely to be interaction between innate immune cells such as macrophages or dendritic cells (DC) and bacterial surface molecules, many of which are lipids or contain lipid moieties. A number of *M. tuberculosis* lipid compounds have been described to interact with receptors on DC or macrophages such as lipomannan (LM), lipoarabinomannan (LAM), phosphatidylinositol mannosides (PIMs), and cord factors trehalose mono- and dimycolate (TMM and TDM) as well as the phthiocerol dimycocerosates (PDIMs) (see Kremer and Besra, 2005, for details on mycobacterial lipids). These surface-bound mycobacterial lipids interact with a variety of innate surface receptors including Toll- (Underhill *et al*., 1999) and C-type lectin receptors such as DC-SIGN (Ehlers, 2010) modulating innate immune responses. For example, it has been shown that monomycolyl glycerol (MMG) modulates host immunity resulting in hypervirulence (Reed *et al*., 2004). However, few data exist which describe the effects of *M. bovis*-derived lipids on bovine innate immune cells with only a few studies using non-target-species animal models or compounds not directly isolated from

M. bovis. This is therefore a field that invites more and detailed work.

In a recent study (Pirson *et al*., 2012), *M. bovis* polar and apolar lipid preparations were prepared and assessed for biological activity in bovine monocytes, monocyte-derived macrophages and monocyte-derived DCs (MoDC). The apolar lipid preparation contained TDM, TMM, PDIMMs, PGL and MMG; while the polar fraction contained, among other lipids, a range of PIMS, penta-acyl trehalose and glucose monomycolate (GMM). These lipid fractions, particularly the polar fractions, altered the cytokine production profile by both fresh and cultured bovine monocytes as well as MoDC inducing significant levels of IL-10, IL-12, MIP-1β, TNFα and IL-6. Further, BoLA class II, CD86 and CD1b expression decreased on all cell types tested after exposure to the polar lipid fraction. Polar lipids also significantly increased CD40 expression only on monocytes and monocyte-derived macrophages but had no effect on its expression on MoDC. Finally, *M. bovis* polar lipid-treated macrophages were less capable as APC than untreated macrophages, while similar treatment of MoDC had no effect on their capability to stimulate allogenic mixed lymphocyte reactions. These data demonstrated that *M. bovis* lipids modulate innate cell responses and in particular may hamper the ability of the host APCs to induce an appropriate immune response. As noted above, differential responses of macrophages and DC may also be relevant to the induction of downstream immune responses. Recent studies (Pirson and Vordermeier, unpublished data) indicate that *M. bovis* lipids also stimulate IFNγ production by NK cells.

Cell-mediated Immune Responses: Correlates of Protection versus Pathology

IFNγ and delayed type hypersensitivity (DTH) responses

A clear feature of the bovine immune response to *M. bovis* infection is an early and persistent production of IFNγ in response to various *M. bovis* antigens, detectable both *in vivo* and within *in vitro* re-stimulation assays (i.e. IGRAs) (Pollock *et al*., 2001). Peripheral IFNγ responses to complex and specific mycobacterial antigens are consistently detectable 2–3 weeks after experimental infection (Waters *et al*., 2010). As with *M. tuberculosis* infection of humans, CD4, CD8 and γδ T cells (as well as NK cells) contribute to the response (Pollock *et al*., 2001; Endsley *et al*., 2009); however, T helper 1 (Th1) CD4 T cells are generally considered the predominant cell type responsible for the robust IFNγ response (Hope *et al*., 2000; Walravens *et al*., 2002). Clearly, CD4 T cells and an intact Th1 response are essential for control of *M. bovis* infection in cattle. These responses, however, are not sufficient for clearance as *M. bovis* infection almost invariably results in persistent and progressive disease. Given the importance of Th1 cells in the adaptive response to tuberculosis, it is not surprising that IFNγ release assays (IGRAs) and DTH (i.e. skin test) responses are useful correlates to infection (reviewed by Schiller *et al*., 2010). The level of these responses, however, do not necessarily correlate with the severity of infection (Waters *et al*., 2012). Most effective tuberculosis vaccines also elicit specific IFNγ and DTH responses (Black *et al*., 2002), but not all vaccines that induce IFNγ and DTH responses are protective against tuberculosis and the amount of IFNγ produced in response to vaccination does not necessarily correlate with the level of protection afforded by the vaccine (Mittrücker *et al*., 2007; Abebe, 2012; Waters *et al*., 2012). Also, protection is not linked with maintenance of a DTH response (Whelan *et al*., 2011a). With that said, non-sensitizing vaccines (e.g. doses of BCG that do not elicit a DTH response) have proven ineffective in vaccine efficacy studies with cattle (Buddle *et al*., 2011). Evaluation of IFNγ responses after experimental infection may also be useful indicators of protection, or lack thereof, in vaccine efficacy studies. Responses with diagnostic potential, such as IFNγ responses, are generally inversely correlated with responses that predict vaccine efficacy, particularly to antigens not included within the vaccine. For instance, increasing ESAT-6/CFP10-specific IFNγ responses after challenge is a negative indicator of vaccine efficacy (e.g. with BCG or *M. bovis* ΔRD1 mutant vaccines) as these responses have been shown

to positively correlate with tuberculosis-associated pathology (Vordermeier *et al*., 2002; Dietrich *et al*., 2005; Waters *et al*., 2009). Thus, failed vaccine approaches resulting in increasing antigen burden and associated pathological changes evoke immune responses such as ESAT-6/CFP10-specific IFNγ responses, indicative of infection.

T-cell effector and central memory (Tcm) subsets

Immunological memory develops naturally as a result of infection and memory T-cell responses are generally considered indispensable for vaccine-elicited protection against most intracellular infectious agents. However, the relationship between effector T-cell responses and long-lasting T-cell memory is not completely understood in humans (Todryk *et al*., 2009) or cattle (Waters *et al*., 2009, 2011). Vaccination strategies are often designed to generate memory cells capable of responding rapidly and efficiently upon subsequent infection. Pathogens and their derivatives are generally transported to lymphoid organs by APCs for initiation of T-cell responses. Initiation of this primary response may take days to weeks to develop, relying on exposure of naïve T cells to antigens in secondary lymphoid organs, expansion of antigen-specific T cells and homing of effector cells to the site of infection (Mackay *et al*., 1990). For instance, with both human and bovine tuberculosis, there is a 2–3 week delay in the response at the primary site of infection that is advantageous for the pathogen, enabling it to define the ensuing response (Ernst, 2012). After initial encounter with cognate antigen and subsequent proliferation/activation, the number of antigen-specific T cells increases up to ~10,000-fold (Hou *et al*., 1994; Murali-Krishna *et al*., 1998; Whitmire *et al*., 1998). In addition to clonal expansion, activated T cells differentiate and exhibit effector functions, expressing important mediators of pathogen control (e.g. IFNγ, TNF-α, IL-17 and cytotoxic granules). If the immune system successfully controls the infection, only a few memory cells remain as 90–95% of the antigen-specific T-cell population undergoes apoptosis (Wilkinson *et al*., 2009; Totté *et al*., 2010). Subsets of memory CD4 T cells in humans include: (i) central memory T cells (Tcm, $CD62L^{+}CCR7^{+}$) that preferentially localize to lymphoid tissues and exhibit great proliferation potential and IL-2 production; and (ii) effector memory T cells (Tem, $CD62L^{-}CCR7^{-}$) that preferentially localize to peripheral tissues and have immediate effector functions (Sallusto *et al*., 1999; Champagne *et al*., 2001; Woodland and Kohlmeier, 2009; Sallusto *et al*., 2010). Tem cells may remain blood associated, either circulating or contained within splenic red pulp or hepatic sinusoids. Upon restimulation, Tem cells undergo relatively little proliferation and secrete minimal IL-2 (Champagne *et al*., 2001; Sallusto *et al*., 2004, 2010). Short-term IFNγ responses to mycobacterial antigens measuring effector and Tem responses are utilized as a correlate to infection with tuberculosis tests for cattle and humans (e.g. IGRAs). Additionally, assessment of long-term IFNγ production (i.e. as a surrogate to Tcm responses) may be used to detect past *M. tuberculosis* infection in humans with negative IGRAs (Butera *et al*., 2009). Thus, measurement of effector and memory T responses may each provide diagnostic benefit.

Upon stimulation by mycobacterial antigens (e.g. rESAT-6:CFP10 or *M. bovis* PPD), bovine peripheral blood CD4, CD8 and γδ T cells from *M. bovis*-infected cattle proliferate and display an activated phenotype (i.e. ↑CD25, ↑CD26, ↑CD44) after 3–6 days in culture (Waters *et al*., 2003; Maue *et al*., 2005). Mycobacterial antigen-activated CD4 cells also decrease expression of CD62L and increase expression of CD45RO (associated with memory T cells) while decreasing expression of CD45R (associated with naïve T cells phenotype) (Maue *et al*., 2005). The variant splices A, B and C of CD45 receptor are not described for cattle (Bembridge *et al*., 1995). These findings demonstrate that cattle exhibit an expected T-cell effector phenotype upon antigen activation within short-term cultures. Additionally, with bovine tuberculosis vaccine efficacy studies, long-term (14 day cultures) T-cell responses (so-called cultured ELISPOT responses, Vordermeier *et al*., 2006) negatively correlate with mycobacterial burden (Waters *et al*., 2009) and tuberculosis-associated pathology and positively with vaccine-induced protection (Whelan *et al*., 2008; Vordermeier *et al*.,

2009b). With this assay, T-cell lines are generated via stimulation of PBMC with mycobacterial antigens including Ag85A, TB10.4 and *M. bovis* PPD. Effector T-cell responses wane over time and memory cells are sustained via addition of IL-2 and fresh medium. After 13 days of culture, cells are washed, transferred to plates containing autologous APCs, cultured overnight and the ensuing response measured by IFNγ ELISPOT. Studies with samples from humans have demonstrated that the responding cells within these long-term cultures (up to 14 days) are mainly Tcms and that this response, in contrast to effector responses, correlates with better infection outcomes (Todryk *et al*., 2009). With cattle, BCG vaccination of neonatal calves induces significant protection against *M. bovis* challenge at 12 months but not at 24 months after vaccination. This loss of efficacy correlates with a significant reduction in the numbers of antigen-specific IFNγ-secreting cells within long-term PBMC cultures (Thom *et al*., 2012). Tcms are likely to contribute to the long-term cultured ELISPOT response to BCG vaccination by cattle, although a lack of an anti-bovine CCR7 antibody has hindered this characterization. In the assessment of the migration pattern of γδ T cells, Vrieling *et al*. (2012) recently demonstrated that an anti-human CCR7 antibody cross-reacts with bovine CCR7 molecules. Recent studies have demonstrated that Tcm cells, with a minor contribution by Tem cells, are the primary cell type responding in long-term cultured IFNγ ELISPOT responses to *M. bovis* infection in cattle (Maggioli *et al*., 2012). Data on the response to vaccination and subsequent challenge to access the correlation between Tcm and Tem responses to protection/pathology are still lacking. However, these data demonstrate the potential for defining a protective signature elicited by vaccination to prioritize vaccine candidates for efficacy testing within calves.

IL-17 and IL-22

There is considerable interest in the role of IL-17 producing cells in the immune response to persistent infection (Cooper, 2010). Significant IL-17 responses are elicited by *M. tuberculosis* infection of mice and humans (Khader and Cooper, 2008; Jurado *et al*., 2012) and *M. bovis* infection of cattle (Vordermeier *et al*., 2009b). Early expression of IL-17 is required for rapid accumulation of protective memory cells in tuberculosis infection of mice (Khader *et al*., 2007); however, the absence of IL-17 during *M. tuberculosis* infection only modestly alters the inflammatory response (Khader *et al*., 2005; Umemura *et al*., 2007). γδ and other non-$CD4^+$ T cells are the primary producers of IL-17 during *M. tuberculosis* infection in mice. Early IL-17 produced by γδ T cells occurs prior to αβ T-cell priming, thus biasing the ensuing adaptive response (Lockhart *et al*., 2006). However, excessive IL-17 responses may be detrimental, resulting in a destructive influx of granulocytes as well as increased amounts of MIP2, TNF-α and IL-6 within affected lungs (Cruz *et al*., 2010). Thus, the timing and amount of IL-17 produced in response to tuberculosis is critical for the balance between control of the bacilli and detrimental inflammatory responses.

With cattle, stimulation of PBMCs with either *M. bovis* PPD or Ag85A elicits IL-17 mRNA expression in samples from *M. bovis*-infected or BCG plus viral-vectored Ag85A vaccinated cattle (Vordermeier *et al*., 2009b). With BCG/Ag85A-vaccinated cattle, IL-17 responses to Ag85A 10 weeks after vaccination and prior to challenge negatively correlate with tuberculosis-associated pathology at post mortem, indicating that vaccine-elicited IL-17 responses are protective (Vordermeier *et al*., 2009b). Likewise, Rizzi *et al*. (2012) recently demonstrated that IL-17 mRNA expression is increased in response to *M. bovis* PPD stimulation in cattle vaccinated with a BCG strain over-expressing Ag85B; and, post-vaccination/pre-challenge IL-17 responses negatively correlated with lesion severity after experimental infection (Rizzi *et al*., 2012). IL-17 responses to *M. bovis* PPD detected after challenge, however, do not correlate with protection as there is a ~20-fold increase in IL-17 gene expression detected in samples from non-vaccinated, vaccinated/protected and vaccinated/non-protected groups (Vordermeier *et al*., 2009b). Additionally, IL-17 mRNA expression (60 and 90 days after experimental infection) in response to *M. bovis* PPD correlates with the presence of gross tuberculous lesions, suggesting that IL-17 may prove useful as a

biomarker of infection (Blanco *et al*., 2011). Using laser capture microdissection followed by qPCR, Aranday-Cortes *et al*. (2013) demonstrated increased IL-17A and IL-22 expression within tuberculous granulomas as compared to non-affected tissues from experimentally infected cattle. Expression was greatest in early lesions with decreasing expression in more advanced lesions, perhaps suggestive of down-regulation of IL-17A and IL-22 associated with expression of other immune-modulating cytokines. Further studies with bovine tuberculosis should prove useful for delineating potential roles for IL-17 and other Th17 cytokines (e.g. IL-22, Bhuju *et al*., 2012) in protective and detrimental responses to *M. bovis* in cattle.

γδ T cells

Bridging innate and adaptive immune functions, γδ T cells play a critical role in the early immune response to various infections of cattle including leptospirosis, anaplasmosis, paratuberculosis, theileriosis and tuberculosis (reviewed in Guzman *et al*., 2012). The frequency of γδ T cells within the peripheral lymphocyte pool in humans and mice is ~5–10% (Kabelitz, 2011) whereas γδ T cells constitute up to 60% of the circulating lymphocyte population in neonatal calves and ~25% in adult cattle (Hein and Mackay, 1991; Jutila *et al*., 2008). Populations of bovine γδ T cells are commonly divided based upon their expression of Workshop Cluster 1 (WC1) (Mackay *et al*., 1989; Clevers *et al*., 1990; Morrison and Davis, 1991; Machugh *et al*., 1997), a transmembrane glycoprotein and member of the scavenger receptor cysteine rich (SCRC) superfamily, which includes CD163, CD5, CD6 and DMBT1 (Sarrias *et al*., 2004). The majority of bovine peripheral blood γδ T cells express WC1, and it is the $WC1^+$ subpopulation that has been shown to respond to *M. bovis* infection, although recent studies indicate that $WC1^-$ γδ T cells are also responsive (McGill *et al*., 2014).

In cattle, γδ T cells undergo dynamic changes in distribution after *M. bovis* infection, with a marked decrease in the circulating pool shortly after infection (Pollock *et al*., 1996; Cassidy *et al*., 1998). The initial decline of peripheral blood γδ T cells has been attributed to trafficking to the site of infection as circumstantially evidenced by the accumulation of γδ T cells within tuberculous lesions of cattle (Cassidy *et al*., 1998; Palmer *et al*., 2007). Following the initial decrease in circulation, percentages of $WC1^+$ γδ cells within the peripheral blood of *M. bovis*-infected cattle increase with a concomitant increase in CD25 expression, indicating an active response to infection (Pollock *et al*., 1996). Similarly, $WC1^+$ γδ T cells are among the first cells to accumulate at the site of DTH responses following PPD injection of *M. bovis*-infected cattle (Doherty *et al*., 1996). And, as with mice (Dieli *et al*., 2003), bovine γδ T cells rapidly infiltrate lungs as well as lung- and head-associated lymphoid structures 1 week after intranasal BCG vaccination (Price *et al*., 2010). These findings demonstrate that bovine γδ T cells traffic to sites of mycobacterial infection, *in vivo*.

With *in vitro* studies, γδ T cells from *M. bovis*-infected cattle proliferate and produce IFNγ in response to stimulation with complex *M. bovis* antigens – including PPDs, whole cell sonicates and culture filtrate proteins (Rhodes *et al*., 2001; Smyth *et al*., 2001) – as well as specific proteins such as Ag85, MPB83, hsp 16.1 and ESAT-6/CFP10 (Rhodes *et al*., 2001; Maue *et al*., 2005). Human and murine γδ T cells proliferate and secrete cytokine in response to both protein and nonprotein phosphoantigens of *M. tuberculosis* (Haregewoin *et al*., 1989; Born *et al*., 1990; Morita *et al*., 1995; Fournie and Bonneville, 1996). Welsh *et al*. (2002) demonstrated that γδ T cells from *M. bovis*-infected cattle respond to pyrophosphates, including isopentyl pyrophosphate (IPP) and monomethyl phosphate. γδ T cells from *M. bovis*-infected cattle also respond to mycolylarabinogalactan peptidoglycan (a complex mycobacterial cell wall component, Vesosky *et al*., 2004) as well as antigens within proteinase-K treated *M. bovis* whole cell sonicates (Welsh *et al*., 2002). Thus, it is evident that bovine γδ T cells respond to a variety of antigens upon *M. bovis* infection; however, the role of this response (i.e. protective versus detrimental) is unclear.

γδ T-cell deficient mice are able to temporarily control BCG (Ladel *et al*., 1995) and

low-dose *M. tuberculosis* infection (D'Souza *et al*., 1997), but exhibit a more severe inflammatory response as compared to control mice, suggesting a role for γδ T cells in granuloma formation and maintenance. In agreement, depletion of $WC1^+$ γδ T cells from SCID-bo mice prior to *M. bovis* infection significantly alters the architecture of the developing granuloma (Smith *et al*., 1999). In contrast, depletion of $WC1^+$ γδ T cells from *M. bovis*-infected cattle has no effect on disease severity or granuloma formation (Kennedy *et al*., 2002). Instead, these animals exhibit a reduction in early IFNγ production and an increased skewing towards a Th2 type response, suggesting a role for γδ T cell-derived cytokines in establishing Th1 immunity. Along these lines, incubation of $WC1^+$ γδ T cells with autologous *M. bovis*-infected dendritic cells induces enhanced expression of MHC II and CD25, as well as increased secretion of IFNγ by the γδ T cells (Price and Hope, 2009). Dendritic cells, in turn, produce increased levels of IL-12 when incubated with γδ T cells, suggesting that reciprocal interactions between dendritic cells and $WC1^+$ γδ T cells may occur *in vivo*. Together, these findings support the notion that early and specific γδ T-cell responses bias the immune response in favour of a Th1 response. Additional functions of γδ T cells in the immune response to mycobacteria include IL-17 production (Lockhart *et al*., 2006; Umemura *et al*., 2007; McGill *et al*., 2014) direct cytotoxicity (Stenger *et al*., 1998; Skinner *et al*., 2003) and – potentially – regulatory functions (Guzman *et al*., 2012). However, further studies are required to fully define these functions with *M. bovis* infection in cattle.

Cytotoxic T lymphocytes (CTLs)

Cytolysis of mycobacteria-infected cells can result in direct killing or release of bacilli for eventual killing via other mechanisms (reviewed by Flynn and Chan, 2001). $CD8^+$ T cells are the primary cell type performing CTL functions with mycobacterial infections; however, other lymphocyte populations such NK cells, $CD4^+$ T cells and γδ T cells also have cytolytic capacity (Stenger *et al*., 1998; Canaday *et al*., 2001). Primary effector functions of $CD8^+$ T cells are production of IFNγ necessary for macrophage activation and lysis of infected macrophages (Einarsdottir *et al*., 2009) and production of perforin and granulysin essential for CTL function via pore formation and antimicrobial functions, respectively. Additionally, IFNγ from $CD4^+$ T cells is required for effective $CD8^+$ T-cell responses (Green *et al*., 2013); thus, CTL functions are essential for the control of mycobacterial infections and $CD4^+$ T-cell responses are supportive of this response.

CTLs are also implicated in the host response to bovine tuberculosis. Antigen-specific CD8 T cells cause release of viable *M. bovis* from infected bovine macrophages, indicating CTL activity (Liebana *et al*., 2000). Activated $CD8^+$ T cells are detectable within the lymphocytic outer core of early-stage bovine tuberculous granulomas, indicating a potential role for these cells in the initial containment of the bacilli (Liebana *et al*., 2007). Bovine T cells also express a homologue of human granulysin, a potent antimicrobial protein stored in association with perforin in cytotoxic granules (Endsley *et al*., 2004). Additionally, antigenic stimulation of peripheral $CD4^+$ T cells from BCG-vaccinated cattle results in enhanced anti-mycobacterial activity against BCG-infected macrophages linked with increased perforin and granulysin transcription (Endsley *et al*., 2007). Expression of the bovine granulysin gene can be induced in $CD4^+$, $CD8^+$ and γδ T cells resulting in anti-mycobacterial activity (Endsley *et al*., 2004, 2007). Granulysin and granzyme A mRNA are detectable within granulomas of *M. bovis*-infected cattle (Endsley *et al*., 2004; Aranday-Cortes *et al*., 2012). Granzymes are a group of serine proteases released by $CD8^+$ T cells and NK cells in cytoplasmic granules along with perforin. Granulysin and perforin gene expression are also up-regulated in peripheral blood $CD4^+$ and $CD8^+$ T cells in both BCG- and *M. bovis* ΔRD1-vaccinated calves (protected) as compared with non-vaccinated (not protected) calves (Capinos Scherer *et al*., 2009). Thus CTLs are involved in the bovine immune response to *M. bovis* infection; however, further studies are required to determine their exact roles in protection and pathogenesis.

Polyfunctional T cells

Polyfunctional T cells simultaneously produce two or more cytokines in response to antigen, and higher frequencies of these cells are associated with control of chronic infections such as HIV, hepatitis C, leishmaniasis, malaria and tuberculosis (reviewed in Wilkinson and Wilkinson, 2010; Caccamo and Dieli, 2012). The majority of studies with human tuberculosis indicate that polyfunctional T-cell responses are associated with active disease (Sutherland *et al*., 2009; Wilkinson and Wilkinson, 2010); however, others indicate a protective role (Geluk *et al*., 2012). Few studies have been performed on the evaluation of polyfunctional T cells in cattle, largely due to the lack of necessary reagents. Whelan *et al*. (2011b) recently described the development of an assay to detect polyfunctional CD4 T cells in *M. bovis*-infected (natural infection) cattle. Bovine polyfunctional CD4 T cells exhibited a characteristic $CD44^{hi}$ $CD62L^{lo}$ $CD45RO^{+}$ TEM phenotype. Interestingly this study, as well as recent as yet unpublished results, suggests that polyfunctional $CD4^{+}$ T cells are associated with pathology rather than protection (Whelan *et al*., 2011b; Whelan, Villarreal-Ramos and Vordermeier, unpublished data). That a more diverse cytokine profile can be reflective of more severe disease has also recently been highlighted by the observation that cattle producing both IL-2 and IFNγ (measured by ELISA after *in vitro* stimulation) are more likely to present with visible pathology at post mortem than those that produce IFNγ only (Rhodes *et al*., 2014). Further studies are warranted to determine the exact role of polyfunctional T cells in the response to *M. bovis* infection as well as vaccination.

B Cells and Antibody Responses

Role for B cells?

Specific roles for B cells in the immune response to tuberculosis are generally considered supportive, rather than essential (reviewed by Maglione and Chan, 2009). Functions attributed to B cells in the response to tuberculosis include antigen presentation, APC regulation via Fc receptors, immune modulatory actions of immune complexes and antibody-dependent cytotoxicity. B cell aggregates are consistently detected in association with tuberculous lesions in mice, cattle, non-human primates, humans and other host species infected with *M. tuberculosis*-complex organisms. These tertiary structures contain naïve, memory and plasma cells as well as intermixed $CD4^{+}$ and $CD8^{+}$ T cells, follicular dendritic cells and mycobacteria-laden APCs (Ulrichs *et al*., 2004). Using immunohistochemistry, Aranday-Cortes *et al*. (2013) demonstrated the presence of B cells ($CD79a^{+}$ cells) within granulomas of tuberculous cattle. In that study, early granulomas (Stages I and II) displayed scattered B cells, whereas more advanced granulomas (Stages III and IV) showed satellite nests of $CD79a^{+}$ cells located peripherally and outside of the fibrous capsule. In mice, formation of B cell follicles within infected lung tissues is dependent upon IL-23 and CXCL13, and CXCL13 production is dependent upon IL-17A and IL-22 in this response (Khader *et al*., 2011). The presence of ectopic germinal centres indicates that the *M. tuberculosis* complex – and the ensuing inflammation – induces active B cell clusters that modulate the host response. Thus, these follicles provide at least a partial framework for coordinated immune control of mycobacterial growth in the affected tissues (Ulrichs *et al*., 2004).

Tuberculin boost

Several antibody-based tests have recently emerged for use in cattle, captive cervids, several wildlife reservoirs of *M. bovis* and various zoo species (most notably elephants). Intradermal tuberculin administration is known to significantly boost antibody responses in tuberculous cattle and cervids, but not in non-infected animals (Lyashchenko *et al*., 2004; Harrington *et al*., 2008). This phenomenon can be observed from 1 to 2 weeks to several months after tuberculin injection (Palmer *et al*., 2006; Chambers *et al*., 2009). The absence of seroconversion in non-infected animals and the short-lived antibody kinetics with features of an anamnestic response strongly suggest

that the tuberculin-boosted antibody response is due to memory B cells originally primed by mycobacterial infection that can be quickly activated upon re-stimulation by tuberculin administration.

Correlations to pathology

Cellular immune responses elicited by mycobacterial infections of cattle generally correlate with infection but not necessarily with the level of pathology (Waters *et al*., 2010). Inoculation of cattle with *M. tuberculosis*-complex strains that are attenuated in cattle (e.g. *M. tuberculosis* $H_{37}R_v$ or *M. bovis* Ravenel results in relatively robust cell-mediated immune responses and persistent colonization with minimal to no lesions. With *M. kansasii* inoculation, cell-mediated immune responses are elicited without detection of the organism or associated lesions. As expected, inoculation of cattle with virulent *M. bovis* (field strains such as 95-1315 or A2122/97) results in robust cell-mediated immune responses, persistent colonization and associated tuberculous lesions. Similar results were observed following the intratracheal inoculation of cattle with a virulent *M. bovis* strain (WAg201) and three attenuated *M. bovis* strains. One attenuated strain had a mutation in the *inh*A gene (WAg405, daughter strain of WAg201), a second strain (ATCC 35721) with a mutation in the principal sigma factor, *rpo*V gene and BCG (Wedlock *et al*., 1999). Extensive macroscopic lesions were found only in cattle inoculated with the virulent strain, while strong antigen-specific IFNγ and IL-2 were induced by all *M. bovis* strains. Interestingly, the virulent strain and two of the attenuated strains (WAg405 and ATCC 35721) elicited strong DTH responses to bovine PPD in the skin test in comparison to BCG and this correlated with rapid *in vitro* proliferation in unstimulated bovine alveolar macrophages and proinflammatory cytokine gene expression. Thus, regardless of the pathological and mycobacterial burden outcome, cell-mediated immune responses are elicited. In contrast, antibody responses generally correlate with levels of pathology associated with mycobacterial infections (Wedlock *et al*., 1999; Waters *et al*., 2010). For instance, mycobacterial-specific antibody is detectable relatively early after *M. tuberculosis* challenge of cattle, yet these responses wane over time, and this is likely to be coincident with the reduction of *M. tuberculosis* colonization. In contrast, with virulent *M. bovis* infection that leads to persistent infection and significant pathology, antibody responses persist, probably due to increasing antigen burden. With less virulent mycobacteria (e.g. *M. kansasii*), antibody responses are elicited and then wane, likely to be coincident with clearance of the pathogen. Regardless of disease expression, mycobacteria-specific antibody responses may be boosted by re-exposure to mycobacterial antigens (e.g. PPD for skin test) or other live mycobacteria, thereby potentially confounding interpretation of serologic tests.

Transmission of *Mycobacterium bovis* from Cattle to Cattle

In most countries with active control programmes, affected cattle herds contain low numbers of infected animals, suggestive of low rates of cattle-to-cattle transmission (Palmer and Waters, 2006). Entry of *M. bovis* into cattle herds may occur via cattle-to-cattle transmission through purchase of infected animals or contiguous spread (Goodchild and Clifton-Hadley, 2001), from wildlife reservoirs (de Lisle *et al*., 2002), or environmental exposure (Phillips *et al*., 2003). Transmission is generally by direct contact with tuberculosis-infected animals as the organism may occur in exhaled droplets, saliva, faeces, milk, urine, vaginal discharges, semen or exudate from tuberculous lesions (e.g. lymph nodes with draining tracts that communicate to the exterior). With *M. tuberculosis*, for example, airborne bacilli can be generated and carried in droplets ranging in size between 0.5 and 2 µm. Droplets of this size are significant in that they are readily dispersed and can remain suspended in air where they are dispersed (Segal-Maurer and Kalkut, 1994). To achieve airborne transmission, *M. bovis* must remain viable inside droplet nuclei. Gannon *et al*. (2007) found that 94%

viability was retained 10 min post-aerosolization, with a half-life of 1.5 h. Infection may also result from ingestion of infected feeds. Housing (e.g. cattle sheds, milking parlours) and crowding increases the contact of naïve animals with infected animals, enhancing the spread of this disease. Lesion distribution in tuberculous farmed cattle indicates aerosol infection, often as a result of exposure to small numbers of viable bacilli (Dean *et al*., 2005). In comparison, significantly larger numbers of bacilli are needed to initiate oral infection (Pollock *et al*., 2006).

M. bovis is also transmitted by indirect contact through contaminated feed and water, equipment or anything that mechanically transfers the organism between locations. Movement of infected animals resulting from transfer of ownership, sharing of breeding animals and fence-line contact with other herds is another common means of transferring the disease between herds and regions (Probst *et al*., 2010). This concept is also supported by Welby *et al*. (2012) where within-herd spread was a factor in herd level breakdowns, with trade in undetected infected animals posing a significant risk. Barlow *et al*. (1998) indicated that cattle movement is the likely cause of transmission in areas where *M. bovis*-infected reservoirs are absent. Also, cattle movements were more likely to be responsible for local breakdowns in isolated areas, rather than spread through wildlife (Bourne, 2007). Studies revealing social interactions also provide insight into the potential for cattle-to-cattle transmission. Sauter and Morris (1995) studying New Zealand cattle identified the herd dominance hierarchy and found that skin-test reactors were likely to be found in the top 50% within that hierarchy. The introduction and use of proximity data logging collars has improved social network tracing (Böhm *et al*., 2009), with up to 26 direct cattle-to-cattle interactions found per day. Cattle prominent in the social hierarchy are also likely to be more inquisitive and are therefore more likely to acquire infection from infectious herd cohorts (Böhm *et al*., 2009). Movement of cattle from TB endemic areas was seen to be a greater risk for TB breakdown compared to other variables (Gilbert *et al*., 2005). Specifically, Gopal *et al*. (2006) investigated herd breakdowns in north-east England and tracked 17 cases where skin-test reactors were traced to herds with the same genotype. Global trade agreements are increasingly being implemented to promote international trade of livestock, thereby increasing risks for inter-regional spread and distribution of TB over great distances.

A number of studies have examined nasal excretion as a source for transmission. Romero Tejeda *et al*. (2006) tested nasal exudates from Mexican cattle using PCR and found excretion from infected cattle. Menzies and Neill (2000) estimated that up to 20% of infected cattle can excrete *M. bovis*. McCorry *et al*. (2005) monitored nasal excretions from cattle infected under controlled conditions and identified two distinct periods of excretion, less than 30 days post-infection and greater than 80 days post-infection. However, transmission from infected cattle to naïve animals held under experimental conditions has not been a common finding; perhaps this was due to stringent airflow management for biological containment. Segal-Maurer and Kalkut (1994) reported that a single change of air reduced airborne *M. tuberculosis* by 67%. More recently, studies using an experimental model of bovine tuberculosis have demonstrated the complexity and unpredictability of cattle-to-cattle transmission (Khatri *et al*., 2012). Sentinel (non-infected) cattle were housed with *M. bovis*-infected cattle (naturally infected and from farms in England or Wales) in ten pens with six sentinels and four donors per pen. After 12 months of direct contact with donors, only 8 of 60 sentinels became infected, demonstrating a relatively low rate of transmission (13.3%) even under conditions of close and continued direct contact. Insights into disease transmission were obtained using molecular typing of *M. bovis* isolates obtained from donor and sentinel animals. Several patterns emerged. In two pens, transmission was likely to be due to an animal with extensive lesions, highlighting the highly infectious nature of animals with advanced disease. In another pen, the only donor with a spoligotype matching one of its infected receptor pen mates had a low pathology score and only a single visible lesion in a mediastinal lymph node, demonstrating that an animal with early-stage infection, and without visible lung pathology, can also be

infectious. In two other pens, the spoligotype pattern of isolates from the receptor cattle did not match spoligotype patterns from any of the lesions from donor animals, indicating either indirect contact via shared airspace (i.e. pen to pen transmission) or through transfer of *M. bovis* by husbandry procedures. Interestingly, in one of these two pens, the spoligotype of the *M. bovis* strain from one of the sentinel animals did not match a spoligotype from any of the donor animals in the study, potentially suggesting transmission from a donor with no visible lesions. This study clearly demonstrates the difficulties in determining the exact nature of transmission events with bovine tuberculosis herd breakdown events. Use of whole-genome sequencing may prove useful for the unravelling of these complex and seemingly unpredictable transmission scenarios (Biek *et al*., 2012; Godfray *et al*., 2013). Additionally, after an animal becomes infected, the period during which it may become infectious will vary (Goodchild and Clifton-Hadley, 2001) as well as the time period that it is detectable as 'infected' – diagnostically. Francis (1946) estimated this period to be between 30 and 50 days although Barlow *et al*. (1998) suggested that 6–20 months is more likely, especially when cattle are tested on a regular basis.

Transmission of *Mycobacterium bovis* from Wildlife to Cattle

The eradication of bovine TB from cattle herds in many countries has been frustrated by the existence of wildlife reservoirs of infection, proving strong evidence of transmission of *M. bovis* from wildlife to cattle. *M. bovis* has a wide host range infecting many wildlife species, but only a limited number including brushtail possums in New Zealand; badgers in the UK and Ireland; white-tailed deer in Michigan, USA; elk and bison in Canada; and wild boar in Spain and Portugal serve as maintenance hosts of *M. bovis* infection (de Lisle *et al*., 2002). Since the 1970s there has been increasing evidence that these maintenance hosts serve as an important source of infection for cattle, supported by *M. bovis* typing studies identifying common strains in wildlife and cattle in the same areas (Collins, 2011).

Studies in New Zealand have shown that a reduction of possum numbers by culling in a locality is followed by a reduction in the annual incidence of TB in cattle (Tweedle and Livingstone, 1994; Caley *et al*., 1999), and infection in livestock increased if infected possums returned (Ryan *et al*., 2006). A characteristic of wildlife as a source of infection was the extreme rarity of severely infected cattle and most herds had only one or two infected animals (Collins, 2011). Large-scale badger culling trials in the UK and Republic of Ireland have provided convincing evidence of transfer of infection from badgers to cattle. In the Randomized Badger Culling Trial (RBCT) undertaken in the UK, proactive culling resulted in a reduction in bovine TB incidence in cattle herds inside culled areas, but a temporary increase in adjacent areas. Reactive culling in response to herd breakdowns was associated with an increase of bovine TB in cattle, possibly as a result of social perturbations in badger social groups (reviewed by Wilson *et al*., 2011). Thus, non-selective culling may have positive and negative outcomes. Based on the results from the RBCT, Jenkins *et al*. (2008) suggested that the contribution of badgers to infection in cattle accounted for 50% in the experimental areas. Studies in the Republic of Ireland indicated that proactive culling had beneficial effects on the bovine TB incidence in cattle and there was no evidence that small-scale culling led to an increase in herd breakdowns (Griffin *et al*., 2005).

A reservoir of *M. bovis* infection in white-tailed deer in five counties in north-east Michigan, USA, has been a source of infection for cattle herds in this region. In a case-control study of farm risk factors for TB, Kaneene *et al*. (2002) found management practices to exclude deer from cattle areas were the most significant factors to reduce farm risk of TB. In Manitoba, Canada, an elk herd was implicated in an outbreak of *M. bovis* in 11 cattle herds surrounding Riding Mountain National Park, where co-mingling elk and cattle were feeding on the same hay bales and a unique strain of *M. bovis* was identified (Miller and Sweeney *et al*., 2013). In the regions of southern

Spain with highest cattle TB prevalence, wildlife species such as European wild boar and red deer showed a high prevalence of TB, which suggested that the disease was shared between domestic and wild hosts (Naranjo *et al*., 2008). In southern and eastern Africa, only four species are suspected to play a role as a maintenance host: buffalo, lechwe, and possibly greater kudu and common warthog. However, there has been no case of bovine spillback from wildlife to cattle confirmed, although indirect contacts between cattle and buffalo do occur at the periphery of several large conservation areas in southern Africa (de Garine-Wichatitsky *et al*., 2013).

Mechanisms to explain routes of transmission of *M. bovis* from wildlife to cattle include both direct and indirect contact. Direct aerosol spread between possums and cattle has been considered the principal route when cattle investigated terminally ill possums, with cattle attracted by the unusual behaviour of ill possums that do not show the avoidance behaviour of healthy animals (Sauter and Morris, 1995). In addition, terminally ill possums often have open draining lesions containing large numbers of organisms. In contrast, indirect contact between badgers and cattle has been considered the principal route for transmission of infection to cattle. Use of automated proximity loggers on badgers and cattle and at badger latrines located on pasture showed that direct contacts between badgers and cattle were very rare, while indirect contacts, visits to badger latrines by badgers and cattle were more common (Drewe *et al*., 2013). The detection of viable *M. bovis* at badger setts and latrines is strongly linked to the frequency of *M. bovis* excretion by infected badgers (Courtenay *et al*., 2006). Badger visits to farm buildings may also be important and badgers have been recorded defaecating and urinating in buildings, sometimes directly on to feed and regularly came within 2 m of housed cattle (Wilson *et al*., 2011). The transmission of infection between wildlife and cattle in Spain was considered most likely from indirect contact based on a study where camera traps were placed around at water spots, food bait stations and pasture on an extensive cattle farm also used for game hunting. Direct interactions between wildlife (deer and wild boar) and cattle were rare, while indirect interactions of wildlife followed by livestock were most frequent at water holes, suggesting water points as a hotspot for indirect interactions (Kukielka *et al*., 2013). Indirect contacts between white-tailed deer and cattle were considered the major route of transmission between the species and it has been demonstrated that experimentally infected deer transmitted *M. bovis* to cattle through sharing of feed in the absence of direct contact between the two groups of animals (Palmer *et al*., 2004).

Special Topics

Mycobacterium bovis-infected cattle with non-visible lesions: latency?

In contrast to *M. tuberculosis* infection in humans, the degree of latency resulting from *M. bovis* infection in cattle is unknown and controversial (Cassidy 2006; Alvarez *et al*., 2009). Most consider the disease as slowly progressive in cattle; however, given the high degree of genetic homology (~99.95%) between *M. bovis* and *M. tuberculosis* it is unclear why latency is a prominent disease stage in humans and has not been clearly defined in cattle. The difference may be host related as reactivation of latent *M. bovis* infection in humans has been demonstrated (Larsen *et al*., 2008) and *M. bovis* infection of Eurasian badgers results primarily in a prolonged non-clinical course of disease with minimal inflammation, suggestive of a latent state (Corner *et al*., 2011). Thus, there is potential for various disease outcomes with *M. bovis* infection that is host contingent.

Dormancy survival regulon (DosR) and enduring hypoxic response (EHR) genes are, in part, responsible for encoding dormancy related functions of *M. tuberculosis* (Lin and Ottenhoff, 2008), of which, many of these genes are conserved between *M. tuberculosis* and *M. bovis* (Alvarez *et al*., 2009). Responses to DosR and EHR antigens are detectable, albeit at low frequency (particularly with DosR antigens), in *M. bovis*-infected cattle (Jones *et al*., 2011). IL-1β responses to the EHR antigen Rv0188 are associated with responses

by infected animals exhibiting low or no pathology whereas responses to other EHR antigens are not correlated with disease status (Jones *et al*., 2011). While DosR and EHR antigens were identified with *in vitro* studies culturing *M. tuberculosis* under conditions mimicking latency (i.e. hypoxia and low levels of NO), responses to these antigens are not restricted to latent versus active *M. tuberculosis* infection of humans (Hinks *et al*., 2009; Schuck *et al*., 2009; Gideon *et al*., 2010). Thus, while *M. bovis* infection of cattle elicits responses to dormancy-associated antigens, it is still uncertain whether these responses are indicative of a latent stage of infection in cattle.

Latency is clinically defined as persistence of viable mycobacteria within a tuberculous lesion in a non-symptomatic host. On rare occasions, *M. bovis* is detected by culture or molecular techniques in tissues from animals without visible tuberculous lesions; however, it is uncertain if infected animals with no visible lesions would eventually develop progressive disease analogous to reactivation in human tuberculosis. Similarly, experimental infection of cattle with laboratory-adapted strains (i.e. *M. tuberculosis* H37Rv, *M. bovis* AN5 or *M. bovis* Ravenel) results in colonization with minimal to no lesions 4–5 months after challenge, despite vigorous cell-mediated immune responses (Whelan *et al*., 2010; Vordermeier, unpublished observations; Waters *et al*., unpublished observations). Using the clinical definition of latency, these animals would be classified as having latent infection; although, as with naturally infected cattle with no visible lesions, it is unknown if active disease would result spontaneously given time or be induced via immune suppression.

It has also been suggested that skin-test or IGRA-positive animals with no visible lesions upon post-mortem inspection may have latent infection (Alvarez *et al*., 2009). Recent studies have clearly demonstrated that IGRA-positive, skin-test negative animals are at increased risk to convert to skin-test positive status over a period of time and present with confirmed *M. bovis* infection upon post-mortem inspection (Cassidy, 2006). With this scenario, animals that are initially IGRA positive, skin-test negative and later confirmed as infected, could be classified as: (i) early-infected with progressive disease; or (ii) latently infected with the potential for subsequent progression to active disease. While IGRAs are a good correlate of infection (median test sensitivity, 87.6%; Schiller *et al*., 2010), the magnitude of the response is not indicative of the severity of lesions or duration of infection in cattle (Waters *et al*., 2012). Thus, animals that are potentially latently infected may have a similar magnitude of response as compared to animals with visible lesions (Waters *et al*., 2010). The possibility of latent *M. bovis* infection in cattle has a profound consequence for both evaluation and use of immune-based diagnostic tests, particularly given the pitfalls of current gold-standard tests. Even with thorough post-mortem examinations, extensive culture of tissues including lymph nodes/lungs with no visible lesions and histopathological assessment including *M. tuberculosis*-complex specific PCR, our ability to detect all infected animals is not 100%. This is likely to be due to the paucibacillary nature of the disease in certain animals, inability to sample all tissues and shortcomings of mycobacterial culture (e.g. harsh decontamination during processing). Fortunately, with more aggressive eradication/control programmes, slaughter surveillance and ante-mortem testing is used to identify tuberculosis-affected herds, of which aggressive measures such as whole-herd depopulation (stamping out) or stringent interpretation of ante-mortem tests may be applied to ensure removal of all infected animals, including those which may be 'latently' infected.

Host genetics: correlations to resistance

Patterns of infection at the population scale are determined largely by relative susceptibility. Animal-level risk factors separate broadly into genetic and non-genetic (environmental) risk factors that act jointly to influence susceptibility. It is biologically untenable that genetic variation in both host and pathogen does not play a role in the outcome of exposure to tuberculous bacteria. There is now compelling evidence from studies in humans,

mice, deer and rabbits that the outcome of infection with *M. tuberculosis*-complex bacteria has a significant genetic component (Allen *et al*., 2010). Species-level differences in susceptibility to bovine tuberculosis have been detected in cattle (Ameni *et al*., 2007); *Bos indicus* animals are more resistant than *B. taurus*. Recent developments suggest that it may be possible to increase the resistance to bovine tuberculosis by genetic selection.

In population-based studies, the proportion of the variance of any trait contributed by host genetic variation (heritability, h_2) can be estimated. Significant heritability is one of the key factors determining the potential success of breeding schemes in livestock production. Recent quantitative genetics studies have demonstrated significant heritability (21%) of susceptibility to bovine tuberculosis in Holstein cattle in Ireland (Bermingham *et al*., 2014) and in the UK (Brotherstone *et al*., 2010; Bermingham *et al*., 2011). For comparison, heritability for milk yield is estimated at around 28%, with substantial genetic gain achieved via selective breeding. Furthermore, field studies are likely to underestimate true heritability for infectious disease predisposition, due to unequal exposure to the pathogen and incomplete sensitivity of the diagnostic tests (Bishop and Woolliams, 2010). One of the significant read-outs of these large-scale quantitative genetics studies is that it should be possible to rank the bovine tuberculosis risk of individual sires (sire relative risk), based on the bovine tuberculosis status that follows in their progeny. Sire relative risk rankings for Holstein–Friesians in Ireland and the UK are currently being developed and are likely to overlap significantly. A recent Irish study also suggests that selective breeding towards bovine tuberculosis resistance should not have a negative impact on other desirable production traits (Bermingham *et al*., 2014).

Molecular genetics seeks to identify the individual genetic variants that explain the heritability of observed resistance/susceptibility phenotypes. Genome-Wide Association Studies (GWAS) have been used in the past in human genetics to find variation underpinning multiple traits, by examining genetic differences between panels of individuals with a phenotype of interest (cases) and individuals without the phenotype (controls). Can cattle exposed to tuberculosis be assembled into such case and control panels? Tuberculosis in cattle, as in all animal models and human studies, presents as a spectrum of infection outcomes or phenotypes (Barry *et al*., 2009; Young *et al*., 2009), a departure from the classical view that tuberculosis infection has a binary outcome. Extrapolation of this spectrum to cattle can clearly define animals as being susceptible (cases) or resistant (controls).

Having been able to classify cattle exposed to tuberculosis as cases or controls, we then must be able to index the differing genetic variation that underpins their phenotypic outcome. Due to the stunning achievements of the bovine genome sequencing project (Bovine Genome Sequencing and Analysis Consortium, 2009; Larkin, 2011) and associated genetic marker discovery (Bovine HapMap Consortium, 2009), cattle GWAS can now index variation at >700,000 genetic markers (SNPs) from across the whole 3 bn letter bovine genome. This development allows for an approach that does not require any *a priori* knowledge of the exact genes/loci causing particular phenotypes, thereby potentially identifying novel networks of genes involved in tuberculosis resistance, and pathways crucial to the host–pathogen interaction. The first of these bovine GWAS has just reported preliminary findings (BBSRC, 2011; Finlay *et al*., 2012; Bermingham *et al*., 2014). The study identified a number of genetic markers associated with the risk of acquiring bovine TB if exposed; some were associated with increased risk and others with reduced risk. Subject to further research and validation, these provisional results suggest that it might be possible to selectively breed cows that are more resistant to bovine tuberculosis.

Additionally, recent advances are taking the field of genetic epidemiology beyond the concept of associating individual genetic polymorphisms with phenotypes. Novel quantitative methodologies can now be employed to associate whole-genome variation with disease phenotypes (Daetwyler *et al*., 2008); such 'genomic selection' can then be used to predict the phenotypic outcome for any animal based on just its whole-genome genotype without the need to undertake lengthy and

costly trait recording or phenotyping exercises. This concept is an extension of genome-wide or genomic selection using SNP arrays (Meuwissen *et al*., 2001), which is now commonplace in most major advanced dairy cattle breeding programmes.

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9 Diagnosis of *Mycobacterium bovis* Infection in Cattle

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Introduction

Bovine tuberculosis (TB) caused by *Mycobacterium bovis* continues to be a major animal health problem, having adverse impacts on socio-economic conditions, public health and trade of animals and animal products. Worldwide it has been estimated that approximately 50 million cattle are infected with *M. bovis* (Hope and Villarreal-Ramos, 2008). The most effective strategy for the control of bovine TB requires identification and removal of infected animals from herds. Cellular immune assays can efficiently identify *M. bovis*-infected animals as they can detect infection at an early stage and have a high relative sensitivity. For more than 100 years the tuberculin intradermal test has been used for this purpose. The application of the tuberculin intradermal test, and removal of test-positive (reactor) cattle, have been responsible for the eradication of bovine TB from many countries including Australia, many European Community countries, Canada and most states in the USA (Cousins, 2001). However, eradication has proved to be more difficult in countries with wildlife reservoirs of *M. bovis* infection, such as the brushtail possum (*Trichosurus vulpecula*) in New Zealand (de Lisle *et al*., 2001), the badger (*Meles meles*) in the UK and Ireland (Delahay *et al*., 2001; Griffin *et al*., 2005) and white-tailed deer in Michigan, USA (O'Brien *et al*., 2011). In these countries, management of the disease must also include control of the disease in the wildlife reservoir of infection.

In a control programme, the early detection of disease is critical. Bovine TB in cattle is a chronic disease and the development of lesions is a consequence of the immune response to the infection. Discernible lesions at post mortem may have taken years to develop and early diagnosis is dependent on the use of ante-mortem immunological assays rather than identification of clinical disease. *M. bovis* is an intracellular pathogen of macrophage/monocyte cell lineages and the immunological response is predominantly affected by T-lymphocytes. Pro-inflammatory cell-mediated responses dominate at the early stage of infection and high levels of circulating antibodies are generally only found during the late stage of the disease or in the presence of extensive lesions (Ritacco *et al*., 1991; Fifis *et al*., 1994). Hence, cellular immune assays are routinely utilized to identify *M. bovis*-infected cattle and the tests used are

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

the *in vivo* tuberculin intradermal test and the *in vitro* (blood-based) interferon-γ (IFNγ) assay.

The choice of the immunological assay and criteria for interpretation of the test is dependent on the population being investigated, whether the animals are apparently healthy or in a herd with a high prevalence of infection, or on the purpose of testing. The purpose of the testing can fall into three major categories:

1. The screening of apparent healthy animals where a high sensitivity of testing is required and some false positives are tolerated.
2. Confirmatory testing, which is often used for re-testing animals that have responded positively or produced an inconclusive result in a screening test. A high specificity is required for this testing and a slightly lower sensitivity could be accepted.
3. Exclusive testing, which is required when an infection is suspected and a high sensitivity is required to ensure all infected animals are identified.

The sensitivity and specificity of testing can be varied depending on the type of immunological test used, whether the test is a response to just purified protein derivative (PPD) prepared from *M. bovis*, a test comparing responses to PPDs prepared from *M. bovis* (bovine PPD) and *M. avium* (avian PPD) or a test using specific mycobacterial antigens, such as ESAT-6 and CFP10. Alternatively, the positive cut-offs for the tests can be varied to introduce a more stringent or severe interpretation or different tests may be used in series or in parallel. No single or combination of tests will provide 100% accuracy. There is a balance between improving the sensitivity of a test, namely the probability of correctly identifying an infected animal, and maintaining a high specificity and reducing the likelihood of false-positive results.

An important component in the control programme is to ensure that the results of ante-mortem testing are closely related to the findings at post mortem. This can be difficult as detectable macroscopic lesion rates are seldom greater than 60%, as cellular assays are more sensitive at identifying infected animals than visual detection of tuberculous lesions at post mortem. Experimental studies have shown that cellular immune assays can detect infection as early as 2 weeks following an intra-tracheal challenge of a low dose of approximately 500 cfu of *M. bovis* (Buddle *et al*., 1994). *M. bovis* infection may also be established in a site that is not closely examined at slaughter or where samples are not easily collected for culture, providing another explanation for the dissociation in the sensitivity of these methods of detection. Corner *et al*. (1990) reported that in a sample of cattle positive in the tuberculin test, abattoir inspection failed to detect an estimated 47% of those with tuberculous lesions which were detected by detailed necropsy. A detailed necropsy examination covering a broad range of anatomical sites was necessary to detect all of the lesioned animals. While further animals can be detected as being infected by culture of *M. bovis* from selected lymph nodes with no visible lesions, practical limitations preclude the identification of all infected animals by laboratory testing.

The aim of this review is not to provide a comprehensive review of ante-mortem and post-mortem diagnosis of bovine TB, as detailed reviews on these topics can be found elsewhere (Monaghan *et al*., 1994; Adams, 2001; de la Rua-Domenech *et al*., 2006; Schiller *et al*., 2010a). Rather, this review is focused on documenting how different tests can be used and on new developments in diagnostic testing.

Tuberculin Intradermal Test

The tuberculin intradermal test for cattle was developed more than 100 years ago and has been shown to be a simple, inexpensive, robust and widely accepted test which can be used for the primary screening of cattle for *M. bovis* infection. The principle of the test is that in *M. bovis*-infected animals, bovine PPD injected intradermally elicits a delayed-type hypersensitivity reaction associated with an influx of sensitized T-lymphocytes and monocytic cells into the site of inoculation (Waters *et al*., 2000). The resulting increase in skin thickness, peaking at 72 h after inoculation, is detected by palpation or measured with callipers. The bovine PPD used in most countries is prepared from

cultures of *M. bovis* AN5 and is a complex mixture of soluble antigens including proteins, but also some carbohydrates and lipids. Some of the antigens in bovine PPD are common to other species of *Mycobacterium*. *M. bovis* AN5 was a field strain isolated from England in 1948 and selected for a high yield of cell mass on glycerinated medium (Paterson, 1948). Although there have been questions raised whether this strain is representative of *M. bovis* strains found worldwide today, recent genome analysis has shown that there is no substantial genetic differences to other *M. bovis* strains (Inwald *et al*., 2003). In addition, intradermal testing with this reagent has been the cornerstone diagnostic test used in countries that have eradicated bovine TB (Cousins, 2001).

The tuberculin intradermal test can be undertaken in the caudal fold at the base of the tail (caudal fold test, CFT) or in the mid-cervical region (single intradermal cervical test, SIT). For both tests a 100 µl volume of bovine PPD is inoculated intradermally and an increase in skin thickness is detected by palpation or measured with callipers at 72 h post-inoculation. The use of CFT for diagnosing *M. bovis*-infected cattle was first described by Moussu and Mantoux (1908) and is currently used for cattle in the southern hemisphere and North America. The test is more cost effective and practical for the yarding conditions that exist in these regions and was used successfully in the eradication of bovine TB from Australia (Cousins *et al*., 1998). It has been suggested from comparative studies that when bovine PPD is inoculated in the cervical region, the test may be more sensitive than when it is inoculated in the caudal fold (Francis *et al*., 1978) and the cervical intradermal test is used in many countries in continental Europe. In the UK and Ireland, the single cervical test was shown to have a poor specificity and a single intradermal comparative cervical test (SICCT) was introduced, where avian PPD prepared from cultures of *M. avium* and bovine PPD are inoculated at sites approximately 12.5 cm apart. Skin thicknesses are measured with callipers immediately prior to inoculation and 72 h later and increases in skin thickness are compared between the two sites. This comparative test is more specific than when bovine PPD is used alone.

The concentrations of the PPDs are expressed in International Units (IU) based on potency testing in Guinea pigs and cattle and the concentrations used vary between countries. The usual interpretation of a positive response in the CFT is any visible or palpable swelling compared to that for the caudal fold on the opposite side, although in regions of low risk, a positive response may be a swelling of ≥4 mm (Buddle *et al*., 2009). In continental Europe, a positive response in the SIT is defined as any detectable increase in skin swelling between 0 and 72 h post-inoculation (Monaghan *et al*., 1994). In the USA, a positive CFT response is defined as any detectable increase in skin swelling at 72 h post-inoculation and a scattergram is used for interpretation of the comparative cervical test to define the reaction as positive, negative or suspect (USDA/APHIS, 2007). In the UK and Ireland, a positive response in the SICCT corresponds to an increase >4 mm in the bovine PPD site compared to that for avian PPD. Implementation of a more stringent interpretation of this test or re-testing of animals that have shown an inconclusive reaction (comparative increase of 1–4 mm) has been introduced in different situations (Monaghan *et al*., 1994). In most countries, tuberculin intradermal tests are undertaken annually in regions that have endemic *M. bovis* infections in livestock and/or wildlife, while this period may be extended in regions free of *M. bovis* infection. In infected herds, the interval may be reduced to as short as 42–60 days.

Estimates of the sensitivity of the tuberculin intradermal test from a number of overseas studies range from 68% to 95% while specificity is estimated to be 96% to 99% (reviewed by Monaghan *et al*., 1994). Data from those studies must be interpreted with caution as the definition of a test-positive response varied between the tests and countries as well as the definition of a TB-infected animal. A major factor in the estimate of sensitivity of the tests may relate to which stage in the control programme the tests are being applied, as the ability to detect disease is influenced by the prevalence of the disease. At the start of an eradication campaign when the prevalence of the disease is high, the positive predictive value is also high. In contrast, at the end of a

country's TB control programme when there are few infected animals, the positive predictive value is low.

A phenomenon known as desensitization has been reported when a second tuberculin intradermal test has been applied to *M. bovis*-infected or sensitized cattle within a short period, which may lead to a false-negative response. Based on this information the recommendation in most countries is that a period of at least 60 days is required between tuberculin intradermal tests, although an interval of at least 42 days between tests has been recommended in the UK (de la Rua-Domenech *et al*., 2006). The optimal interval between tests has not been clearly defined. Radunz and Lepper (1985) used cattle sensitized with killed *M. bovis* to show that suppressed skin reactivity was observed when a second intradermal test was undertaken 4 and 7 days following the initial test, but not when re-tested at 60 days. Doherty *et al*. (1995b) confirmed these results in naturally infected cattle with suppressed skin reactivity when cattle were re-tested with the SICCT 7 days after the first test. One note of caution is that repeated use of the SICCT at 60-day intervals can lead to desensitization. Coad *et al*. (2010) observed a significant progressive reduction in the strength of the comparative PPD response when the SICCT was repeated five times at 60-day intervals. The desensitizing effect was associated with decreased IL-1β and elevated IL-10 responses but, importantly, did not influence antigen specific IFNγ responses. Repeated intradermal testing (five times) of non-infected animals at 8-week intervals was not shown to increase the susceptibility of the animals to a subsequent experimental challenge with *M. bovis* or affect their post-infection intradermal test responses as compared with the previously non-intradermal-tested, challenged animals (Thom *et al*., 2004).

False-negative and false-positive responses to the tuberculin intradermal test

False-negative and false-positive responses occur in the tuberculin intradermal test, similar to any diagnostic test, and the most common causes of these responses are listed in Table 9.1. Francis (1947) reported that reactions in the tuberculin test are not usually apparent in naturally infected animals until 30–50 days following infection, while estimates of the

Table 9.1. Common causes of false-negative and false-positive responses in tuberculin intradermal tests.

False-negative responses	Very early *M. bovis* infection (Francis, 1947)
	Anergy related to generalized *M. bovis* infection or stress (Lepper *et al*., 1977; Pollock and Neill, 2002)
	Early postpartum period (Kerr *et al*., 1946)
	Concurrent exposure to environmental mycobacteria or vaccinated against Johne's disease when SICCT test is used (Hope *et al*., 2005; Coad *et al*., 2013)
	Concurrent bovine viral diarrhoea infection (Charleston *et al*., 2001)
	Concurrent parasitic infections (Flynn *et al*., 2007)
	Use of immunosuppressive drugs (Doherty *et al*., 1995a)
	Poor technique for injection or reading of test, lack of quality control (Monaghan *et al*., 1994; de la Rua-Domenech *et al*., 2006)
	Poor potency PPD (de la Rua-Domenech *et al*., 2006)
	Multiple repetition of tests at short-interval (c. 60 days) testing (Coad *et al*., 2010) or within 7 days of previous skin test (Radunz and Lepper, 1985)
False-positive responses	Exposure to environmental mycobacteria species, including members of the *M. avium* complex and *M. avium* subsp. *paratuberculosis* (Lesslie *et al*., 1975; Thom *et al*., 2008) when the SIT or CFT is used
	Infection with skin tuberculosis (unidentified acid-fast mycobacteria) (Monaghan *et al*., 1994)
	Vaccination against Johne's disease (Coad *et al*., 2013) or with BCG (Whelan *et al*., 2011)

pre-allergic phase in experimentally infected animals have been considerably shorter (Monaghan *et al*., 1994). Anergy to the tuberculin test has been recognized in animals with generalized TB and this may occur when Th2 type responses predominate and increases in antibody responses may be observed (Lepper *et al*., 1977; Pollock and Neill, 2002). Stress of cattle may also temporarily result in anergy. Although a low protein and energy diet has been associated with a lack of tuberculin responsiveness in humans and guinea pigs (Neumann *et al*., 1975; McMurray *et al*., 1989), a study involving feeding *M. bovis*-infected animals on a restricted diet did not show a reduction in the tuberculin intradermal test, IFNγ or lymphocyte proliferation (Doherty *et al*., 1996). Reduced responsiveness in the tuberculin test has been observed in cows in the early postpartum period and Kerr *et al*. (1946) reported that 7 of 20 reactor cows failed to respond to tuberculin immediately after calving, while tuberculin reactivity returned by 4–6 weeks after calving. In the SICCT, false-negative responses can arise when cattle are co-infected with members of the *M. avium* complex and *M. bovis*, and responses to avian PPD may be greater than those to bovine PPD (Hope *et al*., 2005). This situation may also arise when cattle are vaccinated with a Johne's disease vaccine and subsequently infected with *M. bovis* (Coad *et al*., 2013). False-negative responses may also arise temporally when cattle are co-infected with bovine viral diarrhoea virus, resulting from immunosuppression (Charleston *et al*., 2001) or co-infected with parasitic infections such as *Fasciola hepatica*, skewing immune responses towards a Th2-type response (Flynn *et al*., 2007). Incorrect administration of tuberculins or inaccurate reading of the test can result in infected animals being missed as well as the use of low potency tuberculins (de la Rua-Domenech *et al*., 2006). The financial consequences of having a herd classified as *M. bovis*-infected have resulted in some farmers attempting to mask tuberculin reactions in infected animals. Administration of dexamethasone has been shown to reduce responsiveness to tuberculin and infected animals have been misclassified (Doherty *et al*., 1995a).

False-positive responses in the tuberculin test can arise from infection with environmental mycobacteria, members of the *M. avium* complex including *M. avium* subsp. *paratuberculosis* or skin tuberculosis (Lesslie *et al*., 1975; Buddle *et al*., 2003; Thom *et al*., 2008). Many of these infections may result in only a temporary misclassification of animals and the use of comparative tests using avian and bovine PPDs usually overcomes this problem. Skin TB is a clinical condition consisting of a string of nodular swellings following the lymphatics which are most frequently found on the limbs. The lesions mimic tuberculous lesions histologically and may contain a non-culturable acid-fast bacillus. Immunization with BCG or vaccines against Johne's disease can result in false-positive responses to bovine tuberculin (Whelan *et al*., 2011; Coad *et al*., 2013). However, a recent study has shown that responses in the SICCT in BCG-vaccinated cattle reduced markedly by 6–9 months post-vaccination (Whelan *et al*., 2011).

Use of specific reagents in the tuberculin intradermal test

The specificity of the tuberculin intradermal test, especially the caudal fold and single cervical tests, could be markedly improved if specific reagents were identified. This should also allow for the differentiation between BCG or Johne's disease-vaccinated animals from those infected with *M. bovis*. An important recent finding has been that a cocktail of three recombinant proteins from the *M. tuberculosis* complex – ESAT-6, CFP10 and Rv3615c, or a cocktail of peptides derived from these proteins – was able to identify the majority of cattle naturally infected with *M. bovis* (Whelan *et al*., 2010). The sensitivity of the test could be further enhanced with the inclusion of a fourth protein from the *M. tuberculosis* complex, Rv3020c, with the proportion of infected animals increasing from 12 to 14 of the 16 naturally infected animals that had reacted positively in the tuberculin SICCT using the severe interpretation (Jones *et al*., 2012). When these antigens were applied in the skin test to BCG Danish (Staten Serum Institute, Denmark) and/or Johne's disease-vaccinated (Gudair™, CZ Veterinaria, Pontevedra, Spain) cattle only one of 35 animals responded positively.

In contrast, four of the seven cattle vaccinated with BCG alone responded positively in the tuberculin SICCT using the severe interpretation. The optimal concentration of each protein was identified as 10 μg/inoculation and responses were read at 72 h. These results are encouraging as a previous study using ESAT-6 indicated that a concentration of ≥400 μg/inoculation was required (Pollock *et al*., 2003), although the concentration could be slightly reduced if co-administered with a synthetic lipopeptide which had pro-inflammatory properties (Whelan *et al*., 2003). The cost of producing recombinant proteins or peptides for use in an intradermal test may limit their application although there is economy of scale and our calculations based on the use of peptides indicated that production costs would be in the same order of magnitude as tuberculin (A. Whelan and H.M. Vordermeier, unpublished). Recent studies have shown that the display of mycobacterial proteins on biobeads produced in *Escherichia coli* could markedly reduce the concentration of mycobacterial proteins used in an intradermal test (Chen *et al*., 2014), considerably reducing the cost of the reagents. The TB biobead preparation was shown to induce similar intradermal test responses to those for the recombinant proteins or peptides and identified 10 of 11 naturally TB-infected cattle (N. Parlane and B. Buddle, unpublished observations).

Interferon-γ Test

The IFNγ test was developed in Australia in the late 1980s to improve the practicality and accuracy of diagnosis of bovine TB (Rothel *et al*., 1990, 1992). The principle of the test is based on the observation that when sensitized T-lymphocytes from blood of a *M. bovis*-infected animal are re-exposed to antigens from *M. bovis* (such as those in bovine PPD), the cells will release IFNγ, a cytokine (immune messenger). The resulting IFNγ is produced in high quantities *in vitro* and is not readily consumed during short-term culture (Wood and Jones, 2001). Release of IFNγ by T-lymphocytes is regarded as a hallmark of a cellular immune response which is elicited by a TB infection. This release of IFNγ from T-lymphocytes results in the activation of macrophages, enabling these cells to be more effective in killing intracellular pathogens such as mycobacteria. The IFNγ test takes advantage of the profile of immune factors, predominantly IFNγ, which is released in the context of an animal's immune response to infection with *M. bovis*.

The test is carried out in two steps. The first step is the short-term culture of whole blood in the presence or absence of mycobacterial antigens (bovine or avian PPD) and the separation of plasma from these cultures after incubation at 37°C for 16–24 h. This step requires the presence of live white blood cells and this is reflected in the requirement to use heparinized blood and the need to preserve the viability of the cells. By comparing the levels of IFNγ released from blood cultures stimulated with bovine and avian PPD it is possible to differentiate animals infected with *M. bovis* from uninfected animals, including those exposed to or infected with environmental mycobacteria. In the UK and USA, a mitogen control (pokeweed mitogen or staphylococcal enterotoxin B) is often included in a separate blood culture to verify the viability and responsiveness of the cells (Waters *et al*., 2007; Coad *et al*., 2008). Conditions for optimizing the test have been identified (Schiller *et al*., 2009a). The stimulation temperature for the blood cultures needs to be 33°C or higher; carbon dioxide is not required for stimulation; and various plate formats, ranging from 24 to 96 wells per plate, can be utilized. The produced IFNγ is stable at 4°C for 28 days as well as after repeated freeze–thaw cycles.

From a series of trials in various countries, the sensitivity of the IFNγ test has been estimated to vary between 73% and 100%, median of 87.6% and a specificity varying between 85% and 99.6%, median of 96.6% (de la Rua-Domenech *et al*., 2006). The specificity figures need to be interpreted with caution as in some cases they have been based on selected animals, such as those that have responded to a tuberculin intradermal test. The intradermal and IFNγ tests can identify different populations of TB-infected cattle and, when used in parallel, the two tests can identify a higher proportion of infected animals than either test used alone (Neill *et al*., 1994;

Coad *et al*., 2008). The advantages and disadvantages of using the IFNγ test in comparison with the intradermal test are shown in Table 9.2. The IFNγ test is not used as a primary surveillance test for bovine TB due to its higher cost compared to the intradermal test. Nevertheless, a recent European Food Safety Authority review concluded that the PPD-based IFNγ tests can be included in the official tests to grant and retain an officially TB-free herd status, although they also recommended that the test protocols should be harmonized in the EU (www.efsa.europa.eu/en/efsajournal/pub/2975.htm).

The IFNγ test is particularly useful as an ancillary test since it only requires a single visit to a farm and can be performed soon after intradermal testing. The two major applications of the IFNγ test are for 'in series' confirmatory testing of intradermal test reactors for improved specificity and for 'in parallel' testing of intradermal test-negative cattle for improved sensitivity. The value of using 'in series' and 'in parallel' IFNγ testing is readily seen from the 2011–12 year data from New Zealand (Anon., 2012). From a total of 4,321,476 cattle tested in the CFT in 2011–12, 5361 were positive and 5139 of these animals were re-tested in the 'in series' IFNγ test. Only 353 animals were positive in the 'in series' IFNγ test and sent to slaughter, resulting in a major reduction of CFT false positives slaughtered. A total of 28,303 CFT-negative cattle from potentially infected herds were examined with the 'in parallel' IFNγ test. Of these 147 were positive and slaughtered and 30% were found to have TB lesions. Different cut-offs in the IFNγ assay can be used depending on the purpose of the testing. For example, the positive cut-off used in the 'in parallel' IFNγ test in New Zealand is lower than that for the 'in series' to ensure no infected animals are left in potentially uninfected herds.

Factors affecting the efficacy of the IFNγ test

A number of trials have been undertaken to investigate the influence of the intradermal test on the IFNγ test and the results have been contradictory (reviewed by Schiller *et al*., 2010c). In naturally infected cattle, the SICCT neither boosts nor depresses PPD-specific IFNγ production (Coad *et al*., 2007), while studies using experimentally infected animals have produced disparate results. Boosting of the *M. bovis* PPD (bovine PPD) IFNγ production has been observed in naturally infected cattle at 3 days after application of the CFT. In experimentally infected animals, the CFT boosted IFNγ production for up to 7 days, but without influencing test interpretation. In New Zealand, IFNγ testing was previously undertaken 13–33 days after application of bovine PPD in the CFT, but regulations now allow this testing to be undertaken independent of CFT (Buddle *et al*., 2015).

Table 9.2. Practical advantages and disadvantages of the interferon-γ (IFNγ) test over the intradermal test. (Adapted from de la Rua-Domenech *et al*., 2006.)

Advantages	High sensitivity
	Detects *M. bovis* infection earlier than the tuberculin skin test
	No delay in repeat testing
	Only one visit to the farm is required
	Eliminates potential operator bias in reading skin tests
	Cut-offs can be readily altered depending on the purpose of testing
	Defined antigens can be used to differentiate other mycobacterial infections as laboratory test can be highly quality controlled
	Use of positive controls for blood viability
Disadvantages	Specificity may be lower than that for the intradermal test
	Non-specific responses in animals less than 6 months of age
	Blood is perishable and should be transported at temperatures between 10 and 26°C
	Relatively high cost of a laboratory-based test

The IFNγ test can identify TB-infected cattle as early as 2 weeks following infection (Buddle *et al*., 1994; Schiller *et al*., 2010b) and the induction of a positive IFNγ response has been shown to be largely independent of the *M. bovis* infective dose (Dean *et al*., 2005). This provides encouragement that infection with low doses can be detected relatively early, although these findings should be interpreted with caution as they are based on experimental infection of cattle. A potential disadvantage is that calves less than 3–6 months of age can react non-specifically when blood cultures are stimulated with mycobacterial antigens (Olsen *et al*., 2005), possibly as a result of stimulation of natural killer (NK) cells.

A comparison of tuberculin activity in the IFNγ test was recently undertaken, which indicated there were significant differences between sources and concentrations of PPDs used for stimulation in the IFNγ test, suggesting a need for standardization of PPDs (Schiller *et al*., 2010b). Most differences were evident at low concentrations of PPDs, while at high concentrations the differences tended to disappear due to saturation of the system. A novel tool, Relative Potency (RP) 30, was developed to standardize PPDs based on the protein concentration at which a specific PPD has 30% of maximal activity. Generally the PPDs with the highest potency in TB-infected cattle (lowest RP30 values) were the most specific PPDs in uninfected cattle exposed to environmental mycobacteria, due to low cross-reactivity of bovine PPD and/or sufficiently high activity of avian PPD to detect a non-specific IFNγ response. Recently, it was demonstrated that lyophilized PPDs and specific antigens elicit similar responses as compared to standard liquid preparations, thereby affording improved stability of antigens at variable temperatures for use in the assay (Bass *et al*., 2013).

Another factor affecting the strength of IFNγ responses is the delay between taking blood and setting up blood cultures. A reduction in the strength of IFNγ responses has been observed when blood samples are stored overnight before testing compared to setting up cultures on the same day (Ryan *et al*., 2000; Gormley *et al*., 2006; Schiller *et al*., 2009a). Despite this finding, the PPD responder frequency (bovine PPD response minus avian PPD response) for naturally infected cattle for 24 h blood has been shown to be higher than for fresh blood due to resolution of an avian PPD bias for some cattle (Coad *et al*., 2007) and specificity may also be higher following overnight storage (Ryan *et al*., 2000; Schiller *et al*., 2009a). This result is important as in many regions or countries it is not practical to receive all blood samples on the day of collection. For this reason, all blood samples for IFNγ testing in New Zealand or Great Britain (GB) are set up for culture the day after collection, but the maximum time between collection and blood culture has been set at 24 h (GB) or 30 h (NZ) due to a decline in test sensitivity after this time. Increasingly, defined mycobacterial antigens such as ESAT-6 and CFP10 are being used in the IFNγ test to increase specificity. In contrast to the PPD-based test, which is a comparative test, the defined antigen test could be adversely affected by a delay in blood culturing. More stringent cut-offs or time intervals to set up cultures may be necessary to ensure optimal test sensitivity for a defined antigen IFNγ test (Coad *et al*., 2007).

Some animal handling and holding conditions may result in lower IFNγ production. Schiller *et al*. (2009a) reported that in populations of cattle with handling stress (fighting bulls) or difficult holding conditions, a significantly higher number of animals were below the valid cut-off for mitogen stimulation. They also noted that a low mitogen response may not equate to a negative outcome for the TB IFNγ test and 20 of 75 cattle from Mexico with invalid pokeweed mitogen stimulation were positive by the IFNγ test using TB-specific antigens. The effect of clotting and contamination of blood cultures has also been investigated in relation to the TB IFNγ test (G. de Lisle, unpublished observations). Clots may reduce the level of IFNγ production (or detection of IFNγ protein within the supernatant) and the level of reduction is related to the size of the clot. Microbial contamination of blood cultures can either be stimulatory or inhibitory on IFNγ production and the effect is related to the species of contaminating organism.

Use of defined antigens in the IFNγ test

The specificity of the IFNγ test has been enhanced by the replacement of PPDs by specific proteins from the *M. tuberculosis* complex. The two antigens which have shown most promise are ESAT-6 and CFP10. When these antigens are used in the IFNγ test, approximately 90% of TB-infected cattle responding positively to PPDs are identified, while those cattle exposed to the majority of *M. avium* complex strains or *M. avium* subsp. *paratuberculosis* or vaccinated with BCG or Johne's disease vaccines respond negatively (Pollock *et al*., 2000; Vordermeier *et al*., 2001; Buddle *et al*., 2003). Test sensitivity can also be enhanced with use of specific reagents when cattle are co-infected with non-tuberculous mycobacteria and *M. bovis*, as TB diagnosis may be masked by a high response to avian PPD compared to that for bovine PPD (Aranaz *et al*., 2006). Sensitivity of the defined antigen IFNγ test may be enhanced by the addition of other *M. tuberculosis* complex proteins. Recent studies have found that the addition of the mycobacterial proteins, Rv3615c and OmpATb, to a defined antigen IFNγ test identified TB-infected animals which had not responded to ESAT-6 and CFP10 (Sidders *et al*., 2008; Schiller *et al*., 2009b). Also, ESAT-6/CFP-based antigen cocktails are now commercially available from Prionics Ag, Schlieren, Switzerland (Bass *et al*., 2013). Interestingly, it has recently been shown that IL-2 production, while on its own did not display good sensitivity (although it proved to be exquisitely specific) could, together with IFNγ, detect a higher proportion of visibly lesioned cattle compared to those without visible pathology. This could therefore be a useful prognostic marker of disease severity or infectivity (Rhodes *et al*., 2014). Other potential diagnostic cytokine read-out systems such as IL-1β, IP10 or IL-22 have been highlighted recently as potential analytes to be used alongside IFNγ to increase sensitivity, and are undergoing validation (e.g. Jones *et al*., 2010; Aranday-Cortes *et al*., 2012; Waters *et al*., 2012).

Antibody Detection Assays

Antibody detection systems have considerable attraction for use in disease control programmes as these assays are simple, inexpensive and can be run retrospectively on samples; however, they generally have a low sensitivity for diagnosis of *M. bovis* infection in cattle. In cattle, *M. bovis* infection induces a strong early cell-mediated immune response, but a weak antibody response, although as the disease progresses, antibody responses may be easier to detect. Antibody tests could have a role in detection of animals which have anergy in cellular-based assays and likely to be heavily diseased. Alternatively, antibody-based screening could be valuable where 'test and slaughter' programmes are too costly to implement in a country with endemic bovine TB and emphasis should be on cost-effective detection and removal of animals with advanced disease (de la Rua-Domenech *et al*., 2006).

Use of crude mycobacterial extracts in serological tests for detection of bovine TB has proved disappointing due to the low specificity of these assays. In contrast, the recent identification and use of specific immunodominant proteins has shown more promise and an array of new TB serological tests have now become available. These tests include those suited to laboratory-based, automated, large-scale screening including the ELISA format such as the IDEXX *M. bovis* Ab test (IDEXX Laboratories, Westbrook, Maine, USA; Waters *et al*., 2011) and multiplex immunoassay Enfer test (Enfer Scientific, Naas, Ireland; Whelan *et al*., 2008) or a fluorescence polarization assay (Jolley *et al*., 2007). Chembio Diagnostics Systems (Medford, NY, USA) has developed animal-side tests such as the rapid flow immunochromatic (Lyashchenko *et al*., 2008) and dual path platform tests (Greenwald *et al*., 2009), which offer the advantage of conducting assays in the field. Most defined antigen assays are specific, around 98%, while estimates of sensitivity are very variable depending on the source of the animals and stage of infection. This is illustrated from the study of Waters *et al*. (2011) where sera was collected from naturally infected cattle from four countries and assayed in the IDEXX test in a single laboratory. The sensitivities were 77% for sera of animals from GB (n = 184 infected animals), 67% for sera from Irish cattle (n = 130), 46% for those from the USA (n = 122) and 40% for those from New Zealand

(n = 42). The low sensitivities of the test for USA and New Zealand animals were attributed to a low incidence of disease in these countries, with infections detected at an early stage. This was also shown from the results for the Irish animals where animals were subdivided on the basis of disease severity, with test sensitivities ranging from 46% for those with no lesions (n = 50) to 90% for those which were positive in the intradermal and IFNγ tests and had visible lesions (n = 30). With the emergence of these new serological tests for bovine TB diagnosis, it is imperative to determine the comparative sensitivities and specificities from a single set of sera obtained from animals that are confirmed culture positive for *M. bovis*, and those from TB-free regions. An important factor in interpreting the sensitivity differences in different studies is also the timing of the serum collection, as the application of tuberculin as part of an intradermal test has been shown to significantly increase test sensitivities compared to samples from animals taken without prior skin test boosting of the humoral responses.

A recent application for antibody-based assays is the assay of bulk or pooled milk samples for detection of TB-infected animals or herds. A report from Korea indicated that bulk milk samples could be assayed for antibodies to *M. bovis* to detect infected herds as well as herds with tuberculin intradermal test-positive animals (Jeon *et al*., 2010). A study of bulk tank milk samples from 17 Mexican dairy farms which were positive for *M. bovis* DNA using PCR for IS1081 revealed 14 of these dairy farms were positive for bulk milk samples assayed by IDEXX *M. bovis* ELISA, while bulk tank samples from 185 TB-free dairy farms in Michigan, USA, were all negative (Waters *et al*., 2011). However, results from a country with a low incidence of bovine TB and a progressive disease control programme were less rewarding. A study in New Zealand showed no significant differences in antibody levels from bulk tank milk samples from TB-infected and TB-free dairy farms, while the sensitivity and specificity for individual milk samples were 50% and 97.5%, respectively (Buddle *et al*., 2013). The sensitivities for milk and serum samples from the same group of infected animals were very similar. Overall, antibody-based tests do not have the sensitivity for use as primary screening tests in an eradication control programme, although these tests could be useful for identifying infected animals from chronically infected herds where animals have not reacted to cellular-based assays.

Other Ante-mortem Assays

There has been periodic interest in the development and application of assays to detect volatile organic compounds (VOCs) as a means to diagnose various infectious diseases, including human (Phillips *et al*., 2012) and bovine TB (Fend *et al*., 2005). In theory, unique VOC signatures, either host- or pathogen-derived, are elicited by TB infection that could be detected in expired air. Despite promising preliminary results, these tests have not been validated for use with naturally infected cattle and further work is needed to develop a practical assay for use in bovine TB control programmes. Similar to VOCs, there is considerable interest in development of a test to detect biomarkers elicited by *M. bovis* infection and detectable within sera. A recent report by Lamont *et al*. (2014) indicates that both host (e.g. VDPB, alpha-1-antitrypsin and fetuin-A) and pathogen (e.g. MB2515c, MB1895c and Pks5) proteins are specifically increased in sera from *M. bovis*-infected cattle, but not in control samples. While encouraging, further studies are required to validate this approach.

Post-mortem Tests

Post-mortem diagnosis of bovine tuberculosis is a critical part of control/eradication programmes. Accurate determination of *M. bovis* infection status is essential for the establishment of the infection status of individual animals and herds, evaluation of the performance of ante-mortem tests and for surveillance through monitoring of animals at slaughter. In the final eradication phase of TB control programmes post-mortem inspection

becomes the principal surveillance tool and routine ante-mortem testing is reduced and eventually stopped. Knowledge of the pathogenesis and lesion distribution is important for developing protocols for the post-mortem inspection of carcasses (Cassidy, 2006). The predominance of lesions occurring in the head and thorax is consistent with an aerosol route of infection. The lower level of lesions in the mesenteric lymph nodes is indicative of the oral route of infection being less common. The sensitivity of gross post-mortem examination is affected by the method employed and the anatomical sites examined (Corner, 1994).

Macroscopic lesions of TB in cattle have the gross appearance of a tubercle that is a firm white or yellow nodule. On section, a yellowish caseous centre is observed. Calcification of the caseous areas is common. The macroscopic lesions observed in cattle TB are not unique to infection with *M. bovis* and other agents including some mycobacterial species, actinobacillus, fungi or hydatids can produce similar or identical lesions. The histopathological appearance of the lesions caused by *M. bovis* is typically a central area of necrosis surrounded by a granulomatous inflammatory response containing epithelioid macrophages and often multi-nucleated giant cells. Acid-fast staining bacilli can often be seen in the lesions but they are usually low in number and may be absent. Some macroscopic lesions that resemble TB can be clearly shown by histopathology to be due to agents other than *M. bovis*. However, the specificity of histopathological examinations is not sufficient for them to be used for a definitive diagnosis of infection with *M. bovis*, which requires the identification of the organism by either culture or by a DNA amplification procedure such as PCR.

While culture remains the gold standard, care needs to be taken to use a sensitive culture procedure where the decontamination method is optimized and the culture media used is selected for its ability to support the growth of *M. bovis*. Liquid culture procedures have the advantage over the use of solid media in that they are not only sensitive but also quicker. The best liquid culture system was the Bactec 12B system but this medium is no longer available and many laboratories are now using the MGIT™ system (BD Diagnostics, Sparks, MD) (Robbe-Austerman *et al*., 2013). The success of culturing is dependent on the quality of samples and the conditions under which they are transported to the laboratory. In order to avoid problems with overgrowth of contaminating bacteria, samples should be refrigerated during transfer to the laboratory and delays in transport should be avoided. PCR-based tests are being increasingly used for the identification of *M. bovis* in suspect tuberculous lesions. The sensitivity of PCR tests has until recently been less than culture for detecting *M. bovis* in tissues (Taylor *et al*., 2007; Thacker *et al*., 2011) but recent developments such as the use of magnetic bead separation may have enhanced the detection of this organism (Stewart *et al*., 2013). Culture has revealed that cattle may be infected with a wide range of different species of mycobacteria in addition to *M. bovis*. Their potential importance lies in that some of them can produce macroscopic lesions indistinguishable from those caused by *M. bovis* and induce false-positive results in immune-based tests for bovine tuberculosis. For example, *M. kansasii* can infect cattle and induce immune reactivity not only to tuberculins but also the important diagnostic antigens ESAT-6 and CFP10 (Houlihan, 2010).

An important benefit of culture is that it provides isolates of *M. bovis* that can be used for DNA typing which is a valuable procedure for enhancing epidemiological investigations. Early DNA typing procedures included restriction endonuclease analysis, restriction fragment polymorphisms and spoligotyping. They provided an important insight into the epidemiology of bovine tuberculosis where there was a cycle of infection involving cattle and wildlife. Investigations in New Zealand revealed the identical DNA types in cattle and farmed deer and wildlife (brushtail possum, ferret) located in the same area (de Lisle *et al*., 1995). Similarly, the geographical distribution of DNA types of *M. bovis* in Ireland among cattle, deer and badgers was consistent with there being a cycle of infection involving domestic animals and wildlife (Costello *et al*., 1999). To obtain full benefit of DNA typing it is important that it is used in conjunction with other epidemiological investigations. While the

use of variable number tandem repeats is now widely employed for typing (Skuce *et al*., 2010; Price-Carter *et al*., 2011), the recent technological advances in DNA sequencing are likely to result in whole-genome sequencing becoming the method of choice for *M. bovis* DNA typing in the near future (Biek *et al*., 2012).

An interesting development arising from DNA typing has been the identification of members of the *M. tuberculosis* complex other than *M. bovis* causing tuberculous lesions in cattle. In Europe, *M. caprae* has been isolated from tuberculous lesions from cattle, goats, pigs and deer (Rodríguez *et al*., 2011). Direct PCR tests based on DNA sequences from IS6110 and IS1081, which are commonly used for the detection of tuberculosis in cattle, will not distinguish *M. bovis* from *M. caprae*, *M. orygis* and *M. pinnipedii*.

Future Developments

Advances in molecular biology are presenting opportunities to develop new and improved tools to diagnose and control bovine TB in cattle, although it is important to continue to improve the utility of existing tools and strategies. The elucidation of the genome sequences of *M. bovis*, *M. tuberculosis*, *M. bovis* BCG and *M. avium* have allowed comparative genomic analyses to identify additional antigens for inclusion in *M. bovis* specific diagnostic assays. Transcriptome analyses of host gene expression from antigen-stimulated peripheral blood mononuclear cultures from *M. bovis*-infected and non-infected cattle should provide profiles which may more accurately identify infected animals compared to that from a single cytokine response. In addition these profiles may determine the stage of infection to facilitate epidemiological investigations of disease outbreaks. Whole-genome analyses of *M. bovis* clones from TB-infected cattle that are only identified from abattoir surveillance and have responded negatively in tuberculin assays may present a chance to prepare improved tuberculins which may accurately identify infected animals.

If any new test is to replace tuberculin intradermal testing as the primary diagnostic method in a TB control programme it will not only have to be more sensitive and specific, but also be affordable. New diagnostic tests may initially be relatively expensive and have a role as ancillary tests, rather than as replacing intradermal testing as the primary test for TB. For any new diagnostic test, the evaluation should be undertaken in appropriate animal populations and compared to the existing assays. Although the use of more than one type of diagnostic test on animals will increase the chance of identifying an infected animal, this will increase surveillance costs and may only be applicable in certain circumstances. In addition, there needs to be flexibility in determining cut-offs for different assays as these may vary depending on the stage of the control programme and the purpose of testing. However, in countries without government or industry support for compensation of condemned animals, a test identifying animals that are most likely shedding *M. bovis* could reduce the risk of transmission of infection to other animals or humans. Hence, no single test may fit all applications and it is important that a variety of different assays are developed for application in different countries or circumstances.

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10 Vaccination of Cattle Against Tuberculosis

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Introduction

Bovine TB (bTB), mainly caused by *Mycobacterium bovis*, is a significant economic burden to agricultural industries worldwide. It has been estimated that 50 million cattle are infected with *M. bovis* worldwide resulting in around US$3 bn losses annually and this is despite attempts to control the disease. For example, over the last two decades the (tuberculin) test and slaughter strategy failed to prevent a dramatic rise in the incidence of bTB in cattle in England and Wales (https://www.gov.uk/government/statistics/incidence-of-tuberculosis-tb-in-cattle-in-great-britain). Development of new and improved cattle vaccines and diagnostic reagents for cattle as well as other domestic animal species and wildlife has therefore emerged as a research area that could contribute to improved disease control. However, a number of challenges need to be overcome, some scientific, others legal or regulatory. Thus, in countries operating test and slaughter control strategies, differential diagnostic tests are required, particularly when vaccination involves bacille Calmette-Guérin (BCG) or attenuated *M. bovis* vaccines that will compromise the specificity of standard tuberculin-based diagnostic tools. A further complication is that prohibiting cattle vaccination against bTB in the European Union (EU) would necessitate a change of a number of national and EU laws and directives. Vaccine strategies will also require extensive safety studies to allow acceptance by regulators. However, this review will concentrate solely on the scientific aspects of bovine TB vaccine development and not dwell on either legal or regulatory aspects. This chapter will provide a concise review of the state-of-the-art in vaccine and differentiate infected from vaccinated animals (DIVA) test development, concentrating in particular on the contribution of the TBSTEP EU project to the field.

Models for Assessing Vaccine Efficacy in Cattle

Historically, the assessment of vaccines for protection against bovine TB was undertaken by challenging cattle with high doses of *M. bovis* administrated by unnatural routes such as subcutaneously, orally or intravenously (reviewed by Francis, 1947). The resulting pathology was atypical compared to the natural disease, although vaccination with BCG reduced the severity of the disease.

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al.*)

To assess the efficacy of novel TB vaccines and optimize the effectiveness of vaccines, more realistic experimental challenge systems were introduced. Lesions mimicking those observed in the respiratory tract following natural exposure to *M. bovis* have now been reproduced by challenging cattle with relatively low doses of *M. bovis* (10^3 to 10^4 colony-forming units, CFU) by intranasal (Neill *et al*., 1988), intratonsilar (Palmer *et al*., 1999), intratracheal/endobronchial inoculation (Buddle *et al*., 1994) or by aerosol-generating systems (Palmer *et al*., 2002; Rodgers *et al*., 2007). More recent studies to assess TB vaccine efficacy in cattle have focused on using intratracheal/endobronchial inoculation or aerosol challenge, as the resulting lesions have been predominantly found in the lungs and pulmonary lymph nodes, allowing for a presentation of natural disease and a more reproducible scoring of pathology.

Sophisticated end points to measure the qualitative and quantitative effect of vaccination (i.e. protection) have been developed. For example, protection has been measured by determining the proportions of animals with gross lung and pulmonary lymph node lesions, quantitative scoring of gross pathology lesions and assessment of bacterial load in lung lesions and pulmonary lymph nodes. Further refinements include the scoring of granuloma development stages based on histology (e.g. Wangoo *et al*., 2005). The experimental challenge of cattle with *M. bovis* is more severe than that encountered by natural exposure to *M. bovis*. The challenge must be sufficiently severe to induce lesions in the majority of non-vaccinated animals to demonstrate statistically significant differences between vaccinated and control groups as only relatively small numbers of animals can be held in the containment facilities. Due to the severity of the *M. bovis* infection induced by experimental challenge, it has been critical to use lung and pulmonary lymph node lesion scores to assess protection. Scores of 0–5 have been used to quantify lung lesion severity and have been used for individual lung lobes and then pooled (Vordermeier *et al*., 2002) for a score of 0–5 based on total count of lesions in the lungs (Wedlock *et al*., 2011). In addition, lesion scores of 0–3 have been used to assess the severity of lesions in individual pulmonary lymph nodes and scores pooled for all pulmonary lymph nodes. Determination of the percentage of the lungs containing lesions has been determined by radiography (Waters *et al*., 2007). The severity of lesions has also been estimated from histopathological examination and scores of I–IV have been used to classify the types of granulomas present in lesions (Wangoo *et al*., 2005). Example of the application of gross pathology scoring and histological assessment of granuloma development to determine vaccine efficacy are shown in Fig. 10.1.

Mycobacterial counts from lungs have been difficult to measure accurately as *M. bovis* is concentrated in lung lesions and generally in relatively low numbers. In addition, it is not practical to homogenize whole lung lobes. Mycobacterial counts from pulmonary lymph nodes have been estimated by culturing homogenates of samples from lymph nodes and expressing the bacterial count per gram of lymph node (Vordermeier *et al*., 2002). Results can also be shown as the mean number of pulmonary lymph nodes culture positive for *M. bovis*/animal.

An attempt has been made to establish a more natural challenge system by housing vaccinated and non-vaccinated cattle together with naturally infected animals. However, the transmission rate was very low and large group sizes would be necessary to demonstrate a statistically significant effect resulting from vaccination (Khatri *et al*., 2012), although such approaches are feasible in settings that result in higher transmission rates (Ameni *et al*., 2010). The final assessment of protective efficacy should be obtained from natural exposure of vaccinated and non-vaccinated animals to *M. bovis* in field studies. It can be difficult to control the variables associated with studies in the field and these studies can be expensive to undertake particularly when the prevalence of *M. bovis* infection is low in herds. Two recent field studies have been undertaken in countries with a high prevalence of bovine TB and results from BCG vaccination of cattle have been encouraging (Lopez-Valencia *et al*., 2010; Ameni *et al*., 2010).

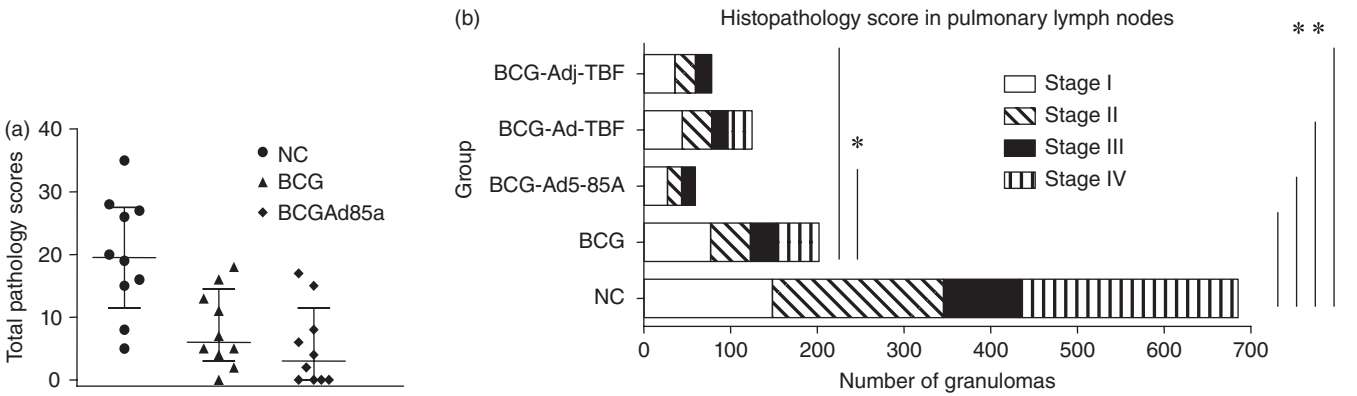


Fig. 10.1. Protective efficacy of BCG and BCG-recombinant adenovirally vectored Ag85A to protect cattle against experimental *M. bovis* challenge. (a) Protection determined by applying a pathology scoring system (modified from Vordermeier *et al*., 2002). (b) Assessment of protection based on granuloma stage scoring (Wangoo *et al*., 2005). NC, unvaccinated controls; BCG, BCG Danish vaccinated group; BCGAd85A, BCG vaccination followed by Ad85A boosting; BCG-Ad-TBF, BCG prime, boosted with different adenoviral vaccine; BCG-Adj-TBF, heterologous prime boost vaccination schedules priming with BCG and boosting with a cocktail of four antigens presented in adjuvant; BCG-Ad5-85A, heterologous prime boost vaccination schedules priming with BCG and boosting with recombinant adenoviruses expressing the antigen Ag85A (from Dean *et al*., 2013).

BCG

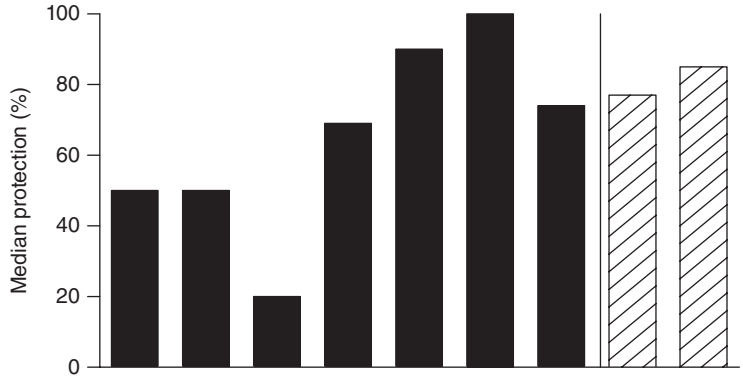
The only TB vaccine potentially available in the short to medium term is BCG, the vaccine used to vaccinate humans against TB. There can be no doubt that BCG will protect cattle against bovine TB – indeed, this has been known since 1912, as evidenced by a large number of field trials since the early 20th century that demonstrated its partial effectiveness. Nevertheless, as in humans, the degree of protection varied considerably between studies (reviewed in Waters *et al*., 2012). However, a range of different strains of BCG exists due to its decentralized propagation after it was distributed around the world in the 1920s and therefore a wide variety of these strains was used in these trials, together with different doses, routes and ways to determine protection. This makes an informative meta-analysis of these trials challenging and possibly misleading.

However, reviewing recent studies of the last two decades following harmonization of the BCG vaccine strains across experiments and many laboratories (initially BCG Pasteur, then BCG Danish SSI) and applying standardized cattle vaccination/challenge models as described in the previous section, one can conclude the following:

1. BCG Pasteur and BCG Danish induce equivalent degrees of protection (Wedlock *et al*., 2007; Hope *et al*., 2011).
2. Neonatal or young calves can be protected at least as well as older calves (Buddle *et al*., 2003; Hope *et al*., 2005).
3. Systemic as well as mucosal (oral or endobronchial) delivery of BCG leads to protection (Buddle *et al*., 2005, 2008).

One contentious attribute of BCG in cattle is how long it will protect them (i.e. its duration of immunity). Recent studies using a stringent experimental challenge model have indicated that protection may last only 1 year (Thom *et al*., 2012), whereas a study undertaken under field conditions in Ethiopia indicated that protection could last at least 22 months (Ameni *et al*., 2010), although the infection conditions in these studies were not directly comparable. This immediately identifies a challenge that one faces to improve BCG vaccine efficacy: to increase its efficacy per se and to extend the duration of the immunity it induces. Last, based on field studies in Ethiopia and Mexico, it is likely that BCG will perform better under field conditions than when tested under the stringent and severe experimentally induced challenge (Lopez-Valencia *et al*., 2010; Ameni *et al*., 2010). To illustrate some of the points made above on BCG, Fig. 10.2 shows the outcome of eight studies performed in our laboratories over the last decade of vaccinating and challenging calves with BCG Danish SSI. This figure depicts the mean protection levels as calculated by the percentage reductions in total pathology scores. It shows that calves of different ages can be equally protected, including very young calves, and that the duration of immunity wanes between 1 and 2 years post-vaccination (Experiments 2 and 3, respectively, with calves vaccinated at the same time, but challenged at different times). It also demonstrates that BCG can be as effective at lower vaccination doses (0.5 human dose equivalent) than the standard cattle dose applied in these studies, which is the equivalent of five human doses (Experiment 8; Buddle *et al*., 2013). A recently described vaccination protocol described to improve the protective efficacy after systemic BCG vaccination is based on the simultaneous systemic and intrapulmonary application of BCG (Tchilian *et al*., 2011). We tested this model in cattle recently and could demonstrate reductions in lung and lung lymph node pathology following simultaneous BCG vaccination compared to either systemic or mucosal vaccination alone, although the differences were not statistically significant (Villarreal-Ramos, Vordermeier, Beverley, unpublished data).

An additional challenge associated with BCG vaccination is that it compromises the specificity of tuberculin PPD-based diagnostic tests such as skin tests for at least 6 months post-vaccination. However, protection is not linked to the maintenance of tuberculin skin test reactivity; that is, skin test-negative animals that were BCG vaccinated are as protected as those that maintain skin test reactivity (Whelan *et al*., 2011a). Thus its use in cattle and other domestic animals will require the development of a diagnostic test



Experiment	1	2	3	4	5	6	7	8	
Age[a]	< 6	< 6	< 6	ca. 36	20–32	< 7	20–32	20–24	
Dose[b]	5	5	5	5	5	5	5	5	0.5
Interval[c]	ca. 3	ca. 12	ca. 24	3	3	6–7	3	4	

Fig. 10.2. Performance of BCG Danish in experimental cattle challenge experiments. Data expressed as mean % protection based on pathology scores ([median total pathology scores in unvaccinated calves minus scores in vaccinated animals divided by median scores established in unvaccinated controls] × 100). The age of the calves at the time of vaccination[a]; the BCG doses applied relative to standard human BCG vaccine doses[b] (2–8 × 10^5 CFU of BCG); as well as the interval between vaccination and challenge[c] are indicated.

that can be used alongside vaccination to differentiate vaccinated and infected cattle (DIVA test). The efficacy of BCG to protect domestic goats will be discussed in later sections of this review.

Improving BCG Efficacy

Conceptually there are two ways of improving BCG efficacy and/or extend its duration of immunity: (i) one could increase immunity (and by inference also its duration) at the time of primary vaccination by amplifying or boosting BCG induced immunity; or (ii) one could boost immunity at the time point of waning immunity (see Fig. 10.1).

Significant progress in developing TB vaccines for cattle has been made. Often these vaccines or vaccine strategies are derived from the close and mutually beneficial collaboration between human and bovine TB vaccine development programmes. It has been shown that one of the most effective vaccination protocols against bTB is based on priming the immune system with BCG followed by boosting with subunit vaccines containing protective antigens that are present in BCG (*heterologous prime-boost strategy*). Subunits have been based on DNA (e.g. Skinner *et al*., 2003, 2005; Maue *et al*., 2007) or virally vectored booster vaccines (Vordermeier *et al*., 2004, 2006, 2009). A variation of this theme is the simultaneous vaccination with BCG and protein subunit vaccines, for example, that has also been shown to improve protection (Wedlock *et al*., 2008). Other approaches that are being taken are employing novel rationally attenuated *M. bovis* strains. For example, two *M. bovis* strains attenuated by chemical UV irradiation (*M. bovis* WAg500 and WAg501) were able to protect cattle pre-exposed to environmental mycobacteria against *M. bovis* when BCG did not (Buddle *et al*., 2002). However, as the mutations were not defined, these vaccines were not developed further. Defined mutants based on *M. bovis* Ravenel after deletion of the RD1 region were as protective in cattle as BCG (Waters *et al*., 2009). The deletion of the RD1 region, apart from being attenuating, also allowed the use of RD1-encoded antigens such as ESAT-6 and CFP10 as DIVA antigens (see below for discussion of DIVA

diagnosis) (Waters *et al*., 2009). Further, a recent cattle study described the improved protective immunogenicity and protection compared to BCG of an *M. bovis* vaccine strain in which the mce2A and mce2B genes were deleted (Blanco *et al*., 2012, 2013). However, this vaccine will not be immediately amenable to DIVA diagnosis based on the RD1 encoded antigens. Another strategy employed is the over-expression of antigens such as Ag85B in BCG, which also lead to improved protection of cattle against tuberculosis (Rizzi *et al*., 2012).

One of the most consistently successful approaches has been the use of virally vectored booster vaccines using Ag85A as antigen (Vordermeier *et al*., 2009; Dean *et al*., 2013). The data generated over the last 6 years at APHA has demonstrated that a replication-deficient recombinant human type 5 adenoviral vaccine expressing the mycobacterial protective antigen Ag85A (Ad-85) consistently improved BCG efficacy when applied in a BCG-prime/adenoviral booster vaccine scenario (Vordermeier *et al*., 2009; Dean *et al*., 2013 and unpublished data). This improvement over BCG was demonstrated by the increase in the proportion of animals that presented without visible tuberculous lesions, reduction in gross pathology or histological pathology compared to BCG vaccination (Fig. 10.1; Vordermeier *et al*., 2009; Dean *et al*., 2013). This strategy (BCG/Ad-85 prime-boost) has also been shown to protect goats against caprine TB (Perez de Val *et al*., 2012) and is in line with current approaches conducted in the human TB vaccine programmes with several of such vaccines being tested in human clinical trials (Ottenhoff and Kaufmann, 2012). We have also demonstrated that intra-pulmonary boosting with Ad-85 boosted both mucosal (lung) and systemic (PBMC) responses following systemic BCG priming (Whelan *et al*., 2012). Recently, we applied Ad-85 intra-pulmonarily at the same time as subcutaneous BCG vaccination (simultaneous vaccination approach as proposed by Tchilian *et al*. (2011) using a murine model) and could demonstrate improved protection compared to BCG alone (Villarreal-Ramos, Vordermeier, Beverley, unpublished data).

DIVA Test

The strategies described above that involved BCG resulted in at least a proportion of animals becoming tuberculin-test positive (both in tuberculin skin and IFNγ tests) after vaccination (Buddle *et al*., 1999; Vordermeier *et al*., 1999; Garnier *et al*., 2003; Brosch *et al*., 2007), necessitating the development of a DIVA test. Thus, the development of complementary diagnostic reagents allowing the discrimination of infected from vaccinated animals remains an essential requirement so that a test and slaughter approach to TB control can be continued in the face of vaccination.

Antigen mining strategies

The elucidation of the genomes of the relevant mycobacterial species (*M. tuberculosis*, *M. bovis*, BCG, *M. avium* subsp. *paratuberculosis*) has revolutionized antigen mining by allowing systematic comparative genome and transcriptome analysis to find antigens suitable for differential diagnosis (reviewed in Vordermeier *et al*., 2011). Strategies to develop DIVA reagents for bTB that would be applicable alongside BCG vaccination are based on a number of approaches (reviewed in Vordermeier *et al*., 2011):

1. *Comparative genomic approaches*. The search for antigens whose genes are deleted from the BCG genome such as the RD1-encoded antigens ESAT-6 and CFP10; individual antigens whose genes were deleted from BCG or mutations in BCG leading to frameshift mutations resulting in modified protein sequences or stop codons, i.e. protein truncations in BCG compared to *M. bovis* (with the protein portions not expressed in BCG being potential DIVA antigens) or antigens that belong to distinct protein classes such as secreted proteins, ESAT-6-like proteins or stage-specific antigens such as latency-associated proteins (Jones *et al*., 2011).

2. *Comparative transcriptomic approaches*. The comparative genomic approaches described above have also been supplemented by comparative transcriptome analysis of mRNA transcription and expression of genes and gene products between *M. bovis* and BCG.

3. *Bacterial cell biology*. Last, we and others have also searched for antigens that are secreted by *M. bovis* but not by BCG (Sidders *et al*., 2008) as secretion has been shown to be a hallmark of immunogenic proteins.

Interferon-gamma (IFNγ) is the prime analyte measured in blood-based TB diagnosis. Due to the easy availability of large blood volumes from cattle, using this test is also an ideal tool to test hundreds of potential antigens simultaneously in the same animal, a feat impossible if one were to use skin testing for mining purposes. Consequently the majority of DIVA tests are based on IFNγ production by blood leukocytes. Two of the major antigenic targets identified in both cattle and humans are ESAT-6 and CFP10 (Pollock and Andersen, 1997a,b; van Pinxteren *et al*., 2000). ESAT-6 and CFP10 are encoded by genes located on the RD1 region of the *M. bovis*/*M. tuberculosis* genome that is deleted from the genome of all BCG strains (Mahairas *et al*., 1996; Garnier *et al*., 2003; Brosch *et al*., 2007). ESAT-6 and CFP10 antigens discriminate between infected and BCG-vaccinated cattle when used as recombinant proteins and as cocktails of synthetic peptides (Buddle *et al*., 1999; Vordermeier *et al*., 1999, 2001). Peptides derived from the sequences of the two proteins were readily identified using blood from *M. bovis*-infected cattle and gave equivalent responses to the recombinant proteins in naturally infected cattle (Vordermeier *et al*., 2001). In contrast, BCG-vaccinated cattle did not respond to this peptide cocktail (Vordermeier *et al*., 2001).

In an extension of the *in silico* comparative genome analysis, the transcriptome of BCG and *M. bovis* under different *in vitro* growth conditions and macrophage infection experiments was also assessed, the transcriptome being the complete gene expression profile of an organism under defined conditions. One protein thus identified, Rv3615c, was recognized at a frequency of about 55% by blood cells from *M. bovis*-infected animals but was not recognized by uninfected or BCG-vaccinated cattle, thus highlighting its potential as a DIVA reagent (Sidders *et al*., 2008). Further analysis of the Rv3615c responses revealed that it could complement ESAT-6 and CFP10 to increase test sensitivity by recognizing a proportion of animals that escaped ESAT-6 and CFP10 detection (Sidders *et al*., 2008). Rv3615c has been shown to be secreted via the esx1 secretion system which is encoded on the RD1 regions although it is not itself located on this region (MacGurn *et al*., 2005). As BCG lacks the RD1 region, Rv3615c cannot be secreted by BCG (Millington *et al*., 2011) which explains why it was not immunogenic in vaccinated animals. The usefulness of Rv3615c as a DIVA diagnostic reagent has also been confirmed by studies of human subjects (Milligan *et al*. 2011).

The results of recent larger trials and experiments to determine the performance of these three antigens are shown in Table 10.1. Using naturally infected animals and uninfected animals in GB, the sensitivity of the ESAT-6/CFP10 cocktail is around 80% (data not shown) which could be increased to around 90% by the inclusion of Rv3615c (Table 10.1), thus confirming in a larger study the results of the pilot study that Rv3615c complemented ESAT-6 and CFP10. This sensitivity level is therefore akin to that seen with tuberculin (Table 10.1). The specificities of both sets of reagents were similar and comparable to those seen with tuberculin (around 97–99%, Table 10.1). Due to the legal prohibition of vaccination of cattle in the field, data obtained from vaccinated/infected or vaccinated/uninfected cattle are based on experimental laboratory-type studies. The numbers of animals studied are therefore of necessity by far smaller than when sampling is possible in the field. Nevertheless, the performance data in Table 10.1 on the sensitivity of this test using the three antigens ESAT-6, CFP10 and Rv3615c, in animals that were BCG vaccinated and infected with *M. bovis* that presented with demonstrable disease at post mortem, were largely comparable to those seen in the naturally infected field animals and on a level that was comparable with tuberculin (Table 10.1). The specificity of the DIVA reagents in BCG-vaccinated calves was also comparable to that observed in the unvaccinated field animals and, as expected, by far superior to the specificity obtained with PPD (Table 10.1). However, one caveat is that the performance characteristics of diagnostic reagents in laboratory studies, as compared to field studies, can be over-estimates as laboratory

Table 10.1. Performance of the interferon-γ DIVA test (ESAT-6/CFP10 and/or Rv3615c).

	DIVA[a]	B-A[b]
BCG vaccinated/experimentally infected		
Number positive/total	42/45	40/45
Sensitivity (95% CI)	93.33 (81.73–98.60)	88.89 (75.93–96.29)
BCG vaccinated		
Number positive/total	8/179	51/179
Specificity (95% CI)	95.53 (91.38–98.05)	71.51 (64.36–78.01)
Field reactors		
Number positive/total	104/115	110/115
Sensitivity (95% CI)	90.43 (83.54–95.12)	95.65 (90.15–98.57)
Controls		
Number positive/total	9/691	10/691
Specificity (95% CI)	98.70 (97.55–99.40)	98.55 (97.35–99.30)

[a]Using the DIVA antigens ESAT-6/CFP10 and/or Rv3615c.
[b]Using bovine and avian tuberculin PPD.

studies are by their nature more controlled. There is therefore an important requirement to assess the performance of these DIVA reagents in field studies testing BCG- vaccinated animals.

While the results discussed in this chapter have been obtained by applying the DIVA test in an *in vitro* blood test format (IFNγ release assay), the same antigens have also been used as skin test reagents (Whelan *et al*., 2010; Jones *et al*., 2012). These studies have demonstrated that, in principle, DIVA skin testing constitutes a practical, specific and sensitive read-out (Whelan *et al*., 2010; Jones *et al*., 2012). Recently, we have added a fourth antigen to the skin test cocktail, Rv3020c (Jones *et al*., 2012), which improved its sensitivity as demonstrated with data generated using naturally infected cattle, while not impacting on specificity (Jones, Buddle, Vordermeier, unpublished; Table 10.2). We have also recently investigated whether skin test reagents based on recombinant proteins or synthetic peptides perform comparably. While both reagent presentations induced potent skin test responses in infected cattle, we could demonstrate significantly higher signal strengths (increased reaction sizes) following protein injection compared to the peptide-based reagent (Jones, Vordermeier, unpublished data). This is an important finding as it will guide the pathway to commercialization for such DIVA skin test reagents. These antigens are also able to differentiate between *M. bovis* infection and *M. avium* subsp. *paratuberculosis* infection or vaccination (Flores-Villalva *et al*., 2012; Coad *et al*., 2013). As a means of reducing the cost of these reagents, a recent study showed that the display of ESAT-6, CFP10 and Rv3615c proteins on biobeads produced in *Escherichia coli* could markedly reduce the concentration of mycobacterial proteins used in an intradermal test (Chen *et al*., 2014). The TB biobead preparation was shown to induce similar intradermal test responses to those for the recombinant proteins or peptides and identified 10 of 11 naturally TB-infected cattle (N. Parlane and B. Buddle, unpublished observation).

Biomarker Discovery: Predictors and Correlates of Vaccine Efficacy

TB vaccine development in both humans and animals has produced a growing portfolio of candidates with potential applicability across species. However, progress has been hampered by the lack of understanding of the underlying biological mechanisms and the lack of correlates of protection that can guide vaccine design or animal experiments, or that can be used as a credible end point in field

Table 10.2. Biomarkers correlating with vaccine efficiency or disease defined in cattle.

Parameter	Category			Reference
	Predictor of vaccine efficacy[a]	Correlate of protection[a]	Correlate of disease/ pathology[a]	
IFNγ (ESAT-6[b])	NA	Inverse[c]	Positive[d]	Vordermeier *et al*., 2002 Skinner *et al*., 2003; Lyashchenko *et al*., 2004
MPB83-specific IgG1	NA	Inverse	Positive	Lyashchenko *et al*., 2004
(MPB70)-specific IL-4 expression	NA	Inverse	Positive	Wedlock *et al*., 2003
IL-2 (PPD)	NA	Inverse	Positive	Rhodes *et al*., 2014
Multifunctional $CD4^+$ T cells based on IFNγ, IL2, TNFα ICS (PPD)	NA	Inverse	Positive	Whelan *et al*., 2011b; Dean *et al*., 2013
micro-RNA miR-155 (PPD-B)	NA	Inverse	Positive	Golby *et al*., 2014
CXCL9, CXCL10, granzyme A (PPD)	Not tested	Not tested	Positive	Aranday-Cortes *et al*., 2012
Ex vivo IL-4δ3 expression	Positive	NA	NA	Rhodes *et al*., 2007
Cultured IFNγ ELISPOT assays (Ag85A or PPD)	Positive	Not tested	Not tested	Vordermeier *et al*., 2006; Vordermeier *et al*., 2009; Waters *et al*., 2009; Thom *et al*., 2012
IL-17 (Ag85A or PPD)	Positive	Inverse	Positive	Vordermeier *et al*., 2009; Aranday-Cortes *et al*., 2012; Rizzi *et al*., 2012
IL-22 (PPD)	Positive	Inverse	Positive	Bhuju *et al*., 2012

[a]For definitions of these parameters see Box 10.1. NA, not applicable.
[b]Stimulating antigen(s) in brackets.
[c]Inverse: lower responses in vaccinated animals compared to controls after *M. bovis* challenge.
[d]Positive: higher responses in control animals correlated with degree of pathology or bacterial load.

Box 10.1. Definition of Terms for Biomarker Categories.

Predictor of protection
Will indicate if vaccine is effective after vaccination but BEFORE infection (i.e. challenge experiments are not required)
Correlate of protection
Will indicate vaccine efficacy after vaccination and infection (i.e. challenge experiments are required)
Correlate of pathology/disease severity
Will increase with increase in disease severity/pathology post-*M. bovis* infection. Will be lower in vaccinated animals that are protected compared to unvaccinated control = inverse correlate of protection

trials. The bottleneck for cattle vaccine development is the expense and paucity of BL3 facilities. Reliable gating criteria to select only the most promising vaccine candidates for testing in BL3 experimentation or field trials would greatly accelerate the pace of vaccine development. IFNγ produced *ex vivo* on its own is a poor predictor of protection because not all vaccines able to induce IFNγ have been found to be protective. However, the absence of vaccine-induced *ex vivo* IFNγ responses could be used to gate out vaccines that are unlikely to protect cattle. Hence, significant research efforts have been directed towards

development and validation of such biomarkers using hypothesis, as well as data-driven approaches including host transcriptome and proteome analysis combined with systems biology. In the next section, we will attempt to summarize recent progress in this area.

To ease the discussion in this biomarker discovery section, it is helpful to introduce the following classification for biomarkers measurable at different time points post-vaccination and post-challenge (see also Box 10.1). Thus, biomarkers can be defined that can predict vaccine efficacy (i.e. those that can be measured post-vaccination but without the need for *M. bovis* challenge), or they can correlate with protective immunity (i.e. those that can be measured after *M. bovis* infection that will give insights into the nature of protective immunity) or they can correlate with disease progression (measured after challenge that can serve also as inverse correlate of protection as these markers are generally of lower magnitude in successfully vaccinated animals post-challenge). Markers of disease progression are often also inversely correlated with vaccination success as they are normally measurable at lower levels in animals that were successfully vaccinated. This is illustrated in Fig. 10.3a that shows results with the 'prototype' marker (i.e. ESAT-6-inducible *in vitro* IFNγ production) correlating with the extent of pathology and bacterial burden in infected cattle. The production of IFNγ is lower in vaccinated animals after *M. bovis* challenge (Vordermeier *et al*., 2002).

The outcome of vaccination experiments in cattle is normally threefold: vaccinated animals are either not protected, or animals are partially protected (defined as presenting significant reductions in pathology) or animals present at post mortem without visible or histological signs of disease ('fully protected') (e.g. Vordermeier *et al*., 2002, 2009). Comparing animals from the same group that were not protected with those that were protected is a very powerful approach to biomarker discovery (Vordermeier *et al*., 2009), and by far more informative than comparing groups that were vaccinated with different vaccines as is very often done. This is shown in Fig. 10.3b using the example of cultured ELISPOT responses that predict the outcome of vaccination (Vordermeier *et al*., 2009).

Applying both hypothesis- and data-driven approaches, a number of biomarkers have been identified that either correlate with disease status and are generally inversely correlated with vaccine efficacy, and others that

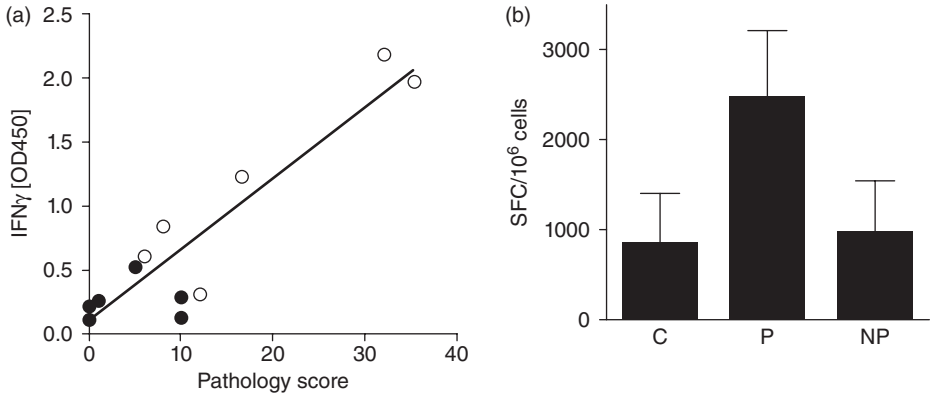


Fig. 10.3. Examples of biomarkers. (a) ESAT-6 inducible IFNγ production as correlate of pathology/bacterial burden and inverse correlate of protection. Open symbols, unvaccinated control calves; closed symbols, BCG vaccinated calves. IFNγ was determined 12 weeks post-*M. bovis* infection (modified from Vordermeier *et al*., 2002). (b) Cultured ELISPOT responses as predictor of vaccine efficacy. Responses were determined after BCG or BCG-Ad85A prime boost vaccination but before *M. bovis* infection. C, unvaccinated controls; P, vaccinated calves that were protected; NP, vaccinated calves that were not protected (Vordermeier *et al*., 2009).

predict vaccine efficacy after vaccination but before *M. bovis* challenge. Table 10.2 provides an overview of the different candidates prioritized. As shown in Table 10.2, a number of markers correlating with pathology and thus indirectly with vaccine efficacy have been identified such as the ESAT-6 induced IFNγ production already mentioned (see Fig. 10.3a and Table 10.2; Vordermeier *et al*., 2002). We have recently investigated the role of *in vitro* IL-2 production as a potential predictor of vaccine-induced protection and memory as well as a potential marker for disease stages in cattle (latency). This approach was based on the data generated in humans that suggested that ESAT-6 and other antigen-specific IL-2 responses were predominant in patients following treatment and latently infected, contrasting with higher antigen-specific IFNγ responses in active TB patients (Millington *et al*., 2007; Casey *et al*., 2010). We developed reagents enabling us to determine bovine IL-2 by ELISA, ELISPOT and cytometry (Whelan *et al*., 2011b; Rhodes *et al*., 2014) and applied them to study IL-2 responses after vaccination and infection. In contrast to the human data cited above, in cattle we found that IL-2 production *in vitro* correlated with the degree of pathology, was undetectable after BCG vaccination before challenge and was after challenge lower than the values determined in unvaccinated and infected animals (Rhodes *et al*., 2014). Interestingly, a similar association of IL-2 with active disease, as compared to latent tuberculosis, was also recently described in human TB patients (Singh *et al*., 2013).

