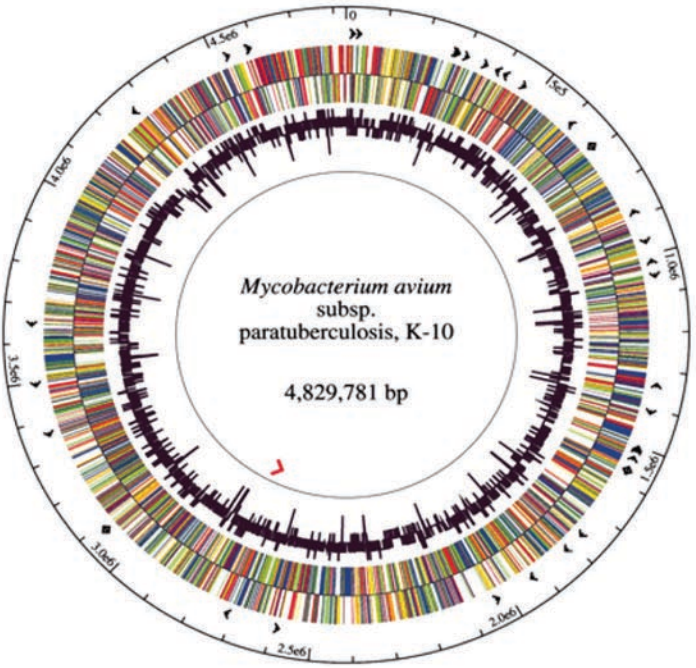
Paratuberculosis

Organism, Disease, Control



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Preface

Almost 100 years ago, Twort and Ingram (1913), in their excellent monograph on Johne's disease (often now called paratuberculosis), reported many of the features that have since been confirmed by much further study. They stated that most farms only have a few cases of the disease a year, that the disease is never seen in very young animals, that cows often develop the disease soon after calving, and that mature, healthy animals put on to an infected farm do not subsequently develop the disease. The economic costs of the disease are calculated and shown to be significant in the currency values of the time. While the word 'supershedder' is not mentioned, there is a clear statement that animals with advanced disease discharge the largest numbers of organisms in their faeces and are the source of the disease for other animals. In the same year as this book appeared, the possibility that the organism that causes Johne's disease might also cause human inflammatory bowel disease was also first suggested (Dalziel, 1913). Plainly, this is not a disease on which fast progress has been made.

There is a tendency to look back at the science landscape years ago and think of it as some sort of golden age. Nothing could be further from the truth. Earlier scientists like Twort, Ingram and others struggled with the same formidable problems the disease presents today but without many of the tools and resources that are available to us. One section of their preface will certainly resonate with many paratuberculosis researchers today:

> Research work on a disease which affects any of the larger domesticated animals is necessarily very costly, not only from the expense of the experimental subjects but also from the cost of feeding and keeping them. On this account, our experiments, though covering a fairly wide field, have not been so numerous in some cases as we should have wished.

They then complain about the difficulty of getting their grants funded and thank the many farmers who have freely helped them, a situation that still prevails today.

Over the many years since Twort and Ingram, gradual advances in controlling the disease have occurred, based substantially on farm practices, sometimes assisted by vaccination. In recent years, with the development and refinements of many scientific disciplines, particularly molecular biology and immunology, our understanding of the organism and host responses to it has dramatically improved. Through these advances, new tools are now available for both study and control of the disease. Despite these advances, paratuberculosis continues to be a challenge in terms of fundamental knowledge of the biology of the disease and the development and operation of programmes to contain it. Moreover, the availability of novel tools poses

its own challenge, which is their validation and appropriate implementation. For instance, rapid tests like the polymerase chain reaction generate data sooner than conventional assays like culture. This distinction may offer an important advantage for clinical laboratories faced with the challenge of providing a prompt, clear-cut result for the client. However, a slower test may yield more pertinent data for a number of research questions, such as quantitative assessment of the bacterial burden in experimental models. The best application of new tools remains a challenge for paratuberculosis, as for other fields of research.

The wide variety of formidable challenges facing researchers, agribusinesses and regulators in trying to control paratuberculosis is clearly outlined in the chapters that follow. In some cases, chapter authors have drawn comparisons to the situation prevailing with other mycobacterial diseases, particularly tuberculosis. Enormous research efforts on tuberculosis over the last 20 years have led to a dramatically improved understanding of that disease, as well as the development of a range of new diagnostic tests and a growing number of potential new vaccines and drugs that are advancing through the development pipeline. Progress has been much slower with paratuberculosis, partly because it attracts much less research funding and partly because of biology (its slower growth when compared with *Mycobacterium tuberculosis* can be an immediate deterrent to new graduate students). Nevertheless, the similarities between *Mycobacterium avium* subsp. *paratuberculosis* and *M. tuberculosis* and the diseases they cause are such that many of the advances in the tuberculosis field have been of considerable assistance to paratuberculosis research, particularly to basic research in the laboratory. A further comparison that has concerned paratuberculosis researchers for many years is the association between *M. avium* subsp. *paratuberculosis* and Crohn's disease in humans. Further improvements in our ability to understand and control paratuberculosis in ruminants should also help resolve the nature of this association.

Driven by these opportunities and the associated concerns, we set out to assemble a single, comprehensive resource on paratuberculosis. While certain advances have been presented in journal reviews, until now there has been no comprehensive coverage of these developments in a single work. We hope this work will facilitate the interaction of scientists already studying paratuberculosis and help make this field more accessible to those on the outside, for whom the existing literature precluded a single, unifying message. Most importantly, we hope that the availability of this resource will serve as a catalyst, to encourage the transformation of this field from one where little has changed in a century to one where the advances are so numerous that no single volume can adequately do them justice. Ultimately, all these endeavours will be judged as successful only when the prevalence of Johne's disease can be steadily reduced across large regions and entire countries. We thank all the contributing authors for their valuable efforts in working towards this shared goal.

Marcel A. Behr
Desmond M. Collins
June 2009

References

Dalziel, T.K. (1913) Chronic interstitial enteritis. *British Medical Journal* ii, 1068–1070.

Twort, F.W. and Ingram, G.L. (1913) *A Monograph on Johne's Disease (enteritis chronica pseudotuberculosa bovis)*. Baillière, Tindall and Cox, London, UK.

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1 History of Paratuberculosis

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1.1 Early Work on Mycobacterial Diseases

1.1.1 A long history of infections

Mycobacteria have caused disease as far back as evidence of illness can be found. Spinal lesions due to mycobacterial infection have been noted in fossilized bones from 8000 BCE; pulmonary lesions are recorded as far back as 1000 BCE (Murray, 2004). As civilizations urbanized, whether in ancient towns along the Nile or in Hellenic city-states of Greece or Imperial Rome, urban dwellers fell victim to the most devastating of mycobacterial diseases, tuberculosis (TB). For centuries, neither its cause nor any hints of its control were grasped by the investigators of the time.

In the late 1800s, industrialization had drawn the populace into cramped and crowded quarters, producing the unsanitary working and living conditions that fuelled spread of disease. One in four deaths recorded in the parishes of England and New England in the 19th century were due to TB (Murray, 1989). But societies of this era would do more than suffer from the contagion; its scientists would begin to comprehend it.

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1.1.2 Villemin (1865)

In 1865, Jean-Antoine Villemin, a French surgeon, mused about the similarity between glanders in horses and TB in humans, noting that glanders could be transferred animal to animal through inoculation. He injected rabbits and guineas pigs with sputum and the caseous components of lesions from human TB patients. The disseminated infections induced in the subject animals clearly proved the infectious nature of TB for the first time (Villemin, 1868). Although other researchers (Edwin Klebs, Julius Cohnheim and Carl Salomonsen) validated his findings, this central insight into one of the scourges of history '. . . for many years was altogether rejected by the fashionable pathology of the day', as noted in Villemin's *British Medical Journal* obituary (Anonymous, 1892).

1.1.3 Koch (1884)

It was not until the elegant, thorough and persuasive work of Robert Koch (1843–1910) that both the aetiological agent of TB and its route of transmission began to be broadly accepted in the scientific community. While Hansen had already demonstrated the presence of leprosy bacillus in 1873, it was Koch's bacteriological techniques and rigorous hypothesis testing that would become the basis of causality criteria still in use today (Koch's postulates) (Brock, 1999).

Koch adopted and improved tools from a variety of fields (microscopy, cell handling and staining, photography, sterilization and pure culture isolation) to systematize analysis of pathogenic bacteria for the first time. His clear demonstration of mycobacterial organisms, along with permanent recording of findings through photography, could not have occurred without his new methods for *in vitro* cultivation of pure organisms. ('C'est un grand progrès' Pasteur was reported to have remarked, and he was right: adoption of Koch's pure culture techniques in the following two decades permitted the isolation and characterization of the major bacterial pathogens affecting humans.) Koch also developed the techniques for production of tuberculin (non-infectious mycobacterial antigens), hoping, in vain, that tuberculin would prove therapeutic (Tomes, 1998).

It was thus that science was given crucial innovative perspectives on and techniques for studying an established and recognized mycobacterial disease. These same techniques and perspectives were then instrumental in identifying and describing what was apparently a new mycobacterial problem, paratuberculosis.

1.2 Paratuberculosis is Described

Reports of a 'wasting' or 'consumptive' disease in cattle had been made as early as 1807 by Edward Skellet (1807), and two decades later by W.A. Cartwright (Cartwright, 1829). While the descriptive phrases reflected terminology used for TB in humans, there was no evidence that the disease was conceptually linked to human TB in the pre-mycobacteriology era. From our perspective today, Hurtrel d'Arboval's description in 1826 sounds particularly fitting for paratuberculosis ('a thickening of the mucous membrane of the large and small intestines associated with chronic diarrhoea') (Twort and Ingram, 1912). These ill animals may in fact represent the first noted cases of paratuberculosis, since the characteristics of the cases did not match any disorder recognized at the time.

1.2.1 Johne and Frothingham (1894)

In 1894, in the Oldenburg region of Germany, a farmer purchased a cow that failed to produce milk or gain weight satisfactorily. Intestinal tuberculosis, caused by *Mycobacterium bovis,* was thought to be the cause, although the cow tested negative by the tuberculin skin test (another scientific contribution of Koch). When the cow died, the intestines, stomach and omentum were sent for examination to the Veterinary Pathology Unit in Dresden. There, the tissues were examined by Drs Heinrich Albert Johne (Fig. 1.1) and Langdon Frothingham, a visiting scientist from the Pathology Unit in Boston, Massachusetts. They noted the thickened intestinal mucosa and

Fig. 1.1. Dr Heinrich Albert Johne.

Fig. 1.2. Dr Herman Markus.

enlarged mesenteric lymph nodes. On histological examination, the intestinal wall was heavily infiltrated with leucocytes and epithelioid cells and occasional giant cells. Using an acid-fast stain, they observed abundant acid-fast (red-staining) bacteria throughout the inflamed tissues. Although the organisms resembled the bacteria that caused TB, a sample of the infected tissue containing the organisms failed to cause disease when injected into guinea pigs. Drs Johne and Frothingham concluded that the disease observed in the cow was caused by the bacterium that causes TB in birds (later called *Mycobacterium avium*) and, in recognition of the pathological similarity to intestinal TB, proposed the name 'pseudotuberculous enteritis' for the condition (Johne and Frothingham, 1895).

In 1902, Dr Herman Markus (Fig. 1.2), a veterinary pathology professor and abattoir meat inspector in the Netherlands, described not one but at least eleven cases of gastrointestinal inflammation in cattle; he tried but failed to isolate the acid-fast organisms he noted in one of the cases (Markus, 1904). When he published the paper, the editor of the German journal (Dr Johne in fact) commented that he and Dr Markus seemed to be describing the same condition. Both authors still believed the enteritis to be due to *M. avium*.

1.3 Paratuberculosis is Clearly Described, Named and a Cause is Found

By the early 1900s, pseudotuberculous enteritis was recognized as a new disease with a well-described morphology. The disease was clearly not limited to one geographical region. Scientists proposed a variety of alternative names for the disease, such as paratuberculosis and hypertrophic enteritis, but no progress was made in clarifying its cause until 1906.

1.3.1 Bang (1906)

In 1906, research on hypersensitivity immune responses by the Danish professor Bernhard Bang (Fig. 1.3) revealed a crucial distinction

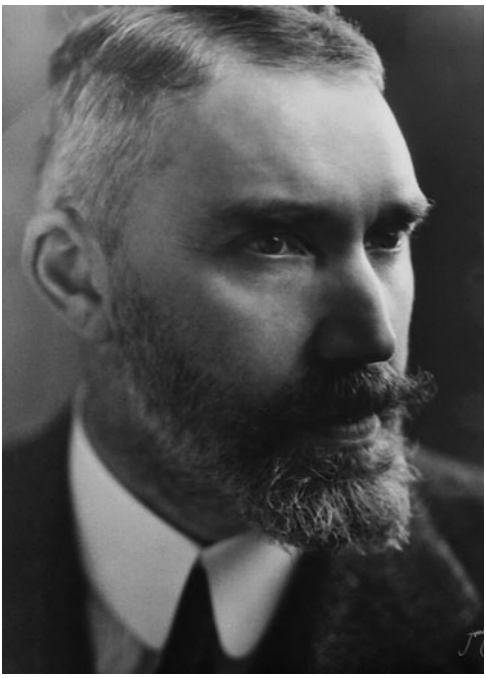

Fig. 1.3. Professor Bernhard Bang.

between infections caused by different species of mycobacteria. He found that cattle with TB responded strongly with fever and a local swelling at the site of injection of *M. bovis* tuberculin (antigens from *M. bovis*) but did not respond to a preparation of antigens from *M. avium*. This finding led to the intradermal tuberculin test, a diagnostic tool used to eradicate bovine TB in Denmark that was adopted throughout the world. A somewhat ancillary finding was that cattle with the condition described by Johne and Frothingham and Markus reacted to the *M. avium* tuberculin but not to the *M. bovis* tuberculin (Bang, 1906). Thus, even before the bacterium that caused this condition was isolated, scientists believed it to be related in some way to the mycobacterium pathogenic to birds. Bang's name for this bovine ailment was 'enteritis chronica bovis pseudotuberculosa'. John McFadyean, a leading figure in British veterinary research and active in TB research, coined the term 'Johne's disease' in the Annual Report for 1906 of the Principal of the Royal Veterinary College (Dunlop and Williams, 1996).

1.3.2 Twort (1912)

A serendipitous observation by the British scientist Frederick William Twort (Fildes, 1951) led to the isolation of the aetiological agent of Johne's disease. Working with laboratory glassware that was not completely cleaned, Twort noted small bacterial colonies growing like satellites around large older colonies he was preparing to discard. The larger colonies were contaminants of the common hay bacillus, *Mycobacterium phlei*. Suspecting that the *M. phlei* bacteria were providing some essential nutrient, given the growth pattern of the smaller colonies, Twort incorporated a heat-killed preparation of *M. phlei* into his culture medium. This new culture medium, he discovered, supported the growth of a new acid-fast bacterium. He named it '*Mycobacterium enteriditis chronicae pseudotuberculosae bovis*, Johne' (Twort and Ingram, 1912).

1.4 Paratuberculosis in the 20th Century

1.4.1 Globalization of paratuberculosis

Awareness of paratuberculosis increased. The disease was recognized based on analysis of dairy cattle evaluated at the University of Pennsylvania in 1908 (Pearson, 1908). It was a primary topic of discussion at the 1913 American Veterinary Medicine Association meeting, where Dr K.F. Meyer presented his doctoral thesis on the topic (Meyer, 1908). Dr Meyer had worked with Dr Koch and his students, and built upon their perspectives on paratuberculosis while at the University of Pennsylvania (Meyer, 1913). Dr Meyer warned '. . . the economic loss will become one of a very serious nature if necessary steps for the control of this disease, which has been brought to this country by importation, are not taken.'

The next several decades were devoted to improved methods for laboratory cultivation of the organism and evaluation of diagnostic tests using these first isolates (Twort and Ingram, 1913). Since the aetiological agent could now be cultivated in the laboratory, a so-called 'johnin' tuberculin was developed for diagnostic

testing. The tuberculin was adopted for skin testing (as was done for TB) and for assays to detect antibodies in serum samples using complement fixation and agglutination techniques.

In the 1920s, paratuberculosis was recognized in other states in the USA and warnings were issued to act promptly to stop spread of this disease. At this early date, some methods for control and prevention were already understood. A University of Wisconsin agricultural experiment station bulletin stated: 'That much can be done to prevent the introduction of the disease into a herd by inquiry concerning the health of every herd from which animals are purchased is self-evident' (Beach and Hastings, 1922). Larson echoed similar warnings stating: 'The disease has, at present, a limited number of sources from which it can spread. These sources are largely the herds of pure-bred cattle, especially those of the Channel Island breeds. These sources of infection will continually increase, unless agencies are operative to offset the constantly increasing commerce in cattle from such herds' (Larson *et al.*, 1924). Not much has changed in the intervening years. Paratuberculosis has spread by animal trade without appropriate biosecurity, within and across country borders.

Paratuberculosis was described in animals on the African and Asian continents in the 1920s, and it was noted in South America and on the Indian continent during the 1930s. The precise introduction of paratuberculosis into Australia is not defined (see Kennedy and Citer, Chapter 28, this volume). By the 1970s, it was clear that the disease was distributed worldwide. Warnings from the US Department of Agriculture (Larsen, 1951) stated: 'Johne's disease may become very prevalent and troublesome in the United States unless more attention is given to its diagnosis and control.' This statement remains pertinent, not just for the USA but for all countries with a domestic agriculture industry.

1.4.2 Key advances of the 20th century

In 1923, the first edition of *Bergey's Manual of Determinative Bacteriology* was published and it officially named the causative agent of Johne's disease *Mycobacterium paratuberculosis*. Much energy was spent in the 1920s on finding a small laboratory animal model of Johne's disease, a goal that remains elusive today (see Talaat, Chapter 20, this volume).

A major achievement of the 1930s was the clear observation of age-dependent resistance of animals to infection by *M. paratuberculosis* (Hagan, 1938). Earlier experimental infection studies had also indicated that '. . . old animals undoubtedly have considerable resistancy against a feeding infection. . . .feeding experiments with large doses of material [often, infected mucosa] on animals from six months to a year remained unsuccessful' (Meyer, 1913).

In the 1940s, paratuberculosis was recognized to be a problem not only in domesticated livestock but in wildlife as well. The hardiness of the organism and its survival in the environment was demonstrated (Lovell *et al.*, 1944). At that time, similarities between paratuberculosis and human leprosy were first noted. Continued evaluation of diagnostic tests led to the discovery of cross-reactivity, resulting in false-positive tests, with bacteria in the genus *Corynebacterium* and other antigenically related bacteria.

Vallée and Rinjard (Vallée *et al.*, 1941), recognizing that the subcutaneous injection of live *M. paratuberculosis* did not cause Johne's disease, evaluated vaccination as a means to control spread of the disease. Vaccination as a means to control paratuberculosis was the subject of many studies in the 1950s and is discussed elsewhere (see de Lisle, Chapter 29, and Huygen *et al.*, Chapter 30, this volume). Additional advances in this decade included recognition of various strains of *M. paratuberculosis*, including pigmented variants and strains that failed to grow on artificial culture media. This observation is addressed in greater detail elsewhere (see Stevenson, Chapter 12, this volume). Also, in 1951, an astute Dutch scientist, Jacob Jansen (1951), observed an association between soil pH and the incidence of Johne's disease. This is the first reported observation that environmental factors may influence occurrence of the disease. The correlation between an animal's age and time of infection, as well as age and onset of clinical disease, was described by

Doyle and Spears (1951). Finally, the new discipline of immunology brought more diagnostic tests, such as the leucocyte migration and fluorescent antibody tests (see Nielsen, Chapter 24, this volume).

The 1960s saw renewed efforts to find an acceptable laboratory animal model of Johne's disease. Important epidemiological observations were made in this era; among these were: (i) the discovery that clinically normal but infected animals (carriers) actively excrete *M. paratuberculosis* in their faeces; and (ii) that *M. paratuberculosis* can be found in the semen of infected bulls and the uterus of infected cows, indicating the possibility of intra- or transuterine infection of fetuses.

As the pace of veterinary research and discovery accelerated in the 1970s and 1980s, knowledge and understanding about many facets of *M. paratuberculosis* and Johne's disease became more complete. It is difficult to single out any one individual or scientific achievement that was of primary importance. Instead, progress was made by a global research effort that advanced the field of paratuberculosis research.

Working at the National Animal Disease Center, Ames, Iowa, USA, Dr Richard Merkal (Fig. 1.4), a pioneer in the field, saw a need to foster closer collaboration and international communication for individuals to address the increasingly numerous aspects of Johne's disease. He organized the First International Colloquium on Paratuberculosis, held in Ames in the summer of 1983 (Fig. 1.5). Out of this grew the International Association for Paratuberculosis and Other Intestinal Mycobacterioses, established by Drs Rod Chiodini and Richard Merkal after the Second International Colloquium on Paratuberculosis in 1988. These colloquia continue to be held every 2 or 3 years in locations around the world and have contributed significantly to the exchange of ideas concerning this challenging disease.

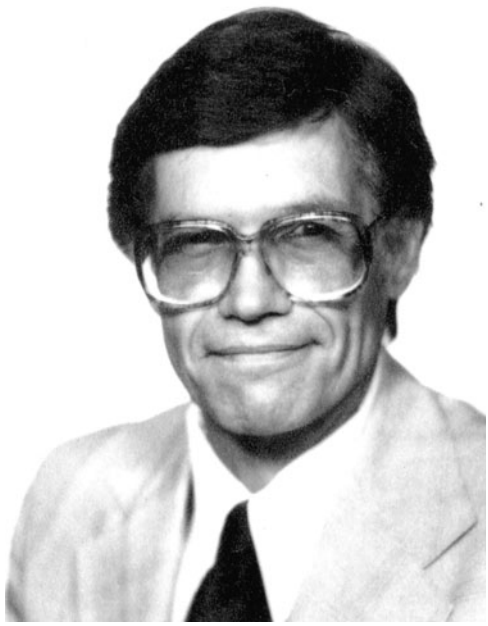

Fig. 1.4. Dr Richard Merkal.

1.5 Paratuberculosis Enters the Genetic and Then the Genomic Era

A new age of paratuberculosis research was ushered in with the discovery of a genetic element unique to *M. paratuberculosis*. This nucleotide sequence in the chromosomal DNA of the organism was simultaneously and independently discovered by Des Collins in New Zealand and the research team led by J.J. McFadden in England in 1989 (see Collins, Chapter 25, this volume). The sequence was found to be an insertion element and was designated IS*900*. This was the first insertion element reported in mycobacteria. The discovery of IS*900* served as the basis for molecular detection of *M. paratuberculosis*, based on the newly described technique of PCR (see Bölske and Herthnek, Chapter 23, this volume). At around the same time, the organism was reclassified from a species (*M. paratuberculosis*) to a subspecies (*M. avium* subsp. *paratuberculosis* (*MAP*)), based on DNA–DNA hybridization analyses (Thorel *et al.*, 1990). The value of this reclassification remains the subject of debate. Some investigators consider that this new name masks the pathogenic nature of *MAP*, leading to the belief that *MAP* is a ubiquitous environmental mycobacteria. Others note that the similarities between *MAP* and other *M. avium* strains provide a natural comparator to understand the uniquely pathogenic

Fig. 1.5. Attendees at the First International Colloquium on Paratuberculosis, Ames, Iowa, 1983.

nature of *MAP* (Turenne *et al.*, 2008). The genome sequence for *MAP* strain K-10 was published in 2005 (Li *et al.*, 2005) and this has ushered in an era of comparative and functional genomics (see Paustian and Kapur, Chapter 8, this volume). Through such studies, it is hoped that the genetic basis for the unique attributes of this pathogenic organism are revealed, leading to new tools to control and prevent this age-old scourge.

1.6 Prospects for the Future

The history of paratuberculosis features many individuals and organizations and their valuable efforts to understand and manage this infection. New tools, rapid and comprehensive communication among colleagues, augmented by global access to and collection of animal and human health data and focused research endeavours, may lead to breakthroughs that match, if not surpass, the discoveries of the last century. Novel resources and tools (such as complete genome sequencing, microarrays, complex immunological assays and molecular genetics – all described elsewhere in this volume) together hold promise towards the development and implementation of improved strategies to contain this infectious scourge. In addition, scientific advances stimulate awareness, which iteratively helps build the needed scientific capacity to address a problem like paratuberculosis. In response to a recommendation made by the US National Academy of Sciences, a comprehensive, multi-institutional, interdisciplinary collaboration termed 'JDIP' was formed (http://www.jdip.org/). An acronym for Johne's Disease Integrated Program, this US-based collaboration has brought together numerous institutions and individuals in support of research and education. A similar endeavour has been established in Europe, called ParaTB Tools (http://www.ucm.es/info/paratbtools/), fostered by the communication network provided by the Internet and

by the needs identified by regional and national control programmes.

Since the first recognition of paratuberculosis roughly 100 years ago, the infection has emerged as a common and troublesome disease of many animal species. Presently there are tools, teams and programmatic support to make important headway in our understanding of the disease and the means of putting it under control. In fact, many of the scientists involved in these efforts are key contributors to this book. With sufficient effort, it should take considerably less time than 100 years to bring this disease under control.

References

Anonymous (1892) J.-A. Villemin obituary. *British Medical Journal* 2, 1091.

Bang, B. (1906) Chronische pseudotuberkulöse Darmentzündung beim Rinde. *Berliner Tierärztliche Wochenschrift,* 50, 759–763.

Beach, B.A. and Hastings, E.G. (1922) Johne's disease a cattle menace. In: *Bulletin 343 of the Madison Wisconsin Agricultural Experiment Station*. University of Wisconsin, Madison, Wisconsin, pp. 1–22.

Brock, T.D. (1999) *Robert Koch: a Life in Medicine and Bacteriology*. ASM Press, Herndon, Virginia, USA.

Cartwright, W.A. (1829) Diarrhoea in a cow. *The Veterinarian* 2, 71–72.

Doyle, T.M. and Spears, H.N. (1951) A Johne's disease survey. *The Veterinary Record* 63, 355–359.

Dunlop, R.H. and Williams, D.J. (1996) In: *Veterinary Medicine: an Illustrated History*. Mosby-Yearbook, St Louis, Missouri, pp. 399–401.

Fildes, P. (1951) Frederick William Twort 1877–1950. *Obituary Notices of Fellows of the Royal Society* 7, 504–517.

Hagan, W.A. (1938) Age as a factor in susceptibility to Johne's disease. *The Cornell Veterinarian* 28, 34–40.

Jansen, J. (1951) Paratuberculosis. *Journal of the American Veterinary Medical Association* 112, 52–55.

Johne, H.A. and Frothingham, L. (1895) Ein eigenthumlicher Fall von Tuberculose beim Rind [A peculiar case of tuberculosis in a cow]. *Deutsche Zeitschrift für Tiermedizin und vergleichende Pathologie* 21, 438–455.

Larsen, A.B. (1951) Johne's disease (paratuberculosis) of cattle (1951). In: *United States Department of Agriculture Circular No. 873*. United States Department of Agriculture, Washington, DC, p. 3.

Larson, V.S., Beach, B.A. and Wisnicky, W. (1924) Problems in controlling and eradicating Johne's disease. *Journal of the American Veterinary Medical Association* 80, 446–467.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S. and Kapur, V. (2005) The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proceedings of the National Academy of Sciences USA* 102, 12344–12349.

Lovell, R., Levi, M. and Francis, J. (1944) Studies on the survival of Johne's bacilli. *Journal of Comparative Pathology* 54, 120–129.

Markus, H. (1904) Eine specifische Darmentzündung des Rindes, wahrscheinlich tuberculöser. *Zeitschrift für Tiermedizin* 8, 68–78.

Meyer, K.F. (1908) Uber die durch saurefeste Bakterien hervogerufene diffuse Hypertrophie der Darmschleimhaut des Rindes (enteritis hypertrophica bovis specifica). PhD thesis, University of Zurich, Switzerland.

Meyer, K.F. (1913) The specific paratuberculosis enteritis of cattle in America. *Journal of Medical Research* 29, 147–191.

Murray, J.F. (1989) The white plague: down and out, or up and coming? *The American Review of Respiratory Disease* 140, 1788–1795.

Murray, J.F. (2004) *Mycobacterium tuberculosis* and the cause of consumption: from discovery to fact. *American Journal of Respiratory Critical Care Medicine* 169, 1086–1088.

Pearson, L. (1908) A note on the occurrence in America of chronic bacterial dysentery of cattle. *American Veterinary Review* 32, 602–605.

Skellet E. (1807) *A Practical Treatise on the Parturition of the Cow, or the Extraction of the Calf, and on the Diseases of Neat Cattle in General; with the Most Approved Methods of Treatment, and Best Forms of Prescription Adapted to Veterinary Practice*. Sherwood, Nely and Jones, London.

Thorel, M.F., Krichevsky, M. and Vincent-Levy-Frebault, V. (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *International Journal of Systematic Bacteriology* 40, 254–260

Tomes N. (1998) The gospel emergent. In: *The Gospel of Germs: Men, Women and the Microbe in American Life*. Harvard University Press, Cambridge, Massachusetts, p. 45.

Turenne, C.Y., Collins, D.M., Alexander, D.C. and Behr, M.A. (2008) *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms. *Journal of Bacteriology* 190, 2479–2487.

Twort, F. and Ingram, G.L.Y. (1912) A method for isolating and cultivating the *Mycobacterium enteritidis chronicae pseudotuberculosae bovis*, Johne, and some experiments on the preparation of a diagnostic vaccine for pseudotuberculosis enteritis of bovines. *Proceedings of the Royal Society, Series B* 84, 517–542.

Twort, F.W. and Ingram, G.L.Y. (1913) *A Monograph on Johne's Disease (enteritis chronica pseudotuberculosa bovis)*. Baillière, Tindall and Cox, London.

Vallée, H., Rinjard, P. and Vallée, M. (1941) Sur la prémunition de l'entérite paratuberculeuse due au bacille de Johne. *Bulletin Académie Médecin* 125, 195–198.

Villemin, J.-A. (1868) *Etudes experimentales et cliniques sur tuberculose*. Ballière et Fils, Paris.

2 Global Prevalence and Economics of Infection with *Mycobacterium avium* subsp. *paratuberculosis* in Ruminants

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2.1 Introduction

Paratuberculosis or Johne's disease (JD) was formally described for the first time in 1895 (Johne and Frothingham, 1895), although cases of chronic diarrhoea with emaciation were described in the Netherlands (Le Franq van Berkhey, 1805) and the UK (Cartwright, 1829). At the beginning of the 20th century, JD was noticed regularly in the Netherlands (Markus, 1904), Denmark (Bang, 1906), France (Leclainche, 1907), Norway (Horne, 1909), the UK (McFadyean, 1906), the USA (Pearson, 1908), Belgium (Lienaux, 1909), and Switzerland (Borgeaud, 1905). Since then, the disease has continued to spread worldwide among cattle populations (Chiodini, 1993). The first prevalence study of infection with *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in cattle was conducted in 1965 in Denmark

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(Jørgensen, 1965). Thereafter, many efforts were made to describe the prevalence of *MAP* in various cattle, as well as other ruminant populations.

Research efforts to determine the prevalence of *MAP* are driven by the wish to develop control and prevention programmes, because of the economic losses caused by the disease and the still unsolved potential zoonotic risk (Benedictus *et al.*, 1987; Chiodini, 1993; Scanu *et al.*, 2007; Groenendaal and Wolf, 2008). Before a prevention and control programme for JD can be designed, both herd- and cow-level prevalence of *MAP* infection should be known. Additionally, in the course of a prevention and control programme, progress towards the goals of the programme should be monitored. Too many JD control programmes have been designed based on the assumption that the prevalence is relatively low, without being based on a valid prevalence study.

Other valid reasons for assessing the prevalence of *MAP* are to determine the burden of disease in a particular population and to determine the need for health services for those animals particularly at risk of contracting the disease. Prevalence studies are also carried out to compare the prevalence of disease in different populations and to follow trends in prevalence or severity over time, so as to better understand the epidemiology of the spread of this disease.

2.2 Tests Used in Prevalence Studies

The slow development of JD and the subsequent prolonged and nearly imperceptible transition between stages of the infection make it impossible to detect all infected animals in a herd with a single test at a defined period in time (Kalis, 2003). Many tests are available, but each individual test essentially only detects a subset of the infected animals. Currently, three approaches are used to diagnose infection with *MAP*: (i) detection of a cell-mediated response; (ii) detection of the organism (or part of it); and (iii) detection of serum antibodies. More information on these diagnostic assays is provided elsewhere (see Whittington, Chapter 22; Bölske and Herthnek, Chapter 23; and Nielsen, Chapter 24, this volume). Owing to the relatively low sensitivity of serological tests, these are more accurate at determining herd-level rather than cow-level prevalence (Clark *et al.*, 2008). Tavornpanich *et al.* (2008) determined that culture of six environmental samples is the most cost-effective test to determine whether a herd is *MAP* positive. However, even with this culture strategy, a significant proportion of low-prevalence herds will not be detected.

In comparing different studies involving prevalence of JD or MAP infection, difficulties partly arise from the variation in the definition of what constitutes a positive case. In some reports, a positive serological result is the case definition, and, in others, a positive case is defined by the presence of bacteria. In studies using an enzyme-linked immunosorbent assay (ELISA) for classification of cow status, the sensitivity of the test is often assumed to be 40–50%. More recent work has estimated that the sensitivity of ELISAs is closer to 10% and that it varies considerably between different ELISAs and even between different batches of the same ELISA (McKenna *et al.*, 2005). Additionally, ELISA positivity is correlated with the level of faecal shedding, a phenomenon known as spectrum effect (Sweeney *et al.*, 1995; Kalis *et al.*, 2002). As a result, studies that estimate the sensitivity of ELISA in animals with a positive faecal culture can be expected to report a high sensitivity with this test. In the case of testing for *MAP* infection, tissue culture is the appropriate gold standard. In studies using tissue culture (lymph nodes, ileum) as the comparator, serum ELISAs had a much lower sensitivity (McKenna *et al.*, 2005). These examples illustrate the difficulties in posing a conceptually simple question: what is the prevalence of paratuberculosis?

2.3 Global Prevalence

Both infection with *MAP* and clinical cases of JD have been reported from all continents that have ruminant populations in whatever degree of husbandry. Although Norway and Sweden report that their cattle are free of

MAP infection, positive cases have been reported in both countries (Tharaldsen *et al.*, 2003; Holstad *et al.*, 2005; Lewerin *et al.*, 2007). A large number of dairy heifers have been exported from Europe and North America to countries in Africa, Asia and Latin America. While it is likely that none of these heifers showed clinical symptoms of JD, a significant proportion of the exported animals can be expected to have been *MAP* infected because the cattle population in the countries of origin was infected with *MAP*. Thus, it is likely that *MAP* infection is present in every country that utilizes ruminants as part of their agricultural base, although the prevalence will differ between regions and countries (Kopecky, 1977; Muskens *et al.*, 2000; Adaska and Anderson, 2003). Moreover, the notion that one is 'free of infection' may be more a function of how rigorously one has looked for the organism than a true lack of prevalence. Knowing that *MAP* infection is at least a potential if not actual concern for agricultural production and food safety, every country should promote a realistic, useful international perspective of the control and prevention of JD. A complete grasp of the extent of *MAP* infection will assist all those affected in the many ways possible by the disease, whether through regulatory activities, management of livestock, research or public health.

2.4 Herd-level Prevalence in Cattle

2.4.1 Dairy cattle

JD is a common disease in all countries with a significant dairy industry, especially in areas with a moderate and humid climate. In a recent extensive summary of JD prevalence in Europe (Nielsen and Toft, 2009), it was concluded that valid prevalence estimates were limited in published data owing to problems with test accuracy in target populations, partly due to study designs unsuitable for accurate estimation of prevalence.

During the early 1990s, the herd-level prevalence of JD in countries with a significant dairy industry was calculated at approximately 10% (Sockett, 1996). More recently, in Belgium (Boelaert *et al.*, 2000), the Netherlands (Muskens *et al.*, 2000), Denmark (Nielsen *et al.*, 2000), Canada (VanLeeuwen *et al.*, 2001) and the USA (Wells and Wagner, 2000), herd-level prevalence has been estimated to be 30–50%. This is based on an increased frequency of clinical disease, occurrence of JD in areas where the disease was formerly unknown and serological prevalence studies. However, all these studies were carried out using an ELISA, and, if the true prevalence could be calculated, it would probably be greater because the sensitivity of the ELISAs was highly overestimated. Although faecal culture is expensive and requires at least 8 weeks for completion, it is still the most accurate test for identifying *MAP* shedders (Kalis *et al.*, 2002). Tissue culture and trace-back to herd of origin would provide the most accurate estimate of herd-level prevalence. However, such a study would be too expensive, not only because of the high number of cultures needed but also because of the labour involved.

In a Dutch study, dairy herds that were closed for at least 3 years with no history of JD were recruited for herd certification, presuming that they would be negative or at least have a very low prevalence (Kalis *et al.*, 2004). In 100 herds, pools of five cows were cultured every 6 months. Although the proportion of infected herds decreased with each round of testing, 61% of the 90 herds that completed 10 rounds of biannual cultures were found to be *MAP* infected.

Recently, culture of faecal samples from the environment of the area where dairy cows live was used to determine whether herds were infected with *MAP*. This method is comparable to pooled faecal culture of a large number of cows per pool, and it detects approximately 70% of infected dairy herds (Lombard *et al.*, 2006). In the USA in 2007, as part of the National Animal Health Monitoring System (2007) in 524 dairy farms in the 17 major dairy states, six environmental samples were collected. In total, 68% of the dairy herds had at least one positive environmental culture. The herd-level prevalence increased with herd size: 63, 75 and 95% of herds with <100 cows, 100–499 and >500 cows were positive, respectively. From this study, along with the

Dutch data, it can be concluded that the prevalence of *MAP* infection is probably a lot higher than 50% in most countries with a significant dairy industry.

2.4.2 Bulk tank milk

Contamination of bulk tank milk can occur through shedding of individual cows in the milk or be the result of contamination of milk with *MAP* from the surface of the teats during the milking process. In only a relatively small proportion of cows that shed *MAP* in the faeces is *MAP* also found in the milk (Sweeney *et al.*, 1992; Streeter *et al.*, 1995; Jayarao *et al.*, 2004). Prevalence of *MAP* in bulk milk is determined using a polymerase chain reaction (PCR) assay (Corti and Stephan, 2002; Bosshard *et al.*, 2006; Haghkhah *et al.*, 2008; Slana *et al.*, 2008) or culture (Jayarao *et al.*, 2004). Prevalence based on culture of bulk tank milk in Pennsylvania dairy herds was 3% (Jayarao *et al.*, 2004), while studies using PCR found a prevalence ranging from 3 to 33% using different targets (F57 and IS*900*). A reason why the apparent prevalence based on culture was lower is that the sensitivity of culture on a sample of milk will be less sensitive than bulk tank PCR (Stabel *et al.*, 2002). Additionally, bulk milk ELISAs have been used to detect infected herds (Nielsen *et al.*, 2007). An ELISA-based study found that 47% of Danish dairy herds were *MAP*-antibody-positive (Nielsen *et al.*, 2000). Geue *et al.* (2007) concluded, however, that a bulk milk ELISA was not suitable for herd diagnosis. Most likely it will only detect herds with a high seroprevalence.

2.4.3 Beef cattle

No studies have been reported on herd-level prevalence of *MAP* infection in beef cattle using faecal culture of samples from individual animals, pooled animals or environmental samples. Rather, studies on beef cattle have used ELISAs, sometimes using confirmation of high ELISA titres with faecal culture in an attempt to correct for the low specificity. In general, the herd-level *MAP* seroprevalence has been lower in beef cattle than in dairy cattle, with studies reporting between 8 and 54% of herds having at least one seropositive animal (Thorne and Hardin, 1997; Dargatz *et al.*, 2001; Waldner *et al.*, 2002; Hill *et al.*, 2003; Roussel *et al.*, 2005; Scott *et al.*, 2007). All studies included a subpopulation of the herd, most commonly including 30 animals per herd. As with studies in dairy cattle, the prevalence of *MAP* infection is likely to be much higher if a more sensitive method is used (i.e. faecal or tissue culture). In countries with an identification and registration system for cattle, samples collected at a slaughterhouse could be used to trace back animals to their herd of origin and estimate the herd-level prevalence of *MAP*.

2.5 Cow-level Prevalence in Cattle

Because tissue culture has the highest sensitivity for detection of *MAP* infection, and because infection occurs early in life, slaughterhouse studies that use tissue culture provide the most reliable estimates of individual cow prevalence of *MAP* infection. Because of their high cost, only a minority of reported studies have used tissue culture (Chiodini and van Kruiningen, 1986; McNab *et al.*, 1991; Cetinkaya *et al.*, 1996; McKenna *et al.*, 2004). These studies predominantly included culled dairy cattle. All studies in beef cattle, except Merkal *et al.* (1987), determined the seroprevalence using an ELISA (Dargatz *et al.*, 2001; Waldner *et al.*, 2002; Hill *et al.*, 2003; Pence *et al.*, 2003; Roussel *et al.*, 2005; Scott *et al.*, 2007).

2.5.1 Dairy cattle

Region- or countrywide prevalence of *MAP* infection ranges from 1.2 to 9.4% if determined using a serum ELISA (Boelaert *et al.*, 2000; Jakobsen *et al.*, 2000; Muskens *et al.*, 2000; Adaska and Anderson, 2003). The apparent herd-level prevalence of *MAP* infection follows a negative binomial distribution, with a large proportion of farms having a relatively low prevalence and some farms, probably

because of the lack of preventive measures, having a high prevalence (Fig. 2.1) (Collins *et al.*, 1994; Muskens *et al.*, 2000; Nielsen *et al.*, 2000; Woodbine *et al.*, 2009). As a result, the mean within-herd prevalence may be overestimated because of the strong influence of these problem herds. On the other hand, the average within-herd prevalence is probably significantly higher than is indicated by ELISA-based studies. Estimates within Canada and the USA using tissue culture have shown that cow prevalence in North America is as high as 16 and 17%, respectively (Chiodini and van Kruiningen, 1986; McKenna *et al.*, 2004). The serum ELISA-based prevalence estimates found in these regions (VanLeeuwen *et al.*, 2001) are not higher than in other regions, and it can therefore be assumed that the true cow-level prevalence of *MAP* infection of dairy cows will also be in the same range. The true herd-level prevalence of *MAP* infection will therefore more likely follow a binomial or even normal distribution (Fig. 2.1).

It is often suggested that both herd- and cow-level prevalence of *MAP* infection are increasing. However, this cannot be confirmed, because no sets of studies have been published using the same sampling strategy and laboratory method in the same region.

2.5.2 Beef cattle

Typically, it has been reported that the prevalence overall is lower in beef cattle than in dairy cattle. The only study using tissue culture in beef cattle, conducted in the USA, found a prevalence of 0.8% in beef cull cows compared with 2.9% in dairy cull cows (Merkal *et al.*, 1987). In this study, ileocaecal lymph nodes, but not ileum, were cultured. Although this probably is one of the body tissues with the highest prevalence of *MAP* infection, the prevalence of *MAP* infection is greater when both sites are cultured (McKenna *et al.*, 2004).

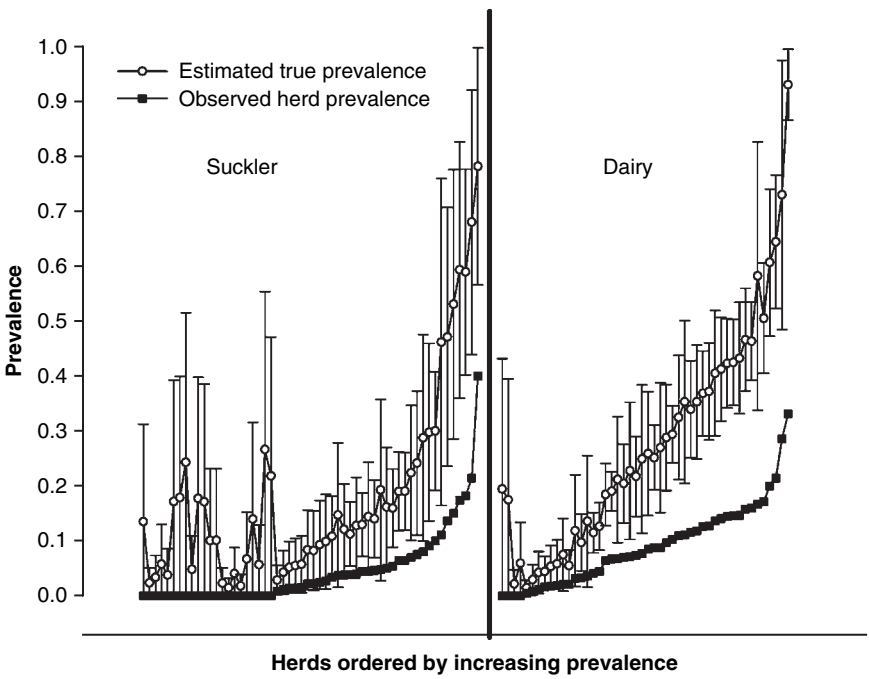

Fig. 2.1. Apparent observed *MAP* seroprevalence (squares) and estimated true prevalence (circles and error lines) for 114 suckler and dairy herds in the UK at the last of three visits (Woodbine *et al.*, 2009). True prevalence was estimated using Markov chain Monte Carlo simulations (Van Schaik *et al.*, 2003).

Estimates of seroprevalence have ranged from 0.4 to 8.0% at the cow level (Dargatz *et al.*, 2001; Roussel *et al.*, 2005; Scott *et al.*, 2007). Different ELISAs were used in these studies, making the results difficult to compare.

2.6 Sheep and Goats

In small ruminants, JD is also considered a disease of worldwide distribution (Smith and Sherman, 1994). Caprine paratuberculosis has been described in numerous countries, including India, Turkey, France, Greece, Norway, Spain, Austria, Switzerland, Portugal, Croatia, Canada, the USA, Australia, New Zealand and Chile (Smith and Sherman, 1994; Mainar-Jaime and Vásquez-Boland, 1998; Muehlherr *et al.*, 2003; Kruze *et al.*, 2007; Nielsen and Toft, 2009). In sheep, paratuberculosis has also spread worldwide (Benazzi *et al.*, 1996; Sergeant, 2003; Bradley and Cannon, 2005; Coelho *et al.*, 2008; Nielsen and Toft, 2009). Unfortunately, the number of prevalence studies carried out among small ruminants is still limited and none of them provide sufficiently accurate and unbiased prevalence estimates (Nielsen and Toft, 2009). Available data show that, throughout Europe, the apparent prevalence estimates are generally low at the animal level. Using faecal culture or ELISA testing, apparent prevalences range from 0.3 to 3.5% (Nielsen and Toft, 2009). In a recent Portuguese study, blood samples were pooled in groups of five and were analysed by PCR (Coelho *et al.*, 2008). The estimated overall individual prevalence of *MAP* in this study ranged from 6.4 to 15.4%.

Estimates of prevalence from sheep at a Canadian slaughter facility were found to be 3% using histology of the ileum (Arsenault *et al.*, 2003). Flock-level prevalence varies significantly in Australia, based upon the region where the flock is. In known low-prevalence areas, the flock-level prevalence can be as low as 0.04–1.5%, while, in known high-risk areas, the flock-level prevalence estimates are dramatically higher, at 29–39% in regions of New South Wales (Sergeant and Baldock, 2002). Studies on a larger number of animals in South Africa and Australia found a prevalence of 2.57 and 0.25%, respectively (Links *et al.*, 1999; Michel and Bastianello, 2000).

2.7 Other Ruminants

Infection with *MAP* has been found in all ruminants that were examined for the pathogen, including bison, buffalo, tule elk, mouflon, chamois, moose, camelids, caribou and reindeer (Deutz *et al.*, 2005) (see Mackintosh and Griffin, Chapter 17, this volume). Proximity of wild ruminants and farmed cattle has been suggested to play an important role in the prevalence in both groups of ruminants (Cetinkaya *et al.*, 1997). Ruminants other than cattle, sheep and goats for which prevalence studies on sufficient numbers of animals have been carried out are deer species such as red deer (Pavlik *et al.*, 2000; Reyes-García *et al.*, 2008), roe deer (Pavlik *et al.*, 2000), fallow deer (Pavlik *et al.*, 2000), white-tailed deer (Shulaw *et al.*, 1986; Davidson *et al.*, 2004) and Florida Key deer (Pedersen *et al.*, 2008), moose (Tryland *et al.*, 2004) and reindeer (Tryland *et al.*, 2004). The studies were carried out in different countries using different diagnostic tests such as faecal or tissue culture, ELISA and restriction fragment length polymorphism analysis. Prevalence of *MAP* infection ranged from 0.3% in white-tailed deer in south-western USA (Davidson *et al.*, 2004) to 30.1% in red deer in Spain (Reyes-García *et al.*, 2008).

2.8 Economic Effects in Dairy and Beef Cattle and Sheep

JD causes economic losses because of a lower milk production (Lombard *et al.*, 2005), reduced slaughter value (Benedictus *et al.*, 1987), increased involuntary culling (Ott *et al.*, 1999), increased calving intervals and infertility (Johnson-Ifearulundu *et al.*, 2000), and diagnosis and possible treatment costs (Benedictus *et al.*, 1987). However, because prevalence estimates are uncertain and the majority of infected animals are subclinical, estimating the true economic effects of JD remains difficult.

Both field studies and simulation studies have been used to determine the economic losses caused by JD. Based on comparisons between herds with and without different levels of JD, total losses due to JD in US dairy herds were estimated at $200–250 million annually

(Ott *et al.*, 1999). The same study also found that the standardized annual losses per animal in five out of six studies ranged between US$20 and US$27. Using a simulation approach, Groenendaal *et al.* (2002) estimated annual losses per animal in an infected herd to be €19 and US$35, respectively, in a Dutch and a Pennsylvanian dairy herd. Caldow and Gunn (2000) estimated annual costs on an infected British dairy herd to be £26 per animal.

In addition to the farm economic losses of JD, an often-considered cost of JD is the risk of a potential link between JD and Crohn's disease in humans. Groenendaal and Zagmutt (2008) performed an economic scenario analysis and concluded that, given the current scientific knowledge about *MAP* and Crohn's disease, it is most likely that the economic consequences of the hypothetical discovery of such a link will be limited, although effects could be large if the consumer's perception of risk is large or if possible risk-mitigation strategies turn out to be ineffective.

In beef cattle, losses due to JD are lower than in dairy cattle, primarily because of the lower prevalence. Annual losses have been estimated at €16–21 per animal in Dutch beef herds (Groenendaal *et al.*, 2003) and £10–18 per animal in UK beef herds (Caldow and Gunn, 2000). Both studies reported losses per animal at the lower end for smaller herds. With regard to the losses caused by JD in sheep, Caldow and Gunn (2000) estimated in a British context the loss of income due to unplanned deaths resulting from JD to range from £24 to £94 per ewe.

In summary, on average the economic loss due to JD in infected dairy herds is relatively low compared with other major diseases such as subclinical mastitis, fertility or lameness. However, because of the right-skewed distribution of individual animal *MAP* prevalence within herds, JD can cause considerable losses in some herds. In beef herds, losses per animal are typically considerably lower.

2.9 Economic Effects of JD Prevention and Control Programmes

Understanding the economic consequences on either a farm or a national level is important in order to support good decision making with respect to the approach in handling the disease. To determine the economic consequence of JD prevention or control, we need to understand three components: (i) JD losses without the prevention and control programme; (ii) JD losses with the prevention and control programme; and (iii) costs of the prevention and control programme. The economic benefits of a prevention and control programme are equal to (i) minus (ii) minus (iii). Because of the slow progression and spread of JD, it is important to take into account long-term costs and benefits since benefits can often only be seen 5–10 years after the start of the effort.

Field studies on JD control and economics are very expensive, lengthy and hence rare. Groenendaal and Wolf (2008) evaluated farm-level economic consequences related to control measures in 40 dairy herds and found that management-related practices to control JD were marginally economically attractive ($3/animal/year) in infected herds.

Simulation studies on JD control and economics have generally shown similar results with regard to the economic effect of management-related practices (Groenendaal *et al.*, 2003; Kudahl *et al.*, 2007). The same two simulation studies also estimated the economic effects of test-and-cull strategies and showed that costs are typically higher than economic benefits. Dorshorst *et al.* (2006) built a decision-tree model to support decisions on JD prevention and control efforts and also concluded that improving herd management practices to control infection spread (hygiene) is often more cost effective than testing. In addition, they found that, for many herds, low-cost tests are more useful than more sensitive, higher-cost tests.

The economic effects of vaccination against JD have been estimated in a field study (Van Schaik *et al.*, 1996) as well as a simulation study (Groenendaal *et al.*, 2002). Both studies concluded that vaccination is attractive from an economic perspective but that it does not lead to a fast reduction in prevalence unless it is combined with calf management improvements.

To our knowledge, only one study (Losinger, 2005) has estimated the economic benefits of control of JD on a national level, taking

into account potential supply and price effects. Not taking into account the costs of prevention and control and assuming immediate eradication of JD, it was estimated that JD results in a total loss to the US economy of $200 million ± $160 million.

2.10 Conclusions

It is essential that the prevalence of a disease at either the herd or animal level is estimated in a justified way when decisions are to be made on whether an infectious disease should be considered important or not and which measures to apply to deal with the disease. Such measures may mean eradication in areas of low prevalence, control in areas of high prevalence and increased surveillance in areas where there is no history of the disease. Prevalence estimates are necessary when modelling or simulating the spread of a disease. However, the prevalence estimates obtained by surveys are affected by the accuracy of the diagnostic test used, and for comparison across studies the apparent prevalence needs to be adjusted according to the test characteristics in order to better estimate the true prevalence.

Considering the reviewed studies on JD, sampling strategies differed along with differences in case definition. Thus the results of these studies and the derived prevalence estimates of *MAP* infection cannot be directly compared. To determine herd- and cow-level prevalence estimates of *MAP* in different countries, we recommend undertaking a supranational standardized study, comparable to the *Neospora caninum* seroprevalence study involving cattle of four countries (Bartels *et al.*, 2006).

It is fair to say that where a serious effort has been made to identify infected animals in countries that have domesticated ruminant populations, some level of *MAP* infection has been found. As to whether or not the prevalence of this disease is rising on a global scale, this remains unclear, owing to differences in study designs and lack of appropriate comparable data and follow-up studies.

The limited numbers of studies on the economic consequences of JD prevention and control have shown economic benefits for management-related measures and vaccination. Test-and-cull programmes have proved to be expensive and only economically beneficial in limited situations.

Further standardization of procedures to monitor the prevalence of *MAP* infection would allow more extrapolation of findings between countries and studies. The design of studies with such standardized testing, along with protocols that include environmental and non-target animal testing surrounding farming operations, would be of great benefit to a global effort in the control and prevention of JD.

References

Adaska, J.M. and Anderson, R.J. (2003) Seroprevalence of Johne's-disease infection in dairy cattle in California, USA. *Preventive Veterinary Medicine* 60, 255–261.

Arsenault, J., Girard, C., Dubreuil, P., Daignault, D., Galarneau, J.R., Boisclair, J., Simard, C. and Bélanger, D. (2003) Prevalence of and carcass condemnation from maedi-visna, paratuberculosis and caseous lymphadenitis in culled sheep from Quebec, Canada. *Preventive Veterinary Medicine* 59, 67–81.

Bang, B. (1906) Chronische pseudotuberculöse Darmentzundung beim Rinde. *Berliner Tierärztliche Wochenschrift* 22, 759–763.

Bartels, C.J., Arnaiz-Seco, J.I., Ruiz-Santa-Quitera, A., Björkman, C., Frössling, J., von Blumröder, D., Conraths, F.J., Schares, G., van Maanen, C., Wouda, W. and Ortega-Mora, L.M. (2006) Supranational comparison of *Neospora caninum* seroprevalences in cattle in Germany, the Netherlands, Spain and Sweden. *Veterinary Parasitology* 137, 17–27.

Benazzi, S., el Hamidi, M. and Schliesser, T. (1996) Paratuberculosis in sheep flocks in Morocco: a serological, microscopical and cultural survey. *Zentralblatt für Veterinarmedizin B* 43, 213–219.

Benedictus, G., Dijkhuizen, A.A. and Stelwagen, J. (1987) Economic losses to farms due to paratuberculosis in cattle. *Tijdschrift voor Diergeneeskunde* 110, 310–319.

Boelaert, F., Walravens, K., Biront, P., Vermeersch, J.P., Berkvens, D. and Godfroid, J. (2000) Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population. *Veterinary Microbiology* 77, 269–281.

Borgeaud, A. (1905) Contribution à l'étude de l'entérite chronique des bovides. *Schweizer Archiv für Tierheilkunde* 47, 221–229.

Bosshard, C., Stephan, R. and Tasara, T. (2006) Application of an F57 sequence-based real-time PCR assay for *Mycobacterium paratuberculosis* detection in bulk tank raw milk and slaughtered healthy dairy cows. *Journal of Food Protection* 69, 1662–1667.

Bradley, T.L. and Cannon, R.M. (2005) Determining the sensitivity of abattoir surveillance for ovine Johne's disease. *Australian Veterinary Journal* 83, 633–636.

Caldow, G. and Gunn, G.J. (2000) Assessment of surveillance and control of Johne's disease in farm animals in GB. Available at: http://www.defra.gov.uk/animalh/diseases/zoonoses/zoonoses_reports/sac2.PDF (accessed 15 May 2009).

Cartwright, W.J. (1829) Diarrhoea in a cow. *The Veterinarian* 2, 71–72.

Cetinkaya, B., Egan, K., Harbour, D.A. and Morgan, K.L. (1996) An abattoir-based study of the prevalence of subclinical Johne's disease in adult cattle in south west England. *Epidemiology and Infection* 116, 373–379.

Cetinkaya, B., Erdogan, H.M. and Morgan, K.L. (1997) Relationships between the presence of Johne's disease and farm and management factors in dairy cattle in England. *Preventive Veterinary Medicine* 32, 253–266.

Chiodini, R.J. (1993) *The History of Paratuberculosis (Johne's Disease): a Review of the Literature 1895 to 1992*. International Association for Paratuberculosis, Providence, USA.

Chiodini, R.J. and van Kruiningen, H.J. (1986) The prevalence of paratuberculosis in culled New England cattle. *The Cornell Veterinarian* 76, 91–104.

Clark, D.L., Jr., Koziczkowski, J.J., Radcliff, R.P., Carlson, R.A. and Ellingson, J.L. (2008) Detection of *Mycobacterium avium* subspecies *paratuberculosis*: comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. *Journal of Dairy Science* 91, 2620–2627.

Coelho, A.C., Pinto, M.L., Coelho, A.M., Rodrigues, J. and Juste, R. (2008) Estimation of the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* by PCR in sheep blood. *Small Ruminant Research* 76, 201–206.

Collins, M.T., Sockett, D.C., Goodger, W.J., Conrad, T.A., Thomas, C.B. and Carr, D.J. (1994) Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. *Journal of the American Veterinary Medical Association* 204, 636–641.

Corti, S. and Stephan, R. (2002) Detection of *Mycobacterium avium* subspecies *paratuberculosis* specific IS*900* insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. *BMC Microbiology* 2, 15.

Dargatz, D.A., Byrum, B.A., Hennager, S.G., Barber, L.K., Kopral, C.A., Wagner, B.A. and Wells, S.J. (2001) Prevalence of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* among beef cow–calf herds. *Journal of the American Veterinary Medical Association* 219, 497–501.

Davidson, W.R., Manning, E.J. and Nettles, V.F. (2004) Culture and serologic survey for *Mycobacterium avium* subsp. *paratuberculosis* infection among southeastern white-tailed deer (*Odocoileus virginianus*). *Journal of Wildlife Diseases* 40, 301–306.

Deutz, A., Spergser, J., Wagner, P., Rosengarten, R. and Köfer, J. (2005) *Mycobacterium avium* subsp. *paratuberculosis* in wild animal species and cattle in Styria/Austria. *Berliner und Münchener Tierarztliche Wochenschrift* 118, 314–320.

Dorshorst, N.C., Collins, M.T. and Lombard, J.E. (2006) Decision analysis model for paratuberculosis control in commercial dairy herds. *Preventive Veterinary Medicine* 75, 92–122.

Geue, L., Köhler, H., Klawonn, W., Dräger, K., Hess, R.G. and Conraths, F.J. (2007) The suitability of ELISA for the detection of antibodies against *Mycobacterium avium* ssp. *paratuberculosis* in bulk milk samples from Rhineland-Palatinate. *Berliner und Münchener Tierarztliche Wochenschrift* 120, 67–78.

Groenendaal, H. and Wolf, C.A. (2008) Farm-level economic analysis of the US National Johne's Disease Demonstration Herd Project. *Journal of the American Veterinary Medical Association* 233, 1852–1858.

Groenendaal, H. and Zagmutt, F.J. (2008) Scenarios analysis of changes in consumption of dairy products due to a hypothetical causal link between *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's Disease. *Journal of Dairy Science* 91, 3245–3258.

Groenendaal, H., Nielen, M., Jalvingh, A.W., Horst, S.H., Galligan, D.T. and Hesselink, J.W. (2002) A simulation of Johne's disease control. *Preventive Veterinary Medicine* 54, 225–245.

Groenendaal, H., Nielen, M. and Hesselink, J.W. (2003) Development of the Dutch Johne's disease control program supported by a simulation model. *Preventive Veterinary Medicine* 60, 69–90.

Haghkhah, M., Ansari-Lari, M., Novin-Baheran, A.M. and Bahramy, A. (2008) Herd-level prevalence

of *Mycobacterium avium* subspecies *paratuberculosis* by bulk-tank milk PCR in Fars province (southern Iran) dairy herds. *Preventive Veterinary Medicine* 86, 8–13.

Hill, B.B., West, M. and Brock, K.V. (2003) An estimated prevalence of Johne's disease in a subpopulation of Alabama beef cattle. *Journal of Veterinary Diagnostic Investigation* 15, 21–25.

Holstad, G., Sigurðardóttir, O.G., Storset, A.K., Tharaldsen, J., Nyberg, O., Schönheit, J. and Djønne, B. (2005) Description of the infection status in a Norwegian cattle herd naturally infected by *Mycobacterium avium* subsp. *paratuberculosis*. *Acta Veterinaria Scandinavica* 46, 45–56.

Horne, H. (1909) Den Lollandse Syke. *Norsk Veterinaermedicin Tidsskrift* 21, 111.

Jakobsen, M.B., Alban, L. and Nielsen, S.S. (2000) A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. *Preventive Veterinary Medicine* 46, 15–27.

Jayarao, B.M., Pillai, S.R., Wolfgang, D.R., Griswold, D.R., Rossiter, C.A., Tewari, D., Burns, C.M. and Hutchingson, L.J. (2004) Evaluation of IS*900*-PCR assay for detection of *Mycobacterium avium* subspecies *paratuberculosis* infection in cattle using quarter milk and bulk tank milk samples. *Foodborne Pathogens and Disease* 1, 17–26.

Johne, H.J. and Frothingham, J. (1895) Ein eigentümlicher Fall von Tuberculose beim Rind. *Deutsche Zeitschrift für Tiermedizin und Vergleichende Pathologie* 21, 438–454.

Johnson-Ifearulundu, Y.J., Kaneene, J.B., Sprecher, D.J., Gardiner, J.C. and Lloyd, J.W. (2000) The effect of subclinical *Mycobacterium paratuberculosis* infection on days open in Michigan, USA, dairy cows. *Preventive Veterinary Medicine* 46, 171–181.

Jørgensen, J.B. (1965) On the occurrence of *Mycobacterium johnei* in the mesenteric lymph nodes of abattoir cattle. *Nordisk Veterinaer Medicin* 17, 97–102.

Kalis, C.H. (2003) Diagnosis and control of paratuberculosis in dairy herds. PhD thesis, Utrecht University, the Netherlands.

Kalis, C.H., Barkema, H.W., Hesselink, J.W., van Maanen, C. and Collins, M.T. (2002) Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 14, 219–224.

Kalis, C.H., Collins, M.T., Barkema, H.W. and Hesselink, J.W. (2004) Certification of herds as free of *Mycobacterium paratuberculosis* infection: actual pooled faecal results versus certification model predictions. *Preventive Veterinary Medicine* 65, 189–204.

Kopecky, K.E. (1977) Distribution of paratuberculosis in Wisconsin, by soil regions. *Journal of the American Veterinary Medical Association* 170, 320–324.

Kruze, J., Salgado, M. and Collins, M.T. (2007) Paratuberculosis in Chilean dairy goat herds. *Archivos de Medicina Veterinaria* 39,147–152.

Kudahl, A.B., Ostergaard, S., Sørensen, J.T. and Nielsen, S.S. (2007) A stochastic model simulating paratuberculosis in a dairy herd. *Preventive Veterinary Medicine* 78, 97–117.

Leclainche, E. (1907) L'entérite chronique hypertrophiante des bovides. *Revue Générale Médicale Vétérinaire* 9, 305–312.

Le Franq van Berkhey, J. (1805) *Natuurlijke Historie van Holland*. P.H. Trap, Leyden, the Netherlands, pp. 217–218.

Lewerin, S.S., Ågren, E., Frössling, J., Bölske, G., Holmström, A., Lindberg, A., Szanto, E. and Viske, D. (2007) Control of paratuberculosis in Sweden. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin. Available at: www.paratuberculosis.org/pubs/proc9/section5.htm (accessed 16 January 2009).

Lienaux, E. (1909) L'entérite hypertrophiante diffuse du boeuf. *Annales de Médecine Vétérinaire* 58, 460–468.

Links, I.J., Sergeant, E., Moloney, B. and Reddacliff, L. (1999) Surveillance for ovine Johne's disease in New South Wales. Update to 31 December 1998. In: Manning, E.J.B. and Collins, M.T. (eds) *Proceedings of the 6th International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin. Available at: http://www.paratuberculosis.org/pubs/proc6/ (accessed 15 May 2009).

Lombard, J.E., Garry, F.B., McCluskey, B.J. and Wagner, B.A. (2005) Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows. *Journal of the American Veterinary Medical Association* 227, 1975–1981.

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B. and Salman, M.D. (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. *Journal of Dairy Science* 89, 4163–4171.

Losinger, W.C. (2005) Economic impact of reduced milk production associated with Johne's disease

on dairy operations in the USA. *Journal of Dairy Research* 72, 425–432.

Mainar-Jaime, R.C. and Vásquez-Boland, J.A. (1998) Factors associated with seroprevalence to *Mycobacterium paratuberculosis* in small-ruminant farms in the Madrid region (Spain). *Preventive Veterinary Medicine* 34, 317–327.

Markus, H. (1904) Eine specifische Darmentzündung des Rindes, wahrscheinlich tuberculöser Natur [A specific intestinal inflammation of cattle, probably tubercular of nature]. *Zeitschrift für Tiermedizin* 8, 68–78.

McFadyean, J. (1906) A new disease of cattle (Johne's disease). In: Annual report for 1906 of the principal of the Royal Veterinary College. *Journal of the Royal Agricultural Society* 67, 230–241.

McKenna, S.L.B., Keefe, G.P., Barkema, H.W., McClure, J., VanLeeuwen, J.A., Hanna, P. and Sockett, D.C. (2004) Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine. *Journal of Dairy Science* 87, 3770–3777.

McKenna, S.L., Keefe, G.P., Barkema, H.W. and Sockett, D.C. (2005) Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. *Veterinary Microbiology* 110, 105–111.

McNab, W.B., Meek, A.H. and Martin, S.W. (1991) An epidemiological study of paratuberculosis in Ontario dairy cattle: study design and prevalence estimates. *Canadian Journal of Veterinary Research* 55, 246–251.

Merkal, R.S., Whipple, D.L., Sacks, J.M. and Snyder, G.R. (1987) Prevalence of *Mycobacterium paratuberculosis* in ileocecal lymph nodes of cattle culled in the United States. *Journal of the American Veterinary Medical Association* 190, 676–680.

Michel, A.L. and Bastianello, S.S. (2000) Paratuberculosis in sheep: an emerging disease in South Africa. *Veterinary Microbiology* 77, 299–307.

Muehlherr, J.E., Zweifel, C., Corti, S., Blanco, J.E. and Stephan, R. (2003) Microbiological quality of raw goat's and ewe's bulk-tank milk in Switzerland. *Journal of Dairy Science* 86, 3849–3856.

Muskens, J., Barkema, H.W., Russchen, E.W., van Maanen, C., Schukken, Y.H. and Bakker, D. (2000) Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. *Veterinary Microbiology* 77, 253–261.

National Animal Health Monitoring System (2007) In: *Part I: Reference of Dairy Health and Management in the United States*. USDA: APHIS: VS, CEAH, Fort Collins, Colorado. Available at: http://nahms.aphis.usda.gov/dairy/index.htm (accessed 14 May 2009).

Nielsen, S.S. and Toft, N. (2009) A review of prevalences of paratuberculosis in farmed animals in Europe. *Preventive Veterinary Medicine* 88, 1–14.

Nielsen, S.S., Thamsborg, S.M., House, H. and Bitsch, V. (2000) Bulk-tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. *Preventive Veterinary Medicine* 44, 1–7.

Nielsen, S.S., Toft, N., Jørgensen, E. and Bibby, B.M. (2007) Bayesian mixture models for within-herd prevalence estimates of bovine paratuberculosis based on a continuous ELISA response. *Preventive Veterinary Medicine* 81, 290–305.

Ott, S.L., Wells, S.J. and Wagner, B.A. (1999) Herd-level economic losses associated with Johne's disease on US dairy operations. *Preventive Veterinary Medicine* 40, 179–192.

Pavlik, I., Bartl, J., Dvorska, L., Svastova, P., du Maine, R., Machackova, M., Yayo Ayele, W. and Horvathova, A. (2000) Epidemiology of paratuberculosis in wild ruminants studied by restriction fragment length polymorphism in the Czech Republic during the period 1995–1998. *Veterinary Microbiology* 77, 231–251.

Pearson, L. (1908) A note on the occurrence in America of chronic bacterial dysentery of cattle. *American Veterinary Reviews* 32, 602–605.

Pedersen, K., Manning, E.J. and Corn, J.L. (2008) Distribution of *Mycobacterium avium* subspecies *paratuberculosis* in the lower Florida Keys. *Journal of Wildlife Diseases* 44, 578–584.

Pence, M., Baldwin, C. and Black, C.C., 3rd (2003) The seroprevalence of Johne's disease in Georgia beef and dairy cull cattle. *Journal of Veterinary Diagnostic Investigation* 15, 475–477.

Reyes-García, R., Pérez-de-la-Lastra, J.M., Vicente, J., Ruiz-Fons, F., Garrido, J.M. and Gortázar, C. (2008) Large-scale ELISA testing of Spanish red deer for paratuberculosis. *Veterinary Immunology and Immunopathology* 124, 75–81.

Roussel, A.J., Libal, M.C., Whitlock, R.L., Hairgrove, T.B., Barling, K.S. and Thompson, J.A. (2005) Prevalence of and risk factors for paratuberculosis in purebred beef cattle. *Journal of the American Veterinary Medical Association* 226, 773–778.

Scanu, A.M., Bull, T.J., Cannas, S., Sanderson, J.D., Sechi, L.A., Dettori, G., Zanetti, S. and Hermon-Taylor, J. (2007) *Mycobacterium avium* subspecies *paratuberculosis* infection in cases of irritable bowel syndrome and comparison with Crohn's disease and Johne's disease: common neural and immune pathogenicities. *Journal of Clinical Microbiology* 45, 3383–3899.

Scott, H.M., Sorenson, O., Wu, J.T., Chow, E.Y. and Manninen, K. (2007) Seroprevalence of agro-ecological risk factors for *Mycobacterium avium* subspecies *paratuberculosis* and *Neospora caninum* infection among adult beef cattle in cow–calf herds in Alberta, Canada. *Canadian Veterinary Journal* 48, 397–406.

Sergeant, E.S. (2003) Estimated flock-prevalence and distribution of ovine Johne's disease in Australia at December 2001. *Australian Veterinary Journal* 81, 768–769.

Sergeant, E.S. and Baldock, F.C. (2002) The estimated prevalence of Johne's disease infected sheep flocks in Australia. *Australian Veterinary Journal* 80, 762–768.

Shulaw, W.P., Gordon, J.C., Bech-Nielsen, S., Pretzman, C.I. and Hoffsis, G.F. (1986) Evidence of paratuberculosis in Ohio's white-tailed deer, as determined by an enzyme-linked immunosorbent assay. *American Journal of Veterinary Research* 47, 2539–2542.

Slana, I., Pralik, P., Kralova, A. and Pavlik, I. (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *International Journal of Food Microbiology* 128, 250–257.

Smith, M.C. and Sherman, D.C. (1994) Wasting diseases. In: *Goat Medicine*. Lea and Febiger, Malvern, Pennsylvania, pp. 307–311.

Sockett, D.C. (1996) Johne's disease eradication and control: regulatory implications. *The Veterinary Clinics of North America. Food Animal Practice* 12, 431–440.

Stabel, J.R., Wells, S.J. and Wagner, B.A. (2002) Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds. *Journal of Dairy Science* 85, 525–531.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P. and Rings, D.M. (1995) Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *American Journal of Veterinary Research* 56, 1322–1324.

Sweeney, R.W., Whitlock, R.H. and Rosenberger, A.E. (1992) *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *Journal of Clinical Microbiology* 30, 166–171.

Sweeney, R.W., Whitlock, R.H., Buckley, C.L. and Spencer, P.A. (1995) Evaluation of a commercial enzyme linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. *Journal of Veterinary Diagnostic Investigation* 7, 488–493.

Tavornpanich, S., Munoz-Zanzi, C.A., Wells, S.J., Raizman, E.A., Carpenter, T.E., Johnson, W.O. and Gardner, I.A. (2008) Simulation model for evaluation of testing strategies for detection of paratuberculosis in midwestern US dairy herds. *Preventive Veterinary Medicine* 83, 65–82.

Tharaldsen, J., Djonne, B., Fredriksen, B., Nyberg, O. and Sigurðardóttir, O. (2003) The national paratuberculosis program in Norway. *Acta Veterinaria Scandinavica* 44, 243–246.

Thorne, J.G. and Hardin, L.E. (1997) Estimated prevalence of paratuberculosis in Missouri, USA cattle. *Preventive Veterinary Medicine* 31, 51–57.

Tryland, M., Olsen, I., Vikøren, T., Handeland, K., Arnemo, J.M., Tharaldsen, J., Djønne, B., Josefsen, T.D. and Reitan, L.J. (2004) Serologic survey for antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in free-ranging cervids from Norway. *Journal of Wildlife Diseases* 40, 32–41.

VanLeeuwen, J.A., Keefe, G.P., Tremblay, R., Power, C. and Wichtel, J.J. (2001) Seroprevalence of infection with *Mycobacterium avium* subspecies *paratuberculosis*, bovine leukemia virus, and bovine viral diarrhea virus in maritime Canada dairy cattle. *Canadian Veterinary Journal* 42, 193–198.

Van Schaik, G., Kalis, C.H.J., Benedictus, G., Dijkhuizen, A.A. and Huirne, R.B.M. (1996) Cost–benefit analysis of vaccination against paratuberculosis in dairy cattle. *The Veterinary Record* 139, 624–627.

Van Schaik, G., Schukken, Y.H., Crainiceanu, C., Muskens, J. and VanLeeuwen, J.A. (2003) Prevalence estimates for paratuberculosis adjusted for test variability using Bayesian analysis. *Preventive Veterinary Medicine* 60, 281–295.

Waldner, C.L., Cunningham, G.L., Janzen, E.D. and Campbell, J.R. (2002) Survey of *Mycobacterium avium* subspecies *paratuberculosis* serological status in beef herds on community pastures in Saskatchewan. *Canadian Veterinary Journal* 43, 542–546.

Wells, S.J. and Wagner, B.A. (2000) Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. *Journal of the American Veterinary Medical Association* 216, 1450–1457.

Woodbine, K.A., Schukken, Y.H., Green, L.E., Ramirez-Villaescusa, A., Mason, S., Moore, S.J., Bilbao, C., Swann, N., Medley, G.F. (2009) Seroprevalence and epidemiological characteristics of *Mycobacterium avium* subsp. *paratuberculosis* on 114 cattle farms in south west England. *Preventive Veterinary Medicine* 89, 102–109.

3 Epidemiology of Paratuberculosis

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3.1 Introduction

This chapter gives a broad overview of the epidemiology of paratuberculosis, recognizing that certain aspects of epidemiology, including the history of paratuberculosis (see Manning and Collins, Chapter 1, this volume) and the global burden of paratuberculosis (see Barkema *et al.*, Chapter 2, this volume), are covered in greater detail elsewhere. As noted in Chapter 1, since the first recognition of this infectious disease in dairy cattle, there has been a steady dispersion of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) over geographical space and host species. As shown in Fig. 3.1, the spread of this chronic insidious disease from its first recognition in Europe to herds of the New World may have coincided with the process of colonization and the subsequent growth of domestic animal agriculture. This pattern of spread continues as countries such as Chile import livestock to foster an expansion of their dairy and meat industries.

Current estimates have over 50% of dairy cattle herds in Europe and North America infected; thus, paratuberculosis is now considered an endemic disease in these regions (USDA-APHIS-VS-CEAH, 2008; Nielsen and Toft, 2009). The herd or flock prevalence in other regions and countries is loosely associated with their history of animal importation, level of industrialization and degree of economic concentration in animal agriculture. No country or region has published sufficient information to claim freedom from *MAP* (Committee on Diagnosis and Control of Johne's Disease, 2003; Nielsen and Toft, 2009).

MAP has a broad host range, as detailed in a series of other chapters (14–18) in this volume. As infection is most commonly found in ruminants, in particular dairy cattle, there have been only limited surveys in non-bovine species, making it tenuous to conclude that prevalence truly is greater in cattle (Committee on Diagnosis and Control of Johne's Disease, 2003). While ruminants appear to be the preferred or natural host for *MAP*, infections in non-ruminants, including horses, pigs, camelids, dogs, non-human primates and humans, have been sporadically reported (Committee

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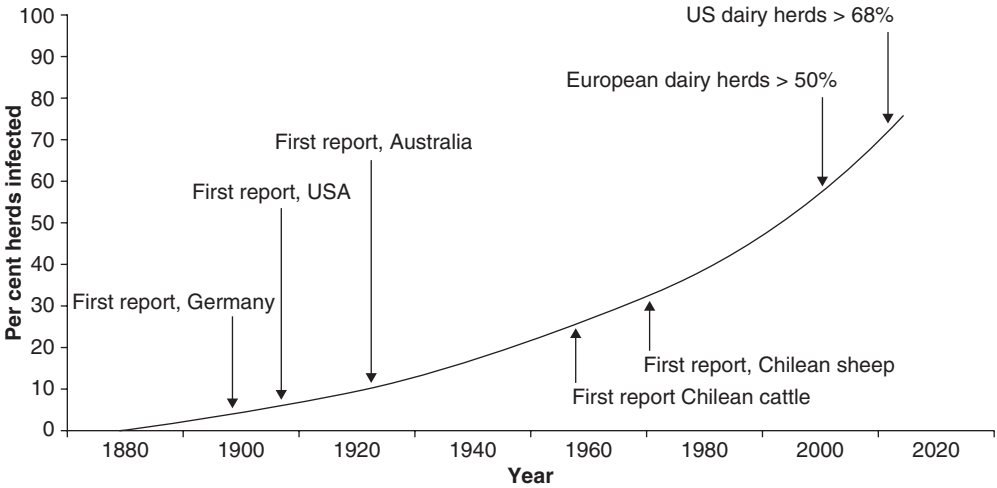

Fig. 3.1. Theoretical global epidemic curve for herd-level paratuberculosis in dairy cattle.

on Diagnosis and Control of Johne's Disease, 2003). *MAP* has also been isolated from a wide range of wildlife species (see Hutchings *et al.*, Chapter 18, this volume). It is difficult to know whether *MAP* infections are more common in certain species due to a true host preference or rather whether the observed between-species differences in infection rates are a function of *MAP* infection opportunity, i.e. animal husbandry and animal trade practices (poor biosecurity, high animal density, adult–newborn manure contact, etc.). There have been no controlled trials to assess the minimal infectious dose of *MAP* among animal species; hence there is no quantification of innate resistance to *MAP* infection or any comparison of pathogenesis patterns across species. The zoonotic potential of *MAP* is controversial (see Behr, Chapter 5, this volume).

3.2 Transmission

Transmission of *MAP* among animals is best studied in those animal species most commonly infected and where the infection has greatest economic importance, i.e. dairy cattle. It is commonly assumed, anecdotally reported, but not proven that similar *MAP* transmission patterns occur in other animal species; this is an open question. Broadly, *MAP* is transmitted much like other faecal–orally transmitted pathogens: by direct ingestion of *MAP*-contaminated faeces or indirectly via *MAP*-faecal-contaminated colostrum, milk, water or feed. A singular aspect of paratuberculosis among mycobacterial infections is the apparent age-dependent infection susceptibility; the infectious dose for neonates is probably low, while that for adults is quite high. The biological basis for this is not known but probably relates to the observation that *MAP* enters the host primarily through intestinal lymphoid tissue known as Peyer's patches (Momotani *et al.*, 1988; Lugton, 1999; Sigurðardóttir *et al.*, 2005), a site that is at peak activity in neonates. Regardless of the mechanism, the notion that infection is age-dependent is so widely held that control programmes to block infection transmission are primarily focused on the neonatal period of an animal's life. Exceptions to this may be in the Cervidae (see Mackintosh and Griffin, Chapter 17, this volume), which seem more susceptible to *MAP*, such that even adults may readily become infected (O'Brien *et al.*, 2006).

Contamination of colostrum, milk, water or feed for neonates with *MAP*-laden manure

facilitates infection transmission (Dieguez *et al.*, 2008; Kudahl *et al.*, 2008; Nielsen *et al.*, 2008; Slana *et al.*, 2008; Stabel, 2008; Tiwari *et al.*, 2009). In addition to faeces-associated transmission, the organism may be found in colostrum and milk in adult cows with advanced, disseminated disease, making *MAP* transmission possible despite the most scrupulous hygienic practices during collection of colostrum or milk (Slana *et al.*, 2008). In late-stage disease, and perhaps even in preclinical stages of *MAP* infection, it is also possible for fetal infections to occur *in utero* (Whittington and Windsor, 2009). Collectively, these multiple transmission modes generally lead to an association between the dam's *MAP* infection status and that of her offspring, with the strength of association being heavily dependent on farm management practices. The greatest association is between older *MAP*-infected females with advanced disease and the infection status of their offspring (Benedictus *et al.*, 2008).

3.3 State of Paratuberculosis Infection

The incubation period for paratuberculosis infection to progress to disease is prolonged. Clinical expression of the infection seems to be associated with sexual maturation and/or parturition. Australian researchers reported a mean incubation period of 5 years in dairy cattle (Jubb *et al.*, 2004). Although less well characterized, the incubation period in goats and Cervidae seems to be shorter, due to their more rapid maturation and/or their greater susceptibility to *MAP*. Of greatest significance to the epidemiology of paratuberculosis is the prolonged period during which some animals are infectious, whether clinically diseased or not. During the preclinical period of infection, unbeknown to the herd or flock owner, some animals can shed *MAP* in faeces, colostrum or milk, contaminating facilities and serving as a direct or indirect source of *MAP* infection for neonates on the farm. Detection and management or culling of such animals is integral to the success of paratuberculosis control programmes (Lu *et al.*, 2008).

Transmission among herds, regions or countries occurs primarily with the trade of *MAP*-infected animals in the preclinical stage of infection. Because *MAP* silently infects animals and is often not detected by laboratory diagnostics, trade of animals based on the test-negative status of individually tested animals cannot provide high-level assurance that the animal is free of *MAP* infection. Trade based on herd-level testing is the only means of providing reasonable assurances that traded animals are not infected. The level of assurance is tied directly to the sensitivity of the test used and the extent of population sampling. National certification programmes are designed to encourage herd testing and herd classification for the purposes of fostering trade of low-*MAP*-risk animals. Detailed information is given elsewhere on control measures in Europe (see Bakker, Chapter 26, this volume), the USA (see Whitlock, Chapter 27, this volume) and Australia (see Kennedy and Citer, Chapter 28, this volume). Globally, it is advisable that the World Animal Health Organization modernize its recommendations for animal testing for *MAP* to limit international movement of this infection using the most cost-effective testing methods, such as enzyme-linked immunosorbent assays for serum antibodies, and herd-level risk classification based on statistical sampling of the population from which the animal originates.

MAP behaves as an obligate parasite of animals. With the possible exception of free-living amoebae, infected host animal cells provide the only ecological niche for *MAP* replication. Inability to produce mycobactin is an example of *MAP*'s adaptation to life inside cells, where mycobactin is not necessary for iron acquisition as it is in the environment (Lambrecht and Collins, 1992). Compensating for the necessity to jump from animal to animal for continuing replication, *MAP* is resistant to environmental factors such as heat, dehydration and sunlight, which may limit its ability to survive the 'waiting period' before infecting its next host. Survival in soils depends on the physical and chemical nature of the soil, content of organic matter, calcium, iron and pH, among other factors. Survival in soils for up to a year has been demonstrated (Johnson-Ifearulundu and Kaneene, 1997; Whittington *et al.*, 2004). Survival in water can be even longer (Whittington *et al.*, 2005). The role of

free-living amoebae in perpetuation or even amplification of *MAP* in water is plausible and worthy of further investigation (Mura *et al.*, 2006; Whan *et al.*, 2006). The degree to which *MAP*-contaminated soil and water are biologically important for infection transmission in domestic agriculture species or wildlife on farms is mostly speculative.

As *MAP* infection spreads in a defined setting, the environmental burden of *MAP* increases and the organism finds its way into myriad other locations, including insects, worms and wildlife (Fischer *et al.*, 2003a,b, 2005). Often the number of *MAP* in these abnormal hosts is low and usually there is limited pathology (Corn *et al.*, 2005). The epidemiology of *MAP* infection in what may be 'dead-end hosts' is not yet understood. Consequently, these abnormal hosts may play no significant role in maintenance of the agent in ecosystems or in passage of the infection back to domestic animals (Corn *et al.*, 2005). A possible exception to this generalization is in the case of wild rabbits in endemic areas (Daniels *et al.*, 2003). *MAP* infections of wildlife are covered in more detail elsewhere (see Hutchings *et al.*, Chapter 18, this volume).

Consistent with its ability to persist in the environment, *MAP* is resistant to heat. With the goal of evaluating survival in the face of pasteurization, *MAP* has been found to be more heat-resistant than other mycobacteria (Grant *et al.*, 2005). Consensus opinion based on data from multiple studies, in particular from surveys of retail milk, is that *MAP* occasionally survives commercial pasteurization and that viable *MAP* can be recovered in low numbers in retail HTST (high-temperature, short-time; 71.7 °C × 15 s) pasteurized milk. This finding is unlikely to affect spread of *MAP* on the farm, but it has potential implications for zoonotic spread to humans. More detailed information on the survival of *MAP* in milk and food is given elsewhere (see Grant, Chapter 4, this volume).

MAP, like other mycobacteria, is also resistant to chemicals, and its chlorine resistance raises speculation about its ability to contaminate and survive in water systems (Whan *et al.*, 2001). If *MAP* were to behave like its close relative *M. avium* subsp. *hominissuis*, colonization and amplification in biofilms in drinking water distribution systems could be a significant problem for human exposure (Falkinham *et al.*, 2001; Norton *et al.*, 2004; Steed and Falkinham, 2006; Torvinen *et al.*, 2007). A recent study in the USA reported over 80% of tap water samples to be culture-positive for *MAP*, with concentrations of 0 to 29,000 colony-forming units/l (Beumer *et al.*, 2008). Again, these data probably speak more towards risk of zoonotic spread than transmission on the farm but highlight the need to define the reservoir of *MAP* bacteria to best establish control strategies.

Sheep strains (also called Type S or Type I/III) and cattle strains (also called Type C or Type II) of *MAP* are readily distinguished (Collins *et al.*, 2002). Detailed information on the differences between these strain types is given elsewhere (see Stevenson, Chapter 12, this volume). While these strain designations may reflect a host preference, the sheep and cattle strains of *MAP* can cross the species barrier (Muskens *et al.*, 2001; Whittington *et al.*, 2001; Moloney and Whittington, 2008). The *MAP* genome is notoriously homogeneous, with one strain representing more than 80% of strains isolated in the USA (Motiwala *et al.*, 2003, 2004). Molecular tools are emerging, however, to allow discrimination among strains. These tools have yet to be applied to large *MAP* strain collections to help understand transmission patterns within and between animal populations. With the caveat that there remains much to learn, the state of the art is that multiple strains of *MAP* appear to move freely among species, with only limited host species barriers. *MAP* strain characterization is described elsewhere (see Collins, Chapter 25, this volume).

The potential for *MAP* to contaminate meat, milk and domestic water supplies and its capacity to infect a broad array of host species raise concerns about the zoonotic potential of *MAP* (see Grant, Chapter 4, this volume). The answer to the zoonosis question affects almost every other issue regarding paratuberculosis diagnosis and control programmes on a regional, national or international level. More importantly, it raises the question of 'Whose problem is it?', meaning 'Who is responsible for paying the costs to fix this growing problem?' If it is solely an economic

Box 3.1. Unanswered questions regarding *MAP* and paratuberculosis.

Are all *MAP* strains equally virulent?
Are there genetic markers for virulence of *MAP* strains?
Are all ruminants equally susceptible to *MAP* infection and disease?
What is the minimal infectious dose by animal species and age?
What is the biological basis for age-dependent susceptibility to *MAP*?
What host factors control infection progression, faecal shedding, dissemination and onset of clinical disease?
What are the most cost-effective control programmes for different animal species and husbandry systems?
To what extent do different animal husbandry practices impact *MAP* transmission?
Will establishment of *MAP* in wild animal populations restrict or even preclude eradication of paratuberculosis?
Can *MAP* survive in biofilms in water distribution systems, much like *M. avium* subsp. *hominissuis*, and become a reservoir for human infection?
Should the national control measures for paratuberculosis be different for countries with endemic bovine tuberculosis?
Given the large-scale efforts to control *MAP* in many countries and the continuing expansion of the epidemic in those same countries, is *MAP* control possible?

issue for animal agriculture, investments to control the problem will largely be the responsibility of animal owners, who will balance the costs of herd infection control versus economic losses due to the disease. If it is a problem affecting both animal and human health, it warrants investment from the public at large, and control measures must be implemented to ensure that milk, meat and domestic water are free of *MAP* or have *MAP* levels as low as reasonably achievable. This highlights the importance of the chapter (see Behr, Chapter 5, this volume) on the association of *MAP* and human disease.

3.4 Knowledge Gaps

There is a long list of knowledge gaps regarding the epidemiology of paratuberculosis, some of which are listed in Box 3.1. These and many other such questions provide fruitful opportunities for the next generation of veterinary microbiologists and epidemiologists.

References

Benedictus, A., Mitchell, R.M., Linde-Widmann, M., Sweeney, R., Fyock, T., Schukken, Y.H. and Whitlock, R.H. (2008) Transmission parameters of *Mycobacterium avium* subspecies *paratuberculosis* infections in a dairy herd going through a control program. *Preventive Veterinary Medicine* 83, 215–227.

Beumer, A., Kind, D. and Pfaller, S.L. (2008) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in drinking water and biofilms using quantitative PCR. *Abstracts of the 108th General Meeting of the American Society for Microbiology*, abstract Q-487. American Society for Microbiology, Washington, DC.

Collins, D.M., De Zoete, M. and Cavaignac, S.M. (2002) *Mycobacterium avium* subsp. *paratuberculosis* strains from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence difference. *Journal of Clinical Microbiology* 40, 4760–4762.

Committee on Diagnosis and Control of Johne's Disease, B.o.A.a.N.R.N.R.C. (2003) *Diagnosis and Control of Johne's Disease*. National Academies Press, Washington, DC.

Corn, J.L., Manning, E.J.B., Sreevatsan, S. and Fischer, J.R. (2005) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging birds and mammals on livestock premises. *Applied and Environmental Microbiology* 71, 6963–6967.

Daniels, M.J., Henderson, D., Greig, A., Stevenson, K., Sharp, J.M. and Hutchings, M.R. (2003) The potential role of wild rabbits *Oryctolagus cuniculus* in the epidemiology of paratuberculosis in domestic ruminants. *Epidemiology and Infection* 130, 553–559.

Dieguez, F.J., Arnaiz, I., Sanjuan, M.L., Vilar, M.J. and Yus, E. (2008) Management practices associated with *Mycobacterium avium* subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. *Veterinary Record* 162, 614–617.

Falkinham, J.O., Norton, C.D. and LeChevallier, M.W. (2001) Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare* and other mycobacteria in drinking water distribution systems. *Applied and Environmental Microbiology* 67, 1225–1231.

Fischer, O.A., Matlova, L., Bartl, J., Dvorska, L., Svastova, P., du Maine, R., Melicharek, I., Bartos, M. and Pavlik, I. (2003a) Earthworms (*Oligochaeta, Lumbricidae*) and mycobacteria. *Veterinary Microbiology* 91, 325–338.

Fischer, O.A., Matlova, L., Dvorska, L., Svastova, P. and Pavlik, I. (2003b) Nymphs of the oriental cockroach (*Blatta orientalis*) as passive vectors of causal agents of avian tuberculosis and paratuberculosis. *Medical and Veterinary Entomology* 17, 145–150.

Fischer, O.A., Matlova, L., Dvorska, L., Svastova, P., Bartos, M., Weston, R.T., Kopecna, M., Trcka, I. and Pavlik, I. (2005) Potential risk of *Mycobacterium avium* subspecies *paratuberculosis* spread by syrphid flies in infected cattle farms. *Medical and Veterinary Entomology* 19, 360–366.

Grant, I.R., Williams, A.G., Rowe, M.T. and Muir, D.D. (2005) Efficacy of various pasteurization time–temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *Applied and Environmental Microbiology* 71, 2853–2861.

Johnson-Ifearulundu, Y.J. and Kaneene, J.B. (1997) Relationship between soil type and *Mycobacterium paratuberculosis*. *Journal of the American Veterinary Medical Association* 210, 1735–1740.

Jubb, T.F., Sergeant, E.S.G., Callinan, A.P.L. and Galvin, J.W. (2004) Estimate of the sensitivity of an ELISA used to detect Johne's disease in Victorian dairy cattle herds. *Australian Veterinary Journal* 82, 569–573.

Kudahl, A.B., Nielsen, S.S. and Ostergaard, S. (2008) Economy, efficacy and feasibility of a risk-based control program against paratuberculosis. *Journal of Dairy Science* 91, 4599–4609.

Lambrecht, R.S. and Collins, M.T. (1992) *Mycobacterium paratuberculosis*: factors which influence mycobactin-dependence. *Diagnosis and Microbiology of Infectious Diseases* 15, 239–246.

Lu, Z., Mitchell, R.M., Smith, R.L., Van Kessel, J.S., Chapagain, P.P., Schukken, Y.H. and Grohn, Y.T. (2008) The importance of culling in Johne's disease control. *Journal of Theoretical Biology* 254, 135–146.

Lugton, I. (1999) Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic mycobacteria. *Immunology and Cell Biology* 77, 364–372.

Moloney, B.J. and Whittington, R.J. (2008) Cross species transmission of ovine Johne's disease from sheep to cattle: an estimate of prevalence in exposed susceptible cattle. *Australian Veterinary Journal* 86, 117–123.

Momotani, E., Whipple, D.L., Thiermann, A.B. and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Veterinary Pathology* 25, 131–137.

Motiwala, A.S., Strother, M., Amonsin, A., Byrum, B., Naser, S.A., Stabel, J.R., Shulaw, W.P., Bannantine, J.P., Kapur, V. and Sreevatsan, S. (2003) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing and identification of unique targets for diagnosis. *Journal of Clinical Microbiology* 41, 2015–2026.

Motiwala, A.S., Amonsin, A., Strother, M., Manning, E.J.B., Kapur, V. and Sreevatsan, S. (2004) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates recovered from wild animal species. *Journal of Clinical Microbiology* 42, 1703–1712.

Mura, M., Bull, T.J., Evans, H., Sidi-Boumedine, K., McMinn, L., Rhodes, G., Pickup, R. and Hermon-Taylor, J. (2006) Replication and long-term persistence of bovine and human strains of *Mycobacterium avium* subsp. *paratuberculosis* within *Acanthamoeba polyphaga*. *Applied and Environmental Microbiology* 72, 854–859.

Muskens, J., Bakker, D., de Boer, J. and van Keulen, L. (2001) Paratuberculosis in sheep: its possible role in the epidemiology of paratuberculosis in cattle. *Veterinary Microbiology* 78, 101–109.

Nielsen, S.S. and Toft, N. (2009) A review of prevalences of paratuberculosis in farmed animals in Europe. *Preventive Veterinary Medicine* 88, 1–14.

Nielsen, S.S., Bjerre, H. and Toft, N. (2008) Colostrum and milk as risk factors for infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. *Journal of Dairy Science* 91, 4610–4615.

Norton, C.D., LeChevallier, M.W. and Falkinham, J.O. (2004) Survival of *Mycobacterium avium*

in a model distribution system. *Water Research* 38, 1457–1466.

O'Brien, R., Mackintosh, C.G., Bakker, D., Kopecna, M., Pavlik, I. and Griffin, J.F.T. (2006) Immunological and molecular characterization of susceptibility in relationship to bacterial strain differences in *Mycobacterium avium* subsp. *paratuberculosis* infection in the red deer (*Cervus elaphus*). *Infection and Immunity* 74, 3530–3537.

Sigurðardóttir, O.G., Bakke-McKellep, A.M., Djønne, B. and Evensen, Ø. (2005) *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. *Comparative Immunology, Microbiology and Infectious Diseases* 28, 223–230.

Slana, I., Kralik, P., Kralova, A. and Pavlik, I. (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *International Journal of Food Microbiology* 128, 250–257.

Stabel, J.R. (2008) Pasteurization of colostrum reduces the incidence of paratuberculosis in neonatal dairy calves. *Journal of Dairy Science* 91, 3600–3606.

Steed, K.A. and Falkinham, J.O., III (2006) Effect of growth in biofilms on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*. *Applied and Environmental Microbiology* 72, 4007–4011.

Tiwari, A., VanLeeuwen, J.A., Dohoo, I.R., Keefe, G.P., Haddad, J.P., Scott, H.M. and Whiting, T. (2009) Risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* seropositivity in Canadian dairy cows and herds. *Preventive Veterinary Medicine* 88, 32–41.

Torvinen, E., Lehtola, M.J., Martikainen, P.J. and Miettinen, I.T. (2007) Survival of *Mycobacterium avium* in drinking water biofilms as affected by water flow velocity, availability of phosphorus and temperature. *Applied and Environmental Microbiology* 73, 6201–6207.

USDA-APHIS-VS-CEAH (2008) *Johne's Disease on U.S. Dairies, 1991–2007*. USDA-APHIS-VS-CEAH, Fort Collins, Colorado. Available at: http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/dairy/dairy07/Dairy2007_Johnes.pdf (accessed 15 June 2009).

Whan, L.B., Grant, I.R., Ball, H.J., Scott, R. and Rowe, M.T. (2001) Bactericidal effect of chlorine on *Mycobacterium paratuberculosis* in drinking water. *Letters in Applied Microbiology* 33, 227–231.

Whan, L., Grant, I. and Rowe, M. (2006) Interaction between *Mycobacterium avium* subsp. *paratuberculosis* and environmental protozoa. *BMC Microbiology* 6, 63.

Whittington, R.J. and Windsor, P.A. (2009) *In utero* infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. *The Veterinary Journal* 179, 60–69.

Whittington, R.J., Taragel, C.A., Ottaway, S., Marsh, I., Seaman, J. and Fridriksdottir, V. (2001) Molecular epidemiological confirmation and circumstances of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. *Veterinary Microbiology* 79, 311–322.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, I.B. and Reddacliff, L.A. (2004) Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Applied and Environmental Microbiology* 70, 2989–3004.

Whittington, R.J., Marsh, I.B. and Reddacliff, L.A. (2005) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. *Applied and Environmental Microbiology* 71, 5304–5308.

4 *Mycobacterium avium* subsp. *paratuberculosis* in Animal-derived Foods and the Environment

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4.1 Introduction

Animals infected by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), whether clinically or subclinically affected, can shed live bacteria in both their faeces and milk. If these animals are farmed for food production, the safety of foods derived from them becomes an important consideration, because *MAP* may also be associated with Crohn's disease in humans (see Behr, Chapter 5, this volume). Infected animals also contaminate their surrounding environment, increasing the risk of spread of Johne's disease at the farm level and potentially contaminating watercourses used for abstraction of drinking water. This chapter summarizes current evidence for the presence of *MAP* in animal-derived foods, describes the effect of various dairy processes on *MAP* survival, and reviews the reservoirs of *MAP* infection in the environment and the various mechanisms potentially aiding its survival for long periods. Shedding of *MAP* by infected animals has implications for food and water safety, as illustrated in Fig. 4.1.

4.2 Evidence of *MAP* in Animal-derived Foods

The current evidence for *MAP* contamination of animal-derived foods, both raw and processed, is summarized in Table 4.1. Results are based on culture or assays using the polymerase chain reaction (PCR). To the best of the author's knowledge, all published food surveillance studies have been included.

4.2.1 Milk and dairy products

Raw cows' milk has been the focus of most surveillance to date because it is recognized

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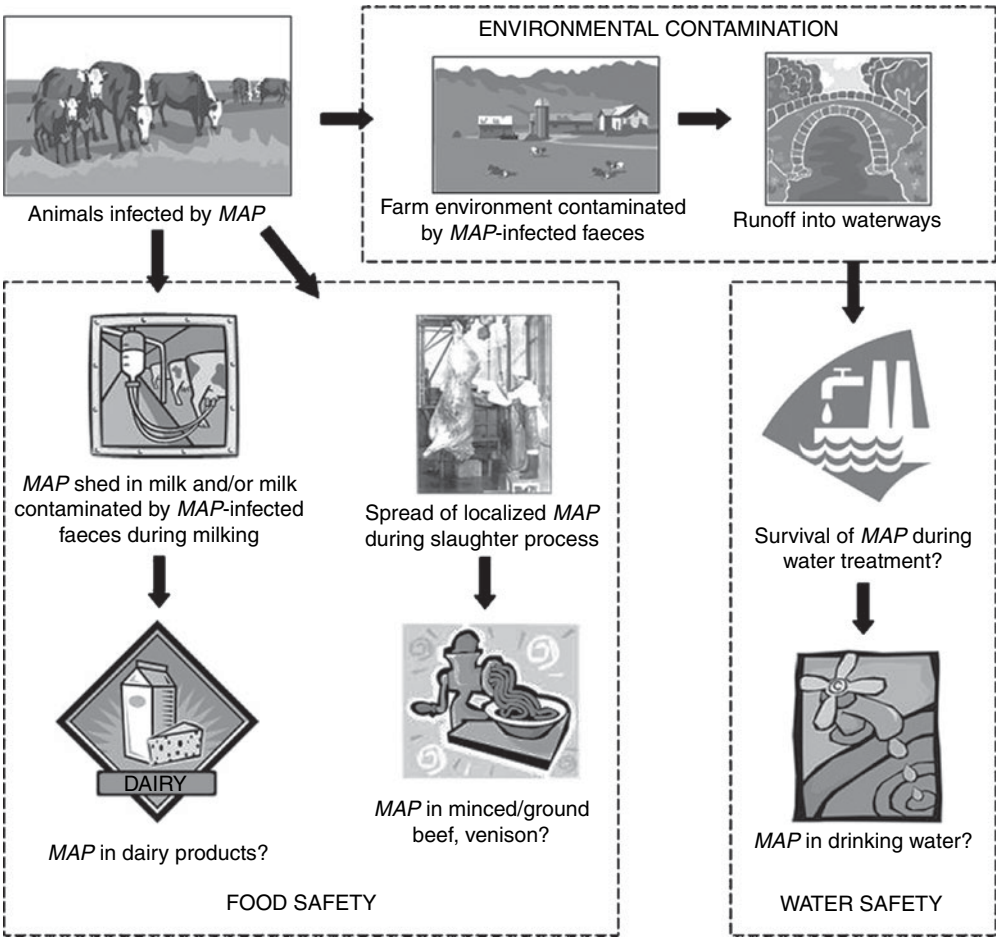

Fig. 4.1. Spread of *MAP* shed by infected animals in faeces and milk, and potential routes of human exposure to *MAP* via animal-derived foods and water.

as a major factor in the transmission of Johne's disease from cow to calf (Nielsen *et al.*, 2008). Published studies have tested raw milk from individual animals, bulk tank at farm level, or bulk silo milk prior to processing. The expectation is that there are two steps in diluting initial levels towards very low or non-detectable levels before processing: first, when milk from individual infected animals is mixed with milk of non-infected herdmates, and, secondly, when milk from one farm is mixed with milk from other farms before dairy processing. Despite this prediction, few data exist on the levels of *MAP* found in raw cows' milk (Table 4.1), and the values reported are likely to be underestimates for two reasons. First, chemical decontamination was applied to milk samples in all but one of these studies (Giese and Ahrens, 2000) and this, depending on the method employed, negatively impacts the estimated counts of *MAP* (Dundee *et al.*, 2001). Secondly, in studies concerning milk from individual animals, the milk tested was obtained after thorough cleaning and disinfection of the exterior of the udder, removing the possibility of faecal contamination. Faecal contamination can and does occur during the milking

Table 4.1. A summary of the evidence for *MAP* contamination of raw and processed animal-derived foods obtained via surveillance studies. Results are separated between milk and dairy products, and beef (bottom of table).

Food	Size of survey	% PCR-positive	% Culture-positive	No. of *MAP* reported	Reference
Milk and dairy products					
Raw cow's milk (individual animals)	26	–[a]	35.0	–[a]	Taylor *et al.* (1981)
	77	–[a]	11.6	2–8 CFU[b]/50 ml	Sweeney *et al.* (1992)
	126	–[a]	8.3	–[a]	Streeter *et al.* (1995)
	11	18.0	45.0	<100 CFU[b]/ml	Giese and Ahrens (2000)
	211	33.0	4.0	–[a]	Pillai and Jayarao (2002)
	1493	13.5	2.8	–[a]	Jayarao *et al.* (2004)
	483	–[a]	18.4	4–20 CFU[b]/50 ml	Ayele *et al.* (2005)
	84	3.6	–[a]	–[a]	Bosshard *et al.* (2006)
	342	32.5	0	10–560 cells/ml[c]	Slana *et al.* (2008)
Raw cows' milk (bulk tank, farm level)	200	9.0	–[a]	–[a]	Sevilla *et al.* (2002)
	52	68.0	0	–[a]	Stabel *et al.* (2002)
	501	22.4	–[a]	–[a]	Stephan *et al.* (2002)
	20	50.0	5.0	–[a]	Pillai and Jayarao (2002)
	29	27.5	20.6	–[a]	Jayarao *et al.* (2004)
	100	3.0	–[a]	–[a]	Bosshard *et al.* (2006)
	110	11.0	–[a]	–[a]	Haghkhah *et al.* (2008)
	5	80.0	0	1–<10 cells/ml[c]	Slana *et al.* (2008)
Raw cows' milk (processing level)	244	7.8	1.6	4–20 CFU[b]/50 ml	Grant *et al.* (2002)
	310	–[a]	0	–[a]	O'Doherty *et al.* (2002)
	389	12.9	0.3	–[a]	O'Reilly *et al.* (2004)
	175	–[a]	0.6	–[a]	Pearce *et al.* (2005)
Pasteurized cows' milk	312	7.0	0	–[a]	Millar *et al.* (1996)
	567	11.8	1.8	–[a]	Grant *et al.* (2002)
	710	15	0	–[a]	Gao *et al.* (2002)
	77	–[a]	0	–[a]	O'Doherty *et al.* (2002)
	357	9.8	0	–[a]	O'Reilly *et al.* (2004)
	702	64.0	2.8	–[a]	Ellingson *et al.* (2005)
	244	–[a]	1.6	–[a]	Ayele *et al.* (2005)
	70	–[a]	2.9	–[a]	Paolicchi *et al.* (2005)
	22	4.5	0	–[a]	Lillini *et al.* (2007)
Raw goat's milk (individual animals)	10	–[a]	10	–[a]	Singh and Vihan (2004)
Raw goats' milk (bulk tank, farm level)	90	1.1	0	–[a]	Grant *et al.* (2001)
	340	7.1	0	–[a]	Djonne *et al.* (2003)

(*Continued*)

Table 4.1. *continued*

Food	Size of survey	% PCR-positive	% Culture-positive	No. of *MAP* reported	Reference
	344	23.0	–[a]	–[a]	Muehlherr *et al.* (2003)
Raw sheep's milk (bulk tank, farm level)	14	0	0	–[a]	Grant *et al.* (2001)
	63	23.8	–[a]	–[a]	Muehlherr *et al.* (2003)
Cheese					
Greek feta cheese	42	50.0	4.7	–[a]	Ikonomopolous *et al.* (2005)
Hard and semi-hard cheeses	42	12.0	2.4	–[a]	Ikonomopolous *et al.* (2005)
Cheese curds	98	5.0	0	–[a]	Clark *et al.* (2006)
Soft, semi-hard and hard Swiss raw milk cheeses	143	4.2	0	–[a]	Stephan *et al.* (2007)
Artisanal raw milk cheeses	19	6.8	20	–[a]	Williams and Withers (2008)
Powdered infant milk formula	51	49.0	2.0	–[a]	Hruska *et al.* (2005)
Spray-dried whole milk powder	190	9.5	0	–[a]	Rowe *et al.* (2007)
Beef					
Minced/ground beef	113	–[a]	0	–[a]	Maher *et al.* (2004)
	200	0	–[a]	–[a]	Jaravata *et al.* (2007)
Beef carcasses	450	4.0–54.0	–[a]	–[a]	Meadus *et al.* (2008)

[a]Not tested or not reported.
[b]CFU, colony-forming units.
[c]counts determined by quantitative real-time PCR.

process, and different cleaning regimes applied to the udder affect the degree of faecal contamination (Gibson *et al.*, 2008).

The most recent study reporting numbers of *MAP* in raw milk (Slana *et al.*, 2008) used quantitative real-time PCR (qPCR) with IS*900* and F57 rather than culture to estimate numbers of *MAP* present in milk from individual cows and in bulk tank samples. *MAP* numbers in the 'tens of cells/ml' (to as high as 560 cells/ml) were reported. This latter value is considerably higher than that reported by Sweeney *et al.* (1992), who estimated two to eight colony-forming units (CFU) of *MAP*/50 ml milk, indicative of a low level of *MAP* in raw milk. The number of *MAP* in bulk tank milk on the farm studied by Slana *et al.* (2008) was in the 'units of cells/ml' (interpreted as 1–<10 cells/ml in Table 4.1). Whilst acknowledging that PCR does not differentiate between viable and dead bacteria, *MAP* cells detected in raw milk by qPCR are likely to be viable, and numbers detected would not be adversely affected by chemical decontamination (as CFU counts are). Therefore, these PCR-based values are possibly more realistic than any of the culture-based values.

Milk from sheep and goats has not been as extensively tested as cows' milk. In the developed world at least, these types of milk would tend to be consumed as dairy products rather than as liquid milk. The situation in developing countries is probably the reverse. The published surveillance data (Table 4.1) mainly relate to bulk tank milk at farm level rather than to milk from individual sheep or goats. *MAP* was not cultured from bulk tank milk of either species in any of the studies but was detected by PCR in all goat milk studies and in one of the sheep studies.

Cheese (particularly raw milk varieties), powdered infant milk formula and whole

milk powder are dairy products that have received some attention as possible vehicles of human exposure to *MAP*. From the published cheese studies there is certainly evidence of *MAP* contamination of the raw milk used for cheese manufacture, as PCR positivity was reported in all studies (Table 4.1). However, of the four published cheese surveys, only two reported the isolation of viable *MAP* (Ikonomopoulos *et al.*, 2005; Williams and Withers, 2008).

4.2.2 Beef

MAP contamination of beef carcasses can arise from three sources: (i) faecal contamination on hide being transferred to carcass during slaughter; (ii) disseminated infection via the bloodstream; and (iii) localized foci of infection, such as lymph nodes, being disrupted, such that the contents are spread to other parts of the carcass or mixed into minced/ground beef. A number of studies report disseminated infection in dairy and beef cattle tested post-mortem (Pavlik *et al.*, 2000; Ayele *et al.*, 2004; Bosshard *et al.*, 2006; Antognoli *et al.*, 2008; Brady *et al.*, 2008; Dennis *et al.*, 2008; Alonso-Hearn *et al.*, 2009). This finding increases the possibility of raw beef products being contaminated by *MAP*. Meadus *et al.* (2008) sampled the hind regions of skinned and dressed beef carcasses at three North American meat-packing plants and tested the samples using IS*900* and F57 nested PCR methods. They found that between 6 and 54% of carcasses were positive for IS*900*, and between 4 and 20% were positive for F57. However, the fact that a nested PCR was needed to detect *MAP* on the carcasses strongly suggested that the amount of *MAP* present was small. Meadus *et al.* (2008) concluded that, although data are limited, beef carcass surfaces may not be a major route of human *MAP* exposure. This conclusion is supported by the findings of two surveys of minced/ground beef, where *MAP* was not detected by IS*900* PCR in minced/ground beef at processing (Maher *et al.*, 2004) or isolated by culture from minced/ground beef at retail (Jaravata *et al.*, 2007).

Johne's disease is an increasing problem in farmed deer (Power *et al.*, 1993, Fawcett *et al.*, 1995; de Lisle *et al.*, 2003; Kopecna *et al.*, 2008) and so there is a risk of venison being contaminated by *MAP* in the same way as beef. Whilst a theoretical risk may exist, no venison surveys have been reported.

4.3 Survival of *MAP* during Dairy Processing

Numerous pasteurization studies involving *MAP* have been conducted. Space does not permit the listing of all these studies here, so readers are directed to published reviews and critiques of these studies by Klijn *et al.* (2001), Lund *et al.* (2002) and Cerf *et al.* (2007). The studies involved different heating apparatus, different *MAP* strains prepared in different ways, and different culture methodologies after heating; consequently, it is very difficult to compare the studies or to reach a consensus opinion on the effect of commercial high-temperature, short-time (HTST) pasteurization conditions (72 °C for 15 s) on *MAP* viability. The findings can best be described as conflicting. Some researchers report inactivation of >7 $\log_{10}$ *MAP*; others consistently report a more modest 4 $\log_{10}$ reduction (Cerf *et al.*, 2007). In light of the fact that several surveys of pasteurized cows' milk at retail level have reported the presence of viable *MAP* in 1–3% of milk samples tested (Table 4.1), it can be inferred that HTST pasteurization of milk is not 100% effective in killing *MAP* all of the time. Given the numbers of bacteria reported above (cells/ml) and inactivation rates by pasteurization between 4 and 7 logs, it is reasonable to infer that low numbers of viable *MAP* are periodically being consumed by people drinking pasteurized cows' milk. The infective dose in humans, and whether this poses a risk of progression from infection to disease, is currently unknown.

When milk is made into cheese, there is an approximately ten-fold concentration of any *MAP* present upon curd formation (Donaghy *et al.*, 2004). Several studies (Sung and Collins, 2000; Spahr and Schafroth, 2001; Donaghy *et al.*, 2004) have shown that the rate of inactivation of *MAP* in cheese is governed by the cheese conditions (specifically pH, a_w

and salt concentration) and duration of ripening. Raw milk cheeses may represent a greater risk of exposure to viable *MAP* (Table 4.1) because numbers have not been reduced by heat treatment (thermization or pasteurization).

In view of the fact that studies have suggested that HTST pasteurization may not completely eliminate viable *MAP*, the effect of novel milk processing techniques has been studied. The use of pulsed electric fields to destroy pathogenic bacteria, as a result of electrical breakdown of the cell membrane and electroporation, has been investigated for *MAP* inactivation. Rowan *et al.* (2001) observed a 5.9 $\log_{10}$ reduction in viable *MAP* when spiked cows' milk was subjected to 2500 pulses at 30 kV/cm in a 25 min period, which represented a greater kill than was achieved by laboratory pasteurization (2.4 $\log_{10}$). Stabel *et al.* (2001) reported that application of 5, 10 or 15 kGy of gamma radiation achieved a 6.0 $\log_{10}$ reduction in *MAP* in raw milk. In contrast, ultraviolet light treatment of *MAP* in milk had minimal effect on viability (0.5–1.0 $\log_{10}$ reduction per 1000 mJ/ml) (Altic *et al.*, 2007). Treatment of *MAP*-spiked milk with high hydrostatic pressure (500 MPa for 10 min) achieved a 4–6 $\log_{10}$ kill (López-Pedemonte *et al.*, 2006; Donaghy *et al.*, 2007), a similar reduction to HTST pasteurization.

4.4 Contamination of the Farm Environment

Infected animals shed *MAP* in their faeces, so they will contaminate their immediate environment, whether housed indoors or grazing outside. Cattle are commonly classified as light, moderate or heavy shedders on the basis of the numbers of *MAP* in their faeces (<300 CFU/g, 300–3000 CFU/g and >3000 CFU/g, respectively (Van Schaik *et al.*, 2003). Hovingh *et al.* (2006) identified a further category of infected animal – the *MAP* supershedder – which can shed >1,000,000 CFU/g faeces. Hovingh *et al.* (2006) found that 10–15% of animals in four infected herds were supershedders. They calculated that a single supershedder would shed more *MAP* than 2000 moderate or 20,000 light shedders. This situation has major implications for levels of *MAP* contamination in bulk tank milk of infected farms, and for environmental transmission of Johne's disease within either dairy or beef herds.

4.4.1 Spread and survival of *MAP* in the environment

Cattle are generally not housed all the time, and movement of animals around the farm results in contamination of outdoor areas. Raizman *et al.* (2004) and Lombard *et al.* (2006) tested environmental samples from various locations around dairy operations in the USA. Farm locations commonly contaminated by *MAP* were parlour exits, floors of holding pens, common alleyways, lagoons, manure spreaders and manure pits. When animals are grazing on pastures, their faeces contaminate soil and grass. Whittington *et al.* (2004) studied survival in faeces in the Australian environment, and *MAP* was cultured for up to 55 weeks from dry, fully shaded locations and for much shorter time periods in unshaded conditions. They postulated that diurnal temperature flux due to infrared radiation, rather than UV inactivation, influenced *MAP* survival. In a subsequent study of survival of *MAP* in dam water in shaded or exposed water troughs, Whittington *et al.* (2005) recorded survival times of up to 48 weeks and 36 weeks, respectively, and for 12–26 weeks longer in the dam sediment. In both studies, Whittington and colleagues obtained results suggestive of dormancy, i.e. *MAP* detection followed by disappearance and then detection again after a period of time. Numerous invertebrate and protozoal species were observed to be present in the dam water (Whittington *et al.*, 2005). It has therefore been suggested that interaction with nematodes, insects or protozoa (Whan *et al.*, 2006) may enable *MAP*, an intracellular pathogen, to survive and/or multiply in the environment. Other potential survival mechanisms of *MAP* in the environment (dormancy, aerosolization and biofilm formation) are reviewed by Rowe and Grant (2006).

MAP on contaminated pasture can run off into watercourses when it rains. Studies

by Pickup *et al.* (2005, 2006) presented evidence of runoff from hills grazed by *MAP*-infected sheep into the Taff and Tywi rivers in South Wales, UK, especially after periods of high rainfall. Viable *MAP* was also isolated from raw water entering water treatment plants in a Northern Ireland survey (Whan *et al.*, 2005). These findings with regard to *MAP* in raw water raise questions about the ability of water treatment processes to remove or inactivate *MAP* before it reaches the consumer. In laboratory simulations, chlorination of *MAP*-spiked water at 2 μg/ml for 30 min resulted in a maximum 2.8 $\log_{10}$ reduction in numbers (Whan *et al.*, 2001), which means that, in common with other mycobacteria, *MAP* is chlorine resistant. Another water treatment process (COCODAFF) physically removes *MAP* along with suspended solids (Pickup *et al.*, 2006). However, the contaminated slurry removed may be disposed of back on to the land, creating a cycle of environmental persistence.

Data from *MAP* testing of drinking water is sparse and conflicting to date. In a UK drinking water survey, no viable *MAP* was isolated (Health Protection Agency, 2003). In a recent US survey, Beumer *et al.* (2008) reported detection of *MAP* in 84–92% of 1 l domestic cold water samples by qPCR and the presence of *MAP* in 89% of biofilm samples from tap gratings. Estimated numbers of *MAP* detected ranged from 1 to 29,000 *MAP*/l, with most samples having <500 *MAP*/l.

4.5 Conclusions

MAP has been cultured from a range of animal-derived food products and drinking water. In addition, *MAP* DNA has been detected in the same range of potential sources of exposure. Whether these studies paint an accurate picture of risk is debatable, as culture-based methods used for the detection of *MAP* are expected to underestimate the true number of organisms present in any particular sample. Factors such as the adverse effect of chemical decontamination on the viability of some of the *MAP* present and overgrowth of cultures by other bacteria potentially masking the presence of *MAP* colonies will also lead to underestimates of *MAP* presence. As detection methods improve and further food and drinking water surveys are undertaken, a clearer picture regarding risk of human and veterinary exposure to *MAP* via these routes will hopefully emerge.

References

Alonso-Hearn, M., Molina, E., Geijo, M., Vazquez, P., Sevilla, I., Garrido, J.M. and Juste, R.A. (2009) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from muscle tissue of naturally infected cattle. *Foodborne Pathogens and Disease* 6, 513–518.

Altic, L.C., Rowe, M.T. and Grant, I.R. (2007) UV light inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk as assessed by FASTPlaqueTB phage assay and culture. *Applied and Environmental Microbiology* 73, 3728–3733.

Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Dennis, M.M., Gould, D.H. and Salman, M.D. (2008) Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results. *Veterinary Microbiology* 127, 4300–4308.

Ayele, W.Y., Bartos, M., Svartova, P. and Pavlik, I. (2004) Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in organs of naturally infected bull-calves and breeding bulls. *Veterinary Microbiology* 103, 209–217.

Ayele, W.Y., Svastova, P., Roubal, P., Bartos, M. and Pavlik, I. (2005) *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic. *Applied and Environmental Microbiology* 71, 1210–1214.

Beumer, A., King, D. and Pfaller, S.L. (2008) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in drinking water and biofilms using quantitative PCR. *Abstracts of the 108th General Meeting of the American Society for Microbiology*, abstract Q-487. American Society for Microbiology, Washington, DC.

Bosshard, C., Stephan, R. and Tasara, T. (2006) Application of an F57 sequence-based real-time PCR assay for *Mycobacterium paratuberculosis* detection in bulk tank raw milk and slaughtered healthy dairy cows. *Journal of Food Protection* 69, 1662–1667.

Brady, C., O'Grady, D., O'Meara, F., Egan, J. and Bassett, H. (2008) Relationships between clinical signs, pathological changes and tissue distribution of *Mycobacterium avium* subspecies *paratuberculosis* in 21 cows from herds affected by Johne's disease. *The Veterinary Record* 162, 147–152.

Cerf, O., Griffiths, M. and Aziza, F. (2007) Assessment of the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in commercially pasteurized milk. *Foodborne Pathogens and Disease* 4, 4433–4447.

Clark, D.L., Anderson, J.L., Koziczkowski, J.J. and Ellingson, J.L.E. (2006) Detection of *Mycobacterium avium* subspecies *paratuberculosis* genetic components in retail cheese curds purchased in Wisconsin and Minnesota by PCR. *Molecular and Cellular Probes* 20, 197–202.

de Lisle, G.W., Yates, G.F. and Montgomery, R.H. (2003) The emergence of *Mycobacterium paratuberculosis* in farmed deer in New Zealand – a review of 619 cases. *New Zealand Veterinary Journal* 51, 58–62.

Dennis, M.M., Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Gould, D.H. and Salman, M.D. (2008) Association of severity of enteric granulomatous inflammation with disseminated *Mycobacterium avium* subspecies *paratuberculosis* infection and antemortem test results for paratuberculosis in dairy cows. *Veterinary Microbiology* 131, 154–163.

Djonne, B., Jensen, M.R., Grant, I.R. and Holstad, G. (2003) Detection by immunomagnetic PCR of *Mycobacterium avium* subsp. *paratuberculosis* in milk from dairy goats in Norway. *Veterinary Microbiology* 92, 135–143.

Donaghy, J.A., Totton, N.L. and Rowe, M.T. (2004) Persistence of *Mycobacterium paratuberculosis* during manufacture and ripening of Cheddar cheese. *Applied and Environmental Microbiology* 70, 4899–4905.

Donaghy, J.A., Linton, M., Patterson, M.F. and Rowe, M.T. (2007) Effect of high pressure and pasteurization on *Mycobacterium avium* ssp. *paratuberculosis* in milk. *Letters in Applied Microbiology* 45, 2154–2159.

Dundee, L., Grant, I.R., Ball, H.J. and Rowe, M.T. (2001) Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk. *Letters in Applied Microbiology* 33, 173–177.

Ellingson, J.L.E., Anderson, J.L., Koziczkowski, J.J., Radcliff, R.P., Sloan, S.J., Allen, S.E. and Sullivan, N.M. (2005) Detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. *Journal of Food Protection* 68, 966–972.

Fawcett, A.R., Goddard, P.J., McKelvey, W.A., Buxton, D., Reid, H.W., Greig, A. and MacDonald, A.J. (1995) Johne's disease in a herd of farmed red deer. *The Veterinary Record* 136, 165–169.

Gao, A., Mutharia, L., Chen, S., Rahn, K. and Odumeru, J. (2002) Effect of pasteurization on survival of *Mycobacterium paratuberculosis* in milk. *Journal of Dairy Science* 85, 3198–3205.

Gibson, H., Sinclair, L.A., Brizuela, C.M., Worton, H.L. and Protheroe, R.G. (2008) Effectiveness of selected premilking teat-cleaning regimes in reducing teat microbial load on commercial dairy farms. *Letters in Applied Microbiology* 46, 295–300.

Giese, S.B. and Ahrens, P. (2000) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. *Veterinary Microbiology* 77, 291–297.

Grant, I.R., O'Riordan, L.M., Ball, H.J. and Rowe, M.T. (2001) Incidence of *Mycobacterium paratuberculosis* in raw sheep and goats' milk in England, Wales and Northern Ireland. *Veterinary Microbiology* 79, 123–131.

Grant, I.R., Ball, H.J. and Rowe, M.T. (2002) Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. *Applied and Environmental Microbiology* 68, 2428–2435.

Haghkhah, M., Ansari-Lari, M., Novin-Baheran, A.M. and Bahramy, A. (2008) Herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* by bulk-tank milk PCR in Fars province (southern Iran) dairy herds. *Preventive Veterinary Medicine* 86, 8–13.

Health Protection Agency (2003) DWI0833. In: *Further Studies on the Incidence of* Mycobacterium avium *Complex (MAC) in Drinking Water Supplies (Including the Detection of* Helicobacter pylori *in Water and Biofilm Samples)*. Drinking Water Inspectorate, London.

Hovingh, E., Whitlock, R.H., Sweeney, R.W., Fyock, T., Wolfgang, D.R., Smith, J., Schukken, Y.H. and Van Kessel, J.S. (2006) Identification and implications of MAP supershedders. *Journal of Animal Science* 84 (Suppl. 1), 134.

Hruska, K., Bartos, M., Kralik, P. and Pavlik, I. (2005) *Mycobacterium avium* subsp. *paratuberculosis* in powdered infant milk: paratuberculosis in cattle – the public health problem to be solved. *Veterinarni Medicina* 50, 8327–8335.

Ikonomopoulos, J., Pavlik, I., Bartos, M., Svastova, P., Ayele, W.Y., Roubal, P., Lukas, J., Cook, N. and Gazouli, M. (2005) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in retail

cheeses from Greece and the Czech Republic. *Applied and Environmental Microbiology* 71, 8934–8936.

Jaravata, C.V., Smith, W.L., Rensen, G.J., Ruzante, J. and Cullor, J.S. (2007) Survey of ground beef for the detection of *Mycobacterium avium paratuberculosis*. *Foodborne Pathogens and Disease* 4, 103–106.

Jayarao, B.M., Pillai, S.R., Wolfgang, D.R., Griswold, D.R., Rossiter, C.A., Tewari, D., Burns, C.M. and Hutchinson, L.J. (2004) Evaluation of IS900-PCR assay for detection of *Mycobacterium avium* subspecies *paratuberculosis* infection in cattle using quarter milk and bulk tank milk samples. *Foodborne Pathogens and Disease* 1, 17–26.

Klijn, N., Herrewegh, A.A.P.M. and De Jong, P. (2001) Heat inactivation data for *Mycobacterium avium* subsp. *paratuberculosis*: implications for interpretation. *Journal of Applied Microbiology* 91, 697–704.

Kopecna, M., Parmova, I., Dvorska-Bartosova, L., Moravkova, M., Babak, V. and Pavlik, I. (2008) Distribution and transmission of *Mycobacterium avium* subspecies *paratuberculosis* in farmed red deer (*Cervus elaphus*) studied by faecal culture, serology and IS900 RFLP examinations. *Veterinarni Medicina* 53, 510–523.

Lillini, E., De Grossi, L., Bitonti, G. and Cersini, A. (2007) MAP in retail pasteurised cows' milk: first report in Italy. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 333–335.

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B. and Salman, M.D. (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. *Journal of Dairy Science* 89, 4163–4171.

López-Pedemonte, T., Sevilla, I., Garrido, J.M., Aduriz, G., Guamis, B., Juste, R.A. and Roig-Sagués, A.X. (2006) Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in cow's milk by means of high hydrostatic pressure at mild temperatures. *Applied and Environmental Microbiology* 72, 4446–4449.

Lund, B.M., Gould, G.W. and Rampling, A.M. (2002) Pasteurization of milk and the heat resistance of *Mycobacterium avium* subsp. *paratuberculosis*: a critical review of the data. *International Journal of Food Microbiology* 77, 135–145.

Maher, M., Rowe, M., Egan, J., Hill, C. and Murphy, P. (2004) *MAP* did not survive pasteurisation and was not found in meat. *Final Update on FIRM Project FS005 Detection and Survival of* Mycobacterium avium *subsp.* paratuberculosis (MAP), Available at www.relayresearch.ie (accessed 18 May 2009).

Meadus, W.J., Gill, C.O., Duff, P., Badoni, M. and Saucier, L. (2008) Prevalence on beef carcasses of *Mycobacterium avium* subsp. *paratuberculosis* DNA. *International Journal of Food Microbiology* 124, 291–294.

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T. and Hermon-Taylor, J. (1996) IS900 PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cows' milk in England and Wales. *Applied and Environmental Microbiology* 62, 3446–3452.

Muehlherr, J.E., Zweifel, C., Corti, S., Blanco, J.E. and Stephan, R. (2003) Microbiological quality of raw goat's and ewe's bulk-tank milk in Switzerland. *Journal of Dairy Science* 86, 3849–3856.

Nielsen, S.S., Bjerre, H. and Toft, N. (2008) Colostrum and milk as risk factors for infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. *Journal of Dairy Science* 91, 4610–4615.

O'Doherty, A., O'Grady, D., Smith, T. and Egan, J. (2002) *Mycobacterium avium* subsp. *paratuberculosis* in pasteurized and unpasteurized milk in the Republic of Ireland. *Irish Journal of Agricultural and Food Research* 4, 117–121.

O'Reilly, C.E., O'Connor, L., Anderson, W., Harvey, P., Grant, I.R., Donaghy, J., Rowe, M. and O'Mahony, P. (2004) Surveillance of bulk raw and commercially pasteurized cows' milk from approved Irish liquid-milk pasteurization plants to determine the incidence of *Mycobacterium paratuberculosis*. *Applied and Environmental Microbiology* 70, 5138–5144.

Paolicchi, F.A., Cirone, K., Morsella, C., Gioffré, A., Cataldi, A. and Romano, M. (2005) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from commercial pasteurized milk. In: Manning, E.J.B. and Nielsen, S.S. (eds) *Proceedings of the 8th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, p. 342.

Pavlik, I., Matlova, L., Bartl, J., Svastova, P., Dvorska, L. and Whitlock, R. (2000) Parallel faecal and organ *Mycobacterium avium* subsp. *paratuberculosis* culture of different productivity types of cattle. *Veterinary Microbiology* 77, 309–324.

Pearce, L.E., Shepherd, J.M., Wiles, P.G., Luo, D. and de Lisle, G.W. (2005) Quantifying the risk of *Mycobacterium avium* subsp. *paratuberculosis* surviving pasteurisation. In: Manning, E.J.B. and Nielsen, S.S. (eds) *Proceedings of*

the 8th International Colloquium on Paratuberculosis. International Association for Paratuberculosis, Madison, Wisconsin, p. 343.

Pickup, R.W., Rhodes, G., Arnott, S., Sidi-Boumedine, K., Bull, T.J., Weightman, A., Hurley, M. and Hermon-Taylor, J. (2005) *Mycobacterium avium* subsp. *paratuberculosis* in the catchment area and water of the River Taff in South Wales, United Kingdom, and its potential relationship to clustering of Crohn's disease cases in the city of Cardiff. *Applied and Environmental Microbiology* 71, 2130–2139.

Pickup, R.W., Rhodes, G., Bull, T.J., Arnott, S., Sidi-Boumedine, K., Hurley, M. and Hermon-Taylor, J. (2006) *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: diverse opportunities for environmental cycling and human exposure. *Applied and Environmental Microbiology* 72, 4067–4077.

Pillai, S.R. and Jayarao, B.M. (2002) Application of IS900PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* directly from raw milk. *Journal of Dairy Science* 85, 1052–1057.

Power, S.B., Haagsma, J. and Smyth, D.P. (1993) Paratuberculosis in farmed red deer (*Cervus elaphus*) in Ireland. *The Veterinary Record* 132, 213–216.

Raizman, E.A., Wells, S.J., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C. and Olsen, K.E. (2004) The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *Journal of Dairy Science* 87, 2959–2966.

Rowan, N.J., MacGregor, S.J., Anderson, J.G., Cameron, D. and Farish, O. (2001) Inactivation of *Mycobacterium paratuberculosis* by pulsed electric fields. *Applied and Environmental Microbiology* 67, 2833–2836.

Rowe, M.T. and Grant, I.R. (2006) *Mycobacterium avium* ssp. *paratuberculosis* and its potential survival tactics. *Letters in Applied Microbiology* 42, 305–311.

Rowe, M.T., Johnston, M., Hitchings, E., Johnston, J. and Donaghy, J. (2007) Lethality of the milk spray-drying process for *Mycobacterium avium* subsp. *paratuberculosis* using a model system. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, p. 348.

Sevilla, I., Aduriz, G., Garrido, J.M., Geijo, M.V. and Juste, R.A. (2002) A preliminary survey of the prevalence of paratuberculosis in dairy cattle in Spain by bulk milk PCR. In: Juste, R.A., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the 7th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 332–336.

Singh, S.V. and Vihan, V.S. (2004) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in goat milk. *Small Ruminant Research* 54, 3231–3235.

Slana, I., Kralik, P., Kralova, A. and Pavlik, I. (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *International Journal of Food Microbiology* 128, 250–257.

Spahr, U. and Schafroth, K. (2001) Fate of *Mycobacterium avium* subsp. *paratuberculosis* in Swiss hard and semihard cheese manufactured from raw milk. *Applied and Environmental Microbiology* 67, 4199–4205.

Stabel, J.R., Waldren, C.A. and Garry, F. (2001) Gamma-radiation effectively destroys *Mycobacterium paratuberculosis* in milk. *Journal of Dairy Science* 84 (Suppl.1), 27.

Stabel, J.R., Wells, S.J. and Wagner, B.A. (2002) Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds. *Journal of Dairy Science* 85, 525–531.

Stephan, R., Buhler, K. and Corti, S. (2002) Incidence of *Mycobacterium avium* subspecies *paratuberculosis* in bulk-tank milk samples from different regions in Switzerland. *The Veterinary Record* 150, 214–215.

Stephan, R., Schumacher, S., Tasara, T. and Grant, I.R. (2007) Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss raw milk cheeses collected at the retail level. *Journal of Dairy Science* 90, 3590–3595.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P. and Rings, M. (1995) Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *American Journal of Veterinary Research* 56, 1322–1324.

Sung, N. and Collins, M.T. (2000) Effect of three factors in cheese production (pH, salt, and heat) on *Mycobacterium avium* subsp. *paratuberculosis* viability. *Applied and Environmental Microbiology* 66, 1334–1339.

Sweeney, R.W., Whitlock, R.H. and Rosenberger, A.E. (1992) *Mycobacterium paratuberculosis* cultured from milk and supramammary lymphnodes of infected asymptomatic cows. *Journal of Clinical Microbiology* 30, 166–171.

Taylor, T.K., Wilks, C.R. and McQueen, D.S. (1981) Isolation of *Mycobacterium paratuberculosis* from the milk of a cow with Johne's disease. *The Veterinary Record* 109, 532–533.

Van Schaik, G., Stehman, S.M., Schukken, Y.H., Rossiter, C.R. and Shin, S.J. (2003) Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. *Journal of Veterinary Diagnostic Investigation* 15, 233–241.

Whan, L.B., Grant, I.R., Ball, H.J., Scott, R. and Rowe, M.T. (2001) Bactericidal effect of chlorine on *Mycobacterium paratuberculosis* in drinking water. *Letters in Applied Microbiology* 33, 227–231.

Whan, L., Ball, H.J., Grant, I.R. and Rowe, M.T. (2005) Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in untreated water in Northern Ireland. *Applied and Environmental Microbiology* 71, 7107–7112.

Whan, L., Grant, I.R. and Rowe, M.T. (2006) Interaction between *Mycobacterium avium* subsp. *paratuberculosis* and environmental protozoa. *BMC Microbiology* 6, 63.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, A.B. and Reddacliff, L.A. (2004) Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Applied and Environmental Microbiology* 70, 2989–3004.

Whittington, R.J., Marsh, I.B. and Reddacliff, L.A. (2005) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. *Applied and Environmental Microbiology* 71, 5304–5308.

Williams, A.G. and Withers, S.E. (2008) Microbiology of some artisanal 'farmhouse' cheeses manufactured in Scotland from unpasteurized milk. *Programme and Abstracts of the 21st International ICFMH Symposium (Food Micro 2008)*, Aberdeen, UK, p. 447.

5 Paratuberculosis and Crohn's Disease

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5.1 Introduction

A critical dilemma for paratuberculosis control is whether *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is strictly a veterinary pathogen or whether it is also a zoonotic agent, associated with Crohn's disease in humans. The hypothesis that *MAP* may present as human inflammatory bowel disease dates to nearly one century ago (Dalziel, 1913), and modern interest in the association of *MAP* with Crohn's disease was stimulated by a review of the subject by Chiodini (1989). However, aside from some minor fluctuations in opinion, this hypothesis has not garnered much support from the mainstream gastroenterology community. In this chapter, I will outline arguments that favour this hypothesis, both epidemiological and fundamental, along with arguments against. The goal will be to provide a current summation of the pros and cons regarding this potential link, in order to define specific research avenues that might help bring this question to resolution. For further in-depth discussion of some of the issues explored in this chapter, the reader is referred to a number of recent reviews on this topic (Greenstein, 2003; Behr and Schurr, 2006; Behr and Kapur, 2008; Lowe *et al.*, 2008).

5.2 Rationale for a Link between Paratuberculosis and Crohn's Disease

Crohn's disease is a chronic, systemic disease with prominent gastrointestinal pathology. The disease is episodic, both over time (relapsing and remitting) and space (anatomical distribution). Crohn's disease is distinguished from ulcerative colitis (UC), as the latter has

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disease limited to the colon, with contiguous pathology and pathology restricted to the mucosa. In contrast, lesions in Crohn's disease can extend from the mouth to the anus and the pathology is transmural. Because *MAP* has been experimentally shown to cause an invasive inflammatory bowel disease in a variety of hosts, there has been a long-standing interest in whether *MAP* is implicated in the pathogenesis of Crohn's disease. Historically, the compelling reason to contemplate this possibility has been the argument of analogy; as *MAP* causes inflammatory bowel disease in ruminants, so too may it cause chronic bowel disease in humans. Although this argument is incomplete, it remains a core observation that has persisted despite changes in the biological understanding of mycobacterial infections and pathophysiological models of Crohn's disease.

The analogy argument draws some support by comparison with other mycobacterial species. Not all organisms cause disease in different hosts. For instance, *Listeria monocytogenes* cannot invade the intestine in mice, because of mutations in the host receptor for internalin A (Lecuit *et al.*, 1999), and *Escherichia coli* O157 causes disease in humans but carriage is asymptomatic in the bovine host (Chase-Topping *et al.*, 2008). In contrast, species-specificity does not appear to be as strict in the case of mycobacterial infections. *Mycobacterium bovis* can cause disease in humans, although it typically does not spread efficiently between people (Evans *et al.*, 2007). Likewise, *M. avium* subsp. *avium*, the pathogenic clone responsible for disease in birds, can cause disease in mammalian hosts such as farmed deer (Mackintosh *et al.*, 2004). As *MAP* has been found to cause disease in a broad range of hosts, it remains possible, but unproven, that this organism also causes disease in humans.

The major issue with analogy is when contradictory data arise. While it may be persuasive to cite veterinary data when arguing towards a role for *MAP* in human disease, there is a logical challenge when human data do not conform to the veterinary paradigm. In other words, if *MAP* causes spillover infections and disease in humans, we should expect that conventional microbiological attributes of this organism would be observable, such as positive results for microscopy and culture from subjects with disease. Otherwise, one risks selective interpretation of data, invoking analogy when results favour an association but advancing exceptional circumstances to explain contrary observations. Moreover, by emphasizing findings that support the association (such as polymerase chain reaction (PCR) results) one risks overlooking the critical missing data (such as culture), which if adequately addressed may ultimately contribute more information to this debate. In summary, the argument of analogy remains compelling but serves mostly as the basis for further investigations, such as human studies looking for evidence of *MAP* infection.

5.3 Epidemiological Data that Implicate *MAP* in Crohn's Disease

Assays to test for *MAP* infection in human subjects include looking for the microbe or looking for a specific immune response. It is the author's contention that mycobacterial diseases in general are diagnosed on microbial grounds, while immunological assays are used to test for infection, rather than disease. In the case of *Mycobacterium tuberculosis*, which is a more virulent organism than *MAP* in model infections, most global estimates have about 2 billion persons infected with the organism contrasted with 'only' 20–30 million active cases. Thus, in a hypothetical point prevalence study, one could expect about 100 individuals with asymptomatic infection for each patient with clinical disease. Assuming that *MAP* can infect humans and cause disease but is less virulent than *M. tuberculosis*, the ratio of asymptomatic infection to disease in a prevalence study could be even higher. From this, it follows that case–control studies using immunological testing should find a large number of positive results in the control group without disease. Furthermore, genetic defects associated with Crohn's disease (discussed below) may further complicate the use of immunological testing. As these defects result in impaired immune responses against microbial stimuli, susceptible individuals who develop disease may have weaker immune responses

than controls, who mount effective immune responses. In this scenario, immunological assays being used to detect evidence of infection in cases and controls may paradoxically show greater evidence of infection in the control group.

In terms of microbiological assays, epidemiological studies have largely used three types of tests: culture, microscopy and PCR. Of these, the most convincing technique (isolation in pure culture) has been the least rewarding. The number of *MAP* isolates (as defined by macroscopic colonies seen on culture media) has been surprisingly low, suggesting to some that human disease due to *MAP* is very uncommon (Shanahan and O'Mahony, 2005). The potential reasons for a low number of positive cultures in a hypothesized infectious disease are many. One issue that has potentially received insufficient attention is the importance of the sample being studied. For reasons probably more related to access than biology, most studies looking for *MAP* in Crohn's disease use mucosal biopsies. However, the pathology of Crohn's disease extends well beneath the mucosa, with characteristic disease extending throughout the bowel wall and to extra-intestinal sites, including the mesenteric fat and fistulas (Pierce, 2009). Despite this, very few samples of deeper tissues, such as mesenteric lymph nodes or lymphatics, have been studied for evidence of *MAP*. Future studies, using validated microbiological methods, are encouraged to consider carefully the tissue being investigated and attempt to analyse the full thickness of the intestinal sample, along with accompanying mesenteric structures.

For microscopy, a number of different techniques have been applied, including tissue Ziehl–Neelsen, and *in situ* hybridization for either ribosomal RNA or IS*900*, a multicopy insertion sequence specific to *MAP* (see Collins, Chapter 25, this volume). As *MAP* organisms are small and coccoid in shape, it is critical that these methods be validated in tissue with defined infections, prior to studying human biopsies. Such validation should not only verify the capacity to detect organisms when they are there in small numbers (sensitivity) but also test whether uninfected tissue and biopsies with other organisms are reported as negative (specificity). Two groups have reported that *in situ* hybridization using IS*900* as a probe is prone to non-specific signals (St Amand *et al.*, 2005; Jeyanathan *et al.*, 2006). Using experimentally infected mouse tissue, Jeyanathan and colleagues found that the most technically specific probes (Ziehl–Neelsen and *in situ* hybridization for ribosomal RNA) were unable to resolve to the subspecies level; therefore they could only report on the presence of *M. avium*. Applying these methods to Crohn's disease and control samples, they reported an odds ratio of 8.6 for detecting *M. avium* in Crohn's disease (Jeyanathan *et al.*, 2007). These findings await independent replication.

In contrast to the paucity of reports on *MAP* infection by culture and microscopy, there is an abundant literature on the detection of *MAP* DNA by PCR. These reports fall under the rubric of 'in-house PCR', with its attendant issues of validation and reproducibility (Flores *et al.*, 2005). None the less, a relatively clear signal has emerged over the past few years, with two meta-analyses finding an overall positive association (Feller *et al.*, 2007; Abubakar *et al.*, 2008). While it is often stated that such studies can be divided into those that do and do not find *MAP* in Crohn's disease (Waddell *et al.*, 2008), an inspection of the Forest plot of findings instead suggests that the large majority of studies have detected an association, with a subset achieving statistical significance (Feller *et al.*, 2007). Whether due to publication bias or other reasons, there are strikingly few studies that have found either a negative association or an odds ratio near unity. If one accepts that there is some association between *MAP* DNA and Crohn's disease, there still remains a fundamental question about the nature of this association. One study found *MAP* DNA in about one-third of control tissues, compared with over 90% of Crohn's disease tissues (Bull *et al.*, 2003). Another study found very few positive results in control tissues, with *MAP* DNA detected in about half of Crohn's disease samples (Autschbach *et al.*, 2005). The former study suggests that *MAP* infection is common but enriched in Crohn's disease. The latter study instead suggests that *MAP* infection is uncommon and that its presence is strongly predictive of disease.

Future studies, using standardized PCR methods, potentially involving interlaboratory comparisons, will be needed to fully elucidate the nature of this association.

Because Crohn's disease is a chronic disease, epidemiological studies have employed a case–control design, where one interrogates samples for evidence of *MAP* once disease is already established. Alternative study designs that would theoretically be more illuminating would include testing biopsy tissue on a large cohort of healthy subjects prior to the diagnosis of Crohn's disease and/or experimentally feeding *MAP* and waiting for evidence of disease after a suitable duration of time. As these designs are neither feasible nor ethical, case–control studies will probably continue to dominate the epidemiological literature on this question. Because of this limitation, there is a need for corroborating biological data to determine the likelihood that an epidemiological association points to a causal role.