Another potential biomarker correlating and predicting vaccine efficacy that has attracted a huge amount of attention in recent years has been the presence of so-called polyfunctional T cells. These cell populations are measured by the intracellular staining for cytokines determined by cytometry. Associations between protection and the numbers of such polyfunctional T cells induced post-vaccination has been described mainly in small animal models (e.g. Aagaard *et al*., 2009; McShane, 2009; Nambiar *et al*., 2012). We found that polyfunctionality in cattle, as defined by the presence of $CD4^+$ T cells producing IFNγ, IL-2 and TNF-α or a combination of at least two of these markers, does not predict vaccine success but correlates strongly with disease progression/increased pathology and was thus an inverse correlate of protection (Table 10.2; Whelan *et al*., 2011b; Dean *et al*., 2013). Other markers of disease progression defined after hypothesis or data-driven experiments using microarrays are listed in Table 10.2 and will not be discussed further in detail in this chapter. The most promising markers that predicted vaccine efficacy after vaccination and before *M. bovis* infection are discussed in the following paragraph.

One predictor of vaccine efficacy is based on T cell memory responses measured by the cultured ELISPOT method (Vordermeier *et al*., 2006, 2009). These responses are significantly elevated in vaccinated/protected animals compared to matched vaccinated calves that were not protected (Waters *et al*., 2007; Vordermeier *et al*., 2009). This marker is applicable to live vaccines such as BCG and BCG-based adenoviral subunit vaccine heterologous prime-boost strategies, as well as to vaccines based on attenuation of *M. bovis* (Waters *et al*., 2007; Vordermeier *et al*., 2009). Interestingly, a recent paper by Thom *et al*. (2012) demonstrated that the maintenance of strong cultured ELISPOT responses correlated with BCG vaccination-induced duration of immunity. Characterization of the cell populations that are responsible for cultured ELISPOT responses defined them as almost exclusively $CD4^+$ but composed of subsets that are, based on their surface phenotype, associated with both central and effector memory phenotypes ($CD45RO + CD62L^{hi}$ and $CD45ROCD62L^-$, respectively) (Villarreal *et al*., unpublished data).

Through the combination of mouse transcriptome studies using DNA microarrays and subsequent validation of interesting biomarkers in cattle by conventional qRT-PCR, we described cytokine responses indicative of the involvement of Th17 lineage cells as predictive biomarkers, such as IL17A and IL23, to be predictive of protection (Aranday-Cortes *et al*., 2010; Aranday-Cortes *et al*., unpublished data). The finding that IL-17 expression after vaccination correlated with protection has been confirmed in a recent cattle study conducted in Argentina (Rizzi *et al*., 2012). The role of IL-17 producing cells in TB has been studied previously in mouse models of human TB. These studies showed that in

vaccinated animals the absence of IL-17 producing memory cells resulted in the loss of Th1 responses and protection (Khader *et al*., 2007; Khader and Cooper, 2008). Thus mixed response of Th1 and IL-17 producing cells (such as Th17, γδ T cells or NK cells) seem to cross-regulate themselves and are both important for protective anti-tuberculous responses (Khader *et al*., 2007; Khader and Cooper, 2008). Our results are therefore confirmatory of these findings.

Recently, we have undertaken whole-genome transcriptome analysis of responses of BCG-vaccinated or BCG-primed adenovirally boosted cattle by deep sequencing (RNASeq) and have identified IL-22 as a major predictor of vaccine success in cattle (Bhuju *et al*., 2012), further suggesting the involvement of Th17 cells, or alternatively Th22 cells (which have not been described yet in cattle to date) in protection together with Th1 responses. We have also developed antibody reagents that recognize bovine IL-22 and used them in cytometry (Steinbach, Jones, Vordermeier, unpublished). We could demonstrate that both $CD4^+$ and $CD4^-$ T cell subsets are producing IL-22 following *in vitro* stimulation with PPD (Steinbach, Jones, Vordermeier, unpublished). The role of IL-22 in protective responses is also not defined although one potential effector mechanism could be the production of beta-defensins. This whole-genome transcriptome approach is likely to yield further and more sophisticated biosignatures that in the future could allow the prediction of vaccine success with less need to undertake resource-intensive challenge experiments.

Lastly, we have also studied the role of micro-RNAs (miR) after vaccination and infection. This approach promises to give insights into epigenetic control of gene expression. We applied a microarray that contained probes to determine bovine micro-RNA expression and found that the expression of miR-155 following *in vitro* stimulation of PBMC with PPD correlated with the degree of pathology but not with vaccine efficacy. This result was confirmed by conventional qRT-PCR (Table 10.2; Golby, Villarreal-Ramos, Vordermeier, unpublished). The application of systems biology suggested that this micro-RNA regulates the expression of genes that result in stronger immune pathology. Interestingly, miR-155 has also been described to be upregulated in human patients with active disease (Wu *et al*., 2012) and in bovine alveolar macrophages following *M. bovis* infection (Vegh *et al*., 2013).

Conclusions and Challenges Ahead

Significant advances have taken place in the development programme for bTB vaccines for domestic ruminants and associated DIVA reagents. The discovery of biomarkers has also progressed with some pace. However, significant challenges exist before cattle vaccination, particularly in the EU, can become a practical option. First, the performance under realistic field conditions of even BCG, much less of the more advanced strategies, is not known, nor is the field performance of the DIVA tests known. Thus, performing large-scale field trials is a priority. There are also important legal and regulatory hurdles to overcome, not least to demonstrate the safety of these new vaccines (and of BCG) in the target species, and also in respect to food safety, to the regulators' satisfaction.

Future research needs

The definition of additional biomarkers predicting vaccine efficacy is required, particularly focusing on gene regulation and epigenetics, as well as the validation of those already described, should be a priority. This will in future allow the definition of rational gating criteria to decide when promising vaccines should be tested in expensive BL3 cattle infection studies or field trials. It is possible that different gating strategies (and different biomarkers supporting these gating decisions) will have to be developed for different types of vaccines. For example, live vaccines could be selected on the basis of small animal experiments demonstrating superior protection compared to BCG for testing in cattle immunogenicity experiments applying relevant predictive biomarkers. Only those vaccines that induce increased levels of these biomarkers compared to BCG would then be tested in BL3 *M. bovis* infection experiments or field experiments (Fig. 10.4). Small animal experimentation, however, will be unlikely to support the gating

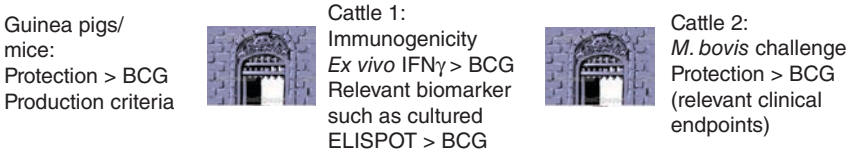


Fig. 10.4. Proposed gating strategy for live attenuated vaccines.

of protein in adjuvant-based vaccines, because adjuvant activity in small animals is often not predictive of activity in cattle.

Can mucosal vaccination improve vaccine efficacy? In this respect, the development of adjuvants in general and mucosal adjuvants and particularly delivery systems that are biologically active in ruminants is a requirement. An overarching need for vaccine development (and also for the further refinement of DIVA reagents) will be high-throughput antigen discovery approaches that combine bioinformatics including algorithms predicting bovine T cell epitopes, MHC peptide elution systems to perform this technique in cattle, and the discovery of non-protein antigens such as lipids and glycolipids. It would also be advantageous to develop vaccines that do not lead to the development of tuberculin skin test reactivity (so-called non-sensitizing vaccines) which would allow the continuation of existing test and slaughter strategies alongside vaccination and thereby avoid potentially expensive DIVA testing. To allow scientists and policy makers to make rational and evidence-based decisions on the shape of ongoing and future development programmes, it is also necessary to develop informative mathematical models of vaccine and DIVA performance.

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11 *Mycobacterium bovis/M. caprae* Infection in Goats and Sheep: Introduction, Epidemiology and Control Measures

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Introduction

Tuberculosis in small ruminants is a chronic infection with a devastating effect in affected flocks; this disease produces a serious economic impact and represents a potential risk for human health. The infection has been largely neglected but awareness of its relevance to animal health has increased in recent years.

The members of the *Mycobacterium tuberculosis* complex (*M. bovis*, *M. caprae* and *M. tuberculosis*) causing infection in small ruminants and the impact of the disease in these species vary depending on geographical areas. Tuberculosis in domestic goats (*Capra aegagrus hircus*) and sheep (*Ovis aries*) has been reported in many countries although there are no official data about the prevalence, except approximate figures in regions where it has been studied in more detail. Tuberculosis in goats can be considered endemic in some countries, with goat populations known to be heavily affected, while in other regions reports are only occasional. The presence of small ruminants infected with tuberculosis may jeopardize the success of the eradication programmes. In some cases, the specific measures applied to cattle are also applied to small ruminants; however, testing of goats or sheep coexisting with cattle may not be mandatory in all countries. This chapter reviews the epidemiology of tuberculosis in small ruminants, the performance of ante-mortem diagnostic tests and control measures that have been used to eradicate infection in affected flocks (Fig. 11.1).

Epidemiology

Initial reports of tuberculosis in small ruminants dating back to the early years of the 20th century were reviewed by O'Reilly and Daborn (1995). In the past the disease was considered uncommon in goats, and it was even thought that this species was naturally resistant to the infection. Sporadic reports described isolated cases affecting animals kept in herds with cattle, reared on cow's milk, grazing with infected cattle (Cousins *et al*., 1993; O'Reilly and Daborn, 1995) or sharing same habitat as infected wildlife, such as feral goats captured from areas in New Zealand where the disease is endemic in the possum population (Anon., 1988).

In recent years tuberculosis has been increasingly identified in terms of reports in some European and African countries and the

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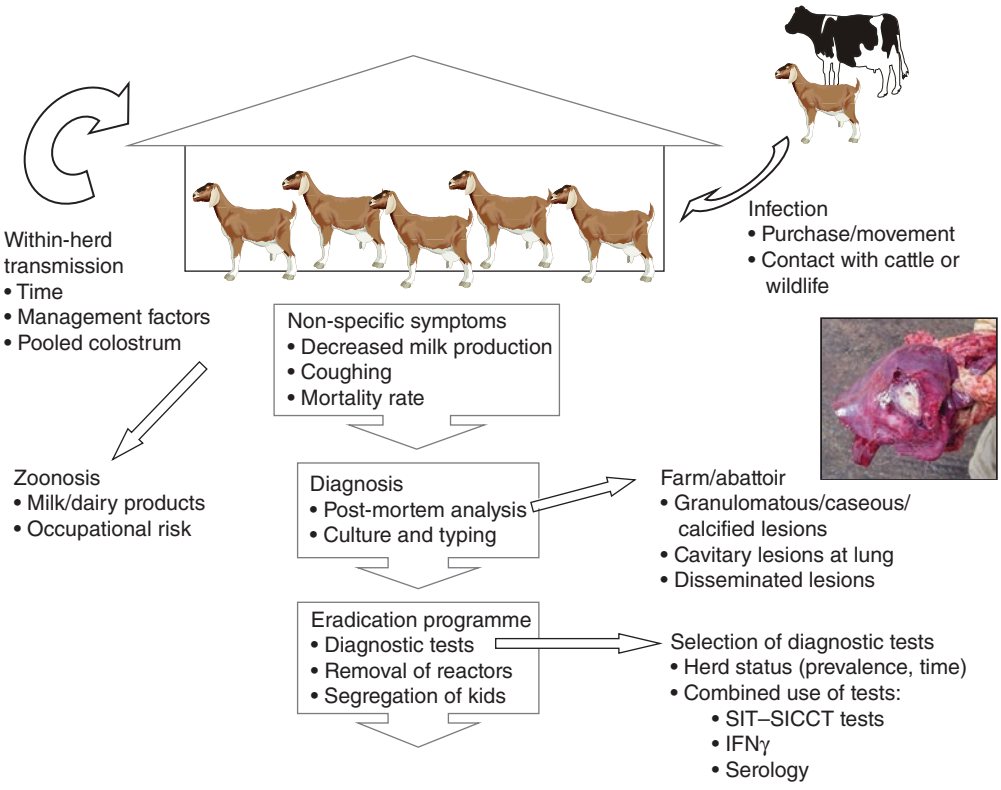

Fig. 11.1. A summary of the main characteristics of tuberculosis in goats.

severity of cases. Whether the increase is related to awareness of the infection and investigation, or to other factors, is unknown. In many cases these findings are a consequence of detailed epidemiological investigation after confirmation of tuberculosis in cattle.

M. bovis is the main etiological agent of the reported cases in the UK, Republic of Ireland, Portugal, France, Italy, Germany, Russia, the USA, Argentina, Brazil, Algeria, Nigeria, Palestinian Territories, India, Australia and New Zealand (Cousins *et al*., 1993; O'Reilly and Daborn, 1995; Zumárraga *et al*., 1999; Haddad *et al*., 2001; Crawshaw *et al*., 2008; Boniotti *et al*., 2009; Cadmus *et al*., 2009; Quintas *et al*., 2010; Higino *et al*., 2011; Sahraoui *et al*., 2011; Shanahan *et al*., 2011; Tschopp *et al*., 2011; Broughan *et al*., 2013; Ereqat *et al*., 2013; Zanardi *et al*., 2013). However, in Portugal, Spain and Greece *M. caprae* is the main pathogen involved (Ikonomopoulos *et al*., 2006; Duarte *et al*., 2008; Rodríguez *et al*., 2011). It has been suggested that *M. caprae* is more adapted to goats than is *M. bovis* (Aranaz *et al*., 1999). This difference could account for the higher prevalence of tuberculosis in goats in Spain, where some goat populations are heavily affected (García-Marín, 1992; Liébana *et al*., 1998) compared to other countries (Broughan *et al*., 2013). However, this theory has not been confirmed in experimental infections.

The presence of tuberculosis has been studied in Africa. Despite continuous close contact, and sharing of pastures with potentially infected cattle, the prevalence based on single intradermal cervical comparative tuberculin (SICCT) test in small ruminants was very low (Tschopp *et al*., 2011). The overall prevalence in small ruminants estimated using SICCT test was 0.5% at cut-off ≥4 mm and 3.8% at cut-off ≥2 mm, but culture resulted in recovery of *M. tuberculosis* and environmental mycobacteria (Kassa *et al*., 2012). The isolation of *M. tuberculosis* in goats suggests a potential

transmission of this agent from humans and would need research into the role of small ruminants in epidemiology of human tuberculosis in this region (Kassa *et al*., 2012). The presence of *M. tuberculosis* has been reported also in Nigeria, likely to be caused by direct transmission from humans (Cadmus *et al*., 2009).

Infection usually occurs at the cattle–small ruminant–wildlife interface. It has been associated with rural traditional management, such as mixed herds, keeping both cattle and small ruminants, extensive grazing that may facilitate contact with wildlife and in some areas with 'village herds' kept communally during the day (Tschopp *et al*., 2011). When infection was studied in Pakistan the use of the SICCT test revealed a low overall percentage of reactor animals to the test at goat and sheep farms and higher at farms when they are kept with cattle and buffalo (Javed *et al*., 2010). These situations of low prevalence may be caused because animals are kept in very small numbers (Javed *et al*., 2010, Tschopp *et al*., 2011).

The contribution of small ruminants to the overall epidemiology of tuberculosis depends on the relative prevalence. Goats may play a role in the maintenance and transmission of tuberculosis. Lack of knowledge of the disease and lack of testing (control programmes and pre-movement tests) may result in dissemination of the infection to other flocks (Crawshaw *et al*., 2008) and also to other animal species. Cross-infection of *M. bovis* between cattle and goats has been reported (Cadmus *et al*., 2009) and also confirmed by molecular epidemiology (Zanardi *et al*., 2013). In some reports wildlife has been considered the most likely source of infection (Daniel *et al*., 2009; Shanahan *et al*., 2011). Goats can also represent a source of infection of *M. caprae* to cattle (Napp *et al*., 2013), not only in mixed herds but also to neighbouring herds.

The susceptibility of goats to tuberculosis has not been investigated. Many studies show wide dissemination of tuberculosis throughout the herd, resulting in high within-herd prevalence, the presence of disseminated gross lesions in a large number of animals and serological response (Crawshaw *et al*., 2008; Napp *et al*., 2013; Zanardi *et al*., 2013). This scenario could be compatible with an increase of severity of tuberculosis in this species, but also with advanced infection, as the infection may go undetected if diagnostic tests are not applied.

Pathology

The route of infection, localization of lesions and appearance of macroscopic lesions are similar to those described in cattle; however, in most animals they are widespread (Liébana *et al*., 1998; Daniel *et al*., 2009). Lesions in goats induced by *M. bovis* or *M. caprae* seem to be more severe than those induced by *M. bovis* in cattle. Diseased animals show granulomatous inflammation characteristic of the infection in different evolutionary stages (Bernabé *et al*., 1991). Common necropsy findings reveal granulomatous/caseous/caseocalcareous lesions of various sizes in the respiratory tract (lung, thoracic lymph nodes), pleura, liver and mesenteric lymph nodes (Liébana *et al*., 1998). Typical caseous lesions in lymph nodes range from pinpoint foci to large lesions (Daniel *et al*., 2009). In the advance stage of infection animals present large areas of cavitation in the lung. These cavitary lesions are optimal for mycobacterial growth; the extensive pneumonic lesions, frequently showing liquefactive necrosis would facilitate the escape of mycobacteria into adjacent airways with potential aerosol spread (Daniel *et al*., 2009). Lesions can be also present in the udder (Crawshaw *et al*., 2008). Similar gross lesions with severe pathology have been described in goats infected with *M. caprae* (Álvarez *et al*., 2008; Bezos *et al*., 2010; Pérez de Val *et al*., 2011).

Microscopically, proliferative granulomas show caseation and central calcification surrounded by specific granulation tissue and encapsulation by connective tissue (Bernabé *et al*., 1991). Detailed description of gross lesions and types of microscopic lesions are depicted in specific reports (Bernabé *et al*., 1991; Sánchez *et al*., 2011; Buendía *et al*., 2013). The presence of acid-fast bacilli in the lesions depends on the reports, some describing the presence of bacilli within epithelioid cells (Daniel *et al*., 2009; Quintas *et al*., 2010), some

describing bacilli difficult to find and few in number (Gutierrez-Cancela and García-Marín, 1993).

Description of histopathological features and associated local immune response of tuberculosis infection in skin-test-positive goats showed marked differences between granulomatous lesions and cavitary lesions in terms of presence of T cells and the ratio; granulomas contain significantly more CD4+ T cells, while cavitary lesions contain significantly more CD8+ T cells and WC1+ γδ cells (Sánchez *et al*., 2011). These changes also occur in peripheral blood of infected animals, and the counts and proportions of subsets of lymphocytes could justify the fluctuations detected in the PPD-specific IFNγ response (Bezos *et al*., 2012c). These changes are similar to those described in advanced *M. bovis* infection in cattle (Bezos *et al*., 2012c).

Diagnosis

Clinical diagnosis

The clinical diagnosis of this disease in small ruminants has the same limitations as in cattle or other animals. Loss of weight and milk production and respiratory distress signs are non-specific and may not alert veterinary practitioners to the possibility of the infection (Bernabé *et al*., 1991; Daniel *et al*., 2009; Quintas *et al*., 2010). Because of these non-specific symptoms, animals may receive unsuccessful treatments with antibiotics and anthelmintics. At an advanced stage, infection leads to progressive emaciation of affected animals, chronic coughing and high mortality rate in caprine herds.

Cell-mediated immune-based tests

Cell-mediated immunity (CMI) is essential in the response to tuberculosis and thus it is the target of the most widely used ante-mortem diagnostic tests. These CMI-based diagnostic tests in small ruminants use the same methods that are applied in cattle: tuberculin tests (single intradermal tuberculin (SIT) test and single intradermal cervical comparative tuberculin (SICCT) test), and the interferon-γ (IFNγ) assay. The biological basis, procedures, reagents, dosages and criteria for interpretation usually follow those described in the World Organisation for Animal Health (OIE) (OIE, 2009) or local guidelines such as European Union (EU) Directive 64/432/EEC[1] (European Commission) for detection of the infection in cattle. Specific details regarding techniques can be consulted in the available reports. Several field trials have been conducted for comparison of the sensitivity and specificity of the diagnostic tests in caprine herds; these have yielded a wide range of data that are likely to reflect the different epidemiological situation of populations under study. A detailed assessment of the current *in vivo* CMI-based and antibody-diagnostic assays is available (Bezos *et al*., 2012d). A wide range of sensitivity values have been obtained (53.2–71% for SIT test, 29.2–83.7% for SICCT test and 71–87.2% for the IFNγ assay).

Few studies have been conducted to evaluate the specificity of these techniques in small ruminants. The evaluation of the specificity of these tests has been satisfactory though this has been performed in small groups of animals or not in the best panel of samples. A recent study on tuberculosis-free accredited flocks revealed overall high specificity for SIT test (93–96% and 97.6–99.2% using severe and standard interpretations, respectively), SICCT test (99.4–100% and 99.6–100%, depending on interpretation) and IFNγ assay (95.1–97.5% and 96.4–98.4% using 0.05 and 0.1 cut-off, respectively) (Bezos *et al*., 2012b).

Intradermal tuberculin tests are the main techniques in use, though there is considerable debate on the performance of the tests. In addition, the tests are difficult to apply in goats because of the fine nature of the skin (Shanahan *et al*., 2011). The SIT test using bovine PPD applied to goats has reported a sensitivity of 82–93.8% (Gutierrez-Cancela and García-Marín, 1993). A later study showed sensitivity was 53.2%; in this case the low rate was attributed to the proportion of young animals at an early stage of infection (Liébana *et al*., 1998). Overall relative sensitivity of SIT, calculated on the number of animals that presented with macroscopic lesions or were *M. caprae* culture-positive of the SIT test (stringent interpretation),

was 71.1%; however, the data showed different sensitivity values between flocks (30–100%), most likely related to infection status of the farms (Bezos *et al*., 2011). Recent data on an extensive study comparing skin tests in infected herds show that overall sensitivity of SIT test was 43.9% (severe interpretation) and 38.8% (standard interpretation) (Bezos *et al*., 2014). A significant weak positive correlation was found between age and skinfold thickness (Bezos *et al*., 2014).

The SICCT test using both bovine and avian PPD is applied to distinguish animals infected with *M. bovis* (or *M. caprae*) to avoid interference caused from sensitization by exposure to environmental mycobacteria. In *M. bovis*-infected herds the percentage of reactors can be high: 71.4% (Zanardi *et al*., 2013) and 82.8% (Quintas *et al*., 2010). Results should be interpreted using the severe (bovine) interpretation, particularly in herds already confirmed as being infected or with close epidemiological links to confirmed infection (Shanahan *et al*., 2011). The SICCT test may show poor results in certain epidemiological situations, such as in herds with a long history of persistent infection with *M. caprae*; in these cases SIT tests and ancillary tests may be applied to improve detection of infected animals (Buendía *et al*., 2013; Bezos *et al*., 2014).

The IFNγ assay has been successfully used for the diagnosis of tuberculosis using the protocol used for cattle and the Bovigam® TB Kit ELISA (Prionics AG LifeTechnologies, Schlieren-Zurich, Switzerland). Standard cut-off values are: (i) bovine PPD (readings at OD_{450}) minus PBS ≥ 0.05, and bovine PPD > avian PPD; and (ii) a less restrictive cut-off point when bovine PPD (readings at OD_{450}) minus PBS ≥ 0.1, and bovine PPD > avian PPD. The IFNγ assay is not included as a routine diagnostic technique in eradication programmes in caprine herds because of logistics and cost.

Cousins *et al*. (1993) were the first to report the use of the IFNγ assay for detection of tuberculosis in goats. Overall sensitivity has been reported as 67.9% (Zanardi *et al*., 2013), 71% (Álvarez *et al*., 2008), 83.7% (Gutiérrez *et al*., 1998) and 87.2% (Liébana *et al*., 1998). A percentage of the avian reactors may also give a high test result with bovine PPD. Examination of these animals demonstrates they can be infected with *M. bovis*, and therefore could be considered to be falsely diagnosed as avian reactors by the IFNγ test. These avianB reactors may produce IFNγ positive results in the subsequent round of tests. These should be interpreted cautiously in situations where great immunoreactivity of the herd occurs. A possible explanation could be the existence of a concurrent sensitivity to *M. avium* (Liébana *et al*., 1998).

According to experimental *M. caprae*-challenge studies, the response to the IFNγ assay is detected 2–4 weeks post-infection, with a peak in the response between 2 and 8 weeks post-inoculation. Responses show individual variability and the subsequent OD values fluctuate beyond that time (Bezos *et al*., 2010; Pérez de Val *et al*., 2011). The sensitivity of the IFNγ assay in goats can be affected by the phenol concentration, the cut-off values and – markedly – by the time delay in blood stimulation. Significant differences were observed when samples were processed within 8 h relative to those stimulated at 16 h and 24 h for both cut-off points. At a cut-off of 0.05 the sensitivity was 100% compared to 76.5% and 52.9%, respectively; at a cut-off of 0.1 the sensitivity was 94.1% compared to 41.2% and 17.6%, respectively (Bezos *et al*., 2012a).

Several field trials have been conducted for comparison of the sensitivity and specificity of the skin tests and the IFNγ assay in goats. Some differences may be attributable to the difference in the concentrations and/or potencies of avian and bovine PPDs (Gutiérrez *et al*., 1998). Sensitivity of the IFNγ assay is usually higher than sensitivity of the intradermal tests and the assay enables detection of the infection at an early stage; however, results differ among reports. In a field trial, repeated IFNγ testing was able to detect 87.2% of the infected individuals within the group of selected goats for the experiment; in the same group the SIT test only detected 44.6% or 53.2% if the inconclusive animals were considered (Liébana *et al*., 1998). In another report, the sensitivities of SICCT and IFNγ tests were similar, at 83.7% (Gutiérrez *et al*., 1998). The relative sensitivities of the CMI diagnostic tests may depend on epidemiological situations such as breed, the strain involved in the outbreak and the time of infection, ranging

from 54.5% to 92.3% depending on herds (Bezos *et al*., 2011). In a herd with persistent infection of goats with *M. caprae*, the IFNγ assay detected 65.3% and the SICCT test detected 44.5% (Buendía *et al*., 2013).

As described in cattle, the comparison between IFNγ assay and SIT test results revealed low-to-moderate agreement, meaning they behave differently, detecting different populations of infected animals (Álvarez *et al*., 2008). Thus, the highest sensitivity is obtained by combining the results of both assays in parallel (considering as positive an animal that is positive to either test). The combination of SIT or SICCT test with the IFNγ assay may reach up to 90.8% (Álvarez *et al*., 2008) or 95.8% of infected goats (Gutiérrez *et al*., 1998), and a slightly lower proportion, 78.2%, in the case of persistent infection (Buendía *et al*., 2013).

Defined specific antigens such as early secretory antigenic target −6 kDa (ESAT-6), culture-filtrate protein-10 (CFP10) and Rv3615c have been evaluated for tuberculosis diagnosis in goats. Overall relative sensitivity of the IFNγ assay with defined antigens was 72.4% using ESAT-6/CFP10 peptides cocktail, compared to 77.7% using PPDs. The overall OD mean values obtained in the IFNγ using ESAT-6/CFP10 peptides and PPDs were 0.246 and 0.446, respectively, although the values were more scattered for PPDs (Bezos *et al*., 2011). In an experimental model the response to ESAT-6/CFP10 and Rv3615c peptide cocktails showed similar kinetics but was weaker than the bovine PPD response (Pérez de Val *et al*., 2011, 2013).

Factors affecting CMI-based tests

A correlation between the degree of pathology found post mortem and immunological response has been proposed. An earlier study showed lower sensitivity for the skin test in the group of goats with severe lesions (animals with cavitary lesions) (75%) compared to the group of goats with minor lesions (86.4%) (Gutiérrez *et al*., 1998). This change in the sensitivity was also observed in the skin test performance in a herd with persistent infection, 49.1% in animals with single lesions and 34.2% in animals with multiple lesions (Buendía *et al*., 2013). In goats experimentally infected with *M. caprae* there is a positive correlation between pathology score (related to severity of lesions) and IFNγ assay result using bovine PPD (Bezos *et al*., 2010, Perez de Val *et al*., 2011) and ESAT-6/CFP10 cocktail and Rv3615c (Pérez de Val *et al*., 2013). In naturally infected goats, a significant yet weak positive correlation was detected with bovine PPD, but lower than values observed in experimentally infected animals (Bezos *et al*., 2011). Correlation between specific responses to ESAT-6/CFP10 in naturally infected goats is controversial, giving positive correlation (Domingo *et al*., 2009) or no correlation (Bezos *et al*., 2011); this difference may be related to different progression of the disease in the animals included in each study.

Several factors that are relatively common in goat farms may have a detrimental impact on the diagnostic tests. Dual infection with tuberculosis and paratuberculosis (*M. avium* subsp. *paratuberculosis*), which can be common in some areas (Bernabé *et al*., 1991; de Juan *et al*., 2002), may interfere with the diagnosis of tuberculosis in goats. This effect has been evaluated by comparing the sensitivity for each test from all tuberculosis-infected animals with those from the subgroup of animals with mixed infection. The SIT test showed a lower sensitivity in animals with the dual infection (54.2%) than that achieved in the overall group (71%); the SICCT test also performed worse in the co-infected group (29.2% compared to 42.7%); therefore, its use is not recommended in mixed infection. IFNγ assay seemed to be more consistent in both groups regardless of the threshold applied (Álvarez *et al*., 2008). Disease progression and this interference were corroborated using an experimental model of trans-thoracic infection; the higher value of IFNγ obtained with avian PPD may mask the response obtained with bovine PPD in the animals with co-infection (Bezos *et al*., 2010).

Vaccination against paratuberculosis may also interfere with diagnostic test results but this is also a controversial issue that has not been studied in detail. Sensitization produced by the vaccine may result in a false positive or

doubtful intradermal reaction, although the SICCT test should be able to discriminate between them. A false response to the IFNγ assay was obtained in kids which had received an inactivated vaccine against paratuberculosis; responses against bovine PPD were higher than those obtained with avian PPD. These responses decreased several months after vaccination, recovering the negative status about 1 year post-vaccination (Aranaz *et al*., 2000). Also, false response of the IFNγ assay using both bovine and avian PPD has been described; the proportion of animals could depend on the adjuvant (Hines *et al*., 2007). Specificity values of the SIT test, and particularly of the IFNγ assay, in a flock vaccinated against paratuberculosis were lower than in non-vaccinated flocks (Bezos *et al*., 2012b). This interference with specificity could easily be solved by using DIVA antigens (Pérez de Val *et al*., 2012a). An additional concern would be the effect of the vaccine in the response to diagnostic tests when animals are infected later on; however, a recent study found only limited interference in the diagnosis of paratuberculosis-vaccinated and *M. caprae*-challenged animals, except for a short period of time after infection (Pérez de Val *et al*., 2012a).

Caseous lymphadenitis (*Corynebacterium pseudotuberculosis*) may produce false positive results in the intradermal tests (Sharpe *et al*., 2010), but this effect has not been fully evaluated. Specificity of the SIT tests in a flock infected with *C. pseudotuberculosis* was lower (Bezos *et al*., 2012b). These false reactions affect mainly young goats and would be expected to decrease over the years, with a very low rate of non-specific reactors over 24 months.

Antibody-based assays

The existence of a percentage of animals that do not react to CMI response tests reinforces the need for the development of antibody-based assays that can maximize detection of infected animals. Animals undetected by CMI-based diagnostic tests represent a great risk as they could maintain the infection in the farm (Álvarez *et al*., 2008). The serological tests could supplement, or even replace, the existing methods. An additional advantage of the ELISA test is its simplicity and low cost. Ideally, these methods should simultaneously detect antibodies for several antigens, covering all stages of infection and the individual differences (Shuralev *et al*., 2012). Also, high sensitivity of an anamnestic ELISA based on bovine PPD as an antigen for detection of infected goats has been reported (Gutiérrez *et al*., 1998), obtaining a sensitivity of 88.6%, higher than that obtained with standard ELISA (54.9%), the SICCT test (83.7%) and the IFNγ (83.7%).

Recently, serological tests based on recognition of specific antigens have been developed. ELISAs based on MPB-70, a major antigen in *M. bovis*, have been successfully used, yielding sensitivity estimates of 93.4% (Acosta *et al*., 2000) and 95.6% (Marassi *et al*., 2009) of *M. bovis*-infected goats. An ELISA based on PPD-B and/or recombinant antigens (MPB70, MPB83, ESAT-6, CFP10) was able to detect animals negative to the other tests (Zanardi *et al*., 2013). The Enfer chemiluminescent multiplex ELISA system has been optimized for detection of antibodies against five antigens for its use in goats infected with *M. bovis*. The test detected 98.3% of SICCT test-positive goats. The test was also evaluated in a small group of goats infected with *M. caprae* (confirmed by culture); 85.7% of infected goats were found positive (Shuralev *et al*., 2012). This preliminary study did not show any clear difference in the antigen recognition patterns between animals infected with *M. bovis* or *M. caprae*. In *M. caprae*-infected goats, a recent study based on the use of an MPB70 ELISA reported higher sensitivity in animals with multiple lesions compared to animals with single lesions (89.4–50.8%) (Buendía *et al*., 2013). Furthermore, a combination of the ELISA and skin test detected 89.1% of goats with tuberculosis, a better result than that obtained by a combination of IFNassay and skin test (Buendía *et al*., 2013). The high figures of sensitivity obtained with serological tests can be caused by a different immune response in these animals or the consequence of the testing of animals at an advanced stage of infection.

Models of Infection

Models of infection using goats as the experimental animal have been developed to study pathogenesis and evolution of immune response. These models have been established using trans-thoracic route (Bezos *et al*., 2010), endobronchial route (Pérez de Val *et al*., 2011) and intratracheal aerosol routes of infection (González-Juarrero *et al*., 2013) to mimic the natural infection. The goat model based on endobronchial infection has been used to evaluate the efficacy of several vaccination strategies comparing *M. bovis* bacillus BCG and *M. bovis* bacillus BCG prime followed by heterologous boosting with a replication-deficient adenovirus expressing the antigen Ag85 (AdAg85A) or adenovirus expressing four antigens (AdTBF) (Pérez de Val *et al*., 2012b, 2013). The vaccines did not prevent mycobacterial infection but promising results were obtained. BCG-AdAg85A- and BCG-AdTBF-vaccinated animals showed reduced pathology and bacterial load compared to BCG and unvaccinated controls. Whole-blood IFNγ responses to ESAT-6/CFP10 and Rv3615c and antibody responses to MPB83 were identified as correlates of disease progression (Pérez de Val *et al*., 2013).

Goats have been proposed as an animal model for human tuberculosis studies of pathogenesis, vaccination and drug therapy (Domingo *et al*., 2009). This suitability is based on several reasons: goats are naturally susceptible, they undergo a similar course of infection to that of humans, they show persistent infection with extracellular bacterial growth, both goat and human lung have intralobular septation by connective tissue and develop fibrosis of tuberculous granulomas; in addition, they are an easy-to-handle model animal, housing in biocontainment condition is more affordable, and animal size is conducive to administration of treatments and X-ray examinations (Domingo *et al*., 2009; González-Juarrero *et al*., 2013 ; Pérez de Val *et al*., 2013).

Tuberculosis in Sheep

Cases of tuberculosis infection in sheep are less frequent compared to descriptions in goats. This has been assumed to be related to sheep husbandry and animal behaviour that limit access to sources of infection, and it has been suggested that sheep are more resistant to bovine tuberculosis than other animals. In fact, a significant association of resistance with some sheep breeds was found (Javed *et al*., 2010). However, some scarce cases of tuberculosis in sheep have been described; most are individual cases or the number of affected animals in the outbreaks is generally low, except the description of two flocks in New Zealand (Cordes *et al*., 1981; Davidson *et al*., 1981) and one in the UK (Houlihan *et al*., 2008). The main etiological agent is *M. bovis*, although some cases in Spain are caused by *M. caprae*, likely to be mirroring the infection in goats; however, the relative number compared to total figures of infected animals is low (Rodriguez-Campos *et al*., 2012; Broughan *et al*., 2013).

Sheep may get infected if they are exposed to infected cattle (same housing, shared pastures) or wildlife (Cordes *et al*., 1981; Houlihan *et al*., 2008), and molecular epidemiology usually detects the same types found in cattle, goats or the environment (Malone *et al*., 2003; Houlihan *et al*., 2008; Muñoz Mendoza *et al*., 2012; van der Burgt *et al*., 2013).

Infected animals can be clinically healthy and detected only during skin testing or abattoir surveillance (Cordes *et al*., 1981; Malone *et al*., 2003; Houlihan *et al*., 2008; Marianelli *et al*., 2010). However, *M. bovis* can also cause clinical disease in sheep. Infected animals from three flocks in north-western Spain showed coughing and dyspnoea (Muñoz Mendoza *et al*., 2012), and an outbreak of bovine tuberculosis infection in sheep was associated with clinical signs consisting of chronic ill-thrift and weight loss but no obvious respiratory signs (van der Burgt *et al*., 2013).

Tubercular lesions in sheep are highly variable in gross appearance, severity and number of affected organs. Post-mortem findings include multiple lung abscesses and caseous/purulent lesions in thoracic lymph nodes, liver, spleen, hepatic, and inguinal and mesenteric nodes. However, some gross lesions may resemble caseous lymphadenitis; an abscess with a thin capsule and copious central sticky mucoid pus, and this makes recognition difficult (Cordes *et al*., 1981; van der Burgt *et al*., 2013). Histopathology was

typical of mycobacterial infection, but there was a distinct paucity of acid-fast bacteria (Muñoz Mendoza *et al*., 2012; van der Burgt *et al*., 2013).

Detection of infected animals is performed with same diagnostic tools. The skin test is applied at the medial thigh and the sensitivity has been reported to be 81.6% (Cordes *et al*., 1981). IFNγ using the same procedure as for other species has been evaluated only in a reduced number of animals, with positive results (Malone *et al*., 2003). The repeated use of the SICCT test at 2-month intervals, followed by removal of reactors, appears to have resulted in apparent elimination of the infection from the flocks (Malone *et al*., 2003; van der Burgt *et al*., 2013).

The contribution of infected sheep to the epidemiology of tuberculosis seems to be limited; however, the presence of lesions in the respiratory tract indicates that sheep may also act as a source of infections for other animals (Malone *et al*., 2003; Broughan *et al*., 2013).

Control Measures

Tuberculosis in small ruminants is not subjected to official eradication programmes except in some countries when they coexist with cattle or a potential epidemiological link is suspected. In some countries they are tested when milk is sold to consumers. Some regions have implemented voluntary tuberculosis control programmes as a result of awareness of the infection.

There is limited official information regarding measures recommended for the control or eradication of tuberculosis infection in small ruminants. As an example, infection in small ruminants is not included in the OIE Listed Diseases and Other Diseases of Importance to International Trade, though goats and sheep are mentioned as potential hosts for *M. bovis* and *M. caprae* in the chapter dealing with bovine tuberculosis. Until relatively recently, goat and sheep farming has remained a traditional farming system with isolated populations, and herds have not been included in health schemes and eradication campaigns. However, tuberculosis in goats and sheep may be a more serious problem than previously recognized. The infection should be kept in mind in the diagnosis of chronic respiratory diseases. A thorough postmortem examination on animals dead on the farm should be performed and inspection should include major organs, serosal surfaces, tonsils and thoracic, mandibular, retropharingeal and portal lymph nodes. A laboratory test should be performed to confirm or rule out the presence of the pathogen(s). The detection of lesions suggestive of tuberculosis at the slaughterhouse is still an essential tool in eradication plans for tuberculosis, because this allows identification of the percentage of animals that could be missed by the diagnostic techniques (Álvarez *et al*., 2008; Muñoz Mendoza *et al*., 2012).

Once infection is suspected, health authorities should establish back and forward tracing and extend the investigations to contact and neighbouring herds (Daniel *et al*., 2009). Access to subsidized diagnosis may contribute to detection of infection and a better knowledge of the prevalence.

Eradication of the tuberculosis infection from the flock is based on prompt detection of infected animals and removal of any potentially infected animal (Daniel *et al*., 2009; Shanahan *et al*., 2011). These can be carried out by use of single or combined techniques for the diagnosis. When within-herd prevalence is high, consecutive rounds of diagnostic methods may be required for detection of infected animals (SICCT or SIT test and IFNγ assay) (Vidal *et al*., 1995; Liébana *et al*., 1998; Shanahan *et al*., 2011). However, the success of this scheme depends fundamentally on the progression of infection within the herd. This progression is linked to time since introduction of infection in the herd and management factors, because as infection progresses the immune response can deteriorate and this should be taken into account when choosing, applying and interpreting the diagnostic techniques. In addition, in advanced cases the removal of reactors is not compatible with economic sustainability of the herd (for instance, a drastic reduction in milk production because of deaths and removal of reactors in a short period of time).

An alternative for herds with a high prevalence is based on the separation of the

new kids from the mothers immediately at birth and feeding on artificial colostrum and milk (Liébana *et al*., 1998; Álvarez *et al*., 2005). This may contribute to maintain the viability of the farm over the medium to long term but requires durable commitment of the farmer. Segregation of tuberculosis-free animals and location in a separated area may ensure that there are no potential sources for contact with infection, and cleaning and disinfection must be applied. In addition, risk factors that should be controlled are: (i) movement involving purchase or sale of animals, contacts by attendance at agricultural shows and natural services (Daniel *et al*., 2009); (ii) direct or indirect exposure with infected wildlife (Daniel *et al*., 2009; Shanahan *et al*., 2011); and (iii) use of pooled colostrum.

Zoonotic Impact

Tuberculosis in small ruminants has a clear public health implication. Humans can get the infection through close contact with animals and consumption of raw milk and unpasteurized dairy products. Tuberculosis lesions can be present in the mammary gland (Crawshaw *et al*., 2008; Quintas *et al*., 2010). *M. bovis* has been cultured from milk specimens from dairy goats with evidence of clinical mastitis and also from pooled bulk tank milk of an infected herd (Shanahan *et al*., 2011) and identified by direct PCR from milk (Ereqat *et al*., 2013). Regarding *M. caprae*, the presence of human isolates displaying the spoligotype signature has been reported, and its origin traced to occupational infection or rural areas where goat farming is common (Gutiérrez *et al*., 1997). Both *M. bovis* and *M. caprae* are zoonotic organisms and should be treated as a risk/hazard Group III organism. This should be taken into account when performing post-mortem inspection and appropriate precautions should be established to prevent occupational infection. Personnel in contact with the infection should be referred to a medical doctor for a Mantoux test and chest radiographs (Shanahan *et al*., 2011).

Conclusion

Infection by pathogens of the *M. tuberculosis* complex produces a devastating infection in small ruminants with serious consequences for farm productivity and also human health impact. The control or eradication of this infection poses a challenge that has not received adequate attention from health authorities. The design of eradication programmes has to take into account the epidemiological background of the farm (recent or persistent infection) and the effect of other infections on the choice of adequate diagnostic tools. The use of combined assays improves detection of infected animals. This control would greatly benefit from research on the combined use of diagnostic techniques, appropriate management measures on the farm and the development of vaccines.

Note

[1] Council Directive of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine (64/432/EEC) (OJ L 121, 29.7.1964, p. 1977) http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1964L0432:20071113:EN:PDF.

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12 Mycobacterial Infections in Camelids

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Introduction

Tuberculosis (TB) is a major infectious disease of mammals caused by infection with bacteria of the *Mycobacterium tuberculosis* complex (MTBC) (Smith *et al*., 2009). The disease is characterized by the formation of granulomas, primarily in the respiratory system and associated lymph nodes, from which the mycobacteria are excreted and infect other susceptible individuals. Most cases of TB in farm animals are caused by infection with *M. bovis*, the member of the MTBC that causes bovine TB. However, TB in camelids caused by infection with *M. microti* (another member of the MTBC), *M. kansasii* and *M. avium* complex has also been reported in Great Britain (GB) and other countries (Johnson *et al*., 1993; Oevermann *et al*. 2004; Lyashchenko *et al*., 2007; Braun *et al*., 2009; Zanolari *et al*., 2009).

There is a growing industry for South American camelids (llamas and alpacas) in GB. They are used for breeding and showing, are kept as producers of fibre, as pets, leisure/trekking (llama) and companion animals. There are an estimated 30,000–35,000 alpacas and 3000–4000 llamas registered with the various British camelid societies (not all camelids are registered), and around half of this GB national herd is located in the south-west of Britain where bovine TB is endemic in cattle and some wildlife species (Eurasian badger – *Meles meles*). Camelids are susceptible to TB, and DNA typing of *M. bovis* isolates from infected camelids to date has largely reflected the locally predominant genotypes of the bacterium found in the neighbouring cattle and wildlife. As there is currently no routine surveillance testing regime for TB in camelids; by the time it has been detected by clinical or post-mortem examination the disease can be advanced and with severe pathology.

This chapter describes the epidemiological, clinical and pathological features of TB in South American camelids (llamas and alpacas) in GB, as well as available diagnostic tests and disease control strategies. The extent of mycobacterial diseases in South American camelids caused by *M. bovis* and other mycobacteria in other countries and reports from Africa and the Middle East on the problem of TB in old world camels are reviewed.

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Frequency and Distribution of TB in the British South American Camelid Population

The first documented episode of TB in British South American camelids caused by *M. bovis* infection was diagnosed in 1999, when a llama from a small pet and showing herd in south-east Wales died, and macroscopic lesions of TB were identified on post-mortem examination. The disease was caused by the predominant strain of *M. bovis* found in cattle and badgers in the surrounding area. Another female llama that had been sold the previous year as a pet from this herd to another neighbouring premises died at the same time and was also found to be infected with *M. bovis* (Barlow *et al*., 1999).

Since then, the annual number of South American camelid herds affected by new incidents (breakdowns) of culture-confirmed *M. bovis* TB infection has risen gradually in GB, reaching a peak of 16 new cases diagnosed in 2010 (Table 12.1). This increase in the incidence of cases detected has coincided with an expansion of the population and the popularity of these species in GB, an increase in the incidence of TB in cattle herds, greater awareness of the disease among veterinary practitioners and herd owners and the introduction of new legislation that made TB a notifiable disease in camelids and other non-bovine domestic animals in 2006.

While the table does provide useful information, such as that virtually all camelid TB breakdowns occur in areas of the country where bovine TB is endemic (west of England and parts of Wales), these statistics probably do not reflect the true prevalence of *M. bovis* infection in the GB camelid population. Although TB is notifiable in camelids in GB there is no compulsory scheme for regular TB testing of camelid herds. In the absence of an active disease surveillance regime, initial detection of TB in camelids primarily relies on the submission by private veterinary surgeons of carcasses or tissue samples for post-mortem examination at government veterinary laboratories, either as routine casework or as suspected clinical cases of TB that die on farm or are euthanized due to a deteriorating body condition. The absence of TB surveillance for camelids also means that the small number of *M. bovis* culture-positives reported in Table 12.1 look very low indeed when compared to the *total* of South American camelid submissions to the APHA, which includes samples for diseases that *do* have well-known surveillance tests, for example parasitic infections. Twomey *et al*. (2014) report that these *M. bovis* culture-positive animals represent just 3.2% of the total of South American camelid submissions for which a positive diagnosis was reached between 2000 and 2011.

Efforts are now in place to provide a more complete data set to supplement the herd incidence figures shown in Table 12.1. Data have recently been made available for 2011–2012 and are shown in Table 12.2. For example, a summary of the numbers of tuberculin skin tests and STAT-PAK® serological tests (Chembio Diagnostic Systems, Inc., Medland, NY) that were carried out are now available (usually for those herds with suspected or confirmed *M. bovis* infection as a result of a voluntary submission), and the numbers of camelids removed as a consequence of TB testing are provided. Importantly, these expanded data begin to provide information on what could be the true extent of camelid TB in GB and the level of TB testing activity in the absence of surveillance testing. For instance, 1261 TB tests were carried out in 2012. If we use our above estimate of ~35,000 South American camelids in GB, with half of these located in the south-west of England and Wales, this amounts to 1261/17,500 camelids, or 7.2% of camelids in the endemic regions of GB tested for TB in 2012. In 2011 this activity was lower, at 5.6% (2012 data included one large herd that was extensively infected and resulted in the removal of large numbers of animals).

Also of interest, Table 12.2 shows that in 2012 a total of 592 camelids were removed as a result of TB (401 reactors plus 191 contacts or clinical cases). At an *ex gratia* compensation payment per camelid of GBP750, that makes a total of GBP444,000 cost to the taxpayer in 2012 for compensation alone going to this relatively small industry. In 2011 this was lower, with 81 animals being removed

Table 12.1. Camelid TB in Great Britain – frequency and geographic distribution of *M. bovis*-infected herds detected between 1999 and 2013 (table information courtesy APHA).

Annual numbers of new incidents of culture-confirmed *M. bovis* identified on South American camelid premises in GB			
Year of disclosure	Infected herds (with >1 dead or culled animal)	Type of herd infected (herd with >1 culled animal)	Location of affected herds
1999–2003	5	2× alpaca 3× llama	Gwent (2 llama holdings), Gloucestershire (alpaca), Herefordshire (llama), Somerset (alpaca)
2004	1*	alpaca	Devon
2005	1	llama	Avon
2006	2 (1)	1× alpaca 1× llama (1)	Sussex (alpaca), Devon (llama)
2007	3 (3)	2× alpaca (2) 1× llama (1)	Carmarthenshire (llama), Powys (alpaca), Dorset (alpaca)
2008	11 (6)	9× alpaca (4) 2× llama (2)	Carmarthenshire (llama), Devon (1 llama, 1 alpaca), Avon (2), Cornwall, Gloucestershire (3), Herefordshire, Worcestershire
2009	12 (6)	all alpacas	Devon (3), Derbyshire, Gloucestershire (2), Shropshire, Somerset (2), Staffordshire, Worcestershire (2)
2010	15 (6)	all alpacas	Devon (4)****, Cornwall (3), Gloucestershire, Hampshire, Monmouthshire, Staffordshire (2), Warwickshire, Worcestershire (2)
2011	6 (4)**	all alpacas	Cornwall, Dorset, Gloucestershire (2), South Gloucestershire (near Bristol)*****, Warwickshire
2012	14 (5)**	11× alpacas (4) 2× llama 1× 'mixed' (1)***	Carmarthenshire, Cheshire, Devon (4), Somerset (2), Staffordshire, Warwickshire***, West Midlands, West Sussex, Wiltshire, Worcestershire
2013	10 (6)	9× alpacas (5), 1 × 'mixed' (1)	Ceredigion, Cornwall, Devon, Gloucestershire (3)***, North Somerset (2), Shropshire, Vale of Glamorgan

(*) No culture possible, but typical histopathology and PCR-positive for *Mycobacterium tuberculosis* complex (MTBC).
(**) The figure in parentheses may increase as some of those incidents have not been closed and ongoing live testing may identify further cases.
(***) A premises on which both alpacas and llamas were kept.
(****) Three of those were epidemiologically linked via movements/purchases of infected animals and could therefore be considered one TB incident.
(*****) Location was incorrectly referred to as Avon in previous updates.

Table 12.2. Summary of camelid TB ante-mortem tests for 2011 and 2012 (table information courtesy APHA).

Calendar year	2011	2012
Total number of ante-mortem (live animal) tests	960	1261
number of ante-mortem blood tests (STAT-PAK)	143	494
number of ante-mortem skin tests	816	767
Number of animals removed as reactors	79	401
Number of other animals removed (e.g. as direct contacts or clinical cases)	3	191

(~GBP61,000 compensation), thus illustrating the difference in costs that one large breakdown can make.

In terms of TB as a serious cause of death for camelids in GB, the British Alpaca Society (BAS) has recently released a summary of reported causes of death among its registered animals to the BAS National Welfare Committee (meeting November 2013). This information is collected from BAS members who are asked to report all deaths of their registered animals via a form on the BAS National Herd Book – in this way the Welfare Committees can be informed of the scale of the issues the national herd is facing. The complete table showing deaths attributed to a number of causes, including parasitic infections, can be found on the web pages of the TB Support & Research Group (www.alpacatb.com). In 2013 (to the end of October) bovine TB was responsible for 31% (203 out of 643) of reported deaths. In 2012 this figure was 28.3% (383/1355), in 2011 it was 8.6% (69/801) and in 2010, 12.5% (63/506) of reported camelid deaths were due to bovine TB. The figures *do not include* certain unregistered camelid categories such as males sold as pets, and cria (TB lesions have been found in a 7 week-old cria). Many breeders only register these animals when they are to be sold, due to the high cost of registration and the mortality rate of cria. This BAS report underlines TB as a major health factor for the camelid industry, with no other single identifiable infectious agent coming close to these percentages – old age was the closest identifiable cause of death in 2013 at 10.8%. A large percentage of recorded deaths for each year (36.9% in 2013; 53.9% in 2012; 68.8% in 2011; 44.9% in 2010) were reported as 'other/not specified', and for these camelids there is no cause of death available.

TB Pathology in South American Camelids in Great Britain

The pathology caused by infection of alpacas and llamas with mycobacteria of the MTBC, which includes *M. bovis* and *M. microti*, can be extensive. The spectrum of lesions seen in these two species of camelid is essentially the same. Most cases of camelid TB in GB are detected following a diagnostic post-mortem examination by the APHA, although some follow post-mortem examination by a private veterinary surgeon and submission of suspect material to the APHA for mycobacterial culture. An incipient market for alpaca meat appears to be developing in GB, with a small number of registered abattoirs (three in England, one in Scotland) approved to slaughter these animals, therefore slaughterhouse surveillance might lead to additional reports in the future.

Clinical signs in infected camelids tend to be vague or non-existent. Subtle changes in behaviour may be detected by observant owners. In some there is a short period of illness terminating with respiratory symptoms. Other signs such as weight loss, loss of appetite, exercise intolerance or an intermittent dry cough are not consistent. Some camelids remain in good body condition until sudden death. As there is no routine surveillance for camelids, it is for the owner or their veterinary surgeon to arrange a post-mortem examination for any dead or moribund animals.

The respiratory system and associated lymph nodes are most frequently affected. The lung lesions may be so extensive that it is surprising that the severe pathology did not prove fatal earlier (Crawshaw *et al.*, 2013). The lesions are white or creamy and caseous.

There may be miliary lesions or multiple foci in the lungs, and in more advanced cases these lesions coalesce to give large areas of caseous necrosis, often involving whole lobes (Plate 3).

Cavitation of the lung parenchyma is sometimes present within large lesions. Cavitation in human TB is recognized as a sign of high infectivity as oxygen entry into partially evacuated lesions supports the expansion of bacterial numbers which then have access to the external environment. Occasional lesions seen in tracheal mucosa are thought to be the result of such extensive pulmonary pathology (Plate 4). The grape-like lesions of tuberculous pleurisy may also be seen in camelids (Plate 5).

The lymph nodes of healthy camelids can be small and difficult to find. By contrast, tuberculous lymph nodes are often massively enlarged and contain multiple white, cream or yellow-tinged caseous foci (Plate 6) and in severe cases the whole node may be replaced by one large caseous lesion (Plate 7).

Multifocal lesions are frequently present in the liver but they are seldom as extensive as those of the lungs of the same individual. Lesions are also seen in other tissues, including the skin (the result of sinus formation from enlarged discharging superficial lymph nodes), the gastrointestinal tract and the mammary gland (Richey *et al*., 2011), all of which can provide additional routes for the excretion of the organism by infectious individuals.

Infection of camelids with *M. microti* (originally identified as a pathogen of voles and shews; Wells, 1937) but probably more widespread than originally envisaged (Kremer *et al*., 1998), has also been described in GB as well as other countries (see below), resulting in tuberculous lesions that are indistinguishable from those caused by *M. bovis*. Non-MTBC mycobacteria may also cause disease in camelids but reports are rare. For example, a llama with a *M. kansasii*-confirmed infection in GB was believed to have contracted it from a river or lake nearby used by water fowl (Johnson *et al*., 1993).

The extensive lesions seen in infected alpacas and llamas in GB, and the high within-herd prevalence of *M. bovis*-infected individuals in some TB outbreaks, suggests that camelids can act as amplifier hosts and vectors of *M. bovis* and could thus spread infection among themselves (Twomey *et al*., 2009) and possibly transmit it to other mammalian species, including humans.

Ante-mortem Diagnostic Tests for Camelid TB

In the European Union (EU) and other countries the single intradermal comparative tuberculin skin test, using tuberculin purified protein derivatives (PPD) extracted from *M. bovis* (PPDB) and *M. avium* (PPDA), remains the primary official TB test for camelids. Although highly specific, this test appears to have low sensitivity in South American camelids (summarized by Alverez *et al*., 2011). In a collective study of 156 alpacas and 175 llamas from GB, Switzerland and the USA that included 44 *M. bovis*-infected and eight *M. microti*-infected animals (Lyashchenko *et al*., 2011) the sensitivity of the single intradermal comparative tuberculin test was estimated to be <5%. However data from the Camelid TB Support & Research Group (www.alpacatb.com), describing a cohort of breakdown herds in GB, provide a higher figure of 9.4% (180 *M. bovis*-infected visibly lesioned alpacas from 16 separate premises, of which 17 were positive to the skin test). This latter sensitivity estimate accords well with skin test sensitivity data from animals undergoing post-mortem examination at the APHA, which suggest around 10% (T. Crawshaw, unpublished observations). A recent publication from Spain (Bezos *et al*., 2013) described higher skin test sensitivities – in their report up to 60% – however, some of the group sizes were small, and in the larger group the 95% confidence interval limits were wide enough to include our estimates for GB camelids above.

One positive consequence of the tuberculin skin test is the boosting effect of specific antibodies (in infected animals), known as an anamnestic antibody response, which was described in an experimental *M. bovis* infection of llamas in 1998 (Stevens *et al*., 1998). Thus the sensitivity of TB-specific antibody tests can be enhanced by a prior skin test, whether the animal is skin-test-positive or not.

In 2006–2007 the APHA began to use the, then new, diagnostic rapid antibody lateral flow STAT-PAK test on South American camelid herd breakdowns in GB on a voluntary basis. This test already had a published track record in the detection of TB-infected camelids that had failed to react to the tuberculin skin test (Lyashchenko *et al*., 2007; Wernery *et al*., 2007; Twomey *et al*., 2010a, 2012). When the STAT-PAK test was deployed in a British llama herd with culture-confirmed *M. bovis* infection, a relative test sensitivity of 69.2% was obtained, and this was increased to 84.6% by taking serum samples at least 10 days after the tuberculin skin test (Dean *et al*., 2009). Thereafter the STAT-PAK test continued to be used on an experimental basis in *M. bovis*-confirmed camelid herds in GB as a voluntary ancillary test to be applied, with the herd owner's consent, ideally between 10 and 30 days post-skin test.

An interesting aspect of the Spanish study mentioned above (Bezos *et al*., 2013) was not only that it addressed antibody boosting by the tuberculin skin test *but also* encompassed skin testing at different sites of the animal. Their data, which also supported the antibody-boosting effect of the skin test, suggested that a prescapular skin test site was certainly as good, if not better, than the currently used axillary site. The axillary site on South American camelids is acknowledged to be a difficult area to use, causing stress to the animals and to their keepers, not to mention the veterinarian conducting the test. This report raises the possibility that the axillary test site could be superseded by a prescapular site, resulting in a less stressful TB test visit to premises that would affect the outcomes of neither the tuberculin skin test nor the antibody test.

In 2009 the APHA investigated the potential for a cell-based interferon-gamma (IFNγ) release assay in South American camelids, which unlike the antibody response would not be dependent upon a prior skin test for boosting. Such an assay would be based upon the *in vitro* stimulation of blood cells, similar to the IFNγ assays that have become commonly used in the diagnosis of TB in cattle and humans in the last 15 years (Wood and Rothel, 1994; Streeton *et al*., 1998; Lalvani *et al*., 2001; Wood and Jones, 2001). The initial in-house data for the camelid IFNγ assay using both llama and alpaca samples appeared promising, and a study funded by the British alpaca and llama industry took place at APHA during 2011–2012 to evaluate the relative performance of the IFNγ assay plus four different serological tests, namely the STAT-PAK® and Dual Path Platform or DPP® (Lyashchenko *et al*., 2011) lateral flow rapid antibody tests (both Chembio Diagnostic Systems, Inc., Medford, NY) and two antibody ELISA tests – the IDEXX bovine tuberculosis antibody test, (IDEXX Laboratories, Inc., Westbrook, ME) (Waters *et al*., 2011a), modified to detect camelid antibodies, and a multiplex ELISA, Enferplex™ (Enfer Scientific, Naas, Ireland) (Whelan *et al*., 2008, 2010).

The tests were applied to diseased alpacas from confirmed TB breakdown herds in GB and TB-free alpacas from non-endemic parts of the country in order to determine test sensitivity and specificity parameters, respectively, for the individual tests. The data were also used to suggest potential test combinations to maximize TB testing efficiency in different testing scenarios (Rhodes *et al*., 2012).

A total of 59 diseased (with gross visible lesions, VL, on post-mortem examination) alpacas from ten distinct confirmed breakdown herds, involving six different *M. bovis* genotypes were available for this study, together with 306 TB-free animals; 257 from 17 distinct GB herds plus 49 serum samples from the USA (courtesy IDEXX Laboratories, Inc.). Not all tests could be performed on all alpacas, but in total, 48 diseased and 257 TB-free alpacas were tested with all tests. A small number of TB-free alpacas reacting positively to the diagnostic tests were removed and investigated to establish their infection status.

The camelid IFNγ test detects MTBC and is not *M. bovis*-specific

The results showed that the IFNγ assay, as a test for *M. bovis* per se, had a low specificity of between 80% and 98%, with the highest (most desirable) specificity (98%) giving a disappointingly low sensitivity (~35%) and the highest sensitivity (80%) giving the lowest specificity (also 80%). Table 12.3 provides the

Table 12.3. IFNγ sensitivity and specificity values (© Crown Copyright 2012; originally published in *Clinical and Vaccine Immunology* 19, 1677–1683).

	VL alpacas (n = 55)			TB-free alpacas (n = 257)		
Test result[a]	n/55	% sensitivity	95% CI	n/257	% specificity	95% CI
PPD+	35	63.6	50.9–76.3	28	89.1	85.3–92.9
EC+	27	49.1	35.9–62.3	26	89.3	86.2–93.6
PPD-EC+	8	14.5	5.2–23.8	21	91.8	88.4–95.2
PPD + EC+	19	34.6	22.0–47.2	5	98.1	96.4–99.8
PPD+ or EC+	44	80.0	69.4–90.6	49	80.9	76.1–85.7

[a]PPD: PPDB-PPDA; EC: ESAT6/CFP10 peptide cocktail. VL, visible lesions.

sensitivity and specificity values of the IFNγ test results supported by Receiver Operator Curve (ROC) analysis of the data to generate positive/negative cut-offs when peripheral blood mononuclear cells were stimulated with PPDB and PPDA (to generate a comparative PPD response as used for cattle IFNγ testing) and the peptide cocktail ESAT6/CFP10 – used for higher specificity bovine TB diagnosis in cattle. The culture-confirmed presence of *M. microti* in two of the PPD-positive TB-free alpacas euthanized and investigated as false positives suggested that the apparent low specificity of the IFNγ assay could be due to its detection of undiagnosed *M. microti* infection, which is a known cause of tuberculous pathology in South American camelids (Pattyn *et al*., 1970; Oevermann *et al*., 2004; Zanolari *et al*., 2009). The comparative PPD IFNγ assay therefore would appear to detect the presence of MTBC mycobacteria, as has previously been described for feline tuberculosis (Rhodes *et al*., 2011), with the presence of an *additional* positive response to ESAT6/CFP10 being the only way to positively differentiate *M. bovis* from other mycobacterial infections.

Serological tests: a way forward for camelid TB

The serological tests did not appear to suffer from the low specificity seen in the IFNγ test. Two of the tests, having a quantitative readout (IDEXX ELISA and DPP rapid antibody test), were amenable to ROC analysis to set appropriate cut-offs for the data, while both the STAT-PAK rapid antibody test and the ENFERplex multispot ELISA provided qualitative positive/negative readouts not amenable to ROC analysis. Nevertheless all four antibody tests presented specificity values of ~97%, moderate sensitivity values of between 60% and 70% (see Table 12.4), and accord well with other (unpublished) STAT-PAK test data from GB breakdown herds that show a sensitivity of 55% (99 out of 180 visibly lesioned South American camelids from confirmed breakdown herds, www.alpacatb.com).

The combination of two or more antibody tests in parallel interpretation provided a significant increase in overall test sensitivity at the expense of a marginal drop in specificity. Table 12.5 shows that the sensitivity of the STAT-PAK test combined with the IDEXX test provided a sensitivity of >80%, with a corresponding small drop in specificity of only 1.9% (Rhodes *et al*., 2012). Thus using two antibody tests in a parallel interpretation (animals positive to *either* test identified as sero-positive reactors) can improve detection of infected camelids in confirmed breakdown herds compared with clinical examination and skin testing alone.

This is in agreement with the findings by Lyashchenko *et al*. (2011) who studied groups of *M. microti*- and *M. bovis*-infected South American camelids across Switzerland, GB and the USA and showed that the use of two antibody tests together (in this case STAT-PAK and DPP tests) with a parallel test interpretation (animals may be positive to *either* test) could exceed the single test sensitivities by >10%, with a small accompanying decrease in specificity.

Table 12.4. Antibody test sensitivity and specificity values (© Crown Copyright 2012; originally published in *Clinical and Vaccine Immunology* 19, 1677–1683).

	VL alpacas			TB-free alpacas		
Test	n/total	% sensitivity	95% CI	n/total	% specificity	95% CI
STAT-PAK	35/52	67.3	54.5–80.8	8/306	97.4	95.6–99.2
DPP	30/52	57.7	44.3–71.1	10/306	96.7	94.1–98.4
IDEXX	36/52	69.2	56.7–81.7	8/306	97.4	95.6–99.2
ENFERplex	32/48	66.7	53.4–80.0	8/257	96.9	94.8–99.0

VL, visible lesions.

Table 12.5. Antibody test combinations for increased sensitivity.

	VL alpacas			TB-free alpacas		
Test combinations	n/48	% sensitivity	95% CI	n/257	% specificity	95% CI
STAT-PAK/IDEXX	39	81.3	71.0–91.6	11	95.8	93.3–98.2
STAT-PAK/ENFERplex	37	77.1	65.2–89.0	14	94.6	91.8–97.4
IDEXX/ENFERplex	37	77.1	65.2–89.0	12	95.3	92.7–97.9

VL, visible lesions.

Interestingly, the very same two antibody tests (STAT-PAK and IDEXX), used in a serial interpretation (animal must be positive to *both* tests to be identified as sero-positive) provided a specificity of 99.7%; only one positive animal was identified from the 306 TB-free cohort in our study, and that animal was *M. microti* culture-confirmed – and thus an MTBC-infected animal, not even a true 'false positive'. The sensitivity of such a serial interpretation was reduced to 56%, but coupled to such a high specificity this latter serial combination of serological tests has potential as a routine surveillance option in non-TB situations (Rhodes *et al*., 2012).

Thus recent studies, supported by the British camelid industry, have provided viable options for TB testing of camelids. Similar to previously published work, it has been demonstrated that a rational combination of serological tests for camelids can be the most promising way forward.

Since this work was published, a pilot study to investigate the use of PCR to detect *M. bovis* DNA in clinical samples (faecal and nasal swabs) from infected South American camelids has suggested an increasing sensitivity in animals with more severe pathology (Crawshaw *et al*., 2014). This test could therefore offer an additional route for the ante-mortem detection of *M. bovis* infection in some cases, but more work will be required, including field validation, to determine the sensitivity and specificity of this test and whether/how it could be applied in the field.

Camelid TB control policy in GB

Under the current animal health legislation, the detection of TB in carcasses of camelids must be immediately notified to the APHA (as is the identification in a laboratory of *M. bovis* in clinical samples from those animals). Herds with suspect cases are placed under movement restrictions pending laboratory culture results. As there is no regular TB testing programme for camelid herds in GB, ante-mortem testing of camelids for TB is currently limited to animals undergoing pre-export certification and in response to TB herd breakdowns that are confirmed by laboratory culture of *M. bovis*. A small number of camelids are also tested for TB every year because they happen to be located on farms with infected cattle herds or on premises adjoining an infected cattle herd. In the EU and many other countries, the tuberculin skin test remains

the primary official test for TB in camelids. In GB the comparative format of the skin test, using PPDB and PPDA injected in the axillary site, has traditionally been used to screen camelids for TB. When necessary, the skin test is repeated at minimum intervals of 90 days. Although highly specific, this test has a low sensitivity in these species, even at a severe interpretation, and is clearly inadequate as a method to resolve confirmed TB breakdowns by repeat test and slaughter, due to the high rates of false-negative results.

In 2006–2007 Defra/APHA began to deploy the rapid antibody lateral flow STAT-PAK test on an experimental basis and with the owner's consent to supplement the comparative skin test in TB breakdown situations. Blood samples are usually collected 10–30 days following a skin test to take advantage of the anamnestic antibody response in infected animals to maximize sensitivity. Based upon the results of the camelid industry-funded study carried out by APHA (above), Defra (with the Scottish and Welsh Governments) is planning to replace the current voluntary blood testing regime of infected camelid herds with a compulsory single intradermal tuberculin skin test followed by a combination of the STAT-PAK and IDEXX tests on all skin test-negative animals, so that animals positive to any of these tests will be removed (parallel interpretation). Completion of at least one combined antibody test of the herd will be a prerequisite for lifting the movement restrictions. Furthermore, any animals traced from herds with a confirmed *M. bovis* infection will undergo the same testing regime on the farm of destination. Negotiations are under way between Defra and the camelid industry bodies to set up a voluntary TB testing scheme for camelids in England, which would include private routine herd surveillance and pre-movement testing.

TB in South American Camelids in Other Countries

TB in South American camelids has been reported in many non-GB countries (Thoen *et al*., 1997; Ryan *et al*., 2008; Zanolari *et al*., 2009; García-Bocanegra *et al*., 2010). Llamas and alpacas have been detected with TB infections caused by *M. bovis* and *M. microti*, and the progressive disease in these animals can lead to high transmission and mortality rates.

In southern Spain, clinical disease caused by *M. bovis* was diagnosed in three alpacas from two related herds (García-Bocanegra *et al*., 2010). A range of signs was observed in these animals including loss of appetite, teeth-grinding, respiratory difficulty, lethargy, fever, anorexia and weight loss. The comparative tuberculin skin test and BOVIGAM® (bovine IFNγ test) (AsureQuality Australia Pty Ltd for Prionics, Switzerland; now part of Thermo Fisher/Life Technologies) assay administered 1 month before the first case was detected and 90 days later yielded negative results (BOVIGAM does not detect camelid IFNγ). On X-ray evaluation the lungs of two of the infected alpacas showed cavitary lesions, and despite an attempt of antibiotic treatment, the disease was increasingly progressive in the three animals, which were later euthanized. Post-mortem examination revealed generalized TB with multifocal-to-coalescing granulomatous, caseous and calcified nodules of various sizes in the lungs, trachea, liver, spleen and various lymph nodes. In the two alpacas with cavitary lesions, abundant acid-fast bacilli (AFB) were detected on Ziehl–Neelsen (ZN)-stained smears of all affected tissues, whereas only paucibacillary disease with AFB staining was occasionally found in certain organs of the third animal.

The more recent study in Spain by Bezos *et al*. (2013) assessed the single and comparative tuberculin skin test and a serological assay (ELISA using MPB83 protein) in an alpaca herd (n = 115) known to be heavily infected with *M. bovis* (>33% TB-related natural deaths during the period of study). Data from this study supported the priming of antibody responses by the skin test for the detection of infected animals, with a parallel use of skin and antibody tests maximizing sensitivity up to 100% in this case.

In the last few decades a number of case reports have been published on *M. microti* infections in various host species including camelids and humans in GB and mainland Europe (Pattyn *et al*., 1970; Kremer *et al*., 1998; van Soolingen *et al*., 1998; Oevermann *et al*.,

2004; Taylor *et al*., 2006; Henrich *et al*., 2007; Xavier-Emmanuel *et al*., 2007; Burthe *et al*., 2008). This slow-growing organism with fastidious requirements for growth on artificial media shows biochemical properties similar to *M. tuberculosis* (Levy-Frebault and Portaels, 1992; van Soolingen *et al*., 1998). Culture and identification of *M. microti* by traditional biochemical methods are difficult; therefore, its prevalence and clinical relevance may have been underestimated in previous studies (Kremer *et al*., 1998; Van Soolingen, 2001).

Generalized TB due to *M. microti* in South American camelids has frequently been reported in Switzerland in the last few years (Oevermann *et al*., 2004; Lyashchenko *et al*., 2007; Zanolari *et al*., 2009). Although the clinical signs were very non-specific, most of the diseased animals showed weight loss, recumbency (tendency to sit) and anorexia in advanced stages of the disease. The comparative tuberculin skin test usually failed to identify infected animals. In contrast, emerging serological tests have shown diagnostic potential for detecting *M. microti* infection in llamas and alpacas (Lyashchenko *et al*., 2011). In these studies, the infected animals predominantly recognized MPB83 protein known to be a major serological target for the antibody response to *M. bovis* in camelids and other host species (Greenwald *et al*., 2003; Lyashchenko *et al*., 2004, 2007; Waters *et al*., 2004; Lyashchenko *et al*., 2011; Waters *et al*., 2011b). Importantly, sero-conversions associated with the *M. microti* infections could be detected long before the onset of clinical disease. At necropsy, AFB-positive lesions were found in multiple tissues. These studies concluded that ante-mortem differential diagnosis remains challenging, particularly during early stages of disease.

Infections with non-MTBC mycobacteria, including *M. kansasii* (Braun *et al*., 2009), *M. avium* (Lucas *et al*., 2003) and *M. avium* subsp. *paratuberculosis* (Belknap *et al*., 1994; Stehman, 1996; Fecteau *et al*., 2009) have also been described. These pathogens may pose a significant confounding factor for specific diagnosis of TB in live animals, as there is no reliable ante-mortem test available to distinguish between the respective infections. The alpaca infected with *M. kansasii* above displayed chronic weight loss and weakness, with the post-mortem examination showing pathology indistinguishable, both macroscopically and histologically, from typical TB. AFB were later identified by PCR analysis as *M. kansasii*.

Clinical signs of Johne's disease (*M. avium* subsp. *paratuberculosis*) in South American camelids are similar to those reported in cattle and small ruminants, including signs of lethargy and chronic weight loss; and in advanced stages, development of diarrhoea or changes in faecal consistency (Belknap *et al*., 1994; Ridge *et al*., 1995; Fecteau *et al*., 2009). Affected animals can present with anaemia of variable severity. The reported age of onset of clinical signs tended to be younger in South American camelids than in cattle; three of the ten diseased alpacas described in one study were between 11 and 14 months of age (Belknap *et al*., 1994). Prevalence of *M. avium* subsp. *paratuberculosis* in alpaca herds remains unknown. A recent study using a commercial RT-PCR assay in four veterinary teaching hospitals in the USA estimated the faecal shedding rate to be 6% (Fecteau *et al*., 2013). Limited information is available regarding epidemiology, pathogenesis, immune responses and testing options for Johne's disease in South American camelids (Kramsky *et al*., 2000; Miller *et al*., 2000).

TB in Old World Camelids

Old World camels (OWCs) comprise the dromedary camel (one-humped *Camelus dromedarius*), historically inhabiting the Middle East and the Horn of Africa; and the Bactrian camel (two-humped *C. bactrianus*), historically inhabiting Central Asia. OWCs have an estimated world population of ~18 million across the arid and semi-arid environments of African and Asian countries. In Africa the dromedary population of about 15 million accounts for ~74% of the world camel population, and of these, 60% are found in East African countries (Somalia, 6.2 million; Sudan, 2.8 million; Ethiopia, 1.7 million, Kenya, 0.9 million). The United Arab Emirates has approximately 360,000 dromedaries. The strength and docility of the camel have been exploited for transport, meat and milk production, as well as sport (racing).

They are extremely important for the livelihood of pastoral communities and cultural life.

Camel TB has been described in Egypt (Mason, 1912, 1917; Mustafa, 1987), the United Arab Emirates (Kinne *et al*., 2006; Wernery *et al*., 2007, Wernery and Kinne, 2012), Pakistan (Zubair *et al*., 2004), Kazakhstan (Elmossalami *et al*., 1971), Somalia, Nigeria (Abubaker *et al*., 2012) and Ethiopia (Mamo *et al*., 2009; Mamo *et al*., 2011; Zerom *et al*., 2013). *M. bovis*, *M. tuberculosis* and NTBC mycobacteria such as *M. kansasii*, *M. aquae*, *M. fortuitum* and *M. smegmatis* have all been isolated from OWCs as causative agents of camel TB (Elmossalami *et al*., 1971; Kinne *et al*., 2006; Mamo *et al*., 2011; Zerom *et al*., 2013).

After the first description of TB in OWCs in the Egyptian Official Gazette in 1888 only sporadic reports were documented until Mason in 1912 published his pathological observations on a series of 20 cases detected during a year's surveillance at a Cairo abattoir. This was followed by a further study of camels slaughtered between 1910 and 1916 which suggested a prevalence of 1.6–5.4% and affecting chiefly old animals (Mason, 1917). In 1987 a brief review (Mustafa, 1987) mentioned that TB was more commonly observed in farmed camels and those in close proximity to cattle but appeared to be rare among pastoral camels, suggesting that close contact facilitates transmission between domesticated animals. In 1991 TB was reported as rare in Somalia, a country which at that time had one of the largest populations of OWC in the world (Abdurahman and Bornstein, 1991; Teka, 1991). The prevalence of TB in dromedaries is also thought to be rare in Dubai, where only four cases have been documented in a 25-year observation period (Wernery *et al*., 2007).

In contrast Elmossalami *et al*. (1971) mention an older study in Kazakhstan that suggested a higher TB prevalence in camels of ~13%, and a more recent study in Ethiopian abattoirs suggested a similar prevalence of ~10%, based on the identification of gross lesions in 906 apparently healthy dromedaries (Mamo *et al*., 2011). However, among those with suspicious lesions, mycobacteria were cultured from 31 of 91 animals, and only two isolates proved to be *M. bovis*. This report suggests that the majority of TB-like pathology in Ethiopian pastoral OWCs may be caused by non-MTBC mycobacteria, and aligns with Elmossalami *et al*. (1971) who isolated *M. kansasii* and *M. smegmatis*, as well as *M. bovis* and *M. tuberculosis* from Cairo abattoir camels in their study.

The pathology in OWCs, first described approximately 100 years ago (Mason, 1912, 1917), is thought to be similar to that seen in South American camelids with the organs most frequently affected in both groups of camelids being the lungs and associated thoracic lymph nodes, where typical caseonecrotic lesions can be particularly extensive. Mason (1912) also mentioned that all cases in OWCs had lesions at these sites, with 60% of cases exclusively involving these sites (other affected tissues including the liver, spleen, kidney, trachea and pericardium). Ethiopian studies have described 54% of infected OWCs with lesions in the lung and associated lymph nodes, and 38% with mesenteric lymph node lesions, followed by retropharyngeal lymph node involvement (Mamo *et al*., 2009), suggesting infection via both inhalation and ingestion. Interestingly, in the latter study 57% of infected OWCs had just a single lesion. Whether this may reflect some sort of latency or immune control of infection in dromedary (as opposed to Bactrian) OWCs is unknown.

TB testing of OWCs should follow the OIE guidelines where the official screening method for camelid trade is the single intradermal comparative tuberculin skin test. For statutory testing PPDB and PPDA are injected into a shaved area of the axilla, and the thickness of the skin measured immediately before and 72 h after injection. However, there is uncertainty over the test sensitivity and specificity (Bush *et al*., 1990), and also the timing of the test reaction – with 5 days being reported as more predictive than the statutory 3 days in one study (Wernery *et al*., 2007). One negative impact of this lack of reliability in the interpretation of the recommended skin test is that testing in OWCs may be discontinued (Bush *et al*., 1990), resulting in a reduced disease surveillance and so posing severe limitations upon any programme to control camelid TB.

Ante-mortem TB testing of Old World Camels in the United Arab Emirates

A new confirmed case of pulmonary TB was reported in a young adult male dromedary bull from a racing herd in Dubai in 2006, nearly 20 years after the last case of camel TB in Dubai had been described (Kinne *et al*., 2006). The bull had shown outward clinical signs of disease and on post-mortem examination was found to have respiratory pathology. The infectious agent was identified as *M. bovis* (antelope clade), and it was suggested that infection may have come from the (then) habit of these coprophagus camels to roam freely in the desert during the day where they could come into contact with the faeces of infected antelopes before returning to their camps in the evenings. As a result of this finding, the entire racing dromedary herd of 57 animals was investigated using two serological tests, the Multi Antigen Print ImmunoAssay (Chembio Diagnostic Systems, Inc., Medford, USA) and the STAT-PAK rapid antibody test together with the tuberculin skin test (Wernery *et al*., 2007). The dromedaries were tested once by the skin test and three times by the serological tests (twice prior to the skin test and once 6 weeks after the skin test). Two camels, plus the index bull case, were found to be consistently reactive to the serological tests. The two new cases were also reactive to the skin test (read at 5 days). The two sero-positive animals were slaughtered and confirmed to have TB at post-mortem examination. Interestingly the skin test appeared not to affect the qualitative readouts of the antibody tests in these two dromedaries. All others in the herd were sero-negative, while the skin test did identify two further inconclusive reactors that since have become skin test-negative and remain apparently healthy. This study highlights the potential of serological testing for MTBC mycobacteria in OWCs where the skin test remains unsatisfactory.

No further TB cases in dromedaries have been reported in the United Arab Emirates (UAE) since that study, probably due at least in part to the active management of dromedary herds (e.g. camels no longer allowed to roam in areas grazed by antelope). While there is no actual policy as such in the UAE for dealing with cases of TB in OWCs, cases of *M. bovis* and human TB strains are reportable to the Ministry of Environment and Water. TB in OWC is not considered a human health risk in the UAE. The Central Veterinary Research Laboratory in Dubai maintains an interest in serological testing for potential other TB cases.

One limitation of the above study was the low number of OWCs involved, and only two (three counting the index case) that were test-positive, and it was recognized that further studies on greater numbers of well-defined samples/camels would be needed to provide more definitive estimates of TB test accuracy.

Old World Camel TB in Ethiopia: more than just *M. bovis*

TB in OWCs of eastern Ethiopia was studied for the first time in 2009 in order to describe its prevalence and to isolate *M. bovis* from infected camels as the causative organism (Mamo *et al*., 2009). The prevalence of the disease in camels, the impact upon the livestock sector of the country and any potential public health significance were all then unknown. The latter was particularly of concern since some pastoral communities retain the habit of eating raw meat and drinking raw milk, providing a potential route for zoonotic spread of disease-causing mycobacteria. An initial study of 276 pastoral camels slaughtered between December 2006 and May 2007 at an abattoir east of Addis Ababa showed 14 camels to have lesions typical of TB infection, providing an estimate of ~5% prevalence in pastoral camels in this area. However, of these 14 lesioned camels, only four had AFB-positive tissue impression smears, and only one of these four samples was PCR-positive for MTBC.

A second study of 906 apparently healthy camels from two further pastoral regions of eastern and southern Ethiopia showed 91 camels to have suspicious TB lesions, providing an estimated prevalence of ~10%. AFB-positive mycobacterial isolates were cultured from 31 (34%) of these lesioned camels, of which 21 provided a positive PCR signal for the genus *Mycobacterium*, but only two were confirmed as MTBC and identified as *M. bovis*

(Mamo *et al*., 2011). Plate 8 shows the tuberculous lesions caused by *M. bovis* in the mesenteric lymph node, on the wall of the intestine, hepatic and cranial mediastinal lymph nodes of one of these camels. The apparently poor recovery of a positive causative agent (i.e. *M. bovis*) from the majority of lesioned camels in these two studies in Ethiopia could be related to a non-optimal culture for non-MTBC mycobacteria, and also reflect the diversity of mycobacteria causing mycobacterial diseases in camels. More recent 16 s rDNA sequencing of these non-MTBC mycobacteria has revealed *M. terrae* complex, *M. flavescens*, *M. brasiliensis*, *M. chelonae* and *M. avium* as causative agents (Mamo *et al*., unpublished data).

Other abattoir studies have similarly highlighted the potential for involvement of non-MTBC as well as MTBC in camel mycobacterioses in Ethiopia. A detailed post-mortem examination of 293 OWCs from eastern Ethiopia showed an estimated prevalence of 12.3% (36/293), with the occurrence of TB lesions significantly associated with female dromedaries. Mycobacteria were isolated from 61% (22/36) of those gross lesions investigated, and molecular characterization of the isolates showed just three to be *M. tuberculosis*, and the majority of the isolates from this study (15/22) to be non-MTBC (Zerom *et al*., 2013). Gumi *et al*. (2012) examined 694 camels slaughtered at Filtu and Addis Ababa abattoirs (mainly camels from southern Ethiopia) and isolated three AFB-positive isolates of which one was *M. tuberculosis* and the other two were non-MTBC. The latter group of organisms have similarly been identified as a significant cause of mycobacterial infections in Ethiopian cattle, with one study describing ~30% (53/171) of isolates as containing 11 non-MTBC species (Berg *et al*., 2009). These findings not only suggest the importance of non-MTBC in OWC TB in Ethiopia, but also highlight a potential role for OWCs in the transmission of *M. tuberculosis* in humans.

These initial studies have prompted a greater interest in camel TB in Ethiopia, and further work is now planned to assess the usefulness of ante-mortem tests (tuberculin skin test and specific antibody ELISA) in camels with defined TB status. Data on the skin test only are currently available for Ethiopia; a study on 305 OWC in the Afar pastoral region of north-east Ethiopia using the comparative intradermal skin test showed 45/305 to react to PPDB (>4 mm cut-off PPDB–PPDA) and 54/305 to react to PPDA (>4 mm cut-off PPDA–PPDB), suggesting a slightly higher proportion of reactions to non-MTBC than MTBC mycobacteria (Mamo, unpublished data).

Old World Camels in the West

There are very few OWC herds kept in GB and mainland Europe. In GB small numbers are kept for tourism/trekking and for sport (racing). The only reported case of TB in an OWC to date, in Europe at least, has been that of an *M. caprae* infection in a dromedary from a zoological collection in Slovenia (Pate *et al*., 2006).

Interestingly, however, there does appear to be an embryonic industry for camel milk products that has recently found its way to GB (Turvill, 2013; O'Leary, 2015). The marketing for camel milk claims health benefits above cow milk (e.g. a higher insulin content; Agrawal *et al*., 2011) and may well find a niche customer base in the near future. OWC milk cheese products are also becoming available following the production by a Danish company (CHR-Hansen) of a pure camel milk-curdling enzyme (notoriously difficult, apparently). As far as TB is concerned, *M. bovis* has in the past been isolated from pooled milk samples from camels in Russia (Donchenko *et al*., 1975a,b). The information available on the camel milk that recently found its way to GB stated that the milk was 'un-processed'. The American-based Camel Milk Association describes its camels as being TB-tested, but no information is provided on the test used, nor is there any information regarding pasteurization of milk.

Dubai opened its first camel dairy industry 5 years ago, and now produces around 5000–6000 l of pasteurized milk, as well as camel milk chocolate (Nagy *et al*., 2013). This herd of 3000 dromedaries is exceptionally well managed and is tested for TB (using the DPP antibody test), brucellosis and other diseases. In the spring of 2013 it received an EU permit to export camel milk and its products to the EU.

Summary

It was not so long ago that camelids were thought to be resistant to TB (Fowler, 2010). However in recent years serious concern has been raised concerning the threat that the disease poses to South American camelids in some of the countries in which they are now reared. We are consequently beginning to realize that any perceived 'resistance' may have more to do with the *lack of exposure to the causative organism*, rather than to any inherent resistance per se, although there may be some as yet unexplored difference in resistance between Bactrian and dromedary camels, not only in the case of TB but in how they deal with other infections also (Wernery, personal communication, 2014), and there is some current scientific interest concerning camel 'nanoantibodies' (Muyldermans, 2013) which may in due course expand our understanding of camelid immune responses generally.

The increase in South American camelid farming in parts of Europe with a high prevalence of TB in cattle and wild mammals, particularly in GB, has shown with no doubt that these animals are susceptible to TB, and moreover they may act as an amplifier host and vectors of infection to other individuals and potentially causing zoonotic spread. This view is supported by the reports of TB in OWCs primarily from the UAE that suggested the opportunistic contact of OWCs with infected antelope faeces as the only potential route of infection with an antelope clade of *M. bovis*. Alternatively, in the more variable climate of Ethiopia there is the opportunity for exposure of OWCs to a range of MTBC and non-MTBC mycobacterial species infecting livestock.

Human cases of TB in GB caused by *M. bovis* have been very rare since the 1990s and mostly occurring in persons over the age of 65 years old, linked to the consumption of unpasteurized *M. bovis*-infected milk during childhood. The systematic culling of cattle reactive to the tuberculin skin test from the 1950s onwards, together with the pasteurization of milk, served to remove the risk of zoonotic *M. bovis* infection from cattle away from the vast majority of the population of GB (reviewed by de la Rua-Domenech, 2006; Mandal *et al*., 2011).

However, the farming of South American camelids in areas of endemic bovine TB in GB now presents a growing industry of livestock that are susceptible to TB, with close contact between animals and humans involved, but with no current systematic TB surveillance. The potential occupational zoonotic risk that TB-infected camelids pose to human contacts is illustrated by the report on *M. bovis* infection of a veterinarian following exposure to a tuberculous alpaca (Twomey *et al*., 2010b), and the infection of an alpaca owner with the same *M. bovis* genotype that was previously isolated from her own alpaca herd (Hosking *et al*., unpublished data).

Human cases of TB caused by *M. microti* are also very rare, and *M. microti* has been described as having a low virulence for humans (Frota *et al*., 2004). However, four cases were reported in Scotland between 1994 and 2005 (Xavier-Emmanuel *et al*., 2007) (with two of the three genotypes isolated displaying a 'llama-type'); three cases were reported in Germany (Niemann *et al*., 2000; Geiss *et al*., 2005); and six cases were reported from France (Panteix *et al*., 2010). Only one individual across these reports was described as immunocompromised, but a concluding line from Xavier-Emmanuel *et al*. (2007) states that *M. microti* remains *a potential threat to the substantial pool of persons with compromised immunity*. There have been two case reports of *M. microti* infection in two immunodeficient individuals, one from Germany (Horstkotte *et al*., 2001) and one from the Netherlands (Foudraine *et al*., 1998). We have seen that South American camelids infected with *M. microti* can develop pathology indistinguishable from that caused by *M. bovis* and that the extent of *M. microti* in the GB camelid population is unknown. Notwithstanding a potential risk of zoonotic spread from camelids, *M. microti* may also of course present a health hazard to the industry and should be a concern for herd health. The final word, then, belongs to Twomey *et al*. (2010b) – reporting on a veterinary surgeon infected with *M. bovis* following the post-mortem examination of an infected alpaca – they conclude that 'Given the increasing population of South American camelids and the potential for them to be infected with members of the MTBC, public health workers should be aware of the zoonotic risk for people in close contact with these animals'.

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13 Tuberculosis in Companion Animal Species

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Introduction

Mycobacteria of importance to companion animals, that is, cats and dogs, include (i) obligate pathogens that can cause tuberculosis; (ii) mycobacteria that are difficult to grow so their environmental niche is difficult to determine, which can cause feline leprosy syndrome (FLS) and canine leproid granuloma syndrome (CLGS); and (iii) facultatively pathogenic opportunistic saprophytes, which can cause non-tuberculous mycobacteriosis (NTM). Regardless of which mycobacteria are involved, most cats present with cutaneous disease which can sometimes progress to pulmonary or systemic disease; only occasional cases present with primary systemic disease. In comparison, most canine cases have disseminated disease at the time of diagnosis.

There are few data on the prevalence of feline and canine tuberculosis around the world. Mycobacterial infections are seen in companion animals more frequently in some countries than others; for example, Australia and parts of Africa and America (North and South) see cases of FLS, CLGS and NTM, while New Zealand used to see tuberculosis (*Mycobacterium bovis*) and still sees FLS and NTM (Malik *et al*., 2006a,b). In Great Britain (GB) these infections are far from rare in cats, being reported in ~1% of all feline tissue samples submitted to diagnostic laboratories for routine histopathology (with ~0.3% being Ziehl Neelsen (ZN)-positive) (Gunn-Moore *et al*., 2013). When cultured, *M. microti* was cultured from 19%, *M. bovis* from 15%, *M. avium* 7%, *M. malmoense* 1%, and unclassified mycobacteria 4% (Table 13.1; Gunn-Moore *et al*., 2011a). A positive culture was only gained in 47% of samples, despite them having histopathological changes indicative of mycobacterial infection. This may have been, in part, because the culture system used was optimized for *M. bovis*, or because some NTM are very difficult to grow, even in optimized systems. In addition, *M. microti* can take many months to grow, and may give a false negative result, especially with paucibacillary samples (Smith *et al*., 2009; Gunn-Moore *et al*., 2011a). Interestingly, canine mycobacterial infections are not common in GB.

Diagnosis and treatment are challenging because there are no pathognomonic histopathological changes for tuberculosis and many mycobacterial species fail to culture, so molecular diagnostics are needed to confirm the cause of the infection. Importantly, until the species has been identified it is not possible to

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Table 13.1. Mycobacterial culture results from cats in Great Britain. The samples had histopathological changes indicative of mycobacterial infection and were submitted to the Animal Health and Veterinary Laboratories Agency (AHVLA, now Animal and Plant Health Agency, APHA) for mycobacterial culture between January 2005 and December 2008 (Gunn-Moore *et al*., 2011a).

Culture results	Number	% of total	% of cultured
Tuberculous mycobacteria; tuberculosis complex group			
M. microti	63	19	40
M. bovis	52	15	33
Non-tuberculous mycobacteria (NTM)			
M. avium	24	7	15
M. malmoense	4	1	3
M. fortuitum	4	1	3
M. celatum	1	<1	<1
M. intracellulare	1	<1	<1
Unclassified	10	3	6
Cultured total	159	47	–
No growth	180	53	–
Grand total	339	100	–

tell whether the infection is tuberculosis, FLS, CLGS or NTM. Treating tuberculosis is contentious because of zoonotic risk and the potential for generating drug-resistant mycobacteria. Where treatment is undertaken, it typically involves giving two or three drugs for ~6 months (Greene and Gunn-Moore, 2012), with a current prognosis that only ~40% of feline cases will achieve an apparent cure (Gunn-Moore *et al*., 2011b).

Epidemiology

Tuberculosis can be caused by a number of different, but closely related, bacteria. Relevant members of the tuberculosis complex group include *M. tuberculosis*, *M. bovis* and *M. microti* (the vole bacillus). *M. tuberculosis* causes over 90% of tuberculosis in man, but rarely infects other mammals, although occasional cases are seen in captive elephants, pet dogs and very rarely, pet cats (Parodi *et al*., 1966; Aranaz *et al*., 1996; Erwin *et al*., 2004; Lobue *et al*., 2010; Mikota and Maslow, 2011; de la Fuente *et al*., 2012; Parsons *et al*., 2012; Martinho *et al*., 2013; Botelho *et al*., 2014; Engelmann *et al*., 2014). *M. bovis* has the broadest host range of the members of the tuberculosis complex group and is globally distributed (Snider, 1971; Lobue *et al*., 2010). It is the main cause of tuberculosis in cattle, but it can also infect other mammals, including humans (where it causes ~1% of cases of tuberculosis), badger, deer, llama, cats, dogs, sheep, goats and pigs, among others (Francis, 1958, 1961; Smith, 1965; Cousins, 2001; de Lisle *et al*., 2001, 2002; Rastogi *et al*., 2001; Delahay *et al*., 2002, 2007; Biet *et al*., 2005; Corner, 2006; Une and Mori, 2007).

M. microti has been found in many countries (GB, Germany, the Netherlands, Switzerland, South Africa and South America), and seen in voles, wood mice, shrews, cats, llama, pigs, ferrets, squirrel monkeys, rock hyrax, a dog, a badger, a bull and humans (Wells and Oxon, 1937; Wells, 1946; Huitema and Jaartsveld, 1967; Pattyn *et al*., 1970; Cousins *et al*., 1994; Gunn-Moore *et al*., 1996; Kremer *et al*., 1998; van Soolingen *et al*., 1998; Deforges *et al*., 2004; Jahans *et al*., 2004; Oevermann *et al*., 2004; Lutze-Wallace *et al*., 2006; Taylor *et al*., 2006; Henrich *et al*., 2007; Lyashchenko *et al*., 2007; Xavier Emmanuel *et al*., 2007; Rüfenacht *et al*., 2011). In cats, *M. microti* infection was previously, rather confusingly, termed *M. microti*-like as it was unclear at the time that it was actually the same organism (Huitema and Jaartsveld, 1967; Gunn-Moore *et al*., 1996; Kremer *et al*., 1998; van Soolingen *et al*., 1998). In addition, a number of reports have discussed cases where the infection was reported to be *M. tuberculosis* (Kipar *et al*., 2003) or *M. tuberculosis* var. *bovis* (Orr *et al*., 1980) which on further investigation were probably *M. microti*.

Historically, tuberculosis used to be common in cats and dogs, with prevalence levels in necropsy studies from Europe and other countries of 1–13% in cats and 0.1–13.5% in dogs (Snider, 1971). In cats, over 95% of tuberculosis was caused by *M. bovis* (Francis, 1961; Orr *et al*., 1980; Pedersen, 1988) with only occasional cases of *M. tuberculosis* (Parodi *et al*., 1966), and *M. microti* (Huitema and van Vloten, 1960; van Dorssen, 1960; Gunn-Moore *et al*., 1996). In dogs, *M. tuberculosis* caused approximately 75% of cases, with most of the rest being due to *M. bovis* (Francis, 1961;

Parodi *et al*., 1966; Liu *et al*., 1980). Most cases of tuberculosis in cats and dogs were believed to result from ingestion of milk from tuberculous cattle (Jennings, 1949; Parodi *et al*., 1966; Snider, 1971) or arose secondary to living in close proximity to *M. tuberculosis*-infected people (Parodi *et al*., 1966; Snider, 1971; Liu *et al*., 1980). Hence, with the reduction of tuberculosis from national herds, the pasteurization of milk and the reduction in human tuberculosis, there has been a marked decline in the prevalence of disease seen in cats and dogs (Jennings, 1949; Smith, 1965; Parodi *et al*., 1966, Snider, 1971; Anon., 2007; Gunn-Moore *et al*., 2010, 2011a).

Currently, tuberculosis in cats and dogs is recognized infrequently (Gunn-Moore *et al*., 2010; Greene and Gunn-Moore, 2012). There has been a handful of case reports of *M. bovis* or *M. microti* infections. Cases of *M. bovis* infection have come from all around the world, including GB, Ireland, mainland Europe (Spain), the USA, Japan, Argentina, Australia and New Zealand (Isaac *et al*., 1983; de Lisle, 1992; Aranaz *et al*., 1996; Kaneene *et al*., 2002; Bauer *et al*., 2004; Ellis *et al*., 2006; Sykes *et al*., 2007; Shrikrishna *et al*., 2009; van der Burgt *et al*., 2009; Zumárraga *et al*., 2009; Gunn-Moore, 2010; Rodríguez *et al*., 2010; Jahns *et al*., 2011; Gunn-Moore, 2014; Murray *et al*., 2015). New Zealand and GB have a high proportion of cases and this is believed to result from bites by infected wild rodents (Gunn-Moore *et al*., 2010) and brushtail possum (de Lisle *et al*., 2002) (see below). Feline *M. microti* infections have been identified mainly in GB (de Bolla, 1994; Blunden and Smith, 1996; Gunn-Moore *et al*., 1996; Xavier Emmanuel *et al*., 2007; Gunn-Moore *et al*., 2011a) and mainland Europe, including Switzerland (Rüfenacht *et al*., 2011) and the Netherlands (van Dorssen, 1960; Huitema and Jaartsveld, 1967; Kremer *et al*., 1998). The one published case of *M. microti* infection in a dog was in France (Deforges *et al*., 2004).

Analysis of the *M. microti* isolates has led to the recognition of two different types of *M. microti* based on their growth characteristics and their molecular specificity (spoligotyping); the vole type and the llama type (van Soolingen *et al*., 1998). The llama type has so far been recognized in humans, llama, a cat and a dog (van Soolingen *et al*., 1998; Horstkotte *et al*., 2001; Deforges *et al*., 2004; Xavier Emmanuel *et al*., 2007); while the vole type has been recognized in voles, cats and a badger (van Soolingen *et al*., 1998; Xavier Emmanuel *et al*., 2007).

In GB and Ireland, tuberculosis is now being seen with increasing frequency in cats (Smith *et al*., 2009; Gunn-Moore *et al*., 2010; Gunn-Moore *et al*., 2011a; Broughan *et al*., 2013a; Murray *et al*., 2015). In GB, infection has a geographical distribution: *M. bovis*-infected cats are found in the south-west of England (co-incident with the areas where cattle, badger, mice and other small rodents are infected with *M. bovis*), while *M. microti* is found in the south-east of England, the north of England and the south of Scotland (where *M. microti*-infected rodents have been detected) (Fig. 13.1; Gunn-Moore *et al*., 2011a). While most infections occur in a single pet within a household, clusters have been seen where a number of animals within the same house or within a close geographical area have been infected with *M. bovis* or *M. microti* (Rüfenacht *et al*., 2011; Gunn-Moore, 2014; Roberts *et al*., 2014; Murray *et al*., 2015). Of 115 recent feline cases in Great Britain, 55% were caused by *M. microti* and 45% by *M. bovis*, with these two infections accounting for 34% of all cases of feline mycobacteriosis in GB (Gunn-Moore *et al*., 2011a). Tuberculosis is seen less commonly in dogs in GB: only seven cases were confirmed by the Animal Health and Veterinary Laboratories Agency (AHVLA, now the Animal and Plant Health Agency, APHA) in the past 7 years, all of which were due to *M. bovis* (Broughan *et al*., 2013a). By way of comparison 116 cats were diagnosed with *M. bovis* in the same time frame (Broughan *et al*., 2013a).

Infection with *M. tuberculosis* is exceedingly rare in dogs and cats, where it occurs as an anthropozoonosis (reverse zoonosis), with pets being infected by their owners. There are very few cases in cats (Aranaz *et al*., 1996; Erwin *et al*., 2004; Posthaus *et al*., 2011), in part because they are naturally resistant to this infection (Soltys, 1958; Smith, 1965). Although dogs are less resistant to it than cats, this disease is now uncommon in the developed world so only occasional case reports have been published; they have come from Switzerland, Germany, Spain, Portugal, South Africa, Brazil and the USA (Aranaz *et al*., 1996;

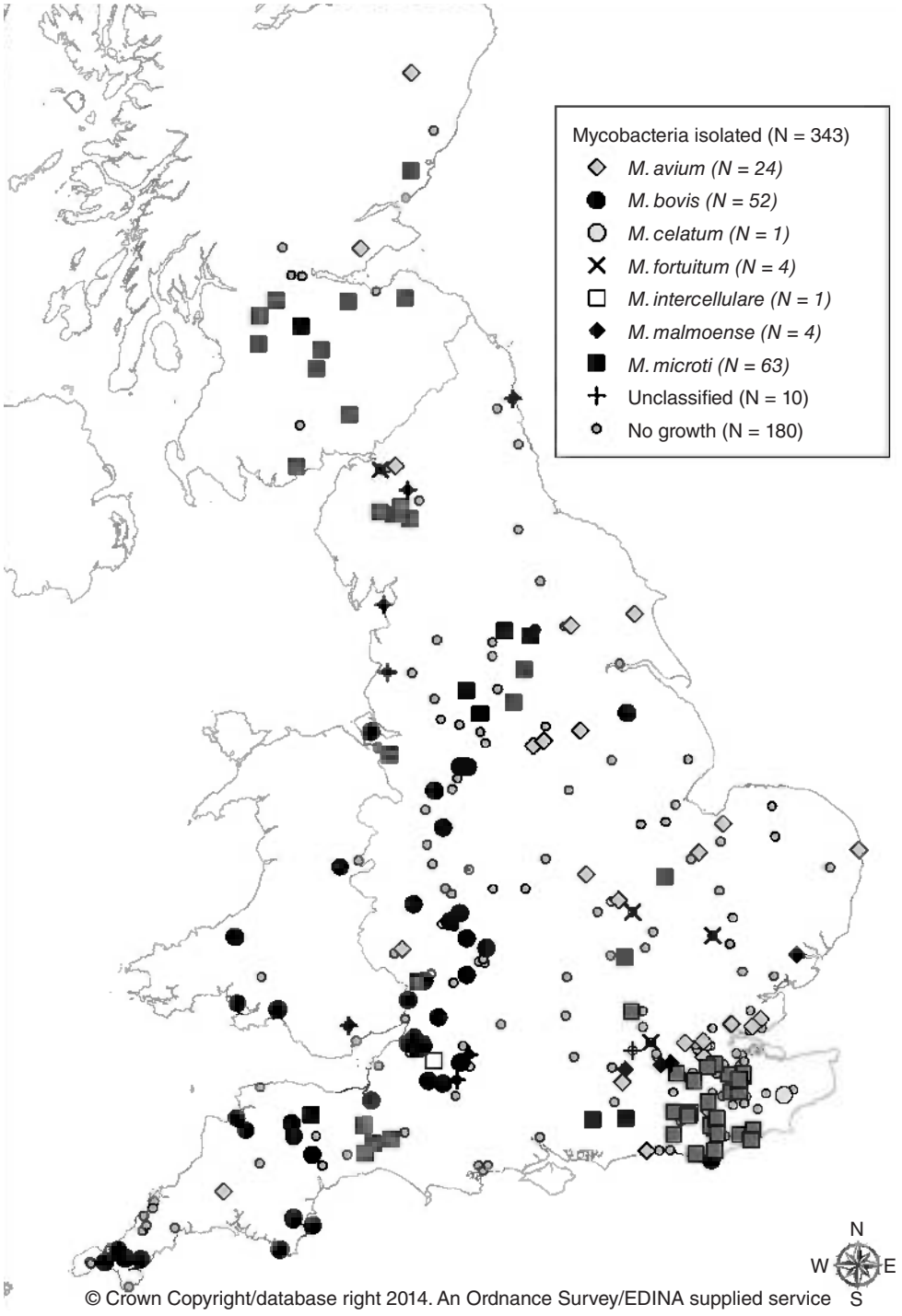

Fig. 13.1. Map of GB showing the location of 339 feline samples from between January 2005 and December 2008 for which the Animal Health and Veterinary Laboratory Agency (AHVLA, now the Animal

Erwin *et al*., 2004; Hackendahl *et al*., 2004; Turinelli *et al*., 2004; Parsons *et al*., 2008, 2012; Posthaus *et al*., 2011; de la Fuente *et al*., 2012; Martinho *et al*., 2013; Botelho *et al*., 2014; Engelmann *et al*., 2014). However, they are of significant concern as a recent report detailed the case of a dog with disseminated tuberculosis which was caused by a multidrug-resistant strain of *M. tuberculosis* (Beijing Strain) (Botelho *et al*., 2014).

Possible routes of infection

Cats and dogs may become infected with *M. bovis* or *M. microti* through a number of different routes:

- Few cases are believed to result from drinking infected cow's milk (Greene and Gunn-Moore, 2012). This is because infection gained by drinking tuberculous milk tends to result in intestinal disease and this form of tuberculosis is now very rare (Gunn-Moore *et al*., 2011a; Greene and Gunn-Moore, 2012). That said, the specific strains (spoligotypes) of *M. bovis* identified in cats and dogs in GB and Ireland are typically the same as those seen in cattle from the same geographic location (Monies *et al*., 2006; Murray *et al*., 2015), so cattle may be responsible for environmental contamination. Since *M. bovis* can survive for extended periods in the environment (Wray, 1975; Morris *et al*., 1994) this could lead to infection of domestic cats and dogs, and/or rodents (see below).
- In some areas of GB and Ireland *M. bovis* is endemic in European badgers (*Meles meles*) (Gallagher and Clifton-Hadley, 2000). While cats and badgers rarely interact directly, there may be potential for cats to become infected via environmental contamination. In support of this, some owners of infected cats have commented that badgers have visited their gardens or that a badger sett was located close to their property (Monies *et al*., 2006). Some dogs will fight with badgers or eat dead badgers (van der Burgt *et al*., 2009) or, if small enough, may go down into badger setts and so may become infected.
- *M. bovis* can also be endemically present in other species of free-ranging wildlife, so the risk of infection will vary in each country dependent on the likely interaction between these species and domestic cats and dogs (de Lisle, 1992; Morris *et al*., 1994). For example, *M. bovis* is endemic in brushtail possums (*Trichosurus vulpecula*) in New Zealand and these have been known to infect cats (de Lisle *et al*., 2002); although an eradication programme is well under way, infection is still a possibility.
- If we look at possible risk factors, we find that most of the cats that develop tuberculosis are keen hunters, reported to be regularly catching small rodents (Gunn-Moore *et al*., 1996). In GB, wild field voles (*Microtus agrestis*), bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) can be infected with *M. microti* (Cavanagh *et al*., 2002; Burthe *et al*., 2008); and common shrews, yellow-necked mice, wood mice, field voles and moles, plus a wide range of other animals (e.g. rats, stoats, polecats, feral mink and ferrets, grey squirrels, foxes, deer, pigs, sheep, llama and alpaca) can be infected with *M. bovis* (Delahay *et al*., 2002, 2007; Biet *et al*., 2005; Une and Mori, 2007; van der Burgt *et al*., 2009; Rodríguez *et al*., 2010; Broughan *et al*., 2013a). It is most likely

Fig.13.1. Continued.

and Plant Health Agency, APHA) tried to culture mycobacteria. Successfully cultured samples were divided into the species isolated or grouped as unclassified mycobacteria (Dr Darren Shaw, from Gunn-Moore *et al*., 2011a). The infections cluster, such that *M. bovis*-infected cats are found in the south-west of England and Wales (coincident with the areas where cattle, badger, mice and other small rodents are infected with *M. bovis*), while *M. microti*-infected cats are found in the south-east of England, the north of England and the south of Scotland (where *M. microti*-infected rodents have been detected). Cat A (the single black circle in the east of England), infected with *M. bovis*, moved from Bristol; while Eastbourne (on the south-east coast of England, and marked with a single black circle) is a known area of *M. bovis*-infected cattle.

that cats become infected when hunting wild rodents. In the case of *M. bovis* the spoligotypes found in cats are the same as those in the cattle, badger and small rodents found in a particular area (Monies *et al*., 2006; Delahay *et al*., 2007); it is likely that small rodents become infected via environmental contamination, possibly around infected badger setts. The *M. microti* spoligotypes are also the same in the rodents and cats from the same geographic location (Smith *et al*., 2009). Damage sustained by cats when hunting accounts for the disease being mainly cutaneous, with lesions frequently affecting the face and legs, that is, the areas most likely to be bitten when playing with prey; plus or minus associated lymphadenopathy (most typically affecting the submandibular lymph nodes) (Gunn-Moore *et al*., 2011a). A canine case of *M. bovis* was caused by being bitten by a squirrel (van der Burgt *et al*., 2009).

- Some authors suggest that other than the European badger, there is currently no evidence for a significant self-maintaining reservoir of *M. bovis* in wild mammals in GB and Ireland (Delahay *et al*., 2002, 2007). That said, whether or not a self-sustaining reservoir is needed is debatable, as new members of these spill-over species are constantly being infected with *M. bovis* from cattle and badger.
- A number of households have been identified where more than one cat or dog has been infected.
    - Most cases have involved cats that had little close contact with each other, and the infections appear to result from hunting the same group of infected prey.
    - A small number of cases have occurred in cats that did not go outside, but were living with another cat (or dog) that was known to be infected and/or did go outside and hunt. These cases appear to represent spread via close contact, particularly sleeping with and/or grooming an infected companion (Fig. 13.1; Isaac *et al*., 1983; Posthaus *et al*., 2011; Murray *et al*., 2014; unpublished data).
- There have now been a small number of nosocomial cases, where cats naturally infected with *M. bovis* have infected other cats via contamination within a veterinary practice, particularly during routine neutering (Fig. 13.1; de Lisle *et al*., 1990; Gunn-Moore, 2014; Roberts *et al*., 2014; Murray *et al*., 2015). This has resulted most frequently, when a cat has had a large lesion draining significant amounts of ZN-positive pus (Gunn-Moore, 2014; Murray *et al*., 2015).
- Pathogenesis: infection is classically believed to occur after protracted exposure, e.g. following repeated exposure to infected small mammals, living on a farm housing tuberculous cattle, or living for prolonged periods with infected humans or poultry. It is therefore likely that there are many sub-clinical cases (Snider *et al*., 1971).

However, *clinical disease can occasionally occur in cats following a single inoculation and disease can then progress very rapidly*. This has been documented on a number of occasions, always with *M. bovis*, with severe disease being seen in under 1 month (Francis, 1958; Isaac *et al*., 1983; Murray *et al*., 2015). Experimentally, subcutaneous inoculation can result in clinical signs in 28–74 days, and peritoneal inoculation can result in clinical signs in 11–36 days (Francis, 1958). This phenomenon has also been seen in naturally occurring nosocomial infections; contamination of a castration site resulted in clinical signs in 3 weeks (Roberts *et al*., 2014), and 42 days (Fig. 13.2; Murray *et al*., 2015), and contamination of an ovariohysterectomy site resulted in clinical signs in just 16 days (Fig. 13.2; Murray *et al*., 2015). When this occurs, disease can progress very rapidly, resulting in death or the need for euthanasia in as little as 8–23 days (Isaac *et al*., 1983; Murray *et al*., 2015). The reason(s) for this rapid progression are unclear, but probably result from high doses of mycobacteria being inoculated and, possibly, the strain of the mycobacteria being inoculated, and/or whether they have been passaged through an atypical host (Aguilar León *et al*., 2009), for example mustelids such as feral mink or ferrets (Murray *et al*., 2015).

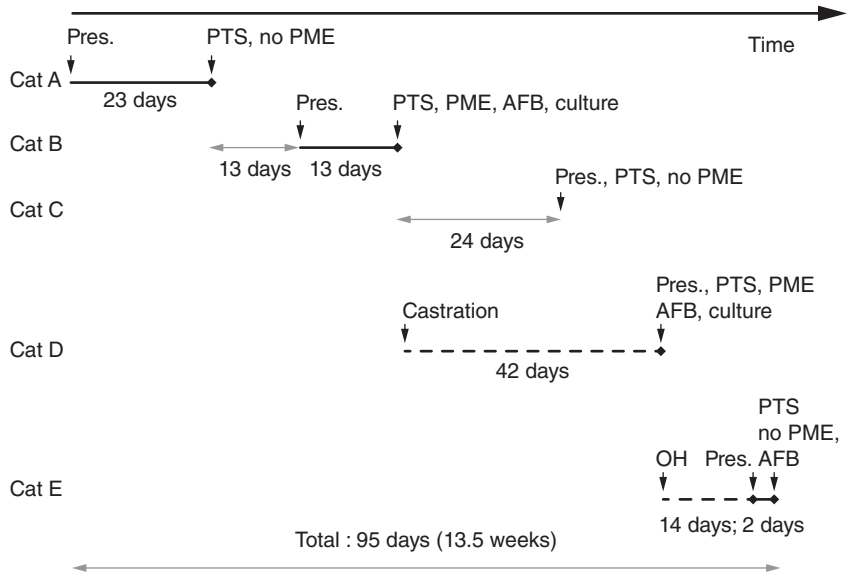

Fig. 13.2. Timeline of infections in Cats A–E. Cats A, B and C lived in the same house, A and B both hunted, developed draining neck lesions and quickly progressed to systemic disease. Cat C did not go outside, but did groom the other two cats and developed respiratory signs. Cat D was castrated on the same day that Cat B was euthanased, then developed draining lesions at the surgical site and extensive intra-abdominal disease. Cat E underwent ovariohysterectomy on the same day that Cat D was euthanased, then developed a draining lesion at the surgical site and intra-abdominal disease. Cats B and D were confirmed to have *M. bovis* of the same spoligotype. The long, grey line at the bottom of the figure indicates the known duration of clinical signs; the pale line indicates the duration between events; and the dashed black line the duration from elective procedure. Pres. – day of clinical presentation; PTS – euthanasia; PME – post-mortem examination; AFB – acid-fast bacteria seen on Ziehl–Neelsen staining; culture – specialist culture looking for mycobacteria; OH – ovariohysterectomy (Murray *et al*., 2015).

Zoonotic and Anthroponotic Risks

Dogs and cats are spill-over hosts for tuberculosis and as such are believed to present a low risk of further dissemination, either to humans or other animals (Francis, 1961). However, all members of the tuberculosis complex pose potential zoonotic risks (Smith, 1965; Isaac *et al*., 1983; Une and Mori, 2007).

Until recently, there had been very few published reports where cats may have infected humans. In a historical case from 1946, a 3-year-old boy was bitten on the arm by a cat in the advanced stages of *M. bovis* tuberculosis, after which he developed generalized infection, and died (Lewis-Jonsson, 1946). In the other case the man involved was working in Australia with a colony of five cats and two possums, all of which had clinical *M. bovis* infection, when he became Mantoux test-positive (he was never clinically ill) (Isaac *et al*., 1983). However, a recent publication detailed a cluster of nine cats with *M. bovis* infection in GB. Of the 24 owners who took up the offer of tuberculosis testing, two were found to have latent infections, and two were found to have active disease (Roberts *et al*., 2014). The spoligotype of the mycobacteria in both owners was the same as their kitten: the kitten had a draining scrotal wound that the owners were bathing daily (without wearing gloves or masks) (Roberts *et al*., 2014; T. Roberts personal communication, 2014). The author (DGM) also knows of a household of three dogs with *M. bovis* infection, where the young son was found to have latent tuberculosis (DGM, unpublished observations). There is also a case where pathology staff were infected with *M. tuberculosis* after they used an electric saw to open the cranium of a dog with tuberculosis in its brain (Posthaus *et al*., 2011).

These cases show that while rare, zoonotic infections can occur from pet cats and dogs to their owners or veterinary surgeons. Since people live in close confines with their pets, often having their pets lick their faces, sit on their knees and/or sleep on their beds, it is important that veterinary surgeons are aware of the potential for zoonotic spread and advise accordingly. When dealing with these cases in the veterinary clinic it is important that veterinary surgeons deal with these cases with suitable care.

While it is important that we take care when dealing with tuberculous pets, we should keep the risks in perspective – *the risk of catching tuberculosis from a pet is very low* (PHE, 2014) – and the greatest risk to humans is spending time with tuberculous humans or, much less frequently, by handling infected cattle.

M. tuberculosis and *M. bovis* can both cause *anthroponotic (reverse zoonotic) infections*. There have been a small number of cases where humans have infected their cats or dogs with *M. bovis* (Hawthorne and Lauder, 1962; Parodi *et al*., 1966; Shrikrishna *et al*., 2009) or their dogs with *M. tuberculosis*, usually by inhalation or ingestion of infected sputum (Erwin *et al*., 2004; Hackendahl *et al*., 2004; Une and Mori, 2007; Parsons *et al*., 2012; Martinho *et al*., 2013).

Predisposition

Age and gender

Tuberculosis is seen most commonly in adult cats and dogs that have outdoor access: median age for *M. bovis* in cats is 3 years, for *M. microti* is 8 years (Fig. 13.3; Gunn-Moore *et al*., 2011a). Unlike tuberculosis in general, where there is no gender bias in cats or dogs (Jennings, 1949), male cats appear to be over-represented with *M. microti* infection (Gunn-Moore *et al*., 1996; Rüfenacht *et al*., 2011).

Immunosuppression

No evidence of classical immunosuppression has been found, and cats tested for FIV and FeLV have usually been negative (de Bolla, 1994; Gunn-Moore *et al*., 1996, 2011a). However, cats with mycobacteriosis are deficient in 25 (OH) vitamin D and 1.25 (OH) vitamin D (Fig. 13.4; Lalor *et al*., 2012; DGM and SL, unpublished observations), which may play a role in the effectiveness of their macrophages to fight these infections. Certainly, in humans with tuberculosis, hypovitaminosis D can prevent the early clearance of infection (Verrall *et al*., 2014) and the efficacy of treatment (Karczmarewicz *et al*., 2013). In addition, an inherited predisposition to tuberculosis in humans has been found to result from defective interferon-gamma (IFNγ) mediated immunity (Altare *et al*., 1998; Remus *et al*., 2001). While we are currently assessing cats with tuberculosis with an IFNγ release assay, we

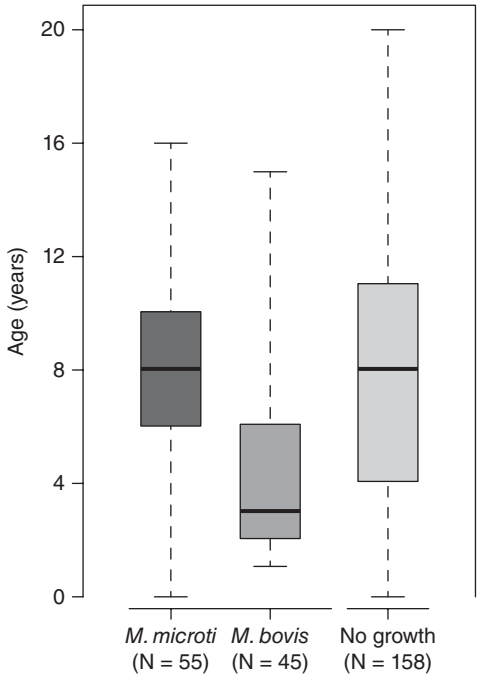

Fig. 13.3. Boxplot of the age distribution (in years) associated with the particular mycobacteria isolated from cats in GB with lesions with histopathological changes indicative of mycobacterial infection. Horizontal thick lines indicate the median age, boxes indicate the interquartile range and whiskers the range. Also included is the number of isolates of each particular *Mycobacterium* species for which the age of the cat was identified (Gunn-Moore *et al*., 2011a).

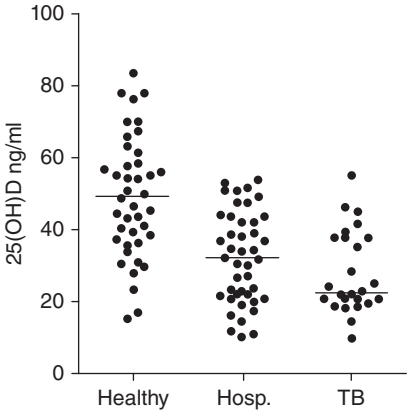

Fig. 13.4. Serum 25(OH)D concentration in healthy cats, hospitalized ill control cats and cats with mycobacteriosis. The horizontal lines represent the median 25(OH)D concentration (Lalor *et al*., 2012).

have not as yet correlated these results with the nature of the cat's immune response (DGM, unpublished observations).