5.4 Fundamental Data that Provide Further Support for a Role of Mycobacteria in Crohn's Disease Aetiopathogenesis

In order to investigate whether mycobacteria contribute to the pathogenesis of Crohn's disease, one can begin by considering what is known from clinical and epidemiological study of Crohn's disease. While there are a number of biomarkers that correlate with disease, including the presence of anti-mannan antibodies (Pineton de Chambrun *et al.*, 2008), there are no simple assays that specifically indicate the presence of Crohn's disease. Therefore, a most compelling starting point is the genetic epidemiology of human disease, especially the genes for which polymorphisms are associated with Crohn's disease but not ulcerative colitis. At the time of writing, there is an ever-increasing list of genes and loci associated with the development of inflammatory bowel disease but only three that are specifically associated with Crohn's disease: *NOD2*, *ATG16L1*, *IRGM* (Cho, 2008).

Both genomic predictions and functional studies have confirmed that each of these genes encodes a protein serving in the innate immune response to intracellular bacterial infection (Shih *et al.*, 2008). NOD2, an intracellular pathogen recognition molecule, senses the muramyl dipeptide (MDP) moiety of bacterial peptidoglycan, implying that defects in NOD2 signalling would predispose to bacterial infection (Girardin *et al.*, 2003; Inohara *et al.*, 2003). ATG16L1 is a protein that is essential for formation of the autophagosome (Cadwell *et al.*, 2008; Saitoh *et al.*, 2008), an intracellular recycling system implicated in the handling of mycobacteria (Gutierrez *et al.*, 2004) and other intracellular pathogens (Rioux *et al.*, 2007). IRGM is a GTPase implicated, through mouse knockout studies, in resistance to infection by intracellular pathogens, including *M. avium* (Feng *et al.*, 2004) and *M. tuberculosis* (MacMicking *et al.*, 2003). An important direction for future research will be determining which bacteria can exploit mutations in these genes to initiate an infection and the ensuing inflammatory host response. As an example, it has now been shown by several groups that persons with permissive *NOD2* mutations have loss of MDP recognition, which is also seen in *Nod2*−/− mice (Ferwerda *et al.*, 2005). *Nod2*−/− mice have defective innate immune recognition of *M. tuberculosis in vitro* and *in vivo* (Gandotra *et al.*, 2007; Divangahi *et al.*, 2008). As NOD2 recognizes MDP and MDP is altered in mycobacteria via an *N*-acetyl muramic acid hydroxylase (Raymond *et al.*, 2005), one critical question is whether this peptidoglycan modification affects NOD2-mediated recognition. In work recently completed, two groups have shown that *N*-glycolyl MDP produced by mycobacteria and related organisms is more potent and more efficacious than *N*-acetyl MDP produced by other bacteria at inducing a variety of NOD2-dependent immune responses, *in vitro* and *in vivo* (Coulombe *et al.*, 2009; Pandey *et al.*, 2009). It remains to be seen whether such specificity applies to the other Crohn's susceptibility genes (*ATG16L1* and *IRGM*) and whether these three genes, which are conceptually linked through a common role in responding to intracellular pathogens, control different infections or, instead, represent distinct checkpoints during the host response to the same intracellular infection.

5.5 Arguments Against a Role for *MAP* in Crohn's Disease

A number of arguments have been presented against a mycobacterial aetiology for Crohn's disease. Although each has merit, none has conclusively refuted this potential link, and these points may be considered opportunities for research and reflection rather than excuses for heated debate. The three most current arguments are: (i) farmers and veterinarians should be at increased risk of a livestock-associated zoonosis; (ii) TNF-α inhibitors used to treat Crohn's disease are incompatible with a mycobacterial aetiology; and (iii) anti-mycobacterial treatment has limited efficacy in treatment of Crohn's disease.

Occupational risk of infectious diseases is well known for agents acquired through direct contact and aerosols. On the other hand, enteric pathogens do not always present the same association between occupational exposure and disease. Farmers are not recognized as a high-risk group for disease due to *E. coli* O157, despite the fact that the cattle intestine is accepted to be the reservoir for this organism. In a study of 8598 *E. coli* O157 cases reported to the Centers for Disease Control in the USA, direct contact with animals was noted in only 11 cases (Rangel *et al.*, 2005). In the case of disease due to *Campylobacter jejuni*, one study of 12,327 cases from Norway found that there was no ecological association between rates of human disease and grazing density (Sandberg *et al.*, 2006). Regarding veterinarians, the evaluation for an increased rate of disease presupposes that there are large enough denominators. In Quebec, Canada, the incidence of Crohn's disease is estimated at about 20 per 100,000 (Lowe *et al.*, 2009), the equivalent of one case per 5000 people per year. As long as the number of veterinarians is lower than 5000, it would be difficult to observe a doubling, or even trebling, of disease in this group. Moreover, the fact that someone is a farmer or a veterinarian at the time that they have Crohn's disease may or may not have any bearing on their risk status at the time of exposure. For *MAP*, time from infection to disease in the natural host can be years. Therefore, epidemiological assessment of surrogate exposures for *MAP* infection would be complicated by ascertainment effects. Indeed, a recent study from the UK concluded that there was no association between Crohn's disease and behaviours likely to be linked with *MAP*, yet they did find an increased risk with consumption of more meat (Abubakar *et al.*, 2007). This example illustrates the challenge in conducting a case–control study that aims to capture epidemiological data as a proxy for *MAP* exposure.

A second argument against an aetiological role for *MAP* is the use of immunosuppressive drugs, including TNF-α inhibitors, in the management of Crohn's disease (Sartor, 2005). These treatments have been introduced based on the prevailing view that Crohn's disease represents an autoimmune disease characterized by excessive host-directed inflammation. While the use of such agents may appear counter-intuitive for a hypothesized infectious disease, it must be recognized that anti-TNF agents were originally developed to reduce severe inflammation during septic shock (Fisher *et al.*, 1993). While there are credible epidemiological data indicating a risk of tuberculosis in persons on these agents (Gardam *et al.*, 2003), it does not appear that this risk is the same in the case of non-tuberculous mycobacteria, where disease is uncommon despite far greater exposure (Winthrop *et al.*, 2008). Moreover, anecdotal evidence exists for a therapeutic benefit with these agents when treating complicated mycobacterial infections: in one case, recurrent leprosy (Faber *et al.*, 2006) and, in another case, tuberculous meningitis (Blackmore *et al.*, 2008). Together, these observations suggest the need for information on the effect of anti-TNF agents during experimental *MAP* infection to convincingly address this point.

The third argument raised is that anti-mycobacterial agents were ineffective in Crohn's disease patients in a large placebo-controlled trial (Peyrin-Biroulet *et al.*, 2007). This argument is based on two premises: (i) the trial was a failure; and (ii) the drugs used were known to be efficacious against chronic *MAP* infection. Although the authors reported a short-term benefit, the original report claimed that this benefit was lost with time (Selby *et al.*, 2007). However, an intention-to-treat

analysis performed on the raw data indicated that the absolute benefit of 15–20% persisted over the 2-year trial (Behr and Hanley, 2008). Therefore, while the effect of antibiotics was less than anticipated, it cannot be stated that the treatment was entirely ineffective. Regarding the degree of efficacy, it is noteworthy that a recent study of pulmonary *M. avium–Mycobacterium intracellulare* infections reported that only 30% of patients treated with multidrug regimens were alive and cured after 5 years (Jenkins *et al.*, 2008). The low treatment success rate may indicate a predominant role of host defects in the pathogenesis of disease and hence a reduced likelihood of cure using antibacterial strategies alone. This may be an important lesson in the treatment of Crohn's disease, itself linked with defective innate immune recognition of microbial products.

5.6 Unproven Hypotheses Regarding *MAP* in Crohn's Disease

In review articles and commentaries, a number of hypotheses have been proposed to explain some of the discrepant observations outlined above. For instance, the high rate of PCR-positive studies in the absence of microscopic detection was compatible with organisms being present in a cell-wall-deficient (Ziehl–Neelsen-negative) form (Greenstein, 2003). These hypotheses represent interesting ideas, which may lead to novel insights into this organism and its ability to engage the susceptible host. However, it is important to distinguish hypotheses that serve as the basis for experimental evaluation from the presentation of these ideas as facts.

Beyond the notion that *MAP* may adopt a cell-wall-deficient or spheroplast form in humans, it has also been suggested that there are humanized strains of *MAP* that are potentially antigenically distinct from classical bovine isolates. If true, then the risk to humans may no longer be from livestock but rather from other humans. Proof of such an assertion would come from demonstrating that human isolates of *MAP* are not only genetically remarkable, in whatever manner defined, but that they are also distinct from organisms circulating in the natural hosts. To date, genetic studies have shown that human isolates have a molecular profile shared with cattle strains (Bull *et al.*, 2000; Ghadiali *et al.*, 2004). The sequencing of a human isolate of *MAP* by the University of Minnesota is expected to provide some definitive data towards resolving this issue.

A third notion is that human *MAP* infection can commonly include a bacteraemic phase. If true, there would be important advantages for epidemiological study, as one could envision sampling a large number of subjects through simple phlebotomy. However, in clinical infectious disease, it is axiomatic that bloodstream infections typically indicate failed control at the site of primary infection. If *MAP* causes bacteraemia that is evident in blood culture vials, one would expect the organism to be readily detectable on culture of diseased intestinal tissue. Importantly, reports on a bacteraemic form of *MAP* have used nested PCR of blood culture vials (Naser *et al.*, 2004) rather than classical microbiological evaluation based on the visualization of bacterial colonies on solid media. Moreover, variations on this method have returned discrepant results; in one study, positive blood PCR was inversely associated with Crohn's disease (Juste *et al.*, 2008) and, in a more recent report, neither live *MAP* nor *MAP* DNA was detected in the blood of 130 patients with Crohn's disease (Parrish *et al.*, 2009). Further studies, including interlaboratory comparisons, will be needed to resolve the question of bacteraemic infection with *MAP*.

5.7 Outstanding Questions and Directions Forward

As the debate about *MAP* and Crohn's disease approaches its centenary, we are equipped with unprecedented knowledge about the bacterium (as detailed in other chapters in this book) but none the less confronted with a confusing debate. For each new study published that supports a role for *MAP* in Crohn's disease, it seems another study appears with negative results, often posing a different question using different methods. The cumulative effect can be disorienting.

For epidemiological studies, the key challenge is to harness advances from veterinary study of *MAP* to define the most appropriate assay. If detection of livestock disease is most sensitive with PCR (and this is not universally accepted) and most specific by culture, then these assays should hold promise in human studies. If cell-wall-deficient forms can be demonstrated as a prominent feature of veterinary disease, then it can be argued that these forms may be important in human disease. However, without validating a method using defined tissue samples, whether from naturally infected hosts or experimentally infected model organisms, it is unlikely that one can truly quantify the operating parameters of assays that aim to detect *MAP* in humans.

For fundamental research, key clues have been offered from the genetics of Crohn's disease. The challenge ahead is not only to test for the role of these genes in control of *MAP* infection but also to determine the specificity of these roles. It has already been shown that *Nod2*–/– mice have impaired resistance to *M. tuberculosis* infection, so it is unlikely that this represents a specific *MAP* resistance gene, as opposed to a mycobacterial resistance gene. Indeed, one study in the USA has reported an association between *NOD2* polymorphisms and human tuberculosis (Austin *et al.*, 2008). None the less, if NOD2 has a critical role in resistance to mycobacterial infection that is not observed during infection with enteric Gram-negative organisms, this may present a valuable clue as to the role of both NOD2 and mycobacteria in the pathogenesis of Crohn's disease.

A key goal of this entire book has been to compile, in one document, a comprehensive resource of what *MAP* is and is not. A proven role of *MAP* in human disease will clearly impact on most other aspects of paratuberculosis control. Even a suspected role of *MAP* may lead to interventions by regulators and governments. Therefore, it follows that researchers interested in examining this particular question should carefully examine other chapters in this text for the development of hypotheses and assays that may bring clarity to this debate. Conversely, developments in Crohn's disease research may help shape the research agenda on Johne's disease. Already one study has shown that mutations in the bovine homologue of *NOD2* are associated with susceptibility to *MAP* (Pinedo *et al.*, 2009). If *MAP* indeed is implicated in the pathogenesis of Crohn's disease, there may be an increasing need for bidirectional research, considering livestock as both a reservoir of infection and a source for biological data on the pathogenesis, epidemiology and diagnosis of *MAP* disease.

References

Abubakar, I., Myhill, D.J., Hart, A.R., Lake, I.R., Harvey, I., Rhodes, J.M., Robinson, R., Lobo, A.J., Probert, C.S. and Hunter, P.R. (2007) A case–control study of drinking water and dairy products in Crohn's disease – further investigation of the possible role of *Mycobacterium avium paratuberculosis*. *American Journal of Epidemiology* 165, 776–783.

Abubakar, I., Myhill, D., Aliyu, S.H. and Hunter, P.R. (2008) Detection of *Mycobacterium avium* subspecies *paratuberculosis* from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. *Inflammatory Bowel Disease* 14, 401–410.

Austin, C.M., Ma, X. and Graviss, E.A. (2008) Common nonsynonymous polymorphisms in the *NOD2* gene are associated with resistance or susceptibility to tuberculosis disease in African Americans. *Journal of Infectious Diseases* 197, 1713–1716.

Autschbach, F., Eisold, S., Hinz, U., Zinser, S., Linnebacher, M., Giese, T., Loffler, T., Buchler, M.W. and Schmidt, J. (2005) High prevalence of *Mycobacterium avium* subspecies *paratuberculosis* IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut* 54, 944–949.

Behr, M.A. and Hanley, J. (2008) Antimycobacterial therapy for Crohn's disease: a reanalysis. *The Lancet Infectious Diseases* 8, 344.

Behr, M.A. and Kapur, V. (2008) The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. *Current Opinion in Gastroenterology* 24, 17–21.

Behr, M.A. and Schurr, E. (2006) Mycobacteria in Crohn's disease: a persistent hypothesis. *Inflammatory Bowel Disease* 12, 1000–1004.

Blackmore, T.K., Manning, L., Taylor, W.J. and Wallis, R.S. (2008) Therapeutic use of infliximab in tuberculosis to control severe paradoxical

reaction of the brain and lymph nodes. *Clinical Infectious Diseases* 47, e83–e85.

Bull, T.J., Hermon-Taylor, J., Pavlik, I., El Zaatari, F. and Tizard, M. (2000) Characterization of IS*900* loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. *Microbiology* 146, 2185–2197.

Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R. and Hermon-Taylor, J. (2003) Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *Journal of Clinical Microbiology* 41, 2915–2923.

Cadwell, K., Liu, J.Y., Brown, S.L., Miyoshi, H., Loh, J., Lennerz, J.K., Kishi, C., Kc, W., Carrero, J.A., Hunt, S., Stone, C.D., Brunt, E.M., Xavier, R.J., Sleckman, B.P., Li, E., Mizushima, N., Stappenbeck, T.S. and Virgin, H.W. (2008) A key role for autophagy and the autophagy gene *Atg16l1* in mouse and human intestinal Paneth cells. *Nature* 456, 259–263.

Chase-Topping, M., Gally, D., Low, C., Matthews, L. and Woolhouse, M. (2008) Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nature Reviews Microbiology* 6, 904–912.

Chiodini, R.J. (1989) Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clinical Microbiology Reviews* 2, 90–117.

Cho, J.H. (2008) The genetics and immunopathogenesis of inflammatory bowel disease. *Nature Reviews Immunology* 8, 458–466.

Coulombe, F., Divangahi, M., Veyrier, F., Gleason, J.L., Yang, Y., Kelliher, M.A., Pandey, A.K., Sassetti, C.M., Reed, M.B. and Behr, M.A. (2009) Increased NOD2-mediated recognition of *N*-glycolyl muramyl dipeptide. *Journal of Experimental Medicine* 206, 1709–1716.

Dalziel, T.K. (1913) Chronic interstitial enteritis. *British Medical Journal* ii, 1068–1070.

Divangahi, M., Mostowy, S., Coulombe, F., Kozak, R., Guillot, L., Veyrier, F., Kobayashi, K.S., Flavell, R.A., Gros, P. and Behr, M.A. (2008) NOD2-deficient mice have impaired resistance to *Mycobacterium tuberculosis* infection through defective innate and adaptive immunity. *Journal of Immunology* 181, 7157–7165.

Evans, J.T., Smith, E.G., Banerjee, A., Smith, R.M., Dale, J., Innes, J.A., Hunt, D., Tweddell, A., Wood, A., Anderson, C., Hewinson, R.G., Smith, N.H., Hawkey, P.M. and Sonnenberg, P. (2007) Cluster of human tuberculosis caused by *Mycobacterium bovis*: evidence for person-to-person transmission in the UK. *The Lancet* 369, 1270–1276.

Faber, W.R., Jensema, A.J. and Goldschmidt, W.F. (2006) Treatment of recurrent erythema nodosum leprosum with infliximab. *The New England Journal of Medicine* 355, 739.

Feller, M., Huwiler, K., Stephan, R., Altpeter, E., Shang, A., Furrer, H., Pfyffer, G.E., Jemmi, T., Baumgartner, A. and Egger, M. (2007) *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. *The Lancet Infectious Diseases* 7, 607–613.

Feng, C.G., Collazo-Custodio, C.M., Eckhaus, M., Hieny, S., Belkaid, Y., Elkins, K., Jankovic, D., Taylor, G.A. and Sher, A. (2004) Mice deficient in LRG-47 display increased susceptibility to mycobacterial infection associated with the induction of lymphopenia. *Journal of Immunology* 172, 1163–1168.

Ferwerda, G., Girardin, S.E., Kullberg, B.J., Le Bourhis, L., de Jong, D.J., Langenberg, D.M., van Crevel, R., Adema, G.J., Ottenhoff, T.H., van der Meer, J.W. and Netea, M.G. (2005) NOD2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. *PLoS Pathogens* 1, 279–285.

Fisher, C.J., Jr., Opal, S.M., Dhainaut, J.F., Stephens, S., Zimmerman, J.L., Nightingale, P., Harris, S.J., Schein, R.M., Panacek, E.A., Vincent, J.L. Foulke, G.E., Warren, E.L. Garrard, C., Park, G., Bodmer, M.W., Cohen, J., Van Der Linden, C., Cross, A.S., Sadoff, J.C. and The Cb0006 Sepsis Syndrome Study Group (1993) Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. *Critical Care Medicine* 21, 318–327.

Flores, L.L., Pai, M., Colford, J.M., Jr. and Riley, L.W. (2005) In-house nucleic acid amplification tests for the detection of *Mycobacterium tuberculosis* in sputum specimens: meta-analysis and meta-regression. *BMC Microbiology* 5, 55.

Gandotra, S., Jang, S., Murray, P.J., Salgame, P. and Ehrt, S. (2007) NOD2-deficient mice control infection with *Mycobacterium tuberculosis*. *Infection and Immunity* 75, 5127–5134.

Gardam, M.A., Keystone, E.C., Menzies, R., Manners, S., Skamene, E., Long, R. and Vinh, D.C. (2003) Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. *The Lancet Infectious Diseases* 3, 148–155.

Ghadiali, A.H., Strother, M., Naser, S.A., Manning, E.J. and Sreevatsan, S. (2004) *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. *Journal of Clinical Microbiology* 42, 5345–5348.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J. and Sansonetti, P.J. (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *Journal of Biological Chemistry* 278, 8869–8872.

Greenstein, R.J. (2003) Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis and Johne's disease. *The Lancet Infectious Diseases* 3, 507–514.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I. and Deretic, V. (2004) Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* 119, 753–766.

Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S.J., Moran, A.P., Fernandez-Luna, J.L. and Nunez, G. (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *Journal of Biological Chemistry* 278, 5509–5512.

Jenkins, P.A., Campbell, I.A., Banks, J., Gelder, C.M., Prescott, R.J. and Smith, A.P. (2008) Clarithromycin vs ciprofloxacin as adjuncts to rifampicin and ethambutol in treating opportunist mycobacterial lung diseases and an assessment of *Mycobacterium vaccae* immunotherapy. *Thorax* 63, 627–634.

Jeyanathan, M., Alexander, D.C., Turenne, C.Y., Girard, C. and Behr, M.A. (2006) Evaluation of *in situ* methods used to detect *Mycobacterium avium* subsp. *paratuberculosis* in samples from patients with Crohn's disease. *Journal of Clinical Microbiology* 44, 2942–2950.

Jeyanathan, M., Boutros-Tadros, O., Radhi, J., Semret, M., Bitton, A. and Behr, M.A. (2007) Visualization of *Mycobacterium avium* in Crohn's tissue by oil-immersion microscopy. *Microbes and Infection* 9, 1567–1573.

Juste, R.A., Elguezabal, N., Garrido, J.M., Pavon, A., Geijo, M.V., Sevilla, I., Cabriada, J.L., Tejada, A., Garcia-Campos, F., Casado, R., Ochotorena, I., Izeta, A. and Greenstein, R.J. (2008) On the prevalence of *M. avium* subspecies *paratuberculosis* DNA in the blood of healthy individuals and patients with inflammatory bowel disease. *PLoS ONE* 3, e2537.

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B. and Cossart, P. (1999) A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *EMBO Journal* 18, 3956–3963.

Lowe, A.M., Yansouni, C.P. and Behr, M.A. (2008) Causality and gastrointestinal infections: Koch, Hill and Crohn's. *The Lancet Infectious Diseases* 8, 720–726.

Lowe, A.M., Roy, P.O., Poulin, M., Michel, P., Bitton, A., St-Onge, L. and Brassard, P. (2009) Epidemiology of Crohn's disease in Quebec, Canada. *Inflammatory Bowel Disease* 15, 429–435.

Mackintosh, C.G., de Lisle, G.W., Collins, D.M. and Griffin, J.F. (2004), Mycobacterial diseases of deer. *New Zealand Veterinary Journal* 52, 163–174.

MacMicking, J.D., Taylor, G.A. and McKinney, J.D. (2003) Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science* 302, 654–659.

Naser, S.A., Ghobrial, G., Romero, C. and Valentine, J.F. (2004) Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *The Lancet* 364, 1039–1044.

Pandey, A.K., Yang, Y., Jiang, Z., Fortune, S.M., Coulombe, F., Behr, M.A., Fitzgerald, K.A., Sassetti, C.M. and Kelliher, M.A. (2009) NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to *Mycobacterium tuberculosis*. *PLoS Pathogens,* Jul 5(7), e1000500.

Parrish, N.M., Radcliff, R.P., Brey, B.J., Anderson, J.L., Clark, D.L., Jr., Koziczkowski, J.J., Ko, C.G., Goldberg, N.D., Brinker, D.A., Carlson, R.A., Dick, J.D. and Ellingson, J.L. (2009) Absence of *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's patients. *Inflammatory Bowel Disease* 15, 558–565.

Peyrin-Biroulet, L., Neut, C. and Colombel, J.F. (2007) Antimycobacterial therapy in Crohn's disease: game over? *Gastroenterology* 132, 2594–2598.

Pierce, E.S. (2009) Where are all the *Mycobacterium avium* subspecies *paratuberculosis* in patients with Crohn's disease? *PLoS Pathogens* 5, e1000234.

Pinedo, P.J., Buergelt, C.D., Donovan, G.A., Melendez, P., Morel, L., Wu, R., Langaee, T.Y. and Rae, D.O. (2009) Association between CARD15/NOD2 gene polymorphisms and paratuberculosis infection in cattle. *Veterinary Microbiology* 134, 346–352.

Pineton de Chambrun, C.G., Colombel, J.F., Poulain, D. and Darfeuille-Michaud, A. (2008) Pathogenic agents in inflammatory bowel diseases. *Current Opinion in Gastroenterology* 24, 440–447.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M. and Swerdlow, D.L. (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases* 11, 603–609.

Raymond, J.B., Mahapatra, S., Crick, D.C. and Pavelka, M.S., Jr. (2005) Identification of the

namH gene, encoding the hydroxylase responsible for the N-glycolylation of the mycobacterial peptidoglycan. *Journal of Biological Chemistry* 280, 326–333.

Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W., Shugart, Y.Y., Griffiths, A.M., Targan, S.R., Ippoliti, A.F., Bernard, E.J., Mei, L., Nicolae, D.L., Regueiro, M., Schumm, L.P., Steinhart, A.H., Rotter, J.I., Duerr, R.H., Cho, J.H., Daly, M.J. and Brant, S.R. (2007) Genome-wide association study identifies new susceptibility loci for Crohn's disease and implicates autophagy in disease pathogenesis. *Nature Genetics* 39, 596–604.

Saitoh, T., Fujita, N., Jang, M.H., Uematsu, S., Yang, B.G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., Tanaka, K., Kawai, T., Tsujimura, T., Takeuchi, O., Yoshimori, T. and Akira, S. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 456, 264–268.

Sandberg, M., Nygard, K., Meldal, H., Valle, P.S., Kruse, H. and Skjerve, E. (2006) Incidence trend and risk factors for campylobacter infections in humans in Norway. *BMC Public Health* 6, 179.

Sartor, R.B. (2005) Does *Mycobacterium avium* subspecies *paratuberculosis* cause Crohn's disease? *Gut* 54, 896–898.

Selby, W., Pavli, P., Crotty, B., Florin, T., Radford-Smith, G., Gibson, P., Mitchell, B., Connell, W., Read, R., Merrett, M., Ee, H. and Hetzel, D. (2007) Two-year combination antibiotic therapy with clarithromycin, rifabutin and clofazimine for Crohn's disease. *Gastroenterology* 132, 2313–2319.

Shanahan, F. and O'Mahony, J. (2005) The mycobacteria story in Crohn's disease. *American Journal of Gastroenterology* 100, 1537–1538.

Shih, D.Q., Targan, S.R. and McGovern, D. (2008) Recent advances in IBD pathogenesis: genetics and immunobiology. *Current Gastroenterology Reports* 10, 568–575.

St Amand, A.L., Frank, D.N., De Groote, M.A. and Pace, N.R. (2005) Use of specific rRNA oligonucleotide probes for microscopic detection of *Mycobacterium avium* complex organisms in tissue. *Journal of Clinical Microbiology* 43, 1505–1514.

Waddell, L.A., Rajic, A., Sargeant, J., Harris, J., Amezcua, R., Downey, L., Read, S. and McEwen, S.A. (2008) The zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: a systematic review. *Canadian Journal of Public Health* 99, 145–155.

Winthrop, K.L., Yamashita, S., Beekmann, S.E. and Polgreen, P.M. (2008) Mycobacterial and other serious infections in patients receiving antitumor necrosis factor and other newly approved biologic therapies: case finding through the Emerging Infections Network. *Clinical Infectious Diseases* 46, 1738–1740.

6 Genetics of Host Susceptibility to Paratuberculosis

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6.1 Introduction

An understanding of genetic variation that contributes to host susceptibility to infection by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is important both to inform animal improvement programmes aimed at reducing susceptibility to infection and for gaining a better understanding of the mechanisms of disease. Creating a more resistant population through breeding programmes should not be considered a complete solution for paratuberculosis but rather one of several tools that could potentially be used to prevent or reduce incidence of infection. Knowledge concerning the genetic basis for susceptibility or resistance to infection may likewise be useful in developing additional tools to this end. This chapter reviews the current state of knowledge concerning evidence for genetic variation in host susceptibility to infection by *MAP* in ruminants.

6.2 Evidence for Differences in Resistance by Breed

Differences between breeds in incidence of infection and/or disease by *MAP* can potentially provide support for a genetic contribution to *MAP* susceptibility. Cetinkaya *et al.* (1997) surveyed cattle producers in the UK and found a higher occurrence of the disease in herds using Channel Island breeds (Jersey, Guernsey) compared with Friesian or other breeds. However, confounding of breed with factors such as location or management must temper any conclusion that these differences are indicative of genetic effects. Similarly, Roussel *et al.* (2005) tested 4579 pure-bred cattle of 14 different breeds in Texas and found highly significant differences between genetic groups in positive ELISA test results. *Bos indicus* pure-breds and crosses (composite breeds) had odds ratios 17-fold and 3.5-fold greater

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than *Bos taurus* breeds for positive test results. However, there was concern that seropositive test results may have been due in some cases to a related organism, as several *B. indicus* herds showed no other evidence (clinical or microbiological) of disease. Alternatively, the authors suggest that these results may suggest a successful host response of the *B. indicus* cattle to exposure. A subsequent study (Elzo *et al.*, 2006) tested 238 cows from an Angus × Brahman diallele cross and also found significantly greater occurrence of seropositive animals with increasing proportion of *B. indicus* breeding. Unlike the study reported by Roussel *et al.* (2005), breed and herd were not confounded in this case, as all animals were from a single herd. Osterstock *et al.* (2008) used microsatellite genotyping to classify cattle in groups of genetically related individuals and then assessed evidence for differences between genetic groups in incidence of infection as shown either by ELISA or by faecal culture of *MAP*. Significantly increased odds of infection were observed for genetic groups comprised of Texas Longhorn and *B. indicus* cattle, although, as noted for other studies, there is confounding of breed with location in this study.

Hickey *et al.* (2003) used necropsy records from a research population of Romney (n = 2348) and Merino (n = 1297) sheep to examine breed effects on ovine Johne's disease incidence. Incidence was higher in Merino (4.78%) than Romney (3.49%) sheep in this research population. Management of the population was such that sheep of the two breeds were co-mingled on most occasions, except for the times of lambing to docking and single-sire mating.

Taken together, these studies provide modest evidence of breed or subspecies effects. In contrast, other studies, such as the epidemiological investigation of ELISA phenotype in culled cattle by Pence *et al.* (2003), are informative for differences in infection between classes of cattle (beef versus dairy) but cannot lead to any conclusion concerning breed effects, given the confounding of breed and management. In summary, there is some evidence for genetic variability in animal susceptibility to *MAP* infection and reason to consider genetic improvement for this trait.

6.3 Estimates of Heritability for *MAP* Infection

A more definitive conclusion can be drawn from studies which examine effects attributable to sire when sires are used across common herds or use similar data in estimation of heritability for *MAP* infection. Nielsen *et al.* (2002) determined infection status in 7410 Danish dairy cattle on the basis of a milk ELISA. Data from the full data set or a subset comprising daughter–dam pairs was analysed for contribution of sire or dam to phenotypic variance. Sire effects accounted for 1.9 and 6.3% of the phenotypic variance, respectively, in the two analyses, providing evidence of a genetic component to susceptibility. Several studies estimated heritability for *MAP* infection using substantial data sets that have included 3000–10,000 cattle. For these, a critical issue has been the different definitions employed for a pertinent phenotype to study as a function of host genetics.

In many studies, infection has been defined by ELISA. Koets *et al.* (2000) used post-mortem analysis of bacterial culture from intestinal tissue and lymph nodes, and histopathology and direct histological examination to determine infection and disease status in 3020 Dutch dairy cattle. Animals testing positive to any of the three measures were considered to be positive for purposes of heritability estimation. Mortensen *et al.* (2004) used ELISA testing of milk samples to determine infection phenotype in a data set comprising records from 11,535 Danish dairy cattle. Gonda *et al.* (2006) used both an ELISA test of blood serum and a faecal culture test for *MAP*, with records obtained from 4603 US Holstein cows. They considered both separate (ELISA versus faecal culture) and combined (positive for either test) assessments in estimating heritability of infection status. Hinger *et al.* (2008) used infection status based on ELISA tests of serum samples from 4524 German Holstein cattle.

Heritability estimates in dairy cattle ranged from <0.01 to 0.18, with most estimates ranging between 0.09 and 0.12 (Table 6.1) (Koets *et al.*, 2000; Mortensen *et al.*, 2004; Gonda *et al.*, 2006; Hinger *et al.*, 2008). Heritability estimates in sheep, although limited to one study with two sheep breeds, are consistent

Table 6.1. Heritability estimates for infection by *MAP* in ruminants.

Study	Species	Phenotype	Model	h^2 Estimate
Koets *et al.* (2000)	Cattle (n = 3020)	ELISA	Threshold	
	All			0.06±0.037
	Vaccinated only			0.09±0.050
	Non-vaccinated			<0.01
	Dam, daughter			0.08±0.095
Mortensen *et al.* (2004)	Cattle (n = 11,535)	ELISA	Linear multivariate with milk yield	0.102
			Univariate; animal model	0.101
			Univariate; sire model	0.091
Gonda *et al.* (2006)	Cattle (n = 4603)	Faecal culture	Threshold	0.153±0.115
		Faecal culture and ELISA	Bivariate threshold	0.125±0.096
		ELISA	Linear	0.159±0.090
		Faecal culture and ELISA	Bivariate linear	0.183±0.082
		ELISA	Ordered threshold	0.091±0.053
		Combined[a]	Threshold	0.102±0.066
Hinger *et al.* (2008)	Cattle (n = 4524)	ELISA	Threshold, Q+[b]	0.062±0.025
			Threshold, Q–	0.052±0.032
			Linear, Q+	0.120±0.024
			Linear, Q–	0.136±0.023
			Linear, ODV_T[c]	0.102±0.043
Hickey *et al.* (2003)	Romney sheep (n = 2348)	Necropsy	Binomial; animal model	0.07±0.14
	Merino sheep (n = 1297)			
	Pure-bred			0.11±0.05
	Cross-bred			0.16±0.23
	All			0.18±0.11

[a]Animals defined as positive if positive to either faecal culture or ELISA.
[b]Q+ denotes that animals with an infection status of 'questionable' were considered as infected for the analysis. Q– denotes that animals with an infection status of 'questionable' were considered as uninfected for the analysis. Results are shown only for animal model analyses.
[c]Phenotype was optical density value from ELISA; a subset of 2084 animals was included in this analysis. Results are shown only for animal model analysis.

with those from cattle. Hickey *et al*. (2003) used necropsy records from research populations of Romney (n = 2348) and Merino (n = 1297) sheep to estimate heritability of ovine Johne's disease incidence. Heritability was higher in Merino (0.18±0.11) than Romney (0.07±0.14) sheep. In summary, heritability estimates varied within and between studies, depending on analytical model and definition of phenotype, but in most cases were significantly different from zero and on the order of approximately 0.10. Clearly there is evidence for genetic variation in host susceptibility to *MAP* infection.

6.4 Candidate Gene Studies

Susceptibility to infection in mice by several mycobacterial species, as well as *Salmonella typhimurium* and *Leishmania donovani*, has been associated with allelic variants of the solute carrier family 11 member 1 gene (*SLC11A1*),

formerly referred to as the natural resistance-associated macrophage protein 1 gene (*NRAMP1*) (Malo *et al.*, 1994). Recently, the effect of mouse *SLC11A1* alleles was examined specifically with regard to *MAP* infection (Roupie *et al.*, 2008); as with other pathogens, profound differences in susceptibility were observed, corresponding to the *SLC11A1* genotype. *SLC11A1* functions as part of the innate immune response, helping block bacterial replication during the early response to infection. While there has been interest in the contribution of *SCL11A1* to innate immune response in cattle, and genetic variants have been identified (Coussens *et al.*, 2004; Martinez *et al.*, 2008), no associations between *SLC11A1* and susceptibility to *MAP* infection have yet been reported.

Tentative associations of the *SLC11A1* gene and MHC region with incidence of ovine Johne's disease (JD) have been reported (Reddacliff *et al.*, 2005), based on genotyping of microsatellite markers closely associated with each. Two flocks of Merino sheep were phenotyped on the basis of clinical assessment, faecal culture and necropsy, with a total of 106 and 92 ewes used. The number of ewes positive for JD varied from 15 to 75 and 2 to 42, respectively, depending on criteria employed. Significance levels reported were nominal *P* values of <0.05 in most cases, uncorrected for multiple hypothesis testing implicit in selecting specific microsatellite alleles for comparison. None the less, the most significantly associated alleles were consistent across the two flocks, increasing the credibility of the results. More recently, positional candidate gene results from work with Crohn's disease in humans have suggested additional genes that have been considered candidates for genetic variation in *MAP* infection and JD in ruminants.

The nucleotide-binding oligomerization domain containing 2 gene (*NOD2*), previously referred to as the caspase recruitment domain 15 protein gene (*CARD15*), is a well-characterized gene that contributes to predisposition to Crohn's disease in humans (Hugot, 2006; Radford-Smith and Pandeya, 2006). Based on similarities between Crohn's disease and JD, *NOD2* has been the subject of study in cattle as a candidate gene. While numerous polymorphisms have been identified in coding or non-coding areas of the bovine *NOD2* gene, association of these with susceptibility to infection has been inconsistent. Taylor *et al.* (2006) sequenced coding regions, 5′ and 3′ flanking regions and partial intronic regions of bovine *NOD2* in a diverse panel of cattle in an effort to identify polymorphisms. A total of 36 polymorphisms was identified in their screening of 42 animals from ten different breeds, which included both *B. taurus* and *B. indicus* subspecies. Association of these polymorphisms with infection could not be adequately tested owing to a paucity of infected animals (n = 11). Subsequently, Pinedo *et al.* (2009a) tested association of three of the *NOD2* polymorphisms identified by Taylor *et al.* (2006) in a case–control study using cattle of dairy (Holstein, Jersey) and beef (Brahman × Angus) types. The study sample size (126 cases, 305 controls) was adequate to detect large single-nucleotide polymorphism (SNP) effects, and an association significant at a nominal $P < 0.01$, after controlling for breed, was found for a non-synonymous SNP in the leucine-rich repeat domain of the gene. Evidence for this association came principally from the Brahman × Angus subset of the data. The same data were subsequently reanalysed considering effects of predicted SNP haplotypes. A haplotype based on two non-synonymous *NOD2* SNPs was found to be significantly associated with infection status (nominal $P < 0.0001$) in an analysis that did not account for breed. The effect attributable to this risk haplotype was due to greater incidence of infection in animals heterozygous for the haplotype (i.e. overdominance). This contrasts with data on *NOD2* alleles and Crohn's susceptibility, where the effects manifest in a partial recessive fashion, as seen by the relative risk increasing exponentially between risk allele heterozygotes to homozygotes or compound heterozygotes (Economou *et al.*, 2004).

Analysis of the *NOD2* locus in US Holstein cattle (B.W. Kirkpatrick, 2009, unpublished results) revealed additional polymorphisms, but none of nine previously or newly identified SNPs genotyped was significantly associated with infection status in a case–control study using 169 case (positive to either ELISA or faecal culture tests or both) and 188 control cows. In addition, only weak evidence of SNP association with infection status was observed

for bovine chromosome 18 (location of *NOD2*) in whole-genome association analyses that genotyped 239 and 285 ELISA-positive cows from two ~5000 cow resource populations (B.W. Kirkpatrick, 2009, unpublished results). Pinedo *et al.* (2009a) point out that the *NOD2* allele that shows association is more frequent in the Brahman × Angus cattle than in Holstein cattle, potentially accounting for the lack of association observed in our work with Holsteins.

Toll-like receptors (*TLR*s) function in the body's recognition of foreign proteins as part of the innate immune system and have been considered as candidate genes for variation in host susceptibility to infection by *MAP*. Mucha *et al.* (2009) examined *TLR1*, *TLR2* and *TLR4* for evidence of polymorphism using infected and uninfected animals from three Slovakian cattle herds. Missense mutations were observed for all three genes, and in one case (*TLR1*) a polymorphism was associated with increased incidence of infection. Results of this study should be interpreted with caution, however, as it is not clear that breed was accounted for in the analysis. Seven different breed groups were represented in the samples drawn from the three herds, and there were large differences in allele frequency and infection status between breeds, a scenario in which spurious association could arise. Pinedo *et al.* (2009b) tested association of three previously identified polymorphisms (White *et al.*, 2003) in *TLR4* with *MAP* infection in cattle. As described above, the infection phenotypes were based on ELISA tests of cows from Holstein and Jersey dairy herds and an Angus × Brahman beef herd. All three *TLR4* polymorphisms tested were missense mutations of the gene. No association of *TLR4* was detected either by examining the SNPs individually or by considering them jointly as haplotypes.

Interferon-γ (*IFN-γ*) has been considered as a candidate gene for susceptibility to *MAP* infection (Pinedo *et al.*, 2009a) owing to its role in innate host response to numerous mycobacterial infections. Pinedo *et al.* (2009a) examined association of *IFN-γ* with susceptibility to *MAP* infection in the same cattle described above, using two SNPs previously described by Schmidt *et al.* (2002). Both SNPs were in the *IFN-γ* coding sequence of exons 1 and 4; the exon 1 polymorphism was non-synonymous, causing an amino acid substitution in the signal peptide region of the gene product, while the exon 4 polymorphism was synonymous. The exon 1 polymorphism had an association individually with infection status at only a nominal $P < 0.05$ level, and *IFN-γ* haplotype analysis did not reveal significant associations. Hinger *et al.* (2007) used a microsatellite (*BMS1617*; Stone *et al.*, 1995) in proximity to *IFN-γ* to test for potential association of this gene with infection. They based infection status on results from ELISA testing in German Holstein cattle and tested association in a case–control design using 594 case and 585 control cows. No association was observed between *BMS1617* and infection status, although this negative result does not rule out a contribution of *IFN-γ* to *MAP* infection in Holstein cattle. Examination of the genomic locations (bovine genome assembly Bta4.0) of *IFN-γ* and *BMS1617* reveals that the two are separated by a distance in excess of 2.5 Mb. Linkage disequilibrium at this distance in cattle should dissipate considerably (Sargolzaei *et al.*, 2008; Kim and Kirkpatrick, 2009), compromising the association test reported by Hinger *et al.* (2007) in regard to inference to effects of *IFN-γ*. In contrast, the remaining seven microsatellites chosen by Hinger *et al.* (2007) to serve as proxies for candidate genes (*IL4, IL10, IL12A, IL12B, IL18, SLC11A1, TNF-α*) were in much closer proximity to the candidate genes, having been subcloned from bacterial artificial chromosomes containing the candidate gene (Buitkamp *et al.*, 1996; Feng *et al.*, 1996; Sonstegard *et al.*, 2000). However, as with *BMS1617*, Hinger *et al.* (2007) found no association between any of these microsatellites and infection status. Candidate gene analyses in the absence of prior mapping of specific genomic regions carry a greater risk of failing to find association compared with positional candidate gene analyses. At the time these studies were conducted, there was little information available from genome-wide scans for *MAP* host susceptibility in cattle.

6.5 Genome-wide Association Analysis

The first genome-wide association study for *MAP* infection in cattle was reported by

Gonda *et al.* (2007). This study used three of the largest half-sib families from a larger Holstein resource population comprising 4586 cows sired by 12 different bulls. Infection status was determined by a combination of ELISA and faecal culture testing; animals positive to either test were deemed positive for infection. The authors conducted the genome-wide scan by first identifying genomic regions of potential interest on the basis of microsatellite genotyping of pooled positive and matching negative samples and then testing the difference between pools in allele frequency. Genomic regions of interest were then examined more closely by genotyping individuals for additional microsatellites in these regions and performing interval mapping analyses. One chromosomal region on bovine chromosome 20 was found to be significant at a chromosome-wise $P < 0.05$. This study lacked power for several reasons, including loss of information in estimating allele frequencies from pooled samples, use of only part of the resource population, analysis of only the paternal genetic contribution (within-family linkage analysis) rather than combined effects of linkage and linkage disequilibrium, and limited marker density. These deficiencies have been remedied in subsequent unpublished work that employed high-throughput SNP genotyping, use of more animals and a more complete genetic analysis. The additional phenotypic and genotypic data in these subsequent analyses provide stronger support for the chromosome 20 QTL. At the time of writing this review, no results from whole-genome association studies of *MAP* infection or JD in cattle have been published, although several studies are ongoing.

Crohn's disease in humans shares similarity with JD in many respects. As a consequence, efforts to map genes and identify allelic variants associated with predisposition to Crohn's disease are of interest for potential inference to genetics of predisposition to JD in ruminants. Genetics of predisposition to Crohn's disease has now been extensively studied through linkage analyses and positional candidate gene analyses (Russell *et al.*, 2004; Newman and Siminovitch, 2005) and, more recently, whole-genome association studies (Lettre and Rioux, 2008; Zhang *et al.*, 2008). Large whole-genome association studies (WGAS) of Crohn's disease provide a recapitulation and validation of numerous preceding linkage studies and have identified additional loci or chromosomal regions associated with this disease. A current listing of results from WGAS of Crohn's disease can be found at www.genome.gov/26525384 (Hindorff *et al.*, 2009). Over 30 susceptibility loci have now been identified (Libioulle *et al.*, 2007; Parkes *et al.*, 2007; Raelson *et al.*, 2007; Rioux *et al.*, 2007; Wellcome Trust Case Control Consortium, 2007; Barrett *et al.*, 2008), and these are listed in Table 6.2, alongside the corresponding genomic location in cattle. In the future, as results become available from cattle WGAS of *MAP* host susceptibility, it will be interesting to see to what extent this corresponds with results for Crohn's disease in humans. As many of the human genes are associated with both ulcerative colitis and Crohn's disease, it will also be interesting to note which ones prove useful in producing candidate genes for study of *MAP* resistance. To date, results from the author's laboratory (B.W. Kirkpatrick, 2009, unpublished results) suggest limited commonality with regard to genomic regions accounting for genetic susceptibility to JD in cattle versus Crohn's disease in humans. A number of factors could account for this, including lesser statistical power in our study relative to the human work, differences in disease mechanisms and definition of phenotype, and the chance occurrence of different functional polymorphisms in the different species.

6.6 Concluding Thoughts

Emerging data from different sources concur that there is clearly some degree of host genetic susceptibility to *MAP* infection. Newly available tools and opportunities, such as complete genome sequences and candidate genes from studies of other hosts, provide a tremendous opportunity to expand this field of investigation in the coming years. While it is premature to conclude that one can exploit genetic variation to control paratuberculosis, insights gained from genetic study of *MAP*

Table 6.2. Validated susceptibility loci identified from human Crohn's disease whole-genome association studies and corresponding bovine genomic locations.

Study[a]	Gene	Human genomic region	Bovine genomic location
1	Intergenic	21q21.1	BTA1, 21.2 Mb
1	*ICOSLG*	21q22.3	BTA1, 147.3 Mb
4	Intergenic	21q22.2	BTA1, 154.9 Mb
1	Intergenic	1q32.1	BTA3, 0.5 Mb
1	*ITLN1*	1q23.2	BTA3, 9.6 Mb
1	*PTPN22*	1p13.2	BTA3, 32.0 Mb
1, 2, 3, 5, 6	*IL23R*	1p31.3	BTA3, 83.8 Mb
1, 3, 5	*ATG16L1*	2q37.1	BTA3, 120.3 Mb
1	Intergenic	7p12.2	BTA4, 5.8 Mb
3	Unknown	7q36.1	BTA4, 116.0 Mb
1	*LRRK, MUC19*	12q12	BTA5, 43.9 Mb
1, 3	Intergenic	5q31.1	BTA7, 20.7 Mb
1, 3, 4	*IRGM*	5q33.1	BTA7, 61.8 Mb
1, 4	*IL12B*	5q33.3	BTA7, 70.7 Mb
1	*JAK2*	9p24.1	BTA8, 41.6 Mb
1	*TNFSF15*	9q32	BTA8, 109.3 Mb
1	Unknown	6q21	BTA9, 46.0 Mb
1	*CCR6*	6q27	BTA9, 105.8 Mb
1	Unknown	13q14.11	BTA12, 13.0 Mb
1	Intergenic	10p11.21	BTA13, 17.7 Mb
1	Unknown	10p15.1	BTA13, 45.3 Mb
1	Intergenic	8q24.13	BTA14, 14.2 Mb
1	C11orf30	11q13.5	BTA15, 55.5 Mb
4	Intergenic	1q31.2	BTA16, 12.4 Mb
1, 4	Intergenic	1q24.3	BTA16, 37.1 Mb
1, 2, 3, 5, 6	*NOD2*	16q12.1	BTA18, 18.1 Mb
1	*ORMDL3*	17q12	BTA19, 41.5 Mb
1	*STAT3*	17q21.2	BTA19, 43.8 Mb
1, 6	*PTGER4*	5p13.1	BTA20, 35.9 Mb
3, 4	Intergenic	5p13.1	BTA20, 36.2 Mb
1, 3, 4	*MST1*	3p21.31	BTA22, 51.4 Mb
3	Unknown	6p21	BTA23, 26.3 Mb
1	*CDKAL1*	6p22.3	BTA23, 37.5 Mb
1, 3, 4	*PTPN2*	18p11.21	BTA24, 44.7 Mb
1, 3, 4	*NKX2-3*	10q24.2	BTA26, 18.8 Mb
1, 3, 5	*ZNF365*	10q21.2	BTA28, 17.4 Mb

[a]Studies cited: 1, Barrett *et al*., 2008; 2, Raelson *et al*., 2007; 3, Wellcome Trust Case Control Consortium, 2007; 4, Parkes *et al*., 2007; 5, Rioux *et al*., 2007; 6, Libioulle *et al*., 2007.

resistance may help at the practical level by guiding diagnostic assays and at the fundamental level by informing on the pathogenesis of disease. Together with studies of the genetic basis of virulence of the responsible organism (see Paustian and Kapur, Chapter 8, this volume), a bi-genomic perspective on paratuberculosis promises to offer a new approach to understanding this disease and to help develop better tools for its control.

References

Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H., Duerr, R.H., Rioux, J.D., Brant, S.R., Silverberg,

M.S., Taylor, K.D., Barmada, M.M., Bitton, A., Dassopoulos, T., Datta, L.W., Green, T., Griffiths, A.M., Kistner, E.O., Murtha, M.T., Regueiro, M.D., Rotter, J.I., Schumm, L.P., Steinhart, A.H., Targan, S.R., Xavier, R.J., NIDDK IBD Genetics Consortium, Libioulle, C., Sandor, C., Lathrop, M., Belaiche, J., Dewit, O., Gut, I., Heath, S., Laukens, D., Mni, M., Rutgeerts, P., Van Gossum, A., Zelenika, D., Franchimont, D., Hugot, J.P., de Vos, M., Vermeire, S., Louis, E., Belgian–French IBD Consortium; Wellcome Trust Case Control Consortium, Cardon, L.R., Anderson, C.A., Drummond, H., Nimmo, E., Ahmad, T., Prescott, N.J., Onnie, C.M., Fisher, S.A., Marchini, J., Ghori, J., Bumpstead, S., Gwilliam, R., Tremelling, M., Deloukas, P., Mansfield, J., Jewell, D., Satsangi, J., Mathew, C.G., Parkes, M., Georges, M. and Daly, M.J. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nature Genetics* 40, 955–962.

Buitkamp, J., Obexer-Ruff, G., Kessler, M. and Epplen, J.T. (1996) A microsatellite (BOBT24) located between the bovine IL4 and IL13 loci is polymorphic in cattle and goat. *Animal Genetics* 27, 212–213.

Cetinkaya, B., Edrogan, H.M. and Morgan, K.L. (1997) Relationships between the presence of Johne's disease and farm and management factors in dairy cattle in England. *Preventive Veterinary Medicine* 32, 253–266.

Coussens, P.M., Coussens, M.J., Tooker, B.Ç. and Nobis, W. (2004) Structure of the bovine natural resistance associated macrophage protein (NRAMP1) gene and identification of a novel polymorphism. *DNA Sequence* 15, 15–25.

Economou, M., Trikalinos, T.A., Loizou, K.T., Tsianos, E.V. and Ioannidis, J.P.A. (2004) Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a meta-analysis. *American Journal of Gastroenterology* 99, 2393–2404.

Elzo, M.A., Rae, D.O., Lanhart, S.E., Wasdin, J.G., Dixon, W.P. and Jones, J.L. (2006) Factors associated with ELISA scores for paratuberculosis in an Angus–Brahman multibreed herd of beef cattle. *Journal of Animal Science* 84, 41–48.

Feng, J., Li, Y., Hashad, M., Schurr, E., Gros, P., Adams, L.G. and Templeton, J.W. (1996) Bovine natural resistance associated macro-phage protein 1 (*Nramp1*) gene. *Genome Research* 6, 956–964.

Gonda, M.G., Chang, Y.M., Shook, G.E., Collins, M.T. and Kirkpatrick, B.W. (2006) Genetic variation of *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins. *Journal of Dairy Science* 89, 1804–1812.

Gonda, M.G., Kirkpatrick, B.W., Shook, G.E. and Collins, M.T. (2007) Identification of a QTL on BTA20 affecting susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins. *Animal Genetics* 38, 389–396.

Hickey, S.M., Morris, C.A., Dobbie, J.L. and Lake, D.E. (2003) Heritability of Johne's disease and survival data from Romney and Merino sheep. *Proceedings of the New Zealand Society of Animal Production* 63, 179–182.

Hindorff, L.A., Junkins, H.A., Mehta, J.P. and Manolio, T.A. (2009) A catalog of published genome-wide association studies. Available at www.genome.gov/26525384 (accessed 6 February 2009).

Hinger, M., Brandt, H., Horner, S. and Erhardt, G. (2007) Association analysis of microsatellites and *Mycobacterium avium* subspecies *paratuberculosis* antibody response in German Holsteins. *Journal of Dairy Science* 90, 1957–1961.

Hinger, M., Brandt, H. and Erhardt, G. (2008) Heritability estimates for antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in German Holstein cattle. *Journal of Dairy Science* 91, 3237–3244.

Hugot, J.P. (2006) CARD15/NOD2 mutations in Crohn's disease. *Annals of the New York Academy of Science* 1072, 9–18.

Kim, E.-S. and Kirkpatrick, B.W. (2009) Linkage disequilibrium in the North American Holstein population. *Animal Genetics* 40, 279–288.

Koets, A.P., Adugna, G., Janss, L.L.G., van Weering, H.J., Kalis, C.H.J., Wentink, G.H., Rutten, V.P.M.G. and Schukken, Y.H. (2000) Genetic variation of susceptibility to *Mycobacterium avium* subsp. *paratuberculosis* infection in dairy cattle. *Journal of Dairy Science* 83, 2702–2708.

Lettre, G. and Rioux, J.D. (2008) Autoimmune diseases: insights from genome-wide association studies. *Human Molecular Genetics* 17, R116–R121.

Libioulle, C., Louis, E., Hansoul, S., Sandor, C., Farnir, F., Franchimont, D., Vermeire, S., Dewit, O., de Vos, M., Dixon, A., Demarche, B., Gut, I., Heath, S., Foglio, M., Liang, L., Laukens, D., Mni, M., Zelenika, D., Van Gossum, A., Rutgeerts, P., Belaiche, J., Lathrop, M. and Georges, M. (2007) Novel Crohn's disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genetics* 3, e58.

Malo, D., Vogan, K., Vidal, S., Hu, J., Cellier, M., Schurr, E., Fuks, A., Bumstead, N., Morgan, K. and Grost, P. (1994) Haplotype mapping and sequence analysis of the mouse *Nramp* gene predicts susceptibility to infection with intracellular parasites. *Genomics* 23, 51–61.

Martinez, R., Dunner, S., Barrera, G. and Cañon, J. (2008) Novel variants within the coding regions

of the *Slc11A1* gene identified in *Bos taurus* and *Bos indicus* breeds. *Journal of Animal Breeding and Genetics* 125, 57–62.

Mortensen, H., Nielsen, S.S. and Berg, P. (2004) Genetic variation and heritability of the antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in Danish Holstein cows. *Journal of Dairy Science* 87, 2108–2113.

Mucha, R., Bhide, M.R., Chakurkar, E.B., Novak, M. and Mikula, I., Sr (2009) Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle. *Veterinary Immunology and Immunopathology* 128, 381–388.

Newman, B. and Siminovitch, K.A. (2005) Recent advances in the genetics of inflammatory bowel disease. *Current Opinion in Gastroenterology* 21, 401–407.

Nielsen, S.S., Grohn, Y.T., Quaas, R.L. and Agger, J.F. (2002) Paratuberculosis in dairy cattle: variation of the antibody response in offspring attributable to the dam. *Journal of Dairy Science* 85, 406–412.

Osterstock, J.B., Fosgate, G.T., Derr, J.N., Cohen, N.D. and Roussel, A.J. (2008) Assessing familial aggregation of paratuberculosis in beef cattle of unknown pedigree. *Preventive Veterinary Medicine* 84, 121–134.

Parkes, M., Barrett, J.C., Prescott, N.J., Tremelling, M., Anderson, C.A., Fisher, S.A., Roberts, R.G., Nimmo, E.R., Cummings, F.R., Soars, D., Drummond, H., Lees, C.W., Khawaja, S.A., Bagnall, R., Burke, D.A., Todhunter, C.E., Ahmad, T., Onnie, C.M., McArdle, W., Strachan, D., Bethel, G., Bryan, C., Lewis, C.M., Deloukas, P., Forbes, A., Sanderson, J., Jewell, D.P., Satsangi, J., Mansfield, J.C., Wellcome Trust Case Control Consortium, Cardon, L. and Mathew, C.G. (2007) Sequence variants in the autophagy gene *IRGM* and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nature Genetics* 39, 830–832.

Pence, M., Baldwin, C. and Black, C.C., 3rd (2003) The seroprevalence of Johne's disease in Georgia beef and dairy cull cattle. *Journal of Veterinary Diagnostic Investigation* 15, 475–477.

Pinedo, P.J., Buergelt, C.D., Donovan, G.A., Melendez, P., Morel, L., Wu, R., Langaee, T.Y., and Rae, D.O. (2009a) Association between CARD15/NOD2 gene polymorphisms and paratuberculosis infection in cattle. *Veterinary Microbiology* 134, 346–352.

Pinedo, P.J., Wang, C., Li, Y., Rae, D.O. and Wu, R. (2009b) Risk haplotype analysis for bovine paratuberculosis. *Mammalian Genome* 20, 124–129.

Radford-Smith, G. and Pandeya, N. (2006) Associations between NOD2/CARD15 genotype and phenotype in Crohn's disease – are we there yet? *World Journal of Gastroenterology* 12, 7097–7103.

Raelson, J.V., Little, R.D., Ruether, A., Fournier, H., Paquin, B., Van Eerdewegh, P., Bradley, W.E., Croteau, P., Nguyen-Huu, Q., Segal, J., Debrus, S., Allard, R., Rosenstiel, P., Franke, A., Jacobs, G., Nikolaus, S., Vidal, J.M., Szego, P., Laplante, N., Clark, H.F., Paulussen, R.J., Hooper, J.W., Keith, T.P., Belouchi, A. and Schreiber, S. (2007) Genome-wide association study for Crohn's disease in the Quebec Founder Population identifies multiple validated disease loci. *Proceedings of the National Academy of Sciences of the USA* 104, 14747–14752.

Reddacliff, L.A., Beh, K., McGregor, H. and Whittington, R.J. (2005) A preliminary study of possible genetic influences on the susceptibility of sheep to Johne's disease. *Australian Veterinary Journal* 83, 435–441.

Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W., Shugart, Y.Y., Griffiths, A.M., Targan, S.R., Ippoliti, A.F., Bernard, E.J., Mei, L., Nicolae, D.L., Regueiro, M., Schumm, L.P., Steinhart, A.H., Rotter, J.I., Duerr, R.H., Cho, J.H., Daly, M.J. and Brant, S.R. (2007) Genome-wide association study identifies new susceptibility loci for Crohn's disease and implicates autophagy in disease pathogenesis. *Nature Genetics* 39, 596–604.

Roupie, V., Rosseels, V., Piersoel, V., Zinniel, D.K., Barletta, R.G. and Huygen, K. (2008) Genetic resistance of mice to *Mycobacterium* paratuberculosis is influenced by *Slc11a1* at the early but not at the late stage of infection. *Infection and Immunity* 76, 2099–2105.

Roussel, A.J., Libal, M.C., Whitlock, R.L., Hairgrove, T.B., Barling, K.S. and Thompson, J.A. (2005) Prevalence of and risk factors for paratuberculosis in purebred beef cattle. *Journal of the American Veterinary Medical Association* 226, 773–778.

Russell, R.K., Nimmo, E.R. and Satsangi, J. (2004) Molecular genetics of Crohn's disease. *Current Opinion in Genetics and Development* 14, 264–270.

Sargolzaei, M., Schenkel, F.S., Jansen, G.B. and Schaeffer, L.R. (2008) Extent of linkage disequilibrium in Holstein cattle in North America. *Journal of Dairy Science* 91, 2106–2117.

Schmidt, P., Kühn, C., Maillard, J.C., Pitra, C., Tiemann, U., Weikard, R. and Schwerin, M. (2002) A comprehensive survey for polymorphisms in the bovine IFN-gamma gene reveals a highly

polymorphic intronic DNA sequence allowing improved genotyping of Bovinae. *Journal of Interferon and Cytokine Research* 22, 923–934.

Sonstegard, T.S., Garrett, W.M., Bennett, G.L., Kappes, S.M., Zarlenga, D.S. and Gasbarre, L.C. (2000) Mapping of seven bovine cytokine genes involved in T-lymphocyte growth, differentiation and immune response. *Animal Genetics* 31, 406–408.

Stone, R.T., Pulido, J.C., Duyk, G.M., Kappes, S.M., Keele, J.W. and Beattie, C.W. (1995) A small-insert bovine genomic library highly enriched for microsatellite repeat sequences. *Mammalian Genome* 6, 714–724.

Taylor, K.H., Taylor, J.F., White, S.N. and Womack, J.E. (2006) Identification of genetic variation and putative regulatory regions in bovine *CARD15*. *Mammalian Genome* 17, 892–901.

Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447, 661–683.

White, S.N., Taylor, K.H., Abbey, C.A., Gill, C.A. and Womack, J.E. (2003) Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. *Proceedings of the National Academy of Sciences of the USA* 100, 10364–10369.

Zhang, H., Massey, D., Tremelling, M. and Parkes, M. (2008) Genetics of inflammatory bowel disease: clues to pathogenesis. *British Medical Bulletin* 87, 17–30.

7 *Mycobacterium avium* Complex

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7.1 Introduction

The *Mycobacterium avium* complex (MAC) comprises several species of slow-growing mycobacteria that are prevalent in environmental, veterinary and clinical settings. The MAC includes professional pathogens of birds and livestock, and opportunistic pathogens of humans, as well as organisms commonly found in soil and water. Historically, classification of MAC organisms was based on phenotypic features, including growth characteristics, source of isolation and virulence in experimental animals. The advent of molecular methods has challenged traditional designations and transformed our view of the MAC. Although the agent of Johne's disease was once considered a separate species, current taxonomy classifies it as *M. avium* subsp. *paratuberculosis*, a pathogenic clone of the MAC.

7.2 History of the *Mycobacterium avium* Complex

7.2.1 Discovery of *Mycobacterium avium*

Avian tuberculosis is a chronic wasting disease of wild and domesticated fowl that is difficult

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to detect in its early phase. Advanced disease is characterized by weight loss, fatigue, ataxia, reduced egg production and ultimately death (Feldman, 1938; Thorel *et al.*, 1997). When Koch first defined the aetiology of tuberculosis, it was assumed that a single type of tubercle bacillus was responsible for all forms of the disease. However, by the early 1890s, there was evidence that the avian tubercle bacillus, originally called '*Bacillus tuberculosis gallinarum*', but generally known as *M. avium*, was distinct from the human (*Mycobacterium tuberculosis*) and bovine (*Mycobacterium bovis*) types (Anonymous, 1891; Maffucci, 1892). Whereas the mammalian isolates produced colonies with a 'dry' morphology that would not grow above 41 °C and were virulent in guinea pigs but not birds, avian isolates typically had a 'moist' morphology, grew at temperatures >42 °C and were virulent in birds and rabbits but not guinea pigs.

By using these criteria to classify isolates from tuberculous animals, it was discovered that infections due to *M. avium* were common in swine. It had long been recognized that avian tuberculosis was contagious among birds and now it appeared that this disease could be spread to hogs. Investigations revealed that afflicted swine had usually been in contact with sick birds and, in several cases, had actually consumed offal from infected fowl (Feldman, 1939). *M. avium* was also isolated from diseased cattle, sheep, deer, marsupials and non-human primates. Although considered relatively resistant, mice, rats, squirrels, dogs, goats and horses could be infected experimentally (Feldman, 1938; Thorel *et al.*, 1997).

7.2.2 *Mycobacterium avium* and human disease

Despite the broad host range of *M. avium*, humans seemed immune to infection. Between 1901 and 1911, the British Royal Commission on Tuberculosis conducted an extensive study to address the possibility of disease transmission between animals and humans. They concluded that *M. bovis* could be transmitted to humans via infected beef and milk. In contrast, the risk posed by *M. avium*-infected eggs and fowl appeared negligible (Miller, 1911). Even so, there continued to be sporadic reports of human disease caused by *M. avium*. Most publications were methodologically flawed and unconvincing, but when Feldman conducted a critical review, he identified 13 probable cases, dating back to 1905 (Feldman, 1938). In another attempt to resolve this issue, Branch (1931) collected strains described in previous studies and subjected them to a uniform set of tests. After assessing both morphological and pathological characteristics, he determined that several isolates were genuine examples of *M. avium* and thus represented authentic cases of human infection. Of the remaining strains, some were typical of *M. tuberculosis*, whereas others did not conform to known types. Branch suggested that these atypical isolates represented novel acid-fast pathogens.

7.2.3 Discovery of *Mycobacterium intracellulare*

Through the 1940s and 1950s, numerous 'atypical' or 'anonymous' acid-fast pathogens were identified, including several responsible for serious human disease. One notable study described a lethal case of disseminated disease in a young girl (Cuttino and McCabe, 1949). Acid-fast bacteria were found in multiple organs, and a mycobacterial infection was suspected. Tuberculosis, leprosy, avian tuberculosis, rat leprosy and, owing to extensive intestinal involvement, Johne's disease were all considered, but none perfectly matched the pathological features of the case. Morphologically the causative agent seemed distinct from known mycobacteria. Also, it failed to produce disease in a veritable zoo of experimental animals (i.e. rats, mice, guinea pigs, rabbits, chickens, frogs, goldfish). Ultimately this novel bacterium was named *Nocardia intracellularis*. Another atypical acid-fast pathogen, dubbed the 'Battey bacillus' because of its prevalence at the Battey State Hospital (Rome, Georgia, USA), was associated with >300 of cases of chronic pulmonary disease (Corpe, 1964). Features of Battey-type pulmonary illness (e.g. cough, weight loss

and lung pathology) closely resembled those of classic tuberculosis. However, the Battey bacillus was morphologically distinct from *M. tuberculosis* and harmless to guinea pigs. Epidemiological studies of the Battey bacillus indicated that it was not transmissible between people. More likely it was acquired from soil (Corpe, 1964). Classification of atypical mycobacteria was a key goal of the Veterans Administration–National Tuberculosis Association Cooperative Study of Mycobacteria (Runyon, 1958). Hundreds of isolates were collected, analysed and eventually divided into four major groups (Runyon, 1958, 1965). Through such work, it was determined that *Nocardia intracellularis* and the Battey bacillus were the same organism. They were renamed *Mycobacterium intracellulare* and assigned to Group III (non-photochromogens) (Runyon, 1965; Wayne, 1966).

7.3 Origins of the *Mycobacterium avium* Complex

In general, disease due to *M. avium* is indistinguishable from that caused by *M. intracellulare* (Iseman *et al.*, 1985). In the clinical setting, both organisms are associated with lymphadenitis in children and chronic pulmonary symptoms in adults. Commonly, the afflicted patients have immunodeficiencies or pre-existing pulmonary conditions. Because *M. avium* and *M. intracellulare* are innately resistant to many antibiotics, treatment is difficult. Antimicrobial therapy requires multiple drugs and may take years to succeed. The same is true in the veterinary setting. Although *M. avium* predominates in birds, both mycobacteria affect swine (Matlova *et al.*, 2005). Before the advent of DNA-based typing methods, it was difficult to differentiate the two species. Even laborious procedures, such as cell wall lipid analysis, serotyping and classical animal infection experiments, could not reliably identify whether an isolate was *M. intracellulare* or *M. avium*. In an attempt to resolve the taxonomy of Group III organisms, the International Working Group on Mycobacterial Taxonomy (IWGMT) employed a panel of 89 isolates, including 47 *M. avium* and *M. intracellulare* strains. For each culture, 292 phenotypic 'characters' were tested, of which 118 proved useful for numerical classification (Meissner *et al.*, 1974). Several Group III species were readily distinguished by this method, but *M. avium* and *M. intracellulare* could not be reliably resolved from one another. Despite suggestions to reclassify *M. intracellulare* as an official subspecies of *M. avium*, the two remained distinct (Wayne, 1966; Meissner *et al.*, 1974; Runyon, 1974). Even so, the concept of a *M. avium–intracellulare* complex did emerge (Meissner *et al.*, 1974). Over time, the precision of the original term has waned. MAC now refers to a group of mycobacteria that exhibit overlapping phenotypic features and cause similar disease syndromes.

7.4 Environmental Reservoir of the *Mycobacterium avium* Complex

MAC disease is not contagious. In a summary of his experiences with Battey-type pulmonary disease, Corpe (1964) comments:

> We have never found a source case. In spite of the fact that well over 95 per cent of the patients we see have been or are married, we have never seen either a husband or a wife also clinically ill with the disease. We have never seen two cases in the same family. This is an entirely different epidemiologic picture than is observed in *Mycobacterium tuberculosis* infections.

The epidemiology of MAC is different because the source of infection is different (Falkinham, 1996). MAC is typically acquired from soil or water. Although some groups have successfully employed molecular techniques to match patient specimens with isolates from an environmental source (von Reyn *et al.*, 1994; Falkinham *et al.*, 2008), the vast environmental reservoir of the MAC confounds such studies. These mycobacteria are abundant in diverse geographical regions, soil types, aquatic ecosystems and urban water distribution systems (Falkinham, 2002). *M. avium* DNA was even detected in samples from the space station Mir (Kawamura *et al.*, 2001). Opportunities for exposure are so extensive

that it is extraordinarily challenging to identify the time and place of infection and then, months or years later, actually isolate the causative MAC clone from that site. Such efforts are further complicated by the fact that infections can be polyclonal (Arbeit *et al.*, 1993; Wallace *et al.*, 1998). The ecological activities of the MAC are largely unknown. In aquatic systems, including swimming pools, hot tubs and municipal pipes, these mycobacteria form biofilms, which enhance resistance to disinfectants and other antimicrobial agents (Vaerewijck *et al.*, 2005). Planktonic cells sloughed from a biofilm can be aerosolized or ingested, and thus contribute to MAC infections. Not all environmental MAC are free-living. Via a process reminiscent of mammalian macrophage infection, *M. avium* can invade and replicate within protozoa (Cirillo *et al.*, 1997; Steinert *et al.*, 1998). The intracellular space is a refuge that provides the mycobacteria with nutrients and protects them from biocides (Steinert *et al.*, 1998). Experiments with tissue culture and animal models even suggest that amoeba-grown bacteria are more virulent than those propagated in standard culture media (Cirillo *et al.*, 1997). As such, MAC-infected protozoans may be an important 'environmental' reservoir for human disease.

7.5 Subspecies of *Mycobacterium avium*

Nucleic acid-based typing methods have changed our view of the MAC. Even early approaches, including DNA–DNA hybridization (Baess, 1983) and commercial 'probes' (Saito *et al.*, 1989, 1990), could readily differentiate *M. avium* from *M. intracellulare*. Conversely, these methods have revealed extensive DNA homology between other, phenotypically dissimilar organisms. Historically, the avian tubercle bacillus, the wood pigeon bacillus and the agent of Johne's disease were classified as separate species (McFadden *et al.*, 1987 ; Saxegaard and Baess, 1988; Yoshimura and Graham, 1988). Current nomenclature identifies them as subspecies of *M. avium* (Thorel *et al.*, 1990). *M. avium* subsp. *avium* refers to the classic avian tubercle bacillus. *M. avium* subsp. *silvaticum*, also known as the wood pigeon bacillus, is an uncommon cause of tuberculosis in wild birds and deer. Although not officially validated, the designation '*M. avium* subsp. *hominissuis*' aims to distinguish human- and pig-derived strains from bird isolates (Mijs *et al.*, 2002). Finally, *M. avium* subsp. *paratuberculosis* (*MAP*) refers to the former *Mycobacterium paratuberculosis* or *Mycobacterium johnei*, the agent of paratuberculosis or Johne's disease. The avian tubercle bacillus (*M. avium* subsp. *avium*) and wood pigeon bacillus (*M. avium* subsp. *silvaticum*) exhibit similar morphological characteristics and both are bird pathogens capable of causing disease in mammals. As such, their genetic similarity is not entirely surprising. The wood pigeon bacillus was traditionally distinguished by a requirement for mycobactin upon primary isolation. Curiously, this siderophore-dependence is lost upon subculture, making *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* almost indistinguishable. Reclassification of *M. paratuberculosis* has met with more resistance. A thorough history of Johne's disease/paratuberculosis is provided elsewhere (see Manning and Collins, Chapter 1, this volume). However, it should be noted that in their initial description of 'pseudo-tuberculous enteritis', Johne and Frothingham postulated that the avian tubercle bacillus was responsible. Also, primary isolation of the organism, called *Mycobacterium enteritidis chronicae pseudotuberculosae bovis* Johne by Twort and Ingram (1912), required media supplemented with a mycobactin-containing extract.

7.6 Taxonomy and Diagnostics for MAC

The following section focuses on diagnostic tools and classification schemes that are typically employed for members of the MAC. How these tests do, and do not, aid in the identification of *MAP* is reviewed. More detailed information on the specific isolation and identification of *MAP* is discussed in greater detail elsewhere (see Whittington, Chapter 22, this volume).

Definitive diagnosis of a mycobacterial infection requires culture of the organism from a clinical specimen, followed by identification using established techniques. Both liquid and solid (agar- or egg-based) media can be used for mycobacterial culture. However, no single medium or growth condition will permit the successful isolation of all mycobacteria and therefore protocols may vary between laboratories. *M. intracellulare* and the classical *M. avium* strains can grow on any standard mycobacterial media, with or without 10% CO_2. While the MAC grows well at 37 °C incubation, *M. avium* strains may grow best at an increased temperature of 40–42 °C (Kent and Kubica, 1985). MAC requires >7 days for growth and 3–4 weeks to reach maturity. Mycobacterial cultures are typically kept up to 6–8 weeks before being considered negative. However, these standard conditions are insufficient for routine isolation of *MAP*. The organism's extremely slow growth rate and requirement for mycobactin supplementation preclude detection. Even when present in immense quantities, it can take several months to detect *MAP* in the clinical setting (Richter *et al.*, 2002).

7.6.1 Traditional classification

Traditionally, speciation of non-tuberculous mycobacteria was based on phenotypic characteristics such as pigmentation, growth rate, growth temperature and biochemical activities. The MAC is considered non-pigmented, although some strains may present with bright yellow pigmentation and ageing cultures may adopt yellow hues. The MAC can also present with various colony morphologies (i.e. smooth or rough) and grow under wide ranges of temperature and pH. The MAC is typically differentiated from other species of the Group III, slowly growing, non-photochromogenic mycobacteria by a positive tellurite test and negative results for urease and Tween hydrolysis (Kent and Kubica, 1985). As discussed above, phenotypic distinction of MAC species is difficult and, owing to their similitude in clinical treatment, identification to the complex level was generally considered sufficient. Notably, the classification algorithms used in clinical microbiology laboratories do not include *MAP*, because it is commonly not considered as a human pathogen.

For decades, serotyping was used for classification of MAC strains. Together, *M. avium* and *M. intracellulare* comprise 28 different serovars (Saito *et al.*, 1990; Wayne *et al.*, 1993). This method relies on the presence of serovar-specific glycopeptidolipids (GPLs) (Brennan *et al.*, 1978; Brennan and Goren, 1979). *MAP* isolates do not produce GPLs and therefore cannot be serotyped. However, this is not a diagnostically useful characteristic since GPL mutants of *M. avium* (Belisle *et al.*, 1993) and other non-serotypeable MAC clones (De Smet *et al.*, 1996) are also encountered. Skilled technicians can also use high-performance liquid chromatography (HPLC) of mycolic acids to successfully separate MAC species (Butler *et al.*, 1992), but differentiation of *MAP* from other *M. avium* subspecies is not possible (Dei *et al.*, 1999).

7.6.2 The *Mycobacterium avium* complex in the molecular era

Genetic methods are less subjective and more reproducible than traditional classification schemes, but results can seem at odds with phenotypic observations. Such is the case with the MAC. Despite the phenotypic similarity of MAC isolates, comprehensive sequence-based studies of MAC involving analysis of the 16S–23S internal transcribed spacer (ITS1) region revealed dozens of distinct sequevars (Frothingham and Wilson, 1993; Mijs *et al.*, 2002) and hinted at the presence of MAC species other than *M. avium* and *M. intracellulare*. Conversely, standard *MAP* isolates are genotypically indistinguishable from the most common *M. avium* lineages. Even so, genetic information can aid in the diagnosis of MAC infections and may eventually facilitate disease treatment. Recent studies suggest that the type and severity of human disease depends upon the MAC species involved (Schweickert *et al.*, 2008). Whereas certain species may pose a minor threat, such that a 'watch and wait' approach to treatment is sufficient, the presence of other MAC organisms may signal a need for more aggressive medical intervention.

The 16S rRNA gene is a robust target for sequence-based identification of bacteria, including the mycobacteria (Rogall *et al.*, 1990). Universal primers have the capacity to amplify this gene from virtually any bacterial isolate. Subsequent sequencing of the hypervariable regions permits species identification. Using this technique, the discovery of new species has skyrocketed, such that two-thirds of currently established mycobacterial species have been described only in the last two decades. As with ITS1 sequencing, the species *M. avium* and *M. intracellulare* are unequivocally distinguished and several novel, albeit closely related, sequences have been observed (Wayne *et al.*, 1996). Nevertheless, all four subspecies of *M. avium* have identical 16S rRNA gene sequences and therefore this target cannot be used to identify *MAP* (Fig. 7.1).

7.6.3 Commercial tests for the identification of MAC

Sequence variation is the basis for a number of commercial assays currently available for the detection and/or speciation of mycobacteria. These tests offer rapid turnaround time and greater accuracy than conventional methods and thus contribute to improved patient care. Some of the first, introduced in the early 1990s and still used today, are the AccuProbe® Culture Identification Tests (Gen-Probe)(Saito *et al.*, 1989). Currently six tests are available. Each targets ribosomal RNA and permits identification of mycobacteria from a positive culture. In addition to tests for the *M. tuberculosis* complex, *M. kansasii*, *Mycobacterium gordonae*, *M. intracellulare* and *M. avium*, there is a MAC ('MAIX') test reputed to identify any

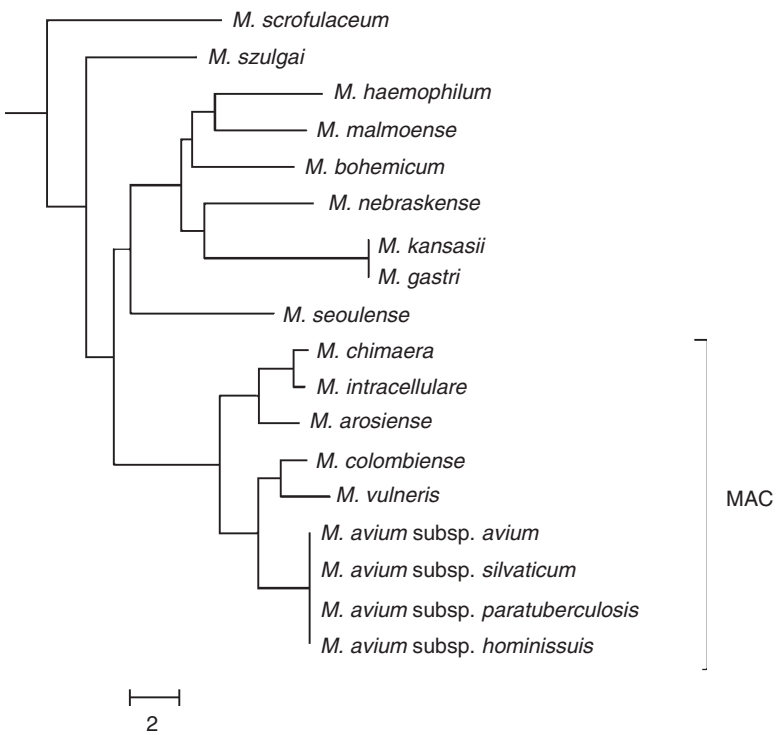

Fig. 7.1. Phylogeny reconstruction of ~1400 bp of the 16S rRNA gene using the neighbour-joining method and performed using the MEGA v4.1 software. The species shown represent the subcluster that included MAC species in the context of a comprehensive 16S rRNA gene sequence alignment of all mycobacterial species. Scale represents the number of base pairs difference.

MAC organism (Lebrun *et al.*, 1992; Viljanen *et al.*, 1993). Because the target of AccuProbe is ribosomal RNA, this assay cannot distinguish *MAP* from other *M. avium* subspecies.

More recently, reverse hybridization line probe assays have become commercially available. These permit detection of multiple mycobacterial species in a single test. The Inno-LiPA® MYCOBACTERIA v2 (Innogenetics) (Tortoli *et al.*, 2001, 2003; Tortoli, 2002) can distinguish *M. avium*, two subsets of *M. intracellulare* and 'MAIS complex' (Lebrun *et al.*, 2003, 2005). However, the target is the ITS1 region, which is not effective for resolution of *MAP*. Hain Lifescience markets several line probe assays for mycobacteria which detect unique signature sequences in the 23S rDNA. The GenoType® Mycobacteria Direct can be used for identification directly from clinical specimens whereas the GenoType® Mycobacterium CM (Common Mycobacteria) and AS (Additional Species) tests require positive cultures. Again, these tests do not differentiate *MAP* from other *M. avium*.

7.6.4 New species of MAC

DNA-based analysis of the MAC has confirmed that the complex includes more than *M. avium* and *M. intracellulare.* In the past 5 years, four new MAC species have been established: *Mycobacterium chimaera* (Tortoli *et al.*, 2004), *Mycobacterium colombiense* (Murcia *et al.*, 2006), *Mycobacterium arosiense* (Bang *et al.*, 2008) and *Mycobacterium vulneris* (van Ingen *et al.*, 2009) (Fig. 7.1). All are slow-growing, are relatively inert with biochemical tests and exhibit HPLC profiles typical of the MAC. The new species are probably less common than *M. avium* or *M. intracellulare*, but, to date, little is known about their true distribution. *M. vulneris* and *M. arosiense* are scotochromogenic (yellow) while the others are non-photochromogenic, as are most MAC. They are often identified by general MAC/MAIS probes but are recognized as novel species primarily due to unique genetic features of 16S rRNA and ITS1 sequences.

A phylogeny based on ITS1 sequevars (Fig. 7.2) hints at the existence of several other novel MAC species that have yet to be officially described. Many good candidates within the MAC can be observed. However, even with the existence of a larger number of MAC species, *MAP* and the other subspecies of *M. avium* remain tightly clustered as a single group (Mav-A to Mav-H). Although eight *M. avium* ITS1 sequevars have been reported, *MAP* strains typically present with the Mav-A sequevar and are indistinguishable from other *M. avium* (Turenne *et al.*, 2006). Unless a sequence-based approach is used, identification of the newly established species and recognition of novel, not-yet-described species is not possible. Depending on the test employed, the clinical laboratory will only be able to identify such organisms as MAC, *M. avium* or *M. intracellulare*.

7.6.5 Phylogeny of *Mycobacterium avium* species

Although the aforementioned gene targets permit species-level identification of MAC organisms, none can distinguish among the various subspecies of *M. avium*. Subspecies-level identification requires other targets, such as insertion sequences. A scheme based on three insertion sequences, IS*1245*, IS*901* and IS*900*, has been used to define the subspecies of *M. avium*: IS*901* is only present in avian strains (*M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*); IS*900* is specific for *MAP*; IS*1245* is absent from *MAP* but present in all other subspecies (Ellingson *et al.*, 2000; Bartos *et al.*, 2006). Hybridization-based methods (e.g. restriction fragment length polymorphisms) that target these elements can be used for subspecies identification as well as strain typing and surveillance. However, PCR-based detection of these insertion sequences should be used with caution and verified via DNA sequencing, since similar elements exist in non-MAC organisms.

Advances in sequence-based methods have improved the characterization of *M. avium* subspecies and now permit the unequivocal identification of *MAP*. The 441-bp region at the 5′ end of the *hsp65* gene is widely used for speciation of mycobacteria (Telenti *et al.*, 1993)

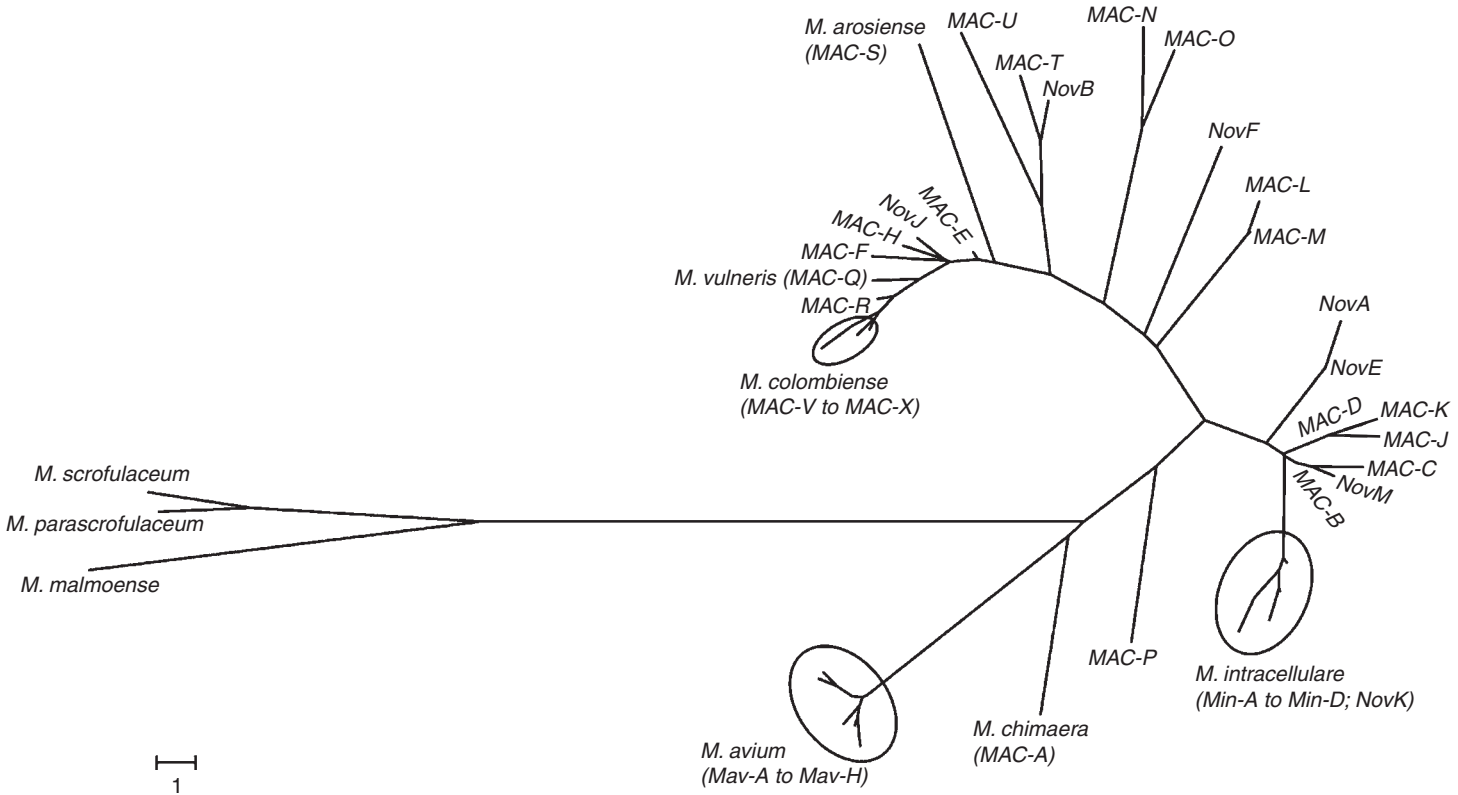

Fig. 7.2. Radial tree of ITS1 sequences of the MAC. Closely related species *Mycobacterium scrofulaceum*, *Mycobacterium parascrofulaceum* and *Mycobacterium malmoense* are included as outgroups. Sequences designated 'Mav', 'MAC' and 'Min' were obtained from GenBank, whereas sequences designated 'Nov' were retrieved from published data (Stout *et al.*, 2008).

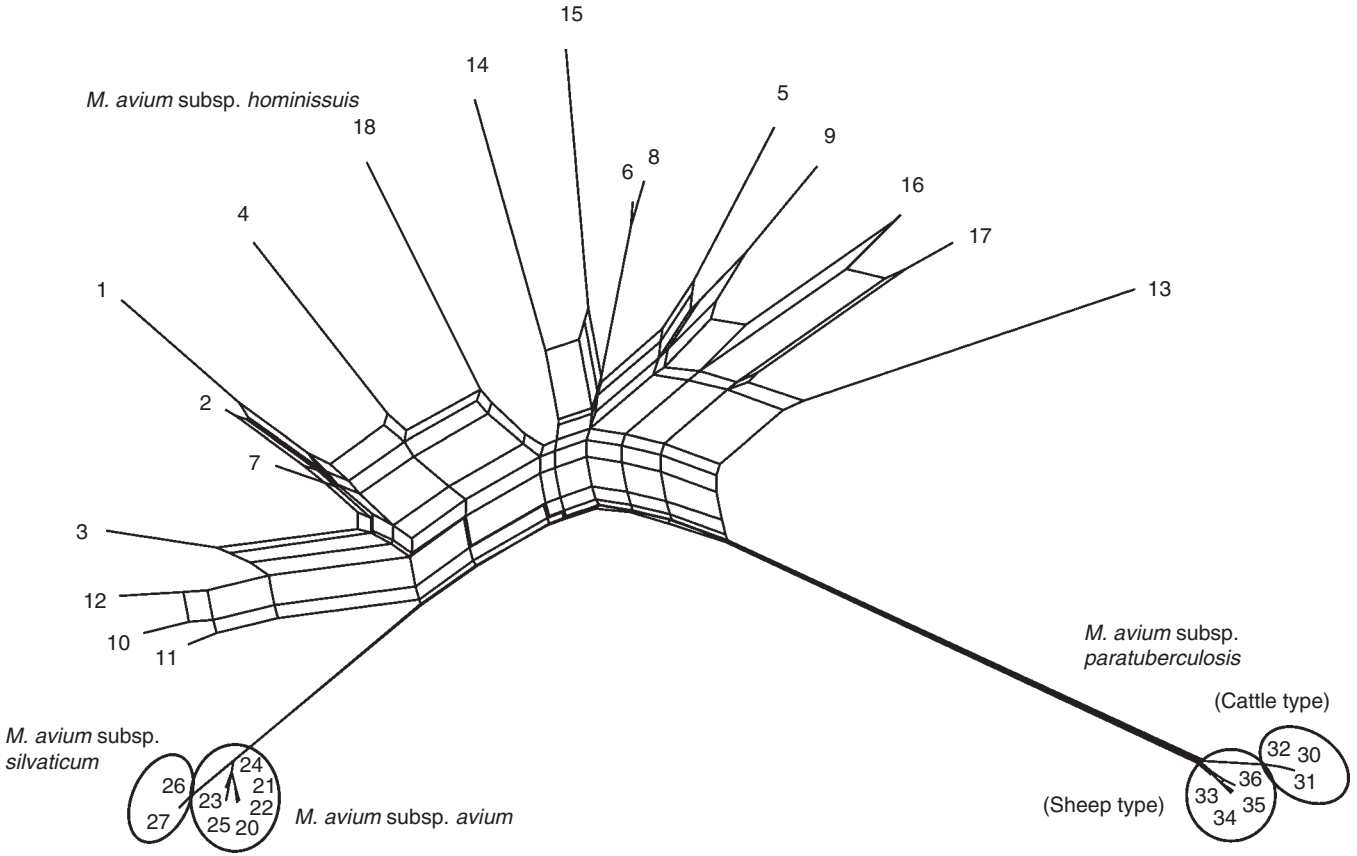

Fig. 7.3. Split network phylogeny of *M. avium* species using the concatenated sequences of ten multilocus sequence analysis (MLSA) genes. Adapted from Turenne *et al.*, 2008, with permission.

but does not effectively differentiate *M. avium* subspecies. In contrast, the 3′ 'tail' end of the *hsp65* gene can simultaneously identify species as well as host-associated subtypes (Turenne *et al.*, 2006). *MAP* is represented by two sequevars, one for each of the sheep (Type I/III or S type) and cattle (Type II or C type) lineages. Another sequevar encompasses both bird-associated pathogens (i.e. subsp. *avium* and *silvaticum* together). *M. avium* subsp. *hominissuis* is heterogeneous, with seven reported sequevars, none of which are present in the other subspecies. Recently described sequence polymorphisms in other genes, including *recF*, *lipT*, *pepB*, *rpoB* and a hypothetical esterase gene, *MAP2698c* (Ben Salah *et al.*, 2008; Turenne *et al.*, 2008), can also separate the *M. avium* subspecies. The *recF* and *lipT* targets even contain polymorphisms with the capacity to separate the host-associated (i.e. sheep and cattle) lineages of *MAP*. By combining the polymorphic gene targets and using multilocus sequence analysis (MLSA), it has been possible to establish the phylogenetic relationships of all recognized *M. avium* subspecies (Turenne *et al.*, 2008). Out of the heterogeneous mix of environmental and opportunistic strains represented by *M. avium* subsp. *hominissuis* emerge two independently evolved pathogenic clones (Fig. 7.3.). One of these comprises the avian subspecies (*M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*) whereas the other only includes *MAP* strains. Closer examination of the *MAP* group reveals separate phylogenetic branches for the sheep and cattle lineages.

7.7 Concluding Remarks

The MAC includes organisms of diverse pathogenic potential. Molecular typing methods have reaffirmed the existence of the complex but also revealed differences between species, subspecies and strains. Proper classification of isolates is essential to a thorough understanding of MAC epidemiology and pathogenesis. Despite its close taxonomic proximity to other *M. avium* organisms, *MAP* stands out as an important pathogen with unique phenotypic and genetic characteristics.

References

Anonymous (1891) The Tuberculosis Congress. *The Lancet* 138, 463–464.

Arbeit, R.D., Slutsky, A., Barber, T.W., Maslow, J.N., Niemczyk, S., Falkinham, J.O., O'Connor, G.T. and von-Reyn, C.F. (1993) Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. *Journal of Infectious Diseases* 167, 1384–1390.

Baess, I. (1983) Deoxyribonucleic acid relationships between different serovars of *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. *Acta pathologica et Microbiologica Scandinavica Section B: Microbiology and Immunology* 91, 201–203.

Bang, D., Herlin, T., Stegger, M., Andersen, A.B., Torkko, P., Tortoli, E. and Thomsen, V.O. (2008) *Mycobacterium arosiense* sp. nov., a slowly growing, scotochromogenic species causing osteomyelitis in an immunocompromised child. *International Journal of Systematic and Evolutionary Microbiology* 58, 2398–2402.

Bartos, M., Hlozek, P., Svastova, P., Dvorska, L., Bull, T., Matlova, L., Parmova, I., Kuhn, I., Stubbs, J., Moravkova, M., Kintr, J., Beran, V., Melicharek, I., Ocepek, M. and Pavlik, I. (2006) Identification of members of *Mycobacterium avium* species by Accu-Probes, serotyping, and single IS*900*, IS*901*, IS*1245* and IS*901*-flanking region PCR with internal standards. *Journal of Microbiological Methods* 64, 333–345.

Belisle, J.T., Klaczkiewicz, K., Brennan, P.J., Jacobs, W.R., Jr. and Inamine, J.M. (1993) Rough morphological variants of *Mycobacterium avium*. Characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. *The Journal of Biological Chemistry* 268, 10517–10523.

Ben Salah, I., Adekambi, T., Raoult, D. and Drancourt, M. (2008) *rpoB* sequence-based identification of *Mycobacterium avium* complex species. *Microbiology* 154, 3715–3723.

Branch, A. (1931) Avian tubercle bacillus infection with special reference to mammals and to man. *Archives of Pathology* 12, 253–274.

Brennan, P.J. and Goren, M.B. (1979) Structural studies on the type-specific antigens and lipids of the *Mycobacterium avium–Mycobacterium intracellulare–Mycobacterium scrofulaceum* serocomplex: *Mycobacterium intracellulare* serotype 9. *The Journal of Biological Chemistry* 254, 4205–4211.

Brennan, P.J., Souhrada, M., Ullom, B., McClatchy, J.K. and Goren, M.B. (1978) Identification of atypical mycobacteria by thin-layer chromatography

of their surface antigens. *Journal of Clinical Microbiology* 8, 374–379.

Butler, W.R., Thibert, L. and Kilburn, J.O. (1992) Identification of *Mycobacterium avium* complex strains and some similar species by high-performance liquid chromatography. *Journal of Clinical Microbiology* 30, 2698–2704.

Cirillo, J.D., Falkow, S., Tompkins, L.S. and Bermudez, L.E. (1997) Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity* 65, 3759–3767.

Corpe, R.F. (1964) Clinical aspects, medical and surgical, in the management of Battey-type pulmonary disease. *Diseases of the Chest* 45, 380–382.

Cuttino, J.T. and McCabe, A.M. (1949) Pure granulomatous nocardiosis: a new fungus disease distinguished by intracellular parasitism. *American Journal of Clinical Pathology* 25, 1–34.

Dei, R., Tortoli, E., Bartoloni, A., Simonetti, M.T. and Lillini, E. (1999) HPLC does not differentiate *Mycobacterium paratuberculosis* from *Mycobacterium avium*. *Veterinary Microbiology* 65, 209–213.

De Smet, K.A., Hellyer, T.J., Khan, A.W., Brown, I.N. and Ivanyi, J. (1996) Genetic and serovar typing of clinical isolates of the *Mycobacterium avium–intracellulare* complex. *Tubercle and Lung Disease* 77, 71–76.

Ellingson, J.L., Stabel, J.R., Bishai, W.R., Frothingham, R. and Miller, J.M. (2000) Evaluation of the accuracy and reproducibility of a practical PCR panel assay for rapid detection and differentiation of *Mycobacterium avium* subspecies. *Molecular and Cellular Probes* 14, 153–161.

Falkinham, J.O. (1996) Epidemiology of infection by nontuberculous mycobacteria. *Clinical Microbiology Reviews* 9, 177–215.

Falkinham, J.O., III. (2002) Nontuberculous mycobacteria in the environment. *Clinics in Chest Medicine* 23, 529–551.

Falkinham, J.O., III, Iseman, M.D., de Haas, P. and van Soolingen, D. (2008) *Mycobacterium avium* in a shower linked to pulmonary disease. *Journal of Water and Health* 6, 209–213.

Feldman, W.H. (1938) *Avian Tuberculosis Infections*. The William & Wilkins Co., Baltimore, Maryland.

Feldman, W.H. (1939) Types of tubercle bacilli in lesions of garbage-fed swine. *American Journal of Public Health* 29, 1231–1238.

Frothingham, R. and Wilson, K.H. (1993) Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *Journal of Bacteriology* 175, 2818–2825.

Iseman, M.D., Corpe, R.F., O'Brien, R.J., Rosenzweig, D.Y. and Wolinsky, E. (1985) Disease due to *Mycobacterium avium–intracellulare*. *Chest* 87, 139S–149S.

Kawamura, Y., Li, Y., Liu, H., Huang, X., Li, Z. and Ezaki, T. (2001) Bacterial population in Russian space station 'Mir'. *Microbiology and Immunology* 45, 819–828.

Kent, P.T. and Kubica, G.P. (1985) *Public Health Mycobacteriology: a Guide for the Level III Laboratory*. US Department of Health and Human Services, Centers for Disease Control, Atlanta, Georgia.

Lebrun, L., Espinasse, F., Poveda, J.D. and Vincent-Levy-Frebault, V. (1992) Evaluation of nonradioactive DNA probes for identification of mycobacteria. *Journal of Clinical Microbiology* 30, 2476–2478.

Lebrun, L., Gonullu, N., Boutros, N., Davoust, A., Guibert, M., Ingrand, D., Ghnassia, J.C., Vincent, V. and Doucet-Populaire, F. (2003) Use of INNO-LiPA assay for rapid identification of mycobacteria. *Diagnostic Microbiology and Infectious Disease* 46, 151–153.

Lebrun, L., Weill, F.X., Lafendi, L., Houriez, F., Casanova, F., Gutierrez, M.C., Ingrand, D., Lagrange, P., Vincent, V. and Herrmann, J.L. (2005) Use of the INNO-LiPA-MYCOBACTERIA Assay (Version 2) for identification of *Mycobacterium avium–Mycobacterium intracellulare–Mycobacterium scrofulaceum* complex isolates. *Journal of Clinical Microbiology* 43, 2567–2574.

Maffucci, A. (1892) Die hühnertuberculose. *Zeitschrift für Hygiene und Infektionskrankheiten* 11, 445–486.

Matlova, L., Dvorska, L., Ayele, W.Y., Bartos, M., Amemori, T. and Pavlik, I. (2005) Distribution of *Mycobacterium avium* complex isolates in tissue samples of pigs fed peat naturally contaminated with mycobacteria as a supplement. *Journal of Clinical Microbiology* 43, 1261–1268.

McFadden, J.J., Butcher, P.D., Thompson, J., Chiodini, R. and Hermon-Taylor, J. (1987) The use of DNA probes identifying restriction-fragment-length polymorphisms to examine the *Mycobacterium avium* complex. *Molecular Microbiology* 1, 283–291.

Meissner, G., Schroder, K.H., Amadio, G.E., Anz, W., Chaparas, S., Engel, H.W., Jenkins, P.A., Kappler, W., Kleeberg, H.H., Kubala, E., Kubin, M., Lauterbach, D., Lind, A., Magnusson, M., Mikova, Z., Pattyn, S.R., Schaefer, W.B., Stanford, J.L., Tsukamura, M., Wayne, L.G., Willers, I. and Wolinsky, E. (1974) A co-operative numerical analysis of nonscoto- and nonphotochromogenic slowly growing mycobacteria. *Journal of General Microbiology* 83, 207–235.

Mijs, W., de Haas, P., Rossau, R., Van der Laan, T., Rigouts, L., Portaels, F. and van Soolingen, D. (2002) Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and '*M. avium* subsp. *hominissuis*' for the human/porcine type of *M. avium*. *International Journal of Systematic and Evolutionary Microbiology* 52, 1505–1518.

Miller, J. (1911) The report of the Royal Commission on Tuberculosis: a critical survey. *British Journal of Tuberculosis* 5, 282–290.

Murcia, M.I., Tortoli, E., Menendez, M.C., Palenque, E. and Garcia, M.J. (2006) *Mycobacterium colombiense* sp. nov., a novel member of the *Mycobacterium avium* complex and description of MAC-X as a new ITS genetic variant. *International Journal of Systematic and Evolutionary Microbiology* 56, 2049–2054.

Richter, E., Wessling, J., Lugering, N., Domschke, W. and Rusch-Gerdes, S. (2002) *Mycobacterium avium* subsp. *paratuberculosis* infection in a patient with HIV, Germany. *Emerging Infectious Diseases* 8, 729–731.

Rogall, T., Flohr, T. and Bottger, E.C. (1990) Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. *Journal of General Microbiology* 136, 1915–1920.

Runyon, E.H. (1958) Mycobacteria encountered in clinical laboratories. *Leprosy Briefs* 9, 21–23.

Runyon, E.H. (1965) Pathogenic mycobacteria. *Advances in Tuberculosis Research* 14, 235–287.

Runyon, E.H. (1974) Ten mycobacterial pathogens. *Tubercle* 55, 235–240.

Saito, H., Tomioka, H., Sato, K., Tasaka, H., Tsukamura, M., Kuze, F. and Asano, K. (1989) Identification and partial characterization of *Mycobacterium avium* and *Mycobacterium intracellulare* by using DNA probes. *Journal of Clinical Microbiology* 27, 994–997.

Saito, H., Tomioka, H., Sato, K., Tasaka, H. and Dawson, D.J. (1990) Identification of various serovar strains of *Mycobacterium avium* complex by using DNA probes specific for *Mycobacterium avium* and *Mycobacterium intracellulare*. *Journal of Clinical Microbiology* 28, 1694–1697.

Saxegaard, F. and Baess, I. (1988) Relationship between *Mycobacterium avium*, *Mycobacterium paratuberculosis* and 'wood pigeon mycobacteria'. Determinations by DNA–DNA hybridization. *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 96, 37–42.

Schweickert, B., Goldenberg, O., Richter, E., Gobel, U.B., Petrich, A., Buchholz, P. and Moter, A. (2008) Occurrence and clinical relevance of *Mycobacterium chimaera* sp. nov., Germany. *Emerging Infectious Diseases* 14, 1443–1446.

Steinert, M., Birkness, K., White, E., Fields, B. and Quinn, F. (1998) *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology* 64, 2256–2261.

Stout, J.E., Hopkins, G.W., McDonald, J.R., Quinn, A., Hamilton, C.D., Reller, L.B. and Frothingham, R. (2008) Association between 16S–23S internal transcribed spacer sequence groups of *Mycobacterium avium* complex and pulmonary disease. *Journal of Clinical Microbiology* 46, 2790–2793.

Telenti, A., Marchesi, F., Balz, M., Bally, F., Bottger, E.C. and Bodmer, T. (1993) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *Journal of Clinical Microbiology* 31, 175–178.

Thorel, M.F., Krichevsky, M. and Levy-Frebault, V.V. (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *International Journal of Systematic Bacteriology* 40, 254–260.

Thorel, M.F., Huchzermeyer, H., Weiss, R. and Fontaine, J.J. (1997) *Mycobacterium avium* infections in animals. Literature review. *Veterinary Research* 28, 439–447.

Tortoli, E. (2002) Identification of mycobacteria by using INNO LiPA. *Journal of Clinical Microbiology* 40, 3111.

Tortoli, E., Nanetti, A., Piersimoni, C., Cichero, P., Farina, C., Mucignat, G., Scarparo, C., Bartolini, L., Valentini, R., Nista, D., Gesu, G., Tosi, C.P., Crovatto, M. and Brusarosco, G. (2001) Performance assessment of new multiplex probe assay for identification of mycobacteria. *Journal of Clinical Microbiology* 39, 1079–1084.

Tortoli, E., Mariottini, A. and Mazzarelli, G. (2003) Evaluation of INNO-LiPA MYCOBACTERIA v2: improved reverse hybridization multiple DNA probe assay for mycobacterial identification. *Journal of Clinical Microbiology* 41, 4418–4420.

Tortoli, E., Rindi, L., Garcia, M.J., Chiaradonna, P., Dei, R., Garzelli, C., Kroppenstedt, R.M., Lari, N., Mattei, R., Mariottini, A., Mazzarelli, G., Murcia, M.I., Nanetti, A., Piccoli, P. and Scarparo, C. (2004) Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 54, 1277–1285.

Turenne, C.Y., Semret, M., Cousins, D.V., Collins, D.M. and Behr, M.A. (2006) Sequencing of *hsp65* distinguishes among subsets of the *Mycobacterium avium* complex. *Journal of Clinical Microbiology* 44, 433–440.

Turenne, C.Y., Collins, D.M., Alexander, D.C. and Behr, M.A. (2008) *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms. *Journal of Bacteriology* 190, 2479–2487.

Twort, F.W. and Ingram, G.L.Y. (1912) A method of isolating and cultivating *Mycobacterium enteritidis chronicae pseudotuberculosis bovis*, Johne, and some experiments on the preparation of a diagnostic vaccine for pseudotuberculosis enteritis of bovines. *Proceedings of the Royal Society of London Series B, Biological Sciences* 84, 517–542.

Vaerewijck, M.J., Huys, G., Palomino, J.C., Swings, J. and Portaels, F. (2005) Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiology Reviews* 29, 911–934.

van Ingen, J., Boeree, M.J., Kosters, K., Wieland, A., Tortoli, E., Dekhuijzen, R.P.N. and van Soolingen, D. (2009) Proposal to elevate *Mycobacterium avium* complex ITS sequevar MAC-Q to *Mycobacterium vulneris* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 59, 2277–2282.

Viljanen, M.K., Olkkonen, L. and Katila, M.L. (1993) Conventional identification characteristics, mycolate and fatty acid composition, and clinical significance of MAIX AccuProbe-positive isolates of *Mycobacterium avium* complex. *Journal of Clinical Microbiology* 31, 1376–1378.

von Reyn, C.F., Maslow, J.N., Barber, T.W., Falkinham, J.O., III and Arbeit, R.D. (1994) Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet* 343, 1137–1141.

Wallace, R.J., Jr., Zhang, Y., Brown, B.A., Dawson, D., Murphy, D.T., Wilson, R. and Griffith, D.E. (1998) Polyclonal *Mycobacterium avium* complex infections in patients with nodular bronchiectasis. *American Journal of Respiratory and Critical Care Medicine* 158, 1235–1244.

Wayne, L.G. (1966) Classification and identification of mycobacteria. III. Species within group III. *The American Review of Respiratory Disease* 93, 919–928.

Wayne, L.G., Good, R.C., Tsang, A., Butler, R., Dawson, D., Groothuis, D., Gross, W., Hawkins, J., Kilburn, J., Kubin, M., Schröder, K.H., Silcox, V.A., Smith, C., Thorel, M.F., Woodley, C. and Yakrus, M.A. (1993) Serovar determination and molecular taxonomic correlation in *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*: a cooperative study of the International Working Group on Mycobacterial Taxonomy. *International Journal of Systematic Bacteriology* 43, 482–489.

Wayne, L.G., Good, R.C., Bottger, E.C., Butler, R., Dorsch, M., Ezaki, T., Gross, W., Jonas, V., Kilburn, J., Kirschner, P., Krichevsky, M.I., Ridell, M., Shinnick, T.M., Springer, B., Stackebrandt, E., Tarnok, I., Tarnok, Z., Tasaka, H., Vincent, V., Warren, N.G., Knott, C.A. and Johnson, R. (1996) Semantide- and chemotaxonomy-based analyses of some problematic phenotypic clusters of slowly growing mycobacteria, a cooperative study of the International Working Group on Mycobacterial Taxonomy. *International Journal of Systematic Bacteriology* 46, 280–297.

Yoshimura, H.H. and Graham, D.Y. (1988) Nucleic acid hybridization studies of mycobactin-dependent mycobacteria. *Journal of Clinical Microbiology* 26, 1309–1312.

8 *Mycobacterium avium* subsp. *paratuberculosis* Genome

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8.1 Introduction

The post-genomic era of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) research began in 2005 with the publication of the complete, annotated genome sequence of isolate K-10, which was isolated from a Wisconsin dairy herd in 1990 (J. Hansen, Iowa, 2009, personal communication). The 4,829,781 base pair sequence was determined by preparing and sequencing a random small insert library and PCR products totalling 66,129 unique sequences (L. Li *et al.*, 2005). The *MAP* K-10 genome was originally annotated as having 4350 predicted open reading frames. Subsequent automated analyses of the genome sequence have annotated up to 4587 total genes (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntma03). The *MAP* genome is characterized by a relatively high proportion of guanine and cytosine nucleotides within the genome (69%), as well as an abundance of insertion sequences and PE/PPE proteins, similar to other sequenced mycobacteria. Genomic regions with altered nucleotide compositions are in many cases associated with mobile genetic elements and have been shown to be sites of large sequence polymorphisms (Fig. 8.1). Recent work using optical mapping technology to compare *MAP* genomes has identified a 648 kb inversion near the origin of replication in the *MAP* K-10 genome that may be due to an error in sequence assembly (Wu *et al.*, 2009).

The completion of the *MAP* K-10 genome sequence has opened the door to many new avenues of research. In the few years since the publication of the genome sequence, the manuscript describing the completed sequence has been cited in the scientific literature more than 85 times. The public availability of a completed genome sequence has allowed researchers to include *MAP* in comparative genomic studies with other mycobacteria, which in turn has facilitated the transfer of information from other fields of research. This has led to new insights into mechanisms of virulence,

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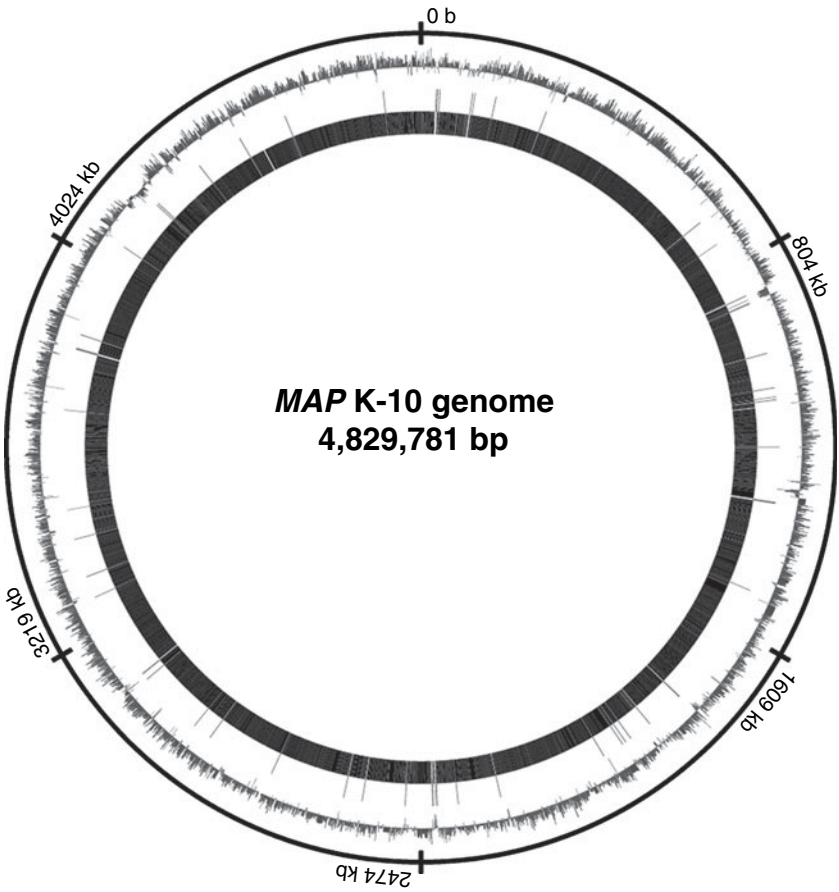

Fig. 8.1. *Mycobacterium avium* subsp. *paratuberculosis* K-10 genome. Beginning at the outer ring, the first circle displays the nucleotide position, starting at the origin of replication. The next circle is a graph of the percentage of guanine and cytosine nucleotides within the genome, while the innermost ring consists of bars representing individual coding sequences, in which those sequences predicted to encode mobile genetic elements are highlighted by moving those bars towards the outside of the ring. The image was generated using the Microbial Genome Viewer (Kerkhoven *et al.*, 2004).

diagnostic targets and the molecular evolution of *MAP* as a pathogen. The genome sequence has facilitated the development of DNA microarrays for use in genome-wide comparative genomic hybridizations and transcriptional analyses. The systematic cloning and expression of a large subset of *MAP* proteins from the annotated genome sequence has been initiated. In addition, the use of proteomic techniques such as tandem mass spectrometry has been enabled by the availability of a reference genome from which to identify the genes that encode observed peptide sequences. The anticipated completion of additional mycobacterial genome projects in the near future (Table 8.1) should further add to the utility of the *MAP* genome sequence.

Other *MAP* genomes have also been subjected to whole-genome sequencing. Draft sequence coverage has been obtained from a human *MAP* isolate (V. Kapur, 2009, unpublished results), while genome closure and annotation are currently under way for a sheep isolate (M. Paustian, 2009, unpublished

Table 8.1. Sequenced mycobacterial genomes.

Completed genomes	Size (Mb)	% G+C	Accession
Mycobacterium abscessus	5.12	64.1	NC_010397.1
Mycobacterium avium subsp. *hominissuis* 104	5.5	69.0	NC_008595.1
Mycobacterium avium subsp. *paratuberculosis* K-10	4.8	69.3	NC_002944.2
Mycobacterium bovis AF2122/97	4.35	65.6	NC_002945.3
Mycobacterium bovis BCG str. Pasteur 1173P2	4.4	65.6	NC_008769.1
Mycobacterium gilvum PYR-GCK	5.96	67.7	NC_009338.1
Mycobacterium leprae TN	3.27	57.8	NC_002677.1
Mycobacterium marinum M	6.62	65.7	NC_010612.1
Mycobacterium smegmatis str. MC2 155	7	67.4	NC_008596.1
Mycobacterium sp. JLS	6	68.4	NC_009077.1
Mycobacterium sp. KMS	6.22	68.2	NC_008705.1
Mycobacterium sp. MCS	5.92	68.4	NC_008146.1
Mycobacterium tuberculosis CDC1551	4.4	65.6	NC_002755.2
Mycobacterium tuberculosis F11	4.4	65.6	NC_009565.1
Mycobacterium tuberculosis H37Ra	4.4	65.6	NC_009525.1
Mycobacterium tuberculosis H37Rv	4.4	65.6	NC_000962.2
Mycobacterium ulcerans Agy99	5.6	65.5	NC_008611.1
Mycobacterium vanbaalenii PYR-1	6.5	67.8	NC_008726.1
Genomes in progress			
Mycobacterium africanum GM041182			
Mycobacterium avium subsp. *avium* ATCC 25291			
Mycobacterium avium subsp. *paratuberculosis* (sheep)			
Mycobacterium avium subsp. *paratuberculosis* (human)			
Mycobacterium bovis BCG (2 isolates)			
Mycobacterium canetti (2 isolates)			
Mycobacterium chelonae CIP 104535			
Mycobacterium chlorophenolicum			
Mycobacterium intracellulare ATCC 13950			
Mycobacterium kansasii ATCC 12478			
Mycobacterium leprae Br4923			
Mycobacterium liflandii 128FXT			
Mycobacterium marinum DL240490			
Mycobacterium microti OV254			
Mycobacterium parascrofulaceum ATCC BAA-614			
Mycobacterium sp. Spyr1			
Mycobacterium tuberculosis (42 isolates)			
Mycobacterium ulcerans (2 isolates)			

results). Preliminary results indicate that the human *MAP* isolate is essentially identical to the bovine K-10 isolate, while the sheep isolate contains several large sequence polymorphisms, some of which have been described previously (Marsh and Whittington, 2005; Marsh *et al.*, 2006; Semret *et al.*, 2006; Wu *et al.*, 2006; Paustian *et al.*, 2008). Phenotypic differences between *MAP* strains are discussed elsewhere (see Stevenson, Chapter 12, this volume). The draft genome sequence of the *MAP* sheep isolate has confirmed the presence of these polymorphisms and provided their precise location. In addition, the genome sequencing project has identified novel features within the genome, such as a 5000 bp segment of which 3500 bp are homologous to sequences from the *Frankia* sp.

EAM1pec genome (M. Paustian, 2009, unpublished results). This region is flanked by a coding sequence homologous to MAP2180c, which itself is annotated as a hypothetical protein with homology only to sequences from the *Frankia* genome. Thus this region may represent a genome insertion event that was later partially excised from the genome of *MAP* cattle isolates. The *MAP* sheep genome sequence has also identified a glycopeptidolipid biosynthesis operon with homology to a similar gene cluster in *M. avium* subsp. *avium* (Paustian *et al.*, 2008), although production of glycopeptidolipids by *MAP* sheep isolates has not yet been experimentally verified.

8.2 Metabolic Pathway Comparison

The complete genome sequence of *MAP* has facilitated functional comparisons of mycobacterial metabolic pathways. Relative to the *M. tuberculosis* genome, *MAP* is lacking the fumarate reductase complex members *frdBCD* as well as the nitrate reductase gene *narX* (Marri *et al.*, 2006). These genes are thought to be important for survival under anaerobic conditions. Three members of the aspartate family (*asnB*, *dapA* and *lysA*) are duplicated in the *MAP* genome (Marri *et al.*, 2006). The genes *dapA* and *lysA* encode proteins involved in lysine biosynthesis and their duplication may therefore be indicative of an increased requirement for lysine, while *asnB* has been shown to facilitate natural resistance to antibiotics in *Mycobacterium smegmatis* (Ren and Liu, 2006). The *MAP* genome is lacking the urease operon (*ureABC* and *ureDFG*), which enables the utilization of ammonia as a nitrogen source (L. Li *et al.*, 2005). Compared with other mycobacteria, the *MAP* genome is lacking the genes *atsBDFH*. These genes are predicted to encode arylsulfatases that are used to generate sulfated glycolipids. Relative to mycobacteria other than *M. avium* subspecies, the *MAP* genome contains an abundance of functionally redundant lipid metabolism genes. *MAP* is missing 19 genes that are present in *M. tuberculosis* but has an additional 35 due to gene duplications (Marri *et al.*, 2006). Overall, there are significant differences in the types of membrane-protein encoding genes present in the *M. tuberculosis* and *MAP* genomes, which probably reflects their distinct differences in pathogenesis. The complete genome sequence of *MAP* has provided a possible explanation for the *in vitro* growth requirement of supplemental mycobactin. The gene cluster encoding the biosynthetic enzymes used to build the mycobactin siderophore (*mbtA-J*) is similar to other mycobacteria, with the notable exception that *mbtA* is truncated by approximately 150 amino acids (L. Li *et al.*, 2005). This observation remains to be experimentally verified. The comparison of the *MAP* genome with other sequenced mycobacteria has enabled researchers to more accurately and efficiently translate research findings from other fields into their studies.

8.3 Virulence Factors

The PE/PPE family of proteins are putative virulence factors originally identified in *M. tuberculosis* (Cole *et al.*, 1998). These proteins are distinguished by their specific domains (Pro-Glu and Pro-Pro-Glu), which often contain polymorphic sequences and are thought to be expressed on the cell surface (Fleischmann *et al.*, 2002). Thus far, PE/PPE proteins have only been found in mycobacteria, where they comprise anywhere from 1% of the genome (*MAP*) to nearly 10% (*M. tuberculosis*). Hypothesized functions for these proteins include host cell binding, antigenic variation, inhibition of antigen processing, macrophage survival and iron uptake. The relative homology between members of this gene family suggests that some perform redundant functions, which would complicate efforts to study individual genes. The genome sequence of *MAP* K-10 has identified 10 PE and 37 PPE homologues, 18 of which are specific to *MAP* (L. Li *et al.*, 2005; Gey van Pittius *et al.*, 2006). A comparative analysis of PE and PPE gene families from sequenced mycobacterial genomes indicates that all but one of the genes present in the *MAP* genome are part of an evolutionary distant group from which other subfamilies later evolved

in other mycobacteria (Gey van Pittius *et al.*, 2006). Experimental data have shown that a *M. avium* subsp. *hominissuis* mutant defective in the production of a PPE protein homologous to MAP1505 was found to be restricted for growth within macrophages (Y. Li *et al.*, 2005). Subsequent analyses of *MAP* PPE proteins have indicated that MAP3420c and MAP1506 are expressed at the cell surface and are immunogenic in cattle (Newton *et al.*, 2009); additionally, MAP1506 sequence polymorphisms were shown to distinguish between different subtypes of *MAP* (Griffiths *et al.*, 2008).

The *MAP* genome contains many homologues of mammalian cell entry (*mce*) genes, which were originally identified in *M. tuberculosis* and have been associated with survival within macrophages and increased virulence (Arruda *et al.*, 1993; Flesselles *et al.*, 1999; Chitale *et al.*, 2001; Haile *et al.*, 2002; Harboe *et al.*, 2002; Kumar *et al.*, 2003; Sassetti and Rubin, 2003; Gioffre *et al.*, 2005). The *MAP* genome encodes 62 proteins containing conserved domains that have been associated with Mce proteins, while the *M. tuberculosis* genome contains 32 (Casali and Riley, 2007). These coding sequences are organized into eight clusters scattered around the *MAP* K-10 genome and share some of the characteristics of ATP-binding cassette (ABC) transport systems. Nonpathogenic environmental mycobacteria also contain *mce* genes, which further suggests that this gene family originally arose as part of a transport system that was later adapted for facilitating cellular invasion. The *mce* genes in pathogenic mycobacteria have been hypothesized to play a role in modulating the cell envelope (Casali and Riley, 2007). Notably, one of the *mce* gene clusters present in *MAP* (MAP2189 to MAP2194) is part of a large sequence polymorphism that is absent from the genomes of other closely related *M. avium* subspecies (Paustian *et al.*, 2005; Semret *et al.*, 2005; Wu *et al.*, 2006). The remainder of this polymorphic region comprises genes homologous to sequences in the *Frankia* strain EAN1-pec genome. The contribution of *mce* genes to *MAP* pathogenesis is not fully understood, but studies on homologous sequences in other mycobacteria suggest that they are likely to be important contributors.

8.4 Repetitive Sequences

The *MAP* genome encodes 12 proteins that are homologous to the REP13E12 family of repetitive elements originally described in *M. tuberculosis* (Gordon *et al.*, 1999). These elements have been shown to be targets of phage integration, and one of the copies (MAP1432) is absent from sheep isolates of *MAP* (Paustian *et al.*, 2008).

Insertion sequences (IS) are short segments of DNA that act as transposable elements. A total of 58 insertion sequences have been identified within the *MAP* genome, including 17 copies of IS*900*, 7 copies of IS*1311* and 3 copies of ISMav2 (L. Li *et al.*, 2005). In addition, two novel insertion sequences were originally identified within the *MAP* genome, although subsequent genome sequence projects have identified homologous sequences in other organisms. IS_MAP02 is present at six locations within the *MAP* genome and is approximately 80% identical to insertion sequences in *Mycobacterium vanbaalenii* and *Mycobacterium gilvum*, although the sequences in these organisms contain a deletion of approximately 100 bp relative to IS_MAP02. ISMAP_04 is present in four copies and is 80% identical to IS elements reported in the genomes of *Mycobacterium* sp. KMS, *Mycobacterium* sp. MCS, *M. avium* subsp. *hominissuis* 104, *Rhodococcus jostii*, *M. smegmatis* and *M. gilvum*.

The *MAP* genome was found to contain 185 mono-, di- and trinucleotide repeats (Amonsin *et al.*, 2004). While no biological implications due to these repetitive sequences have been observed, a subset have proven to be a useful tool for molecular epidemiology studies of *MAP* isolates (Motiwala *et al.*, 2004; Harris *et al.*, 2006) (see Collins, Chapter 25, this volume).

8.5 Unique Genes

The *MAP* K-10 genome contains several genes that are not found in any other organisms for which sequence information is currently available within public databases. Many of these unique coding sequences are grouped together in clusters and some include phage-related

sequences, which points to the route by which they were probably acquired. Initial efforts have been made both to identify these sequences and to determine if any encode proteins that are capable of eliciting an immune response (Bannantine *et al.*, 2002, 2004; Klitgaard Nielsen and Ahrens, 2002; Paustian *et al.*, 2004; Stabel and Bannantine, 2005). The unique nature of these sequences makes them fertile ground for the discovery of novel diagnostic targets and virulence factors. The sequences currently identified as being uniquely present within the *MAP* genome are listed in Table 8.2, and several of these coding sequences are discussed below.

MAP0094 has very low homology to a transcriptional regulator and is immediately adjacent to two genes encoding putative ABC transport proteins (MAP0092 and MAP0093). This suggests that these genes comprise an ABC transport system for a yet-to-be-determined substrate. Near this location is another unique ORF, MAP0099, which has only low homology to a hypothetical protein within the *M. smegmatis* genome.

A large collection of unique genes is located from MAP0851 through MAP0865. This gene cluster contains two genes with homology to phage sequences (MAP0854 and MAP0859c), as well as genes with low homology to an *Acidothermus cellulolyticus* hypothetical protein (MAP0862) and an FtsK-like protein (MAP0865). The remaining genes in this cluster have no homology to any publicly available sequences. Among these unique ORFs, protein domain searches identify functions related to sugar transport (MAP0852) and DNA repair (MAP 0855). The remaining genes encode proteins with no obvious functional domains and thus fail to shed further light on the role of this large cluster of unique sequences. MAP0860c and MAP0862 were cloned and expressed as fusion proteins, but only the latter was found to be immunogenic with sera from animals naturally and experimentally infected with *MAP* (Paustian *et al.*, 2005). This cluster of sequences is flanked on the 5′ end by two insertion sequences (IS*1311* and IS_MAP03) and on the 3′ end by a phage integrase gene (MAP0866) and a tRNA. In addition, the average G+C nucleotide composition of this cluster is 61%, which is significantly lower than the genome average of 69%. These features strongly suggest that this gene cluster was horizontally acquired by a phage-mediated recombination event.

MAP1220c has no homology to currently available sequences. It is part of a cluster of genes that extends from MAP1216c through MAP1219c, which are homologous to genes in the *M. avium* subsp. *hominissuis* 104 genome (MAV_3285 to MAV_3288). These genes encode hypothetical proteins and one putative lipoprotein (MAP1216c).

Another cluster of unique genetic features is present from MAP2149 to MAP2158. This group of sequences includes one with homology to a hypothetical protein from the *Pseudomonas viridiflava* genome (MAP2153). This region also contains four insertion sequences: IS*1311* (MAP2150), ISMAP13 (MAP2155), ISMav2 (MAP2156) and IS*900* (MAP2157), and is flanked on the 5′ end by a phage integrase gene. Similar to other unique gene clusters, the combination of mobile genetic elements and lower average G+C nucleotide composition (61% in this case) suggest a horizontal route of acquisition.

The region between MAP2751 and MAP2767 contains additional coding sequences uniquely present in the *MAP* K-10 genome. Like the other novel gene clusters, this group of sequences contains several phage-related genes (MAP2752, MAP2756c and MAP2766c) and is flanked at the 5′ and 3′ ends by four tRNAs and a phage integrase gene, respectively. The average G+C nucleotide composition is 65%. Several ORFs are homologous to genes encoding hypothetical proteins in other organisms (MAP2755, MAP2758, MAP2759 and MAP2765c), while the remainder have no homology to any publicly available sequences. Purified MAP2751 fusion protein was detected by sera from animals naturally and experimentally infected with *MAP*, while the protein encoded by MAP2753 was not (Bannantine *et al.*, 2004).

Another group of unique sequences is present between MAP3815 and MAP3817c. MAP3817c has sequence features similar to membrane proteins, while the other two ORFs are not homologous to any described sequences. Although a tRNA sequence is nearby, this cluster of sequences does not

Table 8.2. Unique coding sequences present in the *MAP* K-10 genome.

ORF	Size (bp)	Conserved domains
MAP0094	285	None
MAP0852	546	Sugar transport
MAP0853	660	Aldehyde dehydrogenase
MAP0855	945	DNA repair
MAP0856c	1728	None
MAP0857c	318	None
MAP0858	549	None
MAP0860c	891	None
MAP0861	342	None
MAP0863	675	FAD/NAD binding domain
MAP0864	426	None
MAP1220c	336	FAD/NAD binding domain
MAP1636c	474	None
MAP1731c	270	Decarboxylase
MAP2149c	645	None
MAP2151	438	None
MAP2152c	375	None
MAP2154c	576	None
MAP2158	582	None
MAP2751	582	None
MAP2753	759	None
MAP2754	258	Repressor
MAP2757	282	None
MAP2761c	717	None
MAP2762c	441	None
MAP2763c	309	None
MAP2764c	450	None
MAP2767c	552	Ferredoxin
MAP3437c	843	DNA binding
MAP3815	855	None
MAP3816	243	Phage integrase
MAP4267	363	None

display the hallmarks of phage insertion events that were observed for some of the other unique gene groups.

IS_MAP02 is a novel insertion sequence present at six locations within the *MAP* genome (MAP0338c, MAP2416c, MAP2502, MAP2566, MAP3357 and MAP3467c). While the transposase encoded within IS_MAP02 is similar to those found in other insertion sequences, the remainder of the sequence is unique to the *MAP* genome.

Other than the initial identification and immunological characterization, these unique genome features have not been subjected to a

rigorous examination by the scientific community. It remains to be determined whether or not these sequences represent 'genetic baggage' from previous genetic transfer events or keys to understanding the pathogenesis of paratuberculosis. The latter may be true, considering that these sequences distinguish *MAP* from other closely related and less pathogenic *M. avium* subspecies.

8.6 Summary

The *MAP* genome sequence has quickly been utilized in many areas of paratuberculosis research. While great strides have been made with the available sequence information, there is still a large segment of the genome (greater than 30%) that encodes sequences with no identified function. Thus much work remains to be done in order to elucidate further insights into *MAP* virulence and metabolism.

One of the trends observed in the *MAP* genome (and other non-tuberculosis mycobacteria) is the presence of genes with homology to sequences found in other high-G+C, Gram-positive *Actinomycetales*, including species of *Frankia*, *Acidothermus*, *Clavibacter*, *Streptomyces* and *Corynebacterium*. This reflects the shared soil environment inhabited by these organisms and is a reminder that many pathogenic mycobacteria such as *MAP* are not far removed from closely related, non-pathogenic organisms.

Advances in sequencing technology and functional genomics techniques should continue to provide opportunities for new insights into the biology of *MAP*. Additional *MAP* and mycobacterial sequencing projects are under way and should yield valuable information for comparative genomic studies. The challenge for researchers is to wade through the ongoing flood of data in order to identify the critical links that will lead to the development of novel and improved diagnostics, vaccines and management strategies.

References

Amonsin, A., Li, L.L., Zhang, Q., Bannantine, J.P., Motiwala, A.S., Sreevatsan., S. and Kapur, V. (2004) Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis* strains. *Journal of Clinical Microbiology* 42, 1694–1702.

Arruda, S., Bomfim, G., Knights, R., Huima-Byron, T. and Riley, L.W. (1993) Cloning of an *M. tuberculosis* DNA fragment associated with entry and survival inside cells. *Science* 261, 1454–1457.

Bannantine, J.P., Baechler, E., Zhang, Q., Li, L. and Kapur, V. (2002) Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. *Journal of Clinical Microbiology* 40, 1303–1310.

Bannantine, J.P., Hansen, J.K., Paustian, M.L., Amonsin, A., Li, L.L., Stabel, J.R. and Kapur, V. (2004) Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 42, 106–114.

Casali, N. and Riley, L.W. (2007) A phylogenomic analysis of the *Actinomycetales mce* operons. *BMC Genomics* 8, 60.

Chitale, S., Ehrt, S., Kawamura, I., Fujimura, T., Shimono, N., Anand, N., Lu, S., Cohen-Gould, L. and Riley, L.W. (2001) Recombinant *Mycobacterium tuberculosis* protein associated with mammalian cell entry. *Cellular Microbiology* 3, 247–254.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E. 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. and Barrell, B.G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393, 537–544.

Fleischmann, R.D., Alland, D., Eisen, J.A., Carpenter, L., White, O., Peterson, J., DeBoy, R., Dodson, R., Gwinn, M., Haft, D., Hickey, E., Kolonay, J.F., Nelson, W.C., Umayam, L.A., Ermolaeva, M., Salzberg, S.L., Delcher, A., Utterback, T., Weidman, J., Khouri, H., Gill, J., Mikula, A., Bishai, W., Jacobs, W.R., Jr., Venter., J.C. and Fraser, C.M. (2002) Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *Journal of Bacteriology* 184, 5479–5490.

Flesselles, B., Anand, N.N., Remani, J., Loosmore, S.M. and Klein, M.H. (1999) Disruption of the mycobacterial cell entry gene of *Mycobacterium bovis* BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. *FEMS Microbiology Letters* 177, 237–242.

Gey van Pittius, N.C., Sampson, S.L., Lee, H., Kim, Y., van Helden, P.D. and Warren, R.M. (2006) Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (*esx*) gene cluster regions. *BMC Evolutionary Biology* 6, 95.

Gioffre, A., Infante, E., Aguilar, D., Santangelo, M.P., Klepp, L., Amadio, A., Meikle, V., Etchechoury, I., Romano, M.I., Cataldi, A., Hernandez, R.P. and Bigi, F. (2005) Mutation in *mce* operons attenuates *Mycobacterium tuberculosis* virulence. *Microbes and Infection* 7, 325–334.

Gordon, S.V., Brosch, R., Billault, A., Garnier, T., Eiglmeier, K. and Cole, S.T. (1999) Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Molecular Microbiology* 32, 643–655.

Griffiths, T.A., Rioux, K. and De Buck, J. (2008) Sequence polymorphisms in a surface PPE protein distinguish types I, II, and III of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 46, 1207–1212.

Haile, Y., Caugant, D.A., Bjune, G. and Wiker, H.G. (2002) *Mycobacterium tuberculosis* mammalian cell entry operon (*mce*) homologs in *Mycobacterium* other than tuberculosis (MOTT). *FEMS Immunology and Medical Microbiology* 33, 125–132.

Harboe, M., Christensen, A., Ahmad, S., Ulvund, G., Harkness, R.E., Mustafa, A.S. and Wiker, H.G. (2002) Cross-reaction between mammalian cell entry (Mce) proteins of *Mycobacterium tuberculosis*. *Scandinavian Journal of Immunology* 56, 580–587.

Harris, N.B., Payeur, J.B., Kapur, V. and Sreevatsan, S. (2006) Short-sequence-repeat analysis of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* isolates collected from animals throughout the United States reveals both stability of loci and extensive diversity. *Journal of Clinical Microbiology* 44, 2970–2973.

Kerkhoven, R., van Enckevort, F.H., Boekhorst, J., Molenaar, D. and Siezen, R.J. (2004) Visualization for genomics: the Microbial Genome Viewer. *Bioinformatics* 20, 1812–1814.

Klitgaard Nielsen, K. and Ahrens, P. (2002) Putative *in vitro* expressed gene fragments unique to *Mycobacterium avium* subspecies *paratuberculosis*. *FEMS Microbiology Letters* 214, 199–203.

Kumar, A., Bose, M. and Brahmachari, V. (2003) Analysis of expression profile of mammalian cell entry (*mce*) operons of *Mycobacterium tuberculosis*. *Infection and Immunity* 71, 6083–6087.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S. and Kapur, V. (2005) The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proceedings of the National Academy of Sciences of the USA* 102, 12344–12349.

Li, Y., Miltner, E., Wu, M., Petrofsky, M. and Bermudez, L.E. (2005) A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. *Cellular Microbiology* 7, 539–548.

Marri, P.R., Bannantine, J.P. and Golding, G.B. (2006) Comparative genomics of metabolic pathways in *Mycobacterium* species: gene duplication, gene decay and lateral gene transfer. *FEMS Microbiology Reviews* 30, 906–925.

Marsh, I.B. and Whittington, R.J. (2005) Deletion of an *mmpL* gene and multiple associated genes from the genome of the S strain of *Mycobacterium avium* subsp. *paratuberculosis* identified by representational difference analysis and *in silico* analysis. *Molecular and Cellular Probes* 19, 371–384.

Marsh, I.B., Bannantine, J.P., Paustian, M.L., Tizard, M.L., Kapur, V. and Whittington, R.J. (2006) Genomic comparison of *Mycobacterium avium* subsp. *paratuberculosis* sheep and cattle strains by microarray hybridization. *Journal of Bacteriology* 188, 2290–2293.

Motiwala, A.S., Amonsin, A., Strother, M., Manning, E.J., Kapur, V. and Sreevatsan, S. (2004) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates recovered from wild animal species. *Journal of Clinical Microbiology* 42, 1703–1712.

Newton, V., McKenna, S.L. and De Buck, J. (2009) Presence of PPE proteins in *Mycobacterium avium* subsp. *paratuberculosis* isolates and their immunogenicity in cattle. *Veterinary Microbiology* 135, 394–400.

Paustian, M.L., Amonsin, A., Kapur, V. and Bannantine, J.P. (2004) Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's disease. *Journal of Clinical Microbiology* 42, 2675–2681.

Paustian, M.L., Kapur, V. and Bannantine, J.P. (2005) Comparative genomic hybridizations reveal genetic regions within the *Mycobacterium avium* complex that are divergent from *Mycobacterium avium* subsp. *paratuberculosis* isolates. *Journal of Bacteriology* 187, 2406–2415.

Paustian, M.L., Zhu, X., Sreevatsan, S., Robbe-Austerman, S., Kapur, V. and Bannantine, J.P. (2008) Comparative genomic analysis of *Mycobacterium avium* subspecies obtained from multiple host species. *BMC Genomics* 9, 135.

Ren, H. and Liu, J. (2006) AsnB is involved in natural resistance of *Mycobacterium smegmatis* to multiple drugs. *Antimicrobial Agents and Chemotherapy* 50, 250–255.

Sassetti, C.M. and Rubin, E.J. (2003) Genetic requirements for mycobacterial survival during infection. *Proceedings of the National Academy of Sciences of the USA* 100, 12989–12994.

Semret, M., Alexander, D.C., Turenne, C.Y., de Haas, P., Overduin, P., van Soolingen, D., Cousins, D. and Behr, M.A. (2005) Genomic polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* diagnostics. *Journal of Clinical Microbiology* 43, 3704–3712.

Semret, M., Turenne, C.Y., de Haas, P., Collins, D.M. and Behr, M.A. (2006) Differentiating host-associated variants of *Mycobacterium avium* by PCR for detection of large sequence polymorphisms. *Journal of Clinical Microbiology* 44, 881–887.

Stabel, J.R. and Bannantine, J.P. (2005) Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. *Journal of Clinical Microbiology* 43, 4744–4750.

Wu, C.W., Glasner, J., Collins, M., Naser, S. and Talaat, A.M. (2006) Whole-genome plasticity among *Mycobacterium avium* subspecies: insights from comparative genomic hybridizations. *Journal of Bacteriology* 188, 711–723.

Wu, C.W., Schramm, T.M., Zhou, S., Schwartz, D.C. and Talaat, A.M. (2009) Optical mapping of the *Mycobacterium avium* subspecies *paratuberculosis* genome. *BMC Genomics* 10, 25.

9 Molecular Genetics of *Mycobacterium avium* subsp. *paratuberculosis*

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9.1 Introduction

Molecular genetics plays a fundamental role in the study of pathogenic determinants and their potential use in the design of modern tools to prevent, diagnose and treat infectious diseases. As has been demonstrated for other pathogenic mycobacteria, the study of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) pathogenesis requires a genetic system for the introduction of specific mutations into genes in order to define virulence determinants. In addition, satisfaction of the molecular version of Koch's postulates requires a means to restore the wild-type phenotype in the mutant strains (Falkow, 1988, 2004). Comparison of the virulence phenotypes of wild-type, mutant and complemented strains in animal models or ruminant hosts would then provide a means to assess the contribution of any non-essential *MAP* gene to the pathogenicity of this subspecies (Chacon *et al.*, 2004). In this chapter, we will review approaches used for the genetic manipulation of *MAP* and assessment of *MAP* gene expression.

9.2 Development of a Genetic System

MAP is one of the slowest-growing mycobacterial species, with an estimated generation time of approximately 30 h (Lambrecht *et al.*,

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1988). This poses a formidable challenge to the development of an effective genetic transfer system, since the isolation of marker-positive recombinant strains requires maintenance of plate cultures for at least 6–8 weeks. This necessitates incubation of plates under appropriate conditions to prevent dehydration and facilitate the recovery of recombinant strains. Accepting that appropriate culture conditions can be provided to select for successful transformants, the next challenge involves the selection of the appropriate tools for the genetic manipulation of *MAP*.

9.2.1 Shuttle plasmid vectors

As conjugation systems using *Escherichia coli–Mycobacterium* shuttle plasmids and wide host range plasmids had been demonstrated in other mycobacteria (Lazraq *et al.*, 1990; Gormley and Davies, 1991), Foley-Thomas *et al.* (1995) used the *E. coli–Mycobacterium* shuttle plasmid pMV262 to transform several *MAP* strains. This plasmid is a derivative of the *Mycobacterium fortuitum* plasmid pAL5000, which carries the smallest number of nucleotide sequences necessary for mycobacterial replication (*oriM*) and the pUC19 high-copy-number replicon for *E. coli* (Fig. 9.1) (Snapper *et al.*, 1988; Stover *et al.*, 1991; Caceres *et al.*, 1997). The plasmid also carries the kanamycin-resistance gene derived from Tn*903*, which allows selection of recombinants in both *E. coli* and mycobacteria. To obtain transformants, *MAP* bacilli were grown to early exponential phase and concentrated, and plasmid DNA was introduced by electroporation. To obtain a greater number of transformants with a minimum number of spontaneous drug-resistant mutants required selection at high kanamycin concentrations. Further studies have demonstrated that hygromycin and apramycin can also be used for the selection of *MAP* transformants carrying pAL5000-derived plasmids (Rosseels *et al.*, 2006; Alonso-Hearn *et al.*, 2008). In general, transformation efficiencies are low, of the order of 1000 transformants or less per μg of plasmid DNA. These plasmids are maintained at about five copies per cell, though high-copy-number plasmid mutants have been recently developed but not yet tested in *MAP* (Stolt and Stoker, 1996, 1997; Bourn *et al.*, 2007; Mo *et al.*, 2007).

9.2.2 Integrating vectors

A series of vectors carrying the integrase gene and *attP* site of mycobacteriophage L5, but lacking *oriM*, have been developed (Hatfull and Sarkis, 1993). *MAP*, like other mycobacteria, possesses the chromosomal attachment site *attB* for this phage. Vectors carrying the L5 integrase and *attP* can recombine at the *attB* locus, resulting in the integration of plasmid sequences into the mycobacterial chromosome. To date, there are no reports on the use of these vectors in *MAP*. The low transformation efficiency of integrative vectors (100 to 1000 times lower than non-integrative vectors) could pose difficulties for obtaining this type of transformant in *MAP* (Snapper *et al.*, 1990).

9.2.3 Phages and phasmids

Since mycobacterial transformation is notoriously difficult (e.g. low transformation efficiency, scarcity of genetic markers), it is not surprising that researchers have introduced phage-mediated transduction processes as a means for introducing DNA into mycobacteria. The first clear demonstration of mycobacterial recombinant DNA generated by use of mycobacteriophage transduction was achieved by the use of shuttle phasmids (Jacobs *et al.*, 1987). Shuttle phasmids (phagemids) are chimeras of a mycobacteriophage and an *E. coli* cosmid that integrate into a non-essential region of the bacterial genome. These constructs can be readily manipulated in *E. coli* and provide a site for recombinant DNA packaging into *E. coli* bacteriophage lambda heads. The phage replication system present in these vectors allows for multiplication in mycobacteria. Various shuttle phasmids have been developed using several mycobacteriophages, including TM4, D29, L1 and L5 (Snapper *et al.*, 1988; Jacobs *et al.*, 1989a,b). Only TM4 and

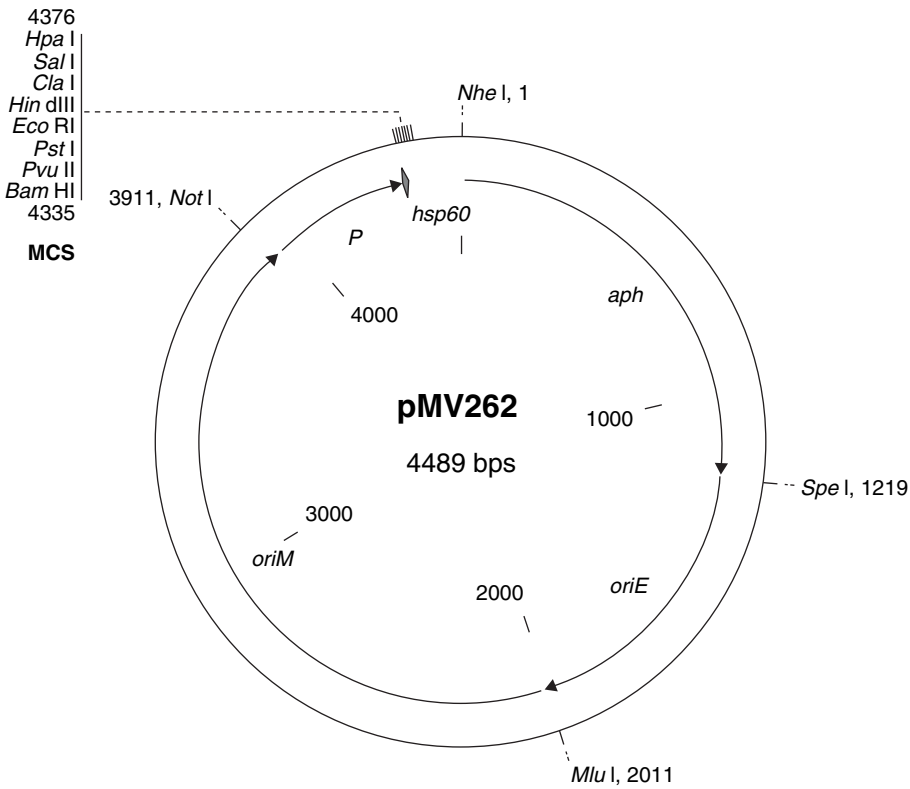

Fig. 9.1. Map of shuttle plasmid vector pMV262. The vector diagram indicates the *Mycobacterium fortuitum* plasmid pAL500 origin of replication (*oriM*) for autonomous replication in *MAP* and many other mycobacterial species, the high-copy-number origin of replication for *Escherichia coli* (*oriE*), a kanamycin-resistance selection marker (*aph*) functional in both *E. coli* and *Mycobacterium*, the *Mycobacterium bovis* BCG heat shock promoter (P), followed by the coding sequence of the first six amino acids of the Hsp60 protein (*hsp60*), and a multiple cloning site (MCS) immediately downstream. Restriction sites outflanking vector components and unique sites at the MCS are indicated. Arrows indicate directions of transcription of the corresponding genes/gene elements shown; or *repA* and *repB* for *oriM*, and *rep(pMB1)* for *oriE*.

shuttle phasmids derived from TM4 form plaques at high efficiency on *MAP* (Foley-Thomas *et al.*, 1995). Thus, these particular phasmids have played a prominent role in *MAP* genetic manipulation (Harris and Barletta, 2001; Chacon *et al.*, 2004).

Despite the fact that TM4 was originally isolated from a *M. avium* lysogen (Timme and Brennan, 1984), this mycobacteriophage replicates in a lytic fashion, forming clear plaques on *MAP* and other mycobacterial lawns (Jacobs *et al.*, 1987; Foley-Thomas *et al.*, 1995), which is helpful for genetic manipulations. An important feature of TM4 is that it infects all tested mycobacterial species, which enables TM4 shuttle phasmids to be propagated in non-pathogenic, fast-growing species, such as *Mycobacterium smegmatis*. Moreover, it has been demonstrated that TM4 propagated on *M. smegmatis* infects *MAP* with the same efficiency as TM4 propagated on *MAP* and vice versa (Foley-Thomas *et al.*, 1995). This further supports the use of TM4-derived shuttle phasmids to introduce a variety of recombination substrates into *MAP* (Fig. 9.2 and following sections).

9.3 Reporter Systems to Study Gene Expression

Gene expression in mycobacteria has been studied using reporting systems including the versatile *E. coli* beta-galactosidase (Barletta *et al.*, 1992; Murray *et al.*, 1992; Timm *et al.*, 1994; Knipfer *et al.*, 1998), green fluorescence protein (GFP) (Dhandayuthapani *et al.*, 1995; Kremer *et al.*, 1995), bacterial (Snewin *et al.*, 1999) and firefly luciferases (Jacobs *et al.*, 1993; Sarkis *et al.*, 1995), and catechol dioxygenase (Curcic *et al.*, 1994; Kenney and Churchward, 1996). So far, stable *MAP* transformants have been described for the GFP and luciferase-based reporter systems. In the studies noted below, the luciferase or GFP genes were expressed under the control of the mycobacterial heat shock promoter P*hsp60*. However, there is no a priori reason why this system could not be tailored for gene regulation studies to a gene of interest, by replacing the P*hsp60* with the promoter from a gene of interest. Transcriptomic analysis using DNA mircoarrays is described elsewhere in this book.

9.3.1 Firefly and bacterial luciferases

The first demonstration of the expression of firefly luciferase in *MAP* was performed with the reporter phasmid phAE39 derived from TM4 (Foley-Thomas *et al.*, 1995). After *MAP* infection, it was shown that the firefly luciferase was transcribed and translated during the phage replication cycle, although the kinetics of luciferase expression in *MAP* were slower than in *M. smegmatis* and *M. avium*. These findings indirectly point to transcription and translation occurring at a lower rate in *MAP*, potentially reflecting the slower rate of *MAP* multiplication. It should be noted that the luciferase expression in *MAP* is intracellular, since the protein is inactivated upon release into low pH assay medium. The same study also reported the stable expression of the firefly luciferase using a shuttle plasmid at sufficiently high levels to enable detection of a minimum of 300 luminescent bacilli. A prototype bioluminescent *MAP* of the sequenced strain K-10 was later used to develop a drug susceptibility assay based on light extinction that had a

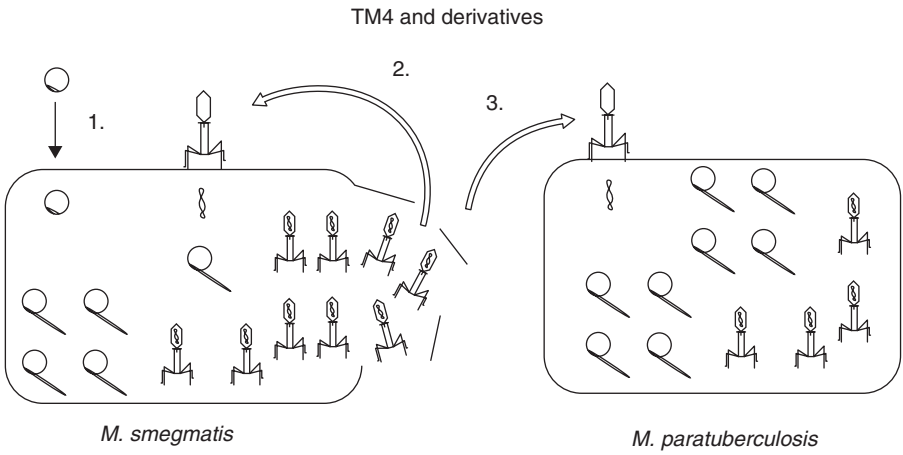

Fig. 9.2. Propagation of broad-range TM4 phage and derivatives. The use of the broad host range phage TM4 and its derivatives to facilitate genetic manipulations is illustrated. 1. Phage DNA is initially introduced in the low-pathogenic, fast-growing *Mycobacterium smegmatis* by transformation with recombinant *Escherichia coli* carrying a plasmid form of the phasmid. 2. High-titre phage stocks are generated in *M. smegmatis*. 3. Phage is used to infect *MAP*. Rolling-circle replication takes place within the mycobacterial host, followed by DNA packaging and release of viable phages upon bacterial lysis.

performance similar to broth macrodilution assays (Williams *et al.*, 1999). This strain may lend itself to automated drug resistance assays.

The two luciferase subunits from the bacterium *Vibrio harveyi* were also introduced into *MAP* using a shuttle plasmid derived from pAL5000 (Rosseels *et al.*, 2006). This recombinant *MAP* expressed levels of luciferase sufficient to estimate bacterial burden during murine infections, representing a significant reduction in time as compared with enumerating *MAP* bacilli by colony counting. Moreover, this bioluminescent *MAP* was used to test the effect of host factors influencing *MAP* survival by infecting various mice strains that carried mutations in genes related to mammalian host survival (Roupie *et al.*, 2008). An overall advantage of bacterial luciferase compared with the firefly luciferase is that it can achieve good levels of detection using less expensive equipment (Rosseels *et al.*, 2006).

9.3.2 Green fluorescence protein

The GFP protein from the algae *Aequorea victoria* was also expressed in *MAP* using a shuttle plasmid (Harris *et al.*, 2002). A plasmid optimized for expression in *M. avium* subsp. *hominissuis* was introduced into *MAP* K-10, yielding a relatively homogeneous population of fluorescent *MAP*. It was demonstrated that GFP-expressing *MAP* could be sorted out from non-fluorescent bacilli using a cell sorter apparatus, even after killing by formalin treatment, a procedure that reduced the fluorescence to approximately 50%, as compared with non-treated viable bacilli. More recently, it was also shown that gene-deletion mutants could be engineered using a strain already expressing optimal levels of GFP (see below) (Park *et al.*, 2008).

9.4 Identification of Gene Expression Signals

Gene expression is a complex process controlled by signals at both the transcriptional and translational levels. This control is mediated by microbial enzymatic and regulatory proteins that bind species-specific DNA or RNA sequences. Identification of these elements is essential to understand how microorganisms respond to environmental cues during infection and disease (Mekalanos, 1992). To identify these elements in *MAP*, Bannantine and colleagues employed a gene-fusion strategy. Chromosomal DNA fragments were cloned upstream from a promoterless truncated beta-galactosidase reporter gene in the fusion vector pYUB76 and introduced into *M. smegmatis* (Bannantine *et al.*, 1997). Activation of beta-galactosidase gene in this vector requires the upstream chromosomal fragment to provide a promoter, a ribosome-binding site and an N-terminal sequence in frame with the truncated reporter gene. Using this strategy, expression signals of different strength were putatively identified and further confirmed by primer extension and sequencing analyses.

Based on these data, a consensus sequence for the '-35 box' (the upstream promoter-binding box for RNA polymerase) was established as 5'-TGMCGT-3' and a weaker consensus of 5'-CGGCCS-3' for the proximal '-10' box, with intervening sequences of 16–20 nucleotides. The weak consensus and relatively high GC content at the -10 box is distinct from other bacteria, such as *E. coli*. Moreover, *MAP* promoters seem rather unique as compared with other mycobacteria, including *M. smegmatis* and *Mycobacterium tuberculosis* (Das Gupta *et al.*, 1993; Agarwal and Tyagi, 2006). None the less, as demonstrated by the gene-fusion strategy, at least a subset of *MAP* promoters is functional in *M. smegmatis*, although the molecular basis for these findings is unknown. In contrast, ribosome binding sites were all proximal to initiation codons of putative ORFs and were complementary to the 3' region of the *Mycobacterium leprae* 16S rRNA. In summary, the subset of *MAP* promoters that has been analysed displays unusual consensus sequences, especially regarding the weaker consensus at the -10 boxes.

9.5 Transposon Mutagenesis

The application of Koch's molecular postulates towards the study of *MAP* has been enabled by the development of an efficient transposition mutagenesis system. In a previous section, we described the use of a shuttle

phasmid derived from mycobacteriophage TM4 to express the firefly luciferase gene in *MAP*. A further modification on this technology was the introduction of a thermosensitive derivative of TM4, such that newly constructed shuttle phasmids were able to propagate at the permissive temperature (30 °C) but unable to replicate at the non-permissive temperature (37 °C) (Bardarov *et al.*, 1997). Moreover, these vectors can be engineered to carry either a transposable element or a recombination substrate (see sections on gene replacement). Since the phasmid cannot replicate, survival of the transposable element at the non-permissive temperature requires its spontaneous insertion into *MAP* genomic DNA (Fig. 9.3). Phasmid phAE94 was engineered with transposon Tn*5367*, which carries the transposase of the *M. smegmatis* insertion element IS*1096* (Cirillo *et al.*, 1991) and the kanamycin-resistance gene from transposon Tn*903* (McAdam *et al.*, 1995). Using phAE94, two independent groups demonstrated that transduction of *MAP* with this phage yielded a large number of transposon mutants at the non-permissive temperature (Harris *et al.*, 1999; Cavaignac *et al.*, 2000).

In one of these studies, a total of approximately 5000 independent transposon mutants of strain K-10 were obtained and verified (Harris *et al.*, 1999). The focus of this study was on the maximization and characterization of the transposition process in *MAP*. Transposon insertions were found to result in unique 8 bp target DNA duplications, with the deduced consensus sequence of the target site being 5′-Py(A/T)A(A/T)-3′. This non-stringent consensus suggested that Tn*5367* can transpose fairly randomly in the *MAP* genome, a property that we designate as macrorandomness for the discussion below. This K-10 transposon library has now been expanded to a comprehensive collection of 13,500 mutants, individually stocked (Harris and Barletta, 2001) for a full representation of the *MAP* genome ($P > 95\%$) under the assumption of random transposition.

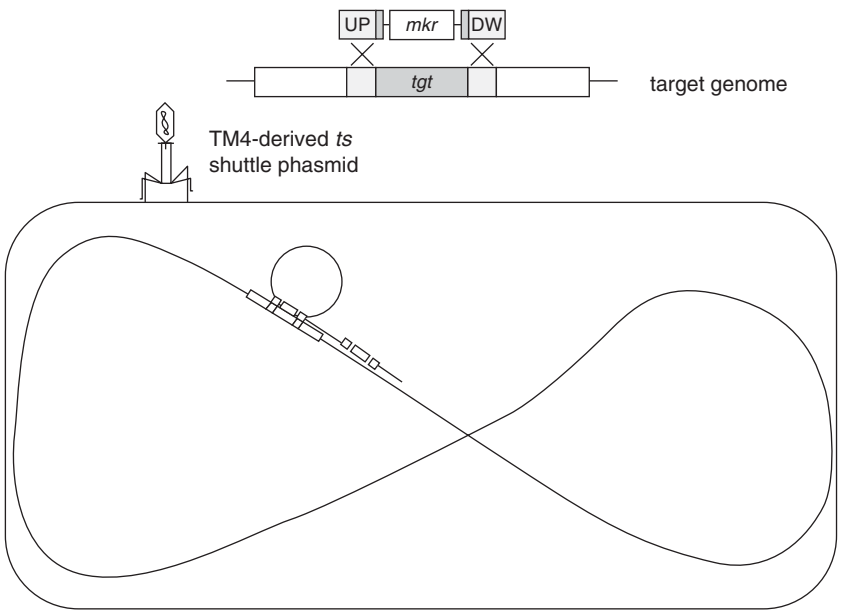

Fig. 9.3. Use of TM4-derived shuttle phasmids for allelic exchange in *MAP*. A thermosensitive (*ts*) derivative of TM4 engineered to carry an allelic exchange recombination substrate is illustrated. The substrate carries a marker gene (*mkr*) outflanked by DNA sequences upstream (UP) and downstream (DW) from the gene targeted for inactivation (*tgt*), with UP and DW usually protruding minimally into the *tgt* gene, as depicted by the darker shading. The phasmid can be propagated at the permissive temperature, as illustrated in Fig. 9.2. At the non-permissive temperature, selection for Mkr^+ transductants results in allelic exchange generating *tgt* deletion mutants.

In the other study, transduction with phAE94 was carried out using a New Zealand cattle strain (989) and a different reference strain (TMC1613) (Cavaignac *et al.*, 2000). The focus in this study was on screening procedures that could result in the identification of attenuated mutants. Mutants were screened by auxotrophy using 7H9 liquid minimal medium, carbon-source utilization and altered Congo red uptake (as an indicator of cell surface alterations). As this study was conducted before the sequence of *MAP* K-10 was published, mutants were determined to be widely distributed in the *MAP* genome, as inferred by positions of the *M. tuberculosis* homologues and the assumed co-linearity of the two mycobacterial genomes.

A more recent development was the use of strain ATCC19698 to create a library of 5000 mutants by transduction with phAE94 (Shin *et al.*, 2006). The focus of this study was the use of high-throughput sequencing of transposon mutant strains. This allowed a more stringent definition of the target consensus as areas of AT or TA repeats such as 5′-TTT(T/A)-3′, 5′-AA(A/T)-3′ or 5′-TTA-3′. Furthermore, completion of the *MAP* genome sequence allowed for a more conclusive demonstration of macrorandomness. However, this study also indicated hot-spot areas for the transposition of Tn*5367*, similar to the results obtained in *M. tuberculosis* for the transposition of the closely related element Tn*5370* (McAdam *et al.*, 2002). This transposition preference may leave genomic regions underrepresented in the corresponding mutant pool. A bioinformatic approach used to classify mutants by functional categories (based on *M. tuberculosis* homologues) provided a first screen for mutants potentially attenuated for survival. To test this, mutants with transposon insertions in *gcpE*, *pstA*, *kdpC*, *papA2*, *impA*, *umaA1* or *fabG2_2* were studied in a murine model, and demonstrated reduced bacterial replication. The attenuated phenotype of the *gcpE* insertion mutant was further confirmed in a surgical calf model of infection (Wu *et al.*, 2007a).

Another approach to transposition mutagenesis is the use of a thermosensitive shuttle plasmid as a vector for the delivery of transposons or recombination substrates (Pelicic *et al.*, 1997). In this approach, a stronger counter-selection pressure is required, namely the bactericidal effect of sucrose and a non-permissive temperature. The bactericidal effect of sucrose requires that the plasmid carry a sucrase gene (*sacB* from *Bacillus subtilis*), whose expression in the presence of sucrose is lethal. Using this approach to deliver Tn*5367* into *MAP*, a library of 2000 mutants of strain ATCC19698 was obtained (Alonso-Hearn *et al.*, 2008). The focus in this study was to screen the library for mutants attenuated in their ability to invade a line (MDBK) of bovine epithelial cells. Screening of a subset of 600 mutants resulted in the identification of five mutants with significantly lower invasion efficiencies. A mutant with an insertion in the ORF MAP3464 was further characterized. This gene encodes an NADH-dependent flavin oxidoreductase. Expression of this gene was shown to be upregulated upon bacterial contact with MDBK cells. In contrast to the wild-type control, the mutant failed to activate the Cdc42 signal transduction pathway involved in actin polymerization.

9.6 Gene Replacement by Homologous Recombination

Shuttle phasmids can also be engineered to enable allelic exchange by cloning regions upstream and downstream from the target gene of interest at either side of a marker gene (e.g. hygromycin) (Bardarov *et al.*, 2002). This technology has been applied to *MAP* to create deletion mutants of strain K-10 in the genes *pknG*, *relA* and *lsr*2 (Park *et al.*, 2008) and *lipN* (Wu *et al.*, 2007b). These studies demonstrated the feasibility of using allelic exchange to generate targeted gene disruption in *MAP*.

Importantly, the application of this technology to *MAP* required intense experimentation to overcome difficulties associated with *MAP*-specific properties, especially those regarding drug susceptibility and slow growth. Use of the standard transduction method (Bardarov *et al.*, 2002) yielded few allelic exchange mutants with the specialized transduction phage used to inactivate the *pknG* gene and no mutants for the inactivation of the *relA* gene (Park *et al.*, 2008). This problem was overcome by carefully controlling the

preparation of *MAP* cells for transduction, to minimize cell clumping and avoid cell damage. In addition, the concentration of hygromycin for selection of transductants was increased, to avoid the isolation of spontaneous drug-resistant mutants. These modifications increaed the frequency of allelic exchange up to 100% and the overall transduction frequency to 3.0×10^{-7}. The method demonstrated the possibility of creating both small and larger deletions in the range of 0.3–1.8 kb. Moreover, similar allelic exchange frequencies were obtained using a recombinant K-10 strain expressing GFP, thus allowing the creation of mutant strains that could be tracked by fluorescence methods. The *lipN* gene knockout mutant was obtained by the standard method described by Bardarov *et al.* (2002). This latter study focused on the transcriptional properties of the mutant strain but no transduction frequencies were provided.

9.7 Complementation Systems

A complementation system is fundamental for the verification that the gene mutated is responsible for the change in phenotype. This is particularly important in the case of transposon mutants, since insertions may have polar effects. Difficulties may arise in complementation experiments. For example, *MAP* mutant strains may transform at lower frequencies than their corresponding wild-type parents. The need for two selectable markers, one to create the mutation and another to select for the complementing plasmid, may also influence, hopefully minimally, the phenotypes of the recombinant strains. A method to unmark the original mutation may ameliorate this problem, allowing the use of one selection marker for the complementation step. This procedure has been applied to *M. bovis*, *M. smegmatis* and *M. tuberculosis*, but the slow growth of *MAP* could make its application to *MAP* more challenging (Bardarov *et al.*, 2002).

Successful integration of single-copy genes into *MAP* has not yet been reported (see section on integrating vectors) and this may be either because of the increased difficulty of carrying out this procedure in *MAP* or because at this stage few people have attempted the procedure. The use of multi-copy shuttle plasmids is currently the main option (see also concluding remarks). As described above, there are three markers that can be used: kanamycin, hygromycin and apramycin.

Currently, there is only one study showing successful complementation of the mutant strain with the wild-type gene. In this case, complementation of a Tn*5367* kanamycin-resistant mutant in MAP3464 was performed with a multi-copy shuttle plasmid carrying an apramycin-resistant marker (Alonso-Hearn *et al.*, 2008). Complementation was shown to fully restore the attenuated invasion phenotype of the mutant strain.

9.8 Concluding Remarks

The developments described in this chapter indicate that *MAP* researchers possess the basic tools to create gene fusions and transposon mutant banks, inactivate a target gene by allelic exchange, and perform complementation and gene expression analyses. However, the development of enhanced molecular genetic techniques has the potential to increase the efficiency of the study of *MAP*. Some of these technologies are either established in other mycobacterial systems or the focus of ongoing research. While promising, their application to *MAP* may not be straightforward.

Among these technologies, we may cite the creation of improved mutant libraries. Currently available libraries are based on Tn*5367*, which carries its own transposase within the integrating element, with the potential for mutant instability. In this context, stable insertion derivatives such as Tn*5370* have been developed for *M. tuberculosis*, including the isolation of signature tagged mutants (McAdam *et al.*, 1990; Cox *et al.*, 1999), but its application to *MAP* has not yet been described. Likewise, a more random transposon may be needed to create mutants in GC-rich regions more likely completely under-represented or absent from current libraries. Mariner-derived transposons and transposon-site hybridization

mutagenesis may help to breach this gap and uncover *MAP* genes that are physiologically essential or conditionally essential to a virulence attribute such as invasion or intracellular survival (Sassetti *et al.,* 2001). In this context, the use of the mariner transposon system has been recently reported in *MAP* and applied to the isolation of mutants attenuated in bovine macrophages (Scandurra *et al.,* 2009). Finally, it would be desirable to develop a more controllable single-copy complementation system and techniques for constructing unmarked in-frame deletion mutants (Bardarov *et al.,* 2002).

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References

Agarwal, N. and Tyagi, A.K. (2006) Mycobacterial transcriptional signals: requirements for recognition by RNA polymerase and optimal transcriptional activity. *Nucleic Acids Research* 34, 4245–4257.

Alonso-Hearn, M., Patel, D., Danelishvili, L., Meunier-Goddik, L. and Bermudez, L.E. (2008) The *Mycobacterium avium* subsp. *paratuberculosis* MAP3464 gene encodes an oxidoreductase involved in invasion of bovine epithelial cells through the activation of host cell Cdc42. *Infection and Immunity* 76, 170–178.

Bannantine, J.P., Barletta, R.G., Thoen, C.O. and Andrews, R.E., Jr. (1997) Identification of *Mycobacterium paratuberculosis* gene expression signals. *Microbiology* 143, 921–928.

Bardarov, S., Kriakov, J., Carriere, C., Yu, S., Vaamonde, C., McAdam, R.A., Bloom, B. R., Hatfull, G.F. and Jacobs, W.R., Jr. (1997) Conditionally replicating mycobacteriophages: a system for transposon delivery to *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the USA* 94, 10961–10966.

Bardarov, S., Bardarov, S., Jr., Pavelka, M.S., Jr., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G. and Jacobs, W.R., Jr. (2002) Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology* 148, 3007–3017.

Barletta, R.G., Kim, D.D., Snapper, S.B., Bloom, B.R. and Jacobs, W.R., Jr. (1992) Identification of expression signals of the mycobacteriophages Bxb1, L1 and TM4 using the *Escherichia–Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusions to the *lacZ* gene. *Journal of General Microbiology* 138, 23–30.

Bourn, W.R., Jansen, Y., Stutz, H., Warren, R.M., Williamson, A.L. and van Helden, P.D. (2007) Creation and characterisation of a high-copy-number version of the pAL5000 mycobacterial replicon. *Tuberculosis* 87, 481–488.

Caceres, N.E., Harris, N.B., Wellehan, J.F., Feng, Z., Kapur, V. and Barletta, R.G. (1997) Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. *Journal of Bacteriology* 179, 5046–5055.

Cavaignac, S.M., White, S.J., de Lisle, G.W. and Collins, D.M. (2000) Construction and screening of *Mycobacterium paratuberculosis* insertional mutant libraries. *Archives of Microbiology* 173, 229–231.

Chacon, O., Bermudez, L.E. and Barletta, R.G. (2004) Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. *Annual Reviews of Microbiology* 58, 329–363.

Cirillo, J.D., Barletta, R.G., Bloom, B.R. and Jacobs, W.R., Jr. (1991) A novel transposon trap for mycobacteria: isolation and characterization of IS*1096*. *Journal of Bacteriology* 173, 7772–7780.

Cox, J.S., Chen, B., McNeil, M. and Jacobs, W.R., Jr. (1999) Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* 402, 79–83.

Curcic, R., Dhandayuthapani, S. and Deretic, V. (1994) Gene expression in mycobacteria: transcriptional fusions based on *xylE* and analysis of the promoter region of the response regulator *mtrA* from *Mycobacterium tuberculosis*. *Molecular Microbiology* 13, 1057–1064.

Das Gupta, S.K., Bashyam, M.D. and Tyagi, A.K. (1993) Cloning and assessment of mycobacterial promoters by using a plasmid shuttle vector. *Journal of Bacteriology* 175, 5186–5192.

Dhandayuthapani, S., Via, L.E., Thomas, C.A., Horowitz, P.M., Deretic, D. and Deretic, V. (1995) Green fluorescent protein as a marker for

gene expression and cell biology of mycobacterial interactions with macrophages. *Molecular Microbiology* 17, 901–912.

Falkow, S. (1988) Molecular Koch's postulates applied to microbial pathogenicity. *Reviews of Infectious Diseases* 10 (Suppl. 2), S274–S276.

Falkow, S. (2004) Molecular Koch's postulates applied to bacterial pathogenicity – a personal recollection 15 years later. *Nature Reviews Microbiology* 2, 67–72.

Foley-Thomas, E.M., Whipple, D.L., Bermudez, L.E. and Barletta, R.G. (1995) Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. *Microbiology* 141, 1173–1181.

Gormley, E.P. and Davies, J. (1991) Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. *Journal of Bacteriology* 173, 6705–6708.

Harris, N.B. and Barletta, R.G. (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clinical Microbiology Reviews* 14, 489–512.

Harris, N.B., Feng, Z., Liu, X., Cirillo, S.L., Cirillo, J.D. and Barletta, R.G. (1999) Development of a transposon mutagenesis system for *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiology Letters* 175, 21–26.

Harris, N.B., Zinniel, D.K., Hsieh, M.K., Cirillo, J.D. and Barletta, R.G. (2002) Cell sorting of formalin-treated pathogenic *Mycobacterium paratuberculosis* expressing GFP. *Biotechniques* 32, 522–527.

Hatfull, G.F. and Sarkis, G.J. (1993) DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. *Molecular Microbiology* 7, 395–405.

Jacobs, W.R., Jr., Tuckman, M. and Bloom, B.R. (1987) Introduction of foreign DNA into mycobacteria using a shuttle phasmid. *Nature* 327, 532–535.

Jacobs, W.R., Jr., Snapper, S.B., Lugosi, L., Jekkel, A., Melton, R.E., Kieser, T. and Bloom, B.R. (1989a) Development of genetic systems for the mycobacteria. *Acta Leprologica* 7 (Suppl. 1), 203–207.

Jacobs, W.R., Jr., Snapper, S.B., Tuckman, M. and Bloom, B.R. (1989b) Mycobacteriophage vector systems. *Reviews of Infectious Diseases* 11 (Suppl. 2), S404–S410.

Jacobs, W.R., Jr., Barletta, R.G., Udani, R., Chan, J., Kalkut, G., Sosne, G., Kieser, T., Sarkis, G.J., Hatfull, G.F. and Bloom, B.R. (1993) Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 260, 819–822.

Kenney, T.J. and Churchward, G. (1996) Genetic analysis of the *Mycobacterium smegmatis rpsL* promoter. *Journal of Bacteriology* 178, 3564–3571.

Knipfer, N., Nooruddin, L. and Shrader, T.E. (1998) Development of an alpha-complementation system for mycobacterial promoter analysis. *Gene* 217, 69–75.

Kremer, L., Baulard, A., Estaquier, J., Poulain-Godefroy, O. and Locht, C. (1995) Green fluorescent protein as a new expression marker in mycobacteria. *Molecular Microbiology* 17, 913–922.

Lambrecht, R.S., Carriere, J.F. and Collins, M.T. (1988) A model for analyzing growth kinetics of a slowly growing *Mycobacterium* sp. *Applied and Environmental Microbiology* 54, 910–916.

Lazraq, R., Clavel-Seres, S., David, H.L. and Rouland-Dussoix, D. (1990) Conjugative transfer of a shuttle plasmid from *Escherichia coli* to *Mycobacterium smegmatis* [corrected]. *FEMS Microbiology Letters* 57, 135–138.

McAdam, R.A., Hermans, P.W., van Soolingen, D., Zainuddin, Z.F., Catty, D., van Embden, J.D. and Dale, J.W. (1990) Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS*3* family. *Molecular Microbiology* 4, 1607–1613.

McAdam, R.A., Weisbrod, T.R., Martin, J., Scuderi, J.D., Brown, A.M., Cirillo, J.D., Bloom, B.R. and Jacobs, W.R., Jr. (1995) *In vivo* growth characteristics of leucine and methionine auxotrophic mutants of *Mycobacterium bovis* BCG generated by transposon mutagenesis. *Infection and Immunity* 63, 1004–1012.

McAdam, R.A., Quan, S., Smith, D.A., Bardarov, S., Betts, J.C., Cook, F.C., Hooker, E.U., Lewis, A.P., Woollard, P., Everett, M.J., Lukey, P.T., Bancroft, G.J., Jacobs, W.R., Jr. and Duncan, K. (2002) Characterization of a *Mycobacterium tuberculosis* H37Rv transposon library reveals insertions in 351 ORFs and mutants with altered virulence. *Microbiology* 148, 2975–2986.

Mekalanos, J.J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. *Journal of Bacteriology* 174, 1–7.

Mo, Y., Quanquin, N.M., Vecino, W.H., Ranganathan, U.D., Tesfa, L., Bourn, W., Derbyshire, K.M., Letvin, N.L., Jacobs, W.R., Jr. and Fennelly, G.J. (2007) Genetic alteration of *Mycobacterium smegmatis* to improve mycobacterium-mediated transfer of plasmid DNA into mammalian cells and DNA immunization. *Infection and Immunity* 75, 4804–4816.

Murray, A., Winter, N., Lagranderie, M., Hill, D.F., Rauzier, J., Timm, J., Leclerc, C., Moriarty,

K.M., Gheorghiu, M. and Gicquel, B. (1992) Expression of *Escherichia coli* beta-galactosidase in *Mycobacterium bovis* BCG using an expression system isolated from *Mycobacterium paratuberculosis* which induced humoral and cellular immune responses. *Molecular Microbiology* 6, 3331–3342.

Park, K.T., Dahl, J.L., Bannantine, J.P., Barletta, R.G., Ahn, J., Allen, A.J., Hamilton, M.J. and Davis, W.C. (2008) Demonstration of allelic exchange in the slow-growing bacterium *Mycobacterium avium* subsp. *paratuberculosis*, and generation of mutants with deletions at the *pknG*, *relA*, and *lsr2* loci. *Applied and Environmental Microbiology* 74, 1687–1695.

Pelicic, V., Jackson, M., Reyrat, J.M., Jacobs, W.R., Jr., Gicquel, B. and Guilhot, C. (1997) Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the USA* 94, 10955–10960.

Rosseels, V., Roupie, V., Zinniel, D., Barletta, R.G. and Huygen, K. (2006) Development of luminescent *Mycobacterium avium* subsp. *paratuberculosis* for rapid screening of vaccine candidates in mice. *Infection and Immunity* 74, 3684–3686.

Roupie, V., Rosseels, V., Piersoel, V., Zinniel, D.K., Barletta, R.G. and Huygen, K. (2008) Genetic resistance of mice to *Mycobacterium paratuberculosis* is influenced by Slc11a1 at the early but not at the late stage of infection. *Infection and Immunity* 76, 2099–2105.

Sarkis, G.J., Jacobs, W.R., Jr. and Hatfull, G.F. (1995) L5 luciferase reporter mycobacteriophages: a sensitive tool for the detection and assay of live mycobacteria. *Molecular Microbiology* 15, 1055–1067.

Sassetti, C.M., Boyd, D.H. and Rubin, E.J. (2001) Comprehensive identification of conditionally essential genes in mycobacteria. *Proceedings of the National Academy of Sciences of the USA* 98, 12712–12717.

Scandurra, G.M., Young, M., de Lisle, G.W. and Collins, D.M. (2009) A bovine macrophage screening system for identifying attenuated transposon mutants of *Mycobacterium avium* subsp. *paratuberculosis* with vaccine potential. *Journal of Microbiological Methods* 77, 58–62.

Shin, S.J., Wu, C.W., Steinberg, H. and Talaat, A.M. (2006) Identification of novel virulence determinants in *Mycobacterium paratuberculosis* by screening a library of insertional mutants. *Infection and Immunity* 74, 3825–3833.

Snapper, S.B., Lugosi, L., Jekkel, A., Melton, R.E., Kieser, T., Bloom, B.R. and Jacobs, W.R., Jr. (1988) Lysogeny and transformation in mycobacteria: stable expression of foreign genes. *Proceedings of the National Academy of Sciences of the USA* 85, 6987–6991.

Snapper, S.B., Melton, R.E., Mustafa, S., Kieser, T. and Jacobs, W.R., Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Molecular Biology* 4, 1911–1919.

Snewin, V.A., Gares, M.P., O'Gaora, P., Hasan, Z., Brown, I.N. and Young, D.B. (1999) Assessment of immunity to mycobacterial infection with luciferase reporter constructs. *Infection and Immunity* 67, 4586–4593.

Stolt, P. and Stoker, N.G. (1996) Functional definition of regions necessary for replication and incompatibility in the *Mycobacterium fortuitum* plasmid pAL5000. *Microbiology* 142, 2795–2802.

Stolt, P. and Stoker, N.G. (1997) Mutational analysis of the regulatory region of the *Mycobacterium* plasmid pAL5000. *Nucleic Acids Research* 25, 3840–3846.

Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., Snapper, S.B., Barletta, R.G., Jacobs, W.R. and Bloom, B.R. (1991) New use of BCG for recombinant vaccines. *Nature* 351, 456–460.

Timm, J., Lim, E.M. and Gicquel, B. (1994) *Escherichia coli*–mycobacteria shuttle vectors for operon and gene fusions to *lacZ*: the pJEM series. *Journal of Bacteriology* 176, 6749–6753.

Timme, T.L. and Brennan, P.J. (1984) Induction of bacteriophage from members of the *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum* serocomplex. *Journal of General Microbiology* 130, 2059–2066.

Williams, S.L., Harris, N.B. and Barletta, R.G. (1999) Development of a firefly luciferase-based assay for determining antimicrobial susceptibility of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 37, 304–309.

Wu, C.W., Livesey, M., Schmoller, S.K., Manning, E.J., Steinberg, H., Davis, W.C., Hamilton, M.J. and Talaat, A.M. (2007a) Invasion and persistence of *Mycobacterium avium* subsp. *paratuberculosis* during early stages of Johne's disease in calves. *Infection and Immunity* 75, 2110–2119.

Wu, C.W., Schmoller, S.K., Shin, S.J. and Talaat, A.M. (2007b) Defining the stressome of *Mycobacterium avium* subsp. *paratuberculosis in vitro* and in naturally infected cows. *Journal of Bacteriology* 189, 7877–7886.

10 Proteome and Antigens of *Mycobacterium avium* subsp. *paratuberculosis*

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10.1 Introduction

In spite of new molecular and immunological methods for diagnosis and vaccine discovery, Johne's disease (JD) remains a major problem, with increasing prevalence worldwide. An important tool in Johne's control would be a simple, rapid and inexpensive assay to detect infected animals or herds. Although enzyme-linked immunosorbent assays (ELISAs) present many of these attributes, currently available tests suffer from limitations in sensitivity and specificity that undermine their potential utility in disease control. Despite this, the inherent benefits of ELISA tests have led to enormous efforts being made for discovery of antigens from *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) that could be used in such a test format. In fact, much of the field of *MAP* proteomics has been driven by the premise that the existing platforms can be readily adapted, using *MAP*-specific antigens, to offer newer and more accurate assays.

This chapter reviews what is known about the proteome of this significant veterinary pathogen by discussing the use of various whole-cell preparations and fractionated components thereof. With improvements in technology, many new modifications to the traditional ELISA assay have been introduced,

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including flow cytometry and assays based on surface-enhanced Raman scattering (SERS). Also, new antigen extracts have been created that appear to improve on the current ELISA test, which has historically used a whole-cell sonicated extract as the antigen. Particularly promising is the EV-ELISA extract, with potential sensitivities above 90%. Recombinant protein expression strategies have gained new momentum as well. The construction and use of the first protein array for *MAP* are one such application that has flowed from this strategy and will be discussed at the end of this chapter.

10.2 Study of *MAP* Proteins

Proteins are generally studied in one of two ways. In function studies, proteins are assessed for a particular property, such as the kinetics of an enzyme or the role it performs in a metabolic pathway. In applied studies, proteins are evaluated for their ability to elicit protection in the host or their utility as a specific antigen in diagnostics. For the ruminant pathogen *MAP*, very few studies have been performed that address the former, yet several studies have been published which fulfil the latter. Because a well-defined and strongly immunogenic antigen can serve both diagnostic needs and vaccination, tremendous efforts have been invested in order to identify the antigen that best serves these purposes for any given pathogen. A survey of the literature shows that no less effort has been put forth to identify antigens of *MAP*, which causes JD. Approximately 250 *MAP* proteins have been evaluated immunologically in some manner. This chapter focuses on applied studies of *MAP* proteins.

Ideally, proteomics is the study of all the proteins produced by an organism. However, in practice, only a subset of these proteins can truly be analysed at any one time, owing to current methodological limitations. Therefore, investigators must divide or fractionate the proteome into more manageable subsets to enable the mass spectrometer to 'see' all the proteins present or enable a polyacrylamide gel to resolve all the proteins in the fractionated sample. From the published genome sequence of *MAP* strain K-10, we now know that there are 4350 open reading frames (Li *et al.*, 2005). If we exclude the 45 tRNA genes and one rRNA sequence, which do not encode proteins, we are left with just over 4300 coding sequences. Therefore, in order to analyse the entire proteome, this is the target number of proteins that investigators should strive to resolve. In more practical terms, technology has not advanced enough to make this ideal a reality. As a result, this chapter will focus on the fractions of the proteome that have undergone analysis and how this information may aid in antigen-based detection of *MAP*.

10.3 Antigen Preparations

Several types of *MAP* antigen preparations have been evaluated and used for serodiagnosis of JD for over two decades (Nielsen and Toft, 2008). These unpurified mycobacterial extracts or fractionated preparations naturally contain multiple antigens. This poses a specificity problem for current serological tests (see Nielsen, Chapter 24, this volume), as crude extracts contain mixtures of *MAP* proteins along with abundant polysaccharide antigens, consisting predominantly of arabinomannan and arabinogalactan. Since these antigenic components are often conserved across the genus, the resulting assays suffer from cross-reactivity. The protoplasmic antigen preparation (PPA) and lipoarabinomannan (LAM), each of which will be described in more detail below, have been adapted to commercial ELISAs (Yokomizo *et al.*, 1983; Sugden *et al.*, 1987). Diagnostic sensitivities of the commercial ELISAs were reported to be around 60% in the early 1990s, but more recent studies report sensitivity as low as 15–20% (Whitlock *et al.*, 2000; Nielsen and Toft, 2008; Shin *et al.*, 2008). Of note, the sensitivity of ELISAs in most of the published reports was evaluated against a positive faecal culture as the reference test, and this apparent decrease in ELISA sensitivity may instead reflect improved sensitivity of faecal culture in the 1990s (Whitlock and Rosenberger, 1990). The true sensitivity of these

ELISA-based tests using PPA and LAM remains an issue for further evaluation.

10.4 Protoplasmic Antigen (PPA)

PPA is a crude antigen mixture prepared by thorough physical disruption of mycobacteria followed by removal of cell debris and cell wall components (Beam *et al.*, 1969). The PPA preparation method described by Beam *et al.* (1969) included pressure disruption, ultracentrifugation, filtration and delipidation steps. Other investigators have prepared PPA by simply disrupting the bacilli with prolonged sonication and removing large, insoluble particles by centrifugation. For this reason, PPA has also been called sonicate antigen (Waters *et al.*, 2003; Bannantine *et al.*, 2008a) or cell extract (Cho and Collins, 2006). Although PPA preparation is commercially available from Allied Monitor and has been used in several reports on JD diagnosis, the preparation protocol is proprietary. Regardless of the differences in PPA preparation protocols, it is likely that PPA contains mostly soluble cell-associated proteins.

In 1983, Yokomizo *et al.* (1983) developed an ELISA test for JD by using PPA, and since then this antigen preparation has been used in commercial ELISA kits and reported widely in the literature (Nielsen and Toft, 2008). In follow-up studies, Yokomizo reported that the ELISA test had false-positive results caused by cross-reactive antibodies and that pre-absorption of serum samples with *Mycobacterium phlei* reduced the false positives (Yokomizo *et al.*, 1985). This pre-absorption step is still being used in the current ELISA tests (Shin *et al.*, 2008). However, a careful and comprehensive search for a better absorbent has not been reported and may be a goal for future research. The cross-reaction of bovine antibodies to PPA seems unavoidable since the preparation contains numerous cytosolic proteins that are involved in well-conserved biochemical processes, such as transcription and lipid biosynthesis.

Some attempts have been made to purify specific antigens in PPA. Bech-Nielsen and colleagues partially purified PPA using SDS-polyacrylamide gel electrophoresis (PAGE), and they observed a major protein band of approximately 34–38 kDa (Bech-Nielsen *et al.*, 1992). An ELISA of the purified PPA showed a higher sensitivity (83%) than that of crude PPA, suggesting that the remaining components of this protein preparation may actually be more antigenic than the intact preparation itself. Also, Abbas *et al.* (1983) isolated an abundant PPA protein that constituted approximately 8% of the total protein in PPA and tested it for antibody binding in an ELISA format. In 44 JD-negative serum samples tested in the study, the ELISA did not show any false-positive reactions; however, the number of tested samples was too small to conclude that the antigen was not cross-reactive. Although the identities of the antigenic proteins have not been discovered in any of these studies, they do suggest that PPA contains strong antigens for the development of an accurate diagnostic test.

10.5 Culture Filtrate Preparations

A well-known antigen preparation derived from the filtrate of a spent culture, termed purified protein derivative (PPD), has been a critical reagent for testing cell-mediated immune responses to mycobacterial infections. It is the antigen of choice for the gamma interferon test (Stabel and Whitlock, 2001; Stabel *et al.*, 2007) as well as skin testing (Robbe-Austerman *et al.*, 2007). Historically, the tuberculin form of PPD was made from autolysed *Mycobacterium tuberculosis* cultured for extended periods of time (Lachmann, 1988). The intact bacilli were removed by centrifugation and the culture supernatant was heated, filtered and precipitated with trichloroacetic acid. This PPD preparation was supplied by the Veterinary Laboratories Agency in Weybridge, UK for many years and is still produced by a number of commercial organizations.

In the case of *MAP*, PPD preparations are difficult to standardize and hence their immunological potency varies widely. In fact, laboratories around the world use different strains of *M. avium* subspecies for producing PPD,

and this confusion has resulted in an effort to characterize the genetic diversity of these strains as an initial step towards standardization (Semret *et al.*, 2006). This study found that some of the Johnin production strains contain a large deletion encompassing just over seven genes, which may result in the poor immunological potency observed with those preparations.

To prepare Johnin PPD, Reid's media is used to culture the bacilli to form pellicles on the surface of the media. The cultures are incubated without shaking and must be handled very gently to prevent the surface pellicle from sinking into the media, which would theoretically render the preparation unusable as an immunological reagent. While this fact is widely acknowledged among producers of PPD, even tuberculin PPD, there is no documented evidence for this conclusion to our knowledge. After a secondary culture has been inoculated into 500 ml Reid's media, it is incubated without shaking for 10 weeks. The cultures are then autoclaved and the cell pellicle is removed with a sterile nylon gauze. The remaining culture filtrate is precipitated with the addition of 40% tricholoroacetic acid, harvested by centrifugation, washed in salt buffer, dissolved in a sodium phosphate buffer, quantitated and stored. Immunological potency is tested in sensitized guinea pigs (Steadham *et al.*, 2002).

It is important to note that the method used by Cho and Collins (2006) to prepare culture filtrate antigens is different from the PPD preparation. The culture filtrate, which is also reported to contain strong antigenic components (Cho and Collins, 2006), seems easier to prepare. A culture of Reid's media is harvested by centrifugation to remove the bacilli and the medium is filtered and concentrated by size exclusion before undergoing dialysis as the final step.

There is tremendous variability in the potency of PPD preparations. Not only is the starting material different for PPD production but the method of preparation is also believed to be a major cause of its variability, begging the question of why Johnin PPD is not prepared using the same method as that used to make tuberculin PPD. There have been some recent, although unpublished, efforts to define the components of PPD through proteomic approaches. One of these studies employed isobaric protein tags to determine quantitative differences between a highly potent preparation of PPD and a preparation of low potency, both produced at the National Veterinary Services Laboratory in Ames, Iowa, USA (J.P. Bannantine, S. Robbe-Austerman and M.L. Paustian, unpublished results). A few studies have evaluated recombinant proteins for performance in the gamma interferon test when compared with Johnin PPD (Griffin *et al.*, 2005; Nagata *et al.*, 2005; Shin *et al.*, 2005). Koets *et al.* (2001) used a PPD-ELISA for detection of 54 *MAP*-infected and 50 uninfected samples and found that an IgG2 response in the infected group, but not an IgG1 response, was significantly higher than in the uninfected group. Caution should be used for vaccine trials, as immunized animals may yield positive reactions in skin tests with either *MAP* PPD (Hines *et al.*, 2007) or *Mycobacterium bovis* PPD (Nedrow *et al.*, 2007).

Two independent research groups recently made major progress in comprehensive proteomic analysis of culture filtrate (CF) proteins and their potential as diagnostic antigens. Leroy *et al.* (2007) were the first to systematically analyse CF proteins of *MAP* cultured in Sauton media. The CF proteins were first separated by 1-D and 2-D SDS-PAGE, and 320 protein spots were excised from these gels and analysed by tandem mass spectrometry. They subsequently identified 25 putative *MAP*-specific proteins through comparative genomic analysis (Leroy *et al.*, 2007). Five of the 25 *MAP*-specific proteins were combined and tested for reactivity with antibodies in sera of *MAP*-infected and uninfected cattle. Based on the results obtained with 19 *MAP*-infected and 48 uninfected serum samples, they reported that the five-protein cocktail had a similar level of diagnostic sensitivity to that of a commercial ELISA test. In a second study, Cho and Collins (2006) prepared both a cellular extract (i.e. PPA) and CF from the JTC303 strain of *MAP* cultured in modified Watson–Reid broth and tested each protein preparation for reactivity with antibodies in sera of *MAP*-infected and uninfected cattle. They found that an ELISA prepared

using CF (JTC-ELISA) was more sensitive than that prepared with a culture extract. In a subsequent study, the diagnostic sensitivity of the JTC-ELISA was compared with that of five commercial ELISAs using 856 bovine sera (Shin *et al.*, 2008). The sensitivity of the JTC-ELISA (56.3%) was significantly higher than that of five current ELISAs (28.0–44.5%). Importantly, the JTC-ELISA showed dramatically higher sensitivity (40%) than that of other ELISAs (20%) in samples from cattle shedding low levels of *MAP* in their faeces. Collectively, these data suggest that secreted proteins of *MAP* appear to contain the stronger antigens for antibody-based tests.

Cho *et al.* (2006) went on to define some of the antigens present in the culture filtrate. They successfully identified 14 proteins, including MAP1569 (ModD), MAP3527 (PepA) and MAP3531c (antigen 85C) among others. Interestingly, ModD was shown to be the most antigenic of the 14 proteins and this same protein was previously identified as a fibronectin attachment protein that mediates uptake by M cells (Secott *et al.*, 2001, 2002, 2004).

10.6 Ethanol Extract

In 1995, Eda *et al.* (2005) used flow cytometry to demonstrate that antibodies in sera of *MAP*-infected cattle bound to the surface of *MAP* but not to that of other mycobacterial species. This observation led to the inference that *MAP* has unique antigens on its outer surface. Furthermore, the antibody–*MAP* binding complexes were detected in natural bovine infections several months earlier than the faecal culture test or commercial ELISA test. The empirical diagnostic sensitivity and specificity of this novel flow cytometric assay were estimated to be 95.2 and 96.7%, respectively. These data suggested that by detecting antibodies to the surface antigens of *MAP* one could develop a diagnostic test to detect early *MAP* infection. In a subsequent study, the same investigators found that an 80% ethanol solution was the most effective solvent for extracting surface antigens that specifically reacted with sera of *MAP*-infected cattle (Eda *et al.*, 2006). An in-house ELISA was developed using the ethanol-extracted preparation and named EVELISA for Ethanol Vortex ELISA. This test showed an improvement over the flow cytometric assay, with an empirical diagnostic sensitivity and specificity of 100 and 96.9%, respectively. The ethanol-extracted mycobacterial antigens were further fractionated by the Folch wash method and acetone precipitation into three fractions: aqueous, interface and chloroform. Each fraction showed reactivity to antibodies in sera of *MAP*-infected cattle; however, empirical sensitivity of the fractions was lower than the EVELISA (unpublished observations), indicating that the complete repertoire of antigens in the ethanol extract was required to maintain the high sensitivity of the EVELISA. Efforts are currently under way to define the components in the ethanol extract preparation. Preliminary data suggest that the preparation contains predominantly carbohydrate and lipid components with a small, but important, protein component.

10.7 Lipid Antigens

Members of the genus *Mycobacterium* produce complex lipid components in their cell wall. Unfortunately, lipidomic studies of *MAP* are still in the initial stages, with very few published reports. Glycopeptidolipids (GPLs) are surface lipids that consist of a lipopeptide core that is N-linked to long-chain fatty acids. O-linked to this same lipopeptide core are mono- and oligosaccharides. These sugar moieties are antigenic and form the basis for the 28 different serovars of the *M. avium* complex (Chatterjee and Khoo, 2001). While GPLs can make up more than 70% of the lipids exposed at the surface of *M. avium* isolates (Etienne *et al.*, 2002), *MAP* are deficient in GPL biosynthesis genes, as revealed by inspection of the K-10 genome sequence (Biet *et al.*, 2008). Interestingly, sheep strains of *MAP* have the genes necessary to synthesize GPL (Paustian *et al.*, 2008), although it remains to be determined if these strains actually do produce GPLs.

Lipoarabinomannan (LAM) is a cell wall lipoglycan found in several species of mycobacteria (Nigou *et al.*, 2003, 2004). It is composed

of a lipid moiety, a highly branched 30–35 mannopyranose core, and around 60 units of arabinopyranose (Nigou *et al.*, 2003). The structures of LAM differ slightly among species of mycobacteria, with the most important difference, in terms of biological activity, being capping motifs attached to the non-reducing end of the arabinan chain (Briken *et al.*, 2004). For instance, LAM of slow-growing *Mycobacterium* species, such as *M. tuberculosis* (Chatterjee *et al.*, 1992), *Mycobacterium leprae* (Khoo *et al.*, 1995) *and M. avium* subsp. *hominissuis* (Khoo *et al.*, 2001) are modified with a capping structure consisting of one to three mannose residues (ManLAM). The fast-growing *Mycobacterium* species *Mycobacterium smegmatis* and *Mycobacterium fortuitum* have been shown to express LAM modified with inositol phosphate caps (PILAM) (Gilleron *et al.*, 1997) or, in the case of *Mycobacterium chelonae*, uncapped LAM (AraLAM) (Guerardel *et al.*, 2002). Although the cap structure of LAM has not been biochemically characterized in *MAP*, its genome sequence is known to contain genes involved in mannose cap synthesis (Li *et al.*, 2005; Dinadayala *et al.*, 2006). It is therefore likely that LAM from *MAP* is decorated by mannosides similar to that observed for other subspecies of *M. avium*.

LAM is capable of inducing strong humoral immune responses (Waters *et al.*, 2003) and, similar to PPA, has been used as an antigen in commercial diagnostic tests for JD (Jark *et al.*, 1997). The drawback is that LAM is conserved among the mycobacteria. Its preparation involves harvesting the mycobacteria, resuspending them in distilled water, passing them through a French press, followed by centrifugation, at which point the supernatant is subject to proteinase K treatment and filtration (Jark *et al.*, 1997). As reviewed by Nielsen and colleagues, reported sensitivities of ELISA tests developed using LAM (LAM-ELISA) were comparable to those of PPA-ELISAs (Nielsen and Toft, 2008). In 1997, Sugden and colleagues tested serum samples using LAM-ELISA and PPA-ELISA, and both tests detected at least 11 of 15 positive *MAP* samples (Sugden *et al.*, 1997). However, LAM does have an advantage over PPA, in that it is a purified, well-characterized antigen and therefore lends itself more readily to production quality control. Large-scale cultures for production of LAM would be needed for commercial development and this could be a drawback. None the less, LAM may still be a good antigen candidate for the development of a *MAP* ELISA when used in combination with other, more specific, *MAP* antigens.

A major cell wall lipopeptide for *MAP*, termed Para-LP-01, has been reported (Eckstein *et al.*, 2006). These investigators were able to show that this lipid is specific and immunoreactive for *MAP*, based on an ELISA assay developed using the lipid as a coating antigen. This same laboratory is separating and analysing a complete set of lipids by thin-layer chromatography using solvents with a wide range of polarity, to identify additional diagnostic and vaccine targets. Unfortunately, Para-LP-01 was not synthesized and produced in large enough quantities to enable further testing and validation. A second *MAP* lipid, termed lipopentapeptide, was successfully synthesized and shown to be unique to *MAP* strains (Biet *et al.*, 2008). This group confirmed that only *MAP* and not other mycobacteria produce this lipopentapeptide. They compared this lipid to *MAP* PPD in an ELISA format and demonstrated similar sensitivity but higher specificity for the lipid. When the lipopentapeptide moieties were synthesized separately, it was determined that the peptidyl moiety was highly immunogenic whereas the lipid moiety was poorly recognized by cattle sera (Biet *et al.*, 2008).

10.8 Recombinant Antigens

There are two important advantages of recombinant proteins in serodiagnostic testing of hosts with *MAP* infection. First, traditional techniques to detect antibodies in animals with JD have used crude extract mixtures, with substantial variability between extracts. Secondly, extracts such as PPD or PPA are prone to cross-reactivity with antibodies generated against different bacterial exposures. The availability of genome sequence data has now made cloning and expression of any coding sequence from this organism straightforward. Furthermore, long culture

periods can be bypassed if antigen production is performed in a faster-growing bacterium. As a side benefit, when *Escherichia coli* is used as the surrogate expression host, striking morphological or physiological changes in the bacterium can sometimes be readily observed, as shown in Fig. 10.1. These changes may supply telling clues as to the function of the protein.

Previous studies with other mycobacteria such as *M. tuberculosis* have provided a foundation such that antigen selection is no longer random but instead based on experimental data. In fact, several *MAP* recombinant proteins have been analysed based on promising immunological studies with *M. tuberculosis* or *M. leprae* (Mullerad *et al.*, 2002; Rosseels *et al.*, 2006; Sechi *et al.*, 2006). One study has shown that recombinant proteins can actually detect a higher percentage of infected deer than PPD or PPA antigens when used in an ELISA format (Griffin *et al.*, 2005). These data suggest that recombinant proteins are better than native proteins, which are not amenable to purification. However, in certain cases, the native protein is more immunogenic than the recombinant one, presumably because of post-translational modifications that only *MAP* can perform. A decrease in immunogenicity has been shown for the MAP1569 (ModD) recombinant when compared with the native protein (Cho *et al.*, 2007). In such cases, unless there is a very easy way to purify that particular protein, it may not be practical as an antigen for a routine diagnostic test.

Nearly all recombinant antigens have been produced using *E. coli* as the host for expression. In only a few studies were *M. smegmatis* (Dupont *et al.*, 2005; Bach *et al.*, 2006; Sechi *et al.*, 2006), a viral vector (Bull *et al.*, 2007) or cell-free methods (Li *et al.*, 2007) used to produce the recombinant proteins. For most of the studies, a single protein was produced and characterized in isolation. More recently, the strategy of analysing more than one protein has emerged. Some investigators have addressed the need to evaluate multiple mycobacterial antigens in parallel by recombinant expression combined with immunological assays via multi-antigen print immunoassays (MAPIA) (Lyashchenko *et al.*, 2000; Waters *et al.*, 2004), immunoblot analysis (Paustian *et al.*, 2004) or ELISA (Olsen *et al.*, 2001; Shin *et al.*, 2004; Willemsen *et al.*, 2006; Cho *et al.*, 2007; Bannantine *et al.*, 2008c). It would be even more informative if dozens of proteins were not only compared with each other but also with known mycobacterial antigens and other *MAP* proteins. A comprehensive analysis of all *MAP* antigens at a whole-genome level in an unbiased assay system is needed to fully determine the antigenic basis of host protective and pathogenic responses to *MAP* infection. The development of a *MAP* protein array (discussed below) aims to address these issues.

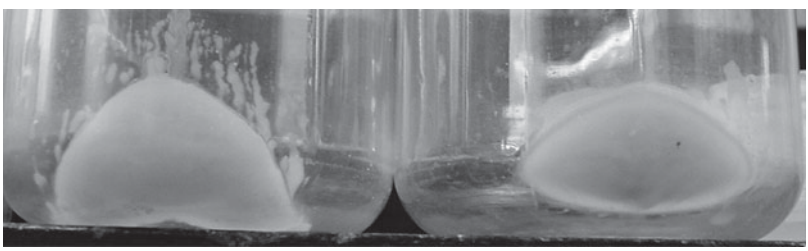

Fig. 10.1. Pellets of *Escherichia coli* at the bottom of a 500 ml centrifuge bottle, which were harvested 2.5 h after induction with IPTG. The pellet on the left was induced to express MAP3532c, which is a membrane protein that contains an ABC type 2 transporter domain, and the pellet on the right was induced to express MAP2226c, a hypothetical protein with a DNA binding domain. Notice that the MAP3532c recombinant *E. coli* has a slimy texture that cannot stick to the side of the centrifuge bottle and therefore is sliding down to the bottom. The *E. coli* on the right, producing MAP2226c, is more typical of a discrete, defined pellet observed for wild-type *E. coli* and most recombinant *E. coli*.

In the gamma interferon (IFN-γ) test, fresh whole blood is collected from the animal and stimulated with antigen, to measure IFN-γ release from sensitized lymphocytes via ELISA. Traditionally, the stimulating antigen used is Johnin PPD. The use of this antigen has contributed to the variable specificities obtained with this test (Kalis *et al.*, 2003). Therefore investigators have examined recombinant proteins in an effort to increase specificity while maintaining the test's high sensitivity. The HSP 70 protein appears to be a promising candidate for eliciting a measurable cell-mediated immune response (Koets *et al.*, 2006), although several other recombinant proteins have been evaluated in the gamma interferon test as one measure of cell-mediated responses (Nagata *et al.*, 2005; Shin *et al.*, 2005).

Unfortunately it is difficult to compare these studies with the goal of determining whether there is a single best stimulating antigen. It would be ideal if investigators would include at least one previously reported antigen in their study design to evaluate whether the antigen they are reporting is better or worse, in terms of sensitivity and specificity, than that previously reported. In this manner, the field can advance incrementally until the best set of antigens for specific tests is established and put into use.

10.9 Proteomic Studies of *M. avium* subsp. *paratuberculosis*

The proteome is the set of proteins expressed by *MAP* under a given condition. By cataloguing the different sets of proteins present in changing conditions, we gain insight into the biology of the organism and initiate large-scale antigen discovery.

Early proteomic efforts to characterize the PPA, PPD and secreted fractions were done using SDS-PAGE gel electrophoresis and agar gel immunodiffusion. However, use of these methods only characterized the sizes of protein and detected the most abundant proteins. For example, in 1985, Bech-Nielsen *et al.* (1985) reported that there are 27 proteins present in the sonicated cell extract of *MAP*. Using the latest proteomic technology, including fractionation methods and two-dimensional gel separation, we now know that there are several hundred proteins present in these types of cellular extracts (Egan *et al.*, 2008). Subsequent proteomic efforts focused on immunoscreening genomic expression libraries for *MAP* antigens using sera from infected cows or other hosts (Bannantine and Stabel, 2001; Gioffre *et al.*, 2006; Willemsen *et al.*, 2006). These studies enabled the first proteome-wide antigen screens of *MAP*, assuming the genomic expression library was representative. Antigens that have been identified from these studies include three secreted antigens (Gioffre *et al.*, 2006; Willemsen *et al.*, 2006) and the major membrane protein encoded by MAP2121c (Bannantine and Stabel, 2001). Thus far no antigen identified from these expression library immunoscreens has been incorporated into a routine diagnostic test.

As an initial foray into modern proteomic studies of *MAP*, two physical disruption methods were compared for their ability to represent the proteome. The results suggested that sonication and bead beating worked equally well, with a similar number of spots visualized on silver-stained, two-dimensional gels. The bead-beating procedure may be more advantageous since the tube remains closed during the procedure (Lanigan *et al.*, 2004). *MAP* proteomes have subsequently been compared with other subspecies under the same conditions (Hughes *et al.*, 2007, 2008; Radosevich *et al.*, 2007), or *in vitro* and *in vivo* conditions have been compared for the same strain (Hughes *et al.*, 2007; Egan *et al.*, 2008). The study by Hughes and colleagues examined the proteome of terminal ileum scrapings from sheep, as compared with *MAP* cultured in Middlebrook 7H9 broth, and was able to resolve approximately 1000 protein spots on two-dimensional gels for each preparation. They successfully identified ten of the proteins present in higher abundance in the sheep-extracted proteome compared with the Middlebrook-extracted proteome. Although they did not use quantitative proteomics in this study, several proteins with similar abundance between the two preparations were used to normalize the data.

10.10 Protein Arrays

Because JD is a multi-stage disease and, unlike *M. tuberculosis* or *M. leprae*, *MAP* survives longer in the environment outside the host, it is critical to understand how protein expression is regulated or modified in the widely variant conditions experienced by the pathogen. From a vaccine or diagnostic standpoint, it is important to know which antigens are expressed at which stage of the disease or life cycle; these types of questions can be addressed using protein array technology.

The power of protein arrays is that equivalent amounts of many proteins are analysed in parallel, thus enabling direct comparison of known antigens with newly identified antigens spotted on the array. Furthermore, this systematic approach enables more comprehensive profiles of the host immune response to the pathogen at any stage of JD. The construction of a protein array for *MAP* was recently undertaken in our laboratories. A 48-spot protein array was initially built to determine the utility of this method as an antigen discovery tool (Bannantine *et al.*, 2008d), and this array was then expanded to include 92 recombinant *MAP* proteins (Bannantine *et al.*, 2008b).

Although protein arrays have only recently been developed, they are already at the forefront of proteomic research (Hoeben *et al.*, 2006; Coleman *et al.*, 2007; Engert *et al.*, 2007; Lee *et al.*, 2007; Bannantine *et al.*, 2008b; Spurrier *et al.*, 2008). High-throughput protein purification and spotting are the most common method of protein array production and the method used to generate the *MAP* protein arrays. However, some investigators have used cell-free translation systems to avoid the labour-intensive process of purifying large sets of proteins, to reduce the storage time and to prevent loss of protein stability or activity (He and Taussig, 2008). We have also investigated this method (Li *et al.*, 2007), but found more consistent expression of *MAP* proteins using the traditional recombinant protein production approach (Bannantine and Paustian, 2006). Purified proteins have the added benefit of ensuring that immunological reactivity is associated with that protein and not due to contaminating proteins from the expression host.

While protein arrays can be used to study protein–protein interactions or quantify antibody levels (Kersten *et al.*, 2003; Coleman *et al.*, 2007), the *MAP* protein array was developed specifically for immune profiling of cattle. This profiling could probably lead to a new diagnostic, but it may be even more valuable in the search for a subunit vaccine. In vaccine strategies, it may be better if one can identify which proteins invoke an immune response within cattle and use only those proteins in a vaccine formulation as opposed to simply immunizing with a whole-cell lysate or killed bacteria.

These studies have shown that single recombinant proteins were much more readily detected than the PPA also spotted on the same array. This suggests that ELISA tests incorporating PPA as the plate-coating antigen might be improved if the assay used one or more recombinant proteins. Why do single antigens react stronger than the PPA? This is most likely due to the heterogeneous nature of the PPA preparation, as described earlier in this chapter. Because so many proteins are present in the preparation and each protein is represented in small quantities, no single antigen can stand out. However, once a dominant antigen is isolated and spotted in a pure form, it should theoretically elicit potent recognition. This observation recalls the work of Bech-Nielsen *et al.* (1992), where a partially purified PPA resulted in a higher ELISA sensitivity than did the crude PPA.

Although only a few cattle could be tested using the protein array, the antibody profiles demonstrated consistency between animals (Bannantine *et al.*, 2008b). In contrast, recombinant proteins tested in sheep showed frustratingly inconsistent results, as no single protein emerged as strongly detected in all sheep (Bannantine *et al.*, 2008c). As there are too many variables between these two studies, it is not possible to make any general conclusions about sheep and cattle humoral immune responses.

10.11 Concluding Remarks and Future Directions

No single method of proteomic analysis of *MAP* antigens is ideal. It would take many

research dollars and several years to assemble a protein array that represents all proteins produced by this ruminant pathogen. Even if that were possible, recombinant production of these proteins will probably affect the antigenicity of at least a subset of them, due to mycobacteria-specific, post-translational modifications. One study has already indicated that certain recombinant proteins are not as antigenically potent as the native proteins (Cho *et al.*, 2007), although this does not appear to be the case for all recombinant proteins (Bannantine *et al.*, 2004, 2008d; Griffin *et al.*, 2005). Traditional proteomic strategies using two-dimensional separation are not without their limitations either. The current state-of-the-art technology remains incapable of separating and resolving a complete proteome. Therefore, only subfractions of the proteome are analysed in any single experiment. Additionally, none of the proteins in the gel are immediately known or consistently located among different experiments. Only through laborious excision of spots and analysis by mass spectrometry can selected proteins be identified. As a result of these and other limitations with each method, the researcher must carefully consider the question to be answered before selecting the method to address a stated hypothesis.

New ideas and methods for the preparation of antigenic proteins have yielded recent breakthroughs that may translate into better diagnostic products to control Johne's disease. This is especially true for the ethanol vortex preparation that has been incorporated into an ELISA test (Eda *et al.*, 2006), and other ELISA tests also show promise (Shin *et al.*, 2008). In addition, the genome sequence has enabled new proteins to be tested in a high-throughput manner (Bannantine *et al.*, 2008a,b). This is encouraging in that the discovery pipeline for *MAP* antigens is now flowing.

Surprisingly little research has applied *MAP* antigens to Crohn's disease studies. In fact, cell-mediated immune responses from Crohn's disease patients have not been reported against *MAP* antigens. This situation may soon change, as a focused research direction is proposed in a new American Academy for Microbiology report on the link between Crohn's disease and *MAP* (Nacy and Buckley, 2008). The report calls for several research priorities, among them improved immunodetection of *MAP*.

As data accrue, it may be worthwhile to conduct a systematic review of the scientific literature and determine whether useful antigens have been described but their potential has not yet been fully exploited. It seems that many described antigens of *MAP* have not been followed up for a variety of reasons. Because detection of antibody or a cell-mediated immune response is simple, rapid and relatively inexpensive, activities leading to the discovery of new antigens with immunodiagnostic potential could easily be intensified.

Although development of antigen-based diagnostics has been the driving force behind many of the advances described, proteomic technologies will be critical in understanding the biology and pathogenesis of *MAP*. For example, blue native gel technology along with the protein array will be instrumental in identifying protein–protein interactions on a genome-wide scale. The blue native technique is excellent at resolving membrane protein complexes (Schagger and von Jagow, 1991), and proteins that participate in these complexes can then be identified by mass spectrometry. Finally, specific interactions of the newly identified membrane protein(s) can be confirmed using the protein array. The protein array can also be used to identify pathogen proteins that may initially interact with the bovine epithelial cells lining the intestine. From these types of experiments, we may finally obtain molecular details surrounding the initial events during infection, beyond what has recently been discovered with the oxidoreductase gene (Alonso-Hearn *et al.*, 2008) and the major membrane protein (Bannantine *et al.*, 2003).

References

Abbas, B., Riemann, H.P. and Lonnerdal, B. (1983) Isolation of specific peptides from *Mycobacterium paratuberculosis* protoplasm and their use in an enzyme-linked immunosorbent assay for the detection of paratuberculosis (Johne's disease) in cattle. *American Journal of Veterinary Research* 44, 2229–2236.

Alonso-Hearn, M., Patel, D., Danelishvili, L., Meunier-Goddik, L. and Bermudez, L.E. (2008) The *Mycobacterium avium* subsp. *paratuberculosis* MAP3464 gene encodes an oxidoreductase involved in invasion of bovine epithelial cells through the activation of host cell Cdc42. *Infection and Immunity* 76, 170–178.

Bach, H., Sun, J., Hmama, Z. and Av-Gay, Y. (2006) *Mycobacterium avium* subsp. *paratuberculosis* PtpA is an endogenous tyrosine phosphatase secreted during infection. *Infection and Immunity* 74, 6540–6546.

Bannantine, J.P. and Paustian, M.L. (2006) Identification of diagnostic proteins in *Mycobacterium avium* subspecies *paratuberculosis* by a whole genome analysis approach. *Methods in Molecular Biology* 345, 185–196.

Bannantine, J.P. and Stabel, J.R. (2001) Identification of two *Mycobacterium avium* subspecies *paratuberculosis* gene products differentially recognised by sera from rabbits immunised with live mycobacteria but not heat-killed mycobacteria. *Journal of Medical Microbiology* 50, 795–804.

Bannantine, J.P., Huntley, J.F., Miltner, E., Stabel, J.R. and Bermudez, LE. (2003) The *Mycobacterium avium* subsp. *paratuberculosis* 35 kDa protein plays a role in invasion of bovine epithelial cells. *Microbiology* 149, 2061–2069.

Bannantine, J.P., Hansen, J.K., Paustian, M.L., Amonsin, A., Li, L.L., Stabel, J.R. and Kapur, V. (2004) Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 42, 106–114.

Bannantine, J.P., Bayles, D.O., Waters, W.R., Palmer, M.V., Stabel, J.R. and Paustian, M.L. (2008a) Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle. *Proteome Science* 6, 5.

Bannantine, J.P., Paustian, M.L., Waters, W.R., Stabel, J.R., Palmer, M.V., Li, L. and Kapur, V. (2008b) Profiling bovine antibody responses to *Mycobacterium avium* subsp. *paratuberculosis* infection by using protein arrays. *Infection and Immunity* 76, 739–749.

Bannantine, J.P., Rosu, V., Zanetti, S., Rocca, S., Ahmed, N. and Sechi, L.A. (2008c) Antigenic profiles of recombinant proteins from *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Veterinary Immunology and Immunopathology* 122, 116–125.

Bannantine, J.P., Waters, W.R., Stabel, J.R., Palmer, M.V., Li, L., Kapur, V. and Paustian, M.L. (2008d) Development and use of a partial *Mycobacterium avium* subspecies *paratuberculosis* protein array. *Proteomics* 8, 463–474.

Beam, R.E., Stottmeier, K.D. and Kubica, G.P. (1969) Purified protoplasmic peptides of mycobacteria: isolation of species-specific peptides from protoplasm of mycobacteria. *Journal of Bacteriology* 100, 195–200.

Bech-Nielsen, S., Burianek, L.L., Spangler, E., Heider, L.E., Hoffsis, G.F. and Dorn, C.R. (1985) Characterization of *Mycobacterium paratuberculosis* antigenic proteins. *American Journal of Veterinary Research* 46, 2418–2420.

Bech-Nielsen, S., Jorgensen, J.B., Ahrens, P. and Feld, N.C. (1992) Diagnostic accuracy of a *Mycobacterium phlei*-absorbed serum enzyme-linked immunosorbent assay for diagnosis of bovine paratuberculosis in dairy cows. *Journal of Clinical Microbiology* 30, 613–618.

Biet, F., Bay, S., Thibault, V.C., Euphrasie, D., Grayon, M., Ganneau, C., Lanotte, P., Daffe, M., Gokhale, R., Etienne, G. and Reyrat, J.M. (2008) Lipopentapeptide induces a strong host humoral response and distinguishes *Mycobacterium avium* subsp. *paratuberculosis* from *M. avium* subsp. *avium*. *Vaccine* 26, 257–268.

Briken, V., Porcelli, S.A., Besra, G.S. and Kremer, L. (2004) Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Molecular Microbiology* 53, 391–403.

Bull, T.J., Gilbert, S.C., Sridhar, S., Linedale, R., Dierkes, N., Sidi-Boumedine, K. and Hermon-Taylor, J. (2007) A novel multi-antigen virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis*. *PLoS ONE* 2, e1229.

Chatterjee, D. and Khoo, K.H. (2001) The surface glycopeptidolipids of mycobacteria: structures and biological properties. *Cellular and Molecular Life Sciences* 58, 2018–2042.

Chatterjee, D., Lowell, K., Rivoire, B., McNeil, M.R. and Brennan, P.J. (1992) Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. *Journal of Biological Chemistry* 267, 6234–6239.

Cho, D. and Collins, M.T. (2006) Comparison of the proteosomes and antigenicities of secreted and cellular proteins produced by *Mycobacterium paratuberculosis*. *Clinical and Vaccine Immunology* 13, 1155–1161.

Cho, D., Sung, N. and Collins, M.T. (2006) Identification of proteins of potential diagnostic value for bovine paratuberculosis. *Proteomics* 6, 5785–5794.

Cho, D., Shin, S.J., Talaat, A.M. and Collins, M.T. (2007) Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. *Protein Expression and Purification* 53, 411–420.

Coleman, M.A., Beernink, P.T., Camarero, J.A. and Albala, J.S. (2007) Applications of functional protein microarrays: identifying protein–protein interactions in an array format. *Methods in Molecular Biology* 385, 121–130.

Dinadayala, P., Kaur, D., Berg, S., Amin, A.G., Vissa, V.D., Chatterjee, D., Brennan, P.J. and Crick, D.C. (2006) Genetic basis for the synthesis of the immunomodulatory mannose caps of lipoarabinomannan in *Mycobacterium tuberculosis*. *Journal of Biological Chemistry* 281, 20027–20035.

Dupont, C., Thompson, K., Heuer, C., Gicquel, B. and Murray, A. (2005) Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Medical Microbiology* 54, 1083–1092.

Eckstein, T.M., Chandrasekaran, S., Mahapatra, S., McNeil, M.R., Chatterjee, D., Rithner, C.D., Ryan, P.W., Belisle, J.T. and Inamine, J.M. (2006) A major cell wall lipopeptide of *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Biological Chemistry* 281, 5209–5215.

Eda, S., Elliott, B., Scott, M.C., Waters, W.R., Bannantine, J.P., Whitlock, R.H. and Speer, C.A. (2005) New method of serological testing for *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) by flow cytometry. *Foodborne Pathogens and Disease* 2, 250–262.

Eda, S., Bannantine, J.P., Waters, W.R., Mori, Y., Whitlock, R.H., Scott, M.C. and Speer, C.A. (2006) A highly sensitive and subspecies-specific surface antigen enzyme-linked immunosorbent assay for diagnosis of Johne's disease. *Clinical and Vaccine Immunology* 13, 837–844.

Egan, S., Lanigan, M., Shiell, B., Beddome, G., Stewart, D., Vaughan, J. and Michalski, W.P. (2008) The recovery of *Mycobacterium avium* subspecies *paratuberculosis* from the intestine of infected ruminants for proteomic evaluation. *Journal of Microbiological Methods* 75, 29–39.

Engert, S., Rieger, L., Kapp, M., Becker, J.C., Dietl, J. and Kammerer, U. (2007) Profiling chemokines, cytokines and growth factors in human early pregnancy decidua by protein array. *American Journal of Reproductive Immunology* 58, 129–137.

Etienne, G., Villeneuve, C., Billman-Jacobe, H., Astarie-Dequeker, C., Dupont, M.A. and Daffe, M. (2002) The impact of the absence of glycopeptidolipids on the ultrastructure, cell surface and cell wall properties, and phagocytosis of *Mycobacterium smegmatis*. *Microbiology* 148, 3089–3100.

Gilleron, M., Himoudi, N., Adam, O., Constant, P., Venisse, A., Riviere, M. and Puzo, G. (1997) *Mycobacterium smegmatis* phosphoinositols-glyceroarabinomannans. Structure and localization of alkali-labile and alkali-stable phosphoinositides. *Journal of Biological Chemistry* 272, 117–124.

Gioffre, A., Caimi, K., Zumarraga, M.J., Meikle, V., Morsella, C., Bigi, F., Alito, A., Santangelo, M.P., Paolicchi, F., Romano, M.I. and Cataldi, A. (2006) Lpp34, a novel putative lipoprotein from *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Medicine series B – Infectious Diseases and Veterinary Public Health* 53, 34–41.

Griffin, J.F., Spittle, E., Rodgers, C.R., Liggett, S., Cooper, M., Bakker, D. and Bannantine, J.P. (2005) Immunoglobulin G1 enzyme-linked immunosorbent assay for diagnosis of Johne's disease in red deer (*Cervus elaphus*). *Clinical and Diagnostic Laboratory Immunology* 12, 1401–1409.

Guerardel, Y., Maes, E., Elass, E., Leroy, Y., Timmerman, P., Besra, G.S., Locht, C., Strecker, G. and Kremer, L. (2002) Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*. Presence of unusual components with alpha 1,3-mannopyranose side chains. *Journal of Biological Chemistry* 277, 30635–30648.

He, M. and Taussig, M.J. (2008) Production of protein arrays by cell-free systems. *Methods in Molecular Biology* 484, 207–215.

Hines, M.E., 2nd, Stiver, S., Giri, D., Whittington, L., Watson, C., Johnson, J., Musgrove, J., Pence, M., Hurley, D., Baldwin, C., Gardner, I.A. and Aly, S. (2007) Efficacy of spheroplastic and cell-wall competent vaccines for *Mycobacterium avium* subsp. *paratuberculosis* in experimentally-challenged baby goats. *Veterinary Microbiology* 120, 261–283.

Hoeben, A., Landuyt, B., Botrus, G., De Boeck, G., Guetens, G., Highly, M., Van Oosterom, A.T. and De Bruijn, E.A. (2006) Proteomics in cancer research: methods and application of array-based protein profiling technologies. *Analytica Chimica Acta* 564, 19–33.

Hughes, V., Smith, S., Garcia-Sanchez, A., Sales, J. and Stevenson, K. (2007) Proteomic comparison of *Mycobacterium avium* subspecies *paratuberculosis* grown *in vitro* and isolated from clinical cases of ovine paratuberculosis. *Microbiology* 153, 196–205.

Hughes, V., Bannantine, J.P., Denham, S., Smith, S., Garcia-Sanchez, A., Sales, J., Paustian, M.L., Mclean, K. and Stevenson, K. (2008) Immunogenicity of proteome-determined *Mycobacterium avium* subsp. *paratuberculosis*-specific proteins in sheep with paratuberculosis.

Clinical and Vaccine Immunology 15, 1824–1833.

Jark, U., Ringena, I., Franz, B., Gerlach, G.F., Beyerbach, M. and Franz, B. (1997) Development of an ELISA technique for serodiagnosis of bovine paratuberculosis. *Veterinary Microbiology* 57, 189–198.

Kalis, C.H., Collins, M.T., Hesselink, J.W. and Barkema, H.W. (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. *Veterinary Microbiology* 97, 73–86.

Kersten, B., Feilner, T., Kramer, A., Wehrmeyer, S., Possling, A., Witt, I., Zanor, M.I., Stracke, R., Lueking, A., Kreutzberger, J., Lehrach, H. and Cahill, D.J. (2003) Generation of *Arabidopsis* protein chips for antibody and serum screening. *Plant Molecular Biology* 52, 999–1010.

Khoo, K.H., Dell, A., Morris, H.R., Brennan, P.J. and Chatterjee, D. (1995) Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. *Journal of Biological Chemistry* 270, 12380–12389.

Khoo, K.H., Tang, J.B. and Chatterjee, D. (2001) Variation in mannose-capped terminal arabinan motifs of lipoarabinomannans from clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *Journal of Biological Chemistry* 276, 3863–3871.

Koets, A.P., Rutten, V.P., De Boer, M., Bakker, D., Valentin-Weigand, P. and Van Eden, W. (2001) Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infection and Immunity* 69, 1492–1498.

Koets, A., Hoek, A., Langelaar, M., Overdijk, M., Santema, W., Franken, P., Eden, W. and Rutten, V. (2006) Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. *Vaccine* 24, 2550–2559.

Lachmann, P.J. (1988) Purified protein derivative (PPD). *Springer Seminars in Immunopathology* 10, 301–304.

Lanigan, M.D., Vaughan, J.A., Shiell, B.J., Beddome, G.J. and Michalski, W.P. (2004) Mycobacterial proteome extraction: comparison of disruption methods. *Proteomics* 4, 1094–1100.

Lee, H.S., Cho, S.B., Lee, H.E., Kim, M.A., Kim, J.H., Park Do, J., Kim, J.H., Yang, H.K., Lee, B.L. and Kim, W.H. (2007) Protein expression profiling and molecular classification of gastric cancer by the tissue array method. *Clinical Cancer Research* 13, 4154–4163.

Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K. and Wattiez, R. (2007) Antigen discovery: a post-genomic approach to paratuberculosis diagnosis. *Proteomics* 7, 1164–1176.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S. and Kapur, V. (2005) The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proceedings of the National Academy of Sciences of the USA* 102, 12344–12349.

Li, L., Munir, S., Bannantine, J.P., Sreevatsan, S., Kanjilal, S. and Kapur, V. (2007) Rapid expression of *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins for antigen discovery. *Clinical and Vaccine Immunology* 14, 102–105.

Lyashchenko, K.P., Singh, M., Colangeli, R. and Gennaro, M.L. (2000) A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *Journal of Immunological Methods* 242, 91–100.

Mullerad, J., Hovav, A.H., Fishman, Y., Barletta, R.G. and Bercovier, H. (2002) Antigenicity of *Mycobacterium paratuberculosis* superoxide dismutase in mice. *FEMS Immunology and Medical Microbiology* 34, 81–88.

Nacy, C. and Buckley, M. (2008) Mycobacterium avium paratuberculosis: *Infrequent Human Pathogen or Public Health Threat?* A Report from the American Academy of Microbiology, Washington DC, USA.

Nagata, R., Muneta, Y., Yoshihara, K., Yokomizo, Y. and Mori, Y. (2005) Expression cloning of gamma interferon-inducing antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 73, 3778–3782.

Nedrow, A.J., Gavalchin, J., Smith, M.C., Stehman, S.M., Maul, J.K., McDonough, S.P. and Thonney, M.L. (2007) Antibody and skin-test responses of sheep vaccinated against Johne's disease. *Veterinary Immunology and Immunopathology* 116, 109–112.

Nielsen, S.S. and Toft, N. (2008) Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. *Veterinary Microbiology* 129, 217–235.

Nigou, J., Gilleron, M. and Puzo, G. (2003) Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 85, 153–166.

Nigou, J., Gilleron, M., Brando, T. and Puzo, G. (2004) Structural analysis of mycobacterial lipoglycans. *Applied Biochemistry and Biotechnology* 118, 253–267.

Olsen, I., Tryland, M., Wiker, H.G. and Reitan, L.J. (2001) AhpC, AhpD, and a secreted

14-kilodalton antigen from *Mycobacterium avium* subsp. *paratuberculosis* distinguish between paratuberculosis and bovine tuberculosis in an enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology* 8, 797–801.

Paustian, M.L., Amonsin, A., Kapur, V. and Bannantine, J.P. (2004) Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's disease. *Journal of Clinical Microbiology* 42, 2675–2681.

Paustian, M.L., Zhu, X., Sreevatsan, S., Robbe-Austerman, S., Kapur, V. and Bannantine, J.P. (2008) Comparative genomic analysis of *Mycobacterium avium* subspecies obtained from multiple host species. *BMC Genomics* 9, 135.

Radosevich, T.J., Reinhardt, T.A., Lippolis, J.D., Bannantine, J.P. and Stabel, J.R. (2007) Proteome and differential expression analysis of membrane and cytosolic proteins from *Mycobacterium avium* subsp. *paratuberculosis* strains K-10 and 187. *Journal of Bacteriology* 189, 1109–1117.

Robbe-Austerman, S., Stabel, J.R. and Morrical, D.G. (2007) Skin test and gamma interferon enzyme-linked immunosorbent assay results in sheep exposed to dead *Mycobacterium avium* subspecies *paratuberculosis* organisms. *Journal of Veterinary Diagnostic Investigation* 19, 88–90.

Rosseels, V., Marche, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K. and Huygen, K. (2006) Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infection and Immunity* 74, 202–212.

Schagger, H. and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Analytical Biochemistry* 199, 223–231.

Sechi, L.A., Ahmed, N., Felis, G.E., Dupre, I., Cannas, S., Fadda, G., Bua, A. and Zanetti, S. (2006) Immunogenicity and cytoadherence of recombinant heparin binding haemagglutinin (HBHA) of *Mycobacterium avium* subsp. *paratuberculosis*: functional promiscuity or a role in virulence? *Vaccine* 24, 236–243.

Secott, T.E., Lin, T.L. and Wu, C.C. (2001) Fibronectin attachment protein homologue mediates fibronectin binding by *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 69, 2075–2082.

Secott, T.E., Lin, T.L. and Wu, C.C. (2002) Fibronectin attachment protein is necessary for efficient attachment and invasion of epithelial cells by *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 70, 2670–2675.

Secott, T.E., Lin, T.L. and Wu, C.C. (2004) *Mycobacterium avium* subsp. *paratuberculosis* fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins. *Infection and Immunity* 72, 3724–3732.

Semret, M., Bakker, D., Smart, N., Olsen, I., Haslov, K. and Behr, M.A. (2006) Genetic analysis of *Mycobacterium avium* complex strains used for producing purified protein derivatives. *Clinical and Vaccine Immunology* 13, 991–996.

Shin, S.J., Yoo, H.S., McDonough, S.P. and Chang, Y.F. (2004) Comparative antibody response of five recombinant antigens in related to bacterial shedding levels and development of serological diagnosis based on 35 kDa antigen for *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Science* 5, 111–117.

Shin, S.J., Chang, C.F., Chang, C.D., McDonough, S.P., Thompson, B., Yoo, H.S. and Chang, Y.F. (2005) *In vitro* cellular immune responses to recombinant antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 73, 5074–5085.

Shin, S. J., Cho, D. and Collins, M.T. (2008) Diagnosis of bovine paratuberculosis by a novel enzyme-linked immunosorbent assay based on early secreted antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Clinical and Vaccine Immunology* 15, 1277–1281.

Spurrier, B., Honkanen, P., Holway, A., Kumamoto, K., Terashima, M., Takenoshita, S., Wakabayashi, G., Austin, J. and Nishizuka, S. (2008) Protein and lysate array technologies in cancer research. *Biotechnology Advances* 26, 361–369.

Stabel, J.R. and Whitlock, R.H. (2001) An evaluation of a modified interferon-gamma assay for the detection of paratuberculosis in dairy herds. *Veterinary Immunology and Immunopathology* 79, 69–81.

Stabel, J.R., Kimura, K. and Robbe-Austerman, S. (2007) Augmentation of secreted and intracellular gamma interferon following johnin purified protein derivative sensitization of cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 19, 43–51.

Steadham, E.M., Martin, B.M. and Thoen, C.O. (2002) Production of a *Mycobacterium avium* ssp. *paratuberculosis* purified protein derivative (PPD) and evaluation of potency in guinea pigs. *Biologicals* 30, 93–95.

Sugden, E.A., Samagh, B.S., Bundle, D.R. and Duncan, J.R. (1987) Lipoarabinomannan and

lipid-free arabinomannan antigens of *Mycobacterium paratuberculosis*. *Infection and Immunity* 55, 762–770.

Sugden, E.A., Stilwell, K. and Michaelides, A. (1997) A comparison of lipoarabinomannan with other antigens used in absorbed enzyme immunoassays for the serological detection of cattle infected with *Mycobacterium paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 9, 413–417.

Waters, W.R., Miller, J.M., Palmer, M.V., Stabel, J.R., Jones, D.E., Koistinen, K.A., Steadham, E.M., Hamilton, M.J., Davis, W.C. and Bannantine, J.P. (2003) Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infection and Immunity* 71, 5130–5138.

Waters, W.R., Palmer, M.V., Bannantine, J.P., Whipple, D.L., Greenwald, R., Esfandiari, J., Andersen, P., McNair, J., Pollock, J.M. and Lyashchenko, K.P. (2004) Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. *Clinical and Diagnostic Laboratory Immunology* 11, 849–855.

Whitlock, R.H. and Rosenberger, A.E. (1990) Fecal culture protocol for *Mycobacterium paratuberculosis*: a recommended procedure. In: *The 94th United States Animal Health Association*. Denver, Colorado, USA.

Whitlock, R.H., Wells, S.J., Sweeney, R.W. and Van Tiem, J. (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Veterinary Microbiology* 77, 387–398.

Willemsen, P.T., Westerveen, J., Dinkla, A., Bakker, D., Van Zijderveld, F.G. and Thole, J.E. (2006) Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets. *Veterinary Microbiology* 114, 337–344.

Yokomizo, Y., Merkal, R.S. and Lyle, P.A. (1983) Enzyme-linked immunosorbent assay for detection of bovine immunoglobulin G1 antibody to a protoplasmic antigen of *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 44, 2205–2207.

Yokomizo, Y., Yugi, H. and Merkal, R.S. (1985) A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. *Japanese Journal of Veterinary Science* 47, 111–119.

11 Host–Pathogen Interactions and Intracellular Survival of *Mycobacterium avium* subsp. *paratuberculosis*

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11.1 Introduction

In *The Art of War*, Sun Tzu states that all warfare is based on deception (Tzu, 2005). Mycobacteria, specifically *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), are no exception to this rule. Mycobacteria represent a group of closely related acid-fast bacilli that encompass a wide range of host tropisms and diseases (Harris and Barletta, 2001; Corn *et al.*, 2005; Motiwala *et al.*, 2006; Behr and Kapur, 2008). In all cases, pathogenic mycobacteria deceive the host immune system by residing within host cells. Among mycobacteria, there are two important pathogenic complexes: the *Mycobacterium tuberculosis* complex and the *M. avium* complex (MAC). The *M. tuberculosis* complex is more readily recognized due to its implications in human health and includes the major pathogens *M. tuberculosis* and *Mycobacterium bovis*. MAC comprises *Mycobacterium intracellulare*, *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis* and *MAP*, all of which share over 90% nucleotide similarity. Despite their genetic similarity, MAC organisms elicit different diseases in both animals and humans,

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including infections of the lung, lymph nodes, bones, skin and gastrointestinal tract (Harris and Barletta, 2001; Behr and Kapur, 2008). Historically, research on MAC organisms has been limited; however, this group is rapidly gaining interest due to associations with opportunistic infections (*M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*) in HIV/AIDS patients and a potential aetiological agent in Crohn's disease (*MAP*) (Prohaszka *et al.*, 1999; Richter *et al.*, 2002; Ghadiali *et al.*, 2004; Behr and Kapur, 2008; Bentley *et al.*, 2008; Waddell *et al.*, 2008) (also see Behr, Chapter 5, this volume).

11.2 Persistence: the Protracted War

Sun Tzu also writes 'There has never been a protracted war from which a country has benefited' (Tzu, 2005). In the case of pathogens and their hosts, one could substitute 'host' for country and 'infection' for war. A hallmark of successful pathogens is the ability to persist within a host for an indefinite period of time. Among the mycobacteria, *MAP* is a leader in this paradigm, often persisting in the ruminant host for 2–5 years before onset of clinical disease. *MAP* succeeds by efficiently invading, replicating and laying siege to the host without being detected or cleared by the immune system (Harris and Barletta, 2001). *MAP* employs several strategies to enter host cells and causes Johne's disease (JD), a chronic inflammatory disorder of the gastrointestinal tract in ruminants (Clarke, 1997; Harris and Barletta, 2001; Whittington and Sergeant, 2001; Tiwari *et al.*, 2006; Zhu *et al.*, 2008).

JD may be categorized into four separate states: silent infection, subclinical infection, clinical disease and advanced clinical disease (Harris and Lammerding, 2001; Chacon *et al.*, 2004). Since *MAP* is primarily transmitted by the faecal–oral route, subclinically infected cattle are of particular concern, as shedding and spread may continue unabated until clinical signs surface (Harris and Barletta, 2001; Harris and Lammerding, 2001; Whittington *et al.*, 2001, 2004; Chacon *et al.*, 2004; Crossley *et al.*, 2005; Grewal *et al.*, 2006; Tiwari *et al.*, 2006). Once ingested, *MAP* gains entrance to subepithelial macrophages by invasion into the lamina propria via microfold cells (M cells) (Momotani *et al.*, 1988; Sigurðardóttir *et al.*, 1999, 2001, 2004; Tiwari *et al.*, 2006; Wu *et al.*, 2007a). Recent studies using a bovine ileal-loop model have shown host upregulation of intestinal trefoil factor (CD73) in response to *MAP* infection (Khare *et al.*, 2009). CD73 expression often marks damage to the glycocalyx and tight junction repair. Therefore *MAP* may also obtain access to subepithelial macrophages by localization and ingress through tight junctions. Once inside macrophages, *MAP* is well adapted to intracellular life and is able to persist and replicate within the phagosome. As is the case with *M. tuberculosis*, this intracellular persistence follows from its ability to efficiently block phagosome–lysosome fusion (Gatfield and Pieters, 2003; Rohde *et al.*, 2007). Interplay between *MAP* and the macrophage may dictate disease progression and outcome; therefore it is of extreme importance to understand early macrophage responses in order to elucidate pathogenesis. These topics will be considered later in this chapter.

The most recognizable feature of JD is the atypical granuloma formation, which is found in the mid- and distal segments of the small intestine (Harris and Barletta, 2001; Tiwari *et al.*, 2006). These atypical granulomatous lesions are thought to represent a late response by the host to control and limit *MAP* spread to the rest of the intestine and draining lymph nodes. However, it has been suggested that mycobacteria may take advantage of granulomas to recruit new macrophages to the site of infection and allow for mycobacterial travel through the granuloma (Davis and Ramakrishnan, 2009). Thus *MAP* may use granulomas as bridges to infect new portions of the intestine as well as other organs, including the mammary gland and mesenteric lymph node (Sweeney *et al.*, 1992a,b, 2006; Patel *et al.*, 2006). Clinical signs, including malabsorption, malnutrition and decreased milk yield, result within 2–5 years of infection, which may lead to death through either a direct cause or culling (Harris and Barletta, 2001; Tiwari *et al.*, 2006).

11.3 Pretend to be Weak: Intestinal Epithelial Cells, Macrophages and *MAP*

Sun Tzu states that in order to evade a strong opponent the best strategy is to appear to be weak (Tzu, 2005). The ability of *MAP* to appear benign and to enter host cells without overtly alarming the immune system may explain why it is so well adapted to its ruminant host (Fig. 11.1). As previously mentioned, *MAP* is spread by the faecal–oral route and gains entry to intestinal walls through the small intestinal mucosa via microfold cells (M cells) or villous epithelial cells overlying Peyer's patches in gut-associated lymphoid tissue (Sigurðardóttir *et al.*, 1999, 2004; Whittington and Sergeant, 2001; Whittington *et al.*, 2004; Crossley *et al.*, 2005; Grewal *et al.*, 2006; Tiwari *et al.*, 2006). M cells represent a primary target for *MAP* infection, which may be due to the lack of lysosomes and hydrolytic enzymes present in these cells (Miller *et al.*, 2007). Therefore many antigenic properties of *MAP* would remain unaltered after passing through M cells. It is well established that fibronectin (FN) attachment proteins present on *MAP* facilitate FN binding of the bacterium, which in turn forms a FN bridge with β1 integrins located on intestinal epithelial cells (Sigurðardóttir *et al.*, 1999; Pieters, 2001; Secott *et al.*, 2001, 2004). Preferential binding of *MAP* to M cells may be explained by the high density of β1 integrin present on the luminal surface of these cells in comparison with other cell types, such as enterocytes. Villous epithelial cell invasion is due to an unknown FN-independent mechanism (Secott *et al.*, 2001, 2004). However, *MAP* preference for M cells appears to require more than just FN–integrin interactions, since the closely related *M. avium* subsp. *avium* enters the intestinal wall by absorptive epithelial cells, despite presence of FN attachment protein genes (Secott *et al.*, 2002). Furthermore, *MAP* construction of a FN bridge is not limited to M cells but has also been shown to occur in other epithelial cell types, such as Caco-2 and T-24 cells (Secott *et al.*, 2002). Notably, both Caco-2 and T-24 cell lines are human derived but *MAP* enters them efficiently. Furthermore, a recent study showed *MAP* invasion and induction of inflammation in human fetal small intestines and colon that had been xenografted in SCID mice (Golan *et al.*, 2009). Together, these data from cell culture and *in vivo* experimentation suggest that *MAP* could infect humans, enhancing the case that *MAP* may be a factor in Crohn's disease onset.

Although *MAP* ultimately resides within macrophage cells, epithelial cell processing of *MAP* may be essential in terms of efficiency of invasion into macrophages. *MAP* exposed to Mac-T cells, a mammary epithelial cell line, displayed increased invasion efficiency in subsequent infections of Madin–Darby bovine kidney cells. Increased invasion efficiency by prior exposure may be due to upregulation of *MAP*3464, an oxidoreductase, which activates host Cdc42 and Rho internalization pathways (Patel *et al.*, 2006; Alonso-Hearn *et al.*, 2008). Although these studies concern invasion into epithelial cells, it can be speculated that a similar mechanism may be at play with the macrophage. Taken together these data suggest an essential role of epithelial cells in processing of *MAP* that not only aids invasion into the subepethelial dome but may also dictate disease progression and outcome.

Although *MAP* preferentially interacts with M cells, *MAP* may invade other epithelial cells. In a recent study conducted by Patel *et al.* (2006), it was suggested that the mammary gland might act as a reservoir for *MAP* within the host. Infection might be sustained within the mammary gland since *MAP* was shown to be capable of invasion from both the apical and basolateral surfaces of Mac-T cells. Increased invasion may be due to a hyperosmolar environment, which *MAP* would naturally encounter in milk. Since *MAP* is isolated from milk and colostrum, it may be first processed by mammary epithelial cells in the host.

DNA microarray analysis of *MAP* 24 h post-infection of Mac-T cells revealed upregulation of 20 *MAP* genes related to regulatory, metabolic and virulence-associated functions compared with *MAP* grown in Middlebrook 7H9 broth cultures. Patel *et al.* (2006) hypothesize that a 35 kDa *MAP* protein, which previously was shown to enhance invasion in epithelial cells, may be upregulated in response

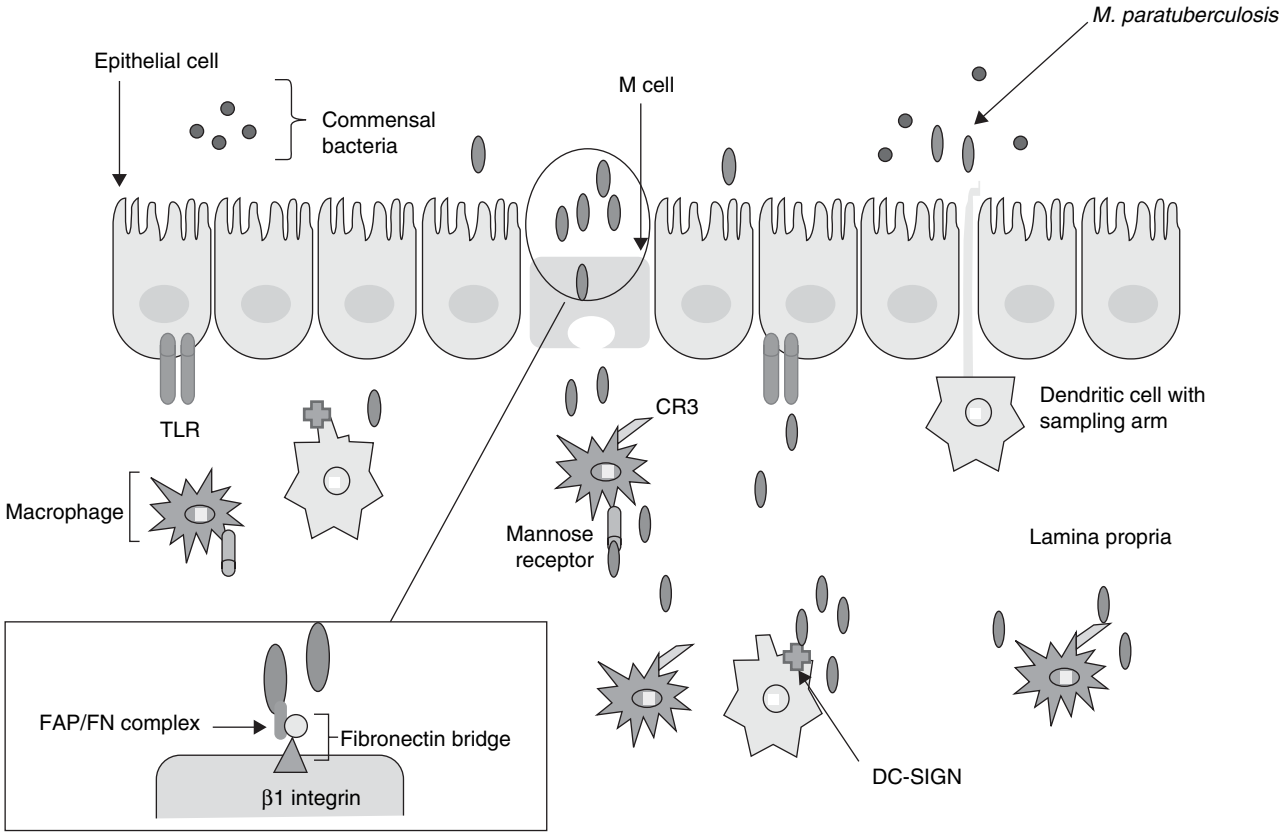

Fig. 11.1. Intestinal cell wall and macrophage invasion tactics used by *MAP*. *MAP* preferentially invades M cells by creating a fibronectin bridge and causes subsequent invasion of subepithelial macrophages. Entry into the macrophage is accomplished by ManLAM binding to CR3 and mannose receptors. *MAP* invasion into the lamina propria may also be gained through intestinal epithelial cells by an unknown mechanism. Dendritic cells may also transport *MAP* inside the lamina propria during sampling through tight junctions. *MAP* interaction with the dendritic cell receptor, DC-SIGN, may prime and promote a Th2 response. TLR, Toll-like receptor.

to prior exposure to Mac-T cells (Bannantine *et al.*, 2003).

Following M cell invasion into the subepithelial dome, *MAP* may encounter dendritic cells and/or macrophages. It is well established that *MAP* interacts with intestinal dendritic cells through its cell wall glycolipid mannosylated lipoarabinomannan (ManLAM) and the dendritic cell receptor DC-SIGN (Jozefowski *et al.*, 2008). *MAP* may use intestinal dendritic cell invasion as a strategic maneuver, since the primary function of intestinal dendritic cells is to sample and present commensal bacteria through tight junctions to the gut-associated lymphoid tissue. Thus, *MAP* would be able to overcome tight junction barriers and be directly transported to the lamina propria to interact with subepithelial macrophages. Furthermore, ManLAM–DC-SIGN interaction may prime *MAP* to direct a Th2 response, which would lead to immune subversion, as suggested for *M. tuberculosis* (Jozefowski *et al.*, 2008).

The uncanny ability of *MAP* and other mycobacteria to invade and survive within macrophages has been suggested as a conserved evolutionary selection for intracellular growth within protozoa (Brown and Barker, 1999; Harb *et al.*, 2000; Mura *et al.*, 2006; Rowe and Grant, 2006). *M. avium* subsp. *avium* has been shown to survive and persist within *Tetrachymena pyriformis* and *Acanthamoeba* species (Cirillo *et al.*, 1997). It appears that prior phagocytosis of *M. avium* subsp. *avium* by *Acanthamoeba castellanii* enhances not only entry of the pathogen into epithelial cells but also virulence, perhaps due to selection for a more virulent phenotype. *MAP* likewise has been shown to invade *A. castellanii* within 180 min of co-culture (Mura *et al.*, 2006). *MAP*–protozoa interactions are extremely provocative in terms of spread of infection and Crohn's disease, since protozoa have been located in dam water contaminated with *MAP* (Whittington *et al.*, 2005). However, whether or not *MAP* invasion in protozoa occurs naturally is still unknown.

As with dendritic cells, ManLAM from *MAP* is capable of interacting with macrophage cell surface receptors. The best-documented interaction is between *MAP* and the mannose receptor, which enhances macrophage phagocytosis of *MAP* (Pieters, 2001; Gatfield and Pieters, 2003; Rowe and Grant, 2006; Souza *et al.*, 2007a). Upon entry into macrophage cells, there is simultaneous replication of *MAP* and bacterial killing by the host, which reflects an initial Th1 response (Rowe and Grant, 2006; Alonso *et al.*, 2007; Woo *et al.*, 2007). Initial killing of *MAP* may be due to a rapid phagosome acidification response from the host, allowing phagosome–lysosome fusion to occur in some cells. The end result of phagosome–lysosome fusion would presumably be destruction of *MAP* and presentation of antigens to T cells via MHC. However, most phagosomes containing *MAP* and other pathogenic mycobacteria fail to mature. Studies of interactions between *M. tuberculosis* ManLAM and macrophages indicate that ManLAM is indispensable for blockage of phagosome maturation (Russell *et al.*, 2002; Yates and Russell, 2005). The ability of *MAP* and other mycobacteria to inhibit phagosome–lysosome fusion is essential to prevent pathogen awareness by the host immune system, thus allowing *MAP* to hijack macrophage resources and persist unabated. An active role for *MAP* in preventing phagosome–lysosome fusion is supported by the observation that live *MAP* is able to persist within phagosomes for 15 days, while phagosome function is not interrupted following uptake of killed *MAP* (Kuehnel *et al.*, 2001). Other macrophage receptors that are important for *MAP* binding include those for complement, immunoglobulin, transferrin, scavengers and surfactant protein A (Pieters, 2001; Souza *et al.*, 2007a). *MAP* binding to one complement receptor prevents activation of an oxygen burst and is abrogated with addition of monoclonal antibodies (Sigurðardóttir *et al.*, 2004).

Invasion efficiency of *MAP* appears to differ with respect to *MAP* genotype, such that species-specific variation occurs (Gollnick *et al.*, 2007). Despite variation in invasion efficiency, *MAP* strains all seem to employ a similar infection protocol or modus operandi. Zhu *et al.* (2008) determined that expression patterns from three *MAP* strains of different types based on short sequence repeats showed upregulation of 27, 22 and 35 genes respectively when isolated from infected bovine monocyte-derived macrophages at 48 and

120 h post-infection. Many of the genes on these lists were similar or had overlapping cellular functions. Pathway analysis categorized gene functions related to small-molecule degradation, energy metabolism, amino acid biosynthesis, lipid biosynthesis, broad regulatory functions, synthesis and modification of macromolecules, cell envelope, transport/binding proteins, virulence, antibiotic production and resistance, and conserved hypothetical proteins. All three *MAP* strains upregulated MAP4041c and MAP4281, which are suggested to play a role in protein transport and act as insertion elements, as well as genes related to lipid degradation, membrane transportation and DNA repair at 48 h. Taken together, this comparative transcriptional analysis suggests that diverse *MAP* genotypes use a similar modus operandi for survival in the host.

Previous studies have investigated the ability of *MAP* strains to regulate expression of major histocompatibility complex molecules during macrophage infection. Although *MAP* infection of J774 murine macrophages did not affect expression of MHC class II molecules, antigen presentation decreased, which may be due to *MAP* limitation of antigen processing (Kuehnel *et al.*, 2001). However, these results conflict with those demonstrating downregulation of MHC class I and class II molecules in bovine macrophages infected with live and killed *MAP* (Weiss *et al.*, 2001). It is exciting to speculate on the potential that one or a number of hypothetical genes identified by Zhu *et al.* (2008) may be responsible for *MAP* regulation of MHC class I and class II molecules and control of antigen processing.

11.4 Strategic Excellence: *MAP* Gene Expression Programmes Following Macrophage Cell Entry

Sun Tzu states 'What is of supreme importance in war is to attack the enemy's strategy' (Tzu, 2005). There is little doubt that *MAP* plays an active role in preventing phagosome maturation and in affecting the outcome of macrophage–T-cell interactions (considered later in this chapter). What does *MAP* do to enhance survival and growth once it arrives inside the macrophage? New genetic and genome-level tools are helping to unravel the initial actions *MAP* takes to ensure survival and victory once inside the macrophage. A recent study by Wu *et al.* (2007b) used DNA microarrays to characterize transcriptional profiles of *MAP* organisms exposed to different stress conditions *in vitro* or shed in cow faeces. Stress conditions were set to mimic environments *MAP* might encounter upon entry into a susceptible host. Expression data analysis revealed unique groups of *MAP* genes that were differentially regulated under *in vitro* stressors, while other groups of genes were differentially regulated in *MAP* from faecal samples. Interestingly, acidic pH induced differential regulation of a large number of genes (n = 597), suggesting high sensitivity of *MAP* to acidic environments, such as in the gut. Generally, responses to heat shock, acidity and oxidative stress were similar in *MAP* and *M. tuberculosis*, suggesting common pathways for mycobacterial defence against stressors. Several sigma factors (e.g. *sigH* and *sigE*) were differentially co-regulated with a large number of genes, depending on the type of stressor applied. While there was little correlation between the identities of genes expressed in *MAP* present in macrophages and *MAP* under induced stress *in vitro*, there was good agreement between the two studies in metabolic pathways induced in *MAP* by macrophages and *in vitro* stressors.

Subsequently, Wu *et al.* (2007b) conducted a functional analysis using a mouse model and identified several *MAP* mutants where differentially regulated genes were ablated. Bacterial and histopathological examinations indicated the attenuation of all gene mutants, especially those selected based on their expression in cow samples (e.g. *lipN*). Overall, this approach profiled mycobacterial genetic networks triggered by variable stressors and identified a novel set of putative virulence genes. Thus, *MAP* uses a common survival theme irrespective of the stressor or the model (*in vitro* stress or macrophage) used. Further analysis of the functional genomes of *MAP* using proteomic approaches and *in vivo* models will help define the mechanisms of disease induction (at the animal level) and phagosome escape (at the cellular level).

During *MAP* replication within the macrophage phagosome, host cell resources and space become limited. Therefore, *MAP* egresses from the cell by inducing apoptosis and is able to infect neighbouring macrophages within the subepithelial dome. Infected macrophages migrate into local lymphatics concurrent with disease progression, resulting in bacterial spread to regional lymph nodes, including the mesenteric lymph nodes (Ayele *et al.*, 2004; Sivakumar *et al.*, 2005; Tiwari *et al.*, 2006; Zhu *et al.*, 2008). *MAP* is able to replicate within regional lymph nodes. Macroscopic lesions develop in the intestine and the mesenteric lymph nodes, causing the intestinal wall to thicken and become corrugated (Sivakumar *et al.*, 2005). Infection may spread to the supramammary lymph node and mammary gland and therefore cause *MAP* contamination in colostrum and milk (Sweeney *et al.*, 1992a,b). Several studies indicate that *MAP* is able to survive the pasteurization process and thus, with respect to the potential zoonotic nature of *MAP*, may pose a significant public health concern for humans susceptible to Crohn's disease (Sweeney *et al.*, 1992b; Grant, 1998).

11.5 Secret Operations: *MAP* Subversion of Macrophage Function and Immune Response

Sun Tzu writes that 'Secret operations are essential in war; upon them the army relies to make its every move' (Tzu, 2005). Once inside the macrophage cell, *MAP* appears to remain invisible to the rest of the immune system, particularly T cells. As with *MAP* gene and protein expression studies, recent advances have allowed us to begin unravelling what effects *MAP* has on the infected host macrophage cell. Macrophages are cells designed to kill invading bacteria and present antigens to other immune effector cells, such as T cells. It is currently not fully known how *MAP* organisms, or other mycobacterial species, survive in macrophages. It is also not clear what effects *MAP* has on infected macrophages and their interactions with other host immune cells (see Stabel, Chapter 21, this volume). From work *in vivo*, it is apparent that infected cattle initially develop an early and effective pro-inflammatory immune response to *MAP*. However, this response typically declines in cattle that progress to clinical disease, favouring a Th2-like response, which leads to antibody production but does not control infection (Stabel, 2000; Coussens, 2001, 2004; Tessema *et al.*, 2001). Why this reduction in pro-inflammatory response occurs and the molecular mechanisms behind it are critical questions that must be addressed if we are to develop effective vaccines and better diagnostics to control Johne's disease. Thus, two of the most pressing questions in *MAP* pathobiology are: (i) how does this organism survive in macrophage cells; and (ii) why does the immune response against *MAP* switch from an appropriate Th1-like response to an ineffective Th2-like response?

11.6 Potential Targets: the Mitogen-activated Protein Kinase (MAPK) Pathway and *MAP*

Recent studies from several laboratories suggest that *MAP* subverts the ability of infected macrophages to react to normal T-cell signalling. This results in macrophages failing to activate and destroy *MAP*, leading to impaired T-cell responses. It has been proposed that *MAP* and other pathogenic mycobacteria alter the ability of macrophages to respond to extracellular signals from T cells, particularly through the CD154–CD40 system (Sommer *et al.*, 2009). This leads to a cytokine response that favours an inappropriate Th2-like activity, including expression of interleukin (IL)-10, and fails to activate macrophages to kill *MAP* organisms in phagosomes. A lack of proper co-stimulatory molecule engagement between *MAP*-infected macrophages and T cells could also lead to development of regulatory T cells (de Almeida *et al.*, 2008). Thus failure of *MAP*-infected macrophages to interact properly with T cells and failure of normal macrophage activation to occur, which leads to *MAP* survival, are central to disease progression. These ideas have recently been developed into models for immune response to *MAP* (Coussens, 2001, 2004; de Almeida *et al.*, 2008).

Many recent studies have focused on examining gene expression patterns and intracellular signalling inside macrophages infected with *MAP*, relative to uninfected macrophages. In other cases, gene expression patterns, particularly those encoding cytokine genes, have been examined in *MAP*-infected intestinal tissues and draining lymph nodes. Specific studies targeting intracellular signalling inside infected macrophages have been conducted. These recent investigations have greatly enhanced our understanding of *MAP* pathobiology, particularly when combined with *MAP* genome sequencing efforts and the recent ability to genetically modify *MAP*.

In one study, it was shown that *MAP*-infected tissues contained high levels of IL-1α and TRAF1 (tumour necrosis receptor associated factor-1) mRNA and protein (Aho *et al.*, 2003). High-level expression of TRAF1 and IL-1α was traced to *MAP*-infected macrophages infiltrating these tissues. Subsequently, it was demonstrated that *MAP* infection of monocyte-derived macrophages (MDM) *in vitro* also enhanced TRAF1 and IL-1α mRNA and protein (Chiang *et al.*, 2007). These results were significant because TRAF proteins are integral intermediates in tumour necrosis factor (TNF) receptor superfamily signalling systems, including CD40, Fas (CD95) and TNF receptors 1 and 2 (Bradley and Pober, 2001). These are all systems critical to macrophage function and activation, cytokine expression, T-cell signalling and induction of apoptosis.

The effect of *MAP* on intracellular signalling in infected macrophages was further highlighted in a study by Murphy *et al.* (2006), who used a bovine immune microarray. They examined differential gene expression in resting macrophages and in macrophages infected with either *MAP* or the closely related *M. avium* subsp. *hominissuis* (*MAH*) strain 104. Although *MAH* can produce serious infections in immune-compromised humans, it is non-pathogenic in ruminants and is readily cleared by an efficient immune response. In general, both mycobacteria activated gene expression in macrophages 24 h post-infection (Murphy *et al.*, 2006). However, macrophage responses to *MAH* were consistently more robust than to *MAP*. Of particular interest, over 41% of the differentially expressed genes in *MAP*-infected cells were members of, regulators of or regulated by the mitogen-activated protein kinase (MAPK) pathway. In keeping with gene expression patterns, *MAH* caused a more robust activation of p38 MAPK and extracellular regulated kinase (ERK1/2), two of the major MAPK family members. This response was also longer lived in *MAH*-infected macrophages relative to *MAP*-infected macrophages (Murphy *et al.*, 2006). Given that activation of p38 MAPK and ERK1/2 occurred within 15 min of *MAP* or *MAH* infection, this response was probably mediated via Toll-like receptor signalling. The robust and sustained activation of macrophages by *MAH* has led to speculation that this may be one reason why infections with this organism are cleared while those with *MAP* are not. Another hypothesis is that *MAP* actively limits its signalling via the MAPK pathway in infected macrophages.

This hypothesis is in line with studies demonstrating that pathogenic mycobacteria phagocytosed by macrophages pre-activated with interferon-γ (IFN-γ) or tumour necrosis factor-α (TNFα), are destroyed through normal phagosome maturation (Flynn *et al.*, 1993; Bonecini-Almeida *et al.*, 1998; Florido *et al.*, 1999). However, treatment of macrophages with these same agents after infection fails to result in efficient destruction of mycobacteria (Denis *et al.*, 1990; Robertson and Andrew, 1991). Therefore, interfering with normal macrophage activation pathways may be a key element in persistent infection and survival of mycobacteria, including *MAP*.

A key question is 'What could *MAP* be doing inside macrophages to enhance bacterial survival and prevent efficient immune clearance?' Clearly, infection of macrophages with *MAP* leads to dramatic upregulation of IL-10 (Weiss *et al.*, 2005; de Almeida *et al.*, 2008). This is significant since IL-10 can severely dampen pro-inflammatory immune responses, which are critical to clearance of *MAP* and other intracellular infections. Activation of IL-10 gene expression in *MAP*-infected macrophages is critically dependent upon rapid signalling through p38 MAPK. This signal is probably mediated via interactions between *MAP* and Toll-like receptor 2 (Souza *et al.*, 2008). Blocking p38 MAPK signalling with

specific inhibitors severely limits *MAP*-mediated increases in IL-10 mRNA (Sommer *et al.*, 2009). Inhibition of p38 MAPK also enhances the ability of macrophages to activate, acidify phagosomes and destroy *MAP* (Souza *et al.*, 2007c). Of interest, inhibition of SAPK/JNK also enhanced the ability of macrophages to destroy *MAP* (Souza *et al.*, 2006), while inhibition of ERK1/2 did not (Souza *et al.*, 2007b).

11.7 Potential Targets: Perturbation of CD40–CD154 Signalling in *MAP*-infected Macrophages

While immediate activation of MAPK signalling by *MAP* is of obvious importance in the response of macrophages to infection, studies focused on this cannot tell us what effect *MAP* might be having on the ability of macrophages to respond to T cells and to activate these cells to respond to infection. One of the most critical components of macrophage–T-cell interactions is engagement of CD40 on macrophages by CD154 (CD40 ligand) on activated T cells. CD40–CD154 binding is one of the major mechanisms leading to macrophage activation via T-cell interactions, and soluble CD154 can mimic many of the processes observed when T cells activate macrophages (Grewal and Flavell, 1996, 1998; Grewal *et al.*, 1997). CD40 is a member of the TNF receptor superfamily and is expressed on numerous cell types, including B cells, monocytes/macrophages, endothelial cells, dendritic cells, fibroblasts and vascular smooth muscle cells (Clark *et al.*, 1996; Van Kooten and Banchereau, 1996). Accordingly, we have recently demonstrated that typically over 70% of bovine MDM stain positive for cell surface CD40 after 7 days of maturation in culture (Chiang *et al.*, 2007). In monocytes and macrophages, CD40 signalling leads to secretion of inflammatory cytokines including IL-12, chemokines including β-chemokines (Stout and Suttles, 1996; di Marzio *et al.*, 2000), and matrix metalloproteinases (Malik *et al.*, 1996); induction of inducible nitric oxide synthase (iNOS); production of nitric oxide (Tian *et al.*, 1995; Van Kooten and Banchereau, 1996); enhanced cell survival; and induction of co-stimulatory molecules (Kiener *et al.*, 1995). T cells derived from CD154-deficient mice are impaired in their ability to induce macrophage effector functions (Stout and Suttles, 1996), and consequently these mice are highly susceptible to intracellular pathogens that would otherwise have been cleared by an appropriate T-cell–macrophage interaction (Soong *et al.*, 1996).

Studies on CD40 signal transduction have resulted in a complex picture of different mediators and pathways involved. Two major signalling pathways are activated downstream of CD40, which both involve activation of latent transcription factors. One pathway involves activation of the inhibitor of nuclear factor kappa B kinase complex, leading to nuclear translocation of active nuclear factor kappa B. The second mechanism is activation of the MAPK pathway, a cascade of phosphorylation events that primarily results in post-transcriptional activation of transcription factors like cAMP-response element binding protein, activating transcription factor, Ets, and AP-1 (Van Kooten and Banchereau, 1996, 2000). Both pathways synergize in inflammatory gene expression, including expression of IL-12p40, iNOS, IL-6, IL-8 and TNFα. Not surprisingly, CD40–CD154 signalling is a target for many intracellular pathogens. For example, Mathur *et al.* (2004) demonstrated that *Leishmania major*, an intracellular parasite causing leishmaniasis in humans, is able to inhibit CD154–CD40-mediated IL-12p40 and iNOS gene expression in murine peritoneal macrophages. This blockade appears to involve interference with activation of two main members of the MAPK pathway, p38 and ERK1/2 (Awasthi *et al.*, 2003; Mathur *et al.*, 2004).

A recent study demonstrated that *MAP*-infected macrophages were defective in some aspects of CD40 signalling (Sommer *et al.*, 2009). In uninfected macrophages, CD40–CD154 binding results in large increases in TNFα, IL-6, IL-10, IL-8, IL-12p40 and iNOS gene expression within 6 h. In *MAP*-infected macrophages, TNFα and IL6 gene expression following CD40–CD154 binding is relatively unaffected. In contrast, *MAP*-infected macrophages fail to activate expression of IL-12p40 and iNOS gene expression. This is a critical

difference, since IL-12 is a major driving force for development of an appropriate Th1-like response and production of IFNγ by T cells. In macrophages, iNOS activity and production of reactive nitrogen species is a major mechanism used to kill phagocytosed bacteria. For an intracellular bacterium such as *MAP*, limiting production of IL-12 and iNOS would ensure survival and development of an inappropriate immune response, particularly in the face of enhanced IL-10 production. It has also been suggested that failure to properly activate and/or engage T cells could lead to development of regulatory T cells, which would further reduce Th1-like immune activity against *MAP* (de Almeida *et al.*, 2008). Results of Sommer *et al.* (2009) are also consistent with observations *in vivo* (Coussens *et al.*, 2004), where *MAP*-infected intestinal tissues contained elevated levels of IL-10 but not IL-12p40 or IL-12p35. These tissues also contain elevated levels of TGFβ (Coussens *et al.*, 2004; Khalifeh and Stabel, 2004). TGFβ can be produced by regulatory Th3 cells and by regulatory γδ T cells, among others.

In summary, many studies have now highlighted the effects of *MAP* on MAPK signalling within infected macrophages. Initial activation of MAPK signalling, particularly through p38 MAPK, is probably responsible for enhanced IL-10 expression in *MAP*-infected macrophages. IL-10 is capable of dampening the response of macrophages to infection and has profound effects on the activity of T cells, particularly limiting production of IFNγ, IL-1α and IL-1β (Buza *et al.*, 2004; de Almeida *et al.*, 2008). Studies of *MAP*-infected tissues also show enhanced expression of IL-10 and of TGFβ, with concomitant absence of IL-12. Although the precise effects of *MAP* on MAPK signalling in infected macrophages have yet to be explained, this remains an exciting research area, which will probably add significantly to our understanding of *MAP* pathogenesis and host immune responses to *MAP*.

What are the possible mechanisms operating in *MAP*-infected macrophages that could alter the MAPK pathway? MAPK activation involves phosphorylation and dephosphorylation of specific substrates, including p38, SAPK/JNK and ERK1/2. Thus, *MAP* could alter the phosphorylation state of one of these proteins. However, within 2–4 h of *MAP* infection, activation of p38, SAPK/JNK and ERK1/2 is quelled, probably through the action of MAPK phosphatases (Sommer *et al.*, 2009). One possibility is that *MAP* alters availability of a particular substrate for one of the MAPK proteins. Since many of the substrates for the main MAPK family members are transcription factors, altering availability of one or more of these could have profound effects on gene expression in infected macrophages. Another explanation is that *MAP* affects the activity of one or more MAPK phosphatases. Decreased phosphatase activity could lead to sustained activation of one or more MAPK target transcription factors, while enhanced phosphatase activity would decrease activation of one or more factors. Either of these last two possibilities could lead to sustained alteration of MAPK signalling, consistent with observations (Weiss and Souza, 2008; Sommer *et al.*, 2009). A final possibility is that *MAP* expresses a kinase or phosphatase that is active on one or more MAPK proteins and therefore *MAP* would directly alter the MAPK signalling system. New methods to genetically alter *MAP* (Foley-Thomas *et al.*, 1995; Harris *et al.*, 1999; Park *et al.*, 2008), combined with the complete genome sequence of *MAP* strain K-10 (Li *et al.*, 2005), could be used to test this final hypothesis directly. *In vitro* methods using purified *MAP*-encoded proteins (Li *et al.*, 2007) and specific host MAPK signalling targets could also address this issue.

11.8 Potential Targets: Other Host Cell Systems Affected by *MAP* Infection

What other host cell systems might *MAP* alter to enhance survival in macrophages and reduce immune activity? A recent study utilized a microarray approach to examine gene expression patterns in MDM infected with ten different *MAP* strains (E.A. Kabara, 2009, unpublished results). These strains represented *MAP* isolates from a diverse range of hosts, including humans, sheep, cattle and bison. Two of the strains were from 'supershedder

cows'. The experiment compared gene expression in *MAP*-infected macrophages from four cows with uninfected macrophages from the same animals. One objective was to identify host macrophage genes whose expression was altered by infection with any strain of *MAP*. Another goal was to identify genes whose expression might be altered by one or more *MAP* strains but not by all, i.e. strain-specific changes in host gene expression. When data were combined across all strains, 91 genes were found to be significantly differentially expressed as a general function of *MAP* infection. These '*MAP* common' host genes included many encoding proteins involved in apoptosis, IL-1 processing and regulation, and several important transcription factors. In general, the trend was for downregulation of pro-apoptotic genes and upregulation of anti-apoptotic genes. This suggests that *MAP*-infected macrophages should be able to survive and resist programmed cell death better than their uninfected counterparts. *MAP*-induced alterations in genes encoding factors involved with IL-1 expression and processing are entirely consistent with a report that *MAP*-infected tissues contain elevated levels of IL-1 (Aho *et al.*, 2003). Indeed, *MAP* infection of MDM cells has also been shown to enhance IL-1 mRNA and protein expression (Chiang *et al.*, 2007).

When gene expression patterns were examined across *MAP* isolates from diverse species using a clustering algorithm, there was no clear division between *MAP* isolates from sheep, cattle or humans. However, there were two distinct clades of *MAP*, each containing four or five separate *MAP* strains. Two supershedder strains clustered tightly together and strain K-10 failed to cluster tightly with any other strain, perhaps reflecting the length of time K-10 has been in culture relative to more recent isolates of *MAP* (Fig. 11.2). As these studies advance, it will be interesting to determine if there are indeed strain-specific effects on macrophage gene expression and if these differences can be related back to differential survival in cells or virulence in animals. The fact that two supershedder strains cluster tightly together indicates that these *MAP* organisms may have unique effects on host macrophage gene expression.

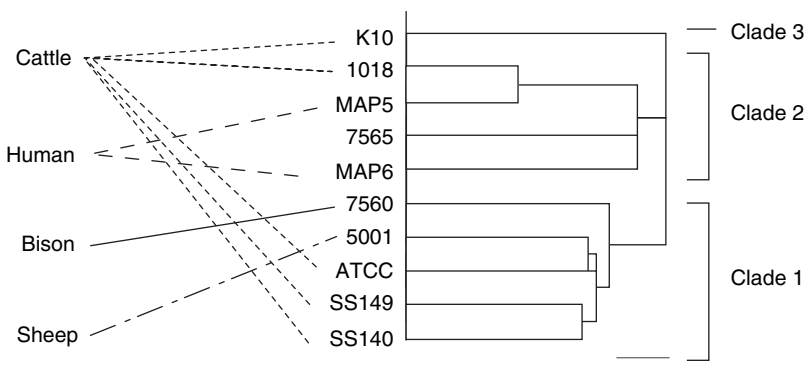

Fig. 11.2. Clustering analysis of macrophage gene expression differences induced by different *MAP* strains. MDM cells were infected at an MOI of 10:1 with *MAP* strains isolated from various host species (left). At 6 h post-infection, RNA was harvested from cells, converted to cDNA and analysed using a bovine immunity microarray (BOTL5) from the Michigan State University Center for Animal Functional Genomics (E.A. Kabara, 2009, unpublished results). A reference design was used in microarray hybridizations, such that MDM cells infected with each strain of *MAP* were compared with uninfected cells from the same cow. For each strain, MDM cells from four separate cows were utilized as biological replicates. Data were analysed using a mixed-models approach and visualized using Cluster 3.0 and Java Treviewer. Dashed lines illustrate the species the various *MAP* strains were isolated from.

11.9 Concluding Remarks and Future Directions

MAP is a highly successful pathogen with diverse survival mechanisms. The secrets to its unique abilities are undoubtedly encoded in its 4.8 megabase genome. Since the *MAP* genome sequence was published in 2005, little research has focused on functional analysis of this fastidious organism. Understanding the functional genome of *MAP* is essential to establish the repertoire of virulence pathways used by this organism *in vivo* or *in vitro*. Thus future studies using systems analysis of its transcriptome and proteomes will significantly aid in improving our understanding of host–pathogen and pathogen–environment interactions of *MAP*. Also encoded in that complex genome are pathways used by *MAP* that will greatly aid in improving *in vitro* culture systems for rapid diagnosis. An additional area of emphasis for future studies in *MAP*–host interactions would be in elucidating their innate responses at calfhood versus adult animal exposures and the mechanisms by which *MAP* traffics to its site of predilection – distal ileum (Peyer's patches) and mesenteric lymph nodes. A clear understanding of its functional genome and its interactions with the host will also aid in establishing good animal models to study this complex but highly successful microbe.

References

Aho, A.D., McNulty, A.M. and Coussens, P.M. (2003) Enhanced expression of interleukin-1alpha and tumor necrosis factor receptor-associated protein 1 in ileal tissues of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 71, 6479–6486.

Alonso, S., Pethe, K., Russell, D.G. and Purdy, G.E. (2007) Lysosomal killing of *Mycobacterium* mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proceedings of the National Academy of Sciences of the USA* 104, 6031–6036.

Alonso-Hearn, M., Patel, D., Danelishvili, L., Meunier-Goddik, L. and Bermudez, L.E. (2008) The *Mycobacterium avium* subsp. *paratuberculosis* MAP3464 gene encodes an oxidoreductase involved in invasion of bovine epithelial cells through the activation of host cell Cdc42. *Infection and Immunity* 76, 170–178.

Awasthi, A., Mathur, R., Khan, A., Joshi, B.N., Jain, N., Sawant, S., Boppana, R., Mitra, D. and Saha, B. (2003) CD40 signaling is impaired in *L. major*-infected macrophages and is rescued by a p38MAPK activator establishing a host-protective memory T cell response. *Journal of Experimental Medicine* 197, 1037–1043.

Ayele, W.Y., Bartos, M., Svastova, P. and Pavlik, I. (2004) Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in organs of naturally infected bull-calves and breeding bulls. *Veterinary Microbiology* 103, 209–217.

Bannantine, J.P., Huntley, J.F., Miltner, E., Stabel, J.R. and Bermudez, L.E. (2003) The *Mycobacterium avium* subsp. *paratuberculosis* 35 kDa protein plays a role in invasion of bovine epithelial cells. *Microbiology* 149, 2061–2069.

Behr, M.A. and Kapur, V. (2008) The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. *Current Opinion in Gastroenterology* 24, 17–21.

Bentley, R.W., Keenan, J.I., Gearry, R.B., Kennedy, M.A., Barclay, M.L. and Roberts, R.L. (2008) Incidence of *Mycobacterium avium* subspecies *paratuberculosis* in a population-based cohort of patients with Crohn's disease and control subjects. *American Journal of Gastroenterology* 103, 1168–1172.

Bonecini-Almeida, M.G., Chitale, S., Boutsikakis, I., Geng, J., Doo, H., He, S. and Ho, J.L. (1998) Induction of *in vitro* human macrophage anti-*Mycobacterium tuberculosis* activity: requirement for IFN-gamma and primed lymphocytes. *Journal of Immunology* 160, 4490–4499.

Bradley, J.R. and Pober, J.S. (2001) Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* 20, 6482–6491.

Brown, M.R. and Barker, J. (1999) Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends in Microbiology* 7, 46–50.

Buza, J.J., Hikono, H., Mori, Y., Nagata, R., Hirayama, S., Aodon-Geril, Bari, A.M., Shu, Y., Tsuji, N.M. and Momotani, E. (2004) Neutralization of interleukin-10 significantly enhances gamma interferon expression in peripheral blood by stimulation with Johnin purified protein derivative and by infection with *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected cattle with paratuberculosis. *Infection and Immunity* 72, 2425–2428.

Chacon, O., Bermudez, L.E. and Barletta, R.G. (2004) Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. *Annual Reviews of Microbiology* 58, 329–363.

Chiang, S.K., Sommer, S., Aho, A.D., Kiupel, M., Colvin, C., Tooker, B. and Coussens, P.M. (2007) Relationship between *Mycobacterium avium* subspecies *paratuberculosis*, IL-1alpha, and TRAF1 in primary bovine monocyte-derived macrophages. *Veterinary Immunology and Immunopathology* 116, 131–144.

Cirillo, J.D., Falkow, S., Tompkins, L.S. and Bermudez, L.E. (1997) Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity* 65, 3759–3767.

Clark, L.B., Foy, T.M. and Noelle, R.J. (1996) CD40 and its ligand. *Advances in Immunology* 63, 43–78.

Clarke, C.J. (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. *Journal of Comparative Pathology* 116, 217–261.

Corn, J.L., Manning, E.J., Sreevatsan, S. and Fischer, J.R. (2005) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging birds and mammals on livestock premises. *Applied and Environmental Microbiology* 71, 6963–6967.

Coussens, P. (2001) *M. avium* subspecies *paratuberculosis* and the bovine immune system. *Animal Health Research Reviews* 2, 141–161.

Coussens, P.M. (2004) Model for immune responses to *Mycobacterium avium* subspecies *paratuberculosis* in cattle. *Infection and Immunity* 72, 3089–3096.

Coussens, P.M., Verman, N., Coussens, M.A., Elftman, M.D. and McNulty, A.M. (2004) Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*: evidence for an inherent proinflammatory gene expression pattern. *Infection and Immunity* 72, 1409–1422.

Crossley, B.M., Zagmutt-Vergara, F.J., Fyock, T.L., Whitlock, R.H. and Gardner, I.A. (2005) Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* by dairy cows. *Veterinary Microbiology* 107, 257–263.

Davis, J.M. and Ramakrishnan, L. (2009) The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136, 37–49.

de Almeida, D.E., Colvin, C.J. and Coussens, P.M. (2008) Antigen-specific regulatory T cells in bovine paratuberculosis. *Veterinary Immunology and Immunopathology* 125, 234–245.

Denis, M., Gregg, E.O. and Ghandirian, E. (1990) Cytokine modulation of *Mycobacterium tuberculosis* growth in human macrophages. *International Journal of Immunopharmacology* 12, 721–727.

di Marzio, P., Mariani, R., Lui, R., Thomas, E.K. and Landau, N.R. (2000) Soluble CD40 ligand induces beta-chemokine production by macrophages and resistance to HIV-1 entry. *Cytokine* 12, 1489–1495.

Florido, M., Goncalves, A.S., Silva, R.A., Ehlers, S., Cooper, A.M. and Appelberg, R. (1999) Resistance of virulent *Mycobacterium avium* to gamma interferon-mediated antimicrobial activity suggests additional signals for induction of mycobacteriostasis. *Infection and Immunity* 67, 3610–3618.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A. and Bloom, B.R. (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *Journal of Experimental Medicine* 178, 2249–2254.

Foley-Thomas, E.M., Whipple, D.L., Bermudez, L.E. and Barletta, R.G. (1995) Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. *Microbiology* 141, 1173–1181.

Gatfield, J. and Pieters, J. (2003) Molecular mechanisms of host–pathogen interaction: entry and survival of mycobacteria in macrophages. *Advances in Immunology* 81, 45–96.

Ghadiali, A.H., Strother, M., Naser, S.A., Manning, E.J. and Sreevatsan, S. (2004) *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. *Journal of Clinical Microbiology* 42, 5345–5348.

Golan, L., Livneh-Kol, A., Gonen, E., Yagel, S., Rosenshine, I. and Shpigel, NY. (2009) *Mycobacterium avium paratuberculosis* invades human small-intestinal goblet cells and elicits inflammation. *Journal of Infectious Diseases* 199, 350–354.

Gollnick, N.S., Mitchell, R.M., Baumgart, M., Janagama, H.K., Sreevatsan, S. and Schukken, Y.H. (2007) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in bovine monocyte-derived macrophages is not affected by host infection status but depends on the infecting bacterial genotype. *Veterinary Immunology and Immunopathology* 120, 93–105.

Grant, I.R. (1998) Does *Mycobacterium paratuberculosis* survive current pasteurization conditions? *Applied and Environmental Microbiology* 64, 2760–2761.

Grewal, I.S. and Flavell, R.A. (1996) The role of CD40 ligand in costimulation and T-cell activation. *Immunology Reviews* 153, 85–106.

Grewal, I.S. and Flavell, R.A. (1998) CD40 and CD154 in cell-mediated immunity. *Annual Reviews of Immunology* 16, 111–135.

Grewal, I.S., Borrow, P., Pamer, E.G., Oldstone, M.B. and Flavell, R.A. (1997) The CD40–CD154 system in anti-infective host defense. *Current Opinion in Immunology* 9, 491–497.

Grewal, S.K., Rajeev, S., Sreevatsan, S. and Michel, F.C., Jr. (2006) Persistence of *Mycobacterium avium* subsp. *paratuberculosis* and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure. *Applied and Environmental Microbiology* 72, 565–574.

Harb, O.S., Gao, L.Y. and Abu Kwaik, Y. (2000) From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. *Environmental Microbiology* 2, 251–265.

Harris, J.E. and Lammerding, A.M. (2001) Crohn's disease and *Mycobacterium avium* subsp. *paratuberculosis*: current issues. *Journal of Food Protection* 64, 2103–2110.

Harris, N.B. and Barletta, R.G. (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clinical Microbiology Reviews* 14, 489–512.

Harris, N.B., Feng, Z., Liu, X., Cirillo, S.L., Cirillo, J.D. and Barletta, R.G. (1999) Development of a transposon mutagenesis system for *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiology Letters* 175, 21–26.

Jozefowski, S., Sobota, A. and Kwiatkowska, K. (2008) How *Mycobacterium tuberculosis* subverts host immune responses. *Bioessays* 30, 943–954.

Khalifeh, M.S. and Stabel, J.R. (2004) Upregulation of transforming growth factor-beta and interleukin-10 in cows with clinical Johne's disease. *Veterinary Immunology and Immunopathology* 99, 39–46.

Khare, S., Nunes, J., Figueiredo, J., Lawhon, S., Rossetti, C., Gull, T., Rice-Ficht, A. and Garry Adams, L. (2009) Early phase morphological lesions and transcriptional responses of bovine ileum infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 46, 717–728.

Kiener, P.A., Moran-Davis, P., Rankin, B.M., Wahl, A.F., Aruffo, A. and Hollenbaugh, D. (1995) Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *Journal of Immunology* 155, 4917–4925.

Kuehnel, M.P., Goethe, R., Habermann, A., Mueller, E., Rohde, M., Griffiths, G. and Valentin-Weigand, P. (2001) Characterization of the intracellular survival of *Mycobacterium avium* ssp. *paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cellular Microbiology* 3, 551–566.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S. and Kapur, V. (2005) The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proceedings of the National Academy of Sciences of the USA* 102, 12344–12349.

Li, L., Munir, S., Bannantine, J.P., Sreevatsan, S., Kanjilal, S. and Kapur, V. (2007) Rapid expression of *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins for antigen discovery. *Clinical and Vaccine Immunology* 14, 102–105.

Malik, N., Greenfield, B.W., Wahl, A.F. and Kiener, P.A. (1996) Activation of human monocytes through CD40 induces matrix metalloproteinases. *Journal of Immunology* 156, 3952–3960.

Mathur, R.K., Awasthi, A., Wadhone, P., Ramanamurthy, B. and Saha, B. (2004) Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses. *Nature Medicine* 10, 540–544.

Miller, H., Zhang, J., Kuolee, R., Patel, G.B. and Chen, W. (2007) Intestinal M cells: the fallible sentinels? *World Journal of Gastroenterology* 13, 1477–1486.

Momotani, E., Whipple, D.L. and Thiermann, A.B. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Veterinary Pathology* 25, 131–137.

Motiwala, A.S., Li, L., Kapur, V. and Sreevatsan, S. (2006) Current understanding of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis*. *Microbes and Infection* 8, 1406–1418.

Mura, M., Bull, T.J., Evans, H., Sidi-Boumedine, K., McMinn, L., Rhodes, G., Pickup, R. and Hermon-Taylor, J. (2006) Replication and long-term persistence of bovine and human strains of *Mycobacterium avium* subsp. *paratuberculosis* within *Acanthamoeba polyphaga*. *Applied and Environmental Microbiology* 72, 854–859.

Murphy, J.T., Sommer, S., Kabara, E.A., Verman, N., Kuelbs, M.A., Saama, P., Halgren, R. and Coussens, P.M. (2006) Gene expression profiling of monocyte-derived macrophages following infection with *Mycobacterium avium* subspecies *avium* and *Mycobacterium avium* subspecies *paratuberculosis*. *Physiological Genomics* 28, 67–75.

Park, K.T., Dahl, J.L., Bannantine, J.P., Barletta, R.G., Ahn, J., Allen, A.J., Hamilton, M.J. and Davis, W.C. (2008) Demonstration of allelic exchange in the slow-growing bacterium *Mycobacterium avium* subsp. *paratuberculosis*, and generation of mutants with deletions at the

pknG, *relA*, and *lsr2* loci. *Applied and Environmental Microbiology* 74, 1687–1695.

Patel, D., Danelishvili, L., Yamazaki, Y., Alonso, M., Paustian, M.L., Bannantine, J.P., Meunier-Goddik, L. and Bermudez, L.E. (2006) The ability of *Mycobacterium avium* subsp. *paratuberculosis* to enter bovine epithelial cells is influenced by preexposure to a hyperosmolar environment and intracellular passage in bovine mammary epithelial cells. *Infection and Immunity* 74, 2849–2855.

Pieters, J. (2001) Entry and survival of pathogenic mycobacteria in macrophages. *Microbes and Infection* 3, 249–255.

Prohaszka, Z., Daha, M.R., Susal, C., Daniel, V., Szlavik, J., Banhegyi, D., Nagy, K., Varkonyi, V., Horvath, A., Ujhelyi, E., Toth, F.D., Uray, K., Hudecz, F. and Fust, G. (1999) C1q autoantibodies in HIV infection: correlation to elevated levels of autoantibodies against 60-kDa heat-shock proteins. *Clinical Immunology* 90, 247–255.

Richter, E., Wessling, J., Lugering, N., Domschke, W. and Rusch-Gerdes, S. (2002) *Mycobacterium avium* subsp. *paratuberculosis* infection in a patient with HIV, Germany. *Emerging Infectious Diseases* 8, 729–731.

Robertson, A.K. and Andrew, P.W. (1991) Interferon gamma fails to activate human monocyte-derived macrophages to kill or inhibit the replication of a non-pathogenic mycobacterial species. *Microbial Pathogenesis* 11, 283–288.

Rohde, K.H., Abramovitch, R.B. and Russell, D.G. (2007) *Mycobacterium tuberculosis* invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host and Microbe* 2, 352–364.

Rowe, M.T. and Grant, I.R. (2006) *Mycobacterium avium* ssp. *paratuberculosis* and its potential survival tactics. *Letters in Applied Microbiology* 42, 305–311.

Russell, D.G., Mwandumba, H.C. and Rhoades, E.E. (2002) *Mycobacterium* and the coat of many lipids. *Journal of Cell Biology* 158, 421–426.

Secott, T.E., Lin, T.L. and Wu, C.C. (2001) Fibronectin attachment protein homologue mediates fibronectin binding by *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 69, 2075–2082.

Secott, T.E., Lin, T.L. and Wu, C.C. (2002) Fibronectin attachment protein is necessary for efficient attachment and invasion of epithelial cells by *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 70, 2670–2675.

Secott, T.E., Lin, T.L. and Wu, C.C. (2004) *Mycobacterium avium* subsp. *paratuberculosis* fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins. *Infection and Immunity* 72, 3724–3732.

Sigurðardóttir, O.G., Press, C.M., Saxegaard, F. and Evensen, O. (1999) Bacterial isolation, immunological response, and histopathological lesions during the early subclinical phase of experimental infection of goat kids with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 36, 542–550.

Sigurðardóttir, O.G., Press, C.M. and Evensen, O. (2001) Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. *Veterinary Pathology* 38, 184–189.

Sigurðardóttir, O.G., Valheim, M. and Press, C.M. (2004) Establishment of *Mycobacterium avium* subsp. *paratuberculosis* infection in the intestine of ruminants. *Advanced Drug Delivery Reviews* 56, 819–834.

Sivakumar, P., Tripathi, B.N. and Singh, N. (2005) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in intestinal and lymph node tissues of water buffaloes (*Bubalus bubalis*) by PCR and bacterial culture. *Veterinary Microbiology* 108, 263–270.

Sommer, S., Pudrith, C.B., Colvin, C.J. and Coussens, P.M. (2009) *Mycobacterium avium* subspecies *paratuberculosis* suppresses expression of IL-12p40 and iNOS genes induced by signalling through CD40 in bovine monocyte-derived macrophages. *Veterinary Immunology and Immunopathology* 128, 44–52.