Clinical Signs

Depending on the route of infection, affected cats and dogs present with clinical signs related to the alimentary, and/or respiratory tracts, and/or with localized disease affecting the skin (Jennings, 1949; Gunn-Moore, 2010). Historically, cats most commonly developed alimentary lesions (secondary to being infected by drinking tuberculous milk) (Jennings, 1949), while dogs developed pulmonary lesions (secondary to inhaling infected droplets of their owner's sputum). In dogs, the initial pulmonary lesions tended to spread very quickly, so the animals typically died with widely disseminate disease (Jennings, 1949; Francis, 1961). Currently, there are really too few cases in dogs to comment; but most appear to present with pulmonary, alimentary and/or systemic signs, although cutaneous or intracranial signs can also be seen (de Lisle, 1992; Bauer *et al*., 2004; Deforges *et al*., 2004; Turinelli *et al*., 2004; Ellis *et al*., 2006; Anon., 2007; van der Burgt *et al*., 2009; Jahns *et al*., 2011; Posthaus *et al*., 2011; de la Fuente *et al*., 2012; Broughan *et al*., 2013a; Martinho *et al*., 2013; Engelmann *et al*., 2014), and one of the authors recently dealt with a dog with a tuberculous tongue lesion (DGM, unpublished observation).

The most usual presentation for tuberculosis in cats is now the cutaneous form, with respiratory and alimentary forms being seen less frequently (Gunn-Moore *et al*., 2010, 2011a; Rüfenacht *et al*., 2011). In cats, the primary complex is often incomplete, especially when the infection gains entry via the mouth or intestines (i.e. granuloma form in the local lymph nodes), but there are no obvious lesions at the site of entry (Snider, 1971; de Bolla, 1994; Gunn-Moore *et al*., 1996, 2011a; Rüfenacht *et al*., 2011; Roberts *et al*., 2014).

Cutaneous disease probably arises from infected bite wounds, local spread, haematogenous dissemination to the skin or, occasionally, contaminated surgical wounds (Jennings, 1949; Smith, 1965; Isaac *et al*., 1983; Gunn-Moore *et al*., 2010; Roberts *et al*., 2014; Murray *et al*., 2015; and unpublished data). The lesions often involve the face, extremities, tail base or perineum (i.e. 'fight and bite sites'). They generally take the form of firm, raised, dermal nodules, ulceration, or non-healing wounds with draining sinus tracts (Figs. 13.5–13.7; Snider, 1971; Pedersen, 1988; Gunn-Moore *et al*., 2011a; Rüfenacht *et al*., 2011). Extension of granulomatous tissue may involve the subcutaneous structures, muscle and/or bone. Skin lesions are commonly associated with either local or generalized lymphadenopathy. On occasion submandibular, prescapular or popliteal lymphadenopathy may be the only clinical finding (Smith, 1965; Blunden and Smith, 1996; Gunn-Moore *et al*., 1996, 2010, 2011a; Rüfenacht *et al*., 2011; Roberts *et al*., 2014).

When the infection spreads to the lungs from other sites, or where it is acquired through inhalation, tubercles arise in the lungs and/or hilar lymph nodes and affected animals present with weight loss, anorexia, dyspnoea and cough. In cats, most pulmonary cases occur secondary to haematogenous spread from cutaneous lesions so the infection is typically diffuse and interstitial (eventually spreading to bronchial), and may include more generalized small focal lesions; the cats are dyspnoeic, sometimes with a soft cough (Jennings, 1949; Smith, 1965; Gunn-Moore *et al*., 1996, 2010; Bennett *et al*., 2011). Occasional cases may

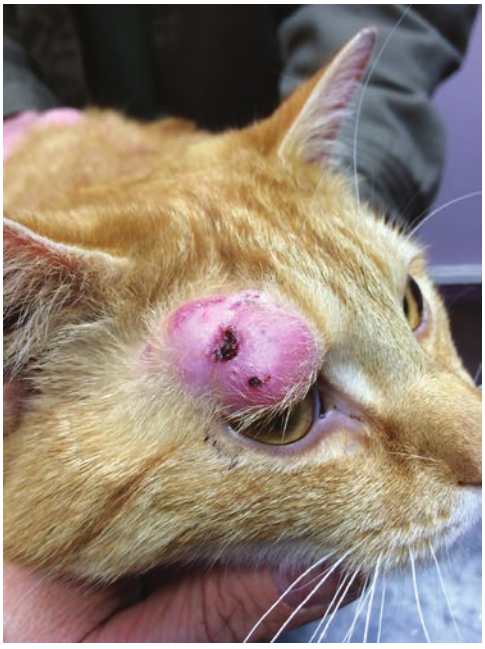

Fig. 13.5. Tuberculous lesion above a cat's eye. *M. bovis*. (Courtesy of Rory Lydon.)

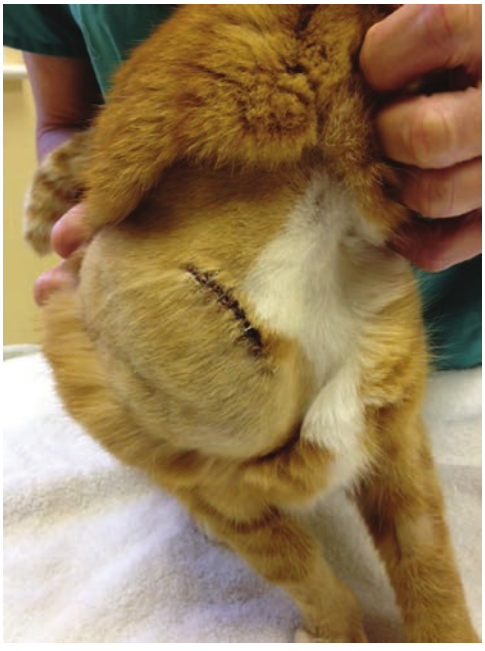

Fig. 13.6. Tuberculous lesion in the pre-sternal lymph node; sutures mark the biopsy site. *M. bovis*. (Courtesy of Emma Coles.)

also develop pneumothorax and/or pleurisy with the accumulation of pleural fluid, and pericardial effusions have also been seen (Snider, 1971). Some cases may sneeze and have a nasal discharge (Jennings, 1949; Huitema and van Vloten, 1960; van Dorssen, 1960; Smith, 1965; Snider, 1971). Only very rare cases develop tubercles which cavitate and break down to communicate with the pleural cavity or bronchii (Jennings, 1949). In dogs, pulmonary cases have occasionally presented with hypertrophic pulmonary osteoarthropathy (Snider, 1971).

In the alimentary form, tubercles arise in the intestines and/or mesenteric lymph nodes. Affected animals develop intestinal malabsorption and present with weight loss, anaemia, vomiting and diarrhoea (Jennings, 1949; Smith, 1965; Monies *et al*., 2006). Occasionally tubercles arise in the tonsils, resulting in signs of oropharangeal disease (Jennings, 1949; Smith, 1965).

A range of clinical signs may be seen with disseminated disease. These include splenomegaly, hepatomegaly, pleural or pericardial effusions, generalized lymphadenopathy, weight loss and fever (Liu *et al*., 1980; Gunn-Moore, 2010). Lameness may result from bone involvement (Jones and Jenkins, 1995; Gunn-Moore *et al*., 1996). Ocular involvement can result in granulomatous conjunctivitis (Fig. 13.8), uveitis, retinal detachment and even signs referable to central nervous system involvement (Formston, 1994; Gunn-Moore *et al*., 1996). Mycobacterial conjunctivitis may be seen on its own (Gunn-Moore *et al*., 1996) or associated with more generalized changes including lymph node and pulmonary involvement (Gow, 2006).

Diagnosis

Unfortunately, many cases of mycobacteriosis look very similar, regardless of which species of mycobacteria is involved (tuberculosis, FLS or NTM). Since different mycobacteria have different zoonotic risks and sources, respond differently to antibiotics and have dissimilar prognoses, further investigation is needed to determine which infection is present. Unfortunately, diagnosis is often challenging.

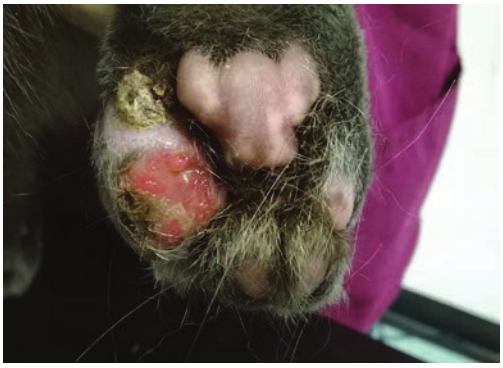

Fig. 13.7. Lesion on a cat's paw. *M. microti*. (Courtesy of Jayne Fisher.)

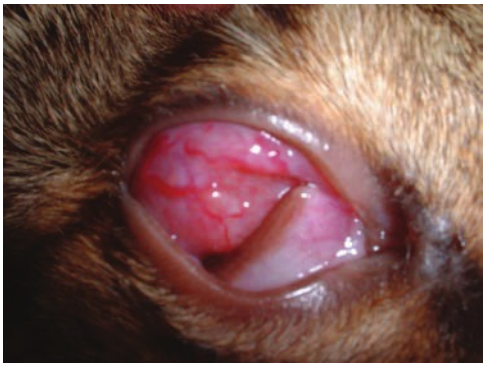

Fig. 13.8. Tuberculous conjunctivitis. *M. bovis*. (Courtesy of Adam Gow.)

This is because many of the mycobacteria do not grow in culture (frequently only ~50% grow) (Gunn-Moore *et al*., 2011a), and even those that do (e.g. *M. microti*) can take 3 months to be identified (Smith *et al*., 2009). In addition, serological tests have historically proved unhelpful and molecular diagnostics are not always available and can be very expensive (Greene and Gunn-Moore, 2012; Broughan *et al*., 2013b).

Non-specific tests

A thorough evaluation of the patient is necessary to assess the extent of local infection and the degree of systemic involvement.

Changes in serum biochemistry and haematology, if present, are non-specific and vary with the severity of disease; for example, hypercalcaemia appears to correlate with disseminated disease (Ellis *et al*., 2006; Gunn-Moore *et al*., 2011a). It is believed to result from vitamin D activation within macrophages during the granulomatous response, and is dependent on the underlying vitamin D status and calcium intake (Chan, 1997; Monies *et al*., 2006). However, how this occurs in the face of serum hypovitaminosis D is unclear (Lalor *et al*., 2012).

Radiography can be useful in the appraisal of lung involvement. However, changes are very variable and include tracheobronchial lymphadenopathy, interstitial or miliary lung infiltration, localized lung consolidation (Figs. 13.9–13.12), or pleural effusion (Bennett *et al*., 2011; Greene and Gunn-Moore, 2012). Since pulmonary involvement is usually via haematogenous spread, this leads to diffuse interstitial (later becoming bronchial) changes being seen most commonly (Bennett *et al*., 2011). Bone lesions tend to consist of areas of bony lysis and sclerosis (Fig. 13.13), osteoarthritis, discospondylitis or periostitis (Fig. 13.14; Bennett *et al*., 2011).

Abdominal radiography and ultrasound examination may reveal hepato- or splenomegaly, abdominal masses, mineralized mesenteric lymph nodes or ascites.

Specific tests

The recently developed interferon-gamma (IFNγ) release assay (IGRA) is showing promise for detecting members of the tuberculosis complex and *M. avium* (Rhodes *et al*., 2008a,b, 2011; Posthaus *et al*., 2011; Parsons *et al*., 2012). It has sensitivity estimates between 70% and

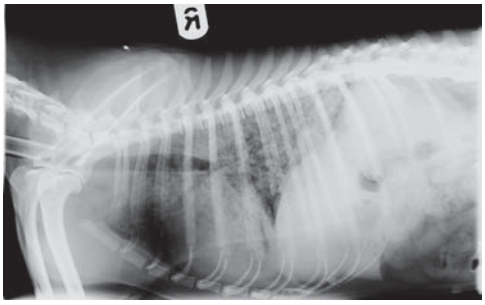

Fig. 13.9. Lateral chest radiograph of a dog showing generalized diffuse infiltration. *M. bovis*. (Courtesy of Kirsten Wilkinson.)

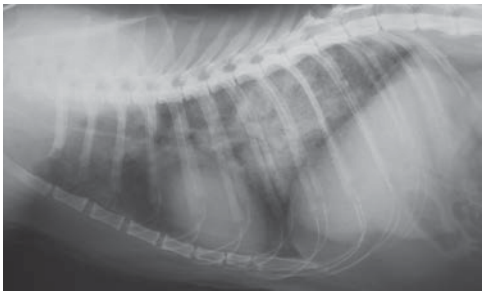

Fig. 13.10. Lateral chest radiograph of a cat showing patchy dense infiltration. *M. microti*.

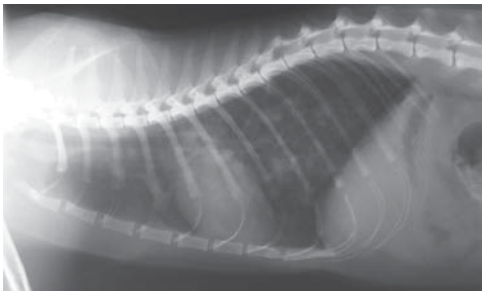

Fig. 13.11. Lateral chest radiograph of a cat showing patchy diffuse infiltration. *M. microti*. (Courtesy of Nico Martinez.)

100% for *M. bovis* (Rhodes *et al*., 2011). The test is also useful when considering a diagnosis of tuberculosis in an animal presenting with only pulmonary changes, but where bronchioalveolar lavage is unrewarding and lung biopsy not possible. Other specific tests have been investigated, but have generally proved unhelpful (Snider, 1971; Kaneene *et al*., 2002; Broughan *et al*., 2013b). However, newer tests for serum antibody responses are still being developed (Rhodes *et al*., 2011). Unlike other species, cats do not react strongly to intradermally administered tuberculin and the results from intradermal skin testing are unreliable (Hawthorne and Lauder, 1962; Snider *et al*., 1971; Kaneene *et al*., 2002). Even in dogs, false positives and false negative can occur (Greene and Gunn-Moore, 2012; Broughan *et al*., 2013b). Other than Rhodes *et al*. (2008b, 2011), no one has looked at responses to *M. microti* infection in particular.

Identification of mycobacteria

Gross pathology may reveal anything from large solid tumour-like masses to multiple small disseminated masses. Lesions are typically greyish white, sometimes with haemorrhagic edges and/or a soft purulent centre. Pulmonary lesions are often greyish red and may be associated with sero-sanguineous pleural fluid. Renal lesions typically occur in the cortex, in the form of infarcts, while intestinal lesions are typically ulcerated Peyer's patches with small submucosal tubercles (Jennings, 1949).

Histopathology of affected tissue generally reveals granulomatous inflammation, with foamy macrophages containing variable numbers of acid-fast bacteria (AFB, Fig. 13.15, see below), and bacilli may also be seen outside degenerating macrophages that border necrotic areas (Jennings, 1949; Snider, 1971; Kaneene *et al*., 2002; Malik *et al*., 2002; Kipar *et al*., 2003; Gunn-Moore *et al*., 2011b). Lymphocytes may be numerous, and fibroblasts may be present, but multinucleate giant cells are usually rare or absent (Snider, 1971; Kaneene *et al*., 2002; Ellis *et al*., 2006). Necrosis and calcification may occur, particularly in larger tubercles, which may be surrounded by zones of histiocytic cells, and a well-defined fibrous capsule may develop (Snider, 1971; Kaneene *et al*., 2002). Pathology can be very suggestive of mycobacterial infection.

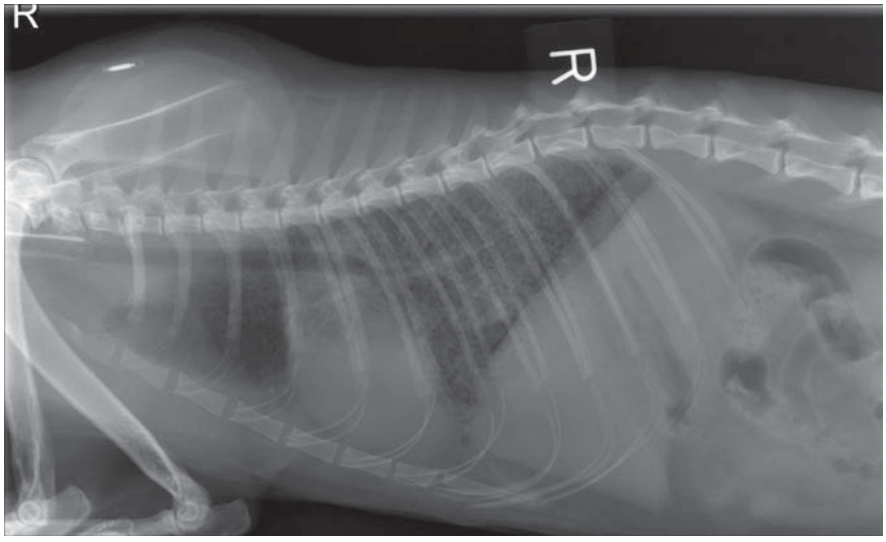

Fig. 13.12. Lateral chest radiograph of a cat showing generalized diffuse infiltration. *M. bovis*. (Courtesy of Barbara Gallagher.)

Aspirates and/or biopsy samples should always be ZN-stained. The number of AFB depends on:

- the species and strain of mycobacteria involved;
- the location of the granuloma; and
- the nature of the cat's immune response (Greene and Gunn-Moore, 2012).

Where the immune response is poor, lepromatous changes are often seen with large numbers of AFB (e.g. lepromatous FLS), while when the immune response is more robust a tuberculous response is more likely, and AFB will be few (tuberculosis, or tuberculous FLS due to *M. lepraemurium* or *M.* sp. strain Tarwin) (Davies *et al*., 2006; Malik *et al*., 2013).

The use of modified Fite's or rapid ZN stains may improve detection of AFB (Malik *et al*., 1994) and Romanowsky stains may sometimes reveal large numbers of macrophages containing negatively stained bundles of organisms and giant cells in lepromatous samples (Studdert and Hughes, 1992; Malik *et al*., 1994, 2002, 2004).

Specialist culture is needed to determine which species of mycobacterium is involved (Gunn-Moore *et al*., 2011a). Unfortunately, many samples that contain AFB fail to culture, including all those with FLS and even some with *M. microti*, particularly when there are few bacteria present (Smith *et al*., 2009; Gunn-Moore *et al*., 2011a).

Molecular PCR and sequencing techniques can be very useful in identifying mycobacteria (Aranaz *et al*., 1996; Brodin *et al*., 2002; Malik *et al*., 2002, 2006a, 2013; Kipar *et al*., 2003; Davies *et al*., 2006; Fyfe *et al*., 2008; Reppas *et al*., 2013). However, they are often expensive and availability may be limited.

Correct handling of biopsy material

In practice, this usually involves taking a biopsy from a case where mycobacterial disease is only one of a number of possible differential diagnoses. If in-house facilities are available for ZN staining, this can be performed on aspirates or biopsy impression smears. However, in most cases biopsy material must be sent to a veterinary diagnostic laboratory. Collect the biopsy, cut it into three or four pieces, fix one in formalin for histopathology and ZN staining and, pending results, place two in a sterile container and freeze them. Where other bacterial infections are suspected, the fourth sample should be sent unfixed for

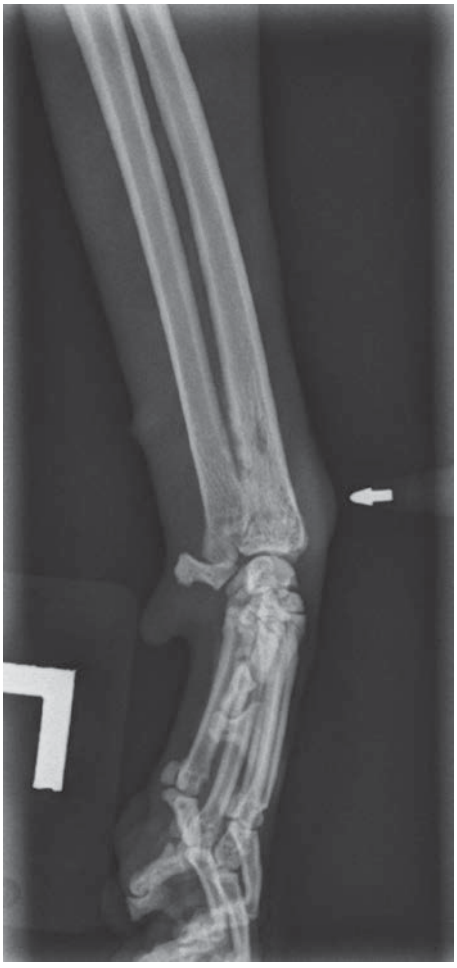

Fig. 13.13. Radiograph of a cat's carpus showing soft tissue and bony changes. The arrow marks the site of the cutaneous bite. *M. bovis*. (Courtesy of Kathryn Ling.)

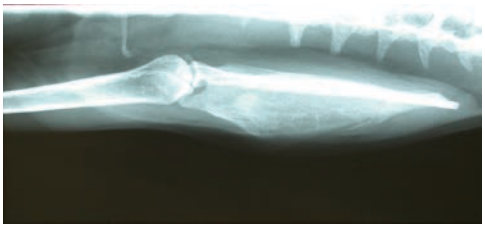

Fig. 13.14. Radiograph of a cat's tuberculous scapular showing marked bony changes. (Courtesy of Claire Lee.)

routine bacterial culture and ZN staining. If the sample is found to have ZN-positive organisms, one of the frozen pieces can be sent for specialist culture (by the APHA and/or a Mycobacterial Reference Laboratory), while the last sample is kept in case further investigation is needed. *This is advisable for all enlarged lymph nodes and cutaneous/subcutaneous lesions in cats*.

Remember: in GB, ~1% of feline tissue samples submitted to diagnostic laboratories for routine histopathology have changes consistent with mycobacterial infection (Gunn-Moore *et al*., 2013).

Until the organism is identified it should be considered a potential human pathogen.

Whenever handling a potentially tuberculous case, wear gloves and use routine aseptic practices when handling the biopsy and the biopsy site. Under the Tuberculosis Orders in England, Wales and Scotland, the identification of *M. bovis* in clinical or pathological samples taken from any mammal (except humans) is notifiable to the APHA. The Orders impose a duty on any veterinary surgeon *who even suspects tuberculosis in a domestic pet* to immediately notify the Divisional Veterinary Manager at the local office of the State Veterinary Service (Defra, 2013). When a confirmed case is euthanased it is advisable to have the body cremated. Unfortunately, until the sample has been cultured (or identified by PCR) it is not possible to know if the infection is caused by *M. bovis*, *M. microti* or one of the other mycobacteria that can cause disease in cats and dogs.

Management

Initial decision making before the species of mycobacteria has been identified

Many feline cases present with a single skin lesion, which is removed and sent for histopathology. It is only when the pathology report comes back suggesting a mycobacterial infection that this differential is considered and the veterinary surgeon has to discuss treatment options with the owner. The decision to treat a case of mycobacteriosis is difficult for many reasons. Most importantly, until

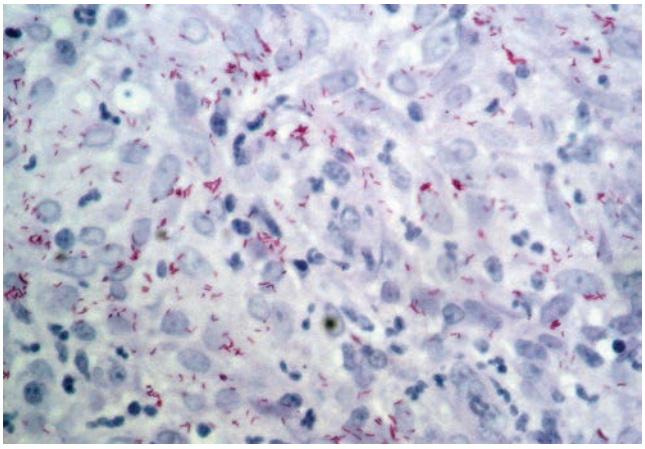

Fig. 13.15. Histopathology of a cat's lymph node revealing intrahistiocytic acid-fast bacteria taken under oil immersion (×100 obj.), ZN-stain. *M. microti*. (Courtesy of Jorge Del Pozo and Richard Fox.)

the species has been identified, it is not usually possible to tell whether the infection is tuberculosis, FLS or NTM (Gunn-Moore *et al*., 2011a). In GB, tuberculosis is likely in 34% of feline cases, with any particular case having a ~15% chance of being caused by *M. bovis* (Table 13.1), although this does depend on where the cat lives in GB (Fig. 13.1; Gunn-Moore *et al*., 2011a). Treating a case of suspected feline tuberculosis is contentious because of the potential zoonotic risk; the need to use drugs that some people feel should be kept for use only in human tuberculosis; and the potential for generating drug-resistant mycobacteria (Masur, 1993).

Before undertaking treatment it is important to consider:

- *Potential zoonotic risk*. The disease may be caused by a member of the tuberculosis complex. All members of the affected cat's household must be considered. It is important to determine if there is anyone with potential immunosuppression (e.g. HIV infection), undergoing chemotherapy or organ transplantation. We strongly advise against treatment where such individuals may be exposed. We also *advise against treatment if the affected animal has generalized disease, significant respiratory tract involvement, or extensive draining cutaneous lesions, as these may increase the risk of transmission*.
- Treatment is almost always long term and can be difficult to maintain given patient non-compliance, the inherent toxicity of some of the drugs and the financial costs involved. In some cases the drugs may at best suppress disease and indefinite treatment may be required (Sieber-Ruckstuhl *et al*., 2007; Greene and Gunn-Moore, 2012). Uncomplicated cutaneous cases carry the most favourable prognosis.
- Tailoring treatment is difficult:
  - Sensitivity testing is recommended as drug sensitivities can vary between different isolates of the same organism (Horne and Kunkle, 2009); however, *in vitro* findings do not always correlate with *in vivo* results.
  - Multiples of drugs are recommended to improve the chance of successful treatment and reduce the risk of generating resistant clones. However, when multiples of drugs are used, interaction is likely, and while some combinations are synergistic others are antagonistic. Unfortunately, the effects can be difficult to predict, even when using the *same* combination of drugs (Choi *et al*., 2012). It can vary: (i) between the species of mycobacteria involved and even the geographical strain of the species; (ii) whether treating intracellular or extracellular bacteria (the location of the bacteria is influenced by the type of pathology present – tuberculous verses lepromatous); and (iii) even with the species of host. Unfortunately,

as yet, we do not know the best drug combinations to use in cats (or dogs), so treatment advice is likely to change with time.
- The drugs need to be effective in the face of an inadequate host immune response (i.e. defective innate ± adaptive immunity) (Malik *et al*., 2013).
- *Drugs used to treat humans with M. tuberculosis may be needed, e.g. rifampicin*, and discussion is needed within the veterinary profession as to the appropriateness of using these drugs if there is a risk of producing resistant clones.
- *Surgical excision of small cutaneous lesions may be considered. However, debulking larger lesions risks wound dehiscence and local recurrence of infection*.

Interim management

Pending a definitive diagnosis, interim therapy with a fluoroquinolone has been recommended. However, this should only be considered in cases of localized cutaneous infection (Gunn-Moore *et al*., 2010). Pradofloxacin is recommended as it is more effective against mycobacteria than the older fluoroquinolones (Govendir *et al*., 2011). With more extensive disease double or triple therapy is advised (Table 13.2) (Greene and Gunn-Moore, 2012; Gunn-Moore, 2014). Giving more than one drug generally gives the best chance of clinical resolution, and decreases the potential for resistance to develop. Resistant clones can be a particular problem when treating tuberculosis, especially when using older fluoroquinolones such as marbofloxacin or enrofloxacin. This is an important consideration since drug resistance will be detrimental, not only to the individual cat, but may also endanger human patients.

Second-line treatments for tuberculosis should be reserved for resistant infections. Drugs licensed for human use can be obtained by veterinary prescription from our pharmacy or larger chemists as long as, in GB, all aspects of cascade prescribing have been considered. In GB, the Royal (Dick) School of Veterinary Studies Pharmacy, Edinburgh, can reformulate and supply these drugs for other veterinary practices for use in animals under their care.[1]

Continuing treatment

It is strongly inadvisable to continue treating a cat or dog once *M. tuberculosis* has been confirmed or *M. bovis* is disseminated; GB and Scottish law dictates that *M. bovis* infection is notifiable. *M. microti* is also potentially zoonotic, although very few human cases have been reported, and none due to feline or canine exposure. Unfortunately, in many cases it is not possible to culture the organisms from tissue samples even when AFB are present. Because of this is it essential to counsel owners carefully so that they know that it might not be possible to identify the causal species, making it difficult to predict potential zoonotic risks and treatment complications.

Ideally, anti-tuberculosis treatment should consist of an *initial* and a *continuation* phase (Greene and Gunn-Moore, 2012). The initial phase usually requires three drugs and lasts for 2 months, while the continuation phase requires two drugs and lasts for perhaps a further 4 months, depending on the extent of disease, and always for at least 3 months following complete resolution of the lesions. In those cases where triple therapy is not feasible, treatment should still involve two drugs and should be given for a minimum of 6–9 months (Greene and Gunn-Moore, 2012).

A combination of drugs needs to be given

First-line treatment for tuberculosis in humans consists of combinations of rifampicin, isoniazid, ethambutol, dihydrostreptomycin and pyrazinamide. However, these drugs are very toxic in cats (their toxicity in dogs is generally unknown), so all but rifampicin are reserved for resistant cases. The newer fluoroquinolones, such as pradofloxacin (or moxifloxacin) have good efficacy against most mycobacteria, and clarithromycin (a macrolide) is useful, especially when given in combination with rifampicin and/or another antibiotic as per culture and sensitivity, e.g. doxycycline. A useful once-daily alternative

Table 13.2. Potentially useful drugs for the treatment of feline and canine tuberculosis (see text for information on potential drug combinations and treatment duration). (Data on toxicity from Gelatt *et al*., 2001; Haburjak and Spangler, 2002; Bennett, 2007; Sieber-Ruckstuhl *et al*., 2007.)

Use	Drug	Cat/Dog	Dose mg/kg	Interval h	Toxicity
1st line Tx	Enrofloxacin[a]	D	5 PO	24	Retinal degeneration in cats
	Marbofloxacin	B	2 PO	24	Retinal degeneration?
	Pradofloxacin	B	3–5 PO	24	Vomiting, hypersalivation
	Moxifloxacin	B	10 PO	24	Vomiting
1st line Tx	Rifamp(ic)in[b]	B	10–15 PO (Max 600 mg/d)	24	Hepatotoxicity, induction of liver enzymes, discolouration of body fluids, generalized erythema + pruritus, poor palatability, nausea, CNS signs, teratogenic
1st line Tx	Clarithromycin	B	5–15 PO	12	Pinnal or generalized erythema, hepatotoxicity? GI signs?
	Azithromycin	B	5–15 PO	24	GI signs?
2nd line Tx	Isoniazid[b]	B	10–20 PO (Max 300 mg/d)	24	Hepatotoxicity, peripheral neuritis, seizures, acute renal failure
Prophylaxis		D±C	10 PO	24	As above
2nd line Tx	Dihydrostreptomycin[b]	B	15 IM	24	Ototoxicity
2nd line Tx	Pyrazinamide[b,c]	B	15–40 PO	24	Hepatotoxicity, GI signs
2nd line Tx	Ethambutol[b]	B	10–25 PO	24	Optic neuritis
2nd line Tx	Doxycycline[d]	B	5–10 PO	12–24	GI signs, oesophagitis
2nd line Tx	Clofazamine[b,e]	C	4–8 (occ. ~10) PO Max 25 total	24	Hepatotoxicity, GI signs, discolouration of body fluids, photosensitization
		D	4–12 PO	24	As above

Tx – treatment; D – dog; C – cat; B – both; PO – per os; IM – intramuscularly; GI – gastrointestinal; CNS – central nervous system.

[a]The authors recommend using a fluoroquinolone that is not enrofloxacin when treating cats as this drug has been associated with retinal degeneration in this species.

[b]These drugs are not licensed for use in pets and may cause potentially serious side effects, e.g. hepatotoxicity or nephrotoxicity. It is advisable to monitor these animals closely, and check routine haematology and serum biochemistry 2 weeks after starting treatment and then if there is any change in the cat's demeanour.

[c]Not effective against *M. bovis* infection.

[d]Give with food or give water after the medication to avoid oesophageal injury.

[e]Can be difficult to obtain.

to clarithromycin is azithromycin. From clinical experience gained over 15 years, we currently recommend an initial phase of rifampicin–pradofloxacin/azithromycin, followed by a continuation phase of rifampicin and either pradofloxacin or azithromycin (Table 13.2) (Greene and Gunn-Moore, 2012). While these combinations are also used when treating human mycobacterial infections, some experimental studies have shown that with *M. tuberculosis* infection, combining the new fluoroquinolones with clarithromycin (or azithromycin) or rifampicin may be at least mildly antagonistic, and combining fluoroquinolones and rifampicin may be antagonistic against extracellular but not intracellular AFB (Balasubramanian *et al*., 2012). What this means for the treatment of feline and canine cases is not yet clear. Rifampicin can cause significant side effects, including hepatotoxicity. It is therefore sensible to monitor cases closely, and check routine haematology and serum biochemistry 2 weeks after starting treatment, every few months if possible, and whenever there is any change in the cat's demeanour and/or appetite.

Ease of administration

All three once-daily medications can be given as liquids and placed in a single syringe prior to oral administration, or given as tablets with all three being placed in a single gelatin capsule and administered together using a pill-popper. Alternately, where oral medication proves difficult, an oesophagostomy tube may be placed (through which the liquid medications can be given) and left in place for the duration of the treatment. It is not recommended that owners put their fingers directly in the animal's mouth when administering medication and they must wash their hands thoroughly after the medication has been given.

Development of resistance

A rifampicin–isoniazid–ethambutol combination may be considered, although toxicity can be severe (Gunn-Moore *et al*., 2010). If necessary, ethambutol can be substituted with dihydrostreptomycin or pyrazinamide. However, *M. bovis* is naturally resistant to pyrazinamide. Rifampicin and isoniazid are more effective and less toxic than ethambutol and dihydrostreptomycin and consequently are more appropriate choices if only two drugs are required.

We know that cats with tuberculosis are deficient in vitamin D (Lalor *et al*., 2012). However, as yet, we do not know if correcting this deficiency will help in their successful treatment.

Prognosis

Prognosis depends on the mycobacteria involved, the extent and severity of the infection and whether treatment involves the appropriate drug(s) for the necessary length of time. In a study of 184 cases of feline mycobacterial disease, ~40% gained apparent cure or long-term remission while ~60% responded temporarily or poorly to treatment or did not respond at all. However, many cats were treated suboptimally, with few receiving more than one drug, many receiving inappropriate drugs (e.g. β-lactam drugs) and most (~60%) being treated for less than 1 month (Gunn-Moore *et al*., 2011b). It is therefore likely that with more appropriate treatment prognosis will be better. Cutaneous tuberculosis caused by *M. microti* or *M. bovis* typically responds well to treatment (even if there is pulmonary involvement), with full treatment currently resulting in ~70–80% of cases gaining long-term remission (D.A. Gunn-Moore, unpublished data). That said, the prognosis should be stated as guarded. Interestingly, one author (DGM) has seen a number of cats which, years after being successfully treated for one mycobacterial infection (e.g. tuberculosis) then contract a different mycobacterial infection (e.g. *M. avium*). This may result from an underlying immunosuppression and/or specific susceptibility to mycobacterial infections.

Very few cases of tuberculosis in dogs have been treated as most present with disseminated disease and are euthanased. However, one dog with anorexia, weight loss and diarrhoea, associated with abdominal masses due to *M. tuberculosis* infection, was treated with rifampicin, clarithromycin and enrofloxacin for 3 months, followed by indefinite treatment with rifampicin and clarithromycin, and was still well 31 months later (Engelmann *et al*., 2014).

Note

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14 Mycobacterial Infections in Elephants

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A Brief History of TB in Elephants

Tuberculosis (TB) is an ancient disease of man and animals, including elephants. TB has also been postulated to have been a factor in the extinction of the mastodon (*Mammut americanum*) during the late Pleistocene (Rothschild and Laub, 2006), as foot lesions identical to those documented in bison and considered as pathognomonic for TB were found in 59 of 113 (52%) mastodon skeletons examined (Rothschild and Laub, 2006). A disease in Asian elephants (*Elephas maximus*) resembling TB was described over 2000 years ago in the ancient Sanskrit text 'Hasthyayurveda' (Iyer, 1937). Case reports in the 19th century include that of an 18-year-old Asian bull that died of TB at Jardin des Plantes in Paris, recorded in the 1802 archives of the European Elephant Group (J. Endres, Germany, 2007, personal communication) as well as a case published in 1875 (Garrod, 1875) concerning TB in an Asian elephant at the London Zoo.

Sporadic case reports of TB in captive Asian elephants appeared in the literature in the early decades of the 20th century (Damman and Stedefeder, 1909; Thieringer, 1911; Narayanan, 1925; Baldrey, 1930 and others). More recently, TB in an African elephant (*Loxodonta africanum*) was reported (Gorovitz, 1962). An early retrospective study found only eight cases of TB-related deaths among 379 elephants in zoos in North America. *Mycobacterium tuberculosis* (*M. tb*) was isolated in three cases; acid-fast organisms were reported in one case, and four cases that occurred prior to 1941 were diagnosed based on gross examination. This study preceded routine diagnostic testing for TB and did not include privately owned or circus elephants (Mikota *et al*., 1994). The first report detailing isoniazid levels in a single elephant was published in 1983 (Devine *et al*., 1983).

Elephant TB 're-emerged' in 1996 in the USA when two travelling circus elephants died 3 days apart, resulting in media coverage and raising public concern (Binkley, 1997). A prior report of TB in a circus elephant may have foreshadowed this event as all three elephants had the same owner (Saunders, 1983). The diagnosis of five new culture-confirmed elephant TB cases the following year in different elephant herds suggested that TB was a more widespread problem than previously thought. TB has since been reported in captive elephants in Europe (Wohlsein *et al*., 2001;

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Gavier-Widen *et al*., 2002; Lewerin *et al*., 2005; Moller *et al*., 2005), Australia (Stephens *et al*., 2013) and Asia (Mikota *et al*., 2007; Abraham, 2008; Mikota *et al*., 2009; Angkawanish *et al*., 2010; Mikota and Maslow, 2011; Ong *et al*., 2013). The first case of *M. tb* in an ex-captive wild African elephant was recently found in Kenya (Obanda *et al*., 2013). *M. tb* has also been isolated from at least two wild Asian elephants in India (Zachariah, 2012).

The initial herd of elephants diagnosed with TB in 1996 in the USA, referred to as the 'Index Herd' in later reports, as well as the finding of TB among multiple other herds in the USA (and later worldwide) awakened interest in the diagnosis, pathogenesis, immunology, natural history and treatment of TB in elephants. The TB Advisory Panel, tasked initially with evaluating the diagnosis and treatment of TB within the Index Herd, merged with the National TB Working Group for Zoo and Wildlife Species which had been formed by the American Association of Zoo Veterinarians to address TB in zoo mammals. The Elephant Subcommittee of this group developed the first edition of the Guidelines for the Control of TB in Elephants in 1998; these guidelines were administered by the Animal Care division of the United States Department of Agriculture (USDA). The United States Animal Health Association currently oversees the scientific content of the guidelines (USAHA Proceedings, 2010).

Epidemiology

Mycobacterial species in elephants

To date, *M. tb* has been recognized as the causative agent in the majority of reported TB cases in elephants worldwide. One case of *M. bovis* has been reported in the USA (Greenwald *et al*., 2009) and four cases in Europe (Wohlsein *et al*., 2001; Pavlik *et al*., 2003; Moller *et al*., 2006). *M. avium* is frequently isolated from elephant trunk wash samples (Payeur *et al*., 2002) but has been associated with pathology in only one case in which disseminated disease was found (Yong *et al*., 2011). Additionally, a variety of non-tuberculous mycobacteria (NTM) have been commonly isolated from elephant trunk wash samples but very rarely with clinical relevance. Two fatal cases of NTM disease were reported in two captive African elephants in which *M. szulgai* was isolated from pulmonary granulomas (Lacasse *et al*., 2007). *M. elephantis* was isolated from a lung abscess in an elephant that died of chronic respiratory disease (Shojaei *et al*., 2000) and from ten human sputum samples and one human lymph node (Turenne *et al*., 2002). The human and elephant cases were unrelated.

USA

Between 1994 and 2013, 57 culture-confirmed cases of TB were diagnosed among a captive population of about 446 elephants in the USA for an estimated lifetime prevalence of 12.4% for both elephant species. The lifetime prevalence is the proportion of a population that at some point in their life have experienced the condition. During 1994–2011, the lifetime prevalence for Asian elephants, which account for almost all of the cases, is estimated at 16.4% (45/274) (Orloski, 2011). Tuberculosis occurs almost six times more frequently in Asian than in African elephants (Orloski, 2011), which may be due to the closer association of Asian elephants with humans during activities rather than a species predilection. Similarly, the higher number of female elephants that have died with TB may simply be due to the larger number of female elephants in USA institutions and/or a greater level of direct contact with female elephants.

Given the low TB burden among humans in the USA, the occurrence of TB in elephants is surprisingly high. Most of the elephants currently in the USA were imported during the 20th century; many elephant handlers were of foreign origin and exchange of animals (and staff) between zoo and circuses was common. Using spoligotyping and variable number tandem repeat (VNTR), 48 elephant isolates obtained between 1997 and 2010 from 33 elephants in 11 states were analysed (Higgins, 2011). Fourteen different spoligotypes were identified that were representative of common lineages such as Beijing, Euro-American and Latino-American and Mediterranean (LAM). Eleven matched spoligotypes in the CDC

database but three had no match, possibly associated with rare or novel lineages. These data would suggest that animal exposures may have occurred outside of the USA.

Europe

Tuberculosis has been found in zoo and circus elephants in Europe. In a survey of human and animal populations in six central European countries between 1990 and 1999, *M. tb* was found in an African elephant in a zoo in Poland (Pavlik *et al*., 2003). *M. bovis* was isolated from a 38-year-old female circus elephant that also had molar malocclusion and urogenital lesions (Wohlsein *et al*., 2001). An outbreak of *M. tb* that involved elephants, giraffes, rhinoceros and buffaloes occurred in a Swedish zoo between 2001 and 2003. Four different TB strains were identified in five elephants and one giraffe. All affected animals were euthanized, in accordance with the Swedish Epizootic Act (Lewerin *et al*., 2005; Moller *et al*., 2006). Subsequently, one of the Swedish elephant strains was implicated in the infection in two African elephants at another zoo (Moller *et al*., 2006).

Australia

A case of *M. tb* occurred in one of five Asian elephants imported from Thailand to an Australian zoo. Retrospective analysis showed that the affected elephant was sero-reactive on the Elephant TB Stat-Pak® and DPP® VetTB Assay for Elephants (Chembio Diagnostics Inc., Medford NY) prior to importation. A chimp housed 110 m from the elephant was affected by the same strain (Beijing). Attempts to determine the mode of transmission were unsuccessful (Stephens *et al*., 2013).

Asia

Sero-surveys together with culture in some cases have been conducted in India, Nepal, Laos, Thailand and Myanmar. In India 15.2% of elephants tested ($n = 387$) were sero-reactive on the Elephant TB Stat-Pak® test. Of these, temple elephants had the highest sero-reactivity (25.4%) followed by privately owned elephants (15%) and forest department elephants (11.6%) (Abraham, 2008). Temple elephants have the greatest human contact and forest elephants the least so these results are not surprising.

In Nepal, a study was undertaken in 2006 to compare four serological techniques (a six-antigen ELISA, a Rapid Test later licensed as the Elephant TB Stat-Pak®, the MAPIA and an immunoblot assay). Trunk wash samples were processed in Nepal and in the USA. Eighteen of 115 elephants reacted on one or more of the serological tests. No *M. tb* complex organisms were isolated on culture by trunk wash (Mikota *et al*., 2007). However, a TB surveillance programme was established and six culture-confirmed cases of *M. tb* were subsequently identified among sero-reactive elephants.

In a separate study, three *M. tb* isolates from elephants in Nepal were found to belong to a specific lineage of Indo-Oceanic clade, EAI5SIT138, based on spoligotyping, TbD1 detection and multi-locus variable number of tandem repeat (MLVA) analysis. One of the elephant isolates also had a new synonymous single nucleotide polymorphism (SNP) T231C in the *gyrA* sequence and the same SNP was also found in human isolates in Nepal. The MLVA results and movement history of two of the elephants suggested that they may have been infected from a common source (Paudel *et al*., 2014).

In Thailand, four Asian elephants were diagnosed with *M. tb* infection. Two isolates were from trunk wash samples and two were from tissues collected post mortem. Genotyping demonstrated that the four isolates were from different sources (Angkawanish *et al*., 2010).

In a preliminary study conducted in Myanmar, 6 of 84 elephants reacted on the ElephantTB STAT-PAK® assay but were non-reactive on the DPP® VetTB Assay for Elephants (U Mar *et al*., 2012).

Elephant-to-elephant transmission

Elephant-to-elephant transmission of *M. tb* is supported by the information provided through outbreak analysis of infected herds.

To date, the Index Herd ('Herd A' from Mikota *et al*., 2001), has an attack rate of approximately 50%. Genetic analysis of infecting strains from this herd demonstrate that they represented the same strain by IS6100 typing, or differed by only one or two bands (Michalak *et al*., 1998; Mikota *et al*., 2001), consistent with clonal spread. An elephant trainer at the same facility was diagnosed with active TB with an organism that matched those cultured from the elephants; outbreak analysis considered this to be more consistent with elephant-to-human transmission than human-to-elephant transmission (Michalak *et al*., 1999). Moreover, individual animals from this herd moved to different facilities a few years later subsequently developed active disease with the same strain of *M. tb*; thus, infection prior to relocation is likely.

Herds B, C and D (Mikota *et al*., 2001) provide additional compelling evidence of direct elephant-to-elephant transmission of infection. The animals from these three herds (based at the Los Angeles Zoo, the San Francisco Zoo and a private facility in California) had been housed together in the past (Mikota *et al*., 2001). Genetic analysis showed that the animals from herds B, C and D were infected with a common strain of *M. tb*, and that this strain differed from those identified in the animals from other herds in the same monograph (Mikota *et al*., 2001). There were no documented cases of human TB known at the zoos and private facility that housed the three herds, and none of the trainers and other staff were known to have worked at more than one facility.

Similarly, animals in Herd F (Mikota *et al*., 2001) were found to be infected with a single strain of *M. tb* that differed genetically from the strains documented in the other herds (Mikota *et al*., 2001). No human cases of active TB were known among staff caring for Herd F (Mikota *et al*., 2001), again suggesting elephant-to-elephant transmission as the most likely route of infection.

Finally, Lewerin and colleagues described an outbreak of *M. tb* among elephants at the Kolmarden Zoo, Sweden (Lewerin *et al*., 2005). Some of the affected elephants produced identical strains. No active TB was diagnosed in zoo staff, although there were cases of latent infection detected during the outbreak investigation (Lewerin *et al*., 2005). Thus, transmission most likely occurred between the animals directly, rather than via a human vector.

Threat to wild populations

The potential for TB to be introduced to wild elephant populations is of great concern for elephant conservation (Mikota, 2008b; Mikota *et al*., 2009; Mikota and Maslow, 2011). In Asia, habitat encroachment has resulted in fragmented elephant populations that might not be able to survive long term if faced with a chronic, insidious disease such as TB. TB due to *M. bovis* has taken its toll in Kruger National Park, first affecting African buffalo and spreading from southern to northern herds over a 20-year period and spilling over into ten other mammalian species (Michel *et al*., 2006). Such a scenario might have an even greater impact on small fragmented elephant populations that might not survive long term.

Other species are also at risk when elephants are used for tourism. In Nepal, for example, elephants transport tourists into protected areas and into close proximity (within spraying distance) of endangered rhinoceros. Successful captive breeding programmes of elephants often depend on captive cows breeding with wild bulls, providing another venue for TB transmission to the wild.

Although only *M. tb* has thus far been isolated from elephants in Asia, infection with *M. bovis* is possible especially where elephants intermingle with domestic livestock for which TB control programmes are generally non-existent and TB status unknown. In critical conservation areas it is essential that TB be controlled at the captive–wild interface. Treatment in the wild would not be feasible.

In some areas, captive elephants are critical to conservation efforts and are used for patrols, anti-poaching operations and scientific research. Rhinoceros censuses in Nepal can only be conducted on elephant back. TB was found in 7 of 15 government elephants that died between 2006 and 2013 (44%). Considering that these elephants were used for breeding or patrol and most were in their

prime, these losses were significant. The time investment to raise and train an elephant for patrol or census work (or scientific studies, for which the elephants are also used) is great.

African elephants do not interface with humans as frequently as Asian elephants. The number of African elephants in captivity in Africa is small compared to the number of Asian elephants in captivity in Asia. Nonetheless the first reported case of *M. tb* in a free-ranging elephant occurred in an ex-captive African elephant in Kenya (Obanda *et al*., 2013).

Two cases of *M. tb* in free-ranging elephants have also been identified in India (Zachariah, 2012), thus demonstrating that transmission to the wild is feasible.

Pathology

Gross pathology

No systematic review of elephant TB lesions has been undertaken and the following has been garnered from the literature as well as the authors' experience and that of pathologist colleagues (Mikota *et al*., 2000; Montali *et al*., 2001; Lewerin *et al*., 2005; Mikota, 2008a; Obanda *et al*., 2013 and others). Although most of our experience has been with Asian elephants, tuberculous lesions in a reported case in an ex-captive African elephant were similar (Obanda *et al*., 2013).

Lesions are mainly found in the lungs and thoracic lymph nodes. They may also be seen in the trachea, retropharyngeal, cervical, axillary and other lymph nodes, or in any organ, including bone and urogenital tract, if the disease is disseminated. In asymptomatic elephants that have died from other causes, tuberculous lesions detected at post mortem have been small and isolated, whereas animals with progressive wasting illnesses have had more extensive lesions with obliteration of normal lung or lymph node architecture. Pulmonary involvement can be extensive, involving 40% or more of the lung tissue even in the absence of clinical respiratory signs (Wohlsein *et al*., 2001). However, it can also be quite localized, necessitating thorough sectioning of all lung fields.

Lesions may be miliary, less than 1 cm in diameter, or there may be large confluent areas of consolidation tens of centimetres across. Small lesions tend to be white to tan, solid, firm and granular, while larger lesions vary from solid, yellow to chalky white, waxy or 'lardaceous', friable, caseocalcareous, to completely mineralized and/or cavitated (Plates 9 and 10). When bronchioles and bronchi are involved, the associated exudate may be surprisingly mucoid or suppurative even in the absence of opportunistic secondary pyogenic bacterial infections (Plate 11). Extension to the pleura and fibrous adhesions to the pericardium have been reported.

Involved lymph nodes are usually enlarged and contain multifocal, nodular to confluent, foci of granulomatous inflammation with an appearance similar to lung lesions ranging from solid to caseous or mineralized and sometimes containing thick yellow-green exudate. In chronic cases, or those that have undergone treatment, there is often regionally extensive fibrosis of the affected areas of lung or lymph node in addition to fibrous encapsulation of granulomas (Plate 12). Tongue ulcerations have been noted in some elephants with TB and may be related or may be due to secondary infections. In one case, *M. tb* was isolated from raised superficial skin lesions on the trunk. It should be noted that, while the above lesions are strongly suggestive of *M. tb* infection, similar lesions have been seen in elephants with NTM infections (Lacasse *et al*., 2007; Yong *et al*., 2011), and with other infectious agents such as *Corynebacterium* spp. and fungi (Lowenstine, unpublished). Saponification and mineralization of fat associated with visceral lymph nodes can also mimic the caseocalcareous granulomas of tuberculosis, but can be differentiated histologically.

Histopathology

On histopathologic examination, TB classically is a granulomatous interstitial or bronchopneumonia. The granulomas usually consist of a centre of caseous necrosis or suppuration surrounded by a thick rim of activated macrophages admixed with neutrophils, and lesser numbers of multinucleated giant cells (Plate 13).

Multinucleated giant cells, particularly classical Langerhans type giant cells, however, are generally infrequent. Dystrophic mineralization (caseocalcareous type of exudate) is variable and most evident in more chronic lesions. Encapsulation by fibrous connective tissue is often present accompanied by varying numbers of lymphocytes and plasma cells within the capsule and at the periphery (Plate 14). Chronic fibrous nodules with mineralized centres and few or no macrophages characterize 'burned out' lesions. In affected areas of lung, oedema and fibrosis of interlobular septa are often prominent and may extend to obliterate normal architecture. Adjacent alveoli are frequently filled with protein-rich oedema and alveolar macrophages. Necrosis of bronchial and bronchiolar epithelium accompanied by histiocytic or mixed histocytic and neutrophilic inflammation is another manifestation (Plate 15). Acid-fast bacilli (AFB) are typically rare and if bacteria are seen there may be as few as three to five organisms per oil immersion field (Terrell, 2011). According to Montali *et al*. (2001), AFB are more frequent in the centres of pulmonary granulomas than in the lymph node lesions. It should be noted that AFB are also rarely, if ever, encountered on impression smears of the lesions, even those from which mycobacteria can be demonstrated by culture or PCR. Thus smears taken during the post-mortem examination to confirm or rule out *M. tb* infection are not reliable. The use of immunohistochemistry for BCG (Bacille de Calmette et Guérin) has also been useful in identifying rare bacilli in lesions (Bonenberger *et al*., 2001; Szeredi *et al*., 2008).

Post-mortem approach and precautions

Conducting an elephant post-mortem examination can be a daunting task. It is highly recommended that facilities holding elephants have a plan and equipment in place including a list of staff or consultants who will participate. Preparation at the last minute can delay the start of the necropsy and may compromise a diagnosis if tissue decomposition is advanced. Packing the body in ice has been used successfully to delay post-mortem deterioration while equipment and personnel are mobilized, which can often be 24 h or longer.

Readers anticipating an elephant necropsy are encouraged to consult McManamon and Terrell (2011) and Montali (2006) for step-by step post-mortem preparation and procedure instructions including special precautions to protect personnel if TB is suspected.

The Elephant Taxon Advisory Group/Species Survival Plan of the American Zoo and Aquarium Association maintains an Elephant Research and Necropsy Protocol. The current version can be accessed online at www.aazv.org (for members of the American Association of Zoo Veterinarians) or at www.elephantcare.org. Post-mortem field procedures for African elephants are also available online (www.elephantcare.org/protodoc_files/afrnecro.pdf).

Human PPD conversion has been associated with attending post-mortem procedures in elephants (Oh *et al*., 2002) and also rhinos (Dalovisio *et al*., 1992). To minimize human exposure, it is strongly advised to begin the examination with the limbs and abdominal viscera, leaving the removal and examination of thoracic viscera until last (Montali, 2006). The lungs of the elephant can then be approached through the diaphragm and assessed by palpation. If granulomas are palpated, samples can be taken without fully opening the chest and removing the thoracic organs (the pluck) en bloc (Montali, 2006). Since access to the anterior aspects of the lungs can be difficult with this approach and small lesions can be missed on palpation, it is advisable to conduct elephant post-mortem examinations with appropriate respiratory protection as described below. In cases in which carcass disposition requires cutting into the thorax, the pluck (heart, liver, lungs and trachea) may have to be removed and should be moved to a tarp or ground cover for examination to contain potentially infectious material. Additionally, enlarged retropharyngeal, cervical and axillary lymph nodes, which might be encountered during initial incisions for reflecting and removing the limbs, are possible indicators of mycobacterial infection and should trigger immediate enforcement of the use of personal protective equipment (PPE) regardless of whether or not pulmonary lesions have been palpated.

PPE including impermeable gowns and aprons, gloves and, most importantly, respiratory

protection are recommended for all elephant post-mortem examinations and are mandatory if the elephant is known or suspected to be TB-infected. Surgical masks do not protect against TB. National Institute for Occupational Safety and Health- (NIOSH-) rated N-95, N-99 and N-100 respirator type masks, cartridge respirators with appropriate particulate filters or powered air-purifying particulate respirators (PAPRs) should be used.

Host Response

There is a paucity of information on the elephant immune system (Lowenstine, 2006). A quantitative, real-time RT-PCR assay for Asian elephants was developed (Landolfi, 2009) and used to compare baseline cytokine mRNA expression in Asian elephants sero-positive or sero-negative on the Elephant TB Stat-Pak® (Chembio Diagnostics, Inc., Medford, New York, USA). Results suggested increased expression of tumour necrosis factor-alpha (TNF-α) and decreased expression of transforming growth factor-beta (TGF-β) in sero-positive elephants. Although not statistically significant, the sero-positive elephants also demonstrated trends toward increased expression of IFNγ and IL-4 and decreased expression of IL-10 and IL-12. IL-2 levels were below the level of detection of the assay (Landolfi, 2010). It was expected that elephants would have cytokine profiles indicative of T_H1/T_H2 imbalance, specifically with decreased T_H1 and increased T_H2 cytokine levels, comparable to humans with active TB. However, significant differences between sero-negative and sero-positive elephants were not identified and the higher T_H1 cytokine (TNF-α, and IFNγ) expression in sero-positive versus sero-negative elephants was contrary to what has been observed in many human studies. Disease stage, which was unknown in elephant cases due to the subclinical nature of this infection, was likely to have impacted results. Characteristic cytokine profiles with T_H1/T_H2 imbalance described in human studies are associated with clinical disease. Parallels to human TB immunopathogenesis are difficult to extrapolate to elephants with subclinical disease. In a subsequent study more closely emulating methodology utilized in human studies, proliferative responses and cytokine mRNA expression were measured in peripheral blood mononuclear cell (PBMC) cultures from TB culture positive/sero-positive, and negative Asian elephants following stimulation with mycobacterial antigens. Results showed that samples from positive elephants had greater proliferation and increased expression of TNF-α, IL-12 and possibly IFNγ in response to stimulation with mycobacterial antigen. These findings indicated that differences in host immune cell function do exist between TB positive and negative elephants, and that measurement of immune cell proliferation and cytokine expression following mycobacterial antigen stimulation could serve as useful diagnostic markers (J. Landolfi, Salt Lake City, 2013, personal communication). Since this chapter was written an excellent review of the lesions and local immune responses in pulmonary tuberculosis in Asian elephants has been published by Landolfi *et al*. (2015).

Diagnosis

The diagnosis of TB in elephants can be challenging. Elephants are the largest land mammals and their size imposes limitations on the diagnostic techniques that can be employed. Chest radiographs are feasible only in very young elephants. The efficacy of the trunk wash, described below, is compromised by a variety of factors not the least of which is the challenge of attempting to obtain a diagnostic lung sample for culture through a 2-m long proboscis. Indirect serological tests have performed remarkably well in elephants and are currently the most reliable tool for early diagnosis in elephants although culture confirmation of disease has limited the validation of some methodologies.

Clinical presentation

In many, if not most, elephant cases, clinical signs of TB are minimal or absent. When present, signs often indicate advanced disease

and may include weight loss, anorexia, coughing, respiratory difficulty and trunk discharge. Exercise intolerance may be seen in working elephants. Ulcerations on the tongue have been observed in a few elephants with other overt clinical signs. In one case *M. tb* was isolated from a small skin nodule on the trunk (Mikota, unpublished) and in another case from a vulvar discharge (Dumonceaux *et al*., 2011).

Intradermal tuberculin test

The intradermal tuberculin test demonstrated poor sensitivity (16.7%) and poor specificity (74.2%) in four infected herds in the USA (Mikota *et al*., 2001). Unreliable skin-test results were also observed in an outbreak in a Swedish zoo (Gavier-Widen *et al*., 2002; Lewerin *et al*., 2005; Moller *et al*., 2005). Intradermal tuberculin testing is not recommended for elephants. Importantly, there was no histologic differentiation of tuberculin-injected sites from saline-injected sites (S. Mikota, unpublished data).

Culture

Although isolation of the organism is the gold standard to diagnose TB, culture has inherent limitations as a primary diagnostic technique in elephants.

The 'trunk wash' has evolved as the preferred method to obtain an elephant respiratory sample for culture (Isaza and Ketz, 1999). The elephant must first be trained to permit handling of the trunk. The process involves instillation of 60 ml of sterile water or saline into the trunk. The trunk is elevated then lowered and the tip directed into a zippered 1- or 2-gallon plastic bag that serves as the collection device. The elephant is asked to forcibly exhale to encourage obtaining a sample from the distal respiratory tract. Not all elephants comply, so sample quality and source are often questionable. This procedure has been particularly problematic in Asia where a modified technique has been developed (Abraham and Davis, 2008).

Elephants use their trunks for a variety of functions and contamination and overgrowth of mycobacterial cultures with mycobacteria other than *M. tb* and/or moulds, both ubiquitous in the environment, commonly occur and compromise results. Some laboratories have developed additional decontamination techniques to address this problem. The Guidelines for the Control of Tuberculosis in Elephants includes the procedures used at the National Veterinary Services Laboratories in the USA. Trunk washes from elephants are collected as three serial samples over a period of 3–7 days, based on human TB diagnosis by sputum smear examination, since shedding of *M. tb* may vary day to day.

Importantly, elephants also are known to shed intermittently over time. In an outbreak among elephants in a Swedish zoo, only seven of 189 trunk wash samples sequentially collected from five elephants diagnosed with TB were culture positive (Moller *et al*., 2005).

Perhaps a more instructive case is of an elephant highly suspected with active TB which was cultured over a period of 17 months prior to treatment initiation. Samples were collected at least monthly and in some months cultures were collected daily (Mikota *et al*., unpublished). *M. tb* was first isolated during the 2nd month of collection. For month 2 through 6 of sample collection, while at least one sample each month yielded *M. tb*, there were individual weeks for which samples were culture negative. It was common during this time that only one or two serial samples would be positive out of groups of three samples. However, for the following 6 months, there was a period of 2 months before the next positive culture followed by a period of 4 months of negative cultures, again ending with *M. tb* shedding. These examples highlight the limitations of culture to detect infected animals as a tool for infection control measures.

Microscopic examination of specimens for acid-fast bacilli (AFB)

Smears of elephant trunk wash samples that yield AFB provide only a presumptive diagnosis of TB as NTM, and organisms such as *Nocardia* spp. are also acid fast. Conversely, direct examination has been very insensitive –

a finding also common in human diagnosis. NTM are commonly isolated from elephants. In one study, 19 trunk wash samples collected from four healthy elephants yielded *M. avium* or NTM including *M. fortuitum*, *M. terrae*, *M. intracellulare* and others (Ball *et al*., 2006).

Serology

Recent advances in understanding the humoral immune response to TB in a wide range of wildlife species including elephants have led to the development of commercial serodiagnostic tests based on innovative immunoassay technologies that can be applied to multiple hosts (Lyashchenko *et al*., 2006; Greenwald *et al*., 2009; Boadella *et al*., 2011; Lyashchenko *et al*., 2011, 2013; Waters *et al*., 2011; Miller *et al*., 2012). The accuracy of antibody tests varies considerably by species (Lyashchenko *et al*., 2008), remarkably, approaching 100% sensitivity and >95% specificity for elephants with use of the ElephantTB STAT-PAK® or Dual Path Platform (DPP) VetTB® assays (Chembio Diagnostics Inc., Medford, New York) or multiantigen print immunoassay (MAPIA) (Greenwald *et al*., 2009; Lyashchenko *et al*., 2012). Antibody responses to *M. tuberculosis* in elephants are relatively strong, particularly during advanced stages of disease (Lyashchenko *et al*., 2006, 2012; Greenwald *et al*., 2009). Elephants appear to be unique among natural host species in developing unusually robust antibody responses to *M. tuberculosis* (Greenwald *et al*., 2009). The serodiagnostic sensitivity for elephants is significantly higher than that found for any other mammalian host (Lyashchenko *et al*., 2008, 2012; Greenwald *et al*., 2009). The reasons for such a robust antibody response in elephant TB remain unknown. One hypothesis is that this may be due to the high level of monocytes in circulation (Greenwald *et al*., 2009).

Serodiagnostic potential for elephant TB detection was first demonstrated by an in-house ELISA using various antigenic preparations from *M. tb* and *M. bovis* (culture filtrates, protein purified derivatives, lipoarabinomannan, MPB70 protein) (Larsen *et al*., 2000). In that study, all seven elephants with culture-confirmed TB showed the presence of circulating antibodies, whereas 95% (38/40) of control elephants were sero-negative. Specific antigens predominantly involved in the elephant immune response have been identified by MAPIA (Lyashchenko *et al*., 2006; Greenwald *et al*., 2009) and included ESAT-6, CFP10 and MPB83 proteins. These antigens are employed in the commercial rapid tests (ElephantTB STAT-PAK® and DPP VetTB®) used in conjunction with a confirmatory MAPIA (Lyashchenko *et al*., 2012), in accordance with the Guidelines for the Control of Tuberculosis in Elephants. To date, out of 44 elephants diagnosed with TB in six different countries (Australia, Germany, France, Nepal, Sweden and USA) in the last decade and tested by MAPIA and/or DPP VetTB®, 43 recognized ESAT-6 and/or CFP10 proteins with or without additional antigens, whereas one elephant reacted to MPB83 protein only (Greenwald *et al*., 2009; Lyashchenko *et al*., 2012; unpublished observations). Interestingly, MPB83 sero-reactivity has been also demonstrated in elephants with fatal mycobacteriosis caused by *M. szulgai* (Lacasse *et al*., 2007). Two additional proteins, Rv0978c and Rv0754, were recently evaluated in Asian elephants by ELISA in India (Verma-Kumar *et al*., 2012). That study used ElephantTB STAT-PAK® Assay as a reference test and utilized a latent class analysis for data interpretation. Unfortunately, the serology findings could not be supported by the gold standard methods (e.g. culture) to determine the true infection status.

Positive predictive value of the antibody tests for elephant TB has been recently validated using a longitudinal design when the true infection status was unknown (Lyashchenko *et al*., 2012). A group of 14 elephants from 11 facilities in five countries were identified as ElephantTB STAT-PAK®-reactive on annual surveillance. The presence of *M. tb*-specific antibodies was confirmed for all of them by MAPIA and DPP VetTB® Assay. At the time of serological testing the elephants were trunk wash culture negative, but all became culture positive in the following months or years, with some animals diagnosed post mortem. This prospective study confirmed the high sensitivity of the antibody tests for elephant TB which was previously demonstrated predominantly by retrospective findings (Larsen *et al*., 2000; Lyashchenko *et al*., 2006; Greenwald *et al*., 2009).

Elephants infected with *M. tb* develop seroconversions months to years prior to culture-based diagnosis (Greenwald *et al*., 2009). Three of the four Asian elephants diagnosed with TB in Thailand produced antibody responses detectable by the ElephantTB STAT-PAK® Assay 10–32 months before isolation of *M. tb* (Angkawanish *et al*., 2010). The antibody levels measured by MAPIA declined following a favourable outcome with antibiotic therapy but they increased shortly before disease recrudescence (Lyashchenko *et al*., 2006, 2012). Similar effects of antitubercular therapy on human immune responses have been described (Bothamley, 1995). The elephant findings demonstrate the potential for real-time serologic monitoring of response to anti-TB therapy as well as disease staging and/or relapse.

Despite the high diagnostic accuracy, antibody tests for elephant TB are unlikely to replace culture methods, as isolation of the pathogen from infected animals will always be necessary to confirm the diagnosis, identify the strain, determine drug susceptibility and collect epidemiology data. However, due to the low cost, relative simplicity and operational advantages of antibody detection methods, management and control of TB in captive elephants and non-domestic species may benefit from rapid antibody assays. This is especially true in range countries where controlling TB at the captive–wild interface is imperative. In addition to serology, background information and medical history – including past exposure and treatments – should be considered. An increased frequency of trunk wash culture testing for antibody-positive elephants may significantly improve confirmation of TB diagnosis (Lyashchenko *et al*., 2012).

Interferon gamma (IFNγ) release assays

In vitro detection of IFNγ release in response to exposure of peripheral mononuclear cells to certain mycobacterial antigens has proven to be highly sensitive and specific for cattle and humans; however, these tests have not demonstrated diagnostic efficacy in other host species.

There is currently no commercially available IFNγ test for elephants. Asian elephant IFNγ has been characterized (Sreekumar *et al*., 2007). The African elephant IFNγ gene has been sequenced, the cytokine was expressed and purified, and a monoclonal antibody produced (Morar *et al*., 2005, 2007). In a recent pilot study, an attempt to design an IFNγ assay was made using blood cells from one *M. tb*-infected elephant confirmed by culture, one suspect and a group of non-infected elephants (Angkawanish *et al*., 2013). However, this development awaits validation in large populations of Asian and African elephants with well-defined infection status.

Nucleic acid amplification techniques (NAAT)

Direct detection of *M. tb* nucleic acids from clinical samples have been attempted in animals; however, NAAT has not been validated for elephants. One study evaluated elephant samples using the Gen-Probe Amplified *M. tuberculosis* Direct Test (MTD; Gen-Probe, San Diego, CA 92121, USA). The MTD was positive in 14 elephants from which *M. tb* or *M. bovis* was cultured, positive in 15 elephants for which TB was not confirmed and negative in six culture-positive elephants (Payeur *et al*., 2002). The presence of non-viable organisms or a low-level infection with shedding below the detection capabilities of culture (100 organisms/ml) was postulated to explain a positive MTD with a negative culture result. Improper specimen collection or transport, variability in sampling, procedural errors, inhibitors, sample misidentification or transcriptional errors could account for the negative result among the six culture-positive, MTD-negative elephants. To allow for epidemiologic tracing and genetic comparison of isolates, as well as the ability to perform susceptibility on infecting isolates, NAAT should be performed in conjunction with culture.

Kay and colleagues (2011) estimated the analytical sensitivity and specificity of three DNA extraction methods to detect *M. tb* complex organisms in trunk wash samples. A ZR Soil Microbe DNA MiniPrep™ (Zymo Research Corporation, Irvine, CA) and a salt and ethanol precipitation (TSEP) technique were evaluated under heat treatment, phenol treatment and contamination with *M. avium*. A column filtration method was tested with soil contaminated samples. Samples were spiked with varying

concentrations of *M. bovis* cells prior to DNA extraction and extracted DNA was amplified using IS6110-targeted PCR analysis. Detection of *M. bovis* for samples prepared using the ZR method was as low as one to five bacteria per 1.5 ml of trunk wash and ten bacteria using the TSEP method under the three conditions. The column filtration method detected as low as 5–50 *M. bovis* cells per 1.5 ml of trunk wash.

A highly sensitive gyrB-based PCR-RFLP assay developed in Nepal successfully differentiated *M. tb*, *M. bovis* and *M. avium* in spiked samples of elephant trunk washes. Subsequent evaluation of 22 TB-suspect elephants did not detect any culture positive animals (Smiley-Wilson *et al*., 2007). The assay was further refined and later used to successfully diagnose *M. tuberculosis* in elephant trunk wash and nasal drip samples (Miller *et al*., 2013).

Compared to culture, NAAT could theoretically provide a more rapid and potentially more sensitive diagnostic technique to diagnose TB in elephants but further research is needed.

Clinical pathology

Clinical pathology associated with TB in elephants has not been widely studied. Culture-positive elephants (n = 5) had significantly lower albumin–globulin ratios (A:G), mean cell haemoglobin concentration and glucose values during active shedding and significantly higher platelets, band neutrophils, eosinophils, calcium and bicarbonate (TCO_2) compared to 20 healthy elephants. Infected elephants had lower A:G ratios associated with a positive trunk wash compared to their baseline values when trunk washes were negative (Harr *et al*., 2001). Low albumin is associated with a poor prognosis in human TB patients (Matos and Moreira Lemos, 2006; Kim *et al*., 2007) and should be evaluated further in elephants.

Acute-phase proteins

As an infectious process, there has been interest in determining whether measurement of acute-phase proteins can assist in either the diagnosis or response to treatment of TB. Human studies have shown that, in general, pulmonary TB is associated with significantly higher levels of C-reactive protein (CRP) and serum amyloid antigen (SAA) whereas serum amyloid protein (SAP), beta-2 microglobulin, ceruloplasmin, α1-acid glycoprotein and transferrin levels were non-predictive (Immanuel *et al*., 1990; Furuhashi *et al*., 2012) and confirmed in more recent studies (Wilson *et al*., 2006). Moreover, treatment has been associated with normalization of the CRP and SAA elevations (Immanuel *et al*., 1990; Wilson *et al*., 2006). While peritoneal and meningeal infection have also been associated with elevated CRP and SAA, differences from uninfected control subjects were not statistically significant (Immanuel *et al*., 1990). Acute-phase proteins have also been demonstrated as having utility in detecting underlying systemic inflammatory processes in animals. In clinical applications, they have been used for screening and prognostication (Cray, 2011). In Asian elephants, threefold-higher levels of SAA were observed during periods of EEHV viraemia (Stanton *et al*., 2013); anecdotal data also support elevations with other infectious and inflammatory stimuli. Unpublished data from a small sample set from one facility found little correlation between mycobacterial infection status and either CRP or SAA levels (Mikota, unpublished data).

Treatment

In the USA, The Guidelines to Control Tuberculosis in Elephants should be consulted for current treatment recommendations (USAHA, 2010.

When TB re-emerged as a problem for captive elephants there was no precedent for treatment in elephants or any other non-human mammalian species. Treatment protocols were therefore based on regimens known to be effective in humans. As a margin of safety, the recommended length of treatment for elephants was 1 year rather than 6 months. Three drugs for 2 months (60 full doses) followed by two drugs for 10 months (300 full doses) composed the treatment regimen, with drug selection ideally based on sensitivity data.

In pan-sensitive cases, isoniazid, rifampin, ethambutol and pyrazinamide have been used. Drug-resistant cases have occurred (Dumonceaux

et al., 2011) necessitating the use of second-line drugs such as ethionimide, ciprofloxacin, enrofloxacin or amikacin.

Oral therapy has proved challenging as elephants often reject bitter-tasting medications. Rectal administration, while labour intensive, has been implemented in the majority of cases.

Pharmacokinetic (PK) analyses of isoniazid (Maslow *et al*., 2005a), ethambutol (Maslow *et al*., 2005b), rifampin (Peloquin *et al*., 2006), and pyrazinamide (Zhu *et al*., 2005) in elephants have guided dosage recommendations. To date, adequate serum drug levels have been achieved with isoniazid, pyrazinamide and ethambutol, but not rifampin. Also, more recent results have suggested that some drugs such as PZA and INH may be more rapidly absorbed via the rectal mucosa than was assumed in the original PK studies (Mikota, unpublished). Therapeutic drug monitoring is recommended during treatment.

Whether serum drug levels known to achieve cure in humans will also achieve cure in elephants is unknown. There is a paucity of information from treated elephants that have been examined post mortem. In some cases the disease appeared to be cured; in others, active lesions were present. Treatment protocols often differed, making comparisons difficult. Several treatment failures have been associated with two-drug regimens; initial treatment with three or four drugs is prudent.

Clinical signs, if present, may improve with treatment; however, there is currently no test to confirm cure. Declining MAPIA reactivity, however, is thought to indicate a response to therapy (Lyashchenko *et al*., 2006).

Elephants may experience TB drug-related side effects (Dumonceaux and Mikota, 2006). Inappetence or anorexia are common and are typically managed by a short drug holiday. Lethargy, pica and anaemia have been reported (Mikota *et al*., 2001). Muscle weakness, ataxia and peripheral neuropathy have also been seen. Some elephants experience excessive epiphora associated with pyrazinamide.

Elevations in aspartate aminotransferase (AST), total bilirubin, gamma glutamyltransferase (GGT), bile acids and lactate dehydrogenase (LDH) have been associated with the administration of TB drugs with a return to baseline after drug withdrawal (Mikota *et al*., 2001; Dumonceaux *et al*., 2011).

Zoonotic Concerns

TB due to *M. tb* as a zoonotic disease with transmission from elephants to humans has been reviewed elsewhere (Maslow, 2006). As a general rule, baseline data of TB status by pre-employment PPD or interferon-gamma release assays have been lacking for most animal keepers, and this has limited epidemiologic investigation into suspected zoonotic transmission of infection. Routine assessment of pre-employment and re-testing on a yearly basis has been adopted at most zoos but is less than universal among other facilities that maintain elephants.

One of the earliest described cases with suspected transmission of TB between elephant and human involved Hazel, a 35-year-old Asian circus elephant that was evaluated at the Audubon Zoo in New Orleans due to illness (Gutter, 1981). Despite attempts at treatment the animal died; at necropsy, there was extensive granulomatous disease of the lungs, liver and spleen with AFB found on direct smear. Culture yielded pan-susceptible *M. tb*. Epidemiologic evaluation noted that one of the elephant trainers was diagnosed with cavitary TB (Greenberg *et al*., 1981); however, subsequent phage typing of the human and elephant isolates showed these to represent distinct strains (Jones and Good, 1982), precluding transmission between elephant and human.

As part of the epidemiologic workup of Herd A (Mikota *et al*., 2001), the Illinois Department of Public Health evaluated each of the staff members at the facility in late summer 1996 and again at 3 months (Michalak *et al*., 1998). Of the 22 individuals evaluated, eight were found to be PPD positive at baseline, 11 were PPD negative at baseline and remained negative when retested at 3 months, and three individuals were negative at baseline but converted to PPD positive at 3 months. Thus, transmission was documented for three individuals. Of the eight PPD individuals positive at baseline testing, most were foreign born

and none had prior testing, so infection prior to work at the facility could not be ruled out. And, while elephant-to-human transmission was considered as most likely for the three PPD skin-test converters, human-to-human transmission could not be precluded as there was one documented case of active TB in a facility worker who had been found to be infected with the same *M. tb* strain as detected in the elephants at the facility (Michalak *et al*., 1998).

Oh and colleagues described potential cases of transmission from elephants to humans and other animals at the Los Angeles Zoo (Oh *et al*., 2002). In 1997 an elephant died of salmonellosis and was found to have granulomatous lung disease at necropsy, culture of which yielded *M. tb*. Evaluation of the staff at the zoo was conducted 3 years later of whom 55 of 310 individuals were found to be PPD positive. Univariate analysis found that risk factors associated with a positive skin test included presence at the elephant necropsy, employment as a groundskeeper, construction workers and male employees. Although the authors postulated causality between a positive skin test and exposure to the elephant or other *M. tb*-infected animals at the zoo, the lack of baseline pre-employment TB screening makes such an assertion impossible to prove.

The most rigorous zoonotic risk investigation was performed in 2009 at an elephant sanctuary that had accepted elephants known to be infected with *M. tb* (Murphree *et al*., 2011). Baseline TB screening in 2006, immediately prior to the transfer of infected animals, and subsequent yearly evaluations were performed on all employees. Of 46 employees tested, 9 demonstrated PPD conversion from negative to positive while working at the facility. While foreign-born workers, those with foreign travel, and those who had previously worked at a health care facility had higher rates of skin-test positive reactions, the only statistically significant association with PPD conversion was work in the quarantine area during the time when a culture positive animal was on premises. In contrast, compliance with N95 respirator usage was protective against skin-test conversion. Transmission of TB among elephants and between elephant and humans was presumed to occur via respiratory droplets and exposure to aerosolized *M. tb* from high-pressure barn washing was considered to be the most significant factor for transmission (Murphree *et al*., 2011).

In some zoo cases involving elephants and other species housed remotely, a mechanism of transmission could not be determined (Oh *et al*., 2002; Stephens *et al*., 2013). Elephants are very social and use their trunks for greeting, one elephant often placing its trunk in the mouth of another. The capacity of the trunk is about 8 l and they can collect and spray water and respiratory secretions a considerable distance. There may be yet undiscovered factors in the transmission of TB from elephants and the possible role of fomites requires further investigation.

Future Research

Our understanding of TB in elephants and how to effectively manage if not cure this disease has only just begun. Our understanding of the elephant's immune system is limited and clearly studies in this area are needed to fully understand the pathophysiology of this disease in elephants. Additional diagnostic tests to definitively identify infections and biomarkers to track the course of disease and response to treatment would be helpful. We lack a test to confirm cure in live elephants.

Pharmacokinetic studies have been published for only four drugs and these were retrospective evaluations of pooled data. These studies should be repeated using larger samples and more time points. Studies of second-line TB drugs are also warranted.

Much knowledge would be gained by performing comprehensive post-mortem examinations of all elephants that have been suspected or diagnosed with and/or treated for TB.

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15 Mycobacterial Infections in Other Zoo Animals

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Introduction

Mycobacterial infections are historical and ongoing concerns in zoological collections worldwide. Due to the chronic nature of mycobacterial disease, individuals and populations can be affected for months to years, sometimes without detection. With the diversity of animal species in zoological collections, mycobacterial infections present diagnostic, epidemiological and other potential challenges for veterinarians, zoological managers, public health and regulatory officials. Tuberculosis (TB) has been recorded as a cause of morbidity and mortality in zoo animals over the last century. This chapter will highlight the information available regarding these infections in zoological collections. Mycobacterial infections reported in zoological taxa are listed in Table 15.1.

The host response to mycobacterial infection is dependent on a number of factors including genetic susceptibility (individual and species related), immune status, infectious dose, virulence of organism and additional confounders such as environment and co-infections (Davies and Grange, 2001). These factors can complicate the understanding of immunopathogenesis, available diagnostics and epidemiology of infection in a variety of susceptible zoo animal species.

Non-human Primates

Old World primates appear to be more susceptible to *M. tuberculosis* and *M. bovis* infection than New World primates (Montali *et al*., 2001). Outbreaks of disease in non-human Asian primates, such as rhesus macaques (*Macaca mulatta*), tend to be rapidly progressive in contrast to African monkeys and great apes which tend to have chronic disease (Baskin, 2014). Typical clinical signs include weight loss, lethargy, weakness, anorexia and respiratory signs such as coughing (Stetter *et al*., 1995; Montali *et al*., 2001). Some infected individuals may be asymptomatic until advanced stages of disease.

Although *M. tuberculosis* is the most common cause of clinical mycobacterial disease in primates, they are susceptible to infection with a variety of other tuberculous (*M. bovis*, *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii*, *M. canetti*) and NTM including *M. avium* complex (MAC), *M. avium-intracellulare*, *M. avium* subsp. *paratuberculosis* and many others (Thorel, 1980; Baskin, 2014). For example, PCR was

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Table 15.1. Pathogenic mycobacterial infections reported in captive wildlife.

Captive wildlife	Mycobacterial species	References
Rhinoceros	*Mycobacterium tuberculosis, M. bovis*, *M. avium* subsp. *paratuberculosis*	Mann *et al*. (1981), Stetter *et al*. (1995), Oh *et al*. (2002), Duncan *et al*. (2009), Espie *et al*. (2009), Bryant *et al*. (2012), Murakami *et al*. (2012)
Tapir	*M. tuberculosis*, *M. pinnipedii*	Montali *et al*. (2001), Michel *et al*. (2003), Redrobe (2003), Moser *et al*. (2008), Kaewamatawong *et al*. (2010), Jurczynski *et al*. (2011a), Murakami *et al*. (2012), Schaftenaar *et al*. (2013)
Onager	*M. bovis*	
Ungulates (bovids, sheep & goats)	*M. tuberculosis*, *M. bovis*, *M. caprae*, *M. kansasii*, *M. avium* subsp. *paratuberculosis*, *M. avium*, *M. intracellulare*	Manning and Collins (2001), Montali *et al*. (2001), Rocha *et al*. (2001a), Kapustin *et al*. (2006), Pate *et al*. (2006), Portas *et al*. (2009), Miller *et al*. (2011)
Giraffe	*M. tuberculosis*	Montali *et al*. (2001), Lewerin *et al*. (2005)
Camelids	*M. bovis*, *M. microti*, *M. pinnipedii*, *M. caprae*, *M. kansasii*, *M. avium* subsp. *paratuberculosis*	Bush *et al*. (1990), Johnson *et al*. (1993), Manning and Collins (2001), Pate *et al*. (2006), Moser *et al*. (2008)
Cervids	*M. bovis*, *M. avium* subsp. *paratuberculosis*	de Lisle *et al*. (2001), Manning and Collins (2001)
Marsupials	MAC, *M. ulcerans*, Runyon group IV	McOrist *et al*. (1985), Mitchell *et al*. (1987), Joslin (1990), Raymond *et al*. (2000)
Carnivores	*M. tuberculosis*, *M. bovis*, *M. microti*, *M. fortuitum*, MAC	Himes *et al*. (1980), Lepper and Corner (1983), Morris and Thoen (1989), Helman *et al*. (1998), Thorel *et al*. (1998), Cho *et al*. (2006), Kapustin *et al*. (2006), Schmidbauer *et al*. (2007), Palgrave *et al*. (2012), Cerveny *et al*. (2013)
Canids	*M. tuberculosis*	
Felids	*M. tuberculosis*, *M. bovis*, *M. microti*, *M. fortuitum*, MAC	
Non-human primates	*M. tb*, *M. bovis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. canetti*, *M. caprae*, *M. avium-intracellulare*, *M. avium* subsp. *paratuberculosis*	Thorel (1980), Thorel *et al*. (1998), Montali *et al*. (2001), Redrobe (2003), Alfonso *et al*. (2004), Amado *et al*. (2006), Rocha *et al*. (2011b), Baskin *et al*. (2014)
Marine mammals		
Pinnipeds	*M. pinnipedii*	Cousins *et al*. (2003), Kiers *et al*. (2008), Moser *et al*. (2008), Kritz *et al*. (2011)
Cetaceans	*M. chelonae*, *M. marinum*, *M. abscessus*	Bowenkamp *et al*. (2001), Calle *et al*. (2007), Wunschmann *et al*. (2008), Clayton *et al*. (2012)
Sirenians	*M. marinum*, *M. kansasii*, *M. fortuitum*	Sato *et al*. (2003)
Small mammals, rodents and lagomorphs	*M. microti*, *M. pinnipedii*, *M. africanum*, MAC	Cousins *et al*. (1994), Harrenstein *et al*. (2006), Gudan *et al*. (2008), Lutz-Wallace *et al*. (2008), Jurcynski *et al*. (2011a), McClure (2012)
Birds	MAC, *M. genovense*, *M. tuberculosis*, *M. bovis*, *M. fortuitum*	Lamberski (1999), Riggs (2012)
Reptiles	*M. marinum*, *M. chelonae*, and *M. thamnopheos*, other NTM	Soldati *et al*. (2004), Jacobson (2007)
Amphibians	*M. ulcerans* (*M. liflandii*), other NTM	Chai (2012)
Fish	*M. marinum*, *M. fortuitum*, *M. chelonae*, other NTM	Gauthier and Rhodes (2009), Francis-Floyd (2011)

MAC, *M. avium* complex.
NTM, non-tuberculous mycobacteria.

used to assess the presence of different mycobacteria in a survey of 68 New World primates. In this group of animals, 65% were positive for mycobacteria, with 11% identified as *M. tuberculosis* (Alfonso *et al*., 2004). However, only 54% of this population was culture positive, similar to recovery rates in other species.

M. bovis has been reported in a captive troop of baboons (*Papio hamadryas*) in a zoo. DNA fingerprinting showed the isolate to contain a single IS6110 copy, suggesting that the origin could be associated with cattle (Thorel *et al*., 1998). Two gorillas have been confirmed with the same spoligotype of *M. pinnipedii* found in two wild-caught fur seals (*Arctocephalus australis*) at the same zoo (Redrobe, 2003). In one gorilla, the infection was discovered as an incidental finding in the spleen and the other detected through ante-mortem testing. *M. africanum* type II infection resulted in signs of depression, coughing and weight loss in an adult mandrill (*Mandrillus sphinx*) and offspring in a Portuguese zoo (Amado *et al*., 2006).

Based on location of lesions, clinical signs and information from experimental infections with *M. tuberculosis* complex, it is assumed that most infections in zoo primates occur through aerosol transmission. The most common pathological changes in these cases are pulmonary granulomas. Lesions may vary from microscopic to disseminated firm grey to yellowish-white nodules. Typical macroscopic findings are cavitary tubercles within the pulmonary parenchyma with involvement of pleura in more severe cases. Tracheobronchial lymph nodes may contain caseous necrotic nodules. Secondary granulomas are commonly found in other lymph nodes, liver, kidney and spleen in advanced disease. Other organ involvement may also occur including vertebrae. Early granulomas consist of epitheloid cells and giant cells with only sporadic acid-fast bacilli (AFB). With progression of disease, granulomas develop a necrotic core surrounded by epitheloid and a few giant cells. Mineralization and development of a fibrous capsule is not common in non-human primates as compared to other species.

Gastrointestinal mycobacteriosis is typically associated with *M. avium* in primates with a rare occurrence of wasting and diarrhoea due to *M. avium* subsp. *paratuberculosis* documented in stump-tailed macaques (*Macaca arctoides*) (Baskin, 2014). Lesions are typically a thickened intestinal mucosa due to a diffuse histiocytic infiltrate and presence of AFB. A mesenteric lymphadenopathy is also present. Features typical of *M. tuberculosis* complex infection are not usually present although tubercles have rarely been reported.

Zoo primates are potentially exposed to mycobacteria from conspecifics, other species (including humans) and environmental sources. In Australia, one male chimpanzee (*Pan troglodytes*) developed progressive disease associated with a TB strain previously isolated from an infected Asian elephant (*Elephus maximus*) in the same zoo. An additional six of the 17 chimps were confirmed positive using the intradermal tuberculin test and were prophylactically treated. Epidemiological studies did not confirm the route of transmission between exhibits although indirect aerosols or fomites (browse) were considered potential sources (Stephens *et al*., 2013). A red-faced black spider monkey (*Ateles paniscus*) was diagnosed with TB due to *M. tuberculosis* in a Brazilian zoo (Rocha *et al*., 2011b). Necropsy and histopathology detected AFB-positive granulomas in lymph nodes, parietal and visceral pleura, lungs, liver, spleen and kidneys. In both zoos, staff and other animals that had had contact with the infected primate were negative to TB diagnostic procedures. Additional routes of mycobacterial transmission in primates include ingestion of infected material or contamination of cuts and abrasions (James, 2012).

Since non-human primates can be infected by *M. tuberculosis* complex as well as by NTM, it is important to definitively identify the causative organism whenever possible. Any animal that is showing clinical signs or has a history that would lead to suspicion of potential infection should undergo a thorough clinical examination including basic diagnostic tests such as complete blood count (CBC), biochemical panel, thoracic radiographs and sampling for mycobacterial organisms and immunoassays (Miller, 2008).

Gastric and/or bronchioalveolar lavage (BAL) can be performed in most primate species to obtain samples for mycobacterial culture and PCR. Additional samples for staining,

culture and/or PCR include faeces, tissue biopsies or other secretions such as sputum or nasal discharge. Molecular techniques such as spoligotyping and MIRU-VNTR are currently being used for epidemiological studies. Although Ziehl–Neelsen staining of diagnostic material can provide preliminary diagnosis of acid-fast organisms, follow-up identification is required to rule out NTM or other bacteria such as *Nocardia* spp.

Initial screening of individuals and troops is usually performed using intradermal testing. Tuberculin doses 1000–10,000 times higher than those used in humans are required to elicit a hypersensitivity response in non-human primates (Calle, 1999). A minimum of 1500 tuberculin units/0.1 ml should be administered intradermally in the upper eyelid, which is examined visually at 24, 48 and 72 h for swelling and erythema. Other sites for comparative injections and potential biopsy include arms, thorax or abdomen, especially in smaller species.

Host responses to mycobacteria in primates appear to vary depending on species. A number of non-human primate species appear to have higher rates of false-positive reactions on intradermal tests including orangutans and leaf-eating primates such as langurs (*Presbytis* spp.) (Calle, 1999). Orangutans in zoos commonly develop responses to intradermal tuberculin possibly due to exposure to NTM. In one study, 68.7% of 171 zoo orangutans had reactions to intradermal tuberculin tests (Wells *et al*., 1990). Gastric lavages were performed in 57 cases and were positive for non-tuberculous mycobacteria in 14 animals (*M. avium*, *M. fortuitum*, *M. gordonae*, *M. terrae*, *M. chelonei*, *M. kansasii*, *M. nonchromogenicum*, *M. paraforuitum*). ELISA testing performed in a small number of these cases produced mixed results (7/12 positives). Similarly, 12 out of 20 orangutans were reactors on skin testing at another zoo. On further testing, mycobacteria were found on culture or microscopic examination in 11 of the 12 individuals with NTM cultured from three individuals. The source of mycobacteria was suspected to be contaminated exhibit drinking water (Calle *et al*., 1988).

In vitro blood tests for detection of mycobacterial infection by interferon gamma release assays (IGRA) have been used in multiple zoo primates including gorillas, orangutans, chimpanzees, gibbons, colobus monkeys, baboons, mandrills, vervet monkeys, guenons, langurs, squirrel monkeys and marmosets (Miller, 2008; Chambers, 2013). A troop of ten mandrills was tested with IGRAs after diagnosis of *M. africanum* type II infection (Amado *et al*., 2006). Three animals (including two clinically affected individuals) tested positive, demonstrating the potential use for detecting *M. tuberculosis* complex infection.

Other immunoassays that have been investigated in primates include serum antigen 85, PrimaTB STAT-PAK® (Chembio Diagnostics Systems, Inc., Medford, NY), multiantigen print immunoassay (MAPIA) and ELISA (Miller, 2008; Chambers, 2013). Preliminary studies on antigen 85 (a mycobacterial-secreted protein) detection in serum have shown promise as an adjunct test for mycobacteriosis in captive orangutans (Kilbourn *et al*., 2001). Key protein antigens of Mtb eliciting specific antibody responses in non-human primates were identified in experimental infection studies on several species (Brusasca *et al*., 2003; Lyashchenko *et al*., 2007). The most seroreactive antigens included ESAT-6, CFP10 and MPT83, which provided the basis for development of PrimaTB STAT-PAK®, a lateral-flow test for primates (Lyashchenko *et al*., 2007). Multi-centre evaluation of this serological test on 243 rhesus (*Macaca mulatta*), 46 cynomolgus (*Macaca fascicularis*) and 133 African green (*Cercopithecus aethiops sabaeus*) monkeys demonstrated 90% sensitivity and 99% specificity. The highest rate of TB detection over the course of infection was achieved when the PrimaTB STAT-PAK® assay was used in conjunction with the skin test. Diagnostic potential of the rapid serology test was suggested in studies on other primate species including silvered langurs (*Trachypithecus cristatus ultima*) that are especially prone to nonspecific skin test reactivity (Georoff *et al*., 2010), spider monkeys and chimpanzees (Stephens *et al*., 2013; Miller, M.A., unpublished data; Yarto, E., personal communication 2011).

The decision to treat non-human primates and other zoo species must be weighed with costs, ethical and biosafety issues. In some cases, valuable specimens have been treated

using multidrug chemotherapy, including isoniazid, streptomycin, ethambutol and/or rifampicin (Montali *et al*., 2001). Due to concerns for zoonotic and anthropogenic transmission, regular screening of staff in contact with zoo primates is strongly recommended.

Carnivores

Unlike primates, carnivores are considered to be relatively resistant to mycobacterial infection, although large cats appear to be more commonly affected.

M. bovis has been detected in lions (*Panthera leo*), leopards (*Panthera pardis*, *P. uncia*), tiger (*Panthera tigris*), jaguar (*Panthera onca*), European lynx (*Lynx lynx*) and fennec fox (*Fennecus zerda*); *M. tuberculosis* in snow leopards (*Panthera uncial*); and *M. microti* in meerkats (*Suricata suricatta*) in zoos (Himes *et al*., 1980; Morris and Thoen, 1989; Helman *et al*., 1998; Thorel *et al*., 1998; Kapustin *et al*., 2006; Schmidbauer *et al*., 2007; Palgrave *et al*., 2012). Rarely, there are case reports of other carnivores infected with *M. tuberculosis* in zoos such as a European otter (*Lutra lutra*) and a polar bear (*Ursus maritimus*) in Europe (Lepper and Corner, 1983).

Clinical signs are usually associated with pulmonary involvement as well as general systemic disease. These include weight loss, unthriftiness, abnormal respiratory signs (cough) and lameness. Radiographic changes of the lungs consistent with pneumonia, tuberculous nodules and cavitary lesions may be present (Helman *et al*., 1998). In addition, lesions due to secondary hypertrophic osteodystrophy have also been reported in an infected tiger (Van de Watering *et al*., 1972). Diffuse pyogranulomatous dermatitis and panniculitis due to *M. fortuitum* infection was reported in a clouded leopard with concurrent B cell lymphoma (Cerveny *et al*., 2013). A rare *M. avium* infection in a Bengal tiger resulted in dyspnoea, anorexia, general malaise and a slow growth rate (Cho *et al*., 2006). Mycobacterial infection of the gastrointestinal system should be included in the differential diagnosis of carnivores with lethargy, inappetance, abdominal distension or other signs of infiltrative disease.

TB infection in large cats often results in cavernous pulmonary lesions with areas of consolidation (Van de Watering *et al*., 1972; Helman *et al*., 1998) (Plate 16). Mediastinal lymphadenopathy may also be present. Granulomas consist of caseous central necrosis surrounded by infiltrated macrophages, lymphocytes and neutrophils and organizing fibrous tissue. Suppurative inflammatory reactions may be associated with bronchi. AFB are often found, especially in early lesions.

In most cases in zoological collections, infection of carnivores is believed to occur due to ingestion of infected meat or carcasses. A jaguar imported from Venezuela died 2 years later in a USA zoo with necrogranulomatous peritonitis, hepatitis and lymphadenitis associated with *M. bovis* infection that was believed to be linked to whole-prey feeding practices at the prior institution (Kapustin *et al*., 2006).

Two snow leopards housed in the same zoo succumbed to *M. bovis* infection after several months of clinical disease (Helman *et al*., 1998). Although both animals had been fed commercial meat and very occasional ungulate carcasses, the source of infection was not determined. A Bengal tiger in a Korean zoo had multifocal nodules in the lungs, liver, kidney and spleen associated with *M. avium* infection (Cho *et al*., 2006). Histopathologic findings were similar to those described for *M. tuberculosis* complex infected felids with few multinucleated giant cells and AFB present. It was speculated that feeding of freshly culled chickens may have been the origin.

Multi-species outbreaks at zoos may complicate understanding of the epidemiology of infection. Four leopards (*Panthera uncia*, *P. pardus*) were infected with *M. bovis*, which was identical to the isolate from a sea lion (*Otaria byrona*) at the same facility (Thorel *et al*., 1998). Bovine TB cases in a European wild animal park included a European lynx (*Lynx lynx*), pot-bellied pigs (*Sus scrofa vittatus*), red deer (*Cervus elaphus*) and buffalo (*Bison bonasus*). Spoligotyping of 17 isolates from various organs of the affected animals revealed an identical pattern indicating a common origin of infection (Schmidbauer *et al*., 2007). The spoligotype was different from the pattern of *M. bovis* strains isolated from the

cattle population in the respective country and the source was not determined.

Diagnosis in carnivores is often made post mortem using histologic examination of tissues, immunohistochemical, culture and PCR, similar to other species (Miller, 2008). PCR from fresh or formalin-fixed tissues can be used to distinguish between mycobacterial species (Helman *et al*., 1998; Cho *et al*., 2006).

Radiographs in carnivores with mycobacteriosis may be useful in triggering other ante-mortem testing. Large felids typically show a bronchointerstitial pattern with cavernous lesions (Morris *et al*., 1989). Ante-mortem techniques for acquiring samples from suspect cases include tracheoscopy with lavage or aspiration of mucus for culture. Positive cultures have been obtained from lions and a tiger using this technique (Lantos *et al*., 2003; Miller *et al*., 2015).

Diagnostic utility of serology has been recently shown for detecting tuberculosis complex infection in meerkats (*Suricata suricatta*), jaguars and lions (Kapustin *et al*., 2006; Drewe *et al*., 2009; Miller *et al*., 2012; Chambers, 2013). Experimental ELISA using *M. bovis* antigens was able to distinguish an infected lion from other intradermal tuberculin-negative lions housed in the same zoo (Morris *et al*., 1989). Retrospective analyses of sera from a *M. bovis*-infected jaguar showed antibody reactivity to MPB83 antigen by MAPIA in samples collected over 1 year prior to death (Kapustin *et al*., 2006). Studies in free-ranging lions diagnosed with bovine TB showed a close association between seropositivity in ElephantTB STAT-PAK® or DPP VetTB® assay (Chembio Diagnostic Systems, Inc., Medford, NY) and disease status (Miller *et al*., 2012). Field evaluation of ElephantTB STAT-PAK® and MAPIA in a free-ranging population of wild meerkats demonstrated that, while each of the tests was of limited diagnostic value when used alone, interpreting the results of these two tests in parallel produced 83% sensitivity and 73% specificity (0.73; 95% CI = 0.62, 0.82) (Drewe *et al*., 2009). These results suggest that rapid blood tests may be useful in the decision-making process for wild carnivores suspected of TB.

Intradermal testing has not been used routinely in captive carnivores, although a comparative test has been evaluated in free-ranging lions (Keet *et al*., 2010). IGRAs have also been investigated in large felids, although a reliable assay has not yet been developed. Sequences for lion and cheetah IFN-gamma have been published and may provide additional diagnostic tools in the future (Maas *et al*., 2010; Morar *et al*., 2013).

Treatment may be attempted in valuable individuals, similar to primates, although disease is often advanced at the time of diagnosis. Concern for transmission to other animals and staff should be considered prior to initiation of therapy.

Small Mammals, Rodents and Lagomorphs

Rock hyrax or dassie (*Procavia capensis*) are small African mammals, taxonomically related to elephants. Imported hyrax with mycobacteriosis in zoos in Australia, the UK, Canada and Croatia had variable signs of muscle weakness/lameness, paralysis and dyspnoea (Cousins *et al*., 1994; Gudan *et al*., 2008; Lutze-Wallace *et al*., 2008). All animals except those in the Croatian zoo were originally imported from South Africa and were shown to be infected with *M. microti*. The animals in Croatia were born in the United Arab Emirates and *M. africanum* was isolated from the female of the pair.

Importation of wild-caught individuals is believed to be the cause of the outbreaks in each of the cases in Australia, the UK and Canada. The isolates from the Canadian and Australian zoos had identical spoligotypes and were similar to an isolate from 1958, suggesting that infection had taken place in South Africa. Since the hyrax pair in the Croatian zoo was captive born but the female died 6 months after importation, it is assumed that infection occurred prior to transport (Gudan *et al*., 2008). Similar findings in the male suggest either intraspecific transmission or exposure to a common source of infection.

Mycobacterial infection in rodents and lagomorphs is rare except for pygmy rabbits (McClure, 2012). The most common cause of mortality in captive adult pygmy rabbits

(*Brachylagus idahoensis*) was disseminated *M. avium* infection (Harrenstein *et al*., 2006). Twenty-nine per cent of this population was diagnosed with mycobacteriosis between 2002 and 2004. Impaired cell-mediated immunity was hypothesized to contribute to the high morbidity and mortality. *M. pinnipedii* has also been isolated from a crested porcupine (*Hystrix cristata*) cared for by the same keeper as infected sea lions (Jurcynski *et al*., 2011a).

To date, diagnosis is typically made post mortem from identification of organisms isolated from tissues by culture with PCR. Spoligotyping is used to compare strains for epidemiological links. Screening of small mammals for mycobacterial infection during quarantine should be performed to prevent introduction to zoological institutions.

Marsupials

Tree kangaroos (*Dendrolagus* spp.) appear to be susceptible to pulmonary, bone and skin infections associated with MAC and other NTM (Joslin, 1990). Osteomyelitis, abscesses, pneumonia and encephalitis have been observed in captive tree kangaroos in several institutions in one survey. Other macropods, particularly wallabies, are also considered susceptible to MAC. *M. ulcerans* has resulted in skin ulcers and respiratory tract infection in koalas (*Phascolarctos cinereus*) (McOrist *et al*., 1985; Mitchell *et al*., 1987). A significant number (24%) of captive tiger quolls (*Dasyurus maculatus*) at one zoological institution have been reported to develop infections with Runyon group IV mycobacteria which present as abscesses and chronic infections of the skin and subcutis (Raymond *et al*., 2000). Lesions were found primarily along the neck and thorax with lymph node involvement resulting in nodular to diffuse pyogranulomatous panniculitis and dermatitis. Although surgical and antibiotic therapy were attempted, the majority of cases were refractory. Lymphocyte proliferation assays performed in tiger quolls were reported to be depressed, possibly making them more susceptible to systemic infections with environmental mycobacteria (Raymond *et al*., 2000).

MAC and Runyon group IV organisms are typically found in the environment and may be contaminants of the soil and substrate in an exhibit. In the case of the tiger quolls, bite wounds associated with breeding in which males inflict injuries to the female's neck could have led to skin infections due to contamination by soil saprophytes (Raymond *et al*., 2000). In one institution with infected tree kangaroos, finches previously housed in the exhibit were believed to be a potential source of infection. On post-mortem examination, four of the eight birds showed histological evidence of lymphocytic hepatitis with three having AFB in the liver (Joslin, 1990), from which *M. avium* was cultured. Although the serotypes of *M. avium* from the birds and tree kangaroos differed, the author mentioned the importance of minimizing potential risk of spread between species as well as the effect of immunosuppression on susceptibility to mycobacterial disease.

Diagnosis of atypical mycobacterial infections in tiger quolls was based on culture of skin lesions in combinations with histopathology and cytology. Similarly, samples from lesions in tree kangaroos should be obtained for culture, cytology and histopathology when possible. Ziehl–Neelsen staining provides a presumptive diagnosis on cytology pending further analyses and allows the clinician to determine if treatment with antituberculosis therapy is warranted. *M. avium-intracellulare* has been cultured from tracheal wash samples in one tree kangaroo with pulmonary lesions (Joslin, 1990); therefore, ante-mortem testing is recommended in suspected cases. Thoracic and skeletal radiographs may reveal suspect lesions.

Ante-mortem diagnostic methods for marsupials are similar to those performed in other mammals (Miller, 2008). Although no validated intradermal tests exist for marsupials, comparative skin tests have been performed using avian and mammalian tuberculins. In one case in which a Matschie's tree kangaroo with a submandibular abscess was drained and treated, the tests were positive at the time of treatment and negative to avian tuberculin 1 month later.

Treatment with isoniazid (INH), ethambutol and amikacin for *M. avium–intracellulare*

osteomyelitis has been attempted in a tree kangaroo (Joslin, 1990). However in most cases, treatment is usually either not attempted or unsuccessful, resulting in euthanasia.

Marine Mammals (including Pinnipeds, Cetaceans and Sirenians)

Marine mammals are susceptible to infection with a variety of mycobacterial species. TB caused by *M. pinnipedii* has been found in captive and wild pinnipeds worldwide (Cousins *et al*., 2003). The most commonly affected species is the Southern sea lion (*Otaria flavescens*) (Kriz *et al*., 2011). Clinical signs include depression, lethargy, dyspnoea and weight loss. Asymptomatic infection and acute mortality are not uncommon in affected populations.

Atypical mycobacteriosis often presents as dermatitis in marine mammals. Disseminated panniculitis due to *M. chelonae* has been diagnosed in a bottlenose dolphin (*Tursiops truncates*) that had been treated with long-term dexamethasone and progesterone (Wunschmann *et al*., 2008). In a white whale (*Delphinapterus leucas*), *M. marinum* infection led to intermittent anorexia and chronic dermatitis (resembling cetacean poxvirus infection) with fatal progression (Bowenkamp *et al*., 2001). Lesions were multicentric and ulcerative (1–3 cm diameter) on the thorax and abdomen. *M. abscessus* also led to dermatitis and mastitis in a beluga whale (Calle *et al*., 2007). In addition to cutaneous infection, mycobacteriosis may present as a respiratory disease. *M. marinum* has been associated with fatal pulmonary infections in two captive Florida manatees (*Trichechus manatus latirostris*) and *M. abscessus* with pneumonia in a bottlenose dolphin (Sato *et al*., 2003; Clayton *et al*., 2012).

Lesions may vary with organism. *M. pinnipedii* has caused nodular granulomatous lesions in lungs, pleura and lymph nodes in an infected sea lion (Kriz *et al*., 2011). Lesions contained AFB. Pathologic findings in a *M. marinum*-infected white whale included multiple cavitated dermal lesions with purulent material extending into the blubber (Bowenkamp *et al*., 2001). Chronic proliferative pleuritis with pleural effusion and multifocal AFB-positive granulomatous pneumonia were also present. Similarly, nodular lung lesions were found in two Florida manatees that were infected with *M. marinum* concurrently with either *M. fortuitum* or *M. kansasii* (Sato *et al*., 2003). *M. abscessus*-associated lesions in a bottlenose dolphin included multiple cavitating pulmonary lesions, fibrinous pleuritis and atelectasis (Clayton *et al*., 2012). Although histopathology revealed diffuse pyogranulomatous pneumonia with rare intracellular bacilli, there were no discrete granulomas. AFB were associated with pyogranulomas of the blubber but not present in lung or lymph nodes of a *M. chelonae*-infected bottlenose dolphin (Wunschmann *et al*., 2008).

M. pinnipedii has been associated with epizootics in captive sea lions. In a European zoo, an outbreak of *M. pinnipedii* infection occurred in a captive colony of 29 sea lions (Kiers *et al*., 2008). Necropsy was positive in 13 of them including three cases with pulmonary involvement. Infected pinnipeds have also been implicated as the source of infection for other animal species in zoos, including a Brazilian tapir (*Tapirus terrestris*), llama (*Lama glama*) and two lowland gorillas (*Gorilla gorilla gorilla*) (Redrobe, 2003; Kriz *et al*., 2011). In addition, spoligotyping and MIRU/VNTR-typing revealed identical molecular characteristics of *M. pinnipedii* isolates from South American sea lions (*Otaria flavescens*), a Bactrian camel (*Camelus bactrianus*) and Malayan tapirs (*Tapirus indicus*) kept in two zoological gardens in Europe (Moser *et al*., 2008).

Diagnostic techniques for marine mammals include sputum/blowhole cytology and culture and haematology. Radiographs and computerized tomography may also detect pulmonary lesions (Jurczynski *et al*., 2011b). Standard culture techniques can be used to isolate mycobacteria; however, molecular methods are required to differentiate *M. pinnipedii* from other *M. tuberculosis* complex organisms. PCR using specific genomic deletions (PiD1, PiD2) and differing genomic regions (RD1mic, RD2seal) permits differentiation of *M. pinnipedii* (Kriz *et al*., 2011).

Tuberculin skin tests using bovine and avian purified protein derivatives (PPDs) have been assessed in several species of pinnipeds

(Needham and Phelps, 1990). Of a group of 40 animals tested, 14 reacted positively to both tuberculins. Ten (of 14) responders had gross lesions at necropsy and/or positive cultures.

Serologic tests have been shown to have ante-mortem diagnostic potential for detection of *M. pinnipedii* infection in sea lions (Moser *et al*., 2008). Furthermore, computed tomographic examination of South American sea lions with suspected *M. pinnipedii* infection demonstrated the added value of this approach, especially if combined with antibody tests such as DPP VetTB assay or MAPIA (Jurczynski *et al*., 2011b). ELISA results using *M. bovis* antigens also appeared to correlate with mycobacterial infection although it is unknown how exposure to NTM may affect results (Cousins, 1987).

Treatment with antimycobacterial drugs and nebulization has been attempted unsuccessfully (Clayton *et al*., 2012).

M. pinnipedii is considered zoonotic, as evidenced by marine mammal handlers becoming tuberculin skin test-positive after exposure to infected animals. In a European zoo, six of the 25 animal keepers who were exposed to *M. pinnipedii* infection in sea lions were skin test-positive; in five of those, infection was confirmed by IGRA (Kiers *et al*., 2008). The most probable route of transmission was nebulization of infectious material during cleaning of enclosures. These findings emphasized the zoonotic potential of pinniped tuberculosis.

Ungulates (including Bovids, Giraffe, Sheep and Goats)

Artiodactylids are typically more resistant to *M. tuberculosis* than *M. bovis*, although all species are considered susceptible to TB. Infection with *M. tuberculosis* and *M. bovis* has been reported more often in bovids and cervids than in sheep and goats, although *M. caprae* has been found in small ruminants. Zoo ungulates can be affected by other pathogenic mycobacteria that mimic *M. bovis* infection, such as *M. kansasii* and *M. szulgai* (Miller *et al*., 2011). Transmission of mycobacterial infection in zoo ungulates is speculated to be through aerosols, fomites or environmental contamination.

Typically, mycobacterial infection is a chronic insidious process that may be asymptomatic until disease is advanced. Clinical signs depend on the organism and site of infection. *M. tuberculosis* infection in an Addra gazelle (*Gazella dama ruficollis*) presented as severe weight loss due to extensive multifocal AFB-positive granulomatous pneumonia (Kapustin *et al*., 2006). *M. bovis* infection manifests with weight loss, respiratory signs (coughing, nasal discharge, exercise intolerance), dull haircoat and lethargy (de Lisle *et al*., 2001; Montali *et al*., 2001). *M. kansasii* and *M. szulgai* infections are often clinically indistinguishable from *M. tuberculosis* complex disease but may also cause lameness or limb swelling due to bone or joint involvement. In one captive herd, two cases of *M. kansasii* infection in bontebok (*Damaliscus pygaragus dorcas*) presented with pneumonia and weakness, or nonresponsive lameness with a draining fistula (Plate 17). Both animals were skin test reactive to bovine PPD and serologically positive on ElephantTB STAT-PAK® (Chembio Diagnostic Systems, Inc., Medford, NY), DPP VetTB and MAPIA (Miller *et al*., 2011). Granulomatous lesions were observed in liver, kidney, spleen, lungs, pleura and multiple lymph nodes on necropsy.

M. avium, *M. parafortuitum* and *M. intracellulare* were isolated from a herd of aoudad (*Ammotragus lervia*) during an epizootic of mycobacteriosis in an Australian zoo (Portas *et al*., 2009). Johne's disease, caused by *M. avium* subsp. *paratuberculosis*, is a recognized problem with domestic goats in petting zoos and all exotic ruminants are considered susceptible with multiple zoos reporting infection (Manning and Collins, 2001). Clinical signs are similar to those in domestic ruminants with chronic wasting and diarrhoea in some species.

Both *M. tuberculosis* and *M. xenopi* have been isolated from bongo antelope (*Tragelaphus eurycerus*), the first causing severe pulmonary disease and the second isolated from a lymph node of a herdmate (Montali *et al*., 2001). Both animals had been housed in a zoo

that a decade later had cases of *M. tuberculosis* in an Asian elephant (*Elephas maximus*), black rhinoceros (*Diceros bicornis*) and three mountain goats (*Oreamnos americanus*). However, strains from these cases were unrelated to the bongo isolate.

In a Brazilian zoo, two waterbucks (*Kobus ellipsiprymnus*) exhibited severe respiratory symptoms and had to be euthanized. AFB-positive granulomatous lesions were found in lungs and mediastinal lymph nodes, from which *M. bovis* organisms were isolated (Rocha *et al*., 2011a). The two animals were born and kept in the same enclosure with the same group, without any contact with other species housed in the zoo. An outbreak of TB due to *M. tuberculosis* in a Swedish zoo involved five elephants and one giraffe (*Giraffa camelopardalis*) (Lewerin *et al*., 2005). Four different strains were isolated from these animals, some of which had more than one strain.

Ungulate TB cases in zoos are typically investigated by herd tuberculin testing of animals that are linked through shared exhibits or staff. In the case of a *M. caprae*-infected camel in a Slovenian zoo, one dromedary camel and six bison were reactors and euthanized (Pate *et al*., 2006). Of these, *M. caprae* was isolated from two bison, whereas *M. scrofulaceum* and *Mycobacterium* spp. were found in another two animals. Using spoliogotyping and MIRU-VNTR, the *M. caprae* isolated from the camel and two bison isolates were shown to be identical. Mtb in a colony of rhesus monkeys resulted in pulmonary disease in an oryx (O*ryx gazelle beisa*) which was spread by water overflow from the moat surrounding the primate enclosure (Lomme, 1976). In a UK zoo, multiple species were infected with *M. pinnipedii* including a pudu (*Pudu puda*) and a llama (*Lama glama*) (Redrobe, 2003).

Culture can be performed from tracheal, gastric or tissue samples either ante or post mortem, although the latter is more common due to logistical difficulties in many species. Molecular techniques for identification such as IS6110 PCR, restriction fragment length polymorphism analysis, spoligotyping and mycobacterial interspersed repetitive units typing have been applied for speciation and epidemiological studies in zoological populations (Pate *et al*., 2006).

The most commonly used diagnostic test for zoo ungulates is the intradermal test (Miller, 2008). This is applied for routine and diagnostic screening for TB. Since zoo species do not fall under most TB regulatory programmes, there is significant variation in frequency of testing, type and sites of tuberculin administration and methods of interpretation. In a survey of tuberculin testing practices for hoofstock at US zoos, respondents listed caudal fold, eyelid and cervical and abdominal skin as routes for injection (Montali and Hirschel, 1990). A similar survey in Swedish zoos found that most ungulates were tested using the cervical site (Sternberg *et al*., 2002). The authors suggested that comparative cervical tests using avian and bovine PPD be used, with results measured at 72 h using calipers. Validation has not been performed and there is significant variability between species. For instance, the intradermal test was shown to be unreliable in gemsbok infected with *M. bovis* (Schaftenaar *et al*., 2013).

Diagnostic potential has been recently suggested for serologic tests in Addra gazelle infected with *M. tuberculosis*, bontebok infected with *M. kansasii* or gemsbok with bovine TB (Kapustin *et al*., 2006; Miller *et al*., 2011; Schaftenaar *et al*., 2013). In particular, ElephantTB STAT-PAK® assay was able to detect antibody in an *M. bovis*-infected gemsbok at the time of euthanasia and 5 months earlier when the animal was negative on the skin test (Schaftenaar *et al*., 2013). An *M. tuberculosis*-infected Addra gazelle seroconverted at least 6 months prior to euthanasia (Kapustin *et al*., 2006), displaying antibody response to multiple antigens (ESAT-6, CFP10, MPB83, F10, Acrl/MPb83 and ESAT-6/CFP10) in MAPIA. Using an ELISA with *M. bovis* extract, serum antibodies have also been detected in *M. bovis*-infected Arabian oryx (*Oryx leucoryx*), wildebeest (*Connochaetes taurinus*) and waterbuck (Haagsma and Eger, 1990).

Treatment of zoo ungulates is of questionable efficacy and may complicate eradication of disease in collections. INH has been unsuccessful in controlling TB in a captive greater kudu (*Tragelaphus strepsiceros*) herd when administered in drinking water (Lyvere, 1975). A three-drug regimen of INH, rifampicin and ethambutol was used to treat six Arabian

oryx in an infected herd (Haagsma and Eger, 1990), with no adverse effects noted. An additional 44 animals were treated for 9 months including one symptomatic individual. No new cases were reported after initiating treatment. Clinical signs resolved in the symptomatic animal, which was euthanized for postmortem examination 6 months post-treatment. Calcified lesions were present throughout the lungs with a smaller number in the spleen and liver. Thoracic lymph nodes were enlarged and contained calcified lesions. Although no AFB were detected, lung lesions demonstrated typical TB histopathology with numerous giant cells. *M. bovis* was cultured from both a lymph node and lung sample.

Cervids

Mycobacterial infection in cervids is predominantly a problem in farmed deer and elk, although sporadic cases of TB have been reported in cervids in zoos (de Lisle *et al*., 2001; Miller, 2008). The single cervical test using bovine PPD has been validated for use in captive cervids in the US. Serological tests (CervidTB STAT-PAK® (Chembio Diagnostic Systems, Inc., Medford, NY), DPP VetTB, MAPIA, ELISA) have variable sensitivity/specificity estimates for different host species but appear to be useful screening tools (Miller, 2008; Chambers, 2013). Cervids residing in zoological facilities are also susceptible to infection with *M. avium* subsp. *paratuberculosis* and should be tested along with other mycobacterial disease during quarantine or in any animal with clinical signs (Manning and Collins, 2001). TB and NTM infections in cervids are covered in more detail in Chapter 19.

Camelids

Camelids have been reported to have disease caused by *M. bovis*, *M. caprae*, *M. microti*, *M. kansasii* and *M. avium* subsp. *paratuberculosis* infection (Bush *et al*., 1990; Johnson *et al*., 1993; Manning and Collins, 2001; Pate *et al*., 2006). These cases typically present with progressive weight loss and emaciation.

In contrast to domestic ruminants, TB in Old World camelids appears as proliferative pulmonary lesions with few Langhan's giant cells and rare AFB. In advanced cases, disseminated pyogranulomatous lesions may be found in multiple organs (Bush *et al*., 1990). In South American camelids, lesions caused by *M. microti* and *M. bovis* were described as multifocal caseous nodules in lungs, lymph nodes and, in some cases, other organs (Robert, 2006; Twomey *et al*., 2007).

A high proportion of camelids reportedly respond to intradermal testing with poor correlation of results with culture (Bush *et al*., 1990; Twomey *et al*., 2007). Studies have shown only 68% of camels with TB have suspect or positive skin tests. Isolation of *M. avium* from a bronchial lymph node in one case indicates that camels may be infected with atypical mycobacteria, resulting in false-positive tuberculin reactions. Antibodies to *M. kansasii*, *M. scrofulaceum* and *M. bovis* in an ELISA suggest that NTM infection may be common in captive camels (Bush *et al*., 1990).

Diagnosis of TB is similar to that in other artiodactyls (Miller, 2008). IGRAs have been used to detect *M. bovis* and *M. microti* infections in South American camelids (Chambers, 2013). Serological assays appear to be useful adjunct tests for diagnosis of mycobacterial infection in camelids. Antibodies have been detected in llamas (*Lama glama*) and alpacas (*Vicugna pacos*) infected with *M. bovis* using rapid immunochromatographic assays (VetTB STAT-PAK®, DPP VetTB) (Twomey *et al*., 2007; Chambers, 2013).

In general, treatment is not recommended. Isoniazid in pelleted feed was administered to an infected herd of Bactrian camels (Bush *et al*., 1990). This resulted in bone marrow suppression and death in six animals.

Mycobacterial infections in camelids are covered in more detail in Chapter 12.

Other Artiodactyls (including Suids and Hippopotamus)

Hippopotamuses are common exhibit animals in zoos, but mycobacterial disease is rarely reported. Due to the risk of immobilization, these animals are not routinely tested. In one

case report, a Nile hippopotamus (*Hippopotamus amphibius*) and two pygmy hippopotamuses (*Choeropsis liberiensis*) in a US zoo were skin tested with mammalian and avian old tuberculin after being exposed to a *M. bovis*-infected black rhinoceros (Mann *et al*., 1981). All animals had indurated swellings at the injection sites; however, sera were negative in an ELISA using bovine and avium PPDs. On necropsy, no evidence of granulomatous disease was present in the Nile and one of the pygmy hippos.

A pygmy hippopotamus arrived in quarantine at a UK zoo and was reactive to bovine tuberculin on comparative skin test (Bouts *et al*., 2009). Sera tested 6 weeks apart with a rapid immunochromatographic assay (VetTB STAT-PAK®) and MAPIA were antibody positive. No gross or histologic signs of TB were found at post-mortem examination. *M. interjectum* was cultured from BAL but not from necropsy tissue samples. Western blot analysis did not show correlation between antibodies to *M. interjectum* and those recognized in MAPIA. These cases demonstrate the difficulty of diagnosing TB in hippopotamus that may be exposed to cross-reactive environmental mycobacteria.

Rhinoceros (and Other Equids)

Although perissodactyls are considered more resistant, *M. tuberculosis* and *M. bovis* have caused clinical infection in African rhinoceros in zoological collections (Mann *et al*., 1981; Valandikar and Raju, 1996; Oh *et al*., 2002; Duncan *et al*., 2009; Espie *et al*., 2009; Murakami *et al*., 2012). TB has been found post mortem in rhinoceros in which there were nonspecific signs of weight loss and lethargy, with a few cases showing respiratory signs such as nasal discharge, coughing or dyspnoea in late stages.

Pathological findings in rhinoceros with TB include lymphadenopathy, lack of fat stores and cavitary or nodular lung lesions (Mann *et al*., 1981) (Plate 18). In one case, multifocal tannish-white small (1–2 mm) foci were also detected in the liver. Microscopically, suppurative granulomas with giant cells and AFB were found in lung lesions.

In South Africa, a black rhinoceros (*Diceros bicornis minor*) was diagnosed with *M. bovis* infection (Espie *et al*., 2009). Genotyping of the isolate revealed limited similarities with *M. bovis* strains detected from South African cattle or wildlife. The source of the infection could not be determined. It has been speculated that cases of TB in wild-caught rhinoceros at zoos outside Africa may have been due to infection acquired prior to importation, although evidence is lacking regarding the source of infection (Mann *et al*., 1981; Stetter *et al*., 1995). These cases illustrate that TB could be a conservation concern for free-ranging rare and endangered species.

Although considered resistant to Johne's disease, there has been one clinical case of weight loss and diarrhoea in a black rhinoceros that had *M. avium* subsp. *paratuberculosis* isolated from faeces (Bryant *et al*., 2012). Signs resolved after treatment with antimycobacterial drugs.

In a rare case of TB in equids, 8 years after an *M. bovis* outbreak occurred in a small herd of gemsbok (*Oryx gazelle gazelle*), an onager (*Equus hemionus onager*) died of bovine TB (Schaftenaar *et al*., 2013). This animal had been kept in the same building as the gemsbok.

Screening of rhinoceros for TB is typically performed when infection is suspected. In a survey of tuberculin testing for rhinoceros in North American institutions, 65% indicated that TB testing was not routinely performed (Godfrey *et al*., 1990). The most common method was an intradermal tuberculin test using bovine PPD (45.5%) in the caudal fold (34.2%), with eyelid, vulva and base of ear as less commonly used sites for injection (21.05%, 21.0% and 18.4%, respectively). Of the 53 reported results for intradermal tests, 81.1% were interpreted as negative with equivocal results in seven animals. In one *M. bovis*-infected black rhinoceros, intradermal testing using mammalian old tuberculin (MOT), bovine and avian PPDs resulted in positive reactions (Mann *et al*., 1981). On biopsy, moderate to marked delayed hypersensitivity was detected in bovine PPD and MOT injection sites. A female rhino bred to this infected individual, along with her calf, skin test converted after the death of the infected male rhino. After 6 months of isoniazid treatment, both animals were subsequently negative on intradermal tuberculin tests. On necropsy, no evidence of granulomatous disease was observed in the female.

Serology appears to be useful for rapid ante-mortem detection of TB in rhinoceros (Moser *et al*., 2008; Duncan *et al*., 2009). In a small sample of black rhinoceros, serum antibodies to *M. bovis* detected by ELISA were correlated with positive intradermal tuberculin reactions (Mann *et al*., 1981). In another study, two culture-confirmed rhinoceroses infected with *M. tuberculosis* and one exposed suspect showed antibody responses to ESAT-6, CFP10 and MPB83 antigens in MAPIA and ElephantTB STAT-PAK® (Duncan *et al*., 2009). When the infected animals were treated with antimycobacterial therapeutics, their serum antibody levels gradually declined, thus suggesting the potential value of semi-quantitative serology for treatment monitoring.

Using ElephantTB STAT-PAK®, MAPIA and DPP VetTB assay, a recent retrospective study revealed specific antibody responses in an onager (*Equus hemionus onager*) that died of bovine TB, but not in three disease-free herd mates. Seroconversion was first noticed in all three immunoassays 5 years before death when the tuberculin skin test was negative (Schaftenaar *et al*., 2013).

Treatment has been empirically performed in rhinoceros. In one case, a black rhinoceros with *M. bovis* isolated from a percutaneous lung biopsy was treated with isoniazid orally (Mann *et al*., 1981). Euthanasia was performed 6 months after treatment was initiated and nodular lesions with suppurative centres were found throughout the lungs. Although cultures were negative, AFB were found in the lung. Isoniazid and rifampin were administered for a total of 41 months to a TB-exposed, skin test-positive black rhinoceros (Duncan *et al*., 2009). Cultures from BAL and gastric lavages were performed ten times during treatment and for 5 years after treatment, all of which were negative for mycobacteria.

Tapirs

Malayan (*Tapirus indicus*) and Baird's (*Tapirus terrestris*) tapirs have been infected with *M. tuberculosis* (Montali *et al*., 2001; Michel *et al*., 2003) or *M. pinnipedii* (Redrobe, 2003; Moser *et al*., 2008; Jurczynski, *et al*., 2011a). Infection in tapirs is often asymptomatic until late in disease. Tapirs exhibited variable courses of disease from days to months prior to death with dyspnoea, tachypnoea, coughing, listlessness, anorexia and loss of condition. Typical tubercular lesions are observed in affected organs such as lung, lymph nodes and liver. Granulomas are characterized by thin fibrous capsule and low numbers of Langhan's giant cells surrounding a caseous necrotic core (Kaewanatawong *et al*., 2010).

Transmission may occur between tapirs, between species in a zoo or possibly from zoo visitors (Moser *et al*., 2008; Kaewamatawong *et al*., 2010; Jurczynski *et al*., 2011a). Isolates of *M. tuberculosis* from infected tapirs in a South African zoo had differing RFLP patterns from one another, indicating different sources (Michel *et al*., 2003). Although other species were infected during the 10-year study, IS6110 heterogeneity between isolates did not indicate interspecies transmission within the zoo. *M. tuberculosis* RD (Rio) strain, a human isolate, was identified in tapirs from a Brazilian zoo, suggesting humans as a source of infection (Murakami *et al*., 2012).

Diagnostic tests for TB in tapirs are similar to those for other ungulates, although nonspecific tuberculin skin test responses are reportedly common in tapirs (Miller, 2008). The inguinal region should be used for intradermal injection of bovine PPD in these species. Nasal, bronchoalveolar or gastric lavages are used to obtain samples for mycobacterial culture. Diagnostic value of serologic tests (ELISA, ElephantTB STAT-PAK®, MAPIA) has been demonstrated for tapirs infected with *M. tuberculosis* or *M. pinnipedii* (Sternberg *et al*., 2002; Moser *et al*., 2008; Jurczynski *et al*., 2011a).

Birds, Reptiles, Amphibians and Fish

Saprophytic mycobacteria are ubiquitous in the environment and may result in infection of multiple taxa including fish, amphibians, reptiles and birds. Many of the mycobacterial species can be potential zoonoses, such as *M. marinum*, *M. ulcerans*, *M. chelonae* and *M. fortuitum*. Treatment of these taxa is not recommended due to poor prognosis, prolonged therapy and zoonotic risks. Management practices should minimize mycobacterial load in

the environment. This section provides a brief overview of mycobacterial infections in the non-mammalian host species that may be important in zoological collections.

In captive avian collections, the most common cause of mycobacterial disease is MAC, although *M. genovense*, *M. intracellulare*, *M. fortuitum*, *M. gordonae*, *M. nonchromogenicin*, *M. simiae*, *M. tuberculosis* and *M. bovis* have also been isolated (Lamberski, 1999; Riggs, 2012). All taxa are susceptible to infection and disease has been detected in anseriforms, columbiforms, gallinaceous birds, gruiforms, passerines, psittacines and raptors. Clinical signs include chronic progressive weight loss. Other signs depend on host or pathogen species and organ system(s) involved, but may include diarrhoea, hepatomegaly, splenomegaly, dyspnoea and lameness associated with osteomyelitis (Lamberski, 1999) (Plate 19).

Mycobacteriosis should be suspected in birds with significant leukocytosis, evidence of pulmonary or air sac masses on radiographs or proliferative osteolytic lesions of long bones. Additional diagnostic tests consist of mycobacterial culture and PCR of samples obtained from biopsy of granulomas or abnormal tissue using laparoscopy or exploratory surgery (e.g. liver biopsy, thickened intestinal tract or air sac granulomas).

In reptiles, NTM species represent one of the most significant causes of infectious granulomatous inflammation (Soldati *et al*., 2004). *M. marinum*, *M. chelonae* and *M. thamnopheos* are the most commonly isolated mycobacteria from reptiles, followed by *M. haemophilum*, *M. kansasii*, *M. agri*, *M. confluentis*, *M. hiberniae*, *M. neoaurum* and *M. nonchromogenicum* (Jacobson, 2007). A variety of reptiles are susceptible to mycobacterial infections including crocodiles, snakes, turtles and lizards. Although clinical signs of chronic infection are often absent or nonspecific, mycobacteriosis should be suspected in animals with loss of condition, skin/scale/plastron ulcerations or discolouration and behaviour abnormalities.

Unlike mammals, mycobacteriosis is a common infectious disease of amphibians (Chai, 2012). Typically, disease is chronic and may manifest with weight loss, varying integumental changes such as pigmentation, ulceration or granulomatous disease, abnormal behaviour or coelomic fluid accumulation. Granulomas may be present in multiple tissues at necropsy. Ante-mortem diagnosis is challenging but should be attempted using aseptic sampling of skin and other tissues via laparoscopy or necropsy.

Disease caused by atypical mycobacteria is commonly found in fish collections in zoos and exhibit aquaria. Although mycobacteriosis has been reported in more than 151 species of fish found in freshwater, marine and brackish water, some species appear to have a greater occurrence such as striped bass (*Morone saxatilis*), cichlids (Cichlidae) and sygnathids (sea horse family) (Gauthier and Rhodes, 2009; Francis-Floyd, 2011). Clinical signs may be nonspecific and include poor body condition, scale loss or skin ulcers, abnormal behaviour or reproductive problems. Coelomic distension and exopthalmia may also be observed. White or greyish nodules (granulomas) may be found in various infected tissue including liver, spleen, kidney, gills, skin, muscle and reproductive organs.

M. marinum, *M. fortuitum* and *M. chelonae* are the most common mycobacterial species affecting fish, although numerous other species have been identified using newer molecular techniques (Gauthier and Rhodes, 2009; Francis-Floyd, 2011). Management of exhibits containing infected fish should include UV or ozone to decrease bacterial load, culling of symptomatic individuals and precautions for staff to prevent zoonotic transmission.

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16 Tuberculosis in Badgers (*Meles meles*)

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Badger Ecology

The European badger (*Meles meles*) is a social mustelid (related to stoats, otters and mink) within the Order Carnivora. It is distributed throughout Europe and parts of the Middle East, but population density varies widely across its range. In Great Britain, where badgers have been associated with *M. bovis* for the last 40 years, they can achieve densities of up to 38 animals per km^2. In the British Isles badgers tend to live in social groups of between 2 and up to 23 adults (Neal and Cheeseman, 1996). They are nocturnally active and spend most daylight hours in their underground burrows (setts) which can be extensive structures. Social groups typically defend a territory which usually contains a single main sett plus a number of less extensive and less frequently used outlier setts (Neal and Cheeseman, 1996). Group living and communal use of underground chambers have clear implications for disease spread.

Usually one, but occasionally more females breed in each social group, producing a mean of three cubs in late winter or early spring (Neal and Cheeseman, 1996). Mating occurs immediately postpartum and again in autumn, coincident with an increase in territorial behaviour (Neal and Cheeseman, 1996). Badgers are unusual in that they delay implantation of the fertile blastocyst (an early stage in embryonic development) until about mid-December to mid-January, which allows cubs to be reared during periods of abundant food availability. Cub mortality can be high, but the annual survival rate of healthy adult badgers is approximately 66% in males and 74% in females (Graham *et al*., 2013).

All adult badgers, but particularly males, scent-mark their territory by defaecating at latrine sites. Territorial defence and mating behaviours can result in bite wounding, particularly among males (Gallagher and Nelson, 1979; Cheeseman *et al*., 1988; Delahay *et al*., 2006), which may have implications for disease spread. In high-density populations, permanent dispersal between territories is relatively uncommon (Rogers *et al*., 1998), and can take a few months with individuals moving between two territories on several occasions before making a final commitment (Roper, 2003). However, some individuals commonly make temporary moves to other social groups (Rogers *et al*., 1998).

Badgers have a wide diet that includes invertebrates, fruit, plant material and carrion, but in parts of the British Isles earthworms

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can form a major component (Neal and Cheeseman, 1996). As badgers frequently forage for earthworms and other food items on pasture this may bring them into direct and indirect contact with cattle. Badgers will also forage on human refuse and livestock feed in farm buildings (Neal and Cheeseman, 1996; Garnett *et al*., 2002).

Pathogenesis

Tuberculosis (TB) is an immuno-pathological disease; the formation of lesions resulting from a cell-mediated immune (CMI) response to the presence of *M. bovis*. TB is a respiratory disease and *M. bovis* gains entry principally through uptake of infectious aerosol particles by alveolar macrophages. Virulence factors act to enhance survival of the bacillus within the host's phagocytic cells and through cytolytic activity that promotes dissemination within the host (Kinhikar *et al*., 2010). Transmigration of infection from the lungs occurs via the lymphatics to the bronchial associated lymphatic tissue and into the circulatory system (Orme and Cooper, 1999). In the case of infection by ingestion, bacilli that survive passage through the stomach may gain access by way of M cells in the epithelium (Khare *et al*., 2009).

Dissemination from the primary site of infection occurs initially during the innate immune response phase and only after the mycobacteria are transported to a lymph node does the CMI response develop (Chackerian *et al*., 2002). The development of lesions commences when the CMI response is generated. The CMI response may be delayed for up to several weeks, depending on the immune competence of the host and the severity of the infectious dose. During this early period infected macrophages may migrate beyond the draining lymph nodes and circulate within the host. When a potent CMI response is generated, infected macrophages become immobilized in lymphoid tissue and lesions then develop wherever infected macrophages are present, including at the initial site of infection in the lungs.

Susceptibility to infection and the minimum infectious dose varies with the route of infection (Gavier-Widen *et al*., 2009). In cattle, the magnitude of the infecting dose influences the rate of disease progression and the severity of disease (Dean *et al*., 2005). It is likely the same applies to badgers. In badgers the primary route of infection is the lower respiratory tract following inhalation of a small infectious aerosol particle and the minimum infectious dose is probably one bacillus. Only particles with an aerodynamic diameter of ≤5 μm, which may contain up to five bacilli, are able to penetrate to the alveolar region where they are deposited by impaction on the alveolar epithelium (Cox, 1986). Tissues higher up the respiratory tract are more resistant to aerosol infection by *M. bovis* because of the presence of ciliated epithelium, a mucus layer and the muco-ciliary escalator (Pantelic *et al*., 2009). The alveolar regions are not covered by ciliated epithelium and clearance of deposited particles occurs by phagocytosis by alveolar macrophages. These macrophages are mobile and, having phagocytosed the droplet, they migrate into the lymphatic system of the host.

Primary infection of the gastrointestinal tract in badgers appears to be rare, as the low pH of gastric secretions acts as an efficient barrier to intestinal infection owing to mycobacteria being killed by acidic conditions of pH <4.5 (Tacquet *et al*., 1962; Giannella *et al*., 1973; Mortatti *et al*., 1987; Vandal *et al*., 2009). To establish infection of the gastrointestinal tract by ingestion, high doses of *M. bovis* were required in cattle, where a dose of 10^7 bacilli was required to reliably establish infection (Palmer *et al*., 2004). The concentrations of *M. bovis* excreted in badger faeces, urine and wound exudates rarely attain these levels (Gallagher *et al*., 1998). Another route of infection is inoculation with contaminated saliva during biting.

Badgers are competent hosts in responding to an *M. bovis* infection as the majority of infection is latent, and the resolution of lesions has been described (Gallagher *et al*., 1998; Gavier-Widen *et al*., 2001), as has the complete elimination of *M. bovis* infection (Clifton-Hadley, cited by Gallagher *et al*., 1998).

Diagnosis

The definitive gold standard for the confirmation of TB is the isolation and culture of

M. bovis from tissues obtained post mortem. However, mycobacterial culture is expensive, labour intensive and slow to generate results, taking up to 12 weeks to confirm a positive result in some cases. Furthermore, the confirmation rate of TB in badgers is proportional to the effort invested and increases as the number and variety of tissues examined increases (Crawshaw *et al*., 2008), as the culture period increases and where efforts are made to limit contamination (Crawshaw *et al*., 2008; Corner *et al*., 2012a). An advantage of isolating *M. bovis* from a badger by culture is that it allows molecular typing to be undertaken, such as spoligotyping or Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeats (MIRU-VNTR) typing, or the sequencing of the whole genome of each isolate (Biek *et al*., 2012), all of which facilitate greater understanding of the epidemiology of the infection.

The presence of gross lesions consistent with TB may be suggestive of disease but the diagnosis requires confirmation. Macroscopic tuberculous lesions must be differentiated from parasitic, fungal, bacterial and foreign body abscesses. *M. bovis* is frequently isolated from tissues with no visible lesions (NVL) but macroscopic lesions are more likely to be culture positive (Crawshaw *et al*., 2008). Infected animals that have NVL pose a particular difficulty in diagnosing TB in both domestic animals and wildlife hosts (reviewed by Gavier-Widen *et al*., 2009). To maximize sensitivity, samples taken for culture from NVL badgers should include not only the lymph nodes from all body compartments – that is, the body, head, respiratory tract and digestive tract – but also the lungs and abdominal organs. In many cases only a single or a few lesions may be present (Gallagher and Clifton-Hadley, 2000). The more intensive and comprehensive the examination, the greater the chances of detecting lesions. The increased intensity should take the form of a greater number of sites examined, thin slicing of lymph nodes and lungs, and close examination of all tissues.

Detection of disease, particularly where only small TB lesions are present, may be improved by histopathology. The finding of granulomas containing acid-fast bacilli in Ziehl–Neelsen stained sections may be taken as strongly indicative of TB in the absence of isolation of the causative organism. However, caution must be exercised in using the presence of acid-fast bacilli to confirm infection as the histological appearance of acid-fast bacilli does not allow differentiation of *M. bovis* from other mycobacteria, such as *M. avium*.

Detection methods based on polymerase chain reaction (PCR) offer the potential for faster and more specific detection of *M. bovis* in tissues, clinical and environmental samples. Genetic probes can be used to reduce the time for confirmation by culture or to avoid culture completely, although most probes are only MTBC group-specific. In general, culture is still more sensitive than PCR for the detection of *M. bovis* from post-mortem samples (Costa *et al*., 2013), although a more recent report suggests this position may soon change (Courcoul *et al*., 2014). A standardized, validated procedure for PCR detection of *M. bovis* is needed.

Recent attempts to validate a real-time PCR assay to detect and quantify *M. bovis* from environmental samples have been encouraging (Travis *et al*., 2011). Field studies are now being undertaken to determine how such assays might be applied for population-level TB surveillance in wildlife. Attempts to isolate viable *M. bovis* from air samples taken from setts known to harbour infected badgers and from the housing of infected badgers were unsuccessful (Jones *et al*., 2013). The frequent isolation of an environmental mycobacterium, *M. gordonae*, by this method demonstrates its feasibility but points to the quantity of viable *M. bovis* detectable in the environment of infected badgers being low.

Diagnosis of TB in the live animal

Culture of mycobacteria from samples taken from live badgers, such as urine, faeces, bite wound exudates or samples from the upper respiratory tract, provides poor diagnostic sensitivity. This is due to low prevalence of excretion in infected badgers, low concentration of bacilli in the samples, contamination of samples and intermittent excretion (Chambers *et al*., 2002). Diagnosis of TB in live badgers is typically based on the detection of

an immune response to *M. bovis* infection either in association with, or as a replacement for, culture of clinical samples. The principal immunological response of the mammalian host to infection with *M. bovis* is the acquired cellular immune response, exemplified by the proliferation of lymphocytes and the production of cytokines such as interferon-gamma (IFNγ). In this respect badgers are no different. Badgers are clearly able to mount a CMI response to *M. bovis* infection as evidenced by their ability to contain infection for several years and mount a normal proliferative response of T-cells to antigenic stimulation, albeit requiring high concentrations of antigen compared with other species, including larger animals such as cattle (Dalley *et al*., 1999).

The cutaneous immune response to the injection of tuberculin in badgers is weak (Higgins, 1985; Mahmood *et al*., 1987) and impractical for free-ranging animals because of the need to examine the skin for a cutaneous reaction at least 24 h after the injection of tuberculin. An increasing number of alternative *ex vivo* immunological tests are becoming available for the diagnosis of TB in badgers and wildlife in general (as reviewed in Chambers, 2009, 2013). The most sensitive test available at present is based on the detection and measurement of IFNγ by enzyme immunoassay following stimulation of whole-blood or isolated peripheral blood mononuclear cells with tuberculin (Dalley *et al*., 2008). The estimated sensitivity and specificity of the test is 80.9% and 93.6%, respectively. Sensitivity in young animals was lower (Chambers *et al*., 2009). The main disadvantages of the test are that blood samples must be processed within 10 h and results are not available until the following day. Specific mycobacterial antigens, ESAT-6 and CFP10, which are not expressed by BCG vaccine, have been incorporated into the IFNγ release assay to differentiate between vaccination with BCG and infection (Chambers *et al*., 2011) but together give a lower sensitivity than when bovine tuberculin is used (Dalley *et al*., 2008). Real-time multiplex PCR assays for a number of badger cytokines have been developed to aid better understanding of the CMI response of badgers (Chambers, unpublished results) and possibly increase diagnostic sensitivity.

Immunological tests based on an antibody (serological) response are available for badgers and are more suitable where low-cost, simple and rapid testing is required. The repertoire of antigens recognized by the humoral immune system of the badger appears restricted compared with some other species (Lesellier *et al*., 2008; Lyashchenko *et al*., 2008). The predominant seroreactivity of infected badgers is to the mycobacterial antigen MPB83, but antibody responses occur in only approximately 50% of infected badgers with the likelihood of seropositivity increasing with disease severity (Chambers *et al*., 2008). A considerable amount of data has now been generated with the STAT-PAK® test (Chembio Diagnostic Systems, Inc., Medford, NY), a rapid chromatographic lateral flow immunoassay similar in format to a human pregnancy test. The test appears particularly suitable for detecting badgers with advanced disease (Chambers *et al*., 2008) but has low sensitivity for detecting infection in badger carcasses (Chambers *et al*., 2010).

A couple of novel approaches to TB detection have also been tried. Positive results were obtained using an electronic nose (e-nose) to discriminate between serum samples obtained from tuberculous versus non-tuberculous badgers on the basis of recognition of a characteristic profile of volatile organic compounds released from serum when heated (Fend *et al*., 2005). The approach has not been taken forwards, largely due to the variability encountered between different e-noses. Most recently, an AC electrokinetic impedance sensing method has shown promise as a diagnostic tool for TB (Cui *et al*., 2013). The method is capable of detecting specific interactions between macromolecules such as antibody binding to antigen and was used to screen serum samples from humans and badgers with TB. Results were consistent with those obtained by a conventional ELISA method, demonstrating that AC electrokinetic impedance sensing can be used for rapid and sensitive detection of specific antibodies in serum samples. With further development, this method might form the

basis of an animal-side diagnostic device for detecting TB infection.

Application of diagnostic tests

For the detection of TB at the level of the badger social group, a combination of diagnostic tests may prove the most sensitive approach. The optimum sampling protocol depends on the sensitivity of detection required, the prevalence of infection and the number of animals sampled (Drewe *et al*., 2010).

The relationship between diagnostic status and infectiousness is not known in detail although animals with more visible lesions post mortem are more likely to be seropositive than those with NVL (Chambers *et al*., 2010) and by inference could be more likely to excrete larger numbers of *M. bovis*. Recently, data collected from longitudinal studies of naturally infected wild badgers have been used to attempt to determine the relationship between the magnitude of the response in the IFNγ release assay and the outcome of infection with *M. bovis* (Tomlinson *et al*., 2015). The magnitude of the early IFNγ response to *M. bovis* antigens was positively correlated with subsequent progression of disease to a seropositive or infectious state. In addition, the IFNγ response declined over time for all badgers, but remained significantly higher for those badgers with evidence of disease progression. These findings are an important first step in describing the progression of immune responses in naturally infected wild badgers, in parallel with clinical markers for disease progression such as excretion of *M. bovis*.

Tests to diagnose *M. bovis* infection in live badgers are not currently suitable for use in a disease-control setting because testing requires capturing and anaesthetising badgers to collect blood samples. However, work is currently under way to address this through the application of novel techniques for the collection of small volumes of blood from conscious badgers. Allied to the STAT-PAK® that produces a result within 15 min, or other rapid tests, it may be possible to quickly obtain a diagnostic test result on an individual animal in the field. Such an approach could open opportunities for targeted disease-control interventions such as vaccination or selective removal at the level of the individual badger or social group.

Epidemiology of *M. bovis* Infection in Badgers

Badgers are an ideal host for *M. bovis* as infected animals can survive for several years, maintaining social interactions and breeding successfully while excreting bacteria (Cheeseman *et al*., 1989; Tomlinson *et al*., 2013b). The majority of infected badgers remain apparently healthy, with a high proportion (33–80%) having latent infection (Gallagher and Clifton-Hadley, 2000).

The risk of transmission of infection from an infected badger is dependent on the stage of disease progression, the route of excretion and the route of exposure. The high prevalence of pulmonary lesions and the high frequency of lung infection in infected badgers strongly supports the lower respiratory tract as the principal site of primary infection and that inhalation of infectious aerosol particles is the principal mode of transmission (Gallagher *et al*., 1976; Dolan, 1993; Fagan, 1993; Gallagher and Clifton-Hadley, 2000; Gavier-Widen *et al*., 2001). The respiratory route is the mode of transmission between captive badgers (Little *et al*., 1982).

For aerosol transmission to occur badgers must be in close proximity, as with increasing distance between source and susceptible host there is a rapid decline in the probability of inhaling an infectious particle (Pantelic *et al*., 2009). There are a variety of environmental factors that influence the physical and biological survival of the airborne pathogen: damage to the bacterial cell during the initial droplet formation, desiccation and rehydration, temperature, relative humidity, ultraviolet (UV) light, ionizing radiation, Open Air Factor (OAF),[1] air ions and pollutants (Donaldson, 1978; Marthi *et al*., 1990; Walter *et al*., 1990; Cox, 1998; Nardell, 2004). Larger droplets (>5 μm) settle rapidly due to gravity; smaller droplets (<1 μm) do not contain bacilli (Wells, 1955).

Infection has been observed in badgers of all ages and there is an accumulated risk of infection with increasing age (Gallagher *et al*., 1998; Jenkins *et al*., 2008a). Generally there is a higher prevalence of infection in male badgers than in females (Wilesmith *et al*., 1986; Cheeseman *et al*., 1989; O'Keeffe *et al*., 1996; Corner *et al*., 2008a), which has been attributed to behavioural differences between the sexes, with males more involved in defence of territory and consequential aggressive behaviour (Gallagher and Clifton-Hadley, 2000). Recent work has shown that female badgers with a known history of clinical excretion of *M. bovis* by two or more routes (i.e. faeces, urine, sputum and pus from wounds: multi-route excretors) have a significantly reduced survival rate (38% per annum) compared to uninfected females, and males appear to have reduced survival associated with any measure of infection (14% per annum in multi-route excretors) (Graham *et al*., 2013). Furthermore, infected females survive longer than males following the detection of bacterial excretion and experience less substantial weight loss once infection has progressed to the point of excretion (Graham *et al*., 2013; Tomlinson *et al*., 2013b). Female badgers may play a critical role in the maintenance of infection at a social group level as heterogeneity in the risk of infection in cubs is linked to the presence of an infectious breeding female in the natal group (Tomlinson *et al*., 2013a).

As outlined above, the detection and diagnosis of infection in live badgers is challenging. Nevertheless, consistent application of diagnostic tests during a long-term study in Gloucestershire, southern England, has identified spatial and temporal trends in *M. bovis* infection (Delahay *et al*., 2000, 2013). The recorded prevalence of infection during this study has remained relatively static within the range of 10–20% of the population despite substantial growth and decline in the total population size. During a large-scale Randomized Badger Culling trial (RBCT) (see below), the initial prevalence of infection determined by post-mortem examination and tissue culture varied from 5.5% to 37.2% over approximately 100 km^2 (Independent Scientific Group on Cattle TB, 2007). Estimates of R_0, based on an intensively studied badger population, varied from 1.1 to 1.2 and increased to an upper value of 1.35 when the geographical population under study was restricted to account for the spatial clustering of *M. bovis* (Delahay *et al*., 2013).

At the population level, there appears to be no simple consistent correlation between prevalence and badger density or social group size (Vicente *et al*., 2007; Delahay *et al*., 2013). However, inter-group movements have been found to correlate locally with the incidence of new cases (Vicente *et al*., 2007), and at a population level, with an increase in the number of recorded cases in the following year (Rogers *et al*., 1998). These findings suggest a mechanism for culling-induced increases in the incidence of disease in badgers because the removal of animals has been shown to enhance inter-group movement among the residual and surrounding population (see below). The results of intensive field studies of badger behaviour indicate that TB test-positive badgers may range further (Garnett *et al*., 2005) and exhibit different patterns of seasonal sett use (Weber *et al*., 2013) compared to test-negative animals. The hypothesis that there are behavioural differences in infected animals compared to non-infected is further corroborated by field investigations of social networks in a badger population, which indicated that TB test-positive animals were more socially isolated from members of their own groups than test-negative animals, but occupied pivotal positions linking social groups (Weber *et al*., 2013).

Disease Transmission to Cattle

It is assumed that *M. bovis* transmission among badgers occurs largely via the respiratory route, with direct inoculation due to biting behaviour being a potential secondary route. Mechanisms of transmission between badgers and cattle are poorly understood although both direct (aerosol) and indirect (environmental contamination) routes have been suggested. A number of studies have shown that removal of badgers can reduce the incidence of *M. bovis* in cattle (see below), thus indicating that badgers contribute to infection in cattle. One recent analysis suggested that

badgers may be responsible for approximately 50% of cases in cattle, including subsequent cattle-to-cattle transmission (although the confidence interval was from 9.1% to 100%), with a contribution of about 6% (95% CI from 1% to 25%) for initial cases (excluding subsequent cattle-to-cattle infection) (Donnelly and Nouvellet, 2013).

Routine cattle testing is not sufficiently regular to detect any seasonal component of transmission from badgers, particularly if 94% of cases are caused by cattle-to-cattle infection (Donnelly and Nouvellet, 2013). The RBCT provided evidence that *M. bovis* transmission between badgers and cattle can occur in both directions (Jenkins *et al*., 2007). The proportion of cases in cattle that occur as a result of direct or indirect contact with badgers at pasture, compared to cases that occur within farm buildings cannot be determined. Cattle do graze grass that has been urinated on by badgers and may graze in close proximity to badger latrines, but direct contact at pasture is two orders of magnitude rarer (Drewe *et al*., 2013). Within farm buildings, cattle feed can be contaminated by visiting badgers and direct contact with housed cattle is much more likely than when cattle are grazing (Garnett *et al*., 2002; Tolhurst *et al*., 2009).

Control Strategies

Since they were first identified as a host for *M. bovis* 40 years ago, various options have been explored to manage the disease in badger populations. Improved biosecurity measures, culling and vaccination are considered as key approaches that can be used, singly or in combination, to either reduce infection rates within badger populations or limit the potential of transmission of infection from infected badgers to cattle. However, options for dealing with the disease in badgers are constrained by economic, conservation and societal reasons. The badger is a species protected by national and international law in Ireland and in the UK. Culling attracts a high level of public scrutiny and is widely reported in the media, resulting in divisive debate. Under the Bern Convention on the Conservation of European Wildlife and Natural Habitats the culling of badgers is only permitted as part of a bovine TB reduction strategy if there is no satisfactory alternative.

Biosecurity measures

The inevitably close association between cattle herds and badgers sharing the same environment provides opportunities for disease transmission either by direct close contact or indirectly through contact with excreta deposited by infected badgers. While it is important to reduce risks wherever possible, it is likely that managing both direct and indirect contact between badgers and cattle in farm buildings will be considerably easier than attempting to manage interactions at pasture. Badger latrines present a potential risk of *M. bovis* transmission to cattle at pasture but may be relatively easy to manage by cordoning off preferred latrine sites to deny access to cattle, although keeping cattle away from excretory products (especially urine) deposited elsewhere on pasture is not likely to be feasible. By contrast, the opportunities for transmission arising in farm buildings should be relatively easy to manage by adopting measures to exclude badgers. Badger visits to farm buildings can be a common occurrence on some farms. In one study, intensive surveillance for 1 year demonstrated that badgers visited buildings at least occasionally on 19 of 32 (59%) farms (Judge *et al*., 2011). On three of the 32 farms (approximately one in ten), visits were very frequent, occurring on more than 60% of nights. Badgers visited both feed stores and cattle housing, with visits to feed stores being more frequent. While badger visits to farmyards occurred all year round, they peaked in late spring/early summer. Badgers were successfully excluded from farm buildings with the use of relatively simple, practical measures, which were 100% effective when adequately applied and maintained. In fact the only recorded incursions into farm buildings during this study occurred when exclusion measures were not employed adequately. Furthermore, the installation of exclusion measures not only stopped entry into buildings but also reduced the level of badger visits to the farmyard as a whole.

Badger culling

The primary goal of culling is to reduce badger numbers in order to reduce the risk of transmission to cattle. Badger culling trials have been undertaken in both the UK and Ireland. The first UK large area study (104 km^2), conducted in 1975 at Thornbury, in Avon, was a non-randomized study with no experimental control or replication. For 6 years the badger population in the area was suppressed by gassing setts with hydrocyanic acid and culling by a variety of methods. The removal of infected badger populations substantially reduced the risk of *M. bovis* infection for cattle in the area and no new cases of TB in cattle were reported in the area until 1991 (Clifton-Hadley *et al*., 1995). The RBCT, directed by the Independent Scientific Group (ISG), commenced in 1998 and involved ten triplicate areas, each consisting of three 100 km^2 areas assigned to either no culling, reactive culling or proactive culling treatments. The reactive culling treatment was suspended in 2003 as the incidence of cattle TB in culled areas increased by 27% compared to the non-cull control areas (Donnelly *et al*., 2003). Proactive culling was completed in October 2005 and analyses showed a 19% reduction in the incidence of cattle herd breakdowns in proactive areas compared with non-cull areas, but a 29% increase in cattle TB incidence in surrounding areas (Donnelly *et al*., 2006). Follow-up analyses found that the beneficial decrease in cattle TB incidence observed within proactive cull areas accrued in subsequent years while the 'edge effect' waned (Jenkins *et al*., 2008b). Previous studies had shown that culling can disrupt the social structure of badger populations and promote enhanced movement among surviving individuals, phenomena collectively referred to as social perturbation (reviewed in Carter *et al*., 2007). Furthermore, it was suggested that these behavioural responses could potentially increase rates of contact among badgers and between badgers and cattle, thereby increasing the risks of disease transmission. Culling-induced spatial disruption and enhanced movement of badgers was observed in the wake of RBCT culls and has been invoked as the most likely cause of enhanced cattle herd breakdown rates in the reactive culling areas and around proactive culls (Woodroffe *et al*., 2006).

In 2011, DEFRA announced that badger culling would be carried out by farmers and landowners in two pilot areas to test the safety, humaneness and efficacy (in terms of badger removal) of this approach. Licences were issued by Natural England (under the Protection of Badgers Act 1992) to enable groups of farmers and landowners in the worst-affected areas to reduce badger populations for the purpose of preventing the spread of disease. Culling aimed to remove at least 70% of badgers across each area over a 6-week period. The target figures were not achieved within the required time frame so extensions of up to 8 weeks were granted, at the end of which both trials had failed to achieve the revised targets.

In Ireland the first formal badger removal study was conducted in east Offaly, and showed that proactive badger removal was associated with a significantly reduced risk of confirmed TB breakdowns in associated cattle herds (Mairtin *et al*., 1998a,b; Eves, 1999). This effect was sustained and continued to fall subsequent to the end of the trial in association with continued, but less intensive, badger removal (Kelly *et al*., 2008). A second trial (Four Area trial) involved intensive, proactive badger culling and led to similar results. Compared to reference areas where badgers were only removed if there was a severe TB outbreak, proactive removal areas experienced substantial reduction in the incidence of TB in each of the four counties every year of the study period (Centre for Veterinary Epidemiology and Risk Analysis, 2004; Griffin *et al*., 2005). These studies provided compelling evidence of the central role played by badgers in the epidemiology of bovine TB (Griffin *et al*., 2005; More, 2009).

The current interim programme being implemented in Ireland aims to reduce the local population of badgers from the starting density of two or more badgers per km^2 to a level in the range of 0.2–0.5 badgers per km^2. The activities are focused in areas of higher disease prevalence in cattle, which also exhibit higher disease prevalence in badgers (Murphy *et al*., 2010, 2011; Corner *et al*., 2012b). In these

high-prevalence areas, badger removal forms the basis of the disease-control strategy by reducing badger numbers and minimizing contact between cattle and infected badgers. An annual culling programme is managed to ensure these lower density levels are maintained. Currently, badgers are culled on 28% of the national area of agricultural land – an upper limit of 30% applies and between 5000 and 6000 badgers are removed per annum.

The available evidence appears to suggest that badger culling has different outcomes in terms of its impact on cattle herd breakdown incidence in the Republic of Ireland compared to the UK. Although culling approaches in the two countries are not directly comparable, such differences could potentially also arise as a consequence of variation in prevailing ecological conditions, particularly in relation to the density and social behaviour of badgers. Further studies on the mechanisms that determine the extent or otherwise of social perturbation in the wake of badger culling may help us to understand why such differences in outcome occur.

Badger vaccination

The aim of vaccination is to reduce the prevalence of infection in the badger population, or to change the expression of the disease and limit the rate of *M. bovis* excretion, thereby reducing transmission between infected badgers and susceptible cattle. Vaccination of badgers as a component of a disease-control strategy may be applied at different scales but the aim in each case is to reduce or remove the risk of transmission from badgers to cattle. There is currently no information on the impact of badger vaccination on TB incidence in cattle although the expectation is that any benefits of vaccination would be likely to accrue over time as infected badgers die off.

Detailed research has been conducted jointly in both Ireland and the UK with a view to the development of an effective badger vaccine and its implementation (Gormley and Collins, 2000; Gormley and Costello, 2003). The BCG vaccine was initially chosen for use based on its availability, safety in a variety of animal species, low production cost and extensive experience of its application in humans. It is presently the only candidate vaccine for badgers and has been shown to be safe when administered parenterally to captive badgers (Lesellier *et al*., 2006). Research on BCG vaccination of badgers against TB was first conducted in captive animals, and involved experiments and associated studies to determine the efficacy of vaccine delivered by a variety of routes. Badgers mount a protective immune response against experimental challenge with *M. bovis* following BCG vaccination by a variety of routes, including parenteral (subcutaneous (s/c) and intramuscular (i/m)) or mucosal (conjunctival and oral) (Corner *et al*., 2008b, 2009, 2010).

Whereas captive badger studies are the most cost-effective way of examining the protective response to vaccination, they cannot be used to predict whether BCG will be protective in free-living badgers subject to natural transmission, nor to estimate vaccine efficacy. Field evaluation of BCG administered i/m to captured badgers demonstrated that the vaccine reduced the incidence of new *M. bovis* seropositive cases in badgers by up to 74% relative to unvaccinated badgers (Chambers *et al*., 2011). Follow-up analysis showed that, in addition to a significant direct benefit of BCG vaccination in individual badgers, there was a significant indirect positive effect of vaccination on unvaccinated cubs born into vaccinated groups; the most plausible explanation being an indirect protective effect through the generation of herd immunity. The results of that field study paved the way for the vaccine to be granted a licence for use in the UK in 2010 (BadgerBCG; the marketing authorization holder is the Animal and Plant Health Agency, Addlestone, Surrey, UK). A subsequent feasibility study (the Badger Vaccine Deployment Project) was set up to investigate practical aspects of the wider implementation of badger vaccination. The project involves trapping and vaccinating badgers in a 100 km^2 area in Gloucestershire, with the primary aims of learning lessons about the practicalities and costs of deploying an injectable vaccine and providing training for others who may wish to apply for a licence to vaccinate badgers.

In 2012 the Welsh Government embarked on a 5-year BadgerBCG vaccination project within their bovine TB 'Intensive Action Area' (IAA) in an effort to support the eradication of bovine TB from Wales. The IAA measures approximately 200 km^2 and is located primarily in a bovine TB endemic area, incorporating north Pembrokeshire and small parts of Ceredigion and Carmarthenshire. Alongside vaccination additional control measures (including enhanced cattle surveillance), improved biosecurity measures and surveillance on non-bovine hosts for TB (goats and camelids) are being carried out. The purpose of the vaccination project is to vaccinate the maximum number of badgers within the area each year for 5 years and to monitor the impact annually on cattle herd breakdowns. It may take additional years to detect any measurable effect of these combined measures in cattle herds. In the first year of the project, 1424 badgers were captured and vaccinated.

Although BCG is currently available for parenteral administration to badgers, the ideal vehicle for wide-scale vaccine deployment is a palatable bait. Hence, a significant amount of research effort in the UK and Ireland has focused on the development of a cost-effective bait that is attractive to badgers and compatible with live BCG vaccine. Several field studies have also been initiated to investigate levels of uptake of candidate baits in wild badger populations.

A 3-year field trial has recently ended in Ireland with the aim of measuring the impact of oral BCG vaccination on disease levels in badgers and to measure the effect of BCG vaccine in badgers with pre-existing *M. bovis* infection. The hope is that it will validate the results of captive badger studies (Corner *et al*., 2010) by showing that the oral BCG vaccine is protective in wild badgers naturally exposed to *M. bovis* infection. In the field trial area in Co. Kilkenny, different proportions (0%, 50% and 100%) of the trappable badger population were vaccinated orally with about 10^8 cfu/ml BCG-Danish strain contained within a lipid formulation (produced by Immune Solutions Ltd, Otago, New Zealand). Badgers were vaccinated individually by administration directly into the pharynx while the animal was under general anaesthesia. Study personnel were 'double-blinded' to which groups of animals received vaccine or a placebo comprising lipid alone. Vaccination was conducted annually and the population examined twice a year. At the end of the 3-year study period the trial site was depopulated and all badgers caught were examined for TB by detailed post-mortem examination. The isolation of *M. bovis* from post-mortem or clinical samples (wound exudates or tracheal swabs) was used to define a case of TB. Analysis of the results is due to be completed in 2015.

Further field vaccination studies have recently commenced in Ireland to vaccinate subpopulations of badgers by means of a capture/vaccinate/release protocol using the injectable vaccine. The targeted badgers are those captured in previously culled, low-density badger areas with a history of high levels of TB in both cattle and badgers. The study comprises a vaccinated area and a control area where culling will be maintained. Levels of bovine TB will be monitored and compared in these areas over 5–10 years.

In 2012, following discussions with industry stakeholders and informed by the views of the external experts who attended an International Vaccination Symposium in Belfast, the Agriculture and Rural Development Minister for Northern Ireland announced a field study to investigate a 'test and vaccinate or remove' (TVR) strategy involving the serological testing of live badgers, vaccinating and releasing the test-negative badgers, and removing test positives. This approach of vaccination combined with selective culling aims to avoid killing presumptive TB-free badgers. The project will monitor for any reduction in transmission of TB to cattle in the study area.

Modelling *M. bovis* Infection in Badgers

Field interventions with sufficient scientific rigour to detect changes in epidemiological dynamics in either badgers or cattle are expensive and time consuming, hence the attraction of using mathematical models to

simulate the likely outcomes of proposed interventions. Numerous models have been constructed to evaluate historical badger culling, predict outcomes of badger vaccination, and combined vaccination and culling scenarios (e.g. White and Harris, 1995; Smith *et al*., 2001; Wilkinson *et al*., 2004). The accuracy and reliability of these models depends on the quality of the information available for model parameterization. Parameters describing aspects of badger biology are well described in the literature, but there is a paucity of quantitative information on epidemiological aspects of infection in badgers and cattle, mainly due to diagnostic limitations (see above). Although models have been useful in helping to inform decision making, they are limited in their accuracy due to the difficulty of describing the transmission dynamics within and between species. Models are generally either density dependent or density independent in their structure. The former are more common but predict that prevalence should be correlated with population size and that there is a clear threshold density below which the disease will not persist. Such models must therefore take account of changes in the behaviour of badgers as their density is reduced by culling (see Carter *et al*., 2007). However, there are very limited empirical data available on this response and so the degree of culling-induced 'social perturbation' in badgers has to be inferred through secondary measures such as cattle herd breakdown rates. The level of increased badger-to-badger contact following culling and the time taken for this to return to normal appear to be critical in determining the optimal approach for managing disease in badger populations (Smith *et al*., 2012). A further challenge in modelling the management of infection in badger populations is simulating realistic responses to vaccination. In models of other disease systems it is commonly assumed that the vaccinated individual is completely protected from disease, whereas the available field data cannot differentiate a vaccine that completely protects vaccinated animals from a vaccine that reduces the rate of disease progression (and thus the proportion of animals that test positive) in all vaccinated animals. These challenges limit the extent to which existing models can identify the most beneficial approach for disease control.

Concluding Remarks

Badgers are clearly an important wildlife reservoir of *M. bovis*, although the extent of their contribution to infection in cattle and the appropriate means of managing transmission risks are hotly debated. TB in badgers has been the subject of a considerable body of research, including several costly large-scale field experiments, yet significant gaps in our knowledge still remain.

In the UK and Ireland, application of comprehensive cattle test and slaughter programmes has served to reduce the incidence of TB in national herds substantially. From a public health perspective, the reduction of disease levels in combination with improved animal husbandry and routine pasteurization of milk has also helped to lessen the risk of zoonotic transmission of infection to herd owners and the general public. Nevertheless, it is unlikely that the disease will be eradicated from cattle without the reservoir of *M. bovis* infection in badgers also being adequately addressed (Gormley and Collins, 2000). There are recognized complex relationships between badgers, cattle and *M. bovis*. Therefore, any strategy devised to control the disease in badgers may result in both beneficial and detrimental effects on the incidence of TB in cattle. The culling of badgers is an emotive subject that is deeply contentious with respect to its likely impact on disease control. The response to public consultation has shown that the majority of the British public (95.6%) are opposed to culling (Defra, 2006). The pilot cull of badgers in Gloucestershire and Somerset in 2013 attracted widespread public protest requiring significant police resources and costs to maintain local public order. A critical area of current data shortfall is how the number of badgers removed and the method of removal relate to the magnitude and duration of the potentially detrimental epidemiological consequences of social perturbation. This makes it difficult to accurately

predict the likely outcome of control measures that include a culling component.

Media coverage of the cattle TB issue is notable for its focus on badgers and the question of whether or not they should be culled. Considerably less attention has focused on other potential factors involved in the spread and maintenance of the disease such as cattle movement, the quality of the testing programmes, or the role of the farmer in improving herd biosecurity. Reducing the potential for direct or indirect contact between badgers and cattle should plausibly lower the risk of disease transmission between the two species, although the most significant route of infection between them is unknown. This lack of fundamental knowledge may explain why previous studies have found that farmers may not always employ practical measures to reduce direct and indirect contact opportunities between badgers and livestock. In surveys, the majority of farmers asked did not favour this approach, the most common reason being that they considered it to be impractical and expensive to implement (Bennett and Cooke, 2005). In order to promote the future uptake of precautionary biosecurity measures on cattle farms it will be important to provide farmers with sufficient information on their potential benefits in terms of reducing disease risks so that they can assess their cost effectiveness.

As a long-term disease-control measure, vaccination of badgers may provide the most attractive option as it has the potential to result in eradication of disease and has no known detrimental effects on badger populations. However, the duration of protective immunity in vaccinated individuals and the effectiveness of disease control by vaccination under field conditions have yet to be assessed. There is no guarantee that successful vaccination of badgers will impact significantly on herd breakdown rates in the absence of other control measures targeted at cattle. It is also most likely that the BCG vaccine will only be protective against new infections, with no anticipated therapeutic effect in badgers that are already infected. The perception that the wide-scale implementation of badger vaccination is likely to be a protracted affair has tended to dampen enthusiasm among farmers and the wider public that this approach can provide benefits in the short to medium term. In order to campaign for and promote vaccination as an alternative to culling, private interest groups combined resources in 2014 to launch the Badger and Cattle Vaccination Initiative (BACVI) with the stated aim to gather donations to sponsor vaccination projects in counties in south-west England. The project is also keen to support cattle vaccination as a disease control measure where possible.

The management of TB in badgers and cattle presents considerable veterinary and ecological challenges, not least because the factors that permit infection to be maintained are likely to vary according to local circumstances. Eradication of the disease will require input from veterinarians, epidemiologists and ecologists, mathematical modellers, economists and others. Public debates will need to progress beyond the reductionist yes/no question on the merits of badger culling, and address broader issues surrounding the underlying spread of TB, its potential causes and sustainable, long-term solutions.

Note

[1] Open-air factor (OAF) (Druett and May, 1969) is one or more pollutants that can kill microorganisms when suspended as aerosols.

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17 Tuberculosis in Pigs and Wild Boar

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Introduction

The Eurasian wild boar (*Sus scrofa*) is a native wild suid with an ancestral range reaching from the Far East to Western Europe and northern Africa. It is the ancestor of the domestic pig. Maintenance of backyard and semi-free-ranging domestic pigs, along with escapes and releases of pigs, wild boar or their crossbreds, have resulted in an almost global distribution of the different forms of *S. scrofa*.

Wild boar and pigs are mostly herbivores obtaining their food often by rooting the ground. They also consume invertebrates such as insects and earthworms, as well as small mammals and birds, or carrion if it is available. Wild boar and feral pig densities can easily reach values of over ten individuals per square kilometre. However, true densities are difficult to assess and most monitoring is based on cull or hunting harvest data. These data indicate increasing populations throughout the ancestral range, as well as in areas with introductions.

Wild boar and feral pigs form matriarchal groups and can reproduce as early as in their first year of life. Litter size is variable between three and eight (with up to three litters produced per year) and life expectancy is high if the hunting pressure is moderate and food plentiful. With high but extremely variable birth and death rates, the exponential rate of increase varies widely (0.25–0.78 under good conditions in Australia; Choquenot *et al*., 1996), resulting in booms and busts in numbers. Thus, wild boar can easily become a nuisance for traffic, agricultural crops, and animal and public health. In addition, feral pigs can be a nuisance for conservation of rare plant species or ground-nesting birds, particularly on islands.

Wild boar and pigs are suitable hosts for members of the *Mycobacterium tuberculosis* complex (MTC), mainly *M. bovis* and *M. caprae*. Suids are of interest in the study of MTC because of their epidemiological role as sentinel and maintenance hosts; because of the usefulness of the pig model for understanding MTC immunology and pathogen–host interaction; and because of the recent developments in TB diagnostics and control that focus on wild boar and pigs and might be extrapolated to other situations.

Epidemiology

Suids, along with a long list of other mammals, are prone to MTC infection. Their status as MTC hosts varies markedly between countries

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

and regions depending on density, dispersion, the levels of infection in other species and ecological factors. Where their densities are low, and the population is widely dispersed as in New Zealand, the rate of intraspecific transmission is too low for feral pigs to independently maintain the disease (Nugent *et al*., 2012). Nonetheless, MTC can persist in such populations (and sometimes reach prevalences of up to 100% in some age classes) if there is frequent spillover transmission from other hosts. Where that occurs, control or eradication of MTC in the other host species has resulted in the disappearance of MTC from pig populations. That has been demonstrated in Australia, where culling or removal of cattle and buffalo resulted in the disappearance of MTC from feral pigs (McInerney *et al*., 1995). Likewise, intensive lethal control of brushtail possums (*Trichosurus vulpecula*; the main wildlife host in New Zealand) results in the rapid decline of MTC prevalence in pigs without any management of the pig population (Nugent *et al*., 2012).

Where pigs are spillover hosts, they may still play some role in overall MTC epidemiology if there is occasional transmission from pigs back to some other species in the overall host complex (Nugent, 2011) by spreading the disease to new, previously uninfected locations. Spillback transmission can potentially also undermine MTC management efforts directed at other hosts because pigs carrying the disease through time may eventually cause re-establishment of the infection in the target host, as has been shown with wild deer (Barron *et al*., 2013). Offsetting those risks, the status of feral pigs as spillover hosts in New Zealand has enabled managers there to use them extensively as sentinels for detecting the continued presence of MTC in other hosts in an area. Their very high utility as sentinels reflects their omnivorous propensity to scavenge the carcasses of most if not all other MTC-infected hosts, and their large home range sizes (Nugent *et al*., 2002).

Where pig or wild boar densities are high, and/or ecological and management factors promote high levels of aggregation, the rate of transmission within the species increases to levels at which MTC can be maintained independently without spillover from other species. This is the main focus of this chapter, and appears to be the case for the Mediterranean habitats of south-western Spain and south-eastern Portugal, where wild boar are regarded as a key component of the wildlife MTC reservoir in the Iberian Peninsula. Feral pigs have also been implicated in *M. bovis* maintenance in Molokai (Hawaii, USA), and semi-free ranging domestic pigs are regarded as a *M. bovis* reservoir in the Mediterranean island of Sicily (Italy). Moreover, wild boar or pig tuberculosis (TB) due to members of MTC is increasingly being reported from many countries all over the world. Australia and New Zealand aside, the true role of wild boar and pigs in MTC epidemiology is still largely unknown for a broad range of settings in Africa, Asia, Europe and America.

Wild boar infection with MTC can occur in the first months of life. That is uncommon in feral pigs in New Zealand (Nugent *et al*., 2015) but in one Spanish study where the overall MTC prevalence was higher, one-third of the 2–3-month-old wild boar piglets were confirmed as infected by culture (Che Amat *et al*., 2015), suggesting different transmission pathways. In heavily infected areas in both countries MTC prevalence increases with age – in Mediterranean Spain an average of 45% prevalence in weaners (6–9 month-old), 60% in yearlings and 70% in adults. There, an increasing temporal trend has been identified over a large area, reaching over 60% prevalence, and is apparently still increasing (Vicente *et al*., 2013). There is no effect of sex on MTC prevalence. Other individual factors modulating wild boar infection with MTC include belonging to an infected social group, genetic traits and possibly co-infections. Genetic traits and dry summers can also determine the likelihood of acquiring generalized TB after infection (Acevedo-Whitehouse *et al*., 2005; Gortázar *et al*., 2011; Vicente *et al*., 2013).

In Mediterranean Spain, almost 50% of all MTC-infected wild boar display lesions in the thoracic region, suggesting a likely route of excretion of mycobacteria (Martín-Hernando *et al*., 2007). Sicilian black pigs also show a high proportion of lung lesions (Di Marco *et al*., 2012). This contrasts with findings from Atlantic Spain and from France, where the proportion of wild boar with thoracic TB lesions

is consistently below 10% (Muñoz *et al*., 2013), and with New Zealand where MTC lesions in feral pigs occur predominantly in the head-associated lymph nodes (Nugent *et al*., 2011). This suggests that infection source and some habitat-mediated factors such as co-infections (or lack of these), resource availability or age at first infection might modulate the likelihood of lesion generalization.

Population factors determining wild boar TB prevalence include density, spatial aggregation, resource concentration (e.g. waterholes or food patches), fencing, low rainfall, TB prevalence in sympatric host species such as cattle and co-infection with e.g. Porcine Circovirus type 2 (Acevedo *et al*., 2007; Risco *et al*., 2013; Vicente *et al*., 2013). Some of these factors are ultimately driven by intensive management for hunting (Vicente *et al*., 2007, 2013). Moreover, TB lesions are not uniformly distributed in wild boar populations with high disease prevalence. Rather, only few individuals (less than 10%) have generalized infection with very high lesion scores. They are often yearlings or young adults, and are probably contributing disproportionately to new infections, acting as so-called 'super-shedders'. Telemetry surveys revealed that MTC is a significant contributor to wild boar mortality in Mediterranean Spain, particularly in yearlings and young adults (Vicente, unpublished data). Wild boar with terminal TB often wander away from their usual home ranges and often die on cattle farms, possibly attracted by the more readily available food resources on them.

The routes of MTC transmission and maintenance within wild boar or pig populations, and the routes of inter-species MTC transmission, are still largely unknown. For feral pigs in New Zealand, and wild boar elsewhere, the almost universal involvement of the mandibular lymph node suggests entry through the oral mucosa but we cannot distinguish between respiratory or oral infection routes.

In southern Spain, approximately a quarter of randomly sampled animals excrete mycobacteria by the oro–nasal route (Vicente, unpublished data), evidencing its potential role as TB spreader. In 2–3-month-old wild boar piglets, transmission between infected and uninfected piglets was practically absent despite keeping weaned piglets in captivity for almost 4 months (Gortázar, unpublished data). This suggests that infected piglets have a very limited contribution to MTC transmission. By contrast, the high proportion of yearlings with severe thoracic lesions suggests that wild boar of this age class (and particularly the proposed 'super-shedders') contribute to most MTC contamination. Since there is a known link between aggregation at waterholes and TB prevalence (Vicente *et al*., 2007), and since MTC contamination has been detected at waterholes (over 50% of the studied sites, Vicente, unpublished), we postulate that indirect (oral) transmission is the most significant route for intra- and inter-species MTC transmission in Spanish wild boar. A recent observational study in the field has documented frequent indirect contacts between wild boar and livestock, especially at waterholes in summer (Kukielka *et al*., 2013). Significant reductions of wild boar numbers have been followed by declines in cattle and deer TB (Boadella *et al*., 2012), and exclusion of cattle from waterholes used by wild boar (and deer) was followed by a decline in cattle TB (Barasona *et al*., 2013). Both observations support the hypothesis of a largely indirect inter-species transmission of MTC, at least in dry Mediterranean habitats. However, wild boar are also carrion consumers, and hence transmission through carrion (and hunting gut pile) consumption may also contribute to MTC maintenance. Of course, respiratory transmission can also not be ignored, at least between closely interacting individuals belonging to the same social group.

A summary of knowledge/technology gaps regarding the epidemiology of MTC infection in wild boar and pigs is provided in Box 17.1.

Immunology

Rooting and wallowing in muddy water, along with a broad dietary spectrum, are likely to bring wild boar and pigs in frequent contact with mycobacteria. Molecular characterization of host–pathogen interactions identified *S. scrofa* genes such as methylmalonyl CoA mutase (MUT), complement component

Box 17.1. Current Knowledge/Technology Gaps Regarding Wild Boar and Pig TB Epidemiology.

- Host status of wild boar outside Mediterranean Spain and Portugal; and of feral pigs and semi-free ranging domestic pigs outside Molokai (USA), Sicily (Italy), Australia and New Zealand.
- Why do wild boar and pigs develop lung lesions more often in dry Mediterranean habitats than in more humid habitats?
- Which factors make an individual develop severe lung lesions and eventually become a super-shedder?
- Which are the routes of MTC transmission within wild boar or pig populations, and which are the routes of inter-species MTC transmission?
- What is the actual contribution of wild boar to MTC maintenance in complex (multi-host) versus simple host systems?
- What are the relative contributions of indirect environmental sources and direct animal-to-animal contact to MTC spread and maintenance?

3 (C3) and other innate and adaptive immune response genes involved in resistance to mycobacterial infection (Naranjo *et al*., 2006a,b, 2007, 2008a; Galindo *et al*., 2009; Pérez de la Lastra *et al*., 2009; de la Fuente *et al*., 2011). Global gene expression profiles in the spleen of European wild boar naturally infected with *M. bovis* showed that immune response was among the biological processes most affected by infection (Galindo *et al*., 2009). Beta-defensin 129, T-cell surface glycoprotein CD8 and B-cell receptor-associated protein 29 were overexpressed in infected animals. Lower expression levels of the immune response genes galectin-1, complement component C1qB and certain HLA class I and class II histocompatibility antigens and immunoglobulin chains were found in infected animals (Galindo *et al*., 2009). The results of the differential expression analysis by suppression-subtractive hybridization in oropharyngeal tonsils and mandibular lymph nodes of field-collected tuberculous and nontuberculous European wild boar showed differences between tonsils and lymph nodes, suggesting that these organs play different roles during MTC infection and progression in this species (Naranjo *et al*., 2006a). Differential gene expression in tonsils and lymph nodes of tuberculous wild boar had some common features evidenced by the inhibition of phagosomal maturation and/or endocytosis and by osteopontin expression, which correlates with pathologic processes. However, evidence of apoptosis in tuberculous animals was found in tonsils only, whereas correlates of Th2 and inflammatory responses were found exclusively in lymph nodes. Tonsils and lymph nodes of nontuberculous animals also presented common differentially expressed genes including C3, as well as other genes that participate in key cellular processes relevant for TB pathogenesis such as Th1 response, antigen presentation and prevention of apoptosis. The genes differentially expressed in nontuberculous wild boar may represent downregulation as a result of mycobacterial infection in tuberculous animals and/or upregulation in nontuberculous individuals as a result of individual resistance and/or protective response to infection. Pathway-focused analysis revealed a role for the Jak-STAT pathway during mycobacterial infection, resulting in downregulation of some of the Jak-STAT effectors such as interleukin 5 (IL-5) and tyrosine kinase 2 (TKY2) (Galindo and de la Fuente, 2012). Additionally, experimental infection of minipigs suggests that the infection induces an initial Th1 response, which is followed by local fibrosis and encapsulation of the granulomas that result in disease control (Gil *et al*., 2010).

Swine infected with *M. bovis* are a useful animal model for elucidating the mechanisms of pathogenesis and host defence to tuberculosis in humans (Bolin *et al*., 1997; Naranjo *et al*., 2008b; Ballesteros *et al*., 2009; Gil *et al*., 2010; Garrido *et al*., 2011). The upregulation of C3 in lymph nodes and tonsils correlates with resistance to tuberculosis in wild boar (Naranjo *et al*., 2006a,b; Pérez de la Lastra *et al*., 2009) and

this mechanism may be enhanced after vaccination with BCG or inactivated *M. bovis* vaccines (Ballesteros *et al*., 2009; Garrido *et al*., 2011). *M. tuberculosis* acquires opsonic C3 peptides by at least two distinct mechanisms and can bind to CR3 at two distinct sites on the receptor (Ernst, 1998). Opsonized *M. tuberculosis* binds CR3 at its C3bi binding domain, and nonopsonized *M. tuberculosis* uses its endogenous capsular polysaccharides to interact with the β-glucan binding site near the C-terminus of CD11b (Ernst, 1998). Higher C3 levels may allow increased opsonophagocytosis and effective bacterial clearance, while interfering with CR3-mediated opsonic and nonopsonic phagocytosis of mycobacteria, a process that could be enhanced by specific antibodies against mycobacterial proteins and/or lipids together with other immune mechanisms (Carroll *et al*., 2009).

In summary, MTC infection induces humoral and cell-mediated immunity in wild boar and swine, but differences exist depending on mycobacterial species and genotypes (Stepanova *et al*., 2012). The upregulation of host innate immune pro-inflammatory genes and signalling pathways constitutes a general antibacterial mechanism in response to intracellular bacteria such as mycobacteria (Galindo and de la Fuente, 2012). The results obtained from molecular studies suggest that mycobacteria induce host innate immune responses while manipulating adaptive immunity to circumvent host-cell defences and establish infection.

A summary of knowledge/technology gaps regarding the immunology of MTC infection in wild boar and pigs is provided in Box 17.2.

Current Methods of Diagnosis and Intervention

Diagnosing MTC infection in carcasses of naturally infected wild boar or pigs is much the same as diagnosing MTC infection in other mammalian taxa. In essence, mycobacterial culture of selected organs (notably the lungs) and lymphoid tissues (mainly the mandibular and left bronchial LNs and the tonsil of the soft palate) in combination with pathology is the most efficient combination. It has been estimated that the proportion of wild boar with no visible lesions is around 20% of the total known-infected individuals.

In live wild boar and pigs, TB due to MTC can be confidently diagnosed by skin testing, gamma interferon testing and serology. Skin testing requires intradermal injection of bovine PPD, usually at the base of the ear (adults) or in the inguinal region (piglets), followed by measuring the increase in skin thickness at 72 h. This technique has a sensitivity of 75–100% and a specificity of 77% but has the disadvantage of being time consuming and, at least with adult wild boar, risky and impractical (Jaroso *et al*., 2010). As an alternative, both plate ELISAs and lateral flow rapid tests are available, with sensitivities ranging from 80% to 90% and a very high MTC specificity (100% in some reports; Boadella *et al*., 2011). One weakness of antibody detection tests is the possibility of false-positive results arising from infections with *M. microti* or other distant members of MTC. Gamma interferon testing has also been shown to be a reliable alternative to skin testing, requiring

Box 17.2. Current Knowledge/Technology Gaps Regarding Wild Boar and Pig Immunology in Relation with MTC Infections.

- Systems biology approach to the characterization of host–pathogen interactions in both infected and uninfected animals exposed to mycobacterial infection.
- Association between MTC strain genotype and host immune response.
- Association between host population genetic structure, microbiome, immune response and mycobacterial infection.
- Role of humoral immunity in control of mycobacterial infection.
- Validation of host- and pathogen-derived biomarkers associated with disease outcome and prevalence.
- Mechanisms responsible for protection against mycobacterial infection.

only one handling of each individual for harvesting blood cells. As in other species, white blood cells are incubated overnight with bovine PPD and the gamma interferon production is later measured by a porcine interferon-specific ELISA test. However, this technique is time consuming and expensive and hence likely to be limited to scientific studies and experimental infection trials.

For large-scale surveys where culture and histopathology are too expensive (as when large numbers of samples are obtained from hunter harvests), serology and gross pathology have proved to represent affordable alternatives. It is hoped that, in the future, developing non-invasive sampling and diagnostic tools will facilitate the monitoring of MTC in non-harvested wild boar and feral pig populations, as well as piglets. Two avenues to follow in this regard are testing faecal samples, and collecting and testing saliva samples for MTC or antibodies.

When considering MTC control in wildlife populations, monitoring is an essential requirement wherever there is need to assess the effectiveness of any MTC management efforts. This should include both population (relative abundance) monitoring and disease monitoring, in parallel. Given the multi-host nature of MTC, monitoring should consider all known hosts in the system. In domestic pigs, population monitoring can easily be performed using the available livestock census. Pig TB can be monitored at the slaughterhouses, but requires aetiological confirmation since lesions can be confounded with those of other mycobacteria. Alternatively, pig sera collected for other disease monitoring purposes can be tested by ELISA. Regarding wild boar and feral pigs, large-scale monitoring of population abundance in Europe occurs most often through hunting harvest results, although specific abundance estimation techniques have become available. For monitoring TB in hunter-harvested wild boar or feral pigs, a combination of gross pathology with serology and culture confirmation of selected cases provides a good balance between cost, effort and results (e.g. Richomme *et al*., 2013; Vicente *et al*., 2013).

The main disease control options include reducing the frequency of intra- and inter-species contact, population reduction, test and cull, and vaccination. These options are described in the following paragraphs.

Reducing the frequency of contact between (potentially infected) pigs and wild boar to other species, particularly cattle, is unlikely to reduce pig and wild boar TB where there are maintenance hosts, since it has been shown that wild boar populations without contact to other hosts are able to maintain MTC circulation. However, this should reduce inter-species transmission to other valuable livestock or wildlife. Methods for reducing inter-species contacts include the manipulation of waterholes, and potentially manipulation of feeding sites. In one experiment carried out in Mediterranean Spain, six waterholes were identified on a cattle farm. Three of these waterholes were fenced to prevent entry by cattle, but allowing wildlife (red deer and wild boar) to access the water (in the case of wild boar, by crossing under the fence). The remaining three waterholes were high-wire fenced and a door installed that provided access to cattle only (a modified bump-gate system). After these modifications, which drastically reduced indirect cattle–wildlife contacts at waterholes, cattle TB incidence declined on the farm, in contrast with the general trend of cattle TB incidence in the study area (Barasona *et al*., 2013). One of the most common approaches to reducing wildlife disease prevalence is to reduce intraspecific contact rates by reducing population density, usually through lethal control. The rationale behind this management tool is that many infectious diseases are density dependent; thus, fewer animals mean lower direct and indirect contact rates, and hence reduced disease transmission. The approach has been very successful in eradicating TB from wildlife over large areas in New Zealand but has required reductions of >95% in the densities of the main host, possums. Reductions of that magnitude are often not acceptable in North America and Europe where the main hosts are native species.

In the specific case of wild boar and feral pigs, increased hunting or culling pressure is likely to affect the age distribution of the population (towards a younger mean age). This change in the age distribution will also contribute to lower TB prevalence, since this

prevalence has been shown to increase with age. A series of observational surveys in Spain has demonstrated that reducing the wild boar density by about 30–90% reduces wild boar TB prevalence by 25–50%, with positive flow-on effects of reduction in TB prevalence in sympatric TB hosts such as deer and cattle (Boadella *et al*., 2012). However, population reduction may not achieve complete disease eradication unless almost all suitable hosts are eliminated from a system and unless new immigration of infected hosts is impeded. Hence, culling is not a sustainable tool for wildlife TB eradication. Even so, lethal control may help to reduce initially high infection rates prior to the start of other interventions such as vaccination.

Test and cull, also known as selective or targeted culling, is how TB has been controlled in cattle in most developed countries. This technique, based on eliminating only test-positive individuals, is also applied to farmed deer. In pigs, test and cull schemes can easily be applied where all or a large proportion of the herd or population can be easily tested, particularly since highly sensitive and specific ELISA tests have become available. This would be the strategy of choice in MTC-infected semi-free ranging pig herds on Sicily and in some other Mediterranean regions, for instance. It is less practical for genuinely free-ranging wild boar because of the high cost of capturing the animals for testing. As a result, a test and cull regime has only been used once, in a still ongoing intervention in a high-prevalence area in southern Spain. There, ~700 wild boar have been live-captured, tested with lateral flow rapid tests and microchipped. Test-negative individuals were released in the capture site. Test-positive animals were released in a different (fenced) site and hunted. The rationale behind this management is involving the hunters in wild boar TB control, without causing a significant loss of hunting-derived income. Since positive animals are culled by hunting there are no losses because of TB control. However, it is still too early to assess the efficacy and practicality of this intervention.

In contrast to lethal control, vaccination has the advantage of being more acceptable to the public and landowners who value wild boar. Wildlife vaccination is regarded as an important component of future TB control strategies in Ireland and the UK, and important steps in developing wildlife vaccination tools have also been made in the USA for white-tailed deer (*Odocoileus virginianus*) and in New Zealand for the Australian brushtail possum. In Spain, successful vaccination and challenge experiments with captive wild boar, along with developments enabling an efficient and selective bait deployment to wild boar piglets, allowed the setting up of the first field vaccination experiments in mid-2012 (Beltrán-Beck *et al*., 2012). Two vaccines are currently used, the attenuated BCG mutant of *M. bovis*, and a new heat-inactivated *M. bovis* vaccine.

M. bovis BCG is the vaccine used in all key wildlife maintenance hosts. A major advantage is its ready availability, since it is widely used for humans in high-prevalence countries. In pigs and in wild boar, as in most other species including humans, BCG does not produce complete immunity from infection, but rather a reduction in lesion development resulting in reductions in clinical disease and infection spread. Single oral doses of about 106 colony-forming units (cfu) of *M. bovis* BCG reduced wild boar mean total lesion scores by 52–56% and thorax lesion scores by 70% (Ballesteros *et al*., 2009; Garrido *et al*., 2011). Re-vaccination apparently boosted the protective response, with 76% reduction in the lesion score (Gortázar *et al*., 2014). Hence, wild boar (and possibly pigs) develop responses to orally administered BCG that limit the consequences of challenge with an *M. bovis* field strain.

However, BCG is a live vaccine and has three main drawbacks:

1. It might be excreted by the vaccinated individuals and contaminate the environment, potentially sensitizing other animals and eventually causing them to test positive in a skin test.
2. BCG could potentially survive in the vaccinated animals and reach the food chain via contaminated meat.
3. BCG deployment under optimal conditions requires avoiding extremely high temperatures, and even at mild temperatures its survival is limited.

These constraints add complexity to the logistics of vaccine deployment under real-life field conditions, making those interventions highly expensive. However, in a controlled study using captive feral swine originating in Texas, USA, BCG could not be recovered from the tissues of animals euthanized at 1, 3, 6 and 9 months post-oral vaccination (n = 4/time point) (Nol, unpublished data). In addition, a study testing BCG excretion in vaccinated wild boar, BCG survival in wild boar carcasses and BCG viability under field conditions also found no serious reasons for concern (Beltrán-Beck *et al*., 2014a). More research is needed in the field of BCG safety in pigs and wild boar.

The second vaccine is a specific heat-killed *M. bovis* field strain. As opposed to BCG this vaccine is not yet commercially available, but it offers the advantages of being completely safe and having no temperature constraints for storage and deployment. This greatly simplifies the logistics and reduces the cost of vaccine deployment. Single oral doses of the heat-inactivated *M. bovis* vaccine elicit responses in wild boar and in pigs that are equivalent to those elicited by live BCG. This heat-inactivated *M. bovis* vaccine reduced mean total lesion scores by 43% and thorax lesion scores by 76% (Garrido *et al*., 2011). As with BCG, re-vaccination also boosted the protective response, achieving an 80% reduction in lesion score (Beltrán-Beck *et al*., 2014b). In pigs, the lesion score reduction achieved in the only known experiment (single vaccination) was 39% (Beltrán-Beck *et al*., 2014b).

The parenteral (intramuscular) version of the heat-inactivated *M. bovis* vaccine is prepared by suspending 10^6 CFU of the killed vaccine strain in Montanide™ (Seppic, Puteaux Cedex, France). This form of delivery of the heat-inactivated vaccine has been tested in controlled vaccination and challenge laboratory trials and is now deployed experimentally on one wild boar farm. On this farm, TB prevalence of the breeding stock has been reduced from 40% to less than 1% in 2 years, after setting up a TB control programme that combines test and cull with vaccination. This study is still ongoing.

The deployment of oral vaccine baits under field conditions faces a number of challenges, including selective delivery to >70% of the target host group. To achieve this target, highly palatable oral bait has been designed and patented, and a portable selective baiting cage, allowing only piglets to access the baits (Fig. 17.1), has also been designed and patented. A preliminary biomarker study without vaccine showed that baits deployed in this way can reach 73% of the wild boar piglets under field conditions (Ballesteros *et al*., 2011; Beltrán-Beck *et al*., 2012).

Fig. 17.1. Photo-trap picture of Eurasian wild boar (*Sus scrofa*) at a selective piglet feeder. Targeted oral bait deployment to wild boar piglets is achieved by using portable cages. This is part of the ongoing field trials to control wild boar TB in Spain through oral vaccination. (Photo courtesy of Jose Angel Barasona, SaBio IREC.)

A field vaccination experiment was begun in 2012 and will last at least until 2015. In this experiment, 10,000 ha of Mediterranean woodlands with high wild boar TB prevalence (~60%) are vaccine-baited every summer. Within this area, two sites with a total area of 5000 ha are baited with BCG and another two sites of similar total area are baited with the heat-inactivated *M. bovis* vaccine. Other sites in the same area remain as unvaccinated controls. Each year ~20,000 vaccine baits are deployed in July and late August, and wild boar are subsequently sampled in autumn and winter during the hunting season. Bait uptake by piglets is measured through biomarkers. This experiment should yield important information on selectivity, efficacy and safety of oral vaccine delivery to wild boar under field conditions, as well as on wild boar response to oral vaccination and vaccine efficacy under field conditions. It will also allow a first estimation of the costs involved in such field-baiting interventions.

Vaccination with BCG or the heat-inactivated *M. bovis* vaccine is also being considered for controlling TB in feral swine on Molokai, where the pigs are thought to be the maintenance host for *M. bovis*. Feral swine are hunted year-round on the island so there is inevitably concern about persistence of live BCG in the animals' tissues, and possible human consumption thereof. Research will soon be conducted, therefore, to test the efficacy of the heat-inactivated *M. bovis* vaccine against TB in Molokai-origin feral swine in a controlled laboratory setting. Use of a killed TB vaccine on Molokai instead of a live vaccine will allow for much greater flexibility in the nature of baits, their storage and deployment, as well as substantially reduce the risk of accidental exposure for non-target wildlife species, livestock and humans on the island.

A summary of knowledge/technology gaps regarding the diagnosis, vaccination and control of MTC infection in wild boar and pigs is provided in Box 17.3.

Wild boar and feral pig populations continue their geographic and numerical expansion. Thus, problems related with diseases are likely to increase in the future. Lessons learned from TB control in wild boar and pigs can become useful for the control of other suid infections, or can eventually be extrapolated to other MTC hosts and different geographic regions.

Box 17.3. Current Knowledge/Technology Gaps Regarding Wild Boar and Pig TB Diagnosis and Control.

- There is a need to assess the effect of infection with *M. microti* on the specificity of antibody-based diagnostic tests.
- There is a technology gap regarding the testing for MTC of non-invasively obtained samples such as faeces or saliva.
- BCG safety and viability of BCG deployment to suids under different environmental conditions require research. Also, safety and sensitization experiments need to be carried out in cattle.
- Ongoing research is addressing the efficacy of BCG/heat-inactivated *M. bovis* combinations in wild boar.
- Most experiments are testing a single intervention tool. Hence, the combined effect of integrated control schemes (for instance combining culling with oral vaccination) needs to be modelled and field tested.
- Results of the ongoing field trial will produce information on safety, selectivity and efficacy of bait deployment; wild boar response to BCG and to the heat-inactivated *M. bovis* vaccine under field conditions; efficacy of oral vaccine deployment for TB control; and the estimated cost of such interventions.
- More field trials should be designed, particularly addressing other situations with lower TB prevalence and different environmental conditions, such as Atlantic habitats.
- Designing, implementation and assessing farm biosafety programmes to mitigate risk of interspecific contact and MTC transmission.

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18 Australian Brushtail Possum: A Highly Susceptible Host for *Mycobacterium bovis*

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Introduction

The Australian brushtail possum (*Trichosurus vulpecula*) is highly susceptible to *Mycobacterium bovis* infection and serves as the principal wildlife reservoir for *M. bovis* infection in New Zealand. Following their introduction from Australia in the mid-1800s to establish a fur trade, possums spread throughout New Zealand and are now a noxious pest. It is now well recognized that possums cause extensive damage to crops, forests and native birdlife as well as serving a vector for the spread of bovine tuberculosis (TB) to both cattle and farmed deer. Epidemiological investigations have estimated that the majority of new breakdowns of TB in cattle and farmed deer herds in New Zealand can be attributed to direct or indirect contact with infected possums (Hutchings *et al*., 2013). The TB–possum problem emerged in the late 1960s (Ekdahl *et al*., 1970) and lethal control of possum populations undertaken since 1994, currently costing the equivalent of more than US$40 million per year, has been a major contributor to the >95% reduction in the number of *M. bovis*-infected cattle and farmed deer herds (Hutchings *et al*., 2013). Infection is fulminating and rapidly lethal in possums, with lesions found predominantly in the lungs and superficial peripheral lymph nodes. The terminal stage of the disease is reached by 2 months following intratracheal delivery of a very low dose of *M. bovis* (Buddle *et al*., 1994) and most possums die from natural infections within 6 months (Jackson *et al*., 1995a; Nugent *et al*., 2013). Valuable information can be obtained from *M. bovis* infection in possums as to why this species is so susceptible to a fulminating mycobacterial disease and, second, how the disease can be controlled by vaccination. Other wildlife species such as badgers in the UK and Ireland; white-tailed deer in northern Michigan, USA; and wild boar in Spain also serve as wildlife reservoirs of *M. bovis* for reinfection of domestic livestock (de Lisle *et al*., 2001) and the development of an oral bait TB vaccine for possums has application for these other animal species. Comparison of the efficacy of BCG vaccine against experimental and natural infection of *M. bovis* in possums provides a useful insight into the extrapolation of findings from experimental challenge

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

studies to the efficacy of TB vaccines in the field. A summary of the unique features of *M. bovis* infection in the possum is shown in Table 18.1.

Biology and Ecology of Possums

Brushtail possums are 2–3 kg nocturnal arboreal marsupials native to Australia. The current possum population in New Zealand is estimated to be about 30 million, which in recent years has been reduced due to culling for TB control and fur harvesting (Warburton *et al*., 2009). The national average is about 1.1 possums/ha (Nugent *et al*., 2015), but the carrying capacity can be over 20/ha in diverse forests (Efford, 2000). Females produce a single young in autumn from 1 to 2 years of age onwards and may produce young in spring as well. Young are dependent on their mothers for about 6 months. The average life expectancy is 3–5 years, although they can survive for 12 years or more (Efford, 2000). Possums are predominantly arboreal folivores but will consume other foods including meat and mammalian carrion (Ragg *et al*., 2000). They den during the day in dark places on the ground or in trees and occupy a range of different dens, though seldom simultaneously sharing dens (Cowan and Clout, 2000).

Tuberculosis

Origins

Despite possums have being sympatric with *M. bovis*-infected cattle in New Zealand since the 1800s, the first case of infection of *M. bovis* in a possum was only detected in 1967 (Ekdahl *et al*., 1970). The implication is that TB did not spillover into possums until the 1960s, but thereafter spillover occurred on a small number of occasions (reviewed by Nugent *et al*., 2015). Although possums are widespread throughout New Zealand, *M. bovis* infection in wildlife, principally in possums, has only been found in 39% of the land area of the country (Livingstone *et al*., 2006). TB was not detected in the millions of possums skinned for fur prior to 1967 (Parkes *et al*., 1996), whereas between 1967 and 1981 TB was found in possums in many different localities (Julian, 1981). An analysis of 83 *M. bovis* isolates recovered from possums between 1982 and 1984 revealed 33 different DNA strain types, with isolates of the same type usually found in the same geographic area (Collins *et al*., 1986). In contrast, TB was not found in possums in Australia, although they were also sympatric with *M. bovis*-infected cattle (Cousins and Roberts, 2001). It has been hypothesized that the spillover of TB to possums in New Zealand was from wild deer rather from domestic

Table 18.1. Summary of unique features of *Mycobacterium bovis* infection in the brushtail possum.

Possums serve as the principal wildlife reservoir of *M. bovis* infection in New Zealand
Control of possum populations in TB endemic regions has had a dramatic effect in reducing cattle and farmed deer herd infection rates
Rapid course of infection to terminal stage of disease
Lesions primarily found in lungs and peripheral superficial lymph nodes following natural infection
Histologically, lesions were characterized by large amounts of necrotic material and many acid-fast bacilli, while multinucleate giant cells, fibroplasia and mineralization were rare
High susceptibility to disease may be associated with a transient activation of lung macrophages following infection
For diagnosis, serology had a low sensitivity, while being higher for the blood lymphocyte proliferation assay
Transmission probably by the respiratory route and less frequently by ingestion and/or percutaneous routes (scratches or abrasions from fighting or mating)
BCG vaccination via a range of different routes including oral baits induced a significant level of protection against *M. bovis* infection
Field BCG vaccine efficacy indicated greater protection than that shown from experimental challenge studies and showed protection against infection

cattle (Morris and Pfeiffer, 1995). Commercial deer hunting began around 1960 and the heads and offal from large numbers of killed deer were left in the field, and it is recognized that possums will scavenge mammalian carrion (Nugent, 2011). Alternatively, possums could have been infected by feeding on tuberculous cattle carcasses. Prior to the introduction of a compulsory control programme, dairy cattle with clinical TB were often slaughtered on farms and occasionally without proper disposal of carcasses, providing a potential source of infection for possums.

Pathology

Natural infection of possums with *M. bovis* results in tuberculous lesions found predominantly in the lungs and peripheral superficial lymph nodes. Frequently, possums have just one or a small number of macroscopic lesions, while in the terminal stage of the disease they become cachectic and infection may become generalized with lesions in lungs, liver, spleen or kidneys (Jackson *et al*., 1995a). Lesions in the superficial lymph nodes may suppurate with sinuses extending to the exterior, discharging purulent material. The lesions are thinly walled and contain large amounts of necrotic material. These lesions are in marked contrast to the caseous and often mineralized lesions observed in the lymph nodes of cattle with TB. The exquisite susceptibility of possums to TB is seen from the very rapid course of infection following experimental aerosol or intratracheal administration of 10–100 colony forming units (CFU) of *M. bovis*, with possums reaching the terminal stage of disease in 8–10 weeks (Buddle *et al*., 1994; Aldwell *et al*., 2003). Extensive lesions are found in the lungs and associated lymph nodes, with bacterial counts of up to 10^7 CFU/g of lung tissue. In the terminal stage of the disease, small macroscopic lesions are commonly found in the spleen, liver and kidneys, along with microscopic lesions in the lymphatics (Cooke *et al*., 1999). Histologically, the earliest lung lesions (2 weeks post-infection, p.i.) consist of aggregates of macrophages and lymphocytes around small blood vessels or in alveolar spaces, while by 4 weeks p.i., there are extensive lesions with necrosis and/or caseation and large numbers of acid-fast bacilli (AFB); while multinucleate giant cells, fibroplasia and mineralization are very rare. A longer course of the disease (2–5 months) can be established in possums following intraconjunctival instillation of 10^3 CFU of *M. bovis* (Corner *et al*., 2003) or subcutaneous inoculation of 20 CFU of *M. bovis* into the interdigital space of the paws (Nugent *et al*., 2013). In these infections, the primary lesions are found in the draining lymph nodes, with secondary spread to the lungs, liver and spleen. While possums are highly susceptible to *M. bovis* this susceptibility does not extend to some of the other pathogenic mycobacteria such as members of the *M. avium* complex.

Pathogenesis

The susceptibility of possums to *M. bovis* is associated with a failure of innate immunity to contain the infection, and following infection there is only a transient macrophage activation. Immunological studies indicated that after intratracheal or aerosol administration with low doses of *M. bovis* (20–100 CFU), peripheral blood lymphocyte proliferation responses to bovine purified protein derivative (PPD) could be detected from 3 weeks p.i. which was 1 week after lymphocyte infiltrations were detected in the lungs (Cooke *et al*., 1999; Denis *et al*., 2005a). Animals developed macroscopic lesions by 4–5 weeks p.i. which coincided with waning in blood lymphocyte responses to mitogen (concanavalin A) and macrophage activation. At 3 weeks p.i., alveolar macrophages from infected possums were producing significant levels of nitric oxide as well as TNFα bioactivity. However, these processes were depressed at 4–5 weeks p.i. when substantial replication of *M. bovis* occurred (Denis *et al*., 2005a). Alveolar macrophages from animals at 3 weeks p.i. blocked the replication of *M. bovis* in part via a nitric oxide-dependent mechanism and were more refractory to *M. bovis* growth than alveolar macrophages from naïve animals or those from animals at 4–5 weeks p.i. Introduction of

autologous lymphocytes from the blood of infected animals in co-cultures rendered infected macrophages more resistant to *M. bovis*. In addition, co-culturing alveolar macrophages, infected *in vitro* with *M. bovis* together with autologous blood mononuclear cells from BCG-vaccinated possums, led to a significant decrease in the metabolic activity of intracellular *M. bovis*, an effect that was contact dependent and nitric oxide independent (Denis *et al*., 2005b).

Diagnosis

The 'gold standard' for diagnosis of TB in possums is mycobacterial culture of *M. bovis*, although a presumptive diagnosis can be based on the characteristic histopathology and presence of AFB (de Lisle *et al*., 2009). Detection of *M. bovis* infection by serology has a low sensitivity, 45–55% (Buddle *et al*., 1995; Lyashchenko *et al*., 2008), while a sensitivity of 70–80% has been estimated for the whole-blood lymphocyte proliferation assay (B. Buddle, unpublished observations). Ante-mortem diagnosis is only used for research purposes, as in the field situation possums are classified as noxious pests and are killed following trapping or by the application of poison baits.

Transmission

Possible routes of transmission of *M. bovis* between possums can be gleaned from the distribution of lesions in these animals, although the exact mechanisms involved are not clear. In an analysis of 73 tuberculous possums with macroscopic or microscopic lesions, lesions were found in 85% of the lungs, 85% of the axillary lymph nodes, 69% of the inguinal lymph nodes and 95% of axillary or inguinal lymph nodes (Jackson *et al*., 1995a). These findings indicated that respiratory transmission is likely, particularly associated with den sharing or close contact such as mating or fighting. Experimental transmission between animals has been very difficult to demonstrate across a wide range of animal species. Attempts to transmit infection from possums experimentally infected by intratracheal inoculation to in-contact possums have generally proved disappointing. Only one of 11 uninfected possums kept individually paired with an experimentally infected possum in a small cage developed a *M. bovis* infection and none of three possums held for 8 weeks with 19 infected possums in a small outdoor pen became infected (Corner *et al*., 2002b). Transmission was achieved in the two outdoor pens when socially dominant possums were infected. Five of 20 in-contact possums in one pen and 12 of 19 in-contact possums in the other became infected following co-habitation with four infected possums in each pen. All possums infected by natural infection in these studies had infection predominantly in the lower respiratory tract. In naturally infected possums, *M. bovis* infection (as determined by histopathology) is widely distributed, a much wider distribution than indicated by the location of gross lesions. From studies in badgers it could be expected that when determined by bacteriology, the distribution of infection would be even wider. When lesions are found only in the superficial peripheral lymph nodes, such as the axillary or inguinal, these may be the result of transmission by percutaneous infection, for example through scratches or abrasions incurred during grooming, mating or fighting or – more probably – the result of dissemination of infection before the animals have developed a potent immunological response (Corner *et al*., 2011). In support of this contention, local superficial wounds infected with *M. bovis* are not found in possums as is the case with *M. bovis*-infected bite wounds in badgers in the UK and Ireland (Corner *et al*., 2012; Jenkins *et al*., 2012). Lesions in the axillary or inguinal lymph node can be produced following subcutaneous injection of a low dose of *M. bovis* into the interdigital space of the paws of possums (Nugent *et al*., 2013), although the distribution of histopathological lesions following this route of infection has not been ascertained.

Macroscopic lesions in the mesenteric lymph nodes are not common, indicating that oral transmission of *M. bovis* is not a major mechanism. Pseudovertical transmission can occur and tuberculous lesions have been observed in the mammary glands of infected

possums (Jackson *et al*., 1995a). Further information on routes of transmission can be obtained from excretion of *M. bovis* from infected possums (Jackson *et al*., 1995b). *M. bovis* was isolated from 36% of tracheal washings, but only from 2% of urine or faecal samples from the tuberculous possums. Large numbers of *M. bovis* can be isolated from discharging fistulae draining from infected superficial lymph nodes.

Despite the high lethality of *M. bovis* for possums, the transmission rate between possums is generally low. When TB is endemic in possum populations, the prevalence of possums with macroscopic lesions is usually <5% and often 1–2%, but can occasionally reach 60% locally (Coleman and Caley, 2000). Local clusters of infection are common and are known as 'hot spots'. The low prevalence of disease in the majority of TB endemic areas may result from possums being principally solitary animals and contacts between possums are usually brief and infrequent (Ji *et al*., 2005). Overall, the presence of disease in a population does not appear to suppress possum densities.

The transmission of *M. bovis* from infected possums to cattle and deer is considered to result from these animals being attracted to and closely investigating, sniffing or licking, terminally ill or dead possums (Sauter and Morris, 1995). Transmission to scavengers including ferrets and wild pigs is presumed to occur from ingestion of tissues from carcasses of *M. bovis*-infected possums and this is based on the predominance of head and gastrointestinal tract infection in these animals (Coleman and Cooke, 2001).

Vaccination

Although possums are very susceptible to *M. bovis* infection, vaccination with BCG via a range of different routes (subcutaneous, intranasal, intraconjunctival and oral) induces a significant level of protection against experimental challenge with *M. bovis* (reviewed by Buddle *et al*., 2009). However, a critical issue for the use of vaccines in wildlife is vaccine delivery, and the only practical and cost-effective means of delivering vaccines to large numbers of wildlife is by oral baits; revaccination of animals is seldom an option. Consequently, single-administration, live vaccines such as BCG are suitable for vaccinating wildlife as opposed to subunit, adjuvanted vaccines which require multiple applications and parenteral administration. A problem encountered with the oral application of BCG vaccine to possums was that vaccine efficacy was reduced due to degradation of the live BCG in the stomach of the possum. This became evident when BCG vaccine efficacy was significantly enhanced when the vaccine was inoculated directly into the duodenum (Buddle *et al*., 1997) and when BCG was administered orally 1 h after treatment with a drug that reduced gastric acidity (Skinner *et al*., 2005). Degradation of BCG during passage through the stomach was overcome by incorporation in a protective novel proprietary lipid matrix, which solidified at temperatures below 30–32°C. This melting temperature had the added benefit of enhancing the 'shelf life' of the vaccine in the environment. The viable counts of BCG suspended in the lipid matrix formulation remained stable for up to 8 months at −20°C, for 7 weeks under room temperature conditions and for 3–5 weeks under field conditions in a forest/pasture habitat, when maintained in weatherproof bait delivery sachets (Cross *et al*., 2009). Possums consuming oral baits containing BCG encapsulated in a lipid matrix and incorporating a bait attractant (chocolate and anise) were shown to have a significant level of protection against an aerosol challenge with virulent *M. bovis*, which was similar to those vaccinated by a subcutaneous injection of BCG (Aldwell *et al*., 2003). Vaccination with BCG did not prevent initial infection with *M. bovis* following challenge, but did reduce the severity of disease. These experimental challenges were relatively severe as non-vaccinated possums displayed clinical signs of disease by 6–7 weeks following aerosol challenge with a low dose of *M. bovis* and were euthanized on ethical grounds by 8 weeks post-infection. Subsequently, oral application of BCG in the lipid matrix has been shown to induce significant protection against TB in badgers, cattle and deer (reviewed by Buddle *et al*., 2011).

The protective effect of the oral administration of BCG to possums was strongest at

2–6 months after vaccination, but still present at lower levels at 12 months (Buddle *et al*., 2006) which concurred with a previous study when BCG was administered intranasally and intraconjunctivally to possums (Corner *et al*., 2001). In a recent study, long-term protection was assessed in possums which had received a single oral application of BCG in a lipid matrix with the animals released back into the field and recaptured 28 months later. The recaptured possums were housed in individual cages in a containment unit and challenged subcutaneously in the interdigital space in the paws (Tompkins *et al*., 2013). The vaccinated possums had a significant reduction in the bacterial counts from the peripheral lymph nodes draining from the challenge sites compared to the control non-vaccinated animals.

A number of other parameters that may influence the effectiveness of orally administered BCG have been investigated in possums (Buddle *et al*., 2006). Vaccine efficacy was not adversely affected by feeding dead BCG 3 months prior to live BCG. Feeding ten pellets of live BCG (10^8 CFU) on one occasion induced a level of protection similar to feeding a single pellet (10^7 CFU) and protection was similar when possums were fed a single pellet containing the Pasteur or Danish strain of BCG.

A finding very relevant to vaccination against TB in both animals and humans is the correlation between protection against experimental challenge and natural exposure to the bacteria. Although BCG vaccination of possums significantly reduced the severity of infection, all vaccinated possums remained infected. In contrast, in two field vaccination trials each extending over 2 years, BCG vaccination produced significant protection against *M. bovis* infection (Corner *et al*., 2002a; Tompkins *et al*., 2009). The trials involved the capture and release of wild possums, with matched non-vaccinated and vaccinated animals from the same areas. The possums were trapped bimonthly, and assessed for tuberculous status by palpation and lesion aspiration; the sites were depopulated after 2 years and post mortems conducted to identify clinical and subclinical infection. In a trial involving oral vaccination of possums with BCG incorporated into the lipid matrix, significantly fewer culture-confirmed tuberculous cases were recorded in the vaccinated possums (1/51) compared to the non-vaccinated animals (12/71), with a vaccine efficacy estimated at 95% (Tompkins *et al*., 2009). These findings concurred with an earlier field trial where BCG vaccine was administered to possums via intranasal spray and intraconjunctival instillation, and again a significant level of protection against natural infection with *M. bovis* was obtained with a vaccine efficacy of 69% (Corner *et al*., 2002a). In these field studies vaccination appeared to have prevented the establishment of infection, an effect that was not seen in the experimental challenge studies. These two field trials have provided encouragement that BCG vaccination could be effective in controlling tuberculosis in wild possum populations. It could also be possible to develop an improved TB vaccine for wildlife and newly attenuated *M. bovis* mutants have shown better protection against experimental *M. bovis* challenge in possums than those vaccinated with BCG (Collins *et al*., 2011).

Another important issue to consider is the efficiency of the uptake of oral baits by wild possums. To address this question, chocolate- and anise-flavoured lipid pellets incorporating a biomarker (rhodamine dye) were placed in weather-resistant possum-bait delivery sachets and the sachets were stapled to trees in areas holding approximately 6–8 possums/ha (Cross *et al*., 2009). At target baiting densities of 40 and 80 sachets/ha, 85% and 100%, respectively, of the captured possums were shown to have accessed the pellets over a 5-day period, indicating that lipid baits distributed in sachets are readily taken up by wild possums.

Research into the development of vaccination against TB in possums was carried out as an alternative approach to the widely used, lethal control of possums using poisons, including the aerial distribution of sodium fluoroacetate (known as the pesticide, 1080). The control of possums with poisons has led to a dramatic decline in the number of infected cattle herds but the use of 1080 in New Zealand is controversial. Furthermore, the goal of the TB programme is now one of total eradication of *M. bovis* from wildlife and this may require measures such as vaccination in addition to the current lethal control of possums (Tompkins *et al*., 2009).

Conclusions

Tuberculosis in the brushtail possum is particularly important as this animal serves as the major wildlife maintenance reservoir for *M. bovis* infection in New Zealand and infected possums are a continual source of infection for cattle and farmed deer. Their exquisite susceptibility to *M. bovis* infection can be seen from the rapid disease progression and from the histopathology of the lesions showing minimal encapsulation, large numbers of AFB and large necrotic areas within lesions. The mechanisms associated with this high susceptibility to infection are not clear, although there is an apparent downregulation of macrophage activity with the progression of infection. One of the most encouraging features of TB studies in possums is the protection induced following vaccination with BCG and the development of an oral bait formulation for wildlife. This formulation serves to protect BCG from degradation in the stomach and enhances shelf-life of the vaccine. There are few studies in target animals comparing protection against TB following experimental challenge and protection following natural exposure to microorganisms from the *M. tuberculosis* complex. The field trials in possums demonstrated that the level of protection against natural exposure to *M. bovis* was considerably greater than that observed following experimental challenge with a low dose of *M. bovis;* experimental challenge studies can be valuable in optimizing vaccine protocols prior to testing in the field.

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19 Tuberculosis in Wild and Captive Deer

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Introduction

Bacteria of the genus *Mycobacterium* are Gram-positive, acid-fast organisms that include several major human and animal pathogens. Although human tuberculosis is generally caused by *M. tuberculosis*, indistinguishable clinical signs and disease can be caused by *M. bovis*. The range of susceptible hosts to *M. bovis* is extremely broad and includes humans, cattle, swine, carnivores and deer.

Deer have played an important role in human history. Excavations of early prehistoric sites in Europe indicate that both deer and wild boar (*Sus scrofa*) were important sources of meat for early humans. Red deer (*Cervus elaphus*) were a particularly important source of meat and other materials for over 5000 years in Northern Europe, and possibly for 50,000 years in warmer southern climates (Clutton-Brock, 1999). Deer are widely distributed and indigenous species can be found in all continents with the exception of Antarctica and Australia.

Today, of the approximately 16 genera and over 50 species of the family Cervidae (Baker, 1984), tuberculosis due to *M. bovis* (bTB) has been reported in at least 14 species. These include red deer, North American elk (*Cervus elaphus nelsoni*), (*C. elaphus manitobensis*), tule elk (*C. elaphus nannodes*), sika deer (*C. nippon*), sambar deer (*C. unicolor swinhoei*), fallow deer (*Dama dama*), white-tailed deer (*Odocoileus virginianus*), mule deer (*O. hemionus*), black-tailed deer (*O. hemionus columbianus*), axis deer (*Axis axis*), roe deer (*Capreolus capreolus*), Chinese muntjac deer (*Muntiacus reevesi*), reindeer (*Rangifer tarandus*) and moose (*Alces alces*) (Table 19.1).

As commonly used terminology varies around the world, some definitions are necessary. In this chapter, the term *deer* is used to represent all members of the family Cervidae. *Captive* refers to settings where deer movement is intentionally restrained (e.g. by fencing), regardless of the geographic size of the restricted area. This includes farms where deer are managed much like livestock, as well as large, expansive 'shooting' enterprises where

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Table 19.1. Tuberculosis in different species of the family Cervidae.

Species	Countries	Captive or wild	References
Elk (*Cervus elaphus nelsoni*)	USA, Canada, New Zealand	Captive, wild	Thoen *et al*. (1992), O'Brien *et al*. (2008), Waters *et al*. (2011)
Red deer (*C. elaphus*)	New Zealand, Australia, USA, England, Hungary, Denmark, Spain, France	Captive, wild	Hellstrom (1979), Beatson (1985), Stuart (1988), Delahay *et al*. (2007), Zanella *et al*. (2008a,b)
Tule elk (*C. elaphus nannodes*)	USA	Captive	Kollias (1980)
Sambar deer (*C. unicolor swinhoei*)	Taiwan	Captive	Chu *et al*. (2012)
Sika deer (*C. nippon*)	USA, Ireland, Taiwan, New Zealand	Captive, wild	Dodd (1984), Mirsky *et al*. (1992), Coleman and Cooke (2001), Chu *et al*. (2012)
Fallow deer (*Dama dama*)	Australia, Ireland, New Zealand, Sweden, USA, Spain, England	Captive	Towar *et al*. (1965), Robinson *et al*. (1989), Paterson (1993), Bolske *et al*. (1995), Delahay *et al*. (2007), Waters *et al*. (2011)
White-tailed deer (*Odocoileus virginianus*)	USA, Canada	Captive, wild	Levine (1934), Ferris *et al*. (1961), Belli (1962), Friend (1963), Schmitt *et al*. (1997), Palmer *et al*. (2000)
Mule deer (*O. hemionus*)	USA	Wild	Rhyan *et al*. (1995)
Black-tailed deer (*O. hemionus columbianus*)	USA	Wild	Kollias (1980)
Axis deer (*Axis axis*)	USA (Hawaii)	Wild	Sawa *et al*. (1974)
Roe deer (*Capreolus capreolus*)	England, Switzerland, Spain, Italy, France	Wild	Bischofgberger and Nabholz (1964), Gunning (1985), Delahay *et al*. (2007), Zanella *et al*. (2008a), Balseiro *et al*. (2009)
Muntjac (*Muntiacus reevesi*)	England	Wild	Delahay *et al*. (2007)
Reindeer (*Rangifer tarandus*)	Soviet Union	Wild	Syroechkovskii (1995)
Moose (*Alces alces*)	Sweden	Wild	Hadwen (1942), Lothian (1981), Bolske *et al*. (1995), Wobeser (2009)

management is less intense. Unrestrained, free-ranging deer will be referred to as *wild*.

Tuberculosis occurs more frequently among captive deer than in wild deer (Griffin and Buchan, 1994; Hunter, 1996). In fact, tuberculosis has been found in every country where captive deer are farmed and managed as livestock (Griffin and Mackintosh, 2000). In wild deer the disease has often 'spilled over' to deer from *M. bovis*-infected cattle. In some regions today, infected wild deer now act as a reservoir of infection, 'spilling back' *M. bovis* to cattle (Palmer, 2013). This is particularly true of white-tailed deer in Michigan, USA (Schmitt *et al*., 1997; O'Brien *et al*., 2002; Palmer *et al*., 2012).

Pathogenesis

Route of infection, pathology and transmission

Severe outbreaks of tuberculosis in deer are uncommon, although occasionally in captive settings a large number of animals are infected or display generalized disease (Robinson *et al*., 1989; Waters *et al*., 2011). This may be the result of one or more animals acting as 'supershedders', efficiently transmitting disease to herd mates (Griffin and Mackintosh, 2000). Initial infection is generally through the mouth or nasal cavity, although cutaneous infection can occur through open or penetrating wounds.

In deer, tuberculosis is a subacute to chronic disease. Even deer with extensive disease may not demonstrate obvious clinical signs. Nevertheless, when present, clinical signs are often non-specific, such as weight loss, poor body condition and rough hair coat. For deer with clinical signs the prognosis is grave (Griffin and Buchan, 1994). In sika (Dodd, 1984), fallow (Towar *et al*., 1965), red (Beatson, 1985; de Lisle and Havill, 1985; Stuart, 1988; Griffin and Buchan, 1994; Nugent *et al*., 2014) and white-tailed deer (Schmitt *et al*., 1997; Palmer *et al*., 2000; O'Brien *et al*., 2001; Fitzgerald and Kaneene, 2013) and in elk (Whiting and Tessaro, 1994) the most common location for tuberculous lesions are the retropharyngeal lymph nodes, followed by the lung and associated lymph nodes (tracheobronchial, mediastinal). This distribution is consistent with exposure through oral and/or aerosol routes (Kollias, 1980). There are exceptions, however, where lungs (Stumpff, 1982; de Lisle and Havill, 1985; Waters *et al*., 2011; Garcia-Jimenez *et al*., 2012), mesenteric lymph nodes (Kollias, 1980; Zanella *et al*., 2008b) or the palatine tonsils (O'Brien *et al*., 2008) have been reported as the most common site for lesion development, although reasons for such differences are unclear.

Generalized disease, defined as lesions in more than one body region (e.g. head and thorax, thorax and abdomen) usually occurs in only a minority of cases (Griffin and Buchan, 1994; Palmer *et al*., 2000; Nugent, 2007). In naturally infected white-tailed deer, roughly 30–35% of deer will exhibit lesions in both the head and thorax (Fitzgerald and Kaneene, 2013). Likewise, in fallow and roe deer in the British Isles, 35.7% and 41.6%, respectively, exhibited generalized disease (Delahay *et al*., 2007). Even so, there have been reports of generalized disease exceeding 50% in some outbreaks in captive and wild, red and fallow deer in Spain (Vicente *et al*., 2006; Martin-Hernando *et al*., 2010). In contrast, lesions have often either been absent or indistinct in the tissues of infected muntjac deer (Delahay *et al*., 2001, 2002; Johnson *et al*., 2008) although one individual exhibited gross lesions in the lungs and pre-scapular lymph nodes (Delahay *et al*., 2007).

Gross lesions vary in size, shape and character; nevertheless, in elk, red, white-tailed, sika and fallow deer they are often recognized as lymphadenopathy and lymphadenitis, the physical characteristics of which may grossly resemble abscesses (Fig. 19.1) (Friend, 1963; Beatson, 1985; Stuart, 1988; Stuart *et al*., 1988; Clifton-Hadley and Wilesmith, 1991; Whiting and Tessaro, 1994; Delahay *et al*., 2007; Garcia-Jimenez *et al*., 2012; Fitzgerald and Kaneene, 2013). So common is this abscess-like appearance that some authors suggest that all abscess-like lesions in deer should be regarded as suspicious and processed by bacteriological culture for mycobacteria (Beatson, 1985; Stuart, 1988). In addition to a liquefactive appearance, lesions may be caseous, with or without dystrophic mineralization (Fig. 19.2). Microscopically, granulomas in deer are not walled off to the degree seen in cattle (Clifton-Hadley and Wilesmith, 1991; Johnson *et al*., 2008). Histologic features of tuberculous granulomas from elk, fallow, sika and red deer have been described previously (Rhyan and Saari, 1995). Species differences in terms of mineralization, presence of neutrophils and the number and morphology of multinucleated giant cells have been observed (Rhyan and Saari, 1995).

In a histological study of tissues from infected red (n = 21), fallow (n = 26), roe (n = 9) and muntjac deer (n = 1) obtained from south-west England and Wales, 70% exhibited granulomas containing relatively high numbers of *M. bovis* bacilli compared to those typically

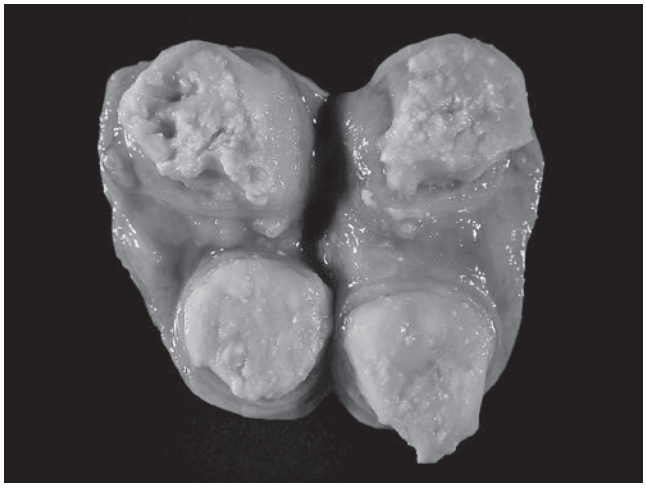

Fig. 19.1. Medial retropharyngeal lymph node from a tuberculous white-tailed deer. Note liquefied, abscess-like nature of lesions.

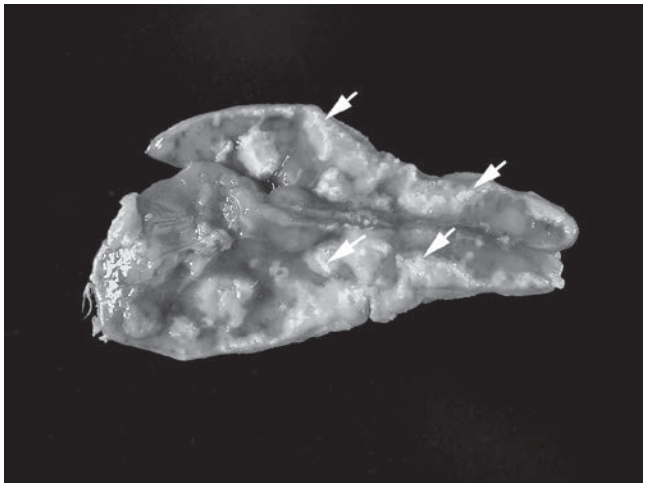

Fig. 19.2. Mediastinal lymph node from a tuberculous white-tailed deer. Lesions are caseous in nature with dystrophic mineralization (arrows).

seen in cattle (Johnson *et al*., 2008). Most lymph node granulomas (97%) showed advanced lesion development, characterized by large, often multifocal areas of necrosis and mineralization, although less distinct multifocal, subpleural nodules were observed in lung tissue from the muntjac deer. A novel lesion scoring system based on the number of bacilli and the degree of encapsulation in each tissue section of a granuloma indicated that 68% of animals (18 red deer, 14 fallow deer, 5 roe deer and the muntjac deer) had a high probability of *M. bovis* excretion. Red and fallow deer had the largest number of poorly encapsulated granulomas, often containing many hundreds of bacilli. These results are consistent with environmental contamination arising from bacilli shed by infected deer, with the potential for onward transmission to other species.

Fawns can easily be infected through the consumption of cow's milk or milk replacer containing *M. bovis* (Hadwen, 1942; Palmer *et al*., 2002b). As a result, shedding of *M. bovis* from the mammary gland of the dam could potentially serve as a means of deer-to-deer transmission. Nevertheless, tuberculous lesions of the mammary gland have rarely been reported (O'Brien *et al*., 2001) and in wild red deer, there was no evidence of transmission to fawns in a population in which half of the adult females were infected (Nugent, 2007). Congenital infection, although sometimes seen with other mycobacterial diseases such as Johne's disease (paratuberculosis), has not been documented in deer (Clifton-Hadley and Wilesmith, 1991; Whiting and Tessaro, 1994).

In human tuberculosis, the combination of granulomas at the initial site of infection and the corresponding draining lymph node (e.g. lung and pulmonary lymph nodes) is known as a primary complex of lesions. Formerly, tuberculosis of the tonsils and cervical lymph nodes in humans was recognized as a primary complex of lesions from which *M. bovis* was often isolated (Cowan and Jones, 1972). As the retropharyngeal lymph nodes are the most common sites for lesions in deer, and efferent lymphatics from the tonsils drain to the retropharyngeal lymph nodes (Saar and Getty, 1975), the tonsils and retropharyngeal lymph nodes may also be viewed as a primary complex in deer. If so, one would expect lesion formation or *M. bovis* colonization of the tonsils. Accordingly, in white-tailed deer, 76% of deer with lesions in the retropharyngeal lymph nodes also had lesions compatible with tuberculosis in the palatine tonsils. Likewise, in deer with palatine tonsilar lesions, 90% had lesions in the retropharyngeal lymph nodes (Palmer *et al*., 2002c). In naturally infected red deer, *M. bovis* was isolated from 61% of palatine tonsil samples (Lugton *et al*., 1998).

The likely route of transmission can be inferred from the distribution of lesions, particularly when only a single lesion or the probable primary complex is present (Biet *et al*., 2005). Hence, it is often presumed that animals with lesions restricted to the respiratory tract have been infected by inhalation of aerosolized *M. bovis*, and that lesions restricted to the alimentary tract arise from infection through ingestion. However, these assumptions may not be entirely reliable. For example, in a study of experimental oral infection of cattle (i.e. infection through consumption of *M. bovis* containing maize), the preponderance of lesions were in the lungs and pulmonary lymph nodes (Palmer *et al*., 2004). Retropharyngeal lymph nodes, a common site for lesion development in cattle, were not affected. In another study it was shown that of 36 bovine calves fed raw milk from *M. bovis*-infected cows, all developed lesions of the lungs and associated lymph nodes with no evidence of infection of mesenteric lymph nodes or intestine (Edwards, 1937).

Experimental infection

Experimental administration of *M. bovis* by aerosol, intranasal, intratracheal, subcutaneous and intravenous routes results in disease in red and white-tailed deer, but the resulting lesion distribution pattern is unlike that seen in naturally infected animals (Mackintosh *et al*., 1993; Palmer *et al*., 2003). In red deer, instillation of 100 colony-forming units (CFU) of virulent *M. bovis* into the palatine tonsilar crypt produced a spectrum of disease centred on the retropharyngeal lymph nodes, which was indistinguishable from that of natural bTB in deer (Mackintosh *et al*., 1993, 1995; Griffin *et al*., 2006a). Not only does intratonsilar inoculation result in disease similar to that of natural infection, but disease can result from inoculation with as few as eight CFU (Mackintosh *et al*., 1995). Intratonsilar inoculations with similar results have been conducted in white-tailed deer (Palmer *et al*., 1999, 2002a). The intratonsilar infection model can be used to study, among other things, disease pathogenesis.

In experimental infections, tuberculous granulomas develop from a collection of epithelioid macrophages, multinucleated giant cells and few neutrophils to a lesion characterized by macrophages, multinucleated giant cells, infiltrates of neutrophils and central necrosis. Multiple granulomas may coalesce to form a large granuloma with multiple centres of necrosis (Palmer *et al*., 2002a). The necrotic

caseum may liquefy, a change not often seen in cattle, and the reason that many lesions in deer resemble abscesses.

Draining fistulas from retropharyngeal, mandibular, parotid, axillary or inguinal lesions are occasional features of tuberculosis in captive red deer, but have not been reported in other species of captive or wild deer (Griffin and Buchan, 1994; Griffin and Mackintosh, 2000), with the exception of rare reports of draining lesions in the abdominal and flank regions of *M. bovis*-infected elk (Stumpff, 1982). In captive red deer, such draining fistulas are thought to be responsible for some of the deer-to-deer transmission (Lugton *et al*., 1998). It has been hypothesized for captive red deer that the practice of licking discharges from draining lesions (where numbers of bacilli are high) facilitates the tonsilar route of infection, thereby resulting in frequent involvement of the retropharyngeal lymph nodes (Nugent, 2007).

Epidemiology

Wild deer

New Zealand

Where deer form part of broader multi-host systems, their epidemiological role(s) can be difficult to discern. This is epitomized by their complex involvement in New Zealand, where bTB has, since the 1960s, become established in a multi-host system encompassing about 40% (10 m ha) of the country (Ryan *et al*., 2006). The wildlife host system in New Zealand comprises a mix of 15 mammalian species (Coleman and Cooke, 2001), all introduced (along with cattle and *M. bovis*) during European colonization in the 1800s. Australian brushtail possums (*Trichosurus vulpecula*) have long been recognized as the main wildlife, and proven maintenance hosts (Morris and Pfeiffer, 1995; Caley *et al*., 1999). However, high prevalences of bTB have also been recorded in wild deer (mostly red deer), feral pigs and feral ferrets (*Mustela furo*) (Nugent, 2011).

As in most developed countries, management of bTB in cattle became progressively more intense during the mid-1900s, but in New Zealand, by the 1970s progress towards eradication of bTB from cattle slowed and eventually stalled after the widespread emergence of bTB in possums (Ryan *et al*., 2006). As a result, from 1990 onwards, the national management programme expanded to include not only livestock, but also lethal control of possums over approximately 8 million ha. This has resulted in more than a 95% reduction in the number of infected cattle and deer herds (Anon., 2013). A key question for this expanded programme was whether management of wild deer, ferrets and feral pigs was also necessary.

Chronologically, bTB was first suspected in wild deer in New Zealand in 1954, confirmed in wild and captive deer in 1970 and 1978, respectively, and had become widespread among wild and captive deer by the 1980s (Beatson, 1985; de Lisle and Havill, 1985; Lugton *et al*., 1998; Livingstone *et al*., 2015). In the late 1900s, the national wild red deer population was estimated at 250,000 (Nugent and Fraser, 1993), and currently there are roughly 1,000,000 captive deer (mostly red) on about 2800 farms. Intraspecific transmission of *M. bovis* has been demonstrated between experimentally infected red deer and uninfected deer housed together in a paddock (Mackintosh and Griffin, 1994). The incidence of bTB can be very high on deer farms where densities are typically several hundreds of deer/km^2 (Robinson *et al*., 1989; Griffin *et al*., 2004) leaving little doubt that captive deer can be maintenance hosts at such high densities.

Surveys in the 1990s indicated that bTB prevalence in wild deer ranged from 8% to 37% in areas where deer were sympatric with uncontrolled tuberculous possums (Nugent, 2007). Most New Zealand wild deer populations are subject to continuous unrestricted year-round hunting (both recreational and commercial), resulting in much reduced densities overall (<10 deer/km^2), with surviving deer widely dispersed in small groups (Nugent *et al*., 2014). Generalized bTB is comparatively uncommon in New Zealand wild deer, with about 25% of deer with culture-confirmed *M. bovis* infection having no visible gross lesions (Nugent, 2007). The anatomical location of infection in wild deer that present with single lesions suggests a predominantly oral route of infection, but sometimes also by inhalation,

and perhaps occasionally via the skin (Nugent, 2007). Excretion of large numbers of tubercle bacilli appears rare among tuberculous wild deer (Lugton, 1997). Although some deer die soon after becoming infected, some animals are able to survive in an infected state for a decade or more (Nugent, 2007).

Early speculation that wild deer could be maintenance hosts of bTB (Morris and Pfeiffer, 1995) was countered by strong indications that bTB transmission among wild deer was rare, specifically that wild fawns were rarely infected even when a high proportion of their dams were infected (Nugent and Lugton, 1995; Lugton *et al*., 1998). This implied that wild deer were predominantly spillover hosts, as was subsequently supported by a 10-year comparison of trends in the prevalence of bTB in deer between areas with and without possum control. In two large (10,000–20,000 ha) forest areas in which possums (but not deer densities) were greatly reduced, bTB prevalence in deer declined to near zero over 8 years, but did not change significantly in two areas where possums were not controlled (Nugent, 2007). The implication was that most, if not all, bTB observed in New Zealand wild deer is acquired from possums. Additionally, as wild deer can become infected in deep forest areas where they have little opportunity to interact with ferrets or livestock, it is likely that feral pigs as well as possums are the principal sources of infection. Since feral pigs are regarded as dead end hosts in New Zealand (Nugent, 2011), this further supports the view that tuberculous possums are the major source of *M. bovis* infection for wild deer.

Observations of captive red deer biting and licking moribund, semi-comatose, *M. bovis*-infected possums provide a plausible mechanism for possum-to-deer transmission (Sauter and Morris, 1995). The absence of infection in young fawns also indicates that indirect transmission to wild deer from feedstuffs, water or other environmental contamination is likely to be rare (Nugent, 2007; Nugent *et al*., 2014). As there is little evidence of either direct or indirect transmission among wild deer, indirect transmission from wild deer to possums, or to captive deer or cattle, is presumably also uncommon. Nevertheless, cattle have occasionally acquired infection from captive deer (Hennessey, 1986); and the detection of bTB in possums, in a previously bTB-free North Island area, soon after captive deer infected with a South Island DNA type of *M. bovis* were moved there provides compelling evidence of transmission from captive deer to possums (Mackereth, 1993).

Although onward transmission of infection from live tuberculous deer to wild conspecifics is rare, post-mortem transmission to scavengers appears to be common, as scavengers such as feral pigs or ferrets often feed upon tuberculous deer carcasses or carcass remnants left by hunters. Importantly, possums, although largely herbivorous, are also occasional scavengers of carrion (Ragg *et al*., 2000), and have been seen feeding on feral pig and deer remains (Nugent *et al*., 2006; Nugent, 2007). This has been suggested as the route by which possums first became infected in New Zealand in the 1960s (Nugent, 2011). Although the risk of spillback of *M. bovis* infection from wild deer to possums is believed to be small, it is of disproportionate epidemiological importance in two ways:

1. Where bTB in possums is unmanaged, deer may amplify the amount of *M. bovis* infection present within the area to a small degree, but more importantly they could translocate infection and transmit it to possums in a new area (i.e. potential spillback from an infected deer that had moved, or had been moved by humans).
2. Where efforts are being made to eradicate bTB from possums, the apparent ability of some deer to survive in an infected state for more than 10 years (far longer than any of the other wildlife hosts in New Zealand), coupled with the risk of spillback to possums once their populations begin to recover, can constrain how quickly possum control can cease and an area declared bTB free (Barron *et al*., 2013).

North America

Characterization of a species as a spillover or maintenance host of *M. bovis* is not fixed but rather a local or regional characteristic that varies with a variety of factors (Nugent, 2011; Nugent *et al*., 2014). Such is the case with white-tailed deer. Evidence from three North

American outbreaks shows that this species can act as maintenance host (Schmitt *et al*., 1997), spillover host (Carstensen and Doncarlos, 2011), or as a component of multi-host maintenance systems (Wobeser, 2009; Shury and Bergeson, 2011), depending on circumstances.

The basic epidemiology of bTB in wild white-tailed deer in Michigan (MI) has been described (O'Brien *et al*., 2002). The likelihood of being bTB-positive increases with age in both males (bucks) and females (does). This tendency plateaus in does such that aged individuals are only slightly more likely to be bTB-positive than 3 year-olds (Fig. 19.3). While risk in bucks is equivalent to that in does during the first 18 months of life, thereafter it continues to rise throughout their lifetimes. This is consistent with both the social structure and behaviour of white-tailed deer. Fawns of both sexes remain with their dams throughout their first year of life, regularly contacting only members of their matrilineal group. Fawns are likely to be exposed only if one or more group members are *M. bovis*-infected. Before parturition the following year, these groups temporarily dissolve and pregnant does segregate themselves for birthing (Conner *et al*., 2008). Subsequently, related does reform matrilineal groups, and previous contact patterns resume. In contrast, bucks do not return, instead forming same-sex groups, the membership of which changes frequently, thereby providing contact with more, and unrelated, deer. Additionally, polygynous breeding behaviours increase the likelihood that bucks will become infected (O'Brien *et al*., 2002; Conner *et al*., 2008). Doe groups exhibit high site fidelity to summer range (Verme, 1973; Tierson *et al*., 1985; Van Deelen *et al*., 1998; Nelson and Mech, 1999) and so are likely to maintain bTB in focal areas, potentially at high prevalence within groups. As such, bTB-free doe groups may tend to remain so, generally having few contacts outside the group.