Soong, L., Xu, J.C., Grewal, I.S., Kima, P., Sun, J., Longley, B.J., Jr., Ruddle, N.H., McMahon-Pratt, D. and Flavell, R.A. (1996) Disruption of CD40–CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. *Immunity* 4, 263–273.

Souza, C.D., Evanson, O.A. and Weiss, D.J. (2006) Regulation by Jun N-terminal kinase/stress activated protein kinase of cytokine expression in *Mycobacterium avium* subsp. *paratuberculosis*-infected bovine monocytes. *American Journal of Veterinary Research* 67, 1760–1765.

Souza, C.D., Evanson, O.A., Sreevatsan, S. and Weiss, D.J. (2007a) Cell membrane receptors on bovine mononuclear phagocytes involved in phagocytosis of *Mycobacterium avium* subsp. *paratuberculosis*. *American Journal of Veterinary Research* 68, 975–980.

Souza, C.D., Evanson, O.A. and Weiss, D.J. (2007b) Role of the MAPK(ERK) pathway in regulation of cytokine expression by *Mycobacterium avium* subsp. *paratuberculosis*-exposed bovine monocytes. *American Journal of Veterinary Research* 68, 625–630.

Souza, C.D., Evanson, O.A. and Weiss, D.J. (2007c) Role of the mitogen-activated protein kinase pathway in the differential response of bovine monocytes to *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. *Microbes and Infection* 9, 1545–1552.

Souza, C.D., Evanson, O.A. and Weiss, D.J. (2008) Role of cell membrane receptors in the suppression of monocyte anti-microbial activity against *Mycobacterium avium* subsp. *paratuberculosis*. *Microbial Pathogenesis* 44, 215–223.

Stabel, J.R. (2000) Transitions in immune responses to *Mycobacterium paratuberculosis*. *Veterinary Microbiology* 77, 465–473.

Stout, R.D. and Suttles, J. (1996) The many roles of CD40 in cell-mediated inflammatory responses. *Immunology Today* 17, 487–492.

Sweeney, R.W., Whitlock, R.H., Hamir, A.N., Rosenberger, A.E. and Herr, S.A. (1992a) Isolation of *Mycobacterium paratuberculosis* after oral inoculation in uninfected cattle. *American Journal of Veterinary Research* 53, 1312–1314.

Sweeney, R.W., Whitlock, R.H. and Rosenberger, A.E. (1992b) *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *Journal of Clinical Microbiology* 30, 166–171.

Sweeney, R.W., Uzonna, J., Whitlock, R.H., Habecker, P.L., Chilton, P. and Scott, P. (2006) Tissue prediction sites and effect of dose on *Mycobacterium avium* subsp. *paratuberculosis* organism recovery in short-term bovine experimental oral infection model. *Research in Veterinary Science* 80, 253–259.

Tessema, M.Z., Koets, A.P., Rutten, V.P. and Gruys, E. (2001) How does *Mycobacterium avium* subsp. *paratuberculosis* resist intracellular degradation? *Veterinary Quarterly* 23, 153–162.

Tian, L., Noelle, R.J. and Lawrence, D.A. (1995) Activated T cells enhance nitric oxide production by murine splenic macrophages through gp39 and LFA-1. *European Journal of Immunology* 25, 306–309.

Tiwari, A., Vanleeuwen, J.A., McKenna, S.L., Keefe, G.P. and Barkema, H.W. (2006) Johne's disease in Canada. Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Canadian Veterinary Journal* 47, 874–882.

Tzu, S. (2005) *The Art of War* (translated by Cleary, T.). Shambhala Publications, Boston, Massachusetts.

Van Kooten, C. and Banchereau, J. (1996) CD40–CD40 ligand: a multifunctional receptor–ligand pair. *Advances in Immunology* 61, 1–77.

Van Kooten, C. and Banchereau, J. (2000) CD40–CD40 ligand. *Journal of Leukocyte Biology* 67, 2–17.

Waddell, L.A., Rajic, A., Sargeant, J., Harris, J., Amezcua, R., Downey, L., Read, S. and McEwen, S.A. (2008) The zoonotic potential of *Mycobacterium avium* spp. *paratuberculosis*: a systematic review. *Canadian Journal of Public Health* 99, 145–155.

Weiss, D.J. and Souza, C.D. (2008) Review paper: modulation of mononuclear phagocyte function by *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 45, 829–841.

Weiss, D.J., Evanson, O.A., McClenahan, D.J., Abrahamsen, M.S. and Walcheck, B.K. (2001) Regulation of expression of major histocompatibility antigens by bovine macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* or *Mycobacterium avium* subsp. *avium*. *Infection and Immunity* 69, 1002–1008.

Weiss, D.J., Evanson, O.A. and Souza, C.D. (2005) Expression of interleukin-10 and suppressor of cytokine signaling-3 associated with susceptibility of cattle to infection with *Mycobacterium avium* subsp. *paratuberculosis*. *American Journal of Veterinary Research* 66, 1114–1120.

Whittington, R.J. and Sergeant, E.S. (2001) Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. *Australian Veterinary Journal* 79, 267–278.

Whittington, R.J., Lloyd, J.B. and Reddacliff, L.A. (2001) Recovery of *Mycobacterium avium* subsp. *paratuberculosis* from nematode larvae cultured from the faeces of sheep with Johne's disease. *Veterinary Microbiology* 81, 273–279.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, I.B. and Reddacliff, L.A. (2004) Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Applied and Environmental Microbiology* 70, 2989–3004.

Whittington, R.J., Marsh, I.B. and Reddacliff, L.A. (2005) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. *Applied and Environmental Microbiology* 71, 5304–5308.

Woo, S.R., Heintz, J.A., Albrecht, R., Barletta, R.G. and Czuprynski, C.J. (2007) Life and death in bovine monocytes: the fate of *Mycobacterium avium* subsp. *paratuberculosis*. *Microbial Pathogenesis* 43, 106–113.

Wu, C.W., Livesey, M., Schmoller, S.K., Manning, E.J., Steinberg, H., Davis, W.C., Hamilton, M.J. and Talaat, A.M. (2007a) Invasion and persistence of *Mycobacterium avium* subsp. *paratuberculosis* during early stages of Johne's

disease in calves. *Infection and Immunity* 75, 2110–2119.

Wu, C.W., Schmoller, S.K., Shin, S.J. and Talaat, A.M. (2007b) Defining the stressome of *Mycobacterium avium* subsp. *paratuberculosis in vitro* and in naturally infected cows. *Journal of Bacteriology* 189, 7877–7886.

Yates, R.M. and Russell, D.G. (2005) Phagosome maturation proceeds independently of stimulation of Toll-like receptors 2 and 4. *Immunity* 23, 409–417.

Zhu, X., Tu, Z.J., Coussens, P.M., Kapur, V., Janagama, H., Naser, S. and Sreevatsan, S. (2008) Transcriptional analysis of diverse strains *Mycobacterium avium* subspecies *paratuberculosis* in primary bovine monocyte derived macrophages. *Microbes and Infection* 10, 1274–1282.

12 Comparative Differences between Strains of *Mycobacterium avium* subsp. *paratuberculosis*

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12.1 Introduction

It was reported as early as the 1930s that Johne's disease (JD) in sheep may be caused by either of two closely related organisms, the classical type (akin to that described in cattle) and an 'ovine type', only differentiated from the first by its resistance to artificial cultivation (Dunkin and Balfour-Jones, 1935; McEwen, 1939; Taylor, 1945). It was not until the 1990s that these two strain types could be distinguished genetically. Since then, a plethora of molecular typing techniques has provided more comprehensive data on *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) strain types. This chapter presents an overview of the comparative differences between *MAP* strains. Details of the various genotyping techniques are described elsewhere (see Collins, Chapter 25, this volume).

12.2 Nomenclature of *MAP* Strain Types

Broadly speaking, *MAP* strains can be divided into two major groups (or strain types), which can be defined according to their growth characteristics, host preferences or host range and pathogenicity. Historically, these strain types were first named according to the species from which they were first isolated and were designated as 'sheep' (S) and 'cattle' (C) types. However, as strain typing became more widely applied it became apparent that *MAP* could be isolated from a broad range of species and that the species of origin was not necessarily an accurate indicator of strain type. It is often not clear when the 'sheep' or 'cattle' designation refers to the host species from which the isolate has been isolated or the strain type to which it belongs. Therefore, to

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avoid confusion, these strain types were subsequently designated as Type I (S type) and Type II (C type) (Stevenson *et al.*, 2002).

The advent of molecular typing techniques made it possible to discriminate genetically between *MAP* isolates, and other strain types have since been described. The application of restriction fragment length polymorphism analysis (RFLP) coupled with hybridization to IS*900* (IS*900*-RFLP) and pulsed-field gel electrophoresis (PFGE) revealed a strain type designated 'intermediate' (Collins *et al.*, 1990) or 'Type III' (de Juan *et al.*, 2005), respectively. Similarly, a 'bison' type has been described following the identification of distinctive small sequence polymorphisms in the genome sequence (Whittington *et al.*, 2001a; Sohal *et al.*, 2009). Recent data from whole-genome comparison studies support the division of *MAP* isolates into the two major strain types I (or S) and II (or C). In this study, there were no distinctions that delineated a third major lineage, such that the authors grouped Type III strains with the Type I strains (Alexander *et al.*, 2009). This grouping is consistent with the observation that Type I and III strains share similar phenotypic traits and host preferences. Similarly, the existence of a distinct 'bison' type is not yet established, and most regarded these isolates as a subtype of Type II strains. Table 12.1 details the various typing techniques used to differentiate these strain types and the nomenclature found in the literature and how this corresponds to the designated types. For consistency with other chapters in this book, Type I and Type II strains are referred to as Type S and Type C, respectively. However, when addressing isolates from specific hosts, the designations Type I and Type II are used to avoid confusion between host provenance and bacterial genotype.

12.3 Phenotypic Differences between *MAP* Strains

The *MAP* strain types differ with respect to the ease with which they can be isolated on artificial media and their respective growth rates. Type C *MAP* strains are comparatively easy to isolate from clinical samples and will grow reasonably well on a range of solid or liquid media supplemented with mycobactin. These strains will typically produce detectable growth in 4–16 weeks, depending on the initial inoculum. Type S *MAP* strains typically grow more slowly and have fastidious requirements for culture on artificial media. Primary isolation of these strain types can take from 4 months to a year. Whittington

Table 12.1. Nomenclature of *MAP* strain types determined by different typing procedures and their relation to the designated Type S and Type C.

		Type S		Type C
Typing method	References	Type I	Type III	Type II
IS*900*-RFLP	Collins *et al.* (1990) Pavlik *et al.* (1999)	Sheep (S)	Intermediate (I)	Cattle (C)
Pulsed-field gel electrophoresis	Stevenson *et al.* (2002) de Juan *et al.* (2005)	Type I	Type III	Type II
IS*1311* PCR-REA	Marsh *et al.* (1999) Whittington *et al.* (2001a)	Sheep (S)	ND	Cattle (C) Bison (B)
DMC-PCR	Collins *et al.* (2002)	Sheep type	ND	Cattle type
RDA-PCR	Dohmann *et al.* (2003)	Type I	ND	Type II
gyr-PCR	Castellanos *et al.* (2007b)	Type I	Type III	Type II
*inh*A-PCR	Castellanos *et al.* (2007a)	Type I	Type III	Type II
PPE-DGGE	Griffiths *et al.* (2008)	Type I	Type III	Type II

ND, not differentiated from Type I by this assay.

et al. (1999) reported that mycobactin J was an essential additive to artificial media for growth of Type S strains, but other researchers have found that these strains will grow independently of mycobactin on Middlebrook-based media (Aduriz *et al*., 1995; M. Behr, Montreal, 2009, personal communication). The addition of egg yolk seems to be beneficial for the primary isolation of Type S strains, although Herrold's egg yolk medium (HEYM), traditionally used for the isolation of *MAP*, does not support good growth of Type S strains (Taylor, 1951; Whittington *et al*., 1999; de Juan *et al*., 2006b; Florou *et al*., 2008a). The failure of HEYM to support the growth of these strains may be due to the inhibitory action of some component of the medium, although inhibition due to malachite green has been ruled out (Whittington *et al*., 1999). Media based on Middlebrook 7H9, 7H10 or 7H11 are often used for primary isolation of Type S strains. The recommended solid media for the isolation of Type S strains are Löwenstein–Jensen and Middlebrook 7H11, both supplemented with mycobactin (de Juan *et al*., 2006b). BACTEC 12B radiometric medium supplemented with mycobactin and egg yolk has been found to be the best liquid medium (Whittington *et al*., 1999; Gumber and Whittington, 2007; Whittington, 2009). In addition to being fastidious in their laboratory growth, Type S strains are also highly sensitive to decontamination procedures and certain antibiotics (Reddacliff *et al*., 2003; Gumber and Whittington, 2007; Whittington, 2009). The addition of ampicillin or vancomycin hydrochloride appears to be detrimental to the recovery of some Type S strains, although this concern must be weighed against the benefits of reducing bacterial contamination.

In general, *MAP* strain types cannot be differentiated according to colony morphology. The exceptions to this are the pigmented strains. These *MAP* strains produce a yellow or orange pigment that is stable during passage both *in vitro* and *in vivo*. They have been isolated from sheep, and the gut mucosa of infected animals shows a typical brilliant yellow colour. To date, all pigmented *MAP* strains that have been typed have been classified as Type I. The phenotypic characteristics of the *MAP* strain types are summarized in Table 12.2.

12.4 Comparison of Epidemiological Characteristics of *MAP* Strains

There appear to be epidemiological trends associated with *MAP* strain types with respect to transmission, host preference and susceptibility to infection. However, the results of many past epidemiological studies need to be interpreted with caution since they often employed media that would not support growth of all *MAP* strain types. This could easily result in a microbiological bias in these reports. Furthermore, many studies did not use molecular typing techniques that differentiated all *MAP* strain types.

Table 12.2. Summary of the major phenotypic and epidemiological characteristics of the *MAP* strain types.

Characteristic	Type S (Type I/III)	Type C (Type II)
Ease of primary isolation	Difficult	Easy
Typical incubation time for primary growth on solid media[a]	4–12 months	5–16 weeks
Typical incubation time for primary growth in liquid media[a]	7–12 weeks	4–10 weeks
Addition of egg yolk to liquid media	Beneficial for primary isolation	Not required
Host range or preference	Predominantly sheep and goats	Common strain in cattle; very broad host range, including both ruminants and non-ruminants

[a]Incubation time depends on type of medium, as described in text.

Type I strains have been isolated predominantly, but not exclusively, from sheep and goats, suggesting a preference for these host species. They include the pigmented *MAP* strains, which have been isolated commonly from animals in the UK (Taylor, 1951; Stevenson *et al.* 2002) and the Faroe islands (F. Saxegaard and B. Hanusson, Norway, 2002, personal communications). Pigmented strains have been reported in Spain (Sevilla *et al.*, 2007) and Morocco (Benazzi *et al.*, 1996) but are relatively uncommon in these settings. Animals infected with these strains exhibit multibacillary paratuberculosis. The production of pigment by this subgroup of strains is intriguing.

Cross-species transmission of Type I strains has been demonstrated experimentally. Calves infected with either pigmented or Icelandic ovine strains developed clinical JD (Taylor, 1953). Deer calves experimentally infected with a Type I strain also developed JD, but it was observed that the deer were less susceptible to the Type I strain than to the Type II strains employed (O'Brien *et al.*, 2006). The pigmented Type I strain isolated by Watt (1954) also could be transmitted experimentally to sheep. However, experimental infections typically involve high doses of *MAP* and therefore may not accurately assess transmission by these different strains.

There are a few reports of cattle naturally infected with Type I strains, including a pigmented *MAP* strain (Watt, 1954). These infections have been associated with bullfighting breeds (de Juan *et al.*, 2006a) or cases where there has been direct or indirect contact of calves with sheep infected with Type I strains (Whittington *et al.*, 2001b). The most convincing demonstration of natural cross-species transmission of Type I strains between sheep and cattle occurred in Iceland. Johne's disease was introduced in 1938 via infected Karakul sheep from Germany and in 1944 spread to the local cattle population, in which it subsequently became endemic (Fridriksdottir *et al.*, 2000). The Icelandic strains were classified retrospectively as Type I strains (Whittington *et al.*, 2001b). Type I *MAP* strains have also been isolated from farmed red deer (de Lisle *et al.*, 1993), but such isolations are rare (O'Brien *et al.*, 2006). Similarly, reports of the occurrence of Type I strains in wildlife are uncommon. One Type I isolate was obtained from a fallow deer (*Dama dama*) (Machackova *et al.*, 2004) and another from a house mouse (*Mus musculus*) (Florou *et al.*, 2008b). The risk of natural transmission of Type I strains from sheep and goats to cattle or deer is believed to be low and probably only occurs when susceptible animals are exposed to high doses. Moloney and Whittington (2008) estimated the prevalence of Type I *MAP* infections in Australian cattle exposed to these strains to be no higher than 0.8%. It will be interesting to see if the prevalence of cross-species transmission of Type I *MAP* strains is revised following surveys that employ appropriate culture conditions to support the growth of all *MAP* strain types and use appropriate genotyping methods.

Type II *MAP* isolates have a very broad host range and are commonly isolated from both domesticated and wildlife species, including non-ruminants (Beard *et al.*, 1999, 2001a; Greig *et al.*, 1999). Non-ruminant species known to have been infected with *MAP* are listed in Table 18.1 (see Hutchings *et al.*, Chapter 18, this volume). Type II is by far the most common *MAP* strain type isolated from cattle. To date, *MAP* isolates from human Crohn's patients that have been typed have all been classified as Type II (Whittington *et al.*, 2000; Bull *et al.*, 2003; Ghadiali *et al.*, 2004; Griffiths, *et al.*, 2008; Paustian *et al.*, 2008). The bison-type strains, as defined by the polymorphism at base 223 of the IS*1311* insertion sequence (Whittington *et al.*, 2001a), have been identified as *MAP* Type II by PFGE and microarray analyses (Sevilla *et al.*, 2007; Paustian *et al.*, 2008). The bison-type strains do not show specificity for bison (*Bison bison*) and have been isolated from Indian sheep and goats (Sevilla *et al.*, 2005), riverine buffalo (*Bubalus bubalis*) (Yadav *et al.*, 2008) and antelope (*Boselaphus tragocamelus*) (Kumar *et al.*, 2008).

Data are accumulating regarding the geographical distribution of *MAP* strains, which has probably been influenced by many factors, including animal movements, strain virulence and farm management systems. Already there are some interesting observations. Type I strains are prevalent in Australian sheep and, despite the fact that Type II

strains have been isolated from Australian cattle, they are rarely, if ever, isolated from Australian sheep (Whittington *et al.*, 2000). This is in contrast to Europe, where both Type I and II strains are isolated from sheep (Stevenson *et al.*, 2009). This difference is possibly linked to differences in management practices between some parts of Europe and Australia, the scale of the farming practices and relative proportions of sheep and cattle co- or sequentially grazing. Furthermore, paratuberculosis is more common in sheep than in cattle in Australia and Type I strains are more virulent for sheep than Type II strains (Verna *et al.*, 2007).

There appear to be two common Type II *MAP* strains across Europe (Stevenson *et al.*, 2009). These are designated [2-1] and [1-1] by PFGE analysis and INMV 1 and 2 by analysis of variable number tandem repeat sequences. B-C1 is the most widespread IS*900*-RFLP type in Europe except in the UK, where the strain type is almost exclusively B-C17 (rarely found elsewhere in Europe).

12.5 Comparison of the Virulence and Pathogenicity of *MAP* Strains

Evidence is accumulating that *MAP* strain types differ in virulence and may be associated with different immunopathological forms of JD. Disease is the result of a complex interplay between host defence mechanisms and attempts by the mycobacterium to circumvent these defences. The phagocyte is the most powerful and important part of the host defences once the epithelial surface of the gut has been breached, and a key determinant of virulence is the ability of the bacterium to enter, survive and replicate within the cells. There is some evidence that different *MAP* strains have different capacities for entry and survival in macrophages. Intracellular studies undertaken by Janagama *et al.* (2006) showed that Type C *MAP* strains persisted in relatively higher numbers in bovine monocyte-derived macrophages (MDMs) when compared with a Type S isolate. This observation was confirmed later by Gollnick *et al.* (2007) using the same *MAP* strains in MDMs originating from both naturally *MAP*-infected and non-infected cows. These studies employed very few strains and more detailed studies are required, using a larger collection of strains.

Zhu *et al.* (2008) undertook transcriptional analysis of the different *MAP* strain types (the same Type S and Type C strains used by Janagama *et al.* (2006) and Gollnick *et al.* (2007)) in MDMs using selective capture of transcribed sequences. Despite variations in the genes identified, the different *MAP* strains responded in a generally similar fashion to oxidative stress, to metabolic and nutritional starvation, in cell survival, and in upregulating genes involved in cell wall biosynthesis. However, transcription of MAP_1728 (YfnB), MAP_1738 (MmpL5), MAP_1729c and MAP_1730 (hypothetical proteins with unknown function) was upregulated only in Type C strains, consistent with their absence from Type S strains examined to date (Table 12.3).

Two studies have presented evidence that different *MAP* strain types may play a role in polarizing the host immune responses, which may determine the different disease pathologies observed. Janagama *et al.* (2006) investigated cytokine responses to different *MAP* strain types in a bovine MDM system using real-time polymerase chain reaction assays, and Motiwala *et al.* (2006) performed a global-scale transcriptional analysis of human macrophages (THP-1 cells) exposed to different *MAP* strains. Common trends emerged from these studies. Both studies reported that Type C *MAP* strains induced anti-inflammatory and anti-apoptotic pathways in the host cells without causing major alterations in the transcription of pro-inflammatory genes, which would favour bacterial survival and persistence. This pattern of gene expression was found to be the same for bovine, bison and human Type C isolates, with different genotypes as defined by short-sequence repeat (SSR) analysis, although the magnitude of the responses varied. In contrast, ovine Type S strains representing distinct SSR genotypes significantly upregulated pro-inflammatory genes. Pro-inflammatory responses are generally associated with protection and elimination of mycobacteria, so this gene expression profile may help to explain why Type S strains

Table 12.3. Polymorphisms in Type C but not in Type S *MAP* strains.

Polymorphism (ORF cluster[a])	Designations	Genes/pathway of particular interest
MAP_1491 to MAP_1484c	LSP[A]20 (Alexander *et al*., 2009) INDEL6 (Castellanos *et al*., 2009)	Pyruvate dehydrogenase complex
MAP_1728c to MAP_1744	Del-2 (Marsh *et al*., 2006; Alexander *et al*., 2009) MAV-14 (Wu *et al*., 2006) INDEL7 (Castellanos *et al*., 2009)	*yfnB* (predicted hydrolase)[b] *mmpS* and *mmpL* genes[bc] MAP_1729c & MAP-1730 (hypothetical proteins)[b] *fabG3* (lipid biosynthesis) *acg, devS*[c]

[a]Exact coordinates of the ORF clusters may vary according to differences in probes used in the various microarray studies.
[b]Transcription upregulated in MDMs in study by Zhu *et al.* (2008).
[c]Also identified by representational difference analysis (Marsh and Whittington, 2005).

rarely cause disease in bovine hosts. However, the results of these studies should be treated with caution since only a few *MAP* strains were investigated. Furthermore, Blumenthal *et al.* (2005) reported different macrophage gene response signatures induced by distinct strains of *Mycobacterium avium* subsp. *avium* and *M. avium* subsp. *hominissuis* (*MAH*) exhibiting differing levels of virulence. Comparative transcriptional analysis of ovine and caprine macrophages infected with different *MAP* strain types has yet to be performed.

Recently, Verna *et al.* (2007) investigated the relationship between *MAP* strains and the pathology of disease in sheep. Infection with Type C isolates of different genotypes resulted in a common pattern, characterized by focal lesions, mainly in the mesenteric lymph nodes, as well as the presence of fibrous tissue and occasionally necrosis and numerous Langhans giant cells in the granulomas. Infection with a Type S isolate induced more severe lesions, occurring mainly in the intestinal lymphoid tissue. There was a conspicuous absence of necrosis, fibrosis and giant cells. Lesions induced by the Type S strain were more severe than those induced by Type C strains, which suggests that Type C strains had a slow, localized development in the early stages of infection. The development of giant cells may be linked to *MAP* strain type rather than host determinants, since giant cells are a feature of natural cases of bovine paratuberculosis and leporine paratuberculosis (Beard *et al*., 2001b) but not ovine paratuberculosis caused by Type S *MAP* strains.

12.6 Genotypic Differences between *MAP* Strains

MAP strain types can be distinguished by genetic polymorphisms, in particular by the presence of large sequence polymorphisms (LSPs), which can be either insertions or deletions. These have been determined by microarray comparisons and *in silico* analysis of whole-genome sequence data from *MAP* strain K-10 and *MAH* strain 104. At the time of writing, a limited number of *MAP* strains have been analysed, especially of Type S, but collective data suggest that there are two major groups of *MAP* strains, corresponding to Types S and C. It has been proposed that *MAP* evolved from a putative *MAH* ancestor by the acquisition of novel genetic material and then into the Type S and C strain types through differential deletion of DNA (Alexander *et al*., 2009). The evolution and phylogeny of *MAP* from *M. avium* is described in more detail elsewhere (see Turenne and Alexander, Chapter 7, this volume).

Type S strains may have retained more ancestral *MAH* DNA, although this requires confirmation by genomic sequencing of more *MAP* strains. They are characterized by the

presence of four clusters of open reading frames (ORFs) that are present in the *MAH* genome but absent from *MAP* K-10 (MAV_1975 to MAV_2008, MAV_2978 to MAV_2998, MAV_3258 to MAV_3270, and MAV_5225 to MAV_5243). These polymorphisms have been consistently detected in all Type S strains analysed to date (Semret *et al.*, 2006; Paustian *et al.*, 2008; Alexander *et al.*, 2009; Castellanos *et al.*, 2009) and details are given in Table 12.4. Castellanos *et al.* (2009) identified additional polymorphisms absent from Type C strains that were not detected by other researchers (MAV_0339, MAV_2223, MAV_2254 and MAV_4125 to MAV_4130). MAV_4351 and MAV_4353 are present in Type S strains but absent from Type C strains (Alexander *et al.*, 2009; Castellanos *et al.*, 2009). These genes may have been deleted as a result of the genomic inversion spanning MAP_3493 to MAP_4280 in Type C strains (Alexander *et al.*, 2009). In the converse direction, Type C strains are differentiated from Type S strains by the presence of two clusters of ORFs (MAP_1490 to MAP_1484c and MAP_1728c to MAP_1744) that are consistently absent from Type S strains (Table 12.3).

Not surprisingly, genomic sequence comparisons of different Type S *MAP* isolates have revealed additional polymorphic regions within Type S strains. Castellanos *et al.* (2009) report the absence of MAP_3584 from Type III *MAP* strains, but this locus has not yet been investigated by other researchers. More Type III strains will need to be analysed to determine if this really is a Type III strain-specific polymorphism. Other heterogenic loci identified in Type S strains include the region MAP_1433c to MAP_1438c, also known as VA-15 (Alexander *et al.*, 2009; Castellanos *et al.*, 2009) and MAP_2325 (Marsh *et al.*, 2006; Paustian *et al.*, 2008; Castellanos *et al.*, 2009).

12.7 Concluding Thoughts

It is abundantly clear that there are at least two, and perhaps more, different strains of *MAP* which are able to cause paratuberculosis in various hosts. The true diversity of *MAP* isolates will only be revealed when adequate culture methods, coupled with appropriate genotyping, are applied to comprehensive and representative collections of organisms.

Table 12.4. Polymorphisms in Type S but not Type C *MAP* strains.

Polymorphism (ORF cluster[a])	Designations	Encoded proteins or pathways of particular interest
MAV_1975 to MAV_2008	PIG-RDA 20 (Dohmann *et al.*, 2003) LSP^{A} 4-II (Semret *et al.*, 2006; Alexander *et al.*, 2009) MAV-7 (Wu *et al.*, 2006) INDEL10 (Castellanos *et al.*, 2009)	Truncates MAP_2178 involved in mycobactin synthesis TetR transcriptional regulator, PPE proteins, HspR protein, PapA2 protein, ABC-2 type transporter, IS*1311*
MAV_2978 to MAV_2998	PIG-RDA 30 (Dohmann *et al.*, 2003) MAV-14 (Wu *et al.*, 2006; Alexander *et al.*, 2009) INDEL5 (Castellanos *et al.*, 2009)	Cytochrome P450, SecD protein, aryl-sulfatase Lipid and energy metabolism
MAV_3258 to MAV_3270	MAV-17 (Wu *et al.*, 2006) INDEL3 (Castellanos *et al.*, 2009) GPL (Paustian *et al.*, 2008; Alexander *et al.*, 2009)	Glycopeptidolipid biosynthesis
MAV_5225 to MAV_5243	PIG-RDA 10 (Dohmann *et al.*, 2003) LSP^{A}18 (Semret *et al.*, 2006; Alexander *et al.*, 2009) MAV-24 (Wu *et al.*, 2006) INDEL16 (Castellanos *et al.*, 2009)	Lipid metabolism

[a]Exact coordinates of the ORF clusters may vary according to differences in probes used in the various microarray studies.

Table 12.5. *MAP* strain-specific polymorphisms that may be associated with differences in virulence and pathogenic traits.

Gene/LSP	Putative role in virulence/pathogenesis	References
MAV_1993 (LSP^{A}4-II)	HspR protein. Global regulator of heat-shock gene expression. Represses *acr*2 involved in virulence and pathogenesis of *Mycobacterium tuberculosis*	Stewart *et al.* (2003, 2005)
MAV_1998 MAV_2006 (LSP^{A}4-II)	PPE family proteins. Elicit increased humoral and cell-mediated response in infected host	Tundup *et al.* (2008)
MAV_2005 (LSP^{A}4-II)	PapA2 protein. Essential for biosynthesis of *M. tuberculosis* virulence factor sulfolipid-1	Kumar *et al.* (2007)
MAV_3258–MAV_3270 (GPL)	Glycopeptidolipids. Promote macrophage activation in a TLR2- and MyD88-dependent manner. Complex cluster with three configurations	Schorey and Sweet (2008); Alexander *et al.* (2009)
MAV_2984 (MAV-14)	Cytochrome P450. Possible involvement in basic cellular processes and virulence	McLean *et al.* (2006)
MAV_2989 (MAV-14)	Aryl-sulfatase. May modulate pathogen–host interactions	Mougous *et al.* (2002)
MAP_1728c–MAP_1744	MmpL proteins: involved in fatty acid transport, associated with cell surface characteristics, biofilm formation and virulence. MmpS proteins: involved in intracellular survival and *in vivo* growth	Recht and Kolter (2001); Domenech *et al.* (2005); Lamichhane *et al.* (2005); Marsh *et al.* (2005)
MAP_1740c (Del-2)	DevS protein. Essential for *in vivo* growth of *M. tuberculosis* and induced during hypoxia	Sherman *et al.* (2001); Sassetti and Rubin (2003)
MAP_1741c (Del-2)	Upregulated during responses to heat shock and hypoxia in *M. tuberculosis*	Sherman *et al.* (2001); Stewart *et al.* (2002)
MAP_1743c (Del-2)	Acg. Associated with detoxification of nitroaromatic compounds in macrophages and granulomas in *M. tuberculosis*	Sherman *et al.* (2001); Purkayastha *et al.* (2002)
MAP_2704 (INDEL11)	Deleted in pigmented S strains. Haemolysin III homologue. Virulence factor for systemic infections of humans with isolates of *M. avium* complex	Maslow *et al.* (1999); Castellanos *et al.* (2009)
MAV_4125 MAV_4126 (INDEL12)	*mce* genes involved in initiation of infection through cell entry and granuloma formation	Gioffre *et al.* (2005); Senaratne *et al.* (2008); Castellanos *et al.* (2009)

There is no doubt that as more discriminatory genotyping methods and 'next-generation' sequencing technologies are applied to a larger number of strains from different geographical regions more genetic polymorphisms will be identified. This should further clarify the phylogeny of *MAP* strains and help to identify differences between Type S isolates. Genomic sequence data should provide a framework to categorize the different phenotypical, epidemiological and pathogenic traits that have been observed between the *MAP* strain types. A number of polymorphisms already identified could have consequences with regard to the virulence and pathogenesis of *MAP* strains, as detailed in Table 12.5. Targeted studies will now be required to determine the functional impact of these natural polymorphisms between strains.

References

Aduriz, J.J., Juste, R.A. and Cortabarria, N. (1995) Lack of mycobactin dependence of mycobacteria isolated on Middlebrook 7H11 from clinical

cases of ovine paratuberculosis. *Veterinary Microbiology* 45, 211–217.

Alexander, D.C., Turenne, C.Y. and Behr, M.A. (2009) Insertion and deletion events that define the pathogen *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Bacteriology* 191, 1018–1025.

Beard, P.M., Henderson, D., Daniels, M.J., Pirie, A., Buxton, D., Greig, A., Hutchings, M.R., McKendrick, I., Rhind, S., Stevenson, K. and Sharp, J.M. (1999) Evidence of paratuberculosis in fox (*Vulpes vulpes*) and stoat (*Mustela erminea*). *The Veterinary Record* 145, 612–613.

Beard, P.M., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Buxton, D., Rhind, S., Greig, A., Hutchings, M.R., McKendrick, I., Stevenson, K. and Sharp, J.M. (2001a) Paratuberculosis infection of non-ruminant wildlife in Scotland. *Journal of Clinical Microbiology* 39, 1517–1521.

Beard, P.M., Rhind, S.M., Buxton, D., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Greig, A., Hutchings, M.R., Stevenson, K. and Sharp, J.M. (2001b) Natural paratuberculosis infection in rabbits in Scotland. *Journal of Comparative Pathology* 124, 290–299.

Benazzi, S., el Hamidi, M. and Schliesser, T. (1996) Paratuberculosis in sheep flocks in Morocco: a serological, microscopical and cultural survey. *Zentralblatt für Veterinarmedizin [B]* 43, 213–219.

Blumenthal, A., Lauber, J., Hoffmann, R., Ernst, M., Keller, C., Buer, J., Ehlers, S. and Reiling, N. (2005) Common and unique gene expression signatures of human macrophages in response to four strains of *Mycobacterium avium* that differ in their growth and persistence characteristics. *Infection and Immunity* 73, 3330–3341.

Bull, T.J., Sidi-Boumedine, K., McMinn, E.J., Stevenson, K., Pickup, R. and Hermon-Taylor, J. (2003) Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subsp. *paratuberculosis* from other species of the *Mycobacterium avium* complex. *Molecular and Cellular Probes* 17, 157–164.

Castellanos, E., Álvarez, J., Aranaz, A., Romero, B., de Juan, L., Bezos, J., Rodríguez, S., Stevenson, K., Mateos, A. and Domínguez, L. (2007a) Use of single nucleotide polymorphisms in *inh*-A gene to characterize *Mycobacterium avium* subsp. *paratuberculosis* into Types I, II and III. In: Nielsen S.S (ed.) *Proceedings of the Ninth International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 6–8.

Castellanos, E., Aranaz, A., Romero, B., de Juan, L., Álvarez, J., Bezos, J., Rodríguez, S., Stevenson, K., Mateos, A. and Domínguez, L. (2007b) Polymorphisms in *gyr*A and *gyr*B genes among *Mycobacterium avium* subsp. *paratuberculosis* Type I, II, and III isolates. *Journal of Clinical Microbiology* 45, 3439–3442.

Castellanos, E., Aranaz, A., Gould, K.A., Linedale, R., Stevenson, K., Álvarez, J., Domínguez, L., de Juan, L., Hinds, J. and Bull, T.J. (2009) Discovery of stable and variable differences in the *Mycobacterium avium* subsp. *paratuberculosis* Types I, II and III genomes by pan-genome microarray analysis. *Applied and Environmental Microbiology* 75, 676–686.

Collins, D.M., Gabric, D.M. and de Lisle, G.W. (1990) Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. *Journal of Clinical Microbiology* 28, 1591–1596.

Collins, D.M., De Zoete, M. and Cavaignac, S.M. (2002) *Mycobacterium avium* subsp. *paratuberculosis* strains from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence difference. *Journal of Clinical Microbiology* 40, 4760–4762.

de Juan, L., Mateos, A., Domínguez, L., Sharp, J. and Stevenson, K. (2005) Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* isolates from goats detected by pulsed-field gel electrophoresis. *Veterinary Microbiology* 106, 249–257.

de Juan, L., Álvarez, J., Aranaz, A., Rodríguez, A., Romero, B., Bezos, J., Mateos, A. and Domínguez, L. (2006a) Molecular epidemiology of Types I/III strains of *Mycobacterium avium* subsp. *paratuberculosis* isolated from goats and cattle. *Veterinary Microbiology* 115, 102–110.

de Juan, L., Álvarez, J., Romero, B., Bezos, J., Castellanos, E., Aranaz, A., Mateos, A. and Domínguez, L. (2006b) Comparison of four different culture media for isolation and growth of Type II and Type I/III *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from cattle and goats. *Applied and Environmental Microbiology* 72, 5927–5932.

de Lisle, G.W., Yates, G.F. and Collins, D.M. (1993) Paratuberculosis in farmed deer: case reports and DNA characterization of isolates of *Mycobacterium paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 5, 567–571.

Dohmann, K., Strommenger, B., Stevenson, K., de Juan, L., Stratmann, J., Kapur, V., Bull, T.J. and Gerlach, G.F. (2003) Characterization of genetic differences between *Mycobacterium avium* subsp. *paratuberculosis* Type I and Type II isolates. *Journal of Clinical Microbiology* 41, 5215–5223.

Domenech, P., Reed, M.B. and Barry, C.E., 3rd (2005) Contribution of the *Mycobacterium*

tuberculosis MmpL protein family to virulence and drug resistance. *Infection and Immunity* 73, 3492–3501.

Dunkin, G.W. and Balfour-Jones, S.E.B. (1935) Preliminary investigation of a disease of sheep possessing certain characteristics simulating Johne's disease. *Journal of Comparative Pathology* 48, 236–240.

Florou, M., Leontides, L., Kostoulas, P., Billinis, C. and Sofia, M. (2008a) Strain-specific sensitivity estimates of *Mycobacterium avium* subsp. *paratuberculosis* culture in Greek sheep and goats. *Zoonoses and Public Health* 56, 1–5.

Florou, M., Leontides, L., Kostoulas, P., Billinis, C., Sofia, M., Kyriazakis, I. and Lykotrafitis, F. (2008b) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from non-ruminant wildlife living in the sheds and on the pastures of Greek sheep and goats. *Epidemiology and Infection* 136, 644–652.

Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S. and Gudmundsdottir, K.B. (2000) Paratuberculosis in Iceland: epidemiology and control measures, past and present. *Veterinary Microbiology* 77, 263–267.

Ghadiali, A.H., Strother, M., Naser, S.A., Manning, E.J. and Sreevatsan, S. (2004) *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. *Journal of Clinical Microbiology* 42, 5345–5348.

Gioffre, A., Infante, E., Aguilar, D., Santangelo, M.P., Klepp, L., Amadio, A., Meikle, V., Etchechoury, I., Romano, M.I., Cataldi, A., Hernandez, R.P. and Bigi, F. (2005) Mutation in *mce* operons attenuates *Mycobacterium tuberculosis* virulence. *Microbes and Infection* 7, 325–334.

Gollnick, N.S., Mitchell, R.M., Baumgart, M., Janagama, H.K., Sreevatsan, S. and Schukken, Y.H. (2007) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in bovine monocyte-derived macrophages is not affected by host infection status but depends on the infecting bacterial genotype. *Veterinary Immunology and Immunopathology* 120, 93–105.

Greig, A., Stevenson, K., Henderson, D., Perez, V., Hughes, V., Pavlik, I., Hines, M.E., McKendrick, I. and Sharp, J.M. (1999) Epidemiological study of paratuberculosis in wild rabbits in Scotland. *Journal of Clinical Microbiology* 37, 1746–1751.

Griffiths, T.A., Rioux, K. and De Buck, J. (2008) Sequence polymorphisms in a surface PPE protein distinguish types I, II, and III of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 46, 1207–1212.

Gumber, S. and Whittington, R.J. (2007) Comparison of BACTEC 460 and MGIT 960 systems for the culture of *Mycobacterium avium* subsp. *paratuberculosis* S strain and observations on the effect of inclusion of ampicillin in culture media to reduce contamination. *Veterinary Microbiology* 119, 42–52.

Janagama, H., il Jeong, K., Kapur, V., Coussens, P. and Sreevatsan, S. (2006) Cytokine responses of bovine macrophages to diverse clinical *Mycobacterium avium* subsp. *paratuberculosis* strains. *BMC Microbiology* 6, 10.

Kumar, P., Schelle, M.W., Jain, M., Lin, F.L., Petzold, C.J., Leavell, M.D., Leary, J.A., Cox, J.S. and Bertozzi, C.R. (2007) PapA1 and PapA2 are acyltransferases essential for the biosynthesis of the *Mycobacterium tuberculosis* virulence factor sulfolipid-1. *Proceedings of the National Academy of Science of the USA* 104, 11221–11226.

Kumar, S., Singh, S.V., Singh, A.V., Singh, P.K. and Sohal, J.S. (2008) Inter-species transmission of 'Bison type' genotype of *MAP* between *Boselaphus tragocamelus* (blue bulls) and small ruminant population in India. *Indian Journal of Animal Sciences* 78, 1186–1191.

Lamichhane, G., Tyagi, S. and Bishai, W.R. (2005) Designer arrays for defined mutant analysis to detect genes essential for survival of *Mycobacterium tuberculosis* in mouse lungs. *Infection and Immunity* 73, 2533–2540.

Machackova, M., Svastova, P., Lamka, J., Parmova, I., Liska, V., Smolik, J., Fischer, O.A. and Pavlik, I. (2004) Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic (1999–2001). *Veterinary Microbiology* 101, 225–234.

Marsh, I.B. and Whittington, R.J. (2005) Deletion of an *mmpL* gene and multiple associated genes from the genome of the S strain of *Mycobacterium avium* subsp. *paratuberculosis* identified by representational difference analysis and *in silico* analysis. *Molecular and Cellular Probes* 19, 371–384.

Marsh, I., Whittington, R. and Cousins, D. (1999) PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* based on polymorphisms in IS*1311*. *Molecular and Cellular Probes* 13, 115–126.

Marsh, I.B., Bannantine, J.P., Paustian, M.L., Tizard, M.L., Kapur, V. and Whittington, R.J. (2006) Genomic comparison of *Mycobacterium avium* subsp. *paratuberculosis* sheep and cattle strains by microarray hybridization. *Journal of Bacteriology* 188, 2290–2293.

Maslow, J.N., Dawson, E.A., Carlin, E.A. and Holland, S.M. (1999) Haemolysin as a virulence

factor for systemic infection with isolates of *Mycobacterium avium* complex. *Journal of Clinical Microbiology* 37, 445–446.

McEwen, A.D. (1939) Investigations on Johne's disease in sheep. *Journal of Comparative Pathology* 52, 69–87.

McLean, K.J., Clift, D., Lewis, D.G., Sabri, M., Balding, P.R., Sutcliffe, M., Leys, D. and Munro, A.W. (2006) The preponderance of P450s in the *Mycobacterium tuberculosis* genome. *Trends in Microbiology* 14, 220–228.

Moloney, B.J. and Whittington, R.J. (2008) Cross species transmission of ovine Johne's disease from sheep to cattle: an estimate of prevalence in exposed susceptible cattle. *Australian Veterinary Journal* 86, 117–123.

Motiwala, A.S., Janagama, H.K., Paustian, M.L., Zhu, X.C., Bannantine, J.P., Kapur, V. and Sreevatsan, S. (2006) Comparative transcriptional analysis of human macrophages exposed to animal and human isolates of *Mycobacterium avium* subsp. *paratuberculosis* with diverse genotypes. *Infection and Immunity* 74, 6046–6056.

Mougous, J.D., Green, R.E., Williams, S.J., Brenner, S.E. and Bertozzi, C.R. (2002) Sulfotransferases and sulfatases in mycobacteria. *Chemistry and Biology* 9, 767–776.

O'Brien, R., Mackintosh, C.G., Bakker, D., Kopecna, M., Pavlik, I. and Griffin, J.F.T. (2006) Immunological and molecular characterization of susceptibility in relationship to bacterial strain differences in *Mycobacterium avium* subsp. *paratuberculosis* infection in the red deer (*Cervus elaphus*). *Infection and Immunity* 74, 3530–3537.

Paustian, M.L., Zhu, X.C., Sreevatsan, S., Robbe-Austerman, S., Kapur, V. and Bannantine, J.P. (2008) Comparative genomic analysis of *Mycobacterium avium* subspecies obtained from multiple host species. *BMC Genomics* 9, 135.

Pavlik, I., Horvathova, A., Dvorska, L., Bartl, J., Svastova, P., du Maine, R. and Rychlik, I. (1999) Standardization of restriction fragment length polymorphism analysis for *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Microbiological Methods* 38, 155–167.

Purkayastha, A., McCue, L.A. and McDonough, K.A. (2002) Identification of a *Mycobacterium tuberculosis* putative classical nitroreductase gene whose expression is coregulated with that of the *acr* gene within macrophages, in standing versus shaking cultures, and under low oxygen conditions. *Infection and Immunity* 70, 1518–1529.

Recht, J. and Kolter, R. (2001) Glycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. *Journal of Bacteriology* 183, 5718–5724.

Reddacliff, L.A., Vadali, A. and Whittington, R.J. (2003) The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. *Veterinary Microbiology* 95, 271–282.

Sassetti, C.M. and Rubin, E.J. (2003) Genetic requirements for mycobacterial survival during infection. *Proceedings of the National Academy of Sciences of the USA* 100, 12989–12994.

Schorey, J.S. and Sweet, L. (2008) The mycobacterial glycopeptidolipids: structure, function and their role in pathogenesis. *Glycobiology* 18, 832–841.

Semret, M., Turenne, C.Y., de Haas, P., Collins, D.M. and Behr, M.A. (2006) Differentiating host-associated variants of *Mycobacterium avium* by PCR for detection of large sequence polymorphisms. *Journal of Clinical Microbiology* 44, 881–887.

Senaratne, R.H., Sidders, B., Sequeira, P., Saunders, G., Dunphy, K., Marjanovic, O., Reader, J.R., Lima, P., Chan, S., Kendall, S., McFadden, J. and Riley, L.W. (2008) *Mycobacterium tuberculosis* disrupted in *mce3* and *mce4* operons are attenuated in mice. *Journal of Medical Microbiology* 57, 164–170.

Sevilla, I., Singh, S.V., Garrido, J.M., Aduriz, G., Rodríguez, S., Geijo, M.V., Whittington, R.J., Saunders, V., Whitlock, R.H. and Juste, R.A. (2005) Molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* strains from different hosts and regions. *Revue Scientifique et Technique – Office International des Epizooties* 24, 1061–1066.

Sevilla, I., Garrido, J., Geijo, M. and Juste, R. (2007) Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiology* 7, 18.

Sherman, D.R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M.I. and Schoolnik, G.K. (2001) Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding alpha-crystallin. *Proceedings of the National Academy of Sciences of the USA* 98, 7534–7539.

Sohal, J.S., Sheoran, N., Narayanasamy, K., Brahmachari, V., Singh, S. and Subodh, S. (2009) Genomic analysis of local isolate of *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* 134, 375–382.

Stevenson, K., Hughes, V.M., de Juan, L., Inglis, N.F., Wright, F. and Sharp, J.M. (2002) Molecular

characterization of pigmented and nonpigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 40, 1798–1804.

Stevenson, K., Álvarez, J., Bakker, D., Biet, F., de Juan, L., Denham, S., Dimareli, Z., Dohmann, K., Gerlach, G-F., Heron, I., Kopecna, M., May, L., Pavlik, I., Sharp, J.M., Thibault, V.C., Willemsen, P., Zadoks, R. and Greig, A. (2009) Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* across host species and European countries with evidence for tansmission between wildlife and domestic ruminants. *BMC Microbiology* 9, 212.

Stewart, G.R., Wernisch, L., Stabler, R., Mangan, J.A., Hinds, J., Laing, K.G., Young, D.B. and Butcher, P.D. (2002) Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. *Microbiology* 148, 3129–3138.

Stewart, G.R., Robertson, B.D. and Young, D.B. (2003) Tuberculosis: a problem with persistence. *Nature Reviews in Microbiology* 1, 97–105.

Stewart, G.R., Newton, S.M., Wilkinson, K.A., Humphreys, I.R., Murphy, H.N., Robertson, B.D., Wilkinson, R.J. and Young, D.B. (2005) The stress-responsive chaperone α-crystallin 2 is required for pathogenesis of *Mycobacterium tuberculosis*. *Molecular Microbiology* 55, 1127–1137.

Taylor, A.W. (1945) Ovine paratuberculosis (Johne's disease of sheep). *Journal of Comparative Pathology* 55, 41–44.

Taylor, A.W. (1951) Varieties of *Mycobacterium johnei* from sheep. *Journal of Pathology and Bacteriology* 63, 333–336.

Taylor, A.W. (1953) The experimental infection of cattle with varieties of *Mycobacterium johnei* isolated from sheep. *Journal of Comparative Pathology* 63, 368–373.

Tundup, S., Pathak, N., Ramanadham, M., Mukhopadhyay, S., Murthy, K.J., Ehtesham, N.Z. and Hasnain, S.E. (2008) The co-operonic PE25/PPE41 protein complex of *Mycobacterium tuberculosis* elicits increased humoral and cell-mediated immune response. *PLoS ONE* 3, e3586.

Verna, A.E., García-Pariente, C., Muñoz, M., Moreno, O., García-Marin, J.F., Romano, M.I., Paolicchi, F. and Pérez, V. (2007) Variation in the immuno-pathological responses of lambs after experimental infection with different strains of *Mycobacterium avium* subsp. *paratuberculosis*. *Zoonoses and Public Health* 54, 243–252.

Watt, J.A.A. (1954) Johne's disease in a bovine associated with the pigmented strain of *Mycobacterium johnei*. *The Veterinary Record* 66, 387.

Whittington, R.J. (2009) Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from faecal and tissue samples in a liquid culture system. *Journal of Clinical Microbiology* 47, 614–622.

Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J. and Fraser, C.A. (1999) Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *Journal of Clinical Microbiology* 37, 1077–1083.

Whittington, R.J., Hope, A.F., Marshall, D.J., Taragel, C.A. and Marsh, I. (2000) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS*900* restriction fragment length polymorphism and IS*1311* polymorphism analyses of isolates from animals and a human in Australia. *Journal of Clinical Microbiology* 38, 3240–3248.

Whittington, R.J., Marsh, I.B. and Whitlock, R.H. (2001a) Typing of IS*1311* polymorphisms confirms that bison (*Bison bison*) with paratuberculosis in Montana are infected with a strain of *Mycobacterium avium* subsp. *paratuberculosis* distinct from that occurring in cattle and other domesticated livestock. *Molecular and Cellular Probes* 15, 139–145.

Whittington, R.J., Taragel, C.A., Ottaway, S., Marsh, I., Seaman, J. and Fridriksdottir, V. (2001b) Molecular epidemiological confirmation and circumstances of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. *Veterinary Microbiology* 79, 311–322.

Wu, C.W., Glasner, J., Collins, M., Naser, S. and Talaat, A.M. (2006) Whole-genome plasticity among *Mycobacterium avium* subspecies: insights from comparative genomic hybridizations. *Journal of Bacteriology* 188, 711–723.

Yadav, D., Singh, S.V., Singh, A.V., Sevilla, I., Juste, R.A., Singh, P.K. and Sohal, J.S. (2008) Pathogenic 'Bison-type' *Mycobacterium avium* subsp. *paratuberculosis* genotype characterized from riverine buffalo (*Bubalus bubalis*) in North India. *Comparative Immunology, Microbiology and Infectious Diseases* 31, 373–387.

Zhu, X., Tu, Z.J., Coussens, P.M., Kapur, V., Janagama, H., Naser, S. and Sreevatsan, S. (2008) Transcriptional analysis of diverse strains of *Mycobacterium avium* subsp. *paratuberculosis* in primary bovine monocyte derived macrophages. *Microbes and Infection* 10, 1274–1282.

13 *Mycobacterium avium* subsp. *paratuberculosis* and Antimicrobial Agents

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13.1 Introduction

There is a paucity of literature on the susceptibility of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) to antimicrobials. This is largely owing to the fact that there has been no compelling reason to attempt treatment of *MAP* disease. In animals, treatment of paratuberculosis is cost-prohibitive. Furthermore, potentially efficacious drugs are not legal for use in food-producing animals, as all meat or milk products from animals treated with such drugs must be excluded from the food chain. In humans, treatment of *MAP* has not been indicated heretofore since *MAP* has not been considered a human pathogen. However, the use with some success of antibiotic agents targeting *M. avium* complex (MAC) infections in Crohn's disease patients has stimulated further work to define the antimicrobial susceptibility profile of *MAP*.

13.2 Veterinary Data

In vitro antimicrobial susceptibility studies are limited to six publications (Chiodini, 1986; Rastogi *et al.*, 1992; Williams *et al.*, 1999; Parrish *et al.*, 2004; Zanetti *et al.*, 2006; Krishnan *et al.*, 2009). Because there are no accepted standards for antimicrobial susceptibility testing (AST) of *MAP*, methods used in these studies varied. Also, until recently, very few human-origin isolates of *MAP* were available for testing; thus most published AST studies were done on animal-origin *MAP* strains, specifically of bovine origin.

In vivo trials of anti-*MAP* therapeutics in animals are few and employed single drugs. Merkal and Larsen (1973) reported treatment of two adult cows with clinical Johne's disease using oral clofazimine at 600 mg/day (roughly 2 mg/kg). One cow treated for 200 days relapsed after treatment was discontinued.

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The other cow, treated for 330 days, remained free of clinical signs but continued to shed *MAP* in faeces. Tissues collected at necropsy had lesions typical of Johne's disease and were *MAP* culture-positive. Rankin (1953) attempted treatment of three clinical cases of bovine paratuberculosis using isoniazid at 12 mg/kg every 12 h for 21–60 days without success (Rankin, 1953). St Jean (1996) has the most extensive report summarizing attempts to treat bovine paratuberculosis, many from personal experience. He concluded that daily isoniazid (20 mg/kg) alone or in combination with rifampin at 20 mg/kg for the duration of the animal's life can forestall progression of *MAP* infections. This application is advocated only for animals of high genetic merit for the purpose of harvesting of germplasm (semen and embryos).

Recently, attention has been given to an ionophore antibiotic, monensin, as a possible therapeutic in adult cattle or as a chemoprophylactic in calves. Monensin is a compound poorly absorbed from the gastrointestinal tract that is used as a feed additive to enhance growth rate and milk production efficiency in cattle. An *in vitro* study using unconventional methods reported that the minimal inhibitory concentration (MIC) of monensin against a single *MAP* strain of animal origin was 0.39 μg/ml (Brumbaugh *et al.*, 2004). Data from *in vivo* studies have been sparse but encouraging. Provision of monensin to adult cattle naturally infected with *MAP* has been associated with modest improvements in histopathology scores, decline in faecal shedding rate, or reduced odds of testing positive on a milk ELISA (Brumbaugh *et al.*, 2000; Hendrick *et al.*, 2006a,b). Whitlock *et al.* (2005) reported that monensin used as a chemoprophylactic in calf milk replacer in dairy calves resulted in reduced tissue colonization and *MAP* faecal shedding.

13.3 Human Data

In humans, there are three independent case reports on treatment of PCR-confirmed *MAP* infections in individual Crohn's patients (Hermon-Taylor *et al.*, 1998; Behr *et al.*, 2004; Chamberlin *et al.*, 2007). In the first report, an adolescent male was treated with clarithromycin (500 mg/day) and rifabutin (300 mg/day) for 32 months, resulting in long-term remission of his Crohn's disease symptoms. In the second report, a 21-year-old Canadian male was treated with clarithromycin and rifabutin (dosages not given) for 12 months, with marked improvement both clinically and endoscopically. In the third report, a 63-year-old male patient with long-standing Crohn's disease experienced clinical remission of his symptoms after a 6-month course of treatment with clarithromycin (1000 mg daily), rifabutin (300–450 mg daily) and levofloxacin (500 mg daily). The patient relapsed, however, after cessation of the antibiotics (W. Chamberlin, Texas, 2008, personal communication). In a small study on *MAP* infection of infectious bowel disease patients and TNF-α, Clancy *et al.* (2007) reported that 14 *MAP* IS*900* PCR-positive patients treated with rifabutin, clarithromycin and clofazimine for at least 3 months had 40% less TNF-α in intestinal biopsy cultures compared with untreated controls.

There have been multiple therapeutic trials involving Crohn's disease patients using antimycobacterial drugs, yielding conflicting and controversial results (Borgaonkar *et al.*, 2000; Hulten *et al.*, 2000; Selby *et al.*, 2007; Behr and Hanley, 2008). However, these cannot be considered *in vivo* anti-*MAP* trials for two reasons: (i) the *MAP* status of patients was not determined in any of these trials; and (ii) most studies used antimicrobials to which *MAP* is resistant, e.g. trials using antituberculous drugs. Hence, data from clinical trials of Crohn's patients are not directly pertinent to this chapter.

13.4 Towards Standardized Methods

Siddiqi *et al.* (1993) and Heifets (1996) established standard methods for *in vitro* determination of minimal inhibitory concentrations (MICs) for drugs against MAC organisms by macrodilution in BACTEC 12B medium. For *MAP* testing, these methods require modification to accommodate its slower replication

rate and fastidious growth requirements. Many technical issues regarding standardization of AST for *MAP* still need to be formally addressed (Van Boxtel *et al.*, 1990; Inderlied, 1994; Rastogi *et al.*, 2000; Krishnan *et al.*, 2009). These issues include drug stability in culture media at 37 °C for up to 2 weeks, drug interaction with the constituents of more complex *MAP* culture media such as Tween 80 and egg yolk, and culture medium pH (the optimal *MAP* growth pH is 6.0). Antimicrobial drug susceptibility data on *MAP* have been reported for a number of agents. However, because of the methodological variation inherent between non-standardized methods, the results of these studies are difficult to evaluate or compare (Rastogi *et al.*, 1992; Williams *et al.*, 1999; Parrish *et al.*, 2004; Zanetti *et al.*, 2006; Beckler *et al.*, 2008).

The use of antimycobacterial agents is not an accepted therapy in Crohn's disease. However, because certain patients fail standard therapies, a number of patients have requested a trial of anti-*MAP* treatment as an alternative approach to attempt to control the course of their chronic disease. In such instances, clinicians and patients require better *in vitro* AST data on *MAP* to guide therapy. While *in vitro* susceptibility does not promise *in vivo* activity, the converse is generally true; an agent that has no activity *in vitro* is unlikely to be beneficial *in vivo*. Towards this end, AST methods have been adapted for testing of *MAP* drug susceptibility using the MGIT ParaTB medium and MGIT 960 instrument. MGIT methods were demonstrated to provide results comparable to better-known methods, such as BACTEC 12B and plate counting (Krishnan *et al.*, 2009). By using the MGIT methods, the MICs of ten antimicrobials against 18 strains of *MAP* (nine human-origin and nine animal-origin) were determined (Table 13.1).

All *MAP* strains were found to be susceptible to the macrolide drugs clarithromycin and azithromycin. No *MAP* strains were found to be resistant to amikacin (three of the 18 strains were classified as having intermediate susceptibility). Rifampicin and rifabutin ranked as the third and fourth most effective drugs, respectively, followed by ciprofloxacin. *MAP* is highly resistant to the anti-tuberculosis drug isoniazid and the anti-leprosy drug dapsone, with intermediate, strain-dependent resistance to ethambutol. These findings are in general agreement with prior publications that have used a variety of methods (Rastogi *et al.*, 1992; Williams *et al.*, 1999; Parrish *et al.*, 2004; Zanetti *et al.*, 2006). The antibiogram of *MAP* is

Table 13.1. MICs[a] for 50% (MIC_{50}) and 90% (MIC_{90}) of 18 *MAP* strains[b] and the percentages of strains classified[c] as susceptible, intermediate or resistant to ten commonly used antimicrobial drugs.

Drug[d]	MIC_{50}	MIC_{90}	Percentage susceptible	Percentage intermediate	Percentage resistant
CIP	1.0	8.0	55.5	5.5	39.0
AZM	2.0	4.0	100	0	0
CLR	0.25	1.0	100	0	0
AMK	3.12	3.12	83.3	16.7	0
RIF	1.5	3.0	44.4	50.0	5.6
RFB	0.5	2.0	11.1	72.2	16.7
EMB	5.0	5.0	27.8	66.7	5.5
CLFZ	0.625	1.25	5.5	16.7	77.8
DAP	>5	>5	0	0	100
INH	>8	>8	0	0	100

[a]MIC is the minimum concentration of a drug required to inhibit more than 99% growth when compared with a 'no drug' control.
[b]Nine strains originating from humans and nine from animals.
[c]Classified based on NCCLS criteria for MAC drug susceptibility testing (National Committee for Clinical Laboratory Standards, 2003).
[d]CIP, ciprofloxacin; AZM, azithromycin; CLR, clarithromycin; AMK, amikacin; RIF, rifampicin; RFB, rifabutin; EMB, ethambutol; CLFZ, clofazimine; DAP, dapsone; INH, isoniazid.

similar to that of another member of the MAC, *M. avium* subsp. *hominissuis* (*MAH*), with the exception that none of the *MAH* strains tested was resistant to rifabutin, and rifabutin was superior to rifampicin based on MIC_{90} values (unpublished data). Only minor differences in drug susceptibility were found between animal- and human-origin strains of *MAP*.

Greenstein *et al.* (2007b) first demonstrated that anti-inflammatory drugs commonly used for treatment of Crohn's disease are capable of inhibiting *in vitro* growth of *MAP*. His group showed that the active metabolite of sulfasalazine (5-aminosalicylic acid at 8–32 μg/ml), the active metabolite of azathioprine (6-mercaptopurine (6-MP) at 2–4 μg/ml), and methotrexate each inhibited *in vitro* growth of *MA*P to varying degrees in mycobactin-supplemented BACTEC 12B medium (Greenstein *et al.*, 2007a). These findings were corroborated for 6-MP in MGIT ParaTB medium™, and 6-MP was shown to be bacterostatic but not bactericidal for *MAP*, with MICs of 1–4 μg/ml using eight strains of animal- and human-origin *MAP* (Shin and Collins, 2008). The inhibitory effects of sulfonamide drugs and their derivatives were not observed in MGIT ParaTB medium™, possibly due to the interference of culture medium components such as thymidine (Ferone *et al.*, 1975; Ferguson and Weissfeld, 1984). BACTEC 12B medium is chemically defined, and MGIT ParaTB medium™ is enriched with egg yolk, a medium component necessary for primary isolation of *MAP* from clinical samples. The effects of anti-inflammatory drugs on *MAP* and the potential for interaction between such drugs and more traditional antimicrobials, coupled with their combined antimicrobial and anti-inflammatory actions in humans, further complicate the design and interpretation of clinical trials on Crohn's disease patients or other patients where *MAP* infection is suspected.

13.5 Concluding Thoughts

If *MAP* is indeed a zoonotic agent and infected humans require antimicrobial therapy, it will take a concerted research effort to establish optimal treatment regimens. No candidate drugs for mycobacterial infections are free of significant side effects and long-term treatment protocols cannot be undertaken lightly. Studies to define optimal therapies must begin with *in vitro* AST, progress to studies in *ex vivo* and/or animal models of *MAP* infection, and culminate in human clinical trials. First, standardized *in vitro* AST methods for *MAP* must be established and validated across laboratories. Clinical trials must be conducted based on a valid diagnostic criterion for *MAP* infection, probably requiring far better *MAP* tests on humans than are currently available. While some indication of potentially beneficial drugs may be inferred from data on MAC infections, the slow growth rate, greater pathogenicity and chronicity of *MAP* infection make any extrapolation of MAC data towards the treatment of *MAP* infections tenuous. Moreover, unlike with *Mycobacterium tuberculosis*, *in vitro* drug susceptibility data are notoriously poor predictors of clinical efficacy in the case of disease due to MAC, perhaps because many patients with MAC disease are immunocompromised (Griffith, 2007). The role of the host immune system may be a critical factor in defining any regimens to treat *MAP* infection, as emerging genetic data on Crohn's disease point to a defect in innate immunity to intracellular bacterial infection. If *MAP* is able to exploit these defects to cause disease in humans, successful therapeutic strategies may require combinations of drugs capable of killing or inhibiting *MAP* in concert with drugs to control the host inflammatory response directed against this infection.

References

Beckler, D.R., Elwasila, S., Ghobrial, G., Valentine, J.F. and Naser, S.A. (2008) Correlation between rpoB gene mutation in *Mycobacterium avium* subspecies *paratuberculosis* and clinical rifabutin and rifampicin resistance for treatment of Crohn's disease. *World Journal of Gastroenterology* 14, 2723–2730.

Behr, M.A. and Hanley, J. (2008) Antimycobacterial therapy for Crohn's disease: a reanalysis. *The Lancet Infectious Diseases* 8, 344.

Behr, M.A., Semret, M., Poon, A. and Schurr, E. (2004) Crohn's disease, mycobacteria, and NOD2. *The Lancet Infectious Diseases* 4, 136–137.

Borgaonkar, M.R., MacIntosh, D.G. and Fardy, J.M. (2000) A meta-analysis of antimycobacterial therapy for Crohn's disease. *The American Journal of Gastroenterology* 95, 725–729.

Brumbaugh, G.W., Edwards, J.F., Roussel, A.J. and Thomson, T.D. (2000) Effect of monensin sodium on histological lesions of naturally occurring bovine paratuberculosis. *Journal of Comparative Pathology* 123, 22–28.

Brumbaugh, G.W., Simpson, R.B., Edwards, J.F., Anders, D.R. and Thomson, T.D. (2004) Susceptibility of *Mycobacterium avium* subsp. *paratuberculosis* to monensin sodium or tilmicosin phosphate *in vitro* and resulting infectivity in a murine model. *Canadian Journal of Veterinary Research* 68, 175–181.

Chamberlin, W., Ghobrial, G., Chehtane, M. and Naser, S.A. (2007) Successful treatment of a Crohn's disease patient infected with bacteremic *Mycobacterium paratuberculosis*. *The American Journal of Gastroenterology* 102, 689–691.

Chiodini, R.J. (1986) Biochemical characteristics of various strains of *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 47, 1442–1445.

Clancy, R., Ren, Z., Turton, J., Pang, G. and Wettstein, A. (2007) Molecular evidence for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease correlates with enhanced TNF-α secretion. *Digestive and Liver Disease* 39, 445–451.

Ferguson, R.W. and Weissfeld, A.S. (1984) Comparison of the suitability of three common bacterial media for susceptibility testing of trimethoprim-sulfamethoxazole. *Journal of Clinical Microbiology* 19, 85–86.

Ferone, R., Bushby, S.R.M., Murchall, J.J., Moore, W.D. and Smith, D. (1975) Identification of Harper–Cawston factor as thymidine phosphorylase and removal from media of substances interfering with susceptibility testing to sulfonamides and diaminopyrimidines. *Antimicrobial Agents and Chemotherapy* 7, 91–98.

Greenstein, R.J., Su, L., Haroutnian, V., Shahidi, A. and Brown, S.T. (2007a) On the action of methotrexate and 6-mercaptopurine on *M. avium* subspecies *paratuberculosis*. *PLoS ONE* 2, e161.

Greenstein, R.J., Su, L., Shahidi, A. and Brown, S.T. (2007b) On the action of 5-amino-salicylic acid and sulfapyridine on *M. avium* including subspecies *paratuberculosis*. *PLoS ONE* 2, e516.

Griffith, D.E. (2007) Therapy of nontuberculous mycobacterial disease. *Current Opinion in Infectious Diseases* 20, 198–203.

Heifets, L. (1996) Susceptibility testing of *Mycobacterium avium* complex isolates. *Antimicrobial Agents and Chemotherapy* 40, 1759–1767.

Hendrick, S.H., Duffield, T.F., Leslie, K.E., Lissemore, K.D., Archambault, M., Bagg, R., Dick, P. and Kelton, D.F. (2006a) Monensin might protect Ontario, Canada dairy cows from paratuberculosis milk-ELISA positivity. *Preventive Veterinary Medicine* 76, 237–248.

Hendrick, S.H., Kelton, D.F., Leslie, K.E., Lissemore, K.D., Archambault, M., Bagg, R., Dick, P. and Duffield, T.F. (2006b) Efficacy of monensin sodium for the reduction of fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in infected dairy cattle. *Preventive Veterinary Medicine* 75, 206–220.

Hermon-Taylor, J., Barnes, N., Clarke, C. and Finlayson, C. (1998) *Mycobacterium paratuberculosis* cervical lymphadenitis, followed five years later by terminal ileitis similar to Crohn's disease. *BMJ* 316, 449–453.

Hulten, K., Almashhrawi, A., El-Zaatari, F.A.K. and Graham, D.Y. (2000) Antibacterial therapy for Crohn's disease: a review emphasizing therapy directed against mycobacteria. *Digestive Diseases and Sciences* 45, 445–456.

Inderlied, C.B. (1994) Antimycobacterial susceptibility testing: present practice and future trends. *European Journal of Clinical Microbiology* 13, 980–993.

Krishnan, M.Y., Manning, E.J.B. and Collins, M.T. (2009) Comparison of three methods for susceptibility testing of *Mycobacterium avium* subsp. *paratuberculosis* to 11 antimicrobial drugs. *Journal of Antimicrobial Chemotherapy*, 64, 310–316.

Merkal, R.S. and Larsen, A.B. (1973) Clofazimine treatment of cows naturally infected with *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 34, 27–28.

National Committee for Clinical Laboratory Standards (2003) *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. M24-A*. NCCLS, Wayne, Pennsylvania.

Parrish, N.M., Ko, C.G., Dick, J.D., Jones, P.B. and Ellingson, J.L.E. (2004) Growth, congo red agar colony morphotypes and antibiotic susceptibility testing of *Mycobacterium avium* subspecies *paratuberculosis*. *Clinical Medicine and Research* 2, 107–114.

Rankin, J.D. (1953) Isoniazid: its effects on *Mycobacterium paratuberculosis in vitro* and its failure to

cure Johne's disease in cattle. *The Veterinary Record* 65, 649–651.

Rastogi, N., Goh, K.S. and Labrousse, V. (1992) Activity of clarithromycin compared with those of other drugs against *Mycobacterium paratuberculosis* and further enhancement of its extracellular and intracellular activities by ethambutol. *Antimicrobial Agents and Chemotherapy* 36, 2843–2846.

Rastogi, N., Goh, K.S., Berchel, M. and Bryskier, A. (2000) *In vitro* activities of the ketolides telithromycin (HMR 3647) and HMR 3004 compared to those of clarithromycin against slowly growing mycobacteria at pHs 6.8 and 7.4. *Antimicrobial Agents and Chemotherapy* 44, 2848–2852.

Selby, W., Pavli, P., Crotty, B., Florin, T., Radford-Smith, G., Gibson, P., Mitchell, B., Connell, W., Read, R., Merrett, M., Ee, H. and Hetzel, D. (2007) Two-year combination antibiotic therapy with clarithromycin, rifabutin and clofazimine for Crohn's disease. *Gastroenterology* 132, 2313–2319.

Shin, S.J. and Collins, M.T. (2008) Thiopurine drugs azathioprine and 6-mercaptopurine inhibit *Mycobacterium paratuberculosis* growth *in vitro*. *Antimicrobial Agents and Chemotherapy* 52, 418–426.

Siddiqi, S.H., Heifets, L.B., Cynamon, M.H., Hooper, N.M., Laszlo, A., Libonati, J.P., Lindholm-Levy, P.J. and Pearson, N. (1993) Rapid broth macrodilution method for determination of MICs for *Mycobacterium avium* isolates. *Journal of Clinical Microbiology* 31, 2332–2338.

St Jean, G. (1996) Treatment of clinical paratuberculosis in cattle. *Veterinary Clinics of North America – Food Animal Practice* 12, 417–430.

Van Boxtel, R.M., Lambrecht, R.S. and Collins, M.T. (1990) Effects of colonial morphology and Tween 80 on antimicrobial susceptibility of *Mycobacterium paratuberculosis*. *Antimicrobial Agents and Chemotherapy* 34, 2300–2303.

Whitlock, R.H., Sweeney, R.W., Fyock, T.L., McAdams, S., Gardner, I.A. and McClary, D.G. (2005) Johne's disease: the effect of feeding monensin to reduce the bio-burden of *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves. In: Nielsen, S.S. (ed.) *Proceedings of the 8th International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, p. 46.

Williams, S.L., Harris, N.B. and Barletta, R.G. (1999) Development of a firefly luciferase-based assay for determining antimicrobial susceptibility of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 37, 304–309.

Zanetti, S., Molicotti, P., Cannas, S., Ortu, S., Ahmed, N. and Sechi, L.A. (2006) 'In vitro' activities of antimycobacterial agents against *Mycobacterium avium* subsp. *paratuberculosis* linked to Crohn's disease and paratuberculosis. *Annals of Clinical Microbiology and Antimicrobials* 5, 27.

14 Paratuberculosis in Cattle

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14.1 Introduction: Prevalence of Paratuberculosis in Cattle

Johne's disease (JD) is widely distributed throughout the world in many ruminant species. The true incidence of JD is hard to estimate for several reasons: (i) most infected cattle are asymptomatic; (ii) clinical cases with decreased milk production may be culled before a final diagnosis can be made; and (iii) there are difficulties in diagnosing cows with early infection. In 1997, the dairy survey of the US National Animal Health Monitoring System (NAHMS) (1997) indicated the dairy herd prevalence to be 30–50%. Reports from slaughterhouse surveys suggest that 7–18% of cattle in the USA are infected (Arnoldi *et al.*, 1983; Chiodini and Van Kruiningen, 1986; Merkal *et al.*, 1987). However, slaughterhouse surveys and most other surveys suffer from imperfect sensitivity, due to both the number of tissues sampled and the test applied. Surveys that collected more tissues from each animal reported a higher prevalence compared with those that sampled only one tissue. For example, a study of 1400 cull dairy cows from the

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north-eastern USA found the apparent prevalence to be 7.2% if any one of three collected samples was culture-positive (Whitlock *et al.*, 1985). Three samples identified 1.4 times as many positive cows as did one sample (Merkal *et al.*, 1987). Culture of 30 or more tissues from each animal will identify many more infected cattle (Sweeney *et al.*, 2006). Culture of tissues proved to be a much more sensitive method for detecting *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) than histopathology (Chiodini and Van Kruiningen, 1986). McKenna *et al.* (2004) reported that histopathology detected only 4.4% of the positive cows detected by culture of the tissues. Interestingly, the vast majority of culture-positive cows at slaughter were in good body condition based on body condition scoring.

14.2 Transmission of Paratuberculosis in Cattle

14.2.1 Infective dose

Early studies suggested that 10^3 bacilli were infectious (Gilmour *et al.*, 1965) and that, with a concentration of 10^6–10^8 *MAP* colony-forming units (CFU) per g of faeces, only a few mg of manure ingested by a young calf would be infectious (Jørgensen, 1982; Whittington *et al.*, 2000b). Another review suggested 50–1000 CFU as infective for young calves (Chiodini, 1996).

Experimental studies have shown that 1.5×10^6 CFU/dose given orally at 21 and 22 days of age reliably induced infection in multiple tissues, yet at a low level (Sweeney *et al.*, 2006). Higher doses at younger ages resulted in greater tissue infection. The results of this study also indicated that, although multiple tissues contained moderate concentrations of *MAP*, tissue culture was much more sensitive than histopathological examination of the tissues at detecting the presence of *MAP*. The highest concentrations of *MAP* were in the jejunal and ileal samples. The number of colonies recovered from the associated lymph nodes was closely correlated but lower than the number of colonies recovered in the adjacent intestinal mucosal tissues. These findings supported those of Momotani *et al.* (1988), who postulated that the M cells were the site of predilection and uptake of *MAP* from the intestinal lumen. The classic predilection sites, the ileocaecal lymph nodes and ileal mucosa, were frequently culture-negative, while jejunal mucosal sites were culture-positive. Similar results have been reported for other experimental infections in calves (Koo *et al.*, 2004). A slightly higher *MAP* challenge dose ($\sim 4 \times 10^6$ CFU/dose), directly inoculated into the tonsillar crypts, resulted in tissue infection and periodic low-level shedding after 146 days (Waters *et al.*, 2003). Again, although multiple tissues were culture-positive in each calf, including the tonsils, all examined tissues were negative by histological examination for both lesions of paratuberculosis and the presence of acid-fast organisms.

The time required for intestinal translocation from the mucosa to adjacent lymph nodes may be as short as 1 h (Wu *et al.*, 2007). Using surgical calf models, *MAP* was inoculated into the ileal lumen and 1–2 h later sections of adjacent lymph nodes were harvested. The samples obtained surgically were positive for *MAP* on culture. Following similar inoculation, the liver was culture-positive for *MAP* 4 days following inoculation, suggesting that *MAP* infections disseminate much earlier than previously thought possible.

14.2.2 Prenatal infection

Earlier papers concluded that approximately 25% of calves born to cattle with clinical signs will be infected *in utero*, with a lower risk in calves born to asymptomatic cows (Seitz *et al.*, 1989; Sweeney *et al.*, 1992a). A recent meta-analysis estimated that 9% (95% confidence limits 6–14%) of fetuses from subclinically infected dams and 39% (20–60%) of clinically affected dams were infected *in utero* with *MAP* ($P > 0.001$) (Whittington and Windsor, 2009). While these findings indicate that the likelihood of fetal infection is dependent on the severity of the dam's infection, more calves born to subclinically infected dams may be infected *in utero* than previously thought. This latter notion is supported by observational data from five calves caught at birth, immediately separated from their dams and fed pasteurized pooled colostrum and

milk replacer. Two of the five calves had positive faecal cultures for *MAP* at or before 12 months of age and all five were culture-positive for *MAP* in one or more tissues (Stabel *et al.*, 2008). From another perspective, calves born to seropositive dams were shown to be 6.6 times more likely to be seropositive themselves compared with calves born to seronegative dams (Aly and Thurmond, 2005). As the infection status of the dam represents a major risk factor for the newborn calf, we advise against keeping a heavily infected and/or symptomatic cow within the herd in the hope of obtaining the calf prior to culling, as the cow will continue to shed billions of organisms into the environment while awaiting a calf that has a very high chance of being infected.

MAP has been isolated from uterine flush fluids of infected cattle, and organisms adhere to embryos *in vitro* (Rhode and Shulaw, 1990). However, while an embryo obtained from an infected cow could result in an infected fetus, embryo transfer from infected cows has rarely resulted in infected calves (Whittington and Windsor, 2009). This practice is also considered safe for the recipient cow (Sweeney, 1996). Semen from bulls kept in commercial bull studs represents a very low risk, as these animals are tested twice yearly for JD and must be test-negative.

14.2.3 Postnatal infection

Most authors agree that the faecal–oral route is the primary mode of transmission from an infected adult to the neonate (Clarke, 1997). Most infections with *MAP* occur in the early neonatal period and are often associated with the calf sucking the manure-contaminated teat and udder when ingesting colostrum (Sweeney, 1996). Multiple-use maternity pens can serve as focal points to spread the infection to the neonates. An uninfected cow may lie on manure from a moderate or high shedder and contaminate her udder. The calf then nurses the cow and may ingest colostrum or milk contaminated with *MAP*. Although calves are most susceptible, older heifers and adult cattle may also become infected from the ingestion of contaminated feed material, as age resistance may be overcome by increased pressure of infection (Taylor, 1953; Larsen *et al.*, 1975).

Colostrum from known infected cows is a much greater risk for the spread of JD to calves than colostrum from known negative dams (Nielsen *et al.*, 2008). *MAP* may be passed through the colostrum and milk of cattle in the later stages of infection (Sweeney *et al.*, 1992b; Streeter *et al.*, 1995). The practice of feeding pooled colostrum or waste milk from cows may help the spread of infection to many calves during their most susceptible stage of life. It is also important to note that *MAP* can survive pasteurization temperatures, so colostrum from known infected cows should not be used (Chiodini and Hermon-Taylor, 1993). The dairy survey of the NAHMS (2002) indicated that 87.2% of dairy farms fed waste milk to their dairy calves.

Beef producers have reported that, as modern breeding practices have favoured docility, this same trait has fostered the ability of newborn calves to 'rob' other dams of both colostrum and milk. This may facilitate the horizontal transmission of *MAP* in beef breeds, even when raised on the open range. The practice of feeding large, round bales of hay on snow-covered fields tends to intensify cattle into one area, increasing the opportunity to contaminate teats and udder with manure from other adult cattle. Since beef calves nurse their dams for several months, faecal–oral transmission of *MAP* is enhanced by these hay feeding practices.

In the case of dairy calves, physical separation to calf hutches, or better yet to another property such as a commercial heifer-raising facility, reduces the risk of *MAP* transmission to the replacement stock. A corollary practice of feeding the manger sweepings from adult cattle to younger heifers has been shown to be a significant risk factor for spreading JD (Rossiter and Burhans, 1996). A recent meta-analysis review to assess age of infection reported that neonatal calves were significantly more susceptible to *MAP* infection than older calves (> 6 months) and adult cattle (Windsor and Whittington, 2009). Adult cattle could be infected with *MAP* if there was a high rate of exposure but were less likely than calves to develop clinical disease. In addition, *MAP*-contaminated forage and water

also represent a significant risk for infection of adult cattle. As noted above, unless massive and repeated doses of *MAP* are consumed, this is likely to be a relatively low risk. Increasingly more dairy herds are switching to intensive grazing of their adult lactating cows. This system relies on moving lactating cows to a new fresh growth of lush pasture every 12–24 h on a rotational basis for 15–30 days. Typically, heifers or steers follow the milking cows to graze the pasture closer to the ground, so the pasture will not need to be clipped or mowed mechanically. This system is an excellent way to provide less expensive lush pasture for the milking herd. However, in herds that have JD, the follower heifers or steers are consuming *MAP* along with the grass left by the milk cows and would have a rather continuous uniform exposure to *MAP* over the months they follow the milking herd, something that veterinarians should be aware of.

In a herd with no previous history of *MAP* infection, introduction of the infection is most likely to occur through the purchase of infected animals. Because of the long incubation period, an infected cow could show no clinical signs of JD and may test negative on both serological and faecal culture tests. The 'carrier' animal could then be purchased, brought into the herd and later serve as a source of infection when she begins shedding the organism. Other breaks in biosecurity, such as farm equipment, boots and clothing contaminated with faeces, could all, in theory, serve as ways of transmission of *MAP* into a new herd. However, the most important way of introducing *MAP* into a susceptible herd is by the addition of apparently uninfected carrier animals.

14.3 Stages of *MAP* Infection

MAP infection has been divided into four stages, depending on the severity of clinical signs, the potential for shedding organisms into the environment and the ease with which the disease may be detected using current laboratory methods. For every cow with advanced JD which is born on a farm, it is likely that 15–25 others are infected (Whitlock, 1992). Only 25–30% of these infected animals will be detected with even the most sensitive molecular testing techniques (Whitlock, 2009). The clinical animal is the 'tip of the iceberg'. As an example, consider a herd with 100 adult cattle and 100 young-stock replacements. Two cows born on the farm several years earlier develop clinical signs, with weight loss and diarrhoea. It is likely that 30–50 other cattle are infected but less than 30% of these will be detectable by faecal culture and/or PCR methodology. It is also reasonable to conclude that, if 25–30 of the adult cattle in a herd of 100 adult cattle are positive on a single herd faecal culture, then most of the herd has been exposed and probably may be infected. An outline of these stages, and their relative importance within an infected herd, is presented in Table 14.1.

14.3.1 Stage I: 'silent' infection (calves, heifers, young stock and adult cattle)

The disease process starts with the initial *MAP* uptake by intestinal phagocytic cells and translocation within the intestinal mucosa. This is followed by spread of the infection to adjacent lymph nodes and eventually into a disseminated infection. Once infection occurs,

Table 14.1. The 'iceberg effect' with different stages of *MAP* infection.

Stage	Type of infection	No. of animals
Stage IV	Advanced clinical disease	1 animal
Stage III	Clinical disease	1–2 cattle
Stage II	Inapparent carrier adults	6–8 cattle
Stage I	Silent infection of calves or young stock	15–25 cattle

the organism proliferates slowly in the jejunal and ileal mucosa and spreads to the regional lymph nodes (Clarke, 1997). While this silent or eclipse phase of infection usually lasts for a minimum of 2 years and sometimes for 10 years or more, cattle in stage I may proceed to stage II or even stage III (clinical JD) by 1 year of age in herds with a high prevalence of JD. From this, it is inferred that the rate of progression of JD is *MAP*-dose dependent, in addition to being dependent on age at infection. Cattle infected in the perinatal period typically progress to stages III and IV over a period of 1–3 years before being culled from the herd.

Stage I-infected cattle may shed *MAP* in their faeces but be below detection levels using current detection methods, including culture and PCR. If these cattle are sampled frequently, *MAP* may occasionally be detected at very low concentrations in their faeces (Waters *et al.*, 2003). At post-mortem examination, the organisms in the tissues may not be visible on microscopic examination but may be detectable by culture of multiple intestinal tissues (Sweeney *et al.*, 2006; Stabel *et al.*, 2008), suggesting that widespread dissemination occurs early in disease development. In contrast to the prevailing dogma, calves infected naturally and experimentally with *MAP* develop both a measurable humoral and cell-mediated immune response to *MAP*-specific proteins and mitogens (Waters *et al.*, 2003; Stabel *et al.*, 2008). However, commercially available enzyme-linked immunosorbent assays (ELISAs) fail to detect an antibody response, suggesting the need for more sensitive tests to detect early-stage infection than those currently available.

14.3.2 Stage II: inapparent carrier adults

Cattle enter stage II disease with higher concentrations of *MAP* in their intestinal tissues. These animals do not manifest weight loss or diarrhoea but may have an altered immune response, with increased gamma interferon production by T cells sensitized to specific mitogens and/or increased antibody response to *MAP* (Bassey and Collins, 1997). It would seem that intestinal mucosal concentrations of *MAP* must reach a critical concentration before organisms are shed in the faeces in sufficient quantity to be detectable by organism detection tests. Most animals in stage II shed *MAP* in their manure, contaminating the environment and serving as sources of infection to other animals on the farm.

The rate of disease progression through stage II is highly variable and is most likely influenced by a wide range of factors, which may include: age at initial exposure to *MAP*, the dose of *MAP* at initial exposure, the frequency of re-exposure over time, genetic factors of both the host and the organism, environmental factors, nutritional factors, production effects and a variety of other stressors. Many, perhaps most, stage II cattle will be culled from the herd for reasons unrelated to documented *MAP* infection status, such as infertility, mastitis, lameness or reduced milk production (Merkal *et al.*, 1975).

14.3.3 Stage III: clinical disease

Animals at stage III have gradual weight loss and diarrhoea. The appetite remains normal and intermittent diarrhoea is often present for weeks. The vital signs, heart rate, respiratory rate and temperature are normal. Emaciation and cachexia develop gradually, with a decrease in milk production (Fig. 14.1). Nearly all animals at stage III are faecal-culture-positive and usually have increased antibody detectable by a commercial JD ELISA test and agar gel immunodiffusion test. Cattle at this stage of infection rarely remain in the herd longer than a few weeks and are culled for weight loss, decreased milk production and unresponsive diarrhoea.

At this stage, the *MAP* population within the intestinal mucosal cells is very high (Fig. 14.2). The normal absorptive capacity of the bowel is abrogated, resulting in weight loss associated with a protein-losing enteropathy. A thickened intestinal mucosa and associated lymphadenopathy along a substantial portion of the small intestinal tract is characteristic of the disease progression (Fig. 14.3). The proliferation of reactive lymphocytes, epithelioid macrophages and giant cells results in infused blunted villi with decreased absorptive

(a)

(b)

Fig. 14.1. Advanced paratuberculosis in Holstein (a) and Guernsey (b) cows. Signs of disease include weight loss, seen as marked reduction of muscle mass and visible skeletal bones such as ribs, pelvis and shoulder girdle (reproduced with the kind permission of Michael Collins).

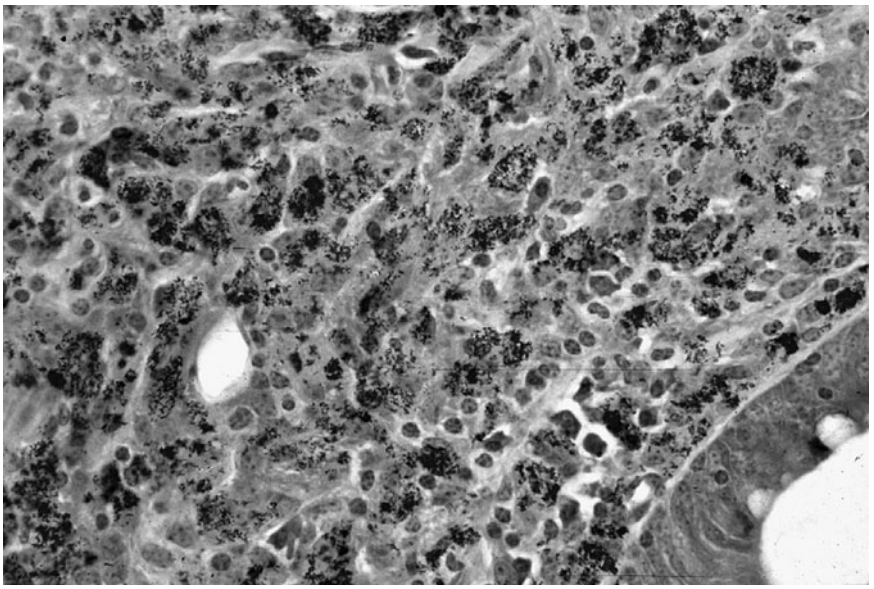

Fig. 14.2. Ziehl–Neelsen stain of ileum from a cow with advanced paratuberculosis. Copious fuchsin-staining organisms throughout the tissue indicate a pluribacillary *MAP* infection (reproduced with the kind permission of Michael Collins).

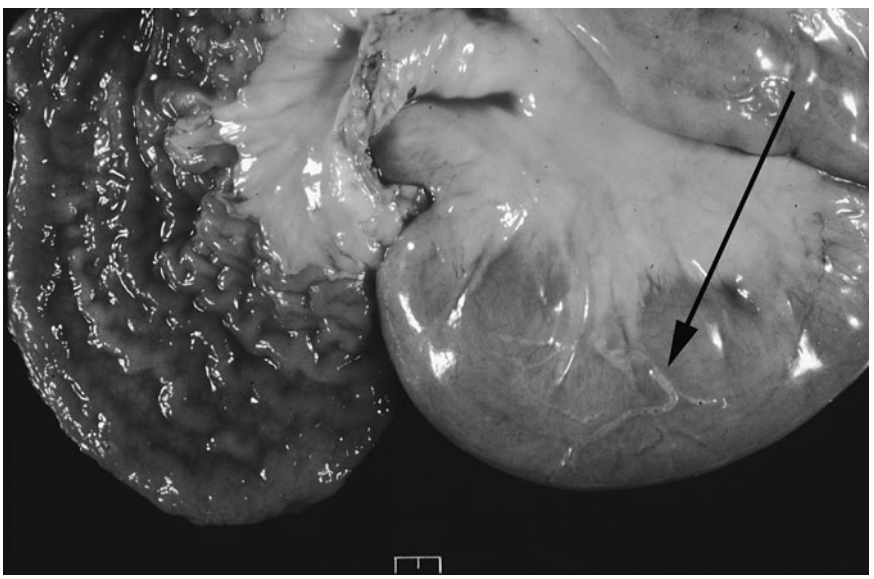

Fig. 14.3. Photograph of ileum manifesting lesions typical of paratuberculosis. To the left, the intestinal wall is thickened due to infiltration by leucocytes and epithelioid cells. To the right, there is mesenteric oedema, with a dilated mesenteric lymphatic channel, shown by the arrow (reproduced with the kind permission of Michael Collins).

capacity. The infection becomes disseminated, with *MAP* detectable in several extra-intestinal sites, including supra-mammary, pulmonary, hepatic and head lymph nodes, if cultured post-mortem. These cows are at higher risk of transmitting the organism *in utero* and have a higher frequency of *MAP* isolated from the milk. Cattle in stage III shed high concentrations of *MAP* in their faeces, contaminating the environment, which often includes feed materials.

14.3.4 Stage IV: advanced clinical disease

Animals in stage IV of the disease are weak, emaciated and usually have chronic, profuse diarrhoea. Intermandibular oedema or bottle jaw is characteristic of this phase of the disease. Animals can progress quickly from stage II to stage IV, sometimes within a few weeks, but a more gradual progression is more typical. Once the diarrhoea is profuse and hypoproteinaemia occurs, the animal's condition deteriorates rapidly, often in a matter of days. Most animals are sent to slaughter for salvage at this point. Otherwise, death occurs as a result of dehydration and cachexia.

14.4 Diagnosis of Paratuberculosis in Cattle

Specific diagnostic assays, including culture (see Whittington, Chapter 22, this volume), PCR (see Bölske and Herthnek, Chapter 23, this volume) and immunological assays (see Nielsen, Chapter 24, this volume), are considered elsewhere. Their application to detection of disease in cattle depends in part on whether diagnostic efforts are aimed at detecting infection of an individual animal or of a herd. As most prevalence studies seek to know the herd prevalence, serological testing of a subset of animals (e.g. 30 second-lactation cows or older) has been used as a sampling strategy, because this is simple with a rapid turnaround time. Over time, less expensive and more sensitive alternatives have been sought, initially in sheep. Since one positive faecal sample from a sheep with multibacillary paratuberculosis could be readily detected when combined with faecal pellets of 49 uninfected sheep (Whittington *et al.*, 2000a), pooled sampling has also been investigated for cows in the USA. In one study, pools of five samples were more sensitive for detecting infected cows than pools of ten samples, and this approach was feasible at a fraction of the cost of whole-herd cultures on all adult cattle (Wells *et al.*, 2002a). As expected, the sensitivity of pooled cultures strongly depends on pool size and the shedding level of the positive sample (low shedder compared with a heavy shedder). Occasionally, culture of the pooled sample was positive, when each individual sample was found to be negative (Kalis *et al.*, 2000; Raizman *et al.*, 2004), which may have been due to sample variation during analysis. Evaluation of pooled faecal samples (1:5) in a range of infected dairy herds detected at least 88% of samples that contained at least one animal with moderate (>10 CFU/tube) to high levels of shedding (Wells *et al.*, 2003). Later studies with pools of ten faecal samples in low-prevalence herds proved cost effective for herd screening and may provide an estimate of *MAP*-infected dairy cows within large herds. Optimal pool size depends on both prevalence and herd size. The optimal number of samples per pool can vary from three for a 500-cow herd with low prevalence to five for a 1000-cow herd with high prevalence (van Schaik *et al.*, 2003).

Composite environmental manure samples (a combination of three or four manure samples from a specific area) from 64 herds known to be infected with *MAP* detected 50 (78%) of the 64 herds with positive faecal pools (Raizman *et al.*, 2004). This provided empirical evidence that environmental manure samples can serve as an excellent proxy to detect herd infection. Refinement of the environmental manure sampling to include composite or pooled manure samples from high cow-traffic areas, manure storage areas and manure from pens/lots representing all cow groups has further increased diagnostic sensitivity for detecting herd infection (Berghaus *et al.*, 2006). Additional investigations have shown that composite environmental manure sampling is more sensitive and less expensive than 30 serum ELISA tests (Raizman *et al.*, 2004; Lombard *et al.*, 2006). In the case of larger herds (350–2500 cows), samples of lagoon water were significantly more likely to give positive

results than composite manure samples from high cow-traffic areas (Berghaus *et al.*, 2006). Because composite environmental samples are collected from areas where cows defaecate daily, the weather or season of collection should not affect the ability to isolate *MAP*.

14.5 *MAP* Supershedders

14.5.1 The supershedder phenomenon

Traditionally, all faecal-culture-positive cattle were judged to be infectious and to represent a threat for spreading the disease to susceptible herd-mates. Over time it was recognized that differences in *MAP* shedding levels existed among culture-positive cattle and that the higher the number of *MAP* colonies detected by culture, the greater the risk for spread of the disease. For the past 20 years, three levels of *MAP* shedding have been recognized, based on results from culture tubes of Herrold's egg yolk medium: low (1–9 CFU/tube), moderate (10–50 CFU/tube) or heavy (>50 CFU/tube) (Crossley *et al.*, 2005). Most North American laboratories report the number of visible *MAP* colonies on each culture tube but most do not enumerate above 50 CFU/tube, the threshold used to define a heavy shedder. During 2004, the laboratory committee of the National Johne's Working Group discussed the need to further quantify heavy shedders. Based on an overwhelming consensus opinion of experienced JD investigators, little justification could be provided to count >50 CFU/tube. As a result, a national policy was developed whereby Johne's testing laboratories using solid media culture were required to report any faecal sample with >50 CFU of *MAP*/tube as a heavy shedder. Heavy shedders represent a four log, or more, spectrum of faecal *MAP* concentration, from 1000 to 10,000,000 CFU/g of faecal sample. We contend that some of those 'too numerous to count' cultures are from supershedders (SSs) and that the consequences to the farm could be disastrous if they are not identified.

The term 'supershedder' (SS) is proposed to describe a cow shedding more than 10,000 *MAP* CFU/g of manure. The SS phenomenon has been reported for other bacterial and viral diseases, including SARS, AIDS and verotoxigenic *Escherichia coli* O157 in cattle (Cray and Moon, 1995; Bach *et al.*, 2005). Most *MAP* SSs are asymptomatic, with no evidence of diarrhoea or weight loss, yet excrete huge numbers of *MAP* organisms into the environment (Whitlock *et al.*, 2005). SS dairy cows have always existed, but this phenomenon was not recognized or accounted for in control programmes such as the USA Voluntary Bovine Johne's Disease Control Program.

Estimates of the frequency of SSs among culture-positive cattle in infected herds have not been published. Preliminary estimates suggest that 10% of heavy shedders (or about 2–3% of all culture-positive cattle at a single time point) may be SSs, excreting >10 billion *MAP* CFU per cow per day. Previous studies in dairy herds found a high proportion (40–60%) of low shedders (Whitlock *et al.*, 2000; Wells *et al.*, 2002b; Crossley *et al.*, 2005), which in the case of many animals probably reflects passive shedding after consumption of feedstuffs or water contaminated by a small number of SSs. Calculations indicate that as little as 1 g of manure (3×10^6 CFU/g) from a SS cow could result in passive shedding of *MAP* in an uninfected cow. If this hypothesis is proven to be correct, passive shedding could explain up to 50% of all culture-positive cattle in the herd when a SS is present. In fact, one SS excretes more *MAP* into the environment than 2000 moderate- or 20,000 low-shedder cows.

SS cattle represent the Trojan horse for Johne's disease. Cows at this later stage of the disease may not initially have clinical disease but represent the greatest threat to spread of *MAP* to other cows in several ways. First, SS cows disseminate *MAP* so effectively on the farm that <1 g ingested by another cow in the ration or on pasture will probably result in transient faecal shedding of *MAP*. It may also induce adult infection if the cow is not already infected and if the re-exposure is frequent. Secondly, footwear, vehicle wheels and other fomites contaminated with just a few grams of manure from these SS cows are potential sources of infection for newborn calves. Moreover, calves born to SS cows have a greater chance of being infected *in utero*. Thirdly, milk

and colostrum from a SS cow are more likely to contain *MAP* and infect the newborn calf. Finally, SS cows serve to contaminate pasture extensively with *MAP*, resulting in exposure of grazing cohorts (as in a leader–follower system) to large numbers of *MAP*.

14.5.2 Active shedding

Cattle shed *MAP* from their gastrointestinal tract by two distinctly different mechanisms. Active shedding occurs when a systemically infected animal excretes *MAP* into the intestinal tract. When an animal becomes infected, most commonly as a neonate, it enters an eclipse phase for 2 or more years, during which time *MAP* is not detectable in faecal samples. Then, as the organisms multiply to produce granulomas in the intestinal tissues and associated lymph nodes, the amount of *MAP* shed in the faeces continues to increase, and, at some point, *MAP* is sufficiently concentrated to be detectable by current diagnostic methods such as routine culture or by PCR.

14.5.3 Passive shedding (pass-through shedding)

Passive shedding occurs when cattle have detectable *MAP* in faecal samples following oral ingestion of *MAP*. Sweeney *et al.* (1992c) described a faecal 'pass-through' phenomenon in which non-infected cows had positive faecal cultures for several days after consuming faeces from *MAP*-infected cattle. Passive shedding routinely occurs following experimental oral *MAP* inoculations in neonatal calves (Sweeney *et al.*, 2006; van Roermund *et al.*, 2007; Stabel *et al.*, 2008), but if detected 14 days or longer following the last *MAP* dose it should be considered evidence of active shedding (Hines *et al.*, 2007).

SS cows contribute most to passive faecal shedding of *MAP* by uninfected cows. That some low shedders and even perhaps moderate shedders might be false positives (caused by passive instead of active shedding) is a major paradigm shift for the diagnosis and management of paratuberculosis. Additionally, it also has implications for evaluation of serological tests for *MAP*, because most investigators have used faecal culture as the gold standard (Collins *et al.*, 2005). Recognition of *MAP* SS has nearly eliminated the need to identify low shedders as early as possible in the course of the disease but has emphasized the necessity to identify SSs that are massively contaminating the environment and exposing other cattle to *MAP*. This is in contrast to the focus of many research efforts for more sensitive detection of organisms in faeces, which will probably result in the detection of more passive shedders.

Passive shedding has been shown to occur in beef cattle when fed hay during the winter months and during other times of the year when they gather in groups. Passive shedding has also been documented in herds positive for Johne's disease where intensive grazing is practised. Grazing systems such as the 'leader–follower' system, in which yearling cattle trail 1 day behind the adult herd and eat the leftover grass, may result in passive shedding (and in some cases lead to active shedding if the infective dose is sufficient) in the yearling cattle.

14.6 Concluding Thoughts

JD remains one of the most important diseases of cattle worldwide. In cattle, the disease is debilitating and is characterized by weight loss and chronic diarrhoea in the later stages of infection. However, cattle in the subclinical stages of the disease often show decreased milk production and are at higher risk for development of other common production diseases such as mastitis. Infections with *MAP* are difficult to control because of long incubation periods (1–10 years), the absence of clinical signs until advanced stages of the disease and the lack of completely reliable diagnostic methods in the preclinical stages of the disease. It is general knowledge that most calves become infected very early in life. Therefore control programmes are based on preventing transmission from adult cattle shedding the *MAP* organisms in faeces to young replacement stock on the farm. Control

programmes specifically focus on calving-pen management, milk- and colostrum-feeding practices and rearing of young stock.

The recent description of SS cattle has been associated with the need to identify these infected cows first within a given herd, as they represent the major source of contamination for the herd. The SS phenomenon has also brought more difficulties in the interpretation of routine diagnostic methods such as faecal culture, as animals that are culture-positive with a low number of *MAP* CFU/g faeces may in fact be passive shedders and not truly infected.

The lack of completely reliable diagnostic tests for the preclinical stages of the disease remains an important problem and is an important area of research. There are no drugs currently approved for the prevention or treatment of JD in cattle. Vaccination, available on a limited basis in the USA, may reduce the incidence of clinical disease, and to a lesser extent the prevalence of infection, but vaccinates are not fully protected from infection and may still shed *MAP*. It appears that *MAP* will not become eradicated from our dairy and beef herds in the near future with currently available tools. Therefore research is needed to identify better drugs and/or vaccines, to prevent infection during the neonatal period or its consequences later in life.

References

Aly, S.S. and Thurmond, M.C. (2005) Evaluation of *Mycobacterium avium* subsp. *paratuberculosis* infection of dairy cows attributable to infection status of the dam. *Journal of the American Veterinary Medical Association* 227, 450–454.

Arnoldi, J.M., Hurley, S.S. and Lesar, S. (1983) Johne's disease in Wisconsin cattle. In: *Proceedings of the Third International Symposium World Association Veterinary Laboratory Diagnosticians*, vol. 2, pp. 493–496.

Bach, S.J., Selinger, L.J., Stanford, K. and McAllister, T.A. (2005) Effect of supplementing corn- or barley-based feedlot diets with canola oil on faecal shedding of *Escherichia coli* O157:H7 by steers. *Journal of Applied Microbiology* 98, 464–475.

Bassey, E.O. and Collins, M.T. (1997) Study of T-lymphocyte subsets of healthy and *Mycobacterium avium* subsp. *paratuberculosis*-infected cattle. *Infection and Immunity* 65, 4869–4872.

Berghaus, R.D., Farver, T.B., Anderson, R.J., Jaravata, C.C. and Gardner, I.A. (2006) Environmental sampling for detection of *Mycobacterium avium* ssp. *paratuberculosis* on large California dairies. *Journal of Dairy Science* 89, 963–970.

Chiodini, R.J. (1996) Immunology: resistance to paratuberculosis. *Veterinary Clinics of North America Food Animal Practice* 12, 313–343.

Chiodini, R.J. and Hermon-Taylor, J. (1993) The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. *Journal of Veterinary Diagnostic Investigation* 5, 629–631.

Chiodini, R.J. and Van Kruiningen, H.J. (1986) The prevalence of paratuberculosis in culled New England cattle. *The Cornell Veterinarian* 76, 91–104.

Clarke, C.J. (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. *Journal of Comparative Pathology* 116, 217–261.

Collins, M.T., Wells, S.J., Petrini, K.R., Collins, J.E., Schultz, R.D. and Whitlock, R.H (2005) Evaluation of five antibody detection tests for bovine paratuberculosis. *Clinical and Diagnostic Laboratory Immunology* 12, 685–692.

Cray, W.C., Jr. and Moon, H.W. (1995) Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Applied and Environmental Microbiology* 61, 1586–1590.

Crossley, B.M., Zagmutt-Vergara, F.J., Fyock, T.L., Whitlock, R.H. and Gardner, I.A. (2005) Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* by dairy cows. *Veterinary Microbiology* 107, 257–263.

Gilmour, N.J., Nisbet, D.I. and Brotherston, J.G. (1965) Experimental oral infection of calves with *Mycobacterium johnei*. *Journal of Comparative Pathology* 75, 281–286.

Hines, M.E., 2nd, Stabel, J.R., Sweeney, R.W., Griffin, F., Talaat, A.M., Bakker, D., Benedictus, G., Davis, W.C., de Lisle, G.W., Gardner, I.A., Juste, R.A., Kapur, V., Koets, A., McNair, J., Pruitt, G. and Whitlock, R.H. (2007) Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Veterinary Microbiology* 21, 197–222.

Jørgensen, J.B. (1982) An improved medium for culture of *Mycobacterium paratuberculosis* from bovine faeces. *Acta Veterinaria Scandinavica* 23, 325–335.

Kalis, C.H., Hesselink, J.W., Barkema, H.W. and Collins, M.T. (2000) Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis. *Journal of Veterinary Diagnostic Investigation* 12, 547–551.

Koo, H.C., Park, Y.H., Hamilton, M.J., Barrington, G.M., Davies, C.J., Kim, J.B., Dahl, J.L., Waters, W.R. and Davis, W.C. (2004) Analysis of the immune response to *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected calves. *Infection and Immunity* 72, 6870–6883.

Larsen, A.B., Merkal, R.S. and Cutlip, R.C. (1975) Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 36, 255–257.

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B. and Salman, M.D. (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. *Journal of Dairy Science* 89, 4163–4171.

McKenna, S.L., Keefe, G.P., Barkema, H.W., McClure, J., Vanleeuwen, J.A., Hanna, P. and Sockett, D.C. (2004) Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine. *Journal of Dairy Science* 87, 3770–3777.

Merkal, R.S., Larsen, A.B. and Booth, G.D. (1975) Analysis of the effects of inapparent bovine paratuberculosis. *American Journal of Veterinary Research* 36, 837–838.

Merkal, R.S., Whipple, D.L., Sacks, J.M. and Snyder, G.R. (1987) Prevalence of *Mycobacterium paratuberculosis* in ileocecal lymph nodes of cattle culled in the United States. *Journal of the American Veterinary Medical Association* 190, 676–680.

Momotani, E., Whipple, D.L., Thiermann, A.B. and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Veterinary Pathology* 25, 131–137.

National Animal Health Monitoring System (1997) In: *Johne's Disease on U.S. Dairy Operations*. USDA: APHIS: SEA, Fort Collins, Colorado. Available at: http://nahms.aphis.usda.gov/beefcowcalf/index.htm (accessed 14 May 2009).

National Animal Health Monitoring System (2002) In: *Part I: Reference of Dairy Health and Management in the United States*. USDA: APHIS: VS, CEAH, Fort Collins, Colorado. Available at: http://nahms.aphis.usda.gov/dairy/index.htm (accessed 14 May 2009).

Nielsen, S.S., Bjerre, H. and Toft, N. (2008) Colostrum and milk as risk factors for infection with *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. *Journal of Dairy Science* 91, 4610–4615.

Raizman, E.A., Wells, S.J., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C. and Olsen, K.E. (2004) The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *Journal of Dairy Science* 87, 2959–2966.

Rhode, R.F. and Shulaw, W.P. (1990) Isolation of *Mycobacterium paratuberculosis* from the uterine flush fluids of cows with clinical paratuberculosis. *Journal of the American Veterinary Medical Association* 197, 1482–1483.

Rossiter, C.A. and Burhans, W (1996) Farm specific approach to paratuberculosis (Johne's disease) control. *Veterinary Clinics of North America Food Animal Practice* 12, 383–415.

Seitz, S.E., Heider, L.E, Heuston, W.D, Bech-Nielsen, S., Rings, D.M. and Splanger, L. (1989) Bovine fetal infection with *Mycobacterium paratuberculosis*. *Journal of the American Veterinary Medical Association* 194, 1423–1426.

Stabel, J.R., Palmer, M.V., Harris, B., Plattner, B., Hostetter, J. and Robbe-Austerman, S. (2008) Pathogenesis of *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves after oral or intraperitoneal experimental infection. *Veterinary Microbiology* 136, 306–313.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P. and Rings, D.M. (1995) Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *American Journal of Veterinary Research* 56, 1322–1324.

Sweeney, R.W. (1996) Transmission of paratuberculosis. *Veterinary Clinics of North America Food Animal Practice* 12, 305–312.

Sweeney, R.W., Whitlock, R.H. and Rosenberger, A.E. (1992a) *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. *American Journal of Veterinary Research* 53, 477–480.

Sweeney, R.W., Whitlock, R.H. and Rosenberger, A.E. (1992b) *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cattle. *Journal of Clinical Microbiology* 30, 166–171.

Sweeney, R.W., Whitlock, R.H., Hamir, A.N., Rosenberger, A.E. and Herr, S.A. (1992c) Isolation of *Mycobacterium paratuberculosis* after oral inoculation in infected cattle. *American Journal of Veterinary Research* 53, 1312–1314.

Sweeney, R.W., Uzonna, J., Whitlock, R.H., Habecker, P.L., Chilton, P. and Scott, P. (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subsp. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. *Research in Veterinary Science* 80, 253–259.

Taylor, A.W. (1953) Experimental Johne's disease in cattle. *Journal of Comparative Pathology* 63, 355–367.

van Roermund, H.J., Bakker, D., Willemsen, P.T. and de Jong, M.C. (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. *Veterinary Microbiology* 122, 270–279.

van Schaik, G., Stehman, S.M., Schukken, Y.H., Rossiter, C.R. and Shin, S.J. (2003) Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. *Journal of Veterinary Diagnostic Investigation* 15, 233–241.

Waters, W.R., Miller, J.M., Palmer, M.V., Stabel, J.R., Jones, D.E., Koistinen, K.A., Steadham, E.M., Hamilton, M.J., Davis, W.C. and Bannantine, J.P. (2003) Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infection and Immunity* 71, 5130–5138.

Wells, S.J., Whitlock, R.H., Lindeman, C.J. and Fyock, T. (2002a) Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 63, 1207–1211.

Wells, S.J., Whitlock, R.H., Wagner, B.A., Collins, J., Garry, F., Hirst, H., Lawrence, J., Saville, W.J. and Naugle, A.L. (2002b) Sensitivity of ELISA test strategies for herd-level detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle herds. *Journal of the American Veterinary Medical Association* 220, 1053–1057.

Wells, S.J., Godden, S.M., Lindeman, C.J. and Collins, J.E. (2003) Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. *Journal of the American Veterinary Medical Association* 223, 1022–1025.

Whitlock, R.H (1992) Diarrhea in cattle. In: Anderson, N.V. (ed.) *Veterinary Gastroenterology*, 2nd edn. Lea & Febiger, Philadelphia, p. 783.

Whitlock, R.H. (2009) Johne's disease. In: Smith, B.P. (ed.) *Large Animal Internal Medicine*, 4th edn. Mosby Elsevier, St Louis, pp. 881–887.

Whitlock, R.H., Hutchinson, L.T., Merkel, R., Glickman, L.T., Rossiter, C., Harmon, S., Spencer, P., Fetrow, J., Bruce, J., Benson, C.E. and Dick, J. (1985) Prevalence and economic considerations of Johne's disease in the northeastern US. In: *Proceedings of the 89th Annual Meeting of the US Animal Health Association*, pp. 484–490.

Whitlock, R.H., Whittington, R.J., Marsh, I.B., Turner, M.J., Saunders, V., Kemsley, P.D. and Rayward, D. (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Veterinary Microbiology* 77, 387–398.

Whitlock, R.H., Sweeney, R.W. and Fyock, T.L. (2005) *Mycobacterium avium* subsp. *paratuberculosis* super-shedders: another factor in the control of Johne's disease. In: Juste, A.R., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, p. 164.

Whittington, R.J. and Windsor P.A. (2009) *In utero* infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. *The Veterinary Journal* 179, 60–69.

Whittington, R.J., Reddacliff, L.A., Marsh, I., McAllister, S. and Saunders, V. (2000a) Temporal patterns and quantification of excretion of *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Australian Veterinary Journal* 78, 34–37.

Whittington, R.J., Fell, S., Walker, D., McAllister, S., Marsh, I., Sergeant, E., Taragel, C.A., Marshall, D.J. and Links, I.J. (2000b) Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep *Journal of Clinical Microbiology* 38, 2550–2556.

Windsor, P.A. and Whittington, R.J. (2009) Evidence for age susceptibility of cattle to Johne's disease. *The Veterinary Journal* (in press).

Wu, C.W., Livesey, M., Schmoller, S.K., Manning, E.J., Steinberg, H., Davis, W.C., Hamilton, M.J. and Talaat, A.M. (2007) Invasion and persistence of *Mycobacterium avium* subsp. *paratuberculosis* during early stages of Johne's disease in calves. *Infection and Immunity* 75, 2110–2119.

15 Paratuberculosis in Sheep

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15.1 Introduction

The prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in domestic sheep worldwide is unknown, as the disease is 'non-notifiable' in many countries. Studying *MAP* in sheep has had a relatively low priority compared with *MAP* in cattle or the more strategically important mycobacterial diseases such as tuberculosis. Reports of Johne's disease (JD) in sheep have been recorded in North and South America, Australasia, the Middle East, Asia, Africa and Europe.

The difficulty of determining the extent of disease has been compounded by the lack of diagnostic tests with high levels of sensitivity and specificity, and the high costs involved with identifying the disease compared with the low value of a sheep. However, the prevalence of *MAP* appears to differ markedly between countries and geographical regions within countries (Sergeant, 2001; Sevilla *et al.*, 2005). Published data on the prevalence of affected sheep flocks range from 2 to 32% between different countries (Bakker *et al.*, 2000; Sergeant, 2001; Nielsen and Toft, 2009). In Spain, the

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estimated prevalence within sheep flocks is 8–10% (Sevilla *et al.*, 2002). In Australia, *MAP* is thought to infect 2.4–4.4% of the flocks, based on data from abattoir surveillance. In New South Wales, Australia, 6–10% of the flocks have ovine JD, while in Western Australia less than 1% of the flocks are thought to be infected (Sergeant, 2001; Sergeant and Baldock, 2002). Within-flock prevalence has not been examined in detail but mortalities of 5–15% per year have been seen in high-prevalence flocks (Reddacliff *et al.*, 2006).

15.2 Host and Pathogen Characteristics that Define Ovine JD

15.2.1 Breed, age and sex of sheep

No study has comprehensively examined whether different breeds of sheep are more or less susceptible to JD, although this point is often mentioned by farmers. In a flock with a low prevalence of *MAP* infection, Merino or Merino–Romney cross ewes were observed to have a significantly higher percentage of clinical ovine JD than Romney sheep (Morris *et al.*, 2006). Although examining breed susceptibility to *MAP* was not the aim of this study, it did highlight the possibility of breed differences. No studies have been published on the relationship between age or sex and susceptibility to infection with *MAP* in sheep.

15.2.2 The strain of *MAP*

The strain of *MAP* that infects sheep will vary with the predominant strain type in the geographical region and whether or not sheep cohabit with other species (Begg and Whittington, 2008). It is becoming clear from experimental infections that the *MAP* strain can have a significant impact on infection rates, the severity of pathology and the number of clinically affected animals (Verna *et al.*, 2007). Sheep develop more severe lesions when experimentally infected with sheep strains rather than cattle strains of *MAP*. Different cattle strains of *MAP* inoculated into sheep also result in different pathological and immunological outcomes (Verna *et al.*, 2007). These factors may be responsible for the differences in disease outcome and prevalence between flocks in different geographical regions or countries. Quarantine authorities in endemic areas should consider excluding animals that harbour particular strains of *MAP* that are not already found in that area. *MAP* strains are described in more detail elsewhere (see Stevenson, Chapter 12, this volume).

15.3 Characteristics of Infection and Disease

15.3.1 Clinical signs and clinical pathology

The clinical signs of JD in sheep are limited to chronic weight loss, which may occur from 2 years of age, with most animals succumbing to disease at 3–5 years of age. Oedema may occur occasionally. In advanced cases there may be hypoalbuminaemia and hypocalcaemia (Jones and Kay, 1996).

Most sheep that die of JD have normal faecal pellets. Diarrhoea is not considered to be a feature of JD in small ruminants, except in the terminal stages of disease. In a study of 50 sheep with clinical JD, most were emaciated; half had normal faecal pellets; 30% had soft-formed faeces; and 20% had severe diarrhoea (Carrigan and Seaman, 1990).

15.3.2 Pathology – gross and microscopic lesions

Advanced cases of JD in sheep typically have thickening of the mucosa of the terminal ileum; the wall may be oedematous and the mucosa thrown up into transverse ridges; there may be cording of the subserosal lymphatics, and these are clearly visible and palpable. The caecum and colon may also be involved. In the cases described by Carrigan and Seaman (1990), lymphadenomegaly was present in 38% of the sheep. Histologically, there was moderate to severe granulomatous enteritis (94% of sheep), typhlitis (74%) and

colitis (14%); the rectum was involved in 2%; lymphocytic infiltrates were also present in most sheep.

In tissue sections from the same animals, 88% of sheep had abundant acid-fast bacilli (AFB) and 12% had few AFB (Carrigan and Seaman, 1990). This dichotomy led others to describe two distinct forms of disease in sheep: paucibacillary and multibacillary (Clarke and Little, 1996). Clarke *et al.* (1996) used a lesion-grading system; sheep with a mean of 0–10 AFB per macrophage were called paucibacillary, and those with >10 were multibacillary. Animals in both groups were emaciated and had carcass oedema; the multibacillary animals were more likely to have detectable gross lesions in the intestine and associated lymph nodes. Histologically, the paucibacillary group tended to have a lymphocytic infiltrate with fewer macrophages compared with the multibacillary group, in which macrophages dominated the infiltrate (Clarke and Little, 1996). A more detailed lesion-grading system with five categories (1, 2, 3a, 3b, 3c) was proposed by Perez *et al.* (1996). Mycobacteria were not visible in most sections from sheep with Type 1 (mild) and Type 3c (severe) lesions but were abundant in Type 3b. Although the authors were far from certain, an ordinal progression of lesions from Type 1 to Type 2 and beyond was implied. However, the time of infection and the influence of a successful immune response in hindering progression and AFB numbers were factors that could not be accounted for (Perez *et al.*, 1996).

The prevalence of the various types of pathology within an infected flock is unclear due to biases in sampling. The progression of lesions is also unknown. It is clear, however, that the first lesions develop in the ileal Peyer's patches and then spread to the surrounding mucosa (Perez *et al.*, 1996).

15.3.3 Route of infection and transmission between individuals

The most common route of infection is the faecal–oral route. Infection can also be spread by intrauterine and transmammary transmission (Lambeth *et al.*, 2004). The intratonsillar route of infection may also play a role (Begg *et al.*, 2005), although this is unlikely to be of significance in the natural infection process, mainly because the large number of bacteria that are ingested and swallowed will heavily outweigh those that may become lodged in the tonsil. Experimental infection data have shown that sheep infected by alternative routes develop altered immunological response profiles compared with those infected orally (Begg *et al.*, 2005). Lambs infected via the intratonsillar route compared with oral-challenged animals had increased gamma interferon (IFN-γ), lymphocyte proliferation and antibody levels. This may partly explain the large variation in immune responses recorded in groups of naturally infected sheep. This has implications for the design of experimental infection models and the outcomes observed from this type of research.

Ileal Peyer's patches in sheep, unlike those in humans, show prenatal maturation, with antigen-independent lymphopoiesis and a rate of lymphocyte production greater than in the thymus. The ileal Peyer's patches in sheep are unique in that they are required for the development of B cells and like the thymus they involute with age (Landsverk *et al.*, 1991). At birth, the Peyer's patches are the single biggest lymphoid tissue, accounting for 1.2% of the lamb's body weight. By 6 weeks of age, the ileal Peyer's patches of a lamb will extend 2.5 m along the terminal ileum. From 12 weeks of age, the ileal Peyer's patches start to involute, with only a few remaining by 18 months of age (Reynolds and Morris, 1983). The ileal Peyer's patch of the neonatal ruminant is considered to be a primary target for *MAP* infection (Sigurðardóttir *et al.*, 2001). The lymphoid follicles of ileal Peyer's patches contain large numbers of B cells but few T cells (Landsverk *et al.*, 1991). While jejunal Peyer's patches persist in adult animals, they contain clusters of B cells and have numerous CD4+ T cells in the associated lymphoid follicle (Landsverk *et al.*, 1991). In sheep, the ileal and jejunal Peyer's patches appear to have different functions. The jejunal Peyer's patches are essential for producing mucosal responses, while the ileal Peyer's patches seed the systemic immune organs with B cells (Mutwiri *et al.*, 1999).

It is generally accepted that the entrance of *MAP* through the intestinal wall is via M cells overlying the Peyer's patches, from where the bacteria are engulfed by macrophages (Momotani *et al.*, 1988). After this, very little is known about the host–pathogen interaction. There is a period of latency or silent infection between when the animal is infected, typically as a lamb, and the time of onset of clinical signs. During the time immediately after oral challenge, most of the bacteria are passively shed into the environment (Reddacliff and Whittington, 2003). Presumably, organisms taken into the intestines by the M cells are in such low numbers that animals may remain unaffected for months or years. The number of bacteria lodged in the intestines and associated lymph nodes can be below the level of detection of tissue culture for months after challenge (Reddacliff and Whittington, 2003; Begg *et al.*, 2005). It is this latency period that creates such a problem for the early diagnosis of ovine JD. Research is currently under way to detect changes in *MAP in vitro* that might reflect the latent stage *in vivo*, as a means to improve the sensitivity of future diagnostic assays (Gumber and Whittington, 2009; Gumber *et al.*, 2009a,b).

15.3.4 Immunopathobiology

Organism dissemination and propagation are possibly due to decreased cellular immunity allowing infection to develop into clinical disease (Stabel, 2000, 2006). Containment and restriction of replication of the bacteria within the macrophage are critical, as these cells can tolerate low numbers of bacteria, whereas higher numbers of *MAP* are cytotoxic and result in apoptosis (Merkal *et al.*, 1968; Bannantine and Stabel, 2002). As bacterial numbers increase within the gut wall, the number of intracellular bacteria sloughed off into the lumen will increase. This results in larger numbers of bacteria within faecal material and an increased chance of detection by faecal culture. It is therefore unsurprising that sheep with multibacillary lesions are more likely to shed *MAP* in faeces than animals with paucibacillary lesions (Whittington *et al.*, 2000a). A sheep with multibacillary disease will shed on average 10^8 bacteria per gram of faeces or up to 10^{10} bacteria per day (Whittington *et al.*, 2000b).

Major histocompatibilty complex (MHC) processing and antigen presentation may be downregulated in *MAP* infections (Berger and Griffin, 2006). Production and expression of MHC class I and II molecules may be reduced in *MAP*-infected macrophages (Alzuherri *et al.*, 1997; Berger and Griffin, 2006). Expression of lymphocyte function-associated antigen, a molecule involved with cell-to-cell interactions, is also reduced in *MAP*-infected macrophages (Alzuherri *et al.*, 1997).

Other innate pathways that are activated during a *MAP* infection include the pattern recognition receptors (PRR). These include the Toll-like receptor family (TLR), involved in recognition of binding of pathogens by macrophages and other cells of the innate immune system. Once these receptors are engaged by microbial ligands, they initiate the innate and adaptive immune response mechanisms. Recent papers indicated that several PRR could be upregulated during *MAP* infection, including TLR1, TLR5, TLR6, TLR8, CARD15, dectin-1 and dectin-2 (Nalubamba *et al.*, 2008; Taylor *et al.*, 2008).

Paucibacillary and multibacillary disease states in sheep correlate broadly with the predominant pathway of immunity (Clarke, 1997). Sheep with paucibacillary lesions are likely to have an associated cell-mediated immune (CMI) response, with large numbers of lymphocytes at the site of disease (Clarke *et al.*, 1996). Lesions found in the small intestine show an increase in the numbers of $CD4^+$ T cells and gamma delta ($\gamma\delta$) T cells, and the ratio of CD4+ to CD8+ is >1 (Little *et al.*, 1996; Reddacliff *et al.*, 2004). Increased antigen-specific lymphoproliferative responses can be seen from blood and gut cells isolated from sheep with paucibacillary disease (Kurade *et al.*, 2004; Kurade and Tripathi, 2008). Increased levels of T helper-1 (Th-1) cytokines are produced within the intestinal tissues by leucocytes in animals with paucibacillary lesions. The predominant cytokines are IFN-γ and interleukin 2 (IL-2) (Clarke *et al.*, 1996; Burrells *et al.*, 1999; Smeed *et al.*, 2007). CMI responses, as measured by lymphocyte transformation and

IFN-γ assays, can be variable along the chain of mesenteric lymph nodes. The lowest level of reactivity is found in leucocytes in the intestinal lamina propria (Burrells *et al.*, 1998; Begg and Griffin, 2005; Begg *et al.*, 2005).

In contrast to paucibacillary lesions, multibacillary lesions typically contain larger numbers of macrophages, and sheep with these lesions typically have a strong Th-2 (humoral antibody) response. Animals with multibacillary lesions have a significantly reduced lymphocyte proliferation response (Burrells *et al.*, 1998; Kurade and Tripathi, 2008). The number of CD4+ T cells in the lesion decreases and the ratio of CD4+ to CD8+ T cells changes from >1 to <1 (Clarke and Little, 1996; Little *et al.*, 1996). The reduction in CD4+ T cells may result in a reduction of the Th-1 CMI response and a lack of macrophage activation and killing (Navarro *et al.*, 1998; Koets *et al.*, 2002). An increase of Type 2 cytokine production is seen in multibacillary lesions; typically the cytokines that increase are the interleukins IL-10, IL-6, IL-8 and IL-18, transforming growth factor-beta 1 (TGF-β1) and tumour necrosis factor alpha (TNF-α) (Alzuherri *et al.*, 1996; Smeed *et al.*, 2007; Munoz *et al.*, 2009). There is a corresponding decrease in the production of IL-2 (Clarke *et al.*, 1996; Burrells *et al.*, 1999). Most studies report a decrease of IFN-γ production in multibacillary or clinically affected animals (Clarke *et al.*, 1996; Burrells *et al.*, 1999; Smeed *et al.*, 2007). Interestingly, increased IL-1α expression in ileal tissues, which has been observed in bovine JD, has not been observed in ovine JD (Smeed *et al.*, 2007).

Only recently have researchers begun to examine an important and often overlooked third group of sheep: animals that appear to contain or overcome *MAP* infection. These sheep may be animals defined as not having a significant infection (no histological lesions) or as animals identified as tissue-culture-negative after experimental infection. These animals have increased expression of the TLR9 and TNF-α genes, and a downregulation of IL-18 (Smeed *et al.*, 2007; Nalubamba *et al.*, 2008). On a cellular level, CD4+, CD8+, CD25+ T cells and B cell populations are increased in the blood, and CD4+, CD25+ T cells and B cells increased in the gut-associated lymph nodes (Begg and Griffin, 2005). Interestingly, the number of B cells in the blood and tissues of the resistant animals was increased relative to diseased and control animals, indicating that the Th-2 humoral response may play a role in immunity to *MAP* infection.

Gamma delta (γδ) T cells may also play a role in *MAP* infections. In young ruminants the γδ T cells make up a large proportion of the T cells, unlike in humans and mice, where alpha beta T cells (CD4+ and CD8+ T cells) predominate (Mackay *et al.*, 1989; Hein and Griebel, 2003). The high numbers of γδ T cells found in the ruminant gastrointestinal tract (Chiodini, 1996) may have a role in the regulation of mucosal immunity to invasion by enteric pathogens (Ferguson, 1990). There is an increase in γδ T cells in the gut-associated lymphoid tissues of lambs exposed to *MAP* (Beard *et al.*, 2000). In multibacillary lesions in *MAP*-infected sheep, the numbers of CD4+ T cells are decreased while the number of γδ T cells stays the same (Little *et al.*, 1996).

15.4 Microbiological Diagnosis

An ideal diagnostic assay would be one that detects responses early after infection and is 100% sensitive and specific. All available diagnostic methods for JD in domestic livestock tend to underestimate true disease prevalence owing to difficulties in identifying subclinically infected animals. Ante-mortem microbial tests for *MAP* infection presently include faecal culture and polymerase chain reaction (PCR). Immunodiagnostic tests include the antibody enzyme-linked immunosorbant assay (ELISA), the agar gel immunodiffusion (AGID) assay, the lymphocyte proliferation or transformation assay, intradermal skin testing and the IFN-γ assay. All of the above assays have associated drawbacks in terms of specificity and/or sensitivity. Post-mortem tests include histopathology, and culture or PCR for *MAP* from tissues.

15.4.1 Culture

Cultivation of *MAP* from intestinal tissues is thought to be the gold standard in the

diagnosis of ovine JD. Prior to 1998, the detection of the sheep strain of *MAP* was unreliable, as available culture media were not appropriate to support its growth. The addition of egg yolk and Mycobactin J to Middlebrook 7H9 broth or 7H10 or 7H11 agar solved this problem (Whittington *et al.*, 1998, 1999).

Where there is subclinical infection with *MAP*, faecal shedding can be intermittent, which lowers the sensitivity of culture. However, sheep with multibacillary lesions shed large numbers of *MAP* that are detectable in culture, even when diluted 100-fold with faeces from uninfected sheep (Whittington *et al.*, 2000a). The sensitivity of pooled faecal culture for flock-level detection is 92%, assuming a sample size of 350 sheep pooled and cultured in lots of 50. Pooled faecal culture has been adopted as the laboratory method of choice for detecting *MAP*-infected sheep flocks in Australia (Sergeant, 2001). *MAP* culture is described in more detail elsewhere (see Whittington, Chapter 22, this volume).

15.4.2 PCR

In recent years, the sensitivities of IS*900*-based PCR assays for the detection of *MAP* from both faecal and tissue samples have greatly improved. This is primarily due to improved DNA extraction techniques and the use of quantitative or real-time PCR assays. The sensitivity of PCR for faecal samples was reported to be equivalent to that of culture with a high specificity due to primers designed to avoid detection of environmental bacteria (Kawaji *et al.*, 2007).

As an alternative, a blood-based PCR assay has been developed on the theory that a bacteraemic phase occurs during *MAP* infections (Juste *et al.*, 2005). Such blood-based assays were designed to overcome the problem of PCR inhibitors in faecal samples, which had caused low sensitivity in the faecal PCR assay. The validation of this assay is pending; in one study, there was a low level of agreement between the blood PCR and an antibody ELISA assay in sheep (Juste *et al.*, 2005). An extensive review of the benefits and disadvantages of PCR is given elsewhere (see Bölske and Herthnek, Chapter 23, this volume).

15.5 Immunodiagnosis

Immunodiagnostic tests such as ELISA, AGID, intradermal skin testing, lymphocyte transformation and IFN-γ assays generally have poor diagnostic sensitivity and can be significantly affected by the stage of infection (Gumber *et al.*, 2006). The sensitivity of the antibody ELISA to detect *MAP* infection appears to depend on the type of kit used. One study of the Parachek™ ELISA reported a sensitivity of 16% at a specificity of 97.5% (Robbe-Austerman *et al.*, 2006a). Another antibody ELISA (Institut Pourquier) had a sensitivity of 34.9% and a specificity of 98.8% (Gumber *et al.*, 2006). The AGID has been found to have an even lower sensitivity than the ELISA (Gumber *et al.*, 2006; Robbe-Austerman *et al.*, 2006a). Again, the sensitivity of the IFN-γ assay is low, ranging from 40 to 75% with specificities ranging from 88 to 98% (Stewart *et al.*, 2002; Robbe-Austerman *et al.*, 2006b). The sensitivity of the intradermal skin test and the lymphocyte proliferation (or transformation) assays have not been examined in detail, with different studies using various cut points for diagnosis of infection (Williams *et al.*, 1985; Reddacliff and Whittington, 2003; Robbe-Austerman *et al.*, 2006a). Validation of the diagnostic assays by defining specificity and sensitivity against the gold standard tests of tissue or faecal culture has not been a feature of many of the studies to date. If validation of the assays is not done, then researchers and clinicians are left with a poor understanding of the assay's operating parameters and how they can use them under different circumstances to diagnose disease.

15.6 Spread of Infection (Epidemiology)

The behaviour of *MAP* in populations of sheep is perhaps best understood through two case studies where the organism was introduced into regions where it was previously unknown, Iceland and Australia. Common features include: (i) the cryptic nature of infection, enabling spread and evasion of normal quarantine practices; (ii) the slow progression of

infection in the population; and (iii) the eventual emergence of paratuberculosis as a very serious disease.

Sheep were introduced to Iceland in the 9th century and remained free of *MAP* until 20 Karakul sheep were imported from Germany in 1933. In fact, three slowly developing infectious diseases evaded quarantine controls due to their long incubation periods: paratuberculosis, maedi/visna and jaagsiekte. Paratuberculosis was diagnosed 5 years later, and 440 farms were infected in the subsequent 20 years. In 1940, the disease appeared in cattle that were grazed with sheep, but fewer cows than sheep were affected. It later occurred in goats and a reindeer (Fridriksdottir *et al.*, 2000). Mortality rates per annum in sheep averaged 8–9% but were as high as 40% on some farms. Sheep and dairy cattle grazed together on pastures during the summer and were housed together during the colder months. Thus there was probably a very high level of cross-species *MAP* contamination. Control measures based on fencing and zoning based on prevalence levels, along with test and cull, failed to impede spread. Destocking of all sheep in some zones was then tried, but the infection was probably maintained in cattle, as healthy sheep introduced 1 year after the destocking programme succumbed to JD. Finally, vaccination of lambs was introduced, and this is still practised to prevent clinical disease. JD in Iceland is caused by an S strain of *MAP* which could not be routinely cultured; it was identified using molecular techniques from archives of histological paraffin blocks (Whittington *et al.*, 2001). This strain of *MAP* appears to be highly virulent for sheep but of low virulence for cattle, as, despite close contact with sheep, the clinical disease is rarely seen in cattle in Iceland; cattle have never been vaccinated in Iceland (Fridriksdottir *et al.*, 2000).

Australia has experienced a similar 'slow epizootic' of paratuberculosis in sheep. The most likely scenario for Australia involved importation of infected sheep from New Zealand prior to 1958 or in the 1970s, as live sheep were not imported in the intervening period (Sergeant, 2001). The first case of ovine JD was diagnosed in New South Wales in 1980 (Seaman *et al.*, 1981), years after its likely introduction. The disease was most unlikely to have been widespread in sheep flocks at that time as there was a strong system of passive surveillance in Australia (provided by regional veterinary laboratories) at no cost to farmers. For this reason, it is likely that the rate of spread of JD increased exponentially after about 1980, associated with the pattern of trade of live sheep in Australia. By the mid-1990s, the disease was of significant economic impact on farms where it had been present for some years. Economic losses of 6.4–8.5% in the gross income margin have been recorded on farms where ovine JD mortality ranged from 6.2 to 7.8% (Bush *et al.*, 2006). By 2000, there were 823 known infected flocks in New South Wales, Victoria, Tasmania and South Australia (Sergeant, 2001). Thousands of other flocks were suspected to be infected, based on purchase of sheep from infected farms and shared farm boundaries. Western Australia, which was geographically isolated, was the last state to notify infection; at the time of detection in 2004, infection was deemed likely to have been present for 7 years or longer; seven flocks with over 82,000 sheep were infected and 144 more were under suspicion (Sunderman, 2004).

A national disease control programme commenced in Australia in 1999; it employed pooled faecal culture and abattoir surveillance to determine infection and set up an assurance programme to identify and protect flocks that did not have the disease (Sergeant, 2001). A killed vaccine was shown to be effective to prevent clinical signs of JD in sheep (Reddacliff *et al.*, 2006), and this is now used widely. Vaccination, in combination with biosecurity and risk management, is now relied on to reduce the rate of spread of the disease. A more detailed description of JD control measures in Australia is given elsewhere (see Kennedy and Citer, Chapter 28, this volume).

Strains of *MAP* from sheep, cattle and many other animals can be clearly divided on genetic grounds into two groups called C and S. A more complete description of these groups, their alternative designations and strain characterization is given elsewhere (see Stevenson, Chapter 12 and Collins, Chapter 25, this volume). The S strain of *MAP*, which was responsible for JD in sheep in Iceland and Australia, is also prevalent in New Zealand.

In other countries it may be displaced by the C strain, where either sheep are uncommon or cattle are prevalent. Sheep are also susceptible to the C strain and it has been a common finding in sheep in Europe (de Juan *et al.*, 2005; Sevilla *et al.*, 2007, 2008; Florou *et al.*, 2009). The difficulty of cultivation of S strain probably leads to underestimation of its distribution and abundance. Cattle, goats and deer can become infected with the S strain (O'Brien *et al.*, 2006; Mackintosh *et al.*, 2007; Moloney and Whittington, 2008; Sevilla *et al.*, 2008), but cattle and deer appear to be more resistant to infection and the associated clincial disease associated with this strain. In Australia, cattle have become infected as calves if exposed to heavily infected sheep, but it is an uncommon infection (Whittington *et al.*, 2001; Moloney and Whittington, 2008).

A detailed investigation on one infected farm in Australia revealed a slow rate of transmission and clustering of infection within age classes of sheep for 7 years. Susceptibility of young sheep to lower levels of *MAP* and the long incubation period before faecal shedding may explain this (Rast and Whittington, 2005). Once contamination rates build up, this pattern may be lost as sheep of any age become infected. This results in increased prevalence rates, with infected sheep possibly shedding *MAP* sooner after infection, but this is yet to be proven. One reason for different levels of prevalence between affected flocks is variation in stocking rate during lambing (Dhand *et al.*, 2007). A higher stocking rate leads to higher levels of contamination, causing a greater risk of exposure of lambs, and this was associated with higher levels of infection (Dhand *et al.*, 2007).

15.7 Concluding Remarks

Ovine JD is a worldwide issue that is overlooked in many countries, due to the low value of sheep and a greater focus on other mycobacterial infections in other species. The spread of the infection has been helped by the slow development of clinical signs, the slow rate of transmission between animals and the low sensitivity of diagnostic tests, especially in the earlier stages of disease. Overall there has been an increase in understanding of the issues surrounding *MAP* infection in sheep. For instance, while previous research predominately focused on diseased animals in comparison to uninfected sheep, the examination of sheep that have overcome a challenge with *MAP* may provide a better understanding of the immune processes implicated in resistance to the disease. Improvements in the focus of current and future research may lead to increased understanding of the disease process and over time may result in improved diagnosis and better control measures.

References

Alzuherri, H.M., Woodall, C.J. and Clarke, C.J. (1996) Increased intestinal TNF-alpha, IL-1 beta and IL-6 expression in ovine paratuberculosis. *Veterinary Immunology and Immunopathology* 49, 331–345.

Alzuherri, H.M., Little, D. and Clarke, C.J. (1997) Altered intestinal macrophage phenotype in ovine paratuberculosis. *Research in Veterinary Science* 63, 139–143.

Bakker, D., Willemsen, P.T. and van Zijderveld, F.G. (2000) Paratuberculosis recognized as a problem at last: a review. *The Veterinary Quarterly* 22, 200–204.

Bannantine, J.P. and Stabel, J.R. (2002) Killing of *Mycobacterium avium* subspecies *paratuberculosis* within macrophages. *BMC Microbiol*ogy 2, 2.

Beard, P.M., Rhind, S.M., Sinclair, M.C., Wildblood, L.A., Stevenson, K., McKendrick, I.J., Sharp, J.M. and Jones, D.G. (2000) Modulation of gammadelta T cells and CD1 in *Mycobacterium avium* subsp. *paratuberculosis* infection. *Veterinary Immunology and Immunopathology* 77, 311–319.

Begg, D.J. and Griffin, J.F. (2005) Vaccination of sheep against *M. paratuberculosis*: immune parameters and protective efficacy. *Vaccine* 10, 4999–5008.

Begg, D.J. and Whittington, R.J. (2008) Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Journal* 176, 129–145.

Begg, D.J., O'Brien, R., Mackintosh, C.G. and Griffin, J.F. (2005) Experimental infection model for Johne's disease in sheep. *Infection and Immunity* 73, 5603–5611.

Berger, S.T. and Griffin, F.T. (2006) A comparison of ovine monocyte-derived macrophage function following infection with *Mycobacterium avium* ssp. *avium* and *Mycobacterium avium* ssp. *paratuberculosis*. *Immunology and Cell Biology* 84, 349–356.

Burrells, C., Clarke, C.J., Colston, A., Kay, J.M., Porter, J., Little, D. and Sharp, J.M. (1998) A study of immunological responses of sheep clinically-affected with paratuberculosis (Johne's disease): the relationship of blood, mesenteric lymph node and intestinal lymphocyte responses to gross and microscopic pathology. *Veterinary Immunology and Immunopathology* 66, 3–4.

Burrells, C., Clarke, C.J., Colston, A., Kay, J.M., Porter, J., Little, D. and Sharp, J.M. (1999) Interferon-gamma and interleukin-2 release by lymphocytes derived from the blood, mesenteric lymph nodes and intestines of normal sheep and those affected with paratuberculosis (Johne's disease). *Veterinary Immunology and Immunopathology* 68, 139–148.

Bush, R.D., Windsor, P.A. and Toribio, J.A. (2006) Losses of adult sheep due to ovine Johne's disease in 12 infected flocks over a 3-year period. *Australian Veterinary Journal* 84, 246–253.

Carrigan, M.J. and Seaman, J.T. (1990) The pathology of Johne's disease in sheep. *Australian Veterinary Journal* 67, 47–50.

Chiodini, R.J. (1996) Immunology: resistance to paratuberculosis. *The Veterinary Clinics of North America – Food Animal Practice* 12, 313–343.

Clarke, C.J. (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. *Journal of Comparative Pathology* 116, 217–261.

Clarke, C.J. and Little, D. (1996) The pathology of ovine paratuberculosis: gross and histological changes in the intestine and other tissues. *Journal of Comparative Pathology* 114, 419–437.

Clarke, C.J., Colston, A., Little, D., Kay, J., Alzuherri, H.M., Sharp, J.M. and Burrells, C. (1996) The immune response in paratuberculosis infection of small ruminants. *Veterinary Immunology and Immunopathology* 54, 1–4.

de Juan, L., Mateos, A., Dominguez, L., Sharp, J.M. and Stevenson, K. (2005) Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* isolates from goats detected by pulsed-field gel electrophoresis. *Veterinary Microbiology* 106, 249–257.

Dhand, N.K., Eppleston, J., Whittington, R.J. and Toribio, J.A. (2007) Risk factors for ovine Johne's disease in infected sheep flocks in Australia. *Preventive Veterinary Medicine* 82, 51–71.

Ferguson, A. (1990) Mucosal immunology. *Immunology Today* 11, 1–3.

Florou, M., Leontides, L., Kostoulas, P., Billinis, C. and Sofia, M. (2009) Strain-specific sensitivity estimates of *Mycobacterium avium* subsp. *paratuberculosis* culture in Greek sheep and goats. *Zoonoses and Public Health* 56, 49–52.

Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S. and Gudmundsdottir, K.B. (2000) Paratuberculosis in Iceland: epidemiology and control measures, past and present. *Veterinary Microbiology* 77, 263–267.

Gumber, S. and Whittington, R.J. (2009) Analysis of the growth pattern, survival and proteome of *Mycobacterium avium* subsp. *paratuberculosis* following exposure to heat. *Veterinary Microbiology* 136, 82–90.

Gumber, S., Eamens, G. and Whittington, R.J. (2006) Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without *Mycobacterium paratuberculosis* infection. *Veterinary Microbiology* 115, 91–101.

Gumber, S., Taylor, D.L., Marsh, I.B. and Whittington, R.J. (2009a) Growth pattern and partial proteome of *Mycobacterium avium* subsp. *paratuberculosis* during the stress response to hypoxia and nutrient starvation. *Veterinary Microbiology* 133, 344–357.

Gumber, S., Taylor, D.L. and Whittington, R.J. (2009b) Evaluation of the immunogenicity of recombinant stress-associated proteins during *Mycobacterium avium* subsp. *paratuberculosis* infection: implications for pathogenesis and diagnosis. *Veterinary Microbiology* 137, 290–296.

Hein, W.R. and Griebel, P.J. (2003) A road less travelled: large animal models in immunological research. *Nature Reviews Immunology* 3, 79–84.

Jones, D.G. and Kay, J.M. (1996) Serum biochemistry and the diagnosis of Johne's disease (paratuberculosis) in sheep. *Veterinary Record* 139, 498–499.

Juste, R.A., Garrido, J.M., Geijo, M., Elguezabal, N., Aduriz, G., Atxaerandio, R. and Sevilla, I. (2005) Comparison of blood polymerase chain reaction and enzyme-linked immunosorbent assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and sheep. *Journal of Veterinary Diagnostic Investigation* 17, 354–359.

Kawaji, S., Taylor, D.L., Mori, Y. and Whittington, R.J. (2007) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater

sensitivity compared to radiometric culture. *Veterinary Microbiology* 125, 36–48.

Koets, A., Rutten, V., Hoek, A., van Mil, F., Muller, K., Bakker, D., Gruys, E. and van Eden, W. (2002) Progressive bovine paratuberculosis is associated with local loss of CD4(+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function. *Infection and Immunity* 70, 3856–3864.

Kurade, N.P. and Tripathi, B.N. (2008) Lymphoproliferative response and its relationship with histological lesions in experimental ovine paratuberculosis and its diagnostic implications. *Veterinary Research Communications* 32, 107–119.

Kurade, N.P., Tripathi, B.N., Rajukumar, K. and Parihar, N.S. (2004) Sequential development of histologic lesions and their relationship with bacterial isolation, fecal shedding, and immune responses during progressive stages of experimental infection of lambs with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 41, 378–387.

Lambeth, C., Reddacliff, L.A., Windsor, P., Abbott, K.A., McGregor, H. and Whittington, R.J. (2004) Intrauterine and transmammary transmission of *Mycobacterium avium* subsp. *paratuberculosis* in sheep. *Australian Veterinary Journal* 82, 504–508.

Landsverk, T., Halleraker, M., Aleksandersen, M., McClure, S., Hein, W. and Nicander, L. (1991) The intestinal habitat for organized lymphoid tissues in ruminants: comparative aspects of structure, function and development. *Veterinary Immunology and Immunopathology* 28, 1–16.

Little, D., Alzuherri, H.M. and Clarke, C.J. (1996) Phenotypic characterisation of intestinal lymphocytes in ovine paratuberculosis by immunohistochemistry. *Veterinary Immunology and Immunopathology* 55, 175–187.

Mackay, C.R., Beya, M.F. and Matzinger, P. (1989) Gamma/delta T cells express a unique surface molecule appearing late during thymic development. *European Journal of Immunology* 19, 1477–1483.

Mackintosh, C.G., Labes, R.E., Clark, R.G., de Lisle, G.W. and Griffin, J.F. (2007) Experimental infections in young red deer (*Cervus elaphus*) with a bovine and an ovine strain of *Mycobacterium avium* subsp. *paratuberculosis*. *New Zealand Veterinary Journal* 55, 23–29.

Merkal, R.S., Monlux, W.S., Kluge, J.P., Larsen, A.B., Kopecky, K.E., Quinn, L.Y. and Lehmann, R.P. (1968) Experimental paratuberculosis in sheep after oral, intratracheal, or intravenous inoculation: histochemical localization of dehydrogenase activities. *American Journal of Veterinary Research* 29, 971–982.

Moloney, B.J. and Whittington, R.J. (2008) Cross species transmission of ovine Johne's disease from sheep to cattle: an estimate of prevalence in exposed susceptible cattle. *Australian Veterinary Journal* 86, 117–123.

Momotani, E., Whipple, D.L., Thiermann, A.B. and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Veterinary Pathology* 25, 131–137.

Morris, C.A., Hickey, S.M. and Henderson, H.V. (2006) The effect of Johne's disease on production traits in Romney, Merino and Merino × Romney-cross ewes. *New Zealand Veterinary Journal* 54, 204–209.

Munoz, M., Delgado, L., Verna, A., Benavides, J., Garcia-Pariente, C., Fuertes, M., Ferreras, M.C., Garcia-Marin, J.F. and Perez, V. (2009) Expression of transforming growth factor-beta 1 (TGF-beta1) in different types of granulomatous lesions in bovine and ovine paratuberculosis. *Comparative Immunology, Microbiology and Infectious Diseases* 32, 239–252.

Mutwiri, G., Watts, T., Lew, L., Beskorwayne, T., Papp, Z., Baca-Estrada, M.E. and Griebel, P. (1999) Ileal and jejunal Peyer's patches play distinct roles in mucosal immunity of sheep. *Immunology* 97, 455–461.

Nalubamba, K., Smeed, J., Gossner, A., Watkins, C., Dalziel, R. and Hopkins, J. (2008) Differential expression of pattern recognition receptors in the three pathological forms of sheep paratuberculosis. *Microbes and Infection* 10, 598–604.

Navarro, J.A., Ramis, G., Seva, J., Pallares, F.J. and Sanchez, J. (1998) Changes in lymphocyte subsets in the intestine and mesenteric lymph nodes in caprine paratuberculosis. *Journal of Comparative Pathology* 118, 109–121.

Nielsen, S.S. and Toft, N. (2009) A review of prevalences of paratuberculosis in farmed animals in Europe. *Preventative Veterinary Medicine* 88, 1–14.

O'Brien, R., Mackintosh, C.G., Bakker, D., Kopecna, M., Pavlik, I. and Griffin, J.F. (2006) Immunological and molecular characterization of susceptibility in relationship to bacterial strain differences in *Mycobacterium avium* subsp. *paratuberculosis* infection in the red deer (*Cervus elaphus*). *Infection and Immunity* 74, 3530–3537.

Perez, V., Garcia Marin, J.F. and Badiola, J.J. (1996) Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. *Journal of Comparative Pathology* 114, 107–122.

Rast, L. and Whittington, R.J. (2005) Longitudinal study of the spread of ovine Johne's disease in a sheep flock in southeastern New South Wales. *Australian Veterinary Journal* 83, 227–232.

Reddacliff, L.A. and Whittington, R.J. (2003) Experimental infection of weaner sheep with S strain *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* 96, 247–258.

Reddacliff, L.A., McClure, S.J. and Whittington, R.J. (2004) Immunoperoxidase studies of cell mediated immune effector cell populations in early *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep. *Veterinary Immunology and Immunopathology* 97, 149–162.

Reddacliff, L., Eppleston, J., Windsor, P., Whittington, R. and Jones, S. (2006) Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Veterinary Microbiology* 115, 77–90.

Reynolds, J.D. and Morris, B. (1983) The evolution and involution of Peyer's patches in fetal and postnatal sheep. *European Journal of Immunology* 13, 627–635.

Robbe-Austerman, S., Gardner, I.A., Thomsen, B.V., Morrical, D.G., Martin, B.M., Palmer, M.V., Thoen, C O. and Ewing, C. (2006a) Sensitivity and specificity of the agar-gel-immunodiffusion test, ELISA and the skin test for detection of paratuberculosis in United States Midwest sheep populations. *Veterinary Research* 37, 553–564.

Robbe-Austerman, S., Stabel, J.R. and Palmer, M.V. (2006b) Evaluation of the gamma interferon ELISA in sheep subclinically infected with *Mycobacterium avium* subspecies *paratuberculosis* using a whole-cell sonicate or a johnin purified-protein derivative. *Journal of Veterinary Diagnostic Investigation* 18, 189–194.

Seaman, J.T., Gardner, I.A. and Dent, C H. (1981) Johne's disease in sheep. *Australian Veterinary Journal* 57, 102–103.

Sergeant, E.S. (2001) Ovine Johne's disease in Australia – the first 20 years. *Australian Veterinary Journal* 79, 484–491.

Sergeant, E.S. and Baldock, F.C. (2002) The estimated prevalence of Johne's disease infected sheep flocks in Australia. *Australian Veterinary Journal* 80, 762–768.

Sevilla, I., Aduriz, G., Garrido, J., Geijo, M.V. and Juste, R.A. (2002) A preliminary survey on the prevalence of paratuberculosis in dairy cattle in Spain by bulk milk PCR. In: Juste, A.R., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 84, 85.

Sevilla, I., Singh, S.V., Garrido, J.M., Aduriz, G., Rodriguez, S., Geijo, M.V., Whittington, R.J., Saunders, V., Whitlock, R.H. and Juste, R.A. (2005) Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions. *Revue Scientifique et Technique* 24, 1061–1066.

Sevilla, I., Garrido, J.M., Geijo, M. and Juste, R.A. (2007) Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiology* 7, 18.

Sevilla, I., Li, L., Amonsin, A., Garrido, J.M., Geijo, M.V., Kapur, V. and Juste, R.A. (2008) Comparative analysis of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle, sheep and goats by short sequence repeat and pulsed-field gel electrophoresis typing. *BMC Microbiology* 8, 204.

Sigurðardóttir, O.G., Press, C.M. and Evensen, O. (2001) Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. *Veterinary Pathology* 38, 184–189.

Smeed, J.A., Watkins, C.A., Rhind, S.M. and Hopkins, J. (2007) Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis. *BMC Veterinary Research* 3, 18.

Stabel, J.R. (2000) Transitions in immune responses to *Mycobacterium paratuberculosis*. *Veterinary Microbiology* 77, 465–473.

Stabel, J.R. (2006) Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. *Animal Health Research Reviews* 7, 61–70.

Stewart, D.J., Stiles, P.L., Whittington, R.J., Lambeth, C., Windsor, P.A., Reddacliff, L., McGregor, H., Dhungyel, O.P., Cousins, D., Francis, B.R., Morcombe, P.W., Butler, R., Salmon, D.D., Roberts, C.F., Sergeant, E., Jasenko, I. and Jones, S.L. (2002) Validation of the interferon-γ for diagnosis of ovine Johne's disease: sensitivity and specificity field trials. In: Juste, A.R., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, p. 48.

Sunderman, F. (2004) Update on the OJD program in Western Australia. *Ovine Johne's Disease – an Update of Australian Research*. Meat & Livestock Australia, Sydney, pp. 102–105.

Taylor, D.L., Zhong, L., Begg, D.J., de Silva, K. and Whittington, R.J. (2008) Toll-like receptor genes are differentially expressed at the sites

of infection during the progression of Johne's disease in outbred sheep. *Veterinary Immunology and Immunopathology* 124, 132–151.

Verna, A.E., Garcia-Pariente, C., Munoz, M., Moreno, O., Garcia-Marin, J.F., Romano, M.I., Paolicchi, F. and Perez, V. (2007) Variation in the immuno-pathological responses of lambs after experimental infection with different strains of *Mycobacterium avium* subsp. *paratuberculosis*. *Zoonoses and Public Health* 54, 243–252.

Whittington, R.J., Marsh, I., Turner, M.J., McAllister, S., Choy, E., Eamens, G.J., Marshall, D.J. and Ottaway, S. (1998) Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR. *Journal of Clinical Microbiology* 36, 701–707.

Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J. and Fraser, C.A. (1999) Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *Journal of Clinical Microbiology* 37, 1077–1083.

Whittington, R.J., Fell, S., Walker, D., McAllister, S., Marsh, I., Sergeant, E., Taragel, C.A., Marshall, D.J. and Links, I.J. (2000a) Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. *Journal of Clinical Microbiology* 38, 2550–2556.

Whittington, R.J., Reddacliff, L.A., Marsh, I., McAllister, S. and Saunders, V. (2000b) Temporal patterns and quantification of excretion of *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Australian Veterinary Journal* 78, 34–37.

Whittington, R.J., Taragel, C.A., Ottaway, S., Marsh, I., Seaman, J. and Fridriksdottir, V. (2001) Molecular epidemiological confirmation and circumstances of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. *Veterinary Microbiology* 79, 311–322.

Williams, E.S., DeMartini, J.C. and Snyder, S.P. (1985) Lymphocyte blastogenesis, complement fixation, and fecal culture as diagnostic tests for paratuberculosis in North American wild ruminants and domestic sheep. *American Journal of Veterinary Research* 46, 2317–2321.

16 Paratuberculosis in Goats

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16.1 Introduction

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) has been detected in goats in most goat-rearing countries, including Austria, Norway, Portugal, Spain, Greece, Great Britain, South Africa, New Zealand and Australia (Stehman, 1996; Nielsen and Toft, 2009). The disease in goats resembles that in sheep in many respects (see Begg and Whittington, Chapter 15, this volume). The prevalence of infection in goats is difficult to estimate in any region or country because of the uncertainty of the diagnosis and the fact that cases are only reported during specific surveys or eradication programmes. It appears that the prevalence has been increasing during the last 10 years, but there are large variations in the estimates from a relatively

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small number of studies that have used different study designs and sampling frames (Nielsen and Toft, 2009).

While the precise burden of *MAP* infection in goats is largely unknown, paratuberculosis in goats is known to cause considerable economic losses due to reduced feed efficiency, reduced milk production and increased culling of infected animals. Moreover, subclinical *MAP* infection has been shown to result in reduced fertility of goats (Kostoulas *et al.*, 2006). Together, these considerations indicate that paratuberculosis in goats leads to decreased revenues and increased costs, including the costs of diagnostic testing and implementing control measures (de Juan *et al.*, 2005).

16.2 Host and Pathogen Factors in Paratuberculosis

16.2.1 Animal risk factors

In cattle, age of the animal at exposure, breed resistance, genetic susceptibility and other diseases have been mentioned as risk factors for development of paratuberculosis (Radostits *et al.*, 2007). Younger animals require a lower infective dose than do older animals, and adult animals are unlikely to get infected unless there is extreme environmental contamination (McKenna *et al.*, 2006). This is generally accepted for other animals as well, but few experimental infections have been conducted in goats (Hines *et al.*, 2007a; Begg and Whittington, 2008). However, based on experimental infections, goats have been found to be less naturally resistant than cattle and sheep for paratuberculosis (Stewart *et al.*, 2007).

16.2.2 Environmental and management risk factors

Conditions predisposing to *MAP* infection include poor hygiene that exposes the young kids to manure from older animals, contaminated milk, inadequate feeding and low body condition (Radostits *et al.*, 2007). Infected goat kids can excrete the bacteria in their faeces for 2–9 months after infection and thereby infect other kids (Stewart *et al.*, 2006). In goats, *MAP* seroprevalence has been associated with a herd size of over 200 animals, the presence of foreign breeds and a high replacement rate (Mainar-Jaime and Vázquez-Boland, 1998). Cross-species infections have been documented between cattle and sheep (Muskens *et al.*, 2001), and are also suspected to occur between cattle and goats (Holstad *et al.*, 2005). Contact with wildlife might pose a risk of *MAP* transmission to domestic animals (Daniels *et al.*, 2003). Surveys indicate that large number of rabbits and access of wildlife to feed supplies are factors which increase the likelihood of a farm having paratuberculosis (Daniels *et al.*, 2002).

16.2.3 Pathogen risk factors

MAP isolates have been classified into two main groups based on culture characteristics and molecular characterization: sheep (also called S or Type I/Type III) and cattle (also called C or Type II) (see Stevenson, Chapter 12, this volume). Goats can be infected by both groups of strains (de Juan *et al.*, 2005), and the isolates of *MAP* obtained from goats manifest genetic variability by a number of different molecular methods (Pillai *et al.*, 2001; Djønne *et al.*, 2005; Sevilla *et al.*, 2007, 2008). Phenotypic differences between these strains of *MAP* have been described, with one group suggesting that isolates from goats have lower pathogenicity for cattle (Saxegaard, 1990). The genetic basis for this observation is not known. In contrast, a study by Gollnick *et al.* (2007) did not find any differences in the ability of different *MAP* strains to survive in bovine monocyte-derived macrophages. At present, there does not appear to be a strain of *MAP* specific to goats, and the importance of strain variability for infection and disease in goats requires further study.

16.3 Characteristics of Infection and Disease

16.3.1 Clinical signs and clinical pathology

Infected animals can be categorized into four groups according to clinical symptoms, faecal

shedding of bacteria and immunological response: (i) silent infection; (ii) subclinical disease; (iii) clinical disease; and (iv) advanced clinical disease (Whitlock and Buergelt, 1996). A similar progression of disease is described for cattle (see Fecteau and Whitlock, Chapter 14, this volume).

In the stage of silent infection, there are no clinical signs, no effect on body weight gain or body condition. There is usually neither bacterial shedding nor detectable circulating antibodies, but cellular immune responses may be detectable by the interferon gamma (IFN-γ) response test.

During the stage of subclinical disease, there are still no clinical signs of paratuberculosis. However, the animals may shed low numbers of bacteria in faeces and there may be evidence of both cellular and humoral immune responses. Goats can become persistent faecal shedders about 1 year post-infection without any clinical signs of paratuberculosis (Storset *et al.*, 2001).

During the third stage of infection, referred to as clinical disease, the only consistent finding is weight loss despite apparently normal food intake (Stehman, 1996). Unlike what is observed in cattle, diarrhoea is rarely seen in goats (Manning and Collins, 2001). In this state, bacteria are typically found in faeces and animals usually have antibodies against *MAP*. Most animals, if not culled, go into stage four.

In advanced clinical disease, animals develop a flaky skin and a poor hair coat, and eventually progressive emaciation, dehydration, anaemia with submandibular oedema and depression are seen (Fig. 16.1). At this stage of the infection, diarrhoea, or more usually a clumping of faeces, can be seen (Stehman, 1996).

16.3.2 Pathology – gross and microscopic lesions

Macroscopic lesions are primarily seen in the intestine and in the draining mesenteric lymph nodes. Intestinal lesions can be segmental or diffuse and are most commonly found in the ileum but can occur throughout the whole length of the intestinal tract. Thickening and folding of the mucosa with transverse folds (Fig. 16.2), and dilated and thickened serosal

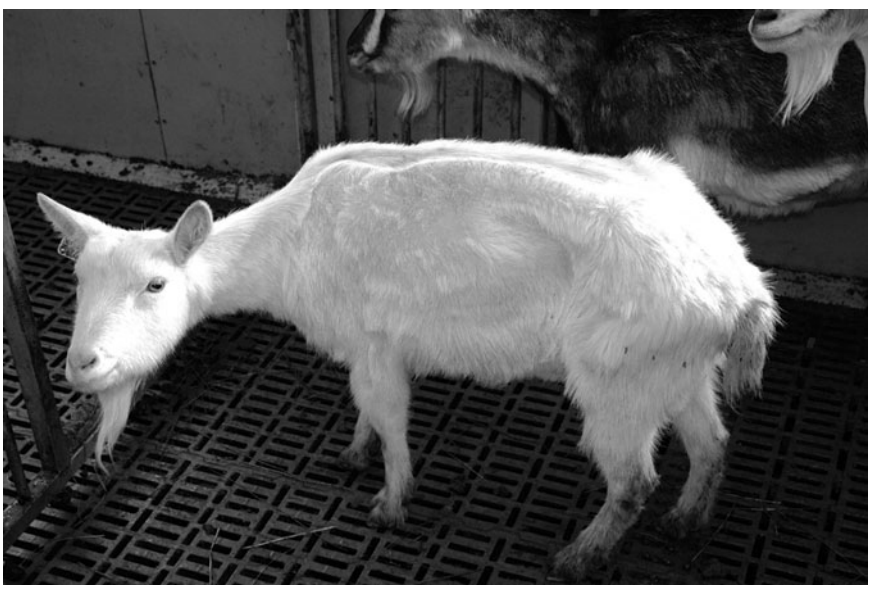

Fig. 16.1. Clinical paratuberculosis in a goat (reproduced with the kind permission of N. Leine).

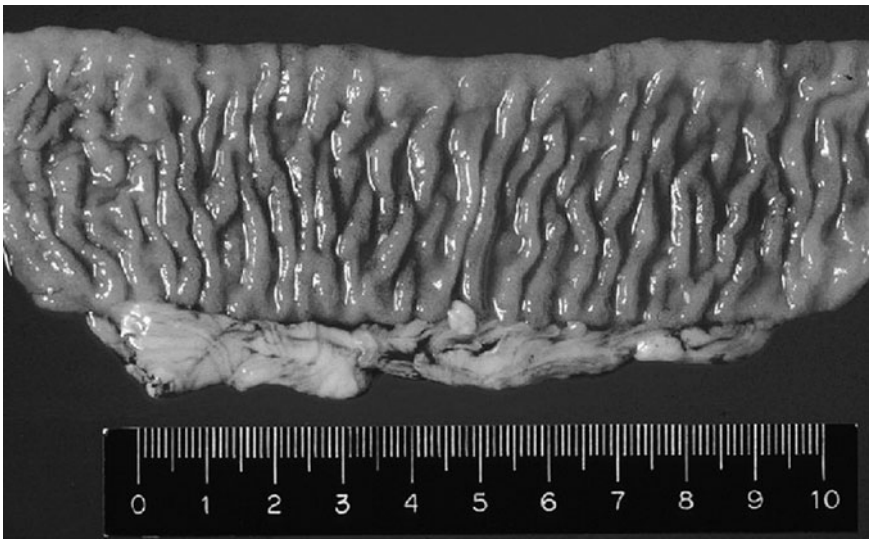

Fig. 16.2. Affected jejunum in a *MAP*-infected goat. Pathological changes observed in this specimen are thickening of the mucosa with transverse folds (reproduced with the kind permission of Ó.G. Sigurðardóttir).

and mesenteric lymphatic vessels are common. The mesenteric lymph nodes are pale, swollen and oedematous. In goats, nodular foci of caseation and mineralization may be present both in the mucosa and in the lymph nodes (Olsen *et al.*, 2002).

The histopathological lesions caused by *MAP* in goats can resemble the spectrum seen in *Mycobacterium leprae* infections. In the tuberculoid form, there are focal aggregates of macrophages surrounded by large numbers of lymphocytes, with few or no acid-fast bacilli. This form is associated with strong cell-mediated immune responses. The lepromatous form is associated with strong humoral immune responses, and the lesions consist of diffuse infiltration of macrophages containing large numbers of acid-fast bacilli. Between these extremes of the spectrum are the so-called intermediary forms and 'borderline forms' (Corpa *et al.*, 2000a). Subclinically infected animals will usually have focal tuberculoid lesions, while individuals with clinical symptoms may present with either the borderline tuberculoid form with only a few bacilli or the borderline lepromatous form with multiple bacilli (Sigurðardóttir *et al.*, 1999).

16.4 Route of Infection and Transmission between Individuals

Animals are most commonly infected through the faecal–oral route: by ingestion of contaminated milk or food products or by accidental ingestion of the organism from contaminated surfaces (Harris and Barletta, 2001). *MAP* can be excreted in colostrum and milk (Streeter *et al.*, 1995), and *MAP* has been detected by PCR in raw goat milk (Djønne *et al.*, 2003). *In utero* infections occur in cattle (Whittington and Windsor, 2009) and have been reported in goats as well (Stehman, 1996). Spreading of *MAP* between flocks is usually due to trading of animals with unknown infection, but spread due to contaminated faeces on pastures also occurs (Radostits *et al.*, 2007).

16.5 Pathogenesis

The ingested bacteria enter the intestinal wall through the small intestinal mucosa. The point of entry in experimentally infected goats has been shown to be through M cells found

in the follicle-associated epithelium lining the domes of the Peyer's patches and also through enterocytes in areas without Peyer's patches (Sigurðardóttir *et al.*, 2001, 2005). The bacteria are subsequently phagocytosed by subepithelial macrophages. Within macrophages, *MAP* resist intracellular degradation and slowly replicate, stimulating inflammatory and immunological responses (Olsen *et al.*, 2002).

Most animals infected with *MAP* are able to control the infection. However, subclinically infected animals will probably remain infected for the rest of their life, and some may intermittently shed the bacteria in faeces, contributing to spread of the infection. Whether infected animals can clear the infection or not remains unclear. In several studies in goats, no lesions were detected after experimental infection, but the presence of latent infections in these animals could not be excluded (Sigurðardóttir *et al.*, 1999; Storset *et al.*, 2001).

16.6 Immune Response

Invasion and inactivation of macrophages activates the host immune system to begin a series of attacks against the *MAP*-infected macrophages, including the rapid development of activated T cells, $CD4^+$ T cells and cytolytic $CD8^+$ cells (Radostits *et al.*, 2007). This cellular immune response is associated with controlled infection in most animals. Animals that are unable to control the disease will develop a humoral immune response along with increased shedding of bacteria in faeces, eventually followed by the onset of clinical symptoms (Olsen *et al.*, 2002).

Several infectious foci in the intestine will develop, and there will be an ongoing battle between the host and the bacteria in these various foci. The local immune responses in the foci may differ, and both humoral and cellular immune responses can sometimes be present at the same time. It has previously been reported that infected animals are able to completely recover from paratuberculosis infection, but experimental infections in goats indicate that reactivation of bacteria in small foci is possible (Storset *et al.*, 2001).

16.7 Diagnosis

The clinical diagnosis of paratuberculosis is challenging in goats; symptoms are vague and non-specific, as numerous other diseases present with weight loss. The disease can be diagnosed by pathology, microbiology (culture and PCR) and immunological methods. None of these methods are very sensitive, especially during the early stages of infection. The sensitivity and specificity of diagnostic tests for *MAP* infections vary significantly, but formal comparison of the different tests that have been reported is difficult. The main reasons are variations in study design, test components and target conditions (Nielsen and Toft, 2008).

16.7.1 Culture-based diagnosis

Cultivation of *MAP* is a highly specific method, but it is costly and generally requires from 8 to 16 weeks of incubation to produce visible colonies on solid media. The sensitivity of culture is difficult to determine, as it is considered to be the 'gold standard' method.

Intermittent faecal shedding is common, and the number of bacteria in faeces may be below detection level. Therefore, in a naturally infected population, probably only about half of the faecal shedders will be detected by culture. Bacteriological culture is effectively 100% specific, as slow-growing, mycobactin-dependent, acid-fast bacteria that harbour the IS*900* element are identified as *MAP* (Olsen *et al.*, 2002).

Different selective and non-selective media containing mycobactin can be used to culture *MAP* from goats, including Löwenstein–Jensen, Herrold's egg yolk medium, Middlebrook 7H11 or Dubos medium (Saxegaard, 1985; de Juan *et al.*, 2006). As goats can be infected by different types of *MAP* strains, different media and an incubation period of 6 months should be used to detect *MAP* in new areas or flocks.

Pooled faecal culture can be used for herd diagnosis if the animals are moderate or high shedders of *MAP*, but this would be unsuitable in herds with only a few low shedders (Eamens *et al.*, 2007).

16.7.2 PCR-based methods

Different methods based on IS*900* PCR have been used to detect *MAP* from different samples of naturally and experimentally infected goats, including faeces (Ikonomopoulos *et al.*, 2007), milk (Djønne *et al.*, 2003) and intestinal tissue (Whittington *et al.*, 1999). The sensitivity and specificity of PCR vary between different methods (see Bölske and Herthnek, Chapter 23, this volume).

16.7.3 Immunological methods

MAP antigens can be detected in paraffin-embedded tissue sections from goats by immunohistochemistry, and this method seems to be more sensitive than staining by the Ziehl–Neelsen technique (Thoresen *et al.*, 1994).

Cell-mediated immune response detected by the *MAP*-specific IFN-γ test can be useful to monitor the paratuberculosis status of non-vaccinated goat herds. The method seems to give fewer false-positive reactions in young goats compared with young cattle, but the method needs to be further evaluated in goats (Storset *et al.*, 2005). The detection of a delayed-type hypersensitivity reaction to johnin has been successfully applied in goats that are naturally infected by *MAP* (Tripathi *et al.*, 2006), but the test results are considered to be unreliable (Kalis *et al.*, 2003).

In contrast, antibody-detection-based tests for *MAP* infection offer speed and economy. Enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion and complement fixation can be used in paratuberculosis control programmes for the goat industries without a major risk of generating large proportions of false-positive test results. Of these methods, the ELISA is most sensitive for detection of infected goats (Whittington *et al.*, 2003; Gumber *et al.*, 2006). All three methods show good specificity, although serological cross-reactions between *MAP* infections and other goat pathogens have been reported. False-positive reactions can occur due to cross-reacting antibodies produced against *Corynebacterium pseudotuberculosis* (Manning *et al.*, 2007) and *Mycobacterium bovis* (Alvarez *et al.*, 2008).

A commercial bovine paratuberculosis ELISA has also been used to test goat milk. Although this appears less sensitive than testing serum, the ELISA on goat milk samples appears to offer a useful, low-cost alternative for detection of goats with paratuberculosis that have progressed to the stage of shedding *MAP* in their faeces (Salgado *et al.*, 2007).

16.8 Treatment

In one study, goats treated with a combination of streptomycin, isoniazid and rifampin had a favourable clinical outcome, but extensive granulomatous inflammatory foci were detected in various organs at necropsy and *MAP* was isolated from lymph nodes (Slocombe, 1982). Consequently, antibiotics are not used to treat paratuberculosis in goats.

16.9 Control

Control of paratuberculosis is challenging and should not be underestimated. Control programmes are rarely successful in eliminating the infection from a herd or flock in the short term without an aggressive and costly programme. Successful prevention and control depend on animal health authorities and livestock industries acquiring a good understanding of the nature and epidemiology of infection, and of the application of tools for diagnosis and control (Kennedy and Benedictus, 2001).

16.9.1 Environment and management

Management is considered to be the most useful tool for controlling paratuberculosis within domestic livestock herds (Harris and Barletta, 2001). Care of newborn kids, separation from older animals, feeding of paratuberculosis-free colostrum and milk, a clean environment and adequate feeding of growing kids are among the most important management tools to control paratuberculosis within a flock. Faecal shedders should be removed,

and goats with lower paratuberculosis status should not be allowed into the flock.

16.9.2 Vaccination

Different paratuberculosis vaccines have been used in goats: live vaccines (Saxegaard and Fodstad, 1985), heat-inactivated vaccines (Corpa *et al.*, 2000b), spheroblastic vaccines (Hines *et al.*, 2007b) or recombinant antigen vaccines (Kathaperumal *et al.*, 2009).

Existing vaccines can delay the onset of clinical symptoms, improve body weight, and reduce mortality and faecal shedding of bacteria, but they do not protect against infection (Rosseels and Huygen, 2008).

Vaccination is generally recommended in young animals, but vaccination of adult goats in infected flocks can reduce the number of animals developing clinical disease (Corpa *et al.*, 2000c). Vaccinated animals develop antibodies that can interfere with existing serodiagnostic tests for paratuberculosis and they can become reactive in the tuberculin skin test, used for the control of bovine tuberculosis (Hines *et al.*, 2007b; Rosseels and Huygen, 2008).

16.10 Concluding Thoughts

Paratuberculosis in goats is documented in many settings, although its prevalence and economic impact are not known. While there have been some advances lately, including a refined understanding of the strains that can cause disease, there remain important issues for control of caprine paratuberculosis, including the absence of sensitive and validated diagnostic assays, especially in the early subclinical stage of infection. Future research priorities are the susceptibility for *MAP* infection and pathogenesis in goats, and further validation of different immunological tests, especially tests based on cell-mediated immune response, on goats.

References

Alvarez, J., de Juan, L., Bezos, J., Romero, B., Sáez, J.L., Gordejo, F.J.R., Briones, V., Moreno, M.A., Mateos, A., Domínguez, L. and Aranaz, A. (2008) Interference of paratuberculosis with the diagnosis of tuberculosis in a goat flock with a natural mixed infection. *Veterinary Microbiology* 128, 72–80.

Begg, D.J. and Whittington, R.J. (2008) Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. *The Veterinary Journal* 176, 129–145.

Corpa, J.M., Garrido, J., García Marín, J.F. and Pérez, V. (2000a) Classification of lesions observed in natural cases of paratuberculosis in goats. *Journal of Comparative Pathology* 122, 255–265.

Corpa, J.M., Pérez, V. and García Marín, J.F. (2000b) Differences in the immune responses in lambs and kids vaccinated against paratuberculosis, according to the age of vaccination. *Veterinary Microbiology* 77, 475–485.

Corpa, J.M., Pérez, V., Sánchez, M.A. and Marín, J.F. (2000c) Control of paratuberculosis (Johne's disease) in goats by vaccination of adult animals. *Veterinary Record* 146, 195–196.

Daniels, M.J., Hutchings, M.R., Allcroft, D.J., McKendrick, J. and Greig, A. (2002) Risk factors for Johne's disease in Scotland – the results of a survey of farmers. *Veterinary Record* 150, 135–139.

Daniels, M.J., Hutchings, M.R., Beard, P.M., Henderson, D., Greig, A., Stevenson, K. and Sharp, J.M. (2003) Do non-ruminant wildlife pose a risk of paratuberculosis to domestic livestock and vice versa in Scotland? *Journal of Wildlife Diseases* 39, 10–15.

de Juan, L., Mateos, A., Domínguez, L., Sharp, J.M. and Stevenson, K. (2005) Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* isolates from goats detected by pulsed-field gel electrophoresis. *Veterinary Microbiology* 106, 249–257.

de Juan, L., Alvarez, J., Romero, B., Bezos, J., Castellanos, E., Aranaz, A., Mateos, A. and Domínguez, L. (2006) Comparison of four different culture media for isolation and growth of type II and type I/III *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from cattle and goats. *Applied and Environmental Microbiology* 72, 5927–5932.

Djønne. B., Jensen, M.R., Grant, I.R. and Holstad, G. (2003) Detection by immunomagnetic PCR of *Mycobacterium avium* subsp. *paratuberculosis* in milk from dairy goats in Norway. *Veterinary Microbiology* 92, 135–143.

Djønne, B., Pavlik, I., Svastova, P., Bartos, M. and Holstad, G. (2005) IS900 restriction fragment length polymorphism (RFLP) analysis of

Mycobacterium avium subsp. *paratuberculosis* isolates from goats and cattle in Norway. *Acta Veterinaria Scandinavica* 46, 13–18.

Eamens, G.J., Walker, D.M., Porter, N.S. and Fell, S.A. (2007) Pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in goats. *Australian Veterinary Journal* 85, 243–251.

Gollnick, N.S., Mitchell, R.M., Baumgart, M., Janagama, H.K., Sreevatsan, S. and Schukken, Y.H. (2007) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in bovine monocyte-derived macrophages is not affected by host infection status but depends on the infecting bacterial genotype. *Veterinary Immunology and Immunopathology* 120, 93–105.

Gumber, S., Eamens, G. and Whittington, R.J. (2006) Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without *Mycobacterium paratuberculosis* infection. *Veterinary Microbiology* 115, 91–101.

Harris, N.B. and Barletta, R.G. (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clinical Microbiology Review*s 14, 489–512.

Hines, M.E., 2nd, Stabel, J.R., Sweeney, R.W., Griffin, F., Talaat, A.M., Bakker, D., Benedictus, G., Davis, W.C., de Lisle, G.W., Gardner, I.A., Juste, R.A., Kapur, V., Koets, A., McNair, J., Pruitt, G. and Whitlock, R.H. (2007a) Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Veterinary Microbiology* 122, 197–222.

Hines, M.E. 2nd, Stiver, S., Giri, D., Whittington, L., Watson, C., Johnson, J., Musgrove, J., Pence, M., Hurley, D., Baldwin, C., Gardner, I.A. and Aly, S. (2007b) Efficacy of spheroplastic and cell-wall competent vaccines for *Mycobacterium avium* subsp. *paratuberculosis* in experimentally-challenged baby goats. *Veterinary Microbiology* 120, 261–283.

Holstad, G., Sigurðardóttir, Ó.G., Storset, A.K., Tharaldsen, J., Nyberg, O., Schönheit, J. and Djønne, B. (2005) Description of the infection status in a Norwegian cattle herd naturally infected by *Mycobacterium avium* subsp. *paratuberculosis*. *Acta Veterinaria Scandinavica* 46, 45–56.

Ikonomopoulos, J., Balaskas, C., Kantzoura, B., Fragiadaki, E., Pavlik, I., Bartos, M., Lukas, J.C. and Gazouli, M. (2007) Comparative evaluation of positive tests to *Mycobacterium avium* subsp. *paratuberculosis* in clinically healthy sheep and goats in south-west Greece using molecular techniques, serology, and culture. *The Veterinary Journal* 174, 337–343.

Kalis, C.H., Collins, M.T., Hesselink, J.W. and Barkema, H.W. (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. *Veterinary Microbiology* 97, 73–86.

Kathaperumal, K., Kumanan, V., McDonough, S., Chen, L.H., Park, S.U., Moreira, M.A., Akey, B., Huntley, J., Chang, C.F. and Chang, Y.F. (2009) Evaluation of immune responses and protective efficacy in a goat model following immunization with a cocktail of recombinant antigens and a polyprotein of *Mycobacterium avium* subsp. *paratuberculosis*. *Vaccine* 27, 123–125.

Kennedy, D.J. and Benedictus, G. (2001) Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species. *Revue Scientifique et Technique* 20, 151–179.

Kostoulas, P., Leontides, L., Billinis, C., Amiridis, G.S. and Florou, M. (2006) The association of subclinical paratuberculosis with the fertility of Greek dairy ewes and goats varies with parity. *Preventive Veterinary Medicine* 74, 226–238

Mainar-Jaime, R.C. and Vázquez-Boland, J.A. (1998) Factors associated with seroprevalence to *Mycobacterium paratuberculosis* in small-ruminant farms in the Madrid region (Spain). *Preventive Veterinary Medicine* 34, 317–327.

Manning, E.J. and Collins, M.T. (2001) *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. *Revue Scientifique et Technique* 20, 133–150.

Manning, E.J., Cushing, H.F., Hietala, S. and Wolf, C.B. (2007) Impact of *Corynebacterium pseudotuberculosis* infection on serologic surveillance for Johne's disease in goats. *Journal of Veterinary Diagnostic Investigation* 19, 187–190.

McKenna, S.L., Keefe, G.P., Tiwari, A., VanLeeuwen, J. and Barkema, H.W. (2006) Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. *Canadian Veterinary Journal* 47, 1089–1099.

Muskens, J., Bakker, D., de Boer, J. and van Keulen, L. (2001) Paratuberculosis in sheep: its possible role in the epidemiology of paratuberculosis in cattle. *Veterinary Microbiology* 78, 101–109.

Nielsen, S.S. and Toft, N. (2008) Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. *Veterinary Microbiology* 129, 217–235.

Nielsen, S.S. and Toft, N. (2009) A review of prevalences of paratuberculosis in farmed animals in Europe. *Preventive Veterinary Medicine* 88, 1–14.

Olsen, I., Sigurðardóttir, Ó.G. and Djønne, B. (2002) Paratuberculosis with special reference to cattle. A review. *Veterinary Quarterly* 24, 12–28.

Pillai, S.R., Jayarao, B.M., Gummo, J.D., Hue, E.C., Tiwari, D., Stabel, J.R. and Whitlock, R.H. (2001) Identification and sub-typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* by randomly amplified polymorphic DNA. *Veterinary Microbiology* 79, 275–284.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W. and Constable, P.D. (2007) Diseases associated with bacteria. In: Rodenhuis, J. and Ball, E. (eds) *Veterinary Medicine: a Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats*, 10th edn. Saunders Elsevier, Philadelphia, Pennsylvania, pp. 1017–1044.

Rosseels, V. and Huygen, K. (2008) Vaccination against paratuberculosis. *Expert Review of Vaccines* 7, 817–832.

Salgado, M., Kruze, J. and Collins, M.T. (2007) Diagnosis of paratuberculosis by fecal culture and ELISA on milk and serum samples in two types of Chilean dairy goat herds. *Journal of Veterinary Diagnostic Investigation* 19, 99–102.

Saxegaard, F. (1985) Isolation of *Mycobacterium paratuberculosis* from intestinal mucosa and mesenteric lymph nodes of goats by use of selective Dubos medium. *Journal of Clinical Microbiology* 22, 312–313.

Saxegaard, F. (1990) Experimental infection of calves with an apparently specific goat-pathogenic strain of *Mycobacterium paratuberculosis*. *Journal of Comparative Pathology* 102, 149–156.

Saxegaard, F. and Fodstad, F.H. (1985) Control of paratuberculosis (Johne's disease) in goats by vaccination. *Veterinary Record* 116, 439–441.

Sevilla, I., Garrido, J.M., Geijo, M. and Juste, R.A. (2007) Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiology* 7, 18.

Sevilla, I., Li, L., Amonsin, A., Garrido, J.M., Geijo, M.V., Kapur, V. and Juste, R.A. (2008) Comparative analysis of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle, sheep and goats by short sequence repeat and pulsed-field gel electrophoresis typing. *BMC Microbiology* 8, 204.

Sigurðardóttir, Ó.G., Press, C.M., Saxegaard, F. and Evensen, Ø. (1999) Bacterial isolation, immunological response, and histopathological lesions during the early subclinical phase of experimental infection of goat kids with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 36, 542–550.

Sigurðardóttir, Ó.G., Press, C.M. and Evensen, Ø. (2001) Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. *Veterinary Pathology* 38, 184–189.

Sigurðardóttir, Ó.G., Bakke-McKellep, A.M., Djønne, B. and Evensen, Ø. (2005) *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. *Comparative Immunology, Microbiology and Infectious Diseases* 28, 223–230.

Slocombe, R.F. (1982) Combined streptomycin–isoniazid–rifampin therapy in the treatment of Johne's disease in a goat. *Canadian Veterinary Journal* 23, 160–163.

Stehman, S.M. (1996) Paratuberculosis in small ruminants, deer, and South American camelids. *Veterinary Clinics of North America – Food Animal Practice* 12, 441–455.

Stewart, D.J., Vaughan, J.A., Stiles, P.L., Noske, P.J., Tizard, M.L., Prowse, S.J., Michalski, W.P., Butler, K.L. and Jones, S.L. (2006) A long-term study in Angora goats experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*: clinical disease, faecal culture and immunological studies. *Veterinary Microbiology* 113, 13–24.

Stewart, D.J., Vaughan, J.A., Stiles, P.L., Noske, P.J., Tizard, M.L., Prowse, S.J., Michalski, W.P., Butler, K.L. and Jones, S.L. (2007) A long-term bacteriological and immunological study in Holstein–Friesian cattle experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis* and necropsy culture results for Holstein–Friesian cattle, merino sheep and angora goats. *Veterinary Microbiology* 122, 83–96.

Storset, A.K., Hasvold, H.J., Valheim, M., Brun-Hansen, H., Berntsen, G., Whist, S.K., Djønne, B., Press, C.McL., Holstad, G. and Larsen, H.J. (2001) Subclinical paratuberculosis in goats following experimental infection. An immunological and microbiological study. *Veterinary Immunology and Immunopathology* 80, 271–287.

Storset, A.K., Berg, I. and Djønne, B. (2005) Evaluation of the gamma interferon test for diagnosis of paratuberculosis in goats. *Veterinary Immunology and Immunopathology* 107, 87–94.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P. and Rings, M. (1995) Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *American Journal of Veterinary Research* 56, 1322–1324.

Thoresen, O.F., Falk, K. and Evensen, Ø. (1994) Comparison of immunohistochemistry, acid-fast

staining, and cultivation for detection of *Mycobacterium paratuberculosis* in goats. *Journal of Veterinary Diagnostic Investigation* 6, 195–199.

Tripathi, B.N., Periasamy, S., Paliwal, O.P. and Singh, N. (2006) Comparison of IS900 tissue PCR, bacterial culture, johnin and serological tests for diagnosis of naturally occurring paratuberculosis in goats. *Veterinary Microbiology* 116, 129–137.

Whitlock, R.H and Buergelt, C. (1996) Preclinical and clinical manifestations of paratuberculosis (including pathology). *Veterinary Clinics of North America – Food Animal Practice* 12, 345–356.

Whittington, R.J., Reddacliff, L., Marsh, I. and Saunders, V. (1999) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in formalin-fixed paraffin-embedded intestinal tissue by IS900 polymerase chain reaction. *Australian Veterinary Journal* 77, 392–397.

Whittington, R.J., Eamens, G.J. and Cousins, D.V. (2003) Specificity of absorbed ELISA and agar gel immuno-diffusion tests for paratuberculosis in goats with observations about use of these tests in infected goats. *Australian Veterinary Journal* 81, 71–75.

Whittington, R.J. and Windsor, P.A. (2009) *In utero* infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. *The Veterinary Journal* 179, 60–69.

17 Paratuberculosis in Deer, Camelids and Other Ruminants

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17.1 Paratuberculosis in Wild and Farmed Deer

17.1.1 Wild and captive deer

There have been a few isolated reports of paratuberculosis in deer prior to 1970, but since then it has been reported frequently in a range of deer species in the wild and in parks, zoos and enclosures in a number of countries (Temple *et al.*, 1979; Pacetti *et al.*, 1994). This includes reindeer in the USSR (Strogov, 1973), axis deer (*Axis axis*) in California (Riemann *et al.*, 1979), Tule elk and white-tailed deer in the USA (Jessup *et al.*, 1981; Chiodini and Van Kruiningen, 1983), fallow deer in Spain (Marco *et al.*, 2002), red, roe and fallow deer in the Czech Republic (Pavlik *et al.*, 2000; Machackova *et al.*, 2004), red deer in the western Alps of Italy (Nebbia *et al.*, 2000) and Austria (Glawischnig *et al.*, 2006) and red and roe deer in Italy (Robino *et al.*, 2008). A serological survey of free-ranging cervids in Norway (Tryland *et al.*, 2004) showed evidence of Johne's disease (JD) in moose, red deer, roe deer and semi-domesticated reindeer, but no evidence was seen in wild reindeer.

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17.1.2 Farmed deer

Since the early 1980s, paratuberculosis has been diagnosed on deer farms in Europe, Asia, North and South America and Oceania. Reported cases include farmed deer in the UK (Gilmour, 1988; Fawcett *et al.*, 1995), Ireland (Power *et al.*, 1993), Denmark (Jorgensen and Jorgensen, 1987), Germany (Commichau, 1982), Belgium (Godfroid *et al.*, 2000), New Zealand (Gumbrell, 1986; de Lisle *et al.*, 1993), Canada (Starke, 1991), Hungary (Pavlik *et al.*, 1994; Machackova *et al.*, 2004), the USA (Manning *et al.*, 1998), Argentina (Mereb *et al.*, 1994) and Australia (Kennedy and Allworth, 2000).

The first case of paratuberculosis in farmed red deer in New Zealand was diagnosed in 1979 (Gumbrell, 1986). Currently, there are approximately 1 million deer on farms in New Zealand and paratuberculosis has been the subject of investigation and research since the mid-1990s. Passive surveillance, principally by the examination of suspect tuberculous lesions identified in deer slaughter plants, subsequently resulted in *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) being identified in over 600 farmed deer on 300 properties (de Lisle *et al.*, 2003). The study suggested that the herd prevalence at that time was approximately 6%. Serological surveillance for paratuberculosis on 627 of New Zealand's deer farms has been carried out since 2000, using blood samples submitted as part of the national tuberculosis eradication scheme (Griffin *et al.*, 2003, 2005). The findings project to a national prevalence of herd infection of approximately 63% (Griffin *et al.*, 2006). A recent non-random sample of 115 New Zealand deer herds of unknown infection status found that 43% had pooled faecal samples culture-positive for *MAP* (Glossop *et al.*, 2006). With time, it appears likely that the majority of deer farms in New Zealand will become infected, due to the movement of deer between farms, the use of cattle and sheep to graze excess pasture on deer farms, and the fact that deer farms were created or are being expanded by taking in land grazed by sheep or cattle, both of which harbour endemic paratuberculosis.

17.1.3 Disease

The pathogenesis of *MAP* infection in cervids is different from that in other ruminant species. Deer appear to be highly susceptible to *MAP* infection and show clinical evidence of disease and severe pathology at an earlier age than other ruminants. Clinical disease has been reported in fawns and yearlings of white-tailed deer, sika and fallow deer (Temple *et al.*, 1979), red deer (Gilmour and Nyange, 1989; Machackova *et al.*, 2004; Mackintosh *et al.*, 2004) and North American elk (Manning *et al.*, 1998). In spite of this, only a small proportion of deer exposed to *MAP* develop clinical disease. Two clinical syndromes have been recognized in heavily infected herds of red deer: sporadic cases in adult deer, which may affect <2% per annum, and outbreaks in 8–27-month-old deer, which generally affect <5% of a group but in severe cases may involve up to 20% (Mackintosh *et al.*, 2004). Adult deer tend to show chronic loss of condition and weight, similar to the disease in sheep and cattle. The clinical disease in young deer tends to be much more acute, with rapid loss of weight and muscle mass over a period of a few weeks rather than months. Affected deer usually have rough or 'moth-eaten' coats and have faecal staining around the tail and hocks as a result of soft or liquid faeces. Blood samples taken for clinical pathology tests typically show low total protein and low serum albumin concentrations, and there may be elevated concentrations of acute inflammatory proteins such as fibrinogen and haptoglobin.

Subclinical paratuberculosis may result in poor growth rates in yearlings, reduced calving percentages in hinds, reduced velvet production in stags, interference with bovine tuberculosis (TB) testing and TB-like lesions in gut lymph nodes at slaughter (Mackintosh, 2008).

17.1.4 Pathology

Post-mortem examination of clinically affected deer typically reveals greatly enlarged jejunal and ileocaecal lymph nodes, occasionally

with white- or cream-coloured caseous lesions. There may be some gross thickening of the jejunum and terminal ileum, but this is usually less obvious than that seen in sheep and cattle. However, the lymphatic drainage vessels from the jejunum to adjacent lymph nodes are often very thickened, and the omentum may be oedematous and in some cases may be adherent to the affected jejunum and lymph nodes (Gilmour and Nyange, 1989; Mackintosh *et al.*, 2004). Subclinically affected deer may also have bovine TB-like caseous lesions in the mesenteric lymph nodes and occasionally these can be found in the retropharyngeal lymph nodes. These paratuberculosis lesions can be indistinguishable from those caused by *Mycobacterium bovis*.

Histopathological examination of gut and lymph node lesions typically shows extensive areas of invasion of affected lymph nodes by macrophages laden with acid-fast organisms (AFOs), i.e. multibacillary disease. In these cases, there are often giant cells, caseation and foci of calcification. The ileocaecal valve may show loss of villous structure and a mixed cellular infiltrate and contain numerous AFOs, and similar lesions are also commonly found proximally in the ileum and/or jejunum. Some deer with less severe disease may show milder lesions and these may have few visible AFOs, i.e. paucibacillary disease (Gilmour and Nyange, 1989; Power *et al.*, 1993; Mackintosh *et al.*, 2004). Histopathological changes due to infection by *MAP*, *M. bovis* and *M. avium* subsp. *avium* can be indistinguishable, and a presumptive microbial diagnosis needs confirmation by culture or PCR. A scoring system has been developed to assist in the objective scoring of the severity of lesions due to paratuberculosis in deer (Mackintosh *et al.*, 2007).

17.1.5 Epidemiology

Although *MAP* strains of both the cattle type (also called C type or Type II) and the sheep type (also called S type or Type I) have been isolated from deer, the cattle type appears to be more pathogenic and is responsible for the outbreaks of serious disease in young farmed red deer. A more detailed discussion of the different strain types of *MAP* is given elsewhere (see Stevenson, Chapter 12, this volume). Clinical disease developed in five out of 16 deer, 21–38 weeks after heavy oral experimental challenge of 4-month-old deer with the C strain, while the S strain was relatively non-pathogenic (Mackintosh *et al.*, 2007). Over 95% of strains of *MAP* isolated from farmed deer in New Zealand have been of the C type (de Lisle *et al.*, 2006).

Young deer appear to be more susceptible to clinical disease than older animals. In a recent study, 30 3-month-old weaners, 20 yearlings and 20 adult red deer were all dosed with 4×10^9 colony-forming units of a C strain of *MAP* and ten of the weaners developed clinical disease in the year following challenge, compared with none of the yearlings or adults (Mackintosh *et al.*, 2008b).

Intrauterine transmission from dam to fetus appears to be more common in red deer than in cattle and sheep. A recent study showed that 90% of clinically affected hinds had an infected fetus (van Kooten *et al.*, 2006). In another study, *MAP* was isolated from 78% of fetuses of a group of 18 subclinically infected red deer hinds (Thompson *et al.*, 2007). In contrast, *MAP* was isolated from only 39% of fetuses from clinically affected dairy cows and 9% of fetuses from subclinically infected dairy cows (Whittington and Windsor, 2007). In sheep, intrauterine transmission is thought to occur in <10% of infected ewes. *MAP*-infected colostrum and milk may play a role in pseudovertical transmission.

As with sheep and cattle, young deer also become infected via the oral route from contaminated pasture and water. On farms, deer-to-deer transmission appears the most common route, although cattle-to-deer and sheep-to-deer transmission may occur. The role of wildlife is not known. Resistance to paratuberculosis has a genetic component, which may be related to resistance against bovine TB (C.G. Mackintosh, unpublished observations).

17.1.6 Diagnosis

A presumptive diagnosis of paratuberculosis can be made in clinically affected deer based

on typical clinical signs. Gross lesions at necropsy and histopathological examination of lesions and tissues can be highly suggestive but may not distinguish between lesions caused by *MAP*, *M. bovis* and *M. avium* subsp. *avium*. The isolation of *MAP* by culture from faeces in living deer or from tissues post-mortem is generally regarded as the diagnostic 'gold standard'. PCR tests can also specifically identify *MAP* in faeces and tissues.

Diagnosis of subclinically infected deer is more difficult. The chronic nature of infection and the relatively low incidence of disease on many deer farms can result in unnoticed *MAP* infection and an underestimate of the effects on production. Similarly, the disease may go unnoticed in wild populations of deer. Thus, *MAP* infection represents an example of a chronic bacterial disease that behaves more like a parasitic disease than a typical acute bacterial infection.

Antibody tests and cell-mediated immune (CMI) tests may provide supporting evidence of infection. Distinct features of these tests as they pertain to JD in deer are discussed below, while a more complete description of the tests and their uses is given elsewhere (see Nielsen, Chapter 24, this volume).

Commercial enzyme-linked immunosorbent antibody assays (ELISAs) (e.g. IDEXX, Portland, Maine, USA) have been used to test deer (e.g. white-tailed deer in North America), but, because they rely on cross-reactivity with cervine antibodies, they need to be recalibrated and their performance evaluated for each deer species that is being tested. An IgG1 antibody ELISA (Paralisa®) has recently been developed specifically for deer. It uses two antigens, Johne's protoplasmic antigen and purified protein derivative johnin, and the responses are read in parallel so that if either is positive the test is considered positive. The Paralisa test is reported to have an estimated sensitivity of >80% and an estimated specificity of >98% (Griffin *et al.*, 2005). There is no commercially available gamma interferon assay marketed for use in deer since the Cervigam® was withdrawn. Some laboratories use an in-house gamma interferon assay.

17.1.7 Prevention and control, including vaccination

Ideally, deer farmers should prevent the introduction of *MAP* on to their farm by acquiring only uninfected livestock, maintaining a closed herd and avoiding cross-grazing with other livestock species. Use of artificial insemination to bring in new bloodlines and control of wildlife on the property may also be worthwhile. However, these measures are not as easy as they may appear. Establishing the infection status of a herd or source of uninfected deer is very difficult, and avoiding cross-grazing, surface water runoff from neighbouring paddocks and controlling wildlife such as rabbits, rodents, roe deer and mustelids may be very difficult. As well as only sourcing deer from 'low-risk' farms, farmers should only purchase deer that are negative to the most sensitive blood tests available and to a faecal culture test.

In the face of a recognized infection, the aim should be to reduce the level of infection in order to eliminate clinical disease and minimize subclinical effects on productivity. Because of limitations in the sensitivity of current tests, it is unrealistic to try to eradicate paratuberculosis from a deer herd. On affected farms, the real cost of paratuberculosis should be estimated by adding some or all of the following production losses: outbreaks of mortality in 8–27-month-old deer, sporadic losses of mixed-age hinds and stags, reduced growth rates in calves and yearlings, increased non-pregnancy rates in hinds, suboptimal antler growth in stags and interference with TB testing. Control measures to reduce these costs are identification and culling of clinically affected deer, as well as culling infected hinds, because their calves will almost invariably be infected *in utero*.

In addition, a number of vaccines have been used for decades, including in deer, where they provide partial protection by reducing faecal shedding (Harris and Barletta, 2001). In Scotland, vaccination was used in deer on a farm that experienced a severe outbreak of paratuberculosis; although infection still occurred, there was a marked reduction in the incidence of clinical disease (Fawcett *et al.*, 1995). There have been a small number

of efficacy studies in deer of various live attenuated and killed *MAP* vaccines (Mackintosh *et al.*, 2003, 2005, 2008a), which showed that oil-adjuvanted killed vaccines gave some protection against clinical disease but did not prevent infection. However, vaccination produced some interference with TB diagnosis, resulting in false-positive reactions to the bovine tuberculin skin test. Because of this interference, it is recommended that these vaccines are not used in breeding animals on deer farms in New Zealand; they may be cost-effective in young deer raised for slaughter as meat animals, as these animals would not need to be tested for TB.

17.2 Paratuberculosis in Camelids and Other Ruminants

Apart from its presence in cattle, sheep, goats and deer, paratuberculosis has also been diagnosed in a wide range of other free-ranging and domesticated ruminants. These include moose (*Alces alces*) (Soltys *et al.*, 1967), bison (*Bison bison*) (Buergelt *et al.*, 2000), riverine buffalo (*Bubalus bubalis*) (Yadav *et al.*, 2008), bighorn sheep (*Ovis canadensis*) (Williams *et al.*, 1983), mouflon (Pavlik *et al.*, 2000) and various antelope species. It has also been described in free-ranging and captive camelids, including camels, llamas and alpacas.

17.2.1 Bison

Approximately 200,000 bison inhabit the USA, with most on ranches or in zoos, or in federal- and state-controlled areas. Paratuberculosis was first described in bison in a breeding herd of 2800 animals. Clinically affected bison showed severe weight loss, failure to shed winter hair and poor body condition (Buergelt *et al.*, 2000). Necropsy revealed mucosal corrugation in the distal small intestine and enlarged mesenteric lymph nodes.

17.2.2 Riverine buffalo

The world population of riverine buffalo is over 160 million and they are spread over 129 countries, with most being in India, which has nearly 100 million. Paratuberculosis has been reported in riverine buffalo in India, where the prevalence of infection was over 40% in some populations (Yadav *et al.*, 2008). A low prevalence was reported in Italy (Lillini *et al.*, 1999).

17.2.3 Camelids

Paratuberculosis has been reported widely in many populations of camels in Asia, the Middle East, Africa and the former Soviet Union (Ivanov and Skalinskii, 1957; Poddubskii *et al.*, 1962; Burgemeister *et al.*, 1975; Feldman *et al.*, 1981; Ovdienko *et al.*, 1985; Gameel *et al.*, 1994; Wernery and Kinne, 2007). There has also been one report from a zoo in North America (Amand, 1974). The disease in camels may have a more rapid course than in cattle, with death occurring after 4–6 weeks' illness (Wernery *et al.*, 2007). In Bactrian camels, the disease was most severe in 3–5-year-old animals (Ovdienko *et al.*, 1985).

Paratuberculosis was first reported in llamas (*Lama glama*) in North America (Belknap *et al.*, 1994), but a review in 2000 considered it was uncommon in llamas and alpacas (*Lama pacos*) in the USA (Miller *et al.*, 2000). It has also been occasionally reported in alpacas in Australia (Ridge *et al.*, 1995; Harkin, 1998), the UK (Davis *et al.*, 1998) and New Zealand (Gibson and Varney, 2007). Clinical disease has occurred in 12–24-month-old alpacas as well as in adults, with signs of diarrhoea, weight loss and hypoproteinaemia. As with other species, necropsy showed emaciation, thickened intestines and enlarged mesenteric lymph nodes (Belknap *et al.*, 1994).

A study conducted in the USA on 84 llamas and 16 alpacas in a research herd believed to be free of paratuberculosis infection was designed to test the specificity of modified antibody-based diagnostic assays (Kramsky *et al.*, 2000; Miller *et al.*, 2000). The specificity of the ELISAs ranged from 48 to 98% and the agar gel immunodiffusion assay had a specificity of 100% on these samples, leading to the recommendation that seropositive results need culture confirmation. A modified commercial bovine ELISA appears

to give a good balance of sensitivity (67%) and specificity (99%) (Kramsky *et al.,* 2000).

Paratuberculosis can be managed and eliminated by combining stringent management with frequent testing and culling, or by combining vaccination (where permitted) with management of faecal–oral transmission (Stehman, 1996). Management of faecal–oral transmission is particularly important to prevent exposure of young stock to the infection. In Australia, a market assurance programme, AlpacaMAP, has been developed for the alpaca industry and it is an integral part of the Australian National JD Program. It provides a herd classification scheme to assure alpaca breeders and their clients that participating herds have been objectively assessed as having a low risk of *MAP* infection. Details may be obtained from the following web site: http://www.animalhealthaustralia.com.au/aahc/index.cfm?0D73269D-AC64-9A65-A26C-76B4AA14E2D4.

References

Amand, W.B. (1974) Paratuberculosis in a dromedary camel. In: *American Association of Zoo Veterinarians Annual Proceedings 1974*, pp. 150–153.

Belknap, E.B., Getzy, D.M., Johnson, L.W., Ellis, R.P., Thompson, G.L. and Shulaw, W.P. (1994) *Mycobacterium paratuberculosis* infection in two llamas. *Journal of the American Veterinary Medical Association* 204, 1805–1808.

Buergelt, C.D., Layton, A.W., Ginn, P.E., Taylor, M., King, J.M., Habecker, P.L., Mauldin, E., Whitlock, R., Rossiter, C. and Collins, M.T. (2000) The pathology of spontaneous paratuberculosis in the North American bison (*Bison bison*). *Veterinary Pathology* 37, 428–438.

Burgemeister, R., Leyk, W. and Gossler, R. (1975) Studies on the occurrence of parasites and bacterial and viral infections in southern Tunisian dromedaries. *Deutsche Tierarztliche Wochenschrift* 82, 352–354.

Chiodini, R. and Van Kruiningen, H. (1983) Eastern white-tailed deer as a reservoir of ruminant paratuberculosis. *Journal of American Veterinary Medical Association* 182, 168–169.

Commichau, C. (1982) Demonstration of paratuberculosis in fallow deer (*Dama dama*). *Berliner und Münchener Tierarztliche Wochenschrift* 95, 248–250.

Davis, R., Keeble, E., Wright, A. and Morgan, K.L. (1998) South American camelids in the United Kingdom: population statistics, mortality rates and causes of death. *Veterinary Record* 142, 162–166.

de Lisle, G.W., Yates, G.F. and Collins, D.M. (1993) Paratuberculosis in farmed deer: case reports and DNA characterization of isolates of *Mycobacterium paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 5, 567–571.

de Lisle, G.W., Yates, G.F. and Montgomery, R.H. (2003) The emergence of *Mycobacterium paratuberculosis* in farmed deer in New Zealand – a review of 619 cases. *New Zealand Veterinary Journal* 51, 58–62.

de Lisle, G.W., Cannon, M.C., Yates, G.F. and Collins, D.M. (2006) Use of a polymerase chain reaction to subtype *Mycobacterium avium* subspecies *paratuberculosis*, an increasingly important pathogen from farmed deer in New Zealand. *New Zealand Veterinary Journal* 54, 195–197.

Fawcett, A.R., Goddard, P.J., McKelvey, W.A.C., Buxton, D., Reid, H.W., Greig, A. and Macdonald, A.J. (1995) Johne's disease in a herd of farmed red deer. *Veterinary Record* 136, 165–169.

Feldman, B.F., Keen, C.L., Kaneko, J.J. and Parver, T.B. (1981) Husbandry and diseases of camels. *Tierarztliche Praxis* 9, 389–402.

Gameel, A.A., Ali, A.S., Razig, S.A., Brown, J., Alhendi, S.A. and El-Sanousi, S.M. (1994) A clinical–pathological study on spontaneous paratuberculosis in camels (*Camelus dromedarius*) in Saudi Arabia. *Pakistan Veterinary Journal* 14, 15–19.

Gibson, I. and Varney, K. (2007) Quarterly review of diagnostic cases – January to March 2007. *Surveillance* 34, 28–31.

Gilmour, N.J.L. (1988) Paratuberculosis. In: Reid, H.W. (ed.) *The Management and Health of Farmed Deer*. Kluwer Academic Publishers, Dordrecht/Boston/London, pp. 113–119.

Gilmour, N.J.L. and Nyange, J. (1989) Paratuberculosis (Johne's disease) in deer. *In Practice* 11, 193–196.

Glawischnig, W., Steineck, T. and Spergser, J. (2006) Infections caused by *Mycobacterium avium* subspecies *avium*, *hominissuis*, and *paratuberculosis* in free-ranging red deer (*Cervus elaphus hippelaphus*) in Austria, 2001–2004. *Journal of Wildlife Diseases* 42, 724–731.

Glossop, J., Wilson, P., Heuer, C., West, D. and Mackintosh, C. (2006) JD in deer: practical steps towards taking control. In: Wilson, P. (ed.) *New Zealand Veterinary Association:*

Deer Branch 2006. New Zealand Veterinary Association, Wellington, New Zealand, pp. 1–5.

Godfroid, J., Boelaert, F., Heier, A., Clavareau, C., Wellemans, V., Desmecht, M., Roels, S. and Walravens, K. (2000) First evidence of Johne's disease in farmed red deer (*Cervus elaphus*) in Belgium. *Veterinary Microbiology* 77, 283–290.

Griffin, J.F.T, O'Brien, R., Spittle, E., Liggett, S., Cooper, M., Crosbie, P. and Rodgers, C. (2003) Immunodiagnostic tests in the management of Johne's disease in deer herds. In: *Proceedings of a Deer Course for Veterinarians, No. 20, Deer Branch of the New Zealand Veterinary Association*. New Zealand Veterinary Association, Wellington, New Zealand, pp. 99–102.

Griffin, J.F.T., Spittle, E., Rodgers, C.R., Liggett, S., Cooper, M., Bakker, D. and Bannantine, J.P. (2005) Immunoglobulin G_1 enzyme-linked immunosorbent assay for diagnosis of Johne's disease in red deer (*Cervus elaphus*). *Clinical and Diagnostic Laboratory Immunology* 12, 1401–1409.

Griffin, J.F.T., Spittle, E., Mackintosh, C.G., Rodgers, C.R., Liggett, S. and Cooper, M. (2006) Serodiagnosis of Johne's disease in red deer (*Cervus elaphus*). In: Manning, E.J.B. and Nielsen, S.S. (eds) *Proceedings of the 8th International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, pp. 496–500.

Gumbrell, R. (1986) Johne's disease in deer. *Surveillance* 13, 15–16.

Harkin, J.T. (1998) Johne's disease and Australian camelids. *Australian Camel Veterinary Association News* 14, 6–11.

Harris, N.B. and Barletta, R.G. (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clinical Microbiology Reviews* 14, 489–512.

Ivanov, B.G. and Skalinskii, E.I. (1957) Pathological changes in paratuberculosis of camels. *Trudy Vsesoyuznogo Instituta Eksperimental'noi Veterinarii* 20, 186–206.

Jessup, D., Abbas, B. and Behymer, D. (1981) Paratuberculosis in Tule elk in California. *Journal American Veterinary Medicine Association* 179, 1252–1254.

Jorgensen, J.B. and Jorgensen, R.J. (1987) Paratuberculosis in two red deer. *Dansk Veterinaertidsskrift* 70, 322–324.

Kennedy, D.J. and Allworth, M.B. (2000) Progress in national control and assurance programs for bovine Johne's disease in Australia. *Veterinary Microbiology* 77, 443–451.

Kramsky, J.A., Miller, D.S., Hope, A. and Collins, M.T. (2000) Modification of a bovine ELISA to detect camelid antibodies to *Mycobacterium paratuberculosis*. *Veterinary Microbiology* 77, 333–337.

Lillini, E., Gamberale, F. and Di Guardo, G. (1999) *Mycobacterium paratuberculosis* infection in a water buffalo (*Bubalus bubalis*) from central Italy. In: Manning, E.J.B. and Collins, M.T. (eds) *Proceedings of the 6th International Colloquium on Paratuberculosis* International Society of Paratuberculosis, Madison, Wisconsin, p. 254.

Machackova, M., Svastova, P., Lamka, J., Parmova, I., Liska, V., Smolik, J., Fischer, O.A. and Pavlik, I. (2004) Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic (1999–2001). *Veterinary Microbiology* 101, 225–234.

Mackintosh, C. (2008) Review: epidemiology of Johne's disease in farmed red deer (*Cervus elaphus*) in New Zealand. In: Sumner, R. (ed.) *Proceedings of the New Zealand Society of Animal Production*. New Zealand Society of Animal Production, Hamilton, New Zealand, pp. 117–121.

Mackintosh, C.G., Labes, R.E., de Lisle, G.W. and Griffin, J.F.T. (2003) Experimental challenge trial of novel Johne's disease vaccine. In: Wilson, P.R. (ed.) *Proceedings of a Deer Course for Veterinarians, Deer Branch of the New Zealand Veterinary Association*. New Zealand Veterinary Association, Wellington, New Zealand, pp. 91–98.

Mackintosh, C.G., de Lisle, G.W., Collins, D.M. and Griffin, J.F.T. (2004) Mycobacterial diseases of deer. *New Zealand Veterinary Journal* 52, 163–174.

Mackintosh, C.G., Labes, R.E., Griffin, J.F. (2005) The effect of Johne's vaccination on tuberculin testing in farmed red deer (*Cervus elaphus*). *New Zealand Veterinary Journal* 53, 216–222.

Mackintosh, C.G., Labes, R.E., Clark, R.G., de Lisle, G.W. and Griffin, J.F.T. (2007) Experimental infections in young red deer (*Cervus elaphus*) with a bovine and an ovine strain of *Mycobacterium avium* subsp. *paratuberculosis*. *New Zealand Veterinary Journal* 55, 23–29.

Mackintosh, C.G., Labes, R.E., Thompson, B.R., Clark, R.G., de Lisle, G.W., Johnstone, P.D. and Griffin, J.F.T. (2008a) Efficacy, immune responses and side-effects of Johne's vaccines in young red deer (*Cervus elaphus*) experimentally challenged with *Mycobacterium avium* subsp. *paratuberculosis*. *New Zealand Veterinary Journal* 56, 1–9.

Mackintosh, C.G., Thompson, B. and Tolentino, B. (2008b) Age susceptibility of deer to Johne's disease – preliminary results. In: Wilson, P.R. (ed.) *Proceedings of a Deer Course for*

Veterinarians, Deer Branch of the New Zealand Veterinary Association. New Zealand Veterinary Association, Wellington, New Zealand, pp. 57–59.

Manning, E.J.B., Steinberg, H., Rossow, K., Ruth, G.R. and Collins, M.T. (1998) Epizootic of paratuberculosis in farmed elk (*Cervus elaphus*). *Journal of the American Veterinary Medical Association* 213, 1320–1322.

Marco, I., Ruiz, M., Juste, R., Garrido, J.M. and Lavin, S. (2002) Paratuberculosis in free-ranging fallow deer in Spain. *Journal of Wildlife Diseases* 38, 629–632.

Mereb, G.C., Bedotti, D.O., Suarez, V.H., Busetti, M.R., Moreira, A.R. and Lorenzo, R.M. (1994) Paratuberculosis in red deer. *Veterinaria Argentina* 11, 107–112.

Miller, D.S., Collins, M.T., Smith, B.B., Anderson, P.R., Kramsky, J., Wilder, G. and Hope, A. (2000) Specificity of four serologic assays for *Mycobacterium avium* ss *paratuberculosis* in llamas and alpacas: a single herd study. *Journal of Veterinary Diagnostic Investigation* 12, 345–353.

Nebbia, P., Robino, P., Ferroglio, E., Rossi, L., Meneguz, G. and Rosati, S. (2000) Paratuberculosis in red deer (*Cervus elaphus hippelaphus*) in the Western Alps. *Veterinary Research Communications* 24, 435–443.

Ovdienko, N.P., Khon, F.K., Sharov, V.A. and Yakusheva, O.V. (1985) Diagnosis of paratuberculosis in camels. *Veterinariya, Moscow, USSR* 4, 65–68.

Pacetti, A., Belletti, G., Gabbi, M., Mutinelli, F. (1994) Paratuberculosis in red deer. *Obiettivie-Documenti-Veterinari* 15, 67–70.

Pavlik, I., Pavlas, M. and Bejckova, L. (1994) Incidence, economic importance and diagnosis of paratuberculosis. *Veterinarni Medicina* 39, 451–496.

Pavlik, I., Bartl, J., Dvorska, L., Svastova, P., du Maine, R., Machackova, M., Ayele, W.Y. and Horvathova, A. (2000) Epidemiology of paratuberculosis in wild ruminants studied by restriction fragment length polymorphism in the Czech Republic during the period 1995–1998. *Veterinary Microbiology* 77, 231–251.

Poddubskii, I.V., Shchurevskii, V.E. and Alikaeva, A.P. (1962) Paratuberculosis among farm animals in the Soviet Union. *Trudy Vsesoyuznogo Instituta Eksperimental'noi Veterinarii* 26, 115–134.

Power, S.B., Haagsma, J. and Smyth, D.P. (1993) Paratuberculosis in farmed red deer (*Cervus elaphus*) in Ireland. *Veterinary Record* 132, 213–216.

Ridge, S.E., Harkin, J.T., Badman, R.T., Mellor, A.M. and Larsen, J.W. (1995) Johne's disease in alpacas (*Lama pacos*) in Australia. *Australian Veterinary Journal* 72, 150–153.

Riemann, H., Zaman, M.R., Ruppanner, R., Aalund, O., Jorgensen, J.B., Worsaae, H. and Behymer, D. (1979) Paratuberculosis in cattle and free-living exotic deer. *Journal of the American Veterinary Medical Association* 174, 841–843.

Robino, P., Nebbia, P., Tramuta, C., Martinet, M., Ferroglio, E. and De Meneghi, D. (2008) Identification of *Mycobacterium avium* subsp. *paratuberculosis* in wild cervids (*Cervus elaphus hippelaphus* and *Capreolus capreolus*) from northwestern Italy. *European Journal of Wildlife Research* 54, 357–360.

Soltys, M.A., Andress, C.E. and Fletch, A.L. (1967) Johne's disease in a moose (*Alces alces*). *Bulletin of the Wildlife Association* 3, 183–184.

Starke, R.K.A. (1991) Paratuberculosis (Johne's disease) in a captive wapiti. In: Renecker, L.A. and Hudson, R.J. (eds) *Wildlife Production: Conservation and Sustainable Development*. University of Alaska, Fairbanks, Alaska, pp. 435–437.

Stehman, S.M. (1996) Paratuberculosis in small ruminants, deer, and South American camelids. *Veterinary Clinics of North America – Food Animal Practice* 12, 441–455.

Strogov, A.K. (1973) [Paratuberculosis in reindeer]. In: *Maloizvestnye Zaraznye Bolezni Zhivotnykh [Little-known Contagious Diseases of Animals]*, 2nd edn. Izdatel'stvo Kolos. USSR, pp. 231–236.

Temple, R.M.S., Muscoplat, C.C., Thoen, C.O., Himes, E.M. and Johnson, D.W. (1979) Observations on diagnostic tests for paratuberculosis in a deer herd. *Journal of the American Veterinary Medical Association*, 914–915.

Thompson, B.R., Clark, R.G. and Mackintosh, C.G. (2007) Intra-uterine transmission of *Mycobacterium avium* subsp. *paratuberculosis* in subclinically affected red deer (*Cervus elaphus*). *New Zealand Veterinary Journal* 55, 308–313.

Tryland, M., Olsen, I., Vikoren, T., Handeland, K., Arnemo, J.M., Tharaldsen, J., Djonne, B., Josefsen, T.D. and Reitan, L.J. (2004) Serologic survey for antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in free-ranging cervids from Norway. *Journal of Wildlife Diseases* 40, 32–41.

van Kooten, H.C., Mackintosh, C.G. and Koets, A.P. (2006) Intra-uterine transmission of paratuberculosis (Johne's disease) in farmed red deer. *New Zealand Veterinary Journal* 54, 16–20.

Wernery, U. and Kinne, J. (2007) An approach to the diagnosis of camel diseases: clinical pathology and post-mortem criteria. In: Gahlot, T.K. (ed.) *Proceedings of the International Camel Conference 'Recent Trends in Camelid Research and Future Strategies for Saving Camels'*. Rajasthan, India, pp. 1–18.

Wernery, U., Kinne, J., Jahans, K.L., Vordermeier, H.M., Esfandiari, J., Greenwald, R., Johnson, B., Ul-Haq, A. and Lyashchenko, K.P. (2007) Tuberculosis outbreak in a dromedary racing herd and rapid serological detection of infected camels. *Veterinary Microbiology* 122, 108–115.

Whittington, R.J. and Windsor, P.A. (2007) *In utero* infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, www.paratuberculosis.org/pubs/proc9/section5.htm (accessed 11 May 2009).

Williams, E.S., Snyder, S.P. and Martin, K.L. (1983) Pathology of spontaneous and experimental infection of North American wild ruminants with *Mycobacterium paratuberculosis*. *Veterinary Pathology* 20, 274–291.

Yadav, D., Singh, S.V., Singh, A.V., Sevilla, I., Juste, R.A., Singh, P.K. and Sohal, J.S. (2008) Pathogenic 'bison-type' *Mycobacterium avium* subspecies *paratuberculosis* genotype characterized from riverine buffalo (*Bubalus bubalis*) in North India. *Comparative Immunology, Microbiology and Infectious Diseases* 31, 373–387.

18 Infection of Non-ruminant Wildlife by *Mycobacterium avium* subsp. *paratuberculosis*

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18.1 Introduction

Paratuberculosis has historically been considered an infection of ruminants. First indications of a wider host range date to the 1970s, but it was not until the late 1990s that non-ruminant wildlife hosts of paratuberculosis were confirmed and further investigated in relation to their significance in the epidemiology of livestock infection.

Numerous theoretical epidemiological studies and practical disease control programmes have demonstrated the added difficulties that a wildlife host can bring to livestock disease control. Ultimately, if the infection can persist in the wildlife host population in isolation (i.e. no infection pressure from other host species) for extended periods of time and there is a viable route of transmission from the wildlife host to the livestock host, then the wildlife species must be included in any disease control strategy. In this chapter we consider the role of non-ruminant wildlife in the epidemiology of paratuberculosis.

 Paratuberculosis: Organism, Disease, Control (eds M.A. Behr and D.M. Collins)

18.2 Host Infection

18.2.1 Known host range

Suspected isolation of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) was reported from a European brown hare (*Lepus europaeus*) in England (Matthews and Sargent, 1977), although the organism responsible was not fully characterized. Lesions attributed to paratuberculosis were subsequently described in a wild rabbit (*Oryctolagus cuniculus*) from Scotland (Angus, 1990). More recently, isolation of *MAP* has been confirmed by a polymerase chain reaction (PCR) assay based on the species-specific IS*900* insertion sequence (Vary *et al.*, 1990). This assay was used in three successive surveys of rabbits from farms in the east of Scotland with a history of paratuberculosis in ruminant livestock (Greig *et al.*, 1997, 1999; Beard *et al.*, 2001a). Following isolation of *MAP* in rabbits, studies were extended to investigate other wildlife species associated with infected farms. *MAP* was isolated from foxes (*Vulpes vulpes*) and stoats (*Mustela erminea*) (Beard *et al.*, 1999), then subsequently from weasels (*Mustela nivalis*), badgers (*Meles meles*), wood mice (*Apodemus sylvaticus*), rats (*Rattus norvegicus*), brown hares, jackdaws (*Corvus monedula*), rooks (*Corvus frugilegus*) and crows (*Corvus corone*) (Beard *et al.*, 2001a). Following the study in Scotland described in Beard *et al.* (2001a), researchers have looked for *MAP* in non-ruminant species around the world. The organism was detected in rodents and wild boar in the Czech Republic (Kopecna *et al.*, 2008), rodents and fox in Greece (Florou *et al.*, 2008), wild boar in Spain, the brown bear and a number of bird species in Slovakia (Kopecna *et al.*, 2006; Gronesova *et al.*, 2008), marsupials in Australia (OIE, 2001), ferrets in New Zealand (de Lisle *et al.*, 2003) and a wide variety of species in the USA (Corn *et al.*, 2005; Palmer *et al.*, 2005; Raizman *et al.*, 2005; Anderson *et al.*, 2007). High prevalence rates and levels of infection in non-ruminant wildlife as found in the UK have not been repeated in Europe or the USA. However, populations of European rabbits in New Zealand have been shown to have similar prevalence of infection as the UK (G. Nugent, New Zealand, 2008, personal communication). See Table 18.1 for a complete list of the known non-ruminant host range of *MAP* to date.

The *MAP* strains that infect non-ruminants are principally of the cattle type (also called C type or Type II), and there has been only one report of a sheep type (also called S type or Type I), which was isolated from a house mouse (*Mus musculus*) (Florou *et al.*, 2008). A more detailed description of the different types of *MAP* is given elsewhere (see Stevenson, Chapter 12, this volume). Generally the *MAP* strains isolated from non-ruminants are the same as the predominant strains infecting ruminants in the same area.

18.2.2 Pathology

In wild ruminants, gross lesions and clinical signs have been reported as similar to those in infected cattle and sheep, where the disease is ultimately fatal (Williams *et al.*, 1979; Buergelt *et al.*, 2000). In contrast, macroscopic lesions are very rare in non-ruminant wildlife and in the authors' experience have been observed only in *MAP*-infected rabbits. To date, there have been no recorded clinical cases of paratuberculosis in non-ruminant wildlife.

The histopathology of non-ruminant *MAP* infections has been studied most in naturally infected rabbits, in which both severe and mild lesions have been observed in the intestines (Greig *et al.*, 1997; Beard *et al.*, 2001b). Severe lesions are characterized by large numbers of infiltrating epithelioid macrophages and giant cells, also a prominent feature of early bovine paratuberculosis and some forms of ovine paratuberculosis. Acid-fast staining reveals numerous intracellular bacilli. In rabbits with severe intestinal lesions, similar histopathological changes are apparent in the mesenteric lymph nodes and gut-associated lymphoid tissue. Some rabbits have been found to exhibit multiple foci of chronic granulomatous inflammation in the liver.

The pathology of *MAP* infections in other non-ruminants is much more subtle, suggesting that these animals may be 'dead-end' hosts for the organism. In foxes, weasels and stoats, small numbers of single, large macrophage-like

Table 18.1. Known non-ruminant wildlife host range of *MAP* by country.

Country	Host type	Species common name	Species Latin name	No. +ve/no. sampled	Location status[a]	Reference
UK	Rodents	Rat	*Rattus norvegicus*	3/35	infected	Beard *et al*., 2001
		Wood mouse	*Apodemus sylvaticus*	3/88	infected	Beard *et al*., 2001
	Lagomorphs	Rabbit	*Oryctolagus cuniculus*	24/110	infected	Greig *et al*., 1999
				100/252	infected	Judge *et al*., 2006
				29/126	infected	Judge *et al*., 2006
		Brown hare	*Lepus europaeus*	1/1	N/K[b]	Matthews and Sargent, 1977
				1/6	infected	Beard *et al*., 2001
	Mustelids	Stoat	*Mustela erminea*	17/37	infected	Beard *et al*., 2001
		Weasel	*Mustela nivalis*	2/4	infected	Beard *et al*., 2001
		Badger	*Meles meles*	1/2	infected	Beard *et al*., 2001
	Birds	Crow	*Corvus corone*	36/60	infected	Beard *et al*., 2001
		Rook	*Corvus frugilegus*	3/53	infected	Beard *et al*., 2001
		Jackdaw	*Corvus monedula*	1/38	infected	Beard *et al*., 2001
	Other	Fox	*Vulpes vulpes*	23/27	infected	Beard *et al*., 2001
Czech Republic	Rodents	Rat	*Rattus norvegicus*	1/17	random	Kopecna *et al*., 2008
		Vole	*Mycrotis arvalis*	1/59	random	Kopecna *et al*., 2008
		Lesser white-toothed shrew	*Corcidura suaveolens*	1/39	random	Kopecna *et al.,* 2008
	Other	Wild boar	*Sus scorfa*	1/805	random	Kopecna *et al*., 2008
Greece	Rodents	House mouse	*Mus musculus*	2/149	infected	Florou *et al*., 2008
		Black rat	*Rattus rattus*	1/55	infected	Florou *et al*., 2008
	Other	Fox	*Vulpes vulpes*	1/10	infected	Florou *et al*., 2008
Spain	Other	Wild boar	*Sus scorfa*	1/65	random	Alvarez *et al*., 2005
Slovakia	Other	Brown bear	*Ursus arctos*	2/20	random	Kopecna *et al.,* 2006
	Birds	Black-headed gull	*Larus ridibundus*	2/11	random	Gronesova *et al*., 2008
		European curlew	*Numenius arquata*	2/2	random	Gronesova *et al.,* 2008
		Ruff	*Philomachus pugnax*	1/9	random	Gronesova *et al*., 2008
		Common cuckoo	*Cuculus canorus*	1/1	random	Gronesova *et al*., 2008
		Savi's warbler	*Locustella luscinioides*	1/3	random	Gronesova *et al*., 2008
		Starling	*Sturnus vulgaris*	1/3	random	Gronesova *et al*., 2008

USA	Rodents	Hispid cotton rat	*Sigmodon hispidus*	1/41	infected	Corn *et al.*, 2005
		Norway rat	*Rattus norvegicus*	1/4	infected	Corn *et al.,* 2005
		South-eastern shrew	*Sorex longirostris*	1/4	infected	Corn *et al.*, 2005
	Lagomorphs	Eastern cottontail	*Sylvilagus floridanus*	1/56	infected	Corn *et al.*, 2005
		Rabbit	*Oryctolagus cuniculus*	1/218	random	Raizman *et al.*, 2005
	Birds	Common snipe	*Gallinago gallinago*	1/1	infected	Corn *et al.*, 2005
		House sparrow	*Passer domesticus*	1/60	infected	Corn *et al.*, 2005
		European starling	*Sturnus vulgaris*	7/40	infected	Corn *et al.,* 2005
	Other	Armadillo	*Dasypus novemcinctus*	4/23	infected	Corn *et al.*, 2005
		Opossum	*Didelphis virginiana*	2/54	infected	Corn *et al.*, 2005
				15/63	random	Anderson *et al.*, 2007
		Racoon	*Procyon lotor*	8/42	infected	Corn *et al.*, 2005
				29/73	random	Anderson *et al.*, 2007
		Striped skunk	*Mephitis mephitis*	1/10	infected	Corn *et al.*, 2005
				3/5	random	Anderson *et al.*, 2007
		Coyote	*Canis latrans*	28/59	random	Anderson *et al.*, 2007
		Fox	*Vulpes vulpes*	5/7	random	Anderson *et al.*, 2007
		Feral cat	*Felis catus*	2/18	infected	Corn *et al.*, 2005
				1/5	random	Anderson *et al.*, 2007
				7/25	infected	Palmer *et al.*, 2005
Australia	Marsupials	Kangaroo	*Macropus fuliginosis fuliginosis*	N/K[b]	N/K[b]	OIE, 2001
		Tammar wallaby	*Macropus eugenii*	N/K[b]	N/K[b]	OIE, 2001
New Zealand	Mustelids	Feral ferrets	*Mustela putorius furo*	8/?	N/K[b]	de Lisle *et al.*, 2003

[a]Location status: infected = sampled from an area with known *MAP* infection; random = sampled from random areas.
[b]Not known: prevalence in both species estimated to be greater than 1.7% but actual figures not reported.

cells or granulomata consisting of ten or fewer cells have been observed in the mesenteric lymph nodes and mucosa-associated lymphoid tissue of the gut (Beard *et al.*, 2001a). Only small numbers of acid-fast organisms (AFOs) have been detected in the cytoplasm of these macrophage-like cells. No histopathological lesions have been observed in the intestines or liver of these host species. Similar mild histopathological lesions have also been observed in a crow and a wood mouse. Beard *et al.* (2001b) observed cells containing fewer than five AFOs scattered throughout the lamina propria of a crow intestine. Multiple granulomata were observed in the liver but did not contain AFOs. In the wood mouse, macrophage-like cells containing AFOs were observed as both single cells and small granulomata within the cortex of the mesenteric lymph node and small intestine.

18.2.3 Prevalence and excretion rates

The contribution made by a species to the amount of *MAP* in the environment is a function of both the numbers of infected animals (prevalence × population size) and the rates of *MAP* excretion. In the UK, prevalences of *MAP* are generally higher in carnivores such as the fox compared with prey species such as lagomorphs and rodents. With an average prevalence of 85%, the fox might be considered as a useful indicator species of on-farm infection (Beard *et al.*, 2001a). Of the herbivore hosts from the Beard *et al.* (2001a) study, prevalences were highest in rabbits (17%) and less than 10% in rats and mice. While prevalences in carnivores tend to be higher than those in herbivores, prevalences in rabbits on a single farm in the UK can be as high as 70% (M. Hutchings, unpublished data). The prevalence of *MAP* in wildlife in the USA ranged from 1.7 to 25%, although the sample sizes for those with a prevalence greater than 19% were small (n = 4). Those species with a prevalence of greater than 10% were all predators or scavengers (armadillo, feral cat, opossum and raccoon) (Corn *et al.*, 2005), which follows the patterns of prevalence found in the UK. Faecal samples were culture-positive from raccoons, armadillos, an opossum and a feral cat, suggesting that these animals shed the bacteria in their faeces (Corn *et al.*, 2005) and therefore have the potential to play a part in the onward transmission of the disease.

There are few published data on *MAP* shedding rates in non-ruminant wildlife. The mean number of colony-forming units (CFU) from infected rabbit faeces was $7.6 \times 10^5 \pm 5.2 \times 10^5$ CFU/g (Daniels *et al.*, 2003a), which is lower than the 10^8 CFU/g reported in faeces from clinically affected cattle (Cranwell, 1997; Whittington *et al.*, 2000). Infected rabbits may also shed *MAP* in their urine (n = 2/17), although the levels of shedding are thought to be far lower than those in faeces (Daniels *et al.*, 2003a). However, as rabbits are asymptomatic, the excretion rates in rabbit faeces is a mean across different levels of infection and as such cannot be compared with the clinically affected cattle excretion rates that are often cited in the literature. Although not directly quantified, pathological comparisons suggest that the shedding rates of other non-ruminant wildlife would be expected to be far lower than for rabbits.

A relative estimate of the input of *MAP* on to pasture suggested that sheep and cattle potentially contributed 4 and 125 times more organisms per hectare per day, respectively, than rabbits. None the less, rabbits were estimated to contribute $>10^6$ CFU of *MAP* per hectare per day (Daniels *et al.*, 2003a).

18.3 Epidemiology

18.3.1 Spatial and temporal patterns of infection in host populations and the environment

Clustering of pathogens in the environment can lead to hot spots of disease at local, regional, national and international levels. Scotland contains regional hot spots of *MAP* in rabbits and this mirrors the distribution in cattle (Greig *et al.*, 1999). At a finer spatial scale within a farm with an overall *MAP* infection prevalence of 40%, the temporal distribution of infection in rabbits followed a cyclical pattern, with a peak in spring of 55.4% and a low in summer of 19.4% (Judge *et al.*,

2005a). Spatially, *MAP*-infected rabbits, and the associated risk of interspecies transmission, were highly clustered in the environment. However, this was mostly due to the clustered distribution of the general rabbit population in the environment.

18.3.2 Inter- and intraspecies routes of transmission within wildlife communities

The faecal–oral route is generally seen as the major route of horizontal transmission in ruminant species, although vertical and pseudovertical (via the ingestion of colostrum or milk contaminated with faeces) have also been shown to occur. Few studies have examined intra- and interspecific routes of transmission within wildlife communities. *MAP* has been isolated from the testes, uterus, placenta and fetuses of rabbits, suggesting the possibility of both sexual and vertical transmission (Judge *et al.*, 2006). *MAP* has also been isolated from the milk of lactating rabbits. However, as rabbits wean their young on their own faeces, the role of milk versus faecal–oral transmission may be difficult to separate. In a population of rabbits with 42% infected adult females, field studies suggest that vertical, i.e. transplacental and/or pseudovertical, transmission can occur in 14% of offspring entering the population at 1 month of age, and this equates to a probability of infection via this route of 0.326. In the same population, an analysis of the prevalence of infection as a function of age estimated that the probability of infection via horizontal transmission (including interspecies transmission) was as high as 3.7% per month. Given an average 18-month lifespan of a wild rabbit, this estimate suggests a high probability of acquiring *MAP* infection in an affected population (Judge *et al.*, 2006).

Carnivores such as stoats, foxes and weasels have relatively little direct contact with contaminated pastures or farm buildings, and it is therefore more likely that they become infected with *MAP* through ingestion of infected prey. *MAP* was isolated from the mesenteric lymph nodes and intestinal tissue of infected rabbits, rats, raccoons, armadillos and feral cats (Greig *et al.*, 1997, 1999; Corn *et al.*, 2005; Florou *et al.*, 2008). Foxes, stoats and weasels ingest entire rodents and large enough proportions of rabbit carcasses to include lymph nodes and intestinal tissue. Lagomorphs and small rodents form the majority of the diet of foxes, stoats and weasels in Scotland (Harris and Lloyd, 1991; King 1991a,b; Leckie *et al.*, 1998). Coyotes and raccoons are also known to feed on rodents and lagomorphs, which may explain the relatively high levels of infection found in these species (Anderson *et al.*, 2007). At the wildlife host community level, there is limited evidence that predation and/or scavenging represents a route of interspecific transmission. As detailed above, the prevalence of infection in carnivore species is generally higher than that in prey species. In one study, 62% of the predators (fox, stoat and weasel) sampled were infected compared with 10% of the prey species (rabbits, rats and mice). Transmission of pathogens through predation has been reported and is explained by carnivores' higher trophic level in the food chain (Zarnke *et al.*, 2000). At least part of the diet of the predators and opportunistic animals such as the opossum and a large part of the diet of corvids (the bird family that includes crows) may consist of scavenging potentially infected prey carcasses (Mason and Macdonald, 1995; Anderson *et al.*, 2007). Therefore scavenging may constitute an additional mode of paratuberculosis transmission.

The importance of carnivores as wildlife hosts of paratuberculosis is more likely to be indirect, since livestock are unlikely to come into contact with large amounts of their faeces. Corvids, on the other hand, may be present on farms at high densities and so cannot be discounted as a potential source of infection. Perhaps more importantly, infected carnivores and corvids could theoretically transport *MAP* over far greater distances than most prey species. For example, in the east of Scotland the home range size of rabbits is 6.3 ha (Hulbert *et al.*, 1996; Daniels *et al.*, 2003c) but is 4000 ha for foxes (Harris and Lloyd, 1991).

18.3.3 Potential livestock-to-wildlife routes of transmission

The association between *MAP* in non-ruminant wild species and paratuberculosis infections

on farms suggests that interspecies transmission may occur between livestock and non-ruminant wildlife. Interspecies transmission has been demonstrated experimentally in the case of rabbits infected with a cattle strain of *MAP* (Mokresh and Butler, 1990; Beard *et al.*, 2001c) and observationally with sheep and feral goats being infected after sharing grazing pasture with infected cattle (Ris *et al.*, 1987, 1988). Given the high rates of shedding by clinically infected cattle and sheep (Cranwell, 1997; Whittington *et al.*, 2000), it is likely that wild herbivores such as rabbits or hares sharing pasture with infected cattle become infected through the ingestion of contaminated grass.

Based on a sample of 20 farms across Scotland, Greig *et al.* (1999) found a statistically significant relationship between farms with an ongoing or recent paratuberculosis problem in cattle and the presence of similar strains of paratuberculosis in cattle and rabbits, providing further support for transmission between the species. In the case of *MAP* infection of granivorous or omnivorous rodents, exposure is more likely to occur in contaminated buildings than pasture (Beard *et al.*, 2001a). Indeed, in one study, rodents from which *MAP* was isolated were all caught in buildings which housed cattle, whereas those captured in adjacent field margins or woodland were negative on culture (M.J. Daniels and M.R. Hutchings, unpublished data).

18.3.4 Persistence of infection in non-ruminant wildlife

Due to the difficulties in experimentally demonstrating the long-term persistence of an infection in a host species, especially a slow-growing pathogen such as *MAP*, mathematical modelling is often used to synthesize known information on the host demography and pathogen transmission to predict if the infection is stable within a host population. Of the known non-ruminant wildlife hosts of *MAP*, this approach has so far only been carried out for rabbits. Judge *et al.* (2007) developed a mathematical model and applied it to combinations of vertical (including pseudovertical) and horizontal rates of transmission, which covered a broad range of plausible values. The long-run averages of the equilibrium prevalences were used to characterize disease persistence for each combination of horizontal and vertical/pseudovertical transmission rates.

This approach predicted that paratuberculosis would persist in rabbit populations at all values of the horizontal and vertical transmission parameters in the range estimated from the field data presented above, and in many cases at all values within 95% confidence intervals around this range (Fig. 18.1). Indeed, the prevalence of *MAP* infection in the study site from which the transmission rates were estimated was 39.7%, falling within the prevalence ranges produced by the model. The persistence of *MAP* infection in rabbit populations suggests that they may act as a reservoir of infection (i.e. self-sustaining) for sympatric livestock. This has significant implications for paratuberculosis control. For example, destocking is often considered as a means of disease control in livestock populations and has been used to attempt to control paratuberculosis in livestock in Australia under the National Ovine Johne's Disease Control and Evaluation Program (Sergeant, 2001; Whittington *et al.*, 2003). However, this type of strategy fails to account for the presence of a wildlife reservoir and may therefore be unsuccessful if wildlife is infected. Furthermore, the possibility of the disease spreading from infected livestock farms to previously uninfected livestock areas through the dispersal of infected wildlife needs to be considered in control strategies. For the UK and other countries with high rabbit populations, it would seem unrealistic to eradicate rabbits at the scale necessary to prevent spillover infection in current hot spots of disease (Judge *et al.*, 2005b). Consequently, management of disease should probably be restricted to rabbit population management (e.g. keeping population densities low), managing livestock–wildlife interactions (e.g. keeping animals, especially the most susceptible animals, away from areas with high rabbit densities) and good livestock management practices in terms of husbandry and movement between herds.

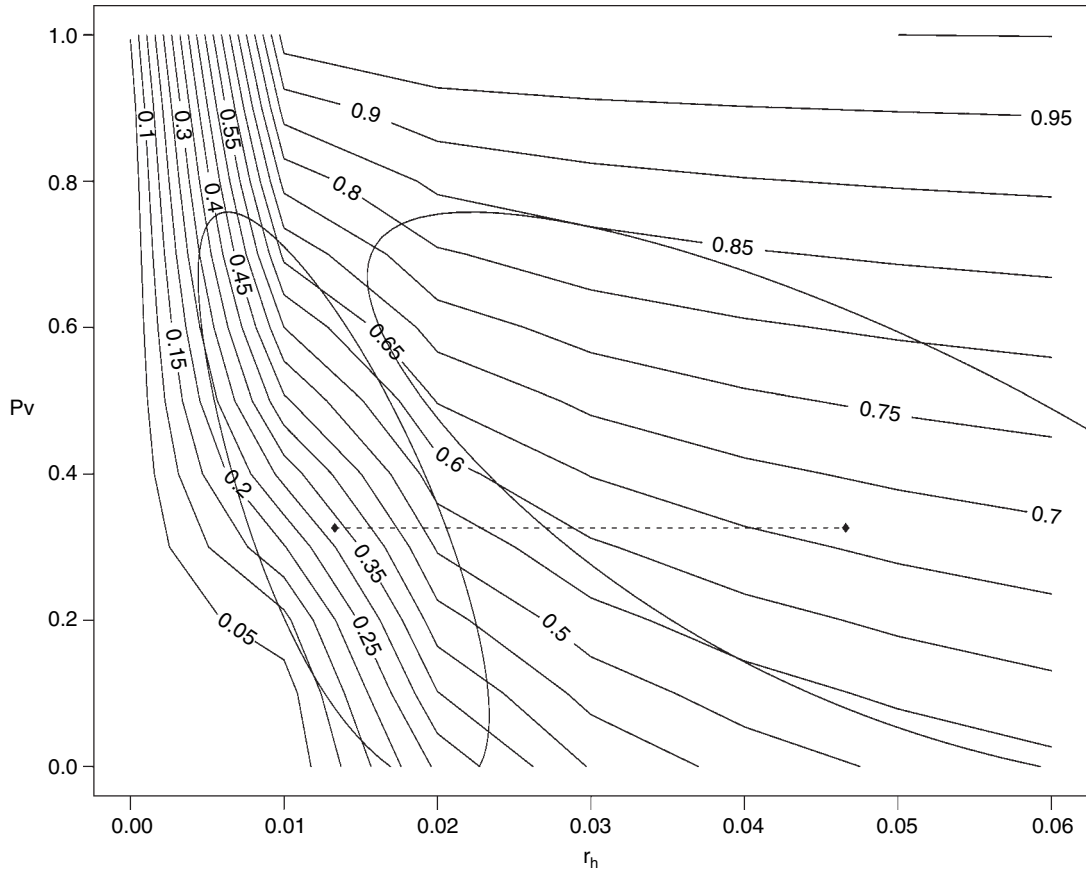

Fig. 18.1. Isopleths of predicted *MAP* prevalence in rabbit populations for all combinations of vertical (Pv) and horizontal (r_h) probabilities of transmission. Field estimates of the values of Pv and r_h are shown with the dashed line. Elipses represent 95% confidence limits around the minimum and maximum r_h estimate.

18.3.5 Risk to livestock

Transmission of *MAP* in livestock is believed to occur mainly through the faecal–oral route (Sweeney, 1996). There are two main ways sheep and cattle can potentially ingest faecal matter from infected wildlife: grazing contaminated pasture and consuming contaminated farm-stored feed.

Grazing livestock tend to avoid pastures contaminated with their own and other species' faeces through: (i) the selection of non-contaminated sward patches; (ii) reduced bite rate when forced to graze contaminated sward patches; and (iii) reduced grazing depth when grazing contaminated swards (Hutchings *et al.*, 1998; Smith *et al.*, 2009b). However, the only known species for which livestock do not use faeces-avoidance behaviour to limit exposure to parasites and pathogens is the rabbit. Sheep and cattle do not avoid rabbit faeces and graze contaminated pastures as they do non-contaminated pastures (Daniels *et al.*, 2001; Judge *et al.*, 2005b; Smith *et al.*, 2009a,b), so the number of rabbit faecal pellets ingested by calves is directly proportional to the level of contamination of the pasture. Given a density of 10–59 rabbits/ha for Scotland, the number of rabbit faecal pellets present at any one time on a grazing pasture is between 30,657 and 290,752 pellets/ha (Daniels *et al.*, 2003a). Cattle ingest 1.27% of the faecal pellets, or between 389 and 3693 pellets/ha grazed. If one-third of such pellets harbour live *MAP* organisms, one calf could ingest 140–1329 infected faecal pellets/ha grazed. Sheep ingest an average of 0.476% of the faecal pellets, giving the potential to ingest between 146 and 1384 faecal pellets/ha grazed, of which 53–498 may be infected. In summary, of the known non-ruminant wildlife host range in the UK, rabbits would be expected to represent the greatest risk to sympatric livestock as they input the greatest amount of *MAP* on to grazing environments through the combination of a relatively high prevalence of infection and high population density of rabbits with access to livestock pasture, and the lack of avoidance of rabbit faeces by grazing livestock.

Livestock are also susceptible to infection through feed. Wildlife excreta, particularly from rodents and birds, can contaminate feed during on-farm storage, after which faeces are likely to be ingested when the feed is given to livestock. Levels of wildlife faecal contamination during on-farm winter feed storage have been quantified in feed stores on four farms in the east of Scotland by Daniels *et al.* (2003b). They reported a mean of 79.9 rodent faeces (95% confidence interval: 37.5–165.9) and 24.9 bird faeces (95% CI: 14.3–41.7) deposited per m^2 of stored feed per month. It was estimated that individual cattle and sheep could encounter 1626 and 814 rabbit faeces, respectively, over the winter. Similarly, infected rodents were found to be associated with livestock premises in Wisconsin (Corn *et al.*, 2005; Anderson *et al.*, 2007), and rodents were trapped in sheds housing goats and sheep in Greece (Florou *et al.*, 2008). It is hypothesized that they became infected through scavenging livestock feed from the floor of the sheds where it has become contaminated with livestock faeces (Florou *et al.*, 2008). Once infected it is likely that the rodents would contribute to contaminating the livestock feed and potentially contribute to the cycle of infection in the host community.

Unlike in grazing systems, livestock that are fed supplementary feedstuffs are often unable to avoid contaminated feed. When presented with contaminated feed such as concentrates, cattle and sheep have some limited ability to actively avoid rat faecal pellets, but this is not the case for mouse faeces (Daniels and Hutchings, 2001). Moreover, the discriminatory power of livestock is limited, and group feeding that increases competition between animals further increases the rates of ingestion of rodent faeces (Daniels and Hutchings, 2001). Given such low levels of avoidance, even with extremely low probabilities of *MAP* transmission, infection may be maintained in livestock populations (Daniels *et al.*, 2003b).

18.4 Control of Paratuberculosis in Wildlife

Reduction in wildlife populations is a common method for the control of livestock infections that have wildlife hosts; its success is

dependent on the characteristics of the infection, as well as on the spatial and social structure of the wildlife host. Few studies have considered wildlife population reduction to control paratuberculosis. However, a recent modelling study suggests that unrealistically high rabbit culls (>95% population reduction) are needed if infection is to be eradicated from local rabbit populations with a single population reduction event, of either individuals or social groups (Davidson *et al.*, 2008). Repeated annual culls are more effective at reducing the prevalence of infection in rabbit populations and eradicating infection. However, annual population reductions of >40% are required over extended periods of time (many years). Thus, this model predicts that *MAP* will persist in rabbit populations without significant and prolonged effort to achieve control (Davidson *et al.*, 2008). Currently, *MAP*-infected rabbit populations may be considered relatively uncommon in the UK and around the world. However, given the ubiquitous distribution of the European rabbit in the UK and New Zealand and the likely persistence of infection in rabbit populations, the use of repeated control strategies may be advisable. Where possible these should be conducted at the landscape scale via landowner cooperation. In settings where other non-ruminant hosts are documented to be infected with *MAP*, similar considerations may apply.

18.5 Conclusions

The high prevalence of *MAP* in some non-ruminant wildlife species and their interaction with susceptible ruminant livestock raise the possibility that they play an active role in the current epidemiology of the disease in livestock and can present a significant challenge to control efforts. The risks of transmission from wildlife to livestock have been frequently raised but are difficult to prove in the field, mainly due to the long incubation period of the disease and the difficulty in excluding other potential sources of infection (Williams *et al.*, 1979; Chiodini and Van Kruiningen, 1983; Greig *et al.*, 1997; Ferroglio *et al.*, 2000; Beard *et al.*, 2001a,b). Nevertheless, a consistent body of evidence implicates the European rabbit as a persistent and significant risk of infection to sympatric livestock. Paratuberculosis in domestic livestock populations is a notoriously difficult disease to control (Stabel, 1998). The participation of non-ruminant wildlife species recently added to the known host range of *MAP* may partially account for these difficulties. Further research is required to quantify the role of rabbits in the epidemiology of *MAP* infection. For example, the relative force of infection of *MAP* from wildlife to domestic livestock and vice versa is currently unknown. This knowledge is needed if we are to improve our approach to *MAP* control and fully appreciate the role of non-ruminant wildlife species in the epidemiology of paratuberculosis.

References

Alvarez, J., de Juan, L., Briones, V., Romero, B., Aranaz, A., Fernandez-Garayzabak, J.F. and Mateos, A. (2005) *Mycobacterium avium* subsp. *paratuberculosis* in fallow deer and wild boar in Spain. *Veterinary Record* 156, 212–213.

Anderson, J.L., Meece, J.K, Koziczkowski, J.J., Clark, D.L., Jr., Radcliffe, R.P., Nolden, C.A., Samuel, M.D. and Ellingson, J.L.E. (2007) *Mycobacterium avium* subsp. *paratuberculosis* in scavenging mammals in Wisconsin. *Journal of Wildlife Diseases* 43, 302–308.

Angus, K. (1990) Intestinal lesions resembling paratuberculosis in a wild rabbit (*Oryctolagus cunicululs*). *Journal of Comparative Pathology* 103, 22–23.

Beard, P.M., Henderson, D., Daniels, M., Pirie, A., Buxton, D., Greig, A., Hutchings, M.R., McKendrick, I., Rhind, S., Stevenson, K. and Sharp, J.M. (1999) Evidence of paratuberculosis in fox (*Vulpes vulpes*) and stoat (*Mustela erminea*). *Veterinary Record* 145, 612–613.

Beard, P.M., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Buxton, D., Rhind, S., Greig, A., Hutchings, M.R., McKendrick, I., Stevenson, K. and Sharp, J.M. (2001a) Paratuberculosis infection of non-ruminant wildlife in Scotland. *Journal of Clinical Microbiology* 39, 1517–1521.

Beard, P.M., Rhind, S., Buxton, D., Henderson, D., Daniels, M.J., Pirie, A., Rudge, K., Greig, A., Hutchings, M.R., Stevenson, K. and Sharp, J.M. (2001b) Natural paratuberculosis infection in

rabbits in Scotland. *Journal of Comparative Pathology* 124, 290–299.

Beard, P.M., Stevenson, K., Pirie, A., Rudge, K., Buxton, D., Rhind, S.M., Sinclair, M.C., Wildblood, L.A. and Sharp, J.M. (2001c) Experimental paratuberculosis in calves following inoculation with a rabbit isolate of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 39, 3080–3084.

Buergelt, C.D., Layton, A.W., Ginn, P.E., Taylor, M., King, J.M., Habecker, P.L., Mauldin, E., Whitlock, R., Rossiter, C. and Collins, M.T. (2000) The pathology of spontaneous paratuberculosis in the North American bison (*Bison bison*). *Veterinary Pathology* 37, 428–438.

Chiodini, R.J. and Van Kruiningen, H.J. (1983) Eastern white-tailed deer as a reservoir of ruminant paratuberculosis. *Journal of the American Veterinary Medical Association* 182, 168–169.

Corn, J.L., Manning E.J.B., Sreevatsan, S. and Fischer J.R. (2005) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging birds and mammals on livestock premises. *Applied and Environmental Microbiology* 71, 6963–6967.

Cranwell, M.P. (1997) Johne's disease: past and present. *Cattle Practice* 5, 217–223.

Daniels, M.J. and Hutchings, M.R. (2001) Response of cattle and sheep to feed contaminated with rodent faeces. *The Veterinary Journal* 162, 211–218.

Daniels, M.J., Ball, N., Hutchings, M.R. and Greig, A. (2001) The grazing response of cattle to pasture contaminated with rabbit faeces and the implications for the transmission of paratuberculosis. *The Veterinary Journal* 161, 306–313.

Daniels, M.J., Henderson, D., Greig, A., Stevenson, K., Sharp, J.M. and Hutchings, M.R. (2003a) The potential role of wild rabbits *Oryctolagus cuniculus* in the epidemiology of paratuberculosis in domestic ruminants. *Epidemiology and Infection* 130, 553–559.

Daniels, M.J., Hutchings, M.R. and Greig, A. (2003b) The risk of disease transmission to livestock posed by contamination of farm stored feed by wildlife excreta. *Epidemiology and Infection* 130, 561–568.

Daniels, M.J., Lees, J.D., Hutchings, M.R. and Greig, A. (2003c) The ranging behaviour and habitat use of rabbits on farmland and their potential role in the epidemiology of paratuberculosis. *The Veterinary Journal* 165, 248–257.

Davidson, R.S., Marion, G., White, P.C.L. and Hutchings, M.R. (2008) Use of host population reduction to control wildlife infection: rabbits and paratuberculosis. *Epidemiology and Infection* 137, 131–138.

de Lisle, G., Yates, G.F., Cavaignac, S.M., Collins, D.M., Paterson, B.M. and Montgomery, R.H. (2003) *Mycobacterium avium* subsp. *paratuberculosis* in feral ferrets – a potential reservoir of Johne's disease. In: Juste, A.R., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 361–362.

Ferroglio, E., Nebbia, P., Robino, P., Rossi, L. and Rosati, S. (2000) *Mycobacterium paratuberculosis* infection in two free-ranging Alpine ibex. *Revue Scientifique et Technique* 19, 859–862.

Florou, M., Leontides, L., Kostoulas, P., Billinis, C., Sofia, M., Kyriazakis, I. and Lykotrafitis, F. (2008) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from non-ruminant wildlife living in the sheds and on the pastures of Greek sheep and goats. *Epidemiology and Infection* 136, 644–652.

Greig, A., Stevenson, K., Perez, V., Pirie, A.A., Grant, J.M. and Sharp, J.M. (1997) Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*). *Veterinary Record* 140, 141–143.

Greig, A., Stevenson, K., Henderson, D., Perez, V., Hughes, V., Pavlik, I., Hines, M.E., McKendrick, I. and Sharp, J.M. (1999) Epidemiological study of paratuberculosis in wild rabbits in Scotland. *Journal of Clinical Microbiology* 37, 1746–1751.

Gronesova, P., Ficova, M., Mizakova, A., Kabat, P., Trnka, A. and Betakova, T. (2008) Prevalence of avian influenza viruses, *Borrelia garinii*, *Mycobacterium avium*, and *Mycobacterium avium* subsp. *paratuberculosis* in waterfowl and terrestrial birds in Slovakia, 2006. *Avian Pathology* 37, 537–543.

Harris, S. and Lloyd, H.G. (1991) Fox (*Vulpes vulpes)*. In: Corbet, G.B. and Harris, S. (eds) *A Handbook of British Mammals*. Blackwell Scientific Press, Oxford, pp. 351–367.

Hulbert, I.A.R., Iason, G.R., Elston, D.A. and Racey, P.A. (1996) Home-range sizes in a stratified upland landscape of two lagomorphs with different feeding strategies. *Journal of Applied Ecology* 33, 1479–1488.

Hutchings, M.R., Kyriazakis, I., Anderson, D.H., Gordon, I.J. and Coop, R.L. (1998) Behavioural strategies used by parasitised and non-parasitised sheep to avoid ingestion of gastrointestinal nematodes. *Animal Science* 67, 97–106.

Judge, J., Kyriazakis, I., Greig, A., Allcroft, D.J. and Hutchings, M.R. (2005a) Clustering of *Mycobacterium avium* subsp. *paratuberculosis* in

rabbits and the environment: how hot is a hotspot? *Applied and Environmental Microbiology* 71, 6033–6038.

Judge, J., Greig, A., Kyriazakis, I. and Hutchings, M.R. (2005b) Ingestion of faeces by grazing herbivores – risk of inter-species disease transmission. *Agriculture Ecosystems and Environment* 107, 267–274.

Judge, J., Kyriazakis, I., Greig, A., Davidson, R.S. and Hutchings, M.R. (2006) Routes of intraspecific transmission of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits (*Oryctolagus cuniculus*): a field study. *Applied and Environmental Microbiology* 72, 398–403.

Judge, J., Davidson, R., Marion, G., White, P.C.L. and Hutchings, M.R. (2007) Persistence of *Mycobacterium avium* subspecies *paratuberculosis* in rabbits: the inter-play between horizontal and vertical transmission. *Journal of Applied Ecology* 44, 302–311.

King, C.M. (1991a) Stoat (*Mustela erminea*). In: Corbet, G.B. and Harris, S. (eds) *A Handbook of British Mammals*. Blackwell Scientific Press, Oxford, pp. 377–387.

King, C.M. (1991b) Weasel (*Mustela nivalis*). In: Corbet, G.B. and Harris, S. (eds) *A Handbook of British Mammals*. Blackwell Scientific Press, Oxford, pp. 387–396.

Kopecna, M., Ondrus, S., Literak, I., Klimes, J., Horvathova, A., Moravkova, M., Bartos, M., Trcka, I. and Pavlik, I. (2006) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in two brown bears in the central European Carpathians. *Journal of Wildlife Diseases* 42, 691–695.

Kopecna, M., Trcka, I., Lamka, J., Moravkova, M., Koubek, P., Heroldova, M., Mrlik, V., Kralova, A. and Pavlik, I. (2008) The wildlife hosts of *Mycobacterium avium* subsp. *paratuberculosis* in the Czech Republic during the years 2002–2007. *Veterinarni Medicina* 53, 420–426.

Leckie, F.M., Thirgood, S.J., May, R. and Redpath, S.M. (1998) Variation in the diet of red foxes on Scottish moorland in relation to prey abundance. *Ecography* 21, 599–604.

Mason, C.F. and Macdonald, S.M. (1995) Corvids feeding on carrion. *Bird Study* 42, 255–256.

Matthews, P.R.J. and Sargent, A. (1977) The isolation of mycobacteria from the brown hare (*Lepus europaeus*). *British Veterinary Journal* 133, 399.

Mokresh, A.H. and Butler, D.G. (1990) Granulomatous enteritis following oral inoculation of newborn rabbits with *Mycobacterium paratuberculosis* of bovine origin. *Canadian Journal of Veterinary Research* 54, 313–319.

OIE (2001) Working Group on Wildlife Diseases report for 2001, OIE, Paris. Available at: http://www.ewda.org/OIE%20Working%20Group%20Rep%202001.pdf (accessed 11 May 2009).

Palmer, M.V., Stoffregen, W.C., Carpenter, J.G. and Stabel, J.R. (2005) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from feral cats on a dairy farm with *MAP*-infected cattle. *Journal of Wildlife Diseases* 41, 629–635.

Raizman, E.A., Wells, S.J., Jordan, P.A., Del Giudice, G.D. and Bey, R.R. (2005) *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging deer and rabbits surrounding Minnesota dairy herds *Canadian Journal of Veterinary Research* 69, 32–38.

Ris, D.R., Hamel, K.L. and Ayling, J.M. (1987) Can sheep become infected by grazing pasture contaminated with Johne's disease? *New Zealand Veterinary Journal* 35, 137.

Ris, D.R., Hamel, K.L. and Weaver, A.M. (1988) Natural transmission of Johne's disease to feral goats. *New Zealand Veterinary Journal* 36, 98–99.

Sergeant, E.S.G. (2001) Ovine Johne's disease in Australia – the first 20 years. *Australian Veterinary Journal* 79, 484–491.

Smith, L.A., Marion, G., Swain, D.L., White, P.C.L. and Hutchings, M.R. (2009a) Inter- and intraspecific exposure to parasites and pathogens via the faecal–oral route: a consequence of behaviour in a patchy environment. *Epidemiology and Infection* 137, 630–643.

Smith, L.A., White, P.C.L., Marion, G. and Hutchings, M.R. (2009b) Livestock grazing behavior and inter- versus intra-specific disease risk via the fecal–oral route. *Behavioral Ecology* (in press).

Stabel, J.R. (1998) Biosecurity and disease – Johne's disease: a hidden threat. *Journal of Dairy Science* 81, 283–288.

Sweeney, R.W. (1996) Transmission of paratuberculosis. In: Sweeney, R.W., *Paratuberculosis (Johne's Disease)*. W.B. Saunders Company, Philadelphia, pp. 305–312.

Vary, P.H., Anderson, P.R., Green, E., Hermon-Taylor, J. and McFadden, J.J. (1990) Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *Journal of Clinical Microbiology* 28, 933–937.

Whittington, R.J., Reddacliff, L.A., Marsh, I., McAllister, S. and Saunders, V. (2000) Temporal patterns and quantification of excretion of *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Australian Veterinary Journal* 78, 34–37.

Whittington, R.J., Marsh, I.B., Taylor, P.J., Marshall, D.J., Taragel, C. and Reddaclife, L.A. (2003) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from environmental samples

collected from farms before and after destocking sheep with paratuberculosis. *Australian Veterinary Journal* 81, 559–563.

Williams, E.S., Spraker, T.R. and Schoonveld, G.G. (1979) Paratuberculosis (Johne's disease) in Bighorn sheep and a Rocky Mountain goat in Colorado. *Journal of Wildlife Diseases* 15, 221–227.

Zarnke, R.L., Dubey, J.P., Kwok, O.C.H. and Ver Hoef, J.M. (2000) Serologic survey for *Toxoplasma gondii* in selected wildlife species from Alaska. *Journal of Wildlife Diseases* 36, 219–224.

19 Experimental Ruminant Models of Paratuberculosis

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19.1 Introduction

In 2006, Johne's Disease (JD) researchers from the USA, the EU, Australia and New Zealand were convened under the sponsorship of the Johne's Disease Integrated Project (JDIP) as a committee to critically review the JD animal model literature and develop international standardized challenge models for cattle, sheep, goats, cervids and mice. The JDIP Animal Model Standardization Committee (AMSC) members consisted of 16 JD researchers, most of whom had successfully completed and published *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) challenge and/or vaccine efficacy studies in various species. The intent was to develop and propose international standard guidelines for models based on animal species that would gain acceptance worldwide. Parameters essential for the development of long-term and short-term infection models were outlined and harmonized to provide 'best fit' JD challenge models. The AMSC findings were published in 2007 (Hines *et al.*, 2007b). Another recent extensive critical review of JD experimental infection models was also published by Begg and Whittington (2008). The conclusions of both extensive reviews were often similar.

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19.2 Bovine Models

19.2.1 Long-term bovine *MAP* challenge model

This model is used typically for studies of vaccination efficacy, preventive or therapeutic interventions, long-term pathogenesis, the immune response and assessment of diagnostic assays. A wide variety of *MAP* strains have been used in experimental infections (Table 19.1). Many reports identify strains only as '*M. johnei*', 'field isolate' or 'clinical isolate', while others provide more specific strain designations. Strain K-10 was selected by the AMSC as the prototype strain for bovine infection models because it is known to be pathogenic and has a well-characterized genotype. However, the laboratory passage status of the K-10 *MAP* strain is currently unknown. Other low-passage virulent strains with a similar short-segment repeat genotype (e.g. 15g–5ggt genotype; Ghadiali *et al*., 2004) or equivalent PFGE or AFLP genotype may also be used (Hines *et al*., 2007b). This allows use of various local *MAP* strains and minimizes regulatory issues concerning the import of infectious organisms.

Types of inocula that have been administered include faeces from an infected animal, intestinal mucosal scrapings, lymph node homogenates or *in vitro*-cultured *MAP* (Table 19.1). In previous studies, *MAP* has been harvested from solid media (Herrold's egg yolk, Middlebrook 7H11, Middlebrook 7H10, Taylor's, Dubos) or from broth (Middlebrook 7H9). Inocula prepared from homogenized intestinal tissue, lymphoid tissue or faeces have been used, but these are difficult to standardize and aliquot for numerous studies. To address these issues, the AMSC has suggested that the chosen strain of *MAP* should be propagated *in vitro* from a master seed stock to mid-log-phase growth. Middlebrook 7H9 broth, supplemented with OADC, mycobactin J and 1% glycerol, was recommended as the culture medium (Hines *et al*., 2007b.).

Storage of the inoculum at 4 °C beyond 4 weeks may result in reduced viability (R. Whitlock, Philadelphia, 2008, personal communication) but storage up to 2 weeks at 4 °C is generally considered acceptable. However, it is recommended that the stored inoculum should be incubated at 37 °C for 2–3 h prior to administration. All samples within a study should be treated in the same manner.

Oral, intragastric and parenteral routes of inoculation have been successfully used (Table 19.1). However, the oral route is recommended by the AMSC as this most closely mimics natural exposure and allows *MAP* uptake by tonsillar tissue as well as the intestinal tract. The calf is induced to suckle from a syringe containing *MAP* mixed with a small volume of milk replacer or pasteurized milk. The mixture can also be gently expressed over the back of the tongue to induce swallowing. Administration by gastric tube is not recommended (Hines *et al*., 2007b).

Published doses range from 10 to 200 mg wet weight and 10^6 to 10^{11} colony-forming units (CFU) (Table 19.1) but were not based on standardized procedures. Doses of 5×10^8 CFU given on 2 consecutive days reliably resulted in infection in calves by 12–14 weeks post-inoculation (Sweeney *et al*., 2006). The AMSC recommends use of a standard bovine challenge dose of approximately 10^9 CFU/dose (100 mg wet weight) given on each of 2 successive days. Quantification of CFU should be by serial dilution and plating on solid medium known to support the organism without added antibiotics. Excessively large doses which produce clinical disease less than 18 months post-inoculation should be avoided as they are not typical in naturally occurring JD (Hines *et al*., 2007b).

Previous studies reported challenge of cattle from 1 day of age to adulthood (Table 19.1). While studies have suggested age-associated reduction in susceptibility (Hagan, 1938; Doyle, 1953, 1956; Larsen *et al*., 1975), a definitive relationship between infectious dose and age has not been established. Some flexibility in age of inoculation is required due to differences in experimental objectives and timing of other interventions, such as vaccination. No published reports provide substantial evidence of breed differences in susceptibility among the common dairy breeds, but some have suggested there may be increased susceptibility of Jersey cattle to natural infection (Koets *et al*., 2000, and unpublished anecdotal data). However, because of the difference in clinical presentation of paratuberculosis in

Table 19.1. Bovine models for *Mycobacterium avium* subsp. *paratuberculosis* infection.

Reference	Age at start	*MAP* strain	Dose(s)	Infection method	Study length	Clinical disease	Faecal culture	Tissue culture	Immune testing	Histopathology
Rankin, 1958	1 month	Clinical isolate (8 subcultures)	100 mg wet wt	IV	4 years	4/6	6/6	6/6	ND[a]	4/6
Rankin, 1961a	3 years	Clinical isolate (8 subcultures)	100 mg wet wt	IV	4 years	0/5	0/5	5/5	ND[a]	1/5
Rankin, 1961b	1, 3, 6 months	Clinical isolate	Unknown	Faecal–oral exposure	5 years	4/9	8/9	9/9	ND[a]	9/9
Payne and Rankin, 1961a	3 months	Clinical isolate	200 mg wet wt	Oral (milk)	Variable up to 14 months	No	ND[a]	Variable	ND[a]	Increased with period of infection
Payne and Rankin, 1961b	3 months or 3 years	Clinical isolate	200 mg wet wt	Oral (milk or water)	Variable up to 6 months	No	ND[a]	6/8 calves 1/8 cows	ND[a]	Increased with period of infection
Rankin, 1962	Variable	Clinical isolate	Unknown	Faecal–oral exposure	4 years	5/6 calves 1/7 cows	6/6 calves 4/7 cows	4/7 cows	Skin test + CFT –	6/6 calves 1/7 cows
Gilmour *et al.*, 1965	3 weeks	Clinical isolate	1×10^8; 1×10^{10} 1 dose $\times$ 10 weeks	Oral (tube)	Variable up to 13 months	No	ND[a]	Increased with period of infection and dose	Skin test +	Increased with period of infection and dose
Stuart, 1965	1 week	Clinical isolate	100 mg wet wt	IV	10 months	Yes	40/40	NR[b]	CFT +	18/40
Larsen *et al.*, 1978	1 month	Naturally infected cows	Unknown	Oral (natural exposure)	Up to 6 years	Yes	20/175	31/175	Skin test +	22/175
Larsen *et al.*, 1973	16 days	Clinical isolate	180 mg wet wt	Oral (milk)	5 months	No	8/8	7/8	Skin test +	8/8
Thorel *et al.*, 1984	4 weeks	Various isolates of *MAP*	1×10^9	IV	12 months	No	ND[a]	23/23	ND[a]	8/23
Krishnappa *et al.*, 1989	NR calves	Clinical isolate from mucosal scrapings	50 g 1 dose $\times$ 10 weeks	Oral	30 weeks	No	1/12	ND[a]	AGID + CIE +	ND[a]

(*Continued*)

Table 19.1. *continued*

Reference	Age at start	*MAP* strain	Dose(s)	Infection method	Study length	Clinical disease	Faecal culture	Tissue culture	Immune testing	Histopathology
Szilagyi *et al.*, 1989	17 days	Strain 5889	1×10^8 2 × 5 days with 15 days between	Oral	400 days	No	NR[b]	NR[b]	NR[b]	ND[a]
Saxegaard, 1990	4 weeks	Clinical goat isolate from tissue	10 mg wet wt; 10 doses over 10 days	Oral (milk)	Variable up to 18 months	No	ND[a]	4/4	ELISA –	No
McDonald *et al.*, 1999	2 months	Clinical isolate	2 g wet wt 3 doses	Oral (gastric tube)	Up to 27 months	No	2/4	1/4	IFN-γ + ELISA –	2/4
McDonald *et al.*, 1999	2 months	Clinical isolate	20 g wet wt 3 doses	Oral (gastric tube)	Up to 27 months	No	4/4	4/4	IFN-γ + ELISA +	4/4
Beard *et al.*, 2001	1 week	Rabbit and bovine isolate	1×10^9 3 doses 1×/week	Oral	6 months	No	5/8 – rabbit 0/4 – bovine	7/8 – rabbit 3/4 – bovine	ND[a]	3/8 – rabbit 2/4 – bovine
Waters *et al.*, 2003	2 weeks	Strain K-10	1.6×10^7 1 × 4 weeks	IT	320 days	No	3/3	3/3	IFN-γ + ELISA +	0/3
Uzonna *et al.*, 2003	28 days	Clinical isolate	1×10^{10} 2 doses	Oral (milk)	49 days	No	ND[a]	15/15	IFN-γ + ELISA –	0/15
Koo *et al.*, 2004	1–2 days	NR	1×10^7 7 doses	Oral	6 months	No	ND[a]	2/3 – PCR	IFN-γ + ELISA –	ND
Simutis *et al.*, 2005	4 weeks	Strain 19698	1×10^8	SC	150 days	No	0/25	1/25	IFN-γ + Skin test +	0/25
Stabel *et al.*, 2003	4 months	Clinical isolate (bovine and bison)	1×10^9 5 doses	Oral (gastric tube)	6 months	No	1/6 – cattle 2/6 – bison	5/6 – cattle 6/6 – bison	IFN-γ + ELISA –	0/6 – cattle 0/6 – bison
Sweeney *et al.*, 2006	2–3 days	Clinical isolate (ATCC 700533)	2.5×10^{10} 2 doses	Oral (milk)	44 days	No	0/6	60/6	ND[a]	06/6
Sweeney *et al.*, 2006	21–22 days	Clinical isolate (ATCC 700533)	H-5×10^9 M-5×10^8 L-1.5×10^6	Oral (milk)	44 days	No	H – 0/8 M – 0/6 L – 0/6	H – 8/8 M – 6/6 L – 6/6	ND[a]	H – 0/8 M – 0/6 L – 0/6

Koets *et al.*, 2006	1 month	Clinical cow faeces (high shedder)	20 g 9 doses	Oral (faeces in milk)	644 days	No	8/10	ND[a]	IFN-γ + ELISA +	ND[a]
Rosseels *et al.*, 2006	2–3 weeks	Strain 19698	1×10^8 10 doses	Oral	875 days	No	NR[b]	ND[a]	Skin test + ELISA –	ND[a]
Wu *et al.*, 2007	3–4 weeks	Strain K-10, strain 19698, Δ*gcpE* mutant	1×10^7–10^8 1 dose	Ileum injection	4 days to 9 months	No	0/5	5/5	Skin test – ELISA – IFN-γ + TNFα + IL12 + IL4 –	5/5
Stewart *et al.*, 2007	6 weeks 14 weeks	Bovine isolate	$1–2 \times 10^{10}$ 15–20 g tissue	Oral	54 months	No No	3/5 5/5	0/3 0/5	IFN-γ + ELISA	0/8
	6 weeks 14 weeks	Ovine isolate	4 weekly doses $1–2 \times 10^{10}$ 15–20 g tissue 4 weekly doses	Oral	35 months	No No	0/5 3/5	0/5 0/4	IFN-γ + ELISA	0/9
Kathaperumal *et al.*, 2008	8 weeks	*MAP*66115-98	1×10^7 daily doses, 2 groups rechallenged 8 weeks later with 5×10^8 for 5 days	Oral	23 weeks PI	No	NR	13/24	IFN-γ + LBT + TNFα + IL12 + IL2 + IL4 – IL10 – FLOW	0/24

[a]ND = not determined.
[b]NR = not reported; AGID = agar gel immunodiffusion test; CFT = complement fixation test; CIE = crossed immunoelectrophoresis; FLOW = flow cytometry; PI = post-inoculation.

Bos indicus breeds, the AMSC has recommended that *Bos taurus* breeds be used exclusively for studies of bovine paratuberculosis (Hines *et al*., 2007b). Current experience suggests that 100 mg pelleted wet weight on 2 successive days results in demonstrable infection in calves less than 8 weeks of age (Hines *et al*., 2007b).

Current diagnostic methods are ineffective for screening of individual calves at or before 8 weeks of age. Calves should be purchased from JD-free farms to ensure lack of prior exposure. In the USA, herds that have achieved the equivalent of Status Level 3 or 4 in the National Voluntary Bovine Johne's Disease Status Control Program should be chosen as source herds (USDA-APHIS, 2002; Hines *et al.*, 2007b). This equates to a closed herd with no history of JD in the previous 5 years, tested annually with at least one whole-herd-negative serological test and one whole-herd-negative faecal test (second lactation and older animals).

Calves should have received adequate quantities of colostrum. Milk replacer, if used, should be high quality and of animal origin (i.e. casein not soy protein) and high-quality rations should be fed. Routine vaccinations may be administered but should not be given on the same days as JD vaccination or *MAP* administration. Anthelmintics and parasiticides should be given to all animals at similar dosages. If individual therapy is necessary (i.e. respiratory infection), an antibiotic known to have minimal effects on *MAP* (i.e. ceftiofur) should be used.

Passive (pass-through) shedding occurs as early as 12 h after oral inoculation. Detection of passive shedding by culture provides additional confirmation of inoculum viability and the sensitivity of the faecal culture method. Positive faecal culture results 14 days after inoculation should be considered shedding due to infection, except in very heavily contaminated environments (R. Whitlock, Philadelphia, 2008, personal communication). The AMSC recommends that animals should have faecal cultures at least monthly during the course of the study. Gross examination and culture of tissues for *MAP* as well as histopathological examination of tissues to identify acid-fast organisms and lesions characteristic of JD should be performed in all studies. At the inoculation dose recommended, colonization of tissues can be detected by culture in most animals by 4–12 weeks after inoculation, although culture of multiple tissues (minimum of three ileum including ileocaecal valve, three jejunum, one duodenum, one spiral colon and three mesenteric lymph nodes including ileocaecal nodes) is necessary. Although tissue samples should be culture-positive by 12 weeks, investigators should not expect to find histological lesions at this early stage. A necropsy and histopathology scoring system should be used (Gonzalez *et al.*, 2005; Hines *et al.*, 2007b). The clinical status of the study animals should, at a minimum, be assessed and recorded monthly and at necropsy (Hines *et al.*, 2007b).

The method of culturing faecal and tissue samples should permit quantification (or semi-quantification, e.g. by counting CFU on solid medium or time to positive detection in automated liquid culture systems). Decontamination by incubation of faecal and tissue samples in 0.6% hexadecyl pyridinium chloride (HPC) for 14–16 h and 3 h, respectively, is strongly recommended (Hines *et al.*, 2007b). There is wide variation in culture methods currently used and batch-to-batch differences occur in media. All samples should be processed on medium from the same batch. Fresh samples may be processed immediately or frozen at –70 °C and thawed only once, with all samples treated in the same manner.

19.2.2 Short-term bovine *MAP* challenge models

Experimental *MAP* infection models that employ direct surgical access to the ileum for administration of *MAP* and collection of intestinal samples have been described (Allen *et al.*, 2005, 2009). In general, these models are most useful for short-term study of host–pathogen interactions. Many parameters should be the same as for long-term challenge, including strain, inoculum preparation and quantification, storage, animal selection criteria and quality control issues. Exceptions are age of administration, dose, experimental end points and sample collection.

Ileal cannulation model

In this model, calves are cannulated at 8 weeks of age under general anaesthesia, using a modification of the method of Streeter *et al.* (1991), as previously described (Hines *et al.*, 2007b; Allen *et al.*, 2009). Calves are inoculated in the ileum with 10^{10} CFU of *MAP* in 20 ml phosphate-buffered saline. *MAP* is taken up by M cells and by dendritic and epithelial cells of the ileum and jejunum within 30 min (Momotani *et al.*, 1988; Sigurðardóttir *et al.*, 1999, 2001). The inoculation procedure is repeated the next day. Ileal mucosal biopsies can be obtained at various time intervals, to test for *MAP* infection and local immune responses.

Invasion/surgical model

Surgical incision and direct deposition of *MAP* into the ileum has been recently employed to establish a model for intestinal invasion to other organs within a few hours of infection (Wu *et al.*, 2007). Although infected calves survive the surgery and repeated biopsy sampling up to 10 months following infection, the focus of this model is to examine early *MAP* intestinal interactions. The movement of *MAP* from intestine to liver, spleen or mesenteric lymph node was shown to differentiate between *MAP* strains with different virulence phenotypes.

Intestinal loop model

An alternative surgical approach is the ligated intestinal loop model with an injected inoculum of 3×10^9 CFU (~ 300 mg wet weight) (Momotani *et al.*, 1988). This is only suitable for studies <12 h in duration and includes end points such as *MAP* detection and histopathology. This model is useful for ultrastructural studies of the early host–pathogen interaction and host immune response as measured by cytokine gene expression.

19.3 Caprine Models

19.3.1 Long-term caprine *MAP* challenge model

The long-term caprine *MAP* challenge model is intended primarily for pathogenesis and vaccine efficacy studies; however, it could also be useful for other studies, including evaluation of diagnostic assays. No particular goat breed has been recommended and any common local breed is generally considered acceptable since no studies have been performed to investigate breed susceptibility.

The AMSC suggests animals should undergo a selection process that includes validation/precertification of infection status. The JD status of the herd of origin is the best method to validate infection status of young kids. Kids should be selected only from closed herds in which all adults have been tested negative by ELISA and faecal culture for at least 1 year and JD vaccination is not practised. All kids should receive sufficient quantities of colostrum. No specific recommendations were made for rations other than they should be of high quality (Hines *et al.*, 2007b).

Goat challenge studies have successfully used *MAP* isolates from clinically infected goats, cattle and sheep, as well as humans (Table 19.2). Twenty-six to 50% of isolates from clinical cases in goats have been of the cattle type (also called C type or Type II) (Motiwala *et al.*, 2004; Sevilla *et al.* 2005, 2007). A more detailed description of the different types of *MAP* is given elsewhere (see Stevenson, Chapter 12, this volume). Motiwala *et al.* (2004) detected little genetic diversity between and within bovine and goat isolates, but Sevilla *et al.* (2007) showed that goat isolates had a high degree of genetic heterogeneity, and Mobius *et al.* (2008) found a high degree of genetic heterogeneity in isolates from German cattle.

In some studies, the inoculum consisted of *in vitro*-cultured *MAP*, while in others it was prepared from homogenized intestinal mucosal tissue scrapings from a clinically diseased animal. The use of a virulent, low-passage goat clinical isolate cultured *in vitro* from a master seed stock, with a genotype identical or closely related to bovine strain K-10, is recommended by the AMSC (Hines *et al.*, 2007b).

The recommended method of inoculum preparation, quantification and storage is the same as stated above for the cattle model. The majority of studies used Middlebrook 7H9 + OADC + mycobactin J + glycerol or Tween 80 for *in vitro* cultivation. This medium with the addition of glycerol (1%), but without Tween

Table 19.2. Caprine models for *Mycobacterium avium* subsp. *paratuberculosis* infection.

Reference	Age at start	*MAP* strain	Dose(s)	Infection method	Study length	Clinical disease	Faecal culture	Tissue culture	Immune testing	Histopathology
Harding, 1957	NR[a]	NR[a]	NR[a]	IH IV IV/Oral Oral	9–16 months	NR[a]	ND[b]	17/24	ND[b]	23/24
Van Kruiningen *et al.*, 1986	2–12 days	Human isolate Linda	3.2×10^7 4.0×10^8 50 mg wet wt	Oral (milk)	Up to 310 days	No	1/4	4/4	ND[b]	4/4
Sigurðardóttir *et al.*, 1999	7–26 days	Clinical goat isolate (3 passages)	10 mg dry wt 1×10 days	Oral (milk)	Up to 49 weeks	No	0/8	2/8	Skin test + ELISA + CFT +	3/8
Sigurðardóttir *et al.*, 2001	18–21 days	Clinical goat isolate	2.365 mg dry wt/3ml 4 loops	Distal ileal ligation	1 h	No	ND[b]	ND[b]	ND[b]	*MAP* in M cells and leucocytes
Sigurðardóttir *et al.*, 2001	23–39 days	ATCC bovine strain 19698	3×10^7 per sleeve	Everted intestine sleeve	1 h	No	ND[b]	ND[b]	ND[b]	*MAP* uptake by M cells and enterocytes
Storset *et al.*, 2001	5–8 weeks	Clinical goat isolate (P173) 2 passages	10 mg 3×10 weeks	Oral (milk)	Up to 117 weeks	Yes	4/7	5/7	IFN-γ + LBT + ELISA +	6/7
Valheim *et al.*, 2002	5–8 weeks	Clinical goat isolate (P173) 2 passages	10 mg 3×10 weeks	Oral (milk)	Up to 117 weeks	Yes	ND[b]	5/7	ND[b]	6/7

Munjal *et al.*, 2005	5–8 weeks	Clinical goat isolate (tissue)	1×10^{10} 7×2 days	Oral	Up to 270 days	Yes	1/10	2/10 (PCR)	LBT + ELISA + AGID +	5/10
Stewart *et al.*, 2006	5 months	Clinical bovine isolate (tissue/ culture)	1×10^{10} 20 g wet wt	Oral	54 months	Yes	10/10	8	IFN-γ + ELISA +	NR[a]
	10 months	Clinical sheep isolate (tissue/ culture)	1×4 weeks		35 months	Yes	9/10	1	IFN-γ + ELISA +	NR[a]
Hines *et al.*, 2007a	3–7 days	Clinical goat isolate	1.5×10^{9} $4 \times$ alternate days	Oral	6–9 months	Yes	Yes	Yes	IFN-γ + Skin test + ELISA + AGID +	50/50
Kathaperumal *et al.*, 2009	5–10 days	*MAP*66115-98	5×10^{8} daily doses, for 7 days	Oral	38 weeks post-vaccination	No	0/25	15/25	IFN-γ + LBT + IL10 + FLOW	12/25

[a]NR = not reported.
[b]ND = not determined; AGID = agar gel immunodiffusion test; CFT = complement fixation test; LBT = lymphocyte blastogenesis test.

or antibiotics, is considered to be the best choice by the AMSC (Hines *et al.*, 2007b). The routes of administration have varied (Table 19.2), and essentially all routes tested have been successful in establishing infection, with even the aerosol route resulting in intestinal pathology (Harding, 1957). The oral route most closely parallels natural exposure and is generally considered the best route of administration.

The size of the challenge inoculum has also varied widely (Table 19.2), ranging from 2.37 to 200 mg and 3×10^7 to 8×10^{10} CFU, and all were generally successful in establishing infection. Two consecutive daily doses of 10^9 organisms (approximately 100 mg pelleted wet weight/dose and ~200 mg total dose) should establish infection in most kids without overwhelming experimental interventions (Hines *et al.*, 2007b).

The age at which to inoculate has varied from the day of birth to 10 months of age (Table 19.2). It is not known whether age-related resistance occurs in goats. The age of administration will depend on the experimental goals and end points, but generally the goats should be less than 4 months of age. Clinical disease is expected to develop in a low percentage of animals at 9–10 months post-inoculation (Hines *et al.*, 2007b).

The experimental end points will depend on the goals of the study. The minimal AMSC recommendations for goat experiments were to determine infection status through a combination of culture, PCR and histopathology, using quantitative or semi-quantitative methods (Hines *et al.*, 2007b). A lesion grading system for gross and histopathological findings (Hines *et al.*, 2007a,b) should be used, with a sufficient range in values to allow statistical analysis. AMSC recommendations for methods of faecal and tissue *MAP* culture and for sample handling are the same as for the bovine model. Like cattle, positive faecal cultures 2 weeks or more post-inoculation should be considered to be due to infection. Faecal cultures should be performed at least monthly from all animals.

19.3.2 Short-term caprine *MAP* challenge models

In general, these models are used for short-term study of host–pathogen interactions. Many parameters should be the same as for long-term challenge, including strain, inoculum preparation and quantification, storage, animal selection criteria and quality control issues.

Intestinal loop model

An intestinal loop model, as previously described in goats by Sigurðardóttir *et al.* (2001), can be used for studying initial JD bacterial–host interactions including bacterial attachment and internalization, bacterial localization, cytokine regulation and early bacterial gene regulation. *MAP* strain, dose, quantification, culture medium, animal selection and sample collection should be similar to the long-term challenge model (Hines *et al.*, 2007b). Strain selection and quantification, animal selection and sample collection should also be similar to those for the long-term challenge model.

Everted intestine sleeve model

An everted intestine sleeve model, as described by Sigurðardóttir *et al.* (2005), is also useful for studying initial host–bacterial interactions, such as bacterial attachment and internalization, bacterial localization and early bacterial gene regulation, but may have limitations when evaluating host gene and early cytokine regulation in response to infection. Approximately 1.0 cm segments of small intestine are excised, everted, washed, maintained in tissue culture and bathed in a suspension of *MAP* for short periods of time. Strain selection and quantification, animal selection and sample collection should be similar to those for the long-term challenge model.

19.4 Ovine Models

19.4.1 Long-term ovine *MAP* challenge model

This model is used primarily for pathogenesis and vaccine efficacy studies but could also be used for evaluating diagnostic assays. The Merino, as well as some dairy sheep breeds, may be more susceptible to *MAP* than other ovine breeds (Frank Griffin, Dunedin, and

Ramon Juste, Derio, 2008, personal communication), but genetic resistance to JD has not yet been identified. While the Merino breed is preferred by members of the AMSC, any breed shown to be susceptible to *MAP* is acceptable (Hines *et al.*, 2007b).

Homogenized lymphoid tissue or intestinal mucosal scrapings from a clinically diseased animal appear to be the best source for reproducing the ovine infection (Table 19.3), but this method is not recommended by the AMSC as a standard for experimental challenge studies in sheep. Successful ovine experimental challenge studies have used *MAP* isolates from cattle, sheep, wildlife and humans (Table 19.3). Until 2000, the majority of experimental studies in sheep did not use characterized ovine *MAP* strains, in contrast to later studies, which have used tissue homogenates or low-passage ovine *MAP* strains grown *in vitro* (Gwozdz *et al.*, 2000; Stewart *et al.*, 2004; Begg *et al.*, 2005). The most common *MAP* isolates from clinical JD in sheep belong to the sheep type (also called S type or Type I). A more detailed description of the different types of *MAP* is given elsewhere (see Stevenson, Chapter 12, this volume). Marked genetic diversity was detected among ovine isolates, as well as between ovine and both bovine and goat isolates (Motiwala *et al.*, 2004). Therefore, to most closely reproduce natural ovine infections, any confirmed-virulent ovine clinical isolate that can be cultivated *in vitro* is considered acceptable (Hines *et al.*, 2007b). Since little information is available on these ovine strains, it is critical that each isolate used be genotyped to allow future comparison. A virulent, low-passage ovine strain cultivated *in vitro* was recommended as the inoculum source (Hines *et al.*, 2007b).

Culture media used in published ovine studies have included Middlebrook 7H11 broth, Middlebrook 7H10 agar and BACTEC™ 460 medium with increased egg concentration. Middlebrook 7H11 broth is the medium preferred by the AMSC for inoculum preparation, but any medium capable of supporting the growth of ovine *MAP* strains is acceptable. The quantification of organisms and handling and storage of the inoculum should be similar to those described for cattle (Hines *et al.*, 2007b).

The usual route of administration has been oral, using either saline or milk suspensions, but other protocols have also been successfully used (Table 19.3). Since the oral route most closely parallels natural exposure, it is generally considered the best route of administration. The challenge inoculum in previous studies has ranged from 15 to 200 mg wet weight of organisms, 2.6×10^1 to 2.6×10^{11} CFU and 0.65 to 80 g of macerated infected tissue from a clinical case (Table 19.3). The lowest doses have generally not been effective in establishing infection (Reddacliff *et al.*, 2004). Route and frequency of dosing have varied widely between studies (Table 19.3). Based on recent experimental evidence, three consecutive daily oral doses consisting of 10^9 organisms per dose (~100 mg pelleted wet weight/dose; 300 mg total) should be used to establish infection (Begg *et al.*, 2005).

The age at inoculation has ranged from day of birth to 10 months (Table 19.3). Experimental challenge at any time up to 4 months of age is considered appropriate. However, age at time of challenge will be influenced by experimental objectives. The AMSC made no recommendation as to when a JD vaccine or other intervention should be administered. As for other species' models, positive faecal cultures 2 weeks or more post-inoculation should be considered to be due to infection. Faecal culture is less reliable in animals infected with sheep *MAP* strains (as compared with cattle strains), and expense is a major consideration in large ovine studies, particularly in the field; none the less, all animals should have periodic faecal cultures using validated methods.