Anthropogenic aggregations of white-tailed deer caused by supplemental feeding disrupt the social segregation that separates matrilineal groups, creating between-group contacts that would have been unlikely otherwise (Blanchong *et al*., 2006), increasing bTB transmission and homogenizing prevalence spatially.

With larger home ranges and more contacts, bucks are likely responsible for most intergroup transmission. This may explain higher prevalence in males and considerably higher disease transmission rates (Cosgrove *et al*., 2012a; Ramsey *et al*., 2014). Older deer and bucks were also more likely to be bTB-positive in Minnesota (MN), USA (Carstensen and Doncarlos, 2011) and Manitoba (MB), Canada

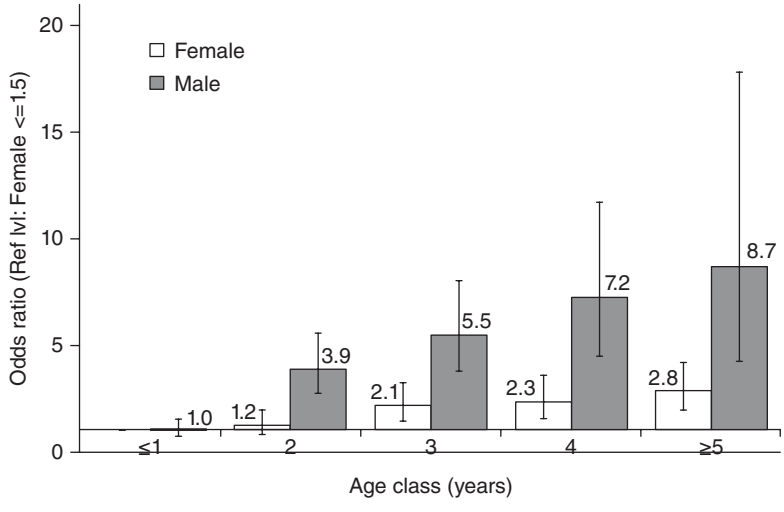

Fig. 19.3. Odds ratio of a white-tailed deer being bTB-positive by age and sex, Michigan, USA, 1975–2013 (n = 198,817). Statistical modelling simultaneously controls for the effects of sex, age, geographic area and survey method.

(Shury and Bergeson, 2011), although comparatively smaller samples limited statistical significance. No obvious adverse population-level effects of bTB on white-tailed deer have yet been observed (O'Brien *et al*., 2006, 2011a).

Whether bTB transmission is frequency dependent or density dependent in white-tailed deer is the subject of active research, and current evidence suggests it may be a function of both gender and spatial scale. Analyses of contact rates at local scales suggest frequency dependence, with greater transmission between members of matrilineal groups (Blanchong *et al*., 2007; Vander Wal *et al*., 2012), but studies have disproportionately focused on does (Schauber *et al*., 2007; Kjaer *et al*., 2008). Others suggest that transmission is intermediate between frequency and density dependence (Habib *et al*., 2011). The complex interplay of social structure, group size, aggregating features, animal movement, season and deer density (Schauber *et al*., 2007; Conner *et al*., 2008; Habib *et al*., 2011; Cross *et al*., 2013) make it difficult to infer the association between locally observed contact rates and the dynamics of transmission at the population level. Moreover, there may be discordance between knowledge of local-scale contacts and the ability to manage bTB at that scale. At broad spatial scales, simulation models of bTB in MI employing simple linear density dependence fit field surveillance data well, both demographically and geographically (Ramsey *et al*., 2014). In that model, transmission rates in bucks were ten times higher than in does (Cosgrove *et al*., 2012a). Field prevalence data in yearling deer also suggest density dependence (Fig. 19.4), consistent with other evidence that density reductions decrease bTB transmission (Lugton *et al*., 1998; Hickling, 2002; Carstensen and Doncarlos, 2011; Nugent *et al*., 2014). Although threshold densities for disease persistence are elusive (Lloyd-Smith *et al*., 2005), MI data suggest that the incidence of bTB in yearling white-tailed deer is likely to be low at densities less than approximately 10 deer/km^2.

Under natural conditions, bTB tends to be highly clustered (O'Brien *et al*., 2004, 2006; Miller *et al*., 2007). While apparent prevalence across the approximately 1500 km^2 core outbreak area in MI has remained relatively stable at just under 2% for the last decade, prevalence at the township (93 km^2) scale has been two to ten times higher in specific areas. Thus, while cattle herd breakdowns and overall prevalence in deer are reduced from historic highs it is not surprising that cattle herds near clusters of higher prevalence in deer continue to

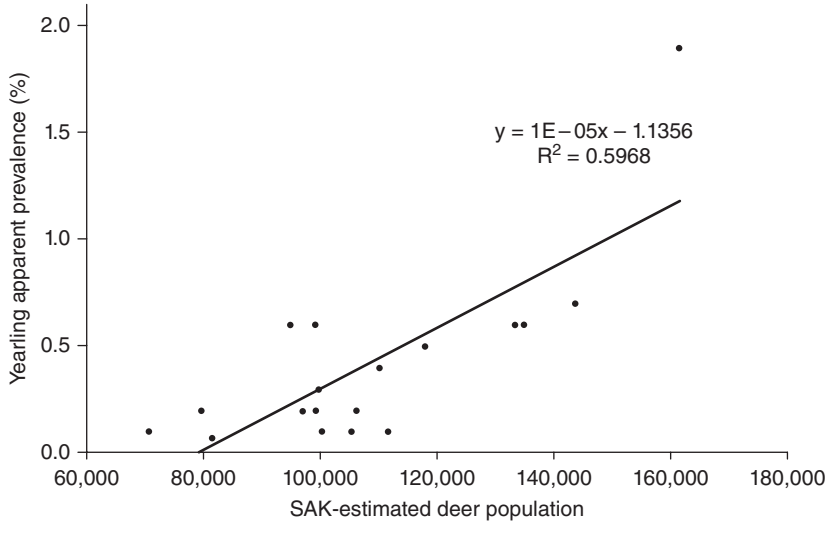

Fig. 19.4. Correlation plot of apparent prevalence of bovine tuberculosis (bTB) in yearling white-tailed deer versus the sex–age kill (SAK) estimated white-tailed deer population, Alcona, Alpena, Montmorency, Oscoda and Presque Isle Counties, Michigan, USA, 1995–2012.

become infected. Spatially-explicit simulation modelling suggests that bTB prevalence in the core area will need to be maintained below 1% before cattle herd infections fall to less than 1/year, and below 0.1% to achieve a 95% probability of having no infected cattle herds in a given year.

A study in MI's bTB endemic area (Berentsen *et al*., 2014) found a majority of radio-collared deer visited feed storage and feeding areas on cattle farms, but only 19% of the deer accounted for nearly 90% of those visits. Visits to such areas on multiple adjacent farms were common. Having localized cattle feeding areas significantly increased deer visits. Direct contacts between deer and cattle are rare (Hill, 2005), therefore, cattle feeding practices provide the most likely route for indirect transmission of *M. bovis* between deer and cattle (Palmer *et al*., 2004; O'Brien *et al*., 2006). Thus, even a small number of infected deer shedding bacilli could be responsible for multiple cattle herd breakdowns in a persistently infected area.

It is important to note that transmission between deer and cattle is two-way. In MN DNA typing of *M. bovis* isolates from cattle and deer were most similar to isolates from Mexican cattle (Milian-Suazo *et al*., 2000). Although in and of itself such typing does not elucidate the direction of interspecies transmission, in this case, because MN is more than 2000 km from Mexico (far beyond dispersal distances documented for white-tailed deer) in all likelihood bTB was introduced by movement of infected cattle, and spilled over into resident deer (Carstensen and Doncarlos, 2011).

Wild elk were maintenance hosts (with white-tailed deer supporting) in an outbreak of bTB in and around Riding Mountain National Park (RMNP) in south-western MB (Wobeser, 2009; Shury and Bergeson, 2011). The two closely related *M. bovis* spoligotypes in the area are unique, and their origin is unknown (Lutze-Wallace *et al*., 2005b), although their introduction pre-dates 1978 (Lutze-Wallace *et al*., 2005a). Elk were first implicated when a hunter harvested a tuberculous elk near a bTB-infected cattle farm. The farm was one of several herd breakdowns occurring between 1950 and 1991 (Lees *et al*., 2003; Lees, 2004; Wobeser, 2009). Systematic surveillance of wild elk and white-tailed deer begun in 1997 has since found 41/4542 (0.9%) elk and 11/7368 (0.1%) white-tailed deer bTB-positive by mycobacterial culture (Shury and Bergeson, 2011). Over 90% of both the bTB-positive elk and white-tailed deer originate from an 1800 km^2 western control zone, suggesting that, like bTB in white-tailed deer in MI, the outbreak is largely clustered. Affected elk are part of a single subpopulation that is largely isolated from other elk within RMNP (Vander Wal *et al*., 2012). Currently, there is no evidence of geographic spread, and the potential for long-distance spread via movement of infected elk is considered low (Vander Wal *et al*., 2012). Population reductions of both elk and white-tailed deer, undertaken for disease management, have been associated with declines in bTB prevalence in both species (Shury and Bergeson, 2011), consistent with density-dependent transmission at a broad spatial scale.

Among the RMNP elk, bTB is more prevalent in males than females. Elk more than 6 years of age are at significantly increased risk (Shury and Bergeson, 2011). No bTB-positive elk younger than 5 years of age has been found since 2003, suggesting the incidence is currently low. All bTB-positive elk in RMNP have had gross lesions, compared to 4/6 (of 3015 total) elk tested in MI (O'Brien *et al*., 2008; D.J. O'Brien, Michigan, USA, 2014, personal communication) but this may be due to more sensitive necropsy protocols used in MB (Shury and Bergeson, 2011). Among positive elk in MI, males (4 versus 2 females) and adults mean age 4.1 ± 1.3 years have been most common. The differing host status of sympatric elk and deer in MB (elk: maintenance; white-tailed deer: spillover) versus MI (white-tailed deer: maintenance; elk: spillover) mirrors bTB prevalence estimates for the two species – in MI: elk 6/3015 (0.2%); white-tailed deer 160/34,321 (0.5%) – over co-occupied range. This highlights the relative segregation of elk and white-tailed deer in the absence of a mutually attractive congregating factor (Miller, 2002). Such factors may be anthropogenic and, in the case of livestock feed, facilitate interspecies transmission of bTB not only to cattle, but also between elk and deer.

Reports of bTB in moose (Wobeser, 2009) and mule deer (Rhyan *et al*., 1995; Wobeser,

2009) also exist, but compared to elk and white-tailed deer, little epidemiological data are available. Historic data from depopulation of Buffalo National Park near Wainwright, Alberta, recorded infection in about 6% (7/113) of moose and 0.8% (2/242) of mule deer sharing range with heavily infected (i.e. approximately 53% with gross lesions) bison (*Bison bison*) (Wobeser, 2009). While this indicates the susceptibility of those species under conditions of high exposure, the relatively solitary habits of free-ranging moose, under more typical conditions (Conner *et al*., 2008), may not lend themselves to maintenance of bTB. In addition, the dietary and habitat preferences of moose have thus far limited their exposure. This may change as moose gradually occupy previously vacant parkland and grassland habitat in western Canada. None of nearly 600 moose tested in the RMNP area has thus far been bTB-positive (Wobeser, 2009). In contrast, the more social behaviour of mule deer (Conner *et al*., 2008) may make them more suitable as maintenance hosts, although their habitat preferences for remote terrain limit their exposure to cattle, which remain the primary source of infection for wild deer.

UK and Republic of Ireland

There are six species of wild deer in the UK and four in the Republic of Ireland. These include the native red deer and roe deer (Great Britain only), and the naturalized fallow deer that were introduced by the Normans. In addition, the non-native Japanese sika, Chinese muntjac and Chinese water deer (*Hydropotes inermis*) were introduced from the late 19th century onwards. All six species can be found in Great Britain, although muntjac and water deer are restricted to England and Wales. Red, fallow and sika deer are also present on the island of Ireland, that is Northern Ireland and the Republic of Ireland (hereafter referred to as ireland), and muntjac have been introduced in several locations in recent years (Carden *et al*., 2011). There is evidence for recent range expansion in all species present in Great Britain (Ward, 2005) and for red, fallow and sika deer in Ireland (Carden *et al*., 2011).

Infection with *M. bovis* has been identified in five of the six species of wild deer in the UK and Ireland. The first reported case in wild deer was a fallow deer found dead in County Wicklow, Ireland (Wilson and Harrington, 1976). Subsequent cases in fallow deer were recorded in Ireland (O'Reilly, 1987) and the UK (Delahay *et al*., 2002). Estimates of prevalence vary widely with location and scale of the study, and include local hotspots of up to 15% (O'Reilly, 1987). An estimate of 18.5% prevalence in a sample of 65 fallow deer collected from throughout southern England and Wales (MAFF, 1997a) was adjusted downwards to 1.2% once farmed and parkland deer were discounted (Simpson, 2000). The largest survey for bTB in fallow deer in the UK to date (n = 504) identified infection in 4.4% (95% CI 2.8–6.5) of carcasses collected from southern England and Wales in areas of relatively high bTB incidence in cattle (Delahay *et al*., 2007).

From 1980 onwards, cases of bTB were recorded in roe deer in south-west England (Gunning, 1985; MAFF, 1997a) with the prevalence of infection in large samples of roe deer estimated at 0.9% (95% CI 0.3–1.9, n = 695) (MAFF, 1997a) and 1.0% (95% CI 0.5–1.9, n = 885) (Delahay *et al*., 2007). In several instances infection in roe deer in south-west England has been recorded in association with bTB outbreaks in local cattle (Gunning, 1985; Proud and Davis, 1998) although no direct links were established.

Early reports of *M. bovis* infection in red deer originated from culled wild deer in a national park in Ireland (Quigley *et al*., 1997) and County Armagh, Northern Ireland, in 1996–97 (Delahay *et al*., 2002) with the prevalence of infection estimated at 2.6% (9/340) and 12.8% (10/78), respectively. During the late 1990s infection was sporadically reported in wild red deer in south-west England (MAFF, 1997a). In a sample of red deer collected from throughout areas of southern England and Wales with a relatively high bTB incidence in cattle (Delahay *et al*., 2007), prevalence of infection was estimated at 1.0% (95% CI 0.1–3.6, n = 196).

M. bovis infection has been confirmed in wild sika deer in the UK and Ireland (Delahay *et al*., 2002). In one area of southern England infection in sika and roe deer (Rose, 1987; MAFF, 1997b) was associated with a serious bTB outbreak in local cattle (MAFF, 1997b). Interestingly,

M. bovis has also been isolated from sika deer in Scotland in the Mull of Kintyre (MAFF, 1997b) and Argyll (MAFF, 1997a) where the incidence of cattle herd breakdowns is extremely low. In County Wicklow, Ireland, infection has also been detected in sika–red deer hybrids (Dodd, 1984).

Since multiple introductions were made to the UK from the late 19th century onwards, muntjac deer have increased in range and abundance (Ward, 2005; Harris and Yalden, 2008) and are now also found in parts of Ireland (Carden *et al*., 2011). The first case of bTB in a muntjac deer was recorded in a female that was shot in south-west England in an area where other deer species were also known to be infected (Delahay *et al*., 2001). A subsequent survey of wild muntjac deer from south-west England identified a prevalence of infection of 5.2% although confidence limits around this estimate are wide (95% CI 1.1–14.4) given the relatively small sample size (n = 58).

The role of deer in the epidemiology of bTB infection in cattle in the UK and Ireland is unclear. The risks of infection of cattle from wildlife are deemed to be greatest from European badgers (*Meles meles*) with supporting evidence for their involvement from large-scale field trials (Griffin *et al*., 2005b; Donnelly *et al*., 2007) although their relative contribution alongside the reservoir of infection in cattle continues to be debated. Spoligotypes of *M. bovis* in wild deer in southern England and Wales were found to broadly reflect those seen in cattle and other wildlife (Delahay *et al*., 2007), consistent with transfer among species. Risk assessments combining data on prevalence, pathology, host biomass (size and abundance) and behaviour (Delahay *et al*., 2007; Ward *et al*., 2009) suggest that, in the UK, risks of cattle exposure to *M. bovis* from deer could be substantial, particularly for fallow deer. However, given the wide geographic variation in deer and cattle abundance across much of the UK (Ward, 2005; Harris and Yalden, 2008; Carden *et al*., 2011) these risks are likely to vary locally (Ward *et al*., 2009). An attempt to evaluate the likely host status (i.e. dead end, spillover or maintenance host) of four deer species by manipulating the reproductive number (Anderson and May, 1991) failed to confidently assign host status for red and roe deer across a wide range of densities, but concluded that infected muntjac and fallow deer were highly likely to act as maintenance hosts at densities considered high in the UK (greater than 56/km^2 for muntjac and 47/km^2 for fallow deer). Moreover, red and roe deer were considered likely to act as spillover hosts, at the most, when present at low to moderate densities (less than 30/km^2 for red deer, less than 1/km^2 for roe deer) (Ward and Smith, 2012). Risk scores and likely host status estimates for deer relative to those for European badgers are consistent with deer playing a secondary role, at most, in the perpetuation of bTB in British cattle. Areas of critical data shortfall regarding the potential role of deer in perpetuating bTB infection in cattle in the UK include the levels of bacterial excretion by deer, the frequency of direct or indirect contact with cattle and local estimates of deer density.

Captive deer

North America

Deer farming in the USA is relatively new compared to New Zealand, where in 1997 there were >1.4 million captive deer (Griffin, 1988; Griffin and Mackintosh, 2000). At the same point, there were roughly 250,000 captive deer in the USA, with whitetails being the most common species. In North America, the captive deer industry has continued to grow, and in many states is regulated by government agencies similar to other livestock production industries (Kaneene *et al*., 2002). Captive deer enterprises produce animals for shooting, venison, antler velvet, breeding and doe urine (an attractant for hunting) (Kaneene *et al*., 2002).

Fox (1923) describes some of the earliest reports of tuberculosis in captive deer in North America. Of 171 deer examined from the Zoological Society of Philadelphia, 19 (11.1%) were considered tuberculous (Fox, 1923). Later, in 1961, two captive white-tailed deer died soon after they were transported from Wisconsin (WI) to Illinois. Necropsy revealed lesions consistent with tuberculosis. Investigations revealed the two tuberculous

deer had originated from a source in MI, were purchased by a WI farmer and sold with another group of deer of WI origin (Ferris *et al*., 1961). Separately, in 1963 in a deer park in MI where red, sika and fallow deer were present, only fallow deer were bTB-positive. Of 55 fallow deer with positive tuberculin skin tests (TST), 41 (75%) had gross lesions consistent with tuberculosis (Quinn and Towar, 1963). In the 5-year period before this discovery, over 200 animals had been sold and transported from this single facility. These two examples serve to illustrate the high risks associated with animal movements between facilities.

In 1980–1981, bTB-positive elk in multiple herds were traced back to a herd in South Dakota. It is believed that *M. bovis* was introduced to the source herd by an elk purchased from a zoo in Iowa (Stumpff, 1982). Importantly, at least three persons associated with these herds (a veterinarian, a herd owner and a rendering plant operator) converted from skin-test negative to skin-test positive status, implying exposure to *M. bovis*.

In 1990, outbreaks in captive elk herds in Canada were traced back to the USA (Essey and Koller, 1994). Between 1991 and 1993 tuberculosis was confirmed in 24 herds in 14 states. By 2003 the number of infected herds had increased to 43. Extensive movement of breeding stock characterized the burgeoning deer-farming industry and was likely to have facilitated widespread distribution of bTB-positive animals (Essey, 1991; Essey and Koller, 1994). The outbreaks of tuberculosis in captive deer in the 1990s prompted the US Department of Agriculture (USDA) to draft uniform methods and rules (UMR) for eradication of bovine tuberculosis in captive deer, initially published in 1994 (Essey and Koller, 1994) and revised in 1999.

In 1997, bTB was identified in a large captive deer-shooting ranch in MI. It is likely that the source of infection in this facility was infected wild deer, which were fenced in upon creation of the ranch (Kaneene *et al*., 2002). Infection was discovered several years later when tuberculous lesions were seen in a deer shot within the facility. Herd depopulation revealed a bTB prevalence of 12% (Palmer *et al*., 2000).

Between 2004 and 2010, bTB was detected in three captive deer facilities in Indiana (elk and other deer species), three in MI, one in New York (red and fallow deer) and one in Nebraska (elk and fallow deer) (Waters *et al*., 2011). Since 2010 no bTB-positive captive deer have been identified, although two facilities remain under quarantine in MI (O'Brien *et al*., 2011a).

UK

The captive deer population in the UK consists largely of farmed red deer and fallow deer in 'park land' enclosures. Also, a semi-domesticated herd of reindeer has been present in the Cairngorms, Scotland, since their introduction in 1952. Reindeer are also found in a number of small zoological collections (Harris and Yalden, 2008). Numbers of captive deer have grown substantially in the UK, with an estimate of approximately 5000 animals on over 100 farms in 1985, rising to about 31,000 in 2012 (DEFRA, 2013a). In 2008 there were estimated to be approximately 400 separate deer farms in the UK (EFSA, 2008). The number of captive deer in parks is less certain but was estimated to be approximately 40,000 in 2005 (DEFRA, 2013a).

The first recorded case of bTB in captive deer in the UK was detected in red deer imported from Hungary (Stuart *et al*., 1988). Official statistics on bTB in captive deer in the UK and Ireland suggest a generally low incidence of infection with the number of deer farms under movement restrictions at the end of 2011 and 2012 at 12 and 9, respectively (DEFRA, 2013b). Post-mortem meat inspection and subsequent notification are the mainstays of surveillance for bTB in captive deer in the UK and there is a legal requirement to report incidents of affected or suspect cases in both captive and wild deer to the veterinary authorities. There is no compulsory routine or pre-movement testing regime for captive deer herds in the UK, and ante-mortem skin testing usually only occurs in response to a bTB incident. Movement restrictions, the isolation and testing of individual animals and the slaughter of reactors can be imposed by the UK's Department for Environment, Food and Rural Affairs (DEFRA).

Zoonotic concerns

M. bovis is well recognized as a pathogen of humans capable of causing disease similar to *M. tuberculosis* (Muller *et al*., 2013). Nevertheless, there have been few reports of humans being infected with *M. bovis* from deer. A major outbreak in captive elk in Canada resulted in one active infection of *M. bovis* in a veterinarian and a number of contacts converted to a positive TST, indicating exposure to *M. bovis* (Fanning and Edwards, 1991). The mode of transmission from these deer to humans was most likely through aerosolized bacilli. In New Zealand, infected captive deer have not been recognized as a source of infection for humans (Baker *et al*., 2006). This is surprising given that captive deer are handled in enclosed sheds that would enhance exposure through aerosolization. An alternative route of infection is through injured skin (i.e. cutaneous). Two cases of *M. bovis* infection in humans have been linked to *M. bovis* found in wild deer in MI (Wilkins *et al*., 2008). One of the two cases was cutaneous tuberculosis in a hunter, the result of an injury sustained during field dressing of a tuberculous white-tailed deer.

Diagnosis

Cell-mediated based tests

Ante-mortem diagnosis of tuberculosis in animals has relied on measurement of delayed-type hypersensitivity (DTH) by intradermal injection of mycobacterial extracts, the most common of which is purified protein derivative (PPD). Early investigation of intradermal skin testing of deer showed the caudal fold test (CFT), as performed in cattle, is unreliable in deer (Norden *et al*., 1996). Skin testing, by injection of PPD in the skin of the lateral neck (i.e. cervical region), was found to be more reliable. DTH reactions resulting from intradermal tuberculin injection appear grossly similar in deer and cattle. Nevertheless, some exceptions have been reported. Reactions in fallow deer have been described as suffuse, soft and up to 12 cm in diameter (Quinn and Towar, 1963), rather than as induration. Alopecia, as well as alterations in hair growth (both loss and increased growth) and pigmentation at the site of injection have also been reported in fallow deer (Towar *et al*., 1965). In one account, after several tuberculin injections, 60 days apart, fallow deer with hair loss and black pigmentation of the skin were euthanized and examined. All had gross lesions consistent with tuberculosis (Quinn and Towar, 1963).

In the USA, captive deer of unknown infection status are tested by the single cervical tuberculin test (SCT) and the results are categorized as negative, suspect or reactor. Suspects and reactors may be re-evaluated using the comparative cervical tuberculin test (CCT). The CCT involves injection of PPD from *M. bovis* (PPD-B) and *M. avium* (PPD-A) at two different sites on the lateral neck. The addition of PPD-A increases the specificity as compared with the SCT. Animals infected with non-tuberculous mycobacteria (NTM) may be suspects or reactors on the SCT; but using the CCT, these animals can often be identified due to a greater change in skin thickness at the PPD-A injection site compared to that at the PPD-B injection site.

There are more species-specific data from red deer on the sensitivity (Se) and specificity (Sp) of the SCT than from any other deer species. In New Zealand, the Se of the SCT in naturally or experimentally infected red deer has been reported to be 82% and 86%, respectively (Corrin *et al*., 1993; Griffin *et al*., 1994). A CCT developed for red deer (Carter *et al*., 1986) showed greater than 90% Se in experimentally infected deer, although under field conditions Se varied from 30% to 80% and Sp ranged from 61% to 88% (Griffin and Buchan, 1994). In a separate study, limited to experimentally infected red deer, the CCT showed a Se and Sp of 91.4% and 98.7%, respectively (Kollias *et al*., 1982). Others have estimated a range of Sp from 46% to 76% in herds of bTB-negative red deer (Norden *et al*., 1996). Regardless of wide-ranging Se and Sp, the SCT and CCT have been used successfully in New Zealand's national test and cull programme to dramatically decrease disease prevalence.

Antibody-based tests

As the prevalence of bTB in captive deer in New Zealand decreased through the use of the TST, Sp became paramount due to the increasing number of false-positive test results. For that reason, the captive deer industry advocated for additional tests to increase Sp. In response to this challenge, a new antibody-based ELISA test (ETB) was developed (Chinn *et al*., 2002) that measured IgG1 antibody levels to PPD-B in animals sampled 14–30 days after the TST. This test had an estimated Se of 89% and Sp of greater than 98% and it was approved as an official serial test for diagnosis of bTB in SCT-positive deer in 2005. During the development of the ETB it became evident that a proportion of herds harboured significant levels of infection of *M. avium* subsp. *paratuberculosis* (*Map*), the cause of Johne's disease. As the specificity of the ETB for bTB was lower in *Map*-infected herds (Griffin *et al*., 2005a), and there were increasing numbers of *Map*-infected herds, a modified ETB was developed for use in herds known to be at low risk of being infected with *M. bovis*. In addition to the antigens PPD-A and PPD-B it includes PPD Johnin (PPD-J) as an additional antigen to identify deer that are *Map*-infected but not *M. bovis*-infected.

In the USA, research on serological tests for bTB in deer (Waters *et al*., 2004; Lyashchenko *et al*., 2008) led to development of a rapid serological test, licensed for use in red, fallow and white-tailed deer and elk (Lyashchenko *et al*., 2013). The dual-path platform (DPP) detects antibodies to two antigens (MPB83 and CFP10/ESAT-6 fusion protein) to which most *M. bovis*-infected deer respond. The Se of the DPP in experimentally or naturally infected deer was 58.1% and 71.9%, respectively. Specificity was shown to be 97.8% (Lyashchenko *et al*., 2013). In a herd of naturally infected elk and fallow deer the Se/Sp of the DPP was 79%/98% and 91%/99%, respectively (Waters *et al*., 2011). In this group of elk the prevalence of disease was unusually high, around 75%. In this scenario, serology greatly outperformed the SCT, with an estimated Se of 11%. The reason for the unusually low Se of SCT is unclear.

Post-mortem diagnosis

A presumptive diagnosis of tuberculosis in deer can be achieved by recognition of the macroscopic appearance of lesions. Caution should be exercised in interpreting the macroscopic and microscopic appearances of lesions because those caused by *M. bovis* in deer are more variable than in cattle. This variability highlights the need to confirm a presumptive morphologic diagnosis of tuberculosis by identification of the causative organism. While classical bacterial culture remains the principal method for detecting *M. bovis*, PCR-based methods have the attraction of providing a rapid answer about whether or not a member of the *M. tuberculosis* complex (MTC) is present. Most PCR tests for detecting tuberculosis in deer use primers from genes such as those for IS1081, which are not only present in *M. bovis*, but also found in other members of the MTC. In the majority of countries, this is of minor importance as *M. bovis* is the only member of the MTC known to infect deer. An exception is in Europe where *M. caprae* infects a wide range of domestic animals and wildlife, including deer (Rodriguez *et al*., 2011). Individual members of the MTC can be identified using DNA typing procedures such as spoligotyping or PCR to identify specific deletions.

While the performance of PCRs has improved, they still are not as sensitive as bacterial culture for detecting *M. bovis* when present in low numbers. The use of a sensitive culture procedure is particularly important when examining tissues that do not have macroscopic lesions of tuberculosis. In MI, the culturing of pooled lymph nodes from up to 20 deer that do not have lesions has proved to be a means of reducing laboratory costs, yet still retaining the ability to identify *M. bovis* in these animals (Schmitt *et al*., 1997). Nevertheless, in New Zealand, the number of bacilli in tissues without gross lesions was found to be low and pooling of tissues from several deer effectively 'diluted' the sample, adversely affecting Se (G.W. de Lisle, New Zealand, 2005, personal communication).

Bacterial culture provides additional information compared to PCR as it can identify other mycobacterial species if present, and it

provides *M. bovis* isolates that can be used for DNA typing. The latter have proved useful to unravel the epidemiology of tuberculosis in deer and determine the role deer play in multi-host infection cycles. Investigations in New Zealand found the same DNA types in cattle, captive deer and wildlife (including ferrets and brushtail possums) in a region of endemic bTB, suggesting a common source of infection (de Lisle *et al*., 1995).

The most common mycobacterial species outside the MTC isolated from suspect tuberculous lesions in deer are members of the *M. avium* complex, including *M. avium* subspecies *avium* and *Map* (Thacker *et al*., 2013). A feature of Johne's disease in captive red deer is the variable appearance of the lesions, usually in mesenteric lymph nodes, ranging from necrotic, liquefied abscesses to granulomas where necrosis is minimal or absent. In contrast to cattle, in captive red deer it has proved difficult to distinguish by microscopic examination whether lesions are caused by *Map* or *M. bovis* (de Lisle *et al*., 2003). For this reason, in New Zealand suspect tuberculosis lesions identified in captive deer at slaughter are routinely examined by PCR and culture and not by histopathology. Members of the *M. avium* complex other than *Map* have been routinely isolated from captive white-tailed deer (Palmer *et al*., 2014). The resulting lesions are usually small and only on rare occasions has generalized clinical disease been observed, as in the case of wild red deer in Scotland and England (Matthews *et al*., 1981), and severe lung disease in an axis deer in a zoo in India (Arora, 1993).

A range of mycobacterial species other than members of the *M. tuberculosis* and *M. avium* complexes has been isolated from deer (de Lisle and Havill, 1985; Palmer *et al*., 2010b, 2014; Thacker *et al*., 2013). Some species, such as *M. kansasii*, are known to be rare causes of bTB-like lesions in deer (Hall *et al*., 2005), while other mycobacterial species have only been isolated from tissues without lesions. The significance of the isolation of these mycobacteria is often unknown, but some have the potential to induce an immune response that causes false-positive responses to immune-based diagnostic tests for tuberculosis.

Disease Control

New Zealand wild deer

The only strategic approach ever considered for bTB control in wild deer in New Zealand has been population reduction. There is a range of well-established management techniques available (including commercial or contracted professional hunting or poisoning) that could be used in New Zealand to reduce deer densities. Furthermore, the national TB management agency, TBfree New Zealand (formerly the Animal Health Board) has the legal power to target wild deer for bTB control if required, but because deer are considered to be spillover hosts, it has not exercised this right (Livingstone *et al*., 2015).

In reality, the only direct control of bTB in deer in New Zealand has resulted from the incidental killing of deer during aerial distribution of poison baits (sodium fluoroacetate (1080)) directed at possums. During intensive possum control operations, about one-third of deer may die incidentally as by-kill (although some estimates have been higher) (Nugent *et al*., 2001; Nugent and Yockney, 2004). This by-kill presumably reduces the number of *M. bovis*-infected deer proportionately, but by itself is insufficient to immediately eradicate bTB from deer. However, the reductions in possum density following control are typically in excess of 90%, presumably causing an immediate proportionate reduction in the rate of spillover from possums to deer. More importantly, this reduction will induce a decline in bTB prevalence to zero in possums that, over a 5–7-year period, also reduces the incidence of new spillback-generated infections in deer to zero (Barron *et al*., 2013). Nevertheless, medium-term survival of previously infected deer (i.e. those that have acquired *M. bovis* prior to possum control and have carried the infection since) would result in only a gradual decline in the prevalence of infection in deer. Taking this mechanism into account, empirical data (Nugent, 2007) and modelling (Barron *et al*., 2013) suggest that it takes 10–15 years for residual *M. bovis* infection to disappear from deer. Modelling predicts that application of practical and affordable additional direct lethal control to reduce the density of deer

would have only a small effect on reducing the time required to eliminate bTB from the deer population (hence supporting the empirical disease management decisions to not directly cull deer for bTB control in isolation).

Crucially, the persistence of *M. bovis* in deer infected a long time previously constrains how quickly bTB can be eradicated from New Zealand wildlife. Likewise, there is a plausible but unquantifiable risk that bTB could spill back from deer to possums, sufficient to establish new disease foci in the possum populations. If possum control was stopped early, and the possum population rebounded to above bTB persistence thresholds, the result could be re-establishment of self-sustaining infection among possums. Thus, while direct management of bTB in wild deer is not an essential prerequisite to eradicating bTB from New Zealand wildlife, the longevity of residual bTB in deer determines the duration of management required to achieve eradication.

New Zealand captive deer

The New Zealand captive deer industry provides a compelling story of the real and perceived threat caused by bTB in a newly domesticated species. Deer farming began in about 1970, but no evidence of bTB infection was seen in captive deer until 1978 (Beatson, 1985) when it was diagnosed in a recently established deer farm. Use of the conventional TST in a test and cull programme from 1978 to 1983 failed to eliminate infection on that farm and when the herd was depopulated in 1983, *M. bovis* was isolated from 22/241 (9%) of TST-negative deer. A voluntary bTB control scheme for captive deer was introduced in 1986, using the SCT. The number of animals tested increased annually until 1995 when a compulsory scheme was introduced for all captive deer herds (Hutchings, 1995). However, sensitization of deer with non-tuberculous environmental mycobacteria produced a significant number of false-positive results, limiting the value of the SCT. A CCT was subsequently developed for deer (Carter *et al*., 1986) and while the test demonstrated greater than 90% Se in experimentally infected deer, Se under field conditions varied from 30% to 80% (Griffin and Buchan, 1994). In 1985, the captive deer industry commissioned the development of alternative laboratory-based diagnostic tests for use in conjunction with the SCT in an attempt to improve the overall accuracy. Griffin and Cross (1986) developed a composite laboratory test, the blood test for bTB (BTB) which was used throughout the captive deer industry for the ensuing 20 years (Griffin and Cross, 1986). The BTB was a complex, cumbersome and expensive test as it embodied multiple parameters of immunity (inflammation-, antibody- and cell-mediated immunity), that when combined and interpreted together, produced an assay with greater than 95% Se and 98% Sp (Griffin *et al*., 1994).

The incidence of bTB in the national captive deer herd decreased from 0.14% in 1989 to 0.07% in 1991 and 0.04% in 1993 (Corrin *et al*., 1993). Systematic use of primary and ancillary herd testing resulted in an incremental decrease in the incidence of bTB from 0.04% in 2002 to 0.005% in 2005. In 2013, a total of ten tuberculous deer (0.0001% of the captive population) was identified and there are currently only five bTB-infected deer herds in New Zealand (Anon., 2013) down from >300 in the late 1980s.

The success of bTB control in New Zealand captive deer represents an excellent example of cooperation between regulators, deer producers and scientists. The proactive deer-farming sector in New Zealand recognized that bTB could compromise the financial viability of this novel farming enterprise. The fledgling industry focused on problem solving rather than levy-based compensation and was motivated to find solutions provided by ancillary technologies. The singular focus of the deer industry in collaborating fully with regulatory authorities and testing agencies has been paramount for the overall success of the programme. Diagnostic tests with improved sensitivity combined with successful efforts to mitigate or eliminate risk of infection from brushtail possums have been essential to guarantee the overall success of this programme.

North American wild deer

Bovine tuberculosis control strategies initiated at the discovery of the MI outbreak assumed

that deer density and aggregations were likely to be the factors most responsible for bTB transmission (Schmitt *et al*., 1997). Subsequent surveillance has largely validated these initial approaches (O'Brien *et al*., 2006). At landscape scale, trends in bTB prevalence have tracked reductions in deer density well over time (Hickling, 2002; O'Brien *et al*., 2011a). Given evidence of density-dependent transmission, three principal approaches have been used to decrease transmission via population reduction: increasing hunter harvest, landowner shooting permits and agency culling.

Hunter harvest provides an efficient means to kill large numbers of deer annually; a repeatable source of specimens for surveillance; recreational and deer management cooperation opportunities for hunters; and salvage of carcasses for consumption. However, disease managers can control harvest intensity only indirectly, and maintaining sufficient harvest pressure for disease control can be difficult. Moreover, efficacy of harvest is contingent upon hunters gaining access to lands where deer are found.

In MI, hunter harvest has been the primary method of population management for both white-tailed deer (Schmitt *et al*., 1997; O'Brien *et al*., 2006) and elk (O'Brien *et al*., 2008). Over 90% of deer tested for bTB have been obtained via hunter harvest, and harvest accounts for about 99% of all deer killed, for both bTB control and generally. In the first decade after bTB management activities were initiated, increased harvest opportunities and plentiful low-cost hunting licences facilitated reduction of the estimated deer population by 51% in the endemic area (O'Brien *et al*., 2011a). That reduction accompanied a 65% decrease in bTB prevalence over the same period. As hunters saw fewer deer, harvest decreased, the population partially rebounded to approximately 106,000 currently, and bTB prevalence is little changed over the last decade. Simulation modelling predicts that current rates of hunter harvest are likely to result in only a modest decrease in bTB prevalence (about 0.3%) over the next 30 years (Ramsey *et al*., 2014). Stable deer harvest in the endemic area over the last decade suggests demand for hunting opportunity has been saturated, and further expansion of opportunities is unlikely to increase harvest sufficiently to eradicate bTB. In addition, hunting restrictions by private landowners have created refuges where deer densities, and bTB prevalence, are often higher (O'Brien *et al*., 2006).

In MN, over 4 years, hunter harvest accounted for 55% of the deer population reduction in the bTB core area (Carstensen and Doncarlos, 2011). Harvest dropped by nearly one-half after the first year, due both to a decrease in deer available for harvest and reduced hunter effort because of perceptions that agency culling had adversely affected deer numbers. Increased harvest opportunities (e.g. extended hunting seasons, inexpensive licences) did not effectively increase harvest, but hunter-harvested deer nonetheless comprised 71% of deer tested for bTB and 44% of the bTB-positives (Carstensen and Doncarlos, 2011).

Landowner permits that allow shooting of deer outside of hunting seasons are often popular with cattle producers as a putative means for controlling deer on their lands. However, evidence suggests that only a small percentage of landowners use them (O'Brien *et al*., 2013). Landowner shooting permits have been used in both MN and MI, but have had minimal effect on deer population density and bTB prevalence. Deer harvested on such permits accounted for just over 2% of MN deer collected for bTB management, and removed only one infected deer (Carstensen and Doncarlos, 2011). In MI, a 3-year programme in which permits were mass-mailed annually to all cattle producers in the endemic area did not significantly increase the overall deer harvest or the number of bTB-positive deer removed. Moreover, it had no demonstrable effect on the rate of cattle herd infections, and created considerable public opposition (O'Brien *et al*., 2011a, 2013).

Culling of deer by government agencies or their contractors enables geographically focal depopulation, and efficient removal of entire deer social groups (Mateus-Pinilla *et al*., 2013), but necessitates gaining ground access to lands where the deer are found. Alternatively, aerial shooting can improve access, but is generally not used because of intense unpopularity. Although agency culling has not been seriously considered in MI (O'Brien *et al*.,

2011b), better public acceptance enabled its effective application in MN (Carstensen *et al*., 2011). The high proportion of publicly owned lands, to which access was unimpeded, facilitated ground shooting. Similarly, aerial shooting was enabled by high-level political support. Culling was aggressive and evidence suggests a high proportion of deer in MN's bTB core area was killed, with numbers removed exceeding population estimates (Carstensen and Doncarlos, 2011). Twenty-seven per cent of deer tested for bTB and 52% of culture-positive deer were obtained via culling. Notably, public support for culling declined markedly as fewer bTB-positive deer were discovered. Yet, it is remarkable that hunters funded most of the culling operations via hunting licence fees, and responded to cessation of culling with the increased hunter harvest requested by wildlife managers. No additional *M. bovis*-infected wild or captive deer or cattle herds have been identified in MN since 2009.

While white-tailed deer occupy distinct home ranges, they overlap extensively and deer do not defend territories (Marchinton and Hirth, 1984). Because they exhibit high site fidelity to their summer range (Verme, 1973; Tierson *et al*., 1985; Van Deelen *et al*., 1998; Nelson and Mech, 1999), it is unlikely that the reduced density brought about by culling would significantly expand or shift home ranges of adjacent deer, thus averting perturbation effects that have complicated bTB management in badgers in the UK (Carter *et al*., 2007; McDonald *et al*., 2008). Indeed, if future surveillance in MN fails to find additional evidence of bTB persistence, it may strengthen the case for culling as the preferred bTB eradication strategy in white-tailed deer.

In the MB bTB outbreak in and around RMNP, variations in regulatory authority over lands has dictated that a variety of population control methods be applied to both elk and white-tailed deer. In RMNP itself sport hunting is prohibited, but extended hunting seasons have been implemented in areas surrounding the park, and First Nations subsistence harvests have been undertaken within RMNP to both gather surveillance samples and decrease population numbers (Shury and Bergeson, 2011). Hunter-harvested animals account for 73% of elk and 91% of deer examined for bTB, but have been significantly less likely to be *M. bovis*-positive than animals that were culled by agencies or as part of a selective capture/test/cull programme. Ground-based shooting of deer by landowners and agencies in areas bordering the park was carried out in 2004, and both elk and deer were culled within the park in 2009 by helicopter capture and captive-bolt euthanasia. Both events aimed to reduce deer densities, but were unpopular with a variety of stakeholders (O'Brien *et al*., 2011b). The decade-old capture/test/cull programme has efficiently removed bTB-positives and faced the least opposition because of its selectivity. While expensive and labour intensive, it has identified and controlled focal aggregations of bTB in elk in areas where hunting is prohibited, and shown some success in reducing prevalence (Shury and Bergeson, 2011).

Genetic resistance and susceptibility

Heritable resistance and susceptibility to bTB was first established in inbred lines of guinea pigs (Wright and Lewis, 1921) and rabbits (Lurie, 1941). Patterns of disease in infected herds of cattle also imply that a proportion of animals are resistant or highly susceptible to *M. bovis* infection (Allen *et al*., 2010). In this respect, use of the intratonsilar inoculation model for deer has provided insights into heritable resistance and susceptibility to bTB (Mackintosh *et al*., 1993, 1995). Experimental infection of a group of 75 males was used to select three groups of animals which displayed extreme phenotypes for either resistance or susceptibility to experimental *M. bovis* infection (Mackintosh *et al*., 2000). Semen from resistant and susceptible animals was used in an artificial insemination programme to produce offspring that were subsequently challenged with *M. bovis* by the intratonsilar route. The results show that resistance or susceptibility to bTB is highly heritable (0.48 ± 0.096). More recent studies confirmed that heritability and selection for resistance to bTB was evident on a deer farm that experienced a catastrophic outbreak of bTB (Griffin *et al*., 2004). Subsequent

studies involving *Map* infection showed that resistance and susceptibility to Johne's disease is also highly heritable in deer (Dobson *et al*., 2013).

Vaccination

Vaccines for wildlife have been used, or considered for use, in diseases that affect public health, impact livestock health/commerce or negatively impact iconic, protected or endangered species (Cross *et al*., 2007). These include rabies in skunks, raccoons, foxes and other mammals (Slate *et al*., 2005); Lyme disease in mice (Tsao *et al*., 2004); plague in black-tailed prairie dogs (Rocke *et al*., 2008); brucellosis in bison and elk (Olsen and Johnson, 2012); classical swine fever in European wild boar (von Ruden *et al*., 2008); anthrax in cheetah and black rhinoceros; and tuberculosis in African buffalo, deer, badgers, brushtail possums and wild boar (Griffin *et al*., 1999; Palmer *et al*., 2007; Ballesteros *et al*., 2009; Ramsey *et al*., 2009; Carter *et al*., 2012). Generally, oral vaccines in the form of baits are considered the most feasible means of vaccinating wildlife. However, under certain circumstances, hand-injected and pneumatic dart-administered vaccines have also been used successfully (Olsen and Johnson, 2012).

In the case of tuberculosis, the human vaccine, *M. bovis* BCG, has been the most widely investigated, having been tested in various species including deer. In all species tested, BCG reduced disease severity and in some trials also protected against infection (Griffin, 2000; Griffin *et al*., 2006b; Palmer *et al*., 2007, 2009). Early efficacy studies demonstrated that a single dose of BCG given to 3 month-old red deer reduced disease severity but was not protective against infection (Griffin and Mackintosh, 2000). By contrast prime-boost with low (10^4 CFU) to moderate (10^6 CFU) doses of BCG protected against both disease and infection. Subsequent studies (Griffin *et al*., 2006b) showed that the interval between prime and boost was important. Animals boosted at 8- or 16-week intervals generated protective immunity, while animals boosted at a 43-week interval showed no protection. The age of animals at primary vaccination may also be important, as the prime-boost effect seen in 3 month-old deer was not evident in cattle, where a single vaccination at 1 day of age provided optimal protection (Buddle *et al*., 2003). Studies in white-tailed deer showed that both oral- and parenterally administered BCG provided the same degree of protection (Nol *et al*., 2008).

In addition to being efficacious, a vaccine must also be safe. This is important for any potential vaccine, but is of special relevance with live vaccines such as BCG. Safety is of particular concern in terms of the animal being vaccinated, other animals (including humans) that may consume a vaccinated animal and animals not intended for vaccination, but nevertheless having contact with vaccine bait (non-target species). *M. bovis* BCG was first used as a human vaccine in 1921. Since that time, billions of doses have been administered, many of which were given to newborn infants. With such a long history there is a great deal of information concerning the safety of BCG in humans. It is considered to have an excellent safety record and to be non-virulent in immunocompetent individuals. Additionally, BCG has been used in many small animal models of human tuberculosis; therefore, much is known concerning safety in mice, rats, guinea pigs and rabbits.

People often consume hunter-killed deer, and so there is potential for humans to contact BCG through consumption of venison. Although disease is not likely to result from human consumption of BCG, false-positive results on TSTs could interfere with public health monitoring for human tuberculosis. BCG has been shown to persist for 3–12 months in lymphoid tissues of deer vaccinated parenterally or orally (Palmer *et al*., 2010b, 2014). Importantly, in studies of vaccinated deer BCG has never been isolated from those tissues commonly consumed by hunters (i.e. muscle). Moreover, cooking meat products at a minimum temperature of 60 °C for at least 10 min has been shown to kill greater than 90% of *M. bovis* that may be present (Merkal and Whipple, 1980). In other circumstances, to avert some risk, heat-killed BCG has been used for vaccination of wild boar (Garrido *et al*., 2011).

Modelling vaccination in the USA

Simulation modelling has examined the potential role that vaccination might play both in the eradication of bTB from free-ranging deer in MI and in control programmes aimed at minimizing cattle herd breakdowns. The frequency of vaccination, the proportion of susceptible deer vaccinated and whether or not baiting was occurring during the hunting season all affected the probability of bTB eradication (Ramsey *et al*., 2014). Annual vaccination of 90% of susceptible deer with a 90% efficacious vaccine was necessary to achieve a 95% probability of eradication within 30 years. Varying vaccine efficacy, duration of guaranteed immunity and the decay of immunity thereafter had only modest effects on times to eradication, with efficacy producing the greatest response. That same level of exposure was predicted to bring about a 95% probability of having no infected cattle herds in the bTB core area within 14 years; exposing 50% (rather than 90%) of susceptible deer reached the same threshold in about 20 years. Vaccinating 50–90% of the susceptible deer within 5 km radius of cattle farms achieved a 95% probability of having zero cattle herd breakdowns in 15–18 years.

Timing vaccination to expose recently born fawns (July–August) was predicted to attain eradication most rapidly, but differences were not marked (D.J. O'Brien, Michigan, USA, 2012, personal communication). Because BCG can persist in lymphoid tissues of vaccinated white-tailed deer for up to 12 months following a single dose (Palmer *et al*., 2010b, 2014), maximizing the period between vaccination and hunting seasons may be desirable to minimize the potential for false-positive TSTs in people consuming vaccinated deer. Hence, for white-tailed deer in the northern hemisphere, January vaccination appears optimal.

Using data from a MI live trapping study (Cosgrove *et al*., 2012a,b), strategies were simulated where individual deer were either uniformly vaccinated for bTB and released; or tested for bTB first, with test-positive deer culled and test-negative deer released, either with or without first being vaccinated. Thirty-two scenarios with varying vaccination frequency, vaccine efficacy, guaranteed period of immunity and subsequent half-life were examined. Over a 30-year period, none of these scenarios achieved 95% probability of eradication. Scenarios including removal of test-positive deer eradicated bTB substantially more often. Annual vaccination of 26% of susceptible deer (the estimated percentage of the population likely to be captured) with a 90% efficacious vaccine, with 1 year of complete immunity was predicted to have a 34% probability of bTB eradication within three decades (Cosgrove *et al*., 2012b) and a projected cost of approximately US$1.7 million annually.

Recent studies provide evidence of secondary transmission of BCG from vaccinated deer to in-contact unvaccinated deer (Palmer *et al*., 2009, 2010a; Nol *et al*., 2013). It is as yet unclear whether such transmissions confer protection from challenge with virulent *M. bovis*. If they do, modelled projections of time to bTB eradication are likely to be overstated. Additionally, in possums, one-off oral BCG vaccination has been shown to provide protection for more than 2 years post-vaccination (Tompkins *et al*., 2013). If duration of immunity is similar in white-tailed deer, progress towards eradication through vaccination may be more rapid than current forecasts suggest.

Vaccination has not been actively discussed as a management strategy for bTB in either elk or white-tailed deer in MB (Shury and Bergeson, 2011). However, genetic evidence in elk suggests that the subpopulation in which the vast majority of bTB-positives has been found has limited interactions with other subpopulations (Vander Wal *et al*., 2012). Consequently, it forms a well-defined target for vaccination should control or prevention plans change.

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20 Tuberculosis in South African Wildlife: Lions, African Buffalo and Other Species

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History of Tuberculosis in South African Wildlife

Tuberculosis has been described in free-ranging wildlife species from many areas of the world with diverse geography and climates (Bengis, 1999). In Africa, tuberculosis caused by *Mycobacterium bovis* (*M. bovis*) has been reported in free-ranging lechwe (*Kobus leche kafuensis*) in the Lochinvar National Park, Zambia (1954) and African buffalo (*Syncerus caffer*) sampled in the Ruwenzori National Park (RNP) in Uganda (1963). In both cases the most likely source of *M. bovis* was shared pastures with infected cattle. The disease was subsequently diagnosed in warthog (*Phacochoerus africanus*) from RNP and infection was thought to occur by scavenging diseased carcasses (de Lisle *et al*., 2001; De Vos *et al*., 2001; Malama *et al*., 2013). More recently, Cleaveland *et al*. (2005) reported the isolation of *M. bovis* from a variety of wildlife species in Tanzania including wildebeest (*Connochaetes taurinus*), topi (*Damaliscus lunatus*), lesser kudu (*Tragelaphus imberbis*), lion (*Panthera leo*) and buffalo (Cleaveland *et al*., 2005). In 2013, the first fatality due to *M. tuberculosis* was reported in an African elephant (*Loxodonta africana*) from Tsavo East National Park, Kenya. The source of infection was not established, although this animal was initially reared as an orphan before rehabilitation into the wild (Obanda *et al*., 2013).

Paine and Martinaglia (1929) reported the first recorded cases of tuberculosis in wildlife in South Africa. *M. bovis* was diagnosed in greater kudu (*Tragelaphus strepsiceros*) and a common duiker (*Sylvicapra grimmia*) in the former Albany district of the Eastern Cape (Paine and Martinaglia, 1929). It was speculated that the animals became infected through drinking from restricted water sources shared with cattle during a preceding drought. The presence of the disease in kudu in this district was confirmed in 1940, and anecdotal reports from farmers in the area suggested that springbok (*Antidorcas marsupialis*), bushbuck (*Tragelaphus scriptus*), hares (*Lepus* spp.) and bushpig (*Potamochoerus larvatus*) had also become infected and died of tuberculosis (Thorburn and Thomas, 1940).

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

In the Hluhluwe-iMfolozi Park (HiP), northern KwaZulu-Natal (KZN) Province of South Africa, BTB was first diagnosed in African buffalo in 1986 and infection later spilled over to lion, chacma baboon (*Papio ursinus*), bushpig (*Potamochoerus larvatus*) and greater kudu (Michel *et al*., 2006, 2009). The original source of *M. bovis* is attributed to communal grazing between buffalo and local cattle before the park was fully fenced in the 1960s. In Spioenkop Nature Reserve, a separate and smaller reserve also situated in the north of KZN Province, it is believed kudus contracted BTB from cattle in adjacent farming areas and transmitted the disease to buffalo (initially test-negative for *M. bovis* and recently introduced into the reserve) during or prior to 1997 (Michel *et al*., 2009).

In the Kruger National Park (KNP), located in the north-eastern region of South Africa, on the border with Mozambique, the first fatal case of mycobacteriosis was found in an impala (*Aepyceros melampus*) carcass located in the south of the park in 1967. A diagnosis of mycobacteriosis was made on the gross and histological appearances of lesions and the presence of acid-fast bacteria (AFB) in multiple organs; however, the presence or species of mycobacteria was not confirmed by culture or typing of the organism. At the time this appeared to be an isolated incident as no further cases were detected despite the inspection of large numbers of carcasses of impala and other species (De Vos *et al*., 1977). The first confirmed case of tuberculosis due to *M. bovis* was in a buffalo near the south-western boundary in July 1990 (Bengis *et al*., 1996). Molecular typing of the organism and retrospective analysis of state veterinary records suggested the disease entered KNP in the late 1950s or early 1960s from a dairy herd located south of the Crocodile River, which demarcates the southern park boundary (Kloek, 1998; Michel *et al*., 2009). Subsequent phylogenetic analysis of isolates has also shown that a single strain of *M. bovis* was introduced into KNP and it was different from at least three distinct strains responsible for the epidemic in HiP (Michel *et al*., 2009; Hlokwe *et al*., 2011). Regular monitoring surveys since the 1990s recorded increases in both incidence and prevalence of infected buffalo herds as the disease spread northwards through the park. In 2006, BTB was detected in a buffalo in the far north of KNP, indicating that it had spread ±350 km from the initial point of entry. Two years later the same strain of *M. bovis* was isolated from a buffalo in the Gonarezhou National Park, showing the disease had crossed the Limpopo River from northern KNP into Zimbabwe (Michel *et al*., 2009; de Garine-Wichatitsky *et al*., 2010).

Bovine tuberculosis (BTB) is a multi-host pathogen and has been diagnosed in over 15 species other than buffalo since its introduction into KNP (Table 20.1). The most significant has been the infection of lions which, as the top predators in the park, are ecologically essential in maintaining biodiversity as well as being an important draw-card for tourists. Additional species in which BTB has been diagnosed include greater kudu, leopard (*Panthera pardus*), cheetah (*Acinonyx jubatus*), spotted hyaena (*Crocuta crocuta*), genet (*Genetta tigrina*), chacma baboon, warthog, honey badger (*Mellivora capensis*), eland (*Taurotragus oryx*) and impala (Michel *et al*., 2006). More recently banded mongoose (*Mungos mungo*) (Brüns, 2014, personal communication, 10 January 2014), giraffe (*Giraffa camelopardalis*), blue wildebeest (*Connochaetes taurinus*) and wild dog (*Lycaon pictus*) (Reininghaus, 2014, personal communication, 10 January 2014) have been added to this list. The last three species were individuals from a private nature reserve located on the south-western boundary of KNP, which is part of the Greater Kruger National Park Complex (GKNPC). Internal fences have been removed from this area, allowing unrestricted movement of animals. Buffalo, and possibly kudu, have proved to be a maintenance host for BTB and all other species in which the disease has been detected are considered spillover hosts (Grobler *et al*., 2002; Renwick *et al*., 2007).

Mycobacterium tuberculosis has been isolated from numerous individuals and species at both the National Zoological Gardens (NZG) in Pretoria and Johannesburg Zoo (mycobacteriosis in zoo animals is covered in more detail in Chapter 15). In semi-free and free-ranging wildlife, the disease has been isolated from vervet monkeys (*Cercopithecus aethiops*) at a Limpopo Province rehabilitation centre, and from a free-living chacma baboon

Table 20.1. *Mycobacterium bovis* in free/semi-free ranging wildlife in South Africa.

Common name	Scientific name	Location	References
Common duiker	*Sylvicapra grimmia*	Agricultural farmland	Paine and Martinaglia (1929)
African buffalo	*Syncerus caffer*	GKNPC[a] and other game parks[b]	Bengis *et al*. (1996)
Lion	*Panthera leo*	GKNPC and other game parks	Keet *et al*. (1996), Michel *et al*. (2006), Hlokwe *et al*. (2011)
Cheetah	*Acinonyx jubatus*	GKNPC	Keet *et al*. (1996)
Leopard	*Panthera pardus*	GKNPC	De Vos *et al*. (2001), Michel *et al*. (2006), Michel *et al*. (2009)
Greater kudu	*Tragelaphus strepsiceros*	Multiple game parks and agricultural farmland	Paine and Martinaglia (1929), Thorburn and Thomas (1940), Keet *et al*. (2001), Michel *et al*. (2009)
Spotted hyaena	*Crocuta crocuta*	GKNPC and other reserves	Michel (2002), Michel *et al*. (2006), Michel *et al*. (2009)
Chacma baboon	*Papio ursinus*	GKNPC and other parks	Keet *et al*. (1996), Keet *et al*. (2000), Michel *et al*. (2006), Michel *et al*. (2009)
Honey badger	*Mellivora capensis*	GKNPC	Michel (2002), Michel *et al*. (2006)
Large spotted genet	*Genetta tigrina*	GKNPC	De Vos *et al*. (2001)
Warthog	*Phacochoerus africanus*	GKNPC and other parks, agricultural farmland	Michel *et al*. (2006), Michel *et al*. (2009)
Bushpig	*Potamochoerus larvatus*	HiP	Michel *et al*. (2006), Michel *et al*. (2009)
Impala	*Aepyceros melampus*	GKNPC	Michel *et al*. (2006)
Bushbuck	*Tragelaphus scriptus*	GKNPC	Bengis *et al*. (2012), Hlokwe *et al*. (2014)
Eland	*Taurotragus oryx*	Other game parks	Michel *et al*. (2006)
Blue wildebeest	*Connochaetes taurinus*	GKNPC	Hlokwe *et al*. (2014)
Banded mongoose	*Mungos mungo*	GKNPC	A. Brüns (unpublished data)
Giraffe	*Giraffa camelopardalis*	GKNPC	T.M. Hlokwe (personal communication, Nov 2013)
Wild dog	*Lycaon pictus*	GKNPC	T.M. Hlokwe (personal communication, Jan 2014)
Nyala	*Tragelaphus angasii*	Other game parks	Hlokwe *et al*. (2014)
Black rhinoceros	*Diceros bicornis*	Other game parks	Keep and Basson (1973), Espie *et al*. (2009)

[a]Greater Kruger National Park Complex.
[b]Other game parks including provincial and private game reserves and game farms.

and a sable antelope (*Hippotragus niger*) on a private game farm. In all cases, the source is believed to be infected humans with transmission facilitated by a high incidence of disease, a growing population and encroachment by people on wildlife habitats (Michel *et al*., 2013).

In recent years a number of *M. tuberculosis* complex species have been identified in free-ranging wildlife in South Africa (van Helden *et al*., 2009). 'Dassie bacillus' caused an extensive necrogranulomatous pneumonia in two apparently healthy rock hyraxes (*Procavia capensis*) harvested in the Western Cape in 2006. Historically, this *Mycobacterium* sp. was first isolated from a hyrax from Nieu Bethesda, Eastern Cape, in the 1950s (Parsons *et al*., 2008). Tuberculosis has been reported in free-ranging suricates (*Suricata suricatta*) in the Kgaligadi desert region of the Northern Cape. Initially, this was thought to be caused by *M. tuberculosis*, *M. bovis* or a 'member of the animal-adapted lineage of the MTC' (Drewe, 2011). Recent analysis of isolates from this suricate population identified a new MTBC species, closely related to the 'Dassie bacillus', which has been named *M. suricattae* (Parsons *et al*., 2013). 'Oryx bacillus', an extremely rare, slow-growing member of the antelope clade of MTBC, was recently isolated from a free-ranging adult female buffalo in KwaZulu-Natal (KZN) (Gey van Pittius *et al*., 2012). Recent genetic typing (spoligotyping and variable number of tandem repeat typing) of isolates from both livestock and wildlife species provide strong evidence of inter- and intraspecies transmission of *M. bovis*. The spatial distribution of BTB is increasing with the detection of the disease in previously uninfected game farms and reserves in five of South Africa's nine provinces. The number of affected wildlife species has also increased over the past 10 years. The spread of *M. bovis* may be facilitated by buffalo as maintenance hosts escaping from conservation areas or the uncontrolled movements of infected but undiagnosed species. As the incidence increases in wildlife it raises concerns as BTB is difficult to control in these populations, future national eradication programmes in domestic stock may be compromised, the zoonotic risk is increased and it may threaten the conservation of certain wildlife species and vulnerable ecological systems (Hlokwe, unpublished data) (de Lisle *et al*., 2002).

Tuberculosis Due to *M. bovis* in Proven and Suspected Wild Maintenance Hosts

By epidemiological definition, for a host to qualify as a maintenance host species it must be able to maintain circulation of infection or perpetuate the disease without introductions from an outside source. The basic requirement for establishment and persistence of any infectious microparasite in a host population is that infected individuals must on average pass the infection on to at least one susceptible individual (Wobeser, 2007). For this to occur, species demographics, spatial distribution, transmission mode, infection duration in the host and survival of the organism outside of the host may individually or collectively all be important factors. The multi-species nature of BTB in free-ranging African mammals illustrates many examples of the complexities of these disease maintenance determinants.

African buffalo (*Syncerus caffer*) have become true maintenance hosts of *M. bovis* in free-ranging populations in Kruger National Park (Bengis *et al*., 1996), in the Hluhluwe/iMfolozi Park (Cooper, 1998), and in Madikwe National Park in South Africa, as well as the Queen Elizabeth National Park in Uganda (Thurlbeck *et al*., 1965; Woodford, 1982), the Kafue National Park in Zambia (Guilbride *et al*., 1963) and in the Serengeti National Park in Tanzania (Cleaveland *et al*., 2005). In buffalo, tuberculous lesions are most commonly found in the head lymph nodes, tonsils, lungs and associated lymph nodes. African buffalo are gregarious bovids and are behaviourally very similar to cattle (Michel and Bengis, 2012). Herds number from tens to thousands, and being 'close contact' animals facilitates aerosol transmission in the herd. Infected buffalo appear to remain persistently infected and infectious, and lesions are generally progressive. Transmission of infection between herds is facilitated by fission/fusion behaviour, whereby large herds break up into smaller foraging groups during the dry season and then re-coalesce with other unrelated groups during the rainy season. In addition, at puberty groups of young heifers and bulls often disperse from their natal herds and join other herds (Cross *et al*., 2009). Persistent circulation

and temporal increases in prevalence and spatial distribution of *M. bovis* infection have been well documented in the Kruger National Park (De Vos *et al*., 2001; Rodwell *et al*., 2001).

Greater kudu (*Tragelaphus strepsiceros*) are social antelopes, but occur in small herds of between two and 25 individuals. They are found in dense woodlands and woodland savannahs, and are shy nervous animals. They are browsers and are partial to the leaves of thorn trees of the genera *Acacia and Ziziphus* (Estes, 1991). Kudu were the first wildlife species to be confirmed with tuberculosis infection on farms in the Eastern Cape Province of South Africa, on which the cattle were heavily infected with tuberculosis (Paine and Martinaglia, 1929; Thornburn and Thomas, 1940). Subsequently BTB was confirmed in kudu in the Spioenkop Nature Reserve in KZN/Natal Province of South Africa, as well as the GKNPC and surrounds (Bengis *et al*., 2001; Keet *et al*., 2001). Typically, kudu present with large abscessed parotid and retropharyngeal lymph nodes, which may rupture and form draining sinuses and the draining purulent material is loaded with acid-fast organisms. Because the parotid lymph nodes generally drain the lymphatics of the skin of the head and the ears, the hypothesis is that thorns contaminated by draining pus may cause percutaneous infection by micro-lacerations of the delicate skin of the large ears of the kudu. Contaminated browse or respiratory aerosols are probably responsible for infection of the retropharyngeal lymph nodes. Kudu also develop multifocal disseminated tuberculous lesions in their lungs and associated lymph nodes, and haematogenous spread to the liver, spleen and kidneys may occur. Kudu are therefore potential super shedders, and infection appears to be maintained in localized populations at least for some considerable time. In the field, where kudu have relatively low population densities, clinical cases are sporadic and it is often difficult to determine whether this is due to horizontal transmission alone, or to exposure to sympatric infected buffalo. Molecular analysis of *M. bovis* isolates from kudu in the aforementioned outbreaks in Spioenkop Nature Reserve and KNP, however, was able to provide the first evidence for the possible maintenance host status of greater kudu. It could be demonstrated that kudu were the most likely source of infection to newly introduced buffaloes in Spioenkop (in the absence of infected cattle herds in the surrounding areas), while in KNP they were shown to maintain a 'kudu- specific' strain of *M. bovis* not found in buffaloes and likely to have been introduced into the park by kudu (Michel *et al*., 2009).

The highest kudu densities in South Africa occur in the Albany thickets and valley bushveld of the Eastern Cape, and this is the region where the possibility of a true kudu maintenance cycle exists, should the disease be reintroduced. Kudu are also among the most common game species traded at game auctions and are widely distributed on game farms in nearly all parts of South Africa. Their long-term role in the BTB epidemiology in wildlife and at the interface between wildlife and livestock remains to be seen in the light of an increasing geographical spread of this disease in South Africa.

Suricates (*Suricata suricatta*) are social diurnal mongooses, endemic to the more arid areas of South Africa's highveld and Karoo; the Kgaligadi thornveld of South Africa and Botswana; and parts of the Namib desert in Namibia. Packs of between ten and 40 animals live communally in a network of underground warrens. They are extremely social and show regular grooming behaviour (Estes, 1991; Kingdon, 1997). In a localized area of the Kgaligadi thornveld in the Northern Cape Province, tuberculosis caused by a newly described species of the *M. tuberculosis* complex named *M. suricattae* (Parsons *et al*., 2013), has become endemic in the suricate population. The lesions appear to start in the lungs or lymph nodes of the head and then spread haematogenously to other organs including liver, spleen, heart, kidney, thoracic lymph nodes, abdominal lymph nodes, mammary glands and skin (Drewe *et al*., 2009). Abscesses of the submandibular and retropharyngeal lymph nodes frequently rupture and form chronically draining sinuses. Thus in suricates it would appear that infection is acquired principally through the alimentary and respiratory routes, whereas excretion (shedding) is most likely via the respiratory tract and suppurating draining sinuses (very similar to

European badgers in the UK and Ireland; Chapter 16). Important maintenance determinants include respiratory shedding of bacteria within the confines of the microclimate of underground warrens, and social grooming of animals with draining tuberculous sinuses. In addition, suricates breed year-round; up to five young are born after an 11-week gestation; and they are weaned after 10 weeks (Kingdon, 1997), so despite the significant mortality among infected individuals, replacement susceptibles are coming on line all the time, allowing for continuous circulation of infection. Circulation of tuberculosis in this subpopulation of suricates has been documented for almost 10 years (Drewe *et al*., 2009).

Tuberculosis in Wild Spillover Hosts of *M. bovis*

M. bovis has one of the broadest host ranges of any known zoonotic pathogen (O'Reilly and Daborn, 1995). Its potential for causing widespread infection and disease is probably highest in a multi-species, free-ranging ecosystem (Renwick *et al*., 2007). In South Africa, *M. bovis* infection has to date been diagnosed in 21 free or semi-free ranging wild mammal species (Table 20.1). Among those, only two (African buffalo and greater kudu) are considered capable of acting as true maintenance hosts for BTB, while the remaining 19 species are considered spillover hosts. According to our current knowledge they lack the potential to establish a persistent intraspecies infection within a locality (de Lisle *et al*., 2001).

Although they are unable to maintain *M. bovis* without transmission from another species, our observations, like those of other investigators elsewhere, indicate that spillover hosts may independently or jointly contribute to persistence of infection in a free-ranging ecosystem such as the KNP (Gortazar *et al*., 2008). Their ability to serve as source of *M. bovis* to secondary spillover species, independent from buffaloes, adds a layer of complexity to the web of interspecies transmissions and the diversity of spillover hosts. Practical examples are cheetah and leopard, for which the source of infection is unclear as they do not prey on buffalo. It is likely that they contract *M. bovis* from a smaller spillover species.

In our experience in the Greater Kruger National Park Complex (GKNPC), and also in other multi-host conservation areas, the case numbers in different spillover hosts can be highly variable and range from a sporadic (e.g. leopard) to an endemic occurrence (e.g. lion), which is likely to be a function of the rate of interspecies transmission to the spillover host (Fenton and Pedersen, 2005). In addition, some species are considered to have maintenance host potential because they develop severe BTB lesions, and may even become super shedders (Keet *et al*., 1996; Keet *et al*., 2000a), but the limiting factors for continuous maintenance and circulation of infection appear to be population size, density, distribution or behavioural ecology.

Spillover species presently known in South Africa include primarily predators, bovidae other than buffalo and kudu, omnivores as well giraffe and black rhinoceros. A more detailed description is given below.

Lion (*Panthera leo*)

Lions are social felids, and pride size varies from three to 40 individuals. Each pride consists of breeding females plus their offspring, and the pride and its territory are protected by a coalition of between one and eight males. Lions preying on infected or dead buffalo and kudu may become infected by ingestion, inhalation or percutaneously. Inhalation (aerosol) infection may occur during social activities such as communal roaring, or growling over choice parts of a carcass. It may also occur during the suffocation of infected prey animals. Percutaneous infection generally follows puncturing of the skin by contaminated claws or teeth during antisocial events such as fighting or squabbling. Most infected lions have pulmonary lesions including bronchiectasis, and these airway lesions are frequently lined with glistening mucoid pus, which microscopically is loaded with large numbers of AFB. Horizontal aerosol transmission is probably efficient, and in some prides a high percentage of the pride members, including cubs, are infected. Infection may also spread from

one pride to another with dispersing young adults or nomadic individuals (de Lisle *et al*., 2001; Keet *et al*., 2010). Clinical signs included emaciation, poorly healing skin lesions, lameness, skeletal and synovial swellings and occasional ocular lesions. Necropsy lesions include nodular and cavitational lesions in the lungs and thoracic and mesenteric lymph nodes, osteomyelitis, polyarthritis/synovitis and amyloid degeneration of the kidneys. The lesions in lions are generally more proliferative and cavitational in nature, with absence of the normal necrosis, caseation and encapsulation seen in other species (Keet *et al*., 2000b).

The high disease prevalence in the buffalo population of the GKNPC provides a constant source of infection to lion prides and ensures the apparent persistence and high serological prevalence of BTB in the lion population (Maas *et al*., 2012). Therefore, lions may be classified as medium-term maintenance hosts, in which infection cannot circulate indefinitely due to the constraints of relatively low population densities and territoriality.

Cheetah and leopard

The spillover host profile seen in both these predator species is consistent with that in lions, although a much lower prevalence has been encountered than in lions in the KNP, which can be ascribed to the solitary nature of both species (Keet *et al*., 1996). It is important to note that the macroscopic lesions in lions, cheetah and leopard do not resemble the classical caseo-necrotic with encapsulation and calcification as observed in bovids. Tuberculous lesions in these wild felids are more proliferative with occasional cavitation, especially in the lungs (de Lisle *et al*., 2001).

Other carnivores

In the KNP *M. bovis* has been cultured from different lymph nodes in the spotted hyaena, especially mesenteric lymph nodes, in the absence of clinical signs (Michel, unpublished data). Most likely hyaenas contract *M. bovis* via the alimentary route during scavenging but it would seem that the infection can be successfully contained within the lymph nodes, minimizing the risk of shedding in this species.

In a recent BTB survey among 81 banded mongooses living and foraging in and around the Skukuza staff village in the KNP, *M. bovis* has been diagnosed by culture of the tracheal washes and multiple thoracic and abdominal lymph nodes and organs of two adult female animals. The animals did not show any clinical signs or compromised behaviour (A.C. Brüns, unpublished data). Most likely banded mongooses become exposed to *M. bovis* while feeding on infected carcasses. The findings also strongly suggest that the animals were capable of at least intermittently shedding *M. bovis*, raising an interesting question regarding the role of horizontal transmission in the epidemiology of BTB in banded mongooses.

Tuberculous lesions were found in the thorax of one large spotted genet during a BTB survey among small mammals in the GKNPC, while passive surveillance was able to detect an isolated case in a honey badger characterized by mesenteric lesions. Both findings are considered incidental and currently of no epidemiological significance as long as the rate of transmission remains low.

The first case of *M. bovis* infection in a wild dog (*Lycaon pictus*), which was killed by lions, was diagnosed in 2013 in the southern part of the GKNPC. Lymph nodes of the head and mesenterium showed suspect lesions which were confirmed as *M. bovis* by culture (T.M. Hlokwe, personal communication, Onderstepoort, 23 January 2014). The transmission route remains speculative, possibly suggesting either an alimentary or respiratory route of infection, or both. In 2012, a wild dog from the uMkhuze area in KwaZulu-Natal exhibiting malaise and loss of condition was misdiagnosed as having an abdominal carcinoma. Histopathology of the mesenteric lymph nodes revealed a tuberculous granuloma and acid-fast organisms.

Chacma baboon (*Papio ursinus*)

Baboons are social primates occurring in troops of 20–50 animals (Estes, 1991). They are omnivores and due to their scavenging

behaviour are probably regularly exposed to *M. bovis*. If one assumes that ingestion of *M. bovis* every so often leads to clinical manifestation and incidentally but unavoidably to intraspecies transmission, one would expect to find a visible number of compromised individuals on tourist roads and inside tourist camps. In reality, BTB cases in baboons have only rarely come to our attention. However, two extensive, possibly epidemiologically related outbreaks of BTB were recorded in Skukuza in the KNP. During the first outbreak in 1996, as well as during the second outbreak in 2010–2011, the infected troop of baboons used man-built structures to sleep at night. In both cases, access ways used by the baboons to enter the facilities, as well as the area where they congregated at night, were severely contaminated with faeces. Infection rates of approximately 50% were diagnosed in both outbreaks (Keet *et al*., 2000a; de Klerk-Lorist *et al*., 2012). BTB appears to be a fulminant disease in baboons and many individuals succumbed to the disease in less than 6 months. Miliary granulomata and tuberculous abscesses are found in most organs, especially lungs, lymph nodes, spleen, liver and kidneys. The multiple granulomas in the spleen are indicative of significant bacteraemia or bacterial embolic showers.

Characteristic of these two outbreaks was that they both remained localized to those troops and there was no evidence of further spread. Although the measures instituted to control the outbreaks included capture and removal of skin test-positive animals, it is believed that the closure of access to the man-made structures to the baboons was the most effective intervention to break the animal-to-animal transmission cycle. Under natural conditions baboons sleep in trees, which limits the faecal–oral and aerosol animal-to-animal transmission.

Wild suids

Warthogs are social porcines occurring in family groups in savannah and open woodland areas. They are diurnal and take refuge in natural or self-dug shelters at night or to escape extreme heat and cold periods. Family groups may socialize while foraging, but rarely share the same burrow (Estes, 1991). In warthogs the lesions are most prominent in the lungs and gastrointestinal tract. As with other communal burrow-dwelling species, the close contact and microclimate inside the confines of a burrow or culvert most probably facilitates aerosol transmission of *M. bovis*. In the Queen Elizabeth National Park in Uganda, and on certain private ranches adjoining the KNP, the paucity of predators and ready availability of burrows has resulted in very high warthog densities, and in these systems warthogs have become true maintenance hosts of TB, with prevalences varying from 5% to 30%. However, in the KNP itself, with its plethora of macro-predators, and species competition for burrows, warthog densities are lower, and cases of tuberculosis in warthogs are sporadic, without any indication of continuous circulation.

Bushpigs (*Potamochoerus larvatus*)

These animals are omnivores and are thought to become infected while scavenging on BTB-infected carcasses of maintenance or spillover hosts. Isolation of *M. bovis* from lung lesions suggests a respiratory route of infection as a result of horizontal transmission (Michel *et al*., 2006; Michel, unpublished data). To date *M. bovis* has only been diagnosed in one bushpig in the HiP.

Bovidae (other than buffalo and kudu)

In South African conservation areas and game farms, spillover infection with *M. bovis* has rarely been diagnosed in wild bovids, other than buffalo and kudu. The few cases diagnosed and confirmed were in common duiker, eland, impala, nyala and bushbuck. In all species lung lesions were described, although the three impalas and a bushbuck developed generalized tuberculous lesions and emaciation (Michel, unpublished data). It is worth mentioning that the impalas originated from a private reserve associated with the GKNPC, where a very high population density

among antelopes was maintained. In contrast, BTB has never been diagnosed in impala in the KNP where the population density is lower. This is despite the fact that it represents the most abundant and widespread antelope species in this park and is often found cohabitating the same environment and sharing water points with infected buffaloes. Furthermore, in the KNP, there were many opportunities to necropsy and sample impalas over the decades, all with negative results. It is tempting to speculate that infection status and disease severity in the few affected impala were driven by the population density and higher inter- and intraspecies contact rates as reported by other investigators (Nugent, 2011).

A different category of spillover infections has been noted in single cases in blue wildebeest (*Connochaetes taurinus*) and black rhinoceros (*Diceros bicornis*) (Keep and Basson, 1973; Espie *et al*., 2009). The abundance of blue wildebeest in the GKNPC, together with the fact that they are extremely gregarious bovids, fulfils critical requirements for interspecies and horizontal transmission. Blue wildebeest usually occur in smaller herds but sometimes congregate in large numbers and are often found co-grazing with buffaloes. It is therefore unexpected that only one incidental finding of *M. bovis* infection in a wildebeest (the animal was clinically healthy and did not show visible lesions) has been recorded in the GKNPC during ongoing surveillance efforts.

Despite their susceptibility to *M. bovis* infection in zoos, rhinoceroses do not presently appear to have been affected by the BTB epidemic in the GKNPC and the HiP. In the latter park, an old black rhino (*Diceros bicornis minor*) cow that died of natural causes was diagnosed with mycobacteriosis at necropsy. Areas of chronic granulomatous reactions were found in the lungs, regional lymph nodes and pleura, and small numbers of AFB were present on histological examination of affected tissues, but no culture for mycobacteria was performed at the time (Keep and Basson, 1973). Outside the GKNPC and HiP, a case of BTB has been reported in an elderly black rhino in Limpopo Province. Two small non- encapsulated granulomas yielding culture-confirmed *M. bovis* were found as an incidental finding at necropsy. This rhino was part of the NZG animal collection and housed under semi-extensive conditions. The source of infection could not be established (Espie *et al*., 2009).

To date one incidental case of *M. bovis* infection was confirmed in a giraffe in the GKNPC suffering from a granulomatous pneumonia (T.M. Hlokwe, personal communication, Onderstepoort, 23 January 2014). In the HiP a second case of tuberculosis was detected histopathologically in bronchial lymph nodes of a giraffe diagnosed with acute pulmonary oedema.

Given the estimated 60-year long history of the BTB epidemic in the southern region of the KNP it may be surprising that the first spillover event was only detected about four decades after the introduction of *M. bovis* into the KNP. An even longer delay between the time of probable spillover from cattle to the first report of BTB in possum was noted in New Zealand (Palmer, 2008). Buffalo are assumed to have been the first and (for some time) sole infected wildlife species, and spillover events were probably limited to their closest interactions. These were presumably predators, and scavenging and omnivorous species, gradually extending to other species sharing their environment such as other herbivores. The actual temporal sequence of spillover events experienced in the GKNPC supports this theory.

For many species with a relatively low susceptibility to *M. bovis*, the threshold for host interactions and pathogen transmissibility is comparably higher but may over time be reached as the number of infected animal species increases, and this may jointly contribute to persistence and widespread transmission in the ecosystem. Ultimately, animal species least at risk of contracting BTB may be seen to succumb sporadically to clinical *M. bovis* disease. It has also been shown for various infected mammalian species in South Africa and elsewhere that their host status may be influenced by the population density. This means that there may not necessarily be a clear distinction between maintenance and spillover hosts in a multi-host system with varying host population densities. A species may be a maintenance host where it is at high density or where transmission rates are elevated

by increased contact (Caley and Hone, 2004; Nugent, 2011). In conclusion, in the GKNPC and other conservation areas in South Africa this concept is believed to be applicable to social host species with high contact rates as well as interspecies transmission rates. Instead of assigning a host status purely based on taxonomy it seems more logical to evaluate the host status, particularly of kudu, lion, warthog and baboon, in the specific context of the affected population and the ecosystem they live in.

While 20 years ago wildlife tuberculosis in South Africa was confined to the GKNPC and the HiP, *M. bovis* has been actively spreading in recent years to smaller game reserves and wildlife ranches, as evidenced by routine diagnostic submissions (Hlokwe, unpublished data).

M. bovis Transmission at the Wildlife–Livestock–Human Interface

M. bovis has been designated a multi-host pathogen, for which open ecosystems provide almost unrestricted opportunities for pathogen exchange between domestic and wild animals (Renwick *et al*., 2007). Cattle have historically played a key role in the introduction of BTB into wildlife conservation areas in many countries, including the KNP in South Africa. Once endemic, the possibility for spillback from wildlife to livestock and humans constitutes an animal and public health risk to communities neighbouring infected wildlife reserves (de Garine-Wichatitsky *et al*., 2013).

M. bovis transmission at the wildlife–livestock–human interface is driven by contact with infected animals or through sharing water and grazing resources, following which the infection in livestock may pose a zoonotic risk to farmers and consumers of infected milk (Michel *et al*., 2010). Interactions between wild and domestic animals and humans are much more limited where wildlife conservation areas are fenced, as is the case in South Africa (Bengis *et al*., 2002). Nevertheless, wildlife–livestock contact cannot be excluded entirely as elephant and human activities and flooding cause ongoing damage to the perimeter fences surrounding the KNP and also the HiP, allowing the maintenance hosts of BTB – buffaloes and greater kudu – to cross into neighbouring livestock farming areas. In addition, greater kudu and warthogs are capable of crossing intact game fences with ease. On the other hand, contact with wildlife is also facilitated by cattle sporadically entering the KNP from communal land for grazing (Jori *et al*., 2011; Brahmbhatt *et al*., 2012).

In 2013, the first spillback of *M. bovis* from KNP wildlife to neighbouring communal cattle was reported, substantiating the potential of infected wildlife to serve as a source of infection to cattle (Musoke *et al*., 2015). The frequency and extent of this spillback is unknown, as is the wildlife host responsible for the transmission. According to recent studies assessing the wildlife species most often observed in contact with cattle, three of the 15 known infected species in the KNP (buffalo, greater kudu and warthog) were identified consistently, though contact rates were low (Jori *et al*., 2011; Brahmbhatt *et al*., 2012; Molefe, unpublished data, 2013).

In general pathogen interspecies transmission is dependent on the interaction rate between host species, whereby closely interacting species may provide a single resource for the pathogen (Bowers and Turner, 1997). Although it is unlikely that large numbers of infected buffalo mingled with communal cattle, close interaction of a single, shedding buffalo over a period of time may have successfully resulted in buffalo-to-cattle transmission.

The wildlife industry in South Africa is a rapidly growing agricultural sector and vast areas previously used for commercial livestock ranching have been transformed into game farms and wildlife conservancies (Michel and Bengis, 2012). Large numbers of wildlife are translocated every year within South Africa, but pre-movement testing for BTB is only mandatory for buffaloes. Hence a new wildlife–wildlife interface has been created between geographically and epidemiologically separate ecosystems.

As a result wildlife tuberculosis in South Africa is no longer confined to the GKNPC and the HiP. Instead we see evidence of *M. bovis* actively spreading to smaller game reserves and game farms as evidenced by routine diagnostic submissions (Hlokwe, unpublished data).

One of the economically most devastating introductions of *M. bovis* occurred in one of South Africa's elite game parks. Madikwe Game Reserve reintroduced buffalo to the park during the early 1990s. These buffalo were sourced from various zoos internationally and other disease-free herds around the country and were tested for BTB prior to introduction. After the buffalo population had been established large numbers of other game species, in particular blue wildebeest, impala and kudu, were introduced into Madikwe for improvement of the predator–prey balance as well as to optimize the tourism product. In the absence of pre-movement testing and a lack of validated tests for the detection of BTB in any of these species, the animals could be transported and offloaded without any quarantine procedures. The disease risk associated with free movement was assumed to be very low. Although the exact source of infection for the Madikwe buffaloes is currently unknown, molecular characterization of the causative *M. bovis* strain indicated a close epidemiological link with infected wildlife, among others kudu, in KZN (Hlokwe, unpublished data). Since a number of kudus in Madikwe originated from KZN one may speculate that newly introduced kudus were the most likely source of BTB to Madikwe. In retrospect, the BTB outbreak was detected about 4–5 years after the first introductions of plains game to Madikwe, and this might have been ample time for kudus and buffalo to interact at artificial waterholes and supplement licks, ultimately leading to the infection of the buffalo population.

This most recent introduction of BTB into a wildlife population signals the potential significance of *M. bovis* as a silent risk factor in game translocations. For kudus in particular it must be taken into consideration that their movement is not restricted by fences (they can jump 3 m high) and therefore kudus might in fact pose a greater risk at the interface with surrounding livestock compared to buffalo, which generally respect the presence of fences.

In certain area, particularly in South Africa, there is often contact between humans and non-human primates. This could either be poverty related where humans and baboons alike are scavenging at large peri-urban rubbish dumping sites (Michel *et al*., 2013), or in the natural environment (picnic sites within large conservation areas) where learned behaviour has taught baboons and monkeys that humans are easy targets for access to free food. Several free-living baboons in the Cape Peninsula in the Western Cape Province, and in one instance in the Limpopo Province, have tested positive for *M. tuberculosis* and the most likely explanation would be the interaction between infected human fomites (including discarded food, plastic bottles and cooking utensils) and the baboons (Parsons *et al*., 2009).

Wildlife Tuberculosis in Intensified Systems

It is generally agreed that mycobacterial diseases have been implicated in causing morbidity and mortality in several zoo collections worldwide (Montali *et al*., 2001). However, it should also be acknowledged that during recent times, wildlife ranching (semi-intensive) and game farming (intensive) have all become more popular, leading to intensified game breeding systems, especially in southern Africa (Bothma and van Rooyen, 2005; Hunt, 2010; Slabbert, 2013). These intensified systems differ from conditions in zoo collections. They usually house larger numbers of animals in rather confined areas, with less access to natural vegetation, and animals experience higher stress levels due to transport, handling and intra-specific competition. Ultimately these conditions will contribute to more direct contact between individuals confined to the same boma or enclosure and therefore a higher risk of possible disease transmission between them.

Some systems worth mentioning with regards to BTB are the disease-free buffalo breeding projects that were initiated in South Africa during the late 1990s (State Veterinary Office, 1999; Hunt, 2010). At the time the widespread *M. bovis* infection and increasing prevalence found in most buffalo herds in the southern region of the KNP had become evident (Michel *et al*., 2006). This was one of the

reasons that led to the request from South African National Parks (SANParks) in 1996 and eventual approval for a breeding project making use of diseased Lowveld buffalo parent stock to breed 'disease-free' calves. The main aim of this breeding project was to preserve the Kruger buffalo genotype if a depopulation decision was made to control BTB in the free-ranging buffalo herds and to supply other national parks with disease-free buffalo of the Kruger genotype (Hunt, 2010).

Breeding projects were generally only registered for parent stock infected with foot-and-mouth disease (FMD) and corridor disease (CD), but free from BTB and contagious abortion (CA). However, one exception was made at a facility where a breeding project made use of BTB-infected buffalo cows to try and produce disease-free offspring (State Veterinary Office, 1999, 2000). In 2000 several consignments of buffalo from various areas with either test-positive or suspect BTB results were moved to these breeding facilities, and the calves born from the infected dams were removed within 48 h of birth and either hand-reared or placed with skin test-negative surrogate Jersey cows.

Although comparisons between different breeding systems were difficult and data sets often incomplete, there was an indication that calves from BTB-infected dams had a higher mortality rate (21%, 17% and 32% from 2002 to 2004, respectively) during the first 3 months after birth, compared to calves born to dams free of BTB. Even calves as young as 1 month of age, and being removed from the dam after birth, still often died of tuberculosis disease and necropsy usually confirmed the presence of lesions, which ultimately stained positive with Ziehl–Neelsen and for which positive culture results were obtained (State Veterinary Office, 2001). Although rarely encountered, tuberculous lesions have been recorded in the endometrium and mammary glands of several bovidae species (Rietkerk *et al*., 1993) and therefore African buffalo could most likely be included. Studies in Pakistan also concluded that the consumption of milk was the most likely cause of infection in young calves (Khan *et al*., 2008). Although the stress of pregnancy, birth and lactation might have reduced the gamma interferon response or even resulted in anergic reactions to intradermal tuberculin testing (Buddle *et al*., 1994), it is also known to have increased the shedding of *M. bovis* in latently infected bovidae (Bondi and Zannino, 1997).

A very strict testing schedule for calves from this breeding project was therefore instituted where the calves had to have five consecutive negative comparative intradermal tuberculin tests (CITT) 3 months apart (State Veterinary Office, 2001; DAFF 2002), before they would be considered to be free of BTB and therefore allowed to be released from the project. Generally disease break-throughs within a calf cohort could be identified within the first three rounds of being tested (thus within the 1st year of life), but during several testing schedules it happened that the first BTB test-positive results were only obtained during the fourth of five BTB testing cycles with some animals being as old as 19 months (State Veterinary Office, 2000). This most probably indicates that although the calf had been removed from the dam shortly after birth, infection could have occurred prepartum via the uterus, during calving, postpartum during close contact grooming between the dam and the calf or through consumption of colostrum or via a contaminated environment, even if the calf was removed within the first 24 h after birth. In particular the chronicity and anergy of infection in these calves were reasons why the process of testing did not achieve the desired outcome, while being very costly.

Even more surprising results were obtained during the fostering of some of these buffalo calves, produced by either BTB-infected or suspect dams, when they were introduced to surrogate Jersey cow mothers. After about 2 months of contact between the fostered calves and their Jersey surrogates, one of the Jersey cows tested positive in a skin test (State Veterinary Office, 2007). It is therefore speculated that even if buffalo calves test negative at a very early age, they could still possibly contaminate the environment through shedding *M. bovis* through faecal, urine or aerosol secretions. Various experiments have also concluded that *M. bovis* can survive in the open environment for several weeks, especially out of direct sunlight (Buffield and Young, 1985; Tanner and Michel, 1999). Boma facilities

generally have large areas of artificial shading for the comfort of the animals during the hotter part of the day (du Toit, 2005). If there were enough shaded areas within a contaminated area there might be a build-up of *M. bovis* in the environment and therefore a greater risk of exposure to other animals within the same facility.

Various experimental *M. bovis* challenge investigations have shown that the nature and extent of disease are largely related to the route and dose of exposure to the causative organism (Thoen and Barletta, 2006). It would then be plausible that a buffalo calf, infected during pregnancy or shortly after birth with a relatively small infectious dose of *M. bovis*, might remain test negative for BTB until the immune system was able to mount an immune response. It is therefore suggested that there is a tangible risk in breeding with buffalo cows infected with BTB, because offspring might often test negative for an extended period of time before infection is detected through the current testing protocols. It could further be speculated that should some of these animals remain test negative for five consecutive tests and then be moved to a clean property, there is still a finite risk (although relatively small) that a latently infected animal can cause a new outbreak at destination. It is therefore strongly suggested that all buffalo calves bred from tuberculosis-positive dams still undergo annual testing for BTB (DAFF, 2015). The buffalo industry in South Africa has evolved into a very lucrative business with some disease-free adult bulls being sold for a multiple of the value of 'diseased' buffaloes (relating to infection with BTB, FMD, brucellosis, CD) with new peak prices being seen at recent auctions (Dayimani, 2013; Slabbert, 2013; Williams, 2013). Unfortunately this has also lead to several irregularities in buffalo movements with obvious attending disease. It also needs to be emphasized that, in some instances, a lapse in biosecurity measures or alteration of test procedures on certain properties has cost some farmers dearly. In the event of any buffalo testing positive for BTB the farm would remain under quarantine and no animals would be allowed to be removed from the property. In effect this would mean that all the buffalo would have to go through the five consecutive negative skin test cycle again before the property could be declared free of BTB (DAFF, 2002). In some instances this testing period can take several years to complete, meaning that no live buffalo can be sold and therefore no revenue generated. BTB could very well be one of the greater risks in causing a collapse of the buffalo industry if biosecurity measures and regular disease testing are not applied.

Management Options

M. bovis infections are notoriously difficult to manage in wildlife. There are numerous examples where eradication proved virtually impossible with only Australia and New Zealand achieving some degree of success. These two countries had the advantage that BTB was found mainly in alien or feral species, and draconian eradication strategies could be applied (de Lisle *et al.*, 2001).

When BTB is diagnosed in an indigenous wildlife species, control and management of the disease becomes significantly more difficult. There are numerous examples where BTB is maintained in wildlife species and where eradication and control have been tried at huge expense, usually without significant success and never achieving eradication, including white-tailed deer (USA), badger (UK), wild boar (southern Europe) and possums (New Zealand) (Palmer *et al.*, 2012). Once BTB becomes self-maintaining in more than one species, no matter how low the prevalence, eradication becomes virtually impossible as involved maintenance hosts will keep reinfecting each other and control measures must be applied indefinitely (O'Reilly and Daborn, 1995).

In the following section we describe briefly the reasons why management options are limited and also consider the limitations of diagnostics, applied monitoring and surveillance systems, and alternative approaches to management of BTB.

Monitoring and surveillance and attempted management of BTB in wildlife in South Africa

Once BTB is diagnosed the dilemma of what to do usually arises as BTB is a controlled disease

in southern African states and requires incidence reporting and a plan of action to eradicate where possible. The economic importance of tuberculosis as a disease of cattle and its zoonotic implications led to the implementation of a national BTB control and eradication scheme in 1969 (Cousins *et al*., 2004). This scheme successfully reduced the incidence of BTB in cattle in South Africa but due to resource limitations the scheme has not been effective since the mid-1990s, and during the following 15 years sporadic outbreaks of BTB have again been found in cattle in all provinces in South Africa (Michel *et al*., 2008; Hlokwe *et al*., 2014) (www.daff.gov.za).

With the outbreaks of BTB in the KNP and HiP, sample culling was initiated by selecting random individuals from a larger group and carrying out complete post-mortem examinations to determine herd or zonal prevalence and spatial spread. In the KNP such sample culls were undertaken in 1991, 1992, 1996 and 1998 (De Vos *et al*., 2001; Rodwell *et al*., 2001). Zonal culling took place in 2005 in the southern part of the KNP. Zonal non-lethal surveillance using capture and gamma interferon testing was also conducted in 2000, 2003, 2006 and 2007 using the technique described in Grobler *et al*. (2002). Similar ante-mortem capture and test surveys have been conducted in Gonarezhou in 2009, 2010 and 2011, where BTB was diagnosed in buffalo in 2009 for the first time (de Garine-Wichatitsky *et al*., 2010).

This *non-lethal* surveillance and monitoring was conducted to avoid unnecessary killing of large numbers of buffalo which may be negative for BTB. Buffalo were immobilized and blood taken, on which a modified gamma interferon test was done. Each buffalo was individually marked and each group radiocollared. Positive animals were located 36 h later and euthanized, and full post-mortem investigations were conducted to confirm macroscopically if BTB was present and to what extent (Grobler *et al*., 2002).

In HiP the high buffalo density and the relatively small average herd size of between 40 and 100 buffalo made it practically feasible to capture and temporarily confine an entire buffalo herd. A management strategy of selective depopulation, whereby only those buffalo infected with tuberculosis are removed from the population, was initiated in 1999. The strategy had a twofold objective: (i) reducing the prevalence of BTB in the HiP buffalo population; and (ii) simultaneously reducing the incremental growth rate of the current buffalo population to ecological levels that can withstand environmental stresses such as drought, which would be damaging to the grazing resource and increase the incidence rate of BTB. If a test and cull approach is applied (as is being conducted in HiP) then all animals in a herd need to be captured, held in a holding facility and culled/released depending on their skin test results.

This entailed utilizing a mobile capture boma method (Openshaw, 1993), where between 40 and 160 buffalo were chased by helicopter at any one time into the site where they were then moved into a funnel area attached to a temporary holding facility constructed of interlocking steel panels. In this facility an entire herd could be segregated into groups, immobilized and the comparative intradermal test carried out. Over a 15-year period this annual control programme has resulted in the capture and testing of over 6000 buffalo and the removal of over 1000 test-positive individuals. It is important to realise that the test and cull programme in HiP is designed merely to reduce prevalence and reduce the risk of spillover rather than complete eradication. A single test on confined buffalo consistently reduced the herd prevalence by 80% which, at a population level (and provided the effort is sustained), is considered sufficient to reduce prevalence over the long term.

Diagnostic challenges

The diagnostic tests currently available have unfortunately not been validated in wildlife (Maas *et al*., 2013). The gamma interferon blood test has been validated in KNP buffaloes but it does not provide consistent specificity and sensitivity values in all buffalo populations (Michel *et al*., 2011). The skin test has been validated in buffalo and lion but not in any other southern African wildlife species (Miller, 2008; Keet *et al*., 2010). Various wildlife species have been tested with an array of

blood-based tests but to date no reliable or validated test exists to determine the BTB status of those animal species (Miller, 2008; Maas *et al*., 2013).

These examples illustrate the difficulty, cost and logistical constraints of effectively carrying out disease monitoring and surveillance in wildlife in the South African context. The cost and logistical challenges of this BTB control method are obvious, and it is not possible to apply it in large areas such as KNP with its large buffalo herds of up to 1000 animals. The same dilemma faces managers trying to determine the status of lion prides using the comparative intradermal tuberculin test – they need to be recaptured after 3 days, which is challenging given the shyness induced by the first round of capture.

In addition, the available ante-mortem diagnostic tests are not specific or sensitive enough to pick up very recent infections or anergic animals. Thus their true disease status and whether they pose a risk to other individuals in the herd or other species may be difficult to determine. This poses challenges about the management or control actions to be adopted. After taking all of these factors into account the manager may realise that management options of BTB in South African wildlife are limited to preventing introduction of the disease, or to containment, test and cull if the disease is diagnosed in a small, contained wildlife reserve (as being implemented in HiP and being contemplated in Madikwe Game Reserve). Delays in decision making early in the course of a recent outbreak may result in the disease spreading to uncontrollable levels. This may result in a test and slaughter scheme (including at least five consecutive negative tests, 3 months apart) becoming necessary to have any sort of certainty that remaining animals are BTB free (DAFF, 2015). It is important to note that this assumption is dependent on annual testing of a minimum percentage of the population, with cost and logistical factors dependent on the total population size.

In extensive systems (e.g. the KNP) test and slaughter would be completely impractical and there would be massive ecological ramifications if a keystone species such as buffalo were to be removed from the system in order to eradicate the disease. As in HiP this approach may be complicated by other maintenance hosts species of which we are not necessarily aware, and which have the potential to act as persistent sources of infection of other populations.

Vaccination is currently not a management option, as found in KNP where a pilot vaccination trial was conducted. Buffalo vaccinated with BCG followed by *M. bovis* challenge did not show significant levels of protection (de Klerk *et al*., 2010). With a greater emphasis on development of veterinary BTB vaccines a targeted vaccination approach can in future be considered for threatened maintenance species or for use in isolated populations.

This becomes more complicated as BTB does not appear to be a threat to large extensive populations (Cross *et al*., 2009). In species that do visibly become diseased, such as lions, their generation turnover time is faster than the slow progress of the disease. This makes draconian intervention difficult to justify and is not supported by most authorities managing wildlife areas, or by the public at large. As our knowledge evolves we have to accept that once BTB is established in a wildlife population it will be impossible to eradicate with the tools currently available, and even controlling the disease will remain a challenge and have very expensive cost implications for the management of the affected wildlife area. This requires us to think more innovatively about how to deal with the management of BTB in wildlife.

Alternative management options for BTB

BTB is a disease that once established in free range wildlife (in the South African experience, multiple species) will most likely be endemically present for the foreseeable future, forcing managers to apply local and specific risk management options primarily to prevent bidirectional infection between livestock and wildlife at the interface. For areas where BTB does not exist, the simple management option of preventing the disease from being introduced will ultimately be the best investment related to the disease in wildlife. The

basic approach of having adequate separation of cattle and buffalo through fencing and/or dedicated herding will mitigate any risk of transmission significantly. Good animal health surveillance in livestock herds in areas where wildlife and livestock overlap is critical to identify BTB infection in livestock, both to avert spillover to and spillback from wildlife populations. Ideally, early detection can then result in quick action to control the disease in livestock through a limited test and cull approach with compensation for culled animals. There is very little evidence that BTB transmits easily between wildlife and livestock, emphasizing that livestock themselves are the main spreader of BTB among other livestock and the zoonotic source for BTB in humans (de Garine-Wichatitsky *et al*., 2013). Recently there has been evidence of spillback from buffalo to cattle on the western boundary of KNP (Musoke *et al*., 2015) so risk management at the interface is an important component of managing BTB in areas where livestock and wildlife can mix and where wildlife is the major maintenance host. In areas where BTB has not been confirmed, very strict selection should take place when introducing wildlife from other areas. Prevention of introduction of BTB should be the ultimate goal of conservation area managers. Apart from the Greater Limpopo Transfrontier Conservation area and Mapungubwe TFCA, most wildlife is kept in fenced areas and movement is assisted through capture (removal) and introduction of captured wildlife elsewhere. It is under these conditions of a new wildlife–wildlife interface that source populations for introductions need to be carefully selected and areas avoided where known BTB-infected animals, livestock or wildlife, occur. The best example of this is, as mentioned above, the recent BTB introduction into Madikwe Game Reserve following large introduction programmes to supplement the wildlife populations.

In conclusion, we recommend consultation with managers and researchers who have learned lessons in areas where BTB has established itself in wildlife, and where surveillance, monitoring or control options have been applied, to prevent unnecessary investment of resources in options with a good likelihood of failing or resulting in poor outcomes. This disease, once established in free-range wildlife (the South African experience), will remain in the area for the foreseeable future, forcing managers to apply local and specific risk management options primarily to prevent bidirectional infection between livestock and wildlife at the interface. For areas where BTB does not exist, the simple management option of preventing the disease from being introduced will ultimately be the best investment related to the disease in wildlife.

Conclusions

Tuberculosis in wildlife (caused by any currently known member of the MTBC) has been present for at least a century and possibly longer in South Africa, and was most probably originally introduced by livestock brought from Europe and the Middle East. Management interventions are limited and often restricted to test and cull control programmes; these are complicated by the limited availability of validated diagnostic tests, which at present are available only for buffalo and lion. A careful multi-disciplinary approach is needed to deal with population and zoonotic risks most effectively and also to reduce the risk of cross infection between wildlife and livestock.

The most important areas of risk are in conservation areas overlapping with communal farming areas and at the other extreme the modern intensification systems being used on game ranches where wildlife have become a valuable economic commodity. Large-scale movement of potentially infected or maintenance hosts may well be a big silent spreader of BTB. There is little doubt that the number of spillover species is increasing and in areas where BTB has been present for a long time it is likely that it will be maintained in a number of species, making control virtually impossible. Fortunately at this stage there is no evidence that BTB is having dramatic effects on populations of species where it has been diagnosed, but continued monitoring will be required to measure this over time.

With the limited tools available to control BTB in wildlife it must be stressed that

vigilant livestock monitoring (especially of bovines) should be conducted regularly, particularly in areas where livestock and wildlife could interact, as the disease is more easily controlled in livestock. Better screening of wildlife species being moved (with an initial focus on kudu) will go a long way to preventing unwanted introductions of BTB into naïve wildlife populations. Prevention remains the most cost-effective management and control approach to BTB in wildlife.

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21 Novel *Mycobacterium tuberculosis* Complex spp. in Group-living African Mammals

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Introduction

Tuberculosis (TB) pathogens of the *Mycobacterium tuberculosis* complex (MtbC) are of global importance in human, domestic animal and wildlife health, and are currently a major concern in conservation, threatening wildlife populations, particularly rare and endangered species (De Lisle *et al*., 2002; Dye, 2006; Renwick *et al*., 2007). Despite the antiquity of this disease (~2700 BC; Galagan, 2014), TB remains a significant health threat with much of the biology of host–pathogen dynamics incompletely understood. In wildlife hosts, TB disease can vary importantly among species with some acting as significant reservoirs of infection while others appear to be only involved in occasional spillover infections. A comparative understanding of how the various MtbC pathogens interact with different wildlife hosts would provide critical insight into the circumstances that might support or reduce the likelihood of pathogen transmission and persistence, and the relative influence of respective pathogens, hosts and environmental characteristics on this process. Advances in this area would greatly contribute to TB disease control in wildlife, but, more importantly, this comparative approach would shed light on the dynamics of TB infection in humans, thus impacting public health control approaches. Here, we review what is known regarding the recent emergence of a number of novel MtbC pathogens among African wildlife with a primary focus on *M. mungi*, a novel TB organism infecting banded mongoose (*Mungos mungo*). We will explore the complexity of factors that appear to influence infectious disease dynamics and host–pathogen systems.

Africa: Geographic Focus for Emerging MtbC Organisms in Wildlife

While TB is a globally occurring disease, the emergence of new wildlife-associated organisms in this pathogen group has largely been restricted to sub-Saharan Africa (Plate 20), specifically Southern Africa. New organisms and their wildlife hosts include: *M. mungi* in banded mongoose (Alexander *et al*., 2010), *M. suricattae* in meerkats (*Suricata suricatta*; Parsons *et al*., 2013), and Dassie bacillus in rock hyrax (*Procavia capensis*; Smith, 1960), with one case in West

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Africa, in a chimpanzee (*Pan troglodytes* ssp., chimpanzee bacillus, Table 21.1; Coscolla *et al*., 2013). This apparent increased identification of novel TB organisms in Africa is consistent with the hypothesis that the MtbC group originally arose in humans on this continent, with geographic spread occurring in association with human immigration out of Africa some 67,000 years ago (reviewed, Galagan, 2014).

Closely related to these organisms is the lineage six, human-associated TB pathogen, *M. africanum* West African-2 (MAF-2). Together with *M. africanum* West African-1, these pathogens are responsible for the majority of human TB infections in West Africa (reviewed in De Jong *et al*., 2010). As with *M. tuberculosis*, MAF-2 infections date as far back as ancient Egypt's Middle Kingdom period (*c*.2000–1600; Zink *et al*., 2003). Despite the close genetic relationship to the wildlife-associated TB organisms in Southern Africa (Plate 22), MAF-2 has never been identified in Southern Africa (Demers *et al*., 2010). Of interest is the fact that the only wildlife-associated TB organism to occur in West Africa infected man's closest relative, the chimpanzee (i.e. chimpanzee bacillus). It has been proposed that there is an unidentified zoonotic reservoir of MAF-2 in West Africa responsible for the persistent infection of humans in the region, driving the spatial and clinical patterns of occurrence (De Jong *et al*., 2010; Demers *et al*., 2010). Despite the persistent pattern of infection with this organism in humans in West Africa, identification of an animal reservoir remains elusive.

With the exception of chimpanzee bacillus, this group of wildlife-associated TB pathogens appears to infect specific host species within geographically restricted regions (Plate 20 and Table 21.1). Pathogen spillover to other wildlife hosts has not been identified, although systematic assessments have not been conducted among other sympatric animal hosts occurring in the same region. Pathogen virulence varies importantly between these organisms, with *M. mungi* and *M. suricattae* being more pathogenic in their respective hosts, and dassie bacillus rarely causing significant disease in the hyrax host (Mostowy *et al*., 2004). The TB organism isolated from a lone chimpanzee appears to represent an isolated spillover event from an unknown reservoir, as there has been no infection detected among other chimpanzees in the region over the last 10 years of intensive study (Coscolla *et al*., 2013).

Clearly of interest is the origin of these novel TB organisms. Are these recently identified TB pathogens newly evolving from some unidentified progenitor? Or, are these pathogens only newly discovered in relation to some ecological perturbation that allowed emergence and detection in the host population? Whole-genome sequencing of chimpanzee bacillus has been completed and work is ongoing on the other members of this group. These efforts will contribute significantly to our understanding of the origin and evolution of these closely related TB pathogens and the MtbC as a whole.

Pathogen Characterization

TB organisms in lineage six that are associated with African wildlife are distinctive in that they contain the single G to A nucleotide change at position 1129 in the gene *Rv1510*, which distinguishes them from all the other members of the MtbC, including the oryx bacillus (Huard *et al*., 2006). The *Rv1510* g1129a SNP (single nucleotide polymorphism) is also found in MAF-2 (Plate 22), which clusters with the African wildlife group as previously discussed. An MtbC-specific multiplex-PCR test (Warren *et al*., 2006) has been useful in differentiating organisms within the MtbC complex including some members of this group, although there is incomplete information available on *M. suricattae* and the chimpanzee bacillus (Table 21.2).

Spoligotyping of these pathogens reveals distinctive results with no known matches to any other MtbC organism previously isolated (Plate 21), including the failure to amplify any direct repeat region spacers for *M. suricattae* (Parsons *et al*., 2013). The full 24 set of MIRU-VNTR types (Supply *et al*., 2006) have also been determined for these organisms (Alexander *et al*., 2002; Parsons *et al*., 2013; Table 21.3). In banded mongoose, MIRU-VNTR analysis identified multiple substrains circulating between years and within the same social group during the same outbreak period, suggesting that there is significant complexity in *M. mungi* transmission and/or evolution of

Table 21.1. Comparison of lineage six wildlife-associated TB pathogens and their respective hosts.

Pathogen						Primary Animal Host			
Mycobacterium strain	First identified	Location	Mode of transmission	Host spectrum	Reservoir	Host	Body size	Diet	Social structure
M. africanum	1968	West Africa	Respiratory	Human, monkey, cow	Unknown, hypothesized non-human primate	Human (*Homo sapiens*)	60–80 kg	Omnivore	Gregarious
M. mungi	2010	Southern Africa (northern Botswana)	Non-respiratory, percutaneous	Banded mongoose	Unknown	Banded mongoose (*Mungos mungo*)	1.0–1.5 kg	Carnivore/ Insectivore	Gregarious
Dassie bacillus	1958	Southern Africa (Eastern cape, South Africa)	Respiratory	Rock hyrax, meerkat	Rock hyrax	Rock hyrax (*Procavia capensis*)	3.6–4.3 kg	Herbivore/ Folivore	Gregarious
M. suricattae	2002	(Kalahari desert, South Africa)	Respiratory	Meerkat	Unknown	Meerkat (*Suricata suricatta*)	0.72–0.73 kg	Carnivore/ Insectivore	Gregarious
Novel chimpanzee isolate	2009	West Africa	Unknown, spillover[a]	Chimpanzee	Unknown	Chimpanzee (*Pan troglodytes*)	26–70 kg	Omnivore	Gregarious

[a]Not confirmed.

Table 21.2. Assessment of genomic regions of difference (RD) in different MtbC members with presence (P) or absence (A) indicated for the region (Warren *et al*., 2006; Alexander *et al*., 2010; Parsons *et al*., 2013).

Species	RD1	RD4	RD9	RD12	RD1$^{mic\ a}$	RD2$^{seal\ b}$
M. canetti	P	P	P	A	P	P
M. tuberculosis	P	P	P	P	P	P
M. africanum	P	P	A	P	P	A
M. microti	P	P	A	P	A	P
M. pinnipedii	P	P	A	P	P	A
M. caprae	P	P	A	A	P	P
M. bovis	P	A	A	A	P	P
M. bovis BCG	A	A	A	A	P	P
M. mungi	P	P	A	P	P	P
M. suricattae	P	P	A	P	?	?

[a]RD1^{mic}, region of difference 1 from *M. microti*
[b]RD2^{seal}, region of difference 2 from *M. pinnipedii*

the organism over that period. Two strain variations were also identified among available M. *suricattae* isolates (Parsons *et al*., 2013).

Epidemiology, Clinical Presentation and Pathology

General features of infection with this pathogen group vary importantly by host. Dassie bacillus and *M. suricattae* have a primary respiratory presentation in their hosts, while *M. mungi* exhibits a non-respiratory mode of transmission (reviewed in more detail below). Predation of the single chimpanzee carcass limited any conclusions regarding clinical or pathological presentations.

M. mungi and banded mongoose

M. mungi invasion of the banded mongoose host differs fundamentally from other pathogens in the MtbC (Alexander *et al*., 2010):

1. Unlike other MtbC organisms, *M. mungi* causes high levels of mortality in mongoose, threatening the persistence of smaller social groups.
2. The progression from clinical presentation to death in affected mongoose is relatively short (2–3 months) in contrast to the other MtbC pathogens where TB disease (in the absence of co-infection) is typically a more chronic condition.
3. Rather than having a primary respiratory transmission route with direct transmission between individuals as is more characteristic of other MtbC species, *M. mungi* appears to infect banded mongoose primarily through a non-respiratory route.

To understand *M. mungi* transmission and persistence dynamics, we have an ongoing, long-term study population of approximately 600 mongooses living in 34 groups (from 2000 to the present). Our work includes range and movement studies, behavioural investigations, gross pathology (n = 117) and histopathology (39 macroscopically positive cases) evaluations, coupled with molecular genetic assessments of both the host and the pathogen. In order to understand mongoose susceptibility to *M. mungi* infection, specifically the potential role of prolonged elevation in stress-related hormone levels, we have also monitored faecal glucocorticoid metabolite production in our study population.

Outbreak dynamics of *M. mungi* in banded mongoose suggest a common pattern of onset in the dry season of each year, with outbreak quiescence largely occurring in the wet season (Fig. 21.1). *M. mungi* is the only pathogen in the group where a seasonal signature is apparent, a unique and intriguing feature of this pathogen. The initial outbreak of TB disease in 2000 appeared to spread as a point-source infection between troops of banded mongoose occupying adjacent home ranges (Fig. 21.1; Alexander *et al*., 2002). As time has progressed, however, the outbreaks have become longer and individuals within and between troops have become infected with decreasing spatial and temporal association between social groups (Fig. 21.1).

Table 21.3. Comparison of the full 24 set MIRU-VNTR results of selected MtbC members (Alexander *et al*., 2010).

Species	MIRU 02 154	VNTR 0424/Mtub04 424	VNTR 0577/ETR-C 577	MIRU 04/ETR-D 580	MIRU 40 802	MIRU 10 960	MIRU 16 1644	VNTR 1955/Mtub21 1955	MIRU 20 2059	VNTR 2163b/QUB1 2163/b	VNTR 2165/ETR-A 2165	VNTR 2347/Mtub29 2347	VNTR 2401/Mtub30 2401	VNTR 2461/ETR-B 2461	MIRU 23 2531	MIRU 24 2687	MIRU 26 2996	MIRU 27 3007	VNTR 3171/Mtub34 3171	MIRU/ETR-E 3192	VNTR 3690/Mtub39 3690	VNTR 4052/QUB26 4052	VNTR 4156/QUB4156 4156	MIRU 39 4348
M. mungi	2	3	2	3	1	5	3	3	2	0^{1}/No^{4}	7^{4}/No^{1}	2	4	4	4	2^{4}/3^{1}	3^{2}/4^{3}	3	3	8 and 9	2^{1}/No^{4}	No	No	2
Dassie bacillus	2	2	4	3	2	7	3	3	2	7	7	2	3	4	4	2	5	4	3	5	5	4	4	2
M. suricattae	2	3^{2}, 2^{1}	5	2	2	6	2	3	2	No	No	3	4^{2}, 5^{1}	5	4	3	4	1	3	5	8	3	1	2
M. africanum	2	4	5	2	2	7	4	4	2	5	6	3	4	4	4	2	4	3	3	5	4	6	3	2
Oryx bacillus	2	2	5	3	2	7	4	3	2	No	3	3	4	2	4	1	4	3	3	4	4	2	3	2
M. microti	2	3	5	4	2	5	6	3	1	6	9	3	4	3	4	2	2	2	3	1	3	9	3	2
M. pinnipedi	2	3	4	5	2	6	4	4	2	9	9	3	4	3	4	2	2	2	3	3	3	7	0	2
M. caprae	2	4	5	3	2	6	2	3	2	4	5	3	4	3	4	2	4	3	2	5	1	3	3	2
M. bovis	2	2	5	4	2	2	4	3	2	4	4	3	4	3	4	1	3	3	3	3	2	5	1	2
M. tuberculosis	2	2	3	2	1	3	2	2	2	5	4	3	2	3	6	1	3	3	3	3	5	5	3	2

Dassie bacillus data supplied by S. Parsons. Oryx bacillus data supplied by Supply *et al*. (2006). *M. africanum* West African 2 strain 8163/02 (ST181), *M. microti* strain 287/99 (ST539), *M. pinnipedii* strain 7739/01, *M. caprae* strain 5358/99 (ST647) and *M. bovis* strain 8490/00 (ST482) data from www.miru-vntrplus.org; no = no amplification. Superscript numbers represent how many individuals that copy number was found in.

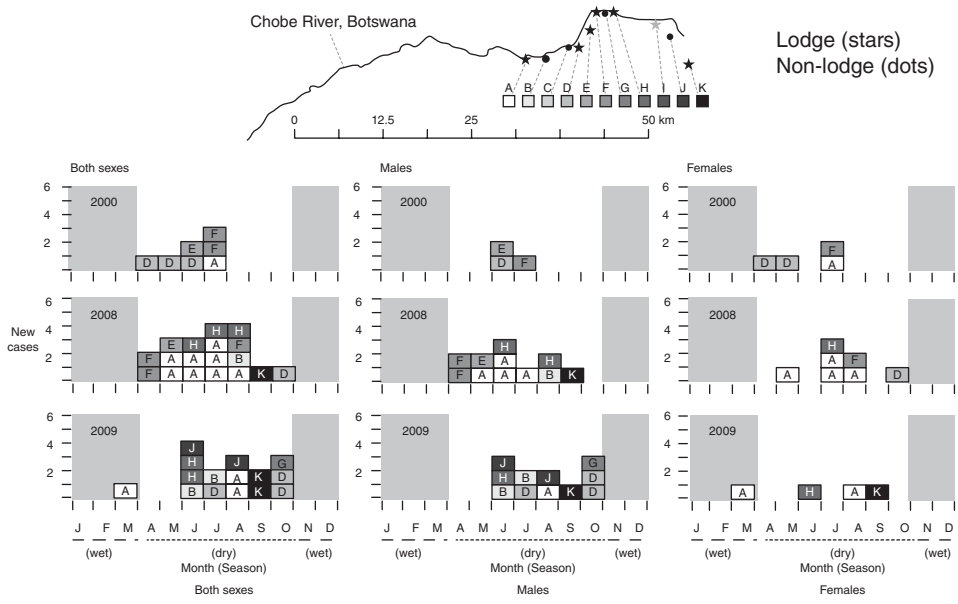

Fig. 21.1. Epidemic curves for outbreaks of *Mycobacterium mungi* in troops of banded mongoose (A–K) along the Chobe River with dots (town- or park-associated) or stars (tourist lodge-associated) for the initial outbreak and two subsequent years. We indicate troops known to have had clinical cases with black dots or stars and those without known cases with grey dots or stars. Each epidemic curve shows the number of new clinical cases by month, colour- and letter-coded by troop location along the Chobe River for 11 regularly observed troops.

Since the first outbreak in 2000, eight of nine (89%) intensively studied troops and 15 of 25 (60%) additional opportunistically surveyed troops are now infected or have had clinical cases at some point. In total, 23 of 34 (68%) troops in the study area have had clinical cases since the first outbreak. Half of known adult and subadult mortalities were attributable to *M. mungi* infection. Period prevalence by troop and by outbreak was generally below 10%, but reached as high as 33%, resulting in some cases with local troop extirpation or fusions. Clinically infected mongoose exhibited cachexia, ataxia, lethargy and lack of fear response, as well as clinically apparent distortion of the *planum nasale* (nasal plane; Fig. 21.2).