Animals to be included in challenge studies should be validated as infection-free based upon the infection-free status of the foundation flock. Lambs for experimental studies should only be selected from closed flocks in which all adults have been negative on ELISA and faecal culture for at least 1 year and JD vaccination is not practised. All lambs should receive adequate quantities of colostrum.

A combination of necropsy with histopathology and bacterial culture to determine levels of tissue colonization is recommended by the AMSC as the minimal requirement to determine infection status of experimental

Table 19.3. Ovine models for *Mycobacterium avium* subsp. *paratuberculosis* infection.

Reference	Age at start	*MAP* strain	Dose(s)	Infection method	Study length	Clinical disease	Faecal culture	Tissue culture	Immune testing	Histopathology
Brotherston *et al.*, 1961	10 weeks	Sheep isolate (var. bovine)	1×10^7	IV	Up to 22 months	No	ND[a]	13/16	ND[a]	ND[a]
Brotherston *et al.*, 1961	1 week		1×10^8 1×3 weeks	Oral	53 weeks	No No Yes	ND[a]	10/16	ND[a]	ND[a]
	7–10 days		1×10^8 1×8 weeks	Oral	53 weeks		ND[a]	9/9	ND[a]	ND[a]
	3 weeks		$1 \times 10^{3–9}$ 1×10 weeks	Oral	Up to 9 months		ND[a]	24/51	ND[a]	ND[a]
Gilmour and Brotherston, 1962	8 months	Sheep isolate (var. bovine)	1×10^9	Oral	Up to 56 days	No	ND[a]	10/12	ND[a]	ND[a]
Nisbet *et al.*, 1962	1 week	Sheep isolate (var. bovine)	1×10^8	Oral	53 weeks	NR[b]	ND[a]	ND[a]	ND[a]	6/12
	7–10 days		1×10^8 1×8 weeks		53 weeks		ND[a]	ND[a]	ND[a]	7/9
	3 weeks		$1 \times 10^{3–9}$ 1×10 weeks		Up to 9 months		ND[a]	23/35	ND[a]	20/35
	3 months		1×10^6 1×10 weeks		Up to 9 months		ND[a]	6/12	ND[a]	5/12
Gilmour and Brotherston, 1962	3 months 10 months	Sheep isolate (var. bovine)	1×10^6 1×10 weeks	Oral	Up to 18 weeks	NR[b]	ND[a]	6/9 9/9	Skin test +	ND[a]
Kluge *et al.*, 1968	3 weeks	Clinical bovine isolate (tissue)	50 mg dry wt 50 mg dry wt 200 mg dry wt	IV IT Oral	Up to 16 months	Yes	NR[b]	NR[b]	NR[b]	Yes
Merkal *et al.*, 1968a,b	3 weeks	Clinical bovine isolate (tissue)	50 mg dry wt 50 mg dry wt 200 mg dry wt	IV IT Oral (milk)	Up to 16 months	Yes	NR[b]	NR[b]	Skin test + CFT + AGID +	Yes
Gilmour *et al.*, 1978	5 months	Sheep isolate (3 passages)	1×10^9 1×10 weeks	Oral	Up to 27 months	Yes	ND[a]	11/22	Skin test +	15/22

Williams *et al.*, 1983a,b	4–5 months	Clinical sheep isolate	50 mg wet wt	Oral	Up to 12 months	No	0/9	5/9	ND[a]	ND[a]
Dukes *et al.*, 1992	1 day	Saiga antelope (tissue)	0.325 g mucosa 2 doses	Oral	18 months	Yes			ND[a]	Yes
Juste *et al.*, 1994	3 months	Bovine isolate (3 passages)	150 mg wet wt 2 doses	Oral	220 days	No	0/5	Yes	ELISA +	Yes
Burrells *et al.*, 1995	1 day	Deer isolate strain JD88/107	1×10^8 1×9 weeks	Oral	NR[b]	No	ND[a]	ND[a]	IFN-γ + LBT +	ND[a]
Begara-McGorum *et al.*, 1998	5–9 days	Deer isolate strain JD88/107	1×10^9 3×2 days	Oral	Up to 41 days	No	1/8	3/8	IFN-γ – ELISA –	4/8
Gwozdz and Thompson, 2002	1–4 weeks	Clinical sheep isolate (tissue)	3.4×10^9	Oral (gastric tube)	108 weeks	Yes	ND[a]	1/10 – PCR	IFN-γ + ELISA + AGID +	1/10
			4.4×10^8		53 weeks			4/9 – PCR	CFT +	3/9
Gwozdz *et al.*, 2000	1–2 months	Clinical sheep isolate (tissue)	4.4×10^8	Oral (gastric tube)	53 weeks	Yes	3/14 – PCR	10/14 – PCR	IFN-γ + ELISA +	9/14
Gwozdz *et al.*, 2001	1–4 weeks	Clinical sheep isolate (tissue)	3.4×10^9	Oral (gastric tube)	108 weeks	Yes	ND[a]	ND[a]	ND[a]	18/28
Reddacliff and Whittington, 2003	12–16 weeks	Sheep isolate (faeces)	2.6×10^1 1×10^4 1×10^8 3×1 week 10×1 week	Oral		No	0/30	0/12 0/12 6/6	IFN-γ + Skin test + ELISA +	0/30
Kurade *et al.*, 2004	8–12 weeks	Clinical sheep isolate (tissue)	1×10^{10} 8×3 days	Oral	Up to 330 days	Yes	3/20	7/20	LBT + ELISA +	20/20
Stewart *et al.*, 2004	6 months	Clinical bovine isolate (tissue/ culture)	1×10^{10} 20 g wet wt	Oral	54 months	Yes	7/10	1/5	IFN-γ +	NR[b]
	10 months	Clinical sheep isolate (tissue/ culture)	1×4 weeks		35 months	Yes	5/10	1/5	ELISA +	

(*Continued*)

Table 19.3. *continued*

Reference	Age at start	*MAP* strain	Dose(s)	Infection method	Study length	Clinical disease	Faecal culture	Tissue culture	Immune testing	Histopathology
Begg *et al.*, 2005	12 weeks	Clinical sheep isolates (JD3 – tissue and W – high- and low-passage culture)	1×10^9 4 × 3 days	Oral	10 months	No	NR[b]	21/30	IFN-γ + LBT + ELISA +	17/30
			5×10^8 1×10^9 1 × 3 weeks	Oral	13 months	Yes	NR[b]	16/30		22/30
			5×10^7	IT		No	NR[b]	8/12		7/12
			5×10^7	Oral						
			1×10^9	Oral	16 months			8/12		9/12
			2 × 1 month					3/12		1/12
Begg and Griffin, 2005	2.5 months	Clinical sheep isolate – JD3	5×10^8 1 × 3 weeks	Oral	Up to 22 months	Yes	NR[b]	NR[b]	IFN-γ + LBT + ELISA +	23/30

[a]ND = not determined.
[b]NR = not reported; AGID = agar gel immunodiffusion test; CFT = complement fixation test; LBT = lymphocyte blastogenesis test.

animals (Hines *et al.*, 2007b). The lesion grading system used for gross and histopathology findings should have a sufficient range in values to allow for statistical analysis (Juste *et al.*, 1994; Hines *et al.*, 2007a,b). Animals should be observed until clinical signs develop in a proportion of the group. The type, quantity and processing of samples collected will vary with the purpose, number of animals, goals and cost of the study. All samples should be collected, handled and processed in the same way to ensure uniformity. Ovine strains apparently grow better in liquid medium, and BACTEC™ 460 was suggested by the AMSC to be the best system by which to recover ovine strains, but no single standard culture medium or method for culture of faeces or tissues was recommended. Decontamination and culture should be performed similarly to those previously described for cattle, providing, at a minimum, semi-quantitative results (Hines *et al.*, 2007b).

19.4.2 Short-term ovine *MAP* challenge models

In general, these models are most useful for short-term study of host–pathogen interactions. Many parameters should be the same as for long-term challenge, including strain, inoculum preparation and quantification, storage, animal selection criteria and quality control issues.

Ileal cannulation model

An ileal cannulation model similar to that previously described for cattle has not been investigated in sheep. This model is considered to have only limited practical use in sheep. However, Hein and co-workers have developed a model for investigating enteric diseases where the afferent lymphatics draining the small intestine are cannulated (Hein *et al.*, 2004). This model allows longitudinal sampling of immune cells and fluids draining the small intestine, which may be useful in evaluating the immunology associated with JD in sheep.

Intestinal loop and everted intestine sleeve models

No study describing an intestinal loop or everted intestinal sleeve model for sheep was found in our literature review. Models similar to that described previously in goats should be equally applicable in sheep for studying the initial bacterial–host interactions *in vivo*.

19.5 Cervid Models

19.5.1 Long-term cervid *MAP* challenge model

No published studies were found in our literature review where intestinal loop, everted intestine or other short-term models have been used in deer. Models similar to that described previously for goats should be equally applicable for deer in studying the initial bacterial–host interactions *in vivo*. In contrast, there is some literature on the long-term cervid challenge model, primarily used for pathogenesis and vaccine efficacy studies. This model may also be useful for evaluation of diagnostic assays and heritable resistance.

High levels of heritable resistance and susceptibility to mycobacterial infection are documented in deer (Mackintosh *et al.*, 2000). Deer appear to be a useful host for disclosing diagnostic markers by which to monitor infection (Griffin *et al.*, 2005), protective immunity or resistance to infection. The majority of studies performed in cervids (Table 19.4) have involved red deer (*Cervus elaphus*), which seem to be more naturally susceptible (Mackintosh *et al.*, 2003) than other cervids (Williams *et al.*, 1983a,b). Experimentally infected deer have a broad spectrum of responses, ranging from limited infection to extremes of pathology and clinical disease. The range of pathology allows clear stratification and analysis of the impact of interventions (Mackintosh *et al.*, 2007). The predominance of bovine strains in naturally infected deer suggests that cervids may provide an alternative experimental challenge model for JD in cattle. Red deer may be naturally infected with either bovine or ovine strains (de Lisle *et al.*, 2006), but they appear more susceptible and develop more severe disease with bovine strains (O'Brien *et al.*, 2006). Clinical signs develop in 25–30% of red deer between 4 and 12 months post-bovine strain challenge (Mackintosh *et al.*,

Table 19.4. Cervid and/or exotic ruminant species models for *Mycobacterium avium* subsp. *paratuberculosis* infection.

Reference	Species	Age at start	*MAP* strain	Dose(s)	Infection method	Study length	Clinical disease	Faecal culture	Tissue culture	Immune testing	Histopathology[c]
Williams *et al.*, 1983a	Bighorn × mouflon	4–5 months	Clinical bighorn sheep isolate	50 mg wet wt	Oral	6 or 12 months	No	NR[a]	9/9	ND[b]	8/9
	Mule deer						Yes		8/8		8/8
	White-tail deer						Yes		2/2		2/2
	Elk						No		8/8		8/8
Williams *et al.*, 1983b	Bighorn × mouflon	4–5 months	Clinical bighorn sheep isolate	50 mg wet wt	Oral	6 or 12 months	No	9/9	9/9	ND[b]	NR[a]
	Mule deer						Yes	2/8	8/8		
	White-tail deer						Yes	0/2	2/2		
	Elk						No	0/8	8/8		
Mackintosh *et al.*, 2003	Red deer	4 months	Clinical deer isolate (bovine var.)	NR 1 × 4 days	Oral	12 months	Yes	NR[a]	NR[a]	Skin test + LBT + ELISA +	39/43
Stabel *et al.*, 2003	Bison	4 months	Clinical isolate (bovine and bison)	1×10^9 5 doses	Oral (gastric tube)	6 months	No	2/6	6/6	IFN-γ + ELISA –	0/6
Mackintosh *et al.*, 2005	Red deer	4 months	Clinical deer isolate (tissue; bovine var.)	1×10^9 1 × 4 days	Oral	12 months	Yes	NR[a]	NR[a]	LBT + ELISA +	42/42
O'Brien *et al.*, 2006	Red deer	4 months	Clinical red deer isolate (tissue; bovine var.)	1×10^9 1×10^7 1×10^3 1 × 4 days	Oral	Up to 44 weeks	NR[a]	NR[a]	40/64	IFN-γ + LBT + ELISA +	Lesions apparent
			Clinical sheep isolate (JD3)	1×10^7 1 × 4 days			NR[a]		11/16	IFN-γ +/– LBT + ELISA –	Lesions apparent

Mackintosh *et al*., 2007	Red deer	4 months	Clinical deer isolate (tissue; bovine var.) Sheep tissue isolate Ovine strain	$10^3 \times 4$ (LB) $10^7 \times 4$ (MB) $10^9 \times 4$ (HB) $10^7 \times 4$ (MO)	Oral	12 months	NR[a]	NR[a]	8/16 LB 16/16 MB 16/16 HB 8/16 MO	LBT + IFN-γ ELISA + (IgG1)	NR[a]
Robinson *et al*., 2008	Red deer	6.5 months	Deer tissue isolate (bovine var.)	1×10^9 4 daily doses	Oral	56 weeks	Yes	NR	NR	IFN-γ + ELISA + FLOW	Lesions apparent
Mackintosh *et al*., 2008	Red deer	4–4.5 months	Deer tissue isolate (bovine var.)	1×10^8 4 daily doses	Oral	14 months	No	21–33%	89–97%	ELISA + Skin test +	Lesions apparent (~20%)

[a]NR = not reported.

[b]ND = not determined; [c]in the cervid model histopathology following necropsy may be used to stratify disease severity; LBT = lymphocyte blastogenesis test; FLOW = flow cytometry.

2005). Red deer are recommended as the model of choice for cervid challenge studies.

Strain, dose, route of inoculation and infection or disease end points used in cattle and goat models appear to apply equally well to cervids (Hines *et al.*, 2007b). In addition, there is evidence that most of the bovine immunological reagents are useful in studies with red deer, with the known exception of TGFβ (Frank Griffin, Dunedin, 2008, personal communication). A dose of 10^3 CFU of a bovine strain produces equivalent infection and pathology to 10^6 CFU of an ovine strain (O'Brien *et al.*, 2006). Thus, bovine strains are preferred, and all challenge parameters in red deer should be the same as those for the bovine and caprine models. A strain with genotype similar to bovine strain K-10 at the bovine dose (10^9organisms, 100 mg) given on 2 consecutive days was recommended as most appropriate for deer challenge studies by the AMSC (Hines *et al.*, 2007b). Age of administration will vary depending on the goals of the study. Deer should be challenged at less than 3 months of age. The relatively early onset of pathology and disease in deer suggest that this model may be cost-effective and informative for study of *MAP* infection. Access to deer bloodlines with resistant or susceptible phenotypes for *Mycobacterium bovis* infection may be valuable in exploring candidate genes which contribute to heritable resistance to other mycobacterial infections.

References

Allen, A.J., Barrington, G.M., Hamilton, M.J., Stabel, J.R., Robbe-Austerman, S. and Davis, W.C. (2005) Development of a calf ileal cannulation model to facilitate testing of existing and candidate vaccines for *M. avium* subsp. *paratuberculosis*. In: Manning, E.J.B. and Nielsen, S.S. (eds) *Proceedings of the 8th International Colloquium for Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, p. 148.

Allen, A.J., Park, K.-T., Abdellrazeq, G.S.A., Rihan, H.M., Barrington, G.M., Lahmers, K.K., Hamilton, M.J. and Davis, W.C. (2009) Development of a bovine ileal cannulation model to study the immune response and mechanisms of pathogenesis of paratuberculosis. *Clinical and Vaccine Immunology* 16, 453–463.

Beard, P.M., Stevenson, K., Pirie, A., Rudge, K., Buxton, D., Rhind, S.M., Sinclair, M.C., Wildblood, L.A., Jones, D.G. and Sharp, J.M. (2001) Experimental paratuberculosis in calves following inoculation with a rabbit isolate of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 39, 3080–3084.

Begara-McGorum, I., Wildblood, L.A., Clarke, C.J., Connor, K.M., Stevenson, K., McInnes, C.J., Sharp, J.M. and Jones, D.G. (1998) Early immunopathological events in experimental ovine paratuberculosis. *Veterinary Immunology and Immunopathology* 63, 265–287.

Begg, D.J. and Griffin, J.F.T. (2005) Vaccination of sheep against *M. paratuberculosis*: immune parameters and protective efficacy. *Vaccine* 23, 4999–5008.

Begg, D.J. and Whittington, R.J. (2008) Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Journal* 176, 129–145.

Begg, D.J., O'Brien, R., Mackintosh, C.G. and Griffin, J.F.T. (2005) Experimental model for Johne's disease in sheep. *Infection and Immunity* 73, 5603–5611.

Brotherston, J.G., Gilmour, N.J.L. and Samuel, J.M. (1961) Quantitiative studies of *Mycobacterium johneii* in the tissues of sheep. I. Routes of infection and assay of viable *M. johneii*. *Journal of Comparative Pathology* 71, 286–299.

Burrells, C., Inglis, N.F., Davies, R.C. and Sharp, J.M. (1995) Detection of specific T cell reactivity in sheep infected with *Mycobacterium avium* subspecies *silvaticum* and *paratuberculosis* using two defined mycobacterial antigens. *Veterinary Immunology and Immunopathology* 45, 311–320.

de Lisle, G.W., Cannon, M.C., Yates, G.F. and Collins, D.M. (2006) Use of a polymerase chain reaction to subtype *Mycobacterium avium* subspecies *paratuberculosis*, an increasingly important pathogen of farmed deer in New Zealand. *New Zealand Veterinary Journal* 54, 195–197.

Doyle, T.M. (1953) Susceptibility to Johne's disease in relation to age. *Veterinary Record* 65, 363–365.

Doyle, T.M. (1956) Johne's disease. *Veterinary Record* 68, 869–878.

Dukes, T.W., Glover, G.J., Brooks, B.W., Duncan, J.R. and Swendrowski, M. (1992) Paratuberculosis in Saiga antelope (*Saiga tatarica*) and experimental transmission to domestic sheep. *Journal of Wildlife Diseases* 28, 161–170.

Ghadiali, A.H., Strother, M., Naser, S.A., Manning, E.J.B. and Sreevatsan, S. (2004) *Mycobacterium avium* subsp. *paratuberculosis* strains

isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. *Journal of Clinical Microbiology* 42, 5345–5348.

Gilmour, N.J.L. and Brotherston, J.G. (1962) Quantitative studies of *Mycobacterium johneii* in the tissues of sheep. IV. The distribution of *M. johneii* shortly after oral dosing. *Journal of Comparative Pathology* 72, 165–169.

Gilmour, N.J.L., Nisbet, D.I. and Brotherson, J.G. (1965) Experimental oral infection of calves with *Mycobacterium johneii*. *Journal of Comparative Pathology* 75, 281–286.

Gilmour, N.J.L., Angus, K.W. and Mitchell, B. (1978) Intestinal infection and host response to oral administration of *Mycobacterium johneii* in sheep. *Veterinary Microbiology* 2, 223–235.

Gonzalez, J., Geijo, M.V., Garcia-Pariente, C., Verna, A., Corpa, J.M., Reyes, L.E., Ferreras, M.C., Juste, R.A., Garcia Marin, J.F. and Perez, V. (2005) Histopathological classification of lesions associated with natural paratuberculosis infection in cattle. *Journal of Comparative Pathology* 133, 184–196.

Griffin, J.F.T., Spittle, E., Rodgers, C.R., Liggett, S., Cooper, M., Bakker, D. and Bannantine, J.P. (2005) Immunoglobulin G1 enzyme-linked immunosorbent assay for diagnosis of Johne's disease in red deer (*Cervus elaphus*). *Clinical Diagnostic Laboratory Immunology* 12, 1401–1409.

Gwozdz, J.M. and Thompson, K.G. (2002) Antigen-induced production of interferon-γ in samples of peripheral lymph nodes from sheep experimentally inoculated with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* 84, 243–252.

Gwozdz, J.M., Thompson, K.G., Manktelow, B.W., Murray, A. and West, D.M. (2000) Vaccination against paratuberculosis of lambs already infected experimentally with *Mycobacterium avium* subspecies *paratuberculosis*. *Australian Veterinary Journal* 78, 560–566.

Gwozdz, J.M., Thompson, K.G. and Manktelow, B.W. (2001) Lymphocytic neuritis of the ileum in sheep with naturally acquired and experimental paratuberculosis. *Journal of Comparative Pathology* 24, 317–320.

Hagan, W.A. (1938) Age as a factor in susceptibility to Johne's disease. *Cornell Veterinarian* 28, 34–40.

Harding, H.P. (1957) Experimental infection with *Mycobacterium johnei*. II. The histopathology of infection in experimental goats. *Journal of Comparative Pathology* 67, 37–52.

Hein, W.R., Barber, T., Cole, S.A., Morrison, L. and Pernthaner, A. (2004) Long-term collection and characterization of afferent lymph from the ovine small intestine. *Journal of Immunological Methods* 293, 153–168.

Hines, M.E., II, Stiver, S., Giri, D., Whittington, L., Watson, C., Johnson, J., Pence, M., Baldwin, C.A. and Aly, S. (2007a) Efficacy of spheroplastic and cell wall competent vaccines for *Mycobacterium avium* subsp. *paratuberculosis* in experimentally-challenged baby goats. *Veterinary Microbiology* 120, 261–283.

Hines, M.E., II, Stabel, J.R., Sweeney, R., Griffin, F., Talaat, A.M., Bakker, D., Benedictus, G., Davis, W.C., de Lisle, G.W., Gardner, I.A., Juste, R.A., Kapur, V., Koets, A., McNair, J., Pruitt, G. and Whitlock, R.H. (2007b) Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Veterinary Microbiology* 122, 197–222.

Juste, R.A., Garcia-Marin, J.F., Peris, B., Saez de Ocariz, C. and Badiola, J.J. (1994) Experimental infection of vaccinated and non-vaccinated lambs with *Mycobacterium paratuberculosis*. *Journal of Comparative Pathology* 110, 185–194.

Kathaperumal, K., Park, S.-U., McDonough, S., Stehman, S., Akey, B., Huntley, J., Wong, S., Chang, C.-F. and Chang, Y.-F. (2008) Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine* 26, 1652–1663.

Kathaperumal, K., Kumanan, V., McDonough, S., Chen, L.-H., Park, S.-U., Moreira, M.A.S., Akey, B., Huntley, J., Chang, C.-F. and Chang, Y.-F. (2009) Evaluation of immune responses and protective efficacy in a goat model following immunization with a cocktail of recombinant antigens and a polyprotein of *Mycobacterium avium* subsp. *paratuberculosis*. *Vaccine* 27, 123–135.

Kluge, J.P., Monlux, W.S., Kopecky, K.E. and Lehmann, R.P. (1968) Experimental paratuberculosis in sheep after oral, intratracheal, or intravenous inoculation: lesions and demonstration of etiologic agent. *American Journal of Veterinary Research* 29, 953–962.

Koets, A.P., Adugna, G., Janss, L.L.G., van Weering, H.J., Kalis, C.H.J., Wentink, G.H., Rutten, V.P.M.G. and Schukken, Y.H. (2000) Genetic variation of susceptibility to *Mycobacterium avium* subsp. *paratuberculosis* infection in dairy cattle. *Journal of Dairy Science* 83, 2702–2708.

Koets, A., Hoek, A., Langelaar, M., Overdijk, M., Santema, W., Franken, P., van Eden, W. and Rutten, V. (2006) Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine

against bovine paratuberculosis. *Vaccine* 24, 2550–2559.

Koo, H.C., Park, Y.H., Hamilton, M.J., Barrington, G.M., Davies, C.J., Kim, J.B., Dahl, J.L., Waters, W.R. and Davis, W.C. (2004) Analysis of the immune response to *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected calves. *Infection and Immunity* 72, 6870–6883.

Krishnappa, G., Jagannath, C. and Rao, B.U. (1989) The specificity of antibody response in experimental and natural bovine paratuberculosis studied by crossed immunoelectrophoresis with intermediate gel. *Veterinary Microbiology* 21, 67–78.

Kurade, N.P., Tripathi, B.N., Rajukumar, K. and Parihar, N.S. (2004) Sequential development of histologic lesions and their relationship with bacterial isolation, faecal shedding, and immune response during progressive stages of experimental infection of lambs with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 41, 378–387.

Larsen, A.B., Merkal, R.S. and Moon, H.W. (1973) Evaluation of a paratuberculosis vaccine given to calves before infection. *American Journal of Veterinary Research* 35, 267–269.

Larsen, A.B., Merkal, R.S. and Cutlip, R.C. (1975) Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 36, 255–257.

Larsen, A.B., Moyle, A.I. and Himes, E.M. (1978) Experimental vaccination of cattle against paratuberculosis (Johne's disease) with killed bacterial vaccines: a controlled field study. *American Journal of Veterinary Research* 39, 65–69.

Mackintosh, C.G., Qureshi, T., Waldrup, K., Labes, R.E., Dodds, K.G. and Griffin, J.F.T. (2000) Genetic resistance to experimental infection with *Mycobacterium bovis* in red deer (*Cervus elaphus*). *Infection and Immunity* 68, 1620–1625.

Mackintosh, C.G., Labes, R.E., de Lisle, G.W. and Griffin, J.F.T. (2003) Experimental challenge trial of novel Johne's disease vaccine. In: *Proceedings of the Deer Branch New Zealand Veterinary Association*. Deer Branch New Zealand Veterinary Association, Wellington, New Zealand, pp. 91–98.

Mackintosh, C.G., de Lisle, G.W. and Griffin, J.F.T. (2005) Experimental challenge model of paratuberculosis in red deer. In: Manning, E.J.B. and Nielsen, S.S. (eds) *Proceedings of the 8th International Colloquium for Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, pp. 115–117.

Mackintosh, C.G., Labes R.E., Clark, G., de Lisle, G.W. and Griffin, J.F.T. (2007) Experimental infections in young red deer (*Cervus elaphus*) with a bovine and an ovine strain of *Mycobacterium avium* subsp. *paratuberculosis*. *New Zealand Veterinary Journal* 55, 23–29.

Mackintosh, C.G., Labes R.E., Thompson, B.R., Clark, R.G., de Lisle, G.W., Johnstone, P.D. and Griffin, J.F.T. (2008) Efficacy, immune responses and side-effects of vaccines against Johne's disease in young red deer (*Cervus elaphus*) experimentally challenged with *Mycobacterium avium* subsp. *paratuberculosis*. *New Zealand Veterinary Journal* 56, 1–9.

McDonald, W.L., Ridge, S.E., Hope, A.F. and Condron, R.J. (1999) Evaluation of diagnostic tests for Johne's disease in young cattle. *Australian Veterinary Journal* 77, 113–119.

Merkal, R.S., Larsen, A.B., Kopecky, K.E., Kluge, J.P., Monlux, W.S., Lehmann, R.P. and Quinn, L.Y. (1968a) Experimental paratuberculosis in sheep after oral, intratracheal, or intravenous inoculation: serologic and intradermal tests. *American Journal of Veterinary Research* 29, 963–969.

Merkal, R.S., Kluge, J.P., Monlux, W.S., Larsen, A.B., Kopecky, K.E., Quinn, L.Y. and Lehmann, R.P. (1968b) Experimental paratuberculosis in sheep after oral, intratracheal, or intravenous inoculation: histochemical localization of hydrolase activities. *American Journal of Veterinary Research* 29, 985–994.

Mobius, P., Luyven, G., Hotzel, H. and Kohler, H. (2008) High genetic diversity among *Mycobacterium avium* subsp. *paratuberculosis* strains from German cattle herds shown by combination of IS*900* restriction fragment length polymorphism analysis and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing. *Journal of Clinical Microbiology* 46, 972–981.

Momotani, E., Whipple, D.L., Thiermann, A.B. and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Veterinary Pathology* 25, 131–137.

Motiwala, A.S., Strother, M., Amonsin, A., Byrum, B., Naser, S.A., Stabel, J.R., Shulaw, W.P., Bannantine, J.P., Kapur, V. and Sreevatsan, S. (2004) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing and identification of unique targets for diagnosis. *Journal of Clinical Microbiology* 41, 2015–2026.

Munjal, S.K., Tripathi, B.N. and Paliwal, O.P. (2005) Progressive immunopathological changes during

early stages of experimental infection of goats with *Mycobacterium avium* subspecies *paratuberculosis*. *Veterinary Pathology* 42, 427–436.

Nisbet, D.I., Gilmour, N.J.L. and Brotherston, J.G. (1962) Quantitative studies of *Mycobacterium johneii* in the tissues of sheep. III. Intestinal histopathology. *Journal of Comparative Pathology* 72, 80–91.

O'Brien, R., Mackintosh, C.G., Bakker, D., Kopencna, M., Pavlik, I. and Griffin, J.F.T. (2006) Immunological and molecular characterization of susceptibility in relationship to bacterial strain differences in *Mycobacterium avium* subsp. *paratuberculosis* infection in the red deer. *Infection and Immunity* 74, 3530–3537.

Payne, J.M. and Rankin, J.D. (1961a) The pathogenesis of experimental Johne's disease in calves. *Research in Veterinary Science* 2, 167–174.

Payne, J.M. and Rankin, J.D. (1961b) A comparison of the pathogenesis of experimental Johne's disease in calves and cows. *Research in Veterinary Science* 2, 175–179.

Rankin, J.D. (1958) The experimental infection of cattle with *Mycobacterium johnei*. I. Calves inoculated intravenously. *Journal of Comparative Pathology* 68, 331–337.

Rankin, J.D. (1961a) The experimental infection of cattle with *Mycobacterium johneii*. II. Adult cattle inoculated intravenously. *Journal of Comparative Pathology* 71, 6–9.

Rankin, J.D. (1961b) The experimental infection of cattle with *Mycobacterium johneii*. III. Calves maintained in an infectious environment. *Journal of Comparative Pathology* 71, 10–15.

Rankin, J.D. (1962) The experimental infection of cattle with *Mycobacterium johneii*. IV. Adult cattle maintained in an infectious environment. *Journal of Comparative Pathology* 72, 113–117.

Reddacliff, L.A. and Whittington, R.J. (2003) Experimental infection of weaner sheep with S strain *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* 96, 247–258.

Reddacliff, L.A., McClure, S.J. and Whittington, R.J. (2004) Immunoperoxidase studies of cell mediated immune effector cell populations in early *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep. *Veterinary Immunology and Immunopathology* 97, 149–162.

Robinson, M., O'Brien, R., Mackintosh, C. and Griffin, F. (2008) Differential immune responses of red deer (*Cervus elaphus*) following experimental challenge with *Mycobacterium avium* subsp. *paratuberculosis*. *Clinical and Vaccine Immunology* 15, 963–969.

Rosseels, V., Marche, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K. and Huygen, K. (2006) Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infection and Immunity* 74, 202–212.

Saxegaard, F. (1990) Experimental infection of calves with an apparently specific goat-pathogenic strain of *Mycobacterium paratuberculosis*. *Journal of Comparative Pathology* 102, 149–156.

Sevilla, I., Singh, S.V., Garrido, J.M., Aduriz, G., Rodríguez, S., Geijo, M.V., Whittington, R.J., Saunders, V., Whitlock, R.H. and Juste, R.A. (2005) Molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* strains from different hosts and regions. *Reviews of Science Technology* 24(3), 1061–1066.

Sevilla, I., Garrido, J.M., Geijo, M. and Juste, R.A. (2007) Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiology* 7, 18.

Sigurðardóttir, O.G., Press, C.M., Saxegaard, F. and Evensen, O. (1999) Bacterial isolation, immunological response, and histopathological lesions during the early subclinical phase of experimental infection of goat kids with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 36, 542–550.

Sigurðardóttir, O.G., Press, C.M. and Evensen, O. (2001) Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. *Veterinary Pathology* 38, 184–189.

Sigurðardóttir, O.G., Bakke-McKellep, A.M., Djonne, B. and Evensen, O. (2005) *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated by the everted sleeve method. *Comparative Immunology, Microbiology and Infectious Disease* 28, 223–230.

Simutis, F.J., Cheville, N.F. and Jones, D.E. (2005) Investigation of antigen-specific T-cell responses and subcutaneous granuloma development during experimental sensitization of calves with *Mycobacterium avium* subsp. *paratuberculosis*. *American Journal of Veterinary Research* 66, 474–482.

Stabel, J.R., Palmer, M.V. and Whitlock, R.H. (2003) Immune responses after oral inoculation of weanling bison or beef calves with a bison or cattle isolate of *Mycobacterium avium* subsp.

paratuberculosis. *Journal of Wildlife Diseases* 39, 545–555.

Stewart, D.J., Vaughan, J.A., Stiles, P.L., Noske, P.J., Tizard, M.L.V., Prowse, S.J., Michalski, W.P., Butler, K.L. and Jones, S.L. (2004) A long-term study in Merino sheep experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*: clinical disease, faecal culture and immunological studies. *Veterinary Microbiology* 104, 165–178.

Stewart, D.J., Vaughan, J.A., Stiles, P.L., Noske, P.J., Tizard, M.L.V., Prowse, S.J., Michalski, W.P., Butler, K.L. and Jones, S.L. (2006) A long-term study in Angora goats experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*: clinical disease, faecal culture and immunological studies. *Veterinary Microbiology* 113, 13–24.

Stewart, D.J., Vaughan, J.A., Stiles, P.L., Noske, P.J., Tizard, M.L.V., Prowse, S.J., Michalski, W.P., Butler, K.L. and Jones, S.L. (2007) A long-term bacteriological and immunological study in Holstein–Friesian cattle experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis* and necropsy culture results for Holstein–Friesian cattle, Merino sheep and Angora goats. *Veterinary Microbiology* 122, 83–96.

Storset, A.K., Hasvold, H.J., Valheim, M., Brun-Hansen, H., Berntsen, G., Whist, S.K., Djonne, B., Press, C.M., Holstad, G. and Larsen, H.J.S. (2001) Subclinical paratuberculosis in goats following experimental infection: an immunological and microbiological study. *Veterinary Immunology and Immunopathology* 80, 271–287.

Streeter, M.N., Barron, S.J., Wagner, D.G., Hibberd, C.A., Owens, F.N. and McCollum, F.T. (1991) Technical note: a double L intestinal cannula for cattle. *Journal of Animal Science* 69, 2601–2607.

Stuart, P. (1965) Vaccination against Johne's disease in cattle exposed to experimental infection. *British Veterinary Journal* 121, 289–318.

Sweeney, R.W., Uzonna, J., Whitlock, R.H., Habecker, P.L., Chilton, P. and Scott, P. (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subsp. *paratuberculosis* organism recovery in a short-term bovine experimental infection model. *Research in Veterinary Science* 80, 253–259.

Szilagyi, M., Kormendy, B., Suri, A., Tuboly, S. and Nagy, G. (1989) Experimental paratuberculosis (Johne's disease) – studies on biochemical parameters in cattle. *Archives of Experimental Veterinary Medicine Leipzig* 43, 463–470.

Thorel, M-F., Pardon, P., Irgens, K., Marly, J. and Lechopier, P. (1984) Paratuberculose expérimentale: pouvoir pathogène chez le veau de souches de Mycobactéries mycobactine-dépendantes. *Annales Recherche Vétérinaire* 15, 365–374.

USDA APHIS (2002) *Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program*. Available at: http://www.johnesdisease.org/Uniform%20Program%20Standards%20for%20the%20Voluntary%20Bovine%20National%20Johne's%20Disease%20Program.pdf (accessed 12 May 2009).

Uzonna, J.E., Chilton, P., Whitlock, R.H., Habecker, P.L., Scott, P. and Sweeney, R.W. (2003) Efficacy of commercial and field-strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* 21, 3101–3109.

Valheim, M., Storset, A.K., Aleksandersen, M., Brun-Hansen, H. and Press, C.M. (2002) Lesions in subclinical paratuberculosis of goats are associated with persistent gut-associated lymphoid tissue. *Journal of Comparative Pathology* 127, 194–202.

Van Kruiningen, H.J., Chiodini, R.J., Thayer, W.R., Coutu, J.A., Merkal, R.S. and Runnels, P.L. (1986) Experimental disease in infant goats induced by a *Mycobacterium* isolated from a patient with Crohn's disease: a preliminary report. *Digestive Disease Science* 31, 1351–1360.

Waters, W.R., Miller, J.M., Palmer, M.V., Stabel, J.R., Jones, D.E., Koistinen, K.A., Steadham, E.M., Hamilton, M.J., Davis, W.C. and Bannantine, J.P. (2003) Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infection and Immunity* 71, 5130–5138.

Williams, E.S., Synder, S.P. and Martin, K.L. (1983a) Experimental infection of some North American wild ruminants and domestic sheep with *Mycobacterium paratuberculosis*: clinical and bacteriological findings. *Journal of Wildlife Diseases* 19, 185–191.

Williams, E.S., Synder, S.P. and Martin, K.L. (1983b) Pathology of experimental infection of North American wild ruminants and domestic sheep with *Mycobacterium paratuberculosis*. *Veterinary Pathology* 20, 274–290.

Wu, C., Livesey, M., Schmoller, S.K., Manning, E.J.B., Steinberg, H., Davis, W.C., Hamilton, M.J. and Talaat, A.M. (2007) Invasion and persistence of *Mycobacterium paratuberculosis* during early stages of Johne's disease in calves. *Infection and Immunity* 75, 2110–2119.

20 Experimental Small Animal Models of Paratuberculosis

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20.1 Introduction

Small rodent models of paratuberculosis are normally utilized to reduce the cost associated with larger models of infection (e.g. cattle or goat models). In addition, the use of small animal models can successfully reproduce certain signs of paratuberculosis in a shorter period compared with ruminant models. Mice represent the forefront small animal model for paratuberculosis. The other small animal model, the white New Zealand rabbit (Beard *et al.*, 2001), is infrequently used and will be only briefly discussed in this chapter. The rabbit model is usually utilized to produce antibodies useful for researchers focused on Johne's disease (JD). As for many infectious agents, mice provide a convenient model to study certain aspects related to the immunopathogenesis of infection with *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). Given the impressive repertoire of immunological reagents and genetically distinct breeds, mice can serve in testing the virulence of *MAP* mutants, examining vaccine candidates and dissecting the host–pathogen interaction during infection (Mullerad *et al.*, 2002a; Shin *et al.*, 2006; Bull *et al.*, 2007; Chen *et al.*, 2008).

20.2 The Mouse Model and the Genetics of *MAP* Pathogenesis

Because *MAP* infection can require prolonged times from infection until clinical signs of JD appear, mice offer the advantage that *MAP*-specific granuloma formation can be observed within 3–6 weeks of infection (Tanaka *et al.*, 1994; Shin *et al.*, 2006). Using immunocompromised (SCID) mice, intestinal lesions characteristic of JD in cattle were obtained, including luminal infiltration with mononuclear cells containing acid-fast bacilli (Mutwiri *et al.*, 2001). Also, infected intestines show increased ion

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secretion and damage to the epithelial cell lining of intestinal villi, both potential reasons for the chronic wasting syndrome associated with JD in infected cows. Factors associated with *MAP* invasion and attachment to intestinal cells, particularly M cells, were initially examined using the ileal loop of mice, where mycobacterial fibronectin attachment protein was analysed (Secott *et al.*, 2002, 2004). Furthermore, the antigenicity of *MAP*-specific novel antigens was tested first in mice (and rabbits) as a prelude to further analysis on naturally infected cattle (Paustian *et al.*, 2004). This stepwise approach provides a paradigm for examining the functional significance of novel antigens and may also serve in the development of novel diagnostic reagents.

Another attractive aspect of the mouse model is its usefulness for studying a large number of animals in an experiment on a relatively low budget. This economy with numbers is especially marked in comparison to ruminant models. In our experience, we have been able to assay the virulence of 11 *MAP* transposon mutants in BALB/c within a relatively short time (12 weeks of infection), enabling us to identify seven virulence genes in *MAP*, including *pstA* and *papA2* (Shin *et al.*, 2006). In a subsequent experiment, we assayed six additional mutants, again in a short time frame, permitting us to document the contribution of transcriptionally regulated genes (e.g. *lipN*, *prrA*) to *MAP* survival during infection. Further validation of the attenuation of some of these mutants was successfully attempted in the cattle model of paratuberculosis for a *gcpE* mutant (Wu *et al.*, 2007a) as well as a *pstA* mutant (Wu *et al.*, 2009). Results from the cattle model validated the strategy for testing the virulence of *MAP* mutants in the mouse model first, before testing in any ruminant model of infection. It will be interesting to see whether mutants attenuated in ruminants are found for which no phenotype is detected in currently used mouse strains.

20.3 The Mouse Model and Immunology of *MAP* Infection

The role and type of immune responses against *MAP* can be dissected in specific strains of mice that are either naturally of different susceptibility to mycobacterial infection or engineered to have specific gene disruptions. Studies of macrophages derived from mice with specific mutations of pathogen recognition receptors (e.g. TLR-2, TLR-4, NOD2) have pointed to a role for innate immunity in the recognition and immune response directed against *MAP* (Ferwerda *et al.*, 2007). Regarding adaptive immunity, the role of γδ T cells was studied in BALB/c mice with a knockout of γδ T cells to determine their role in granuloma formation (Tanaka *et al.*, 2000). Another study, using mice on a C57BL/6 background, demonstrated a major role for αβ cells compared with γδ cells, by quantifying tissue lesions in mice deficient for the αβ T-cell receptors (Stabel and Ackermann, 2002). Reconstitution of *MAP*-infected SCID mice with spleen cells from immunocompetent BALB/c mice significantly reduced lesions caused by *MAP* infection, indicating that protection against *MAP* is dependent on cell-mediated immunity (Mutwiri *et al.*, 2002). The involvement of this type of immunity in responding to infection with *MAP* was further documented by studying T-cell responses specific to the 35 kDa protein of *MAP* (Basagoudanavar *et al.*, 2006). Finally, mouse models can also inform on the quality of adaptive immune responses. Both Th1 and Th2 responses were observed in mice inoculated with recombinant antigens (e.g. superoxide dismutase) (Mullerad *et al.*, 2002a), opening the door for testing these antigens as vaccine candidates against *MAP* infection.

Several acellular, protein-based subunit vaccines have been developed against *MAP* and tested in the mouse model of paratuberculosis. Recombinant *MAP* proteins of Ag85B, superoxide dismutase and thiol peroxidase elicit strong immune responses in mice (Mullerad *et al.*, 2002a,b, 2003). In these models, subunit vaccine inoculation triggers strong levels of cytokines (e.g. IFN-γ, TNF-α, IL4, IL6) in the presence of the Ribi adjuvant. Interestingly, a polypeptide encoding three open reading frames (ORFs) of *MAP* antigens provided protective responses against challenge with the virulent strain of *MAP* (Chen *et al.*, 2008). In another challenge system with *Mycobacterium tuberculosis*, the polypeptide vaccine construct gave a similar protection to DNA immunization

of mice (Skeiky *et al.*, 2004), by an unknown mechanism of protection. Because DNA immunization does not require protein preparations, this facilitates the screening of *MAP* ORFs in a mouse model, using a protocol of expression library immunization (Huntley *et al.*, 2005; Talaat and Stemke-Hale, 2005). A total of 26 protective antigens have been identified in this way. Recently, an adenovirus-based vaccine (Ad5) encoding four fused antigens elicited strong immune responses against *MAP* using a strategy based on vaccination with DNA constructs followed by Ad5 inoculation of mice (Bull *et al.*, 2007). All these vaccine candidates will now require further assessment and validation in a ruminant model of paratuberculosis. In the present scenario, the mouse model can act as a preliminary screen of vaccine candidates to eliminate non-protective constructs. To determine if the mouse model faithfully serves this purpose will require comparative experiments in different hosts.

20.4 Parameters of the Mouse Model of Paratuberculosis

Depending on the specific goal of mouse *MAP* infections, different protocols can be employed. In immunization studies, candidate vaccines can be inoculated by the specified route and then protection can be measured 12 weeks following challenge with a fully virulent strain of *MAP*. To evaluate the mouse model, sections from the liver, spleen and intestine are usually analysed for mycobacterial colonization and histological lesions. In addition, immunological assessment can be done at the time of challenge with the virulent strain, as an attempt to uncover potential correlates of protection. Generally a significant reduction compared with controls of organ load with *MAP* (at least 1 log) and granuloma formation are the major parameters for evaluation. These two readouts depend on the genetic background of the mouse and the virulence of the mycobacterial strain (Tanaka, *et al.*, 1994; Mullerad *et al.*, 2002b; Shin *et al.*, 2006). Some studies have used BALB/c or C57Bl/6 mice, which are immunocompetent but susceptible to mycobacterial infection (Veazey *et al.*, 1995). Others have used C3H mice, which are more resistant to *MAP* infection (Tanaka *et al.*, 1994). Despite the presence of granulomatous lesions in both susceptible and resistant breeds of mice, the number of lesions and bacterial colonization levels declined dramatically in the resistant breed. Athymic nude mice provide another alternative model. When these mice were inoculated orally with *MAP*, there was bacterial shedding in the faeces (Hamilton *et al.*, 1991), a rarely reported event in other mice.

Another important parameter of the mouse model is the dose of infection and the route of administration. Oral inoculation of 10^{11} colony-forming units (CFU)/animal produces granulomatous lesions in 58% of the animals; however, lesions are limited to the mesenteric lymph node (Veazey *et al.*, 1995). On the other hand, intraperitoneal (IP) injection of a low dose of *MAP* (10^6 CFU) induces a small number of epithelioid granulomas, as compared with the multifocal granulomas composed of macrophages and epithelioid cells that are observed after a higher dose of infection (10^8 CFU) (Tanaka *et al.*, 2000). In our hands, an IP dose of 10^8 CFU/animal of virulent strains of *MAP* typically produces enough granulomatous responses to record by 3 weeks post-infection, and these lesions are well formed by 6 and 12 weeks post-infection (Shin *et al.*, 2006; Wu *et al.*, 2007b). In several reports, IP injection produced the highest reproducible infection rate (100%) compared with other routes of delivery (Mutwiri *et al.*, 1992). In a high-throughput format, analysis is focused on the liver and intestine of inoculated animals. Both organs are analysed for histological lesions as well as mycobacterial colonization over a period of 12 weeks (Shin *et al.*, 2006) (see Fig. 20.1).

Despite the several advantages for the mouse model of *MAP*, several shortcomings limit the utility of this model. For example, there are known anatomical, physiological and immunological differences between cattle and mice, which could affect the outcome of infection. Also, some of the typical features of JD in cattle (e.g. diarrhoea, severe intestinal lesions) cannot be reproduced in mice. None the less, the mouse model for paratuberculosis may play an important role in vaccine development

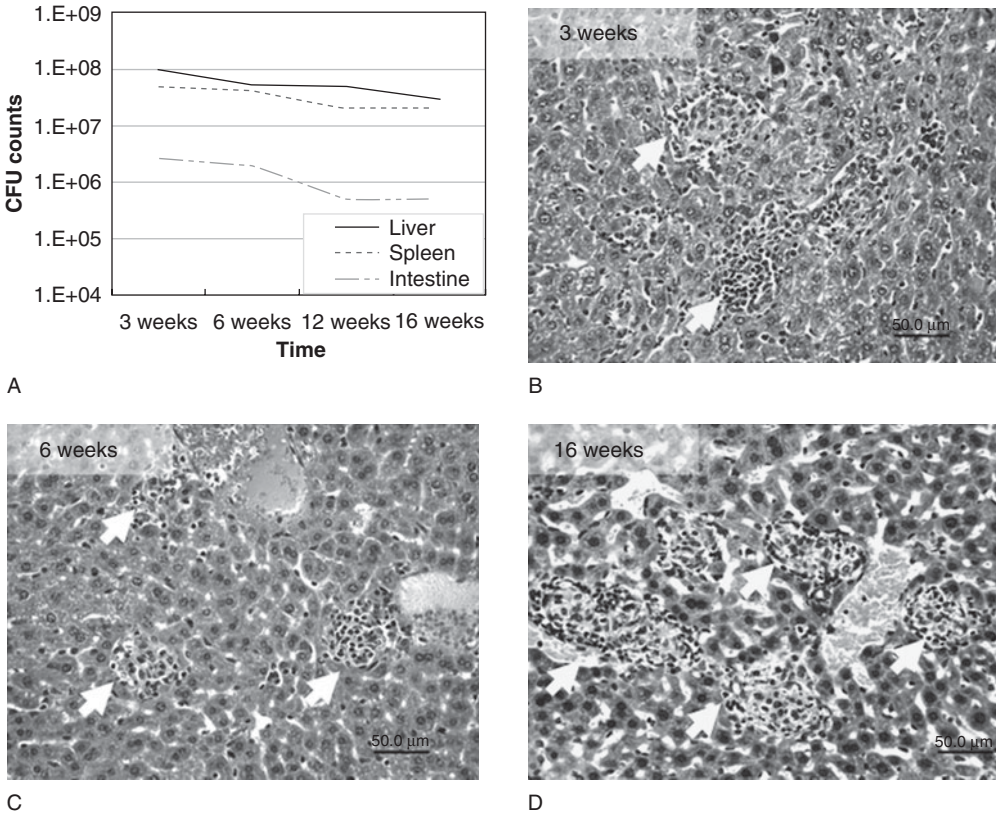

Fig. 20.1. Readouts used to monitor the murine model following infection with *MAP*. A. Colonization levels of *MAP* in liver, spleen and intestine of mice after infection with 10^8 CFU/mouse by intraperitoneal injection. B–D. Histopathology of liver, at 3 weeks (B), 6 weeks (C) and 16 weeks (D) post-infection. Features of granulomas (white arrows) associated with *MAP* infection include aggregates of macrophages and lymphocytes, which increase in size with time. Haematoxylin and eosin stain was used to examine liver sections.

against JD. Unlike the bovine, goat or sheep models of JD, many immunological reagents are well developed for mice, permitting a more mechanistic assessment of the histopathological outcomes observed. Also, mice with variable genetic background are readily available, allowing the dissection of host–pathogen interactions on a molecular level.

20.5 The Rabbit Model of Paratuberculosis

Rabbits can be naturally infected with *MAP* (Judge *et al.*, 2005) and provide an attractive model to study the immunopathogenesis of *MAP* infection. Historically, rabbits were used to generate polyclonal antibodies against *MAP* antigens, as both live and heat-killed *MAP* elicit strong immune responses in rabbits (Stabel *et al.*, 1996). Several clones of a genomic library of *MAP* were reactive to rabbit antibodies, including Csp1 (also called 25 kDa) and Pks7 (Bannantine and Stabel, 2001). One of these antigens (Csp1) was shown to be expressed during macrophage survival. Recently, rabbits were also used to generate antibodies against specific *MAP* antigens (34 kDa) that are expressed in naturally infected cows (Malamo *et al.*, 2007). All of these reports suggest that rabbits can provide a valid model for generating

Table 20.1. Main features of the mouse and rabbit models of paratuberculosis.

	Model	
Feature	Mouse	Rabbit
Breeds	C57/BL6, BALB/c, SCID	White New Zealand
Route of administration	Oral, intraperitoneal	Oral
Infectious dose	10^7–10^8 CFU[a]/animal	10^5–10^9 CFU[a]/animal
Sample collection	Liver, spleen, intestine, lymph nodes, serum	Liver, spleen, intestine, lymph nodes
Diarrhoea	Never	Frequent
Faecal shedding	Rarely	Occasionally
Experimental end points	CFU[a], histopathology, immunological evaluations	CFU[a], histopathology, clinical signs
Shortcomings	Rare development of clinical signs of JD	Lack of reagents, some clinical signs of JD

[a]CFU: colony-forming units.

reagents useful for the investigation of *MAP* pathogenesis. With the success of the rabbit model in immunodiagnostics of *MAP*, this model was also investigated to study the pathogenesis of paratuberculosis.

All rabbit models of *MAP* pathogenesis reported so far include oral administration of *MAP*, with variable success in inducing infection in all of the animals (Mokresh *et al.*, 1989; Mokresh and Butler, 1990; Vaughan *et al.*, 2005). In one report, almost 50% of the inoculated animals did not show any sign of infection, despite the high dose of infection (10^7 CFU/animal) (Mokresh *et al.*, 1989). None the less, higher inocula (10^8 CFU) resulted in a higher infection rate (~70%) of inoculated rabbits (Mokresh and Butler, 1990). Interestingly, rabbits showing signs of the disease suffered from granulomatous enteritis and diarrhoea (Vaughan *et al.*, 2005), hallmarks of JD in cattle. Additionally, signs of body weight loss and clinical emaciation were also evident in a considerable number of inoculated rabbits (Vaughan *et al.*, 2005). In all of these reports, *MAP* was isolated from variable tissues, especially the intestinal tract and mesenteric lymph nodes. However, faecal shedding of *MAP* was not always detectable in infected animals (Vaughan *et al.*, 2005).

Overall, rabbits provide an important small animal model to study key aspects of JD that cannot be investigated in mice. Aspects related to the induction of diarrhoea, weight loss or intestinal colonization could be better investigated in rabbits than in mice. In most reports, rabbits developed signs within the first year post-infection, a time period that is much shorter than calf infections. Despite all of these advantages, the use of the rabbit model in *MAP* research is restricted because of the limited repertoire of immunological reagents and the expenses associated with housing rabbits compared with mice. Table 20.1 presents key aspects of the mouse and rabbit models of paratuberculosis in a comparative format.

References

Bannantine, J.P. and Stabel, J.R. (2001) Identification of two *Mycobacterium avium* subspecies *paratuberculosis* gene products differentially recognised by sera from rabbits immunised with live mycobacteria but not heat-killed mycobacteria. *Journal of Medical Microbiology* 50, 795–804.

Basagoudanavar, S.H., Goswami, P.P. and Tiwari, V. (2006) Cellular immune responses to 35 kDa recombinant antigen of *Mycobacterium avium paratuberculosis*. *Veterinary Research Communications* 30, 357–367.

Beard, P.M., Rhind, S.M., Buxton, D., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Greig, A., Hutchings, M.R., Stevenson, K. and Sharp, J.M. (2001) Natural paratuberculosis infection in rabbits in Scotland. *Journal of Comparative Pathology* 124, 290–299.

Bull, T.J., Gilbert, S.C., Sridhar, S., Linedale, R., Dierkes, N., Sidi-Boumedine, K. and Hermon-Taylor, J. (2007) A novel multi-antigen virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis*. *PLoS ONE*, 2, e1229.

Chen, L.H., Kathaperumal, K., Huang, C.J., McDonough, S.P., Stehman, S., Akey, B., Huntley, J., Bannantine, J.P., Chang, C.F. and Chang, Y.F. (2008) Immune responses in mice to *Mycobacterium avium* subsp. *paratuberculosis* following vaccination with a novel 74F recombinant polyprotein. *Vaccine* 26, 1253–1262.

Ferwerda, G., Kullberg, B.J., de Jong, D.J., Girardin, S.E., Langenberg, D.M.L., van Crevel, R., Ottenhoff, T.H.M., Van der Meer, J.W.M. and Netea, M.G. (2007) *Mycobacterium paratuberculosis* is recognized by Toll-like receptors and NOD2. *Journal of Leukocyte Biology* 82, 1011–1018.

Hamilton, H.L., Cooley, A.J., Adams, J.L. and Czuprynski, C.J. (1991) *Mycobacterium paratuberculosis* monoassociated nude mice as a paratuberculosis model. *Veterinary Pathology* 28, 146–155.

Huntley, J.F., Stabel, J.R., Paustian, M.L., Reinhardt, T.A. and Bannantine, J.P. (2005) Expression library immunization confers protection against *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infection and Immunity* 73, 6877–6884.

Judge, J., Kyriazakis, I., Greig, A., Allcroft, D.J. and Hutchings, M.R. (2005) Clustering of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits and the environment: how hot is a hot spot? *Applied and Environmental Microbiology* 71, 6033–6038.

Malamo, M., Okazaki, K., Sakoda, Y. and Kida, H. (2007) Carboxyl terminus of the 34 kDa protein of *Mycobacterium paratuberculosis* shares homologous B-cell epitopes with *Mycobacterium avium* and *Mycobacterium intracellulare*. *The Veterinary Record* 161, 853–857.

Mokresh, A.H. and Butler, D.G. (1990) Granulomatous enteritis following oral inoculation of newborn rabbits with *Mycobacterium paratuberculosis* of bovine origin. *Canadian Journal of Veterinary Research* 54, 313–319.

Mokresh, A.H., Czuprynski, C.J. and Butler, D.G. (1989) A rabbit model for study of *Mycobacterium paratuberculosis* infection. *Infection and Immunity* 57, 3798–3807.

Mullerad, J., Hovav, A.H., Fishman, Y., Barletta, R.G. and Bercovier, H. (2002a) Antigenicity of *Mycobacterium paratuberculosis* superoxide dismutase in mice. *FEMS Immunology and Medical Microbiology* 34, 81–88.

Mullerad, J., Michal, I., Fishman, Y., Hovav, A.H., Barletta, R.G. and Bercovier, H. (2002b) The immunogenicity of *Mycobacterium paratuberculosis* 85B antigen. *Medical Microbiology and Immunology* 190, 179–187.

Mullerad, J., Hovav, A.H., Nahary, R., Fishman, Y. and Bercovier, H. (2003) Immunogenicity of a 16.7 kDa *Mycobacterium paratuberculosis* antigen. *Microbial Pathogenesis* 34, 81–90.

Mutwiri, G.K., Butler, D.G., Rosendal, S. and Yager, J. (1992) Experimental infection of severe combined immunodeficient beige mice with *Mycobacterium paratuberculosis* of bovine origin. *Infection and Immunity* 60, 4074–4079.

Mutwiri, G.K., Kosecka, U., Benjamin, M., Rosendal, S., Perdue, M. and Butler, D.G. (2001) *Mycobacterium avium* subspecies *paratuberculosis* triggers intestinal pathophysiologic changes in beige/scid mice. *Comparative Medicine* 51, 538–544.

Mutwiri, G.K., Rosendal, S., Kosecka, U., Yager, J.A., Perdue, M., Snider, D. and Butler, D.G. (2002) Adoptive transfer of BALB/c mouse splenocytes reduces lesion severity and induces intestinal pathophysiologic changes in the *Mycobacterium avium* subspecies *paratuberculosis* beige/scid mouse model. *Comparative Medicine* 52, 332–341.

Paustian, M.L., Amonsin, A., Kapur, V. and Bannantine, J.P. (2004) Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's disease. *Journal of Clinical Microbiology* 42, 2675–2681.

Secott, T.E., Lin, T.L. and Wu, C.C. (2002) Fibronectin attachment protein is necessary for efficient attachment and invasion of epithelial cells by *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 70, 2670–2675.

Secott, T.E., Lin, T.L. and Wu, C.C. (2004) *Mycobacterium avium* subsp. *paratuberculosis* fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins. *Infection and Immunity* 72, 3724–3732.

Shin, S.J., Wu, C.-W., Steinberg, H. and Talaat, A.M. (2006) Identification of novel virulence determinants in *Mycobacterium paratuberculosis* by screening a library of insertional mutants. *Infection and Immunity* 7, 3825–3833.

Skeiky, Y.A.W., Alderson, M.R., Ovendale, P.J., Guderian, J.A., Brandt, L., Dillon, D.C., Campos-Neto, A., Lobet, Y., Dalemans, W., Orme, I.M. and Reed, S.G. (2004) Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or

recombinant protein. *Journal of Immunology* 172, 7618–7628.

Stabel, J.R. and Ackermann, M.R. (2002) Temporal *Mycobacterium paratuberculosis* infection in T-cell receptor (TCR)-α and TCR-δ-deficient mice. *Veterinary Immunology and Immunopathology* 89, 127–132.

Stabel, J.R., Ackermann, M.R. and Goff, J.P. (1996) Comparison of polyclonal antibodies to three different preparations of *Mycobacterium paratuberculosis* in immunohistochemical diagnosis of Johne's disease in cattle. *Journal of Veterinary Diagnostic Investigation* 8, 469–473.

Talaat, A.M. and Stemke-Hale, K. (2005) Expression library immunization: a road map for discovery of vaccines against infectious diseases. *Infection and Immunity* 73, 7089–7098.

Tanaka, S., Sato, M., Taniguchi, T. and Yokomizo, Y. (1994) Histopathological and morphometrical comparison of granulomatous lesions in BALB/c and C3H/HeJ mice inoculated with *Mycobacterium paratuberculosis*. *Journal of Comparative Pathology* 110, 381–388.

Tanaka, S., Itohara, S., Sato, M., Taniguchi, T. and Yokomizo, Y. (2000) Reduced formation of granulomata in γδ T cell knockout BALB/c mice inoculated with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology Online* 37, 415–421.

Vaughan, J.A., Lenghaus, C., Stewart, D.J., Tizard, M.L. and Michalski, W.P. (2005) Development of a Johne's disease infection model in laboratory rabbits following oral administration of *Mycobacterium avium* subspecies *paratuberculosis*. *Veterinary Microbiology* 105, 207–213.

Veazey, R.S., Tayor, H.W., Horohov, D.W., Krahenbuhl, J.L., Oliver, J.L.I. and Snider, T.G.I. (1995) Histopathology of C57BL/6 mice inoculated orally with *Mycobacterium paratuberculosis*. *Journal of Comparative Pathology* 113, 75–80.

Wu, C.W., Livesey, M., Schmoller, S.K., Manning, E.J.B., Steinberg, H., Davis, W.C., Hamilton, M.J. and Talaat, A.M. (2007a) Invasion and persistence of *Mycobacterium paratuberculosis* during early stages of Johne's disease in calves. *Infection and Immunity* 75, 2110–2119.

Wu, C.W., Schmoller, S.K., Shin, S.J. and Talaat, A.M. (2007b) Defining the stressome of *Mycobacterium avium* subspecies *paratuberculosis in vitro* and in naturally infected cows. *Journal of Bacteriology* 189, 7877–7886.

Wu, C.W., Schmoller, S.K., Bannantine, J.P., Eckstein, T.M., Inamine, J.M., Livesey, M., Albrecht, R. and Talaat, A.M. (2009) A novel cell wall lipopeptide is important for biofilm formation and pathogenicity of *Mycobacterium avium* subspecies *paratuberculosis*. *Microbial Pathogenesis* 46, 222–230.

21 Immunology of Paratuberculosis Infection and Disease

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21.1 Introduction

The study of host immune responses to *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is complicated by a number of factors, including the protracted nature of the disease and the stealthy nature of the pathogen. Improved tools for the measurement of immunological responses in ruminant species, particularly the bovine, a key target species for *MAP* infection, have allowed the dissection of host immunological responses to infection to some extent. Noted as one of the more fastidious mycobacteria, *MAP* produces a chronic infection characterized by periods of subclinical infection extending for 3–5 years. Many animals clear the infection during this period, but it is almost impossible by current methods to distinguish animals that clear infection from those that remain infected but control progression of disease. Escalation of paratuberculosis to a more clinical state marked by diarrhoea and weight loss is thought to be caused by immune dysfunction. This chapter is focused on host immunity after infection with *MAP* and draws upon published studies in bovine tuberculosis and other mycobacterial pathogens in order to fill knowledge gaps. The goal is to develop an improved understanding of the disease process, which in turn can guide the development of diagnostic assays and new vaccines. More information on host–pathogen interaction at the molecular level is given elsewhere (see Coussens *et al.*, Chapter 11, this volume).

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21.2 Bacterial Uptake

The major route of *MAP* infection in ruminants is via ingestion, so uptake through mucosal surfaces is the first step in the infective process. The gut-associated lymphoid tissue (GALT) in ruminants is organized into lymphoid aggregates known as Peyer's patches. Bacterial antigens are transported on a selective basis into GALT by associated mucosal cells. The portal of entry for *MAP* into the lymphoid tissue is the M cell, a specialized epithelial cell that lacks the brush-border microvilli, digestive enzymes and surface mucus commonly associated with enterocytes (Featherstone, 1997). The disrupted surface of the M cell allows microorganisms to transcytose through the cell, followed by phagocytosis by macrophages or dendritic cells (DCs) on the basolateral side of the cell. A landmark study elucidating the temporal pattern of *MAP* uptake into the Peyer's patches of ileal loops of neonatal calves found that the bacilli were first taken up by the M cells and then secondarily passed to subepithelial and intraepithelial macrophages within the domes of the Peyer's patches (Momotani *et al.*, 1988). In agreement, after exposure of ligated intestinal loops from goat kids to a solution containing a clinical isolate of *MAP* for 1 h, bacilli were most frequently seen in the cytoplasm of the M cells (Sigurðardóttir *et al.*, 2001). Furthermore, transcellular movement of *MAP* across M cells was demonstrated within 30 min after inoculation of the intestinal loops. Studies conducted using the everted sleeve method concluded that uptake of *MAP* by the intestinal mucosa of goat kids is not restricted to M cells but can also include regional enterocytes (Sigurðardóttir *et al.*, 2004, 2005).

It has been suggested that preferential uptake by M cells may be mediated by B1 integrin receptors, which have been demonstrated in high density on the apical side of M cells (Sigurðardóttir *et al.*, 2005). Although little is known about the role that receptors may play in the internalization of *MAP* by M cells, Secott *et al.* (2004) demonstrated that invasion of M cells was increased 2.6-fold following pretreatment of *MAP* with fibronectin. Alternatively, uptake by M cells was inhibited 52–73% following treatment of fibronectin-opsonized *MAP* with monoclonal antibodies directed against integrin subunits a5, aV, b1 and B3 (Secott *et al.*, 2004). Other receptors on M cells, including Toll-like receptor-4 (TLR4) and platelet activating factor receptor, are classified as microbial pattern recognition receptors involved in the recognition of bacterial pathogens (Tyrer *et al.*, 2006). The role of bacterial factors and host receptors in the initial uptake of *MAP* by M cells requires further study, as it conceptually represents a point in the infection process amenable to intervention.

21.3 Innate Response to *MAP* Infection

Once *MAP* is phagocytized, the host cells home to other areas of the body, including the lamina propria of the small intestine, the mesenteric lymph nodes and the peripheral circulation (Lugton, 1999). Organisms may remain intact, thwarting the bactericidal mechanisms of the antigen-presenting cell (APC), or they may be processed and presented to T lymphocytes, engendering a domino effect on immunological responses. As an intracellular pathogen, *MAP* is able to survive within the macrophage by inhibiting maturation of the phagosome. Hostetter *et al.* (2003) demonstrated higher levels of transferrin receptor, an early phagosome marker, and reduced levels of lysosome-associated membrane protein one, a late maturation marker, on phagosomes containing live versus killed *MAP*. This attenuation of phagosome maturation has been documented for other mycobacteria and has been linked to an inhibition of phagosome acidification (Kuehnel *et al.*, 2001). Since acidification of the phagosome–lysosome is associated with the production of bactericidal agents such as nitric oxide, reactive oxygen species (H_2O_2 and OH^-) and lysosomal hydrolases, it correlates with reduced intracellular replication of mycobacterial pathogens. The production of these cellular components may be significantly altered upon exposure to mycobacteria, thereby preventing effective killing of the intracellular organism (Akaki *et al.*, 1997; Miller *et al.*, 2004; Daniel *et al.*, 2006; Souza *et al.*, 2007).

In one study, diminished ATPase expression in bovine macrophages 18 h after infection with *MAP* was positively correlated with reduced phagosome acidification (Weiss *et al.*, 2004). Further investigation determined that the mitogen-activated protein kinase (MAPK) pathway favours bacterial survival by preventing acidification of phagosomes in bovine monocytes (Souza *et al.*, 2006). Inhibition of the MAPK pathway resulted in increased interleukin (IL)-12 expression and decreased expression of IL-10 by infected macrophages. Previously, neutralization of IL-10 was shown to increase killing of *MAP* by bovine monocyte-derived macrophages, an effect correlated with increased phagosome acidification and nitric oxide production (Weiss *et al.*, 2005). These data suggest that the MAPK pathway may be an important mediator in mycobactericidal activity, perhaps through its role in cytokine secretion.

Activation of macrophages upon ingestion of *MAP* is probably dependent upon the interaction of the pathogen with surface receptors of the macrophage, including TLRs, which are pattern recognition receptors present on macrophages and DCs. TLRs are important in the initiation of the adaptive immune response via activation of MyD88-dependent and independent signalling pathways (Krutzik and Modlin, 2004; Nguyen and Pieters, 2005). Several mechanisms of action have been proposed for TLRs, including regulation of bactericidal agents such as reactive nitrogen and oxygen species, and lysosomal peptides (Sieling and Modlin, 2001). In addition, the expression of TLRs on DCs serves to link the innate and adaptive immune systems through secretion of IL-12, supporting a Th1-mediated immune response (Krutzik and Modlin, 2004). Of the 11 TLRs that have currently been identified in mammals, TLR2 has received the most attention for mycobacterial recognition, as cell wall lipoproteins of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG activate TLR2-mediated host responses (Quesniaux *et al.*, 2004).

Only one comprehensive study evaluating the association of TLRs with *MAP* infection has been conducted to date (Taylor *et al.*, 2008). Gene expression of TLRs was assessed in ileal and jejunal tissues and their associated lymph nodes and correlated with infection status (uninfected, paucibacillary and multibacillary). Higher levels of TLR1, TLR2 and TLR4 expression were observed in tissues from naturally infected sheep compared with uninfected controls, suggesting a potential role for these TLRs in immune regulation. However, in contrast to other mycobacterial disorders, a significant upregulation in gene expression of TLR3, TLR5 and TLR8 was also noted in the infected groups. Although each of these TLRs have broadly defined roles, it is not clear if the upregulation noted in the present study can be specifically associated with pathogenesis of *MAP* infection or merely reflects localized inflammatory responses.

It was shown recently that activation of the MAPK pathway in bovine monocytes was mediated through TLR2 during infection with *MAP* but not *M. avium* subsp. *avium* (Weiss *et al.*, 2008). Treating the monocytes with anti-TLR2 resulted in increased acidification of phagosomes, phagosome–lysosome fusion and bacterial survival in *MAP*-infected cells, suggesting that TLR2 signalling through the MAPK pathway is critical to macrophage bactericidal activities. This is further substantiated by work characterizing the involvement of TLR2, TLR4 and NOD2 (nucleotide-binding oligomerization domain 2) receptors in recognition of *MAP* by macrophages isolated from TLR knockout mice (Ferwerda *et al.*, 2007). In this study, stimulation of peritoneal macrophages obtained from *Tlr2*–/– mice with *MAP* resulted in substantial reductions in cytokine secretion compared with macrophages from wild-type controls. Similar but less marked results were noted for *Tlr4*–/– mice. In addition, activation of TLR4-mediated responses was highly dependent upon the presence of intact versus sonicated preparations of *MAP*, indicating that components of the cell wall may be necessary to engage TLR4. These results suggest that TLRs are important mediators of the innate immune response to mycobacterial pathogens and that TLR2 may be of primary importance for cellular activation and subsequent induction of cytokines. Additional receptors on macrophages that have been implicated in *MAP* infections as potential modes of entry into the cell are the complement receptors, CR1, CR3 and CR4; mannose receptors that bind

lipoarabinomannan; Fc receptors; and CD14 (Coussens, 2001; Hostetter *et al.*, 2005; Souza *et al.*, 2007, 2008).

21.4 Early Infection – Macrophage–T-cell Interaction

Macrophages activated by mycobacteria produce a number of cytokines, such as IL-1, TNF-α and IL-12 (Wang *et al.*, 1999; Hope *et al.*, 2004). Infection with *MAP* initiates the upregulation of the aforementioned cytokines, in addition to other pro-inflammatory cytokines, IL-6, IL-8 and IL-10 (Adams and Czuprynski, 1994; Coussens *et al.*, 2004; Weiss *et al.*, 2004; Motiwala *et al.*, 2006). Presentation of major histocompatibility complex (MHC) class II antigens on the surface of the macrophage, along with IL-1 secretion, results in the activation of T cells. Activated T cells produce IL-2, which aids in the clonal expansion of specific CD4+ T helper cells and CD8+ cytolytic T cells. Upon activation, the naive CD4+ T cell can differentiate into either Th1 or Th2 subpopulations, based upon the nature of the antigen presented. Differentiation of the CD4+ T-cell population is skewed towards a Th1 T-cell subpopulation in the early stages of *MAP* infection, characterized by the secretion of the Th1-associated cytokines, IFN-γ, IL-2 and TNF-α (Burrells *et al.*, 1999; Stabel, 2000). The strong bias towards a Th1-mediated immune response in the early stages of infection is dominated by the key effector cytokine, IFN-γ. Studies have reported higher expression of IFN-γ in peripheral blood mononuclear cells (PBMC) of cattle infected with *MAP* (Coussens *et al.*, 2003, 2004; Khalifeh and Stabel, 2004a), and this was correlated with higher levels of IFN-γ secreted by PBMC isolated from animals in the subclinical or early stages of infection, whether it be natural or experimental (Stabel, 2000; Waters *et al.*, 2003; Khalifeh and Stabel, 2004a; Stewart *et al.*, 2006). IFN-γ plays a crucial role in the activation of T cells and macrophages, DC maturation, upregulation of MHC class I and II molecules, and production of reactive oxygen and nitrogen species by macrophages (Delvig *et al.*, 2002; Shankar *et al.*, 2003). CD4+ T cells appear to be the primary source of IFN-γ in mycobacterial infections including *MAP*, but CD8+ and γδ T cells also produce IFN-γ (Bassey and Collins, 1997). Quantities of IFN-γ secreted by γδ T cells in response to *MAP* antigens appear to be lower than amounts produced by CD4+ or CD8+ T cells, but this may be antigen-dependent (Shin *et al.*, 2005). IFN-γ not only induces the secretion of IL-12 by APCs, resulting in Th1 induction via a paracrine pathway, but also acts to directly augment Th1 polarization via an autocrine mechanism that does not involve IL-12 (Teixeira *et al.*, 2005).

IL-12 and IL-18 are both important mediators of anti-mycobacterial immunity and appear to induce IFN-γ synergistically through activation of Th1 cells (Kohno *et al.*, 1997; O'Donnell *et al.*, 1999; Sugawara *et al.*, 1999). Although they share some biological properties, these two cytokines utilize different signal transduction pathways in the induction of IFN-γ, suggesting a unique regulatory process in T-cell activation (Nembrini *et al.*, 2006). Very little information has been published on the roles of IL-12 and IL-18 in *MAP* infections to date. Gene expression studies have generated variable results (Coussens *et al.*, 2004; Tanaka *et al.*, 2005; Smeed *et al.*, 2007). Interestingly, when used as an adjuvant for paratuberculosis vaccines, IL-12 has not demonstrated increased IFN-γ secretion or reduced *MAP* colonization in tissues of vaccinated animals (Uzonna *et al.*, 2003; Kathaperumal *et al.*, 2008). This is in contrast to a study that demonstrated that co-immunization with a plasmid containing IL-12 and *M. tuberculosis* Ag85B enhanced IFN-γ secretion and protection against *M. tuberculosis* infection (Triccas *et al.*, 2002). It has been suggested that induction of a strong T-cell receptor (TCR) response is responsible for the majority of IFN-γ production and that IL-12 and IL-18 play supporting roles, perhaps becoming more critical when a TCR signal is weak or misaligned (Nembrini *et al.*, 2006). Since mycobacterial cell wall components are highly antigenic, it is likely that IL-12 and IL-18 are both used by the host as compensatory mediators of IFN-γ production.

21.5 Transition from Early to Late Infection – T-cell Subpopulations

Activated CD4+, CD8+ and γδ T-cell subpopulations are recognized sources of IFN-γ, but CD4+ T cells appear to be most highly reactive to mycobacterial antigens in the early stage of infection (Flynn *et al*., 1993). Naturally infected cows with paratuberculosis have an increased frequency of CD4+ and CD8+ T cells in the total PBMC population compared with healthy controls, with equivalent percentages of both within the subclinical and clinical infection groups (Stabel *et al*., 2007). Further delineation of the CD4+ subset demonstrated increased expression of CD25 and CD45RO, suggesting that these cells were highly activated and that prior exposure to *MAP* via natural infection fostered an effector–memory cell phenotype within the CD4+ subpopulation. This was in agreement with an earlier study evaluating cellular reactivity to antigens over a 320-day infection period in calves experimentally infected with *MAP* (Koo *et al*., 2004). CD4+ T cells with a memory cell phenotype (CD4+CD45RO+) are the predominant population of cells responding to antigens in the first year of infection, with an increase in the expression of CD25 and CD26 activation markers observed within the CD4+ subpopulation. In contrast, CD8+CD45RO+ T cells have low levels of CD25 and CD26 expression initially, but after 18 months of infection the cell phenotype begins to shift towards increased expression of these activation markers on CD8+ T cells (Koo *et al*., 2004). In a study conducted on naturally infected cows, greater intracellular IFN-γ was noted for CD8+CD45RO+ cells compared with CD4+CD45RO+ cells for clinical cows after stimulation of cells with johnin PPD (Stabel *et al*., 2007). Higher levels of secreted and intracellular IFN-γ were also observed for CD8+ cells after stimulation of PBMC with BCG-infected DCs in cattle vaccinated with BCG (Hope *et al*., 2004). These results suggest that, although CD4+ T cells may be critical in early responses to infection, CD8+ T cells may play a more significant role in late infection. The role that CD8+ T cells fulfil may be influenced by the maturity of the host immune system or the number of exposures to a particular pathogen.

Since γδ T cells produce IFN-γ in response to mycobacteria, it is believed that they contribute to the protection of the host early in the infection process, in a way that is distinct from that of αβ T cells (Kaufmann, 1996). This role was evaluated experimentally by subjecting TCR-δ, TCR-α and TCR-β gene deletion mutant mice to challenge with *Listeria monocytogenes* (Mombaerts *et al*., 1993). The mutant mice were able to control infection after challenge, although concomitant treatment of TCR-α and TCR-β mice with an anti-TCRγ/δ antibody resulted in colonization of the spleen (Mombaerts *et al*., 1993). In a *MAP* challenge study, TCR-α-deficient mice had higher levels of *MAP* colonization in their tissues compared with TCR-δ-deficient mice or C57BL/6 control mice (Stabel and Ackerman, 2002). Lesions were located predominantly in the liver or the ileum, depending upon the period of infection, and lesion scores were higher for TCR-α-deficient mice. Further suggestion of a unique role for γδ T cells stems from evidence that during experimental infection with intracellular pathogens such as *Listeria* and *M. avium* subsp. *avium*, TCR-γδ-deficient mice formed atypical lesions in their tissues instead of the granulomatous lesions seen in wild-type mice (Mombaerts *et al*., 1993; Saunders *et al*., 1998). Similarly, Tanaka *et al*. (2000) presented evidence of reduced granulomatous lesions in TCR-γδ-deficient mice after challenge with *MAP*. Results from these studies suggest that γδ T cells play a role in clearance of the pathogen after infection, which either harmonizes with or compensates for αβ T cells. One mechanism of action for γδ T cells in mycobacterial infections may be involvement in the formation of granulomas, either through increased IFN-γ production or by signalling the influx of macrophages to the lesion site.

21.6 Late Infection – T-cell Responses

The progression of paratuberculosis from a subclinical to clinical state is associated with a switch from Th1 to Th2 immune response. The production of Th2 regulatory cytokines, IL-4, IL-5 and IL-10, supports a humoral immune

response characterized by the expansion of B lymphocytes, immunoglobulin secretion and control of Th1-mediated responses. Both IL-4 and IL-10 have been noted to play specific roles in the suppression of IFN-γ production by CD4+ Th1 cells (Peleman *et al.*, 1989; Larner *et al.*, 1993; Ito *et al.*, 1999). There is little evidence to suggest a specific role for IL-4 in mediating protective immunity against *MAP*, as expression of IL-4 in tissues does not appear to be associated with the infection status of the host animal (Sweeney *et al.*, 1998; Coussens *et al.*, 2004). However, two reports have documented reduced expression of IL-4 in PBMC from subclinically and clinically infected cows as compared with control cows after stimulation with *MAP* (Coussens *et al.*, 2004; Karcher *et al.*, 2008). In contrast, IL-4 secretion by PBMC stimulated with a whole cell sonicate of *MAP* was greater in clinical cows compared with control and subclinical cows, fitting the paradigm shift to Th2-mediated immunity in clinical disease (Karcher *et al.*, 2008). IL-10 seems to play a more significant role in mycobacterial infections in general, and specifically during *MAP* infection. An upregulation of IL-10 in tissues, including ileum, mesenteric lymph nodes and cultured PBMC, from naturally infected cattle and sheep with paratuberculosis has been observed (Coussens *et al.*, 2004; Khalifeh and Stabel, 2004b; Smeed *et al.*, 2007). Increased expression of IL-10 was observed in bovine monocyte-derived macrophages infected with *MAP* as compared with *M. avium* subsp. *avium* (Weiss *et al.*, 2002). Further understanding of a role for IL-10 was gained through studies demonstrating that the addition of exogenous IL-10 to bovine cell cultures prior to infection with live *MAP* reduced IFN-γ secretion (Khalifeh and Stabel, 2004a). Alternatively, neutralization of IL-10 in johnin-PPD-stimulated whole blood increased IFN-γ production 23-fold in cattle with subclinical paratuberculosis (Buza *et al.*, 2004). The effects of IL-10 on IFN-γ may be mediated through IL-12, as the addition of exogenous IL-10 to human monocyte cultures reduces secretion of IL-12, whereas neutralization of IL-10 activity results in increased IL-12 secretion (Fulton *et al.*, 1998). The same has been observed with bovine T cells, where sensitization of a cow to mycobacteria with PPD and IL-12 resulted in an IL-12-mediated increase in IFN-γ secretion associated with decreased IL-10 expression (Tuo *et al.*, 1999). Moreover, neutralization of IL-10 activity in bovine monocyte-derived macrophage cultures resulted in increased acidification of phagosomes, increased expression of IL-12, IL-8, TNF-α and MHC class II molecules, and increased killing of *MAP* in culture (Weiss *et al.*, 2005). These studies provide evidence of the importance of interplay between Th1 and Th2 cytokines in the regulation of host responses to mycobacterial pathogens, allowing a balance to be maintained between protective and pathogenic responses.

21.7 Late Infection – T Regulatory Cells

A subpopulation of T cells known as T regulatory (Treg) cells further mediates host immune responses to mycobacterial pathogens. The characteristic phenotype of Tregs is CD4+CD25+, and induction of these cells is favoured by a Th2 environment. CD4+CD25+ naive Tregs are produced in the thymus and are associated with autoimmunity (Beissert *et al.*, 2006). CD4+CD25+ T cells comprise approximately 5–10% of the total peripheral CD4+ T-cell population in humans, yet only a small proportion of these represent Treg cells (< 2% of CD4+ T cells). Treg cells are often distinguished by expression of the forkhead box P3 (FoxP3) transcription factor and a variety of cell-surface molecules such as CD25, CD45RB, intracellular cytotoxic T-lymphocyte-associated antigen-4 and glucocorticoid-induced tumour necrosis factor receptor family-related protein (Hori *et al.*, 2003; Morgan *et al.*, 2005).

In distinction from naive Tregs, another class of Tregs is induced upon exposure to antigens in the periphery, resulting in differentiation of CD4+CD25− to adaptive Tregs. These Tregs are referred to as either Tr1 or Th3 cells and are responsible for controlling immune responses during infection via production of immunosuppressive cytokines, IL-10 (Tr1) and TGF-β (Tr1 and Th3). The immunosuppressive effects of IL-10 on IFN-γ were

previously discussed, but TGF-β also plays a regulatory role in host immunity through inhibition of T-cell activation and proliferation that is associated with reduced IFN-γ production (Lúdvíksson *et al.*, 2000). Several studies have demonstrated that the number of CD4+/CD25+ T cells is increased in the peripheral blood and at the site of infection in human patients with active tuberculosis (TB) (Chen *et al.*, 2007; Li *et al.*, 2007). Depletion of CD4+CD25+ T cells from CD4+ T-cell cultures results in higher IFN-γ production by TB patients compared with controls after *ex vivo* stimulation of cells with *M. tuberculosis* (Ribeiro-Rodrigues *et al.*, 2006). After 4 weeks of antibiotic treatment, production of IFN-γ in supernatants of whole blood stimulated with *M. tuberculosis* increased, associated with a decline in IL-10 secretion and declining numbers of CD4+CD25+ Tregs (Ribeiro-Rodrigues *et al.*, 2006). In mice infected with *M. tuberculosis*, addition of anti-IL-10 or anti-TGF-β to CD4+ T-cell cultures resulted in an increase in IFN-γ production, with greater production elicited after neutralization of TGF-β (Mason *et al.*, 2007). How this information actually correlates with disease severity is not entirely known, yet infection of mice experimentally depleted of CD4+CD25+ Tregs resulted in a tenfold reduction in bacterial load in the lung (Scott-Browne *et al.*, 2007). In one study of patients with TB, FoxP3 expression was increased 2.3-fold in patients with extrapulmonary TB compared with patients with infection confined to the lung and was 2.6-fold higher at the disease site compared with peripheral blood (Guyot-Revol *et al.*, 2006). The increase was associated with increased secretion of IL-10 and TGF-β.

More recently, it was shown that IL-10 production by PBMC isolated from cows with subclinical paratuberculosis was associated with reduced secretion of IFN-γ (de Almeida *et al.*, 2008). Depletion of IL-10-producing T cells from the PBMC of infected cattle revealed the characteristic phenotype of T regulatory cells, CD4+CD25+. Previously, a highly significant reduction in IFN-γ production was noted with the addition of exogenous IL-10 and TGF-β to PBMC cultures, with and without the presence of live *MAP*, an effect that was independent of the infection status of the cows (Khalifeh and Stabel, 2004a). Interestingly, an increase in IL-10 secretion was observed after the addition of TGF-β to cell cultures. Similarly, exogenous IL-10 and TGF-β suppressed the production of IFN-γ by PBMC from TB skin-test-positive individuals, with greater suppression in the presence of both IL-10 and TGF-β (Othieno *et al.*, 1999). The least suppression was noted with the addition of IL-10 alone, indicating that TGF-β mediates the suppression of IFN-γ both directly and indirectly through IL-10.

A recent association of IL-23 and IL-17 with mycobacterial infections has been made (Khader and Cooper, 2008). Interleukin-23 (IL-23) is a heterodimeric cytokine consisting of two subunits, p40 (similar to IL-12) and p19. IL-23 is an important part of the inflammatory response to mycobacterial infections through activation of IFN-γ. IL-23 stimulates naive CD4+ T cells to differentiate into a novel subset of cells called Th17 cells, a response that appears to be co-dependent upon TGF-β (Aggarwal *et al.*, 2003). Th17 cells produce IL-17, a pro-inflammatory cytokine that induces other pro-inflammatory cytokines, including IL-1, IL-6, TNF-α, TGF-β and GM-CSF, as well as mediating recruitment of macrophages and neutrophils to the site of infection. Although IL-23 and IL-17 appear to play roles throughout infection, the most critical period may be in the control of inflammation in late-stage TB (Khader and Cooper, 2008). It has been suggested that Th1 and Th17 responses provide balance within the host and that disease progression may be related to the loss of balance between them. This suggestion is substantiated by studies conducted with IFN-γ-deficient mice challenged with *M. bovis* BCG, which found that both IFN-γ and IL-17 were induced within 21 days of infection (Cruz *et al.*, 2006). Comparing the responses of IFN-γ-deficient and wild-type mice to BCG challenge, it was also shown that IFN-γ regulates the induction of IL-17 (Cruz *et al.*, 2006). Although studies have yet to be conducted to evaluate participation of IL-17 and IL-23 in the immunopathology of *MAP* infections in cattle and other ruminants, it can be speculated that this type of regulatory activity may be similar.

21.8 Late Infection – B-cell Responses

The role that B cells play in mycobacterial infections is not well understood. However, in addition to the secretion of antibody, B cells act as antigen-presenting cells and play a role in the activation of CD4+ Th2 cells. A recent report documented a regulatory role for B cells in chronic inflammatory pathogenesis that is mediated through IL-10 secretion (Lampropoulou *et al.*, 2008). Upon aerosol infection with *M. tuberculosis*, B cell−/− mice had higher IL-10 production, bacterial burden and increased immunopathology in the lungs (Maglione *et al.*, 2007). Previously, a significant increase in B-cell numbers was noted in naturally infected cows demonstrating clinical signs of paratuberculosis as compared with subclinical cows or healthy controls (Waters *et al.*, 1999). Recent work described the expansion of a $CD5^{bright}$ subpopulation of B cells in the peripheral blood of cattle subclinically infected with *MAP*, which probably represents the B1a population (Stabel and Khalifeh, 2008). This finding suggests that a shift in the B-cell subpopulations is taking place during infection, which may directly or indirectly affect pathogenesis induced by inflammatory T cells. Further, a delay in the dissemination of *M. tuberculosis* to the spleen and liver and the development of pulmonary lesions was noted in knockout mice deficient in mature B cells (Bosio *et al.*, 2000). In contrast, infection of *Rag*−/− mice (deficient in both B and T cells) with *M. tuberculosis* did not affect granuloma formation in the lungs (Chackerian *et al.*, 2002). These observations suggest that the interaction of B cells and T cells is an integral component in the host response to mycobacterial infection and that B cells play a role in regulating pathogenesis of infection.

21.9 Immunopathology

Granulomatous lesions in the intestine are a hallmark characteristic of *MAP* infection, and the composition of cell types within the lesions is correlated with stage of infection. The localized immune response at the site of lesions has been shown to change in accordance with lesion severity. The progression of disease from asymptomatic to clinical has been associated with decreased CD4+ T-cell and increased γδ T-cell populations in the lamina propria of the ileum, with no differences in cell frequency noted between asymptomatic infected and non-infected cattle (Koets *et al.*, 2002; Valheim *et al.*, 2004). An increase in CD8+ T cells, concomitant with reduced numbers of CD4+ T cells, was also noted in goats with clinical paratuberculosis (Navarro *et al.*, 1998). The pattern of cytokine expression in intestinal tissue is also influenced by the state of infection (Sweeney *et al.*, 1998; Lee *et al.*, 2001; Coussens *et al.*, 2004; Khalifeh and Stabel, 2004b; Tanaka *et al.*, 2005). Major findings include an upregulation of pro-inflammatory cytokines such as IFN-γ, IL-1, TNF-α, TGF-β, IL-5 and IL-8 in tissues from infected animals compared with non-infected controls. Studies contrasting cytokine expression in the asymptomatic and symptomatic stages of disease have demonstrated that subclinically infected animals generally have increased levels of IFN-γ and IL-18 in tissues compared with clinically infected cows (Sweeney *et al.*, 1998; Khalifeh and Stabel, 2004b; Tanaka *et al.*, 2005). In contrast, clinically infected animals have greater expression of pro-inflammatory cytokines, IL-1 and TNF-α; Th2 cytokines, IL-4 and IL-10; and Th3 cytokine, TGF-β (Alzuherri *et al.*, 1996; Lee *et al.*, 2001; Khalifeh and Stabel, 2004b; Tanaka *et al.*, 2005). More recently a comprehensive evaluation of cytokine gene expression in the ileal tissues of sheep that were classified into one of three groups – paucibacillary, multibacillary or asymptomatic – was performed (Smeed *et al.*, 2007). Expression of many of the pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α and IL-12, increased progressively according to stage of disease, from lowest for non-infected animals to highest for multibacillary sheep. Secretion of pro-inflammatory cytokines may trigger a sequence of events that contributes to the formation of lesions in the tissues. Although pro-inflammatory cytokines are critical for the recruitment of immune cells to fight infection, protracted activation may result in tissue

damage and lesion formation (Clarke, 1997). An increase in TGF-β is one mechanism of protection invoked by the host to suppress production of Th1 and Th2 cytokines. A linear increase in TGF-β expression was correlated with increased pathology in the intestinal tissue of infected sheep (Clarke, 1997), suggesting that TGF-β was responding to the need to control tissue damage potentiated by the secretion of pro-inflammatory cytokines (Smeed *et al.*, 2007). The pattern of cytokine expression in target tissues of infected animals has shown a clear Th1–Th2 paradigm correlating with infection status, which parallels that of other mycobacterial diseases such as leprosy and TB (Modlin, 1994; Flynn and Chan, 2001).

21.10 Summary

The stealthy nature of *MAP* is rewarded by a chronic infection within the host, characterized by long periods of latency and asymptomatic disease. This ensures that the disease can spread quietly and efficiently within and between groups of animals. Even animals that are able to mount robust responses to *MAP* infection are at risk of the pathogen circumventing normal control measures and eventually overtaking the host. Negative feedback mechanisms designed to control immunopathological responses may, in fact, be contributing to a loss of protective immunity in the host. The complicated nature of these responses suggests that we have only scratched the surface of our understanding of host responses to mycobacterial pathogens.

References

Adams, J.L. and Czuprynski, C.J. (1994) Mycobacterial cell wall components induce the production of TNF-alpha, IL-1, and IL-6 by bovine monocytes and the murine macrophage cell line RAW 264.7. *Microbial Pathogenesis* 16, 401–411.

Aggarwal, S., Ghilardi, N., Xie ,M.H., de Sauvage, F.J. and Gurney, A.L. (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *Journal of Biological Chemistry* 278, 1910–1914.

Akaki, T., Sato, K., Shimizu, T., Sano, C., Kajitani, H., Dekio, S. and Tomioka, H. (1997) Effector molecules in expression of the antimicrobial activity of macrophages against *Mycobacterium avium* complex: roles of reactive nitrogen intermediates, reactive oxygen intermediates, and free fatty acids. *Journal of Leukocyte Biology* 62, 795–804.

Alzuherri, H.M., Woodall, C.J. and Clarke, C.J. (1996) Increased intestinal TNF-alpha, IL-1 beta and IL-6 expression in ovine paratuberculosis. *Veterinary Immunology and Immunopathology* 49, 331–345.

Bassey, E.O. and Collins, M.T. (1997) Study of T-lymphocyte subsets of healthy and *Mycobacterium avium* subsp. *paratuberculosis*-infected cattle. *Infection and Immunity* 65, 4869–4872.

Beissert, S., Schwarz, A. and Schwarz, T. (2006) Regulatory T cells. *Journal of Investigative Dermatology* 126, 15–24.

Bosio, C.M., Gardner, D. and Elkins, K.L. (2000) Infection of B cell-deficient mice with CDC 1551, a clinical isolate of *Mycobacterium tuberculosis*: delay in dissemination and development of lung pathology. *Journal of Immunology* 164, 6417–6425.

Burrells, C., Clarke, C.J., Colston, A., Kay, J.M., Porter, J., Little, D. and Sharp, J.M. (1999) Interferon-gamma and interleukin-2 release by lymphocytes derived from the blood, mesenteric lymph nodes and intestines of normal sheep and those affected with paratuberculosis (Johne's disease). *Veterinary Immunology and Immunopathology* 68, 139–148.

Buza, J.J., Hikono, H., Mori, Y., Nagata, R., Hirayama, S., Aodon-geril, Bari, A.M., Shu, Y., Tsuji, N.M. and Momotani, E. (2004) Neutralization of interleukin-10 significantly enhances gamma interferon expression in peripheral blood by stimulation with johnin purified protein derivative and by infection with *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected cattle with paratuberculosis. *Infection and Immunity* 72, 2425–2428.

Chackerian, A.A., Alt, J.M., Perera, T.V., Dascher, C.C. and Behar, S.M. (2002) Dissemination of *Mycobacterium tuberculosis* is influenced by host factors and precedes the initiation of T-cell immunity. *Infection and Immunity* 70, 4501–4509.

Chen, X., Zhou, B., Li, M., Deng, Q., Wu, X., Le, X., Wu, C., Larmonier, N., Zhang, W., Zhang, H., Wang, H. and Katsanis, E. (2007) CD4+ CD25+FoxP3+ regulatory T cells suppress *Mycobacterium tuberculosis* immunity in patients with active disease. *Clinical Immunology* 123, 50–59.

Clarke, C.J. (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. *Journal of Comparative Pathology* 16, 217–261.

Coussens, P.M. (2001) *Mycobacterium paratuberculosis* and the bovine immune system. *Animal Health Research Review* 2, 141–161.

Coussens, P.M., Colvin, C.J., Rosa, G.J., Perez Laspiur, J. and Elftman, M.D. (2003) Evidence for a novel gene expression program in peripheral blood mononuclear cells from *Mycobacterium avium* subsp. *paratuberculosis*-infected cattle. *Infection and Immunity* 71, 6487–6498.

Coussens, P.M., Verman, N., Coussens, M.A., Elftman, M.D. and McNulty, A.M. (2004) Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*: evidence for an inherent proinflammatory gene expression pattern. *Infection and Immunity* 72, 1409–1422.

Cruz, A., Khader, S.A., Torrado, E., Fraga, A., Pearl, J.E., Pedrosa, J., Cooper, A.M. and Castro, A.G. (2006) Cutting edge: IFN-γ regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. *Journal of Immunology* 177, 1416–1420.

Daniel, D.S., Dai, G., Singh, C.R., Lindsey, D.R., Smith, A.K., Dhandayuthapani, S., Hunter, R.L., Jr. and Jagannath, C. (2006) The reduced bactericidal function of complement C5-deficient murine macrophages is associated with defects in the synthesis and delivery of reactive oxygen radicals to mycobacterial phagosomes. *Journal of Immunology* 177, 4688–4698.

de Almeida, D.E., Colvin, C.J. and Coussens, P.M. (2008) Antigen-specific regulatory T cells in bovine paratuberculosis. *Veterinary Immunology and Immunopathology* 125, 235–245.

Delvig, A.A., Lee, J.J., Chrzanowska-Lightowlers, Z.M. and Robinson, J.H. (2002) TGF-beta1 and IFN-gamma cross-regulate antigen presentation to CD4 T cells by macrophages. *Journal of Leukocyte Biology* 72, 163–166.

Featherstone, C. (1997) M cells: portals to the mucosal immune system. *Lancet* 350, 1230.

Ferwerda, G., Kullberg, B.J., de Jong, D.J., Girardin, S.E., Langenberg, D.M., van Crevel, R., Ottenhoff, T.H., Van der Meer, J.W. and Netea, M.G. (2007) *Mycobacterium paratuberculosis* is recognized by Toll-like receptors and NOD2. *Journal of Leukocyte Biology* 82, 1011–1018.

Flynn, J.L. and Chan, J. (2001) Immunology of tuberculosis. *Annual Review of Immunology* 19, 93–129.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A. and Bloom, B.R. (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *Journal of Experimental Medicine* 178, 2249–2254.

Fulton, S.A., Cross, J.V., Toossi, Z.T. and Boom, W.H. (1998) Regulation of interleukin-12 by interleukin-10, transforming growth factor-beta, tumor necrosis factor-alpha, and interferon-gamma in human monocytes infected with *Mycobacterium tuberculosis* H37Ra. *Journal of Infectious Disease* 178, 1105–1114.

Guyot-Revol, V., Innes, J.A., Hackforth, S., Hinks, T. and Lalvani, A. (2006) Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *American Journal of Respiratory Critical Care Medicine* 173, 803–810.

Hope, J.C., Thom, M.L., McCormick, P.A. and Howard, C.J. (2004) Interaction of antigen presenting cells with mycobacteria. *Veterinary Immunology and Immunopathology* 100, 187–195.

Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057–1061.

Hostetter, J., Steadham, E., Haynes, J., Bailey, T. and Cheville, N. (2003) Phagosomal maturation and intracellular survival of *Mycobacterium avium* subspecies *paratuberculosis* in J774 cells. *Comparative Immunology, Microbiology and Infectious Diseases* 26, 269–283.

Hostetter, J., Kagan, R. and Steadham, E. (2005) Opsonization effects on *Mycobacterium avium* subsp. *paratuberculosis* – macrophage interactions. *Clinical and Diagnostic Laboratory Immunology* 12, 793–796.

Ito, S., Ansari, P., Sakatsume, M., Dickensheets, H., Vazquez, N., Donnelly, R.P., Larner, A.C. and Finbloom, D.S. (1999) Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma-induced genes by suppressing tyrosine phosphorylation of STAT1. *Blood* 93, 1456–1463.

Karcher, E.L., Beitz, D.C. and Stabel, J.R. (2008) Modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Immunology and Immunopathology* 123, 277–288.

Kathaperumal, K., Park, S.U., McDonough, S., Stehman, S., Akey, B., Huntley, J., Wong, S., Chang, C.F. and Chang, Y.F. (2008) Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine* 26, 1652–1663.

Kaufmann, S.H. (1996) Gamma/delta and other unconventional T lymphocytes: what do they see and what do they do? *Proceedings of the National Academy of Sciences of the USA* 93, 2272–2279.

Khader, S.A. and Cooper, A.M. (2008) IL-23 and IL-17 in tuberculosis. *Cytokine* 41, 79–83.

Khalifeh, M.S. and Stabel, J.R. (2004a) Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of *Mycobacterium avium* subsp. *paratuberculosis* in monocyte-derived macrophages from naturally infected cattle. *Infection and Immunity* 72, 1974–1982.

Khalifeh, M.S. and Stabel, J.R. (2004b) Upregulation of transforming growth factor-beta and interleukin-10 in cows with clinical Johne's disease. *Veterinary Immunology and Immunopathology* 99, 39–46.

Koets, A., Rutten, V., Hoek, A., van Mil, F., Muller, K., Bakker, D., Gruys, E. and van Eden, W. (2002) Progressive bovine paratuberculosis is associated with local loss of CD4(+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function. *Infection and Immunity* 70, 3856–3864.

Kohno, K., Kataoka, J., Ohtsuki, T., Suemoto, Y., Okamoto, I., Usui, M., Ikeda, M. and Kurimoto, M. (1997) IFN-gamma-inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. *Journal of Immunology* 158, 1541–1550.

Koo, H.C., Park, Y.H., Hamilton, M.J., Barrington, G.M., Davies, C.J., Kim, J.B., Dahl, J.L., Waters, W.R. and Davis, W.C. (2004) Analysis of the immune response to *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected calves. *Infection and Immunity* 72, 6870–6883.

Krutzik, S.R. and Modlin, R.L. (2004) The role of Toll-like receptors in combating mycobacteria. *Seminars in Immunology* 16, 35–41.

Kuehnel, M.P., Goethe, R., Habermann, A., Mueller, E., Rohde, M., Griffiths, G. and Valentin-Weigand, P. (2001) Characterization of the intracellular survival of *Mycobacterium avium* ssp. *paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cellular Microbiology* 3, 551–566.

Lampropoulou, V., Hoehlig, K., Roch, T., Neves, P., Calderon Gomez, E., Sweenie, C.H., Hao, Y., Freitas, A.A., Steinhoff, U., Anderton, S.M. and Fillatreau, S. (2008) TLR-activated B cells suppress T cell-mediated autoimmunity. *Journal of Immunology* 180, 4763–4773.

Larner, A.C., Petricoin, E.F., Nakagawa, Y. and Finbloom, D.S. (1993) IL-4 attenuates the transcriptional activation of both IFN-alpha and IFN-gamma-induced cellular gene expression in monocytes and monocytic cell lines. *Journal of Immunology* 150, 1944–1950.

Lee, H., Stabel, J.R. and Kehrli, M.E., Jr. (2001) Cytokine gene expression in ileal tissues of cattle infected with *Mycobacterium paratuberculosis*. *Veterinary Immunology and Immunopathology* 82, 73–85.

Li, L., Lao, S.H. and Wu, C.Y. (2007) Increased frequency of CD4(+)CD25(high) Treg cells inhibit BCG-specific induction of IFN-gamma by CD4(+) T cells from TB patients. *Tuberculosis* 87, 526–534.

Lúdvíksson, B.R., Seegers, D., Resnick, A.S. and Strober, W. (2000) The effect of TGF-beta1 on immune responses of naive versus memory CD4+ Th1/Th2 T cells. *European Journal of Immunology* 30, 2101–2111.

Lugton, I. (1999) Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic mycobacteria. *Immunology and Cell Biology* 77, 364–372.

Maglione, P.J., Xu, J. and Chan, J. (2007) B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with *Mycobacterium tuberculosis*. *Journal of Immunology* 178, 7222–7234.

Mason, C.M., Porretta, E., Zhang, P. and Nelson, S. (2007) CD4+ CD25+ transforming growth factor-beta-producing T cells are present in the lung in murine tuberculosis and may regulate the host inflammatory response. *Clinical and Experimental Immunology* 148, 537–545.

Miller, B.H., Fratti, R.A., Poschet, J.F., Timmins, G.S., Master, S.S., Burgos, M., Marletta, M.A. and Deretic, V. (2004) Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. *Infection and Immunity* 72, 2872–2878.

Modlin, R.L. (1994) Th1–Th2 paradigm: insights from leprosy. *Journal of Investigative Dermatology* 99, 336–341.

Mombaerts, P., Arnoldi, J., Russ, F., Tonegawa, S. and Kaufmann, S.H. (1993) Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. *Nature* 365, 53–56.

Momotani, E., Whipple, D.L., Thiermann, A.B. and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Veterinary Pathology* 25, 131–137.

Morgan, M.E., van Bilsen, J.H., Bakker, A.M., Heemskerk, B., Schilham, M.W., Hartgers, F.C., Elferink, B.G., van der Zanden, L., de Vries, R.R., Huizinga, T.W., Ottenhoff, T.H. and Toes, R.E. (2005) Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Human Immunology* 66, 13–20.

Motiwala, A.S., Janagama, H.K., Paustian, M.L., Zhu, X., Bannantine, J.P., Kapur, V. and Sreevatsan, S. (2006) Comparative transcriptional analysis of human macrophages exposed to animal and human isolates of *Mycobacterium avium* subspecies *paratuberculosis* with diverse genotypes. *Infection and Immunity* 74, 6046–6056.

Navarro, J.A., Ramis, G., Seva, J., Pallares, F.J. and Sanchez, J. (1998) Changes in lymphocyte subsets in the intestine and mesenteric lymph nodes in caprine paratuberculosis. *Journal of Comparative Pathology* 118, 109–121.

Nembrini, C., Abel, B., Kopf, M. and Marsland, B.J. (2006) Strong TCR signaling, TLR ligands, and cytokine redundancies ensure robust development of type 1 effector T cells. *Journal of Immunology* 176, 7180–7188.

Nguyen, L. and Pieters, J. (2005) The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. *Trends in Cellular Biology* 15, 269–276.

O'Donnell, M.A., Luo, Y., Chen, X., Szilvasi, A., Hunter, S.E. and Clinton, S.K. (1999) Role of IL-12 in the induction and potentiation of IFN-gamma in response to bacillus Calmette-Guérin. *Journal of Immunology* 163, 4246–4252.

Othieno, C., Hirsch, C.S., Hamilton, B.D., Wilkinson, K., Ellner, J.J. and Toossi, Z. (1999) Interaction of *Mycobacterium tuberculosis*-induced transforming growth factor beta1 and interleukin-10. *Infection and Immunity* 67, 5730–5735.

Peleman, R., Wu, J., Fargeas, C. and Delespesse, G. (1989) Recombinant interleukin 4 suppresses the production of interferon gamma by human mononuclear cells. *Journal of Experimental Medicine* 170, 1751–1756.

Quesniaux, V., Fremond, C., Jacobs, M., Parida, S., Nicolle, D., Yeremeev, V., Bihl, F., Erard, F., Botha, T., Drennan, M., Soler, M.N., Le Bert, M., Schnyder, B. and Ryffel, B. (2004) Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes and Infection* 6, 946–959.

Ribeiro-Rodrigues, R., Resende Co, T., Rojas, R., Toossi, Z., Dietze, R., Boom, W.H., Maciel, E. and Hirsch, C.S. (2006) A role for CD4+CD25+ T cells in regulation of the immune response during human tuberculosis. *Clinical and Experimental Immunology* 144, 25–34.

Saunders, B.M., Frank, A.A., Cooper, A.M. and Orme, I.M. (1998) Role of gamma delta T cells in immunopathology of pulmonary *Mycobacterium avium* infection in mice. *Infection and Immunity* 66, 5508–5514.

Scott-Browne, J.P., Shafiani, S., Tucker-Heard, G., Ishida-Tsubota, K., Fontenot, J.D., Rudensky, A.Y., Bevan, M.J. and Urdahl, K.B. (2007) Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *Journal of Experimental Medicine* 204, 2159–2169.

Secott, T.E., Lin, T.L. and Wu, C.C. (2004) *Mycobacterium avium* subsp. *paratuberculosis* fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins. *Infection and Immunity* 72, 3724–3732.

Shankar, G., Pestano, L.A. and Bosch, M.L. (2003) Interferon-gamma added during bacillus Calmette-Guérin induced dendritic cell maturation stimulates potent Th1 immune responses. *Journal of Translational Medicine* 1, 7.

Shin, S.J., Chang, C.F., Chang, C.D., McDonough, S.P., Thompson, B., Yoo, H.S. and Chang, Y.F. (2005) *In vitro* cellular immune responses to recombinant antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 73, 5074–5085.

Sieling, P.A. and Modlin, R.L. (2001) Activation of Toll-like receptors by microbial lipoproteins. *Scandinavian Journal of Infectious Diseases* 33, 97–100.

Sigurðardóttir, O.G., Press, C.M. and Evensen, O. (2001) Uptake of *Mycobacterium avium* subsp. *paratuberculosis* through the distal small intestinal mucosa in goats: an ultrastructural study. *Veterinary Pathology* 38, 184–189.

Sigurðardóttir, O.G., Valheim, M. and Press, C.M. (2004) Establishment of *Mycobacterium avium* subsp. *paratuberculosis* infection in the intestine of ruminants. *Advanced Drug Delivery Reviews* 56, 819–834.

Sigurðardóttir, O.G., Bakke-McKellep, A.M., Djønne, B. and Evensen, O. (2005) *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. *Comparative Immunology, Microbiology, and Infectious Diseases* 28, 223–230.

Smeed, J.A., Watkins, C.A., Rhind, S.M. and Hopkins, J. (2007) Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis. *BMC Veterinary Research* 3, 18–28.

Souza, C.D., Evanson, O.A. and Weiss, D.J. (2006) Mitogen activated protein kinase p38 pathway

is an important component of the anti-inflammatory response in *Mycobacterium avium* subsp. *paratuberculosis*-infected bovine monocytes. *Microbial Pathogenesis* 41, 59–66.

Souza, C.D., Evanson, O.A., Sreevatsan, S. and Weiss, D.J. (2007) Cell membrane receptors on bovine mononuclear phagocytes involved in phagocytosis of *Mycobacterium avium* subsp. *paratuberculosis*. *American Journal of Veterinary Research* 68, 975–980.

Souza, C.D., Evanson, O.A. and Weiss, D.J. (2008) Role of cell membrane receptors in the suppression of monocyte anti-microbial activity against *Mycobacterium avium* subsp. *paratuberculosis*. *Microbial Pathogenesis* 44, 215–223.

Stabel, J.R. (2000) Cytokine secretion by peripheral blood mononuclear cells from cows infected with *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 61, 754–760.

Stabel, J.R. and Ackermann, M.R. (2002) Temporal *Mycobacterium paratuberculosis* infection in T-cell receptor (TCR)-alpha and TCR-delta-deficient mice. *Veterinary Immunology and Immunopathology* 89, 127–132.

Stabel, J.R. and Khalifeh, M.S. (2008) Differential expression of CD5 on B lymphocytes in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Immunology and Immunopathology* 126, 211–219.

Stabel, J.R., Kimura, K. and Robbe-Austerman, S. (2007) Augmentation of secreted and intracellular gamma interferon following johnin purified protein derivative sensitization of cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 19, 43–51.

Stewart, D.J., Vaughan, J.A., Stiles, P.L., Noske, P.J., Tizard, M.L., Prowse, S.J., Michalski, W.P., Butler, K.L. and Jones, S.L. (2006) A long-term study in Angora goats experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*: clinical disease, faecal culture and immunological studies. *Veterinary Microbiology* 113, 13–24.

Sugawara, I., Yamada, H., Kaneko, H., Mizuno, S., Takeda, K. and Akira, S. (1999) Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. *Infection and Immunity* 67, 2585–2589.

Sweeney, R.W., Jones, D.E., Habecker, P. and Scott, P. (1998) Interferon-gamma and interleukin 4 gene expression in cows infected with *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 59, 842–847.

Tanaka, S., Itohara, S., Sato, M., Taniguchi, T. and Yokomizo, Y. (2000) Reduced formation of granulomata in gamma(delta) T cell knockout BALB/c mice inoculated with *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Pathology* 37, 415–421.

Tanaka, S., Sato, M., Onitsuka, T., Kamata, H. and Yokomizo, Y. (2005) Inflammatory cytokine gene expression in different types of granulomatous lesions during asymptomatic stages of bovine paratuberculosis. *Veterinary Pathology* 42, 579–588.

Taylor, D.L., Zhong, L., Begg, D.J., de Silva, K. and Whittington, R.J. (2008) Toll-like receptor genes are differentially expressed at the sites of infection during the progression of Johne's disease in outbred sheep. *Veterinary Immunology and Immunopathology* 124, 132–151.

Teixeira, L.K., Fonseca, B.P., Vieira-de-Abreu, A., Barboza, B.A., Robbs, B.K., Bozza, P.T. and Viola, J.P. (2005) IFN-gamma production by CD8+ T cells depends on NFAT1 transcription factor and regulates Th differentiation. *Journal of Immunology* 175, 5931–5939.

Triccas, J.A., Sun, L., Palendira, U. and Britton, W.J. (2002) Comparative affects of plasmid-encoded interleukin 12 and interleukin 18 on the protective efficacy of DNA vaccination against *Mycobacterium tuberculosis*. *Immunology and Cell Biology* 80, 346–350.

Tuo, W., Estes, D.M. and Brown, W.C. (1999) Comparative effects of interleukin-12 and interleukin-4 on cytokine responses by antigen-stimulated memory CD4+ T cells of cattle: IL-12 enhances IFN-gamma production, whereas IL-4 has marginal effects on cytokine expression. *Journal of Interferon and Cytokine Research* 19, 741–749.

Tyrer, P., Foxwell, A.R., Cripps, A.W., Apicella, M.A. and Kyd, J.M. (2006) Microbial pattern recognition receptors mediate M-cell uptake of a Gram-negative bacterium. *Infection and Immunity* 74, 625–631.

Uzonna, J.E., Chilton, P., Whitlock, R.H., Habecker, P.L., Scott, P. and Sweeney, R.W. (2003) Efficacy of commercial and field-strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* 21, 3101–3109.

Valheim, M., Sigurðardóttir, O.G., Storset, A.K., Aune, L.G. and Press, C.M. (2004) Characterization of macrophages and occurrence of T cells in intestinal lesions of subclinical paratuberculosis in goats. *Journal of Comparative Pathology* 131, 221–232.

Wang, J., Wakeham, J., Harkness, R. and Xing, Z. (1999) Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *Journal of Clinical Investigation* 103, 1023–1029.

Waters, W.R., Stabel, J.R., Sacco, R.E., Harp, J.A., Pesch, B.A. and Wannemuehler, M.J. (1999) Antigen-specific B-cell unresponsiveness induced by chronic *Mycobacterium avium* subsp. *paratuberculosis* infection of cattle. *Infection and Immunity* 67, 1593–1598.

Waters, W.R., Miller, J.M., Palmer, M.V., Stabel, J.R., Jones, D.E., Koistinen, K.A., Steadham, E.M., Hamilton, M.J., Davis, W.C. and Bannantine, J.P. (2003) Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infection and Immunity* 71, 5130–5138.

Weiss, D.J., Evanson, O.A., Moritz, A., Deng, M.Q. and Abrahamsen, M.S. (2002) Differential responses of bovine macrophages to *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. *Infection and Immunity* 70, 5556–5561.

Weiss, D.J., Evanson, O.A., Deng, M. and Abrahamsen, M.S. (2004) Sequential patterns of gene expression by bovine monocyte-derived macrophages associated with ingestion of mycobacterial organisms. *Microbial Pathogenesis* 37, 215–224.

Weiss, D.J., Evanson, O.A., de Souza, C. and Abrahamsen, M.S. (2005) A critical role of interleukin-10 in the response of bovine macrophages to infection by *Mycobacterium avium* subsp. *paratuberculosis*. *American Journal of Veterinary Research* 66, 721–726.

Weiss, D.J., Souza, C.D., Evanson, O.A., Sanders, M. and Rutherford, M. (2008) Bovine monocyte TLR2 receptors differentially regulate the intracellular fate of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. *Journal of Leukocyte Biology* 83, 48–55.

22 Cultivation of *Mycobacterium avium* subsp. *paratuberculosis*

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22.1 Introduction

Cultivation and identification of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is the definitive diagnostic test for Johne's disease. The pathology induced by the organism in the intestinal tract is characteristic, but, even where acid-fast bacilli (AFB) are visualized in

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tissues associated with the granulomatous infiltrate, specificity is not assured because other mycobacterial species sometimes infect the gut. Immunohistochemistry and *in situ* hybridization reactions conducted on tissue sections containing AFB are not specific for *MAP* and neither are serological tests such as ELISA. *MAP* is defined as an obligate parasite and pathogen; for this reason its precise identification by cultivation is the benchmark for diagnosis of paratuberculosis at individual animal and herd/flock levels: if *MAP* is detected there must be – or must recently have been – an infected animal somewhere nearby. Thus it is standard practice to use culture to confirm a presumptive diagnosis in individual animals. Furthermore, culture of samples pooled from more than one animal, or the environment, is now accepted for herd and flock surveillance. One area where the specificity of culture is yet to be used widely is in the investigation of the proposed link between *MAP* and Crohn's disease in humans.

Important aspects in the culture of *MAP* are the analytical sensitivity of the particular method, the contamination rate, the cost of the test and the time taken to report results. Unfortunately, this information is lacking for many culture protocols. As culture of *MAP* is used as a gold standard to evaluate other types of diagnostic tests, there is an element of uncertainty about the performance of all diagnostic tests for paratuberculosis, including polymerase chain reaction (PCR), serology and histopathology (Whittington and Sergeant, 2001). For example, a report of outstanding diagnostic performance of an enzyme-linked immunosorbent assay (ELISA) or a faecal PCR test in early-stage disease might be due to the use of a relatively insensitive faecal culture protocol.

22.2 Historical Perspective

The first report of reliable cultivation of *MAP* was published in 1912 and was both detailed and rigorous (Twort and Ingram, 1912). The study was stimulated by the challenge to grow an organism that was obviously present in the lesions of affected livestock but clearly different from tuberculous mycobacteria, which were prevalent at the time. Twort and Ingram deduced that there must be a nutrient missing from *in vitro* culture media that precluded growth of the Johne's disease bacillus. They speculated that it had lived a pathogenic existence for long enough to lose an attribute possessed by its wild ancestor and retained by tuberculous mycobacteria. To compensate for this loss, they added heat-killed human tubercle bacilli to a Dorset egg medium base, and *MAP* then grew from clinical samples of cows with Johne's disease. They tested many strains of tubercle bacilli for their ability to support the growth of *MAP* and found this property to be quite variable, so they evaluated other mycobacterial species. The timothy grass bacillus, *Mycobacterium phlei*, was superior to other mycobacteria, and inclusion of this organism, or mycobactin derived from it or other mycobacteria, has enabled the culture of *MAP* ever since. Twort and Ingram also showed that inclusion of egg was beneficial, that extracted liver broth and agar media were less suitable than a clotted egg medium, that albumin was not needed for growth and that glycerol was beneficial. *MAP* grew at 28–43 °C. They described the morphology of *in vitro*-grown *MAP*, showed that it was non-spore forming, non-motile and acid fast, and was a strict aerobe when growing but was not killed in the absence of oxygen for 3 months at 39 °C. They reported a slow rate of growth, with tiny, discrete colonies forming in 3–5 weeks. Cultures were not killed by light and were resistant to disinfectants.

In summary, much of the useful knowledge about cultivation of *MAP* was provided in one paper in 1912. This breakthrough enabled additional improvements to occur, including the evaluation of alternative culture media, assessment of decontamination solutions and improvements to sample preparation protocols (Minett, 1942; Merkal *et al.*, 1982). Another important advance was the realization that there were two types of *MAP* (Type C and Type S), with distinct culture requirements (see Stevenson, Chapter 12, this volume). A further advance in the molecular era was the

introduction of PCR to identify growth of *MAP* in cultures.

22.3 General Principles for the Cultivation of *MAP*

The cultivation of *MAP* is based on traditional methods for the culture of slowly growing mycobacteria from clinical samples (Merkal *et al.*, 1964; Gillespie, 1999). There are four critical steps, described in more detail in the sections that follow.

1. Decontamination of clinical samples to destroy or suppress irrelevant, mostly rapidly growing microbes, which include both bacteria and fungi. These are present in vast numbers in faeces.
2. Prolonged incubation in appropriate media containing antimicrobial agents to suppress any remaining contaminants for long enough for *MAP* to emerge.
3. Recognition of *MAP* colonies on solid media or a particular sign of growth in broth media.
4. Identification of *MAP* by phenotypic and/or genotypic means.

22.4 Culture Media

22.4.1 Composition of solid and liquid media

Both solid and liquid media can be used to cultivate *MAP*. Solid media are probably more commonly used as they are cheaper, less instrumentation is required and identification of the organism can be simpler. Culture media for *MAP* must include essential nutrients, often include antimicrobials to discourage growth of contaminants and may include dyes to assist recognition of colonies. The ingredients or composition of the most commonly used contemporary culture media are provided in Tables 22.1 to 22.3.

Many media evaluated by early workers in mycobacteriology led to progressive refinement of simple egg-based slants such as Dubos medium, but these have fallen out of favour and do not seem to have been used for more than 20 years (Saxegaard, 1985). Instead, a consensus was reached that two media were most suitable: Lowenstein Jensen medium (LJ) in some European countries and Herrold's egg yolk medium (HEYM) elsewhere

Table 22.1. Comparison of contemporary media suitable for cultivation of Type C strains of *MAP*. Amounts are per litre.

Ingredient	HEYM[a]	LJ[b]
LJ base (Difco)		23.25 g
Sodium chloride	4.5 g	
Beef extract	2.7 g	
Peptone	9.0 g	
Agar	15.3 g	
Whole egg homogenate		625 ml
Egg yolk	120 ml	
Malachite green	100 mg	250 mg
Glycerol	34 ml	7.5 ml
Sodium pyruvate	4.1 g[c]	4.0 g
Mycobactin		2.0 mg
Cycloheximide		0.75 g
Chloramphenicol		0.20 g
Penicillin G		2×10^5 U

[a]Herrold, 1931; Merkal et al., 1964; Merkal and Curran, 1974. Most animal health laboratories in the USA currently include vancomycin, amphotericin B and nalidixic acid in HEYM (Robbe-Austerman, pers. comm., 2009.).
[b]Kalis et al., 2000.
[c]optional.

Table 22.2. Composition of Middlebrook media, which are suitable for cultivation of both Type C and S strains of *MAP*, and the chemically defined Watson Reid medium, which supports the growth of some laboratory-adapted strains.

Ingredient	Modified Middlebrook 7H9 (BACTEC 12B)[e]	Modified Middlebrook 7H10 agar[e]	Modified Middlebrook 7H11 agar[e]	Watson Reid medium[f]
Base medium				
Casein digest	667 mg[a]	1.0 g[b]	1.0 g	–
Ammonium sulfate	333 mg	500 mg	500 mg	–
Monopotassium phosphate	667 mg	1.5 g	1.5 g	2 g
Disodium phosphate	1.7 g	1.5 g	1.5 g	–
Sodium citrate	67 mg	400 mg	400 mg	–
Magnesium sulfate	33 mg	25 mg	50 mg	1 g
Calcium chloride	0.33 mg	0.5 mg	–	20 mg
Zinc sulfate	0.67 mg	1 mg	–	10 mg
Copper sulfate	0.67 mg	1 mg	–	–
Arginine	–	–	–	5 g
L-glutamic acid	333 mg	500 mg	500 mg	–
Cobalt chloride	–	–	–	2 mg
Sodium chloride	–	–	–	2 g
Ferric ammonium citrate	27 mg	40 mg	40 mg	75 mg
Pyridoxine	0.67 mg	1 mg	1 mg	–
Biotin	0.33 mg	0.5 mg	0.5 mg	–
Malachite green	–	0.25 mg	1 mg	–
Bacto agar	–	15 g	15 g	–
Enrichment		ADC	OADC	
Oleic acid	–	–	50 mg	–
Albumin fraction V, Bovine	3.3 g[a]	5.0 g	5.0 g	–
Dextrose	–	2.0 g	2.0 g	10 g
Catalase	32,000 U[a]	3 mg	4 mg	–
Sodium chloride	–	–	850 mg	–
C-14 palmitic acid	667 µCi[a]	–	–	–
Additives				
Egg yolk	167 ml[c]	250 ml	250 ml	–
Mycobactin J	0.83 mg[c]	1.25 mg	1.25 mg	–
PANTA PLUS[d]	33.3 ml[c]	50 ml	50 ml	–
Glycerol	–	5 ml	5 ml	60 ml

[a]Amounts are per 1000 ml for BACTEC 12B and Watson Reid medium and per 1330 ml for 7H10 agar and 7H11 agar. Added by manufacturer to Middlebrook 7H9 broth to form BACTEC 12B medium; [b]added as Casitone; [c]added to the enriched BACTEC 12B media at a rate of 1 ml egg yolk, 5 µg (100 µl) Mycobactin J, 200 µl PANTA PLUS and 700 µl water per 4 ml vial; final volume 6 ml/vial (data from Becton Dickenson BACTEC 12B package insert); [d]consists of polymyxin B 1000 U/ml, amphotericin B 100 µg/ml, nalidixic acid 400 µg/ml, trimethoprim 100 µg/ml, azlocillin 100 µg/ml and polyoxyethylene stearate 4 mg/ml; [e]adapted from Whittington *et al.* (1999); [f]from Merkal and Curran (1974).

(Table 22.1). Middlebrook 7H10 or 7H11 agar and 7H9 broth are also suitable base media, but, for optimal growth of *MAP*, egg yolk must be added (Table 22.2). In most circumstances mycobactin is an essential component of media for primary culture of *MAP*, but there may be sufficient carry-over of mycobactin to new media to disguise this need during subculture (Lambrecht and Collins, 1992). Laboratory adaptation of some strains of *MAP* has enabled their propagation in chemically defined media such as Watson Reid broth without mycobactin or egg (Table 22.2) (Watson, 1935; Morrison, 1965). Such media are unsuitable for cultivation of field strains (Merkal and Curran, 1974).

Table 22.3. Comparison of composition of BACTEC 12B and MGIT ParaTB media.

	Amount/litre		Amount/vial	
Ingredient	BACTEC	MGIT	BACTEC	MGIT
Base medium				
Middlebrook 7H9 broth			4.0 ml	7.0 ml
Fluorescent indicator			–	110 µl
Bovine albumin[a]	3,300 mg	4,820 mg	19.8 mg	40 mg
Casein[a]	667 mg	48.2 mg	4.0 mg	0.4 mg
Catalase[a]	32,000 U	4,627 U	192 U	38.4 U
Oleic acid[a]	–	9.64 mg	–	0.08 mg
Egg yolk[b]	167 ml	30.1 ml	1.0 ml	0.25 ml
Mycobactin J	0.83 mg[b]	Included[e]	5 µg[b]	Included[e]
Amphotericin B[c,d]	3.33 mg	7 mg	20 µg	60 µg
Nalidixic acid[c, d]	13.3 mg	18 mg	80 µg	150 µg
Vancomycin hydrochloride[c]	–	18 mg	–	150 µg
Polymyxin B[d]	33,300 U	–	200 U	–
Trimethoprim[d]	3.33 mg	–	20 µg	–
Azlocillin[d]	3.33 mg	–	20 µg	–
Ampicillin (optional)	100 mg	100 mg	0.6 mg	0.83 mg

[a]Added by manufacturer to standard Middlebrook 7H9 broth to form BACTEC 12B medium; added by user as MGIT ParaTB supplement to each MGIT ParaTB medium vial; [b]added by user to each BACTEC 12B medium vial (100 µl mycobactin J, 600 µl water, 1 ml egg yolk), resulting in a final volume of 6 ml medium; egg yolk (0.25 ml plus 0.25 ml water); added by user to each MGIT ParaTB medium vial, resulting in a final volume of 8.3 ml; BD recommends Egg Yolk Enrichment, a 50% egg yolk solution, at a rate of 0.5 ml per MGIT ParaTB medium vial; [c]added by user to each MGIT ParaTB medium vial from stock antibiotic solutions; [d]added by user to each BACTEC 12B medium vial as 0.2 ml PANTA PLUS, which also includes 4 mg/ml polyoxyethylene stearate; [e]amount not specified by the manufacturer.

The inclusion of C^{14}-labelled palmitic acid as a carbon source in liquid medium to enable radiometric detection of C^{14}-labelled carbon dioxide produced through microbial respiration was a breakthrough in medical microbiology (Middlebrook *et al.*, 1977; Reggiardo and Tigertt, 1977). The growth signal is not specific but triggers an examination of the broth for the pathogen of interest. In practice, the culture bottles are incubated and transferred periodically to a semi-automated ion-chamber reader (BACTEC 460), which pierces a rubber seal with a sterile needle and samples the gas phase. This method was adapted for *MAP* by adding egg yolk and mycobactin to commercial BACTEC 12B medium (Table 22.3) (Damato *et al.*, 1987; Damato and Collins, 1990).

Other liquid culture systems designed for medical microbiology have been used to culture *MAP* (Grant *et al.*, 2003; Ellingson *et al.*, 2004; Stich *et al.*, 2004). All utilize a proprietary medium that is based on Middlebrook 7H9 broth. The BACTEC MGIT 960 system (Becton Dickinson) relies on detection of a fluorescent signal which develops from an indicator at the base of the culture vial as oxygen is consumed during microbial respiration; the amount of egg yolk that can be added is limited as it interferes with measurement of the growth signal. ESP culture technology (Trek Diagnostics) relies on detection of a change in pressure in the gas phase of a sealed culture vial. The MB/BacT system (BioMerieux) is based on a colorimetric indication of carbon dioxide production. Each of these culture formats enables early identification of microbial growth by incubating culture vials within a machine that regularly monitors the growth signal. Like the original BACTEC 460 system, these newer methods require the formal identification of *MAP* when a growth signal is detected. While the MGIT system is gaining acceptance for detection of *MAP* in clinical samples (Grant *et al.*, 2003; Shin *et al.*,

2007), two studies have shown that vancomycin, an ingredient in commercial MGIT ParaTB medium for paratuberculosis, is inhibitory to some common strains of *MAP* (Thornton *et al.*, 2002; Gumber and Whittington, 2007). A comparison of the composition of BACTEC12B and MGIT ParaTB medium for paratuberculosis is provided in Table 22.3. Caution should be exercised in use of MGIT ParaTB medium unless it can be modified and proven to support the growth of all common strains of *MAP*.

The benefits of including egg or egg yolk in culture media for isolation of *MAP* have been noted since 1912 (Twort and Ingram, 1912), but the reasons it is needed are still not clear. Some *MAP* strains can be grown in some media without egg, while it is vital in other circumstances (Cousins *et al.*, 1995; Whittington *et al.*, 1999; Grant *et al.*, 2003). When benzalkonium chloride (BAC) or hexadecylpyridinium chloride (HPC) is used to decontaminate faecal samples, egg yolk is required in 7H10 or 7H9 media to enable growth of *MAP*. However, simply washing the *MAP* pellet once in water enables growth in these media without addition of egg yolk (Whipple and Merkal, 1983), suggesting that egg yolk may play a role in neutralizing disinfectants carried over into the culture medium with the inoculum (Merkal and Curran, 1974). This may explain why egg yolk is necessary for primary isolation but not for subculture (Cousins *et al.*, 1995).

Sodium pyruvate was reported to stimulate the growth of *MAP* and is included in both the LJ and the HEYM media used by some laboratories (Merkal and Curran, 1974; Jorgensen, 1982; Kim *et al.*, 1989; Juste *et al.*, 1991; Eamens *et al.*, 2000). Pyruvate enables *MAP* colonies to grow larger in LJ medium, compensating for the inhibitory effects of antibiotics that are included in this medium (Jorgensen, 1982).

Twort and Ingram (1912), and later Merkal *et al.* (1982), investigated many fundamental properties of media and incubation conditions. Shaking a liquid culture reduced growth of *MAP*; a CO_2 atmosphere was not required; and pH 6 was beneficial for growth compared with a higher pH.

Antimicrobials such as malachite green, cycloheximide, amphotericin B, vancomycin, nalidixic acid, chloramphenicol, penicillin G, polymyxin B, trimethoprim, azlocillin and ampicillin have been included in various media but thorough evaluation of their potential to inhibit growth of *MAP* is mostly lacking (Shin, 1989; Whitlock and Rosenberger, 1990; Stabel, 1997; Kalis *et al.*, 1999; Whittington *et al.*, 1999; Gumber and Whittington, 2007).

Dyes such as brilliant green (Minett, 1942) may be added to solid media to improve visibility of colonies. Methylene blue becomes concentrated in the colonies as they develop (Fig. 22.1) (Whittington *et al.*, 1999). Dye uptake by colonies can vary between strains and within a culture (Parrish *et al.*, 2004). Colonies are readily observed on media containing malachite green, particularly LJ medium, which has a smooth surface; the surface of HEYM slopes is often finely pitted, which can make recognition of small colonies quite challenging, despite the inclusion of the dye. Small colonies are relatively difficult to see on modified Middlebrook 7H10 and 7H11 agars, which are pale yellow due to their egg yolk content.

22.4.2 Culture requirements of different strains of *MAP*

The *MAP* strains that cause Johne's disease in most cattle are not phenotypically representative of the entire taxon. Different strains of *MAP* occur commonly in sheep but are also found in goats, deer and sometimes cattle in many countries. Molecular genetic differences between *MAP* isolates led to the naming of so-called Type C (cattle) and Type S (sheep) strains based on the hosts of origin of the isolates (D.M. Collins *et al.*, 1990). Other types of *MAP* include yellow-orange pigmented strains in the UK and a so-called bison strain in the USA. Compared with Type C strains, other strains grow less well, poorly or not at all on HEYM (D.M. Collins *et al.*, 1990; Juste *et al.*, 1991; Whitlock *et al.*, 1999; Whittington *et al.*, 1999; Stevenson *et al.*, 2002). Furthermore, Type S strains do not grow readily in MGIT ParaTB medium (Gumber and Whittington, 2007). More detailed information on *MAP* strain types is given elsewhere (see Stevenson, Chapter 12, this volume).

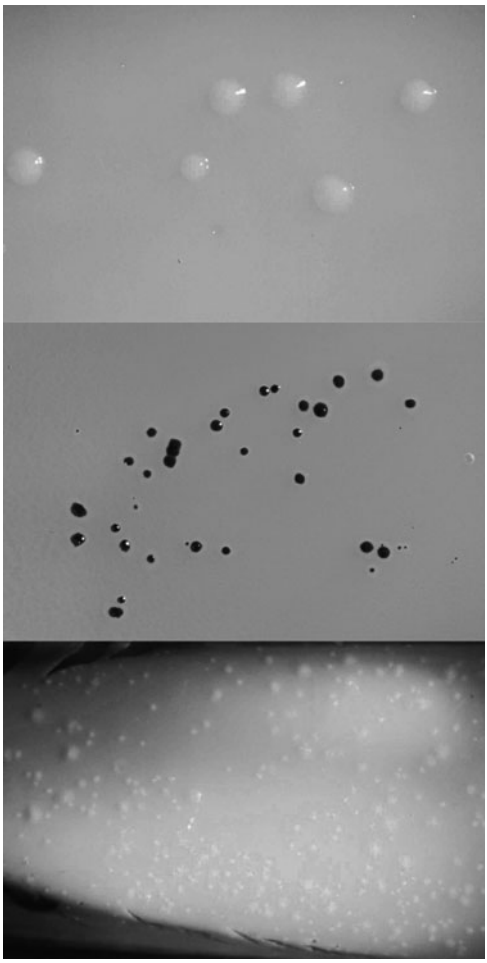

Fig. 22.1. Colonies of Type S *MAP* growing on modified Middlebrook 7H10 agar. Upper panel, subculture after 13 weeks' incubation; middle panel, subculture after 13 weeks' incubation with methylene blue incorporated in the agar; lower panel, primary isolate after 20 weeks' incubation.

22.4.3 Incubation period

MAP grows very slowly; generation times in liquid media range from 1.3 to 4.4 days and are inversely proportional to the inoculum size (Lambrecht *et al.*, 1988; Kim *et al.*, 2002). When grown on the most suitable media, colonies of *MAP* typically do not appear for several weeks and sometimes not for several months. Incubation periods of 12–20 weeks are standard for solid media before negative culture results are reported from livestock. Growth in liquid media is more rapid (Damato and Collins, 1990), and incubation is usually terminated after 8–12 weeks. The reasons for the very slow rate of growth of *MAP* compared with its close relative *M. avium* subsp. *hominissuis* are unclear (Bannantine *et al.*, 2003).

22.5 Specific Applications of Culture

22.5.1 Faecal samples

Decontamination of samples

The first attempts to cultivate *MAP* from clinical samples used decontamination methods available for tuberculosis. Sodium hypochlorite was once popular (Dunkin, 1928) but trials were also undertaken with sodium hydroxide (NaOH), sulfuric acid, hydrochloric acid, phenol, benzalkonium chloride (BAC) and oxalic acid (OA) (Minett, 1942; Merkal *et al.*, 1964; Gunnarsson, 1979; Gunnarsson and Fodstad, 1979). By 1980, a combination of NaOH and OA was adopted in protocols used in northern Europe (Jorgensen, 1982). The cationic quaternary ammonium compound hexadecylpyridinium chloride (HPC) was used first in the USA, replacing BAC (Merkal, 1984), and is now the basis for current protocols in many countries, including North America, Europe and Australasia (Table 22.4). Antimicrobial dyes such as malachite green and/or one or more antibiotics have often been included in decontamination solutions (Cameron, 1956; Shin, 1989; Whitlock and Rosenberger, 1990; Kalis *et al.*, 2000; Ellingson *et al.*, 2005).

Faecal samples present a particular problem due to their high load of enteric bacteria, and most protocols require removal of the particulate matter with which many microbes associate. This is achieved by suspending faeces in water or decontamination solution followed by filtration through cheesecloth (Cameron, 1956), by light centrifugation (Merkal *et al.*, 1968) or more commonly by sedimentation. The filtrate or supernatant is removed, and if it is already a decontamination solution it is inoculated directly into culture media. Alternatively, a water suspension is added first to a

Table 22.4. Common methods for cultivation of *MAP* from faeces.

Method	Decontamination	Medium and inoculum	Incubation	Notes and references[a]
North American methods				
1. Sedimentation (One-step HPC)	Faeces 1g added to 25 ml 0.75% HPC, shaken 30 min and allowed to stand for 2 h; 4 ml supernatant aspirated into a transfer pipette and allowed to settle overnight in the upright pipette. Drops from pipette used as inoculum.	HEYM, sodium pyruvate 4.1 mg/ml; 3 slopes/sample; 3 drops of inoculum/tube allowed to dry on surface of loosely capped tubes 1 week, then tubes sealed.	16 weeks	(Kim *et al*., 1989) Modified 7H9 liquid media may be used in place of HEYM (M.T. Collins *et al*., 1990; Cousins *et al*., 1995)
2. Sedimentation (Two-step water–HPC)	Step 1. Faeces 1 g added to 35–40 ml water, shaken 30 min at 22 °C then allowed to settle 30 min. Step 2. 5 ml of upper part of supernatant added to 25 ml 0.9% HPC (final conc. 0.75%), mixed and allowed to stand overnight (16–24 h) at room temperature. The sediment is used as inoculum.	HEYM;4 slopes per sample; 1 may have no MJ; 0.1 ml of inoculum per tube allowed to dry on surface of loosely capped tubes for 1 week then tubes sealed.	12 weeks; examined with dissecting microscope	(Whipple and Merkal, 1983; Whipple *et al*., 1991) HPC replaced BAC in the original method of Merkal in 1982
3. Sedimentation centrifugation (Two-step double incubation centrifugation)	Step 1 as above except faeces 2–5 g, water 35 ml. Step 2 as above except 0.9% HPC made up in half-strength BHI, incubated at 35–37 °C, then centrifuged at 900 × g for 30 min. The pellet is resuspended in 1 ml half-strength BHI containing VAN and incubated overnight or longer (may be stable for 3 days at 35–37 °C). This suspension is used as inoculum.	HEYM; 4 slopes per sample; 0.25 ml of inoculum per tube allowed to dry on surface of loosely capped tubes for 1 week then tubes sealed. Modified 7H9 liquid or 7H10 agar may be used instead.	16 weeks	(Shin, 1989; Whitlock and Rosenberger, 1990) inclusion of BHI and VAN at Cornell by Shin. Method used in most countries. In Australia, 10–12 ml saline nstead of water; incubation in VAN for 3 days; (Whittington, 2009).

(*Continued*)

Table 22.4. *continued*

Method	Decontamination	Medium and inoculum	Incubation	Notes and references[a]
4. Similar to 3. above but with NADC modification	Step 1 as above except faeces 1–2 g, water 35 ml. Step 2 as above except entire 25–30 ml supernatant is removed, centrifuged 1700 × g 20 min, the pellet resuspended in 30 ml 0.9% HPC–BHI, mixed and incubated overnight at 37 °C, centrifuged 1700 × g 20 min, the pellet resuspended in VAN overnight 37 °C. This suspension is used as inoculum.	HEYM with nalidixic acid 50 µg/ml, vancomycin 50 µg/ml; 4 slopes per sample; 0.2 ml inoculum/tube allowed to dry on surface of loosely capped tubes for 1 week then tubes sealed.	12 weeks; examined with dissecting microscope	(Stabel, 1997)
North European culture method				
5. Three-step sedimentation centrifugation	Faeces 5 ml or 2 g mixed with 8 ml 4% NaOH, shaken 15 min, centrifuged 1000 × g 15 min. Pellet added to 5 ml 5 mg/ml OA and 1 mg/ml malachite green solution, mixed 15 min, centrifuged 1000 × g 15 min. Pellet mixed with 4 ml 0.5 mg/ml neomycin and 50 mg/ml amphotericin B and allowed to stand overnight at 20 °C. Clear upper layer just above the sediment is used as inoculum.	LJ. 3–4 drops of inoculum allowed to dry on surface of loosely capped tubes for 24 h then tubes sealed.	26 weeks	(Jorgensen, 1982; Kalis *et al.*, 1999). Jorgensen's decontamination method was based on Beerwerth (1967).

[a]The first reference is the earliest mention of the original method. Abbreviations: VAN, vancomycin 100 µg/ml plus amphotericin B 50 µg/ml plus nalidixic acid 100 µg/ml; BHI, brain heart infusion; OA, oxalic acid; NaOH, sodium hydroxide; HPC, hexadecylpyridinium chloride; BAC, benzalkonium chloride; MJ, mycobactin J.

decontamination solution and the sediment from this is inoculated (Merkal *et al.*, 1968).

Concerns about the failure to inactivate contaminants that were not in a vegetative growth phase led to incubation of the faecal suspension in antimicrobial solutions made up in nutrient media, in the hope that spores would germinate and then be killed. This approach has been used for preparation of milk samples and is commonly applied to faecal samples, where HPC and antibiotics are constituted in half-strength brain heart infusion (BHI) broth (Shin, 1989; Whitlock and Rosenberger, 1990; Ellingson *et al.*, 2005).

Culture protocols have evolved independently in different laboratories over the years (Table 22.4). In some European countries, the NaOH–OA decontamination protocol is used in conjunction with LJ medium. In the USA and most other countries, sedimentation methods with HPC are combined with HEYM or liquid media. In a small study, 20 AFB smear-positive bovine faecal and tissue samples were processed using either NaOH–OA or HPC and inoculated on to LJ media with resulting sensitivity of 70 and 85%, respectively; all samples were positive by culture in modified BACTEC 12B medium after HPC decontamination (M.T. Collins *et al.*, 1990). In the absence of data from a larger study, it is unclear whether the decontamination protocol or medium can be interchanged without loss of sensitivity or an increase in the contamination rate. A comparison of the two most popular types of solid media – HEYM and LJ – in a large study in Denmark using only the NaOH–OA decontamination protocol found the HEYM medium had slightly greater sensitivity (Nielsen *et al.*, 2004).

Concentration of MAP from faecal samples

To increase the analytical sensitivity of faecal culture, *MAP* may be concentrated during or after decontamination of the sample. This was first reported by Merkal *et al.* (1964), who allowed coarse particles to settle, then removed and centrifuged the faecal water suspension before decontamination. Later modifications included centrifuging the decontamination solution (Kim *et al.*, 1989), combining both of these earlier methods (Stabel, 1997) and incorporating three decontaminants (Kalis *et al.*, 2000). As an alternative to centrifugation, 3 µm filtration to trap *MAP* was tried (M.T. Collins *et al.*, 1990). These approaches were all successful in concentrating *MAP* from a sample, although the penalty may be an increased contamination rate from other microbes concentrated in the inoculum (Eamens *et al.*, 2000).

Pooled faecal culture

The pooling of faecal samples from more than one animal for culture is a logical way to reduce the cost of detection of *MAP* at herd or flock level. Initial attempts were not encouraging due to loss of sensitivity (Vialard *et al.*, 1993). However, pooled culture was shown to be highly sensitive in sheep, provided that an animal with multibacillary disease was present, as these shed about 10^8 *MAP*/g of faeces (Whittington *et al.*, 2000b). For practical application, a pooling rate of 50 was selected and was shown to provide higher flock-level sensitivity than serological examination of the same animals (Whittington *et al.*, 2000a; Sergeant *et al.*, 2002). By 2002, this test, together with abattoir surveillance for gross lesions of paratuberculosis, had replaced other screening tests in Australia. Research in cattle showed similar benefits, but a lower pooling rate was required, generally five (Kalis *et al.*, 2000; Wells *et al.*, 2002, 2003; Eamens *et al.*, 2007b, 2008). Both computer modelling and empirical results showed that pooled faecal culture is practical and cost effective for cattle (van Schaik *et al.*, 2003; Kalis *et al.*, 2004). The same approach is applicable in goats and probably most other species (Eamens *et al.*, 2007a). Pooled faecal culture provides aggregate results but the data can be used to estimate the prevalence of infection within a population, provided that some pools yield negative results. There are several published methods for calculating within-herd prevalence from pooled samples (Toribio and Sergeant, 2007). Apart from ensuring the thorough mixing of the pooled sample, there are no additional technical considerations. The individual samples used to create a pool can be stored if it is desired to determine later which individual animals contributed to the positive pooled culture result.

22.5.2 Tissue samples

MAP is readily isolated from the intestinal tissues of infected animals and has sometimes been cultured from human tissues (Chiodini, 1989). Less effort has been devoted to development of protocols for culture of tissues than there has been to the culture of faeces, probably because contamination rates have not caused concern (Taylor, 1950; Smith, 1953). Methods described for tissues are simpler, usually involving only one decontamination step. Incubation of homogenized tissues overnight or for up to 3 days in HPC is sufficient to remove most contaminants from the intestinal wall or lymph nodes (Cousins *et al.*, 1995; Whittington *et al.*, 1999). Usually fat is trimmed away, and the remaining intestine or lymph node is finely divided with scissors, ground, blended mechanically or disrupted using a stomacher machine in solutions that may include proteases to disrupt tissue structure (Merkal *et al.*, 1964; Merkal, 1973; Sweeney *et al.*, 1992; Aduriz *et al.*, 1995). The homogenate is decontaminated by suspension in an antimicrobial solution, and the sediment is inoculated. As for faeces, *MAP* can be concentrated by centrifugation to improve analytical sensitivity, but this tends to increase the contamination rate (Reddacliff *et al.*, 2003b). An alternative approach used the Zwitterionic detergent CB-18 to release *MAP*, combined with a cocktail of lytic enzymes to destroy contaminants; this was tried on intestinal tissues from cattle and bison but has not been compared with traditional methods independently (Thornton *et al.*, 2002).