Histological studies of *M. mungi* infection in banded mongoose indicate that the organism invades primarily from an environmental source through cuts and abrasions in the *planum nasale* and skin of the mongoose (Alexander *et al.*, 2002; Fig. 21.2). Following invasion, *M. mungi* infects local macrophages and proliferates in the sub-epidermis/dermis. Extensive examination of lymph nodes by individuals indicates that the size and progression of lesions in the lymph nodes are similar, suggesting that the pathogen simultaneously spreads to all nodes on entry into the lymphatic system. In some instances, TB lesions have been noted on the skin of the legs or scrotum with limited invasion of the underlying tissue. Organ involvement occurs later in the disease process after dissemination of the pathogen into the nodes. We found few pulmonary lesions, and there were no cases of infection in the mesenteric or hilar lymph nodes, independent of infection in other peripheral lymph nodes. Tissue involvement varied greatly among affected individuals but was highest in the regional lymph nodes, spleen and liver. Histologically, tuberculosis pneumonia was determined to be haematogenous rather than bronchogenous (i.e. by inhalation). This was consistent with the presentation of pneumonic TB being present in only a minority of

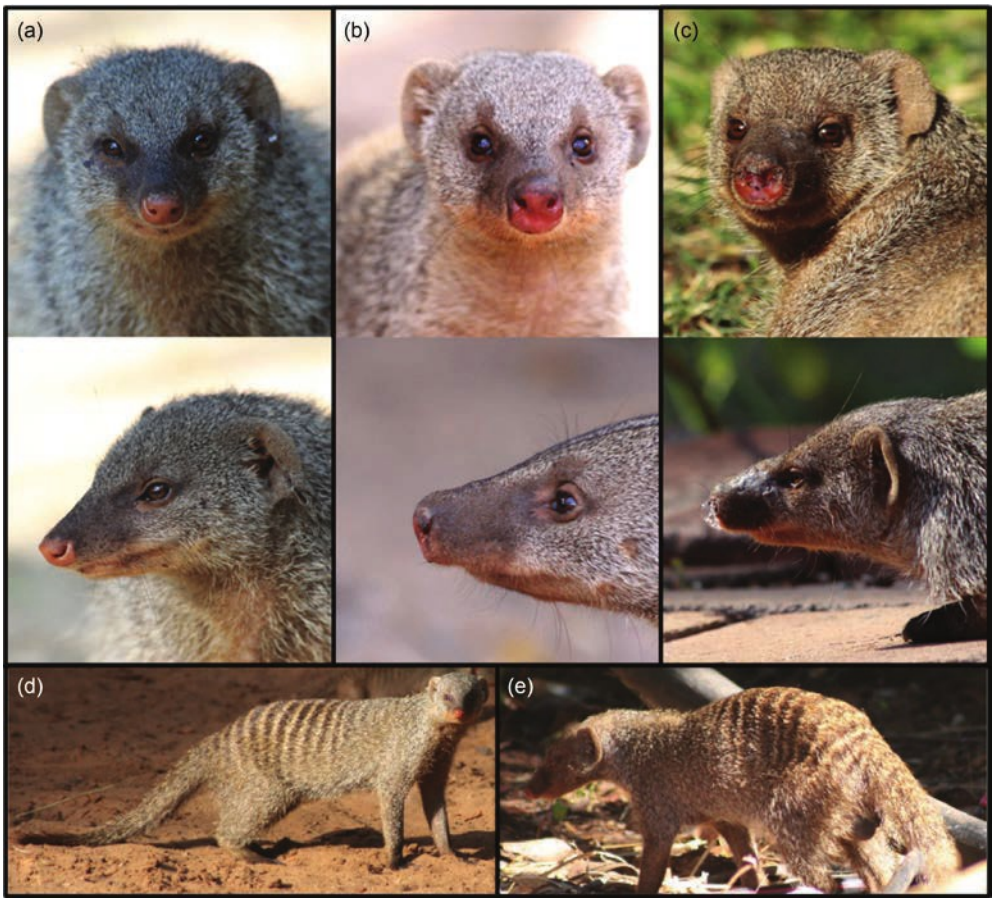

Fig. 21.2. *M. mungi* nasal distortion in the banded mongoose. (a and d) Healthy adult mongoose; (b) adult mongoose with initial clinical signs of *M. mungi* invasion with enlargement and distortion of nose; (c) adult mongoose with advanced stages of *M. mungi* invasion, including extreme nasal distortion, necrosis of nasal planum and the excretion of nasal mucous; (e) clinically infected mongoose exhibiting cachexia, ataxia and lethargy.

advanced cases with disseminated TB. This was a consistent finding among all animals examined post mortem during the study period. In August of 2011, however, we identified our first case of primary respiratory transmission, with macroscopic lesions found in the lung and no other lesions noted – a completely new presentation detected in our study population. The appearance of primary respiratory infection suggests that the transmission mechanisms of the pathogen may be changing. It is presently unknown if this is related to changes in the pathogen and/or host, or is simply so infrequent an event that it had not been identified previously.

Banded mongooses are fossorial animals, meaning they are adapted to digging and living underground. They will readily cohabit with humans in towns and around lodges in protected areas. While they den in termite mounds and other natural environments, they will use man-made structures where available including toilet drains, septic tanks and foundations. The banded mongoose diet comprises soil-associated macroinvertebrates, small mammals and reptiles (Rood, 1974). These foraging and denning behaviours could provide the necessary abrasions to the nose allowing pathogen invasion to occur in the mongoose host. Mongoose scent marking

behaviour involves the frequent use of faeces, urine and anal gland secretions (Brown *et al*., 1985; Müller and Manser, 2008). This might provide a pathway for exposure during scent inspections of these secretions. However, *M. mungi* has not been identified to date in banded mongoose faeces evaluated for the presence of pathogen specific DNA (n = 120, Alexander, unpublished data).

Host and Pathogen Ecology

Mammalian hosts of the African mammal group in the MtbC are all social or group-living mammalian species, with no identification to date of endemic TB disease in any solitary species. This is also true for wildlife reservoirs of bovine tuberculosis across the globe (e.g. brushtail possum, *Trichosurus vulpecula*; semi-social, group-denning behaviour (Ramsey *et al*., 2002); badger, *Meles meles* (Krebs *et al*., 1998); bison, *Bison bison* (Joly and Messier, 2004); African buffalo, *Syncerus kaffer* (Rodwell *et al*., 2001) and other herbivores). TB transmission is generally associated with close and prolonged contact (Lalvani *et al*., 2001). These criteria may only be identified in social or group-living wildlife species where there is group stability, necessary contact characteristics are obtained, and conspecific transmission can occur at a sufficient rate for pathogen persistence in the host population. Solitary species might then only be affected by spillover events from social wildlife host reservoirs. It is possible that TB infections, particularly in small solitary animals, are simply not detected as the host succumbs to predation pressure that otherwise is subverted for a period in social species due to group vigilance and defence activities. However, this pattern provides support for the important role of group-living and social structuring in TB disease dynamics, persistence and species association.

Fossorial behaviour, silicosis and TB disease

All of the wildlife hosts endemically infected with lineage six TB engage in fossorial behaviour. Between foraging and digging in the soil, and sleeping in groups in dens and caves, these wildlife species can inhale large quantities of soil containing crystalline silica dust. Silica is a common constituent of soil and rocks, and exposure to and inhalation of silica particles can cause a host of diseases including silicosis, lung cancer and silica-associated pulmonary TB (Rees and Murray, 2007). Silicosis has also been identified in a variety of mammalian species, primarily in captive and domestic animals (Suedmeyer *et al*., 1999). Silica dust has been found in the lungs of wild, fossorial badgers susceptible to bovine tuberculosis (Higgins *et al*., 1985) as well as in banded mongoose (Alexander, unpublished data). It has been proposed that silica dust may act as an immunosuppressant, increasing susceptibility to TB (Higgins *et al*., 1985). Indeed, humans with silicosis have three times the chance of contracting TB in a high TB risk area compared to healthy individuals (Cowie, 1994), with the chance of TB contraction increasing where risk of TB contraction increases with the severity of silicosis disease (American Thoracic Society Committee of the Scientific Assembly on Environmental and Occupational Health, 1997). Although silicosis has yet to be identified in these lineage six wildlife-associated hosts where pulmonary transmission is identified, the primarily fossorial/cave dwelling nature common to these TB hosts may provide insight into conditions conducive to the emergence and persistence of these novel TB organisms.

Human-modified Landscape – Influence on Host Behaviour and Implications for TB Disease Dynamics

Human alteration of landscapes is increasingly seen as an important influence on wildlife populations across the globe (Vitousek *et al*., 1997; Daszak *et al*., 2001; Brooks *et al*., 2002). The population of banded mongooses in north-eastern Botswana infected with *M. mungi* live along a gradient of anthropogenic disturbance and provides an important opportunity to assess the influence of environmental factors on host behaviour and TB transmission (Fig. 21.3,

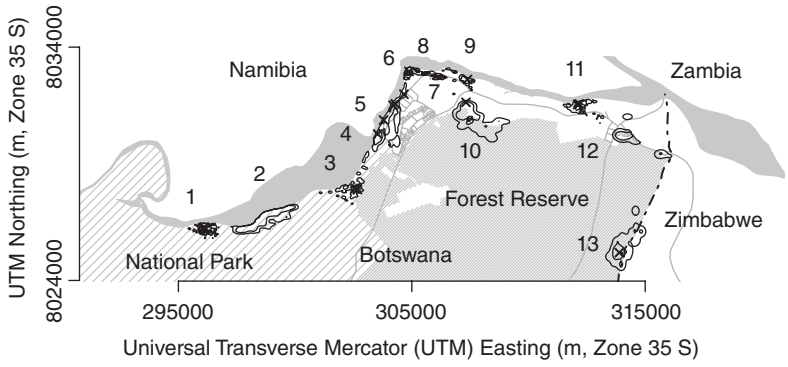

Fig. 21.3. Banded mongooses (*Mungos mungo*) lived along the Chobe River (solid grey polygon) in north-eastern Botswana (2008–2011). Our core study troops (polygons of 95% kernel density home ranges, 1–13) lived in the Chobe National Park (troops 1, 2, 3 and 4), Kasane Forest Reserve (troops 3, 10, 12 and 13) and in the towns of Kasane and Kazungula (troops 5, 6, 7, 8, 9, 11 and 12). Synanthropic troops (i.e. those living with humans) lived at lodges (troops 1, 3, 5, 6, 7, 8, 9, 11 and 13) or in towns (troop 12) or in close association with a military camp (troop 4). Two (apoanthropic) troops had no access to anthropogenic resources (troops 2 and 10). Troop 10 lived near the town refuse site but did not forage in the site. Black crosses indicate substantial lodge or town refuse sites.

Alexander *et al*., 2010; Laver, 2013). Mongoose troops live along a continuum of environmental change from those troops in relatively pristine habitats (protected areas such as a national park and forest reserve) with negligible anthropogenic perturbation to urban habitats where environmental change can be extensive. Troops living in human-modified environments where levels of anthropogenic mortality are high appear to be heavily impacted by the pathogen, with increased disease-mediated mortality threatening troop survival. In contrast, large troops living outside of these areas appear to be little affected by endemic *M. mungi* infection. With such dramatic differences in anthropogenic perturbation among these groups, it is plausible to hypothesize that anthropogenic drivers may play an important modulating role in the dynamics of *M. mungi*-infection of banded mongooses.

We hypothesize that for group-living species, these differences are created by the complex interaction that can occur between infectious disease, Allee Effects (AEs) and areas of increased mortality associated with anthropogenic influences (Sanderson *et al*., 2014). In group-living species, such as the banded mongoose, population dynamics and group persistence can be influenced by group size (Courchamp *et al*., 1999b). Fitness benefits accrue from the aggregation of conspecifics due to positive fitness effects at the individual and group levels (Jennions and Macdonald, 1994; Courchamp *et al*., 1999a). AEs arise when group size is decreased below some threshold and these positive fitness effects are lost, increasing the risk of group extinction.

Mongoose stress physiology and TB

Stress hormones such as glucocorticoids may play a role in mediating trade-offs between energetics and immune function (Elenkov and Chrousos, 1999; Sapolsky *et al*., 2000; Moore and Hopkins, 2009). However, when glucocorticoids are elevated for prolonged periods, they may induce homeostatic overload (Mcewen and Seeman, 1999) and, hence, suppress cell-mediated immunity in general (Dhabhar and Mcewen, 1999; Dhabhar, 2000), and resistance to mycobacteria in particular. We are currently investigating the role of host stress physiology in *M. mungi* infection in banded mongooses. Our work in this area indicates that, late in the dry season, banded mongooses in northern Botswana experience prolonged elevation of glucocorticoids (assayed using faecal glucocorticoid metabolites, FGMs; Laver *et al*., 2012; Laver, 2013). We postulate that in

some animals this may cause homeostatic overload and impair immune function, specifically cell-mediated immunity, changing host pathogen interactions and creating seasonal disease dynamics observed in *M. mungi* infections in the population (Fig. 21.4; Laver, 2013).

Mongoose TB – perfect storm?

In the late dry season, banded mongooses in our population may face a 'perfect storm' of nutritional limitation, agonistic encounters at concentrated food resources, aggressive evictions, oestrus, competition for mates, parturition and predation pressure on pups. This amalgamation of stressors may push glucocorticoid responses into homeostatic overload and impair cell-mediated immunity, resulting in the high prevalence of *M. mungi* observed in the dry season.

Group- and Individual-level Behaviour and TB Transmission

Often, consideration of infectious disease dynamics is based on the assumption that all individuals within a population have identical levels of susceptibility and exposure to the disease agent in question. In many cases, however, this assumption will not provide adequate and essential detail necessary to understand disease transmission dynamics (Alexander *et al*., 2012). TB disease in many wildlife species appears to be influenced by individual-level behaviour, potential key to understanding exposure, transmission, persistence dynamics and, ultimately, control. Hosts infected with the lineage six wildlife-associated TB organisms have important behavioural characteristics that may influence pathogen exposure and transmission dynamics as well as survival of infected hosts.

Group Stability, Group Structure and TB Disease

Increased contact between infected and susceptible members of a group can result in higher infectious disease rates (Alexander, 1974; Schmid-Hempel and Crozier, 1999; Altizer *et al*., 2003). With TB, while other factors influence transmission (e.g. individual susceptibility, environmental factors), the occurrence of prolonged and close contact with infected individuals is seen to be a primary risk factor. In wildlife, this would be determined by social group stability and group structure. Banded mongooses are highly social animals and have

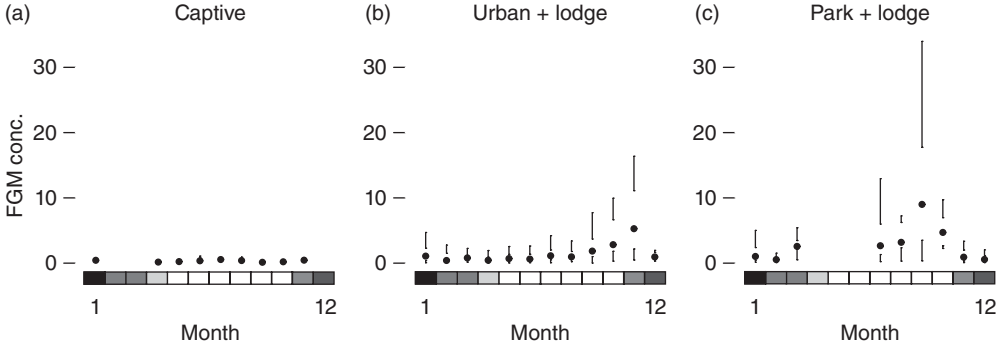

Fig. 21.4. Monthly faecal glucocorticoid metabolite (FGM, μg g^{-1} org. content) levels (median: dots; variability: quartile plots) of banded mongooses (*Mungos mungo*) in north-eastern Botswana (2008–2011), grouped according to level of synanthropy: (a) captive, (b) urban habitat with tourist lodges, (c) national park with tourist lodges. Rainfall is depicted on the lower *x*-axis with shading (black: 100%; white: 0%) relative to the highest median monthly rainfall (January: 184 mm) and reproductive events are depicted on the upper *x*-axis with shading (black: 100%; white: 0%) relative to the month with the highest number of reproductive events. In (a), workers fed the captive group a constant food supply and suppressed reproduction (black quartile plots) and then released the group from reproductive suppression (grey quartile plots).

sustained contact with conspecifics within a troop (Rood, 1975). Once these troops are formed, they remain structurally stable with very little between-group movement (Cant *et al*., 2013). Stable social structures are also characteristic of meerkat, rock hyrax and chimpanzee groups, despite differences in sex ratios and group size, the introduction of dispersing males and/or the birth of individuals and the loss of individuals (Drewe *et al*., 2009b; Carne *et al*., 2013; Ilany *et al*., 2013). The highly stable groups formed by these species would support the long-term and prolonged contact thought necessary for TB transmission between or within group members (Carne *et al*., 2013).

Structural differences between species' social groups, however, may lead to important variation in transmission dynamics. For example, in meerkats, increases in group size led to individuals interacting more with a local subgroup of individuals as opposed to maintaining interactions at a group-wide level (Drewe *et al*., 2011). Due to this, TB may be transmitted among these locally, socially connected subgroups but is not likely to spread to the remainder of the larger group due to a lack of social connections (Drewe *et al*., 2011). Similarly, while there is no evidence that the novel chimpanzee bacillus is transmitted between chimpanzees, if this were to happen, social structure and contact networks would also influence disease dynamics. For example, chimpanzees were found to interact more commonly with related individuals and those individuals occurring in other similar-sized family groups (Rushmore *et al*., 2013). Individuals from chimpanzee families inhabiting the periphery of the community were also less likely to be involved in pathogen transmission (Rushmore *et al*., 2013). These individuals, however, may have an increased likelihood of exposure to disease from neighbouring chimpanzee communities (Rushmore *et al*., 2013).

Affiliative and Agonistic Social Behaviours

Social interactions within a group can determine the outcome of disease at the individual and group level. In highly gregarious mammals, such as the wildlife hosts infected by lineage six wildlife-associated TB, the direction of social interactions can be particularly important in determining TB transmission and, in the future, may play an important role in developing surveillance and vaccination programmes.

Social interactions between conspecifics can be defined as affiliative; helpful and supportive, or agonistic; or aggressive and competitive. One particularly important affiliative behaviour is allogrooming, integral to building and maintaining social bonds between conspecifics. While this social behaviour has been known to reduce the threat of ectoparasites (Ueckermann *et al*., 2011), it may increase the risk of infectious disease transmission (Drewe, 2010). Allogrooming in meerkats has been linked to increased *M. suricattae* transmission rates, with meerkats that frequently allogroom having an increased likelihood of being infected, compared to conspecifics that allogroom less or not at all (Drewe, 2010). In contrast, individuals being groomed were less likely to be infected with TB (Drewe, 2010). In banded mongoose troops, individuals infected by *M. mungi* were less active and less vigilant than healthy mongooses, but were allogroomed at an increased rate (Fairbanks, 2013). If *M. mungi* can be transmitted horizontally, and allogrooming increases the exposure rate as seen in meerkats, then the increased rate of allogrooming of TB-infected individuals may increase TB transmission rates among banded mongoose (Fairbanks, 2013). In chimpanzees, oestrus events have been shown to significantly increase pairwise subgroup associations in social networks, most likely due to increased allogrooming and mating during this time. In relation to these group dynamics, chimpanzee communities are thought to be at an increased risk of disease outbreaks when multiple females share oestrus cycles and, subsequently, subgroup associations are magnified (Rushmore *et al*., 2013).

Important agonistic social behaviours in gregarious mammals include aggressive behaviours, which are primarily used to establish and assert dominance, as well as to protect resources and breeding opportunities. Elevated aggression levels in banded mongooses have been correlated with *M. mungi* transmission (Fairbanks *et al*., 2012). However, instead of increasing horizontal transmission

of TB, elevated levels of aggression within troops appears to have an important indirect effect by increasing injury rates and TB disease in a group. It is suspected that environmental transmission of *M. mungi* occurs through open wounds incurred while fighting over food waste or other items (Fairbanks *et al*., 2012). Despite grooming in meerkats being a primary behaviour associated with TB transmission, aggression was also shown to influence the incidence of TB infection among individuals (Drewe *et al*., 2011). Meerkats who were recipients of aggression were more likely to be infected with TB, as were individuals in the middle of a 'chain of aggressive interactions' (Drewe, 2010). In a more recent paper, however, contrary results were obtained suggesting that aggressive meerkats have a higher risk of contracting TB than those receiving aggression (Drewe *et al*., 2011). Despite these contrasting outcomes, it is clear that aggression can play an important role in TB transmission among meerkats. Although rarely investigated and lacking data concerning TB risk, aggression in chimpanzees may also aid in pathogen transmission more generally (Calvignac-Spencer *et al*., 2012).

The position of an individual within the dominance hierarchy of a social group can also have significant impacts on TB contraction and transmission. In meerkats, dominant individuals, despite being socially prominent, were not infected by TB (Drewe, 2010). This observation coincides with dominant meerkats having an increased likelihood of being groomed rather than grooming others, and also an increased likelihood of being the aggressor, rather than receiving aggression (Drewe, 2010).

Dispersal/Roving and Inter-group Transmission

Dispersal is extremely important in animal communities, aiding in gene flow, and increasing population fitness and species distribution (Stenseth and Lidicker, 1992). Dispersal, however, can also aid in pathogen transmission through the emigration and immigration of diseased individuals between populations, groups and areas. For example, *M. bovis* infections appeared higher in badgers that had immigrated and no longer resided in their natal social group (Woodroffe *et al*., 2009). Furthermore, badgers in smaller social groups had a higher prevalence of TB (Woodroffe *et al*., 2009). It is hypothesized that this may be due to increased interactions between members of these smaller groups with neighbouring social groups (Woodroffe *et al*., 2009). Likewise, 'roving' meerkats (mature male meerkats which leave their social group to seek breeding opportunities in neighbouring meerkat groups) were more likely to be infected with TB and may provide an important link for TB transmission between social groups (Drewe, 2010). In banded mongooses, however, TB-infected individuals appeared to have reduced dispersal rates and, although limited, data suggest that healthy individuals are more likely to disperse given a greater number of sick individuals in the group (Fairbanks *et al*., 2014). In chimpanzees, males tend to stay in their natal groups while females disperse at adolescence. Likewise, male juvenile rock hyraxes are required to disperse from their natal groups and remain primarily solitary (Ilany *et al*., 2013). Given this, males in these divergent behavioural settings will have different impacts on the spread of TB in the population. Clearly, it is essential to understand individual and group behaviour for a particular species in order to correctly characterize important elements of TB transmission dynamics in a population.

Public Health Significance

While human infection from these TB organisms have not been recorded, this may be related to the fact that many TB organisms are misidentified, with accurate identification requiring more sophisticated molecular approaches (Alexander *et al*., 2010; Parsons *et al*., 2013). Indeed, both *M. mungi* and *M. suricattae* were originally identified as *M. tuberculosis* and *M. bovis*, respectively (Alexander *et al*., 2002; Drewe *et al*., 2009a; Alexander *et al*., 2010; Parsons *et al*., 2013). A limited survey was conducted among patients presenting with TB disease in northern Botswana at the local hospital within the home range of the affected mongoose troops. *M. mungi* was not

identified in the sputum sample of any patient although all were clinically identified as being TB infected (n = 54, Alexander and Gey van Pittius, unpublished data). In the case of *M. mungi*, and possibly others, TB disease may not present in the human host as a primary respiratory infection. Failure to identify the organism may arise as a consequence of inappropriate sample type.

Conclusion

The diversity of TB organisms and their hosts provides us with a unique comparative window to assess and begin to understand the dynamics of this group of highly successful pathogens. The complex ecology of newly emerging TB pathogens reviewed here suggests that host, environment and pathogen biology are important to disease dynamics and that successful control will only be identified when we begin to understand these interactions. There is an urgent need to encourage human and animal health professionals to look across both systems as the commonalities and divergent characteristics of these host–pathogen–environment systems are likely to provide the information essential to TB control, something that has remained largely elusive in much of the developing world.

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22 Rabbit Model of Mycobacterial Diseases

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Introduction

Understanding host–pathogen interactions is an important step in developing efficient intervention strategies to eliminate infectious diseases, such as tuberculosis (TB), in humans. Due to significant ethical and practical considerations associated with studying infectious diseases in humans, cost-effective and tractable surrogate animal models that can produce similar disease pathology have been developed and evaluated. Early approaches to the systematic selection and evaluation of animal models of human infectious diseases started during the early 19th century with the development of bacteriological research, including the pathogenesis and transmission of TB. In fact, one of Robert Koch's postulates mandates that 'inoculation of the isolated human pathogen to animals must reproduce the same disease conditions' to prove that a pathogen is the cause of an infectious disease (Koch, 1882). Since then several animal species, including mouse, rat, guinea pig, rabbit, non-human primate (NHP) and other vertebrates, have been explored as infectious disease models. Although each of these experimental models provides valuable insight into disease pathogenesis, it is important to select the model that most closely mimics the pathological sequelae of human disease in order to accurately predict outcomes of interventions in humans.

Advantages of the Rabbit Model in Biomedical Research

The following are some of the reasons why rabbits have been used extensively in biomedical research to study various human diseases:

1. Striking similarities have been reported between humans and rabbits in several anatomical, physiological and biochemical aspects; for example, the lipid-lowering drugs to improve cardiovascular mortality in humans were developed from observations in rabbits fed with a high-cholesterol diet, which produced vascular deposits and coronary plaques similar to humans (Besterman, 1970).
2. Similar to humans, the polymorphonuclear cells (PMN) from rabbits treated with fMLP showed identical production kinetics of reactive oxygen species (Opdahl *et al*., 1987).
3. Phylogenetically, the rabbit is closer to humans than are mice, rat and guinea pigs. Similar to humans, the lungs represent the target

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

of the anaphylactic response in rabbits. Both species contain fewer capillaries per unit volume of the tracheobronchial capillary bed; have identical symmetrical branching of the lung airways; and similar growth rate of lung expansion from birth to adulthood, compared to other animal species (Kamaruzaman *et al*., 2013).
4. The antigen-processing machinery in rabbit and human immune cells function similarly, and are strikingly different from the mouse. The nucleotide sequence of a β-chain variable region ($V_T\beta$) of the human T cell receptor (TcR) gene is more conserved in rabbits than in mice (Marche *et al*., 1985). Moreover, an identical pattern of TcR-mediated antigen recognition by the major histocompatibility complex (MHC) molecules and conserved coevolution of MHC and TcR were reported between rabbits and humans.
5. Unlike the case in mice, in which samples must often be pooled, individual rabbits provide sufficient organ tissue and blood samples for various analyses. In addition, rabbits are more economical to purchase and house than NHP.
6. Rabbits are more docile than rats and mice, easy to handle and are multiparous with a shorter gestational period than NHP.

Despite these advantages, development of the rabbit model has been hampered mainly due to lack of commercially available immunological reagents and cellular markers, as well as to the higher cost of conducting rabbit experiments compared to experiments on mice. In addition, genetic manipulations in rabbits are difficult and the molecular tools for such studies have not been developed for high-throughput screening.

The Rabbit Model of Pulmonary Tuberculosis

In the mid-1900s, extensive research involving rabbits was conducted by Max Lurie, Ratcliffe, Wells, Yamamura and Dannenberg, who established the basic principles of various aspects of the host response, including resistance/susceptibility to infection following inoculation with *Mycobacterium bovis* (Mbo) or *M. tuberculosis* (Mtb). Details of experimental designs from these studies, including infection of inbred and outbred rabbits with pathogenic mycobacteria and relevance to the understanding of human TB, have been well described previously and will not be discussed in detail here (Lurie, 1964; Dannenberg, 2006).

Active pulmonary tuberculosis by *M. bovis infection*

The granulomatous process and cavity formation in humans with TB are probably the most important aspects of human disease to be studied in animal models. The presence of cavitary lung disease in humans increases the risk for TB relapse by tenfold, despite completion of standard therapy. In addition, the presence of massive numbers of replicating bacilli within such cavities, which are not easily penetrated by most anti-TB drugs, increases the risk of drug-resistant TB. Importantly, rabbits have been shown to be a reproducible model for human cavitary TB and dissemination. Rabbits are relatively more susceptible to progressive disease following infection by pathogenic Mbo strains than to various Mtb strains. Therefore, pulmonary infection of rabbits with virulent Mbo strains has been used to model advanced disease stages in human TB, including liquefaction, cavitation and dissemination.

In a model reported by Converse *et al*., exposure of rabbits to aerosolized Mbo Ravanel S strain produced progressive disease in a dose-dependent manner (Converse *et al*., 1996, 1998). At low doses of infection (~500 inhaled bacilli) with Mbo Ravanel S strain, at least 3.5 times more bacilli were required to form one grossly visible lesion, compared to high-dose infection (~5000 inhaled bacilli). Implantation of 200–500 CFU (low dose) into rabbit lungs produced few grossly visible primary granulomas with caseous centres at 5 weeks post-infection. Most of these lesions liquefied and formed small cavities by 13 weeks. With time, although cavitation was commonly noted, many of the lesions were either slowly progressing or non-progressing and showed variable maturation states, with most of them being fibrocaseous nodules. Consistently, bacillary replication at the centre of the lesions

also varied between different lesion types. In these rabbits, tubercles were also found in the tracheobronchial lymph nodes, ileocaecal junction, appendix and kidneys, suggesting haematogenous spread. Tuberculin skin tests were positive for all the infected rabbits and the reaction sizes ranged from ~1.0 to 4.7 cm^3 after 4 weeks of infection. Significant variations among infected rabbits were observed in this model. Thus, although some animals did not show signs of progressive disease, others developed severe cavitary disease.

Since aerosol infection yields a variable numbers of cavities, a novel bronchoscopic technique was developed to infect animals (Nedeltchev *et al*., 2009). This model was shown to consistently produce cavities in infected rabbit lungs by 6–15 weeks post-infection. In this model, rabbits were sensitized by multiple, subcutaneous injections (each at 3–4 days apart) of heat-killed Mbo in incomplete Freund's adjuvant. At 40 days post-sensitization, pathogenic mycobacterial strains (Mbo Ravenel, AF2122, Mtb H37Rv and CDC1551) or Bacillus Calmette-Guerin (BCG) were bronchoscopically instilled into the sensitized and non-sensitized rabbit lungs and animals were monitored for 105 days. In this study, most of the sensitized rabbits showed tuberculin skin test positivity and produced cavitary lung lesions irrespective of the infecting mycobacterial strain. The largest cavities were noted at the site of bacillary inoculation; similar gross pathology and cellular composition of cavities were noted for a wide range of infectious inoculation doses (5000–18,000 CFU). In addition, cavities formed by instillation of Mbo were larger, featuring necrosis, caseation, liquefaction and cavitation, although individual lesions showed heterogeneity in their evolution/maturation state. Only rabbits infected with Mbo uniformly showed extra-pulmonary dissemination, which was independent of sensitization state. Interestingly, a higher bacillary burden (by 1.5 $\log_{10}$) was noted in the lungs of sensitized animals relative to non-sensitized rabbits. Although granulomatous lesions of variable sizes and degrees of maturation were observed in the lungs of both the sensitized and non-sensitized rabbits, they did not contribute to the increased bacillary load observed between these two groups of rabbits. Thus, presence of cavitation accounted for the increased bacterial CFU in the infected rabbit lungs in the sensitized rabbits. Consistently, increased bacillary growth was observed within the liquefied core of cavities, reaching 10^6–10^9 CFU per lesion. This number is comparable to the bacillary load found in similar human lung cavities. Experimental findings from this infection model are consistent with earlier intrathoracic and intravenous Mbo infection studies, which have shown collectively that rabbits sensitized either with heat-killed or live (low-dose) Mbo form lung cavities upon subsequent infection with virulent mycobacteria as early as 30 days post-infection (Wells and Lurie, 1941; Ratcliffe and Wells, 1948; Yamamura, 1958).

Active pulmonary tuberculosis established by various strains of *Mycobacterium tuberculosis*

In general, rabbits, like humans, are considered more resistant to Mtb infection than are mice and guinea pigs. Mbo and Mtb produce distinct spectra of pathology in infected rabbit lungs. Pulmonary infection with Mbo produces more severe disease, characterized by greater numbers of liquefying, cavitary lesions, ultimately leading to animal death. On the other hand, the outcome of infection in rabbits has been shown to be dependent on the nature of the infecting Mtb strain as observed in experimental infection with Mtb CDC1551, Erdman and HN878, as well as the common laboratory strain Mtb H37Rv (Bishai *et al*., 1999; Manabe *et al*., 2003; Subbian *et al*., 2011c, 2012). Rabbits infected with less virulent Mtb strains, at a similar infectious inoculum, rarely develop cavities and the bacillary load is effectively contained over time in most of the lung lesions, which manifest as fibrotic nodules. Using aerosol infection, Manabe *et al*. (2003) evaluated the pathogenic potential of Mtb strains CDC1551, Erdman, and H37Rv. In this study, virulence end points included the number and characteristics of grossly visible lung tubercles after 5 weeks of infection. In this model, the Erdman strain showed the most virulent phenotype, producing one

grossly visible lesion for 320–827 inhaled CFU; this number ranged from 586 to 1889 for H37Rv and to 1667 for CDC1551. After normalization for inhalation dose, both the average number of tubercles and their sizes were found to be greater in rabbit lungs infected with the Erdman strain, followed by H37Rv and CDC1551, in descending order of virulence (Manabe *et al*., 2003). Similar findings were reported earlier in a rabbit aerosol infection model (Bishai *et al*., 1999) that compared the virulence of Mtb CDC1551 to H37Rv based on the lung tubercles produced by these strains. In this study, an average inhaled inoculum of 1580 CFU for Mtb H37Rv and 1810 CFU for CDC1551 were required to produce one visible tubercle. Despite the similar number of grossly visible tubercles produced by Mtb CDC1551 and H37Rv in the rabbit lungs after aerosol infection with 10^4 bacilli, the average size and volume of tubercles, as well as number of bacillary CFU present per lesion, were found to be significantly lower in the lungs of rabbits infected with CDC1551 relative to those in H37Rv-infected lungs (Bishai *et al*., 1999).

We have established a rabbit model of progressive pulmonary TB using Mtb HN878 strain, a member of the hyper-virulent Beijing lineage, for infection in a 'snout-only' aerosol exposure system (Tsenova *et al*., 2006b; Via *et al*., 2008; Subbian *et al*., 2011c). In our model, 10^3 CFU of Mtb HN878 implanted in the lungs grow exponentially to reach 10^7 CFU by 4 weeks. Thereafter, the lung bacillary load remained relatively constant until 12 weeks, and a 1 $\log_{10}$ increase in the bacterial CFU was noted at 16 weeks post-infection. The lung morphology of Mtb HN878-infected rabbits at 2 weeks showed no grossly visible lesions, although histological examination revealed increased cellularity in the lungs, with more PMN, as well as the presence of peribronchial and perivascular lymphoid aggregates at the site of infection. However, at 4 weeks post-infection, ~80 small (1–3 mm diameter) solid granulomas of homogenous size and architecture were noted. Almost all of these lesions showed signs of central necrosis and associated inflammation. At this time, the granulomas predominantly comprised lymphocytes, PMN and histiocytes. The centre of these necrotic lung granulomas stained positive for a hypoxia-specific probe, pimonidazole (Via *et al*., 2008). Although the number of rabbit lung granulomas did not change significantly until 16 weeks, considerable heterogeneity in size and maturation was noted among these lesions over time, beginning at 8 weeks post-infection. While some of the necrotic granulomas increased in size, developing liquefaction to variable degrees, few suppurative granulomas underwent cavitation by 16 weeks post-infection. Lung histology of HN878-infected rabbits at 12 weeks post-infection revealed the presence of multinucleated giant cells and foamy macrophages in the granulomatous areas enriched with histiocytes. At 16 weeks post-infection, few of the lesions appeared to be resorbing (i.e. fibrotic encapsulation with a central area of calcification and mineralization surrounded by layers of collagen and fibrin), while others were large, confluent and/or coalescing, tending towards cavitation. However, most of the lesions were at various maturation states (caseation and liquefaction) and of variable sizes, ranging from 4 to 8 mm diameter (Kaplan and Tsenova, 2010). Acid-fast staining of Mtb HN878-infected rabbit lungs at 8 weeks showed many bacilli within central areas of necrosis surrounded by histiocytes. At this time, extrapulmonary dissemination of bacilli to spleen and liver was also observed. Greater numbers of bacilli were noted in these organs in animals that exhibited lung cavities. Although the calcified and fibrotic lesions at 16 weeks post-infection had very few acid-fast bacilli, increased bacillary load was noted at the surface of cavitary lesions, resulting in a net increase in the number of CFU at this time. The presence of many bacilli at the surface of lung cavities is a unique feature shared by rabbit and human TB.

Enumeration of immune cells at the site of infection from Mtb HN878-infected rabbits at 4 weeks post-infection showed that 54% and 45% of total viable mononuclear cells were of non-lymphoid and lymphoid origin, respectively. This pattern of cellular distribution was maintained until 16 weeks post-infection. Of the non-lymphoid cell populations determined from 4 to 16 weeks after infection, 60–82% were CD14+ monocytes and/or macrophages, whereas elevated numbers of

CD4+ T lymphocytes were noted at 4 weeks (63%), gradually decreasing to ~18–35% by 16 weeks after infection. However, the proportion of CD8+ T lymphocytes did not change significantly from 4 to 16 weeks, remaining at ~8–12%. While the percentage of CD4−/CD8− lymphocytes increased gradually from 27% at 4 weeks to 71% at 12 weeks, the majority of these cells were IgG+ B cells. Based on these findings, there appears to be a delay and/or inadequacy in the recruitment/accumulation, as well as activation of immune cells of the host innate and adaptive responses, ultimately driving the initial infection into progressive, cavitary disease in HN878-infected rabbit lungs.

Latent infection established by clinical strains of *M. tuberculosis*

Compared to mice and guinea pigs, rabbits show considerable variation in host responses following infection with different Mtb strains. Lurie and colleagues infected outbred rabbits intravenously with human-type bacilli (Mtb), which led to progressive lung infection with a high bacillary load by 1 month, followed by a culture-negative state in all animals after 4 months (Lurie, 1964). However, lung homogenates from the majority of the Mtb-infected rabbits at 6 months post-inoculation produced viable colonies without requiring any immune suppression treatment, suggesting that although the infection in the lungs was achieved, complete latency was not established. Since then, at least two more rabbit models of control of infection have been reported in the literature (Manabe *et al*., 2008; Subbian *et al*., 2012).

In a model reported by Manabe *et al*. (2003) aerosol infection of rabbits with Mtb H37Rv at either a high dose (average of about 1.6 $\log_{10}$ CFU in the right upper lobe; RUL) or low dose (average of 1.2 $\log_{10}$ CFU in the RUL) inoculum resulted in bacillary multiplication until 5 weeks post-infection, reaching 3.7 and 3.8 $\log_{10}$ CFU in the RUL in high- and low-dose-infected rabbit lungs, respectively. While the average number of lung granulomas in these two groups of infected rabbits at this time was 187 and 47, respectively, none of the tested animals were culture negative at this time. Histological observation of lung sections showed small lesions with central necrosis surrounded by PMN, epithelioid macrophages, lymphocytes and plasma cells. The number of lung CFU and the granulomatous lesions decreased remarkably in the majority of infected rabbits from 5 weeks until either 15 weeks (high dose) or 10 weeks (low dose). At this time, the RUL had 0–3.4 $\log_{10}$ (high dose) or 0–1.9 $\log_{10}$ (low dose) CFU with an average number of 9 and 7 small granulomas in the high- and low-dose categories, respectively. In addition, the homogenates from the majority of infected rabbits at this time were culture negative. After 15 weeks, a few rabbits from each infection group had large, caseous granulomas. While rates of culture negativity did not change significantly at 20 (high dose) and 36 (low dose) weeks post-infection, RUL CFU increased to 2.5 $\log_{10}$ (high dose) and 2.2 $\log_{10}$ (low dose), respectively. Although all the infected rabbits had positive tuberculin skin tests, none of the animals reactivated disease spontaneously. When a subset of infected rabbits was given dexamethasone for 5 weeks starting at 10 weeks post-infection, an increased number of granulomas and bacilli were noted in the lungs of treated, relative to untreated, animals. However, 3 of 18 rabbits did not show any culture positivity after dexamethasone treatment. Although only a portion of lung was plated for CFU and grossly visible lesions were observed, the authors concluded that the reduction in RUL CFU was sufficient to classify the disease as 'persisting' in a proportion of infected rabbits and that dexamethasone treatment resulted in reactivation of the disease (Manabe *et al*., 2008).

In another study, Kesavan *et al*. (2009) have shown that aerosol infection of rabbits with ~1.5 $\log_{10}$ CFU of H37Rv produced about 200 granulomas with a peak bacillary load of 3.5 $\log_{10}$ after 5 weeks of infection. This was followed by a gradual decrease in the number of granulomas and lung CFU at 10 and 15 weeks, resulting in complete absence of lung granulomas and CFU by 20 weeks post-infection. Administration of dexamethasone for 5 weeks beginning 10 weeks post-infection caused reactivation, manifested by increased granuloma number and lung bacillary load. However, in this model immunosuppressive treatment

was initiated while the lung bacillary load was still high (~3 $\log_{10}$) and histopathological analysis was not reported in this study.

The salient pathophysiological features of human latent TB infection are faithfully reproduced in our novel Mtb CDC1551-infected rabbit model (Flynn *et al*., 2008; Kaplan and Tsenova, 2010; Subbian *et al*., 2012). Unlike the model described by Subbian *et al*. (2012), no cultivable bacilli or lung lesions are noted in these latently infected rabbits. However, infection can be reactivated following treatment with immunosuppressive drugs. Rabbits were infected via aerosol with the hyperimmunogenic strain Mtb CDC1551 (Manca *et al*., 2001) using a high (10^4 CFU) or low (10^3 CFU) dose inoculum. Active bacillary growth occurred up to 4 weeks, reaching a peak lung burden of about 10^6 (high dose) and 10^5 (low dose) CFU. Thereafter, a significant reduction in the number of lung bacilli was noted at 8 weeks, and bacilli could not be cultivated at 12–16 (low dose) or 20–24 (high dose) weeks post-infection. Importantly, the kinetics of bacillary growth (i.e. rapid growth followed by gradual but complete clearance of cultivable bacteria) did not change significantly between high- or low-dose infections. However, no extra-pulmonary dissemination of bacilli from the lungs to the liver or spleen was noted, even in those rabbits infected with a high-dose inoculum. The absence of cultivable bacilli in the lungs at 3–5 months post-infection was not due to complete sterilization of the bacterial population by host immunity, but rather represents a true latent infection with a paucibacillary population of viable organisms. This has been demonstrated following immune suppression of latently infected rabbits, leading to reactivation and resumed bacillary growth to a lung load matching that observed at 4 weeks after infection. Although the sub-pleural lesions were microscopic at 4 and 8 weeks, no obviously visible lesions were noted once latency was established in the Mtb CDC1551-infected rabbit lungs. In addition, no caseation, liquefaction or cavitation was noted in any of the lung granulomas at any time during the establishment of latency in these rabbits. However, histological study of the rabbit lung sections at 4 weeks revealed multiple foci of cellular aggregates, comprising macrophages and lymphocytes that formed into well-defined granulomas with more immune cell accumulation, some of which had signs of central necrosis, at 8 weeks post-infection. At 12 weeks post-infection, most of the granulomas were small and less cellular, tending to regress and resorb. At later time points (20–24 weeks), the lung parenchyma appeared normal, without any granulomas or fibrosis. In contrast, rabbits treated for 4 weeks with immune suppressive therapy, starting at 20 weeks post-infection, had extensive, diffuse cellular accumulation of mainly PMN and macrophages. Though no necrosis, caseation, liquefaction or cavitation was noted in these dense cell aggregates, the environment appeared to be conducive to prolific bacterial growth. Interestingly, at 20 weeks post-infection, one of 17 Mtb CDC1551-infected rabbits had a single, grossly visible sub-pleural lesion in the lung. This persistent or percolator lesion had ~3 $\log_{10}$ bacilli and contained large aggregates of epithelioid macrophages and PMN surrounded by layers of lymphocytes. Analysis of immune cell distribution in the rabbit lungs at different stages of infection revealed a gradual increase in the percentage of non-lymphocyte monocytes from 4 weeks (~33%) to peak at 8 weeks and plateau at similar elevated levels (about 70%) until 12 weeks. However, the percentage of lymphocytes peaked at 4 weeks (67%) before gradually decreasing to ~30% at 8 and 12 weeks. Among the lymphocytes, the percentage of $CD4^+$ and $CD8^+$ T cells gradually increased from 4 to 8 weeks and stabilized at similar numbers until 12 weeks; in contrast, B cells comprised more than 70% of the lymphocytes enumerated at 4, 8 and 12 weeks. In summary, we characterized a rabbit model of LTBI and reactivation, which recapitulates key features of the human condition, including reactivation disease in the setting of immune compromise.

Molecular Correlates of Host Immune Response During Active and Latent Tuberculosis

The rabbit genome sequencing project initiated by the Broad Institute has 7× coverage of

the genome in their latest (OryCun2.0) version released in August 2009 (www.broadinstitute.org/science/projects/mammals-models/rabbit/rabbit-genome-sequencing-project). According to this version, the diploid rabbit genome is organized into autosomes (21 pairs) and sex chromosomes (X and Y), and the size of rabbit genome is estimated to be 3500 Mb (haplotype) with ~45% GC% content (www.ncbi.nlm.nih.gov/genome/316). Currently, the rabbit genome is predicted to contain 19,293 coding genes, 3375 short non-coding genes, 1001 pseudo genes and 24,964 gene transcripts (http://useast.ensembl.org/Oryctolagus_cuniculus/Info/Annotation#assembly). We took advantage of the whole genome microarray, which contains 44,000 probe sets representing the entire rabbit genome, to explore the kinetics of transcriptional changes occurring in rabbit lungs after infection with Mtb HN878 (active TB) or Mtb CDC1551 (latent TB) (Subbian *et al*., 2011a, 2013b). We have also used global lung gene expression analysis to decipher the very early (3 h post-infection) changes in the host response to Mtb infection, which determine the outcome of infection as either active disease or latent infection (Subbian *et al*., 2013a).

Early host determinants of differential response to *M. tuberculosis* infection

The ability of host innate immune cells, such as alveolar macrophages, to determine the fate of infecting Mtb either to proliferate or to perish, was reported in studies by Lurie and colleagues using susceptible and resistant rabbits (Lurie, 1964). These observations were later extended in rabbit studies by Dannenberg and colleagues (Dannenberg, 2006). The activation state of macrophages and their production of various proteases, anti-microbial enzymes and chemokines before and after Mtb engulfment have been shown to be associated with their differential ability to handle the phagocytosed bacilli. We characterized the very early events at the site of Mtb infection at the cellular and molecular level, and showed for the first time that these events determine the outcome of TB infection. In our rabbit model of pulmonary TB, aerosol infection with Mtb HN878 produces progressive disease with cavities, while Mtb CDC1551 infection is efficiently controlled, leading to latent TB infection.

To understand the early immunological events following infection with each of these strains, we infected separate groups of rabbit with either Mtb HN878 or CDC1551 using a similar inoculum (~3.5 $\log_{10}$ CFU). After 3 h, lungs were harvested from the infected animals and analysed for changes in histology, bacillary load and gene expression (Subbian *et al*., 2013a). A significant increase in the numbers of leukocytes and PMN were noted in the HN878-infected rabbit lungs, compared to those infected with CDC1551. The early accumulation of PMN was also associated with increased myeloperoxidase activity in the lungs of HN878-infected rabbits. Lung gene expression analysis revealed a higher number of significantly upregulated genes in the HN878 group relative to the CDC1551 group (982 versus 923), when compared to uninfected controls. These transcriptional changes were more striking when analysed in the context of specific networks and pathways; most of the genes in the host inflammatory response network were induced in the HN878- infected rabbit lungs, compared to those infected with CDC1551, as early as 3 h post-infection. In addition, compared to CDC1551 infection, a greater number of genes involved in other cell function networks and pathways associated with inflammation, such as macrophage activation, f-Met-Leu-Phe-stimulation and PMN activation were significantly upregulated and activated by HN878 infection of rabbit lungs. The differential gene expression pattern also suggests that the upregulated networks and pathways are regulated by the transcriptional factor STAT1, which was also significantly induced in HN878-infected rabbit lungs. Taken together, these findings show that differential recruitment and activation of immune cells, particularly PMN, occurs as early as 3 h in rabbit lungs after infection with different Mtb strains, and these processes impact the recruitment and function of adaptive immune cells, ultimately dictating the course of TB infection.

Rabbit lung transcription profiling of active and latent tuberculosis

The gene expression profile of Mtb HN878-infected rabbit lungs (active disease) was consistent with the kinetics of disease progression with time (Subbian *et al*., 2011a). The number of significantly differentially expressed genes (based on a false discovery rate of 5%) gradually increased from 4 (about 5300 genes) to 8 (about 8600 genes) and 12 weeks (about 13,800 genes) post-infection. The majority of these differentially expressed genes were upregulated during the course of infection/disease at 4 (about 58%) and 8 (about 62%) weeks after infection. The top cellular pathways affected by the perturbations in individual gene expression in the rabbit lungs include immune cell growth and proliferation, cell morphology and movement, cell death/apoptosis, immune cell trafficking and hematologic development and function. In addition, expression of several host genes involved in macrophage activation, Th1 and Th2 type immunity, including cytokines/chemokines and their receptors (*IFNG*, *IL10*, *IL13*, *IL6*, *TNFA*, *TNFB*, *CXCR3*, *GMCSF*, *MIF* and *CCL4*) and signalling molecules and their receptors (*NFkB*, *APRIL1*, *PI3K3* and *TLR2*), as well as intracellular trafficking and apoptosis-related genes (*RAB7*, *LAMP2*, *BCLX*, *FAS* and *RAB11*) showed a gradual increase, particularly of macrophage activation and the Th2 immune response genes, from 4 to 8 weeks that remained at similar levels at 12 weeks of infection. Overall, our data reveal a deterioration of host immunity during Mtb HN878 infection of rabbit lungs and predominance of Th2 immunity. Based on these findings, we proposed that suboptimal activation and/or function of innate immune cells in HN878-infected rabbits, in association with an early and rapid recruitment and activation of cells that exacerbate inflammation, lead to a slow and inadequate onset of innate and adaptive immunity, thereby failing to control bacillary growth, and instead promoting a chronic active infection with cavity formation.

Global gene expression analysis of rabbit lungs infected with Mtb CDC1551 revealed a profile that contrasts with the one observed during progressive TB in Mtb HN878-infected rabbits (Subbian *et al*., 2013b). The number of differentially expressed genes in Mtb CDC1551-infected rabbit lungs peaked as early as 2 weeks to ~25,000 genes, gradually decreasing to ~15,700 and ~13,800 genes at 4 and 8 weeks, respectively, post-infection. The differentially expressed genes were involved in host cell growth and proliferation, cellular assembly and movement, cell-to-cell signalling and cell death pathways. Gene expression analysis of natural killer cell activation, dendritic cell maturation, antibacterial activities, activation of macrophages and T cells, as well as autophagy, were significantly activated in the lungs of Mtb CDC1551-infected rabbits. Moreover, real-time measurement of transcripts from a list of genes comprising cytokines/chemokines (*IL2*, *IL18*, *IFNG*, *CXCR3*, *CSF2*, *TNFA*, *IL13*, *IL5*, *IL4*, *CCL11* and *CCR3*), cell surface receptors (*TLR4*, *TLR6*, *CD4*, *CD80*, *CD86*, *CD28*, *IL1RA*, *IL4R* and *PTPRC*) and regulators (*IRF1*, *SOCS5*, *STAT4*, *CEBPB*, *GATA3*, *IRF4*, *JAK1*, *NFATC4* and *STAT6*) involved in the host Th1 and Th2 type immune response showed an early (2 weeks) and robust activation of T cell-based adaptive immunity, particularly a Th1 response in Mtb CDC1551-infected rabbit lungs. Another important aspect emerging from cumulative observations from the bacterial CFU, histopathology and immune cell count, as well as from gene expression analysis, is that many of the cellular activation networks that were activated at 2 and 4 weeks were downregulated with their gene expression insignificant, at 12 weeks, when the bacillary load declined significantly, tending to complete clearance. This was also evidenced by the absence of histopathological features at this time in the CDC1551-infected rabbit lungs.

The Rabbit Model of Tuberculous Meningitis

Tuberculous meningitis (TBM) is one of the most serious manifestations of Mtb infection of the central nervous system (CNS), and is responsible for about a 50% mortality rate among affected children. Moreover, most of the cured patients have permanent neurological

complications associated with substantial disability. We have successfully established the only rabbit model of TBM that clinically, immunologically and pathologically resembles human disease (Tsenova *et al*., 1998, 1999, 2006a, 2007). In our model, rabbits were intracisternally infected with Mbo Ravenel, Mtb H37Rv or HN878. The animals reproducibly developed TBM of different degrees of disease severity, ranging from acute to subacute. Rabbits inoculated with 2×10^7 CFU (high dose) of Mbo Ravenel exhibited acute inflammation marked by elevated PMN numbers in the cerebrospinal fluid (CSF) within 24 h of infection, followed by a shift to mononuclear cells that cumulatively increased the total number of infiltrated leukocytes by more than fivefold 6 days after infection. The breach in the blood–brain barrier, resulting in the accumulation of inflammatory cells in the CSF, was observed as early as 2 h post-infection, as marked by a gradual increase in CSF protein levels through day 8 post-infection. Consistent with elevated protein and leukocyte levels, the bacillary load was also increased as early as day 2, peaking at about 4 $\log_{10}$ CFU, which remained stable at similarly high levels up to day 8. In addition, the level of TNF-α, a pro-inflammatory cytokine, peaked in the CSF after 2 h of infection and was maintained at quantifiable but low levels until 5 days. Just before the rabbits became moribund at day 8 post-infection, there was a second peak in TNF-α level noted in the CSF. In contrast, elevated levels of TNF-α in plasma, comparable to the levels found in the CSF at 2 h, were noted at day 2, with a plateau until 6 days post-infection. The immunopathology correlated with the human clinical course of TBM, including loss of coordination, irritability, lateral recumbency, ophthalmitis and hemiparesis. Histologically, granulomatous lesions, as well as meningitis in the Mbo-infected rabbits, began with leptomeningeal thickening noted as early as the day after infection, and arachnoiditis characterized by macrophage and lymphocyte infiltration. Progressive vasculitis of blood vessels within the meninges, characterized by infiltration of the adventitia, was noted as the disease progressed. At 7 days post-infection, the inflammation extended transmurally with fibrinoid necrosis of the smooth muscle of the vessel wall, with few rabbits showing signs of encephalitis. By the peak of TBM, infection had disseminated to other organs, as bacilli could be cultured from the lungs and spleens of infected rabbits.

Intracisternal infection of rabbits with 5×10^5 CFU (low dose) Mbo Ravenel produces a subacute TBM disease, which is pathologically and immunologically similar to the human disease at identical disease severity. Although the kinetics of disease progression and symptoms were similar between the acute and subacute models of rabbit TBM, the onset of disease pathology, inflammatory response and death of animals were significantly delayed in the latter compared to the former (Tsenova *et al*., 2002).

The rabbit model of TBM established with infection by the human-type tubercle bacilli, H37Rv and HN878, showed similar pathological and immunological responses as observed in rabbits infected with Mbo Ravenel. However, compared to H37Rv, intracisternal inoculation of HN878 resulted in greater than 1.5 $\log_{10}$ CFU increase in the CSF and lungs, despite a similar initial inoculum used for infection. The proportional difference in tissue bacillary growth, a measure of the host response to infection, was also noted in the infected rabbits that were previously vaccinated with BCG. Though HN878 and H37Rv were both controlled in the brain and lungs of BCG-vaccinated rabbits, only HN878 disseminated rapidly to the liver and spleen, while H37Rv was effectively contained from dissemination. However, BCG vaccination did not eliminate the pathological manifestations associated with HN878 infection. The inflammatory response elicited by HN878 correlated with the clinical manifestation of TBM, including loss of coordination, pareses and paralysis of limbs after 3 weeks of infection, whereas none of the eight rabbits infected with H37Rv showed any neurological deficits. In summary, a significant difference in overall disease burden was noted between BCG-vaccinated, H37Rv-infected and HN878-infected rabbits.

Rabbit Models of Non-tuberculous Mycobacterial Diseases

Although it is thought that spontaneous mycobacteriosis is rare in rabbits, recent studies have found evidence for mycobacterial infections occurring naturally in lagomorphs. Disseminated mycobacteriosis, caused by *M. avium*, was associated with weakened cell-mediated immune responses and increased morbidity and mortality in rabbits (Harrenstien *et al*., 2006). Johne's disease or paratuberculosis, a progressive enteric disease of ruminants, is caused by *M. avium* subspecies *paratuberculosis* (MAP) infection. Recently, several investigators have reported that rabbit populations in the wild can be infected by MAP and that the infection can exist as an asymptomatic/sub-clinical reservoir of MAP. As many as 1 million bacilli may be excreted per gram of rabbit faecal pellet, which can infect and cause clinical disease in sympatric farm livestock (Greig *et al*., 1997; Shaughnessy *et al*., 2013). In addition, MAP isolated from naturally infected rabbits was capable of causing active disease in calves (Beard *et al*., 2001). Importantly, the histopathology of granulomatous lesions observed in the lymph nodes and intestines of ruminants with active paratuberculosis were similar to the MAP-infected rabbit tissues (Maio *et al*., 2011). These studies not only highlight the transmission chain of paratuberculosis between wild rabbits and farm animals, but also the similarities in pathological changes between these two animal species, despite manifesting divergent outcomes following MAP infection.

M. genavense, a member of the non-tuberculous mycobacteria (NTM), has been reported to cause disseminated mycobacteriosis in HIV-infected individuals. In these patients, granulomatous lesions and associated inflammation were observed in several organs, including lung, liver, spleen, kidney and lymph nodes (Bottger *et al*., 1992). Recently, *M. genavense* infection has been reported in a rabbit with lung granulomatous lesions, resembling the human counterpart (Ludwig *et al*., 2009). Histologically, both the human and rabbit lung consisted of foamy macrophages, multinucleated giant cells and lymphocytes (Ludwig *et al*., 2009).

Though *M. chelonae* and *M. fortuitum* are considered opportunistic NTM pathogens, they can cause pulmonary disease through bloodstream infections in HIV-positive patients and skin disease in immune-competent individuals (Wolinsky, 1979; Aldabagh and Tomecki, 2009; Weiss and Glassroth, 2012). These strains can also cause mycobacterial keratitis, an infectious eye disease, in humans who are undergoing laser *in situ* keratomileusis surgery to fix refractive errors of the eyes (Turner, 1970; Chandra *et al*., 2001). Due to their similar eye structure, cornea size and the feasibility of slit-lamp examination similar to humans, rabbit models of human mycobacterial keratitis have been developed to study the pathological features as well as to test the efficacy of various antibiotic treatments (Prince, 1964). Importantly, the clinical features of mycobacterial keratitis in rabbit eyes closely resemble the human counterpart. While experimental rabbits infected intrastromally with *M. fortuitum* produced self-limited stromal keratitis, inoculation of *M. chelonae* under the corneal flaps of rabbits produced corneal abscess with progressive opacities of variable size, structure and appearance similar to those observed in human keratitis (Paschal *et al*., 1992; Adan *et al*., 2004). In addition, it has been suggested that rabbits in the wild are exposed to environmental mycobacteria from soil, water and food sources. These ingested mycobacteria can exist as a part of the rabbits' intestinal flora in the cecum and intestine. Exposure to environmental mycobacteria can sensitize the rabbit immune system and impact the vulnerability of these animals during subsequent exposure to virulent, disease-causing strains of mycobacteria (Shield, 1983). This hypothesis is further justified by the observations that the sensitivity of rabbits to tuberculin was at least 100 times and 50% lower compared to that seen in humans and guinea pigs, respectively (Converse *et al*., 1998).

Tuberculosis Vaccine Studies in Rabbits

Considerable efforts have been expended in developing an improved vaccine for TB since the only currently available vaccine, BCG, has

been shown to have variable efficacy against pulmonary TB among ethnically and geographically divergent human adult populations. Currently, the efficacy of TB vaccines in animal models is measured in terms of reduction in the number of lung bacillary load and granulomatous lesions, extended period of survival, improvement in disease pathology and prevention of bacillary dissemination from the lungs to other organs (McMurray, 2001). However, most of the animal models used for vaccine evaluation fail to achieve all these parameters. Therefore, development of appropriate animal models to test new and improved vaccines to protect against tuberculosis is needed urgently. The primary purpose of any TB vaccine is to prevent progression of infection to active disease. In this context, choosing the appropriate animal model to evaluate the efficacy of potential TB vaccines is crucial for their success in human clinical trials. Rabbits, similar to humans, show higher innate and acquired resistance to Mtb infection than do mice and guinea pig, culminating in restriction of bacillary growth and control of early tubercles. In fact, studies have shown that model animals such as some genetically susceptible mouse strains, guinea pigs and Lurie's 'susceptible' rabbits, which are more vulnerable to Mtb infection and active disease, produced only weak protective immunity following vaccination, compared to Mtb-resistant species such as outbred rabbits and NHP (Lurie, 1964; Medina and North, 1999). Thus, outbred rabbits can be a better model than other animals in evaluating the efficacy of potential vaccine candidates. These conclusions are derived from the observations that while mice can produce strong cell-mediated immunity (CMI), they mount a weak delayed-type hypersensitivity response (DTH). Conversely, guinea pigs elicit a strong DTH response, but a poor CMI response. However, rabbits exert strong DTH and CMI responses, resembling their human counterparts upon Mtb infection (Dannenberg, 2010). The combined DTH and CMI responses contribute to the early and effective bacillary control in the lungs of immunized, relative to non-immunized, rabbits (Dannenberg, 2006). In contrast to humans and rabbits, inhalation of relatively few virulent Mtb invariably produces a progressive pulmonary disease in mice and guinea pigs, which ultimately kills these hosts. In contrast, the alveolar macrophages (AM) of outbred rabbits contain the growth of most inhaled bacilli. Similarly, more than 90% of infected humans do not develop active disease. This innate resistance to Mtb infection is a unique advantage of the rabbit model, which can be critical for vaccine efficacy studies. In addition, the number of primary lesions (tubercles) formed in the rabbit lungs following infection is dependent on the nature of the Mtb strain and independent of the inoculum dose used for infection. Thus, to produce one visible lesion in rabbit lungs, an average of 300–3000 bacilli are needed. Therefore, the ability of a vaccine to reduce the number of primary lung tubercles can be used as a measure of its efficacy in preventing progression to primary TB.

The tubercle count method has been used by Lurie and Dannenberg in several rabbit model studies to investigate the pathogenesis of TB (Lurie, 1964). Accordingly, the number and size of pulmonary tubercles in infected rabbit lungs are counted after 5 weeks post-infection as a measure of both the host response (disease pathology) and the virulence of the infecting bacilli. Dannenberg *et al*. (2000) evaluated the protective efficacies of four BCG strains (Danish, Japanese, Tice and Pasteur), as well as six *M. microti* strains (NCO 8712, ATCC 35781, ATCC 35782, OV 254, ATCC 11152 and ATCC 19422) in a rabbit model of pulmonary TB using Mtb H37Rv for infection. In this study, the ability of these vaccines to reduce the number of visible tubercles in the lungs after 5 weeks of infection was compared with the unvaccinated control rabbits. Results from this study showed variable efficacy among members of the same group of vaccines. While rabbits vaccinated with BCG Pasteur, *M. microti* OV254 and ATCC 11152 showed ~75% fewer lung tubercles, those vaccinated with BCG Danish, Japanese and Tice strains, as well as *M. microti* NCO 8712, ATCC 35781 and ATCC 35782, had about 40% fewer tubercles compared to unvaccinated controls. These findings were consistent with the protective efficacy of BCG vaccines observed in human clinical trials. Thus, the tubercle-counting method could serve as an

early, effective and facile technique to assess the protective efficacy of vaccine candidates.

The impact of the nature of infecting bacilli on vaccine efficacy was reported in a rabbit model of TB meningitis (Tsenova *et al*., 2007). In this study, the protective efficacy of BCG Danish 1331 strain was evaluated after the vaccinated rabbits were challenged intrathecally with either H37Rv or a hyper-virulent Mtb HN878 strain, a member of the W-Beijing family, at similar infectious dose. To assess the efficacy of BCG vaccination, the clinical disease pathology, bacillary load and histology of the brain, lung, liver and spleen were compared between the vaccinated and unvaccinated Mtb-infected rabbits. Though BCG vaccination reduced the disease pathology in the Mtb-infected rabbit brain and lungs, compared to unvaccinated controls, the protective efficacy was strikingly different between those challenged with HN878 and H37Rv. A better protective response, marked by significant reduction in the organ bacillary load, disease pathology, inflammation, number and size of granulomatous lesions, was observed in the vaccinated rabbits challenged with H37Rv compared to HN878. In addition, BCG vaccination prevented dissemination of the infecting bacilli to the spleen and liver only in the H37Rv-challenged rabbits. Thus, the extent of protection offered by BCG is highly affected by the nature of the infecting Mtb strain in a reciprocal fashion, with least protective efficacy against infection by more virulent strains.

In addition, a new vaccine candidate for TB was also tested for its protective efficacy in rabbits compared to the standard BCG vaccine. Here, too, vaccine efficacy of Mtb72F, a recombinant polyprotein of Mtb comprising Mtb32 and Mtb39, was compared with BCG for their protective efficacy in a rabbit model of TB meningitis. This was evaluated both as a single vaccine and as a prime-boost vaccine with BCG (Tsenova et al., 2006a). Intrathecal infection of rabbits with H37Rvat 10 weeks post-vaccination with either BCG or Mtb72F significantly reduced the bacillary load in the CSF, diminished the dissemination of bacilli from the brain to the lungs and liver, and prevented body weight loss, compared to the unvaccinated controls. In addition, both vaccines significantly reduced disease pathology characterized by reduced inflammation and smaller granulomatous lesions in the lungs and brain of infected rabbits. A similar level of protective efficacy was conferred by BCG and Mtb27F on these Mtb-infected animals, though rabbits vaccinated with Mtb27F were better protected from weight loss, compared to BCG-vaccinated animals. Moreover, relative to unvaccinated Mtb-infected rabbits, a significant increase in the proliferation of Mtb antigen-specific splenic T cells was noted after vaccination with either BCG or BCG followed by boosting with Mtb27F. However, Mtb27F used as a booster vaccine with BCG did not confer additional protection to the Mtb-infected rabbits than that offered by vaccination with either of the individual vaccines.

Anti-tuberculosis Drug Testing Studies in Rabbits

The currently available standard multidrug (Isoniazid, INH; Rifampicin, RIF; Pyrazinamide, PZA; and Ethambutol, EMB) regimen for human TB, while efficacious in preventing relapse if taken properly, is cumbersome and requires a minimum of 6 months. This prolonged therapy has been attributed to the presence of non-replicating 'persister bacilli', which are relatively tolerant to the activity of the standard anti-TB drugs, as well as to the reduced ability of antibiotics to penetrate and accumulate within the necrotic cores of granulomas in which these persister bacilli reside (Vandiviere *et al*., 1956). The heterogeneity of lesion size, maturation state (ranging from necrotic to caseating, liquefying and cavitating lesions), cellular composition and distribution in the lungs and other organs of Mtb-infected rabbits closely resembles the spectrum of human TB histopathology. The intra-lesion variation, combined with the number of thriving bacilli in these lesions within the same animal, makes the rabbit model of pulmonary TB a vital tool for drug distribution, and pharmacokinetic (PK) and pharmacodynamic (PD) evaluation of

well-established, as well as for novel anti-TB drugs, such as fluoroquinolones (Kjellsson *et al*., 2012; Padilha *et al*., 2012). In a rabbit model of pulmonary TB, Kjellsson *et al*. (2012) reported the PK of INH, RIF, PZA and moxifloxacin (MXF) in the plasma and within lung granulomas using liquid chromatography–mass spectrometry (LC/MS) techniques. Results from this study showed that INH, RIF, PZA and MXF have variable penetration into rabbit lung granulomas. While MXF had good distribution and penetration into rabbit lung parenchyma and TB granulomas, the concentrations of INH, RIF and PZA were significantly lower in the granulomas compared to their levels in the plasma. These observations were further validated in a more advanced high-sensitivity matrix-assisted laser desorption/ionization combined with mass spectrometry imaging study of rabbit lung granulomas treated with MXF, which showed a lower accumulation of MXF in the caseum at the centre of the granulomas, compared to highly cellular, peripheral areas of the lesions (Prideaux *et al*., 2011).

The rabbit model has also been used for TB drug toxicity studies. Hepatotoxicity, associated with INH and its metabolites, is the most common side effect of anti-TB drugs in humans. Similar INH-mediated liver toxicity and necrosis were observed in rabbits; this pathological feature was not found in identical studies performed using other model animals, including rats (Sarich *et al*., 1995). In addition, the synergistic hepatotoxic effect observed in humans co-treated with INH and RIF were reproduced only in a rabbit model; no such hepatotoxicity was noted in other animal models (Sarich *et al*., 1995; Shen *et al*., 2008). Recently, Padilha *et al*. (2012) have shown that co-treatment with ciprofloxacin (CIP), a fluoroquinolone used as a second-line anti-TB drug, and INH/RIF did not increase the risk of hepatotoxicity in rabbits, compared to INH/RIF alone. However, addition of CIP to INH and RIF can increase the efficiency and/or reduce the duration of treatment. Based on the results of these studies, rabbits (with or without Mtb infection) can serve as a useful model to study the PK/PD and drug safety of standard, as well as novel, anti-TB drugs.

The Role of Rabbits in Novel Anti-TB Drug Discovery

One of the long-term complications in some pulmonary TB and/or TBM patients is that, even after successful completion of treatment, severe organ dysfunction persists, including structural lung damage in pulmonary TB patients, and irreversible neurological deficits in TBM. These sequelae are in part due to the exacerbated inflammatory response by the host, driven mainly by pro-inflammatory cytokines, including TNF-α, to Mtb infection before and during anti-TB drug treatment. Treatment of TB patients with immune modulatory drugs including corticosteroids, in combination with standard antibiotics, dampens the production of pro-inflammatory molecules and alleviates associated inflammatory responses, especially in the case of TBM (Alzeer and FitzGerald, 1993; Cisneros and Murray, 1996; Thwaites *et al*., 2004).

Rabbit models of pulmonary TB and TBM have been used as a platform to test novel immune modulator compounds that can improve the clinical outcome and potentially reduce the duration of current treatment (Tsenova *et al*., 1998, 2002; Subbian *et al*., 2011a,b). In a rabbit model of acute TBM, which resembles human disease at the immunological, clinical and pathological levels, Tsenova *et al*. (1998) showed that despite a significant reduction in bacillary load, Mbo-infected rabbits treated with standard anti-TB drugs alone had persisting lesions and associated inflammation of the brain that also resulted in 50% mortality rate of infected and treated rabbits. In contrast, infected rabbits treated with a combination of standard anti-TB drugs plus thalidomide, an immune modulatory compound that inhibits TNF-α production, showed significant reduction in brain pathology and all the animals were protected from TBM-associated death. Similarly, in a subacute model of rabbit TBM, in which the establishment of disease pathology is delayed relative to the rapid disease progression observed in an acute TBM model, treatment with an adjunct immunomodulatory drug 3 (IMiD3), an analogue of thalidomide that is under development for potential use in the treatment of inflammatory conditions in humans, in

combination with standard anti-TB drugs, significantly reduced disease manifestations and improved the survival of infected rabbits, compared to animals treated with standard antibiotics alone (Corral and Kaplan, 1999; Tsenova *et al*., 2002).

Recently, the effect of a phosphodiesterase-4 (PDE4) inhibitor combined with INH on improving the treatment outcome was evaluated in our rabbit model of HN878-induced active, pulmonary TB (Subbian *et al*., 2011a,b). PDE4 inhibitors (PDE4i) belong to a class of immune modulators which, in contrast to immune suppressors, inhibits mononuclear cell-derived TNF-α by blocking PDE4, an enzyme involved in cyclic AMP homeostasis, ultimately dampening Mtb-induced macrophage activation (Muller *et al*., 1996). In the rabbits with chronic pulmonary TB established by Mtb HN878 infection, 8 weeks of treatment with a PDE4i plus INH, starting from 4 weeks post-infection, enhanced the response to INH treatment as demonstrated by significantly reduced lung bacillary load and the number of granulomatous lesions, compared to animals treated only with INH. Importantly, treatment of infected rabbits with the PDE4 inhibitor alone did not increase the lung bacillary load or the extent of lung involvement by granulomas significantly, and the compound was not bactericidal *in vitro*. In addition, histological analysis of Mtb-infected rabbit lungs, treated with INH plus PDE4i, showed a striking improvement in the resorption of the lung granulomas with less fibrosis and inflammation. Normal lung function was restored in these rabbits, relative to animals treated with INH alone, which had residual lung damage despite bacterial clearance. Consistent with these findings, the transcript levels of several host matrix metalloproteases (*MMP1*, *3*, *12* and *MMP14*) and pro-inflammatory molecules (*TNFα*, *CRP*, *SPP1*, *ARG1*, *IL4* and *IL8*) involved in fibrosis and tissue remodelling, and inflammatory response, respectively, were significantly reduced in the infected rabbit lungs treated with INH plus PDE4i, compared to INH alone (Subbian *et al*., 2011b). In addition, the expression pattern of several of the Mtb genes in the infected and co-treated rabbit lungs resembles that observed during bacillary survival within resting macrophages (Homolka *et al*., 2010). Thus, using a rabbit model of active pulmonary TB, we have shown that altering the physiology of the infecting bacilli by modulating the host immune response can significantly affect their vulnerability to antibiotic therapy within granulomatous lesions.

Conclusions

The rabbit has been used to model various clinically relevant mycobacterial diseases, including TB. The kinetics of Mtb infection followed by progression to disease or containment are similar to the situation observed in infected humans. The nature of various granulomatous lesions, their cellular composition and their maturation processes are similar between humans and rabbits. However, more extensive work in the rabbit model of mycobacterial diseases has been impeded by the lack of molecular tools for genetic manipulation and immunological exploration that allow us to better understand the host determinants involved in infection and/or the disease processes. Release of the complete and curated rabbit genome sequence, in addition to the development of species-specific antibodies and markers for cell surface, cytoplasmic and nuclear proteins should accelerate the usage of this model animal in the future, facilitating our gaining more insight into the host–pathogen interactions underlying mycobacterial diseases. Such vital efforts would ultimately benefit the mission of global TB eradication programmes.

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23 Laboratory Models of Tuberculosis: Guinea Pigs

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Introduction

The guinea pig has contributed much to our collective understanding of tuberculosis in the almost two centuries since it was first used as a laboratory animal model. Guinea pigs have been used extensively to gain a better understanding of tuberculosis pathogenesis, factors that influence host susceptibility to *Mycobacterium tuberculosis* infection as well as for preclinical testing of new tuberculosis prevention and treatment strategies. Guinea pigs continue to be widely used to test experimental vaccines, adjuvants and antimicrobial drugs intended to control the spread of tuberculosis in humans and animals. Recent studies have highlighted the value of the guinea pig model in gaining a better understanding of the alterations in host and pathogen metabolism associated with active tuberculosis and the search for novel pathogen- as well as host-direct tuberculosis therapies. In this chapter, we review the major historical as well as more recent advances attributed to the guinea pig tuberculosis model. Besides reviewing the basic host responses to *M. tuberculosis* infection, we highlight how the guinea pig model continues to contribute significantly to the ongoing search for new strategies to control the global spread of tuberculosis.

Historical Significance

Decades before Robert Koch in 1882 cultured, isolated and identified the tubercle bacillus as the causative agent of human tuberculosis, other investigators recognized that experimental *M. tuberculosis* infection in guinea pigs resulted in a clinical disease that closely mimicked that of humans (Sanderson, 1867; Klein, 1874). They recognized that, besides being highly susceptible to infection, the pathology of guinea pig tuberculosis resembled human tuberculosis, more so than any other laboratory or domestic animal species. Surgical implantation of inflammatory exudate obtained from human patients into guinea pigs, resulted in a chronic granulomatous disease in the lungs and other parts of the body. In particular, the formation of well-organized granulomatous inflammatory lesions (granulomata) with caseous necrosis bore a striking resemblance to that of humans both grossly and microscopically. In addition to this groundbreaking work, Koch also used guinea pigs to develop Koch–Henle Postulates (Koch, 1982) an experimental approach still widely used today to establish the direct relationship between a microbial pathogen and the expression of an infectious disease. Guinea pigs were

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also used by Bangs in the early development of tuberculin and are still used as the most reliable determinate of purified protein derivative (PPD) potency (Rangel-Frausto *et al*., 2001). By the end of the 19th century, even though the use of laboratory animals including guinea pigs continued, basic research interests shifted away from the study of pathology to the rapidly expanding fields of microbiology, immunology and then molecular biology of tuberculosis. However, in recent years there has been renewed interest in tuberculosis pathology and basic pathogenesis of tuberculosis, in particular how human granuloma formation influences the efficacy of antimicrobial drugs and how lesion morphology contributes to the alterations in host and pathogen metabolism. The need for a better understanding of the *in vivo* host–pathogen interaction necessitates the use of animal models such as the guinea pig, which more closely resembles the naturally occurring disease, to aid in the search for novel tuberculosis prevention, and diagnostic and therapeutic strategies.

Pathology

Historically, outbred strains of guinea pigs have been used to model human and animal tuberculosis; however, inbred strains are also available and are of particular value in vaccine trials or studies of fundamental immune responses to *M. tuberculosis* infection. It was the formation of well-structured granulomata with caseous necrosis in outbred guinea pigs that led early investigators to conclude that the response to experimental *M. tuberculosis* infection was similar to that of humans. Aerosol exposure of guinea pigs to laboratory and clinical *M. tuberculosis* strains results in the development of a characteristic inflammatory lesion referred to as a granuloma, which most often forms at the site of the primary infection in the lung. The early lesions consist of a mixture of mononuclear cells, primarily lymphocytes and macrophages and fewer multinucleated giant cells granulocytes and plasma cells (Ulrichs and Kaufmann, 2006). The distinct spatial organization of these different cell types, combined with fibrous encapsulation and central caseous necrosis, are the most relevant features shared by both humans and guinea pigs infected with *M. tuberculosis* (Turner *et al*., 2003). Smith *et al*. (1970) were the first to explain how the immune status of guinea pigs impacted the morphologic features and rate of tuberculosis granuloma progression in guinea pigs. Aerosol exposure of immunologically naïve guinea pigs to *M. tuberculosis* results in the formation classical granulomata with central caseous necrosis as well as post-primary lesions consisting of ill-defined, coalescing foci of mononuclear cell inflammation (tuberculosis pneumonia), which resulted from haematogenous reinfection of the lung following extra-pulmonary dissemination of bacilli from the primary site of infection. However, in guinea pigs that were rendered partially immune through vaccination with *M. bovis* BCG (BCG) prior to *M. tuberculosis* challenge, primary granuloma formation was not only delayed but also had different morphological features distinct from that of naïve animals. Primary lesions in BCG-vaccinated guinea pigs failed to develop necrosis and closely resembled post-primary lesions in immunologically naïve animals. The differences in the cellular make-up of post-primary tuberculosis in guinea pigs has been interpreted to be due to the combination of haematogenous reinfection of the lung which also coincides with the development of adaptive cellular immunity (McMurray, 2001, 2003a). The distinct morphological differences in granuloma morphology in guinea pigs, which is dependent on the immune status or the stage of infection, has helped improve our understanding of basic tuberculosis pathogenesis, the morphological changes associated with immune protection and the dynamic changes in immune cell phenotypes, cytokine and chemokine expression during experimental tuberculosis.

The pathogenesis of primary granuloma necrosis is poorly understood but as mentioned above is indicative of an unfavourable immune response to *M. tuberculosis* infection. Necrosis may be in the form of a single, expanding focus within the centre of primary granulomata or cavitation of lesions and surrounding lung parenchyma, which is the most severe manifestation of active tuberculosis

disease. Granuloma necrosis accounts for the establishment of a low-oxygen microenvironment in guinea pigs and other established tuberculosis animal models (Lenaerts *et al*., 2007; Via *et al*., 2008). The *in vivo* and *in vitro* expression of hypoxia has been linked to the expression of phenotypic drug tolerance of *M. tuberculosis* and is currently a topic of active investigation (Ahmad *et al*., 2009; Ryan *et al*., 2010). Cavitary lesions in the apical lung lobes are a frequent manifestation of post-primary tuberculosis in humans resulting from re-exposure to infectious bacilli or reactivation of latent tuberculosis (Balasubramanian *et al*., 1994b). Cavity formation is associated with a poor clinical outcome and contributes significantly to the spread of tuberculosis when they communicate with conducting airways. The extensive and often irreversible lung damage is associated with the proliferation of large numbers of drug-tolerant bacilli, which respond poorly to combination antimicrobial drug therapy. Cavitary disease in guinea pigs is rare but, in a model of natural transmission of *M. tuberculosis* from humans to guinea pigs, a proportion of animals that developed active tuberculosis disease also developed cavitary lesions (Dharmadhikari *et al*., 2011). Perhaps one of the most intriguing findings from the human to guinea pig natural transmission model was the low rate of active infection in guinea pigs and the reversion from a positive to a negative tuberculin skin test in a significant proportion of animals. These findings suggest that under certain experimental conditions guinea pigs are able to clear bacilli effectively with greater frequency than previously thought possible (Balasubramanian *et al*., 1994a). Moreover, the failure of this highly susceptible host species to develop active disease uniformly under these experimental conditions suggests that conventional guinea pig challenge models may benefit from further refinement to better mimic the naturally occurring disease in humans (Escombe *et al*., 2007; Garton *et al*., 2008). This natural exposure model not only offers an opportunity to gain a better understanding of the basic pathogenesis of *M. tuberculosis* transmission but also an improved strategy to test new prevention and treatment strategies. The guinea pig natural transmission model has already proved valuable in evaluating strategies to reduce transmission of the bacilli to health care workers in the clinical setting (Dharmadhikari *et al*., 2012).

Cavitary lung lesions in guinea pigs, as in humans, involved extensive regions of lung parenchyma and contained large numbers of extracellular bacilli that were suspended in a matrix derived in part from necrotic cellular debris and degenerate granulocytes. Our poor understanding of the pathogenesis of cavity formation is largely due to our inability to consistently reproduce these lesions in laboratory animals. To date, sensitized rabbits infected with highly virulent *M. tuberculosis* develop pulmonary abscesses that often communicate with conducting airways to form cavities (Converse *et al*., 1996). Evidence from guinea pig and other animal studies implicate an unregulated pro-inflammatory response to *M. tuberculosis* in the pathogenesis of both central granuloma necrosis and cavity formation. The infiltration of granulocytes prior to the development of granuloma necrosis in guinea pigs, combined with the predominance of neutrophils within cavitary lesions, supports their role in the pathogenesis of tuberculosis lesion necrosis (Eum *et al*., 2010). Moreover, BCG vaccination of guinea pigs prior to virulent *M. tuberculosis* challenge, a treatment that prevents lesion necrosis in guinea pigs, is associated with significantly fewer circulating and tissue-infiltrating granulocytes measured by flow cytometry in guinea pigs (Ordway *et al*., 2007). The association of granulocytes with the *in vivo* persistence of extracellular bacilli in *M. tuberculosis*-infected guinea pigs recently inspired the development of a novel *in vitro* assay to screen antimicrobial drugs specifically targeting drug-tolerant bacilli (Ackart *et al*., 2014a).

The rate of bacilli dissemination and the severity of post-primary tuberculosis disease severity in guinea pigs have been shown to correlate with *in vivo* virulence of different *M. tuberculosis* challenge strains (Balasubramanian *et al*., 1992a,b). More recent studies have shown that besides the rate of bacilli dissemination and severity of post-primary disease, the severity of granuloma necrosis in experimentally infected guinea pigs correlated with *in vivo* virulence and in some cases antimicrobial

drug susceptibility and human-to-human transmission (Palanisamy *et al*., 2008, 2009). The relationship between challenge strain and the rate of disease and lesion progression in guinea pigs is unknown but may be directly linked to the ability of bacilli to better evade innate immune defences through the expression of unique cell wall lipids (Tsenova *et al*., 2005). The ability of specific *M. tuberculosis* cell wall lipids to induce inflammation also supports the direct link between bacterial products and *in vivo* virulence and granuloma formation. The mycolic acid trehelose dimycolate (TDM), in particular, has been show to induce a potent pro-inflammatory response in a variety of animal models including guinea pigs (Hunter *et al*., 2006a,b; Sugawara *et al*., 2007).

Another unique pathologic feature of *M. tuberculosis* infection that was first described in guinea pigs is the early involvement of pulmonary lymphatics by granulomatous inflammation. This early microscopic feature was first described by Klein in 1865 (Klein, 1874) and was more recently confirmed in guinea pigs infected by low-dose aerosol exposure (Basaraba *et al*., 2006). The early involvement of pulmonary lymphatics and lymph nodes has recently suggested that tuberculosis is primary a lymphoid tissue disease, and that spread to other parts of the body is in large part mediated by lymphatic spread (Behr and Waters, 2014). Recent unpublished data studies suggest that the delay in the development of adaptive immunity in diabetic guinea pigs infected with *M. tuberculosis* may be related to a delayed delivery of specific antigen from the primary site of infection in the lung to the draining regional lymph nodes (Podell, unpublished). The migration of antigen presenting cells may be impaired in part by mechanical or functional obstruction of pulmonary lymphatic vasculature by granulomatous inflammation (Basaraba *et al*., 2006).The guinea pig model of tuberculosis has contributed much to our understanding of granuloma formation and the host and pathogen factors that contribute to *in vivo* progression of active tuberculosis disease. Many questions about granuloma and granuloma necrosis pathogenesis remain unanswered, in particular the host and pathogen factors that contribute to tuberculosis cavity formation. Even though cavitary disease cannot be consistently reproduced in the guinea pig, exposure of guinea pigs to particular strains of *M. tuberculosis* through natural aerosol transmission from humans results in cavitary disease (Dharmadhikari *et al*., 2011); therefore, it is feasible to manipulate the guinea pig model to study this unique and clinically important manifestation of human tuberculosis.

Metabolism

Progressive *M. tuberculosis* infection is associated with poorly understood alterations in host systemic metabolism. Like humans (Oluboyo and Erasmus, 1990) guinea pigs infected with *M. tuberculosis* develop non-diabetic hyperglycaemia, impaired glucose tolerance and dyslipidaemia (Podell *et al*., 2012). Besides the alterations in systemic metabolism, metabolomics studies using guinea pigs demonstrate profound alterations in cellular metabolism at the site of primary infection in the lung. Using a nuclear magnetic resonance-based metabolomics analysis of lung tissue and serum obtained from *M. tuberculosis*-infected guinea pigs, Somashekar *et al*. (2011, 2012) characterized the dynamic changes in the host metabolic profile, which was consistent with a metabolic shift to glycolysis and glutaminolysis associated with activated host immune cells within an increasingly hypoxic microenvironment as lung lesions progressed. Moreover, the increased expression of antioxidant tripeptide glutathione within lung lesions in the early stages of infection was consistent with the generation of reactive oxygen and nitrogen species as a consequence of progressive disease (Venketaraman *et al*., 2008; Guerra *et al*., 2011; Palanisamy *et al*., 2011).

Lipid and cholesterol metabolism in guinea pigs is similar to that of humans and is characterized by a higher ratio of the pro-atherogenic, low-density lipoproteins to high-density lipoproteins in blood (Fernandez and McNamar, 1989; Fernandez and Volek, 2006). Guinea pigs also express high systemic lipoprotein lipase and cholesterol transport activity, therefore are able to mobilize cholesterol rich lipid particles between blood and tissues like humans

in response changes in dietary fat content (Fernandez *et al*., 1992; Fernandez and Volek, 2006). The use of guinea pigs to study altered host metabolism during *M. tuberculosis* infection is relevant given the increasing interest in gaining a better understanding of how bacilli acquire and utilize host metabolic intermediates as a source of energy (Lee *et al*., 2013). Recent evidence suggests that intracellular and extracellular bacilli form intracellular lipid bodies derived from host lipids, which are an important morphological feature of persistent, non-replicating bacilli (Garton *et al*., 2008; Russell *et al*., 2010; Griffin *et al*., 2012; Lee *et al*., 2013). The significance of these data illustrates the important physiological similarities guinea pigs share with humans, and which are absent in other rodent species.

The alterations in systemic and cellular metabolism in guinea pigs in response to *M. tuberculosis* infection make this species valuable for evaluating novel therapeutic strategies aimed at restoring host metabolic homeostasis during active tuberculosis or directly targeting bacilli metabolic pathways therapeutically (Sharma *et al*., 2000), which can be used alone or in combination with conventional antimicrobial drug therapy.

Tuberculosis Risk Factors

Besides differences in *M. tuberculosis* strain virulence, there are a number of infectious and non-infectious diseases in humans that significantly increase the risk of contracting tuberculosis. The most common are HIV co-infection, smoking, air pollution, chronic lung and kidney disease, malnutrition and diabetes (Lopez *et al*., 2006). Among the established tuberculosis risk factors, the profound immunosuppression associated with HIV infection is the best known and most extensively studied. To date, the only animal model that resembles tuberculosis in HIV-positive individuals is infection of non-human primates with simian immunodeficiency virus (SIV) (Diedrich *et al*., 2010; Mehra *et al*., 2011). Much less is known about how other risk factors increase the susceptibility to *M. tuberculosis* and few have been modelled in animals. Guinea pigs have been used to study the effects of protein malnutrition on immunity conferred by BCG vaccination prior to virulent challenge (McMurray and Bartow, 1992; McMurray *et al*., 1999; Sugawara *et al*., 2007). More recently, Podell *et al*. (2014) developed a novel model *M. tuberculosis* infection in guinea pigs with type 2 diabetes to mimic the emerging epidemic of diabetes tuberculosis co-morbidity in humans. This model system represents the first model of type 2 diabetes in guinea pigs, as well as the first model of tuberculosis in an animal model with a clinical syndrome that closely resembles type 2 diabetes, the most common form in humans. In common with humans, diabetic guinea pigs developed a higher bacterial burden, more severe pulmonary and extra- pulmonary pathology, a rapidly progressive pro-inflammatory response in response to *M. tuberculosis* infection and poor response to antimicrobial drug therapy (Restrepo *et al*., 2008, 2014; Jeon *et al*., 2010; Baker *et al*., 2011, 2012; Ferrara *et al*., 2012). These early studies of *M. tuberculosis* infection in diabetic guinea pigs suggest that a delay in adaptive immunity combined with rapid bacterial growth contribute to more severe and rapidly progressive infection compared to non-diabetic guinea pigs. This model not only supports the previous observations that glucose and lipid metabolism in guinea pigs closely resemble that of humans but that diabetes is a significant tuberculosis risk factor. It should prove valuable not only in gaining a better understanding of basic pathogenesis of tuberculosis in diabetics, but also in the testing of therapeutic strategies to better control diabetes and to treat tuberculosis when the two diseases occur together.

Immunology

The pathological and clinical manifestations of tuberculosis in humans and animals are the consequence of the initial innate and subsequent and persistent adaptive cellular immune response to *M. tuberculosis* infection. The persistence of viable bacilli and/or mycobacterial antigens drives the progressive immunopathology that results in the manifestations

of active tuberculosis disease. As in humans, the expression of the adaptive cellular immune response to *M. tuberculosis* infection in guinea pigs is reflected by a strong delayed-type hypersensitivity response to an intradermal injection of crude preparations of PPD. The tuberculin skin test is used extensively in humans as an immunologic indicator of recent exposure as well as latent or active *M. tuberculosis* infection. Recent studies using BCG-vaccinated and *M. tuberculos*is-infected guinea pigs demonstrated that the immunological response to a defined preparation of three mycobacterial proteins was indistinguishable from that of PPD and was mediated by CD4+ and CD8+ T lymphocytes and pro-inflammatory cytokines at the site of intradermal injection (Yang *et al*., 2011). A breakthrough in the ability to characterize the cellular immune response of guinea pigs infected with *M. tuberculosis* came with the development and validation of antibodies that recognize cell surface markers of guinea pig immune cells and cytokine array (Tan *et al*., 1985; Kraal *et al*., 1986; Takizawa *et al*., 2004, 2006; Tree *et al*., 2006). Subsequently, these reagents were used to characterize the dynamic changes in immune cell phenotypes in response to experimental *M. tuberculosis* infection as well as the responses to BCG vaccination in guinea pigs by flow cytometry and cytokine gene transcript analysis (Ordway *et al*., 2007, 2008; Grover *et al*., 2009; Tree *et al*., 2010).

Preclinical Vaccine and Adjuvant Testing

Guinea pigs have an important role in vaccine research and development. They are a more stringent means of determining the potential of a novel vaccine to prevent the development of severe disease than other small animals such as mice. The similarities to humans described above also make the data on vaccine efficacy more relevant and the guinea pig model has provided essential preclinical efficacy and safety information on many vaccine candidates (Orme *et al*., 2001; McMurray, 2003b; Orme, 2005, 2006; Gupta *et al*., 2007; Williams *et al*., 2009). Demonstration of efficacy in this model is currently the main 'gate-keeper' to progression of a vaccine towards clinical development and this is measured either by a reduction in the bacterial load in lungs and spleens at 4–5 weeks post-infection or by the mean survival time over a prolonged follow-up period. The bacterial load read-out has increased statistical power to discriminate vaccines and it is more rapid and less costly than survival studies but the latter are preferred to test the long-term impact of vaccination to prevent severe disease. Subjective or semi-quantitative assessment of the pathology at the time of necropsy is also made and, in some studies, improved weight gain over the course of the post-challenge period has also been used to define protection (Horwitz *et al*., 2000). Guinea pigs are also used to demonstrate the safety of live attenuated vaccines according to EU regulations and guidelines, and as illustrated in the development of novel live vaccine candidates (Walker *et al*., 2010; Arbues *et al*., 2013; Grode *et al*., 2013).

Lessons learned from the first human efficacy trial of a new vaccine performed in infants, BCG-MVA85A, indicate a strong need for identification of protective correlates in animal models that mirror human data as well as the more careful design of preclinical studies to improve statistical power and a demonstration of efficacy equivalent to that expected from vaccines in human clinical trials (Tameris *et al*., 2013; McShane and Williams, 2014). Despite the results of the phase IIb trial, some protective efficacy from this vaccine was apparent in preclinical models including guinea pigs, where the read-out of efficacy was enhanced survival compared to BCG alone (McMurray, 2003b). It is important to note that the percentage improvement over BCG was relatively small in this and other preclinical studies. Such small improvements are feasible to detect in experimental settings because it is possible to control for variables such as exposure dose and underlying factors, which influence susceptibility to TB disease. In a clinical trial setting, the studies are powered to detect much larger differences, and it may be time to set more stringent criteria for efficacy in the animal models. The guinea pig model offers a number of opportunities for 'raising the bar' in terms of providing evidence of efficacy greater than BCG. As previously stated, the outcome of infection is dependent on the

challenge strain that is used and the use of clinical strains may provide a more stringent basis for evaluation of vaccines and their improvement upon BCG vaccination (McShane and Williams, 2014). Guinea pigs infected with both Beijing and non-Beijing clinical strains develop more rapidly progressive pulmonary and extra-pulmonary disease, with those of higher pathogenicity tending towards greater induction of regulatory T-cell responses and reduced T-cell activation over the disease course (Kato-Maeda *et al*., 2012). The challenge of guinea pigs with a variety of clinical *M. tuberculosis* isolates has raised the potential of strain specificity not only on the basic pathogenesis but also on vaccine efficacy. Most of the studies of vaccine efficacy have been performed using a limited number of laboratory strains of *M. tuberculosis*, similar to preclinical drug testing. The impact that strain differences has on vaccine efficacy in the guinea pig and other models remains an important and understudied area of research (Ordway *et al*., 2011).

Guinea pigs are also an important model in the testing of improved vaccine adjuvants and subunit vaccines. Adjuvant choice and formulation are important considerations in the preclinical testing not only of tuberculosis vaccines but also of improved adjuvants. Recently the incorporation of a host protein, High Mobility Box Group 1 (HMGB1), a member of the alarmin group of immunostimulatory proteins, was tested in mice and subsequently in guinea pigs (A. Grover and A.A. Izzo, unpublished data) as a fusion protein with the immunodominate *M. tuberculosis* protein ESAT-6 (HMGB1-ESAT-6). In the mouse model this novel fusion protein generated a strong cell-mediated immune response, which correlated with significant immune protection (Grover *et al*., 2014). The guinea pig model was also used recently to determine the biological function and potential benefit of a subunit vaccine expressing the *M. tuberculosis* latency associated protein alpha-crystallin (Acr, HspX) in a BCG boost vaccine strategy. The native and two recombinant variants of HspX failed to provide protection when given alone in animals challenged with wildtype *M. tuberculosis* but were protective in an *M. tuberculosis* strain lacking hspX, which was less virulent *in vivo*. The data from these guinea pig studies not only demonstrated the importance of hspX as a virulence gene but suggested immune protection was driven by *M. tuberculosis* antigens co-purifying with HspX. Multiple candidate *M. tuberculosis* genes were identified to have altered expression which may account for the loss of virulence in this subunit vaccine candidate that may prove beneficial as a boost to BCG (Wieczorek *et al*., 2014).

The guinea pig remains an important model in the ongoing search for new tuberculosis vaccines and adjuvants. Continual improvement in the availability of immunological reagents is further contributing to the value of this model in identifying the next generation of tuberculosis diagnostic and prevention strategies.

Preclinical Drug Testing

While the mouse has been used most often in the preclinical evaluation of new antituberculosis drugs, the guinea pig offers distinct advantages in evaluating new therapeutic approaches intended for use in humans. Historical studies have demonstrated the importance of differences in lesion morphology in antimicrobial drug treatment responses in humans and guinea pigs (Barclay *et al*., 1953a, 1953b; Manthei *et al*., 1954). These early studies suggested that not only are guinea pigs an excellent model for preclinical testing of anti-tuberculosis drug therapy, but also that lesion morphology has a significant impact on the antimicrobial penetration and potentially efficacy. More recent studies using advanced imaging techniques have further confirmed these studies in the rabbit model (Prideaux *et al*., 2011; Kjellsson *et al*., 2012; Via *et al*., 2012). The direct relationship between antimicrobial drug efficacy and lesion types was also highlighted using one of the most recently FDA-approved anti-tuberculosis drugs bedaquiline (TMC207), which was shown to highly effective in significantly shortening antimicrobial drug treatment intervals (Lenaerts *et al*., 2007; Shang *et al*., 2011) yet failed to completely eradicate bacilli within granulomatous lesions in guinea pigs (Lenaerts *et al*., 2007; Hoff *et al*., 2011). These studies again highlighted the importance of lesion morphology

and the persistence of extracellular, drug-tolerant bacilli in the *in vivo* expression of phenotypic drug tolerance (Ahmad *et al*., 2009; Shang *et al*., 2012). To test the hypothesis that preventing primary granuloma necrosis would significantly improve antimicrobial drug therapy by eliminating the persistence of extracellular bacilli, Shang *et al*. (2012) investigated vaccination with BCG before challenge with *M. tuberculosis*. BCG vaccination of guinea pigs prior to antimicrobial drug therapy failed to completely prevent the relapse of active disease; however, fewer animals relapsed compared to non-vaccinated guinea pigs (Shang *et al*., 2012). The most significant finding of this study was that drug-treated guinea pigs harboured drug-tolerant bacilli for up to 500 days before there was clinical or pathological evidence of disease relapse.

The persistence of extracellular, drug-tolerant bacilli within residual foci of granuloma necrosis, and calcification in guinea pigs, inspired the development of an *in vitro* model to screen new therapeutic strategies that specifically target drug-tolerant *M. tuberculosis*. The observation that extracellular bacilli survived aggressive combination antimicrobial therapy suggested that besides lesion hypoxia and poor drug penetration, other factors may be in play in the expression of antimicrobial drug tolerance. Ackart *et al*. (2014a) demonstrated that bacilli maintained *in vitro*, attached to an extracellular matrix derived from lysed human leukocytes, expressed phenotypic drug resistance as complex microbial communities when exposed to high doses of first-line antimicrobial drugs alone or in combination. Moreover, these investigators used this assay system to test the ability of anti-biofilm drugs to disperse attached communities to render bacilli again drug susceptible (Ackart *et al*., 2014b). These studies demonstrate not only the potential host contribution to the expression of antimicrobial drug tolerance by *M. tuberculosis*, but also a novel therapeutic strategy in which small anti-biofilm compounds can be used to potentiate the *in vitro* and *in vivo* activity of existing and future anti-tuberculosis drugs.

A recent, important refinement of antimicrobial studies in guinea pigs has been the use of pharmacokinetics to establish human-equivalent doses of anti-tuberculosis drugs to develop appropriate guinea pig doses for isoniazid, rifampin, pyrazinamide, moxifloxacin and streptomycin, as well as other investigational compounds (Ahmad *et al*., 2010a,b; Dutta *et al*., 2013a,b). The increasing emergence of multi-drug resistant strains of *M. tuberculosis* highlights the importance of developing not only new antimicrobial compounds but also new drug combinations and effective drug treatment strategies, while at the same time minimizing the toxic side effects that significantly impact patient compliance. The value of the guinea pig in chemotherapy studies is reflected in the recent comparisons of efficacy between rifampicin and rifapentine. It has been suggested that rifapentine, as a substitute for rifampicin, may eliminate toxicity due to reduced activation of hepatic microsomal metabolic pathways. The lack of therapeutic advantage demonstrated in the guinea pig model is consistent with recent human studies that demonstrated no significant advantage of this drug substitution (Dorman *et al*., 2012). Guinea pigs also demonstrate differences in their response to treatment with pyrazinamide. Although there is synergy with rifampin, as is also the case in humans, when higher PZA doses are administered alone they provide a greater bacillary reduction in guinea pigs compared to mice (Ahmad *et al*., 2011). If this translates to humans, further investigation of higher PZA dosing may be warranted, using the guinea pig model.

The guinea pig tuberculosis model has contributed much to our understanding of the host–pathogen interactions that influence *in vitro* and *in vivo* susceptibility to antimicrobial drug therapy. The importance of lesion type has an important influence on the ability to deliver therapeutic drug concentrations. Moreover, the association of extracellular bacilli with a complex mixture of macromolecules derived from necrotic host cells may also contribute to the shift in bacilli metabolism as a strategy to survive antimicrobial drug therapy. The topic of drug toxicity remains an important, under-investigated area of tuberculosis research. The pathogenesis of antimicrobial drug toxicity is still poorly understood and represents an important area

of research in which animal models, including the guinea pig, will prove beneficial.

Conclusions

The guinea pig model of tuberculosis has contributed much to our understanding of the mechanisms by which *M. tuberculosis* causes disease and has helped to identify therapeutic and prevention strategies for over 200 years. The preclinical testing of new drugs and vaccines intended for use in humans benefit from the use of guinea pigs as a relevant model of naturally occurring tuberculosis in humans. Not only do guinea pigs recapitulate important features of active human tuberculosis but they also develop a host response to experimental infection, vaccination and drug treatment similar to that of human patients. The development of new immunological tools and the use of guinea pigs as recipients of naturally transmitted *M. tuberculosis* are further increasing the relevance of this model in the ongoing search for new tuberculosis prevention, diagnostic and treatment strategies.

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24 Of Mice and Mycobacteria: Lessons from a Manipulatable Model

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Introduction

While the mouse is not recognized as a natural host for *Mycobacterium tuberculosis*, it has become a highly useful tool for investigating the interaction between *M. tuberculosis* and a mature vertebrate immune system. Unlike any other host, the mouse has been sculpted and probed over the decades and has provided extensive and in-depth knowledge of how the mammalian immune response handles this inflammatory yet slow-growing bacterium which is difficult to eradicate. The key features that have prompted the use of the mouse over the years are that it is easy to house humanely, it is relatively economical to use in significant numbers, it is highly tractable and it can be used to generate reproducible and definitive data sets. While there is resistance to the use of the mouse as a tool to select drugs and vaccines for human use, its capacity to define immune pathways and mechanisms is undeniable.

The statement that the mouse is a flawed model of TB is heard often among researchers focused on overcoming the tuberculosis (TB) public health crisis. This statement is debatable but is certainly true if one assumes that one mouse strain (i.e. the commonly used C57BL/6) represents the entirety of the human response to *M. tuberculosis* infection. The power of the mouse model lies not in mimicking all forms of human disease but rather in that it is uniquely malleable and thereby capable of mimicking specifically defined conditions. The variety of responses to mycobacterial infection available within the mouse model means that the choice of the specific mouse model is critical to a successful outcome. It is also critical that interpretation, expectations and extrapolation of data sets from the mouse model are appropriately managed. Specifically, data from one mouse strain do not automatically apply to another mouse strain and certainly data from the mouse model do not automatically apply to humans. In the realm of translation studies, the mouse provides a tool with which to probe basic mechanistic pathways which, once identified, can be investigated in the human using sophisticated experimental medicine.

As is the case for other animals, there is a wide variety of health-associated outcomes following delivery of *M. tuberculosis* to mice. These outcomes depend on route of delivery, dose of delivery, genetic make-up of the mouse and potentially the environment within which the mouse is housed. The route of delivery is an important aspect of any mouse model of

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mycobacterial exposure. While the intravenous route provides for a reproducible, potentially high-dose challenge model with which to probe the extremes of what is required to control mycobacterial infection, it completely omits the role of the lung as a portal of entry. In this regard, the delivery of bacteria directly to the lung can be very useful, but even here the difference in outcomes between intranasal, intratracheal and aerosol delivery can be quite extensive. Dose can substantially impact the outcome of infection regardless of the route of exposure. Indeed, it is clear that while *M. tuberculosis* can induce a strong protective acquired immune response, if the initial dose of bacteria is too high then the mouse will succumb regardless of the level of acquired immunity expressed (Collins, 1982; Cooper, 2009). The lethal dose for the mouse also depends upon its genetic make-up and there is a wide range of resistance to morbidity and mortality within the available mouse strains, even following a low-dose aerosol infection (Medina and North, 1998; Turner *et al*., 2003). Finally, while the importance of the above factors in disease progression is quite well defined, there is renewed interest in factors such as the way we house and maintain the mouse models used so extensively in biomedical research. Specifically, the constituents of the mouse microbiome are now known to influence the immune system (Molloy *et al*., 2012); in addition, the temperature at which we house mice may alter their immune responses (Karp, 2012) and even the sex of the scientist handling the mice may impact the outcome (Sorge *et al*., 2014). Despite this wide variety of factors capable of impacting the outcome of mycobacterial infection in the mouse there is a common thread throughout the mouse studies which indicates that macrophage activation, acquired T-cell immunity and a regulated inflammatory response are critical to survival (North and Jung, 2004; Cooper, 2009).

Origins of the Mouse as a Host for Mycobacteria

Mice have been bred for coat colour and unusual behaviours for several centuries in a manner similar to the domestication of dogs and agricultural species. This activity has led to an unusual genetic make-up for the mice now used in laboratory studies. Rather than being a general representation of *Mus musculus* wild mice from around the world, the current in-bred strains are derived from a small population of 'fancy' mouse strains initially generated in Europe for the pet trade (Yang *et al*., 2011). These 'fancy' mice are largely *M. m. domesticus* with significant contribution from *M. m. musculus* which are likely to be of Asian origin (Nagamine *et al*., 1992). In a recent analysis comparing a wide range of in-bred strains to natural populations of *M. musculus* wild-caught mice from around the world, it appears that laboratory mice have limited haplotype diversity and non-randomly distributed genetic diversity (Yang *et al*., 2011).

The 'fancy' mice that formed the basis of the majority of the in-bred mice infected experimentally with mycobacteria were generated at the beginning of the 20th century and were adopted early on by investigators looking for animals that were easy to handle, small enough to house relatively inexpensively and which would provide some level of reproducibility within control groups. Early breeders for the pet trade soon found large orders coming from scientists and, becoming intrigued, they became partners with the scientists in their investigations into the mechanisms of disease. Miss Abby Lathrop was one such breeder whose initial lines contributed to the C57BL/6 strain which has become the cornerstone of much mycobacterial research. Other lines were developed more specifically for research, and in particular research into infectious disease, including the Swiss mice developed by Dr Clara Lynch who brought the nine progenitors of her strain into the USA from Switzerland in a shoebox. These tales illustrate not only the inventiveness of the original breeders of these lines but also the rather random nature of the origins of these mouse strains.

One aspect of these early breeding events is that they occurred in barns and in direct contact with humans and domestic animals exposed to a fairly high incidence of tuberculosis; there were almost 200 deaths per 100,000 in the USA in 1900 (Gordon, 1953). One wonders whether the combined long association

of the mouse with humans and the intensive exposure of the 'fancy' mice and the early inbred colonies with human populations harbouring high rates of TB may have resulted in accidental selection for TB resistance in the strains. For example, the C57BL/6 strain which derives from the activities of Miss Lathrop in breeding barns in Granby, MA, (Potter and Lieberman, 1967) is a relatively resistant strain (Musa *et al*., 1987; Medina and North, 1998) as were the Swiss mice which travelled via ocean liner with Dr Lynch to the Rockefeller Institute in New York in 1926 (Lynch *et al*., 1965; Potter and Lieberman, 1967).

Once established lines became readily available in the 1930s and 1940s, they were used extensively in studies of cancer and infectious disease. Most of the earliest work on *M. tuberculosis* infection in animal models was performed in rabbits and guinea pigs; however, from the 1940s to the 1960s the mouse came into its own as a reproducible model of mycobacterial infection. A careful paper during this time showed that following inhalation of droplet nuclei containing either *M. bovis* Ravenel or *M. tuberculosis* H37Rv (at quite a high initial dose of about 500/mouse) the bacteria were found in largely isolated alveolar macrophages during the first 6–9 days post-infection (Ratcliffe and Palladino, 1953). The bacteria apparently grew over the following 9–12 days and the alveolar macrophages accumulated together with increasing numbers of blood monocytes and polymorphonuclear leucocytes to occupy 2–3 alveolae while the surrounding alveolar walls remained normal. By 15 days, the bacteria were greatly increased in number (determined by histology) with the alveolar macrophages being compacted at the centre of the lesion and also being outnumbered by influxing mononuclear and polymorphonuclear cells. By days 18–21, the bacteria were observed to be reduced in number and the alveolar macrophages had largely been replaced by monocytes from the bloodstream (Ratcliffe and Palladino, 1953). An intriguing aspect of this work is that the mouse infection described above was at the same time being directly compared to the rat, guinea pig and hamster response to the same two pathogens. The description paraphrased above, which covered the first 21 days post-infection, was considered so similar between all these small animal models that there was no distinction made between the rats, mice, guinea pigs and hamsters. After day 21, however, the models were described independently with the rats, mice and hamsters showing similar development of 'irregularly outlined foci within which the alveolar walls provide a type of skeleton' with 'monocytes and lymphocytes accumulating in the perivascular and intralobular tissues enclosed by the lesions and filling the alveolar spaces', while the guinea pigs developed 'tubercles that were sharply circumscribed with some slight growth of fibrous tissue' (Ratcliffe and Palladino, 1953). The development of the lesions in the lungs of the mouse was followed further and necrotic centres of lesions were found to communicate with the bronchioles between the 5th and 7th weeks; spread of the bacteria via the bronchi was considered to have occurred after week 7. There was a difference between *M. bovis* and *M. tuberculosis* in the mouse model, with the *M. bovis* Ravenel lesions growing more rapidly and causing the death of the animals earlier than the H37Rv (Ratcliffe and Palladino, 1953). While this report predates all of the antibody-based phenotyping of cells and the live imaging that is used to follow the development of inflammatory responses, it provides a fairly clear and concise description of what can occur in the mouse model early following a high-dose aerosol infection. It also serves to show that the early response to mycobacterial infection is similar between small mammals, as also reported recently for rabbits, guinea pigs and mice (Dannenberg and Collins, 2001).

In addition to studying the results of aerosol infection, there were extensive studies on bacterial characterization which involved the mouse model. In 1947 Pierce, Dubos and Middlebrook developed a method to grow mycobacteria from a variety of sources including 'human tuberculous material' in liquid media and to use the axenically grown bacteria to inoculate mice via different routes (Middlebrook *et al*., 1947; Pierce *et al*., 1947). Using this technique they found that the intravenous and intracerebral routes were more reproducible and more lethal than the intraperitoneal route (Pierce *et al*., 1947) and that

virulence within mice was associated with morphological characteristics of the bacteria seen on solid media (Middlebrook *et al*., 1947). The growth of various mycobacterial isolates was also followed in the organs of mice infected via different routes using organ homogenates on bacterial plates (Pierce *et al*., 1953), thus providing for the first time a clear picture of the population dynamics of mycobacterial infection in the mouse. These same growth and enumeration techniques are the basis of those used today (Ordway and Orme, 2011). Using these culture and plating techniques these investigators were also able to determine that the number of bacteria was reduced in the organs of mice that had been vaccinated with live (Dubos *et al*., 1953a) or dead (Dubos *et al*., 1953b) mycobacteria. Another important development which resulted from the ability to culture bacteria from infected mice was the observation that drug-susceptible *M. tuberculosis* H37Rv can be apparently cleared from the host by treatment with isoniazid and pyrazinamide (McCune and Tompsett, 1956; McCune *et al*., 1956). In these studies the effect of treatment was more readily detected as changes in bacterial burden rather than as changes in the histological analysis; however, it became clear that treatment resulted in the inability to detect bacteria by microscopy, culture or sub-inoculation into guinea pigs (McCune and Tompsett, 1956; McCune *et al*., 1956). What was even more exciting about this work was the observation that 3 months after treatment, *M. tuberculosis* would grow back in a subset of mice and that the bacteria which grew out remained susceptible to the drug cocktail used (McCune and Tompsett, 1956; McCune *et al*., 1956). The model was also useful for the study of the interaction between the *M. tuberculosis*, the drugs and the host as, in subsequent studies, this 'Cornell' model (named for the location of the work) was formalized. Using a range of interventions it was determined that recrudescence of disease could be promoted only by the delivery of cortisone beginning at the third month of treatment (McCune *et al*., 1966a); it was also determined that there was a specific need for both isoniazid and pyrazinamide and that they need be delivered in specific sequence (McCune *et al*., 1966b). These studies identified the ability of *M. tuberculosis* to enter a persistent phase in the face of drug-induced stress and demonstrated that these persistent bacteria were almost impossible to detect. These observations have informed the use of multi-drug cocktails and support the prolonged treatment of humans in order to avoid the development of these persistent mycobacteria within the TB patient.

The mouse model developed further in the 1970s to the 1990s when in-bred mouse strains were investigated using the infection and plating techniques developed in earlier decades to begin to identify quantifiable susceptibility and resistance traits. As the capacity to genetically manipulate the mouse and the bacteria has developed over the last two decades the ability to investigate host–pathogen interactions has expanded dramatically; however, the observations made over the first part of the 20th century largely hold true today.

Modern Mouse Models

In much of the work discussed in the previous section the mice are described as being from the Rockefeller Institute (now Rockefeller University) or 'commercial farms' and the strain information is sometimes limited to 'albino' or 'Rockefeller Swiss' or simply a 'variety of strains' and it would be difficult to retroactively assign the specific strain relationships between these mice without close attention to the original laboratory notebooks. Despite the lack of genetic information on these strains of mice, investigators were able to categorize mice for susceptibility and resistance to some degree (Middlebrook *et al*., 1947; Pierce *et al*., 1947; Donovick *et al*., 1949). As molecular tools improved, the ability to identify and develop strains of mice with known and quantifiable resistance and susceptibility traits to mycobacterial infection became possible. Despite the similarities of origin (i.e. being derived from pet strains in Europe which were then expanded and established as in-bred lines in the UK and the USA) the mouse strains that became the stock-in-trade for biomedical science exhibit widely different outcomes to infection with *M. tuberculosis*.

Using an intravenous challenge model it was shown that while CBA, C3H, DBA/2 and 129/SvJ mice succumbed within the first 150 days post-infection, BALB/c and C57BL/6 and their F1 crosses with the susceptible DBA/2 strain survived out to 200–250 days; the F1s of the C57BL/6 and the 129/SvJ and the BALB/c and C57BL/6 showed even greater survival past 300 days (Medina and North, 1998). In this study, the bacteria were fully quantitated prior to inoculation and were probably given at a dose much lower than used in the earlier resistance studies which had described the C57BL/6 mice dying within 20–30 days (Lynch *et al*., 1965). At the turn of the 21st century, the strains that exhibited widely different susceptibility to *M. tuberculosis* infection were used to generate F2 crosses and the phenotype and genotype of these crosses investigated. These breeding studies showed that susceptibility to disease following infection with *M. tuberculosis* is a quantifiable trait and is clearly under multigenic control (Lavebratt *et al*., 1999; Kramnik *et al*., 2000; Mitsos *et al*., 2000). Using this type of genetic analysis, several independent susceptibility loci have been identified including *sst1* which contains the *lpr1* gene (Pan *et al*., 2005); loci on chromosomes 3 and 9 which contribute to the differences between susceptible I/St and resistant A/Sn strains (Sánchez *et al*., 2003); and the *Trl1–4* loci which contribute to the susceptibility of the DBA/2 strain (Marquis *et al*., 2009). The variety of outcomes seen with these studies illustrates the fact that there are multiple immune components required to control *M. tuberculosis* infection. Indeed, the variable outcomes depend upon a variety of immune parameters including innate ability of macrophages to control intracellular bacteria, the kinetics of expression of acquired responses and the ability of the mouse strain to regulate the mononuclear nature of the inflammatory response (Cooper, 2009).

In addition to the currently available inbred strains of mice, which came about somewhat stochastically, and which have limited and non-random genetic diversity (Yang *et al*., 2011) a new mouse resource has been developed over the last 10 years called the Collaborative Cross. This new breeding programme is a collaborative effort designed to support analysis of quantitative trait loci (QTL) (such as contribute to the measurable health outcomes following *M. tuberculosis* infection in mice (Lavebratt *et al*., 1999; Kramnik *et al*., 2000; Mitsos *et al*., 2000)) to a near single gene level (Churchill *et al*., 2004). By community discussion, eight founder strains from existing inbred and wild-derived strains that represent the genetic diversity of the three major *Mus musculus* subspecies were used to initiate the project. Genetic analysis of these strains, which comprise A/J, C57BL/6, 129Sv/ImJ, NOD/LtJ, NZO/H1J, CAST/EiJ, PWK/PhJ and WSB/ EiJ, indicate that over 90% of the genetic diversity of the laboratory strains are represented and that the variation is randomly distributed across the genome (Threadgill and Churchill, 2012). Use of this new resource within the mycobacterial research field is likely to provide a more rapid determination of QTL which contribute to the specific measurable health outcomes following *Mycobacterium tuberculosis* infection in the mouse.

In contrast to the 'forward' genetic approaches discussed above, a more targeted approach has been to use 'reverse' genetics wherein a specific gene is targeted and its function in resistance to *M. tuberculosis* studied. It is in this area of highly mechanistic analysis that the mouse contributes significantly, as the availability and flexibility of the genetic tools in the mouse are currently unparalleled in mammalian models. Early studies in the 1990s used simple gene-deletion studies wherein the susceptibility of mice to infection was compared between gene-deleted mice and their wild-type or heterozygote litter mate controls. Using this tool, the critical role of the cytokines IFNγ, TNF and IL-12 in mediating survival following any type of exposure to *M. tuberculosis* was determined (Cooper *et al*., 1993, 1997, 2002b; Flynn *et al*., 1993, 1995). These tools also allowed for more detailed analysis of the role of route and dose to be investigated such that while susceptibility to *M. tuberculosis* aerosol infection is not dramatically increased by lack of B cells, MHC class I or γδ T cells (D'Souza *et al*., 1997, 2000; Johnson *et al*., 1997; Mogues *et al*., 2001), absence of these molecules results in susceptibility to an intravenous challenge (Flynn *et al*., 1992; Vordermeier *et al*., 1996; D'Souza *et al*., 1997).

Extensive studies using mice lacking specific genes ensued through the 2000s and while early studies showed the gross need for specific molecules and cells, they also highlighted the potential for unforeseen complexity. As an illustration of this, it became apparent early in genetic deletion studies that subunits of the IL-12 family of cytokines were differentially required for the mouse to resist *M. tuberculosis* infection. In particular, the absence of the gene for IL-12p40 subunit (*il12b*) resulted in rapid sensitivity of mice to *M. tuberculosis* while absence of the other subunit of the IL-12p70 cytokine, IL-12p35 (*il12a*), did not result in such acute susceptibility (Cooper *et al*., 2002b). In addition, while the IL-23p19 (*il23a*) subunit (which binds to IL-12p40 to generate the cytokine IL-23) does not confer resistance to a low-dose aerosol infection in otherwise intact mice, it is required for protection in the absence of IL-12p35 (Khader *et al*., 2005). These data illustrate the power of the mouse model in dissecting the hierarchical roles of specific molecules during the complex mammalian response to mycobacterial infection.

More recently it has become possible to remove single genes from specific cell types at defined times throughout infection. The expression of exogenous diphtheria toxin receptor (DTR) can be genetically engineered into mice under the control of cell-specific promoters (Saito *et al*., 2001) and delivery of diphtheria toxin to the mouse can then substantially deplete cells expressing the receptor at specific times. Using this model, it was found that cells positive for the C-C chemokine receptor 2 (CCR2) are required to deliver *M. tuberculosis* bacteria that entered via the lung to the draining lymph node (Samstein *et al*., 2013). However, it is the dendritic cells, not the CCR2-expressing transport cell, that are responsible for driving T-cell activation (Samstein *et al*., 2013). In addition, use of the DTR model on cells that express CD11c (Jung *et al*., 2002) demonstrates that T cells are compromised in their ability to be activated if CD11c-expressing cells lack one of the subunits of the IL-12 receptor, IL-12Rβ1 (*il12rb1*) (Robinson *et al*., 2010). In addition to the DTR system, the cre/loxP system (Kühn and Torres, 2002) provides a very powerful way to induce or reduce expression of single genes within specific cells and at defined time points. One other model which has gained popularity over recent years is the bone marrow chimera model (Shizuru *et al*., 1996). In these chimeras, bone marrow is transferred into an irradiated host and repopulates the radio-sensitive portions of the haematopoietic system (Shizuru *et al*., 1996; Torrado *et al*., 2013). In this way, it is possible to create a mouse wherein just the CD4 T cells lack a specific gene or to compare the response of CD4 T cells expressing different gene sets (i.e. intact or gene-deleted) within the same host (Mayer *et al*., 2008). The ability to compare cells within the same host greatly improves the ability to define cell-specific function to a single gene during *M. tuberculosis* infection. Recent use of this tool allowed for an age-old question to be addressed – the need for direct T cell interaction with macrophages in the control of bacterial growth. Specifically, it was found that in a bone marrow chimera wherein some of the macrophages did not express MHC class II, it was only those macrophages that were MHC class II-positive that were able to reduce bacterial burden (Srivastava and Ernst, 2013). That this MHC class II dependent event was mediated by CD4 T cells was confirmed by the fact that depletion of CD4 T cells ablated the MHC class II dependent bacterial control (Srivastava and Ernst, 2013).

While all of the above tools are powerful, each comes with its own caveats. Specifically, total gene deletion from birth can result in the development of compensatory mechanisms which then hide the impact of gene deletion. The depletion of cells using molecules such as diphtheria toxin depends upon the ability to deliver depleting/stimulating molecules effectively to *M. tuberculosis*-induced granulomata. Similarly, induction of large amounts of cell death by the use of toxins or antibodies can provide signals to the innate response which can impact the progression of inflammation. Further, in the bone marrow chimera models, radiation treatment does not deplete non-dividing cells of the haematopoietic system and so tissue macrophages and memory T cells within chimeras could be derived from the host rather than the donor bone marrow. It is important, therefore,

to be aware of the specific caveats associated with each model and to consider using a variety of methods to confirm the role of specific molecules or pathways within the mouse model.

The T-cell Response of the Mouse Following Aerosol Exposure to *M. tuberculosis*

Despite the variety of outcomes to mycobacterial exposure in various mouse strains, there are canonical features of the immune response to *M. tuberculosis*. These include the fact that *M. tuberculosis* is inflammatory in most organs, that it induces either a mononuclear and/or polymorphonuclear inflammation and that the acquired cellular response is induced and critical to control of bacterial growth (Cooper, 2009). Despite the commonality between mouse strains, the immune response is not universally protective and the consequences for the mouse depend upon the rapidity of the acquired response and the efficacy of the response once it arrives at the site of infection (Cooper *et al*., 2011; Cooper and Torrado, 2012).

The outcome of infection depends upon the interplay between bacterial growth, the development of a mononuclear granuloma and the effective expression of acquired cellular responses. It is therefore critical to define the factors mediating initiation and regulation of these events *in vivo*. One of the useful aspects of the mouse model is that the earliest events following aerosol infection can be followed in the lung and the role of specific cell types and effector molecules in combating this infection addressed. As detailed above, by following the progression of infection in the lung following a high-dose aerosol infection, it was possible to determine that alveolar macrophages are the first target of *M. tuberculosis* infection and that inflammatory cells later dominate the lesion (Ratcliffe and Palladino, 1953). More recent work utilizing antibodies, microscopy and flow cytometry to characterize the cell types involved in the early response to *M. tuberculosis* infection echo this early work (Guilliams *et al*., 2013). Alveolar macrophages, dendritic cells, neutrophils and inflammatory monocytes can all take up *M. tuberculosis* and all can be found within granulomata in the mouse. The importance of each cell type in the control of bacterial growth has not been fully outlined; however, it is likely that while alveolar macrophages initially take up bacteria and may indeed contribute to the success of the infection (Leemans *et al*., 2001), inflammatory monocytes, dendritic cells and neutrophils may also play a role (Gonzalez-Juarrero and Orme, 2001; Wolf *et al*., 2007; Skold and Behar, 2008; Blomgran and Ernst, 2011; Samstein *et al*., 2013).

Perhaps the most significant observation over the past decade in terms of early events following exposure to an aerosol infection is the fact that T-cell activation does not occur until bacteria arrive in the draining lymph node (Chackerian *et al*., 2002; Gallegos *et al*., 2008; Reiley *et al*., 2008; Wolf *et al*., 2008). While the exact mechanisms leading to the delayed migration of bacteria to the draining lymph node are not yet defined, the behaviour of macrophages and neutrophils to initial infection has been implicated in this delayed initiation. Specifically, *M. tuberculosis* preferentially drives necrosis in both neutrophils and macrophages upon infection and if this process is compromised then there is accelerated T-cell activation which is associated with apoptosis of these infected cells (Divangahi *et al*., 2010; Blomgran and Ernst, 2011; Blomgran *et al*., 2012). This delay in initiation of acquired T-cell immunity is important because bacteria do not arrive in the lymph node until 7–9 days post-aerosol infection and this delay results in the primary lesions in the lung being predominantly composed of an innate inflammatory response to bacterial infection rather than a T-cell regulated response (Fig. 24.1). In contrast peripheral organs, which are populated by bacteria disseminating around 9–12 days post-infection, elaborate an inflammatory response to the bacteria which can be regulated by the rapidly arriving acquired immune response (Fig. 24.1). The implications of this slowed induction of T-cell activation for vaccine-induced control are evident in that if circulating memory T cells do not see antigen until 7–9 days post-infection then they are unable to express rapid control of the infection. This delay in recognition of

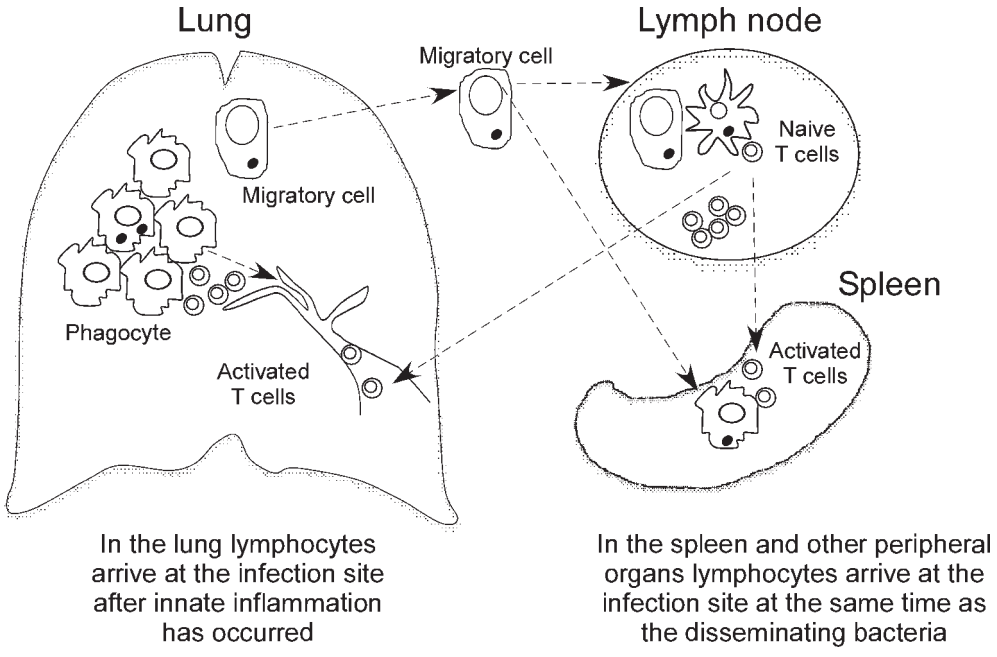

Fig. 24.1. Induction of protection in the mouse lung following aerosol delivery of *Mycobacterium tuberculosis*. *M. tuberculosis* is recognized as a pathogen by alveolar macrophages but persists and grows within these cells. At the same time an inflammatory infiltrate occurs in the lung in response to the invading pathogen; this response precedes development of the acquired T-cell response. Inflammatory monocytes take the invading bacteria to the draining lymph node but not until 7–9 days post-infection. Once in the draining lymph node, bacteria and antigen are taken up by dendritic cells within the node and these cells begin to activate naïve antigen-specific T cells. The activated T cells then divide, differentiate and migrate to inflamed sites throughout the body. Bacteria in the lung proliferate within the innate inflammatory site which has been developing since initial infection and they thus occupy a unique niche relative to bacteria that disseminated to other organs around 9–12 days post-infection. Bacterial dissemination outside the lung coincides closely with the beginning of the acquired T-cell response and therefore the response to bacteria in these organs is rapidly regulated by the antigen-specific T cells both at the level of bacterial growth and in the type of inflammatory response that ensues. The inflammatory lesion in the lung is dominated by activated non-infected macrophages through which antigen-specific T cells must travel to make contact with the *M. tuberculosis*-infected phagocytes.

infection when *M. tuberculosis* enters via the aerosol route is likely to be the reason that most mouse vaccine models result in only 1–1.5 logs of protection in the lung (Cooper, 2009).

Once T cells have been activated, they differentiate and migrate to sites of inflammation. At the site of infection they must function for a prolonged period of time and persist within a fairly toxic environment (Fig. 24.1). Examination of T-cell activation and function within the *M. tuberculosis*-infected mouse has been greatly enhanced by the use of two tools: the generation of mice that carry a single T-cell receptor (TCR) specific for mycobacterial antigens, and multimer stains which bind to CD4 and CD8 T cells that express TCR's specific for mycobacterial antigens. Using these two tools it is possible to determine when and where antigen is available for T-cell activation and also to define the function, location and phenotype of endogenous antigen- specific T cells within the mouse (Kearney *et al.*, 1994; Altman *et al.*, 1996). TCR transgenic CD4 T cells specific for *M. tuberculosis* antigen can be purified from naïve mice and transferred into infected congenically marked mice and the location and phenotypic state can be assessed

by flow cytometry and live imaging (Gallegos *et al*., 2008; Reiley *et al*., 2008; Wolf *et al*., 2008; Egen *et al*., 2011). Using these techniques it appears that there is very little antigen-specific stimulation occurring within mycobacterial lesions. Specifically, there is little migration arrest or cytokine production occurring within the mycobacterial lesion (Egen *et al*., 2008, 2011) and this is associated with reduced antigen availability (Bold *et al*., 2011). While this suggests that the T cells within the lesion are not responding optimally, it is also clear that if antigen-specific CD4 T cells are unable to migrate into the lung lesions then growth control does not occur in the lung (Mogues *et al*., 2001). Indeed, when the expression and distribution of the chemokine CXCL13 is limited within mice then T cells accumulate within the lung parenchyma but do not enter the macrophage-dominated lesion and growth arrest is compromised (Khader *et al*., 2009, 2011). Similarly, when CD4 T cells lack the CXCR5 molecule that mediates responses to the chemokine CXCL13, the migration of these cells into the granuloma is compromised and they also fail to mediate effective growth arrest (Slight *et* al., 2013).

More recently, the importance of different functional sub-groups of antigen-specific CD4 T cells, induced by *M. tuberculosis* and classified using the cell surface markers PD-1 and KLRG1, has been more fully appreciated; these markers have become established as a way to group antigen-specific T cells into functional groups (Kaech and Wherry, 2007). Use of these markers has provided insight into the variety of T cells developing at the *M. tuberculosis*-induced inflammatory site. Specifically, when CD4 T cells from *M. tuberculosis*-infected mice were sorted based on expression of PD-1 and KLRG1 it was found that these markers described functional sub-groups of cells during *M. tuberculosis* infection. Thus, while the PD-1 expressing CD4 T cells can proliferate and persist within an infected mouse, they produce only limited cytokine. In contrast those expressing the KLRG1 marker produce a lot of cytokine but fail to proliferate or persist in the lung (Reiley *et al*., 2010). It is also clear that while recently activated and proliferating CD4 T cells are able to persist within the macrophage-dominated mycobacteria-induced granuloma, highly activated CD4 T cells are eliminated by the toxic products of the activated macrophages (Pearl *et al*., 2012). More recently it has been shown that expression of the chemokine receptor CXCR3 on antigen-specific CD4 T cells is associated with migration of these cells from the vasculature into the lung parenchyma and that a large number of these cells, which do not express CXCR3 but do express the phenotypic marker KLRG1, are located in the blood vasculature (Sakai *et al*., 2014). Importantly, while the cells that are within the blood vasculature are capable of producing high levels of protective cytokine, the CXCR3 expressing antigen-specific CD4 T cells migrate rapidly into the lung and are better at mediating bacterial growth arrest (Sakai *et al*., 2014). Taken together, these data suggest that highly activated effector T cells are unable to accumulate effectively within the infected lung and that a less activated, proliferation-competent antigen-specific T cell, which is able to migrate and persist within the macrophage-dominated lesion, may be a preferable target for vaccine- induced memory T-cell populations.

Pathological Response of the Mouse to *M. tuberculosis* Exposure

It is apparent from the discussion throughout this chapter that describing the response to *M. tuberculosis* in a single strain of mouse and presenting it as representative of all mice would result in a skewed view of the range of pathologic responses that the mouse can express. That being said, most of the studies involving the pathological consequences of specific gene deletion in the *M. tuberculosis* infection of the mouse have been undertaken in the C57BL/6 mouse and so some description of the response of this specific strain to infection is provided here.

Dose and route of infection are important to the pathological consequences seen in the mouse, as is the delivery system even when the lung is used as a portal of entry. Thus, while intravenous injection will result in deposition of *M. tuberculosis* throughout the capillary bed of the lung, it will also deposit bacteria within the spleen and other lymphoid

organs at exactly the same time. In the intravenous model, therefore, initiation of infection and of the acquired cellular response begin almost at the same time and the pathological response is also widespread throughout the lung. In contrast, aerosol delivery to the lung of bacteria, within droplet sizes of 5 μm, results in the deposition of a few bacteria into the alveolar tissue and the initiation of the response is delayed (see Fig. 24.1). Following the aerosol route of infection, the inflammatory site is discrete and the lesions in the lung remain largely circumscribed throughout (Fig. 24.2). Early examination of the lung following low-dose aerosol of a suitably prepared inoculum should reveal very few pathological consequences over the first 20 days aside from a mild interstitial pneumonia (Khader *et al*., 2009). If the particle size is too large or there are too many dead bacteria or bacterial products within the inoculum then there will be diffuse early inflammation within the lung. Over the first 20 days, the bacteria grow in the lung exponentially with little apparent immune-mediated control; however, when antigen-specific T cells accumulate to a sufficient degree then phagocytes in the lung become activated and bacterial growth ceases (Khader *et al*., 2007; Reiley *et al*., 2008).

It is following this early cessation of bacterial growth that the inflammatory response begins to develop in the lung (Rhoades *et al*., 1997; Khader *et al*., 2009). The response develops differently in different animal models (despite similar bacterial growth kinetics) (Dannenberg and Collins, 2001) and in different mouse strains. In the C57BL/6 mice there is a progressive chronic interstitial fibrosing response which remains discretely confined (Fig. 24.2) (Rhoades *et al*., 1997). The cellular constituents of the lesion are largely mononuclear, consisting of phagocytes and lymphocytes (Gonzalez- Juarrero *et al*., 2001), with a variety of cell surface markers indicating the presence of macrophage and dendritic-like cells (Gonzalez- Juarrero and Orme, 2001; Wolf *et al*., 2007; Skold and Behar, 2008). Mononuclear phagocyte accumulation occurs within alveolar spaces and lymphocytes migrate from the blood vessels to accumulate within the phagocyte areas (Fig. 24.3a and c); the amount of inflammation induced in the lung is quite extensive relative to the bacterial burden (Fig. 24.3e). If pathways that are critical for control of inflammation and bacterial control are absent in the C57BL/6 mouse, then the mononuclear response is lost in favour of a florid, predominantly polymorphonuclear accumulation, which can break through to the airways (Fig. 24.3b and d) (Cooper *et al*., 2002a, Desvignes and Ernst, 2009; Nandi and Behar, 2011); this is largely accompanied by bacterial growth (Fig. 24.3f). Eventually, even in the intact C57BL/6 mouse, bacterial regrowth occurs and this is fatal and associated with accumulation of polymorphonuclear cells and loss of lymphocyte follicles (Rhoades *et al*., 1997).

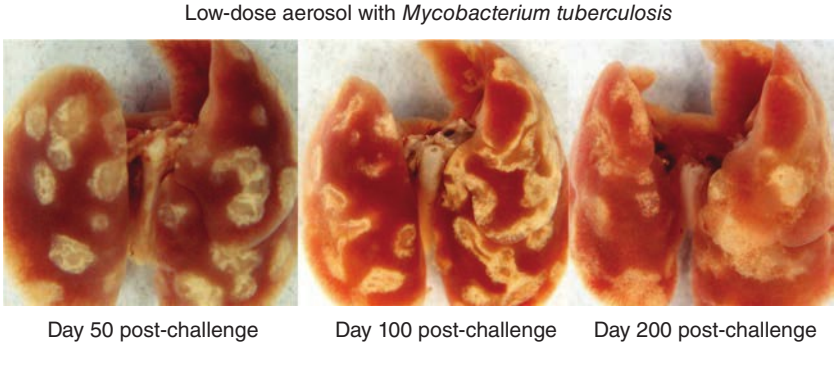

Fig. 24.2. The discrete development of lesions within the mouse lung over time following aerosol infection with *Mycobacterium tuberculosis*. The images show whole lungs taken from mice infected via the aerosol route with approximately 100 colony-forming units of *M. tuberculosis* H37Rv. The lesions are discrete but coalesce over time. Bacterial burden remains constant over the time frame illustrated here. (Photos courtesy of R.J. North and L. Ryan.)

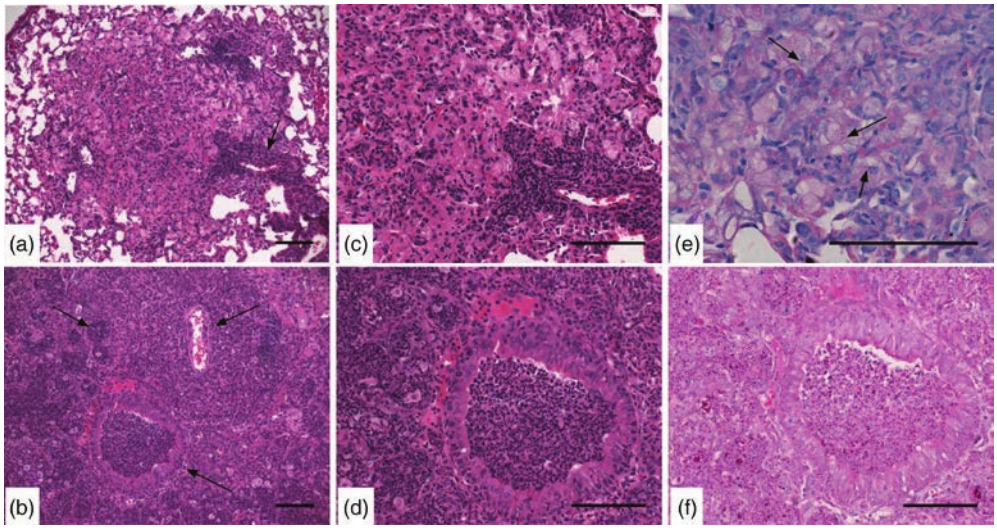

Fig. 24.3. The pathological outcome of infection with *Mycobacterium tuberculosis* differs depending on the mouse. (a) Sixty days following a low-dose aerosol infection of the C57BL/6 mouse mononuclear inflammatory cells accumulate in the alveolar spaces and lymphocytes migrate from the blood vessels (arrow) into the parenchyma in response to the chemokine signals generated by the inflamed infection site. The response is limited and normal lung tissue remains but the lesions do not develop a classical tuberculous, circumscribed phenotype. (b) C57BL/6 mice (lacking the gene for *il12a* which codes for the IL-12p70 subunit protein IL-12p35) exposed to the same aerosol infection develop a neutrophilic response, again filling the alveolar spaces (top left arrow). Inflammatory cells again exit into the lesion from the blood vessels (top right arrow). In further contrast to the intact mouse, neutrophillic exudate can be detected in the airways (bottom right arrow). (c) At higher power the lesion in the C57BL6 can be seen to be largely mononuclear while in (d) it is clear that the cellular infiltrate is polymorphonuclear in the mice lacking *il12a*. (e) Despite the relatively high level of mononuclear accumulation in the C57BL/6 mouse there are very few acid-fast bacteria (arrows) even at high power. (f) In contrast there are high numbers of acid-fast bacteria throughout the lesion and within the airway exudate in the mice lacking *il12a*. In all images the scale bar represents 100 μm. (Photos courtesy of M. Tighe.)

In contrast to the C57BL/6 mice, SWR/J mice exposed to the same aerosol inoculum develop a more rapid inflammatory response and despite generation of cytokine-producing CD4 T cells in the lung they fail to effectively limit bacterial growth between days 20 and 60 post-infection. Bacterial numbers reach a high level in the SWR/J and the mice succumb to this bacterial burden in the context of a florid inflammatory response resulting in necrosis and consolidation of the lung by 100 days of infection (Turner *et al*., 2003). In contrast to both the C57BL/6 and the SWR/J mice, the CBA/J mouse exhibits an intermediate phenotype wherein it expresses an effective acquired cellular response which quite rapidly fails to contain bacterial growth and thereby undergoes early recrudescence of disease (Turner *et al*., 2001). This early recrudescence is associated with high expression of the immunoregulatory cytokine IL-10 in the macrophage population (Turner *et al*., 2002; Beamer *et al*., 2008). Importantly, if IL-10 is absent in the CBA/J mouse then the rather diffuse consolidating pulmonary inflammation seen in this mouse is replaced by organized structures with limited involvement of the surrounding tissue. These organized granulomata have centres containing foamy macrophages surrounded by distinct layers of macrophages further surrounded by lymphocytes and collagen (Cyktor *et al*., 2013). It is interesting in this model that IL-10 needs only be inhibited during the early part of the infection to have profound later pathological consequences (Cyktor *et al*., 2013).

As discussed above, there are several loci associated with susceptibility to *M. tuberculosis* infection, and some of these are associated with immunopathological consequences. The phenotype of the *sst1* locus is expressed at the phagocyte level and the resistance allele promotes apoptotic cell death over necrotic cell death and this is associated with reduced bacterial growth (Pan *et al*., 2005). The resistance allele of *sst1* is also associated with reduced necrosis in the lung regardless of route or dose of infection; however, the final outcome in terms of immunopathological consequences is dependent upon other non-linked mediators of inflammation (Pichugin *et al*., 2009). Mice with the *sst1* susceptibility locus are being used quite extensively in drug testing as a result of the pronounced necrotic activity within the lung.

An important development in our understanding of the mouse response to pulmonary *M. tuberculosis* infection has been the realization that acquired, innate and stromal cells exquisitely regulate each other during the immunopathological response to chronic *M. tuberculosis* infection. In this respect lung epithelial cells have also been shown to produce the chemokine CXCL5 in response to *M. tuberculosis* infection and thereby promote neutrophil influx (Nouailles *et al*., 2014). In addition, it is clear that excessive antigen burden (delivered as repeated BCG delivery to *M. tuberculosis*-infected mice) results in increased neutrophil accumulation within lung lesions (Cruz *et al*., 2010). Further, the role of IFNγ, once considered primarily to act as a macrophage-activating cytokine, is now appreciated to be a key player in maintaining the mononuclear granuloma (Cooper *et al*., 2002a). Specifically, this cytokine acts on stromal cells and T cells to limit IL-17-mediated recruitment and survival of neutrophils (Desvignes and Ernst, 2009; Nandi and Behar, 2011). It also acts in concert with nitric oxide (a product of IFNγ activated phagocytes) to limit the accumulation of activated T cells within mycobacterial granulomata (Gomes *et al*., 1999; Pearl *et al*., 2012) and to limit pathological levels of IL-1 by inhibiting inflammasome activation (Mishra *et al*., 2013).

Current Uses of the Mouse Model

While the main purpose of the mouse model is the delineation of mechanisms whereby a mammalian host responds to a chronically persisting, highly inflammatory bacterial infection, it can also be used as a tool to probe specific questions related to translational studies. There is a great deal of intellectual resistance to the use of the mouse as a tool to screen vaccine and drug candidates, but it still remains a widely used tool in these fields. The critical point about the use of any animal model in both translational and basic studies is that it should provide greater insight into the question being asked than could be achieved from experimental medicine in humans. Simply using the mouse to perform translational studies because it is economical or easier than experimental medicine studies is pointless. What is required for the use of the mouse in translational studies is that it provides the opportunity to yield a defined result which can then be used as a basis for focused experimental medicine studies. It is of course critical that the feedback from the experimental medicine studies informs future translational studies in the mouse and that this process is iterative in nature. Within these confines of utility, the extensive capacity to manipulate the mouse model provides a sizeable resource which should not be ignored simply because 'the mouse is not human'.

One example of effective use of the mouse model lies in making careful selection of the mouse strains and by using the variety of strain-related responses to *M. tuberculosis* infection as a tool to probe the efficacy of specific drugs and or vaccines within different environments. For example, low-dose aerosol infection of the immunocompetent C57BL/6 mouse allows for screening of drugs within macrophage-dominated discrete lung lesions. In contrast, if the focus is the action of drugs within an unregulated environment wherein there is rapid bacterial growth, one could use an immunodeficient version of the C57BL/6, such as one missing the IFNγ gene which is critical for bacterial growth control (Lenaerts *et al*., 2003). If the focus of the work is addressing the function of drugs or vaccines

within an environment dominated by neutrophils rather than macrophages, and which progresses to necrosis, then mice lacking the ability to respond to IFNγ within specific cell populations could be useful (Desvignes and Ernst, 2009; Nandi and Behar, 2011). For a simpler model of neutrophil-dominated lesions and necrosis, strains such as the I/St (Eruslanov *et al*., 2005) or those with the *sst1* susceptibility locus (Pichugin *et al*., 2009) can also be used.

One concern about the mouse is that while many animal models develop hypoxic lesions, the C57BL/6 mouse infected via the aerosol route does not do so (Via *et al*., 2008). However, there are other strains that may develop hypoxia under normal aerosol-delivery conditions, such as the C3HeB/FeJ (Harper *et al*., 2012) and other routes of infection can also induce hypoxic lesions such as a low dose of bacteria via the ear dermis into mice genetically deficient in the ability to produce large amounts of nitric oxide (Reece *et al*., 2010). While some of these models may appear to be highly manipulated, the purpose of a model is not necessarily to mimic nature; rather, it is to define the role of a specific component in the outcome of an experiment and, as stated before, the mouse is exquisitely suited to this purpose.

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25 Non-human Primate Laboratory Models of Tuberculosis

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Introduction

Non-human primates (NHPs) are phylogenetically similar to humans and are the logical animal to model human *Mycobacterium tuberculosis* (Mtb) infection. NHPs can be divided into Old World (e.g. macaques) and New World monkeys (e.g. marmosets) and represent a diverse group of animals that have varying susceptibility to *Mycobacterium* infection. To the best of our knowledge, there is no established literature describing the diagnosis of tuberculosis (TB) in wild, free-ranging animals, leaving it unclear if macaques are truly natural hosts after Mtb infection. Yet, outbreaks of TB in animals kept in captivity is well documented and has been predominantly characterized by infection from *M. tuberculosis*, *M. bovis*, *M. avium* and *M. kansasii* (Benson *et al*., 1955; Kaufmann *et al*., 1975; Sesline *et al*., 1975; McLaughlin *et al*., 1976; Thoen *et al*., 1977; Zumpe *et al*., 1980; Mayhall *et al*., 1981; Holmberg *et al*., 1982; Wilson *et al*., 1984; Goodwin *et al*., 1988; Centers for Disease and Prevention, 1993; Thorel *et al*., 1997; Panarella and Bimes, 2010; Parsons *et al*., 2010; Payne *et al*., 2011). TB in an NHP facility is a substantial concern and, when it is identified, very often more than one animal is found to be affected. Although it cannot be excluded that these cases could have been derived from exposure to a (single) human index case, it seems very likely that transmission between NHPs does occur either by aerosol (as NHPs can cough with experimental infection) or ingestion. When human and NHP populations live in close proximity to each other, the opportunity for anthroponotic transmission increases though this has not been well established (Wilbur *et al*., 2012). While Mtb infection in macaques has been associated with human TB (K. Maetz-Rensing *et al*., personal communication), cases of NHP transmission to humans have not been documented.

Tuberculosis is a serious global health problem as well as a formidable challenge for the TB research and vaccine development community. As discussed below, NHPs provide an important experimental model to address issues in pathogenesis of Mtb infection, protective immunity, vaccine-induced protection, drug treatment and novel approaches to therapy. These models provide important translational information that complements human clinical research efforts in the TB field.

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

Macaque Model

For the purposes of this discussion, macaques discussed in this chapter will refer to either the rhesus (*Macaca mulatta*) or the cynomolgus (*M. fascicularis*) macaque. As in humans, the diagnosis of Mtb infection in macaques can be difficult and with serious consequences if missed. Generally, overt behavioural signs of disease are often instinctively masked by these wild animals and only become apparent when TB disease is advanced. Clinical measures are rarely, if at all, specific unless exploited under controlled experimental conditions (Osburn *et al*., 1977; Verreck *et al*., 2009), and conventional radiographic imaging by chest X-ray by a low intrinsic resolution reveals disease only at more advanced stages (Lewinsohn *et al*., 2006). Macaques with Mtb infection can be asymptomatic (mimicking latent human infection). Diagnosis relies on both detection of immunologic responses against *Mycobacterium* spp. and/or microbiologic detection from biological samples (Lin *et al*., 2008). Because many human immunologic reagents can be used in macaques, reagents are readily available to detect pathogen-specific host immune responses. Probably the most widely applied immune diagnostic is the tuberculin skin test (TST) using Old Mammalian Tuberculin. This test can reveal a typical cellular type IV hypersensitivity response within 72 h after *in vivo* challenge by intradermal antigen injection in the infected primate host, reminiscent of the human skin test diagnostic. For monkey TSTs palpebral injection is chosen mostly for practical reasons of readout and better sensitivity (Barclay *et al*., 1970). However, as in man, this immune diagnostic test has several drawbacks including a questionable sensitivity and a higher frequency of false-negative test results, in particular when disease is severe (Bywater *et al*., 1962). By experimental challenge in (rhesus) macaques it has been demonstrated that the DTH response (like lymphocyte proliferation) is suppressed at higher infection doses (Chaparas *et al*., 1975; Gormus *et al*., 2004). Also in natural outbreaks in (rhesus) macaque populations in captivity, animals with negative TST-DTH tests have been subsequently identified with high(est) disease burden (with speculation that this could have been the index case) (Garcia *et al*., 2004; Vervenne *et al*., 2004). Whole blood or peripheral blood mononuclear assays for mycobacterial specific production of interferon-gamma (IFNγ) release assays (IGRA) similar to those that are being used in clinical TB diagnosis, have been suggested to complement traditional TST TB screening procedures (Garcia *et al*., 2004; Vervenne *et al*., 2004; Bushmitz *et al*., 2009). In the experimental setting, a positive IGRA is generally seen within the first 8 weeks of infection (exposure) but can wane over time (Walsh *et al*., 1996; Capuano *et al*., 2003; Lin *et al*., 2006, 2009). Lymphocyte proliferation assays have also been used as a measure of detecting mycobacterial specific immune (Capuano *et al*., 2003; Lin *et al*., 2006; Lin *et al*., 2008) responses but are more labour intensive than other immunologic assays. Mycobacterial specific immunoglobulins (e.g. in serum or plasma) have also been suggested as an adjunctive diagnostic test and for colony maintenance purposes (Brusasca *et al*., 2003; Lyashchenko *et al*., 2007; Sharpe *et al*., 2010; Khan *et al*., 2011; Min *et al*., 2011; Ravindran *et al*., 2014). Among both cynomolgus and rhesus macaques, clinical symptoms and signs associated with the development of active TB include decreased activity, weight loss, cough, and fever, anaemia and increases in inflammatory markers (erythrocyte sedimentation rate, C-reactive protein) (Capuano *et al*., 2003; Lin *et al*., 2006; Lin *et al*., 2009; Verreck *et al*., 2009; Sharpe *et al*., 2010).

Unlike other animal models of human TB, granulomas seen in the macaque model of Mtb infection are identical to humans both in their histopathological architecture and spectrum of granuloma types, including cavity formation (Canetti, 1955; Basaraba, 2008; Via *et al*., 2008; Flynn and Klein, 2011; Kaushal *et al*., 2012). This is particularly important as the bacterial susceptibility to various drug regimens varies based on the differing microenvironments of each granuloma (Connolly *et al*., 2007; Kjellsson *et al*., 2012). The macaque model is among the few animal models that develop true asymptomatic infection to mimic human latent infection (Capuano *et al*., 2003; Gormus *et al*., 2004). This phenomenon facilitates the ability to understand what factors are

critical in the initial control of Mtb infection and maintaining long-term control to prevent reactivation as it relates to human infection.

Susceptibility to Mtb infection between rhesus versus cynomolgus macaques

Based on natural outbreak records, there is no established literature supporting differential susceptibility to infection with *Mtb* between rhesus and cynomolgus monkeys (Centers for Disease and Prevention, 1993; Garcia *et al*., 2004). Yet experimental infection studies do suggest that rhesus appear more susceptible to disease and develop acute progressive disease more rapidly than cynomolgus macaques. In general, rhesus macaques have higher rates of haematogenous spread, more severe clinical disease, higher frequency of pneumonia, greater numbers of lung lesions and more gross pathology although these findings are less dramatic between cynomolgus and rhesus macaques when higher (i.e. >1000 colony-forming units (CFU)) doses are used (Langermans *et al*., 2001; Motzel *et al*., 2003; Sharpe *et al*., 2009). Despite this generalization, the breeding origin of the animals is an important factor in the relative TB disease susceptibility of the two monkey species. After humans, these macaques are the most globally widespread primates and their geographic barriers are associated with gene linkage disequilibrium between populations (Hernandez *et al*., 2007). These populations can be genetically distinguished by polymorphism and haplotypes in the Major Histocompatibility Complex (MHC), single nucleotide polymorphism (SNP) in the genome and distinctive mitochondrial DNA sequences (Viray *et al*., 2001; Smith and McDonough, 2005; Ferguson *et al*., 2007; Kanthaswamy *et al*., 2009; Doxiadis *et al*., 2013). For rhesus macaques, grossly Indian-, Chinese- and Burmese-type animals (so-called spectrotypes) are distinguished. Cynomolgus macaques can be classified into populations that are grossly associated with their geographic origin – the great Asian islands, the South-east Asian mainland and Mauritius (with the latter evolved from only a small imported founder population). Even within the same species of rhesus or cynomolgus macaques, animals from different spectrotypes or different geographic areas can display differing susceptibility upon controlled experimental infection (personal communication, F. Verreck and S. Sharpe, unpublished). By prospective head-to-head comparison, Indian rhesus macaques were more susceptible to TB disease following *Mtb* strain Erdman infection than Chinese rhesus based on pathology, bacteriology and several clinical measures of disease (F. Verreck, unpublished). Preliminary data suggest that Mauritius cynomolgus macaques are more susceptible to Mtb infection (Erdman strain) than Chinese cynomolgus macaques after aerosol exposure to an estimated dose of 230 CFU (personal communication, S. Sharpe, unpublished). Thus, regarding macaques and their use as preclinical models for TB and therapy evaluation, the breeding origin of the animals must be taken into account for study design/stratification and data interpretation. Apart from the genetic divergence between populations, separate cohorts of either species may also respond differently to mycobacterial infection due to environmental factors between breeding groups and/or research centres. For example, Perry *et al*. (2010) found that cynomolgus macaques with a natural *Helicobacter pylori* infection were less likely to progress to active TB disease upon experimental Mtb infection. The epidemiological spectrum present in human TB is also likely to be reflected in the outbred NHP macaque populations.

Rhesus macaque-specific Mtb infection

As early as the 1950s (Schmidt, 1955, 1956, 1966; Good, 1968) rhesus macaques were infected with Mtb by intra-tracheal inoculation (1500 CFU), developed TB and were subsequently treated with anti-tuberculous drugs. Rhesus macaques develop disease that closely mimics human TB; in particular, that seen in children and immunosuppressed adults (Lewinsohn *et al*., 2006). Following infection, rhesus macaques develop pulmonary disease, common manifestations of which include pulmonary granulomas with calcification, caseation, cavitation and infection of tracheobronchial lymph nodes, followed by haematogenous spread to the liver, spleen and kidney

(Good, 1968; Barclay *et al*., 1970). The severity of the changes in clinical parameters, rate of disease progression, extent and pattern of pulmonary disease and extra-pulmonary spread are all influenced by the route of delivery, the Mtb strain, the infectious dose and the genetic background of the macaque.

Experimental infection of rhesus macaques is typically achieved either through exposure to aerosols of Mtb bacilli (Barclay *et al*., 1970, 1973; Baram *et al*., 1971; Ribi *et al*., 1971; Anacker *et al*., 1972; Janicki *et al*., 1973; Chaparas *et al*., 1975; Shen *et al*., 2002b; Sharpe *et al*., 2009, 2010; Cepeda *et al*., 2013), or through instillation of liquid suspensions of Mtb into the trachea (Langermans *et al*., 2001; Verreck *et al*., 2009) or the bronchi (Schmidt, 1956, 1959, 1966, 1972; Good, 1968; Lewinsohn *et al*., 2006; Cepeda *et al*., 2013). To date, studies suggest that changes in clinical parameters and behaviour caused by infection are consistent and not affected by the route of infection. Similarly, the types of pulmonary lesions that develop following infection do not appear to be influenced by the route of inoculation; however, the pattern of pulmonary disease that develops is altered, with the development of more diffuse lesions following aerosol delivery compared to delivery by the intra-bronchial route (Cepeda *et al*., 2013). This is not unexpected, as aerosol exposure delivers bacilli evenly throughout the lung, while intra-tracheal and intra–bronchial delivery targets the inoculum to specific areas of the lung. Cepeda provided the only report to directly compare aerosol and bronchoscopic delivery; however, the comparison of the outcome is hampered because the doses given by aerosol were higher than those given by bronchoscope (Cepeda *et al*., 2013). What is clear is that the dose of Mtb administered strongly affects the outcome of infection and the higher the dose the greater the pulmonary disease burden that develops and the higher the likelihood that animals will develop progressive disease (Sharpe *et al*., 2009; Zhang *et al*., 2011).

In addition to the amount of Mtb delivered, the strain of Mtb used is a major influence on the outcome of infection. The two most commonly used strains are Erdman and H37Rv, with Erdman considered to be the more virulent of the two (Gormus *et al*., 2004). Since the turn of the 21st century, the Erdman strain has been used in studies in rhesus macaques, all of which have reported that infection leads to the development of active and progressive disease irrespective of the delivery route (Gormus *et al*., 2004; Sharpe *et al*., 2009, 2010; Verreck *et al*., 2009) even when low doses are used. The H37Rv strain has been used extensively since the establishment of the rhesus infection model in the 1970s. When high doses (500–1000 CFU) have been delivered, either by the intra-tracheal (Lewinsohn *et al*., 2006; Sugawara *et al*., 2009), or the aerosol (Shen *et al*., 2002b) routes, active and often rapidly progressive disease has been reported to develop. The outcome of infection following delivery of lower doses is a more mixed picture, studies using low doses (<50 CFU) by aerosol (Barclay *et al*., 1970, 1973; Ribi *et al*., 1971; Janicki *et al*., 1973) or <200 CFU (Gormus *et al*., 2004) showed that animals control the infection for up to 4 months post-inoculation study. However, the development of active disease has been reported following bronchoscopic delivery of doses of 20–100 CFU (Zhang *et al*., 2011). The studies reporting infection with H37Rv were conducted over a 40-year period and in different laboratories across the globe which raises the possibility that the differing levels of virulence seen could be attributed the use of different isolates or preparations. Although there are only limited reports of subclinical infection following infection with H37Rv, the strain CDC1551 has been reported to be less virulent than other strains of Mtb, as aerosol infection with 500 CFU resulted in the development of subclinical infection in 11 of the 12 exposed animals (Mehra *et al*., 2011). To date, the experimental model of human latent infection in rhesus macaques is based on infection with CDC1551 that differs with the latent model in cynomolgus macaques (see below). Both are important contributions to the TB field.

Cynomolgus macaque-specific Mtb infection

Unlike studies in rhesus macaques, far fewer studies investigating the effects of differing

Mtb strains, inoculum dose and infection modalities have been published with cynomolgus macaques. Most experimental studies in cynomolgus macaques have used the Erdman strain of Mtb and no published studies have directly compared the use of different bacterial strains, although attenuated vaccine strains have been used (Sugawara *et al*., 2007; Larsen *et al*., 2009).

Walsh *et al*. (1996) published the first experimental infection of cynomolgus macaques (Erdman strain, intra-tracheal) showing that animals infected with high doses (10^4–10^5) developed severe disease whereas low-dose infection (10^1–10^2) could develop in asymptomatic infection akin to latent infection seen in humans. The development of both active and latent infection outcomes after infection without modification of immunity or Mtb strain provides an opportunity to examine the factors that predict outcome with limited variability. Adult cynomolgus macaques infected with low-dose Mtb (~25 CFU, Erdman strain) via bronchoscopic instillation results in an equal proportion of animals with active TB and latent infection (LTBI) based on clinical, microbiologic and radiographic criteria (Capuano *et al*., 2003; Lin *et al*., 2009) based on human clinical definitions. At 6 months post-infection, animals with LTBI are defined as those without clinical signs or symptoms of active TB, normal X-ray, normal inflammatory markers and the absence of Mtb growth by either broncho-alveolar lavage (BAL) or gastric aspirate (GA) beyond the first 2 months post-infection. An estimated 5% of cynomolgus macaques infected with Mtb will be clinically stable and have normal inflammatory markers and X-ray but intermittently shed Mtb by GA or BAL and are called 'percolators'. This group is likely to represent a spectrum of LTBI as its immunologic, microbiologic and gross pathologic features are similar to LTBI animals (Lin *et al*., 2009). This spectrum of LTBI has been reported in humans (Houk, 1980; Barry *et al*., 2009; Berry *et al*., 2010). Active animals have clinical signs (e.g. weight loss, anorexia) or symptoms (e.g. cough) consistent with TB, elevated inflammatory markers either as ESR or C-reactive protein (Capuano *et al*., 2003; Langermans *et al*., 2005; Lin *et al*., 2009) and growth of Mtb by either GA or BAL at least 2 months after infection. While extra-pulmonary disease does occur in cynomolgus macaques, it is relatively uncommon, even in animals with active TB (Lin *et al*., 2009), and haematogenous dissemination is rare unless under conditions of experimental immune compromise (Lin and Flynn, unpublished). Animals with active TB also develop a wide range of manifestations seen in humans such as Pott's disease, extra-pulmonary disease, brain tuberculoma and meningitis (Capuano *et al*., 2003; Lin *et al*., 2009; Lin and Flynn, unpublished). Monkeys with LTBI can be stable for years whereas animals with active TB can have either acute deterioration or a chronic pattern of disease (Capuano *et al*., 2003). Animals with active TB have more gross tuberculous pathology, higher total bacterial burden and greater bacterial dissemination compared to those with LTBI (Lin *et al*., 2009).

Modelling human Mtb infection in macaques

Many human immunologic reagents (e.g. *in vitro* and *in vivo* reagents, transcriptional assays) cross react with macaques and are readily available for immunologic studies. This facilitates the ability to identify and investigate the immunologic factors critical to various stages of Mtb infection and vaccine-induced protection. For example, CD4 T cells play an important role in controlling early infection that is independent of IFNγ production, with a suggestion that CD8 T-cell function is impaired without CD4 T-cell help (Lin *et al*., 2012c, Yao *et al*., 2014). The role of regulatory CD4 T cells has not been fully defined, but early during the course of infection these cells decrease in the periphery with an increase in regulatory T cells in the airways, suggesting migration from the blood to the lung compartment (Green *et al*., 2010). Tumour necrosis factor–alpha (TNF) also plays a role in the early control of Mtb infection. Loss of TNF resulted in bacterial dissemination in most, but not all, animals with normal granuloma formation as has been reported in humans (Lin *et al*., 2010). This is in contrast to reports in the murine model in which loss of TNF function

was associated with loss of granuloma structure (Flynn *et al*., 1995; Mohan *et al*., 2001; Chakravarty *et al*., 2008). There is also strong evidence that Vγ2Vδ2+ T cells contribute to adaptive immune responses in mycobacterial infections (Shen *et al*., 2004; Chen *et al*., 2013). While studies to understand the immunologic factors that control early infection are fairly straightforward, understanding what factors maintain latent infection are difficult. This is, in part, because of the limited understanding of the spectrum of latent infection that likely affects the variable risk of reactivation observed in humans (Barry *et al*., 2009; Lin and Flynn, 2010; Zumla *et al*., 2013). TNF and CD4 T cells play important roles in the maintenance of latent infection but not all latently infected macaques (and humans) reactivate with TB after TNF neutralization or depletion of CD4 T cells (Lin *et al*., 2010; Lin *et al*., 2012c). With regard to vaccine-protective immune responses, CD8 T cells have been shown to play a key role in BCG-induced protection (Chen *et al*., 2009). Transcriptional studies have shown that vaccine-derived protection against Mtb challenge involves the expression of lung specific indole-amine 2,3-dioxygenase and IL10 genes (Mehra *et al*., 2013; Roodgar *et al*., 2013) as well as systemic induction of IL2, IL17, IL21, IL22 and IFNγ (Wareham *et al*., 2014). Thus immunologic studies can be performed in the macaque model to recapitulate human Mtb infection.

The resurgence of TB as a global epidemic was attributed to the HIV epidemic, showing the important relationship between the two pathogens. The impacts of HIV on both recent and remote Mtb infection poses essential questions that are not understood yet epidemiologically important (Getahun *et al*., 2010; Lawn *et al*., 2011; Zumla *et al*., 2013; Churchyard *et al*., 2014). The macaque model is the only model that can naturally sustain both simian immune deficiency virus (SIV) and Mtb infection, thereby modelling HIV–TB co-infection. Since the 1990s rhesus macaques have been used to model HIV infection and revealed key aspects in its transmission and immunologic control. Rhesus macaques have been used extensively in SIV research and, as a result, their MHC homologues have been more thoroughly identified and SIV strains characterized for experimentation. Cynomolgus macaques have been used less frequently in SIV research and much less characterization has been performed on them, specifically with respect to SIV-specific behaviour (with the exception of Mauritius cynomolgus macaques that have very limited genetic diversity) (Hatziioannou and Evans, 2012). In general, the most common SIV strains appear to be less pathogenic in cynomolgus macaques compared to rhesus. Nonetheless, macaques infected with both SIV and Mtb can serve as important tools to examine pathogen–pathogen interaction and immunological consequences of HIV–Mtb co-infection in humans (Shen *et al*., 2002a; Safi *et al*., 2003; Diedrich *et al*., 2010, 2013; Diedrich and Flynn, 2011; Mattila *et al*., 2011). Recently a neonatal non-human primate model of HIV–TB co-infection has been developed (Cepeda *et al*., 2013). The full potential of this model has not been fully exploited and is likely to warrant further development (Diedrich and Flynn, 2011).

The ability to perform *in vivo* imaging in Mtb-infected NHPs drastically improves the ability to monitor disease progression under experimental conditions. Methods of *in vivo* imaging include high-resolution computed tomography (CT) (Lewinsohn *et al*., 2006), magnetic resonance imaging (MRI) (Sharpe *et al*., 2009; Rayner *et al*., 2013) and positron emission tomography (PET). PET CT imaging with 18-fluorodeoxyglucose (18F-FDG), a probe that functionally detects areas of increased metabolic activity and correlates to tuberculous granulomas in macaques, has shown that lung granuloma patterns of disease progression during the first 8 weeks of infection differ between animals that would later develop active TB compared to those with LTBI (Coleman *et al*., 2014). Specifically, animals that would later develop LTBI had fewer granulomas and with very few new granulomas occurring after 3 weeks post-infection in contrast to animals that would later develop active TB. These data correlate with previously published data in which active disease was associated with higher IFNγ responses, and more frequent elevations in ESR and detection of Mtb during the first 8 weeks post-infection (Lin *et al*., 2006; Lin *et al*., 2009).

The fact that fewer granulomas were observed in LTBI animals as early as 3 weeks post-infection, before the adaptive immune system is known to occur, suggests that both innate and very early adaptive immune responses are likely to play a critical role in the infection. Thus, imaging studies can provide informative data about disease progression during early infection (Sharpe *et al*., 2009; Rayner *et al*., 2013; Coleman *et al*., 2014) and even during drug treatment (Lin *et al*., 2013). The potential of how these modalities can contribute to the TB field is still being developed.

The diversity of granuloma types in this large animal model makes it accessible to focus on bacterial properties that may play important roles during infection. For example, through the use of serial PET CT, these granulomas appear to have very independent and dynamic functions during the course of infection (Lin *et al*., 2013; Coleman *et al*., 2014) that are consistent with the spectrum of granuloma types (Capuano *et al*., 2003; Lin *et al*., 2009) and range of bacterial burden of each granuloma in both active and LTBI animals at necropsy (Lin *et al*., 2014). Using a set of individually characterized Mtb bacteria to infect cynomolgus macaques, it was determined that each granuloma originates from a single bacterium, which is also likely to contribute to the individual nature of the granulomas (Lin *et al*., 2014). Bacterial growth within individual granulomas peak at 4 weeks post-infection and decrease thereafter as the adaptive immune response develops. The ability to infect with mutant strains of Mtb has also been exploited in this model with differing results between mice and macaque (Dutta *et al*., 2010), suggesting that the microenvironments of macaque granulomas (and presumably humans) may differ from other animal models.

Vaccine testing against TB in macaques has a long history. From the 1970s, vaccine evaluation studies of rhesus macaques were commonly challenged with low-dose (less than 100 CFU/dose) *Mtb* strain H37Rv delivered by aerosol and followed for 2–3 months post-infection (Barclay *et al*., 1970; Ribi *et al*., 1971). BCG (or experimental mycobacterial cell wall preparations) could confer a protective effect in rhesus monkeys as measured by histopathology and chest X-ray analysis. BCG's effect was strongest upon intravenous or intrapulmonary delivery as compared to the intradermal immunization as it is routinely applied in clinical vaccination (Anacker *et al*., 1972; Barclay *et al*., 1973). Since that time, variable protection of BCG in rhesus macaques has been reported that may be attributed to changes in the dose (3000 versus <100 CFU), route of infection (intra-tracheal versus aerosol), strain (Erdman versus H37Rv), genetic background and environmental exposures to each colony of rhesus used (Langermans *et al*., 2001; Verreck *et al*., 2009). BCG vaccination in cynomolgus macaques has shown some protection under high-dose inoculation circumstances (Reed *et al*., 2009), but variable protection in lower doses, which may be attributed to differences in method of infection and strain (Lin *et al*., 2012b, Wareham *et al*., 2014).

Conducting vaccine studies in macaques is a huge investment, not only for the researcher but also for the vaccine developers. NHP studies are generally the next milestone to human trials and the most recent failure of MVA85A (Tameris *et al*., 2013) serves as a reminder that the current standards used to measure success in animal models do not necessarily predict outcome in human trials (McShane and Williams, 2014). Because an immunologic surrogate of protection does not yet exist, a broad range of clinical, bacterial and pathologic parameters are generally chosen. Pathological, radiographic and bacteriological measures have been used in addition to clinical parameters (e.g. survival, weight loss, inflammatory markers, anaemia) that generally correlate with disease pathology and severity of disease in the rhesus model (Verreck *et al*., 2009; Sharpe *et al*., 2010). Immunogenicity in the blood and BAL can be easily measured in NHPs as they would in humans but the biggest obstacle remains the lack of a surrogate marker for protection. In the few recent studies using rhesus macaques, vaccine effect has been evaluated primarily at a fixed study end point between 2 and 4 months post-infection; a time frame in which the non-vaccinated controls of this highly susceptible species presented with substantial progressive disease (Sugawara *et al*., 2009;

Verreck *et al*., 2009; Sharpe *et al*., 2010). The majority of vaccine studies performed on cynomolgus macaques have been performed using moderate to high doses of Mtb (Erdman strain) via intra-tracheal or intra-bronchoscopic methods to induce active TB (severe in most cases) (Sugawara *et al*., 2007, 2009; Reed *et al*., 2009; Okada *et al*., 2009, 2011; Coler *et al*., 2013). Less dramatic signs of protection are unlikely to be detected when severe disease is experimentally generated, especially in studies where survival is used as an end point. More recent studies using low-dose Mtb in the cynomolgus macaques showed that a vaccine candidate reduced the rate of active TB and prevented reactivation from latent infection (Lin *et al*., 2012b). Such studies are also difficult to conduct as the range of outcomes (equal numbers of active and latent animals in non-vaccinated controls) requires a larger sample size. Such a wide variety of experimental designs (differing in dose, method of infection, type of macaque with variable genetic background) and measurement methods make it almost impossible to compare and interpret any comparative data between vaccine studies. Realistically the number of vaccines anticipated is likely to outpace the practical ability to conduct human trials in high-endemic areas. Thus methods to prioritize vaccines going forward into humans are critical. New readouts of vaccine protection such as recently reported advanced imaging modalities may provide valuable tools to support the rhesus macaque model for TB research (Sharpe *et al*., 2009).

NHP use for testing against TB in NHPs has existed over several decades (Schmidt, 1955, 1956, 1966) and is particularly important as the search for shorter treatment regimens is a priority. The spectrum of granuloma types among NHP models is particularly attractive and overcomes the potential disadvantages of small animal models for drug testing (Koul *et al*., 2011). Newer methods assessing the biodistribution of drugs through the body and directly into the various types of granulomas are ongoing and will improve our understanding of drug treatment in TB (Prideaux *et al*., 2011; Kumar Verma *et al*., 2012). Immune modulation as an adjunct to drug treatment, especially in the context of multidrug-resistant TB treatment, is another strategy that has been performed in the NHP model of TB (Lin *et al*., 2012a, 2013; Coler *et al*., 2013) and is likely to continue.

Macaque model: challenges and future potential

Without question, NHPs are an expensive model to maintain, particularly in the appropriate biosafety level containment. Animals are outbred, of limited resource and therefore expensive. NHP housing, especially biosafety level 3 housing after Mtb infection, requires very specific containment and adequate space for animal caging (in many cases, single animal housing) as animals can cough on each other. The chronic nature of Mtb infection and the ability to establish latent infection requires long experiments which are often 3–6 months' minimum duration of infection, depending on the model, and this incurs added expense. Specialized veterinary staff is required for the care and enrichment of NHPs in addition to experiment-specific procedures such as blood draws, BAL, imaging or drug treatment. Specialized research technicians are also required for handling samples and performing the appropriate immunologic and bacteriologic assays required for the study. Importantly, procedures at the time of necropsy can be quite labour intensive and generally include a team of both veterinary and research staff. Bacterial burden is among the more important measurements to detect differences between experimental groups, especially among vaccine studies. Various methods have been used to measure differences in burden that include homogenizing the entire lung, individual lobes, random sections of the lobes (stereology), individual granulomas or some variation thereof for growth of live Mtb or by bacterial staining. Bacterial burden from mediastinal lymph nodes and extra-pulmonary sites is estimated in similar fashion (Lin *et al*., 2009, 2014; Reed *et al*., 2009; Sharpe *et al*., 2009; Luciw *et al*., 2011). Because of the large size of the animal, harvesting each individual granuloma from every lobe is quite labour intensive and must be balanced with the other assays required

for the experiment. Similar to the variations in macaque species, Mtb strain, dose and method of infection, each laboratory has its own established methodology of estimating gross pathology, bacterial burden and disease dissemination that can make it difficult to know how generalizable one study is to another species or strain and ultimately to human infection.

Improved methods of making the NHP more efficient are likely to be on the horizon. Using 18F-FDG-PET CT before and during the course of anti-TB treatment, reductions in metabolic activity and lesion size correlate with decreased bacterial burden, indicating that PET CT may eventually be useful in predicting drug efficacy for new drug regimens (Lin *et al*., 2013). One potential challenge, however, is the large size of these animals such that generating adequate quantities of new compounds against TB becomes prohibitive. The marmoset model (see below) addresses this issue. Yet another potential improvement in the model is the finding that early patterns of disease progression can predict outcome (i.e. active TB and latent infection) (Coleman *et al*., 2014) that suggests that some studies could be shortened and outcome predicted early, thus limiting the cost of NHP studies substantially. The complex interactions of both host and pathogen within the granuloma, and how they contribute to overall outcome, is challenging but these complexities provide important opportunities for more innovative methods of investigation such as computational modelling (Fallahi-Sichani *et al*., 2011; Marino *et al*., 2011; Garijo *et al*., 2013) and other new perspectives.

Common Marmoset Model

The common marmoset (*Callithrix jacchus*) is a 350–400 g New World primate belonging to the Callitrichidae family native to the coastal forest of Brazil. The animals are easily bred and a number of breeding colonies have been established in the USA to provide marmosets for research use. The common marmoset has long been used in modelling ageing, reproduction and toxicology (Ludlage and Mansfield, 2003; Zuhlke and Weinbauer, 2003; Carrion and Patterson, 2012). More recently the marmoset has been employed as a model of respiratory viral and bacterial diseases, but it was uncertain if it would be susceptible to Mtb infection (Mansfield, 2003; Nelson *et al*., 2010; Carrion *et al*., 2011; Omatsu *et al*., 2011). It has been thought that Old World primates are more susceptible to *M. tuberculosis* complex (MTBC) than are New World primates although the reasons for this are unclear. Studies questioning this difference have implicated the structure of regulatory regions of the TLR2 gene as one possible explanation but it is also possible that there is just a scarcity of data (Yim *et al*., 2006). There are relatively few reports of neotropical primates with confirmed infection with MTBC, and the source is usually not known, but colonization with non-tuberculous mycobacteria has been reported more commonly (Leathers and Hamm, 1976; Soave *et al*., 1981; Heard *et al*., 1997; Alfonso *et al*., 2004; Henrich *et al*., 2007; Rocha *et al*., 2011).

Colonies of NHP are routinely administered TSTs to monitor for MTBC outbreaks. There were no reports of TST-positive marmosets with MTBC infection, but a colony was found to contain TST-positive individuals with subcutaneous granulomatous lymphoid tissue that had culture evidence of *M. kansasii* (Wachtman *et al*., 2011). The same colony had TST-positive animals with faecal samples that were positive for *M. gordonae* DNA, but necropsies revealed no pathology associated with *M. gordonae* positivity, suggesting the test was detected apathogenic intestinal colonization. Published reports document only a single case of zoonotic infection of a common marmoset with Mtb (Michel and Huchzermeyer, 1998). The initial studies show marmosets are highly susceptible to experimental infection with Mtb but that subclinical disease is also possible (Via *et al*., 2013; personal communication, J. Flynn and F. Verreck).

Experimental infection characteristics

Low-dose aerosol delivery, as well as intrabronchial instillation, has been used for experimental infection of the marmoset (Via *et al*., 2013). The presentation of the resulting

disease is somewhat different depending on the route, with aerosol delivery resulting in lesions located throughout the parenchyma and distal air spaces, whereas instillation produces a more localized disease. The Mtb strains that have been used for infection include a recent Beijing clade clinical isolate and Mtb Erdman, both of which caused rapidly progressing disease and morbidity within 4–6 weeks. Mtb CDC1551 also caused rapidly progressing disease but mortality was delayed to 8–12 weeks with low-dose exposures of 25 CFU and very low-dose infection resulted in a durable case of subclinical disease (J. Flynn, personal communication). Marmosets were also susceptible to infection with *M. africanum* (MAF) and respond with a greater volume of radiologic lung disease than might be expected from the recovered CFU from the lung, similar to the reported aggravated disease presentation in humans (de Jong *et al*., 2010). The rapidity of disease progression was correlated loosely with the infectious dose and the virulence of the infecting strain.

Disease development was monitored by [^{18}F] FDG-PET/CT, changes in body weight, temperature, food consumption and behaviour. Onset of weight loss was observed between 3 and 6 weeks post-infection and was directly correlated with the infectious dose. Increases in the lung field density coincided with the onset of weight loss and distinct lesions in the lung became apparent by CT scans 4–6 weeks post-infection. Animals infected with a recent Beijing isolate showed the most rapid progression as measured by the increase in abnormal density of the lung and had a median survival time of only 37 days. MAF infection resulted in a slower rate of disease development but both it and the Beijing strain showed increased extra-pulmonary dissemination of disease that could be detected by an increase in ^{18}F-FDG activity in the liver and spleen by PET/CT scanning. Marmosets infected with CDC1551 showed the slowest rate of disease progression and were the only animals to develop cavitary disease. Quantitative assessment of disease burden by FDG-PET/CT allowed an accurate assessment of disease progression in these animals that was highly correlated with pathology findings at necropsy.

Pathology presentation

At necropsy, all Mtb-infected marmosets showed evidence of pulmonary nodules and regions of consolidation consistent with active tuberculosis. Adhesions between lung lobes and with other adjacent structures including the thoracic wall were common. Enlarged lymph nodes with central caseation were common in the marmosets infected with MTBC strains other than CDC1551; those infected with CDC1551 typically showed enlargement only. Visible lesions in the spleen, liver and pericardium were observed in some Beijing- and MAF-infected animals. Histologically, both necrotizing and non-necrotizing granulomas with either a multifocal or coalescing distribution were present, usually embedded in regions of tuberculous pneumonia, as has been observed in other NHP models. Necrotizing granulomas had a central area of necrosis surrounded by epithelioid macrophages, and variable numbers of lymphocytes and neutrophils. In the Beijing- and MAF-infected animals, many lesions had poorly developed marginal fibrosis and showed direct contiguous extension of inflammation between adjacent alveolar airways. Fibrosis was more prominent in the CDC1551-infected animals and centrally cavitated lesions were observed in 50% of the animals, sometimes clearly connecting to a pulmonary airway, with bordering fibrosis. Expansion of the peribronchiole/perivascular interstitium by inflammatory cells was also common, producing thickened airways throughout the lung.

Use in therapeutic evaluation

We have examined the response to experimental chemotherapy in Mtb-infected common marmosets initially by examining two drug regimens that had been utilized clinically and for which human relapse rates were known (Via *et al*., 2015). After infection, the marmosets were allowed to develop extensive disease for approximately 6–8 weeks with monitoring by PET/CT. Marmosets were easily habituated to oral administration of paediatric syrups containing anti-TB drugs and began regaining weight within 2 weeks of

administration of INH-containing regimens. The PET/CT images clearly showed both a reduction in disease volume by CT and a reduction of FDG uptake in the lesions of treated animals. The model is being developed further for assessment of the sterilizing potential of new drug combinations and to prioritize lead compounds. Given the limited resources, it is more efficient to produce smaller quantities of lead compound for testing in a smaller animal model that better recapitulates human TB than the current existing ones. If such results are promising, these lead compounds can then be synthesized in greater quantities for large-scale, large animal models.

Challenges and future potential

These NHPs reach maturity by 1.5 years and enter senescence by 8 years. They typically give birth to twin or triplet offspring, twice per year, and are best housed in family groups or as pairs (Bullock *et al*., 1969). This means that a small colony of ten breeding pairs can provide roughly 30–40 animals for experiments per year. Because of group housing the space requirements are modest and marmosets can be adapted to high-containment laboratories (Carrion and Patterson, 2012). Marmosets may be hand caught and do not require sedation for blood draws, drug administration or other simple manipulations. With weights less than 0.5 kg, these NHP require only moderate amounts of preclinical compound for efficacy testing yet yield reasonable amounts of tissue for determination of bacterial burden, pathology, pathogen gene expression, tissue drug concentration and blood products all from the same individual. The marmoset is tolerant to anaesthesia (Ludlage and Mansfield, 2003) and fits in to most small animal scanners used for mice, reducing the need for additional highly expensive equipment if imaging is desired. Finally, the species is an FDA-accepted toxicology model and a contributor to fulfilling the two-species rule for efficacy demonstration of the European Union.

Marmosets in captivity are susceptible to developing diarrhoea when stressed, and may also develop inflammatory bowel disease or wasting syndrome, perhaps caused in part by the laboratory diet (Tucker, 1984; Schroeder *et al*., 1999; Gore *et al*., 2001). One suggestion has been that eliminating gluten from the diet might alleviate the problem; another is that the diet is lacking in protein (Tucker, 1984; Kuehnel *et al*., 2013). Another challenge is that because the body weight of the marmoset is low, the blood volume that can be drawn is limited, making pharmacokinetic and immunological studies more challenging. In addition, while some human immunological reagents are cross-reactive with the marmoset tissue, the number is more limited than with many Old World primates (Kap *et al*., 2009).

Summary of NHP Models of Human TB

While difficult and expensive, NHPs provide an important niche of human TB that cannot be addressed in the other animal models. NHPs recapitulate early infection, active TB and latent infection similar to humans. The architectural structure of the granulomas and spectrum of granuloma type are almost identical to humans, providing a critical background for investigations in drug treatment, vaccine efficacy, immunology and host–pathogen interaction. Last, the phylogenetic similarly between humans and NHPs cannot be dismissed, as responses in the NHPs are more likely to reflect human Mtb infection.

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26 *Mycobacterium leprae* in Humans

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Leprosy: A Chronic Disease of Skin and Nerves

Leprosy is a chronic mycobacterial infection caused by the obligate intracellular pathogen *Mycobacterium leprae* that mainly damages skin and peripheral nerves, causing a broad array of skin lesions, peripheral neuropathy and anaesthesia with related disfigurement, deformity and disability along with the social stigma intimately associated with this ancient disease (Scollard *et al*., 2006). The remarkable inter-individual variability in clinical manifestations of leprosy closely parallels the hosts' abilities to mount effective immune responses to *M. leprae*, dependent on the interplay of both cell-mediated and humoral responses. This is evidenced by the overall resistance toward developing leprosy in the majority of individuals who are infected, accompanied by high cellular immune reactivity against *M. leprae*. In addition, the variability in immune responses to *M. leprae* is also clear from the well-known immunological and clinical leprosy spectrum in those who progress to disease, ranging from tuberculoid (TT) or borderline tuberculoid (BT), or paucibacillary (PB) leprosy to lepromatous (BL/LL) or multibacillary (MB) leprosy. The concept that the range of clinical and histopathological manifestations represented a spectrum of disease states was first proposed by Skinsnes (1964). The division of the forms of disease was categorized using the Madrid system (Davison *et al*., 1960), followed by the more commonly used Ridley–Jopling classification scheme (Ridley and Jopling, 1966). The Ridley–Jopling classification system is divided into five distinct forms bounded by two immunological extremes. At the polar tuberculoid end of the spectrum (TT), patients have a very strong cell-mediated immune response, usually having a single hypopigmented lesion with loss of sensation within the border. Histologically, there is a clear-cut well-organized granulomatous response consisting of epithelioid cells and lymphocytes with no or few acid-fast bacilli detectable in stained tissue sections. TT and borderline tuberculoid (BT) patients in general show high cellular responses to *M. leprae* antigens *in vitro* as measured by the production of Th1 cytokines, particularly IFNγ, and have low antibody titres to *M. leprae*-specific antigens. At the polar lepromatous end of the spectrum (LL), patients have essentially lost all capacity to respond to *M. leprae* antigens due to T cell anergy and have high antibody titres to *M. leprae* antigens, particularly the *M. leprae*-specific

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals:The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

glycolipid, PGL-I (Hunter and Brennan, 1981; Spencer and Brennan, 2011), and with disseminating progressive infection that manifests itself with numerous poorly defined raised or nodular lesions that can occur anywhere on the body. Histological examination reveals a disorganized granuloma filled with 'foamy' macrophages in the dermis filled with huge numbers of acid-fast bacilli and rafts of bacilli called globi. These distinctive cells are filled with vesicles containing mainly host-derived lipids organized as lipid bodies (Mattos *et al*., 2010; de Mattos *et al*., 2012), a likely nutritional source for *M. leprae* and first described in lepromatous lesions by Virchow (1863). The borderline states (BT/BB/BL) are positioned in-between, and reflect immunologically rather unstable phenotypes. The different outcomes of *M. leprae* infection are assumed to be caused by host defence mechanisms, which are still not completely understood in terms of their pathogenic effect.

Archaeological Evidence

Leprosy is one of the oldest diseases known to be associated with man. It is one of the few infections that exhibits characteristic skeletal changes that have been found in archaeological sites. Radiological examinations have been performed in living individuals who had characteristic changes, notably degraded bone formations in the anterior nasal spine and in the alveolar process of the maxilla that occurs in late-stage lepromatous disease (Møller-Christensen, 1961), but is absent in tuberculosis, syphilis, by injury or by trauma. In particular, there were numerous gravesites associated with monasteries and hospitals that specifically cared for those afflicted with leprosy (Mercer, 1915; Møller-Christensen, 1953). In some cases, those who died of the disease were buried in a prescribed area of the cemetery only for those afflicted with leprosy, separate from others interred, as if this feared disease could contaminate the deceased even after death. Analysis of ancient bones that showed evidence of pathological lesions, including bone pitting and bone resorption, indicate that leprosy was present in north-west India in 2000 BC (Robbins *et al*., 2009), and was confirmed by molecular sequencing methods in 1st-century CE bones from the Tomb of the Shroud burial site, the earliest known date for the occurrence of this disease in Israel (Matheson *et al*., 2009). Analysis of rare single-nucleotide polymorphisms (SNP) found in *M. leprae* samples throughout the world from modern times to ancient burial sites has led to theories about the origins of the disease and the migration routes based on the location and distribution of these types in each part of the world (Monot *et al*., 2005, 2009). Recent analysis of five medieval *M. leprae* isolates from the UK, Sweden and Denmark revealed remarkable genomic conservation of each SNP subtype based on comparisons with modern strains (Schuenemann *et al*., 2013). The SNP subtype 2F was found from one sample from each of these countries, whereas the current geographic distribution of modern branch 2 subtypes is found only in Iran and Turkey, suggesting a Middle-Eastern origin of this medieval subtype. The other two ancient DNA samples from the UK and Denmark were both 3I, the latter isolated from a 14th-century tooth sample named Jorgen_625. The *M. leprae* DNA inside the tooth was remarkably preserved due to very high levels of mycolic acids, a hydrophobic mycobacterial cell wall constituent that was found within the tooth pulp. Both of these samples were extremely close in sequence to all of the branch 3 strain sequences reported previously from modern patient isolates and armadillos from the southern USA (Truman *et al*., 2011), consistent with the European origin of leprosy in North America. Thus the archaeological evidence found in bone lesions coming from many different ancient burial sites throughout Europe, the UK and the Middle East indicates that the subtypes found at that time had settled there with known human migration patterns, and that there have been very few changes in the *M. leprae* sequence for more than 1000 years, indicating a very stable genome.

Historical Stigma Associated with Leprosy

The stigma associated with leprosy has been a part of the history of this disease from

antiquity, although many of the images that have been used to conceptualize the fear and abhorrence of the disfigurement and disability that comes to most people's minds come from writings and images depicted from Biblical and Medieval times, or from movies in the modern era. The oldest known written references about leprosy are in the Egyptian Ebers papyrus dated to around 1550 BC (Hulse, 1972). Historians have also suggested that texts describing characteristics of the disease, including skin lesions and bone deformity or loss along with forms of treatment, are found in the Sanskrit translations of the *Atharva Veda*, hymns that were composed in India before the 1st millennium BC (Bloomfield, 2004). Another ancient Indian medical text written around 600 BC, the *Sushrata Samhita*, is considered to have the earliest descriptions of different forms of leprosy based on signs and symptoms of disease (Dharmendra, 1947). Three forms were described: one form showed sensory deficits in the skin in the absence of skin lesions; and in two forms skin lesions showed sensory loss or ulceration (Kunjlal, 1907, 1911, 1916). These three forms would be consistent with the signs and symptoms of: (i) pure neural leprosy; (ii) cutaneous skin lesions with sensory loss, as in the case at the tuberculoid end of the spectrum; and (iii) lepromatous skin lesions, respectively. Biblical references to leprosy in the Old Testament, particularly from Leviticus 13 and 14, have been discounted as being actual leprosy due to accumulated errors in translations from the Hebrew *tsara'ath*, where the original meanings of the names for a variety of skin diseases, both acute and chronic, were aggregated together and called *lepra*, a Greek term recognized by physicians at the time as a dry, scaly skin condition such as that found in psoriasis. Nevertheless, it is apparent from biblical writings that disfiguring skin diseases were viewed as a particular concern, requiring elaborate cleansing rituals for individuals to be accepted back into the community and, in some cases, extreme measures that included banishment of the individual or burning their belongings or dwelling in attempts to rid the source of contamination. The establishment of modern leprosy in Western civilization is likely to have occurred in Greece about the turn of the 3rd century BC when soldiers of Alexander the Great returned from India around 327 BC with slaves from that region (Andersen, 1969; Browne, 1985). This new skin disease was accurately described by Straton, a disciple of a physician from Alexandria, Egypt, around 300–250 BC, and given the name *elephantiasis Graecorum*, the name for leprosy at that time. It was not until much later (777–857 AD) that Johannes Damascenus first used the term *lepra* to describe a medical term for this disease (Andersen, 1969) that by this time had become well established in Greece and Rome and was beginning to slowly spread northward into Europe and the UK.

The Spread of Leprosy to Europe and the UK

As leprosy spread through Europe and into the UK, the first laws were enacted that specifically dealt with those afflicted by leprosy in the Code of Laws in Wales that took effect during the reign of Hywel the Good (born *c*.880–died 950 AD) (Browne, 1985). A short time later, during the reign of Edgar the Peaceful in England, a law was passed that made leprosy a valid reason for divorce. In 1346, Edward III issued a proclamation that expelled those afflicted by leprosy from the city of London. Leprosy had increased tremendously with the return of the Crusaders, many of whom contracted leprosy from countries of the Ottoman Empire, where the disease was much more prevalent. The return of the Crusaders from 1100 AD onward brought a change in how people viewed those with the disease, and thousands of almshouses, hospices and lazarets (the latter a term that was a house of quarantine in France specifically for those with leprosy and other communicable diseases) were established by Christian charitable organizations to deal with the increasing number of cases that peaked during the 13th and 14th centuries. The medieval Orders of Chivalry, in particular, were established to care for those afflicted by leprosy. The Order of St Lazarus of Jerusalem, founded in 1050 AD, established itself in England and became very rich and powerful with the number

of endowments of land and donations from various religious groups that wished to help those suffering from this disease. One of the largest of these organizations still functioning today is the Order of Malta, whose full title is 'The Sovereign Military Hospitaller Order of St John of Jerusalem, of Rhodes and Malta', founded prior to the taking of Jerusalem by the Crusaders in 1099 (Browne, 1985). This order has been primarily concerned with the care of leprosy sufferers, and to this day provides funding for scientific research devoted to the diagnosis, treatment and cure of leprosy.

Estimates of prevalence rates of leprosy at its peak in Western Europe are unknown, but could have been as high as 30–40 per 10,000 population, which is now considered a level highly endemic in parts of northern Brazil. Several events are thought to have led to its gradual decline in England, foremost being that there was a serious famine in 1325, which was followed by the Black Death (bubonic plague) in 1349, which wiped out up to one-third of the total population in the UK and most of Europe. Those poor who were afflicted with other diseases such as leprosy probably had much higher death rates. At the same time, the general standards of living were increasing, and there were improvements in nutrition and a lowering of overcrowding conditions, factors which even today are known to decrease the risk of individuals living in leprosy-endemic areas.

Gerhard Hansen and the Discovery of *M. leprae*

As the numbers of cases declined in Europe, the spread of leprosy northward resulted in prevalence rates peaking in Scandinavian countries, particularly Norway, Finland and Iceland, in the middle of the 19th century. In Norway, two prominent Norwegian dermatologists, Daniel Danielssen (1815–1894) and Carl Boeck (1808–1875) published an illustrated book entitled 'Om Spedalsked' in 1848 (Danielssen and Boech, 1848) showing in great detail all of the skin manifestations of lepromatous disease and realized the importance of nerve damage causing lagophthalmos (inability to close the eyelids completely due to injury to the facial nerve), atrophy of muscles to the hands and other neurological impairments causing loss of sensation. Danielssen mentored a young physician who was studying leprosy, Gerhard Henrik Armauer Hansen (1841–1912), who noted that he consistently saw rod-shaped bodies (bacteria) with a microscope from scrapings of skin lesions of patients diagnosed with leprosy. He initially submitted a report in 1873 on his findings, and published his beliefs that what he had discovered was the causative agent of this disease (Hansen, 1874, 1875). It was not until 1880 that Albert Neisser was able to successfully stain the rod-shaped bacilli from lepromatous patient lesion material supplied by Hansen with some new analine dyes he had begun using, at which point he claimed to have discovered the disease-causing bacillus. Hansen was unable to cultivate the bacterium, not knowing that to this day there is no axenic medium capable of growing *M. leprae in vitro*. His failed attempts to infect a human subject with material transferred from a leprosy patient resulted in his being fired from his position at the hospital and banned from practising medicine for years due to ethical issues. Nevertheless, he is attributed with having discovered *M. leprae*, the first bacterial pathogen linked to a specific human disease. These seminal findings in Norway opened the door for future scientific research on the possibility that bacteria were the causative agents of other human diseases such as plague, tuberculosis and cholera, paving the way for the germ theory of disease to develop, and destroyed centuries-old beliefs that leprosy was due to hereditary traits, the result of some punishable sin by the person infected, or bad air caused from decaying organic material spreading epidemics of disease.

The initial response of Norwegian health officials to confront the spread of leprosy was to gather all of those afflicted, regardless of what form of disease they had, and board them in many farmsteads, which is likely to have increased the persistence of the disease. A better solution came in 1856 when a number of newly built hospitals opened to receive more than 700 leprosy patients. With the changes

in patient care, lowering the density of infected individuals and improvements in nutrition and economics, the prevalence decreased from 300/10,000 population in certain cities in 1857 to almost none by the early 1920s, despite there being no available antibiotic therapy during this period. Similar dramatic declines in leprosy had already occurred in Europe and the UK, and despite thousands of cases of leprosy being diagnosed in immigrants coming from Africa and Asia, cases of endemic leprosy arising in Europe, the UK and Scandinavian countries have been extremely rare. Since the *M. leprae* genome has been shown to be essentially unchanged for the last 1000 years; the impressive decrease in prevalence has been attributed almost exclusively to improvements in socio-economic conditions in these areas, mainly in the last 100 years (Schuenemann *et al*., 2013).

Isolation of Leprosy Patients

One of the more controversial aspects of leprosy control by governments in almost every country in the world was the forced segregation and confinement of people afflicted with leprosy to leprosaria, institutions or hospitals, many for most of their lives. Governments and health authorities passed laws allowing the arrest and relocation of people thought to be infected to what was essentially a prison in the mistaken belief that leprosy was highly infectious and that isolation was the only way to control the spread of disease. Two places in the USA served as sites where those who were diagnosed with leprosy were sent (and there were many cases where individuals were mistakenly diagnosed with leprosy, but were sent there anyway): Kalaupapa, on the island of Molokai, Hawaii; and Carville, Louisiana. A brief description of the history of each place follows.

Kalaupapa, Molokai, Hawaii

In 1865, the Hawaiian king, Kamehameha V, signed into law 'An Act to Prevent the Spread of Leprosy'. Health authorities paid bounty hunters on all of the Hawaiian islands to arrest and detain anyone suspected of having leprosy. In the first year, 1866, 142 people were forcibly transported by ship and left on an isolated peninsula of land on Molokai that was surrounded on three sides by the ocean and on the fourth side by a 2000 foot lava cliff. There were only crude shelters, no medical care and food was infrequently delivered. It was a hideous, dehumanizing and lawless place, where only the strong survived. New arrivals were sometimes greeted with a warning, 'In this place there is no law'. At its peak in 1890, the Settlement of Kalaupapa had 1174 residents, including patients from a dozen countries who had the misfortune of being caught by health authorities in Hawaii. Mortality in the first 5 years was a staggering 46% (Tayman, 2006). Of the estimated 8000 people who died on the peninsula, only 1500 gravesites mark where former residents of the settlement are buried. The situation only improved gradually with the arrival of Father Damien deVeuster, a Belgian priest who came to Hawaii in 1863, and who volunteered to minister to the outcasts at the settlement in 1873. He organized the building of shelters, an infirmary, a church and other buildings, and treated the patients' wounds while tending to their spiritual needs. Despite repeated warnings, he refused to take precautions while changing bandages and applying ointments to the patients' wounds. Besides ministering to the sick, it is estimated that he personally buried about 1300 of the residents who succumbed to their disease. In 1884, it was confirmed that he had leprosy, and he was joined by Brother Dutton and several Franciscan sisters headed by Mother Marianne Cope to carry on his work. He died in 1889 at the age of 49, and was buried next to St Philomena's, the church that he had built at Kalaupapa. In October 2009, Father Damien was canonized by the Catholic Church and became a saint. The last law restricting travel of former leprosy patients was repealed in 1969, 103 years after the first group arrived and, for the first time, residents could come and go without travel restrictions. About 20 former patients still reside at their homes at Kalaupapa, most in advanced age, who receive appropriate medical care for their residual neurologic, skin or organ damage caused by their disease.

Carville, Louisiana and the National Hansen's Disease Program (NHDP)

The site that later became the sole treatment and research centre for all leprosy patients in the continental USA was a former plantation, first established in the early 1800s and originally known as Indian Camp. Led by Dr Isadore Dyer, a dermatologist and leprologist from Tulane University medical school, the abandoned farm, former slave quarters and administration buildings were acquired by the Louisiana State Legislature in 1894 to establish the Control Board for the Louisiana Leper Home at Carville, 'a place of refuge, not reproach; a place of treatment and research, not detention' (Krahenbuhl, 2013). The first seven leprosy patients were transported by river barge from New Orleans, as laws at that time prohibited them from using any public transport. Shortly thereafter, care of the leprosy patients was contracted to four Catholic Daughters of Charity of St Vincent de Paul (Stein and Blochman, 1974). The state of Louisiana purchased the property in 1905, thus creating the first state-funded 'Home' for leprosy patients in the USA. In 1916, John Early, a patient at the Louisiana Home, testified before Congress concerning the need for a hospital and research centre for leprosy, proposing that a facility could offer patients hope instead of only custodial care in an almost inaccessible location segregated from society. In 1917, Senate Bill 4086 was passed with support from the American Leprosy Missions, the Surgeon General and the US Public Health Service to establish a Leprosarium in Carville. The 'Home' was sold to the US Federal Government and the US Public Health Service (USPHS) officially took over control in 1921, renaming it the US Marine Hospital Number 66, The National Leprosarium of the US (Krahenbuhl, 2013).

In spite of more enlightened care and treatment of leprosy patients at this facility, the fact remained that Federal and State laws allowed health authorities to physically detain, arrest and transport any person in the USA who was diagnosed with this disease, without their consent, to Carville. At the turn of the 20th century, there was still such enormous stigma attached to the disease that laws allowed husbands or wives to divorce their spouse and declare them legally dead. New arrivals to Carville were asked to choose a new name (since many of their families had disowned them or declared them deceased) and were given a patient identifier number that was with them at the facility until they died or were considered cured and discharged.

In 1931, 'Stanley Stein' (the alias taken by a former Texan pharmacist) began publishing the *Sixty-Six Star*, an in-house patient news sheet that later became *The STAR*, an internationally recognized newsletter with the mission to advocate for the rights of people affected by Hansen's Disease (he preferred using this name instead of the term leprosy, which was viewed by many as pejorative). The patient rights movement to afford dignity to those affected by leprosy, educate the public and lower the stigma attached to the disease became stronger from the 1950s onwards, due in a large part to this publication. In 1963, *The STAR* first published a book written by Stein about his life at Carville, *Alone No Longer* (Stein and Blockman, 1974).

Scientific research concerning the immunology, genomics, proteomics and metabolomics of the *M. leprae* bacillus, the development of new drugs to treat leprosy patients and their reactions (rifampicin was introduced in 1971; thalidomide, found to decrease reactions and nerve damage, was also supplied by the USPHS at Carville for US and Canadian patients) and pioneering surgical techniques to restore the use of nerve damage to fingers and hands, were all conducted at the NHDP clinical and research laboratories during the golden age of leprosy research beginning in the 1960s. This coincided with the development of the mouse footpad model for the growth of *M. leprae* by Charles Shepard in 1960 (Shepard, 1960, 1962), facilitating the testing of new anti-mycobacterial drugs against the pathogen (Shepard and Chang, 1962; Shepard *et al*., 1971). In the 1970s, Kirchheimer and Storrs (Kirchheimer and Storrs, 1971) found that armadillos could be infected by *M. leprae*, which then became a source of large quantities of the purified bacillus that could not be cultivated *in vitro*. The reagents that were developed from these bacilli (whole cells, native protein subcellular fractions, DNA, lipids, the

M. leprae-specific phenolic glycolipid I antigen (PGL-I), lipoarabinomannan (LAM), and monoclonal and polyclonal antibodies recognizing these constituents) were provided free of charge to leprosy researchers worldwide through a National Institutes of Health (NIH) Leprosy Research Materials Contract run by NHDP and Colorado State University for more than 30 years. In 1985, the National Hansen's Disease Center was renamed and dedicated in honour of the late Congressman Gillis W. Long, who had successfully fought to prevent the transfer of patient care from Carville by the Federal government to outside private contractors during the Reagan administration. This centre continues to this day to provide inpatient and outpatient referral care to individuals living with this disease throughout the USA.

Host genetic factors influencing disease susceptibility

The continuum of disease symptoms is a direct result of interactions between the host's immune response and the bacterium (Quintana-Murci *et al*., 2007) and reflects the complexity of host–pathogen interactions. Lepromatous and tuberculoid manifestations of leprosy represent host immune responses at opposite ends of the spectrum, both being rather stable responses. 'Borderline' cases (BT, BB and BL) represent a variety of clinical responses and underscore a mostly unstable immune response to the bacillus (Ridley and Jopling, 1966). Since *M. leprae* is a product of extreme reductive evolution (Drosos *et al*., 1986; Cole *et al*., 1998, 2001), having an exceedingly low diversity of genomic sequences among all strains worldwide, it is unlikely that differences in susceptibility or presentation of clinical symptoms are determined by bacillary strain variation (Monot *et al*., 2005). Thus, hosts' immunological composition and the resulting integrity of immune response are thought to play a critical role in ultimately determining the outcome of the course of infection. Multibacillary infection is associated with a mainly T helper type 2 (Th2) cell response, whereas paucibacillary infection is associated with an immune response mediated by T helper type 1 (Th1) cells (Modlin, 1994). The implication that susceptibility is a consequence of host genetic factors has arisen from studies involving twins, complex segregation, tests of association with human leukocyte antigen (HLA) genes and familial clustering (Shields *et al*., 1987; Zhang *et al*., 2009). There are a limited number of studies that have investigated the impact of genetic variants in mitigating the susceptibility or resistance to leprosy. Many results are inconclusive because they lack thorough replication studies but have provided critical advances toward understanding the pathogenesis of leprosy. Here we present a brief summary of a select group of genetic studies that have been published.

HLA

Several studies have consistently reported the involvement of HLA alleles and haplotypes, chiefly those of Class II genes, as important genetic factors controlling susceptibility to various forms of leprosy (Mira, 2006). The clinical manifestation of leprosy depends on the type of immune response that is initiated by the host and the balance of Th1- versus Th2-mediated mechanisms that may be partially controlled by the operation of antigen presentation involving HLA molecules. The chief role of HLA is to present peptide derived from *M. leprae* to host T cells (Rani *et al*., 1992). Simply put, a host that fails to express an HLA allele that is capable of presenting *M. leprae* peptides in a manner that will generate an appropriate T cell response will be more susceptible to infection. An individual whose HLA system offers protection against *M. leprae* is one that can present antigen to appropriate T cells that are subsequently stimulated to multiply and eliminate the bacilli by signalled secretion of cytokines that destroy infected cells as is the case in a protective Th1 response. The pattern of pro-inflammatory cytokine expression/secretion ultimately dictates the type of immune response that will be elicited by the host and, in turn, determine whether the infection will be cleared, kept at bay or allowed to proliferate.

HLA Class I

Several studies have demonstrated an association of certain HLA Class I alleles with polar forms of leprosy or with leprosy disease per se (all forms ranging from TT to LL). HLA Class I allelic associations with leprosy were often dependent on in which countries and geographical locations the surveys were made. For example, the HLA-Aw21 allele was shown to be a factor of susceptibility to TT in Ethiopian patients whereas HLA-A9 and HLA-A2 were factors in resistance to leprosy in Thailand and Korea, respectively. In Iran there was a preponderance of HLA-B35 and a decrease of HLA-A1 in LL patients. HLA-B40, HLA-A2-B40, HLA-A11-B40 and HLA-A24-B4 were frequent among Indian leprosy patients. With the advent of molecular genotyping, HLA Class I alleles were determined for multibacillary leprosy patients that were positively associated with *HLA-A*02:06*, *A*11:02*, *B*18:01*, *B*51:10*, *C*04:07 and C*07:03* and negatively associated with *HLA-C*04:11* in Indian patients. Recent work has shown a positive association with leprosy per se with *HLA-A*11*, *HLA-B*38* and *HLA-C*12* and a negative association with *HLA-C*16* in a Brazilian population. In a Vietnamese and Indian study population, the allele *HLA-C*15:05* was positively correlated with disease. Although several studies have been completed comparing HLA Class I frequencies in leprosy cases to endemic controls and there has been some association found either with polar forms of leprosy or leprosy per se, results have been rather inconsistent; all work reviewed in Jarduli *et al*. (2013).

HLA Class II

Collectively, studies of Class II have shown that the main determinants for *M*. *leprae* reside on DR and not DP or DQ molecules (Ravikumar *et al*., 1999; Ohyama *et al*., 2001). In Indian, Brazilian, Chinese and Japanese patients HLA-DR2 (specifically *DRB1*15* and *DRB1*16* as determined by molecular genotyping) is primarily associated with leprosy (Jarduli *et al*., 2013). In Indian patients *DRB1*15:01* and *DRB1*15:02* were associated with LL and TT, respectively. *HLA-DRB1*15:01* and **15:02* differ from each other by a single amino acid at codon 86. Codon 86 resides in the polymorphic peptide-binding pocket 1 of the Class II molecule. In another Indian study, both **15:01* and **15:02* were found to be associated with tuberculoid leprosy and thus indicated that pocket 1 may not be involved in determining the outcome of leprosy. It was subsequently shown that mutations that result in net neutral or negative charge within pocket 4 of the DRB1 molecule might cause poor binding of *M*. *leprae* peptides (Uko *et al*., 1999). Thus it is hypothesized that HLA molecules with the highest affinity to *M*. *leprae*-derived peptides produce the greatest T cell proliferative and IFNγ response, whereas presentation with low affinity Class II will likely result in a more muted cell-mediated response. Alternative presentation of peptide by specific Class II molecules may result in activation of suppressor/regulatory T cells (Mutis *et al*., 1994).

Other studies involving HLA-DRB1 indicate a link between innate and T cell-mediated immunity and showed that the presence of a VDRE (vitamin D response element) in the proximal promotor region of the *HLA-DRB1* gene increases gene expression imparting vitamin D sensitivity to the *DRB1*15:01* allele (Ramagopalan *et al*., 2009). It is possible that vitamin D influences leprosy either directly or indirectly because of the association between these variants and leprosy pathogenesis. In separate studies involving Nigerian and Javanese patients, peptide pocket motifs containing a variety of charged and uncharged amino acids were both negatively and positively associated with leprosy. Surveys of HLA-DRB1 alleles among Turkish, Vietnamese, Brazilian, Korean and Argentinean leprosy cases also defined certain haplotypes that were curiously restricted to within those study demographics.

Despite numerous studies comparing HLA Class I or Class II gene frequencies with various polar forms of leprosy cases, the results have been, at best, inconsistent; for review see Jarduli *et al*. (2013). Though genetic epidemiology data for HLA in leprosy are extensive, interpretive caution must be exercised due to strong linkage disequilibrium for

all the alleles in this chromosomal region. It is also common to encounter numerous cases of weak study designs and a publication bias for positive results. Functional data will be necessary to prove that any HLA alleles positively or negatively correlate to host susceptibility to leprosy.

PARK2-PACRG

PARK2 and *parkin co-regulated gene (PACRG)* are co-regulated genes sharing a bi-directional promotor element and are expressed in all immune tissues and Schwann cells located on chromosome 6q25-q27. *PARK2* encodes the protein parkin, an E3 ubiquitin ligase that is part of the ubiquitin–proteasome system that mediates targeting proteins for degradation. An ubiquitin ligase is a ligase enzyme that interacts with an ubiquitin-containing E2 ubiquitin-conjugating enzyme which allows recognition of target proteins slated to be ubiquitinated. E3 ligases target specific protein substrates for degradation by the proteosome and is thought to be subsequently involved, at some level, in antigen processing. Mutations in the PARK2 gene have been previously shown to be the cause of autosomal recessive early onset Parkinson's disease (Kitada *et al*., 1998). The *PACRG* product forms a large molecular complex with chaperones, such as the heat shock proteins hsp70 and hsp90. In Parkinson's patients, PACRG forms Lewy bodies that interfere with the suppression of unfolded parkin-associated endothelin receptor-like (PAEL) receptor-induced neuronal cell death (Takahashi and Imai, 2003; West *et al*., 2003; Schurr *et al*., 2006).

In a study of leprosy cases in a Vietnamese cohort, investigators identified a locus within this gene that was significantly associated with leprosy, irrespective of the subtype of the disease (Mira *et al*., 2003, 2004). This was the first example of the use of positional cloning to identify a human gene associated with susceptibility to an infectious disease (Buschman and Skamene, 2004). The specific locus is within the shared promotor region of the *PARK2/PACRG* genes. These results were confirmed in a second study of Brazilian families having one or more cases of the disease (Mira *et al*., 2004).

The precise mechanism(s) of how the ubiquitin–proteosome pathway that is involved in intracellular protein degradation regulates the processing and subsequent presentation of antigen to lymphocytes is currently unknown, but is likely to play a role in susceptibility to leprosy, perhaps because proper antigen processing is required for appropriate initiation of adaptive immunity (Mueller, 2004; Watts, 2004). The discovery that *PARK2* is a leprosy susceptibility gene was completely unexpected and the role of parkin in leprosy pathogenesis remains unknown. Further support for the role of *PARK2/PACRG* in infectious disease susceptibility has been obtained by the identification of promotor variants as risk factors in typhoid fever (Ali *et al*., 2006).

Downregulating parkin using STEALTH *si*RNA duplexes in THP-1 cells differentiated with TPA to macrophages, human monocyte-derived macrophages and human Schwann cells, resulted in a consistent and specific decrease in two key mediators of innate immunity, interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1/*CCL2*) in response to mycobacteria or LPS (de Leseleuc *et al*., 2013). Production of IL-6 at 6 h by THP-1 cells stimulated with live *M. leprae* or *M. bovis* BCG was dependent on pretreatment with 1,25-dihydroxyvitamin D_3 (VD). Parkin knock-down cells treated with VD failed to induce IL-6 by mycobacteria. Toll-like receptor-(TLR)-mediated transduction (via LPS treatment) required for IL-6 induction such as phosphorylation of IκB-α and induction of levels of nuclear IκB-ζ were not affected by parkin silencing. Other signalling events such as phosphorylation of MAPK ERK1/2 and p38 were unaffected by parkin silencing, while JNK activation was increased but failed to explain the altered cytokine production. Finally, the genetic risk factors of leprosy within the *PARK* promoter correlated significantly with *M. leprae* sonicate-triggered *CCL2* and *IL6* transcript levels in whole-blood assays (de Leseleuc *et al*., 2013). Although these results would indicate that parkin does not affect LPS-induced NF-κB and is independent of the mitogen-activated (MAP) kinase pathway,

they were the first to associate genetically controlled changes in the production of MCP-1/*CCL2* and IL-6 with known factors for leprosy susceptibility.

Recent work designed to assess the overall combined contribution of significantly associated SNPs has suggested that epistatic SNP–SNP interactions involving PARK2 and cytokine genes added an additional dimension to leprosy susceptibility. *In silico* protein–protein interactions of PARK2 and crucial pro-inflammatory molecules indicate that PARK2 is a key component of immune regulation, modulating the production of different cytokines on infection (Chopra *et al*., 2014). This underscores the immense complexity of host immune components that need to be unravelled in order to accurately ascribe a genetic composition that defines a phenotype of leprosy susceptibility.

SLC11A1/NRAMP

The gene encoding the solute carrier family 11 member 1 (*SLC11A1*, formerly *NRAMP1*) is located on chromosome 2q35. *SLC11A1* is the human homologue of the mouse gene *Slc11a1*. Mice with a naturally occurring Gly169Asp mutation are susceptible to a range of intracellular pathogens including *Leishmania donovani*, *Salmonella typhimurium*, some strains of *M. bovis*, *M. lepraemurium*, *M. intracellulare*, *Toxoplasma gondii*, *Candida albicans* and *L. infantum*, but probably not *M. tuberculosis* (Fitness *et al*., 2002). This substitution of a charged amino acid in one of the putative transmembrane domains may cause misfolding of the Slc11a1 protein, resulting in it being targeted for degradation at the endoplasmic reticulum. Null mutants have the same susceptibility phenotypes as those mice carrying the naturally occurring point mutation, indicating that the point mutation results in a completely non-functional gene product.

Antigen-presenting cells (APC) containing the mutant Slc11a1 are defective in antigen processing for presentation to T cells, most likely because of a metal ion requirement for metalloprotease activity and/or endosomal fusion events. The function of Slc11a1 appears to be involved in depletion of divalent cations in phagosomes that are essential for microbial survival therein (Cellier *et al*., 1994; Jabado *et al*., 2000). In addition, Slc11a1 appears to influence MHC Class II molecules and cytokines regulating antigen presentation, such as TNFα and IL1β. Such effects may account for the polarity of Th1 versus Th2 responses observed in mice with wild-type Slc11a1 compared to those with the mutant gene after infection with *L. donovani* or exposure to typhoid toxin.

The evidence that *SLC11A1* is associated with leprosy susceptibility is not clear-cut. The fact that non-random segregation of the haplotype occurs among South-east Asian leprosy pedigrees has implicated *SLC11A1* in leprosy susceptibility; however, while this haplotype allotment was more pronounced in Vietnamese cohorts it was not shared among four Chinese families, suggestive of the possibility of ethnic heterogeneity (Abel *et al*., 1998). Ethnic heterogeneity may explain why studies in other populations have failed to find *SLC11A1* linkage or association with leprosy susceptibility per se (Shaw *et al*., 1993; Roger *et al*., 1997; Roy *et al*., 1999; Meisner *et al*., 2001). In studies of a cohort in Mali, heterozygocity for an insertion or deletion of a microsatellite sequence (TGTG) in the 3′ untranslated region of SLC11A was common among multibacillary cases (Meisner *et al*., 2001). It is currently unknown whether the microsatellite has any functional effect.

The *SLC11A1* region has been found to be in linkage with the Mitsuda response among both healthy and affected members of 20 South-east Asian leprosy families (Alcais *et al*., 2000). The Mitsuda response measures the size and extent of a reaction obtained between 28 and 30 days after intradermal injection of *M. leprae* lepromin antigen. It is regarded as a measure of the *M. leprae*-induced granuloma-forming capacity. Thus, this finding is in agreement with the hypothesis that, in addition to, or instead of being important in control of mycobacterial infection at the macrophage level, *SLC11A1* may play a role in the development of acquired antimycobacterial immune responses in humans. Further evidence will be required to formulate a definitive correlation of genetic composition with disease susceptibility.

NOD2

The nucleotide-binding oligomerization domain 2 receptor (NOD2) is an intracellular sensing molecule that recognizes bacterial cell wall petidoglycan and muramyl dipeptide motifs (Franchi *et al*., 2009). It is expressed in macrophages and epithelial cells. Ligand-bound NOD2 initiates signalling cascades mediated by RIPK2 through an ubiquitination process involving the recruitment of TAK1 (transforming growth factor β-activated kinase 1) and NEMO (nuclear factor κB (NF-κB) essential modulator) to the NOD-RIPK2 complex (Hitotsumatsu *et al*., 2008). Iκb proteins encoded by *NFKBIA* and *NFKBIB* (nuclear factor of kappa light chain polypeptide gene enhancer in B cells inhibitor alpha and beta, respectively) become degraded, leading to the migration of various combinations of subunits of the NF-κB complex to the cell nucleus and subsequent activation of NF-κB target genes such as TNFSF15 (Gonsky *et al*., 2013).

Four single-nucleotide polymorphisms have been associated with leprosy susceptibility: two SNPs are located intragenically and two SNPs (rs9302752 and rs7194886) are located between *NOD2* and its neighbouring 5′ gene *SNX20* (which encodes the sorting nexin 20 protein). The two intergenic SNPs are more strongly associated with leprosy. Rs9302752 and rs7194886 are thus likely to be regulatory variants on *NOD2* expression.

TLRs

TLRs are single-pass transmembrane proteins that mediate cell signalling during innate immune responses. They are key mediators of immediate host responsiveness to a wide range of potential pathogenic structures known as pathogen-associated molecular patterns (PAMP), mediating the activation of transcription factors and determining the pattern of subsequent secretion of pro-inflammatory cytokines and chemokines. Aberrancy in the TLR pathway is associated with disease and inflammation. It has been shown that TLR1 and 2 and TLR2 and 6 form heterodimers and are involved in *M. leprae* antigen recognition (Krutzik *et al*., 2003). TLR2 is involved in monocyte/macrophage activation and cytokine production in leprosy (Krutzik *et al*., 2005). TLR2 is also expressed on Schwann cells, a primary target of *M. leprae*, and has been associated with increased apoptosis, indicating a possible mechanism by which activation of innate immunity may contribute to nerve injury (Oliveira *et al*., 2003).

TLR 1, 2 and 6 are related phylogenetically. TLR1 and 6 have high sequence homology suggestive of a recent gene duplication event. While TLR1/TLR2 has been shown to be involved in *M. leprae* antigen recognition, TLR 6 plays a role in *M. leprae* persistence in Schwann cells (Mattos *et al*., 2011). Hence, variation in any of these genes is likely to modulate the host's innate immune response which may predispose an individual to mounting an ineffective or excessive inflammatory response. A *TLR1* polymorphism (rs5743618; I602S) showed a protective effect for the type 1 reaction involving 933 Nepalese leprosy cases when compared to a control group comprising a mixture of type 2 reactions and reaction-free patients with all forms of leprosy. Given that this allele drives a lower production of pro-inflammatory mediators, this association is not surprising in the least. In a study of two independent samples from India, the rs5743681 allele associates with a protective factor against leprosy per se. Another *TLR1* allele (rs4833095; N248S) was shown to be associated with protection factor against type 2 reactions. However, problems with this work are that although the sample size was large (842 cases), the analysis of type 2 reactions focused on only 11 cases. Additionally, the control group was mostly composed of paucibacillary leprosy cases which are not at risk for type 2 reactions.

The studies reviewed herein are consistent with those of the two-step model for the development of leprosy, in which successful infection of *M. leprae* is first established in genetically predisposed persons, and the subsequent clinical manifestation of disease is influenced by other host factors and environmental factors (Casanova and Abel, 2002). Genome-wide association studies that directly test for a genetic association with the multibacillary or the paucibacillary form may uncover additional host genetic factors involved in the second step of disease development.

In summary, *M. leprae* can cause very different disease phenotypes in humans, probably

due to individual variation in genetic profile and, consequently, in immune responses. Of the many reports of genes associated with leprosy, relatively few have been replicated in additional study populations. Further studies, involving a large number of genetic factors in populations from different parts of Brazil and other parts of the world, should be conducted to elucidate the interactions between these factors, which may be useful in the prognosis and clinical evolution of leprosy patients and provide further insight into the host immune mechanisms and dynamics necessary to provide immunity to *M. leprae*. The combination of environmental, microbial and nonmicrobial (socio-economic) factors and host genetic and nongenetic factors ultimately determines the outcome of exposure and infection. Whatever the relative contributions of these factors, the occurrence of clinical disease implies that the host defence to *M. leprae* has failed.

Antibiotic Therapy for Leprosy

Prior to the 1940s, there were few options for treating leprosy. One treatment that was used for decades was an oil extracted from *Hydnocarpus wightiana*, or chaulmoogra oil, for oral ingestion or injection into the lesions of the skin. Although it may have had a slight mycobacteriostatic effect, it mainly stimulated mild inflammation and fibrosis at the injection site. In the 1940s several antibiotic compounds, including sulfonamides and chemically related sulfones, were tested in patients residing at Carville. These antibiotics had very little effect, but in 1941 Dr Guy Faget gave a sulfone derivative, called Promin, to another group of 22 patients. The results were slow but dramatic, with clearance of nodules in early cases within 6 months, and remarkable improvement in patients with longstanding disfiguring disease in 2–3 years (Faget *et al*., 1943, 1946). In five patients, the bacterial index (BI) was reduced to zero, and in the remainder, the progression of the disease was completely stopped. The drug therapy was hailed as the 'Miracle at Carville', and soon there were other sulfone derivatives including Diasone® (developed by Abbott Laboratories) and solapsone (developed by Burroughs Wellcome) that were tested and found to be effective. By 1947, 179 Carville patients were taking Promin, while 162 were on Diasone (Stein and Blochman, 1974). Within 6 years of beginning treatment with this class of drugs, 35 of the patients on Promin were well enough to be discharged, while another ten on Diasone were discharged. Another related drug, called dapsone, had been used successfully to treat streptococcal mastitis in cows, but was initially thought to be too toxic for use in humans. However, studies in several countries using a lower dose of 100 mg per day was found to be just as effective, and soon it became the standard monotherapy treatment drug of choice for leprosy. Although it is now realized that the use of a single antibiotic for long-term treatment of mycobacterial disease (including leprosy, which for some individuals lasted for more than 10 years) was inherently risky and would eventually generate drug-resistant strains, it was not until 1964 that reports of drug resistance to dapsone were recognized (Pettit and Rees, 1964). Fortunately, two new classes of drugs for the treatment of leprosy came on the scene, rifampicin, a very effective bacteriocidal mycobacterial drug, and clofazimine, a fat-soluble dye now known to have marked anti-inflammatory and immunomodulatory effects. These three drugs were incorporated into the standard recommended World Health Organization (WHO) multidrug therapy (MDT) regimen to treat all forms of leprosy beginning in 1982, and the drugs have been provided free of charge to any person diagnosed with leprosy worldwide for many years by WHO. The drugs were donated previously by The Nippon Foundation and the Sasakawa Memorial Health Foundation (1995–1999) and currently by Novartis and the Novartis Foundation for Sustainable Development (2000–2015).

Current Worldwide Leprosy Situation

The most recent statistics on global leprosy showed that there were 232,857 new cases detected in the world in 2012, an actual increase of 2.7% over the number of new cases detected in 2011 (WHO, 2013). There were only 16 countries remaining that reported more

than 1000 new cases (95% of the total), indicating that the disease is slowly being limited to a smaller number of countries. The three countries with the highest numbers of new cases were India (134,752), Brazil (33,303) and Indonesia (18,994), which combined represent 80% of the total global burden. The proportion of grade 2 disabilities (G2D) among new cases is seen as an indicator of awareness within the community and the capacity of the public health system to detect new cases early enough to prevent the development of deformities. *The Enhanced Global Strategy for further reducing the disease burden due to leprosy (2011–2015)* (WHO, 2015b) set as a target the reduction of the G2D rate by one-third of that present in 2010 (0.23 per 100,000 population). However, with 14,409 cases of G2D reported in 2012, this was actually an increase to 0.25 per 100,000 population. Thus, the trends indicate that the numbers of new cases and those with G2D are either stagnating or increasing.

Focus on leprosy detection efforts in Brazil

Currently, Brazil has a new case detection rate of 1.5 per 10,000 population, meaning that it is the only country remaining in the world that has not met the WHO goal of less than one new case per 10,000 population. Leprosy prevalence in Brazil varies tremendously regionally. Using data supplied by SINAN, the national database for reportable diseases[1], we have determined the new case detection rates for all 5567 Brazilian municipalities (Fig. 26.1), and the detection rate per 100,000 population for each state (Fig. 26.2), showing this regional clustering. As can be seen, there are few cases detected in the southern states of Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS). However, there is high new case detection in the central-western regions – Mato Grosso (MT) and Goiás (GO); the north – Amazonas (AM), Acre (AC), Rondônia (RO), Tocantins (TO) and Pará (PA); and the north-east – Maranhão (MA), Piauí (PI), Ceará (CE), Pernambuco (PE) and Bahia (BA). Pará, the second largest state in Brazil, has had a historically documented huge burden of untreated or asymptomatic hidden cases in many poor cities, with surveys revealing that 4% or more of schoolchildren and household contacts can be diagnosed with clinical signs and symptoms of disease (Barreto *et al*., 2011, 2012, 2014; Salgado *et al*., 2012) (Fig. 26.3). Those states with the highest new case detection rates have only 17% of the total population of Brazil, but have 53.5% of all of the new cases of leprosy (Penna *et al*., 2009a,b). In addition, the number of cases of G2D (2234, 6.7%) and the number of cases reported in children (2246, 6.7%) were both relatively high. Leprosy in children is correlated with community-level factors, including the recent presence of disease and active foci of transmission in the community.

Use of a Geographic Information System (GIS) and spatial analysis tools to track leprosy

We have coupled the use of a GIS to locate areas of high and low prevalence and identify 'hot pockets' within these hyperendemic settings (Barreto *et al*., 2014) in the northern state of Pará. The use of GIS has been emphasized by the WHO (2015a) as one of the tools for leprosy elimination that can 'provide a graphical analysis of epidemiological indicators over time, the spatial distribution and severity of the disease, identify pockets of high endemicity and indicate where there is a need to target extra resources'. We have been able to map index cases and those with the highest anti-PGL-I titres in schoolchildren and household contacts (therefore, those with the greatest risk to developing disease) and are beginning to understand how the disease is spread from multiple foci of infection in space and time. One of the main findings of our studies is that children with leprosy or those with subclinical infection were in close proximity to spatial and temporal clusters of leprosy cases in surveyed cities (Barreto *et al*., 2014). We believe that these findings can be applied to guide and enhance leprosy control programmes to target intervention to these specific areas more systematically.

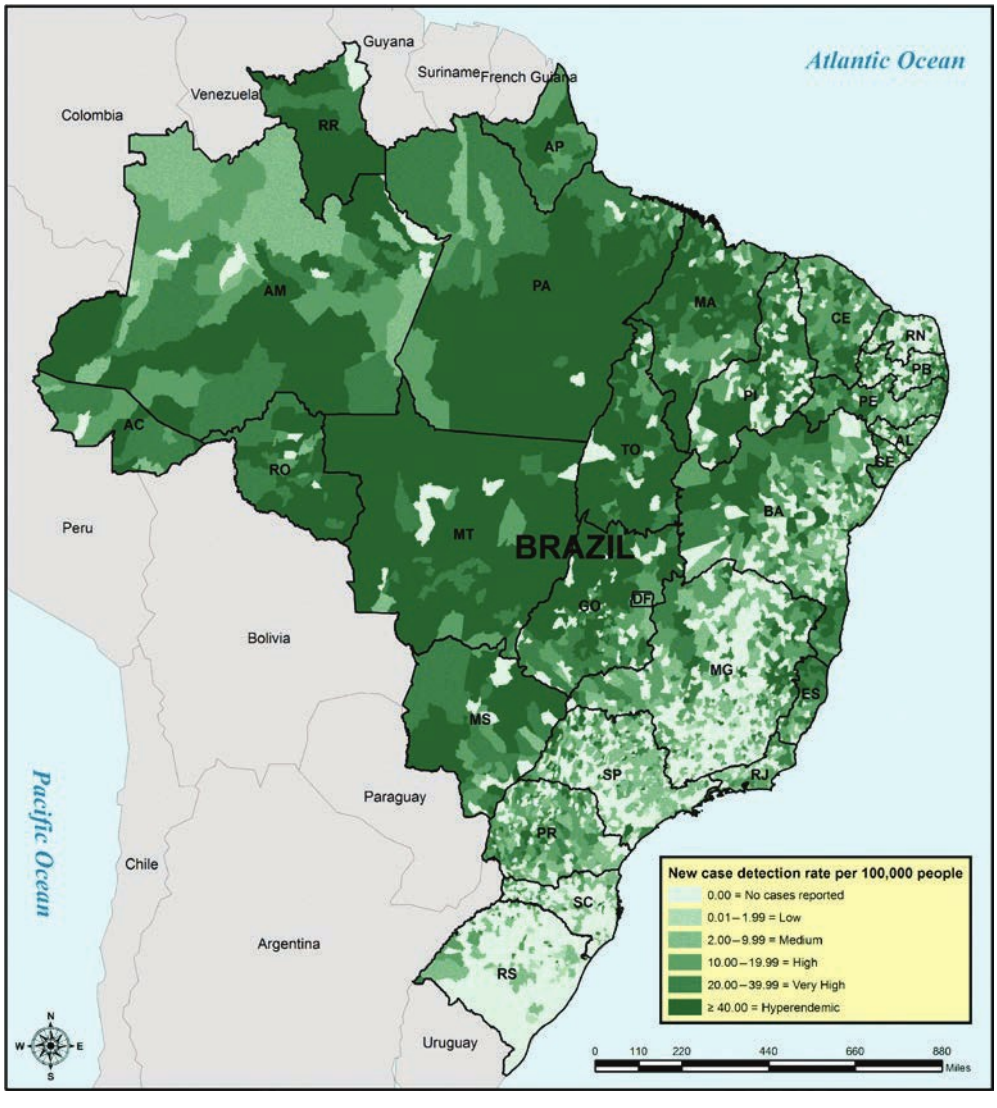

Fig. 26.1. Leprosy distribution in Brazil by municipality, 2012. New case detection rate per 100,000 population (NCDR) for each municipality of the country in 2012. Of the 5567 Brazilian municipalities, 3345 (60%) reported at least one new case in 2012. The NCDR ranged from 0 to 506.9 (Marcelândia – MT). The Brazilian Ministry of Health classifies those municipalities with a NCDR ≥40 as hyperendemic. (From data collected from SINAN/SVS-MS in April 2014.)

Future Prospects: Development of Tests for the Early Diagnosis of Leprosy

Since the discovery of the *M. leprae*-specific glycolipid antigen PGL-I in the early 1980s (Brennan and Barrow, 1980; Hunter and Brennan, 1981), native or synthetic PGL-I has been used in the development of many different serological tests to test for leprosy infection. Assessment of antibody titres to PGL-I have been shown to be a useful tool in surveys of leprosy prevalence, and correlate closely with the bacterial load in patients. In endemic countries such as Brazil, simple ELISA assays

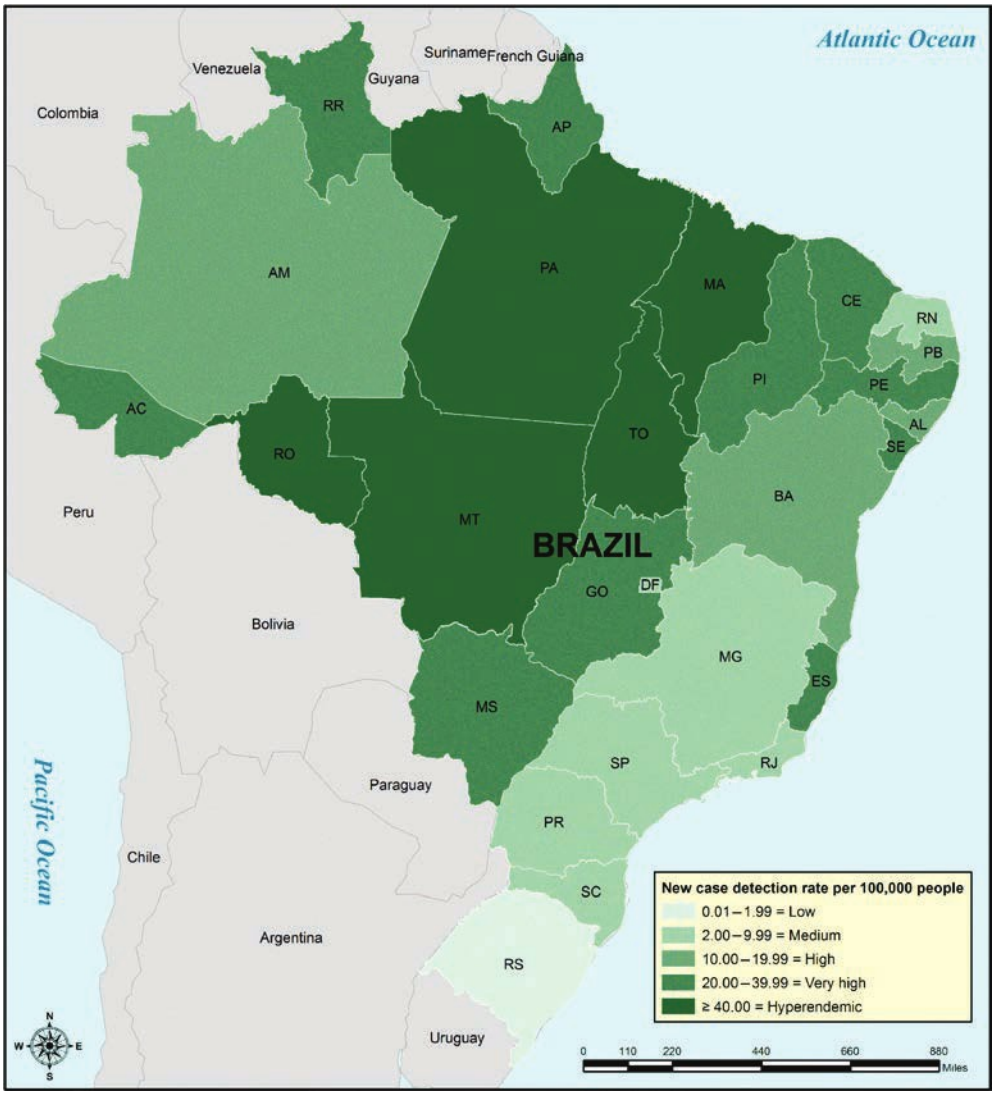

Fig. 26.2. Leprosy distribution in Brazil by state, 2012. New case detection rate per 100,000 population (NCDR) for each state in the country in 2012. All states reported new cases in 2012, but there is an important regional heterogeneity in the spatial distribution of the cases. The NCDR ranged from 1.36 (RS) to 80.34 (MT). Five states were classified as hyperendemic according to the Brazilian Ministry of Health parameters (NCDR ≥40), including Pará (PA), Maranhão (MA), Tocantins (TO), Mato Grosso (MT) and Rondônia (RO). (From data collected from SINAN/SVS-MS in April 2014.)

are used in many leprosy reference centres to assist in the classification of patients for correct treatment (MB versus PB), to monitor the titre during and after treatment, and to determine which household contacts of index cases are most at risk by having a high titre to PGL-I (Buhrer-Sekula *et al*., 2008; de Moura *et al*., 2008). In endemic settings, studies suggest that the true number of leprosy cases may be six times higher than officially reported (Moet *et al*., 2008). The state of Pará, Brazil, has very high new case detection rates in the general population (51.1/100,000 population) and among children less than 15 years

Fig. 26.3. Performing surveys of schoolchildren and household contacts in a rural area in Oriximiná, Pará, Brazil. Clockwise from upper left: Dr Claudio Salgado and his survey team visit a rural school to examine schoolchildren; a visit to the home of a diagnosed child from a school to examine household contacts; physiotherapist Layana Guimarães assessing muscle strength of fingers of the hand of a child who was diagnosed with multibacillary disease; Dr Salgado writing up a prescription for MDT, which will be provided to the child at her school free of charge; close-up of many hypopigmented anaesthetic lesions on the shoulder and arm of a child; nurses at the school with blister packs of MDT medication.

old (18.3/100,000 population). These rates can be considered an underestimate of the real situation because only 42% of the population is covered by the primary health care service responsible for leprosy control implementation and active case finding. Still, one issue that arises in hyperendemic areas is that when 4% or more of a population are diagnosed with clinical signs and symptoms of leprosy, upwards of 40–50% of the general population in

these areas will have a positive titre to PGL-I (Barreto *et al*., 2011). Since the majority of these positive individuals will not succumb to disease, the prognostic value of a positive PGL-I ELISA test is debatable. For this reason, active case finding in these communities and follow-up of household contacts of index cases and those with demonstrated high titres to PGL-I, as well as coordinating the finding of new cases with the local community health agents, are all key components of a functional leprosy control programme.

Other protein antigens are being examined to determine if there are better prognostic indicators of biomarkers of infection and disease progression. These include the fusion protein antigen LID-1 developed by the Infectious Disease Research Institute (IDRI) in Seattle, WA. It has been shown that those who progress to disease can show a positive titre to this antigen up to 1 year or more prior to being diagnosed with leprosy (Duthie *et al*., 2007; Spencer *et al*., 2012). We have actually seen high antibody titres to LID-1 in a family member living with a multidrug-resistant index case that had no signs or symptoms of disease for 3 years, but then was diagnosed with severe LL disease. The high titre to LID-1 3 years prior to diagnosis was likely to be due to a high BI that existed somewhere (possibly in the nerves); it just took years for obvious skin lesions to develop. These are the kinds of hidden cases of leprosy that some clinicians in the leprosy community believe should be treated prophylactically to prevent the spread from asymptomatic individuals. Working with Drs Steven Reed and Malcolm Duthie of IDRI, we are evaluating a new antigen, LID-NDO® (Infectious Disease Research Institute (IDRI), Seattle, WA), which couples the synthetic disaccharide of PGL-I to the fusion protein LID-1 (Paula Vaz Cardoso *et al*., 2013; Duthie *et al*., 2014). This composite glycoprotein appears to be more sensitive at detecting antibodies in leprosy patients than the use of either antigen alone, and has been used in a rapid lateral flow diagnostic test developed by OrangeLife, a Brazilian company, and In-Bios International, Seattle, WA, for evaluation in field conditions. It is hoped that a simple, rapid, field-friendly test can be used to assist clinicians in diagnosing leprosy early before the development of nerve damage and disability.

Note

[1] SINAN (Sistema de Informação de Agravos de Notificação) is Brazil's national database for reporting infectious diseases in Brazil, including leprosy. Their website can be found at http://dtr2004.saude.gov.br/sinanweb.

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27 Animal Models for Leprosy Research

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Introduction

Although *Mycobacterium leprae*, discovered by Armauer Hansen in 1873, was one of the first microorganisms to be associated with a human infectious disease, it has yet to be cultured in axenic medium. For nearly a century, attempts by numerous investigators to cultivate the organism outside the human host, or to develop an animal model that accurately recapitulates leprosy, were met with frustration and failure. Animals infected (reviewed in Johnstone, 1987) included the typical laboratory species such as various strains of mice, rats, guinea pigs, gerbils, hamsters, rabbits and monkeys, as well as an assortment of other mammals (e.g. dogs, cats, pigs, armadillos, chinchillas, fruit bats, lemmings, voles, possums, chipmunks and hedgehogs), birds (e.g. pigeons, chickens, paddy birds, canaries, parrots and love-birds) and cold-blooded animals (e.g. eels, fresh and salt water fish, tadpoles, frogs, turtles, snakes, lizards and alligators). The variability seen in success rates for cultivation in these animals may have been due partly to the low quality of the *M. leprae* inoculum that was used, as it was largely crude patient-derived biopsy material of unknown concentration and viability. In addition, early investigators used a myriad of experimental protocols which often did not take into account the prolonged growth cycle of *M. leprae* or its preference for cooler temperatures for optimum growth. The seminal reports by Shepard (1960), describing the reproducible cultivation and passage of *M. leprae* in the foot pads of mice, and of Kirchheimer and Storrs (1971), detailing the development of lepromatous leprosy in the nine-banded armadillo, initiated approaches for research in leprosy that were previously unattainable. This chapter will focus on the advances and opportunities offered by the mouse and armadillo models of *M. leprae* infection.

Mouse Model

Mice (*Mus musculus*) are undoubtedly the most widely used experimental animal in medical research. They are small, relatively inexpensive mammals which are easy to house and maintain. They are prolific breeders with a short gestation time. There are hundreds of well-defined strains, including outbred, inbred and genetically engineered mutants, which can be purchased from several commercial vendors worldwide. Perhaps most importantly, there is an ever-growing multitude of

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

sophisticated biological reagents available that enable complex and detailed examination of physiological and immunological processes.

Infection Strategies

Foot pad infection

The cultivation and passage of *M. leprae* in the foot pads of immunocompetent mice were first reported by Shepard (1960) and subsequently confirmed by him and others (Shepard, 1962; Rees, 1964; Pattyn, 1965). The foot pad was chosen for infection primarily because of its cool temperature and other mycobacteria had been successfully cultured in this site (Fenner, 1956). Shepard found that if a low number of *M. leprae* ($\leq 10^4$) were inoculated into the foot pad, the bacilli would multiply locally with a doubling time of ~13 days until reaching a peak of ~10^6 organisms at 6 months post-infection. The growth in the foot pad was limited by the host immune system (Welch *et al*., 1980), and inoculation with a larger number of *M. leprae* immunized the mouse. Although time-consuming and expensive, this technique was highly reproducible and enabled the establishment and maintenance of isolates of *M. leprae*. Moreover, drug testing, detection of drug resistance, experimental vaccine evaluation and immunological studies became feasible.

In efforts to develop murine models more representative of human lepromatous leprosy (LL), various investigators applied this 'mouse foot pad (MFP) assay' to immunodeficient strains. The importance of T cells in host defence against leprosy was firmly established upon infection of thymectomized and irradiated mice (Rees, 1966; Ebenezer *et al*., 2002), congenitally athymic mice (Colston and Hilson, 1976; Chehl *et al*., 1983; Dawson *et al*., 1983) and SCID mice (Yogi *et al*., 1991; Azouaou *et al*., 1993; Ishaque and Sticht-Groh, 1994). In these immunosuppressed strains, multiplication of *M. leprae* in the foot pad can reach 10^{10} or more bacilli. Athymic *nu/nu* mice are now routinely used for culture of highly viable *M. leprae* for experimental use (Truman and Krahenbuhl, 2001).