22.5.3 Milk

The shedding of *MAP* into the milk of infected cows was recognized many years ago (Alexejeff-Goloff, 1935; Taylor *et al.*, 1981), while its isolation from the milk of two Crohn's disease patients has fuelled the debate about its role in humans (Naser *et al.*, 2000). There has been a great deal of research on ways to detect the organism in samples of milk from individual cows, from bulk tank milk and from pasteurized milk, including retail samples (Millar *et al.*, 1996; Grant *et al.*, 2002). Pragmatically, reliable culture from milk is vital to study the effects of pasteurization on the viability of *MAP* (Stabel *et al.*, 1997). Most culture protocols involve centrifugation of milk at about 2500 × g for 15 min to produce a pellet containing *MAP*; to increase the chance of detection, the cream layer (where *MAP* also partitions) may be collected and pooled with the pellet for culture (Gao *et al.*, 2005). Samples of 40–50 ml of milk are necessary as the efficiency of recovery is <20% and there may be <10 colony-forming units per 50 ml (Sweeney *et al.*, 1992; Gao *et al.*, 2005). Immunomagnetic separation of *MAP* from milk using specific antibody-coated magnetic beads has been reported but does not appear to have found wide application (Grant *et al.*, 1998); this approach was ineffective for faecal samples (Mason *et al.*, 2001).

Several groups have shown that decontamination in 0.75% HPC in a one-step sedimentation protocol is appropriate to minimize contamination and is less damaging to *MAP* than double incubation methods (Sweeney *et al.*, 1992; Dundee *et al.*, 2001; Gao *et al.*, 2005). As milk ages, the population of contaminants increases, so that by 8 days decontamination in HPC is ineffective (Gao *et al.*, 2005). Raw milk should be processed within 2 days.

22.5.4 Blood

Isolation of *MAP* from blood is not undertaken routinely and there have been no systematic studies on suitable methodologies for culture or on the prevalence of bacteraemia. To the author's knowledge there has been only one study involving blood culture from livestock (Koenig *et al.*, 1993); heparinized buffy coat-plasma preparations were decontaminated in 0.75% HPC and cultured on HEYM, with positive results from one of seven cows that had advanced disease. Despite the paucity of data on this method, there have been at least two studies looking for *MAP* in the blood of Crohn's disease patients, with various methods, different levels of stringency in microbiological assessment and conflicting results (Naser *et al.*, 2004; Parrish *et al.*, 2009).

22.5.5 Environmental samples

It is not technically difficult to culture *MAP* from environmental samples, including soil, water and pasture, using methods based on those for faeces (Whittington *et al.*, 1998, 2004, 2005; Pickup *et al.*, 2005). Concentration of water samples by centrifugation or filtration is required prior to culture. Culture of faecal samples collected from the environment is an application of pooled faecal culture. It is applicable particularly for dairy cattle, where manure collects in drains adjacent to the dairy parlour. Faeces from most cows will collect there over time, become at least partially mixed with faeces from many other cows and is readily available for sampling. It is the cheapest method of sampling for determination of herd infection status (Lombard *et al.*, 2006; Tavornpanich *et al.*, 2008). The method has also been applied for extensively grazed sheep, but few positive cultures were obtained from pastures on affected farms (Whittington *et al.*, 2003). As the number of animals contributing to a particular sample is unknown, environmental sampling cannot be used to reliably estimate within-herd prevalence. Other applications of culture include the search for possible environmental transport vectors of *MAP*, including blowflies, earthworms and parasitic nematode larvae (Whittington *et al.*, 2001; Fischer *et al.*, 2003, 2004).

Observations of *MAP* in the environment using culture revealed an important phenomenon (dormancy) whereby the organism was able to survive for an extended period in a non-cultivable state (Whittington *et al.*, 2004). Further evidence of dormancy was an extended lag phase when stressed *MAP* were inoculated into liquid media (Gumber *et al.*, 2008). *MAP* possesses genes and proteins that are known from homology searches with other mycobacteria to be dormancy-associated.

22.5.6 Enumeration of *MAP*

Estimation of the number of *MAP* organisms that are present in a clinical sample can be used to determine the level of risk posed by livestock through contamination of the environment. Accurate enumeration of *MAP* is often needed in experimental infection models to enable a repeatable inoculum between trials, and in vaccine efficacy experiments to study faecal shedding rates between vaccinates and controls (Reddacliff *et al.*, 2006; Begg and Whittington, 2008).

Direct microscopic counts measure the total number of *MAP* bacteria, while PCR-based methods measure total DNA levels (which can be related back to the total number of *MAP* cells); these methods tend to overestimate counts by including both live and dead cells. In contrast, culture-based methods provide an estimate of number of viable cells. Direct colony counts on HEYM slopes have often been used. Methods applicable to liquid cultures include turbidimetric estimates, most probable number (MPN) estimates or time to growth. As *MAP* tends to clump when in suspension, leading to underestimation of count, considerable effort is required to achieve thorough dispersion of cells.

Turbidimetric estimates are only suitable for high concentrations of cells; 1 absorbance unit at 600 nm corresponds to a concentration of about 10^8 cells/ml (Shin *et al.*, 2007). MPN methods are applicable over a wide range of cell numbers but require the culture of replicates of a serial dilution through to an end point, which is the highest dilution before growth no longer occurs; thus they are very costly in terms of media (Whittington *et al.*, 2000b; Reddacliff *et al.*, 2003a). Estimation of count by the time to growth in radiometric BACTEC 12B medium is based on the observation that generation time is proportional to inoculum size; this is less susceptible than other methods to error through bacterial clumping, is highly sensitive, can be done with a single culture vial and is applicable to a range of sample types and strains of *MAP* (Lambrecht *et al.*, 1988; Reddacliff *et al.*, 2003a). The same approach has been validated recently in MGIT media (Shin *et al.*, 2007). Time to detection in the Trek ESP system is also related to inoculum size (Kim *et al.*, 2002), but its reliability for enumeration is not yet known. Colony counts are likely to substantially underestimate the actual count relative to MPN counts or time to growth in liquid media (Reddacliff *et al.*, 2003a), although there was close correlation

between counts on HEYM, BACTEC 12B medium and MGIT medium in a recent study (Shin *et al.*, 2007).

22.6 Contamination and Survival of *MAP* during Culture

22.6.1 Contamination rate

An important characteristic of any *MAP* culture protocol is its capacity to prevent growth of irrelevant microbes which may be present in a clinical sample. On solid media, colonies of *MAP* may not be visible among colonies of other organisms, but sometimes *MAP* can still be detected by PCR from the surface of the slope (Secott *et al.*, 1999; Whittington, 2009). There is lack of consistency in the literature in the use of the term 'contamination', which makes it difficult to compare rates between different studies. It can mean a mixed culture of *MAP* with other organisms, a light growth of irrelevant organisms or complete overgrowth of the medium, requiring cultures to be repeated.

The reported contamination rates in *MAP* faecal culture are extremely variable and there is still a need for a better culture protocol (Table 22.5). Contamination rates for culture on solid media in human clinical mycobacteriology are also highly variable, and in one study ranged from 0.4 to 41% (van Griethuysen *et al.*, 1996).

Contamination of faecal cultures is due to the presence of microbes that resist decontamination. Not unexpectedly, this can vary with the diet of livestock and their geographical location, and contamination is typically clustered within groups of samples from certain farms or localities (Whitlock *et al.*, 1989; Whittington, 2009). In one study of 2599 faeces from 137 farms, 1.7–11% of samples among farms yielded growth of irrelevant organisms (Whittington, 2009). Culture protocols may need to be modified, for example by inclusion of additional antibiotics, in order to obtain satisfactory results from samples from some farms.

Contamination of tissue cultures is less problematical. Contamination for 2577 intestinal tissues and associated lymph nodes from sheep was <0.2% using HPC decontamination and growth in modified BACTEC 12B medium (Whittington, 2009).

22.6.2 Analytical sensitivity and reduction in *MAP* counts during processing

There is a dramatic loss of *MAP* when samples are prepared for culture. The antimicrobials that are used to prepare samples or included in culture media may have a deleterious effect on viability, recovery or growth of *MAP*. A progressive loss of viable organisms occurs with each step in sedimentation and centrifugation protocols, as only part of the material from one step is taken forward to the next step. Protocols with NaOH or OA reduced the concentration of *MAP* from cattle by 1–2 logs in 4 h and BAC reduced it by 1 log; HPC did not affect the viability of *MAP* over a 5-day incubation at concentrations of up to 1% (Jorgensen, 1982; Merkal *et al.*, 1982; Whipple and Merkal, 1983). The double incubation protocol (method 3 in Table 22.4), which involves both HPC and VAN antibiotics, is associated with a 2.7 log loss of Type S strains of *MAP*, of which 1.3–1.7 log was due to the VAN step and little was due to HPC (Reddacliff *et al.*, 2003b). Thus, about 99% of the viable organisms in a sample can be killed or lost during processing. When these factors are taken into account, several researchers have reported the analytical sensitivity of faecal culture to be no better than 10^2 viable organisms per 1–2 g of faeces (Jorgensen, 1982; Reddacliff *et al.*, 2003b). Obviously there will be false-negative culture outcomes for samples that contain few organisms due to the loss of *MAP* during the culture process.

The situation with tissue samples is similar or worse; there was a 3.1 log loss with the HPC protocol (Reddacliff *et al.*, 2003b). There is a striking deleterious impact of HPC on *MAP* in milk: the temperature (22 °C optimal) and duration of incubation (2–5 h optimal) in this decontaminant can be critical to minimize inactivation of *MAP*. After 5 h incubation in HPC, losses are such that analytical sensitivity in liquid media is no better than 10^2–10^3 *MAP* cells per ml of milk (Grant *et al.*, 2003). Conflicting data for the impact of HPC

Table 22.5. Reported contamination rates of faecal cultures for *MAP* using contemporary culture methods.

Species	Decontamination protocol[a]	Media	No. of samples (no. of slopes per sample)	Contamination rate	Definition of contamination
Cattle	3. Sedimentation, centrifugation, HPC	HEYM	4688 (4)	9.6% of samples 58% of slopes	Overgrowth of contaminants on ≥2 slopes (Whitlock *et al.*, 1989)
Cattle	1. and 2. Sedimentation or centrifugation, HPC	HEYM	131 (3)	26% and 60%	Presence of contaminants (Kim *et al.*, 1989)
Cattle	1. Sedimentation, 3 μm filtered, HPC	BACTEC 12B	603	3.9%	Uncertain (M.T. Collins *et al.*, 1990)
Cattle	1. Sedimentation, HPC	BACTEC 12B	453	7.7%	Presence of contaminants (Cousins *et al.*, 1995)
Alpaca	1. Sedimentation, HPC	BACTEC 12B	137	7.3%	Presence of contaminants (Cousins *et al.*, 1995)
Cattle	5. Centrifugation, NaOH–OA	LJ	2989 (4)	0.13% of samples 7.0% of slopes	Overgrowth (Kalis *et al.*, 1999)
Cattle	3. Sedimentation, centrifugation, HPC	HEYM	463 (4)	30%	Overgrowth of contaminants on ≥1 slope (Secott *et al.*, 1999)
Cattle	5. Centrifugation, NaOH–OA	LJ and HEYM	2513 (4 HEYM; 2 LJ)	13–14% of samples	Overgrowth (Nielsen *et al.*, 2004)
Sheep	3. Sedimentation, centrifugation, HPC	BACTEC 12B	5066	11.7%	Presence of contaminants (Whittington, 2009)

[a]The number corresponds to a method in Table 22.4.

on the recovery of *MAP* from faeces, tissues and milk suggest that there might be complex interactions between this chemical, the type of substrate, the strain of *MAP* and the culture media.

22.7 Identification of *MAP* in Cultures

22.7.1 Fundamental characteristics

When colonies that consist of AFB are recognized on solid media or when growth is reported in broth culture, the next challenge is to identify *MAP*. A presumptive identification can be made based on slow growth (colonies develop after ≥3 weeks) and host tissue predilection, as cases of granulomatous enteritis associated with AFB in livestock are most likely to be Johne's disease.

Twort and Ingram (1912) were the first to describe *MAP* colonies. They were initially round, smooth and white, then tended to heap up slightly and become dull light yellow with wrinkling of the surface; pigmentation of colonies was influenced by the colour of the *M. phlei* or egg that was added to the medium.

Colony morphology is dependent on the medium, and addition of supplements can dramatically alter it: Tween compounds, which may be hydrolysed and become a source of oleic acid for the organism, lead to otherwise irregular granular colonies on 7H9 agar appearing instead as entire, smooth and domed (van Boxtel *et al.*, 1990). In contrast, colonies of Type S *MAP* growing on 7H10 agar are white, circular, shiny, raised and convex. They reach ≤1 mm in diameter by 6 weeks (Whittington *et al.*, 1999) (see Fig. 22.1 for photos of *MAP* colonies).

22.7.2 Mycobactin dependency

MAP isolates generally require an exogenous source of mycobactin for growth on commonly used media, and this attribute has become the primary feature used to distinguish *MAP* from other mycobacteria (Morrison, 1965; Thorel *et al.*, 1990). Demonstration of mycobactin dependency involves subculture of a colony from the primary slope or from broth to two media, one with and the other without mycobactin, then comparing the degree of growth on the two media (Whipple *et al.*, 1991). Demonstration of mycobactin dependency is not infallible and the result is influenced by the pH of the medium, the concentration of iron and the carry-over of mycobactin attached to the cell wall of *MAP* from the primary culture medium (Lambrecht and Collins, 1992). *MAP* that were cultured from the intestinal tissues of sheep in Spain were not dependent on mycobactin for primary isolation on 7H11 agar but were on LJ (Aduriz *et al.*, 1995). To further complicate matters, some strains of *M. avium* appear to be mycobactin dependent (Matthews *et al.*, 1977; Thorel, 1984).

22.7.3 Molecular confirmation using IS*900*

The discovery of IS*900*, an insertion element thought to be specific for *MAP*, provided a molecular basis for identification (Green *et al.*, 1989). PCR is now used alone or in combination with mycobactin dependency for the identification of *MAP* in cultures. This is sufficient for well-accepted epidemiological associations between *MAP* and a particular host in regions where *MAP* is endemic. IS*900* is a member of a family of insertion sequences, some of which closely resemble IS*900* and so positive results can be obtained from other mycobacterial species using probes and primers that are commonly applied (Cousins *et al.*, 1999; Englund *et al.*, 2002; Kim *et al.*, 2002). Strategies to resolve closely related IS*900*-like insertion sequences include restriction endonuclease analysis of the PCR product, sequencing of the PCR product, use of internal probes in assays based on technology such as Taqman and high-stringency real-time PCR protocols with specific primers, and the assessment of melting temperatures (Cousins *et al.*, 1999; Englund *et al.*, 2002; Kim *et al.*, 2002; Kawaji *et al.*, 2007). Because the identification of *MAP* has been shown to be less than 100% specific on several counts, and regardless of assurances from laboratories, microbiological diagnoses should be questioned if they do not make epidemiological sense. This is how the organisms that carry IS*900*-like sequences were discovered (Cousins *et al.*, 1999). Thus, for confirmation of an index case in any species, it is critical that IS*900*-based PCR does not serve as a stand-alone test used for culture confirmation. In such investigations, additional microbiological data to confirm the identification of *MAP* are needed.

PCR analysis is readily conducted after release of DNA from bacterial cells using simple methods such as boiling a suspension of the colony (Whittington *et al.*, 1999). However, it can be quite difficult to obtain suitable samples for identification of *MAP* from modified BACTEC 12B medium cultures, because egg yolk inhibits PCR amplification of IS*900*. The first protocols for PCR confirmation required subculture in BACTEC 12B medium without egg yolk and incubation for a few weeks, which delayed the diagnosis (Cousins *et al.*, 1995). Later, a simple extraction of the BACTEC 12B broth in ethanol was developed to remove the egg yolk, followed by heating of the supernatant to lyse *MAP* cells and release DNA (Whittington *et al.*, 1998). In some cases, particularly where the culture contained other types of bacteria as well as *MAP*, purification of DNA

from the lysate using a silica column was required to remove residual inhibitors of PCR. This method has been used for more than 10 years with great success (Whittington, 2009). Several other methods for harvesting DNA from broth cultures that contained egg yolk were compared recently using ten samples; simply boiling the broth to release DNA was almost as successful as a commercial column kit (Sweeney *et al.*, 2006).

22.7.4 Effect of contaminants on the identification of *MAP*

Many cultures of *MAP* are mixed cultures that also contain irrelevant microbes. The latter confound the identification of *MAP* in liquid culture by inhibiting IS*900* PCR, increasing the number of samples for which purification of DNA extracts is required prior to PCR. On solid medium, contaminants have been shown to inhibit the growth of *MAP* or to obscure *MAP* colonies (Secott *et al.*, 1999; Whittington, 2009). Thus contaminants complicate, delay and increase the costs of culture of *MAP*.

22.8 Comparison of Culture Methods

Methods for cultivation of *MAP* from clinical samples ideally should have the following characteristics: capacity to support the growth of all strains of *MAP*, high analytical sensitivity, low contamination rate, short incubation period, ease of identification of *MAP*, low overall cost, and low occupational health and environmental impact. Not all media support the growth of all strains of *MAP*. However, modified 7H10 agar, 7H11 agar and BACTEC 12B medium appear to do this and within reasonable incubation periods. Common Type S strains of *MAP* do not grow well or even at all on HEYM, LJ or in MGIT ParaTB medium. Not surprisingly, HEYM selects for a narrower range of genetic types of *MAP* than does liquid culture (Cernicchiaro *et al.*, 2008).

The various protocols used for culture of *MAP* on solid media have different sensitivities (Jorgensen, 1982). Seemingly small differences between protocols can affect both sensitivity and contamination rate. For example, sensitivity for several methodological variations using HPC and HEYM ranged from 39 to 68% in one study (Eamens *et al.*, 2000) and varied by tenfold in another (Stabel, 1997).

Liquid culture methods have greater sensitivity than solid culture, regardless of the strain of *MAP* (Table 22.6). The lower sensitivity of solid media is disguised by the fact that protocols often specify inoculation of multiple (up to four) solid medium slopes. A reduction from four to three HEYM slopes reduced sensitivity by 8% (Whitlock *et al.*, 1989). The sensitivity of culture of *MAP* from milk in BACTEC 12B and MGIT media was similar (Grant *et al.*, 2003).

Table 22.6. Comparative sensitivity of various faecal culture methods.

			Sensitivity (no. slopes inoculated)				
			Solid media		Liquid media		
Species	No.	No. positive	HPC[a] HEYM	HPC 7H10	HPC BACTEC 12B	HPC MB/BacT	Reference
Cattle	603	75	60% (2)		92%		M.T. Collins *et al.*, 1990
Cattle	179	38	39–68% (3)		89%		Eamens *et al.*, 2000
Cattle	25[b]	17	82%			88%	Stich *et al.*, 2004
Cattle	240	81	46–65% (2)			80%; 94%[c]	Motiwala *et al.*, 2005
Sheep	1535	202		34% (1)	98%		Whittington, 2009

[a]Various decontamination protocols were used; [b]selected interlaboratory check test samples; [c]identification by: subculture; molecular detection.

MAP grows at different rates on different types of media. *MAP* colonies took 3 weeks longer to appear on HEYM than on 7H10 agar, and the organism was evident 5 weeks sooner in BACTEC 12B medium than on HEYM slopes (Damato and Collins, 1990). Thus, time to reporting a result is shorter with liquid media than with solid media. Provided contamination rates are minimized, *MAP* can be identified equally well from solid or liquid media.

There are no contemporary cost–benefit analyses for any of the culture methods, but incubation time and the time taken to clarify the status of contaminated cultures are costly to laboratories. In regulatory applications, costs to the users of test results include business risk associated with the delay between submission of samples and receipt of results and the costs of false-negative test results. There is a high level of demand from industry for the development of quicker culture methods.

BACTEC 12B medium is a low-level radioactive source, which creates a waste disposal problem in some jurisdictions, whereas the other liquid systems have fewer environmental and occupational health and safety constraints. All methods create substantial laboratory waste through use of disposable plasticware, antibiotics and other chemicals.

22.9 Quality Control

Good laboratory practice dictates that positive and negative control samples are included with each batch of samples to be processed. Although several international studies have been conducted, there are few published reports of the results of interlaboratory quality control tests for culture of *MAP* (Sockett *et al.*, 1992). Programmes to evaluate the accuracy and consistency of culture test outcomes between laboratories are conducted in several countries. In Australia, annual interlaboratory proficiency tests for paratuberculosis are conducted under guidance from the OIE reference laboratory in Melbourne (Gwozdz, 2006) and are managed by the Australian National Quality Assurance Program for veterinary laboratories; participation is required for laboratory accreditation under the National Association of Testing Authorities. Faecal culture tests are evaluated less often than serological tests. The degree of difficulty in procuring samples to represent the full spectrum of species and levels of *MAP* burden, standardizing samples and shipping faecal samples in good condition to multiple laboratories is a disincentive to the regular conduct of such tests for *MAP* cultivation.

22.10 General Recommendations and Conclusions

If better control of paratuberculosis through detection of infected herds is desired, liquid culture methods, which are much more sensitive than solid culture, will need to be used more widely. International standards for culture protocols are required, as there are many variations of protocols, which have significant effects on sensitivity. This variability prevents the meaningful analysis of new diagnostic assays for which culture results serve as the gold standard. Contamination of faecal cultures remains a major hurdle to timely and economical culture of *MAP*, and improved decontamination protocols would be a major advance, enabling greater use of liquid culture. It remains to be seen whether an all-purpose liquid culture system can be developed in time to replace BACTEC 12B medium, which may soon be discontinued by the manufacturer as the MGIT 960 system takes over from it in the medical microbiology market. Modified BACTEC 12B is currently the only liquid culture medium that has been proven to be capable of growing all common strains of *MAP*. The widespread global use of media that do not support the growth of all strains has slowed appreciation of the true diversity, distribution and prevalence of *MAP* and biased understanding of the taxon in favour of the most common strains from cattle.

References

Aduriz, J.J., Juste, R.A. and Cortabarria, N. (1995) Lack of mycobactin dependence of

mycobacteria isolated on Middlebrook 7H11 from clinical cases of ovine paratuberculosis. *Veterinary Microbiology* 45, 211–217.

Alexejeff-Goloff, N.A. (1935) The pathogenesis and excretion of the bacilli in bovine paratuberculosis (Abstract). *Journal of Comparative Pathology* 48, 81–82.

Bannantine, J.P., Zhang, Q., Li, L.L. and Kapur, V. (2003) Genomic homogeneity between *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* belies their divergent growth rates. *BMC Microbiology* 3, 10.

Beerwerth, W. (1967) Culture of mycobacteria from faeces of domestic animals and their significance for the epidemiology of tuberculosis [Die Zuchtung von Mycobakterien aus dem Kot der Haustiere und ihre Bedeutung für die Epidemiologie der Tuberkulose]. *Praxis der Pneumologie* 21, 189–202.

Begg, D.J. and Whittington, R.J. (2008) Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. *The Veterinary Journal* 176, 129–145.

Cameron, J. (1956) Isolation of *Mycobacterium johnei* from faeces. *Journal of Pathology and Bacteriology* 71, 223–225.

Cernicchiaro, N., Wells, S.J., Janagama, H. and Sreevatsan, S. (2008) Influence of type of culture medium on characterization of *Mycobacterium avium* subsp. *paratuberculosis* subtypes. *Journal of Clinical Microbiology* 46, 145–149.

Chiodini, R.J. (1989) Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clinical Microbiology Reviews* 2, 90–117.

Collins, D.M., Gabric, D.M. and de Lisle, G.W. (1990) Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. *Journal of Clinical Microbiology* 28, 1591–1596.

Collins, M.T., Kenefeck, K.B., Sockett, D.C., Lambrecht, R.S., McDonald, J. and Jorgensen, J.B. (1990) Enhanced radiometric detection of *Mycobacterium paratuberculosis* by using filter-concentrated bovine fecal specimens. *Journal of Clinical Microbiology* 28, 2514–2519.

Cousins, D.V., Evans, R.J. and Francis, B.R. (1995) Use of BACTEC radiometric culture method and polymerase chain reaction for the rapid screening of faeces and tissues for *Mycobacterium paratuberculosis*. *Australian Veterinary Journal* 72, 458–462.

Cousins, D.V., Whittington, R., Marsh, I., Masters, A., Evans, R.J. and Kluver, P. (1999) Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS*900*-like sequences detectable by IS*900* polymerase chain reaction: implications for diagnosis. *Molecular and Cellular Probes* 13, 431–442.

Damato, J.J. and Collins, M.T. (1990) Growth of *Mycobacterium paratuberculosis* in radiometric, Middlebrook and egg-based media. *Veterinary Microbiology* 22, 31–42.

Damato, J.J., Knisley, C. and Collins, M.T. (1987) Characterization of *Mycobacterium paratuberculosis* by gas–liquid and thin-layer chromatography and rapid demonstration of mycobactin dependence using radiometric methods. *Journal of Clinical Microbiology* 25, 2380–2383.

Dundee, L., Grant, I.R., Ball, H.J. and Rowe, M.T. (2001) Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk. *Letters in Applied Microbiology* 33, 173–177.

Dunkin, G.W. (1928) A diagnostic agent for the detection of Johne's disease and its method of preparation. *Journal of Comparative Pathology and Therapeutics* 41, 94–108.

Eamens, G.J., Whittington, R.J., Marsh, I.B., Turner, M.J., Saunders, V., Kemsley, P.D. and Rayward, D. (2000) Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. *Veterinary Microbiology* 77, 357–367.

Eamens, G.J., Walker, D.M., Porter, N.S. and Fell, S.A. (2007a) Pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in goats. *Australian Veterinary Journal* 85, 243–251.

Eamens, G.J., Whittington, R.J., Turner, M.J., Austin, S.L., Fell, S.A. and Marsh, I.B. (2007b) Evaluation of radiometric faecal culture and direct PCR on pooled faeces for detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle. *Veterinary Microbiology* 125, 22–35.

Eamens, G.J., Walker, D.M., Porter, N.S. and Fell, S.A. (2008) Radiometric pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in low-shedder cattle. *Australian Veterinary Journal* 86, 259–265.

Ellingson, J.L.E., Koziczkowski, J.J. and Anderson, J.L. (2004) Comparison of PCR prescreening to two cultivation procedures with PCR confirmation for detection of *Mycobacterium avium* subsp. *paratuberculosis* in U.S. Department of Agriculture fecal check test samples. *Journal of Food Protection* 67, 2310–2314.

Ellingson, J.L.E., Anderson, J.L., Koziczkowski, J.J., Radcliff, R.P., Sloan, S.J., Allen, S.E. and Sullivan, N.M. (2005) Detection of viable

Mycobacterium avium subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. *Journal of Food Protection* 68, 966–972.

Englund, S., Bolske, G. and Johansson, K.E. (2002) An IS*900*-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiology Letters* 209, 267–271.

Fischer, O.A., Matlova, L., Bartl, J., Dvorska, L., Svastova, P., Maine, R.D., Melicharek, I., Bartos, M. and Pavlik, I. (2003) Earthworms (Oligochaeta, Lumbricidae) and mycobacteria. *Veterinary Microbiology* 91, 325–338.

Fischer, O.A., Matlova, L., Dvorska, L., Svastova, P., Bartl, J., Weston, R.T. and Pavlik, I. (2004) Blowflies *Calliphora vicina* and *Lucilia sericata* as passive vectors of *Mycobacterium avium* subsp. *avium*, *M. a. paratuberculosis* and *M. a. hominissuis*. *Medical and Veterinary Entomology* 18, 116–122.

Gao, A., Odumeru, J., Raymond, M. and Mutharia, L. (2005) Development of improved method for isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bulk tank milk: effect of age of milk, centrifugation, and decontamination. *Canadian Journal of Veterinary Research* 69, 81–87.

Gillespie, S.H. (1999) Microbiological diagnosis of granulomatous infections. In: James, D.G. and Zumla, A. (eds) *The Granulomatous Disorders*. University Press, Cambridge, UK, pp. 122–131.

Grant, I.R., Ball, H.J. and Rowe, M.T. (1998) Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. *Applied and Environmental Microbiology* 64, 3153–3158.

Grant, I.R., Ball, H.J. and Rowe, M.T. (2002) Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. *Applied and Environmental Microbiology* 68, 2428–2435.

Grant, I.R., Kirk, R.B., Hitchings, E. and Rowe, M.T. (2003) Comparative evaluation of the MGIT™ and BACTEC culture systems for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from milk. *Journal of Applied Microbiology* 95, 196–201.

Green, E.P., Tizard, M.L., Moss, M.T., Thompson, J., Winterbourne, D.J., McFadden, J.J. and Hermon-Taylor, J. (1989) Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Research* 17, 9063–9073.

Gumber, S. and Whittington, R.J. (2007) Comparison of BACTEC 460 and MGIT 960 systems for the culture of *Mycobacterium avium* subsp. *paratuberculosis* S strain and observations on the effect of inclusion of ampicillin in culture media to reduce contamination. *Veterinary Microbiology* 119, 42–52.

Gumber, S., Taylor, D.L. and Whittington, R.J. (2008) Growth pattern and partial proteome of *Mycobacterium avium* subsp. *paratuberculosis* during the stress response to hypoxia and nutrient starvation. *Veterinary Microbiology* 133, 344–357.

Gunnarsson, E. (1979) Isolation of *Mycobacterium paratuberculosis* from sheep and cattle in Iceland. *Acta Veterinaria Scandinavica* 20, 191–199.

Gunnarsson, E. and Fodstad, F.H. (1979) Cultural and biochemical characteristics of *Mycobacterium paratuberculosis* isolated from goats in Norway. *Acta Veterinaria Scandinavica* 20, 122–134.

Gwozdz, J.M. (2006) Paratuberculosis. In: *Annual Reports of OIE Reference Laboratories and Collaborating Centres*. World Organisation for Animal Health, Paris, pp. 125–127.

Herrold, R.D. (1931) Egg yolk agar medium for the growth of tubercle bacilli. *The Journal of Infectious Diseases* 48, 236–241.

Jorgensen, J.B. (1982) An improved medium for culture of *Mycobacterium paratuberculosis* from bovine faeces. *Acta Veterinaria Scandinavica* 23, 325–335.

Juste, R.A., Marco, J.C., Saez-de-Ocariz, C. and Aduriz, J.J. (1991) Comparison of different media for the isolation of small ruminant strains of *Mycobacterium paratuberculosis*. *Veterinary Microbiology* 28, 385–390.

Kalis, C.H.J., Hesselink, J.W., Russchen, E.W., Barkema, H.W., Collins, M.T. and Visser, I.J.R. (1999) Factors influencing the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bovine faecal samples. *Journal of Veterinary Diagnostic Investigation* 11, 345–351.

Kalis, C.H.J., Hesselink, J.W., Barkema, H.W. and Collins, M.T. (2000) Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis. *Journal of Veterinary Diagnostic Investigation* 12, 547–551.

Kalis, C.H.J., Collins, M.T., Barkema, H.W. and Hesselink, J.W. (2004) Certification of herds as free of *Mycobacterium paratuberculosis* infection: actual pooled faecal results versus certification model predictions. *Preventive Veterinary Medicine* 65, 189–204.

Kawaji, S., Taylor, D.L., Mori, Y. and Whittington, R.J. (2007) Detection of *Mycobacterium avium*

subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. *Veterinary Microbiology* 125, 36–48.

Kim, S.G., Shin, S.J., Jacobson, R.H., Miller, L.J., Harpending, P.R., Stehman, S.M., Rossiter, C.A. and Lein, D.A. (2002) Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 14, 126–131.

Kim, Y.G., Bech-Nielsen, S., Slemons, R.D. and Spangler, E. (1989) Comparison of two methods for isolation of *Mycobacterium paratuberculosis* from bovine fecal samples. *American Journal of Veterinary Research* 50, 1110–1113.

Koenig, G.J., Hoffsis, G.F., Shulaw, W.P., Bech-Nielsen, S., Rings, D.M. and St-Jean, G. (1993) Isolation of *Mycobacterium paratuberculosis* from mononuclear cells in tissues, blood, and mammary glands of cows with advanced paratuberculosis. *American Journal of Veterinary Research* 54, 1441–1445.

Lambrecht, R.S. and Collins, M.T. (1992) *Mycobacterium paratuberculosis*. Factors that influence mycobactin dependence. *Diagnostic Microbiology and Infectious Disease* 15, 239–246.

Lambrecht, R.S., Carriere, J.F. and Collins, M.T. (1988) A model for analyzing growth kinetics of a slowly growing *Mycobacterium* sp. *Applied and Environmental Microbiology* 54, 910–916.

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B. and Salman, M.D. (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. *Journal of Dairy Science* 89, 4163–4171.

Mason, O., Marsh, I.B. and Whittington, R.J. (2001) Comparison of immunomagnetic bead separation-polymerase chain reaction and faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in sheep faeces. *Australian Veterinary Journal* 79, 497–500.

Matthews, P.R.J., McDiarmid, A., Collins, P. and Brown, A. (1977) The dependence of some strains of *Mycobacterium avium* on mycobactin for initial and subsequent growth. *Journal of Medical Microbiology* 11, 53–57.

Merkal, R.S. (1973) Laboratory diagnosis of bovine paratuberculosis. *Journal of the American Veterinary Medical Association* 163, 1100–1102.

Merkal, R.S. (1984) Paratuberculosis: advances in cultural, serologic, and vaccination methods. *Journal of the American Veterinary Medical Association* 184, 939–943.

Merkal, R.S. and Curran, B.J. (1974) Growth and metabolic characteristics of *Mycobacterium paratuberculosis*. *Applied Microbiology* 28, 276–279.

Merkal, R.S., Kopecky, K.E., Larsen, A.B. and Thurston, J.R. (1964) Improvements in the techniques for primary cultivation of *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 25, 1290–1294.

Merkal, R.S., Larsen, A.B., Kopecky, K.E. and Ness, R.D. (1968) Comparison of examination and test methods for early detection of paratuberculous cattle. *American Journal of Veterinary Research* 29, 1533–1538.

Merkal, R.S., Lyle, P.A.S. and Whipple, D.L. (1982) Decontamination, media and culture methods for *Mycobacterium paratuberculosis*. In: *Proceedings of the 86th Annual Meeting of the United States Animal Health Association*. United States Animal Health Association, Richmond, Virginia, pp. 519–523.

Middlebrook, G., Reggiardo, Z. and Tigertt, W.D. (1977) Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *American Review of Respiratory Disease* 115, 1066–1069.

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T. and Hermon-Taylor, J. (1996) IS*900* PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cow's milk in England and Wales. *Applied and Environmental Microbiology* 62, 3446–3452.

Minett, F.C. (1942) The diagnosis of Johne's disease of cattle by cultural means. *Journal of Pathology and Bacteriology* 54, 209–219.

Morrison, N.E. (1965) Circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*. *Journal of Bacteriology* 89, 762–767.

Motiwala, A.S., Strother, M., Theus, N.E., Stich, R.W., Byrum, B., Shulaw, W.P., Kapur, V. and Sreevatsan, S. (2005) Rapid detection and typing of strains of *Mycobacterium avium* subsp. *paratuberculosis* from broth cultures. *Journal of Clinical Microbiology* 43, 2111–2117.

Naser, S.A., Schwartz, D. and Shafran, I. (2000) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. *American Journal of Gastroenterology* 95, 1094–1095.

Naser, S.A., Ghobrial, G., Romero, C. and Valentine, J.F. (2004) Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *The Lancet* 364, 1039–1044.

Nielsen, S.S., Kolmos, B. and Christoffersen, A.B. (2004) Comparison of contamination and growth of *Mycobacterium avium* subsp. *paratuberculosis* on two different media. *Journal of Applied Microbiology* 96, 149–153.

Parrish, N.M., Ko, C.G., Dick, J.D., Jones, P.B. and Ellingson, J.L. (2004) Growth, Congo Red agar colony morphotypes and antibiotic susceptibility testing of *Mycobacterium avium* subspecies *paratuberculosis*. *Clinical Medicine and Research* 2, 107–114.

Parrish, N.M., Radcliff, R.P., Brey, B.J., Anderson, J.L., Clark, D.L., Jr., Koziczkowski, J.J., Ko, C.G., Goldberg, N.D., Brinker, D.A., Carlson, R.A., Dick, J.D. and Ellingson, J.L. (2009) Absence of *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's patients. *Inflammatory Bowel Diseases* 15, 558–565.

Pickup, R.W., Rhodes, G., Arnott, S., Sidi-Boumedine, K., Bull, T.J., Weightman, A., Hurley, M. and Hermon-Taylor, J. (2005) *Mycobacterium avium* subsp. *paratuberculosis* in the catchment area and water of the River Taff in South Wales, United Kingdom, and its potential relationship to clustering of Crohn's disease cases in the city of Cardiff. *Applied and Environmental Microbiology* 71, 2130–2139.

Reddacliff, L.A., Nicholls, P.J., Vadali, A. and Whittington, R.J. (2003a) Use of growth indices from radiometric culture for quantification of sheep strains of *Mycobacterium avium* subsp. *paratuberculosis*. *Applied and Environmental Microbiology* 69, 3510–3516.

Reddacliff, L.A., Vadali, A. and Whittington, R.J. (2003b) The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. *Veterinary Microbiology* 95, 271–282.

Reddacliff, L., Eppleston, J., Windsor, P., Whittington, R. and Jones, S. (2006) Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Veterinary Microbiology* 115, 77–90.

Reggiardo, Z. and Tigertt, W.D. (1977) Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *American Review of Respiratory Disease* 115, 1066–1069.

Saxegaard, F. (1985) Isolation of *Mycobacterium paratuberculosis* from intestinal mucosa and mesenteric lymph nodes of goats by use of selective Dubos medium. *Journal of Clinical Microbiology* 22, 312–313.

Secott, T.E., Ohme, A.M., Barton, K.S., Wu, C.C. and Rommel, F.A. (1999) *Mycobacterium paratuberculosis* detection in bovine feces is improved by coupling agar culture enrichment to an IS*900*-specific polymerase chain reaction assay. *Journal of Veterinary Diagnostic Investigation* 11, 441–447.

Sergeant, E.S., Whittington, R.J. and More, S.J. (2002) Sensitivity and specificity of pooled faecal culture and serology as flock-screening tests for detection of ovine paratuberculosis in Australia. *Preventive Veterinary Medicine* 52, 199–211.

Shin, S. (1989) Report of the Committee on Johne's Disease. In: *Proceedings of the 93rd Annual Meeting of the United States Animal Health Association*. United States Animal Health Association, Richmond, Virginia. pp. 380–381.

Shin, S.J., Han, J.H., Manning, E.J. and Collins, M.T. (2007) Rapid and reliable method for quantification of *Mycobacterium paratuberculosis* by use of the BACTEC MGIT 960 system. *Journal of Clinical Microbiology* 45, 1941–1948.

Smith, H.W. (1953) Modifications of Dubos's media for the cultivation of *Mycobacterium johnei*. *Journal of Pathology and Bacteriology* 66, 375–381.

Sockett, D.C., Carr, D.J. and Collins, M.T. (1992) Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of *Mycobacterium paratuberculosis* infection in cattle. *Canadian Journal of Veterinary Research* 56, 148–153.

Stabel, J.R. (1997) An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. *Journal of Veterinary Diagnostic Investigation* 9, 375–380.

Stabel, J.R., Steadham, E.M. and Bolin, C.A. (1997) Heat inactivation of *Mycobacterium paratuberculosis* in raw milk: are current pasteurization conditions effective? *Applied and Environmental Microbiology* 63, 4975–4977.

Stevenson, K., Hughes, V.M., de Juan, L., Inglis, N.F., Wright, F. and Sharp, J.M. (2002) Molecular characterization of pigmented and non-pigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 40, 1798–1804.

Stich, R.W., Byrum, B., Love, B., Theus, N., Barber, L. and Shulaw, W.P. (2004) Evaluation of an automated system for non-radiometric detection of *Mycobacterium avium paratuberculosis* in bovine feces. *Journal of Microbiological Methods* 56, 267–275.

Sweeney, R.W., Whitlock, R.H. and Rosenberger, A.E. (1992) *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *Journal of Clinical Microbiology* 30, 166–171.

Sweeney, R.W., Whitlock, R.H. and McAdams, S.C. (2006) Comparison of three DNA preparation

methods for real-time polymerase chain reaction confirmation of *Mycobacterium avium* subsp. *paratuberculosis* growth in an automated broth culture system. *Journal of Veterinary Diagnostic Investigation* 18, 587–590.

Tavornpanich, S., Munoz-Zanzi, C.A., Wells, S.J., Raizman, E.A., Carpenter, T.E., Johnson, W.O. and Gardner, I.A. (2008) Simulation model for evaluation of testing strategies for detection of paratuberculosis in midwestern US dairy herds. *Preventive Veterinary Medicine* 83, 65–82.

Taylor, A.W. (1950) Observations on the primary isolation of *Mycobacterium johnei* in primary culture. *Journal of Pathology and Bacteriology* 62, 647–650.

Taylor, T.K., Wilks, C.R. and McQueen, D.S. (1981) Isolation of *Mycobacterium paratuberculosis* from the milk of a cow with Johne's disease. *The Veterinary Record* 109, 532–533.

Thorel, M.F. (1984) Review of the occurrence of mycobactin dependence among mycobacteria species. *Annales de Recherches Vétérinaires* 15, 405–409.

Thorel, M.F., Krichevsky, M. and Levy-Frebault, V.V. (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. *nov.*, *Mycobacterium avium* subsp. *paratuberculosis* subsp. *nov.*, and *Mycobacterium avium* subsp. *silvaticum* subsp. *nov. International Journal of Systematic Bacteriology* 40, 254–260.

Thornton, C.G., MacLellan, K.M., Stabel, J.R., Carothers, C., Whitlock, R.H. and Passen, S. (2002) Application of the C(18)-carboxypropylbetaine specimen processing method to recovery of *Mycobacterium avium* subsp. *paratuberculosis* from ruminant tissue specimens. *Journal of Clinical Microbiology* 40, 1783–1790.

Toribio, J.A. and Sergeant, E.S. (2007) A comparison of methods to estimate the prevalence of ovine Johne's infection from pooled faecal samples. *Australian Veterinary Journal* 85, 317–324.

Twort, F.W. and Ingram, G.L.Y. (1912) A method for isolating and cultivating the *Mycobacterium enteritidis chronicae pseudotuberculosae bovis*, Johne, and some experiments on the preparation of a diagnostic vaccine for pseudo-tuberculous enteritis of bovines. *Proceedings of the Royal Society of London (Series B) (Biology)* 84, 517–543.

van Boxtel, R.M., Lambrecht, R.S. and Collins, M.T. (1990) Effect of polyoxyethylene sorbate compounds (Tweens) on colonial morphology, growth, and ultrastructure of *Mycobacterium paratuberculosis*. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* 98, 901–908.

van Griethuysen, A.J., Jansz, A.R. and Buiting, A.G.M. (1996) Comparison of fluorescent BACTEC 9000 MB system, septi-chek AFB system, and Lowenstein–Jensen medium for detection of mycobacteria. *Journal of Clinical Microbiology* 34, 2391–2394.

van Schaik, G., Stehman, S.M., Schukken, Y.H., Rossiter, C.R. and Shin, S.J. (2003) Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. *Journal of Veterinary Diagnostic Investigation* 15, 233–241.

Vialard, J., Lacheretz, A., Thiercy, A., Richard, Y. and Prave, M. (1993) Detection of bovine paratuberculosis by culture of pooled feces [Détection de l'infection paratuberculeuse chez les bovins par une technique de coproculture de groupe]. *Revue de Médecine Vétérinaire* 144, 527–533.

Watson, E.A. (1935) Tuberculin, Johnin and Mallein derived from non-protein media. *Canadian Public Health Journal* 26, 268–275.

Wells, S.J., Whitlock, R.H., Lindeman, C.J. and Fyock, T. (2002) Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 63, 1207–1211.

Wells, S.J., Godden, S.M., Lindeman, C.J. and Collins, J.E. (2003) Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. *Journal of the American Veterinary Medical Association* 223, 1022–1025.

Whipple, D.L. and Merkal, R.S. (1983) Modifications in the technique for cultivation of *Mycobacterium paratuberculosis*. In: Merkal, R.S. (ed.) *Proceedings of the International Colloquium on Research in Paratuberculosis*. International Association for Paratuberculosis, Ames, Iowa, pp. 82–92.

Whipple, D.L., Callihan, D.R. and Jarnagin, J.L. (1991) Cultivation of *Mycobacterium paratuberculosis* from bovine fecal specimens and a suggested standardized procedure. *Journal of Veterinary Diagnostic Investigation* 3, 368–373.

Whitlock, R.H. and Rosenberger, A.E. (1990) Fecal culture protocol for *Mycobacterium paratuberculosis*. A recommended procedure. In: *Proceedings of the Annual Meeting of the United States Animal Health Association*. US Animal Health Association, Richmond, Virginia, pp. 280–285.

Whitlock, R.H., Rosenberger, A.E. and Spencer, P.A. (1989) Laboratory culture techniques for Johne's disease: a critical evaluation of contamination and incubation times. In: *Proceedings of the 93rd Annual Meeting of the United States Animal Health Association*. United States Animal Health Association, Richmond, Virginia. pp. 382–386.

Whitlock, R.H., West, S.R., Layton, B., Ellingson, J., Stabel, J., Rossiter, C., Buergelt, C., Ginn, P., Pavlik, I., Collins, M.T., Juste, R. and Habecker, P. (1999) Paratuberculosis in bison: a comparison of PCR, culture, serology and histopathology. In: Manning, E.J.B. and Collins, M.T. (eds) *Proceedings of the Sixth International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 424–438.

Whittington, R. (2009) Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from faecal and tissue samples in a liquid culture medium. *Journal of Clinical Microbiology* 47, 614–622.

Whittington, R.J. and Sergeant, E.S. (2001) Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. *Australian Veterinary Journal* 79, 267–278.

Whittington, R.J., Marsh, I., Turner, M.J., McAllister, S., Choy, E., Eamens, G.J., Marshall, D.J. and Ottaway, S. (1998) Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR. *Journal of Clinical Microbiology* 36, 701–707.

Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J. and Fraser, C.A. (1999) Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *Journal of Clinical Microbiology* 37, 1077–1083.

Whittington, R.J., Fell, S., Walker, D., McAllister, S., Marsh, I., Sergeant, E., Taragel, C.A., Marshall, D.J. and Links, I.J. (2000a) Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. *Journal of Clinical Microbiology* 38, 2550–2556.

Whittington, R.J., Reddacliff, L.A., Marsh, I., McAllister, S. and Saunders, V. (2000b) Temporal patterns and quantification of excretion of *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Australian Veterinary Journal* 78, 34–37.

Whittington, R.J., Lloyd, J.B. and Reddacliff, L.A. (2001) Recovery of *Mycobacterium avium* subsp. *paratuberculosis* from nematode larvae cultured from the faeces of sheep with Johne's disease. *Veterinary Microbiology* 81, 273–279.

Whittington, R., Marsh, I., Taylor, P.J., Marshall, D.J., Taragel, C. and Reddacliff, L. (2003) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from environment samples collected from farms before and after destocking sheep with paratuberculosis. *Australian Veterinary Journal* 81, 559–563.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, I.B. and Reddacliff, L.A. (2004) Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Applied and Environmental Microbiology* 70, 2989–3004.

Whittington, R.J., Marsh, I.B. and Reddacliff, L.A. (2005) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. *Applied and Environmental Microbiology* 71, 5304–5308.

23 Diagnosis of Paratuberculosis by PCR

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23.1 Introduction

In earlier reviews of assays based on the polymerase chain reaction (PCR) for detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), it was concluded that PCR alone for direct detection had not performed well enough to make it a practical alternative to

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other diagnostic tools (Nielsen *et al.*, 2001). When PCR was applied to confirm the identity of cultured acid-fast bacteria, the sensitivity of the assay was 100% (Manning and Collins, 2001), but when applied directly to biological samples the sensitivity was low (Grant *et al.*, 1998). Application of PCR directly to the sample material had been plagued with interference by components of the sample, which usually resulted in inhibition of the PCR reaction and could lead to false-negative results (Stevenson and Sharp, 1997). Research to surmount this obstacle was promising; immunomagnetic separation (IMS), a technique where specific antibody-coated magnetic beads are used to selectively separate *MAP* from the sample matrix (Grant *et al.*, 1998), was regarded as the best method for milk (Grant and Rowe, 2001) but did not work so well for faecal samples (Whittington, 2002). Another method tried for pre-treatment was hybridization capture (Marsh *et al.*, 2000). Since that time, there have been considerable advances in PCR techniques and DNA extraction methods, such that PCR holds more promise now for the diagnosis of paratuberculosis.

23.2 PCR Techniques

PCR has been of monumental importance for the field of molecular biology (Mullis and Faloona, 1987; Mullis, 1990), with a vast range of both clinical and research applications in biology and medicine. PCR is the temperature-guided enzymatic replication of a specific DNA sequence, where the product is used as a template for the next round of replication, thus allowing for an exponential amplification of the DNA product – a chain reaction yielding millions of DNA copies. PCR has revolutionized molecular diagnostics, not least in the field of infectious disease. Slow-growing or non-culturable bacteria and viruses have become easier to identify, and suspected pathogen growth by culture methods has become very easy to confirm.

In diagnostic PCR, the DNA sequence used for amplification needs to be chosen with an appropriate degree of specificity. The target for detection may be as wide as an entire phylum or as narrow as a certain strain and is defined by the design of DNA oligonucleotides, so-called primers, which bind to the target and initiate the reaction. After an appropriate number of reaction cycles, the high number of copies produced renders the DNA detectable and amenable to further analysis, such as visualization on a gel or sequencing.

23.2.1 Conventional PCR

The most basic PCR set-up, often referred to as conventional PCR, uses only a single primer pair (often referred to as forward and reverse primers), a DNA polymerase, dNTPs (deoxynucleoside triphosphates), which are the building blocks for DNA replication, a buffer solution and usually Mg^{2+} ions. DNA template is added to this mixture in tubes or wells. The reaction takes place in a thermal cycler, which heats and cools the mixture through 20–40 cycles of denaturation, annealing and elongation.

As illustrated in Fig. 23.1, the DNA strands are separated in the denaturation step (usually at 94–96 °C). During the annealing step (at around 50–65 °C), the primers anneal to the complementary sites on the 3′ end of the respective single DNA strands. The polymerase binds to the 3′ end of the primer, and DNA polymerization is initiated. The elongation step (often set to 70–72 °C) is at the optimum temperature for DNA polymerization. Here, the primer is extended to form a new DNA strand, complementary to the target DNA. In the second round of temperature cycling, primers and DNA polymerase will anneal not only to the original template but also to the copy from the last cycle. The product created by the forward primer will be the template for the reverse primer, and vice versa, limiting the size of the new product to that of the sequence between both primers. In subsequent cycles, exponential amplification of the sequence will result in a product defined in size by the interval between the primers. Eventually, the activity of the DNA polymerase decreases and limiting reagents are exhausted. The product yield levels off and eventually the reaction stops.

In conventional PCR, products are separated by gel electrophoresis, most often on

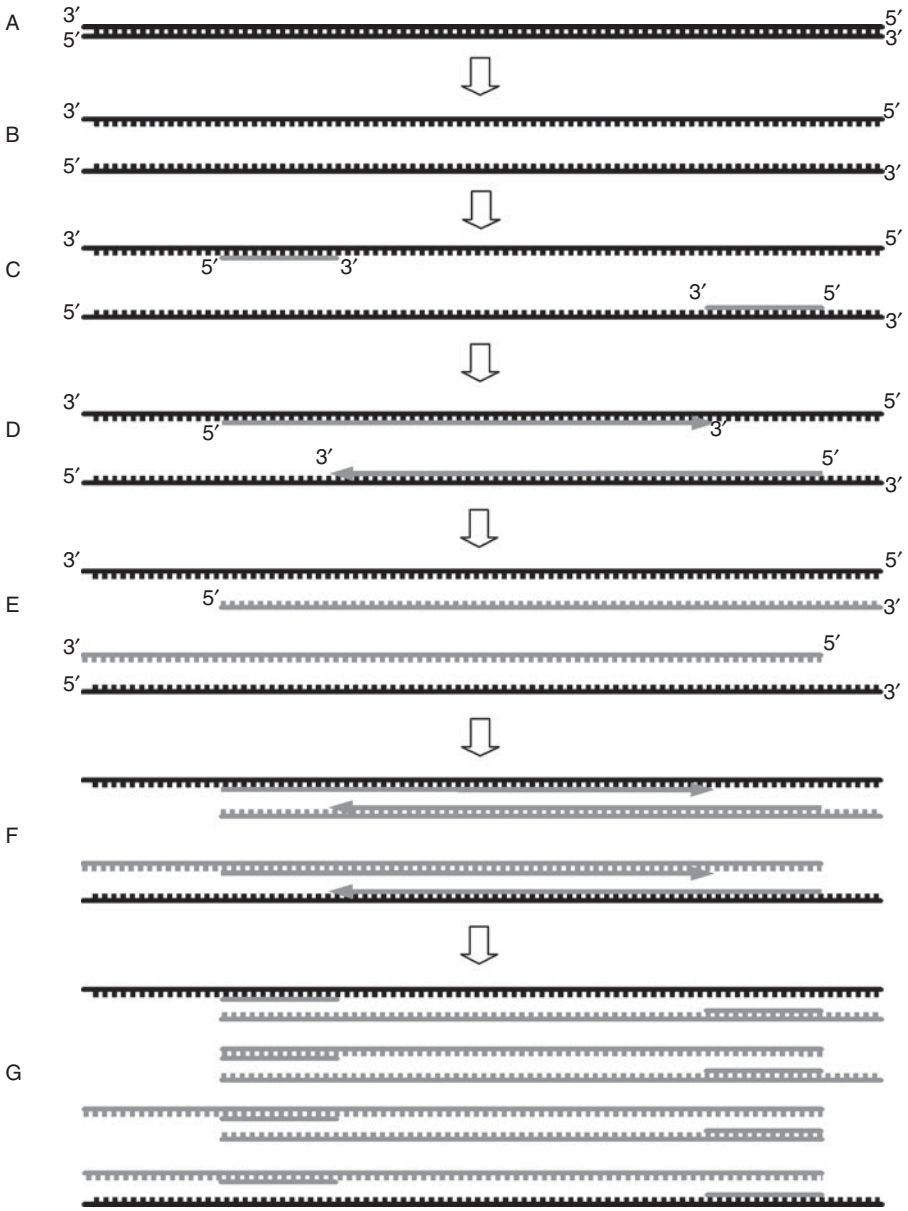

Fig. 23.1. Illustration of the PCR reaction. Template DNA is shown in black, primers and newly synthesized DNA in grey. Labels for 3′ end and 5′ end are omitted for clarity in illustrations F and G. A. Template DNA; B. cycle 1, denaturation; C. annealing; D. elongation; E. cycle 2, denaturation; F. annealing and elongation, which produces the product specified by the primers; G. cycle 3, denaturation and annealing. Increasingly more of the product is produced in each following cycle.

agarose gels. The electric potential attracts the negatively charged DNA towards the positive terminal with a speed inversely proportional to the size of the molecules. By staining the gel with ethidium bromide, which binds to double-stranded DNA and becomes strongly fluorescent, DNA can be visualized as bands under UV light. The observed distances the bands have travelled are compared with bands from a mass standard (a so-called ladder) to determine the product size. This step serves to confirm that the product, if present, is the size expected from the pathogen of interest, rather than a non-specific amplification product. Also, the intensity of the bands can give a rough quantification of the original amount of infectious agent. With a limited amount of target DNA, these bands can be difficult to detect. Recent reports on other amplicon detection methods include using spot hybridization and colorimetric detection (Halldórsdóttir *et al.*, 2002; Taddei *et al.*, 2004).

For detection of *MAP*, the most popular target gene is the multi-copy element IS*900*. It was first described by Green *et al.* (1989), but also independently isolated by Collins *et al.* (1989), and was first used as a target in a diagnostic PCR by Vary *et al.* (1990). Some of the most cited studies where conventional PCR was applied in *MAP* research include investigations of the presence of *MAP* in tissues from patients with Crohn's disease (Moss *et al.*, 1992; Sanderson *et al.*, 1992) and screening of retail milk (Millar *et al.*, 1996). The basic PCR technique has been elaborated into better and somewhat more complex methods, some of which are described in the following sections. Problems and limitations of conventional PCR are outlined in Table 23.1.

23.2.2 Nested PCR

The running of two consecutive PCR reactions with two different sets of primers, using the product of the first reaction as template in the second, enables a number of potential advantages to be achieved, including specificity and sensitivity. The first set of primers is outside the second set, resulting in progressively shorter amplicons. As all four primers have to match their target in the same DNA region to obtain a final product, specificity is enhanced. In addition, because new reagents are added in the secondary reaction, sensitivity is also increased (Englund *et al.*, 2001; Ikonomopoulos *et al.*, 2004). In the case of insufficient product for visualization after the first PCR, there may none the less be enough DNA to yield a detectable product in the nested PCR. In addition, any PCR inhibitors in the first run will be diluted when a volume of product is transferred as template to the second PCR; if inhibition is incomplete, this dilution may now permit the PCR reaction to proceed.

Table 23.1. Issues with the use of conventional PCR.

Issue	Explanatory notes
Limited sensitivity	As bands must be visible to the naked eye, sufficient starting DNA must be used in the PCR for a positive result. This is more often a problem with direct PCR (PCR on DNA from prepared clinical samples) than colony PCR
Relatively high time and labour consumption	Gels must be cast, loaded, operated and analysed
Quantification	Unreliable and imprecise
Risk of cross-contamination	For diagnostic purposes, PCR tubes should be opened for electrophoresis in a separate room to limit the spread of PCR product to the rest of the laboratory. A minute amount of product in a new sample may cause a reaction and false-positive result (Noordhoek *et al.*, 1994)
Inhibition	PCR inhibitors, such as certain salts, ionic detergents, alcohols, etc. can inhibit the reaction. Most direct PCR techniques suffer from this problem to varying degrees

Nested PCR is even more demanding of time and labour than conventional PCR. The transfer of products from the first run to the second is extremely critical and poses an even greater risk for cross-contamination, which is the main disadvantage with nested PCR. Positive reactions from the first run may contain millions of amplicons (DNA copies), and an involuntary transfer of the slightest amount to a negative tube may cause a false positive. In this case, specificity will be greatly compromised. A solution to this is one-tube nested PCR, which is difficult to design but none the less circumvents these problems.

The high sensitivity of nested PCR is generally not needed for detection of *MAP* in liquid culture, as the amount of *MAP* should be sufficient after growth. Rather, because of the considerable risk of cross-contamination in nested PCR, one should refrain from using it for this purpose.

The first reported use of nested PCR to detect *MAP* was in a comparison between faecal culture and PCR (Collins *et al.*, 1993). Many of the most cited reports where nested PCR has been used to detect *MAP* are investigations of Crohn's disease (Lisby *et al.*, 1994; Ryan *et al.*, 2002; Bull *et al.*, 2003; Autschbach *et al.*, 2005). Other important studies include a method comparison (Fang *et al.*, 2002) and a study of *MAP* in pasteurized milk (Gao *et al.*, 2002). Among recent publications of international veterinary importance are two method comparisons (Möbius *et al.*, 2008; Pinedo *et al.*, 2008) and a screening of beef carcasses (Meadus *et al.*, 2008).

23.2.3 Real-time PCR

In conventional PCR, only the amount of final DNA product is measured, a process referred to as end point analysis. In real-time PCR or quantitative PCR (qPCR) the instrument monitors the reaction progress throughout every temperature cycle by measuring the light emitted from a fluorophore incorporated with the DNA product, by using either a fluorescent dye that intercalates with all double-stranded DNA (SYBR Green being the most widely used) or target-specific fluorescent probes. The progress of the reaction is viewed on a computer. Since the SYBR Green signal will increase by either the desired product or non-specific double-stranded products, a melting curve analysis is done after the PCR reaction. In contrast, fluorescent probes bind to a specific site within the desired product, therefore increasing the specificity of the assay. There are different probe technologies, such as Scorpions, Molecular Beacons and FRET probes, but the most widely used is the so-called TaqMan probe.

On the 3′ end of the TaqMan probe, there is a so-called quencher dye attached, which prevents the fluorescent reporter dye on the 5′ end from emitting light. Due to the exonuclease activity of the Taq polymerase, the probe is digested during the elongation process and the dyes are separated, allowing the reporter dye to fluoresce. When the amount of DNA product increases, so does the fluorescent signal from the liberated reporters.

The number of cycles needed for the fluorescence to reach a certain threshold level, often referred to as the Ct value, is a concrete measure that lends itself to comparison across samples and quantification of target DNA. The lower the Ct value, the more target DNA in the sample. If used with a standard curve of known amounts of target, absolute quantification can be achieved. Not only can qPCR be more specific than conventional PCR but it may also be more sensitive, due to the sensitive detection of fluorescent signal by the instrument. However, direct comparisons of nested PCR versus qPCR report a similar sensitivity of the methods (Fang *et al.*, 2002; Christopher-Hennings *et al.*, 2003; Schönenbrücher *et al.*, 2008). An important advantage is that no more laboratory work has to be performed on the DNA product, as the recorded reaction data are sufficient for analysis.

The first reported use of qPCR for *MAP* detection was made by Eishi *et al.* (2002) in a screening of sarcoidosis patients for a number of pathogens. The first report of veterinary relevance was the previously mentioned method comparison by Fang *et al.* (2002). Other frequently cited reports include the first development of a SYBR Green qPCR for *MAP* (O'Mahony and Hill, 2002) and application of IMS in milk (Khare *et al.*, 2004). In addition to

its usefulness for pathogen detection, qPCR can also be combined with reverse transcriptase PCR for mRNA detection and expression analysis, as Basler *et al.* (2008) demonstrated on *MAP*.

23.2.4 Multiplex PCR

The use of several sets of primers designed for different targets enables simultaneous testing of a sample for several target sequences. In conventional PCR, the different targets are identified as bands of different size on the gel. In qPCR, the fluorophores in the respective Taqman probe can be chosen to emit light of different wavelengths, measured in separate channels.

The first reported application of multiplex PCR for *MAP* was the PCR identification of mycobacterial growth in BACTEC medium by using primers targeting both IS*900* and the 16S rRNA gene, specific for the *Mycobacterium* genus (Cousins *et al.*, 1995). Another duplex PCR based on the genes f57 and p34, developed for discrimination of a number of different mycobacteria in formalin-fixed, paraffin-embedded tissues, was described by Coetsier *et al.* (2000). This method was later modified to allow for differentiation of single from mixed infections in cultures (Godfroid *et al.*, 2005). Bull *et al.* (2000) reported the development of a multiplex PCR fingerprinting method for *MAP*. By targeting different IS*900* loci, ten *MAP* types (referred to as MPIL types) could be discriminated by different gel patterns. Stanley *et al.* (2007) used multiplex PCR together with the FASTPlaqueTB assay for identification of viable *MAP*. Moravkova *et al.* (2008) developed a multiplex PCR for detection and differentiation of different *M. avium* subspecies, using primers for IS*900*, IS*901*, IS*1245* and the *dnaJ* gene.

23.2.5 Internal amplification control (IAC)

Often multiplex PCR is used to co-amplify an internal amplification control (IAC) molecule (sometimes referred to as a 'mimic'), to monitor PCR inhibition, as described by Ballagi-Pordány and Belak (1996). Application of IAC for *MAP* PCR includes methods developed by Englund *et al.* (1999), Halldórsdóttir *et al.* (2002) and Rodriguez-Lazaro *et al.* (2004). Tasara *et al.* (2005) developed a PCR system including primers for the genes IS*900*, f57 and 16S rRNA as well as an IAC. Brey *et al.* (2005) described a PCR system with primers for IS*900*, *hspX* and an IAC. More recent publications have applied qPCR with an IAC detected in its own channel (Herthnek *et al.*, 2006; Schönenbrücher *et al.*, 2008; Slana *et al.*, 2008a).

23.3 Quality Control of PCR Assays

The great risk of cross-contamination in routine diagnostic PCR testing was demonstrated in early collaborative quality control studies among tuberculosis laboratories; problems with both lack of specificity and lack of sensitivity were documented (Noordhoek *et al.*, 1996). The study underlined the need for inclusion of appropriate controls and adequate protocols to prevent cross-contamination.

False-negative results can be caused by inhibition of the PCR reaction from substances present in the sample. To monitor for negative test results not being caused by PCR inhibition, an IAC should be used. An alternative but more laborious way is to spike parallel samples that did not yield a reaction with a positive control and carry out an additional PCR (Lecouvet *et al.*, 2004; Irenge *et al.*, 2009).

To avoid false positives, measures to prevent cross-contamination should include routines such as performing the different tasks of PCR testing in separate laboratory rooms. Another way for control of cross-contamination is the use of uracil N-glycosylase (UNG) (Longo *et al.*, 1990). This enzyme incorporates dUTP instead of dTTP in the PCR products. Treatment of starting reactions with UNG will degrade all previously formed dUTP-containing DNA and prevent amplification. For nested PCR in the two-tube format, these two methods of preventing cross-contamination are not fully reliable and rigorous quality controls and participation in proficiency testing programmes are needed. Proficiency-testing panels are available for *MAP* testing of faecal samples (Johne's Disease Faecal Proficiency Panel)

from the National Veterinary Services Laboratories, Ames, USA (Payeur and Capsel, 2007). Recommendations for validation and quality control of PCR methods can be found in the OIE Terrestrial Manual (Belak and Thorén, 2008).

23.4 Choice of Target Genes for PCR

The IS*900* element has been used extensively as a specific target for *MAP*, for both PCR and typing (see Collins, Chapter 25, this volume). Although IS*900* has only been found in *MAP*, it has been reported that other, sometimes distantly related, mycobacteria could give a positive reaction with some of the PCR protocols (Cousins *et al.*, 1999; Englund *et al.*, 2002; Kim *et al.*, 2002; Taddei *et al.*, 2008). Several new PCR systems, targeting the IS*900* of *MAP*, have been designed with the aim of trying to avoid cross-reaction with these IS*900*-like elements (Bull *et al.*, 2003; Herthnek and Bölske, 2006; Kawaji *et al.*, 2007). This is difficult, and certainly there is no guarantee that one can avoid cross-reaction with unknown IS*900*-like elements. Other presumed *MAP*-specific genes have therefore been chosen as targets for PCR assays. It may be unwise to simply abandon IS*900* for another presumed specific target gene that has been less exhaustively evaluated; IS*900* as a target for PCR systems is generally better validated than the new alternatives and may well continue to be used as a first choice, being complemented with suitable confirmation methods. However, new IS*900* PCR systems will need further validation regarding specificity. Strain 2333, harbouring an IS element at the present time known to be the most similar to IS*900* (Englund *et al.*, 2002), can be obtained from the Pasteur Institute strain collection (CIP 107487). Target genes used in PCR systems for *MAP* detection are shown in Table 23.2.

As can be expected, the use of targets with multiple copies translates into a PCR with higher sensitivity than is achieved with single-copy targets (Stabel *et al.*, 2004; Herthnek and Bölske, 2006; Irenge *et al.*, 2009). ISMap*02*, an element with six copies (Stabel and Bannantine, 2005), was evaluated in comparison to IS*900*. IS*900* was one log more sensitive on spiked samples, and, in an evaluation performed on low-level faecal samples, more samples were positive for IS*900* than ISMap*02*.

23.5 Confirming PCR Identification

When using culture, an isolate can be subcultured and studied for several characteristics, permitting confirmation via the results of additional tests. However, in the case of PCR-based detection, cultured bacteria are not available and some other form of independent confirmation has to be applied. In a laboratory that uses PCR, a convenient alternative is to do a second PCR, targeting another gene when the primary PCR system is positive (Herthnek and Bölske, 2006) or by doing a multiplex PCR of

Table 23.2. *MAP* genes used for PCR in paratuberculosis diagnostic testing.

Gene	Copy no.	References about validation and use in PCR assays
IS*900*	15–20	Recent papers: Möbius *et al.*, 2008; Schönenbrücher *et al.*, 2008; Irenge *et al.*, 2009
F57	1	Vansnick *et al.*, 2004; Tasara and Stephan, 2005; Bosshard *et al.*, 2006; Herthnek and Bölske, 2006; Glanemann *et al.*, 2008; Herthnek *et al.*, 2008; Möbius *et al.*, 2008; Schönenbrücher *et al.*, 2008; Irenge *et al.*, 2009
IS*Mav2*	3	Strommenger *et al.*, 2001; Stratmann *et al.*, 2002; Shin *et al.*, 2004; Glanemann *et al.*, 2008; Möbius *et al.*, 2008; Schönenbrücher *et al.*, 2008
hspX	1	Ellingson *et al.*, 1998, 2000; Stabel *et al.*, 2004
Gene 251	1	Bannantine *et al.*, 2002; Sibley *et al.*, 2007
Gene 255	1	Bannantine *et al.*, 2002; Möbius *et al.*, 2008
ISMap*02*	6	Stabel and Bannantine, 2005; Irenge *et al.*, 2009

two or three *MAP* targets (Schönenbrücher *et al.*, 2008; Irenge *et al.*, 2009).

Confirmation can also be obtained by sequencing the PCR amplicon. To get an unambiguous confirmation of IS*900* as a target, there should be no sequence difference from the reference strain K-10 (Semret *et al.*, 2006). Since many PCR methods target a *MAP*-specific gene, it is expected that most amplicons will be consistent with *MAP* upon sequence analysis. An alternative approach is to target a gene that is shared among a number of organisms but for which *MAP* has distinct polymorphisms. For example, primers for the 3′ variable region of the *hsp65* gene will amplify any isolate of *M. avium* or *Mycobacterium intracellulare*, and sequence analysis will then distinguish which species or subspecies was present (Turenne *et al.*, 2006).

23.6 Pretreatment and Extraction Procedures

23.6.1 Immuno-magnetic separation (IMS)

IMS involves the capture of whole *MAP* cells by magnetic beads coated with specific antibodies and separation from the sample material by magnets. It was first applied for culture of *MAP* in milk (Grant *et al.*, 1998) and later in combination with PCR, referred to as IMS-PCR (Grant and Rowe, 2001). Phenol–chloroform extraction can be coupled with IMS for DNA purification. A method similar to IMS, where a peptide is used to capture *MAP*, has also been described (Stratmann *et al.*, 2006).

23.6.2 Bacterial lysis

Lysis of the tough cell wall of *MAP* is a prerequisite to get DNA accessible for extraction and purification, and it requires harsh methods. Simple methods such as enzymatic digestion or boiling alone have proved inadequate, but effective lysis of *MAP* can be achieved by combining chemical methods with bead beating (Herthnek, 2009). Bead beating is a general term for using small beads mixed with the sample to disrupt tissues or tough cell walls by forceful shaking in a machine. Different combinations of enzymatic treatment, freeze–thaw/boiling, bead beating or kits for plant DNA purification have been compared for pretreatment of samples (Garrido *et al.*, 2000; Odumeru *et al.*, 2001; Zecconi *et al.*, 2002; Chui *et al.*, 2004). Inclusion of bead beating in the protocol gave, in most cases, the best sensitivity for the test.

23.6.3 Hybridization capture

In the hybridization/sequence capture technique, oligonucleotide sequences chosen from specific genes, such as IS*900*, are bound to magnetic beads and used to capture *MAP* DNA and separate it from non-specific DNA and inhibitory substances present in crude, complex samples like faeces and tissue (Marsh *et al.*, 2000; Englund *et al.*, 2001; Halldórsdóttir *et al.*, 2002; Vansnick *et al.*, 2007).

23.6.4 Spin column extraction

Spin columns are designed for centrifugation, which increases the flow rate compared with that of gravity-flow columns. DNA is purified by binding to a silica membrane in the column and washing away impurities before elution with a buffer solution. This technique is commercially available both in diagnostic kits for *MAP* and in separate extraction kits.

23.6.5 Organic solvent extraction

Solvent extraction is a simple way to purify DNA from a mixture of proteins, lipids and nucleic acids. Often the mixture is extracted with phenol followed by chloroform. The effects of organic solvents are to dissolve hydrophobic molecules and to denature proteins, which makes them insoluble in water. As a result, cell membranes and cellular proteins are either dissolved in the phenol–chloroform, which is discarded, or trapped in the interface between the two phases. DNA and RNA remain

in the aqueous phase and are easily separated. Because hazardous substances are used, this activity should be performed in a chemical hood.

23.7 Sample-specific Modifications

23.7.1 Faeces

The PCR tests for detection of *MAP* in faecal samples have vastly improved in recent years, leading to an increased sensitivity of detection of low shedders. This improvement is due to improved DNA extraction and purification procedures and, to a lesser extent, the more sensitive PCR systems now available. Recent DNA extraction procedures and PCR systems applied to faeces are presented in Table 23.3. Extraction methods for faeces have also been modified for environmental samples (soil and manure) (Cook and Britt, 2007).

23.7.2 Milk

Because of the low concentrations of *MAP* in milk, most DNA extraction methods employ centrifugation of a relatively large volume of milk, typically 10 ml (Slana *et al.*, 2008b). IMS, which is often applied on milk (Grant and Rowe, 2001; Khare *et al.*, 2004; O'Reilly *et al.*, 2004), can be used on small volumes of uncentrifuged milk, but more often large volumes are used. It is, however, not trivial which fractions to discard after centrifugation. In many methods, including IMS, both whey and cream are discarded (Tasara and Stephan, 2005; Pinedo *et al.*, 2008; Slana *et al.*, 2008a). As first shown by Millar *et al.* (1996), the cream fraction of raw milk can sometimes contain most of the *MAP*, which is why inclusion of the cream fraction should be considered. The pellet and cream fractions can be pooled (Gao *et al.*, 2007; Herthnek *et al.*, 2008) or the complete sample volume can be processed, as with peptide-mediated capture (Stratmann *et al.*, 2006) and unspecific surface capture by magnetic beads (Donaghy *et al.*, 2008). Other recent publications include a method comparison (Alinovi *et al.*, 2009) and a diagnostic method for screening (Herthnek *et al.*, 2008).

23.7.3 Tissues

Extraction from human intestinal biopsy samples and *MAP* detection with nested PCR are described by Bull *et al.* (2003). Glanemann *et al.* (2008) applied a multiplex PCR targeting the F57 and IS*Mav2* genes for biopsies from dogs. Tissues from cattle and sheep with clinical paratuberculosis were tested with an IS*900* real-time PCR. Extraction was performed with the QIAamp Mini kit (Qiagen). The researchers found *MAP* in most of the faeces, blood and tissue samples. In muscle samples, there were up to 9.5×10^6 *MAP*/g (Nelli *et al.*, 2008). No confirmation of *MAP* was performed. Although *MAP* in animals with clinical signs seems likely, confirmation of *MAP* should have been done from unusual PCR findings (samples from muscle and blood).

Bosshard *et al.* (2006) investigated 101 slaughtered dairy cattle with a real-time PCR targeting the F57 gene and found *MAP* in 8.9% of faecal samples, 4.9% of mesenteric lymph nodes, 0.9% of ileum tissue, 3.6% of milk samples and 2.9% of samples of diaphragmatic muscle. Confirmation was by sequencing F57 amplicons.

Detection of *MAP* in formalin-fixed tissues is described by Coetsier *et al.* (2000) and Miller *et al.* (2002). Ikonomopolous *et al.* (2004) compared different IS*900* PCR assays on formalin-fixed, paraffin-embedded tissue samples from cattle and poultry.

23.7.4 Semen

Ayele *et al.* (2004) detected *MAP* in semen batches from a faecal-shedding bull with a conventional IS*900* PCR, after extraction with a QIAamp DNA Mini Kit (QIAGEN, Germany). Buergelt *et al.* (2004) reported detection of *MAP* in bull semen with a nested PCR. Khol *et al.* (2007), using a duplex IS*900*/F57 real-time PCR, found *MAP* in nine semen samples from a bull with the IS*900* PCR and in six of the nine also with F57 PCR. Herthnek *et al.* (2006) developed a real-time PCR based on IS*900* and assessed the sensitivity on spiked semen to ten *MAP* per sample of 100 µl

Table 23.3. Summary of recently reported direct faecal PCR assays and comparison with faecal culture.

Samples (no.) Sample storage	Lysis method	DNA extraction	Target gene	PCR	Faecal culture (decontamination and media)	PCR results compared with culture (no. pos/ sample size)	References
Bovine faeces (63) No storage information	Bead beating	a) Column purification (HerdChek®)[a] b) In-house	IS*900*	qPCR (TaqMan) Nested	DI-C, HEY	a) qPCR (41/63) b) qPCR (43/63) Nested (44/63) Culture (44/63)	Christopher-Hennings *et al*., 2003
Bovine faeces (23) No storage record	Bead beating	IMS, solvent extraction	IS*900*	qPCR (TaqMan)	DI-C, HEY	PCR (17/23) Culture (17/23)	Khare *et al*., 2004
Bovine faeces (310) Some frozen –20 °C	Boiling/ freezing	Spin column (Roche High Pure PCR Kit®)[b]	IS*900*	qPCR (SYBR Green), semi-nested	NaOH, LJ	PCR (31, 32 doubtful/310) Culture (20/310)	Bögli-Stuber *et al*., 2005
Bovine faeces (81) No storage record	Heating 100 °C for 10 min	Centrifugations and washings in Tris-EDTA buffer	IS*900* ISMap*02*	qPCR (SYBR Green) Nested	DI-DC, HEY	qPCR IS*900* (60/81) qPCR ISMap*02* (59/81) Nested IS*900* (59/81) Nested ISMap*02* (59/81) Culture (60/81)	Stabel and Bannantine, 2005
Bovine faeces (1808) Cultured fresh	Bead beating	Spin column (QIAamp® DNA Stool Mini Kit)[c]	IS*Mav2*	qPCR (TaqMan)	DI-C, HEY	PCR (7.6% of 1808) Culture (18.4% of 1808)	Wells *et al*., 2006
Bovine faeces (47) Frozen –70 °C × 2	Bead beating	Spin column (QIAamp® DNA Stool Mini Kit)[c]	IS*900* F57	qPCR (TaqMan) IAC for IS*900*	NaOH, HEY, LJ DI-C, HEY (NVSL)	PCR IS*900* (47/47) PCR F57 (47/47) Culture (47/47)	Herthnek and Bölske, 2006
Ovine faeces (68) Some frozen	Bead beating	Solvent extraction (JohnePrep™ kit)[d]	IS*900* F57, ISMap*02*	Multiplex qPCR	DI-C, BACTEC	PCR (52/68) Culture (28/68)	Kawaji *et al*., 2007
Bovine faeces (328) No storage record	Bead beating	Spin column (PowerSoil™ Kit)[e]	IS*900*	Nested	DI-C, HEY	PCR (27/328) Culture (11/328)	Pinedo *et al*., 2008
Bovine faeces (143) Cultured fresh	Bead beating	Spin column (Tetracore kit)[f]	*hspX*	qPCR	DI-C, HEY	PCR (31/143) Culture (27/143)	Alinovi *et al*., 2009

[a]HerdChek® *Mycobacterium paratuberculosis* DNA Test Kit, IDEXX Laboratories, Westbrook, Maine, USA; [b]High Pure PCR Template Preparation Kit®, Roche Diagnostics, Mannheim, Germany; [c]QIAamp® DNA Stool Mini Kit, QIAGEN; [d]JohnePrep™ kit, Kyoritsu Seiyaku Co., Tokyo, Japan; [e]PowerSoil™ DNA Isolation Kit, Mo Bio Laboratories, Carlsbad, California; [f]MAP Extraction System and VetAlert™ Johne's Real-Time PCR kit, Tetracore®, Rockville, Maryland. Abbreviations: IAC, internal amplification control; NVSL, National Veterinary Services Laboratories, USDA, APHIS, Veterinary Services, Ames, Iowa; qPCR, real-time PCR; DI-C, double-incubation, centrifugation method (Whitlock and Rosenberger, 1990); DI-DC, double-incubation, double-centrifugation method (Stabel, 1997); NaOH, NaOH/oxalic acid method (Beerwerth, 1967); HEY, Herrold's egg yolk medium; LJ, Löwenstein–Jensen medium.

semen after bead-beating and extraction with phenol–chloroform.

23.7.5 Liquid culture medium

DNA preparation from liquid cultures is usually easier than from clinical samples. However, media components, especially egg yolk enrichment, used in the automated culture systems may cause inhibition of the PCR reaction. Kim *et al.* (2004) used a column purification kit for DNA preparation from ESP II cultures. Naser *et al.* (2004) applied boiling/cooling lysis and solvent extraction and a nested PCR for *MAP* detection in MGIT cultures. Sweeney *et al.* (2006) tried a simple method comprising only boiling, and Whittington *et al.* (1999) used differential centrifugation with ethanol and boiling. These two protocols were, in most cases, successful in detecting *MAP* in liquid culture despite their lack of an effective lysis method, which would have increased sensitivity. However, when working with positive cultures, there are large numbers of bacteria and therefore the high sensitivity of nested PCR should not be needed to reveal a *MAP*-specific amplification product.

23.8 Comparison of PCR with Culture

23.8.1 Enumeration of *MAP* cells

The analytical sensitivity of a PCR method is often given as colony-forming units (CFU) per weight or volume unit. The viable count on solid media underestimates the amount of *MAP* for several reasons: (i) *MAP* cells tend to aggregate so that a colony often does not represent a single cell but rather an aggregate of several cells; (ii) one single cell of a poorly growing strain may not be able to grow a colony, so a colony will represent several cells from an aggregate; and (iii) dead cells will not be measured by culture-based counting, but PCR may detect them if the DNA is intact. Other methods for enumeration of cells in a *MAP* culture that might be more accurate than CFU are, for example, counting of cells in a Bürker chamber (Herthnek *et al.*, 2006) and solid-phase cytometry (Vansnick *et al.*, 2007).

An underestimation of numbers of *MAP* by culture will lead to an overestimation of the sensitivity of the PCR method when the two methods are compared. To make a figure for sensitivity meaningful for diagnostic purposes, it should be expressed as *MAP* per volume or weight of the sample. The different ways to measure and express sensitivity of different tests in the literature often makes a comparison difficult. Additionally, the detection of *MAP* in clinical samples by culture is influenced by the decontamination procedure, which lowers the sensitivity of the culture methods further. These aspects are covered in more detail elsewhere (see Whittington, Chapter 22, this volume).

23.8.2 Comparison of sensitivity for culture and PCR

An alternative approach to estimating the relative sensitivity of culture and PCR is to compare the number of animals positive in a defined study by both methods (Table 23.3). However, factors that can influence viability of *MAP* in a sample may also influence sensitivity of the culture method. Freezing and storage of the samples may decrease sensitivity of the culture method. Giese *et al.* (1996) found that samples with low numbers of *MAP* (<10 colonies/tube) did not become positive when cultured again after freezing at –20 °C. Freezing and storage of bovine faecal samples at –20 °C lowered the viability of *MAP* significantly (Khare *et al.*, 2008). Freezing faecal samples at –70 °C lowered the viability of *MAP* according to Richards and Thoen (1977), although extended storage did not lead to further losses. In contrast, Khare *et al.* (2008) found little loss of viability from freezing at this temperature. PCR results are little affected by freezing the samples, but the lowered viability of *MAP* when samples are stored at –20 °C influences the culture results and should be accounted for when evaluating a comparison. Freezing at –70 °C probably has little effect, perhaps depending on how the freezing was done, but repeated freezing and thawing might influence the viability of

MAP. It is therefore important to know in each study how the samples were stored. While it is not possible to resolve all discordant results between two assays without a third assay, one should none the less take into account the overall proportion of animals positive by each of these tests.

23.9 Concluding Thoughts

In the past decade, there have been significant improvements in PCR-based detection of *MAP*. These include improvements spanning sample processing, amplification and detection methods. The precise analytical sensitivity of this method is still difficult to ascertain, but some promising data suggest that PCR may achieve a comparable sensitivity to that of culture. While culture may remain the definitive gold standard in research applications, the improved turnaround time with PCR makes this an attractive diagnostic modality for clinical applications, especially where adequate safeguards are in place to ensure accurate and reliable results.

References

Alinovi, C.A., Ward, M.P., Lin, T.L., Moore, G.E. and Wu, C.C. (2009) Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. *paratuberculosis*. *Veterinary Microbiology* 136, 177–179.

Autschbach, F., Eisold, S., Hinz, U., Zinser, S., Linnebacher, M., Giese, T., Loffler, T., Buchler, M.W. and Schmidt, J. (2005) High prevalence of *Mycobacterium avium* subspecies *paratuberculosis* IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut* 54, 944–949.

Ayele, W.Y., Bartos, M., Svastova, P. and Pavlik, I. (2004) Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in organs of naturally infected bull-calves and breeding bulls. *Veterinary Microbiology* 103, 209–217.

Ballagi-Pordány, A. and Belák, S. (1996) The use of mimics as internal standards to avoid false negatives in diagnostic PCR. *Molecular and Cellular Probes* 10, 159–164.

Bannantine, J.P., Baechler, E., Zhang, Q., Li, L. and Kapur, V. (2002) Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. *Journal of Clinical Microbiology* 40, 1303–1310.

Basler, T., Geffers, R., Weiss, S., Valentin-Weigand, P. and Goethe, R. (2008) *Mycobacterium avium* subspecies induce differential expression of pro-inflammatory mediators in a murine macrophage model: evidence for enhanced pathogenicity of *Mycobacterium avium* subspecies *paratuberculosis*. *Immunobiology* 213, 879–888.

Beerwerth, W. (1967) Die Züchtung von Mycobakterien aus dem Kot der Haustiere und ihre Bedeutung für die Epidemiologie und Bekämpfung der Tuberkulose. *Praxis der Pneumologie* 21, 189–202.

Belak, S. and Thorén, P. (2008) Validation and quality control of polymerase chain reaction methods for the diagnosis of infectious diseases. In: OIE Biological Standards Commission (ed.) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Office International des Epizooties, Paris, pp. 46–55.

Bögli-Stuber, K., Kohler, C., Seitert, G., Glanemann, B., Antognoli, M.C., Salman, M.D., Wittenbrink, M.M., Wittwer, M., Wassenaar, T., Jemmi, T. and Bissig-Choisat, B. (2005) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by real-time PCR and culture: a comparison of the two assays. *Journal of Applied Microbiology* 99, 587–597.

Bosshard, C., Stephan, R. and Tasara, T. (2006) Application of an F57 sequence-based real-time PCR assay for *Mycobacterium paratuberculosis* detection in bulk tank raw milk and slaughtered healthy dairy cows. *Journal of Food Protection* 69, 1662–1667.

Brey, B.J., Radcliff, R.P., Clark, D.L., Jr. and Ellingson, J.L. (2005) Design and development of an internal control plasmid for the detection of *Mycobacterium avium* subsp. *paratuberculosis* using real-time PCR. *Molecular and Cellular Probes* 20, 51–59.

Buergelt, C.D., Donovan, G.A. and Williams, J.E. (2004) Identification of *Mycobacterium avium* subspecies *paratuberculosis* by polymerase chain reaction in blood and semen of a bull with clinical paratuberculosis. *International Journal of Applied Research in Veterinary Medicine* 2, 130–134.

Bull, T.J., Hermon-Taylor, J., Pavlik, I.I., El-Zaatari, F. and Tizard, M. (2000) Characterization of IS900 loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. *Microbiology* 146, 3285–3297.

Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R.

and Hermon-Taylor, J. (2003) Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *Journal of Clinical Microbiology* 41, 2915–2923.

Christopher-Hennings, J., Dammen, M.A., Weeks, S.R., Epperson, W.B., Singh, S.N., Steinlicht, G.L., Fang, Y., Skaare, J.L., Larsen, J.L., Payeur, J.B. and Nelson, E.A. (2003) Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *Journal of Veterinary Diagnostic Investigation* 15, 87–93.

Chui, L.W., King, R., Lu, P., Manninen, K. and Sim, J. (2004) Evaluation of four DNA extraction methods for the detection of *Mycobacterium avium* subsp. *paratuberculosis* by polymerase chain reaction. *Diagnostic Microbiology and Infectious Disease* 48, 39–45.

Coetsier, C., Vannuffel, P., Blondeel, N., Denef, J.F., Cocito, C. and Gala, J.L. (2000) Duplex PCR for differential identification of *Mycobacterium bovis*, *M. avium*, and *M. avium* subsp. *paratuberculosis* in formalin-fixed paraffin-embedded tissues from cattle. *Journal of Clinical Microbiology* 38, 3048–3054.

Collins, D.M., Gabric, D.M. and de Lisle, G.W. (1989) Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. *FEMS Microbiology Letters* 60, 175–178.

Collins, D.M., Stephens, D.M. and de Lisle, G.W. (1993) Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. *Veterinary Microbiology* 36, 289–299.

Cook, K.L. and Britt, J.S. (2007) Optimization of methods for detecting *Mycobacterium avium* subsp. *paratuberculosis* in environmental samples using quantitative, real-time PCR. *Journal of Microbiological Methods* 69, 154–160.

Cousins, D.V., Evans, R.J. and Francis, B.R. (1995) Use of BACTEC radiometric culture method and polymerase chain reaction for the rapid screening of faeces and tissues for *Mycobacterium paratuberculosis*. *Australian Veterinary Journal* 72, 458–462.

Cousins, D.V., Whittington, R., Marsh, I., Masters, A., Evans, R.J. and Kluver, P. (1999) Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis. *Molecular and Cellular Probes* 13, 431–442.

Donaghy, J.A., Rowe, M.T., Rademaker, J.L., Hammer, P., Herman, L., De Jonghe, V., Blanchard, B., Duhem, K. and Vindel, E. (2008) An inter-laboratory ring trial for the detection and isolation of *Mycobacterium avium* subsp. *paratuberculosis* from raw milk artificially contaminated with naturally infected faeces. *Food Microbiology* 25, 128–135.

Eishi, Y., Suga, M., Ishige, I., Kobayashi, D., Yamada, T., Takemura, T., Takizawa, T., Koike, M., Kudoh, S., Costabel, U., Guzman, J., Rizzato, G., Gambacorta, M., Du Bois, R., Nicholson, A.G., Sharma, O.P. and Ando, M. (2002) Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *Journal of Clinical Microbiology* 40, 198–204.

Ellingson, J.L.E., Bolin, C.A. and Stabel, J.R. (1998) Identification of a gene unique to *Mycobacterium avium* subspecies *paratuberculosis* and application to diagnosis of paratuberculosis. *Molecular and Cellular Probes* 12, 133–142.

Ellingson, J.L., Stabel, J.R., Bishai, W.R., Frothingham, R. and Miller, J.M. (2000) Evaluation of the accuracy and reproducibility of a practical PCR panel assay for rapid detection and differentiation of *Mycobacterium avium* subspecies. *Molecular and Cellular Probes* 14, 153–161.

Englund, S., Ballagi-Pordany, A., Bölske, G. and Johansson, K.E. (1999) Single PCR and nested PCR with a mimic molecule for detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Diagnostic Microbiology and Infectious Disease* 33, 163–171.

Englund, S., Bölske, G., Ballagi-Pordány, A. and Johansson, K.-E. (2001) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples by single, fluorescent and nested PCR based on the IS900 gene. *Veterinary Microbiology* 81, 257–271.

Englund, S., Bölske, G. and Johansson, K.E. (2002) An IS900-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiology Letters* 209, 267–271.

Fang, Y., Wu, W.H., Pepper, J.L., Larsen, J.L., Marras, S.A., Nelson, E.A., Epperson, W.B. and Christopher-Hennings, J. (2002) Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. *Journal of Clinical Microbiology* 40, 287–291.

Gao, A., Mutharia, L., Chen, S., Rahn, K. and Odumeru, J. (2002) Effect of pasteurization on

survival of *Mycobacterium paratuberculosis* in milk. *Journal of Dairy Science* 85, 3198–3205.

Gao, A., Mutharia, L., Raymond, M. and Odumeru, J. (2007) Improved template DNA preparation procedure for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by PCR. *Journal of Microbiological Methods* 69, 417–420.

Garrido, J.M., Cortabarria, N., Oguiza, J.A., Aduriz, G. and Juste, R.A. (2000) Use of a PCR method on fecal samples for diagnosis of sheep paratuberculosis. *Veterinary Microbiology* 77, 379–386.

Giese, S.B., Huda, A., Wedderkopp, A., Klausen, J. and Ahrens, P. (1996) Cultivation of *Mycobacterium paratuberculosis* in Dubos broth combined with PCR. In: Chiodini, R.J., Hines, M.E., II and Collins, M.T. (eds) *Proceedings of the 5th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 302–306.

Glanemann, B., Schonenbrucher, H., Bridger, N., Abdulmawjood, A., Neiger, R. and Bulte, M. (2008) Detection of *Mycobacterium avium* subspecies *paratuberculosis*-specific DNA by PCR in intestinal biopsies of dogs. *Journal of Veterinary Internal Medicine* 22, 1090–1094.

Godfroid, J., Delcorps, C., Irenge, L.M., Walravens, K., Marche, S. and Gala, J.L. (2005) Definitive differentiation between single and mixed mycobacterial infections in red deer (*Cervus elaphus*) by a combination of duplex amplification of p34 and f57 sequences and Hpy188I enzymatic restriction of duplex amplicons. *Journal of Clinical Microbiology* 43, 4640–4648.

Grant, I.R. and Rowe, M.T. (2001) Methods for detection and enumeration of viable *Mycobacterium paratuberculosis* from milk and milk products. *Bulletin of the International Dairy Federation* 362, 41–50.

Grant, I.R., Ball, H.J. and Rowe, M.T. (1998) Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. *Applied and Environmental Microbiology* 64, 3153–3158.

Green, E.P., Tizard, M.L.V., Moss, M.T., Thompson, J., Winterbourne, D.J., McFadden, J.J. and Hermon-Taylor, J. (1989) Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Research* 17, 9063–9073.

Halldórsdóttir, S., Englund, S., Nilsen, S.F. and Olsaker, I. (2002) Detection of *Mycobacterium avium* subsp. *paratuberculosis* by buoyant density centrifugation, sequence capture PCR and dot blot hybridisation. *Veterinary Microbiology* 87, 327–340.

Herthnek, D. (2009) Molecular diagnostic methods for *Mycobacterium avium* subsp. *paratuberculosis*. PhD thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden. Available at http://diss-epsilon.slu.se/archive/00001954/ (accessed 11 June 2009).

Herthnek, D. and Bölske, G. (2006) New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*. *BMC Microbiology* 6, 87.

Herthnek, D., Englund, S., Willemsen, P.T. and Bölske, G. (2006) Sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine semen by real-time PCR. *Journal of Applied Microbiology* 100, 1095–1102.

Herthnek, D., Nielsen, S.S., Lindberg, A. and Bölske, G. (2008) A robust method for bacterial lysis and DNA purification to be used with real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *Journal of Microbiological Methods* 75, 335–340.

Ikonomopoulos, J., Gazouli, M., Pavlik, I., Bartos, M., Zacharatos, P., Xylouri, E., Papalambros, E. and Gorgoulis, V. (2004) Comparative evaluation of PCR assays for the robust molecular detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Microbiological Methods* 56, 315–321.

Irenge, L.M., Walravens, K., Govaerts, M., Godfroid, J., Rosseels, V., Huygen, K. and Gala, J.L. (2009) Development and validation of a triplex real-time PCR for rapid detection and specific identification of *M. avium* subsp. *paratuberculosis* in faecal samples. *Veterinary Microbiology* 136, 166–172.

Kawaji, S., Taylor, D.L., Mori, Y. and Whittington, R.J. (2007) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. *Veterinary Microbiology* 125, 36–48.

Khare, S., Ficht, T.A., Santos, R.L., Romano, J., Ficht, A.R., Zhang, S., Grant, I.R., Libal, M., Hunter, D. and Adams, L.G. (2004) Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and feces by a combination of immunomagnetic bead separation–conventional PCR and real-time PCR. *Journal of Clinical Microbiology* 42, 1075–1081.

Khare, S., Adams, L.G., Osterstock, J., Roussel, A. and David, L. (2008) Effects of shipping and storage conditions of fecal samples on viability of *Mycobacterium paratuberculosis*. *Journal of Clinical Microbiology* 46, 1561–1562.

Khol, J.L., Beran, V., Kralik, P., Slana, I., Aurich, C., Pavlik, I. and Baumgartner, W. (2007) *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in semen and organs of a breeding bull.

In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Congress on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin. Available at http://www.paratuberculosis.org/pubs/proc9/abst154e.htm (accessed 11 June 2009).

Kim, S.G., Shin, S.J., Jacobson, R.H., Miller, L.J., Harpending, P.R., Stehman, S.M., Rossiter, C.A. and Lein, D.A. (2002) Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 14, 126–131.

Kim, S.G., Kim, E.H., Lafferty, C.J., Miller, L.J., Koo, H.J., Stehman, S.M. and Shin, S.J. (2004) Use of conventional and real-time polymerase chain reaction for confirmation of *Mycobacterium avium* subsp. *paratuberculosis* in a broth-based culture system ESP II. *Journal of Veterinary Diagnostic Investigation* 16, 448–453.

Lecouvet, F., Irenge, L., Vandercam, B., Nzeusseu, A., Hamels, S. and Gala, J.L. (2004) The etiologic diagnosis of infectious discitis is improved by amplification-based DNA analysis. *Arthritis and Rheumatism* 50, 2985–2994.

Lisby, G., Andersen, J., Engbæk, K. and Binder, V. (1994) *Mycobacterium paratuberculosis* in intestinal tissue from patients with Crohn's disease demonstrated by nested primer polymerase chain reaction. *Scandinavian Journal of Gastroenterology* 29, 923–929.

Longo, M.C., Berninger, M.S. and Hartley, J.L. (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93, 125–128.

Manning, E.J.B. and Collins, M.T. (2001) *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. *Revue Scientifique et Technique OIE* 20, 133–150.

Marsh, I., Whittington, R. and Millar, D. (2000) Quality control and optimized procedure of hybridization capture-PCR for the identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces. *Molecular and Cellular Probes* 14, 219–232.

Meadus, W.J., Gill, C.O., Duff, P., Badoni, M. and Saucier, L. (2008) Prevalence on beef carcasses of *Mycobacterium avium* subsp. *paratuberculosis* DNA. *International Journal of Food Microbiology* 124, 291–294.

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T. and Hermon-Taylor, J. (1996) IS*900* to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cow's milk in England and Wales. *Applied and Environmental Microbiology* 62, 3446–3452.

Miller, J.M., Jenny, A.L. and Payeur, J.B. (2002) Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin-fixed tissues from culture-negative ruminants. *Veterinary Microbiology* 87, 15–23.

Möbius, P., Hotzel, H., Rassbach, A. and Köhler, H. (2008) Comparison of 13 single-round and nested PCR assays targeting IS900, ISMav2, f57 and locus 255 for detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* 126, 324–333.

Moravkova, M., Hlozek, P., Beran, V., Pavlik, I., Preziuso, S., Cuteri, V. and Bartos, M. (2008) Strategy for the detection and differentiation of *Mycobacterium avium* species in isolates and heavily infected tissues. *Research in Veterinary Science* 85, 257–264.

Moss, M.T., Sanderson, J.D., Tizard, M.L.V., Hermon-Taylor, J., El-Zaatari, F.A.K., Markesich, D.C. and Graham, D.Y. (1992) Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum* in long-term cultures from Crohn's disease and control tissues. *Gut* 33, 1209–1213.

Mullis, K.B. (1990) The unusual origin of the polymerase chain reaction. *Scientific American* 262, 56–61, 64–65.

Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology* 155**,** 335–350.

Naser, S.A., Ghobrial, G., Romero, C. and Valentine, J.F. (2004) Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet* 364, 1039–1044.

Nelli, R.K., Graham, E., Dunham, S.P. and Taylor, D.J. (2008) Real-time PCR identification of *Mycobacterium avium* subspecies *paratuberculosis* in ovine and bovine tissues. *The Veterinary Record* 163, 422–423.

Nielsen, S.S., Nielsen, K.K., Huda, A., Condron, R. and Collins, M.T. (2001) Diagnostic techniques for paratuberculosis. *Bulletin of the International Dairy Federation* 362, 5–17.

Noordhoek, G.T., Kolk, A.H., Bjune, G., Catty, D., Dale, J.W., Fine, P.E., Godfrey-Faussett, P., Cho, S.N., Shinnick, T., Svenson, S.B., Wilson, S. and van Embden, J.D.A. (1994) Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. *Journal of Clinical Microbiology* 32, 277–284.

Noordhoek, G.T., Van Embden, J.D. and Kolk, A.H. (1996) Reliability of nucleic acid amplification

for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. *Journal of Clinical Microbiology* 34, 2522–2525.

Odumeru, J., Gao, A., Chen, S., Raymond, M. and Mutharia, L. (2001) Use of the bead beater for preparation of *Mycobacterium paratuberculosis* template DNA in milk. *Canadian Journal of Veterinary Research* 65, 201–205.

O'Mahony, J. and Hill, C. (2002) A real time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the light cycler. *Journal of Microbiological Methods* 51, 283–293.

O'Reilly, C.E., O'Connor, L., Anderson, W., Harvey, P., Grant, I.R., Donaghy, J., Rowe, M. and O'Mahony, P. (2004) Surveillance of bulk raw and commercially pasteurized cows' milk from approved Irish liquid-milk pasteurization plants to determine the incidence of *Mycobacterium paratuberculosis*. *Applied and Environmental Microbiology* 70, 5138–5144.

Payeur, J.B. and Capsel, R.T. (2007) Evaluation of different organism based methods for the detection and identification of *Mycobacterium avium* subspecies *paratuberculosis* from bovine feces. In: Nielsen, S.S. (ed.) *9th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin. Available at http://www.paratuberculosis.org/pubs/proc9/abst43b_o2.htm (accessed 11 June 2009).

Pinedo, P.J., Rae, D.O., Williams, J.E., Donovan, G.A., Melendez, P. and Buergelt, C.D. (2008) Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of paratuberculosis in dairy cows. *Transboundary and Emerging Diseases* 55, 125–133.

Richards, W.D. and Thoen, C.O. (1977) Effect of freezing on the viability of *Mycobacterium paratuberculosis* in bovine feces. *Journal of Clinical Microbiology* 6, 392–395.

Rodriguez-Lazaro, D., D'Agostino, M., Pla, M. and Cook, N. (2004) Construction strategy for an internal amplification control for real-time diagnostic assays using nucleic acid sequence-based amplification: development and clinical application. *Journal of Clinical Microbiology* 42, 5832–5836.

Ryan, P., Bennett, M.W., Aarons, S., Lee, G., Collins, J.K., O'Sullivan, G.C., O'Connell, J. and Shanahan, F. (2002) PCR detection of *Mycobacterium paratuberculosis* in Crohn's disease granulomas isolated by laser capture microdissection. *Gut* 51, 665–670.

Sanderson, J.D., Moss, M.T., Tizard, M.L.V. and Hermon-Taylor, J. (1992) *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut* 33, 890–896.

Schönenbrücher, H., Abdulmawjood, A., Failing, K. and Bulte, M. (2008) New triplex real-time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *Applied and Environmental Microbiology* 74, 2751–2758.

Semret, M., Turenne, C.Y. and Behr, M.A. (2006) Insertion sequence IS*900* revisited. *Journal of Clinical Microbiology* 44, 1081–1083.

Shin, S.J., Chang, Y.F., Huang, C., Zhu, J., Huang, L., Yoo, H.S., Shin, K.S., Stehman, S. and Torres, A. (2004) Development of a polymerase chain reaction test to confirm *Mycobacterium avium* subsp. *paratuberculosis* in culture. *Journal of Veterinary Diagnostic Investigation* 16, 116–120.

Sibley, J.A., Woodbury, M.R., Appleyard, G.D. and Elkin, B. (2007) *Mycobacterium avium* subspecies *paratuberculosis* in bison (*Bison bison*) from northern Canada. *Journal of Wildlife Diseases* 43, 775–779.

Slana, I., Kralik, P., Kralova, A. and Pavlik, I. (2008a) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *International Journal of Food Microbiology* 128, 250–257.

Slana, I., Paolicchi, F., Janstova, B., Navratilova, P. and Pavlik, I. (2008b) Detection methods for *Mycobacterium avium* subsp. *paratuberculosis* in milk and milk products: a review. *Veterinarni Medicina* 53, 283–306.

Stabel, J.R. (1997) An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. *Journal of Veterinary Diagnostic Investigation* 9, 375–380.

Stabel, J.R. and Bannantine, J.P. (2005) Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. *Journal of Clinical Microbiology* 43, 4744–4750.

Stabel, J.R., Bosworth, T.L., Kirkbride, T.A., Forde, R.L. and Whitlock, R.H. (2004) A simple, rapid, and effective method for the extraction of *Mycobacterium paratuberculosis* DNA from fecal samples for polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation* 16, 22–30.

Stanley, E.C., Mole, R.J., Smith, R.J., Glenn, S.M., Barer, M.R., McGowan, M. and Rees, C.E.

(2007) Development of a new, combined rapid method using phage and PCR for detection and identification of viable *Mycobacterium paratuberculosis* bacteria within 48 hours. *Applied and Environmental Microbiology* 73, 1851–1857.

Stevenson, K. and Sharp, J.M. (1997) The contribution of molecular biology to *Mycobacterium avium* subspecies *paratuberculosis* research. *The Veterinary Journal* 153, 269–286.

Stratmann, J., Strommenger, B., Stevenson, K. and Gerlach, G.F. (2002) Development of a peptide-mediated capture PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *Journal of Clinical Microbiology* 40, 4244–4250.

Stratmann, J., Dohmann, K., Heinzmann, J. and Gerlach, G.F. (2006) Peptide aMptD-mediated capture PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bulk milk samples. *Applied and Environmental Microbiology* 72, 5150–5158.

Strommenger, B., Stevenson, K. and Gerlach, G.F. (2001) Isolation and diagnostic potential of ISMav2, a novel insertion sequence-like element from *Mycobacterium avium* subspecies *paratuberculosis*. *FEMS Microbiology Letters* 196, 31–37.

Sweeney, R.W., Whitlock, R.H. and McAdams, S.C. (2006) Comparison of three DNA preparation methods for real-time polymerase chain reaction confirmation of *Mycobacterium avium* subsp. *paratuberculosis* growth in an automated broth culture system. *Journal of Veterinary Diagnostic Investigation* 18, 587–590.

Taddei, R., Barbieri, I., Pacciarini, M.L., Fallacara, F., Belletti, G.L. and Arrigoni, N. (2008) *Mycobacterium porcinum* strains isolated from bovine bulk milk: implications for *Mycobacterium avium* subsp. *paratuberculosis* detection by PCR and culture. *Veterinary Microbiology* 130, 338–347.

Taddei, S., Robbi, C., Cesena, C., Rossi, I., Schiano, E., Arrigoni, N., Vicenzoni, G. and Cavirani, S. (2004) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples: comparison of three polymerase chain reaction-based diagnostic tests with a conventional culture method. *Journal of Veterinary Diagnostic Investigation* 16, 503–508.

Tasara, T. and Stephan, R. (2005) Development of an F57 sequence-based real-time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *Applied and Environmental Microbiology* 71, 5957–5968.

Tasara, T., Hoelzle, L.E. and Stephan, R. (2005) Development and evaluation of a *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) specific multiplex PCR assay. *International Journal of Food Microbiology* 104, 279–287.

Turenne, C.Y., Semret, M., Cousins, D.V., Collins, D.M. and Behr, M.A. (2006) Sequencing of hsp65 distinguishes among subsets of the *Mycobacterium avium* complex. *Journal of Clinical Microbiology* 44, 433–440.

Vansnick, E., De Rijk, P., Vercammen, F., Geysen, D., Rigouts, L. and Portaels, F. (2004) Newly developed primers for the detection of *Mycobacterium avium* subspecies *paratuberculosis*. *Veterinary Microbiology* 100, 197–204.

Vansnick, E., De Rijk, P., Vercammen, F., Rigouts, L., Portaels, F. and Geysen, D. (2007) A DNA sequence capture extraction method for detection of *Mycobacterium avium* subspecies *paratuberculosis* in feces and tissue samples. *Veterinary Microbiology* 122, 166–171.

Vary, P.H., Andersen, P.R., Green, E., Hermon-Taylor, J. and McFadden, J.J. (1990) Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *Journal of Clinical Microbiology* 28, 933–937.

Wells, S.J., Collins, M.T., Faaberg, K.S., Wees, C., Tavornpanich, S., Petrini, K.R., Collins, J.E., Cernicchiaro, N. and Whitlock, R.H. (2006) Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. *Clinical and Vaccine Immunology* 13, 1125–1130.

Whitlock, R.H. and Rosenberger, A.E. (1990) Fecal culture protocol for *Mycobacterium paratuberculosis*. A recommended protocol. In: *94th Annual Meeting of the US Animal Health Association*. US Animal Health Association, Richmond, Virginia, pp. 280–285.

Whittington, R.J. (2002) An overview of paratuberculosis diagnostic tests. In: Juste, R.A., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 131–135.

Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J. and Fraser, C.A. (1999) Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *Journal of Clinical Microbiology* 37, 1077–1083.

Zecconi, A., Mosca, A., Piccinini, R. and Robbi, C. (2002) A comparison of seven different protocols to extract *Mycobacterium avium* subsp. *paratuberculosis* DNA from bovine faeces. In: Juste, R.A., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 270–273.

24 Immune-based Diagnosis of Paratuberculosis

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24.1 Introduction

Immune-based diagnosis of paratuberculosis relies on the occurrence of an immune response to infection by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). Temporal aspects of the immune responses are currently inadequately characterized, adversely affecting the use and interpretation of immune-test results. The utility of these tests can be improved if one considers the context of the diagnostic test. This chapter discusses immune-based diagnostics for various purposes of testing and decision making.

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24.2 Purposes of Diagnosis

MAP infections have a long and variable incubation period. During this period, a number of outcomes can occur: (i) establishment of the infection; (ii) shedding of *MAP* in faeces; (iii) bacteraemia, which can lead to shedding of *MAP* in milk or transfer of *MAP* to a fetus *in utero*; and (iv) decreased milk production, weight loss, diarrhoea or death. These outcomes are of variable interest to different decision makers (politicians, farmers, etc.) in various settings (no reported occurrence, low prevalence or high prevalence). Consequently, the reason for performing a diagnostic test will vary as well. The following are examples of purposes for testing: (i) certification, i.e. a herd, animal or region certified free of *MAP* infection; (ii) establishment of a prevalence, i.e. the proportion of *MAP*-infected animals in a target population, to determine the need for intervention; (iii) reduction of transmission to prevent future *MAP* infections; (iv) increase of production parameters (e.g. milk and beef); and (v) increase of animal welfare, e.g. avoiding having animals suffer from diarrhoea. These distinct purposes will serve as the background for further discussion of immune-based diagnosis. In addition to these different aims of testing, the target conditions can range across: (i) non-infected (for certification); (ii) *MAP* infected (for prevalence estimation); (iii) *MAP* infectious (for reduction of transmission); and (iv) *MAP* affected (for increased production parameters and animal welfare). The term '*MAP* affected' will be used in this chapter instead of 'clinical disease' because of the variable definition and use of the term 'clinical' in the literature and among decision makers. A clinical diagnosis can depend on the observer's ability to detect changes in the cow's condition. The term '*MAP* affected' also includes the non-observable negative effects on the cow, e.g. reduced milk production or body weight.

Purposes of testing can change for a decision maker as a control scheme progresses, and target conditions can therefore also change. However, the four target conditions – (i) non-infected, (ii) *MAP* infected, (iii) *MAP* infectious and (iv) *MAP* affected – cover most phases in many of the control and certification programmes currently in place around the world.

24.3 Test Characteristics

A test can be characterized by its sensitivity (Se) and specificity (Sp). Se describes the probability that a test can detect an animal with a given target condition, and Sp describes the probability that a test can rule out the target condition. Target conditions vary with purpose of testing and time, and the World Organization for Animal Health (OIE) has endorsed the 'fitness for purpose' criterion in test validations (Anonymous, 2003). This concept has not yet gained full awareness in test evaluations for *MAP* infections. In future test evaluations, the purpose of testing should be specified, and Se and Sp estimated in relation to a target condition fulfilling this purpose. Currently Se and Sp estimates are inconsistently reported, with highly variable target conditions. A summary of Se and Sp estimates for antemortem tests, stratified by target condition, is given by Nielsen and Toft (2008). Because these estimates relate to the purpose of testing and rely on the occurrence of immune responses, these aspects will be discussed further.

24.4 Immunity and Immune-based Diagnostic Tests

24.4.1 Immunological responses

Specific immune responses to *MAP* infections can be divided into pro-inflammatory and anti-inflammatory reactions (see Stabel, Chapter 21, this volume). The pro-inflammatory process is characterized by production of gamma interferon (IFN-γ) and other cytokines involved in the cell-mediated responses, which attempt to control the infection by destroying *MAP* in activated macrophages (Coussens, 2001). Shedding of *MAP* occurs sporadically and IFN-γ stimulates the production of immunoglobulin G2 (IgG2). The pro-inflammatory reactions may gradually be replaced by anti-inflammatory processes, characterized by the occurrence of IgG1 antibodies. Therefore,

IFN-γ and IgG2 are mainly associated with pro-inflammatory responses and IgG1 with anti-inflammatory responses. Both responses may occur simultaneously due to a gradual shift between the two responses. IgG2 generally occur at lower concentrations than IgG1 (Koets *et al.*, 2001). The immune-based diagnostics are primarily based on detection of IFN-γ, IgG1 and IgG2 antibodies using antigens derived from *MAP*.

The speed of progression between disease stages is poorly characterized. Some infected cows produce antibodies several years prior to continuous shedding of detectable amounts of *MAP*. However, in other animals, antibodies may not be detectable during the early stages of infection, when *MAP* shedding is minimal. This phenomenon is illustrated in Fig. 24.1. It should be noted that this example cannot be considered a definitive understanding of the antibody response, because the appearance of antibodies remains to be fully elucidated.

24.4.2 Antigens and antibodies

The antigen choice for an immune-based test has a major impact on the test result. Antibody reactivity is not well characterized for the majority of *MAP*-specific antigens available. Koets *et al.* (2001) characterized the ability of IgG1 and IgG2 to react with four antigens: two cytosolic antigens (heat-shock proteins), a cell wall component (lipoarabinomannan) and a *MAP*-derived protein purified derivative (PPD-P), also called johnin, which is the *MAP* tuberculin. IgG1 reactivity to all four antigens was elevated in cows classified as *MAP* shedders compared with non-infected animals. Only PPD-P was associated with high levels of IgG2 in 'non-shedders', '*MAP* shedders' and animals with 'clinical paratuberculosis'. PPD-P was also associated with very high levels of IgG1 in *MAP* animals with clinical disease. These results are complex and underline the need to know the central components of a given immune-based test.

24.4.3 Types of tests and sample material

One of the most widely used immune-based tests is the indirect antibody enzyme-linked immunosorbent assay (ELISA), which detects antibodies in serum and milk samples. The accuracies of different ELISAs are similar, irrespective of whether they are used with

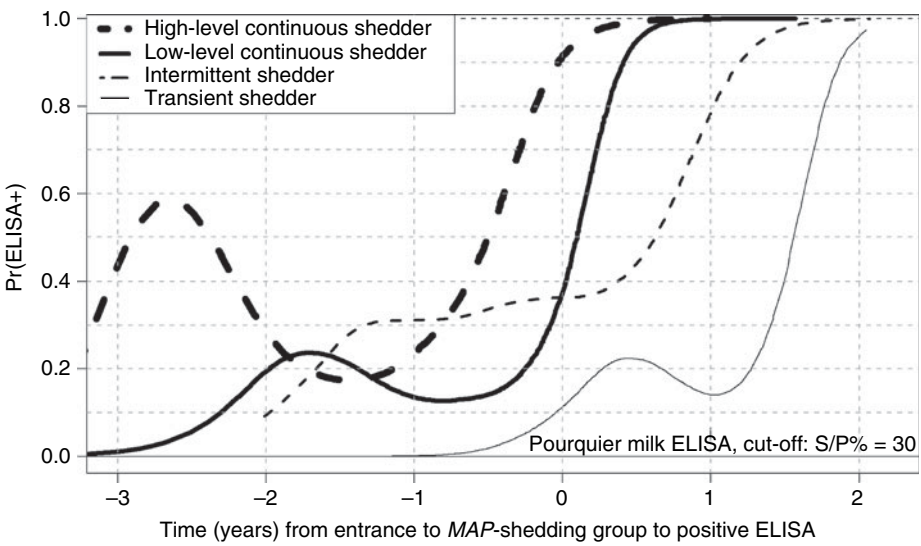

Fig. 24.1. Probability of testing positive by a commercial antibody ELISA at various time points relative to the start of *MAP* shedding.

milk or serum samples (Sweeney *et al.*, 1995; Nielsen *et al.*, 2002b). However, there are differences in the probability of testing positive during lactation between the two sample types. A serum ELISA is more likely to yield a positive reaction towards the end of lactation, whereas a milk ELISA is more likely to be positive at the beginning of lactation (Nielsen *et al.*, 2002a). These differences can be used to optimize sampling but will not be discussed further in this chapter.

The IFN-γ response after stimulation of a whole-blood sample with *MAP*-specific antigens, e.g. PPD-P, has also been used in an ELISA formatted test. Detection of IFN-γ depends on the processes for successful antigen presentation and presence of viable T cells, which are stimulated by the antigen to secrete IFN-γ. Antigen presentation is reduced within 8 h after sampling. However, Jungersen *et al.* (2005) have developed a method to rescue the co-stimulatory signals required for antigen presentation and T-cell reactivity, so sample processing can occur up to 24 h after sampling.

24.4.4 Advantages and disadvantages of immune-based tests

Among the main advantages of immune-based diagnostic tests are that they are relatively inexpensive, can easily be adapted to high-throughput testing and the results can be available in 1–2 weeks in routine settings. Since antibody ELISA testing is cheaper than most agent-detecting tests, farmers are more likely to test their animals frequently, if frequent testing is required (see Section 24.6.2). The advantage of sampling milk over serum is that the milk is often already collected for other purposes in milk recording schemes and is easy to obtain from the animal. Since immune-based tests detect host responses to a *MAP* infection, they should be able to determine that an infection has already occurred and may have persisted. A prerequisite is that the test is characterized for various stages of infection. Repeated testing makes determination of stage of infection easier.

A major disadvantage of immune-based tests is that they do not provide a direct measure of a *MAP* infection, infectiousness or being affected by a *MAP* infection (see Section 24.2, Purposes of Diagnosis). Therefore, there is a need to correlate the immune response profiles to relevant stages of infection. False-positive reactions occur because the positive test results in some cases do not relate to the purpose of testing. For example, antibodies can occur prior to shedding of *MAP*. If testing is done to determine if the animal is infectious prior to this shedding, the detection of antibodies will be considered false positive, while the animal is actually infected.

False-positive reactions may also occur due to cross-reacting antibodies or laboratory errors. It should be noted that vaccinated animals can be reactors in immune-based tests for a considerable period of time (Muskens *et al.*, 2002), without necessarily having *MAP* infection. These disadvantages make communication of test results complex. None the less, with appropriate interpretation, immunological tests may be more reliable than microbiological tests, depending on the purpose of testing.

24.5 Cell-mediated Tests

There are two tests available for the detection of a cell-mediated immune (CMI) response to *MAP* infection: the intradermal tuberculin test or skin test, using johnin or avian PPD, and the IFN-γ test. The skin test was used more frequently in the past but its current use is limited (Kalis *et al*., 2003). The introduction of the *in vitro* IFN-γ assay has replaced the skin test in current studies using the CMI response in the diagnosis of *MAP* infection.

Se and Sp estimates for IFN-γ tests have not been reported for detection of *MAP*-infected animals. It has been suggested that some animals with CMI reactions may be able to control and eradicate *MAP* (Mikkelsen *et al.*, 2009). If true, a test-positive reaction may be an indicator of a past infection, and the test can be expected to have a specificity of less than 100%, regardless of the antigens used. For animals deemed to be *MAP* infectious, Se estimates for IFN-γ tests have been between 0.13 and 0.85, with Sp estimates between 0.88

and 0.94 (Nielsen and Toft, 2008). There are limited data available to support the use of CMI diagnostics for purposes other than determining if animals have been exposed to *MAP*. If IFN-γ tests are to be used, animals should be >15 months of age to avoid false-positive reactions (Jungersen *et al.*, 2002) caused by innate IFN-γ production by NK cells.

24.6 Antibody ELISAs

Multiple antibody ELISAs have been evaluated, and Se and Sp estimates vary greatly within and between tests (Nielsen and Toft, 2008). It is therefore not possible to provide point estimates characterizing these tests. They should preferably be evaluated prior to their specific use in a specific population.

24.6.1 Detection of *MAP*-infected animals

Overall, the Se of antibody ELISA for detection of *MAP*-infected animals is low (~5–30%), but it increases with increasing age (Nielsen and Toft, 2006). Sp estimates are generally above 95% for commercial ELISAs.

24.6.2 Detection of *MAP*-infectious animals

The ability of antibody ELISAs to detect *MAP*-infectious animals depends on the test frequency (Fig. 24.1), the test make and the cut-off chosen to deem the ordinal ELISA response 'positive' or 'negative'. For example, assuming that only animals with high-level continuous shedding can infect a susceptible animal, one can test such animals on different schedules to reduce risk of transmission. As shown in Fig. 24.1, using the Pourquier milk ELISA (IDEXX, Montpellier, France) to test such animals daily, one can expect to detect 91% of infectious animals (time = 0 in Fig. 24.1). However, if the test was performed once per year, and this test date was 1 year prior to the start of the high-level continuous shedding, the Se would only have been 24% (Fig. 24.1, time = −1 year). In a third scenario, if such infectious animals were tested four times per year, 70–91% would be detected (Fig. 24.1, time interval −0.25 to 0 years). This example illustrates the major impact of test frequency on the Se. The cumulative Se from repeated testing would be higher if at least one positive test result from repeated test dates was considered sufficient to deem the animal positive.

Sp for the target condition 'infectious' can be interpreted as the probability that an animal tests negative given that it is not shedding *MAP* in doses deemed likely to cause infection in a susceptible animal. It should be noted that a 'non-infectious' animal can be infected with *MAP*. Therefore, animals with *MAP*-specific antibodies that are negative by a sensitive microbiological assay can be considered to be *MAP* infected but not *MAP* infectious.

Some decision makers want to confirm that test-positive animals are *MAP* infected or *MAP* infectious, but it is not possible to exclude infection from an ELISA-positive animal by use of ante-mortem tests. For example, in cows positive by the Pourquier milk antibody ELISA, faecal samples would only be positive by culture in 50–60% within the first year after testing positive for antibodies (Fig. 24.2, time interval 0 to 1 year). Some of the cows were therefore not *MAP* infectious at the time of occurrence of antibodies. However, the majority of cows would shed detectable amounts of *MAP* within 3.5 years after testing positive in the ELISA (Fig. 24.2, time interval 0 to 3.5 years). Therefore, cows that were antibody-positive but negative in culture would be considered *MAP* infected but non-infectious. In practice, a microbiological test for infectious animals should only be used to determine if an animal is shedding *MAP* at the time of testing. As illustrated by this example, a microbiological test for infectious animals should not be used to rule out *MAP* infection in an antibody-positive animal.

24.6.3 Detection of *MAP*-affected animals

In an animal with a high pre-test suspicion of disease (e.g. persistent diarrhoea), ELISAs tend to perform well in confirming the presence of

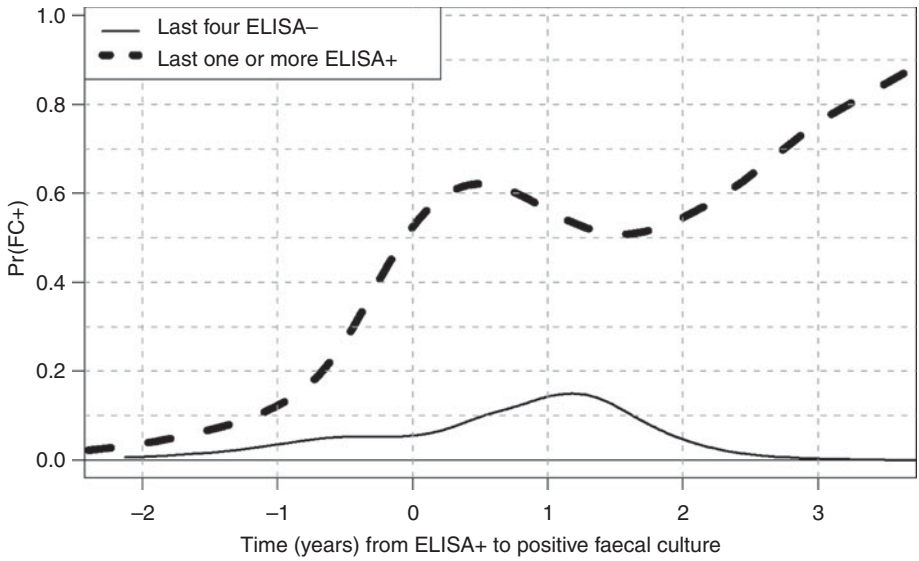

Fig. 24.2. Probability of testing positive in a faecal culture test relative to the time of testing positive in the commercial Pourquier milk ELISA (at cut-off sample to positive ratio of 30%).

MAP infection. For example Weber *et al.* (2009) reported that the Pourquier serum ELISA had a Se of 0.83–0.92, at a Sp of 0.998. However, not all cows affected by *MAP* infection will experience diarrhoea but they may experience reduced productivity. Therefore a combination of milk production data and antibody results can be used to increase the diagnostic accuracy (Wang *et al.*, 2006). Cows with fluctuating antibodies (fluctuating between test-positive and test-negative) may have increased milk production, whereas those with last-positive or repeated-positive test results can experience decreased milk production (Nielsen *et al.*, 2009). This decline may start 300 days prior to the occurrence of antibodies in some cows. To determine the *MAP* status of an animal, it may be necessary to evaluate both production data and ELISA results.

24.6.4 Use of ELISA results on an ordinal scale

The results from antibody ELISAs are measured on an ordinal scale but are often dichotomized to antibody-positive or antibody-negative results. In this manner, communication from the laboratory is more straightforward. However, this simplification comes at the cost of a decrease in information, if the ELISA values correlate with the probability of infection. Toft *et al.* (2005) estimated that cows with an optical density (OD) value of 0.3 had 4% probability of shedding *MAP*, whereas if the cows had an OD value of 1.0 the probability of *MAP* shedding was 80% (Fig. 24.3). Therefore, there may be considerable gains in using the values on the ordinal scale, whether it is OD values or sample-to-positive ratios (S/P values) (Collins, 2002).

24.6.5 Predictive values of antibody ELISA

Single antibody ELISA results may be of limited value, except for prevalence estimates and herd certification, because animals with various antibody profiles are in different stages of *MAP* infection. Instead, the results from frequent testing can better reveal the stage of infection, by following trends in results with time. However, there is limited information on test evaluation in the context of repeated testing. Current information suggests that the

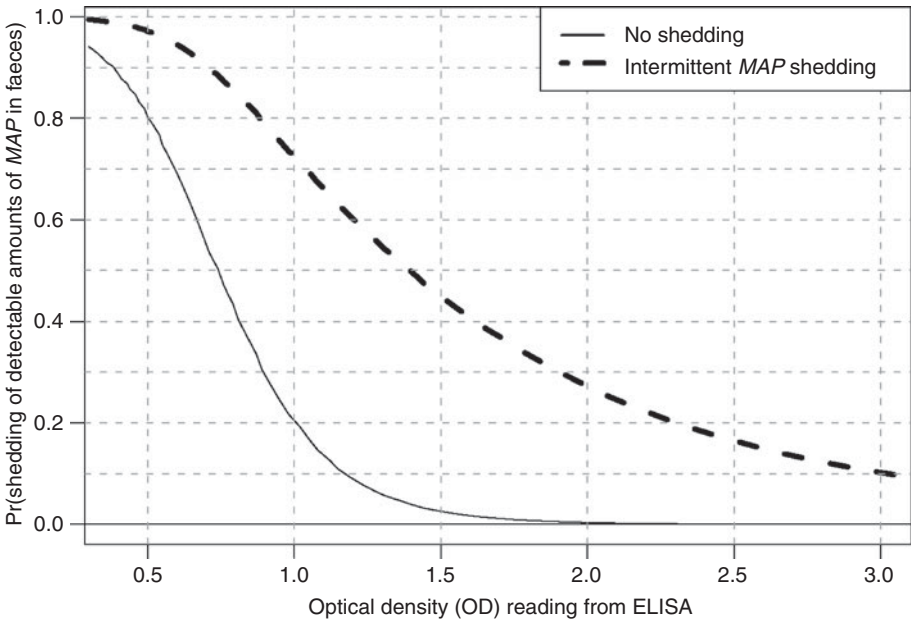

Fig. 24.3. Probability of detectable shedding of *MAP* in faeces. Values below full line show probabilities of no *MAP* shedding at a given OD value. Values between full and dashed line are probabilities of 'intermittent' *MAP* shedding, and values above dashed line are probabilities of continuous *MAP* shedding (modified from Toft *et al.*, 2005).

Table 24.1. Prognostic properties of antibody ELISA to predict *MAP*-affected, *MAP*-infectious, *MAP*-infected and non-infected adult cows.

	Predicted condition			
ELISA results	Non-infected	*MAP* infected	*MAP* infectious	*MAP* affected
Repeated negative	Depending on test history of herd	Possible in infected population	Rare	Rare
Fluctuating but last negative	Rare	Likely	Possible but likely to be low	Rare, a potential future event
Last sample positive	Rare	Very likely	Likely	Likely
Repeated positive	Rare	Very likely	Very likely	Likely, may only be in near future

predictive properties of antibody ELISA are as shown in Table 24.1.

24.7 Decision Making

Diagnostic test results are usually part of decision making related to specific purposes, such as those specified in Section 24.2. Therefore it is difficult to have a single recommendation for each test result. The following are suggestions related to different purposes. These suggestions are not the only decision options.

24.7.1 Certification

Animal herds cannot be certified free of *MAP* infection if insufficient numbers of animals are tested or if the animals tested are too

young. The number of animals required to be tested depends on the Se and Sp of the specific antibody ELISA used, in addition to the herd demography. For example, Sergeant *et al.* (2008) suggested that a minimum of 80 adult animals need to be tested to suggest that a herd has a low (but not zero) prevalence. An approach to correct for false-positive reactions caused by antibodies not specific for *MAP* was also suggested. Follow-up testing of ELISA-positive animals using a microbiological test cannot be recommended, because ELISA-positive animals not shedding *MAP* are frequent (see Section 24.6.2).

The probability that a herd is free of *MAP*-infected animals (P_{Free}) depends on the number of animals tested. P_{Free} is an expression that a herd has a lower prevalence than a predetermined prevalence, usually referred to as the design prevalence. Herds with a sufficient number of animals tested and with a high P_{Free} can be classified as 'free of *MAP* infection'. A requirement for certifying herds 'free of *MAP* infection' is that the herd is closed, or animals purchased originate from 'free' herds. Herds which are not 'free of *MAP* infection' may be classified as *MAP* infected, and a control plan can be established.

A special case of 'certification' could be to determine if an action plan has been effective. An action plan to reduce transmission with *MAP* is pivotal to control of *MAP* infections in general. Testing using a cell-mediated diagnostic test such as IFN-γ in animals >15 months of age could be used to determine if this action plan is working. If there are test-positive animals born after the establishment of the action plan, and the test is considered 100% specific, then the action plan should be revised, because test-positive reactions would suggest that the animals have been exposed to *MAP*.

24.7.2 Reduction of transmission

An action plan to reduce transmission could include testing using antibody ELISA. Animals that are test-positive have a high risk of either being *MAP* infectious or becoming *MAP* infectious (Fig. 24.2). Therefore either these animals should be culled or measures to avoid transmission of *MAP* to susceptible animals should be established. Animals that are repeatedly negative in antibody ELISA tests generally have a low probability of shedding *MAP* (Fig. 24.2). However, there is a chance that these animals might shed *MAP*, particularly if the ELISA used has a low Se for detection of *MAP*-infectious animals. An increased test frequency can increase the overall probability of detecting infectious animals prior to the start of high bacterial shedding. A 'sufficient' proportion of the infectious animals can be detected in this way (Kudahl *et al.*, 2008), but it is unlikely that all infectious animals will be detected. Test-negative animals should be tested repeatedly, because they might become *MAP* infectious at some stage in their life. A more detailed description of a possible approach is described by Nielsen (2009).

24.7.3 Increase of production parameters and animal welfare

Production parameters can be evaluated on either a herd or animal level. Consequently, productivity can be related to both the individual and the herd. Usually, decisions relating to the individual would be culling prior to the animal becoming affected by *MAP* infection, whereas decisions relating to the herd could also include decisions leading to reduction of transmission (see above). On the cow level, single antibody ELISA results may be insufficient to determine if a cow is *MAP* affected. The test information needs to be combined with production data or clinical observation of the cow. A positive ELISA result in combination with a decline in milk production or diarrhoea should lead to immediate culling to avoid further production losses. Animals with production losses or diarrhoea potentially related to *MAP* infection can be confirmed using ELISA.

24.8 Recommendations and Concluding Thoughts

Summary recommendations based on currently available test modalities are presented

Box 24.1. Summary recommendations.

An immune-based diagnostic test should be evaluated for its ability to detect *MAP*-infected and *MAP*-infectious animals in the population on which it will be used.

The purpose of testing should be clear, and the interpretation of the test results should be done in relation to the purpose. In particular, special consideration should be given to definition and communication of results, which can be 'false positive' for one purpose and 'true positive' for other purposes.

Cell-mediated immunodiagnostics may be used to determine if a population has been exposed to *MAP* but cannot be used to take actions on the individual animal.

Humoral immunodiagnostics can be used to establish relative prevalence estimates, which can be used for comparison of estimates obtained previously with the same test.

Antibody ELISAs can be used for certification only if a sufficient number of animals are tested. A test-positive animal should not be confirmed by an agent-detecting test in a certification scheme. False-positive reactions can be excluded via mathematical formulas or retesting with other immune-based tests.

Antibody ELISA can be used for risk-based management of *MAP*-infectious animals, since it will generally detect *MAP*-infected animals before they become *MAP* infectious. However, frequent testing is a prerequisite if this approach is used.

in Box 24.1. Priorities for future research include characterization of immune responses in prospective studies that last the lifetime of animals and factors that are involved in variation in immune responses. In addition, performance of diagnostic tests used in repeated testing should be characterized, and CMI detecting tests should be evaluated for their use with respect to the purpose of testing. Current diagnostic tests have potential if they are used and the test results interpreted appropriately (Box 24.1). However, the use of tests detecting humoral immune responses could have greater utility with repeated testing, whereas CMI tests might be useful for early detection of *MAP*-infected or *MAP*-exposed animals.

References

Anonymous (2003) Validation and certification of diagnostic assays. Resolution no. XXIX by the International Committee to the OIE. Available at:http://www.oie.int/vcda/eng/en_background_vcda.htm (accessed 27 December 2008).

Collins, M.T. (2002) Interpretation of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay by using likelihood ratios. *Clinical and Diagnostic Laboratory Immunology* 9, 1367–1371.

Coussens, P.M. (2001) *Mycobacterium paratuberculosis* and the bovine immune system. *Animal Health Research Reviews* 2, 141–161.

Jungersen, G., Huda, A., Hansen, J.J. and Lind, P. (2002) Interpretation of the gamma interferon test for diagnosis of subclinical paratuberculosis in cattle. *Clinical and Diagnostic Laboratory Immunology* 9, 453–460.

Jungersen, G., Grell, S.N., Clemensen, A., Roust, T. and Howard, C.J. (2005) Interleukin-12 potentiation of the interferon-gamma test rescues day-old blood samples for the diagnosis of paratuberculosis. In: Manning, E.J.B. and Nielsen, S.S. (eds) *Proceedings of the 8th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 501–506.

Kalis, C.H., Collins, M.T., Hesselink, J.W. and Barkema, H.W. (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. *Veterinary Microbiology* 97, 73–86.

Koets, A.P., Rutten, V.P., de Boer, M., Bakker, D., Valentin-Weigand, P. and van Eden, W. (2001) Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infection and Immunity* 69, 1492–1498.

Kudahl, A.B., Nielsen, S.S. and Østergaard, S. (2008) Economy, efficacy, and feasibility of a risk-based control program against paratuberculosis. *Journal of Dairy Science* 91, 4599–4609.

Mikkelsen, H., Jungersen, G. and Nielsen, S.S. (2009) Association between milk antibody and

interferon-gamma responses in cattle from *Mycobacterium avium* subsp. *paratuberculosis* infected herds. *Veterinary Immunology and Immunopathology* 127, 235–241.

Muskens, J., van Zijderveld, F., Eger, A. and Bakker, D. (2002) Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. *Veterinary Microbiology* 86, 269–278.

Nielsen, S.S. (2009) Use of diagnostics for risk-based control of paratuberculosis in dairy herds. *In Practice* 31, 150–154.

Nielsen, S.S. and Toft, N. (2006) Age-specific characteristics of ELISA and fecal culture for purpose-specific testing for paratuberculosis. *Journal of Dairy Science* 89, 569–579.

Nielsen, S.S. and Toft, N. (2008) Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. *Veterinary Microbiology* 129, 217–235.

Nielsen, S.S., Enevoldsen, C. and Gröhn, Y.T. (2002a) The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. *Preventive Veterinary Medicine* 54, 1–10.

Nielsen, S.S., Grønbæk, C., Agger, J.F. and Houe, H. (2002b) Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. *Preventive Veterinary Medicine* 53, 191–204.

Nielsen, S.S., Krogh, M.A. and Enevoldsen, C. (2009) Time to the occurrence of a decline in milk production in cows with various paratuberculosis antibody profiles. *Journal of Dairy Science* 92, 149–155.

Sergeant, E.S., Nielsen, S.S. and Toft, N. (2008) Evaluation of test-strategies for estimating probability of low prevalence of paratuberculosis in Danish dairy herds. *Preventive Veterinary Medicine* 85, 92–106.

Sweeney, R.W., Whitlock, R.H., Buckley, C.L. and Spencer, P.A. (1995) Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. *Journal of Veterinary Diagnostic Investigation* 7, 488–493.

Toft, N., Nielsen, S.S. and Jørgensen, E. (2005) Continuous-data diagnostic tests for paratuberculosis as a multistage disease. *Journal of Dairy Science* 88, 3923–3931.

Wang, C., Turnbull, B.W., Gröhn, Y.T. and Nielsen, S.S. (2006) Estimating receiver operating characteristic curves with covariates when there is no perfect reference test for diagnosis of Johne's disease. *Journal of Dairy Science* 89, 3038–3046.

Weber, M.F., Verhoeff, J., van Schaik, G. and van Maanen, C. (2009) Evaluation of Ziehl–Neelsen stained faecal smear and ELISA as tools for surveillance of clinical paratuberculosis in cattle in the Netherlands. *Preventive Veterinary Medicine* 92, 256–266.

25 Strain Characterization of *Mycobacterium avium* subsp. *paratuberculosis*

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25.1 Introduction

Before the mid-1980s, the ability to differentiate strains of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) was extremely limited. There were reports of a pigmented and a non-pigmented strain of *MAP* in sheep, and evidence from primary *in vitro* culture that the strains that often infected sheep were different from those that infected cattle (Taylor, 1951). It was also reported that the strain infecting Icelandic sheep was different from that infecting Norwegian goats (Gunnarsson, 1979). However, these differences were not sufficiently clear-cut for determining the genetic stability or geographical distribution of even these few strains. Until the advent of DNA techniques, there was no way of reliably characterizing *MAP* strains into different types. Because strain typing is very useful for answering a number of epidemiological questions, absence of such a technique limited the range of epidemiological studies on paratuberculosis that could be performed.

This chapter examines the history and current use of DNA typing techniques applied

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to *MAP*. The chapter is devoted largely to describing the techniques themselves but also briefly outlines some of the information that has been elucidated from their use. While typing can be used to search for markers of functional strain differences, for quality control and for investigation of laboratory cross-contamination, its major use is for epidemiological studies. The greatest benefits from strain typing occur when typing results are used together with the results of classical epidemiological studies.

Typing techniques for *MAP* can be broadly divided into two categories: total genomic DNA methods, which with little alteration can be employed to type any bacterial species; and specific sequence methods, which rely on knowledge of certain DNA sequences in the *MAP* genome. The current specific sequence methods being used are based on the number of copies of different repetitive DNA sequences identified in the *MAP* genome, and these methods have been the most popular for typing *MAP* strains.

Most of the typing systems for *MAP* clearly distinguish strains into at least two groups. These groups are designated by several somewhat confusing nomenclatures, described in more detail elsewhere (see Stevenson, Chapter 12, this volume). The group of *MAP* strains that, with the notable exceptions of those from Icelandic cattle (Whittington *et al.*, 2001a) and from fighting bulls in Spain (de Juan *et al.*, 2006), appears to be the overwhelming cause of paratuberculosis in cattle in most countries is called Type C in this chapter; the group of strains that is the overwhelming cause of paratuberculosis in sheep in New Zealand, Australia, Morocco, South Africa and Iceland is called Type S (de Lisle *et al.*, 1993; Whittington *et al.*, 2000). Type S also includes strains originally described as I (Collins *et al.*, 1990). The trend over the last 10 years of combining groups of strain types originally called S and I into a single type has been justified by recent studies based on large-sequence polymorphism analysis (Alexander *et al.*, 2009). Use of the terms Type C and Type S does not imply absolute host specificity, as both types have been isolated, at least occasionally, from all the common ruminant hosts (Whittington *et al.*, 2000), but the designation has epidemiological usefulness in many situations.

25.2 Total Genomic DNA Methods

25.2.1 Restriction endonuclease analysis (REA)

The first DNA typing system to be applied to *MAP* strains was restriction endonuclease analysis (REA) (Collins and de Lisle, 1986). In this conceptually simple technique, purified genomic DNA from a selected strain is digested with a suitable restriction endonuclease, and the fragments produced are separated on the basis of size by agarose gel electrophoresis. While moderate fragment separation can be obtained on gels <300 mm long (Whipple *et al.*, 1989), strain differences can be better detected if the DNA fragments are separated on 400-mm-long cooled gels for 24 h with buffer recirculation. Since, with the enzymes used (BstEII, PvuII and BclI) under these conditions, the informative fragments range in size from 5 to 15 kb, stringent methods to prepare very high molecular weight DNA are not required. This approach had already been successfully applied to typing strains of the related species *Mycobacterium bovis* (Collins and de Lisle, 1985) and continues to be a highly useful tool for epidemiological studies involving *M. bovis* (Collins, 1999). When REA was first applied to three reference *MAP* strains and 23 *MAP* strains from New Zealand cattle, two of the reference strains and all but one of the cattle strains had identical patterns (Collins and de Lisle, 1986). The remaining cattle strain had one fragment difference with one enzyme pattern, indicating the very close genetic similarity of all these strains. The third reference strain, US Department of Agriculture strain 18, had very different restriction patterns from the other *MAP* strains. This was the first genetic evidence, later confirmed (Chiodini, 1993), that strain 18 had been misclassified.

Subsequently, when REA was applied to a very diverse collection of 50 *MAP* strains from a range of host species in five countries, the strains were clearly separated into two

groups (Collins *et al.*, 1990). Strains isolated predominantly from cattle formed one group (Type C), and strains isolated predominantly from sheep formed a second group (Type S). This established for the first time a clear genetic basis for the long-held belief, based largely on *in vitro* culture characteristics, that many isolates from sheep were different from those from cattle. This had important epidemiological implications as cattle and sheep graze the same pastures in some countries, and the result indicated that infection between sheep and cattle might be much less frequent than infection within the same host species.

Interestingly, REA also revealed a single fragment difference between a human isolate of *MAP* and the most common Type C strain found in several countries. While only a modest number of strains were analysed in this study, the result raised the possibility that successful infection of humans with *MAP* might only occur with strains that were slightly different genetically from those commonly found in cattle. Overall, REA divided the 50 strains into eight different types. However, the technique has not been further used for *MAP* strains because it is technically difficult to perform, and because the same 50 strains could be separated into ten types with a technically easier method based on restriction fragment length polymorphisms (RFLPs) with the insertion sequence IS*900* (Collins *et al.*, 1990).

25.2.2 Pulse-field gel electrophoresis (PFGE)

Pulse-field gel electrophoresis (PFGE) is a form of REA but differs from that described above in several respects. In PFGE, restriction endonucleases are used that cut genomic DNA infrequently, so that 10–20 large fragments of 20–700 kb in size are produced; very high molecular size DNA is obtained by lysing the bacteria and digesting the DNA in agarose; and the large fragments are separated through agarose gels over 12–24 h by continually changing the direction of the electrical field during electrophoresis. While the technique has become the gold standard for food-borne pathogens (Gerner-Smidt *et al.*, 2006), it has only been used to a moderate extent to type *MAP* strains. In the first report of its application to *MAP*, six strains were characterized into three types (Coffin *et al.*, 1992). Although the differences seen were not as clear as more recent improvements of the technique achieve, they were sufficiently encouraging for others to use the approach on a variety of *MAP* strains, mainly from cattle, sheep and goats (Feizabadi *et al.*, 1997; Grant *et al.*, 2002; Stevenson *et al.*, 2002; de Juan *et al.*, 2005; Sevilla *et al.*, 2007). PFGE clearly distinguishes Type C and Type S strains but, like most other typing techniques, does not distinguish Type C strains from bison strains (Sevilla *et al.*, 2007). On the occasions when PFGE has been compared with other typing methods, it has shown moderately better discrimination than RFLP analysis with IS*900* for both Type S and Type C strains isolated from sheep and goats (Stevenson *et al.*, 2002; de Juan *et al.*, 2005) but only slightly better discrimination for Type C strains isolated from cattle (Grant *et al.*, 2002; de Juan *et al.*, 2005). Thus it supports the results found in a larger number of RFLP typing studies: that there is a low degree of diversity of Type C strains of *MAP* in cattle throughout the world. On the basis of PFGE patterns, Type S strains, which are common in sheep, group closely with each other. The main reasons for the limited use of PFGE for *MAP* studies are because the method is expensive and time consuming, and some strains cannot be successfully typed (Grant *et al.*, 2002; Sevilla *et al.*, 2007).

25.2.3 Amplified fragment length polymorphism (AFLP) analysis

Whereas PFGE and REA define types based on large- and moderate-size DNA restriction fragments respectively, amplified fragment length polymorphism (AFLP) analysis defines types based on small-sized (50–700 bp) DNA restriction fragments. Because of the small amount of DNA in each fragment, the fragments are amplified by attaching primers and performing a polymerase chain reaction (PCR) in order to be visualized. Different restriction enzymes can be used to digest the DNA and, through design of the primers, usually only a subsection

of fragments from the digest are selected for analysis. Because of these variations in technique, it is often difficult to compare the results from different AFLP studies. Three studies from the USA have used AFLP to type local *MAP* strains, mainly isolated from cattle (Motiwala *et al.*, 2003; O'Shea *et al.*, 2004; Kiehnbaum *et al.*, 2005). The two larger studies, which between them looked at 128 *MAP* isolates from cattle, found that 73% (Kiehnbaum *et al.*, 2005) and 90% (Motiwala *et al.*, 2003) of the isolates analysed were characterized into only two types. However, because different isolates as well as different techniques were used in the two studies, it is not clear whether the two types referred to are the same. One of the studies also applied AFLP to four strains from sheep, which were classified as Type S strains on the basis of a well-described polymorphism in the insertion sequence IS*1311* (Motiwala *et al.*, 2003). By AFLP, one of these strains had the most common type found for Type C strains isolated from cattle, and another had a type that was more closely related to other Type C strains than it was to the other Type S strains. This inability of AFLP to clearly distinguish Type S strains from Type C strains may partly account for the technique not being widely adopted.