Intra-nerve infection

Although *M. leprae* does not readily infect murine Schwann cells *in vivo* and does not manifest neurological involvement in a natural infection, bacilli can be injected directly into the nerve. Shetty *et al*. inoculated 10–20 × 10^6 viable *M. leprae* into the sciatic nerve of immunocompetent and immunosuppressed mice (Shetty, 1993; Birdi *et al*., 1995; Shetty *et al*., 1995, 1999). They saw little change in the number of *M. leprae* recovered over time and a striking decrease in bacterial viability in the organisms recovered. The lack of survival and limited multiplication of the organisms was likely to be due to the warm temperature of the mouse sciatic nerve. Although Schwann cells were not infected, a tuberculoid-like granulomatous response was elicited in normal, unsensitized, Swiss white mice whereas unsensitized immunosuppressed thymectomized and irradiated mice developed a macrophage response. In later studies using live and dead *M. leprae* or whole cell wall fraction in either immunocompetent mice or *Rag1*−/− mice (which lack both T and B cells), Rambukkana *et al*. (2002) reported substantial demyelination compared to mice inoculated with buffer alone. Therefore, although intraneural inoculation does not represent a natural infection with *M. leprae*, this route of administration has potential for investigation of both immunologically and non-immunologically mediated nerve damage in leprosy.

Ear infection

Similar to foot pads, ear pinnae in mice are cooler than the rest of the body and investigators studying cutaneous leishmaniasis have shown that they have similar utility to the foot pad for modelling immune responses (Baldwin *et al*., 2003). Mice supported limited growth of *M. leprae* in ears over 30 weeks, and the draining lymph nodes showed marked cellular infiltration (Duthie *et al*., 2007). There was also an increase in the numbers of IFNγ secreting CD4+ T cells at the site of inoculation in the ears over time, suggesting a local inflammatory response.

Applications of the Mouse Model

Studies of the granuloma

The primary cell populations that respond to infection with *M. leprae* are T cells and macrophages. However, the dynamics of their interactions within the microenvironment of the lesion are not fully understood. With a chronic disease such as leprosy, which has an extremely slow progression and can remain asymptomatic for years between initial infection and diagnosis, it is particularly difficult to define the early cellular interactions in the immune response. Murine foot pad infection is the most accessible method for investigating the *M. leprae*-induced granuloma.

Immunocompetent mice

In addition to permitting only limited growth of *M. leprae*, the histopathological changes that occur in the foot pad of conventional, immunocompetent mice are rather minimal (Shepard, 1960). The cellular infiltration into the foot pad is composed largely of macrophages and epithelioid cells which become more vacuolated as infection progresses and bacterial replication ceases, suggesting macrophage activation (Evans and Levy, 1972). Numerous lymphocytes are present but neutrophils are rare and there is no necrosis in the tissue. Nerves are not infected although there have been reports of neural damage very late (>2 years) in infection (Rees *et al*., 1969). Bacilli are primarily intracellular, often in clumps, and become increasingly more beaded as the infection proceeds past the peak of bacterial growth.

Immunosuppressed mice

In contrast, athymic *nu/nu* mice exhibit features of LL disease. As infection progresses over the course of several months there is an influx of macrophages into the foot pad to accommodate the multiplying bacilli, eventually becoming a huge 'leproma' consisting of heavily infected foamy macrophages that replaces almost all of the normal tissue (Chehl *et al*., 1983). These macrophages can contain hundreds of bacilli, emphasizing the innocuous nature of this organism (Hagge *et al*., 2004). Bacilli are also found in striated muscle cells and eventually in perineural cells and fibroblasts. Dissemination is rather slow, but late in infection (>1 year) bacteria can be found in virtually all tissues except the central nervous system.

Genetically engineered mice

The availability of genetically engineered mice has greatly enhanced the utility of the mouse model for biological research. Currently, hundreds of targeted gene knockout (KO) mouse strains are commercially available, as well as strains with conditional KO, tissue-specific KO, multiple KO and knockin mutations. Mice with defects in pathways important in host defence, especially those with deletions in specific cytokines, chemokines, cytokine and chemokine receptors, various immune modulators and cell surface markers, have been particularly useful in the study of a variety of infectious diseases. With leprosy, where the manifestations along the spectrum are largely due to the immune response of the infected host and also perhaps to the compensatory mechanisms evoked during an inadequate response, mice with deletions at specific points of the immune cascade have been especially enlightening for improving understanding of *M. leprae* pathogenesis. Furthermore, these murine strains have enabled immunological investigations that had heretofore been problematic due to the long, protracted course of *M. leprae* infection. Finally, additional modifications can be induced in the mice. Double KO can be generated either by treating with various inhibitors or antibodies or even crossbreeding KO strains (Hagge *et al*., 2014). Conversely, a KO function can be restored after infection using cytokine therapy or adoptive transfer of competent immune cells. Thus, these mouse models have been used to explore cell-mediated immunity to *M. leprae* both *in vivo* and *in vitro* and to study the microenvironment of the granuloma in leprosy pathogenesis.

Two foot pad infection protocols have been utilized to examine the host response to

the bacilli. The Shepard MFP assay allows bacterial growth to be monitored as well as granuloma development, both histopathologically and by immunohistochemical staining, for up to 18 months' infection. An alternate higher-dose protocol permits the cellular composition of the leprosy lesion to be explored more thoroughly. Mice are injected in the foot pad with $>10^6$ *M. leprae* and foot pad induration can be measured for changes throughout infection. Foot pad tissues can be harvested at key intervals and examined for cytokine and chemokine expression by real-time RT-PCR, and for cell phenotypes by histopathological and immunohistochemical analyses. Cells can also be isolated directly from the granulomas and analysed by flow cytometry and ELISA for cell surface marker expression and/or cytokine production after *in vitro* culture and stimulation with *M. leprae* antigens. Consequently, the long-term effects of chronic *M. leprae* infection on the granulomatous response have been evaluated (Krahenbuhl and Adams, 2000; Cooper *et al*., 2002; Adams *et al*., 2012).

The host cell-mediated immune response to foot pad infection with *M. leprae* has been evaluated *in vivo* and *in vitro* in >10 KO strains of mice. Since a strong cell-mediated immune response is important for controlling leprosy, in contrast to the humoral response, most studies are in mice with deficiencies in cytokines and products important in Th1 development (Table 27.1). Interestingly, when comparing the findings with intact immunocompetent control mice with high cell-mediated immunity and athymic *nu/nu* mice with virtually no cell-mediated immunity, it was found that no KO of a single cytokine, T cell type or antimicrobial mechanism transformed any of these strains into a totally immunocompromised model towards *M. leprae* like the athymic *nu/nu* mouse. However, based on their unique characteristic profiles, most KO strains could be placed along the leprosy spectrum. These findings insinuate the presence of alternative or compensatory mechanisms of host resistance in these animals, the very mechanisms which may be occurring in the borderline region of the leprosy spectrum.

Compared to wild type mice, IL12/23p40 KO mice exhibited a decreased ability to control *M. leprae* growth and evidenced reduced foot pad induration with altered CD4+ and CD8+ T cell composition due to the lack of protective IL-12 and proinflammatory IL-23, respectively (Adams *et al*., 2012). IFNγ KO also allowed augmented growth of *M. leprae*, a massive, non-invasive cellular infiltration into the foot pad, and a TH2-type cytokine response (Adams *et al*., 2002). Mice lacking TNF and LTα, two closely related cytokines, gave very contrasting responses (Hagge *et al*., 2009). TNF KO and TNFR KO demonstrated extensive lymphocytic infiltration, primarily of activated CD4+ T cells, yet growth of *M. leprae* was augmented throughout the infection period. In contrast, LTα-deficient mice were unable to recruit lymphocytes into the infected foot pad, develop and maintain granulomas or optimally control the growth of *M. leprae* in the chronic stage. Mice that have a mutation in the respiratory burst oxidase *phox91* gene exhibit responses to *M. leprae* infection which are similar to wild type mice (Hagge *et al*., 2007). In contrast, NOS2 KO controlled growth of *M. leprae* similarly to control mice but developed a large, destructive granulomatous response and a concomitant strong Th1 cytokine and chemokine response (Adams *et al*., 2000; Cooper *et al*., 2002).

In addition, of great interest was the disparity in the T cell populations between the foot pad granuloma and the draining lymph node (Hagge *et al*., 2007; Adams *et al*., 2012). Upon infection with *M. leprae*, the granuloma that developed in the foot pads of immunocompetent mice had a very characteristic lymphocyte profile, consisting primarily of T cells of the activated CD4+ phenotype. Lymph nodes, in contrast, generally contained more B cells than T cells and equal numbers of CD4 and CD8 cells which were of the naïve phenotype. Therefore, cell populations in the foot pad were distinct from those in the draining lymph node, underscoring the differences in systemic responses from that of the leprosy lesion itself. However, examining lymph node profiles has been valuable with strains that yielded little granulomatous response in the foot pad (e.g. LTα-deficient mice (Hagge *et al*., 2009)).

Table 27.1. Attributes that have been assessed to classify KO mouse strains along the leprosy spectrum.

	B6	Cybb	IL10	NOS2	LTα	TNF	TNR1	CD8	CD4	IFNγ	p40	*nu/nu*
ML growth	+	+	+	+	++	+++	+++	++	+++	+++	+++	++++
Granuloma/Induration	+/–	+	+	++++ destruc	+/–	+++ destruc	+++ destruc			+++ unorg	+/–	++++ leproma
Lymphoid	CD4> CD8	CD4> CD8	CD4 > CD8	↑CD4	few	↑CD4	↑CD4	CD4	CD8	↑CD4	↑CD8	–
Myeloid	Epith MΦ	Epith MΦ	Epith MΦ	Epith MΦ	Spindle MΦ			Epith MΦ		↑PMN	Epith MΦ	Foamy MΦ
Cytokine	Th1	Th1	Th1	↑Th1	↓Th1	↑Th1	↑Th1			Th2	↓Th1	
Class	Ind	Ind		BT		BT	BT	BT	BB	BB-BL	BL	LL

Enumeration and Viability Assays

Acid-fast bacilli (AFB) count versus repetitive sequence in *M. leprae* (RLEP)

The inability to culture *M. leprae* in artificial medium not only impedes the understanding of vital molecular and cellular events in the pathogenesis of leprosy, it also prevents researchers from easily and definitively distinguishing between live and dead bacilli. Until recently multiplication of *M. leprae* could only be determined by direct microscopic counting of AFB over time (Shepard and McRae, 1968). Although this method has numerous drawbacks, such as bacterial clumping, problems with incomplete tissue homogenization, and lack of sensitivity and specificity, analyses of foot pad harvests have shown that, if done by well-trained personnel using a large number of samples, reliable and reproducible results can be obtained (Krushat *et al*., 1976). Theoretically, this method allows counting of ~5000 bacteria/ml of tissue homogenate. There is no way, however, to differentiate *M. leprae* from other acid-fast staining bacilli. Therefore, additional tests must be conducted on clinical samples if a mixed infection is suspected.

A *M. leprae*-specific real-time PCR-based method can increase the sensitivity of *M. leprae* enumeration in tissue samples by at least tenfold. The *M. leprae* chromosome contains a minimum of 28 copies of a dispersed repetitive sequence, RLEP, which contains an invariant 545-bp core flanked by variable structures of 100-bp at the left and 44- to 47-bp at the right (Woods and Cole, 1990). The 545-bp core sequence is highly specific for *M. leprae* and a TaqMan®-based (Life Technologies, Grand Island, NY, USA) PCR assay has been developed to amplify this region for enumeration of *M. leprae* from MFPs, armadillo tissues and clinical samples (Truman *et al*., 2008). This assay can detect as few as 300 bacteria/ml of tissue homogenate and is gaining popularity over direct counts.

Determination of *M. leprae* viability

Shepard's MFP assay and RLEP PCR do not distinguish live and dead bacilli and only ascertain relative viability via titration of *M. leprae* suspensions in large numbers of mice over time. Thus, researchers have been eager to find alternate methods to determine *M. leprae* viability.

One of the earliest indices of bacterial viability was the morphological index, which expresses the ratio of solid staining (i.e. viable) bacteria to beaded (i.e. dead) bacteria in a given sample (Ridley, 1971). Researchers also tried, with some success, to score for percentage of viability on the basis of *M. leprae* ultrastructure and correlated this with growth of *M. leprae* in the MFP assay (Silva *et al*., 1984). However, these methods were not particularly reliable as they were highly subjective and dependent on the quality of fixation and staining. Uptake of radiolabelled purines and pyrimidines (e.g. adenosine, hypoxanthine and thymidine) has been reported to be a good indicator for *M. leprae* viability within macrophages, but this method is of little value with *M. leprae* in axenic culture (Harshan *et al*., 1990). Determination of the Na+/K+ ratio in individual bacteria appears to be an indicator of *M. leprae* viability in axenic cultures (Wiese *et al*., 1994) but requires expensive mass spectrometry set up and has never been validated using the MFP assay or other concurrent methods. Measurement of ATP content via bioluminescence also has been reported to have a good overall correlation with growth in MFPs by some groups (Katoch *et al*., 1988; Gupta *et al*., 1997).

The most reliable biochemical method for determining *M. leprae* viability is measuring the rate of palmitic acid oxidation. In this radiorespirometry assay ^{14}C-palmitic acid, as the sole carbon source, is oxidized to $^{14}CO_2$ which is captured and measured daily for 7 days (Franzblau, 1988). The cumulative count correlates extremely well with MFP data. Radiorespirometry is now the most commonly used biochemical method for determining *M. leprae* viability in axenic (Franzblau, 1988), macrophage (Ramasesh *et al*., 1991) and mouse (Truman and Krahenbuhl, 2001) studies.

Differential staining of live and dead bacteria with fluorescent vital dyes is a simple way to score for percentage of viable *M. leprae* in a suspension. The underlying principle is to use two dyes, one of which is able to penetrate bacteria with intact membranes while the other cannot; therefore, the second dye will

only stain bacteria with damaged membranes. Assuming that bacteria with damaged membranes are not viable, one can easily score bacterial viability by differential staining. Fluorescein diacetate and ethidium bromide is one such dye combination which has been found to be satisfactory, although there have been some background staining issues (Kvach *et al*., 1984; Odinsen *et al*., 1986). Another dye combination that has been used extensively and validated with radiorespirometry as well as the MFP assay is Syto9 and propidium iodide (Lahiri *et al*., 2005; Davis *et al*., 2013).

Most of the above methods require a high number of organisms for accurate results. In addition, these tests must be carried out soon after acquisition of purified viable bacilli. These parameters inherently compromise the sensitivity and utility of these methods for clinical studies. The most sensitive method for determining viability of non-cultivable organisms is currently by quantitating transcripts of target genes by RT-PCR. *sodA* message, when normalized using RLEP counts, was a good indicator of *M. leprae* viability in short-term experiments which makes it a suitable viability marker for *in vitro* studies (Martinez *et al*., 2009). A recent study by Davis *et al.* (2013) showed that expression levels of *esxA* and *hsp18* were excellent indicators of *M. leprae* viability *in vivo*. Importantly, these molecular viability assays were performed on nucleic acids purified from ethanol-fixed tissues, making their adaptability to clinical and field samples plausible.

Drug testing

The MFP assay is still the 'gold standard' for evaluation of chemotherapeutic agents against *M. leprae*. There are three different variations in which it is used in this regard, each with its advantages and limitations. The most simple and straightforward among the three protocols is the 'continuous' method (Shepard and Chang, 1962). Foot pads of immunocompetent mice are inoculated with low numbers of live *M. leprae*. Drugs are administered daily throughout the infection period, and drug activity is measured by the percentage or fold inhibition of *M. leprae* multiplication in treated mice compared to placebo controls. The second and most tedious method is the 'proportional bactericidal' method (Colston *et al*., 1978). Groups of mice are inoculated with serial tenfold dilutions of *M. leprae* and drug is administered from day 0 for various lengths of time. Enumeration of bacteria from treated and control mice 12 months post-treatment is used to calculate the proportion of surviving *M. leprae*. The 'proportional bactericidal' method can also be used to determine the efficacy of a particular drug or treatment regimen in leprosy patients. *M. leprae* is obtained from fresh skin biopsies of patients before, during and after treatment and serial tenfold dilutions are inoculated into MFPs. The mice are sacrificed 12 months post-inoculation (without any treatment) and *M. leprae* are enumerated to ascertain the proportion of viable *M. leprae* that was present in the inocula, which in turn will be proportional to the efficacy of the treatment (Ji *et al*., 1993, 1996, 1998). While the 'continuous' method fails to distinguish between bactericidal and bacteriostatic activity, the 'proportional bactericidal' method cannot detect bacteriostatic activity. Thus, these shortcomings are overcome by using the 'kinetic' method (Shepard, 1969; Shepard *et al*., 1971). MFPs are inoculated as in the 'continuous' method but administration of drugs does not start until day 60 when *M. leprae* are assumed to be in their exponential growth phase in the foot pads. Efficacy of a drug is measured by the time lag between treated and control mice in reaching 10^6 *M. leprae*/foot pad. Growth will not resume (or will be extremely slow) after cessation of treatment with a bactericidal drug, whereas with a bacteriostatic drug *M. leprae* growth will resume giving rise to a measurable 'growth delay'.

Recently a variation of the kinetic method was described using athymic *nu/nu* mice instead of immunocompetent mice, since athymic *nu/nu* mice are a more suitable model for LL patients who have very little or no cell-mediated immunity against *M. leprae*. In addition, instead of using 'growth delay' as the measure of drug efficacy, a variety of molecular and biochemical tests was used to determine *M. leprae* viability in treated and control mice at 30 days post-completion of drug treatment. The results clearly showed that a single or even five daily doses of rifampin was not enough to

achieve significant killing of *M. leprae* in athymic *nu/nu* mice (Davis *et al*., 2013), unlike immunocompetent animals. Studies with *M. tuberculosis* have also shown that the host immune response plays a significant role in the outcome of chemotherapy (Chapuis *et al*., 1994; Zhang *et al*., 2011).

Since multidrug therapy (MDT) is the only means of leprosy control currently available, determination of emerging trends of drug resistance in leprosy endemic areas is extremely important, especially with regard to rifampicin which is the most important component in both MDT and ROM therapy. The 'continuous' method for drug evaluation can also be used for drug susceptibility testing of *M. leprae* (Shetty *et al*., 2003), but large quantities of *M. leprae* from patient material are required for the months-long MFP assay. Fortunately, rifampicin resistance can be attributed to specific mutations in the *rpoB* gene of *M. leprae*, as are dapsone and fluoroquinolone (ofloxacin) resistance to that of *folP* and *gyrA* genes, respectively. These mutations occur in specific regions of the target genes called drug resistance determining regions (DRDR) (Williams and Gillis, 2012). Consequently, drug resistance can be rapidly assessed in biopsies of suspected cases by PCR amplification of the target gene DRDR followed by mutation detection by various methods (Cambau *et al*., 2012; da Silva Rocha *et al*., 2012; Williams *et al*., 2013). Direct PCR sequencing of target gene DRDRs is the method of choice for the ongoing global surveillance of drug resistance in leprosy (Williams and Gillis, 2012). However, the MFP assay is still the only method available to ascertain resistance to antibiotics such as clofazimine, a first-line leprosy drug for which the mechanism of action is not yet understood and no target gene(s) is known.

Evaluation of vaccine candidates

Shepard (1965) was the first to use the MFP assay to experimentally test vaccines against *M. leprae* infection, and it is still the animal model of choice to initially screen and evaluate leprosy vaccine candidates. In a typical experiment, groups of mice are vaccinated either subcutaneously or intradermally followed by a low dose (≤10^4) *M. leprae* challenge. Foot pads are harvested from these mice at approximately 6 months post-challenge for enumeration of AFB either by direct AFB counting or RLEP PCR, and protection is calculated as a percentage or fold inhibition of *M. leprae* growth. A single intradermal injection of heat-killed *M. leprae* (10^7) is used as the positive control vaccine for most protection studies (Ngamying *et al*., 2003; Duthie *et al*., 2013). Recently the mouse ear pinnae infection model was also used to evaluate potential vaccine candidates (Raman *et al*., 2009; Duthie *et al*., 2013). In these studies, inhibition of cellular infiltration in the draining lymph node or infiltration of CD4+ cells at the site of inoculation was taken as the end point rather than inhibition of actual growth of *M. leprae*.

Diagnostic assays: insights into antigen responsiveness

Development of a reliable laboratory diagnostic test for early or preclinical leprosy is a top priority as early treatment is essential to prevent nerve damage, deformity and to block transmission. However, sensitivity is a problem because preclinical leprosy presents a minimum immune stimulation. Furthermore, the unknown duration since exposure and the variability in human immune responses confound results. An important advantage of the experimental MFP assay is that it eliminates many of these issues, especially the problems of unknown length of exposure to *M. leprae* and of cross-reactivity with other mycobacterial species that are common in natural hosts. Hence, MFP studies have been exploited in an effort to develop standardized protocols for immunodiagnosis. One such study involved evaluation of the cell-mediated immune response, gauged in terms of IFNγ secretion by splenic lymphocytes, from infected mice stimulated *in vitro* with *M. leprae* antigens (Lahiri *et al*., 2011). In an attempt to model 'subclinical' or early leprosy, various doses of *M. leprae* and lengths of infection were titrated in foot pads. The aim was to establish the minimum dose at the earliest time

point required for the development of a measurable cell-mediated immune response. It was found that for a consistent and significant response 3 months after inoculation, a minimum dose of 1×10^5 live *M. leprae* per foot pad was required. Both BALB/c and C57Bl/6 mice responded similarly though the response was more robust in C57Bl/6 mice. This model was able to detect most, though not all, of the recombinant *M. leprae* antigens recognized by patient sera or peripheral blood mononuclear cells (Lahiri *et al*., 2011; Sampaio *et al*., 2011; Spencer *et al*., 2011). This 'early' disease model may be used to screen *M. leprae* antigens for immunodiagnostic purposes that may then be further evaluated in the natural hosts of *M. leprae*. T cells isolated from the site of *M. leprae* infection (i.e. *M. leprae*-induced foot pad granuloma) are also being examined for their antigen responsiveness, and KO mice are proving increasingly useful in this regard for screening proteins for selective diagnosis (Hagge *et al*., 2007, 2009).

Limitations of the mouse model

Although mice are an invaluable resource, they possess some immunological differences from humans which investigators must keep in mind when using them to study immunity, especially with regard to mycobacterial diseases. For example, they lack a homologue for granulysin, a key anti-mycobacterial protein generated by human NK and CD8+ T cells (Liu *et al*., 2006). Their repertoire of CD1 cell surface marker expression, which is important for lipid antigen recognition, is different from that of humans (Moody *et al*., 1999). In addition, NOS2-generated reactive nitrogen intermediates, which are toxic radicals of activated macrophages that have antimycobacterial properties (Adams *et al*., 1991; Chan *et al*., 1992; Hagge *et al*., 2014), are produced much more robustly by murine macrophages compared to human macrophages (Weinberg *et al*., 1995; Jung *et al*., 2013). Mouse strains also vary in their ability to recognize certain mycobacterial antigens such as CFP10 (Kamath *et al*., 2004; Lahiri *et al*., 2011). Finally, mice are highly resistant to infection with *M. leprae* unless they are immunosuppressed in some fashion, and they are not an ideal model for the study of nerve infection with the bacilli.

Armadillo Model

In addition to humans, nine-banded armadillos (*Dasypus novemcinctus*) are the only other natural host of *M. leprae*. Free-ranging armadillos in the southern USA are known to harbour high rates of *M. leprae* infection and zoonotic transmission of *M. leprae* from armadillos to humans has been established (Truman *et al*., 2011). With high rates of enzootic infection, a compressed infection cycle and the ability to examine post-mortem reticuloendothelial (RES) tissues to confirm truly positive infections, wild armadillos can be effective population models for evaluating new diagnostic techniques and intervention measures (Duthie *et al*., 2011; Pena *et al*., 2011). However, the armadillo's primary use has been as laboratory animal hosts for experimentally induced leprosy and *in vivo* propagation of *M. leprae*.

M. leprae infection in the armadillo closely recapitulates many of the structural, physiological and functional aspects of leprosy seen in humans; and armadillos are the only non-human hosts that develop extensive neurological involvement with *M. leprae* as seen with humans. Because of the heavy burdens of bacilli they harbour (up to 10^{12} *M. leprae* per animal), nine-banded armadillos have become the hosts of choice for propagating large quantities of *M. leprae*, and they are advancing now as important models for the pathogenesis of nerve injury in leprosy. Although armadillos are not commonly used in laboratory studies outside of leprosy, and there is a paucity of armadillo-specific research reagents, the newly completed whole-genomic sequence for the nine-banded armadillo has enabled researchers to undertake more sophisticated molecular studies in recent years, and an array of new armadillo-specific reagents are now being developed. These animals are likely to play a central role in piloting new therapies and diagnostic regimens in the future, and will help provide new insights into the oldest known infectious neurodegenerative disorder, leprosy.

Natural history and husbandry

Armadillos are exotic-looking mammals about the size of housecats with short legs, strong claws and a hard but flexible segmented carapace armouring most of their body. Members of the order Xenarthra, they are evolutionarily related to sloths and anteaters. The term 'armadillo' can be applied to 21 different species in nine different genera (Talmage and Buchanen, 1954). However, the armadillo of greatest importance in leprosy research is *Dasypus novemcinctus* (i.e. the long-nosed, southern or nine-banded armadillo), although a few reports also suggest that *D. septemcinctus* (seven-banded armadillo) and *Euphractus sexcinctus* (six-banded armadillo) may be partially susceptible to *M. leprae* (Balina *et al*., 1985).

The armadillo's long lifespan (12 years) and cool body temperature (32°C–35°C – optimal for *M. leprae*) are the main physiological traits that first attracted the attention of leprosy researchers. Armadillos are found only in the Americas and range from northern Argentina to the central USA. They can adapt to many diverse habitats but are most abundant in low-lying and coastal areas. Their population has undergone extensive geographic expansion in recent years (Taulman and Robbins, 1996); however, they do not hibernate and a poor tolerance of cold temperatures is the main factor limiting their ultimate range. Armadillos began expanding their range into the USA from Mexico only since 1880. They continue to colonize new areas and are expected to eventually roam over more than 50% of the entire geographic area of the USA. Armadillos in parts of Texas are reported to reach densities of up to 50/km^2, but much lower population densities are reported among armadillos in South America (MacDonald, 1985; Truman *et al*., 1991; Loughry *et al*., 1998; McDonough, 2000; Cullen *et al*., 2001; Naughton-Treves *et al*., 2003) and pioneering armadillo populations show less genetic diversity than their counterparts elsewhere in Central and South America (Arteaga *et al*., 2012; Loughry and McDonough, 2013).

Armadillos can be maintained by any laboratory capable of housing rabbits or other medium-sized mammals. Modified rabbit cages with soft plastic flooring inserts are used in our laboratory, and the animals are adapted to using cat-litter pans. Individual units can be ganged together with a tunnel to separate the sleeping and feeding areas, and a small plastic trash can with shredded paper functions as a sham burrow to enrich their environment.

The reproductive cycle of the armadillo is characterized by diapausic development and polyembryony (Talmage and Buchanen, 1954). Females typically mate in the summer, but the embryos do not implant in the uterine wall until late autumn, when they then divide into identical quadruplicates sharing the same haemochorial placenta. The genetically identical quadruplicates show a heritable component in the armadillo's response to *M. leprae* that can be seen among litter-mates (Storrs and Williams, 1968; Alter *et al*., 2011). Gravid females taken from the wild will litter in captivity and the genetically identical offspring can be useful models to study the role of host genetics and genomic factors on disease susceptibility (Misch *et al*., 2010). However, armadillos are not reliably bred in captivity and must be obtained from the wild for investigative purposes.

Walsh *et al*. (1975) discovered that free-ranging armadillos in Louisiana harboured a natural infection with *M. leprae*, and subsequent investigations confirmed that the disease is widespread among North American armadillos (Truman *et al*., 1986; Walsh *et al*., 1986). Leprosy was not present in the New World during pre-Colombian times and armadillos must have acquired *M. leprae* from humans sometime in the last 300–400 years. The specific origins of *M. leprae* infections among wild armadillos, its geographic range and what risks infection in these animals might present to humans have remained topics of interest. Recent studies indicate that *M. leprae* can be transmitted zoonotically between humans and wild armadillos in the southern USA (Truman *et al*., 2011). While the role of armadillos in the ecology of leprosy elsewhere in the Americas is less clear, biomarkers of *M. leprae* infection have been reported among wild armadillos in Brazil, Colombia and Argentina, and armadillos may be contributing to the transmission of leprosy across their natural range (Truman, 2005; Truman and Fine, 2010).

Manifestations of leprosy in armadillos

In humans, leprosy lesions manifest mainly in cooler body regions, including the skin and mucous membranes of the upper respiratory tract (Yassin *et al*., 1975; Sato *et al*., 2007). For nearly a century, investigators attempted to grow *M. leprae* in various different animals with cool body temperatures (Couret, 1911). Following the discovery that the foot pads of mice (~32°C) would support limited replication of *M. leprae* (Shepard, 1960), Kirchheimer and Storrs began experimentally infecting nine-banded armadillos (Kirchheimer and Storrs, 1971). The heavy burdens of bacilli that manifested in armadillos became a boon to leprosy research and made these animals the hosts of choice for propagating *M. leprae*. Concentrations of 10^{9-11} *M. leprae*/g of liver, spleen or lymph node are not uncommon among armadillos (Job, 2000; Truman *et al*., 2008).

Infection and clinical manifestations of disease

Armadillos are susceptible to infection with *M. leprae* by a variety of routes, including intravenous, percutaneous and respiratory instillations (Truman and Sanchez, 1993). As few as 10^3 *M. leprae* are sufficient to establish infection (Job et al., 1985). However, incubation periods are shortest when high doses of bacilli ($0.1–4.0 \times 10^9$) are delivered intravenously (Prabhakaran *et al*., 1984). These bacilli are taken up in nerves and RES tissues where they slowly proliferate and disseminate to other parts of the body. Intermittent low-level bacteraemia leads to a generalized dissemination of bacilli in the late stages of the infection. No organ system is spared, but cooler body regions such as ears, nose, tongue, footpads, bronchi and lungs tend to exhibit greater involvement; adrenal glands, bladder, heart, intestine, kidneys, ovaries and testes (which are internal in armadillos) are involved less commonly (Job *et al*., 1985; Job *et al*., 1993). Most animals develop heavy infections, with up to 10^{12} recoverable bacilli in the liver and spleen within 18–24 months of experimentally induced infection (Job *et al*., 1985; Truman and Sanchez, 1993). Lower challenge doses require correspondingly longer periods to manifest full dissemination (Truman *et al*., 2008). Of course, studies addressing events of preclinical leprosy do not require fully disseminated infections and can be initiated immediately following challenge.

Armadillos exhibit few overt signs of clinical disease. A large portion of the armadillo's body is occluded from view by its carapace (Truman, 2008). Abrasions around the eyes, nose and feet are the most common signs evidencing an evolving insensitivity in the skin, but are also somewhat non-specific. In the laboratory, plantar ulceration is common in the later stages of infection and involvement of neural tissues is demonstrable at even the earliest stages of disease. Classical foot drop or malformation is not commonly observed in armadillos and the animals probably best represent early clinical stages of active leprosy uncomplicated by secondary injury or long-term therapy.

As the infection progresses, a number of abnormalities associated with a severe hypochromic microcytic anaemia appear in the haemogram. Although *M. leprae* has no toxins and is not life-threatening in man, infected armadillos develop profound anaemia following invasion of their bone marrow and through extra-vascular haemolysis of RBC. Serum LDH concentrations >2000 IU are highly correlated with finding $>1 \times 10^9$ bacteria per g of liver or spleen. AST and ALT are similarly altered. Liver and renal functions become compromised late in infection, and the animals eventually will succumb to secondary complications of persistent bacteraemia if not humanely sacrificed (Truman and Sanchez, 1993).

Immunological and histopathological spectrum

In both humans and armadillos, leprosy exhibits a wide immunological and histopathological spectrum, classifiable over a range from the polar extremes of tuberculoid (TT) and lepromatous (LL), with three indistinct borderline forms in-between (Ridley and Jopling, 1966). The TT form is characterized by well-organized

granulomatous lesions containing few bacilli, whereas the LL form manifests as numerous poorly organized, diffuse lesions containing high numbers of bacilli (Walker and Lockwood, 2006). Multi-bacillary hosts also have a strong antibody response to *M. leprae* while paucibacillary hosts exhibit greater cell-mediated immune responses. The majority of armadillos (70%) exhibit a lepromatous-type of response to *M. leprae*, but the full spectrum of disease can be observed among individuals within the overall population (Job *et al*., 1983; Job and Truman, 2000). The histopathological response spectrum of individual animals to infection can be predicted by intradermal injection of heat-killed *M. leprae* (prepared as lepromin) in a Mitsuda reaction (Mitsuda, 1949; Krotoski *et al*., 1993). The type of leprosy that each animal might manifest seems to have no relationship to their differing environmental exposures or geographic origins and the response appears to be innate.

Among laboratory-infected animals, IgM antibodies to the *M. leprae*-specific phenolic-glycolipid-1 antigen (PGL-1, Hunter *et al*., 1982) arise in about one-third of the time required for bacilli to become detectable in skin scrapings and ear biopsies (Truman *et al*., 1986). The appearance of these antibodies and general level is highly correlated with the bacterial load in the animal's RES tissues (Truman *et al*., 1986). First detection is generally associated with a 1+ BI (Bacteriological Index, approximating 10^4 bacilli/g) in some RES tissues (Job *et al*., 1990; Job *et al*., 1991). Levels of IgM antibodies to PGL-1 increase with increasing bacterial load in the animal and persist over the course of the disease (Truman *et al*., 1986).

Neurological involvement and pathogenesis

Beyond sharing a unique susceptibility to *M. leprae*, the most salient feature of leprosy in both humans and armadillos is extensive neurological involvement with *M. leprae*. Little is known about the pathophysiology of leprosy nerve damage, although it is likely to involve a complicated interplay of both host inflammatory and bacterial-mediated events (Scollard, 2008; Wilder-Smith and Van Brakel, 2008). A major impediment to a better understanding of nerve injury in leprosy is the lack of available tissues for studies. Leprotic lesions are highly focal and usually distributed asymmetrically over the body and in nerves (Nations and Barohn, 2002; Rodrigues and Lockwood, 2011). Ethical and practical limitations make it almost impossible to biopsy affected human nerves, and when fresh fibres can be obtained, they rarely contain a lesion. Human nerves derived from amputated limbs reflect only the end-stages of pathogenesis, and are generally not suitable for detailed molecular analysis or intervention studies (Antunes *et al*., 2006). Only the nine-banded armadillo reliably exhibits extensive neurological involvement upon infection with *M. leprae*, and these animals are the most abundant source of leprotic neurological fibres for research purposes.

M. leprae manifests in armadillos with similar structural and pathological changes as observed in the skin and nerves of human leprosy patients. There is marked inflammation with bacilli attached to the progressively demyelinating Schwann cells (SCs), and a functional neural deficit can be demonstrated in leprotic nerves using electrophysiology (Scollard, 2008). Leprosy in man can only be diagnosed clinically, and all patients already exhibit some degree of nerve damage by the time their disease is first discovered. A notable advantage of the armadillo is the opportunity to examine the pathogenesis of infection at preclinical stages that have never been observed in humans, and which are more likely to be effective targets for therapeutic intervention.

Immediately upon intravenous experimental infection of armadillos, *M. leprae* begins to populate the peripheral nerves, and continues to disseminate from these early foci. The post-tibial branch of the armadillo sciatic nerve (PT) runs just beneath the skin surface of the medial hind limb for approximately 5 cm between the knee and the ankle. This nerve has a high frequency of involvement with *M. leprae* in both armadillo and human infections. It is easily accessible in the armadillo and a useful target for study. Although the duration of experimental infection in armadillos (4–24 months) is highly

compressed as compared to the many years involved in human disease, bacillary loads of ≥10^6 *M. leprae*/cm are common in armadillo PT nerves even during this short time period.

Anti-leprosy drug therapies must have good neural penetration in order to kill bacilli sequestered in nerves. However, even once the bacilli are killed by the antimicrobial drugs, clearance of bacilli from nerves and skin lesions is slow. In one study, 10 *M. leprae*-infected armadillos were allowed to incubate their infections for 12 months before five of them were treated with 10 mg/kg rifampin once monthly for an additional 12 months. Although each of the treated animals showed clinical improvement in skin lesions and ulcers as a result of the antimicrobial therapy, examination of their PT nerves showed residual presence of *M. leprae* with bacterial counts averaging 10^4–10^5 bacilli per cm of PT nerve even after the conclusion of a full year of treatment. This heavy burden of (dead) bacilli provides a rich substrate for continued immunological interaction with the host and enables chronic insidious injury to nerves involved with *M. leprae*.

Although there are no comparable human studies, armadillo nerve segments can be used effectively for gene expression profiling and analysis of cell signalling pathways. Molecular markers for neurodegeneration and regeneration, along with the gene expression profile of inflammatory cytokines, and enumeration of the bacterial load of *M. leprae* in the nerve, are useful therapeutic end points for laboratory studies. They also highlight the importance of developing new therapies to enhance clearance of bacilli from the host in conjunction with antibacterial treatment to limit the progress of insidious neuropathy.

Electrophysiological studies

Armadillos do not reliably respond to thermal, light or tactile stimulants, but electrophysiological measurements of nerve conduction can be used effectively to assess function of armadillo motor nerves. Their hard carapace and thick skin limit the number of nerves that can be examined, but electrophysiology is a convenient, non-invasive means to assess functional characteristics of peripheral nerves. Demyelination of axons results in decreased nerve conduction velocity (NCV) and loss of axons causes decreased compound motor action potential (CMAP) (Franssen, 2008). Normal armadillos exhibit nerve conduction profiles similar to those seen in humans (mean NCV 62.09 ± 10.72 m/s, mean CMAP 1.55 ± 0.33 mV). Among *M. leprae*-infected armadillos, peripheral conduction deficit begins early in the course of their disease and progresses over time. Decreased CMAP (<0.9 mV) is most common and may be measurable within 120 days of infection. Abnormal NCV (<40 m/s) also can be observed among longer-term infected armadillos. Approximately 75% of experimentally infected armadillos develop a demonstrable conduction deficit in their PT nerves over the course of infection, and nearly all of the animals that develop conduction deficit also eventually exhibit other clinical signs of neuropathy (Sharma *et al*., 2013).

Epidermal nerve fibre and Schwann cell density

Defective nociceptive perception, especially hot and cold stimulation, is recognized as an early indicator of neuropathy in leprosy. Such techniques are not suitable among armadillos, but the morphological and quantitative study of nerves in skin biopsy can offer an effective alternative tool to assess thin nerve fibre structure related to the thermal sensitivity function. Immunostaining of punch skin biopsies for protein gene product 9.5 (PGP9.5), a neuronal pan axonal marker, has been used by several investigators to visualize the intraepidermal nerve fibres, dermal nerves and SCs in lieu of nerve conduction tests which may fail to detect small nerve fibre impairment in humans and a variety of animal species. The small fibre innervation is length dependent and robust normative data for epidermal nerve fibre densities (ENFD) in the distal limb have been developed for humans (McArthur *et al*., 1998). In small fibre sensory neuropathies associated with diabetes, HIV and idiopathic small fibre sensory neuropathies, a decrease in epidermal density in the distal leg has been demonstrated (Holland *et al*., 1997;

Periquet *et al*., 1999; Polydefkis *et al*., 2004; Goransson *et al*., 2006). Abnormalities have been demonstrated in cutaneous innervations even in individuals with normal tendon reflexes at the ankles, normal sural nerve action potential amplitudes and normal quantitative sensory tests (Gibbons *et al*., 2006).

Quantitation of epidermal fibres in skin biopsies of ears, abdomen and a distal leg of naïve armadillos has shown a length-dependent innervation similar to humans, and *M. leprae*-infected animals show a degenerative lower mean ENFD compared to naïve animals (Truman *et al*., 2014). Double staining of cutaneous axons and SCs in naïve armadillos also mimics the human cutaneous nerve network pattern. SCs of dermal cutaneous nerves in infected armadillos tend to increase in density and thus provide indirect evidence that during early infection SCs undergo proliferation while harbouring *M. leprae* (Ebenezer *et al*., 2013).

Impairment of muscle architecture and function in infected armadillos

A common pathological hallmark of human leprosy and *M. leprae*-infected armadillos is the involvement of extremities. In the foot, the lumbrical muscles are innervated by the medial and lateral plantar nerves and these nerves are similarly involved in some leprosy patients. Muscle paralysis can result from injury to these nerves. The organization of intact muscle architecture can be evaluated by labelling muscle tissues with antibodies to adult myosin, a family of ATP-dependent, actin-binding and highly conserved muscle motor protein. Labelling of transverse sections of control armadillo muscles showed a highly organized architecture of muscle fibres with clear endomysium and perimysium, similar to human and rodent skeletal muscle (Truman *et al*., 2013). In contrast, lumbrical muscles from infected armadillos can display a markedly disorganized pattern of muscle fibres with disorganized endomysium and perimysium. Analysis of transverse sections of lumbrical muscles with antibodies specific for basal lamina components, laminin and collagen, may also show markedly disrupted and abnormal extracellular matrix expression in infected muscles as compared to control animals. Nuclear labelling may also reveal an increased accumulation of cells in the muscle, most likely mononuclear inflammatory cells, and the distribution of *M. leprae* within the lumbrical muscles in infected animals can be seen using antibody to *M. leprae* PGL-1 that specifically detects whole *M. leprae* (Masaki *et al*., 2013).

Similarly, Brand *et al*. (1981) showed that the PCSA (cross-sectional area/mass) of muscles in the hands of leprosy patients could be used as a surrogate measure of grip strength and index muscle atrophy. Examining the PCSA of armadillo small (intrinsic) lumbrical and flexor muscles also shows a qualitative reduction of muscle mass among infected armadillos, with PGL1 IgM-positive animals having an average of 20% less muscle mass than naïve normal animals (Truman *et al*., 2013). Detailed histopathological studies showed that long-term infection in the armadillo also has discernible effect on the morphological and molecular composition of skeletal muscle fibres. These features in skeletal muscles in infected armadillos resemble muscle pathology and function impairment documented in patients with LL leprosy (Werneck *et al*., 1999) and suggest the potential of using the armadillo model not only for neuropathies but also myopathies associated with human leprosy.

The use of armadillos as vaccine models

Since armadillos so closely recapitulate leprosy in humans there has long been an interest in using them to test specific interventions, especially anti-leprosy vaccines. Earliest vaccination studies in armadillos used increased lymphoblast transformation to assess effective vaccine sensitization and then monitored animal survival as end points. In those studies, heat-killed preparations of *M. leprae* alone, or *M. leprae* potentiated with BCG, showed increased survival in challenged animals. However, more than 3 years was required for the studies to culminate (Kirchheimer *et al*., 1978). Later, Job *et al*. (1993) vaccinated armadillos

with BCG and examined the animal's histopathological response to lepromin as an early indicator of effective vaccination. They showed that 20–40% of the animals had an intensified lepromin reaction in their skin following vaccination, but these results also showed poor correlation with long-term survival rates.

Since leprosy is an infectious neurological disorder in man – not a life-threatening disease – a more appropriate end point for clinical interventions is likely to be the onset and progress of neuropathy rather than survival rates of animals. *M. leprae* can be found localized in peripheral armadillo nerves within days of experimental inoculation, and motor nerve injury can be demonstrated with electrophysiology within just a few months of experimental inoculation. In a recent study using BCG vaccine and a standard high dose *M. leprae* challenge, we saw that 75% of the vaccinated armadillos survived to 19 months post-challenge compared to only 30% of the non-vaccinated animals. More importantly, both vaccinated and sham-vaccinated control armadillos showed nerve involvement as early as 6–8 months post-challenge. In addition, by 7 months after challenge about 70% of the armadillos in both groups had detectable PGL-1 IgM antibodies levels. In this study, BCG was seen as partially protective in armadillos with regard to survival, but ineffective at preventing or delaying nerve injury caused by *M. leprae* infection. Importantly, nerve injury can be used as an effective early end point in vaccine and other intervention studies, which might better reflect the most salient features of leprosy. Additional studies on this are ongoing.

Limitations of the armadillo model

The primary limitation in use of armadillos is the paucity of reagents, especially antibodies, to facilitate investigations. However, with recent completion of the armadillo whole-genomic sequence investigative reagents can be generated more easily, and specific antibodies and molecular probes and primers (Adams *et al*., 2005; Pena *et al*., 2008; Pena *et al*., 2011) can be designed. Of course, all of these reagents require independent development and verification of their quality.

Armadillos are not available from standard commercial vendors and must be obtained from the wild for investigative purposes. Some Institutional Animal Care and Use Committees or animal facilities may not be equipped to deal with wild animals. In addition, such wild animals are highly outbred and may exhibit wide variations in response to challenge. Armadillos do not breed reliably in captivity. However, female armadillos routinely give birth to monozygotic quadruplicates, and gravid females captured from the wild will litter in captivity, making it possible to conduct studies on matched sets of identical twins (Truman, 2008).

Conclusions

Effective animal models can help provide pivotal new understandings about the mechanisms involved in complex disease processes such as those manifested in leprosy. Both the mouse and armadillo models provide large numbers of highly viable *M. leprae* for experimental use from a controlled and known infection status. Mice, with their ease of use, availability of an abundance of biological reagents, numerous genetically defined strains and readily assessable granulomatous foot pad lesion, are ideal for studying basic immunological parameters of infection. For many years they have also proved useful for the evaluation of potential new anti-leprosy drugs and to determine experimental vaccine efficacy. With armadillos, comparative pathological studies have shown that many of the physiological and structural aspects of human leprosy are closely recapitulated in a highly compressed disease duration that exhibits equivalent functional defects in the animals. Other than humans, the nine-banded armadillo is the only animal that develops extensive neurological involvement with *M. leprae*, and this species is an abundant source of leprotic neurological fibres for basic science investigations. Rare neurological events in both normal and leprotic tissues, from time periods and in material quantities that cannot be obtained from human subjects, can be studied only with these animals. Developing techniques to effectively detect and monitor the onset and

progress of leprosy neuropathy could have significant benefit to leprosy patients. Both mouse and armadillo models are complementary to scientific investigation. Each system can better inform the other and together can provide new insights for developing effective intervention strategies and ameliorating the human suffering caused by leprosy.

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28 *Mycobacterium avium* subsp. *paratuberculosis* Infection, Immunology and Pathology of Livestock

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) infection in ruminants leads to a chronic and progressive enteric disease (Johne's disease) that results in loss of intestinal function, poor body condition and eventual death. Transmission is primarily through a faecal–oral route in neonates but contaminated colostrum, milk and *in utero* routes have also been described (Aly and Thurmond, 2005). An interesting and challenging feature of MAP infection is that it traverses both subclinical and clinical stages. The subclinical period is prolonged, often lasting years, and animals are often asymptomatic, making detection of infected animals difficult. Initially after infection and throughout the subclinical period the bacterial load is low and enteric lesions are absent or difficult to identify. Stressors such as parturition and concurrent disease have been incriminated in progression to clinical disease (Stabel, 2000). It has been suggested that an immunologic disruption occurs to push the animal towards a more clinical presentation of disease but the exact mechanism(s) for this is unclear. During the clinical period animals will lose weight and milk production will decrease. Diarrhoea is common and is often intermittent. Large numbers of bacteria are shed in the faeces during this phase of infection, which can heavily contaminate the environment.

Clinical Aspects and Pathology of MAP Infection

Prominent gross and microscopic lesions are usually present in animals that have progressed to the clinical stage of disease. Generally these lesions are present in the small intestine, and most consistently in the ileum near the ileo-caecal valve (Clarke, 1997). Lesions consist of widely scattered to diffuse disorganized infiltrations of macrophages, lymphocytes and eosinophils with occasional multinucleate giant cells into the mucosa and submucosa. Macrophages often extend around and within lymphatic vessels and lymphangitis often continues into the mesentery. Mucosal macrophage infiltration is often substantial and replaces much of the normal mucosal structures, imparting a thickened and corrugated appearance to the small intestine grossly. Granulomatous inflammation usually extends further into the mesenteric lymph nodes as well. The morphology of the granulomas varies from small discrete aggregates of macrophages (focal type) to widespread macrophage infiltration affecting much

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

of the mucosa and submucosa (diffuse type) (Hostetter *et al*., 2005; Tanaka *et al*., 2005). Macrophages in granulomas often contain numerous acid-fast bacilli that are prominently demonstrated with acid-fast stains and this is termed multibacillary granuloma. In sheep and in cattle to some extent, MAP infection can lead to focal type granulomas that contain fewer bacteria. These have been termed paucibacillary granulomas (Clarke and Little, 1996; Tanaka *et al*., 2005). The density of bacteria within granulomatous lesions may reflect the stage of disease progression and has been demonstrated to correlate to local cytokine expression patterns. Multibacillary granulomas correlated to high TGF-β and low IFNγ expression (Munoz *et al*., 2009). MAP is also a pathogen of wildlife with infection identified in wild cervid and bovid populations, again typically with granulomatous inflammation of the small intestine and mesenteric lymph nodes (Carta *et al*., 2013). In red deer both multibacillary and paucibacillary granulomatous lesions have been identified (Clark *et al*., 2010).

Macrophage–MAP Interaction

Mucosal macrophages are a key component of intestinal mucosal defence and homeostasis. However, despite an array of killing mechanisms for invading pathogens, macrophages serve as the cellular niche for MAP within the intestine. The ability of MAP to persist and replicate within the macrophage involves deficits in both innate and adaptive arms of the immune system and is an area of intense interest. During initial infection, M cells in the ileal domes have been shown to be a site of MAP translocation into the mucosa (Momotani *et al*., 1988). Additionally, in experimental models translocation through surface enterocytes of the small intestine has been demonstrated (Sangari *et al*., 2001; Wu *et al*., 2007; Bermudez *et al*., 2010). MAP entry into epithelium has been shown to lead to IL-1β secretion by epithelial cells. This in turn promotes macrophage recruitment. This may be one mechanism whereby MAP establishes intestinal infection through concentrating the target cell type near the site of entry (Lamont *et al*., 2012).

Mycobacterial binding to macrophage surface receptors initiates the process of establishing intracellular survival. A number of receptor–ligand interactions have been described for MAP, which includes complement receptors, integrin receptors, CD14 and mannose receptors. It is likely that MAP entry is mediated by a combination of surface receptors (Souza *et al*., 2007a). One of the key MAP surface lipids, mannosylated lipoarabinomannan, binds with surface mannose receptors and promotes IL-10 production, thereby dampening downstream macrophage killing mechanisms (Souza *et al*., 2013). Ligation of the pattern recognition receptor TLR2 on bovine monocytes has also been shown to promote IL-10 production and interference with phagolysosome maturation (Weiss *et al*., 2008).

The intracellular location for MAP within macrophages is a phagosome that fails to acidify and mature into a functional phagolysosome (Kuehnel *et al*., 2001; Hostetter *et al*., 2003). Woo *et al*. (2007) reported that intracellular MAP survival is not 100% and a percentage of intracellular bacteria are killed. There are multiple factors that facilitate intracellular survival for MAP, many of which are shared by other pathogenic mycobacteria. Interference with macrophage activation is a strategy used by MAP to avoid depredation. Arsenault *et al*. (2012) have shown that MAP-infected macrophages are resistant to IFNγ activation through inhibition of the JAK-STAT signalling pathway. Souza *et al*. (2007b) have reported that there is activation of the MAPK-ERK pathway that leads to increased gene expression of IL-12 and TNF-α, suggesting that this pathway is involved in a protective response. However, activation of MAPK-ERK signalling did not correlate with increased bacterial killing in infected monocytes (Souza *et al*., 2007b). Apoptosis is one mechanism that promotes killing of intracellular pathogens in macrophages. It has recently been shown that MAP-infected macrophages are resistant to apoptosis via inhibition of caspase activation (Kabara and Coussens, 2012). IL-1α and TRAF1 have shown high expression in MAP-infected macrophages *in vitro* and within ileal granulomas. This may suggest tissue injury by IL-1 α and interference with apoptosis signalling by TRAF1 as mechanisms that promote progression of the infection (Aho *et al*., 2003; Chiang *et al*., 2007).

The variation in reported expression of macrophage activation markers may be in

part related to the stage of the infection. In the late stages of natural infection multibacillary granuloma macrophages did not express the classical activation marker inducible nitric oxide synthase (iNOS) (Hostetter *et al*., 2005). In contrast, Delgado *et al*. (2010) found iNOS-positive macrophages in the ileum and mesenteric lymph nodes of infected cattle. A decrease in iNOS in cultured monocytes infected with MAP has been found to correlate with decreased IL-12p40 expression and impaired CD40 signalling (Khalifeh and Stabel, 2013). CD40 ligand expression also has been shown to be downregulated in PBMC recovered from cattle in the clinical stage of disease after culture with MAP. In this study, infected macrophages secreted IL-10 and TGF-β (Sommer *et al*., 2009). Osteopontin is an acidic glycoprotein that is expressed by multiple cell types and has a diverse set of known actions. This protein is often secreted, but an intracellular localization also exists. Immune cells including macrophages, dendritic cells and lymphocytes produce osteopontin and this promotes increased IL-12 and decreased IL-10 production (Inoue and Shinohara, 2011). MAP antigen stimulation of PBMC from MAP-infected cattle led to osteopontin expression that correlated with IFNγ and IL-12 production, suggesting a protective role against MAP (Karcher *et al*., 2008a). Osteopontin has also been detected within the granulomatous lesion in the ileum of naturally infected cattle and was identified within macrophages, lymphocytes and plasma cells in the lesion (Karcher *et al*., 2008b). This may suggest a role for this protein in granuloma regulation and disease pathogenesis (Karcher *et al*., 2008b).

Dendritic Cell–MAP Interaction

Dendritic cells (DC) are key antigen-presenting cells that can promote development of strong cell-mediated immune responses. Immature DC reside in strategic mucosal sites where they are positioned to sample antigens. When foreign antigens are encountered DC undergo maturation whereby phagocytic responses are diminished and antigen presentation activity increases. This corresponds to increased expression of MHC II and costimulatory molecules as well as increased IL-12 secretion. Mature DC migrate to local lymph nodes where antigen is presented to T cells (Reis e Sousa, 2006). Interference with antigen presentation is a survival strategy employed by pathogenic mycobacteria including MAP. In experimental infection DC are recruited to sites of MAP inoculation into the intestine (Charavaryamath *et al*., 2013). Bovine DC have also been shown to be permissive to MAP infection in culture; however, maturation of bovine DC was incomplete as demonstrated by reduced phagocytic ability, as well as low expression of MHC II, CD40 and CD80. In addition, infected bovine DCs secreted high IL-10 and little IL-12. These data suggested that MAP-infected DC had a semi-mature phenotype (Lei and Hostetter, 2007; Lei *et al*., 2008). This is important because semi-mature DCs have been shown to favour a tolerogenic T cell phenotype, and this may be a mechanism of disease pathogenesis. Bermudez *et al*. (2010) recently have shown that DC take up high numbers of MAP that have translocated across the intestinal epithelium and that there was bacterial survival within DC. Infected DC may then act as couriers for bacterial spread to the mesenteric lymph nodes (Bermudez *et al*., 2010). However, MAP antigens have been identified including fibronectin attachment protein, a 70-kDa heat shock protein and CobT, all of which have been shown to promote strong DC maturation through activation of TLR4 signalling and development of protective T cell responses (Lee *et al*., 2009; Hoek *et al*., 2010; Byun *et al*., 2012). These proteins have potential to be used for generation of cell-mediated immune responses via vaccination.

Gamma Delta T Cells and MAP

There is growing interest in the roles of gamma delta (γδ) T cells in the pathogenesis of mycobacterial infections. γδ T cells are a diverse group of T lymphocytes that vary in phenotype, density and function by species. However, they are often concentrated at mucosal surfaces and their functions span innate and adaptive immune responses (Nanno *et al*.,

2007; Plattner and Hostetter, 2011). A diverse group of mycobacterial antigens that includes both protein and non-protein antigens are recognized by γδ T cells, including multiple phosphoesters that are collectively termed 'phosphoantigens'. In most animal species, γδ T cells are a minor component of the T cell population. However, in calves γδ T cells comprise nearly 40% of the circulating T cells, with a progressive decrease in this population as the animal matures (Wilson *et al*., 1996), making this T cell subset an attractive area of research for MAP infection in the bovine. Understanding the role that γδ T cells play in driving and sustaining adaptive immunity may provide novel insight into strategies to maintain protective responses in ruminants against MAP. In the bovine, γδ T cell subsets have been characterized based upon cysteine-rich scavenger receptor molecule workshop cluster 1 (WC1) with WC1+ and WC1− subsets. The WC1+ subset is further divided into WC1.1+ and WC1.2+ cells (Rogers *et al*., 2005; Guzman *et al*., 2012). Bovine γδ T cell have diverse functions including antigen presentation, immune effector function, chemokine/cytokine secretion and regulatory functions (Collins *et al*., 1998; McGill *et al*., 2013). A role in the pathogenesis of mycobacterial infection has been proposed. Tanaka *et al*. (2000) demonstrated that mice deficient in the γδ TCR had fewer and smaller granulomas than wildtype mice infected with MAP. This suggested that γδ T cells are involved in granuloma formation during MAP infection. More recently, Plattner *et al*. (2009) showed that in highly organized granulomas induced by a killed bacterin, WC1+ cells were recruited initially and then WC1− cells became the dominant γδ T cell subset in the mature granuloma. Interestingly, this sequence was reversed in poorly organized granulomas that developed in response to live MAP infection. This suggests that γδ T cells play a role in driving the development and organization of granulomas that develop in response to MAP infection (Plattner *et al*., 2009). γδ T cells arrive at MAP infection sites earlier than CD4+ αβTCR cells and generate significant amounts of IFNγ, again suggesting an early role for this cell type in control of MAP infection (Plattner *et al*., 2013). Stabel *et al*. (2013) have recently shown that peripheral blood γδ T cells increased in MAP-infected calves, but not in *M. bovis*-infected calves. This suggests unique components of the immune response to MAP infection, including the role of γδ T cells.

Adaptive Immune Responses

While the target cell type for MAP infection is the macrophage, adaptive immunity is critical for driving macrophage activation and control of bacterial replication. Classically it has been described that following MAP infection calves will develop a strong T helper type 1 (Th1) immune response, which persists into adulthood. This cellular immune response holds pathogen replication and spread in check, preventing disease progression (Chiodini, 1996). In a certain percentage of adult animals an immune transition period occurs. The Th1 response progressively wanes and an ineffective Th2 response develops. It is during this period that significant amounts of MAP antibodies are generated. Progression to a Th2-dominated immune response often correlates with clinical progression and is associated with heavy bacterial shedding and development of prominent granulomatous lesions in the small intestine and mesenteric lymph nodes (Stabel, 2000).

It is likely, however, that this view of the adaptive immune response to MAP infection is oversimplified and that there is significant overlap of Th1/Th2 immune responses (Stabel, 2006). The Th1 response to MAP infection is characterized by generation of IFNγ, IL-2, TNF-α and IL-12 (Burrells *et al*., 1999). IFNγ is important in activating macrophages to kill intracellular MAP and to further drive development of the Th1 response (Yang *et al*., 1998). However, recent evidence suggests that there is also an early antibody immune response in calves infected with MAP. Maattanen *et al*. (2013) have shown that protein kinases associated with cell-mediated and humoral immunity are activated within 1 month after experimental infection (Maattanen *et al*., 2013). Waters *et al*. (2003) identified development of antibody responses in MAP-infected calves within months after infection (Waters *et al*., 2003). Stabel *et al*. (2009) have found using

multiple routes of inoculation that CD25+/CD45RO+ B cells were present in peripheral blood in the early months after experimental infection (Stabel *et al*., 2009). Moreover, in adult cattle with natural MAP infection there is often a lack of clear polarization of Th1/Th2 immune responses (Vazquez *et al*., 2013). Begg *et al*. (2011) have questioned the dominance of the Th1 type response early after infection in sheep. In these studies antibody and IFNγ responses were detected early after infection and were present in sheep with both the paucibacillary and multibacillary type intestinal granulomatous lesions. This study indicates that both cell-mediated and humoral responses are involved in control of disease progression. The authors suggest that clinical disease is related to progressive immune dysfunction rather than to an abrupt shift in immune polarity (Begg *et al*., 2011).

In the clinical stages of MAP infection there are changes in the cytokine profiles. IFNγ has been reported to be higher in subclinical animals, whereas IL-10, TGF-β and IL-4 expression has been identified in animals that have progressed to clinical disease (Sweeney *et al*., 1998; Khalifeh and Stabel, 2004; Tanaka *et al*., 2005; Stabel and Robbe-Austerman, 2011). However, high IL-10 production has been identified early in MAP infection as well. Mesenteric lymph node cells taken from cattle during the subclinical period produced significant amounts of IL-10 after stimulation with MAP antigen (Subharat *et al*., 2012). These stimulated cultures also produced high levels of IFNγ and TNF-α. The conclusion from this study was that IL-10 may play an immunomodulatory role limiting early tissue injury (Subharat *et al*., 2012).

Recently a role for T regulatory cells (Treg) has been proposed for MAP pathogenesis. Regulatory T cells are important negative inhibitors of inflammatory responses. T regulatory cells have been described in the bovine by coexpression of CD4/CD25 and Foxp3, along with a cytokine profile that includes IL-10 and TGF-β (Seo *et al*., 2007; Coussens *et al*., 2012). In addition, γδ T cells, including WC1.1 and WC1.2 subsets, may also have regulatory function with similar cytokine secretion profiles (Hoek *et al*., 2009). Weiss *et al*. (2006) found that ileal lymphocytes from subclinical animals were hyporesponsive to MAP antigens and mitogens. This group also found that CD4/CD25+ T cells were higher in the ileal mucosa of infected versus noninfected animals. Their conclusion was that Tregs may play a role in suppressing lymphocyte function in the ileum during MAP infection (Weiss *et al*., 2006). T regulatory cells may play a role in shifts in cytokine responses late in disease. Khalifeh and Stabel (2004) demonstrated that PBMC from late stage clinical animals produced high levels of IL-10 and TGF-β. Coussens (2004) have also shown that PBMC from MAP-infected animals generate IL-10. Using depletion studies they determined that CD25+ cells were significant producers of IL-10 in PBMCs, suggesting a Treg phenotype. Collectively, this information may suggest that suppressor lymphocytes including Tregs may play a role in shifts in cytokine profiles during the progression of MAP infection from subclinical to clinical periods.

Diagnostics

To date definitive diagnosis of MAP infection on an individual animal basis remains a challenging issue. The biology of Johne's disease presents unique problems when choosing the appropriate diagnostic tools to detect infection. Most commonly, animals become infected as neonates, exposed to either faecal material or milk contaminated with MAP. This point is critical since neonatal immune systems are immature and ill equipped to respond to an infectious pathogen. The MAP pathogen takes advantage of this immunologic immaturity and hides itself within the macrophages of the host. The long latent period after infection and preceding the onset of clinical disease is associated with negligible humoral immune responses, resulting in a lack of sensitivity in the detection of antibody using standard ELISA tests. Generally speaking, consistent and reliable detection of antibody in the serum and milk is associated with end-stage clinical disease. Despite this, the ELISA test remains a popular diagnostic tool format due to its relative ease, speed and inexpensive nature. Several commercial ELISA

kits are available for measurement of MAP antibodies in serum and milk, including Herdchek® (IDEXX, Westbrook, ME, USA), Parachek® (Prionics, Omaha, NE, USA), Pouquier (Institut Pourquier, Montpellier, France), Serelisa® (Synbiotics; Zoetis, Florham Park, NJ, USA) and ID Screen Paratuberculosis® (ID Vet, Montpellier, France), as well as ELISA tests that are formatted solely for milk such as AntelBio® (Lansing, MI, USA). Many studies have been conducted to compare the efficacy of these ELISA tests in the detection of MAP infection with variable results. Köhler *et al.* (2008) evaluated five ELISA test kits with bovine serum, finding that two of the test kits achieved specificities of >99%. Sensitivity of detection was dependent upon the stage of disease but kit sensitivities ranged from 40% to 70%, using cut-offs suggested by the manufacturer. Other reports also suggest that the sensitivity and specificity of detection can differ between ELISA tests (Collins *et al*., 2005; McKenna *et al*., 2005; Nielsen and Toft, 2008; Dieguez *et al*., 2009). However, one comprehensive comparison of four commercial ELISA kits demonstrated little difference in their ability to detect infected cows (30.2–41.5%) (Fry *et al*., 2008). The basic premise of all of these ELISA assays is the use of a whole-cell antigen preparation (sonicate or protoplasmic) as a capture antigen. Theoretically this should increase the sensitivity of detection since the assays are inclusive of any antigen-specific antibodies that could bind. However, using a whole-cell antigen preparation can also lead to issues with specificity, since mycobacteria are genetically highly conserved. The genetic homology between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*, a closely associated subspecies found in the environment, is greater than 99% (Bannantine *et al*., 2003). Since this can lead to false-positive results, many ELISA tests have included an absorption step with *M. phlei* to bind nonspecific epitopes (Bech-Nielsen *et al*., 1992). This technology has improved the specificity of many ELISA assays for detection of MAP antibodies to greater than 99% but has not rendered them foolproof. Three paratuberculosis ELISA tests (two commercial and one in-house) were evaluated for cross-reactivity to bovine TB (Lilenbaum *et al*., 2009). All three ELISA tests demonstrated cross-reactivity with sera from bovine tuberculosis cows (21.8% of TB-positive animals), a major concern in the field. Therefore, development of novel ELISA tests for the detection of MAP antibodies has continued in recent years, with many of these tests evaluating recombinant MAP proteins as the capture antigens. The use of singular MAP proteins or cocktails of proteins, which have been selected based upon antigenicity and lack of cross-reactivity with closely related mycobacteria, may provide greater specificity in the assays. In one study, a cadre of 18 recombinant MAP proteins was evaluated but no single MAP protein was immunodominant among all of the infected animals and antigenicity of the proteins was highly variable between infected animals and within animals over time (Bannantine *et al*., 2008). One of the recombinant proteins, MAP0862, is uniquely expressed by MAP and was able to detect 9 of 11 infected sheep but it also demonstrated some reactivity in control animal sera. Other proteins, MAP2737, MAP0862, MAP0865 and MAP0852, demonstrated strong reactivity that may show promise for an antigen mixture for a serologic assay. Additional studies have been undertaken with degrees of success to evaluate various MAP recombinant proteins in the ELISA format (Hughes *et al*., 2008; Kawaji *et al*., 2012), all of which demonstrated some potential for use of the proteins for the detection of MAP antibodies in serum and/or milk. Mon *et al*. (2012) tested 54 recombinant proteins in an immunoblot format before selecting seven antigenic proteins for an antigen cocktail to evaluate in the ELISA format. They found that the seven-protein cocktail was able to detect 18 of 25 infected animals with little cross-reactivity to sera from healthy control animals. However, reactivity with sera from TB-infected animals was just marginally below the absorbance cut-off of 0.1. More recently, a new ELISA test for paratuberculosis using enoyl coenzyme hydratase as the capture antigen demonstrated greater sensitivity but also greater specificity than the Pourquier ELISA test when tested against sera from animals challenged with other mycobacterial species including *M. bovis*, *M. scrofulaceum*, *M. intracellulare* and

M. avium subsp. *hominissuis* (Nagata *et al*., 2013). In addition, detection of antibodies was possible as early as 2 months post-infection in MAP-challenged calves. Based upon a small sample set (30 sera each from infected and healthy cows), the sensitivity of detection averaged 96.7% and specificity was 96.7%.

Most studies incorporating MAP recombinant proteins in the ELISA format have reported sensitivities of detection higher than that of commercial ELISA kits. With some exceptions, these studies have focused on the reactivity in sera from clinically affected animals, discounting the presence of negligible reactivity in subclinical animals, thereby inflating sensitivities of detection. Although the use of MAP recombinant proteins remains a viable alternative for new commercial ELISA tests, some additional promising approaches in recent years have instead focused on modifying methods to prepare the capture antigens. Shin *et al*. (2008) recovered the culture filtrate from *in vitro* grown MAP in early to mid-log phase and used the filtrate as the capture antigen in an ELISA platform (JTC-ELISA). Upon evaluation of sera from 444 cows in various stages of infection, the JTC-ELISA performed significantly better than any of the commercial kits tested for cows shedding even the lowest level of MAP in their faeces (1–9 cfu/slant). At a cut-off value of 0.15 (sample/positive ratio), the JTC-ELISA had an overall sensitivity of 74% and a specificity of 99%, with other ELISA test kits averaging a 50% sensitivity of detection on the same sample set. Additionally, Eda *et al*. (2006) showed that an ethanol–vortex extraction of MAP antigen used to coat ELISA plates (EVELISA) yielded greater sensitivity (97%) and specificity (100%) when compared to a commercial ELISA test. Again, positive test results were obtained for cows classified as low shedders (4 cfu/slant) and even experimentally infected calves as early as 174 days post-infection, suggesting that this method of antigen preparation was conducive to greater exposure of epitopes for binding MAP-specific antibodies. The EVELISA was further improved with the addition of an *M. phlei* absorption step to reduce cross-reactivity with nonspecific antibodies (Scott *et al*., 2010).

Accurate assessment of early MAP infection is rather more tenuous as the long latent period is associated with strong cell-mediated immunity and weak to negligible humoral immunity. Historically the common field diagnostic test to measure cell-mediated immunity in mycobacterial infections has been the skin test. However, this test is labour intensive, making it impractical for continuous herd surveys, and also suffers a lack of specificity for the diagnosis of paratuberculosis. Strides have been taken in recent years to define and improve protocols to quantitate antigen-specific interferon-γ (IFNγ) in plasma as an alternative measure of cell-mediated immunity. Kalis *et al*. (2003) demonstrated that specificity of the johnin skin test and IFNγ assay were comparable, supporting the use of the latter in the early diagnosis of paratuberculosis. The earliest reports of application of the IFNγ test for the diagnosis of paratuberculosis appeared in 1992 with a publication comparing the efficacy of the IFNγ ELISA test with an IFNγ bioassay and an absorbed serum antibody ELISA in six cattle herds (Billman-Jacobe *et al*., 1992). The IFNγ ELISA yielded a sensitivity of detection up to 93.3% of subclinical cows and 100% of clinical cows in the herds, compared to average sensitivities of 33.3% and 80% for subclinical and clinical cows, respectively, for the competing assays. Subsequent reports further defined sensitivity and specificity values for this assay, suggesting that the IFNγ assay demonstrated promise in identifying subclinically infected animals (Stabel, 1996; Huda *et al*., 2003; Kalis *et al*., 2003). Accurate detection does not appear as much age-dependent (Huda *et al*., 2004), but more likely related to infectious dose and maturity of the animal's immune system. Experimentally infected calves have demonstrated robust antigen-specific IFNγ responses as early as 90 days post-infection but interpretation of responses under 30 days of infection is tenuous (Waters *et al*., 2003; Koo *et al*., 2004; Stabel and Robbe-Austerman, 2011). As with antibody-mediated diagnostic tests, false-positive reactions in the IFNγ test due to mixed infections within the herd or vaccination may confound results. Waters *et al*. (2004) demonstrated that the recombinant ESAT-6:CFP10 fusion protein could

differentiate IFNγ responses of MAP- and *M. bovis*-infected animals. However, additional studies have been conducted to identify MAP antigens that would invoke greater specificity while maintaining high levels of sensitivity for identification of animals with paratuberculosis (Olsen and Storset, 2001; Huntley *et al*., 2005a; Nagata *et al*., 2005; Rosseels *et al*., 2006). A recent review summarized research reports evaluating MAP antigens in cell-mediated assays and concluded that there are no obvious antigen candidates at this point (Mikkelsen *et al*., 2011). However, this group later evaluated 14 novel recombinant antigens in the IFNγ assay and demonstrated that three latency proteins, LATP-1, LATP-2 and LATP-3, exhibited high immunogenicity and specificity in the IFNγ assay (Mikkelsen *et al*., 2011). More recently, a cadre of 30 recombinant MAP proteins was evaluated for IFNγ responses in subclinically infected sheep (Hughes *et al*., 2013). Four proteins (MAP1297, MAP1365, MAP3651c and MAP0268c) were selected for further evaluation with mean differences between infected and control groups noted for the latter three proteins. Other recombinant MAP proteins that have demonstrated promise as singular antigens for elicitation of IFNγ responses in cattle are MAP2077c, MAP1204, MAP1272c and MAP1087 (Stabel *et al*., 2012). These proteins invoked responses similar in robustness and in diagnostic value to johnin PPD and a whole-cell sonicate preparation of MAP. Besides antigen preparations, an alteration of host immunity or manipulation of culture conditions may augment antigen-specific IFNγ responses. Intradermal sensitization of cattle with johnin PPD prior to sampling, as well as addition of anti-IL-10 antibody, recombinant IL-12 and both recombinant IL-7 and IL-12 to cultures, all served to enhance IFNγ responses in animals with paratuberculosis (Buza *et al*., 2004; Stabel *et al*., 2007; Mikkelsen *et al*., 2012; Plain *et al*., 2012). These alterations to the testing procedure or *in vitro* culture conditions may have significant impact on the sensitivity of the assay, enabling earlier detection of infection.

Greater than 95% of infected animals do not exhibit outward signs of disease, yet a high percentage of these animals shed MAP in their faeces. Detection of faecal shedding of the MAP organism via culture or PCR is the still the most reliable test that can traverse the subclinical and clinical stages of infection. However, the high cost of faecal culture or PCR can be prohibitive for some producers, leading to pooling of faecal samples as a cost-effective alternative for herd screening (Tavornpanich *et al*., 2004). One major difficulty in microbiologic culture of MAP is due to the fastidious growth pattern of the bacterium and the long periods of incubation required. Solid medium methods of culture generally require 8–12 weeks for growth, whereas incubation periods are reduced to 2–8 weeks using the liquid medium methods of culture such as Bactec™ (Becton Dickinson, Franklin Lakes, NJ, USA), MGIT™ (Becton Dickinson, Franklin Lakes, NJ, USA), Trek ESP® (Thermo Fisher Scientific, Oakwood Village, OH, USA) (Collins *et al*., 1990b; Rajeev *et al*., 2006; Kawaji *et al*., 2013). Since infected animals may shed the organisms intermittently in their faeces, use of faecal culture alone as a diagnostic test may result in a misrepresentation of infection within the herd with between 25% and 50% of infected animals detected by faecal culture (Whitlock *et al*., 2000; Nielsen *et al*., 2002). Detection of MAP by PCR is a useful alternative to culture and has improved detection time to mere days, depending upon the assay. PCR assays have been developed utilizing gene targets such as IS900, ISMav02, ISMAP02, 251, F57 and HspX (Ellingson *et al*., 1998; Motiwala *et al*., 2003; Shin *et al*., 2004; Stabel and Bannantine, 2005; Bosshard *et al*., 2006). Specificities of detection are quite high, averaging >99%, but sensitivities can vary depending upon sample, DNA extraction protocol, conventional versus real-time PCR methodology and single versus double PCR amplification reactions (Christopher-Hennings *et al*., 2003). More recently, a phage-based detection assay was developed by coupling an immunomagnetic capture of MAP with a phage amplification assay, followed by detection of the phage in an ELISA test (Stewart *et al*., 2013). This assay may be useful as a screening tool for detection of MAP in faeces in the herd but needs further field evaluation.

Vaccines

Vaccination has been a successful tool for the management of many infectious diseases in livestock, and paratuberculosis is no exception. Vaccines have been available for paratuberculosis since 1926, with administration resulting in reduced faecal shedding of MAP and reduced clinical signs in infected animals, and further evidence suggesting a reduction in the incidence of disease within herds (Kalis *et al*., 2001). Paratuberculosis vaccine studies have demonstrated the induction of both cellular and humoral immune responses; however, it is widely accepted that vaccination will not prevent infection. Most commercial formulations of paratuberculosis vaccines consist of a heat-killed whole-cell preparation in an oil emulsion. In the USA, only one such vaccine (Mycopar™ (Boehringer Ingelheim Vetmedica, Fort Dodge, IA, USA)) is licensed for use and it is known to be a strain of *M. avium*, not MAP. Paratuberculosis vaccines available outside the USA are Gudair® (Zoetis Australia, Rhodes, NSW, Australia), a heat-killed whole-cell vaccine based upon Weybridge strain 316F that is approved for use in small ruminants, and, Silirum® (Zoetis, Zoetis Australia, Rhodes, NSW, Australia), a newer candidate using the 316F strain that is being evaluated for use in cattle (Juste *et al*., 2009). Although these commercial vaccines provide benefit in the management of paratuberculosis, there are disadvantages associated with them such as reactions at the site of injection, including severe inflammation and granuloma formation. Another major drawback in using whole-cell vaccines is the interference with bovine tuberculosis skin testing and accurate serologic detection of MAP-infected cattle (Bastida and Juste, 2011). Newer serologic diagnostic tools for bovine tuberculosis using immunochromatography are demonstrating promise in distinguishing between *M. bovis*-infected animals from either MAP-infected or vaccinated animals (Stabel *et al*., 2011; Lyashchenko *et al*., 2013).

Developing subunit or DNA vaccines would significantly reduce or eliminate some of the troubling aspects of whole-cell vaccines without sacrificing beneficial properties. A number of putative antigens have been identified from MAP and their immunogenicity has been examined by serodiagnostic and lymphocyte stimulation assays (Huntley *et al*., 2005a; Rigden *et al*., 2006; Kadam *et al*., 2009; Newton *et al*., 2009; Gillan *et al*., 2010; Hoek *et al*., 2010; Platt *et al*., 2010; Bannantine *et al*., 2011a). Several of these immunogens have been evaluated for use as subunit vaccines, including a 70 kDa heat shock protein (Hsp70), a novel 74F polyprotein and a mixture of Ag85/SOD proteins. These proteins have demonstrated success in protection against MAP challenge in mice, cattle and goat models, resulting in reduced tissue colonization and faecal shedding of MAP (Koets *et al*., 2006; Chen *et al*., 2008; Kathaperumal *et al*., 2008). Each of the aforementioned subunit vaccines also invoked cell-mediated and humoral immunity in the respective animal model, suggesting immune-mediated protection. More recently, vaccination with a leuD mutant of MAP demonstrated excellent protection in goats after oral challenge (Faisal *et al*., 2013a). Goats vaccinated with the leuD mutant had markedly reduced tissue burdens compared to nonvaccinates and goats vaccinated with the killed vaccine (Mycopar). Further, leuD-vaccinated goats had higher and more sustained levels of IFNγ, IL-2, IL-1β and IL-17 during the study, suggesting that host protection may have been modulated by enhanced Th1/proinflammatory responses.

More importantly, vaccination of cattle with the Hsp70 subunit vaccine did not result in interference with the comparative cervical skin test for bovine tuberculosis. Positive responses to AvPPD were noted in calves vaccinated with either Gudair or Hsp70 but responses to BoPPD were only noted in Gudair vaccinates, indicating promise for more accurate discrimination between paratuberculosis and bovine tuberculosis (Santema *et al*., 2009). It is interesting to note that vaccine candidates are not always equally effective among species of animals. An example of this is the *Salmonella*-vectored Ag85/SOD vaccine that demonstrated protection by reduced tissue colonization in a mouse model but was not highly protective in goats, suggesting that differences in host immunity may play a role in vaccine efficacy (Faisal *et al*., 2013b).

DNA vaccines are an alternative format that has proved successful for many mycobacterial pathogens, including *M. tuberculosis*, *M. bovis*, *M. leprae* and *M. avium* (Lowrie *et al*., 1997; Martin *et al*., 2000; Hu *et al*., 2009). DNA vaccines are attractive alternatives as they can stimulate both the endogenous (MHC class II restricted) and exogenous (MHC class I restricted) antigen presentation pathways. This results in activation of both CD4+ and CD8+ T cells, resulting in an immune response that more closely mimics that of the host animal in natural infection (Romano and Huygen, 2009). The development of DNA vaccines for MAP has been attempted thus far by very few laboratories. Roupie *et al*. (2008) demonstrated that DNA encoding the MAP0586c gene was protective in Balb/c mice but not C576BL/6 mice. Vaccination with this plasmid resulted in high levels of IL-2 and IFNγ secretion in both strains of mice when compared to immunization with control DNA. Further work was performed by vaccination of mice with plasmid DNA encoding eight unique MAP antigens (Roupie *et al*., 2012). None of the DNA vaccine candidates successfully conferred protection although BCG vaccination reduced MAP colonization in the spleen and liver. Immunization with a cocktail consisting of five plasmids encoding Ag85A, Ag85B, Ag85C, SOD and MAP2121c successfully attenuated the number of viable MAP recovered from the spleen and liver of vaccinated mice (Park *et al*., 2008). This protection correlated with increased CD4+ and CD8+ T cell responses, lymphocyte proliferation and IFNγ secretion. Earlier protection was reported using a pool of MAP antigens in a randomized expression library immunization approach (Huntley *et al*., 2005b). In two of the protective libraries, sequenced genes were found that encode for transport/binding, membrane and virulence proteins and mycobactin/polyketide synthases. Further exploration in this area of study is warranted to identify reasonable candidates that elicit protective immunity in the host. Clearly, a safe and effective vaccine must be developed that not only prevents infection, but also eliminates shedding of MAP through the faeces, colostrum and milk of infected cows. If this is accomplished, the spread of Johne's disease will be significantly curtailed.

Genomics of MAP and Relatedness to Other *M. avium* Subspecies

M. avium subsp. *paratuberculosis* (MAP) is a member of the *M. avium* complex (MAC). Other members of the MAC group include at least three subspecies: *M. avium* subsp. *avium*; *M. avium* subsp. *silvaticum*; *M. avium* subsp. *hominissuis*; and a second species, *M. intracellulare*. Before 1988, MAP was a species by itself. The proposal of a subspecies designation for MAP was based on DNA hybridization studies (Saxegaard *et al*., 1988; Yoshimura and Graham, 1988; Thorel *et al*., 1990). This reclassification was met with some resistance due to the distinct phenotype of MAP (Chiodini, 1990), but genetically the subspecies designation was justified. There are no naturally occurring plasmids or extrachromosomal elements in MAP or other members of the MAC. In 1989, the insertion sequence IS*900* was discovered (Collins *et al*., 1989; Green *et al*., 1989) and its characterization was the subject of several papers (Tizard *et al*., 1992; Doran *et al*., 1994, 1997) because it was considered a MAP-specific element. Only much later was it revealed that some sequences in the IS*900* element were conserved among non-paratuberculosis members of the MAC (Cousins *et al*., 1999; Semret *et al*., 2006).

Foundational research that includes genome sequencing and screening for unique or variable regions that may be used for molecular epidemiology has been explored in detail for MAP. Subsequent studies building on this foundation have led to a better understanding of the genomic diversity of this bacterium along with its evolutionary position within the MAC. For example, we now know what genetic regions distinguish MAP from all other mycobacteria (Turenne *et al*., 2007; Alexander *et al*., 2009) and we can readily distinguish between cattle and sheep isolates of MAP. Researchers are still working to correlate these genetic differences to defined phenotypes. This section discusses how genomics was used to identify strain differences within the *M. avium* complex and then focuses specifically on differences among MAP strains.

Genomic Diversity Among the *Mycobacterium avium* Complex

All members of the MAC are highly genetically similar, although small distinctions between *M. avium* subspecies and MI were noted long ago (Boddinghaus *et al*., 1990; De Smet *et al*., 1996; Feizabadi *et al*., 1997; Thorel *et al*., 2001; Paustian *et al*., 2008). The genome sequences available for members in the MAC group include bovine (Li *et al*., 2005), ovine (Bannantine *et al*., 2012), camel (Ghosh *et al*., 2012) and human isolates of MAP (Wynne *et al*., 2011; Bannatine *et al*., 2014), as well as two MAH isolates, designated strain 104 and strain TH135 (Uchiya *et al*., 2013). These genomes clearly demonstrate the close genetic similarity between the subspecies (Bannantine *et al*., 2003) and support the early DNA homology studies that initially suggested this similarity (Saxegaard *et al*., 1988; Yoshimura and Graham, 1988; Hurley *et al*., 1989). The genome sequences have become important tools to define additional genetic variations among MAP isolates, especially differences between the cattle and sheep isolates (Dohmann *et al*., 2003; Marsh *et al*., 2006) or differences among vaccine strains (Bull *et al*., 2013). Unfortunately, studies thus far have not explained why members of the MAC group show preference for diverse hosts such as MAP in ruminants, *M. avium* subsp. *hominisuis* in pigs and in immunocompromised humans, and *M. avium* subsp. *avium* in birds. These host range differences have led to the search for a genetic underpinning, but so far these differences have only been explored for diagnostics (Stabel and Bannantine, 2005) or targets for molecular epidemiology (Bannantine *et al*., 2013). MAC strains can be distinguished based upon presence or absence of large sequence polymorphisms (LSPs) (Paustian *et al*., 2008), presence or absence of insertion elements (Green *et al*., 1989; Johansen *et al*., 2005), or single-nucleotide polymorphisms (SNP) (Turenne *et al*., 2007; Alexander *et al*., 2009). MAP, in particular, can be distinguished from other MAC strains by an SNP in the MAP1025 gene (Bannantine *et al*., 2011b) or by the absence of LSP^{A}8 (Semret *et al*., 2005).

A significant source of diversity lies in mobile genetic elements. Most of these elements have been identified in MAC strains, and some have been found to be useful in subtyping isolates. The genome sequence of MAP has revealed a total of 19 different insertion elements in the cattle strain K-10 (Li *et al*., 2005) and these elements are important factors contributing to the diversity of MAC members. Olsen *et al*. (2004) discovered the ISMpa1 element and observed that three copies were present in the MAP genome. The genome project designation of ISMpa1 is IS_MAP12 and sequence data analysis confirms that it is present in three copies in the K-10 genome. This element was found in all MAP isolates examined and in selected porcine isolates of *M. avium* subsp. *avium* (Olsen *et al*., 2004), but absent in other *M. avium* subsp. *hominissuis* isolates. Johansen *et al*. (2005) examined two insertion sequences, IS1311 and IS1245, which share 85% homology, and discovered that IS1245 could be misidentified in MAP when using an extended IS1245 probe. However, after designing a shorter, more specific probe they demonstrated that the element was in fact not present in MAP. This discrepancy was attributed to the strong sequence similarity between IS1245 and IS1311, an element that is represented seven times in MAP. This study serves to further highlight that even the known differences between MAP and other MAC strains are subtle and can lead to confusion. A few MAP insertion elements, discovered only by genome sequencing, are present in MAP isolates and absent from MAC isolates. One such element, ISMAP02, which has already been used to develop a novel PCR-based test for faecal samples (Stabel and Bannantine, 2005) and colostrum (Pithua *et al*., 2010), is present in six copies in the genome.

The most widely known IS element is IS*900*, which is present in 17 copies in both strain K-10 and S397 genomes and has been used for decades as a diagnostic target in addition to the strain typing method mentioned above. Sequencing of other MAP genomes has shown that the number of IS*900* elements is stable across strains except for the human strain MAP4, which has 16 copies (Bannatine *et al*., 2014). However, analysis of MAP vaccine strains has shown that IS*900* copy number can vary depending on the existence

of duplicated regions or deletions in the specific strain analysed (Bull *et al*., 2013). Also, this element has similar sequence regions known to exist in non-MAP strains (Englund *et al*., 2002; Motiwala *et al*., 2004).

Some regions of the MAP genome have been discovered that are not present in other MAC strains. Using RDA technology Sheridan *et al*. (2003) studied the 6.5-kb GS element of MAP that was previously reported to be absent in MAA (Tizard *et al*., 1998). This large region was analysed using bioinformatic tools, which predicted that coding sequences are involved in GDP-fucose biosynthesis and modification of the oligosaccharide moiety of GPL. Stratmann *et al*. (2004) also used the RDA technique to discover a novel 7-kb ABC-transporter operon located within a 38-kb segment that is flanked by insertion sequences. Several gene clusters thought to be involved in iron uptake are also present on this genomic island. This is the first pathogenicity island reported in MAP. Since these studies, several other genomic insertions and deletions have been discovered that represent a significant source of MAP genetic diversity (Marsh *et al*., 2006; Alexander *et al*., 2009). Some of these LSPs are quite useful in strain identification, as discussed later.

By hybridizing genomic DNA from MAC strains to DNA microarrays, a total of 24 and 18 genomic islands was identified belonging to *M. avium* subsp. *hominissuis* and MAP, respectively (Wu *et al*., 2006) suggesting the mosaic nature of genomes among MAC strains (Semret *et al*., 2005; Alexander *et al*., 2009). Interestingly, large regions of genomic inversions were found among MAP and *M. avium* subsp. *hominissuis* strains (Wu *et al*., 2006), another indication of the presence of a common ancestor between members of the MAC group. These genomic islands, along with the genes contained within each island and the evolutionary implications for these regions, have been reported and summarized (Paustian *et al*., 2005; Alexander *et al*., 2009). A total of six genomic insertions specific to MAP comprise 125 kb and two of these are putative prophages (Alexander *et al*., 2009). Analysis of these genomic islands as insertion or deletion events led to the proposal of a biphasic evolution of MAP (Alexander *et al*., 2009). Added to these data was an extensive multilocus sequencing study that suggested *M. avium* subsp. *hominissuis* is the ancestor strain from which MAP and *M. avium* subsp. *avium* independently evolved (Turenne *et al*., 2008). Finally, it appears that MAP itself is genetically homogenous with primary differences observed between cattle and sheep strains. Therefore, genome sequencing of a sheep isolate was recommended to close this knowledge gap.

Differences Between Sheep and Cattle Strains of MAP

Since 2003, genetic diversity among MAP isolates has been examined extensively using genomic approaches such as identification of sequence repeats in the genome (Bull *et al*., 2003; Amonsin *et al*., 2004; Overduin *et al*., 2004) or gel-based assays including amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) analysis (O'Shea *et al*., 2004; de Juan *et al*., 2005). The MAP lineage can generally be divided into two phenotypically distinct branches, cattle (C) strains and sheep (S) strains. The C strains are generally more robust and grow faster than the fastidious S strains. Human and wildlife isolates generally fall into the cattle lineage whereas the sheep isolates group separately, although camel isolates have recently been shown to be in the S strain lineage (Ghosh *et al*., 2012). The initial efforts to distinguish among MAP strains using the IS*900* element also occurred in the early 1990s using BstEII restriction fragment length polymorphism analysis (Collins *et al*., 1990a; Whipple *et al*., 1990). While sheep and cattle were strains known to be phenotypically different in growth rate and appearance, the RFLP experiments were the first to demonstrate genetic differences.

Several MAP DNA microarrays have been built for genomic hybridization studies to better define genes or regions that are present or absent between strains and can yield clues to host phenotype. The initial MAP microarray consisted of 4110 spotted PCR products representing 95% of the predicted coding sequences in the K-10 genome and

was designed to examine genomic diversity among MAC member strains (Paustian *et al*., 2005). Second-generation oligonucleotide arrays represented every predicted open reading frame in the strain K-10 genome, but they also contained MAH probes (Semret *et al*., 2004; Wu *et al*., 2006; Paustian *et al*., 2008; Castellanos *et al*., 2009). Many of the common regions of divergence revealed among the non-paratuberculosis MAC isolates were grouped into clusters of adjacent genes. This same approach was later used to identify three segments comprising 24 genes that were missing in the Australian sheep strain, Telford 9.2, when compared to the bovine strain K-10 (Marsh *et al*., 2006). These genomic differences may account for the host specificity observed in sheep strains of MAP. Two of these three segments are conserved among USA and Australian sheep isolates (Bannantine *et al*., 2012).

Whittington *et al*. (2000, 2001) and Marsh *et al*. (1999) developed the IS1311 amplification-restriction enzyme analysis to distinguish C strains from S strains at a genotype level. This technique could also be used to distinguish other subspecies of *M. avium*, but was not able to further subdivide MAP C and S isolates. Likewise, analysis of genomic LSPs quickly distinguished C strains from S strains by the presence of $LSP^{A}20$ and absence of $LSP^{A}4$, but failed to further discriminate the S strains (Biet *et al*., 2012). However, PFGE was able to further divide the S strains into type I and type III (Stevenson *et al*., 2002; de Juan *et al*., 2006). The presence of unique strains had been previously suggested when IS*900* RFLP analysis (Pavlik *et al*., 1999) failed to classify some isolates into either the type I (S) or type II (C) classification, leading to a new type III classification (de Juan *et al*., 2005). IS*900* RFLP and SNP analysis of the gyrAB genes (Castellanos *et al*., 2007) and MAP1506 (Griffiths *et al*., 2008) also clearly divided MAP into the type II, type I and type III lineages, whereas MIRU-VNTR does not provide such definitive results (Biet *et al*., 2012). In the case of MAP1506, a single-nucleotide polymorphism at nucleotide position 344 is used to differentiate type I and type III S strains. A 'G' nucleotide at this position signifies a type I strain and an 'A' signifies a type III strain (Griffiths *et al*., 2008). Based upon these results, the genome sequenced sheep strain S397 is a type III strain isolated from the USA (Bannantine *et al*., 2012). The type III strains are considered more heterogeneous than type I strains.

Little has been done to directly correlate genetic differences with phenotype, but S strains appear to have a narrow host range compared to C strains. Furthermore, some pathogenic differences between these lineages have been noted in terms of their survival within macrophages (Motiwala *et al*., 2004; Gollnick *et al*., 2007; Borrmann *et al*., 2011). Macrophages infected with sheep strains produced different cytokine profiles and levels of phagocytic efficiency compared with cattle strains. One study was successful in correlating a genotype difference to an observable phenotype. Differences in the length and binding characteristics of a heparin-binding haemagglutinin adhesion protein between C and S strains have recently been reported for heparin binding (Lefrancois *et al*., 2013). The C-terminal domain of this protein is smaller and its binding to heparin is weaker in the C strains compared to the S strains.

Strain Differences Applied to Strain Typing Methods

Short sequence repeats (SSR) analysis (Amonsin *et al*., 2004) can further reveal genetic diversity and can discriminate among MAP isolates. SSR requires DNA sequencing and the ability to accurately read long poly G tracts, particularly at locus 2, which can be subject to sequencing errors (Thibault *et al*., 2008; Fritsch *et al*., 2012), but it appears that despite these deficiencies, SSR typing has recently emerged as the most commonly used technique and the most discriminatory (Harris *et al*., 2006; Pradhan *et al*., 2011). Of the 11 discriminating loci originally discovered for MAP SSR, locus 1 is always included in published studies, suggesting its importance. It was the only locus used by Motiwala *et al*. (2004) whereas most other studies included both loci 1 and 8 in some combination (Ghadiali *et al*., 2004; Corn *et al*., 2005; Motiwala *et al*., 2005; Sevilla *et al*., 2008). The four most discriminating SSR

loci are 1, 2, 8 and 9 (Douarre *et al*., 2011). However, recent evidence suggests that locus 2 may be unstable, showing changes even after one subculture (Kasnitz *et al*., 2013). Despite increased reliance upon SSR typing, historically IS*900* RFLP has been the most widely used method and has been standardized for ease of interpretation (Pavlik *et al*., 1999).

Application of Molecular Epidemiology to Johne's Disease

There are still significant gaps in knowledge about how MAP strains are transmitted within a herd and the role wildlife plays in spreading MAP from one farm to another. Only recently have molecular epidemiological studies emerged that applied strain typing techniques beyond the laboratory to track MAP strains in farm settings (Motiwala *et al*., 2004; Forde *et al*., 2012). Using the molecular techniques described, investigators can determine if one strain of MAP predominates on a farm or if multiple circulating strains exist at similar levels (Pradhan *et al*., 2011). MAP is prevalent in the environment, not only on farms, but also in zoo settings. One recent study detected MAP in a variety of zoo animals by IS*900* semi-nested PCR (Munster *et al*., 2013). The application of genotyping methods to paratuberculosis will enable the tracking of MAP isolates/subtypes on a single dairy farm or other wildlife settings. This will allow researchers to identify and track movements of the most successful/widespread isolates as well as to obtain clues to prevent the transmission of MAP.

Several studies have used one or more of the typing methods described here to survey MAP strains within a given region. For example, 17 sheep isolates from Spain showed considerable diversity among type III strains (Sevilla *et al*., 2008). They further showed through SSR and PFGE methods that multiple strain types are present on a single farm in 20 of 33 bovine farms analysed. They observed that new strains emerged while others disappeared over a 6-year time frame from 2000 to 2005. Interestingly, the type III strains seem to be predominant in Spain while type I strains appear more common in Australia, New Zealand and the USA. In Germany, MIRU-VNTR genotypes 1 and 2 found commonly in Europe were present in 44% and 28%, respectively, of the 91 isolates examined in dairy cattle (Stevenson *et al*., 2009; Douarre *et al*., 2011; Fernandez-Silva *et al*., 2012). In a second study, genotype 1 was more dominant at 52% of all 71 bovine isolates examined among 14 dairy herds in Germany (Mobius *et al*., 2008). The SSR method was further used to track strains shed in faeces and present in tissues from three dairy herds over time in north-eastern USA to determine transmission dynamics (Pradhan *et al*., 2011). A total of 15 different strains among 142 were catalogued within and between the three herds. Seven strain types were present on more than one farm while the eight others were farm specific. One strain (SSR type 2) predominated, present in 89% of the infections in a single herd, while multiple strains were present within animals in the other herds. The researchers further found that at least one-half of the low-shedding cows had the same strain as that of high-shedding cows (termed 'super shedders'), suggesting that super-shedder cows may readily transmit MAP to their herd mates. These methods can also determine if super-shedder cows are infected with a particular genotype of MAP or if clinical animals shed multiple strains in their faeces (Harris *et al*., 2006).

In conclusion, MAP infection elicits a complex and problematic series of responses within the host. Although in recent years a more studied approach to infection and pathogenesis has evolved, resulting in progress in our understanding of host immunity to infection, much remains to be elucidated.

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29 Nontuberculous Mycobacterial Infections

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Introduction

Nontuberculous mycobacteria (NTM) are opportunistic pathogens that share environments with animals, poultry and humans. The causative agent of Johne's disease in cattle, *Mycobacterium avium* subspecies *paratuberculosis*, is the only classic pathogen of the group; all other subspecies are opportunistic pathogens. For the opportunists, disease follows exposure to the portion of the population that is transiently susceptible. Quite possibly the major sources of NTM infection for humans are drinking water distribution systems and premise plumbing (Falkinham *et al*., 2001; Falkinham, 2011). As NTM are natural inhabitants of soils (Iivanainen *et al*., 1997; De Groote *et al*., 2006), soil is a source of infection for both humans and animals (via dusts). NTM are quite hardy; their wax-rich outer membrane contributes to their resistance to disinfection and antibiotics (Brennan and Nikaido, 1995). As the NTM are innately resistant to anti-tuberculosis agents, drug therapy is problematic, even in humans and companion animals. For agronomic animals, for example pigs, it is more cost effective to reduce levels of NTM in the animal's environment.

Although NTM grow slowly (one generation per day) relative to other microorganisms, they are disinfectant resistant, able to grow on low concentrations of organic matter and form biofilms (George *et al*., 1980). Biofilm formation allows the slowly growing NTM to persist in water distribution systems without threat of being washed out. Depending on the pipe surface, NTM biofilm can numbers reach $2 \times 10^5/cm^2$. Once established in a drinking water distribution system or building, NTM are quite difficult to eradicate, although hot water temperatures above 130°F (55°C) are associated with fewer NTM (Falkinham, 2011).

NTM are transmitted either via aerosols or via dusts. Aerosols can be generated either naturally from bodies of water (Parker *et al*., 1983); or from sprays, perhaps used to cool animals in a mist. Infections in humans have been traced to showers (Falkinham *et al*., 2008). NTM in dusts generated from NTM-rich potting soils have also been linked to infection in humans (De Groote *et al*., 2006).

Over the past 20 years there has been a terrific increase in the number of *Mycobacterium* species (>150; Tortoli, 2003). Speciation of isolates of the genus *Mycobacterium* is important, as the species differ in virulence, antibiotic susceptibility, host range and ecology (Thorel *et al*., 1990; Tortoli, 2003). DNA-based methods (e.g. PCR) are available for mycobacterial identification

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 Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria (eds H. Mukundan *et al*.)

(Tortoli, 2003). In Table 29.1, I have listed the major *Mycobacterium* species infecting humans and animals. As there has been radical revision of taxonomy of the genus *Mycobacterium*, care should be taken in reviewing data published prior to 2003 (Tortoli, 2003).

The virulence of the NTM is low and it is likely that infection is due to either a physiologic or genetic predisposition in hosts that makes them unusually susceptible. To date there have been few papers identifying genes and proteins that have an impact on virulence of NTM. Within a single species, virulence in animal models varies widely, yet no genes have been assigned to the increased virulence of some isolates relative to others. There is, however, one subspecies that is of high predictable virulence, *M. avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease in cattle and other ruminants (Biet *et al*., 2005).

Nontuberculous Mycobacterial Infections in Humans

Nontuberculous mycobacteria cause pulmonary, dermal and disseminated infections in humans. At the outset, it is important to point out that it is likely that everyone with an NTM infection has some predisposing risk factor that makes them considerably more susceptible than the rest of the human population. The incidence of infections (principally pulmonary disease) caused by NTM increased from 1–2 per 100,000 in 1990 to 8–10 per 100,000 in 2005 (Marras *et al*., 2007). In the USA, that translates to 30,000 active cases. Disease presentations include: pulmonary disease, cervical lymphadenitis (Wolinsky, 1995), skin infections, furunculosis (Winthrop *et al*., 2002) and disseminated disease in individuals with AIDS or who are immunosuppressed due to cancer, chemotherapy, or coincident with transplantation (Wayne and Sramek, 1992).

Table 29.1. *Mycobacterium* spp. infecting humans and animals.

Mycobacterium species	Human disease	Animal disease
M. avium		
M. avium subsp. *hominissuis*	Yes	Yes (pigs)
M. avium subsp. *avium*	No	Yes (birds)
M. avium subsp. *silvaticum*	No	Yes (wild animals)
M. avium subsp. *paratuberculosis*	Perhaps	Yes
M. intracellulare	Rare	Unknown
M. chimaera	Yes	Unknown
M. haemophilum	Yes	Unknown
M. kansasii	Yes	Unknown
M. marinum	Yes	Yes (fish)
M. malmoense	Yes	Unknown
M. xenopi	Yes	Unknown
M. abscessus	Yes	Unknown
M. chelonae	Yes	Unknown
M. fortuitum	Yes	Unknown

Risk Factors for Nontuberculous Mycobacterial Infection in Humans

Risk factors for mycobacterial pulmonary disease among immunocompetent individuals include lung damage such as pneumoconiosis, black lung, smoking, or alcoholism (Wolinsky, 1979). Patients with cystic fibrosis can be infected with *M. avium* or *M. abscessus* (Jonsson *et al*., 2007). Young children (18 months to 5 years) can present with *M. avium* cervical lymphadenitis (Wolinsky, 1995). Quite possibly, these children are relatively susceptible as they have erupting teeth, providing a direct route of lymph node infection due to trauma to the gums. Last, it has been shown that individuals with chronic rhinosinusitis caused by NTM are infected with the same NTM species and strain as isolated from the drinking water they use to irrigate their sinuses (Tichenor *et al*., 2012). Individuals with gastroesophaegeal reflux disease (GERD) are also at higher risk for pulmonary disease caused by NTM (Koh *et al*., 2007; Thomson *et al*., 2007). A new presentation of NTM pulmonary disease is found in slender, elderly men and women who lack any of the classic risk factors (Prince *et al*., 1989; Reich and Johnson, 1991; Kennedy and Weber, 1994). This group of patients makes the largest contribution to the current increase in NTM disease. One study of such elderly patients

identified that some had mutations in the chloride membrane transport protein, CFTR (Kim *et al*., 2005).

Individuals who are immunosuppressed as a consequence of HIV infection, cancer, chemotherapy or transplantation are also at risk for NTM infection (Wayne and Sramek, 1992). These patients present with NTM bacteraemia. Fortunately as a result of highly active anti-retroviral treatment (HAART) for HIV-infected individuals, NTM infection, primarily *M. avium* subsp. *hominissuis*, in that group of patients has disappeared.

Two NTM species are associated with skin infections: *M. marinum* and *M. haemophilum* (Dobos *et al*., 1999). Unsurprisingly, both species have a growth temperature optimum of 30°C and cannot be recovered on laboratory medium if incubated at 37°C. An important risk factor for acquisition of *M. marinum* infection is superficial cuts and exposure to infected fish (Wolinsky, 1979). Thus, fishermen and individuals keeping aquaria are at risk for infection. *M. haemophilum* skin infections are found in immunosuppressed individuals and have been associated with kidney dialysis (Dobos *et al*., 1999).

Surgery-associated, nosocomial infections of skin and surgical wounds have been shown to be due to *M. abscessus*, *M. chelonae* and *M. fortuitum* (Wallace *et al*., 1998). In a number of instances the NTM isolate in the patient has been traced to contaminated disinfectant solutions, drinking water or ice (Wallace *et al*., 1998).

In addition to pulmonary infection, NTM exposure can lead to hypersensitivity pneumonitis. Hypersensitivity pneumonitis is produced in rabbits exposed to cell envelope fractions (Richerson *et al*., 1982) and mycobacterial cells can elicit hypersensitivity reactions in macrophage cells (Huttunen *et al*., 2000). Recently, inhalation of mycobacterial-containing aerosols generated from hot tubs was linked to hypersensitivity pneumonitis (Rickman *et al*., 2002; Marras *et al*., 2005). It has been hypothesized that exposure to aerosols of metal recovery fluids in the automobile industry is responsible for outbreaks of hypersensitivity pneumonitis. Mycobacteria, including the novel species *M. immunogenum*, have been isolated from metal recovery fluids linked to hypersensitivity pneumonitis (Moore *et al*., 2000). Mycobacteria are likely to be introduced during mixing of the organic-based metal recovery fluid with water to produce the emulsion that cools the working surfaces of cutting and grinding tools and removes metal fragments. Mycobacteria can metabolize many of the hydrocarbon constituents of metal recovery fluid (Krulwich and Pelliccione, 1979) and survive addition of disinfectant used to inhibit microbial growth. Thus, like drinking water distribution systems and household plumbing, this engineered environment selects for mycobacterial persistence and growth.

Nontuberculous Mycobacterial Infections in Animals

Although animals are surrounded by NTM, the slow development of symptoms following infection means that disease symptoms are not seen in a variety of agronomic important animals such as beef cattle, broiler chickens and turkeys, due to their short lifetimes. Thus, reports of animal NTM infection are predominantly in dairy cattle, breeder pigs and companion animals.

Johne's Disease in Cattle and *M. avium* subsp. *paratuberculosis*

Johne's disease is a multibacillary diarrhoeal disease of cattle and other ruminants caused by infection by *M. avium* subsp. *paratuberculosis* (MAP). In cattle it is characterized by chronic diarrhoea, progressive weight loss, reduced milk production and high numbers (10^9 colony-forming units/g) of *M. avium* subsp. *paratuberculosis* in faeces (Biet *et al*., 2005). If untreated, infection leads to emaciation and death. In sheep there is emaciation, but no profuse diarrhoea.

Johne's disease can be found among ruminant populations in countries with developed agriculture. A relatively high proportion of US dairy farms (50%) have at least one to two infected cows. Unfortunately, many breeders may not be aware of MAP infection

in their herds. It is thought that in herds transmission is from infected mothers and the appearance of Johne's disease in a herd without a prior history of Johne's disease is usually due to importation of infected cows. Infection could also occur via consumption of MAP-contaminated pasture grass, silage or water.

As high numbers of MAP are excreted, it is not surprising that MAP DNA can be detected in drinking water (Pickup *et al*., 2005). MAP presence in drinking water is important as it relates to the hypothesis that *M. avium* subsp. *paratuberculosis* is also the etiologic agent of Crohn's disease in humans.

Animals and *M. avium* subsp. *hominissuis* Infection

Pigs and humans share susceptibility to *M. avium* subsp. *hominissuis*, although there is no evidence that *M. avium* subsp. *hominissuis*-infected pigs are a threat for human infection. Horses have also been reported with *M. avium* subsp. *hominissuis* infections (Blahutkova *et al*., 2011). This same subspecies is also possibly responsible for the rare, dermal *M. avium* infections in companion animals (Kazda *et al*., 2009). Systemic *M. avium* subsp. *hominissuis* disease in dogs or cats is often a sign of an underlying immune deficiency.

M. avium subsp. *hominissuis* infection in pigs is difficult to detect at its onset owing to the slow progression of disease symptoms. Often disease is only recognized upon inspection post mortem, namely the appearance of tuberculous lesions (granulomas) in cervical or mesenteric lymph nodes. Symptoms include loss of weight, an intermittent cough, lack of appetite, digestive system disorders and reduced activity. However, it is important to point out that pigs displaying that spectrum of symptoms may have rather advanced disease. In the absence of animal-to-animal transmission, it is logical to proceed on the hypothesis that infection is due to exposure to *M. avium* subsp. *hominissuis* from water or soil. Exposure of pigs to bedding material containing *M. avium* subsp. *hominissuis* (e.g. sawdust) and inadequate nutrition contribute to appearance of disease (Kazda *et al*., 2009).

Poultry and *M. avium* subsp. *avium* Infections

Avian tuberculosis is characteristic of production practices involving large flocks of chickens (*Gallus domesticus*). As *M. avium* subsp. *avium* is rarely associated with infection in humans, avian tuberculosis does not pose a risk for infection in humans. Development of clinical disease is only seen in 2-year-old and older hens and cocks (Kaevska *et al*., 2010). This suggests that changing the stock of laying hens can prevent avian tuberculosis development. *M. avium* subsp. *avium* disease cannot get established in a broiler fattening system lasting only 6–8 weeks. In addition to poultry, a variety of bird species kept in captivity is susceptible to *M. avium* subsp. *avium* infection, but waterfowl are resistant. *M. avium* subsp. *avium* infections are also found in a variety of wild animals (Biet *et al*., 2005).

Early clinical signs of *M. avium* subsp. *avium* infection are lethargy, reduced egg laying and loss of appetite, followed by emaciation; and, in the case of intestinal tract infection, also diarrhoea (Moravkova *et al*., 2011). Many infected birds can live for an extended period and maintain good nutritional status during which time they can be indistinguishable from normal healthy birds. Antemortem diagnosis of *M. avium* subsp. *avium* infection can employ the intradermal tuberculin test, using an avian tuberculin or faecal culture. Unfortunately, the tests lack a high level of sensitivity and specificity, as exposure without disease can trigger a positive reaction. Post-mortem diagnosis involves microscopic examination, culture or PCR detection of MAC in either the liver or spleen.

Wild Animals and *M. avium* subsp. *silvaticum* Infection

M. avium subsp. *silvaticum* is the most common *M. avium* subspecies isolated from NTM-infected wild animals. Tuberculous lesions were detected in mesenteric lymph nodes of two (0.2%) wild boars, from which *M. avium* subsp. *avium* was isolated (Trcka *et al*., 2006). As the wild boars were killed near a farm

where pheasant were infected with the same subspecies, the boars could have been infected from eating infected pheasants or from water, soil or bedding material on the farm. A study of one infected herd of red deer (*Cervus elaphus*) found four related RFLP types, indicative of a single environmental source of infection (Machackova-Kopecna *et al*., 2005).

The most striking features identified in *M. avium* subsp. *silvaticum*-infected animals are loss of weight accompanied by intermittent cough, lack of appetite, digestive system disorders, lesions of the mammary gland and reduced movement. *M. avium* subsp. *silvaticum*-infected wild boars and ruminants usually do not show any clinical signs of disease. However, if the animal is displaying clinical signs, it always implies that the disease has progressed to the point where anatomical structure and function of the infected tissues and organs are deteriorating. Common sites of tuberculous lesions are in head and intestinal lymph nodes. A range of factors may predispose wild animals to NTM infection: stress, injury and inadequate nutrition (Biet *et al*., 2005).

Fish and *M. marinum* Infections

The main causative agent of *Mycobacterium* infection in fish is *M. marinum*, although many other NTM species have been linked to infection in a variety of fish (Gauthier and Rhodes, 2009). Exposure to *M. marinum*-infected fish through fishing or aquaculture is a risk factor for skin granulomas in humans (Gauthier and Rhodes, 2009).

Clinical signs of mycobacteriosis in fish are usually non-specific and include abnormal behaviour including: evidence of fright, loss of appetite, progressive wasting, ascites, raised scales or their loss, changes in cutaneous pigmentation, dermal ulcerative lesions and skeletal deformities manifested as spine curvature disorders making smooth swimming impossible (Gauthier and Rhodes, 2009). Systemic organ infection is common and may be difficult to recognize in its early stages until manifested by superficial changes, namely granulomatous gills. The acute form of the disease, in which the animal dies rapidly after onset of symptoms, occurs rarely.

Post-mortem examination of infected fish reveals small white lesions (tubercles) in grossly swollen organs. Microscopic examination of the lesions shows classic tuberculous granulomas characterized by central necrosis surrounded by epithelioid cells and histiocytes (Gauthier and Rhodes, 2009).

M. marinum can be introduced into any aquaculture facility through a number of routes: infected stock, input water, plants or feed. *M. marinum* infection in pompano were traced to the presence of *M. marinum*, sharing the same DNA fingerprint pattern, in aquarium filters and aquarium water and biofilms (Yanong *et al*., 2010). The main infection entry sites are the gills and injured skin of fish. Most injuries occur in fighting fish defending their territories, and at the time of sexual activity, incautious handling and in the presence of sharp objects in aquaria (e.g. unsuitable decorative items). Stress caused by inadequate diets, unfavourable conditions and transfer between natural and artificial environments are considered predisposing factors.

When fish display symptoms of *M. marinum* infection, they should be removed from the aquarium as soon as possible. Care must be taken by staff to avoid infection; removal is best accomplished with gloves. Following removal of infected fish, the aquarium and its associated water delivery system should be cleaned and sterilized to the best degree possible to prevent more fish from becoming infected.

Characteristics of Nontuberculous Mycobacteria

The major determinant of the physiology, metabolism and ecology of the NTM is the hydrophobic, thick, long-chain lipid (C_{60}–C_{80})-containing outer membrane (Brennan and Nikaido, 1995). It provides a hydrophobic, impermeable barrier to the diffusion or transport of hydrophilic compounds (metals, oxyanions, nutrients, antibiotics and disinfectants) (Rastogi *et al*., 1981). Unsurprisingly, NTM are antibiotic and disinfectant resistant. The characteristic slow growth of mycobacteria

(one generation per day) also contributes to disinfectant- and antibiotic-resistance. Unlike rapidly growing bacteria that do not have sufficient time to induce survival mechanisms when challenged with exposure to antimicrobials, the slowly growing NTM have time to transcribe genes and thereby induce functions designed to protect cells from a variety of environmental insults. A summary of the physiologic characteristics that are determinants of their ecology are listed in Table 29.2.

As all NTM cells are hydrophobic – in fact the highest among bacteria (van Oss *et al*., 1975) – cells prefer surface attachment to suspension; namely to pipes in water distribution systems (Falkinham *et al*., 2001) and household plumbing (Falkinham, 2011) and to soil particles (Falkinham *et al*., 2001). Therefore, studies to detect the presence of NTM are best served by sampling biofilms in water taps and shower heads in households (Falkinham, 2011) and particulate samples in surface water and distribution systems. In fact, reduction of turbidity during drinking water treatment has the added benefit of reducing NTM numbers (Falkinham *et al*., 2001). NTM cell surface hydrophobicity also results in their preferential aerosolization water to air in droplets that are 1000- to 10,000-fold enriched relative to the concentration in the bulk suspension (Parker *et al*., 1983).

Although neither thoroughly investigated nor appreciated, NTM are likely to be important agents of environmental biodegradation of two classes of compounds: the naturally occurring humic and fulvic acids and hydrocarbon pollutants. High numbers of NTM are found in humic and fulvic acid rich habitats such as eastern US coastal brown water swamps (Kirschner *et al*., 1992) and peat-rich, boreal forest soils of Finland (Iivanainen *et al*., 1997). In addition, mycobacteria have been detected in samples collected from toxic waste dumps and there is a relatively extensive literature documenting the ability of mycobacteria to degrade a wide range of hydrocarbons and chlorinated hydrocarbons (Krulwich and Pelliccione, 1979).

Table 29.2. Physiologic determinants of nontuberculous mycobacteria.

Habitat	Determinant NTM trait
Natural soils	Hydrophobicity drives particle attachment
	Growth and survival in phagocytic amoebae and protozoa
	Humic and fulvic acid growth stimulation
	Growth at low (6%) oxygen levels
Natural waters	Oligotrophic
Drinking water distribution systems	Chlorine and disinfectant resistance
	Hydrophobicity drives surface attachment – biofilm formation
	Metabolism of humic and fulvic acids
Household plumbing	Heat resistance
	Growth under conditions of stagnation (low oxygen)
	Pipe adherence – biofilm formation
Building plumbing	Heat resistance
	Recirculating hot water systems
	Pipe adherence – biofilm formation
Granular activated carbon (GAC) filters	Disinfectant and metal resistance
Polluted waste dumps	Metal resistance and acid tolerance
	Hydrocarbon and chlorinated hydrocarbon degradation
	Growth at reduced oxygen levels (6%)

Sources of Nontuberculous Mycobacteria

NTM are widely distributed in the natural and human-engineered environment: (i) it should be noted that those habitats are shared with humans as well as with important agronomic plants and animals; and (ii) it should be understood that the NTM are not contaminants, but rather normal inhabitants able to survive, grow and persist in natural soils, natural waters and engineered water systems including those in buildings and homes. As pointed out, the NTM are ideally adapted to

coexist with humans in a number of habitats, particularly in drinking water distribution systems and household plumbing. Table 29.3 lists those habitats and the NTM adaptations and determinants supporting their persistence.

Routes of Transmission of Nontuberculous Mycobacteria

Pulmonary infection can occur via the inhalation of NTM-laden aerosols or soil particles (dusts) or as a result of swallowing NTM (in water or food) and aspirating the bacteria because of gastric reflux. NTM are readily transferred from water to air because of their high surface hydrophobicity. Any occupational or household aerosol-generating system (e.g. humidifiers) whose source is piped drinking water should be viewed as a potential source of NTM aerosols. For example, exposure to aerosols from hot tubs and spas with their artificial bubble-generating apparatus has been linked to NTM pulmonary disease (Mangione *et al*., 2001); to hypersensitivity pneumonitis (Marras *et al*., 2005); or to hypersensitivity pneumonitis resulting from exposures to either hot tubs and spas (Marras *et al*., 2005) or aerosolized metal-recovery fluid used in automobile manufacturing (Moore *et al*., 2000).

Table 29.3. Habitats occupied by nontuberculous mycobacteria.

Habitat
Soils, especially peat-rich forest soils and brown water, coastal and estuarine soils
Natural waters, especially drainage from peat-rich forest soils and brackish estuaries
Drinking water distribution systems (biofilms)
Plumbing in buildings, hospitals and households (biofilms)
Showerheads, spas and hot tubs, humidifiers, therapy baths and swimming pools
Granular activated carbon (GAC) filters in water lines and refrigerators
Water-damaged building materials
Polluted waste dumps
Aquaria and fish (*M. marinum*)
Pasteurized and unpasteurized milk (*M. avium* subsp. *paratuberculosis*)

Environmental Sensitization to Nontuberculous Mycobacteria

A long-standing problem associated with recognition that humans and animals are continually exposed to environmental mycobacteria is the use of mycobacterial antigens for differential diagnosis of infection. As environmental *Mycobacterium* NTM species share antigens, it is to be expected and found that humans and animals show evidence of infection by the environmental NTM. Skin tests with a purified protein derivative antigen prepared from a strain of *M. intracellulare* (PPD-B) showed that between 50% and 80% of 18–25 year-old single county residents of the south-eastern USA had positive skin tests (Edwards *et al*., 1969). The development of positive skin tests for mycobacteria due to environmental exposure to NTM (called 'nonspecific sensitivity') also obviates the utility of skin testing humans and animals for evidence of mycobacterial disease. Positive skin tests could be due to disease, vaccination or environmental exposure.

Finally, NTM can cause granulomatous, 'tuberculous' lesions in cervical and mesenteric lymph nodes. This is a particular problem in examination of carcasses in pig abattoirs. If a tuberculous cervical lymph node is found, the head cannot be used for human food consumption, only the body. If a tuberculous mesenteric lymph node is found, then the entire carcass cannot be used for food for human consumption. As the frequency of pigs with 'tuberculous' lymph nodes at slaughter is approximately 5% in the USA, the USDA decision directly affects the profitability of pig farming.

Agricultural and Engineering Practices Influencing Nontuberculous Mycobacteria

NTM are poor competitors in the environment as they grow slowly. However, human

engineering practices such as water disinfection select for NTM. Chlorine and other disinfectants kill off other waterborne microorganisms, allowing the NTM to proliferate and grow on available nutrients in the absence of competition. Numbers of NTM increase at least twofold between the treatment plant and distal regions of the distribution system (Falkinham *et al*., 2001). As NTM enter water treatment plants from surface water attached to particulates, it follows that reduction in water turbidity will reduce NTM numbers (Falkinham *et al*., 2001). Because granular activated carbon (GAC) filters do not prevent the passage of bacteria (i.e. the pores are too big) and the filters trap nutrients and metals, NTM numbers are increased in water flowing out of GAC filters (Rodgers *et al*., 1999). Finally, we have measured the highest numbers of NTM in buildings, hospitals, apartments and condominiums with recirculating hot water systems (Tichenor *et al*., 2012). This is likely to be due to the growth of the NTM as they are circulated throughout the building, coupled with their relative heat resistance.

Reducing Nontuberculous Mycobacterial Disease in Humans and Animals

Table 29.4 lists suggested measures to reduce numbers of NTM in the human and animal environment. The recommendations are not based on trials, but rather on evidence gathered concerning numbers of NTM in different types of samples. For example, households of NTM patients whose hot water temperature was greater than 55°C (130°F) had a lower frequency of samples containing NTM compared with households whose hot water heater temperature was equal to or less than 50°C (125°F) (Falkinham, 2011). Likewise, households with well (ground water) sources had a lower frequency of samples with NTM, compared with those in which water came from a piped (utility) source.

Table 29.4. Recommendations to reduce NTM exposure.

Recommendation	Rationale
Drain the hot water heater	NTM accumulate and grow
Raise hot water heater temperature	Low NTM in hot water
Use a well source	Low NTM in ground water
Install filters that remove bacteria	Prevent passage of NTM with 0.2 µm pores
Avoid GAC water filters	GAC filters promote NTM growth
Reduce showerhead aerosols	Showerhead NTM linked with disease
Remove and clean showerheads	Showerheads have NTM, soak in bleach
Avoid humidifiers, hot tubs, spas	Generate NTM aerosols
Avoid dusts from potting soil	Potting soil is rich in peat and NTM

Summary

Nontuberculous mycobacteria (NTM) are opportunistic pathogens of animals, poultry and humans. They are normal inhabitants of natural waters, drinking water distribution systems and premise plumbing, and so companion and agronomic animals, poultry, birds and fish are at risk of NTM infection. NTM infections occur in the lungs, skin and bloodstream and in humans are almost invariably associated with some risk factor, either genetic or physiologic. These NTM are resistant to disinfectants used in water treatment and to most anti-tuberculosis drugs. The appearance of NTM infection in agronomic animals and poultry causes considerable economic loss to animal producers. NTM infection occurs through exposure to habitats containing NTM, such as water and soil; there is no evidence of transmission of infection between persons or animals. Remediation and prevention of NTM infection is difficult due to their resistance to disinfection and their ubiquitous presence in the environment.

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Index

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