25.2.4 Random amplified polymorphic DNA (RAPD) analysis

Random amplified polymorphic DNA (RAPD) analysis is based on PCR of genomic DNA using one or two short random sequence primers that are selected empirically. Once suitable primers have been identified, the technique is simple to perform. However, because the primers do not exactly match most target sites, the technique is very sensitive to small changes in reaction conditions and results can be difficult to reproduce exactly. Two studies have applied RAPD analysis to *MAP* strains isolated from cattle in Germany (Scheibl and Gerlach, 1997) and the USA (Pillai *et al.*, 2001) and both report some differentiation. In the larger study, 208 field isolates from cattle were divided into six types, with two types representing 58% of the strains. In general, this supports the result found by other typing methods that there is a low degree of diversity of Type C strains of *MAP* in cattle. Because of its limited reproducibility, the RAPD technique is unlikely to be applied on a wide enough scale to make more detailed comparisons with other typing methods possible.

25.3 Insertion Sequence Analysis

25.3.1 IS*900*

Characterization of *MAP* strains by RFLP analysis with the insertion sequence IS*900* has been the most commonly used typing system for *MAP* strains (Whittington *et al.*, 2000; Motiwala *et al.*, 2006). This repetitive DNA sequence, which is 1451 bp long and present in 15–18 copies, was identified independently in two laboratories (Collins *et al.*, 1989; Green *et al.*, 1989) and rapidly applied to the typing of *MAP* strains (Collins *et al.*, 1990). In this technique, genomic DNA is digested with a restriction enzyme that does not cut within IS*900*; the DNA fragments produced are separated by agarose electrophoresis, blotted on to nylon and hybridized to a probe made from part of the IS*900* DNA sequence (Fig. 25.1). In some cases, the differences identified appear to be due to the presence or absence of a copy

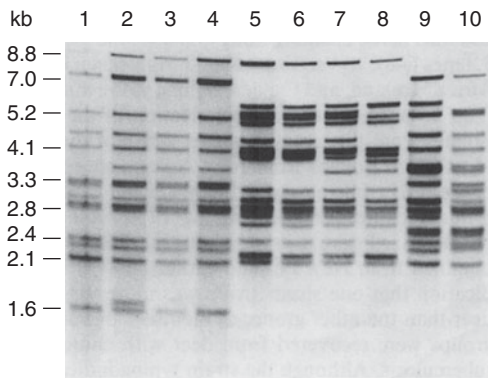

Fig. 25.1. IS*900* RFLP patterns of genomic DNA digested with BstEII from *MAP* strains: lanes 1–4, common Type C strains; lanes 5–7, Type S strains from New Zealand; lanes 8–10, Type S strains from the Faeroe Islands, South Africa and Iceland respectively (from de Lisle *et al.*, 1993, with permission).

of IS*900* in a strain, while in other cases they appear to be a result of DNA sequence changes resulting from genomic rearrangements between adjacent IS*900* loci (Whipple *et al.*, 1990; de Lisle *et al.*, 1993; Bull *et al.*, 2000). As with other typing techniques that involve digestion of genomic DNA, the choice of restriction enzyme is important. Most studies have used one or more of the three enzymes BstEII, PvuII and PstI. By itself BstEII gives slightly better discrimination than the other two enzymes (Whipple *et al.*, 1990; Pavlik *et al.*, 1999; Stevenson *et al.*, 2002; Machackova *et al.*, 2004), but the best discrimination is obtained by combining the results of two or three enzymes. For example, Whipple *et al.* (1990) characterized 34 mostly USA bovine isolates into five BstEII types, four PvuII types and four PstI types and achieved a combined characterization of eight types. However, even with this combined characterization, 62% of the isolates had the same type. Analysis of 96 *MAP* isolates from wild ruminants in the Czech Republic found 82% of isolates had a single IS*900* RFLP type (Machackova *et al.*, 2004), while analysis of 47 isolates of Type C from farmed cattle, deer and goats in New Zealand found 94% had the same IS*900* RFLP type (D.M. Collins, 2009, unpublished results); this type was common in Australia (Cousins *et al.*, 2000) and was also the predominant type in the Czech Republic and elsewhere in Europe (Pavlik *et al.*, 1999). In another study, analysis of 61 *MAP* isolates from farmed cattle and deer in Argentina found that 75% had a single IS*900* RFLP type (Moreira *et al.*, 1999). In this case, the predominant *MAP* type in Argentina was the same as that in the UK (Greig *et al.*, 1999), from which many Argentinian cattle were originally sourced. The same type also occurred in a collection of *MAP* strains mostly from Europe, but at a much lower frequency (Pavlik *et al.*, 1999). International standardization of the IS*900* RFLP designations enabled results from many different countries to be compared (Pavlik *et al.*, 1999). This large comparative study drew together results from typing 998 isolates of Type C. In most cases, the isolates had been typed using both BstEII and PstI. While more than two-thirds (696) of the isolates analysed came from the Czech Republic, isolates from 16 other countries were also included. A total of 28 different subtypes of Type C were identified, two-thirds of which (19) were represented by only one to five isolates. While detailed information on which types came from which countries is not given in this study, it is evident from the overall results and from the published studies on which this comparison is based that a small number of IS*900* RFLP types predominates globally. The widespread global dispersal of these types, and stability studies on a few types (Pavlik *et al.*, 1999), indicates that the types are very stable and therefore very reliable for long-term broad-brush epidemiological studies.

Because IS*900* typing is simpler to implement than total genomic methods and gives similar or only slightly less discrimination of isolates, its use has been investigated in many countries. Its widespread application has enabled it to confirm the results from REA and other methods, that *MAP* isolates can be characterized into Type S and Type C. However, because the variation in IS*900* RFLP patterns that occurs within Type C and Type S strains has been relatively modest, IS*900* typing has been of only minor help in studying between- or within-herd transmission (Thibault *et al.*, 2008), or transmission from wildlife to farmed animals (Greig *et al.*, 1999). In common with the total genomic techniques, the method is also slow as it requires subculture of an isolate in order to obtain sufficient DNA of good quality to achieve acceptable results. While the speed of typing *MAP* strains based on IS*900* can be improved by using a multiplex PCR for IS*900* integration loci (MPIL), which requires much smaller amounts of DNA, MPIL does not detect all the differences found by the IS*900* RFLP technique (Bull *et al.*, 2000; Motiwala *et al.*, 2003).

25.3.2 IS*1311*

The insertion sequence IS*1311* is present in seven copies in *MAP* strains of Type C and nine copies in *MAP* strains of Type S. Not surprisingly, RFLP analysis with IS*1311* very clearly distinguishes *MAP* strains of Type C from those of Type S (Collins *et al.*, 1997), but the method has been little used as a typing

system. This is because, although it clearly divided six strains of Type S into four subtypes, it did not differentiate between strains of Type C in three separate studies (Collins *et al.*, 1997; Whittington *et al.*, 1998; Johansen *et al.*, 2005).

While RFLP analysis with IS*1311* has not been found to be useful, PCR restriction analysis utilizing small sequence differences between some copies of IS*1311* in Type C strains, Type S strains and strains from bison has been used as a method to distinguish these strains from each other (Marsh *et al.*, 1999; Whittington *et al.*, 2001b; Sibley *et al.*, 2007). With the recent availability of much more DNA sequence information from *MAP* strains, this method of distinguishing between strains of Type C and Type S may be replaced by simpler PCR methods based on DNA sequences that uniquely define each of these types (Collins *et al.*, 2002; Alexander *et al.*, 2009).

25.4 Other Repetitive Sequences

25.4.1 Variable number tandem repeats (VNTRs)

A variety of tandemly repeated DNA sequences, most of them 50–80 bp in length, have been found to be very useful for typing strains of the *Mycobacterium tuberculosis* complex over the last 10 years. The typing is based on detecting the number of copies of a particular repeat, as the copy number sometimes differs between strains. The copy numbers are sufficiently stable at most loci to be used for typing, and the best discrimination is obtained by analysing 24 different loci in each strain (Supply *et al.*, 2006). The sequences are referred to as variable number tandem repeats (VNTRs). Based on particular characteristics, some VNTRs are also designated as mycobacterial interspersed repetitive units (MIRUs); for simplicity, the term MIRU will not be further used in this chapter. Short-sequence repeats (SSRs), a special class of very small tandem repeats, are discussed separately in the next Section (25.4.2). The great attraction of VNTR typing is that, because it is based on PCR amplification, it requires only small amounts of sheared DNA, which can potentially be obtained from primary cultures. VNTRs were identified in *MAP* when sufficient DNA sequence of the genome became available for searching, and were immediately investigated for their typing potential (Bull *et al.*, 2003). The four or five VNTR loci selected in early studies gave only very limited discrimination of *MAP* strains (Bull *et al.*, 2003; Overduin *et al.*, 2004). More recent studies used six (Romano *et al.*, 2005), seven (Möbius *et al.*, 2008) or eight (Thibault *et al.*, 2007) VNTR loci for typing *MAP* strains of Type C. The additional VNTR loci were selected from more than 30 different VNTR loci in the *MAP* genome (Romano *et al.*, 2005; Thibault *et al.*, 2007). In two of the studies (Thibault *et al.*, 2007; Möbius *et al.*, 2008), the overall discrimination of VNTR typing was similar to that of IS*900* typing, and combination of the two typing systems gave much better discrimination than either system separately. The third study (Romano *et al.*, 2005) obtained no discrimination of *MAP* strains by VNTR typing. These three studies cannot be compared in detail, as in each case different *MAP* strains were used and each laboratory employed a different combination of VNTR loci; but taken together they provide an excellent guide to the VNTR loci that have most potential to provide discrimination in other situations. The total number of VNTR loci in the *MAP* genome varies depending on the computer program used (Romano *et al.*, 2005; Thibault *et al.*, 2007) as well as the chosen selection criteria, such as minimum and maximum length, copy number and per cent sequence matching (Institut de Génétique et Microbiologie, 2008). While it appears that not all possible VNTR loci have yet been assessed for their typing potential, sufficient work has been done to make it unlikely that the employment of novel VNTRs would greatly improve discrimination. However, the ability of VNTR typing to further subdivide *MAP* strains that have been typed by other methods (Thibault *et al.*, 2008) is likely to ensure continuation of its use in at least the medium term.

25.4.2 Short-sequence repeats (SSRs)

SSRs consisting of simple tracts of a single nucleotide or multimers of di- or trinucleotides

have been investigated for typing a number of bacterial species including *M. tuberculosis* (Amonsin *et al.*, 2004). Examination of 78 such loci in the genome of *MAP* revealed 11 loci that were polymorphic, and these were reported to be more discriminatory for a collection of 33 *MAP* strains than were AFLP and MPIL (Amonsin *et al.*, 2004). A potential disadvantage, acknowledged but not quantified in this seminal study, was the need to take account of sequence errors due to strand slippage during either PCR or sequence reactions, by sequencing each locus in both directions as well as testing duplicate samples. Later studies refer to the need for visual inspection of sequence traces to correct ambiguities (Motiwala *et al.*, 2005) and the need for consensus between two readers of the traces (Thibault *et al.*, 2008). These caveats are particularly directed at the two loci with tracts of G nucleotides that, because of their discrimination ability for *MAP* strains of Type C in both the USA and Europe, would probably be included in any panel of four or five loci used for SSR typing. The difficulties apply to G repeats of greater than 11 and led a European laboratory (Thibault *et al.*, 2008) to conservatively assign all alleles greater than 11 as the same type. In other studies, the cut-off value used is greater than 13 and, if the cut-off of 11 had been used instead, the SSR technique would have been significantly less discriminating than reported in several studies (Ghadiali *et al.*, 2004; Motiwala *et al.*, 2005; Harris *et al.*, 2006; Sevilla *et al.*, 2008). Difficulties in amplifying the loci with G repeats in a few strains have also been reported (Ghadiali *et al.*, 2004; Cernicchiaro *et al.*, 2008). A further possible disadvantage of SSR typing is strand slippage occurring too frequently during chromosomal duplication. All DNA typing systems rely on genetic changes occurring with time, but if the changes occur at a locus more frequently than the length of time over which epidemiological studies are carried out then that locus becomes of limited use. Extreme examples are recorded for *Campylobacter jejuni*, where slippage of some single nucleotide tracts occurs at such a high frequency that all subcultures of a single clone contain several polymorphic variations in almost equal proportions (Parkhill *et al.*, 2000). There is no indication that this occurs for *MAP* strains, and in fact recent studies reported that the four most discriminatory loci in the USA, which included two tracts of G nucleotides, were stable in three *MAP* strains tested over ten subcultures (Harris *et al.*, 2006). A very recent study also reports that two of the most commonly used SSR sequences are invariant in 98 isolates of the bison type from different parts of north India (Singh *et al.*, 2009). The most recent comparisons of SSR typing with other methods reported that using six SSR loci gave considerably better discrimination than VNTR typing, which itself was more discriminating than typing based on IS*900* (Thibault *et al.*, 2008), and that using two SSR loci for typing gave a similar discrimination to PFGE (Sevilla *et al.*, 2008). A further minor disadvantage of using only SSR for typing *MAP* strains is that although some strains of Type S show clustering by SSR typing they are in other cases more similar to strains of Type C than they are to each other (Amonsin *et al.*, 2004; Motiwala *et al.*, 2004).

25.5 Comparison of Techniques and Future Outlook

A comparison of aspects of the different methods is shown in Table 25.1. Four methods of typing *MAP* strains predominate. IS*900* RFLP typing has been the most used method. It very clearly separates *MAP* strains into Type S and Type C but gives insufficient discrimination between strains within each type for it to be useful for detailed epidemiological studies. It employs simple DNA techniques that are available in many laboratories but it is slow because of its requirement for moderate amounts of high-quality DNA. PFGE electrophoresis is also a slow technique but it gives somewhat better discrimination than IS*900* typing. PFGE has not been as popular as IS*900* typing because it requires a level of skill and equipment that is not available in many laboratories and the discrimination of strains is still not sufficiently good for detailed epidemiological studies. VNTR typing is simpler and faster than IS*900* and PFGE typing and is easily performed in most laboratories, but it

Table 25.1. Comparison of various aspects of the typing methods.

Methodology	Ease of use	Discrimination	Reproducibility	Time
REA	Difficult	+	++++	Slow
PFGE	Moderate	+++	+++	Slow
AFLP	Moderate	+	+++	Moderate
RAPD	Very easy	++	+	Fast
IS*900*	Easy	++	++++	Slow
VNTR	Very easy	+++	++++	Fast
SSR	Very easy	++++	+++	Fast

does not by itself give better discrimination than these earlier methods. SSR typing appears at first sight to be a more discriminating typing method than any of the other three methods by themselves, although much of this extra discriminating power depends on the ability of a laboratory to reliably designate the longer tracts of G nucleotides, and this can be a problem. Since these four methods reflect different features of the *MAP* genome, it is perhaps not completely unexpected that they are complementary to each other; in every case where a reasonably diverse collection of *MAP* strains was typed, a combination of any two of these methods always gave significantly better discrimination than one method alone. One recent paper from a laboratory with experience in many typing methods recommends first typing strains by VNTR as this technique is more accessible to most laboratories, followed by SSR typing with one or more loci and, for even better discrimination, IS*900* typing (Thibault *et al.*, 2008). Laboratories that are better resourced may start with SSR typing of four to six loci before employing other methods.

From the comparisons of using two or three of the predominant methods that have so far been reported, it appears unlikely that any laboratory would find the small increase in discrimination achieved by performing all four methods to be justified for the time and expense incurred; in fact few laboratories are likely to use even three methods for typing large numbers of strains. Since a combination of at least two of the four most common typing methods appears necessary for most epidemiological studies, this is likely to become the accepted approach in the medium term.

In the longer term, and probably in the near future for well-resourced laboratories, the ability to sequence ever-larger amounts of DNA will inevitably lead to higher-resolution typing systems being developed for *MAP* strains. Single nucleotide polymorphisms have already been demonstrated in a small number of genes surveyed in *MAP* strains (Turenne *et al.*, 2008) as well as in IS*900* (Castellanos *et al.*, 2009a), and wider surveys will undoubtedly reveal more differences. Microarrays based on major genomic insertions and deletions are already being used to characterize *MAP* strains (Castellanos *et al.*, 2009b), and detection of suitable single nucleotide polymorphisms could also be incorporated in a microarray system. The new generation of sequencing techniques even raises the real possibility of total genome sequencing being used as a typing method (Medini *et al.*, 2008). While such extreme characterization is probably not necessary for most purposes, some improvement on the current typing systems would undoubtedly lead to a more precise understanding of *MAP* epidemiology.

References

Alexander, D.C., Turenne, C.Y. and Behr, M.A. (2009) Insertion and deletion events that define the pathogen *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Bacteriology* 191, 1018–1025.

Amonsin, A., Li, L.L., Zhang, Q., Bannantine, J.P., Motiwala, A.S., Sreevatsan, S. and Kapur, V. (2004) Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis*

strains. *Journal of Clinical Microbiology* 42, 1694–1702.

Bull, T.J., Hermon-Taylor, J., Pavlik, I., El-Zaatari, F. and Tizard, M. (2000) Characterization of IS*900* loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. *Microbiology* 146, 2185–2197.

Bull, T.J., Sidi-Boumedine, K., McMinn, E.J., Stevenson, K., Pickup, R. and Hermon-Taylor, J. (2003) Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. *Molecular and Cellular Probes* 17, 157–164.

Castellanos, E., Aranaz, A., de Juan, L., Álvarez, J., Rodríguez, S., Romero, B., Bezos, J., Stevenson, K., Mateos, A. and Domínguez, L. (2009a) Single nucleotide polymorphisms in the IS*900* sequence of *Mycobacterium avium* subspecies *paratuberculosis* are strain type-specific *Journal of Clinical Microbiology* 47, 2260–2264.

Castellanos, E., Aranaz, A., Gould, K.A., Linedale, R., Stevenson, K., Alvarez, J., Dominguez, L., de Juan, L., Hinds, J. and Bull, T.J. (2009b) Discovery of stable and variable differences in the *Mycobacterium avium* subsp. *paratuberculosis* Type I, II, and III genomes by pan-genome microarray analysis. *Applied and Environmental Microbiology* 75, 676–686.

Cernicchiaro, N., Wells, S.J., Janagama, H. and Sreevatsan, S. (2008) Influence of type of culture medium on characterization of *Mycobacterium avium* subsp. *paratuberculosis* subtypes. *Journal of Clinical Microbiology* 46, 145–149.

Chiodini, R.J. (1993) Abolish *Mycobacterium paratuberculosis* strain 18. *Journal of Clinical Microbiology* 31, 1956–1958.

Coffin, J.W., Condon, C., Compston, C.A., Potter, K.N., Lamontagne, L.R., Shafiq, J. and Kunimoto, D.Y. (1992) Use of restriction fragment length polymorphisms resolved by pulsed-field gel electrophoresis for subspecies identification of mycobacteria in the *Mycobacterium avium* complex and for isolation of DNA probes. *Journal of Clinical Microbiology* 30, 1829–1836.

Collins, D.M. (1999) Molecular epidemiology: *Mycobacterium bovis.* In: Ratledge, C. and Dale, J. (eds) *Mycobacteria: Molecular Biology and Virulence*. Blackwell Science, Oxford, UK, pp. 123–135.

Collins, D.M. and de Lisle, G.W. (1985) DNA restriction endonuclease analysis of *Mycobacterium bovis* and other members of the tuberculosis complex. *Journal of Clinical Microbiology* 21, 562–564.

Collins, D.M. and de Lisle, G.W. (1986) Restriction endonuclease analysis of strains of *Mycobacterium paratuberculosis* isolated from cattle. *American Journal of Veterinary Research* 47, 2226–2229.

Collins, D.M., Gabric, D.M. and de Lisle, G.W. (1989) Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. *FEMS Microbiology Letters* 60, 175–178.

Collins, D.M., Gabric, D.M. and de Lisle, G.W. (1990) Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. *Journal of Clinical Microbiology* 28, 1591–1596.

Collins, D.M., Cavaignac, S. and de Lisle, G.W. (1997) Use of four DNA insertion sequences to characterise strains of the *Mycobacterium avium* complex isolated from animals. *Molecular and Cellular Probes* 11, 373–380.

Collins, D.M., De Zoete, M. and Cavaignac, S.M. (2002) *Mycobacterium avium* subsp. *paratuberculosis* from cattle and sheep can be distinguished by PCR based on a novel DNA sequence difference. *Journal of Clinical Microbiology* 40, 4760–4762.

Cousins, D.V., Williams, S.N., Hope, A. and Eamens, G.J. (2000) DNA fingerprinting of Australian isolates of *Mycobacterium avium* subsp. *paratuberculosis* using IS*900* RFLP. *Australian Veterinary Journal* 78, 184–190.

de Juan, L., Mateos, A., Domínguez, L., Sharp, J.M. and Stevenson, K. (2005) Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* isolates from goats detected by pulsed-field gel electrophoresis. *Veterinary Microbiology* 106, 249–257.

de Juan, L., Alvarez, J., Aranaz, A., Rodríguez, A., Romero, B., Bezos, J., Mateos, A. and Domínguez, L. (2006) Molecular epidemiology of Types I/III strains of *Mycobacterium avium* subspecies *paratuberculosis* isolated from goats and cattle. *Veterinary Microbiology* 115, 102–110.

de Lisle, G.W., Yates, G.F. and Collins, D.M. (1993) Paratuberculosis in farmed deer: case reports and DNA characterization of isolates of *Mycobacterium paratuberculosis*. *Journal of Veterinary Diagnostic Investigation* 5, 567–571.

Feizabadi, M.M., Robertson, I.D., Hope, A., Cousins, D.V. and Hampson, D.J. (1997) Differentiation of Australian isolates of *Mycobacterium paratuberculosis* using pulsed-field gel electrophoresis. *Australian Veterinary Journal* 75, 887–889.

Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytiä-Trees, E., Ribot, E.M., Swaminathan, B. and PulseNet Taskforce. (2006) PulseNet: a five-year update. *Foodborne Pathogens and Disease* 3, 9–19.

Ghadiali, A.H., Strother, M., Naser, S.A., Manning, E.J. and Sreevatsan, S. (2004) *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. *Journal of Clinical Microbiology* 42, 5345–5348.

Grant, I.R., Ball, H.J. and Rowe, M.T. (2002) Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. *Applied and Environmental Microbiology* 68, 2428–2435.

Green, E.P., Tizard, M.L., Moss, M.T., Thompson, J., Winterbourne, D.J., McFadden, J.J. and Hermon-Taylor, J. (1989) Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Research* 17, 9063–9073.

Greig, A., Stevenson, K., Henderson, D., Perez, V., Hughes, V., Pavlik, I., Hines, M.E., 2nd, McKendrick, I. and Sharp, J.M. (1999) Epidemiological study of paratuberculosis in wild rabbits in Scotland. *Journal of Clinical Microbiology* 37, 1746–1751.

Gunnarsson, E. (1979) Isolation of *Mycobacterium paratuberculosis* from sheep and cattle in Iceland. *Acta Veterinaria Scandinavica* 20, 191–199.

Harris, N.B., Payeur, J.B., Kapur, V. and Sreevatsan, S. (2006) Short-sequence-repeat analysis of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* isolates collected from animals throughout the United States reveals both stability of loci and extensive diversity. *Journal of Clinical Microbiology* 44, 2970–2973.

Institut de Génétique et Microbiologie (2008) Query in the tandem repeats database (bacteria). Available at http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php (accessed 3 December 2008).

Johansen, T.B., Djønne, B., Jensen, M.R. and Olsen, I. (2005) Distribution of IS*1311* and IS*1245* in *Mycobacterium avium* subspecies revisited. *Journal of Clinical Microbiology* 43, 2500–2502.

Kiehnbaum, L.A., Amonsin, A., Wells, S.J. and Kapur, V. (2005) Amplified fragment length polymorphism to detect clonal diversity and distribution of *Mycobacterium avium* subspecies *paratuberculosis* in selected Minnesota dairy cattle. *Journal of Veterinary Diagnostic Investigation* 17, 311–315.

Machackova, M., Svastova, P., Lamka, J., Parmova, I., Liska, V., Smolik, J., Fischer, O.A. and Pavlik, I. (2004) Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic (1999–2001). *Veterinary Microbiology* 101, 225–234.

Marsh, I., Whittington, R. and Cousins, D. (1999) PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* based on polymorphisms in IS*1311*. *Molecular and Cellular Probes* 13, 115–126.

Medini, D., Serruto, D., Parkhill, J., Relman, D.A., Donati, C., Moxon, R., Falkow, S. and Rappuoli, R. (2008) Microbiology in the post-genomic era. *Nature Reviews. Microbiology* 6, 419–430.

Möbius, P., Luyven, G., Hotzel, H. and Köhler, H. (2008) High genetic diversity among *Mycobacterium avium* subsp. *paratuberculosis* strains from German cattle herds shown by combination of IS*900* restriction fragment length polymorphism analysis and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing. *Journal of Clinical Microbiology* 46, 972–981.

Moreira, A.R., Paolicchi, F., Morsella, C., Zumarraga, M., Cataldi, A., Fabiana, B., Alicia, A., Piet, O., van Soolingen, D. and Isabel, R.M. (1999) Distribution of IS*900* restriction fragment length polymorphism types among animal *Mycobacterium avium* subsp. *paratuberculosis* isolates from Argentina and Europe. *Veterinary Microbiology* 70, 251–259.

Motiwala, A.S., Strother, M., Amonsin, A., Byrum, B., Naser, S.A., Stabel, J.R., Shulaw, W.P., Bannantine, J.P., Kapur, V. and Sreevatsan, S. (2003) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis. *Journal of Clinical Microbiology* 41, 2015–2026.

Motiwala, A.S., Amonsin, A., Strother, M., Manning, E.J., Kapur, V. and Sreevatsan, S. (2004) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates recovered from wild animal species. *Journal of Clinical Microbiology* 42, 1703–1712.

Motiwala, A.S., Strother, M., Theus, N.E., Stich, R.W., Byrum, B., Shulaw, W.P., Kapur, V. and Sreevatsan, S. (2005) Rapid detection and typing of strains of *Mycobacterium avium* subsp. *paratuberculosis* from broth cultures. *Journal of Clinical Microbiology* 43, 2111–2117.

Motiwala, A.S., Li, L., Kapur, V. and Sreevatsan, S. (2006) Current understanding of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis*. *Microbes and Infection* 8, 1406–1418.

O'Shea, B., Khare, S., Bliss, K., Klein, P., Ficht, T.A., Adams, L.G. and Rice-Ficht, A.C. (2004) Amplified fragment length polymorphism reveals genomic variability among *Mycobacterium avium* subsp. *paratuberculosis* isolates. *Journal of Clinical Microbiology* 42, 3600–3606.

Overduin, P., Schouls, L., Roholl, P., van der Zanden, A., Mahmmod, N., Herrewegh, A. and van Soolingen, D. (2004) Use of multilocus variable-number tandem-repeat analysis for typing *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 42, 5022–5028.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Whitehead, S. and Barrell, B.G. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668.

Pavlik, I., Horvathova, A., Dvorska, L., Bartl, J., Svastova, P., du Maine, R. and Rychlik I. (1999) Standardisation of restriction fragment length polymorphism analysis for *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Microbiological Methods* 38, 155–167.

Pillai, S.R., Jayarao, B.M., Gummo, J.D., Hue, E.C., Tiwari, D., Stabel, J.R. and Whitlock, R.H. (2001) Identification and sub-typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* by randomly amplified polymorphic DNA. *Veterinary Microbiology* 79, 275–284.

Romano, M.I., Amadio, A., Bigi, F., Klepp, L., Etchechoury, I., Llana, M.N., Morsella, C., Paolicchi, F., Pavlik, I., Bartos, M., Leão, S.C. and Cataldi, A. (2005) Further analysis of VNTR and MIRU in the genome of *Mycobacterium avium* complex, and application to molecular epidemiology of isolates from South America. *Veterinary Microbiology* 110, 221–237.

Scheibl, P. and Gerlach, G.F. (1997) Differentiation of *Mycobacterium paratuberculosis* isolates by rDNA-spacer analysis and random amplified polymorphic DNA patterns. *Veterinary Microbiology* 57, 151–158.

Sevilla, I., Garrido, J.M., Geijo, M. and Juste, R.A. (2007) Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiology* 7, 18.

Sevilla, I., Li, L., Amonsin, A., Garrido, J.M., Geijo, M.V., Kapur, V. and Juste, R.A. (2008) Comparative analysis of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle, sheep and goats by short sequence repeat and pulsed-field gel electrophoresis typing. *BMC Microbiology* 8, 204.

Sibley, J.A., Woodbury, M.R., Appleyard, G.D. and Elkin, B. (2007) *Mycobacterium avium* subspecies *paratuberculosis* in bison (*Bison bison*) from northern Canada. *Journal of Wildlife Diseases* 43, 775–779.

Singh, S.V., Sohal, J.S., Singh, P.K. and Singh, A.V. (2009) Genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* isolates recovered from animals, commercial milk, and human beings in north India. *International Journal of Infectious Diseases* 13, e221–e227.

Stevenson, K., Hughes, V.M., de Juan, L., Inglis, N.F., Wright, F. and Sharp, J.M. (2002) Molecular characterization of pigmented and non-pigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Clinical Microbiology* 40, 1798–1804.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C. and van Soolingen, D. (2006) Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* 44, 4498–4510.

Taylor, A.W. (1951) Varieties of *Mycobacterium johnei* isolated from sheep. *The Journal of Pathology and Bacteriology* 63, 333–336.

Thibault, V.C., Grayon, M., Boschiroli, M.L., Hubbans, C., Overduin, P., Stevenson, K., Gutierrez, M.C., Supply, P. and Biet, F. (2007) New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* strains: comparison with IS*900* and IS*1245* restriction fragment length polymorphism typing. *Journal of Clinical Microbiology* 45, 2404–2410.

Thibault, V.C., Grayon, M., Boschiroli, M.L., Willery, E., Allix-Béguec, C., Stevenson, K., Biet, F. and Supply, P. (2008) Combined multilocus short sequence repeat and mycobacterial interspersed repetitive unit variable-number tandem repeat typing of *Mycobacterium avium* subsp. *paratuberculosis* isolates. *Journal of Clinical Microbiology* 46, 4091–4094.

Turenne, C.Y., Collins, D.M., Alexander, D.C. and Behr, M.A. (2008) *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium*

avium subsp. *avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms. *Journal of Bacteriology* 190, 2479–2487.

Whipple, D.L., Kapke, P.A. and Andrews, R.E., Jr. (1989) Analysis of restriction endonuclease fragment patterns of DNA from *Mycobacterium paratuberculosis*. *Veterinary Microbiology* 19, 189–194.

Whipple, D., Kapke, P. and Vary, C. (1990) Identification of restriction fragment length polymorphisms in DNA from *Mycobacterium paratuberculosis*. *Journal of Clinical Microbiology* 28, 2561–2564.

Whittington, R., Marsh, I., Choy, E. and Cousins, D. (1998) Polymorphisms in IS*1311*, an insertion sequence common to *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species. *Molecular and Cellular Probes* 12, 349–358.

Whittington, R.J., Hope, A.F., Marshall, D.J., Taragel, C.A. and Marsh, I. (2000) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS*900* restriction fragment length polymorphism and IS*1311* polymorphism analyses of isolates from animals and a human in Australia. *Journal of Clinical Microbiology* 38, 3240–3248.

Whittington, R.J., Taragel, C.A., Ottaway, S., Marsh, I., Seaman, J. and Fridriksdottir, V. (2001a) Molecular epidemiological confirmation and circumstances of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. *Veterinary Microbiology* 79, 311–322.

Whittington, R.J., Marsh, I.B. and Whitlock, R.H. (2001b) Typing of IS*1311* polymorphisms confirms that bison (*Bison bison*) with paratuberculosis in Montana are infected with a strain of *Mycobacterium avium* subsp. *paratuberculosis* distinct from that occurring in cattle and other domesticated livestock. *Molecular and Cellular Probes* 15, 139–145.

26 Paratuberculosis Control Measures in Europe

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26.1 Introduction

Johne and Frothingham (1895) were the first to officially describe paratuberculosis in Europe as a chronic enteritis in cattle. The disease was, because of the size and shape of the acid-fast bacteria, thought to be a case of an intestinal form of avian tuberculosis in cattle. A literature review of the history of paratuberculosis in Europe by Goudzwaard (1971) shows that, not long after this first description, identical observations were made across Europe: the Netherlands (1903), Belgium (1905), Switzerland (1905), France (1906), Denmark (1906), the UK (1907), Norway (1908) and Russia (1911). However, even earlier descriptions of a chronic enteritis in cattle with comparable symptoms, e.g. by Cartwright (1829), most likely refer to paratuberculosis as well. Therefore, paratuberculosis must have, even at the time of its first description, already been widespread across Europe, not only in cattle but also in other ruminants.

In 1906, using experimental infections in guinea pigs and calves, Bang (1906) was able to differentiate the disease from avian

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tuberculosis, leading to the isolation and culture of the fastidious aetiological agent designated *Mycobacterium enteritidis chronicae pseudotuberculosae bovis johne* by Twort and Ingram (1912). Shortly thereafter, the complement fixation test was developed by the younger of the Twort brothers (Twort, 1912), and later optimized in the Bang laboratory by Bang's son (Bang and Anderson, 1913). Both assays not only resulted in the improvement of the diagnosis of Johne's disease (JD), as it was named by M'Fadyean (1907), but also allowed initiation of the first attempts to control the disease in Europe.

26.2 Why Control Programmes Are Needed

Despite uncertain estimates of the burden of paratuberculosis in livestock in different European countries, the prevalence is thought to be significant (Nielsen and Toft, 2009) and may also be increasing. Even though this increase is not supported by reliable data from prevalence studies, it is strongly supported by modelling studies (Kudahl *et al.*, 2008). Moreover, model-based estimates project that if no control measures are taken the prevalence of JD will further increase, seriously affecting the quality of domestic livestock in Europe and elsewhere.

The effects of paratuberculosis at the animal level, leading to a reduced milk yield, reduced slaughter value and premature culling of animals, and hence significant financial losses, have been well documented (Benedictus *et al.*, 1987), However, due to the fact that disease in domesticated livestock is largely subclinical and non-notifiable, JD is likely to be significantly under-reported (Kennedy and Benedictus, 2001). Therefore the financial losses can be expected to be significantly higher than reported in literature, further supporting the need for effective control programmes. Economic costs are described in more detail elsewhere (see Barkema *et al.*, Chapter 2, this volume).

Herd owners generally choose to keep infected animals as long as possible if the milk yield is high enough, implying that animals are culled very close to the clinical stage (Buergelt and Duncan, 1978). Despite the introduction of education programmes (Benedictus *et al.*, 2000) for farmers on management changes needed to support the programmes to control paratuberculosis, possibly provoked by the high milk price in the years 2007–2008, this is still the case. For instance, when the Dutch Central Veterinary Institute wanted to purchase high shedders of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) to obtain samples for test development, three animals, recently detected as high shedders, were selected from the records of the Dutch Animal Service for purchase. One of the animals was not allowed to be put on transport by the veterinarian because of its poor overall condition, while the other two animals were euthanized within 3 weeks of arrival because of their rapidly deteriorating condition. While this example is anecdotal, it is noteworthy that both animals were shedding 10^6–10^7 *MAP* per g of faeces and probably represented sources of significant *MAP* transmission, both within their herds of origin and potentially to the milk supply. Therefore, for both animal welfare and disease control, animals infected with *MAP* should not be kept as long as possible and development of advanced stages of disease should be avoided. It follows that a control programme using diagnostic methods aimed at early detection and slaughter of infected animals will result in improved animal welfare and reduced infection pressure within the herd.

Even though animal welfare is becoming increasingly important for the consumer, an even more compelling reason for both consumers and stakeholders from industry to initiate and support control programmes is the much-debated possible link between *MAP* and Crohn's disease in humans. Thus far, conflicting results using various approaches to prove or disprove the existence of a possible link have led to lack of clarity on the role of *MAP* in the aetiology of Crohn's disease (see Behr, Chapter 5, this volume). Recently, in order to give more direction to the ongoing and future research efforts, Lowe *et al.* (2008) identified crucial research questions required to be answered to be able to assess the possible causal role of *MAP* (or other microbes) in

the aetiology of Crohn's disease. Since answering these questions unambiguously will not be easy and can be expected to take some time, there is a risk that funding of the needed control measures might await the answers. In the interim, advocates of the precautionary principle submit that there is sufficient reason to support control programmes aimed at improving animal health and welfare, as part of supporting a sustainable agriculture. At the same time, such programmes would reduce the exposure of the food chain to *MAP*. Furthermore, the European Union (EU) 'White Paper on Food Safety', published by the European Commission (2000), outlined the 'Farm to Table' concept as the cornerstone for the EU policy on food safety, which was envisaged as an integrated policy covering all sectors of the food chain, including production. The White Paper also recognized the importance of animal health and welfare to public health and consumer protection and established the principle that feed and food operators have primary responsibility for food safety. Fortunately, paratuberculosis has been recognized as a problem by many of the stakeholders involved (Bakker *et al.*, 2000) and this has resulted in new initiatives to develop control schemes for paratuberculosis in Europe. Several of those schemes and their backgrounds will be discussed in the sections below, including some of the pitfalls encountered.

26.3 Options for Controlling Paratuberculosis

Any conceivable control programme for paratuberculosis will be based on reducing within-herd transmission of *MAP*. Therefore, control measures will be based on a combination of management changes reducing the risk of transmission between animals that shed *MAP* and other non-infected (young) animals, and the removal of infectious animals by early detection (test and cull) or vaccination strategies (Benedictus *et al.*, 2000).

Within the EU, there is no mandatory programme for the control of paratuberculosis directed by the European Commission. Since government funding of control schemes by national or local authorities would result in an unfair competition between EU member states, the funding of such schemes is restricted to other stakeholders such as dairy and meat industry and farmers' organizations. Government involvement is, in general, restricted to supporting experimental programmes and fundamental research.

Animal husbandry in Europe is characterized by diversity of farming systems, both between and within countries. As a consequence, the options for introduction of control schemes not only depend on the difference in farming systems, e.g. the feasibility of introducing optimal management changes in dairy versus beef herds, but are also dictated by financial criteria. The possible options can differ significantly between regions as well as between animal species. For this reason, economic modelling plays an important role in the design and feasibility studies of control programmes, based on diagnostic tests (Groenendaal *et al.*, 2002) as well as on vaccination (van Schaik *et al.*, 1996).

Since test-and-cull programmes will have to be based on repetitive testing, involving moderate to high costs for longer periods, such programmes will hardly ever be an option for small ruminant owners, because of the number of animals in those herds and the small profit margin per animal. Therefore, the test-and-cull approach will be restricted mainly to dairy cattle. Thus, control by vaccination is the only viable option for small ruminants.

While options for the control of paratuberculosis are dictated by financial constraints and farming practices, the overall disease status of the herd, region or country will also be an important determinant of what options are applicable, if any. In Great Britain, for example, where the incidence of bovine tuberculosis is again increasing, it will be difficult to motivate farmers and their organizations to embark on an expensive and long-lasting control programme for paratuberculosis before bovine tuberculosis is under control. Similar situations occur in other countries where the available resources are needed to fulfil the requirements of the already ongoing mandatory control programmes for diseases ranking higher on the list for eradication.

On the other hand, in Sweden and Finland, the situation is completely the opposite. With an estimated herd prevalence of less than 0.3% (Viske *et al.*, 1996), these countries are almost free of paratuberculosis, motivating the use of extensive control schemes to protect this status (Sternberg and Viske, 2003). Measures include a whole-herd slaughter policy in the case of the detection of a single infected animal, back-tracing of animals bought and sold from that farm, testing of all contact herds by faecal culture and disinfection of buildings and farming equipment as well as manure. In Finland, paratuberculosis is not even regarded as a problem; in the past decades only a few cases have been detected in beef cattle and none in dairy cattle, lessening the need for a certification or control programme (Seuna and Seppänen, 2003). Because of these different situations and the variable amount of resources available to address this problem, a wide array of different approaches and control schemes in Europe have been developed, as detailed below.

26.4 The Use of Vaccination to Control Paratuberculosis

Vaccination against paratuberculosis was first introduced in France by Vallee and Rinjard (1926) using a subcutaneous injection of a living, non-virulent strain of *MAP*. Since then, many different formulations of the vaccine have been evaluated in a large number of countries worldwide. In Europe, two of the oldest control programmes for paratuberculosis are based on vaccination: in sheep in Iceland and in goats in Norway.

Paratuberculosis was introduced in Iceland in 1933 from Germany, first into sheep and subsequently into cattle, and almost ruined sheep farming. Using hundreds of kilometres of fences, the country was divided into infected and non-infected zones, and attempts were made to control paratuberculosis by using test-and-cull methods (Fridriksdottir *et al.*, 2000). Not until the development of an improved heat-killed vaccine by Sigurdsson (1952) and the subsequent introduction of a vaccination programme was control of paratuberculosis successful, leading to a 94% reduction of mortality in sheep. Since 1996, vaccination has been compulsory in endemic areas and losses have been reduced considerably (Fridriksdottir *et al.*, 2000).

In Norway, two vaccines have been used in the control of paratuberculosis in goats: the Icelandic heat-killed vaccine and the live, attenuated vaccine (Saxegaard and Fodstad, 1985). Vaccination was mandatory in all goat kids from 1978 to 1985 and resulted in a rapid reduction of infection. Paratuberculosis seemed to be under control until a new case was diagnosed in 1991, followed by the diagnosis of new cases in 23 other herds. Therefore, in 1993, obligatory vaccination was again introduced in endemic areas, on the west coast and in the central part of southern Norway (Djønne, 2003).

In Spain, vaccination has been the chosen strategy for control of paratuberculosis in sheep since the first cases in the early 1970s. Early analysis of this strategy showed a better cost–benefit ratio when compared with a test-and-cull strategy (Juste and Casal, 1993). Initially, the Spanish Ministry of Agriculture provided free vaccine for replacer lambs, but farmers have had to pay for the vaccine and its administration since the 1990s. Vaccination is widely used; e.g. in the most severely affected region, Castilla-Leon, vaccination has become a standard practice for replacer lamb rearing.

In general, the same criteria applied for sheep are used for goats, but in some regions the use of vaccination is restricted because of its interference with the single intradermal test for tuberculosis (R.A. Juste and J.M. Garrido, Bilbao, 2009, personal communication).

In the Netherlands, an experimental vaccination programme operated from 1984 until 2000. Dairy herds with at least 5% clinical cases were selected for this study. In two vaccinated herds, *MAP* was not demonstrated by faecal culture (Muskens *et al.*, 2002). However, even though the number of clinically, as well as subclinically, affected animals was rapidly reduced after vaccination, the number of infected animals could not be shown to be reduced (Wentink *et al.*, 1994). Despite the fact that an economic analysis of the effect of vaccination was shown to be highly profitable (van Schaik *et al.*, 1996), the

vaccination trial was halted because of its interference with the tuberculin intradermal test as well as the gamma-interferon assay (Muskens *et al.*, 2002). Remarkably, within a few years of the vaccination trial ending, serious problems with clinical paratuberculosis and loss of profitability were reported in goat dairy farms (D.P. Dercksen, Deventer, 2004, personal communication). Apparently, the 'off-label' use of the cattle vaccine in goat herds had been quite significant during the vaccination trial, and the vaccine had been quite effective in the control of paratuberculosis. Given the urgency of the problem and the fact that bovine tuberculosis is not endemic in those herds, the Gudair vaccine was exempted from registration and vaccination of dairy goats has since then become accepted practice. The Dutch Ministry of Agriculture, Nature and Food Quality granted the request for exemption so quickly that no follow-up studies on the efficacy of the vaccination could be initiated in time. However, given the fact that the complaints by the herd owners have subsided, vaccination has again demonstrated its effectiveness.

26.5 The Use of Test and Cull to Control Paratuberculosis

Control schemes for paratuberculosis based on a test-and-cull approach depend on the availability of suitable diagnostic assays to detect infected animals as early as possible, preferably before transmission of *MAP* to other non-infected animals. However, due to the slow pathogenesis of paratuberculosis, a measurable response to many existing tests is most reliable during the later stages of infection (see Whittington, Bölske and Herthnek, and Nielsen, Chapters 22–24, this volume). A critical review of the literature on ante-mortem diagnostic tests available for the detection of *MAP* infection in livestock (Nielsen and Toft, 2008) showed that the sensitivity of most tests is lowest in the infected but not yet infectious or affected animals. As a consequence, multiple rounds of testing over longer periods are required to acquire reliable information on the herd status. In addition, the average herd size in Europe is relatively small, further affecting the reliability of the surveys (Groenendaal *et al.*, 2002). In Norway, combining the small herd sizes with the assumed low prevalence precludes the use of serology for a reliable national prevalence study (Tharaldsen *et al.*, 2003).

An excellent illustration of problems that can be encountered during herd certification for freedom from paratuberculosis is the study by Kalis *et al.* (2004) of closed dairy herds with no history of paratuberculosis. These herds were selected for certification using a pooled faecal culture method on animals 2 years of age and older and at 6-month intervals; for a schematic presentation of the programme see Fig. 26.1. Herds with one or more culture-positive pools were regarded as infected. Starting with 90 qualifying non-suspected herds, only 35 remained culture-negative after nine rounds of testing; thus 61% of the non-suspected herds were found to be *MAP* infected. The majority of the positive herds were detected in the first rounds of testing and most of them had only one positive pool. This confirms observations made during the first Dutch national prevalence study, using an absorbed ELISA, where 73.5% of positive herds had only one or two reactors, indicating a low within-herd prevalence in infected herds (Muskens *et al.*, 2000). In addition, this study illustrates why repeated rounds of diagnostic testing over a long period are required to obtain a reliable indication of the infection status of a herd. The most important result of this study is the observation that in a group of closed dairy herds where *MAP* infection was unsuspected the majority were found to be positive. Such a result might discourage farmers from joining such a programme on a voluntary basis and raises the question of whether such programmes are viable, unless a low herd prevalence has been confirmed in advance.

Lessons learned from this study (Kalis *et al.*, 2004) were applied to the Dutch Paratuberculosis Program, launched in 1998. The aim of the programme was to achieve a high level of assurance for the participating herds to be free of paratuberculosis and was supported financially by the Department of Agriculture, dairy industry and farmers (Benedictus *et al.*, 2000). A schematic presentation of the

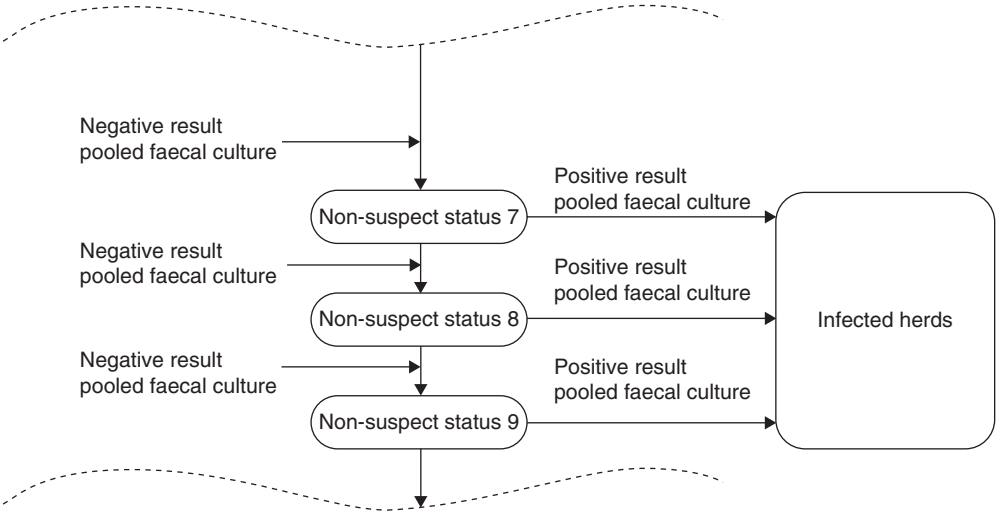

Fig. 26.1. Schematic representation of the experimental control scheme based on pooled faecal culture (Kalis *et al.*, 2004).

programme is shown in Fig. 26.2. As with the earlier study, participation was restricted to closed herds with no history of paratuberculosis and no signs of infection. As a first step, a negative absorbed ELISA result of all animals older than 2 years was required to qualify for Level 6. Following this, negative results of a pooled faecal culture of five faecal samples at 12-month intervals over a period of 4 years enabled a herd to attain Level 10. An important difference from the study by Kalis *et al.* (2004) was that positive faecal pools were investigated further by individual culturing. When the result of these individual cultures was negative, the herd was allowed to return to the programme, despite the positive result of the pooled culture. However, if one or more of the individual samples was culture-positive, the herd owner removed the positive animal(s) for slaughter within 30 days, in order to return to the programme. As a result of this policy, herds that acquired the Level 10 status cannot be guaranteed to be paratuberculosis-free; however, the level of infection is clearly very low. In follow-up, surveillance of Level 10 herds using pooled faecal culture results in occasional positive findings. Importantly, herd owners that acquired this level were satisfied with this approach and have been willing to maintain this level, at their own cost, after the subsidized programme ended (G. Jensma, Lelystad, 2007, personal communication). The above illustrates not only the dilemmas encountered during a certification programme but also the need to develop programmes to assist herds found to be infected to regain their *MAP*-free status, such that participation in a voluntary programme does not lead to punitive consequences.

26.6 Current Control Schemes and New Initiatives

In recent years, a number of new control schemes have been developed in different regions and countries across Europe, in response to an increasing concern with regard to the paratuberculosis situation. In addition to the programmes already in place in the Nordic countries, e.g. Norway (Tharaldsen *et al.*, 2003), new programmes have been developed and new initiatives are being considered. Due to the fact that several initiatives are locally organized and not widely published, a non-exhaustive listing is shown below.

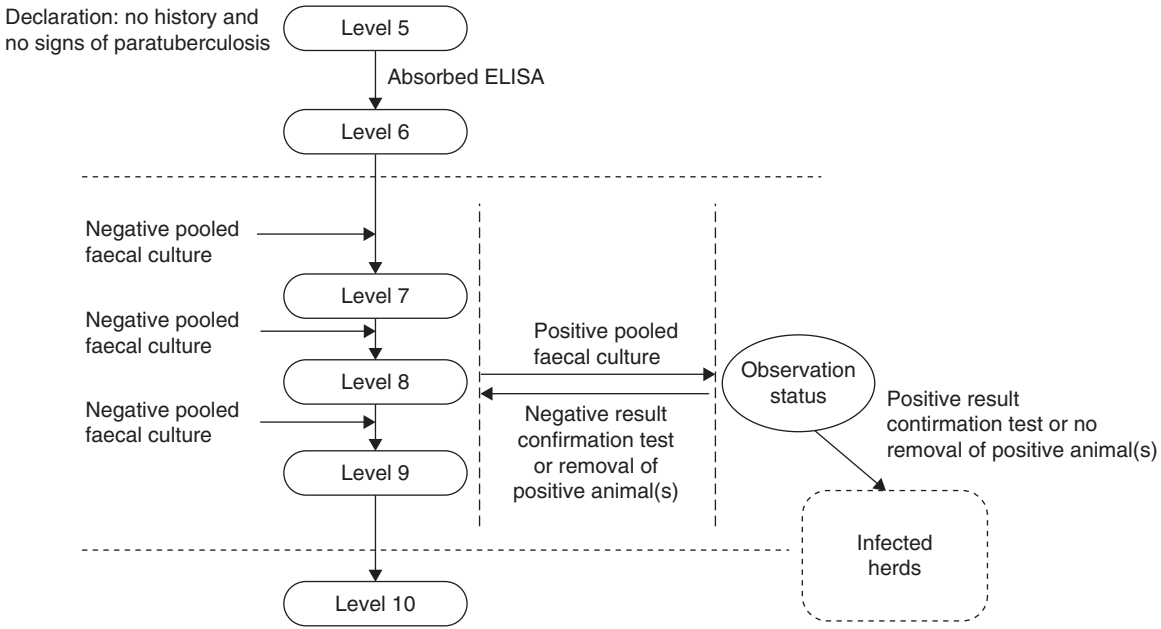

Fig. 26.2. Schematic representation of the Dutch paratuberculosis control scheme, including the observation status (Benedictus *et al.*, 2000).

26.6.1 The Netherlands

In January 2006, a new programme for paratuberculosis control was launched. Its aim is not the certification of herds for freedom from paratuberculosis. Instead, its focus is on milk quality and it aims to reduce the amount of *MAP* present in milk entering factories. This bulk milk quality-assurance programme is carried out by the Dutch Animal Health Service and is based on testing milk samples of all lactating cows or serum samples of all cattle more than 3 years of age, at 24-month intervals by ELISA. At the request of the herd owner, positive ELISA results can be confirmed by individual faecal culture (Weber and van Schaik, 2009). This latter approach has the inherent danger of a low overall sensitivity of the combined diagnostic tests, potentially resulting in significant misclassification of animals that are infected but not yet high shedders of *MAP*. These animals will then remain in the herd for another 2 years (Muskens *et al.*, 2003).

Herds with no positive results are assigned 'Status A'; herds in the control procedure from which the test-positive cattle have been removed are assigned 'Status B'; and herds with test-positive cattle remaining in the herd are assigned 'Status C' (Weber and van Schaik, 2009). The programme received a boost in January 2008, when the Dutch Dairy Board started paying for the first round of testing for all Dutch dairy herds. This resulted in >80% participation of Dutch dairy herds in the programme. The ultimate goal is mandatory participation after 2011, restricting delivery of milk to the factory to herds of Status A or B only.

26.6.2 Denmark

In Denmark, a voluntary control programme was started in 2006, aimed at providing tools for farmers to control infection with *MAP* and ultimately at reducing prevalence in the country towards a long-term goal of eradication. The initiative for this programme stems from the Danish Cattle Federation, a combination of stakeholders in the cattle industry. Approximately 85% of the 4403 dairy herds are estimated to be infected and many farmers are experiencing economic losses. Paratuberculosis infection status is established using a milk ELISA every 3 months, but, in contrast to other programmes, no status or infection levels are assigned to participating farmers and the farmers are informed that the programme will last 6–8 years. A crucial part of the programme is the training of local health advisers to assist farmers in drafting an action plan for their herd: e.g. establish aims for the within-herd control, provide a risk assessment and give interpretation of test results. Since the removal of test-positive animals is on a voluntary basis, the results of subsequent diagnostic tests will be used in combination with milk production data to persuade the farmer to remove the animal. This approach is strongly supported by the initial data, which show an economic benefit to herds following this advice (S.S. Nielsen, Copenhagen, 2008, personal communication). Even though all costs for the frequent testing and herd advisory services have to be paid by the farmer, the level of participation has increased since the start of the programme; in early 2009, approximately 29% of the herds, representing 40% of the Danish dairy cattle population, were participating. There are multiple reasons for farmers to participate, including benefits to animal health, production losses already experienced or to be prevented, and certification for the sale of live animals (S.S. Nielsen, Copenhagen, 2009, personal communication).

26.6.3 Belgium

In Belgium, the dairy industry, in close collaboration with the farmer organizations, started a voluntary control programme for paratuberculosis in 2008. The initiation of the programme was motivated by animal health and welfare, and economic reasons (export protection), as well as the application of the precautionary principle because of possible human health concerns. The programme is based on annual testing of all animals more than 30 months old, using the milk ELISA for dairy cattle and the serum ELISA for dry cattle. Positive animals are to be removed for

slaughter within 6 months of a positive finding. Testing is carried out by the Animal Health Service and paid for by the farmer. However, the costs are in part refunded by the National Animal Health Foundation (Sanitair Fonds) (M. Govaerts, Brussels, 2009, personal communication).

26.6.4 Luxembourg

Luxembourg recently developed a new control programme for paratuberculosis, with assignment of different levels (status) based on absorbed ELISA test results of animals older than 24 months. The status categories are assigned according to the percentage of test-positive animals in a herd:

- Status A, 0% ELISA-positive animals
- Status B, <5% ELISA-positive animals
- Status C, 5–10% ELISA-positive animals
- Status D, >10% ELISA-positive animals
- Status O, no sampling or no slaughter of positive animals.

Following the assignment of the herd status, management recommendations are made to the farmer according to the herd status. These include keeping a closed herd (Status A), applying sanitary measures (Status B), being obliged to apply sanitary measures (Status C and D), and restricting animal movement to the abattoir or fattening farms only (Status O). In addition, recommendations are being made for the introduction of animals into a herd while maintaining the original status (C. Dahm, Luxembourg, 2009, personal communication).

26.6.5 France

In France, Groupements de Défense Sanitaire (Sanitary Defence Groups) have now harmonized activities under a National Federation. Since 2004, different local measures are now unified into two national programmes: the control of paratuberculosis in infected holdings and a monitoring programme of negative herds.

In addition to management changes aimed at reducing the transmission of *MAP* within the herd, different testing regimes have been developed for the control of paratuberculosis in infected holdings: for herds with one recently purchased infected animal and for herds in which paratuberculosis is endemic. ELISA, faecal culture or PCR can be used, although the latter two are recommended for herds in which paratuberculosis is endemic. Herds are allowed to leave the programme provided that: (i) the herd has shown no clinical signs in the last 3 years; (ii) all tests have been negative for 2 years; (iii) no animal with a positive test has been sold in the past 2 years; and (iv) no previously positive animals remain in the herd (Petit, 2006).

The monitoring and certification programme for negative herds requires participating herds to perform two series of testing, using ELISA, faecal culture or PCR, on all animals older than 24 months at an interval between 9 and 30 months. In order to maintain the negative status, all animals between 24 and 72 months of age should be tested at intervals of 21–27 months. Cattle from other qualified herds can be purchased without specific requirements (Petit, 2006).

26.6.6 Germany

In the Federal Republic of Germany, there is currently no national paratuberculosis control scheme. However, the guidelines or 'Leitlinien' for control of paratuberculosis issued by the federal government (Anonymous, 2005) form the basis for the voluntary control programmes initiated in an increasing number of federal states or 'Bundesländer', e.g. in Brandenburg, Lower Saxony, North Rhine–Westphalia, Saarland and Thuringia.

In Thuringia, for example, a voluntary programme has been initiated by the local veterinarians with the support of Animal Health Insurance and the Animal Health Service. The focus is on the identification of infected herds and removal of shedders in infected herds, complemented by hygienic measures. The identification of infected animals is based on the absorbed ELISA on animals older than 24 months, and confirmed by faecal culture, PCR or histology. Negative herds are only allowed

to purchase animals from other non-suspected herds. The costs for the control measures are paid for by the farmer but are in part refunded by the Animal Health Insurance (H. Koehler, Jena, 2009, personal communication).

26.6.7 Austria

Austria has a compulsory control programme for clinical paratuberculosis, which came into force in 2006. The programme includes cattle, sheep, goats and farmed deer. The programme is based on a surveillance of slaughter animals and dead animals and on regular clinical inspections of the herds by state veterinarians. Samples of suspected animals are tested by the national reference laboratory for confirmation, and clinically ill, *MAP*-positive animals have to be culled within 3 days. In addition, the use of milk from clinical cases is prohibited. For the herd of origin, a whole-herd test (using serology or PCR) and hygienic measures are recommended. Compensation for culled animals, depending on age and value of the animal, is paid by the government (Khol *et al.*, 2009).

26.6.8 Spain

There is no official nationwide programme for paratuberculosis control in Spain, but in some autonomous communities there are specific schedules based on voluntary testing and culling within the frame of the so-called Animal Health Protection Groups (Asociacion de Defensa Sanitaria). These are local associations of farmers that agree to comply with certain regulations in order to protect their livestock from infectious diseases. Farmers are advised to cull positive animals, but no indemnity is given and therefore culling depends on the farmer's decision.

After approval by the Spanish Veterinary Drug Agency and the Ministry of Agriculture, an experimental paratuberculosis control programme for cattle was launched in the Basque country, based on vaccination using the Silirum vaccine. Currently, there are six dairy and two beef herds in the vaccination programme, and all controlled variables indicate beneficial effects. No adverse effects of the vaccine have been observed and there is an increasing number of farmers that want to enrol in the programme (R. Juste, Bilbao, 2009, personal communication).

26.6.9 Italy

In Italy, there is currently no nationwide control scheme for paratuberculosis. The Italian reference laboratory provides guidelines for voluntary paratuberculosis control schemes in dairy and beef cattle. Farmers and veterinarians are leading training and education programmes to implement management changes and introduce voluntary control measures. Based on the experiences from experimental programmes, e.g. in the Veneto region (Pozzato *et al.*, 2009), new control programmes are being developed in several regions of the country. In two northern provinces, Milano and Lodi, certification programmes based on the use of the absorbed ELISA resulted in progressive levels of certification. This approach has now been approved for the Veneto region, where a new programme will start in 2010 (N. Arrigoni, Podenzano, 2009, personal communication).

26.7 Concluding Thoughts

For reasons of animal health and welfare as well as for potential zoonotic aspects of the disease, paratuberculosis control in Europe is currently characterized by a new series of initiatives. In general, vaccination is used for small ruminants, where it contributes to control but not eradication of paratuberculosis (Saxegaard and Fodstad, 1985). Although experimental use of cattle vaccination has been effective in controlling paratuberculosis (Muskens *et al.*, 2002) as well as profitable (van Schaik *et al.*, 1996), interference with the tuberculin intradermal test for bovine tuberculosis restricts its use in cattle. For beef herds, where the options for the introduction of management changes such as the rapid separation of cow and calf after calving are limited, the use of vaccination might be one of the few viable

options. However, control in cattle through vaccination will require an effective vaccine that does not interfere with the diagnosis of bovine tuberculosis.

In addition to the use of vaccination, numerous new schemes have been developed for the control of paratuberculosis in cattle, as shown in the previous section. Except for the Nordic countries, where strict control measures are in place to ensure a low prevalence of infection, most control schemes are on a voluntary basis. Combined with the fact that the initiatives are locally organized and the approaches and funding depend on the local situation, this has resulted in a wide variety of control schemes.

A common factor in all approaches is acknowledgement of the need to introduce management changes to reduce transmission and the need to warn participating herd owners that controlling paratuberculosis will take considerable time and effort. Since programmes have to be voluntary and are, in most schemes, paid by the farmer, costs have to be reduced. For example, a gradual shift from faecal culture to absorbed ELISA on milk helps to make testing affordable and thus more attractive for participating farmers. As demonstrated by studies (Muskens *et al.*, 2000; Kalis *et al.*, 2004), a large proportion of the infected herds are low-prevalence herds, complicating the certification for freedom from infection in the face of tests with low sensitivity. New test strategies might have to be developed, in particular for smaller herds, to counter this problem (Sergeant *et al.*, 2008).

Despite a large number of similarities between Dutch and Danish farming and a similar history in their respective disease control programmes, the current control schemes have evolved quite differently, due to the different goals that have been set by the stakeholders. In the Danish approach, the milk ELISA is used at a high frequency to achieve a maximum sensitivity, with the ultimate goal of eradication. The Dutch approach, also based on the milk ELISA, tests at a lower frequency, with the option to investigate positive results by faecal culture. This approach may result in a lower overall sensitivity, but the goal is different. This scheme aims at disease control rather than eradication, by removing the highest shedders of *MAP*. Moreover, when using subtly different programmes in different jurisdictions, it is important that farmers are aware of these distinctions, as the setting of realistic expectations is a critical prerequisite for long-term cooperation in such ventures.

One of the opportunities afforded by the many European programmes is the comparison of these 'natural experiments' in paratuberculosis control. As both the Dutch and Danish programmes are closely monitored, it will be interesting to determine the success of these different strategies in achieving their stated goals. Conversely, the introduction of control schemes in different European countries, in the apparent absence of coordination, may at times complicate comparisons, both of the effectiveness of programmes and, at a more basic level, in the terminology of certification levels. Ironically, increased participation in the respective programmes could complicate free trade, one of the main goals of the EU. Combined with the fact that the present recommended diagnostic tests are still based on the inadequate testing of individual animals and not on whole-herd testing (Anonymous, 2004), the development of a common theme in the control schemes, e.g. at least one annual whole-herd ELISA test, has the potential to improve this situation considerably.

References

Anonymous (2004) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paratuberculosis (Johne's disease). Available at http://www.oie.int/eng/normes/mmanual/a_00045.htm (accessed 11 June 2009).

Anonymous (2005) Leitlinien für den Umgang mit Paratuberkulose in Wiederkäuerbeständen. Bekanntmachungen Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft. Available at http://www.animal-health-online.de/drms/rinder/paraleit.pdf (accessed 11 June 2009).

Bakker, D., Willemsen, P.T. and van Zijderveld, F.G. (2000) Paratuberculosis recognized as a problem at last: a review. *Veterinary Quarterly* 22, 200–204.

Bang, B. (1906) Chronische pseudotuberkuloese Darmentzuendung beim Rinde. *Berliner Tierarztliche Wochenschrift* 42, 759–763.

Bang, O. and Andersen, C.W. (1913) Einige Untersuchungen über komplement-bindende Antistoffe bei experimenteller und spontaner Tuberkulose sowie paratuberkulöser Darmentzündung des Rindes *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abteilung Originale A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie* 69, 517–538.

Benedictus, G., Dijkhuizen, A.A. and Stelwagen, J. (1987) Economic losses due to paratuberculosis in dairy cattle. *The Veterinary Record* 121, 142–146.

Benedictus, G., Verhoeff, J., Schukken, Y.H. and Hesselink, J.W. (2000) Dutch paratuberculosis programme history, principles and development. *Veterinary Microbiology* 77, 399–413.

Buergelt, C.D. and Duncan, J.R. (1978) Age and milk production data of cattle culled from a dairy herd with paratuberculosis. *Journal of the American Veterinary Medical Association* 173, 478–480.

Cartwright, W.A. (1829) Diarrhæa in a cow. *The Veterinarian* 2, 71–72.

Djønne, B. (2003) Paratuberculosis in goats – a special focus on the Nordic countries. *Acta Veterinaria Scandinavia* 44, 257–259.

European Commission (2000) White paper on Food Safety. Available at http://ec.europa.eu/dgs/health_consumer/library/press/press37_en.html (accessed 11 June 2009).

Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S. and Gudmundsdottir, K.B. (2000) Paratuberculosis in Iceland: epidemiology and control measures, past and present. *Veterinary Microbiology* 77, 263–267.

Goudzwaard, J. (1971) Johne's disease in goats. PhD thesis, Faculty of Veterinary Medicine, Utrecht, the Netherlands.

Groenendaal, H., Nielen, M., Jalvingh, A.W., Horst, S.H., Galligan, D.T. and Hesselink, J.W. (2002) A simulation of Johne's disease control. *Preventive Veterinary Medicine* 54, 225–245.

Johne, H. A. and Frothingham, L. (1895) Ein eigenthümlicher Fall von Tuberculose beim Rind. *Deutsche Zeitschrift für Tiermedizin und vergleichende Pathologie* 21, 438–455.

Juste, R.A. and Casal, J. (1993) A simulation of different strategies for control of ovine paratuberculosis. *Preventive Veterinary Medicine* 15, 101–115.

Kalis, C.H., Collins, M.T., Barkema, H.W. and Hesselink, J.W. (2004) Certification of herds as free of *Mycobacterium paratuberculosis* infection: actual pooled faecal results versus certification model predictions. *Preventive Veterinary Medicine* 65, 189–204.

Kennedy, D.J. and Benedictus, G. (2001) Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species. *Revue Scientifique et Technique* 20, 151–179.

Khol, J.L., Duenser, M., Damoser, J. and Baumgartner, W. (2009) A new approach in the compulsory fight against paratuberculosis – eradication of clinical cases. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 297–299.

Kudahl, A.B., Nielsen, S.S. and Østergaard, S. (2008) Economy, efficacy, and feasibility of a risk-based control program against paratuberculosis. *Journal of Dairy Science* 91, 4599–4609.

Lowe, A.M., Yansouni, C.P. and Behr, M.A. (2008) Causality and gastrointestinal infections: Koch, Hill, and Crohn's. *Lancet Infectious Diseases* 8, 720–726.

M'Fadyean, J. (1907) Johne's disease: a chronic bacterial enteritis in cattle. *Journal of Comparative Pathology and Therapeutics* 20, 48–60.

Muskens, J., Barkema, H.W., Russchen, E., van Maanen, K., Schukken, Y.H. and Bakker, D. (2000) Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. *Veterinary Microbiology* 77, 253–261.

Muskens, J., van Zijderveld, F., Eger, A. and Bakker, D. (2002) Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. *Veterinary Microbiology* 86, 269–278.

Muskens, J., Mars, M.H., Elbers, A.R., Van Maanen, K. and Bakker, D. (2003) The results of using faecal culture as confirmation test of paratuberculosis-seropositive dairy cattle. *Journal of Veterinary Medicine Series B* 50, 231–234.

Nielsen, S.S. and Toft, N. (2008) Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. *Veterinary Microbiology* 129, 217–235.

Nielsen, S.S. and Toft, N. (2009) A review of prevalences of paratuberculosis in farmed animals in Europe. *Preventive Veterinary Medicine* 88, 1–14.

Petit, H. (2006) Approaches to farm-level control of paratuberculosis in France. In: *Proceedings of the Cattle Consultancy Days 2006*. Nyborg, Denmark, pp. 146–152.

Pozzato, N., Stefani, E., Bottazzari, M., Benini, N., Cestaro, F., Passarini, G. and Vicenzoni, G. (2009) Experimental control program for paratuberculosis in dairy cattle in the Veneto region. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*.

International Association for Paratuberculosis, Madison, Wisconsin, pp. 315–316.

Saxegaard, F. and Fodstad, F.H. (1985) Control of paratuberculosis (Johne's disease) in goats by vaccination. *The Veterinary Record* 116, 439–441.

Sergeant, E.S., Nielsen, S.S. and Toft, N. (2008) Evaluation of test-strategies for estimating probability of low prevalence of paratuberculosis in Danish dairy herds. *Preventive Veterinary Medicine* 85, 92–106.

Seuna, E. and Seppänen, J. (2003) National control strategies and ongoing research in Finland. *Acta Veterinaria Scandinavica* 44, 251–253.

Sigurdsson, B. (1952) Vaccination against paratuberculosis (Johne's disease). *Journal of Immunology* 68, 559–565.

Sternberg, S. and Viske, D. (2003) Control strategies for paratuberculosis in Sweden. *Acta Veterinaria Scandinavica* 44, 247–249.

Tharaldsen, J., Djønne, B., Fredriksen, B., Nyberg, O. and Siguroardóttir, O. (2003) The National Paratuberculosis Program in Norway. *Acta Veterinaria Scandinavia* 44, 243–246.

Twort, C.C. (1912) The agglutination and complement fixation reactions in animals experimentally inoculated with Johne's bacillus, with special reference to the relation of this bacillus to other acid-fast bacilli. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abteilung Originale A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie* 66, 316–318.

Twort, F.W. and Ingram, G.L.Y. (1912) A method for isolating and cultivating the *Mycobacterium enteritidis chronicae pseudotuberculosae bovis johne* and some experiments on the preparation of a diagnostic vaccine for pseudotuberculous enteritis of bovines. *Proceedings of the Royal Society of London* 84, 517–543.

Vallee, H. and Rinjard, P. (1926) Etudes sur l'entérite paratuberculeuse des bovides (note préliminaire). *Revue Générale de Médécine Vétérinaire* 35, 1–9.

van Schaik, G., Kalis, C.H., Benedictus, G., Dijkhuizen, A.A. and Huirne, R.B. (1996) Cost–benefit analysis of vaccination against paratuberculosis in dairy cattle. *The Veterinary Record* 139, 624–627.

Viske, D., Larsson, B., Engvall, A. and Bölske, G. (1996) Paratuberculosis in Sweden. In: Chiodini, R.J., Hines, II, M.E. and Collins, M.T. (eds) *Proceedings of the Fifth International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 143–146.

Weber, M.F. and van Schaik, G. (2009) Results of the Dutch bulk milk quality assurance programme for paratuberculosis. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 324–327.

Wentink, G.H., Bongers, J.H., Zeeuwen, A.A. and Jaartsveld, F.H. (1994) Incidence of paratuberculosis after vaccination against *M. paratuberculosis* in two infected dairy herds. *Journal of Veterinary Medicine* B 41, 517–522.

27 Paratuberculosis Control Measures in the USA

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27.1 Introduction

Johne's disease (JD) has been recognized in the USA since its first description in North America by Pearson (1908). Research to better understand the incubation period, methods of transmission and economic aspects progressed gradually over the years. Cornell University conducted relatively intensive investigations during the 1930s (Hagan and Zeissig, 1933; Hagan, 1938), and later there were ongoing research efforts at the National Animal Disease Laboratory in Ames, Iowa, for several decades after 1960 (Whipple and Merkal 1983; Chiodini *et al.*, 1984; Merkal *et al.*, 1987).

From a national perspective, during the 1980s, the focus of JD management was at the state level, with some states developing their own control programmes, often focused on quarantining infected herds (Chiodini *et al.*, 1984). States such as Wisconsin, Iowa, Indiana and Pennsylvania began implementing vaccination to control JD in heavily infected herds. Pennsylvania was one of the only states to implement an indemnity programme designed to provide partial financial compensation for farmers for sending culture-positive cattle to slaughter. At peak activity of this programme, the state was paying more than $150,000 per year to farmers, in addition to providing laboratory testing at no cost (Whitlock *et al.*, 1994).

Despite these efforts, over time, the prevalence of JD in the USA appears to be gradually increasing. The reasons for this increase are incompletely understood but may relate to an increase in herd size, the purchase of infected herd replacements and ineffective control programmes. The goal of this chapter will be to describe the state of JD control in the USA and outline priorities for future research.

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27.2 First National US JD Programme

During the early 1990s a national JD task force of state and federal government, extension personnel and university researchers, and animal agriculture organizations collaborated under the auspices of the Johne's Committee of the United States Animal Health Association to develop the first national JD certification programme for cattle (Whipple, 1993). Briefly, a comprehensive programme was outlined that developed criteria for cattle herds to be certified at low risk for JD. Following negative results of testing of adult cattle in the herd by either enzyme-linked immunosorbent assay (ELISA) or faecal culture, the herd was granted a test-negative status. Sequential annual testing with negative results gave a higher probability of being at low risk for JD. The testing had to be completed in laboratories that participated and passed annual ELISA and faecal culture check tests conducted by the National Veterinary Services Laboratory in Ames, Iowa. Although the certification programme was constructed for both beef and dairy herds, the greatest interest and participation in the programme was from dairy herds. Prior to development of the first national JD certification programme, only four states had their own state-level certification programme: New York, Maryland, Wisconsin and Pennsylvania, while ten other states were actively considering a JD certification programme. Eleven states had their own control programmes (Whitlock, 1993).

The JD certification programme expanded slowly over the next few years as more states became involved, but enhanced value of sale of cattle from herds enrolled in the certification programme was never fully realized. The demand for replacement dairy cattle to expand herd size proved to be a major factor outweighing producer concerns regarding JD infection. The National Animal Health Monitoring Study (1997) reported that 55% of US dairy farmers were fairly knowledgeable about JD, while 35% did not consider themselves knowledgeable and another 10% had not heard of the disease. This lack of knowledge about JD resulted in an extensive educational effort by the educational subcommittee of the National Johne's Working Group (NJWG) (Whitlock *et al.*, 2000).

Although an intense national educational effort was implemented by the NJWG to enhance cattle producers' and veterinarians' knowledge about JD, market forces prevailed, especially with large herd expansions (herds >500 dairy cattle). The first national Johne's certification programme was based on alternate-year testing by either ELISA or faecal culture and replaced several earlier state programmes based entirely on annual faecal culture. Although the new certification programme was scientifically sound, it attracted less than an estimated 600 herds in total to participate from all states over a period of 4 years. Consequently, in 1997, the NJWG appointed a committee to develop a more affordable and flexible, yet scientifically sound, Johne's herd certification programme. The committee was charged with developing a voluntary programme, with flexibility for the owners to remain at any level, and costing less than the 1993 certification programme.

The NJWG developed a new Johne's programme, called the 'United States Voluntary JD Herd Status Program for Cattle' (VJDHSP) (Bulaga, 1998). This programme is based on herd-level diagnostics, not individual animal diagnostic criteria, and now serves as the foundation programme for JD in the USA. The VJDHSP programme is focused entirely on test-negative herds, with no consideration given for infected herds. To assist states, herd veterinarians and producers dealing with JD, the NJWG developed a follow-up programme for infected herds. This document, entitled 'Minimum Recommendations for Administering and Instituting State Voluntary JD Programs for Cattle' is found in the 1999 Johne's Committee report (Whitlock and Rossiter, 1999). Uniform Program Standards for the Voluntary Bovine JD Control Program are available at a website of the US Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS).

27.3 Current JD Programme in the USA

The current Voluntary National JD Control Program has three major elements: (i) education; (ii) management; and (iii) herd testing and classification. Education about JD takes many forms, including group workshops, producers discussing JD with the herd veterinarians, lay publications about JD and via the World Wide Web. An excellent website for producers and veterinarians, called the Johne's Information Center, is maintained at the University of Wisconsin. This multifaceted educational effort includes 20 PowerPoint slide presentations for both lay and professional agricultural staff, including veterinarians. Much other information and educational material on JD are available at the Johne's Information Central website, a cooperative effort of the National Institute for Animal Agriculture and USDA-APHIS Veterinary Services, in association with the National Johne's Working Group and US Animal Health Association.

The management component is accomplished with a herd risk assessment completed by a veterinarian trained and certified to do Johne's risk assessments, followed up with a herd management plan. Risk assessment scores are based on visual assessment of each environment and owner responses. The risk for faecal–oral and colostrum disease spread or gaps in a farm's biosecurity are estimated for each management practice. The assessment ranges from calf risks to adult cow risks, with a range of possible scores (Figures 27.1– 27.6). Veterinarians consider the impact of JD prevalence on the ability to reduce risks and estimate whether the risk is very low, low, moderate, high or very high. The total score for each type of animal decreases as the proportion of risk of transmitting Johne's disease diminishes. A total score for the final assessment (Figure 27.7) is determined by summing the scores from Figures 27.1–27.6. The herd risk assessment and management plan are the key elements of the current US Johne's control programme. The risk assessment is done only by veterinarians trained by state and federal officials approved by the designated state coordinator. Once certified to perform risk assessment and design a management plan, veterinarians must obtain additional training every 3 years for new information about JD.

Once the risk assessment is completed, the same certified veterinarians prepare the herd management plan, which outlines management and biosecurity changes that will reduce the transmission of JD and the prevalence of JD on that farm. The herd management plan is not mandatory for the producer. Laboratory testing is voluntary and may be subsidized by state and/or federal funds. The exact type of testing is entirely at the discretion of the veterinarian and farmer. However, if the testing is

Risk factors (Place an X in the box to the right of the management practice that most closely signifies the risk for that item.)	0	1 V. Low	2 Low	3	4	5 Moderate	6	7	8 High	9	10 V. High
1. Multiple animal use (single pen → dense crowded group)											
2. Manure build-up risk for calf ingestion (clean dry → dirty, wet)											
3. Area also used for sick cows (never → always)											
4. Presence of JD clinical animals / suspects (never → always)											
5. Manure-soiled udders / legs (never → always)											
6. Calves born in other areas (never → always)											
7. Time calves stay with dam (<30 minutes → >24 hours)											
8. Calves nurse dam (never → most or all)											

Fig. 27.1. Calving area risk factors.

Risk factors	0	1 V. Low	2 Low	3	4	5 Mod.	6	7	8 High	9	10 V. High
1. Fed pooled colostrum (never or JD negative → high-risk cows)											
2. Fed colostrum from individual cow to several calves (as 1. above)											
3. Fed unpasteurized pooled milk (JD negative → high risk)											
4. Possible manure contamination of colostrum or milk: at harvest, or by utensils, traffic or people (none any source → frequent many sources)											
5. Possible manure contamination of calf feed or water by cows, traffic splatter, equipment or people (as 4. above)											
6. Direct cow contact or potential manure contamination of calf pen by cows, traffic splatter, equipment or people (as 4. above)											

Fig. 27.2. Pre-weaned heifer risk factors.

Risk factors	0	1 V. Low	2	3	4 Mod.	5	6	7 V. High
1. Direct contact or pen contamination with cows' manure (none → always)								
2. Possible manure contamination of feed: refused cow ration, stored feed, equipment, cows, traffic splatter, people or runoff (never → frequently)								
3. Potential for contamination of water: shared with cows, traffic splatter, runoff or people (never → frequently)								
4. Share pasture with cows (never → frequently)								
5. Manure spread on forage grazed / harvested same season (never → frequently)								

Fig. 27.3. Post-weaned heifer risk factors.

Risk factors	0	1 V. low	2	3 Mod.	4	5 V. high
1. Direct contact or contamination with cows' manure (none → always)						
2. Possible manure contamination of feed: stored feed, equipment, cows, traffic splatter, people or runoff (never → frequently)						
3. Potential for contamination of water sources: shared with cows or by cows, traffic splatter, runoff or people (never → frequently)						
4. Share pasture with cows (never → frequently)						
5. Manure spread on forage and fed same season (never → frequently)						

Fig. 27.4. Bred heifer and yearling bull risk factors.

Risk factors	0	1 Low	2	3	4 High
1. Possible manure contamination of feed: when fed or stored, by equipment, traffic splatter, runoff or people (never → frequently)					
2. Possible manure contamination of water: by cows, traffic splatter, people or runoff (never → frequently)					
3. Direct access to accumulated or stored manure (never → frequently)					
4. Manure spread on forage grazed / harvested same season (never → frequently)					

Fig. 27.5. Cow and bull risk factors.

Additions and replacements	Number of Animals				
	1–5	6–12	13–20	21–50	>50
1. Get additions or replacements from level 2-4 status herd	0	2	4	6	8
2. From low-risk herds, level 1 or pre-tested herds	10	11	12	13	14
3. From single-source non-tested or non-programme herds	20	22	23	26	28
4. From multiple sources, non-tested or non-programme herds or markets	30	34	36	38	40

Fig. 27.6. Risk factors from additions and replacements.

Risk factor area	Maximum score	Herd score	Each Area Herd Score / Each Area Max Score (%)	Each Area Herd Score / Your Total Herd Score (%)
Calving	80			
Pre-weaned heifers	60			
Post-weaned heifers	35			
Bred heifers	25			
Cows and bulls	16			
Additions and replacements	60			
Total	276			

Fig. 27.7. Total risk factor for final assessment.

federally supported it must be performed in a USDA-approved laboratory.

If the herd has JD, then it is placed in the management tract; testing proceeds as best fits the farm. If the herd does not have JD, then the farmer may elect to enter the test-negative or status programme. The status programme has four levels (1 to 4), each with increasingly lower risk of JD present in the herd. The test-negative component must include a herd management plan based on the requirements in the management element. Each higher level represents a greater probability that the herd is free of JD. However, this does not certify that a herd is free of JD. Herds in the test-negative component may remain at any given level by doing monitoring testing or may advance to a higher status with additional testing. A website is available describing 'How to Do Risk Assessments and Management Plans for Johne's Disease'.

27.4 Biosecurity Practices and Herd Management Plans

The key to preventing, controlling and eliminating paratuberculosis in a herd is implementation of a rigorous herd management plan designed to reduce exposure of young calves to *MAP* (Rossiter and Burhans, 1996). Factors such as finances, movement of cattle on the farm, maternity and sick cow pen locations, feed delivery to adult cattle, location and structure of feed bunks, and personnel issues are a few of the specific issues that are reviewed with a focus on how to best limit transmission of *MAP* to young calves. Farm managers are encouraged to adopt two fundamental control principles: (i) prevent highly susceptible newborn calves and young animals from ingesting manure from infected adults; and (ii) reduce total farm environmental contamination of *MAP* by culling infected animals shedding the highest concentrations of *MAP*. Calves should be separated from their dams at birth and fed single-source colostrum from culture-negative and/or ELISA-negative cows. The same management factors that reduce the risk for JD also reduce the risk of other faecal–oral diseases, such as those caused by salmonella, cryptosporidia, *Escherichia coli* and campylobacter (McKenna *et al.*, 2006).

Faecal culture testing of the whole herd, followed by aggressive culling of infected animals, is very effective in reducing the prevalence of paratuberculosis in the herd (Rossiter and Burhans, 1996). The risks of transmission of *MAP* within both dairy and beef herds have been compiled into three major documents, entitled 'How to Do Risk Assessments for Johne's Disease', a 'Handbook for Veterinarians and Dairy Producers' and a 'Handbook for Veterinarians and Beef Producers'. These documents are available at the website of Johne's Information Central.

In herds with low to moderate infection levels (≤1% clinical cases per year), wise use of a combination of testing, culling and biosecurity measures may eliminate clinical disease within 1–3 years and most infected adults in 5–7 years, as the adult cattle are culled over time. Complete elimination of infected cattle is likely to take many years after clinical Johne's disease is no longer apparent. Biosecurity measures should remain in place, or Johne's disease is likely to recur. Managers of herds at low risk for Johne's disease need to be reminded that a major risk factor for JD is the purchase of replacement cattle from herds of unknown Johne's status. As herd owners continue to expand herd size with the acquisition of purchased animals, JD is often introduced via the purchased cattle.

Herds with more severe, widespread infection require aggressive control programmes and many years to eliminate clinical JD. These herds should consider vaccination, as noted above. However, a practical control programme and sound herd management may eliminate clinical disease in these herds and reduce the economic impact of JD to a minimum. Feeding monensin to heifers and all adult cows should reduce the *MAP* bioburden on the farm (Whitlock *et al.*, 2005) and therefore may reduce transmission to young susceptible calves. Rather than focusing attention to detect all *MAP* shedders, the diagnostic efforts should concentrate on eliminating cattle shedding the highest concentration of *MAP*, i.e. *MAP* supershedders (Whitlock *et al.*, 2007).

27.5 Environmental and Pooled Faecal Samples

JD continues to be considered a herd disease, with most diagnostic efforts designed to detect infection at the herd level rather than the individual level. Initially, serum ELISA tests on 30 cows (second lactation or older) was designed to fill this need, since this sample subset was relatively inexpensive with a rapid turnaround time. Over time, less expensive and more sensitive alternatives were sought, especially for sheep. An Australian report showed that one positive faecal sample from a sheep with multibacillary paratuberculosis could be readily detected when combined with faecal pellets of 49 uninfected sheep (Whittington *et al.*, 2000). Later this approach to testing sheep flocks became the standard approach as a sensitive, efficient

means to detect *MAP* infection (see Whittington, Chapter 22, this volume). Subsequently, reports from the USA with cattle manure samples showed that pools of five samples were sensitive to detect infected cows and could be done at a fraction of the cost of whole-herd cultures on all adult cattle (Wells *et al.*, 2002). In this experimental study, 1:5 and 1:10 pools were tested. The sensitivity of pooled cultures ranged from 30 to 100% and was strongly dependent on pool size and the shedding level of the positive sample (low shedder compared with a heavy shedder). Occasionally culture of the pooled sample will be positive while each individual sample is negative (Wells *et al.*, 2003; Raizman *et al.*, 2004). Evaluation of pooled faecal samples (1:5) in a range of JD-infected dairy herds detects at least 87% of samples that contain at least one animal shedding moderate (>10 colony-forming units/tube) to high numbers of *MAP* (Kalis *et al.*, 2000). Later studies with pools of ten faecal samples in low-prevalence herds proved cost effective for herd screening and may provide an estimate of *MAP*-infected dairy cows within large herds (van Schaik *et al.*, 2003; Tavornpanich *et al.*, 2004). Optimal pool size depends on both prevalence and herd size and varies from three samples per pool for a 500-cow herd with low prevalence to five samples per pool for a 1000-cow herd with high prevalence (van Schaik *et al.*, 2003).

Composite environmental manure samples (a combination of three to four manure samples from a specific area) from high cow traffic areas in 64 herds known to be infected with *MAP* detected 50 (78%) of the 64 herds with positive faecal pools, thus providing evidence that environmental manure samples serve as an excellent proxy to detect herd infection (Lombard *et al.*, 2006). With refinement of the environmental manure sampling, by taking composite or pooled manure samples from high cow traffic areas, manure storage areas and pens/lots representing all cow groups within the herd, the diagnostic sensitivity to detect herd infection is further increased (Berghaus *et al.*, 2006). Additional investigations have shown that composite environmental manure samples from high dairy cow traffic sites and manure storage area samples have a greater sensitivity and would be less expensive for detecting herd *MAP* infection than 30 serum ELISA tests of second-lactation and older cattle (Raizman *et al.*, 2004; Lombard *et al.*, 2006). Samples of lagoon water from larger herds (350–2500 cows) were significantly more likely to give positive results than composite manure samples from high cow traffic areas (Berghaus *et al.*, 2006). Because composite environmental samples are collected from high cow traffic areas where cows defaecate daily, the weather or season of collection should not affect the ability to isolate *MAP*. Additionally, *MAP* has been shown to remain viable for long periods in the environment (Jorgensen, 1977; Richards and Thoen, 1977; Whittington *et al.*, 2003, 2004).

27.6 Vaccination

The only JD vaccine approved for use in the USA is a killed *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) product with a mineral oil adjuvant. First used on a research basis to collect efficacy data, this vaccine has been employed to help control JD in heavily infected herds where husbandry and management changes have been insufficient. This vaccine has been approved by the United States Department of Agriculture (USDA) Center for Veterinary Biologics on a conditional basis, as is likely for any future JD vaccine considered for use in the USA. The conditional approval requires secondary approval by the state veterinarian of any state where the vaccine might be used. If approved for a specific herd, the vaccine is sold by the manufacturer through the state veterinarian's office to the herd veterinarian. In most states where the vaccine is used, the herd owner, herd veterinarian and state veterinarian sign a three-way 'Memorandum of Understanding' about the conditions of its use on that farm. These memorandums typically require implementation of management changes in addition to an agreement by the owner that the herd veterinarian will administer the vaccine only to calves less than 35 days of age. Vaccinated animals must be properly eartagged and have an ear tattoo, in addition to

completed paperwork, copies of which must be filed with the state veterinarian's office. If the state veterinarian approves the use of JD vaccine in a state, then the vaccine can only be used in herds on a case-by-case basis. In most states, the state veterinarian's office requires laboratory confirmation of JD diagnosis by an organism-based test (typically culture or histopathology) and a high-prevalence herd.

The vaccine is currently used in 18 states. Many state veterinarians remain concerned about the potential increase in bovine tuberculosis (TB), as the JD vaccine may complicate detection of *Mycobacterium bovis* infection in TB-exposed herds. This concern stems from the fact that the JD vaccine can cause a positive tuberculin test, necessitating a costly and labour-intensive comparative cervical test for TB. As an example, a 600-cow dairy herd vaccinated for JD recently held a dispersal sale. Since the owner wanted to sell cows to owners in other states, this required that all adult cows be TB tested, resulting in 120 positive caudal-fold TB tests. Comparative cervical tests had to be done on each of these cows. While all were fortunately negative for TB, the costs were significant, illustrating the economic reality of this concern.

Currently an estimated 25 vaccine candidates are being evaluated for potential efficacy by being put through a series of tests (microbiological and immunological) and with a reduced number being evaluated in goats then cattle. This joint project, sponsored by the JD Integrated Program (a consortium of JD researchers centred in the USA) and the US Department of Agriculture Animal and Plant Health Inspection Service, will require upwards of 4 years, and probably longer. It is anticipated that only JD vaccines that do not induce a tuberculin skin response will be approved in the future.

27.7 Summary

The National Johne's Control Program in the USA has implemented a significant educational effort directed towards both producers and veterinarians, which resulted in a much greater awareness of JD by the veterinary profession and farmers, especially dairy producers. Currently, laboratory infrastructure and national check tests for both serology and organism detection are in place to meet the demand for diagnostic testing for JD. New testing formats, including pooling of manure samples and testing of composite environmental manure samples, are becoming more common, and both procedures have been incorporated in the national standards. A summary of some aspects of JD control in the USA is given in Table 27.1.

A major disappointment of most paratuberculosis investigators has been the lack of full endorsement of the herd status or test-negative programme. Although more herds have qualified for the status programme, the sale of low-risk cattle from these herds has

Table 27.1. Summary of aspects of JD control in the USA.

	2003	2004	2005	2006	2007
No. of advisory committees	42	44	47	48	49
No. of states in compliance with VBJDCP[a] at year start	34	36	43	48	49
No. of herds in VBJDCP[a]	4,722	6,189	7,876	8,736	8,674[b]
Total no. of status herds	543	993	1,472	1,779	1,760[b]
No. of ELISA tests	549,810	673,299	697,264	784,978	130,178[b]
No. of cultures	97,057	101,786	105,685	125,336	23,312[b]
No. of pooled samples				3,093	1,124[b]
No. of environmental samples				1,700	89[b]

[a]Voluntary Bovine JD Control Program.
[b]Courtesy of Dr Michael Carter, National Johne's Coordinator, USDA-Veterinary Services, 28 March 2007.

not commanded significant additional sale premiums compared with cattle from non-status herds. Cattle sold from status herds in Minnesota have brought higher prices, but the overall demand for these cattle has not increased to the point that participation in the status programme is being valued to the extent necessary to expand the programme. This is especially true for the larger dairy herds with more than 1000 cows. When those herds need replacement cattle for expansion, JD has been of little concern.

Federal funding of the national Johne's programme has decreased over the past 5 years, from a high of nearly $22 million per year to $12 million for the 2007 fiscal year. With decreased federal funding, fewer funds are available to support each state JD programme, resulting in diminished participation by farmers and veterinarians who were paid for the professional time necessary to perform risk assessments and produce herd management plans. If the federal funds continue to decrease, then producer participation will also probably decrease. It is the author's contention that, unless *MAP* is perceived to have a good probability of causing Crohn's disease in humans, then federal and state funds for JD will be nominal and our current national programme will continue to have little participation. To the best of the author's knowledge, no herd in the USA has completely eradicated JD, even over 25 years of excellent management, semi-annual testing and culling of most positive cows. It therefore appears likely that only with an effective and extensively used vaccine can Johne's ever be well controlled.

References

Berghaus, R.D., Farver, T.B., Anderson, R.J., Jaravata, C.C. and Gardner, I.A. (2006) Environmental sampling for detection of *Mycobacterium avium* ssp. *paratuberculosis* on large California dairies. *Journal of Dairy Science* 89, 963–970.

Bulaga, L.L. (1998) U. S. Voluntary Johne's Disease Herd Status Program for Cattle. In: *Proceedings of the 102nd Annual Meeting, United States Animal Health Association*. United States Animal Health Association, Minneapolis, Minnesota, pp. 420–433.

Chiodini, R.J., Van Kruiningen, H.J. and Merkal, R.S. (1984) Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *The Cornell Veterinarian* 74, 218–262.

Hagan, W.A. (1938) Age as a factor in susceptibility to Johne's disease. *Cornell Veterinarian* 28, 34–40.

Hagan, W.A. and Zeissig, A. (1933) Six years' experience with a herd experimentally infected with Johne's disease. *Cornell Veterinarian* 23, 1–15.

How to Do Risk Assessments and Management Plans for Johne's Disease. Available at http://www.johnesdisease.org/Risk%20Assessment%20&%20Management%20Plans%20for%20Johne's.pdf (accessed 9 June 2009).

Johne's Information Center. Available at http://www.johnes.org/ (accessed 9 June 2009).

Johne's Information Central. Available at http://www.johnesdisease.org/ (accessed 9 June 2009).

Jorgensen, J.B. (1977) Survival of *Mycobacterium paratuberculosis* in slurry. *Nordisk Veterinaermedicin* 29, 267–270.

Kalis, C.H., Hesselink, J.W., Barkema, H.W. and Collins, M.T. (2000) Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis. *Journal of Veterinary Diagnostic Investigation* 12, 547–551.

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B. and Salman, M.D. (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. *Journal of Dairy Science* 89, 4163–4171.

McKenna, S.L., Keefe, G.P., Tiwari, A., VanLeeuwen, J. and Barkema, H.W. (2006) Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. *Canadian Veterinary Journal* 47, 1089–1099.

Merkal, R.S., Whipple, D.L., Sacks, J.M. and Snyder, G.R. (1987) Prevalence of *Mycobacterium paratuberculosis* in ileocecal lymph nodes of cattle culled in the United States. *Journal of the American Veterinary Medical Association* 190, 676–680.

National Animal Health Monitoring Study (1997) Available at http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/dairy/#dairy1996 (accessed 6 June 2009).

Pearson, L. (1908) A note on the occurrence in America of chronic bacterial dysentery of

cattle. *American Veterinary Review* 32, 602–605.

Raizman, E.A., Wells, S.J., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C. and Olsen, K.E. (2004) The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *Journal of Dairy Science* 87, 2959–2966.

Richards, W.D. and Thoen, C.O (1977) Effect of freezing on the viability of *Mycobacterium paratuberculosis* in bovine feces. *Journal of Clinical Microbiology* 6, 392–395.

Rossiter, C.A. and Burhans, W. (1996) Farm specific approach to paratuberculosis (Johne's disease) control. *The Veterinary Clinics of North America – Food Animal Practice* 12, 383–415.

Tavornpanich, S., Gardner, I.A., Anderson, R.J., Shin, S., Whitlock, R.H., Fyock, T., Adaska, J.M., Walker, R.L. and Hietala, S.K. (2004) Evaluation of microbial culture of pooled fecal samples for detection of *Mycobacterium avium* subsp. *paratuberculosis* in large dairy herds. *American Journal of Veterinary Research* 65, 1061–1070.

Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program. Available at http://www.aphis.usda.gov/animal_health/animal_diseases/johnes/downloads/johnes-umr.pdf (accessed 6 June 2009).

van Schaik, G., Stehman, S.M., Schukken, Y.H., Rossiter, C.R. and Shin, S.J. (2003) Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. *Journal of Veterinary Diagnostic Investigation* 15, 233–241.

Wells, S.J., Whitlock, R.H., Lindeman, C.J. and Fyock, T. (2002) Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis. American Journal of Veterinary Research* 63, 1207–1211.

Wells, S.J., Godden, S.M., Lindeman, C.J. and Collins, J.E. (2003) Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. *Journal of the American Veterinary Medical Association* 223, 1022–1025.

Whipple, D. (1993) National paratuberculosis certification program. In: *Proceedings of the 97th Annual Meeting of the US Animal Health Association*. Cummings Corporation and Carter Printing Co., Richmond, Virginia, pp. 311–316. Available at http://www.livestocktrail.uiuc.edu/dairynet/paperDisplay.cfm?ContentID=200 (accessed 9 June 2009).

Whipple, D.L. and Merkal, R.S. (1983) Modifications in the technique for cultivation of *Mycobacterium paratuberculosis*. In: Merkal, R.S. (ed.) *Proceedings of the International Colloquium on Research in Paratuberculosis*. International Association for Paratuberculosis, Ames, Iowa, pp. 82–92.

Whitlock, R.H. (1993) Johne's disease control programs and diagnostic testing by individual states. In: *Proceedings of the Livestock Conservation Institute*. St Louis, Missouri, pp. 48–54.

Whitlock, R.H. and Rossiter, C.A. (1999) Report of the Committee on Johne's Disease. In: *Proceedings of the 102nd Annual Meeting of the United States Animal Health Association*. United States Animal Health Association, St Joseph, Missouri, pp. 420–433.

Whitlock, R.H., van Buskirk, M., Sweeney, R.W. and Hutchinson, L.T. (1994) Pennsylvania Johne's disease control program (1973–1993): a review of the 20-year program. In: Chiodini, R.A., Collins, M.T. and Bassey, E.O.E. (eds) *Proceedings of the Fourth International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Ames, Iowa, pp. 102–110.

Whitlock, R.H., Adams, J. and Weber, G. (2000) National Johne's Working Group: five year review with path forward. In: *Proceedings of the 104th Annual Meeting, United States Animal Health Association*. United States Animal Health Association, Richmond, Virginia, pp. 400–415.

Whitlock, R.H., Sweeney, R.W., Fyock, T., McAdams, S., Gardner, I.A. and McClary, D.G. (2005) Johne's disease: the effect of feeding monensin to reduce the bioburden of *Mycobacterium avium* subspecies *paratuberculosis* in neonatal calves. In: Smith, R.A. (ed.) *Proceedings of the Thirty Eighth Annual Convention, American Association of Bovine Practitioners*. American Association of Bovine Practitioners, Stillwater, Oklahoma, pp. 191–192.

Whitlock, R., Mangrove, B., Sweeney, R.W., Smith, J., Van Kessel, J., Hovingh, E., Karns, J., Wolfgang, D., Johnson, T., Fyock, T., Schukken, Y. and Gardner, I. (2007) RT-PCR and serial dilution of fecal samples to detect MAP supershedders (SS). In: *Proceedings of the 3rd Annual Conference of the Johne's Disease Integrated Program*, pp. 34–35. Available at http://www.jdip.org/index.php?option=com_content&task=view&id=69&Itemid=119 (accessed 8 June 2009).

Whittington, R.J., Fell, S., Walker, D., McAllister, S., Marsh, I., Sergeant, E., Taragel, C.A., Marshall, D.J. and Links, I.J. (2000) Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium*

subsp. *paratuberculosis* infection in flocks of sheep. *Journal of Clinical Microbiology* 38, 2550–2556.

Whittington, R., Marsh, I., Taylor, P.J., Marshall, D.J., Taragel, C. and Reddacliff, L. (2003) Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from environment samples collected from farms before and after destocking sheep with paratuberculosis. *Australian Veterinary Journal* 81, 559–563.

Whittington, R.J., Marshall, D.J., Nicholls, P.J., Marsh, I.B. and Reddacliff, L.A. (2004) Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Applied and Environmental Microbiology* 70, 2989–3004.

28 Paratuberculosis Control Measures in Australia

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28.1 Rationale for Control

Australia is a major producer and exporter of livestock and their products, and the Australian livestock industries and governments aim to assure domestic and international markets of the high quality and health status of these animals and products. These organizations work together with other stakeholders through Animal Health Australia, under a National Johne's Disease (JD) Control Program. Animal Health Australia was formed in 1996 as a not-for-profit company funded by the Australian government, state and territory governments, the livestock industries and associated service organizations and institutions to facilitate and coordinate national animal health programmes (Animal Health Australia, 2009).

Within Australia, paratuberculosis is not uniformly distributed across the livestock production regions, or among all sectors of the affected livestock industries. It is rare or absent across very large areas of northern and western Australia and in the alpaca and beef industries. The national programme aims to secure and protect the favourable status of these livestock populations and regions from incursions of paratuberculosis.

A third driver for controlling paratuberculosis in Australia is to reduce the economic and social impacts on owners of infected herds and flocks in the endemically infected regions of southern Australia, by providing the knowledge and tools to assist farmers to manage the disease.

28.2 History

Paratuberculosis was almost certainly introduced with ruminants and other livestock following British settlement from the late 18th century. For the next 150 years, imports of large numbers of susceptible animals continued both from Europe and from Asia. Following the establishment of the Australian Federation in 1901, quarantine restrictions were progressively tightened to protect Australia's freedom from many major infections and the imports of animals decreased. However, two distinct types of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) have become endemic in southern Australia (Cousins *et al.*, 2000; Whittington *et al.*, 2000a).

Paratuberculosis was diagnosed in cattle imported from Europe on several occasions in the early 20th century and in Australian-bred cattle in 1925 (Albiston, 1965). This disease in cattle, caused by Type C strains of *MAP*, was subsequently found in goats (Lenghaus *et al.*, 1977) and, relatively recently, during the 1990s in alpaca (Ridge *et al.*, 1995) and red deer. The presence of Type S strains of *MAP* in sheep was first diagnosed in 1980 (Seaman *et al.*, 1981; Seaman and Thompson, 1984), having probably been introduced with imported sheep from New Zealand some 20–30 years earlier (Sergeant, 2001). More detailed information on the different types of *MAP* strains is given elsewhere (see Stevenson, Chapter12, this volume).

28.3 Distribution and Prevalence

Australia occupies a large land mass between latitudes 10° and 45° south. Most Australian livestock are grazed outdoors all year round, on pasture in environments that range from tropical to cool temperate and arid. Bovine JD caused by Type C *MAP* strains is now endemic in the dairy cattle population in temperate south-eastern Australia (as reflected by the map in Fig. 28.1). Most dairy farms in south-eastern Australia are irrigated or in high rainfall areas and are relatively heavily stocked, but the within-herd prevalence of paratuberculosis is relatively low (Jubb and Galvin, 2004b).

In contrast, beef breeding herds are usually run at low stocking rates on non-irrigated land. Bovine JD is rare in the beef cattle sector, which has had little or no contact with dairy cattle. The within-herd prevalence of bovine JD in beef herds is also usually very low (Jubb and Galvin, 2004a), but high rates of subclinical and clinical infection have been recorded in a small number of herds in which cows and calves have been run intensively. Infection with Type C strains of *MAP* has been found occasionally in sheep flocks, but the balance of evidence is that sheep are not important in the epidemiology of bovine JD in Australia.

Paratuberculosis caused by Type S strains of *MAP* has probably been spreading in south-eastern Australia for about 50 years and, more recently, in temperate south-western Australia. It primarily infects sheep but has been detected occasionally in cattle (Whittington *et al.*, 2000b; Moloney and Whittington, 2008) and goats (Whittington and Taragel, 2000; Western Australia Department of Agriculture, 2004). There is no evidence in Australia that Type S strains have spread among cattle and goats.

European rabbits are an introduced pest that is widespread in Australia, and detection of infection in Scottish rabbits (Greig *et al.*, 1997) raised concerns about their role in the spread of paratuberculosis in Australia. Fortunately, studies in endemically infected environments have not detected *MAP* in over 600

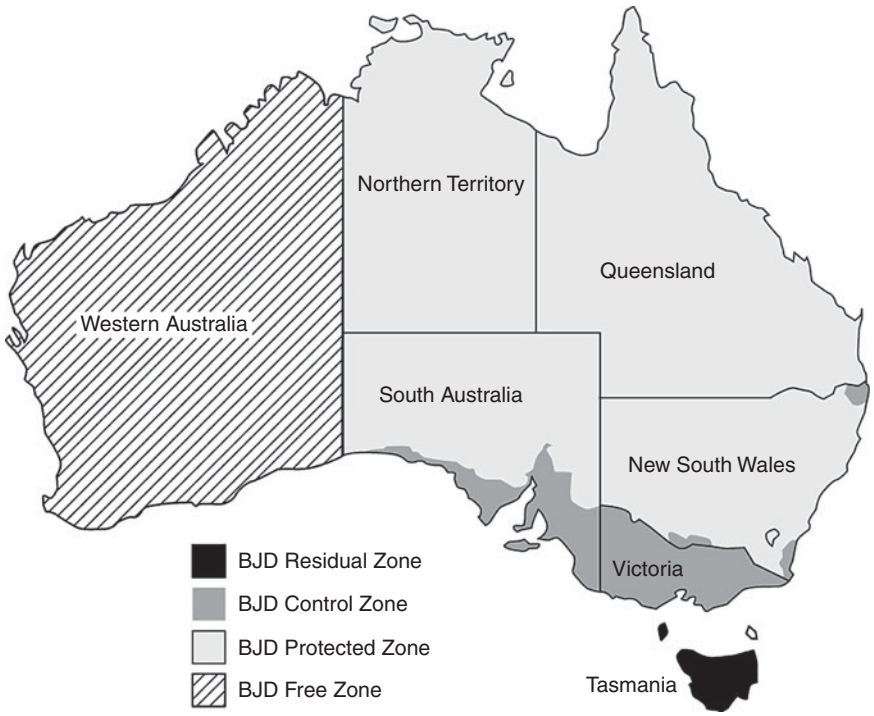

Fig. 28.1. Bovine JD (BJD) zones in Australia, 2001 (source: NSW Department of Primary Industries).

rabbits (Abbott, 2000; Kluver, 2005) and they are considered not to be important in the epidemiology of paratuberculosis in Australia.

28.4 Early Control of Paratuberculosis in Australia

Endemic disease control in animals is the constitutional responsibility of the states and territories in Australia. A range of control programmes, such as exclusion, vaccination, destocking, test-and-cull programmes or minimal intervention, had been implemented at various times in one or more states and territories during the latter half of the 20th century. It was recognized that many of these programmes focused on known infected herds and flocks that were subject to regulatory control, while most infected herds and flocks continued to trade and spread the disease. Interstate movement restrictions into northern and western Australia, where paratuberculosis was unknown or uncommon, were onerous on south-eastern producers as these regions attempted to protect themselves. These programmes have been reported by Albiston (1965) and Milner and Wood (1989). During this time, the dairy industry in particular was actively researching improved methods of detection and control. A national programme began in 1995 because of concerns about:

- ongoing spread to non-infected regions, herds and flocks
- significant productivity effects and mortalities in some herds and flocks
- restrictions on sales of livestock from known infected herds and flocks and the farms on which they grazed
- increasing speculation about a possible link between *MAP* infections in animals and Crohn's disease in people.

Australia has a proud recent history of national disease control, having successfully eradicated contagious bovine pleuropneumonia, bovine tuberculosis and bovine brucellosis in the latter half of the 20th century. The cattle industries in particular had been strong partners in the later programmes, and they led the national livestock organizations to establish a National JD Coordinating Committee in 1995, with the Australian government, state governments and the dairy and red-meat research and development organizations.

One of the key decisions taken at the start of this programme was to recognize that, although Type S and Type C strains of *MAP* both cause paratuberculosis, the infections are epidemiologically distinct. This had profound implications for the management of both infections, as beef cattle and sheep are commonly grazed on the same land in southern Australia. The genotypic differences between Type C and Type S, later confirmed in Australia (Whittington *et al.*, 1998), supported this policy decision, as did the ability to grow Type S strains in radiometric culture (Whittington *et al.*, 1999).

Another key decision was to shift the focus from known infected herds and flocks and to develop programmes that would allow herds and flocks to transparently demonstrate a low-risk status through nationally agreed Market Assurance Programs. The first such programme was launched for cattle in 1996 (Kennedy and Neumann, 1996) and was followed by similar programmes for sheep, alpaca and goats over the next 3 years (Allworth and Kennedy 2000; Kennedy and Allworth, 2000). These high-level assurance programmes still cater largely to pedigree and large commercial herds and flocks that sell breeding animals. The programmes have been updated and improved to adapt to changing needs, improved understanding of risk and technical advances in tests and vaccines.

Two national standards were developed early in the programme to underpin a national approach. The process to agree on standard diagnostic tests and their quality control interpretation commenced in 1995 (Tennent *et al.*, 1998; Walker *et al.*, 1999). These standards, now called the Australian and New Zealand Standard Diagnostic Procedures for JD (SCAHLS, 2009a), and the associated quality control testing (SCAHLS, 2009b) have been reviewed and updated with new tests from time to time under the supervision of the laboratory standards subcommittee of the national committee of chief veterinary officers.

The third national standard was the Standard Definitions and Rules (SDRs) for regulatory control of JD. These were developed and approved by the national committee of chief veterinary officers, commencing with those for Type C infection in 1997 (Anonymous, 1997) and subsequently for Type S infection (Anonymous, 1998). These have also been updated as needed, the cattle SDRs most recently in 2008. The sheep SDRs were substantially modified to support a major shift from a regulatory approach for the control of ovine JD in 2004 and have been updated to reflect improvements in the programme in 2008.

28.5 National Management Model

Paratuberculosis is managed under two separate subprogrammes of the National JD Control Program: the National Bovine JD Strategic Plan and the Ovine JD Management Plan. These plans have evolved over the past decade to have greater industry ownership and financial input. In the endemically infected regions, they have also transferred much of the focus and responsibility from government regulatory programmes to individual risk assessment and management. In the free and low-prevalence areas and industry sectors, however, government is still actively supporting industry to reduce the risk of entry of paratuberculosis, to conduct surveillance and to control or eradicate infection when detected.

Programme policy development is typically undertaken jointly by national industry and government representatives on programme management committees and technical working groups. Where required, for official disease control or certification standards, policies are submitted to the national committee of chief veterinary officers for official endorsement. Stakeholder committees are also active in most states and territories.

The livestock industry research and development corporations have been actively involved in supporting these activities through collaboration in prioritizing and funding research and extension (Dairy Australia, 2009; Meat and Livestock Australia, 2009).

For the first decade, research was largely of an applied nature, to answer key questions for the control and assurance programmes. This resulted in improved diagnostic tests, including less expensive pooled faecal culture techniques (Whittington *et al.*, 2000c; Eamens *et al.*, 2007, 2008), and understanding of the epidemiology and impacts of JD in Australia, for instance by assessing risk factors (Dhand *et al.*, 2007), modelling risk of spread (Sergeant, 2003) and assessing the effect of ovine JD on sheep farm gross margins (Bush *et al.*, 2006). More recently, larger research projects on more fundamental research on pathobiology and immunology have been funded for both cattle and sheep infections.

Because paratuberculosis is a challenging infection to prevent and control at a national, regional and farm level, there has been a strong emphasis on extension and training. Animal Health Australia and/or the states train and approve veterinarians to supervise farms involved in the Market Assurance Program and state disease control programmes. The Market Assurance Program approval course and assessment for veterinarians is now Web-based. Animal Health Australia, research and development bodies and the states conduct complementary extension activities on the various aspects of the disease and its programmes, with web sites also linked to international resources (Animal Health Australia, 2009).

28.6 Assessing the Cattle JD Situation

After 5 years of the national programme, the understanding of paratuberculosis in cattle in Australia had matured, and it was appreciated that one, largely regulatory, control model did not suit the needs of both dairy and beef industries in the control and residual zones of south-eastern Australia. Paratuberculosis was well established in the dairy industry, which was contracting to the cool temperate south-east. On the other hand, the evidence was that infection was uncommon in the beef industry (Fig. 28.2), which was penalized by the same movement restrictions into the northern protected zones.

Animal Health Australia contracted a suite of review projects in 2000, under the banner of National Bovine JD Evaluation, in order to assess current tools and knowledge with a view to future management approaches. Surveillance and occasional eradication activities in the northern regions in Queensland (Pitt *et al.*, 1999) and the Northern Territory and in Western Australia (Ellis *et al.*, 1998; Martin, 2008) justified their continued protected or free zone status.

Furthermore, the concept of protecting the beef industry in the endemic regions was considered both desirable and possible. Subsequently, a partial survey of beef herds in the control and residual zones detected only six ELISA reactors and one infected animal among the 9000 cattle tested in South Australia, Victoria and Tasmania. Three infected herds were detected in an isolated pocket in northern NSW, where the local dairy industry had largely been replaced by beef production in recent years. Although incomplete, the survey increased confidence that beef cattle herds that had little or no contact with the dairy industry had a low risk of being infected with paratuberculosis and deserved both protecting and being given concessions in movements into the protected zones. In 2002, fewer than 150 out of an estimated 170,000 beef herds were known to be infected with paratuberculosis. The comparative prevalence of officially known infection in beef and dairy herds by zone is illustrated in Fig. 28.2.

28.7 National Bovine JD Strategic Plan

After considerable consultation, Animal Health Australia was charged with managing

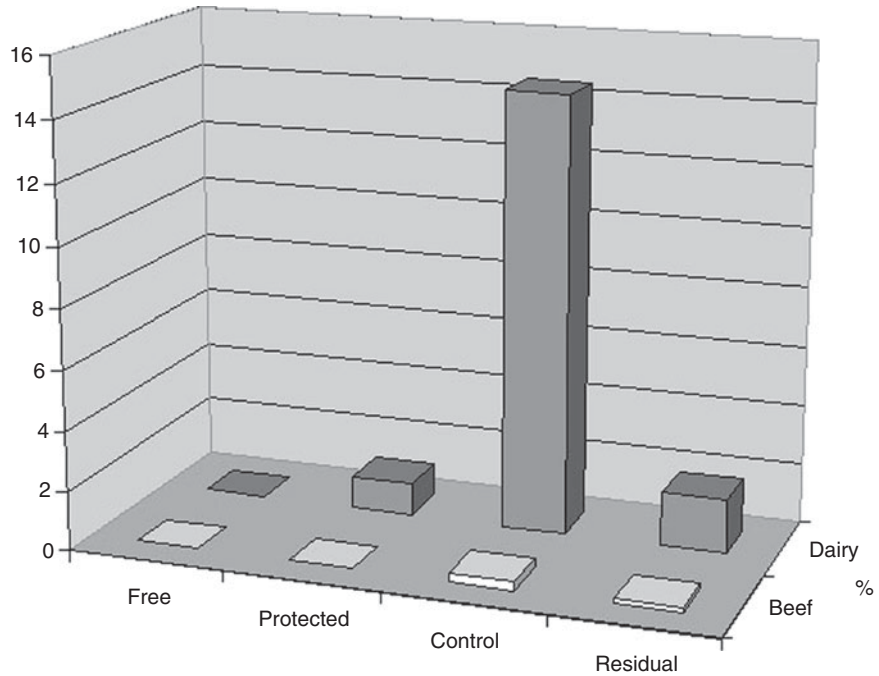

Fig. 28.2. The percentages of beef and dairy herds officially classified as infected by zone in December 2002.

the National Bovine JD Strategic Plan in 2003, with three goals:

1. to reduce contamination of farms and farm products by *MAP*
2. to protect the status of non-infected herds and regions
3. to reduce the social, economic and trade impact of bovine JD at herd, regional and national levels.

The rationale for these goals reflects the current situation in Australia.

- Australia is in a favourable situation in comparison with other developed producers of livestock and animal products, as endemic JD is restricted to south-eastern Australia, predominantly in dairy herds.
- The beef cattle industry wants to retain maximum domestic market access without compromising the free status of the majority of Australian herds and possibly jeopardizing international market access for live cattle in particular.
- Producers in free and protected zones want to maintain their favourable situation in order to retain market access (without testing costs) and, in some cases, to avoid the regulatory disease control programmes that would be required if bovine JD occurred in their zones.
- Producers with infected herds want pathways to ease the stigma of known infection and enable progression towards regaining market access for cattle and land without price penalties.
- The dairy industry wants to control bovine JD and its impact, and to protect market access for its products, particularly on the international market.

It was envisaged that risk assessment and management and commercial drivers would play a major role in achieving these goals. Furthermore, the plan recognized that

the beef and dairy sectors and different regions would have varying objectives and strategies, depending on the prevalence and epidemiology of infection, management practices and market needs.

The original 2003 plan has been updated each year but has essentially retained the same philosophy and approach. The 2008/9 annual programme has a budget of A$1.7 million, funded from producer levies. State government animal health services provide additional operational support for the programme. The main strategies for this programme are developing and applying risk-based management methods to minimize infection, developing new tools and knowledge through research and development, improving understanding of the management of the disease through communication and training and assistance to affected producers.

28.8 Key Projects under the National Bovine JD Strategic Plan

28.8.1 Beef Only

The means of identifying and protecting the pure beef sector in south-eastern Australia is a scheme called Beef Only. It is based on a written owner declaration in a standard format that a herd is not known or suspected to be infected and has little or no direct or indirect contact with dairy cattle. The declaration is used primarily at sales of young replacement breeding cattle, where pens of Beef Only cattle are clearly identified. States audit a sample of declarations and have found high compliance rates. Beef Only cattle can move into the protected zones.

28.8.2 National financial and non-financial assistance package

The Cattle Council of Australia has provided substantial assistance since 2004 to encourage owners of infected beef herds to eliminate JD, and owners who suspect infection to have suspect cases investigated. Animal Health Australia contracts two rural counsellors, who work with official veterinarians and/or private veterinarians to develop a plan on how to eradicate infection, usually by total depopulation. However, in herds in which the source of infection has been recent, partial depopulation of high-risk groups and monitoring the testing of the balance are often undertaken. As well as financial assistance with culling and testing costs, farmers in the scheme are encouraged and assisted to engage a financial adviser to assess that aspect of eradication options. To date, infected or suspect status has been lifted from about half of the 135 herds involved. An external review in 2007 found that it was making a major contribution to meeting the beef industry's goals regarding JD.

28.8.3 National Bovine JD Dairy Assurance Score

As JD is endemic in the south-eastern dairy industry, the new approach to control encourages voluntary implementation of management strategies to reduce the prevalence of infection within herds and the risk of spread between herds. The key tool in this approach is the National Bovine JD Dairy Assurance Score, which was launched in 2007 (Dairy Australia, 2009). The score provides a ranking of risk, with 0 for herds that do not test negative or do not implement auditable calf-rearing programmes to reduce the incidence of infection in replacement heifers. That is, herds that do not engage in the programme have the lowest score. Herds that have calf rearing in place score 3, and infected herds can progress from scores of 1 to 6 by successful control that is also demonstrated by testing. Tested negative herds score 7 and herds in the Market Assurance Program score from 8 to 10. In endemic regions, approved calf-rearing schemes designed to reduce the incidence of infection are emphasized, and calves reared under such schemes receive additional credits. However, assumptions about the application and effectiveness of such practices have been questioned (Ridge *et al.*, 2005) and are due for formal evaluation by the dairy industry by 2012.

The Queensland Protected Zone and Western Australian Free Zone require scores of 8 and 10 respectively for introduced dairy cattle. Queensland surveyed its cattle herds in 2008 and found no evidence of JD (J. Berry, Brisbane, personal communication, 2008). In South Australia and New South Wales, where herd prevalences are low and the state dairy industries and governments have developed locally adapted programmes within the national plan to encourage control, farmers must declare their cattle's score when selling dairy stock. About 95% of South Australia's herds had enrolled in the Dairy ManaJD programme by 2008, with industry-funded testing of dairy herds and audited calf rearing resulting in the great majority of these having tested negative to attain high dairy scores. Using the score is voluntary in Victoria and Tasmania.

28.9 Other Species Affected by Type C Strains of *MAP*

Goats and South American camelids are also susceptible to infection with Type C strains and have complementary programmes to provide assurance and manage risk. JD has also been detected in red deer herds in the past decade, but little progress has been made in developing cost-effective tests for deer and a draft Market Assurance Program has not been implemented.

28.9.1 Goats

In conjunction with Animal Health Australia and the states, the goat industry developed a risk rating system and a National Goat Health Declaration form in 2008 (Animal Health Australia, 2009). Infected herds have a rating of 0, with high-assurance herds in the Goat Market Assurance Program (which has been running since 1999) having a rating of 8. As in the cattle industry, contact with dairy animals is regarded as high risk. Risk rating can be improved by testing negative, by vaccinating with the only registered paratuberculosis vaccine in Australia (Gudair™) and by implementing an approved kid-rearing programme.

28.9.2 Alpaca

A JD outbreak in alpaca in south-eastern Australia was contained in the mid-1990s (Ridge *et al.*, 1995) and no cases were detected for over a decade. Uptake of the Alpaca Market Assurance Program from 1998 had been strong, but costs and the low likelihood of herds being infected led to agreement that, like beef cattle, alpaca deserved to be regarded as a protected population. To encourage biosecurity in the industry and to monitor for occurrences of paratuberculosis and other important infections, the alpaca industry developed the Q-Alpaca programme in 2005 (Australian Alpaca Association, 2009). Among other practices, all deaths in Q-Alpaca herds should be investigated.

28.10 Ovine JD Management Plan

The rate and geographical distribution of detections of JD in sheep increased in New South Wales during the early 1990s (Fig. 28.3), and from 1995 onwards the infection was detected in the other south-eastern states of Australia (Sergeant, 2001). Endemic infection was detected in Western Australia in 2003, although there had been earlier isolated cases (Western Australia Department of Agriculture, 2004). These detections, largely of clinical disease, reflected much earlier spread and establishment of the infection itself.

A sheep Market Assurance Program was endorsed in 1997 to help sheep breeders demonstrate a low-risk status, and national Standard Definitions and Rules were agreed shortly afterwards, with zones defined (Anonymous, 1997). The various disease control responses in the states relied heavily on quarantine and movement controls, in some cases depopulation of infected flocks. Failure to obtain national sheep industry support for infected producers resulted in a A$40 million national Control and Evaluation Program from 1998 to 2004. The aim of this programme was to better understand the disease while controlling its spread by regulatory means, but with little assistance to owners of infected flocks and of flocks in the restricted zones. The disease was

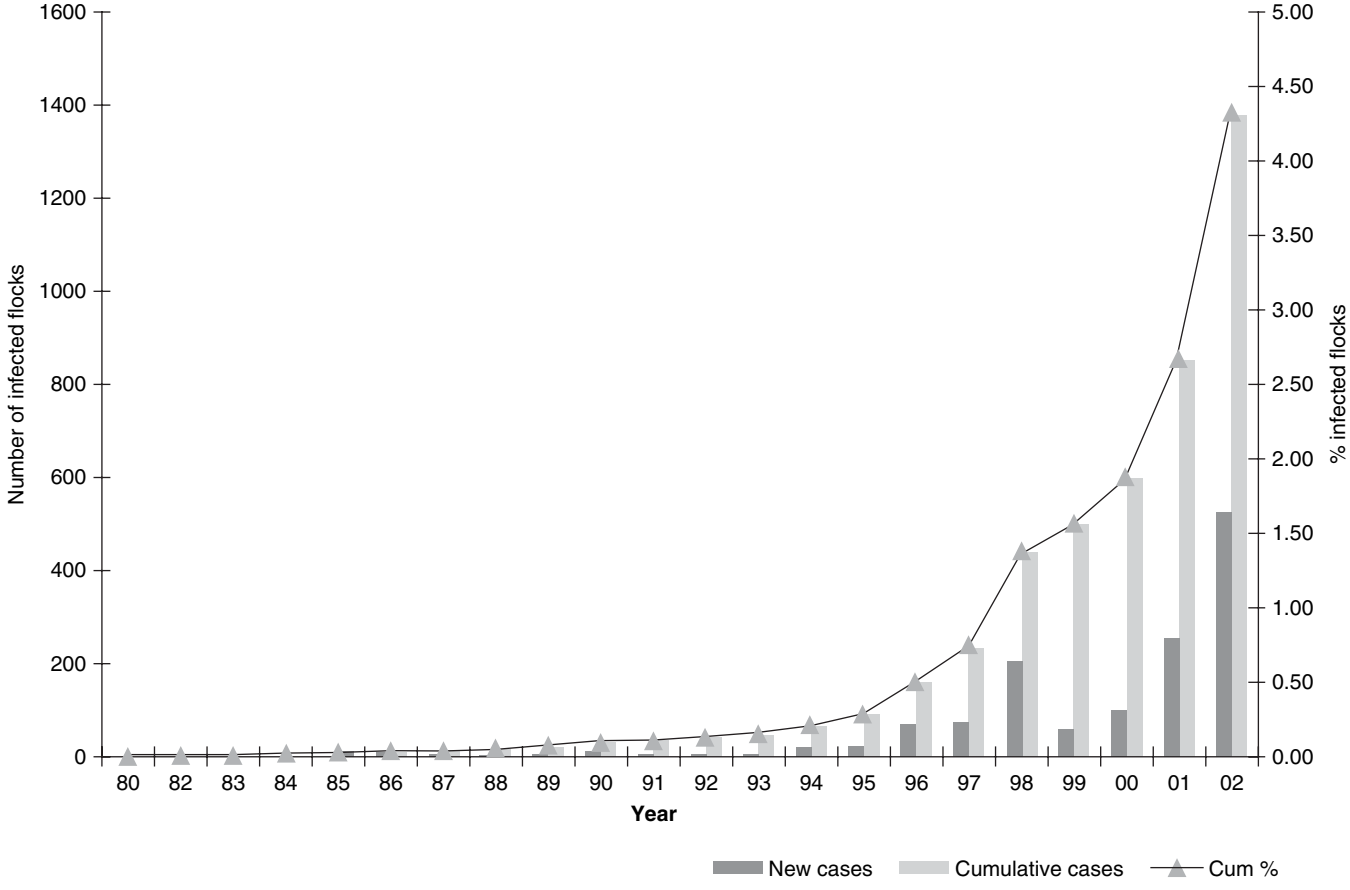

Fig. 28.3. The numbers of sheep flocks known to be infected with ovine JD, 1980–2002 (source: NSW Department of Primary Industries).

largely driven underground and continued to spread, as many producers objected to and, inadvertently or deliberately, did not comply with the regulatory programme.

The success of the concurrent research programme, as detailed by Meat and Livestock Australia (2009), and the obvious failure of the regulatory control programme in most areas led to a major review and revision of the national programme. The sheep industry's vision under the new approach from 2004 was that areas currently with little or no ovine JD will remain unaffected while infected areas would contain or reduce the within- and between-flock prevalence.

The approach was largely based on vendor declaration of assessed risk and risk management by individual farmers when buying sheep and on wider use of Gudair™ vaccine. A comprehensive, but relatively simply communicated, risk assessment system, called the Assurance Based Credit (ABC) scheme, was agreed nationally in 2003 and remains the foundation of the Australian approach to managing ovine paratuberculosis. It is based largely on a quantitative risk assessment (Sergeant, 2003), and flock owners can claim from 0 to 10 credits based on the background flock prevalence in their region or prevalence area (Fig. 28.4), and the particular risk-reduction measures that have been undertaken in the flock, such as being in the Market Assurance Program, flock testing or vaccination coverage (Animal Health

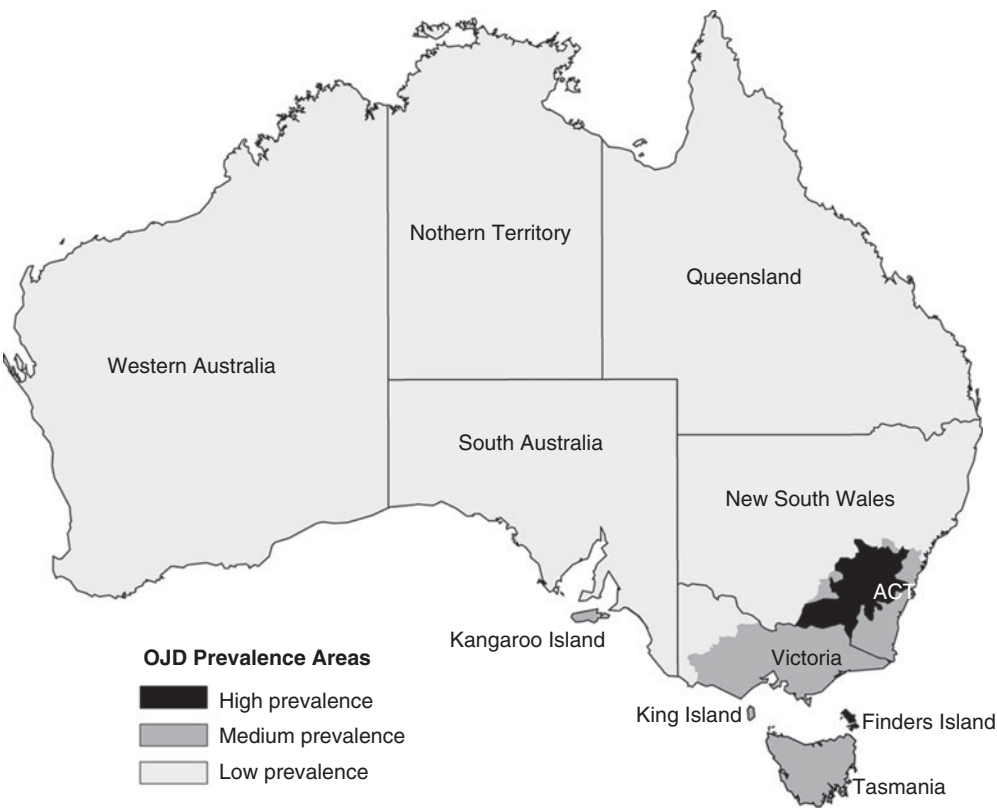

Fig. 28.4. Revised high-, medium- and low-prevalence areas for ovine JD, 2008 (source: NSW Department of Primary Industries).

Australia, 2009). The effectiveness of vaccination is being monitored to better assess its capacity to reduce excretion of organisms and transmission of infection (Eppleston *et al.*, 2005; Reddacliff *et al.*, 2006). Nearly 3 million doses of Gudair™ vaccine were sold in 2007.

Prevalence areas were defined by the flock prevalence estimated by a Bayesian model (Sergeant and Baldock, 2002) using data from post-mortem inspection of the abdominal viscera of adult sheep at key abattoirs (Bradley and Cannon, 2003). This voluntary approach to encourage disease control and declaration of ABC credits when selling sheep was supported financially in some states from levies that were used to subsidize testing and vaccination.

The programme was improved in 2008 with the national sheep industry organizations financing abattoir surveillance that not only monitored the flock prevalence but provided data for the states for feedback to producers on detection of disease and negative surveillance data that could be used for ABC testing credits. The budget for the programme in 2008/9 was AUS$0.8 million. Approximately 6500 directly consigned lines, comprising 1.65 million adult sheep, were inspected in 2008. Inspection was also expanded to report on several other important diseases of sheep that could be detected grossly at post-mortem inspection. The meat inspection branch of the Australian Quarantine and Inspection Service provided the assurance of quality of abattoir monitoring. Complementing this service in 2008, the Australian Livestock and Property Agents Association led the move to develop and use a standardized Sheep Health Statement for sheep sales in Australia (Animal Health Australia, 2009). It not only facilitates the declaration of the ABC score and flock history but includes declarations on other key transmissible diseases, such as ovine foot rot and sheep lice.

28.11 Achievements

Eight governments and eight national livestock industry organizations are involved in the control of paratuberculosis in Australia. Although the occurrence of Type C and Type S infections is different and the goals of diverse regions and industry sectors vary, these governments and industry organizations collaborate in developing and implementing complementary and mutually recognized paratuberculosis control programmes. This relationship is not maintained easily but depends on formal consultation mechanisms, open and respectful communications and national coordination. The previously dominant role of government has been curtailed over the years by reduced government resourcing, but this has facilitated a maturing of the industries' roles and ownership of the programmes. The sheep industry's financing of abattoir surveillance and reporting and the beef industry's funding of the Financial and Non-Financial Assistance Scheme are prime examples.

From a technical perspective, the epidemiology and impacts of paratuberculosis caused by Type C and Type S strains in Australia are now well understood, thanks to a large body of applied research, field investigations and critical reviews of the effectiveness of operational programmes. Programmes have been adapted accordingly. Regulatory control and eradication are maintained in the officially free and apparently free areas and sectors.

The goals of the cattle and sheep programmes are largely being met. The cattle populations in the northern and western parts of Australia are still either officially or apparently free of infection and the small numbers of known infected beef herds in south-eastern Australia are being assisted to eradicate the infection. JD is now virtually unknown in alpaca.

Reducing the spread of infection to new farms in the endemically infected dairy cattle and sheep industries remains a significant challenge. Ovine paratuberculosis did spread to the former Western Australian Free Zone and the estimated flock prevalences in south-eastern Australia have been slowly increasing over the past 5 years, although to a large extent these reflect spread several years ago. However, producers in these sectors and the goat industry now have standardized tools to help them understand, assess and manage their risk and manage their farm biosecurity. The Dairy Bovine JD Assurance Score, the ABC scheme for sheep and the risk rating for goats

give all herd and flock owners the means to make a vendor declaration of the risk that their stock poses. In these industries, the simple biosecurity messages to stock buyers are to ask for a signed declaration, to buy stock with the same or a higher score as their own herd or flock and to remember that the higher the score is the better. Combined with rearing management and/or vaccination of replacement stock, these procedures should be lowering the incidence of infection.

A related achievement of the programmes is that the increasing promotion and use of vendor declarations of risk for paratuberculosis is increasing appreciation of the need for farm-level biosecurity, which should lead to reduced spread of endemic pests and diseases generally and help prevent the spread of exotic agents should they be introduced.

28.12 Lessons Learned

Australia successfully eradicated three endemic diseases of cattle by regulatory test-and-cull programmes and movement restrictions in the second half of the 20th century. Similar approaches have been shown not to be appropriate for endemic paratuberculosis, given the largely subclinical nature of the infection and the relative lack of sensitivity of currently available diagnostic tests at the individual animal level. The Australian experience has demonstrated that regulatory programmes that focus on known infected herds and flocks discriminate against and penalize their owners and, understandably, discourage compliance. As a result, the majority of infected herds and flocks had remained outside official control programmes in the past and paratuberculosis continued to spread. Understanding the two types of *MAP* infections in the Australian environment and developing and communicating appropriate assurance and risk assessment schemes that apply to all producers have been critical steps towards successful control in the endemic regions and livestock sectors. Regulatory action against those who make false or misleading vendor declarations remains an important aspect of the programme.

In those regions and livestock sectors, such as the beef and alpaca industries, where the disease is rare or absent, appropriate surveillance, investigations and tracing must be undertaken and reported to maintain confidence in their status. However, support programmes must provide producers with the confidence that, if they report suspicion of infection, they will be assisted technically, financially and emotionally to deal with it.

28.13 Future Directions

Australia has a long-term interest in maintaining its favourable animal health status for many pests and diseases, including paratuberculosis. Although a causal association between *MAP* and Crohn's disease has not been established, Australian livestock industries want to assure domestic and international customers and food authorities that not only is the infection absent from large parts of the livestock populations but it is also being energetically controlled in those sectors where it does occur.

The approaches will continue to focus on voluntary risk assessment, declaration and management in the endemic areas and be largely driven and funded by livestock industries, with targeted technical and regulatory support from governments. Although these schemes are maturing, the technical basis for these voluntary risk assessment schemes, such as the effectiveness of vaccination in infected sheep flocks and of calf rearing in infected dairy herds, will continue to be scrutinized and challenged, especially by producers and regions that are risk averse. In the free areas, governments will be expected to remain actively involved in movement controls, surveillance and eradication for the occasional detections.

References

Abbott, K.A. (2000) *Prevalence of Johne's Disease in Rabbits and Kangaroos*. Research Project TR.050. Meat and Livestock Australia, Sydney, Australia.

Albiston, H.E. (1965) Johne's disease. In: Sneddon, H.R. *Diseases of Domestic Animals in Australia, Part 5, Vol. I, Bacterial Diseases*, 2nd edn, revised by Albiston, H.E. Commonwealth Department of Health, Canberra, pp. 150–162.

Allworth, M.B. and Kennedy, D.J. (2000) Progress in national control and assurance programs for ovine Johne's disease in Australia. *Veterinary Microbiology* 77, 415–422.

Animal Health Australia (2009) Johne's disease. Available at http://www.animalhealthaustralia.com.au/programs/jd/jd_home.cfm (accessed 29 January 2009).

Anonymous (1997) *Standard Definitions and Rules for Johne's Disease in Cattle*. Standing Committee on Agriculture and Resource Management, Canberra, Australia.

Anonymous (1998) *Standard Definitions and Rules for Johne's Disease in Sheep*. Standing Committee on Agriculture and Resource Management, Canberra, Australia.

Australian Alpaca Association (2009) Quality Alpaca. Available at http://www.alpaca.asn.au/pub/AAA/qa/intro.shtml (accessed 13 February 2009).

Bradley, T. and Cannon, R.M. (2003) Determination of individual animal-level sensitivity of abattoir surveillance for ovine Johne's disease. In: Trengove, C., Larsen, J.L. and Allworth, B. (eds) *Proceedings of the Australian Sheep Veterinary Society 2003 – Cairns Conference*, Volume 13. Australian Sheep Veterinary Society, Brisbane, pp. 61–62.

Bush, R.D., Windsor, P.A. and Toribio, J.A. (2006) Losses of adult sheep due to ovine Johne's disease in 12 infected flocks over a 3-year period. *Australian Veterinary Journal* 84, 246–253.

Cousins, D.V., Williams, S.N., Hope, A. and Eamens, G.J. (2000) DNA fingerprinting of Australian isolates of *Mycobacterium avium* subsp. *paratuberculosis* using IS*900* RFLP. *Australian Veterinary Journal* 78, 184–190.

Cox, J.C., Drane, D.P., Jones, S.L. and Milner, A.R. (1991) Development and evaluation of a rapid absorbed immunoassay test for the diagnosis of Johne's disease in cattle. *Australian Veterinary Journal* 68, 157–160.

Dairy Australia (2009) Bovine Johne's disease. Available at http://www.dairyaustralia.com.au/bjd (accessed 29 January 2009).

Dhand, N.K., Eppleston, J., Whittington, R.J. and Toribio, J.A. (2007) Risk factors for ovine Johne's disease in infected sheep flocks in Australia. *Preventive Veterinary Medicine* 82, 51–71.

Eamens, G.J., Walker, D.M., Porter, N.S. and Fell, S.A. (2007) Pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in goats. *Australian Veterinary Journal* 85, 243–251.

Eamens, G.J., Walker, D.M., Porter, N.S. and Fell S.A. (2008) Radiometric pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in low-shedder cattle. *Australian Veterinary Journal* 86, 259–265.

Ellis, T.M., Norris, R.T., Martin, P., Casey, R.H. and Hawkins, C.D. (1998) Evidence for freedom from Johne's disease in cattle and goats in Western Australia. *Australian Veterinary Journal* 76, 630–633.

Eppleston, J., Reddacliff, L., Windsor, P., Links, I. and Whittington, R. (2005) Preliminary observations on the prevalence of sheep shedding *Mycobacterium avium* subsp. *paratuberculosis* after 3 years of a vaccination program for ovine Johne's disease. *Australian Veterinary Journal* 83, 637–638.

Greig, A., Stevenson, K., Perez, V., Pirie, A.A., Grant, J.M. and Sharp, J.M. (1997) Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*). *The Veterinary Record* 140, 141–143.

Jubb, T.F. and Galvin, J.W. (2004a) Effect of a test and control program for Johne's disease in Victorian beef herds 1992–2002. *Australian Veterinary Journal* 82, 164–166.

Jubb, T.F. and Galvin, J.W. (2004b) Effect of a test and control programme for bovine Johne's disease in Victorian dairy herds 1992–2002. *Australian Veterinary Journal* 82, 228–232.

Kennedy, D.J. and Allworth, M.B. (2000) Progress in national control and assurance programs for bovine Johne's disease in Australia. *Veterinary Microbiology* 77, 443–451.

Kennedy, D.J. and Neumann, G.B. (1996) The Australian national Johne's disease Market Assurance Program. In: Chiodini, R.J., Hines, M.E. and Collins, M.T. (eds) *Proceedings of the 5th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 121–131.

Kluver, P. (2005) A survey of potential wildlife reservoirs for *Mycobacterium paratuberculosis*. In: *Proceedings MLA OJD Harvest Year Conference*. Meat and Livestock Australia, Sydney, Australia.

Lenghaus, C., Badman, R.T. and Gillick, J.C. (1977) Johne's disease in goats. *Australian Veterinary Journal* 53, 460.

Martin, P.A. (2008) Current value of historical and ongoing surveillance for disease freedom: surveillance for bovine Johne's disease in Western Australia. *Preventive Veterinary Medicine* 84, 291–309.

Meat and Livestock Australia (2009) Ovine Johne's disease. Available at http://www.mla.com.au/TopicHierarchy/InformationCentre/AnimalHealthandWelfare/Animalhealth/Diseases/

Ovine+Johnes+Disease.htm (accessed 29 January 2009).

Millner, A.R. and Wood, P.R. (eds) (1989) *Johne's Disease: Current Trends in Research, Diagnosis and Management*. CSIRO Publications, East Melbourne, Australia.

Moloney, B.J. and Whittington, R.J. (2008) Cross species transmission of ovine Johne's disease from sheep to cattle: an estimate of prevalence in exposed susceptible cattle. *Australian Veterinary Journal* 86, 117–123.

Pitt, D., Baldock, C., Black, P., Roberts, J., MacKenzie, S. and Houston, E. (1999) Evaluation of Johne's disease ELISA testing in north Queensland cattle and its application in proving 'freedom' in Queensland – an historically free state. In: Manning, E.J.B. and Collins, M.T. (eds) *Proceedings of the 6th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 388–394.

Reddacliff, L., Eppleston, J., Windsor, P., Whittington, R. and Jones, S. (2006) Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Veterinary Microbiology* 115, 77–90.

Ridge, S.E., Harkin, J.T., Badman, R.T., Mellor, A.M. and Larsen, J.W.A. (1995) Johne's disease in alpacas (*Lama pacos*) in Australia. *Australian Veterinary Journal* 72, 150–153.

Ridge, S.E., Baker, I.M. and Hannah, M. (2005) Effect of compliance with recommended calf-rearing practices on control of bovine Johne's disease. *Australian Veterinary Journal* 83, 85–90.

SCAHLS (2009a) Australian and New Zealand Standard Diagnostic Procedures. Available at http://www.scahls.org.au/standardprocedures/standards.htm (accessed 29 January 2009).

SCAHLS (2009b) SCAHLS Quality Plan for Johne's disease testing. Available at http://www.scahls.org.au/standardprocedures/terrestial/JD.pdf (accessed 29 January 2009).

Seaman, J.T. and Thompson, D.R. (1984) Johne's disease in sheep. *Australian Veterinary Journal* 61, 227–229.

Seaman, J.T., Gardner, I.A. and Dent, C.H.R. (1981) Johne's disease in sheep. *Australian Veterinary Journal* 57, 102–103.

Sergeant, E.S. (2001) Ovine Johne's disease in Australia – the first 20 years. *Australian Veterinary Journal* 79, 484–491.

Sergeant, E.S.G. (2003) *Quantitative Assessment of the Risk of Ovine Johne's Disease in Sheep Flocks. Final Report.* Australian Wool Innovation, Melbourne, Australia.

Sergeant, E.S.G. and Baldock, F.C. (2002) The estimated prevalence of Johne's disease infected sheep flocks in Australia. *Australian Veterinary Journal* 80, 762–768.

Tennent, J.M., Cousins, D.V., Condron, R.J. and Eamens, G.J. (1998) Johne's disease. In: *Australian Standard Diagnostic Techniques for Animal Diseases*. CSIRO Information Services, Melbourne, Australia.

Walker, S., Rohde, M. and Condron, R.J. (1999) The Australian National Quality Assurance Program. In: *Proceedings of the 6th International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 565–570.

Western Australia Department of Agriculture (2004) *Ovine Johne's Disease – the Disease*. Bulletin 4630. Western Australia Department of Agriculture, Perth, Australia.

Whittington, R.J. and Taragel, C.A. (2000) *Cross Species Transmission of Ovine Johne's Disease – Phase 1 – Final Report, Project OJD.005*. Meat and Livestock Australia, Sydney, Australia.

Whittington, R., Marsh, I., Choy, E. and Cousins, D. (1998) Polymorphisms in IS1311, an insertion sequence common to *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species. *Molecular and Cellular Probes* 12, 349–358.

Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J. and Fraser, C.A. (1999) Evaluation of modified BACTEC 12B radiometric medium and solid media for the culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *Journal of Clinical Microbiology* 37, 1077–1083.

Whittington, R.J., Fell, S., Walker, D., McAllister, S., Marsh, I., Sergeant, E., Taragel, C.A., Marshall, D.J. and Links, I.J. (2000a) Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. *Journal of Clinical Microbiology* 38, 2550–2556.

Whittington, R.J., Hope, A.F., Marshall, D.J., Taragel, C.A. and Marsh, I. (2000b) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS*900* restriction fragment length polymorphism and IS*1311* polymorphism analysis of isolates from animals and a human in Australia. *Journal of Clinical Microbiology* 38, 3240–3248.

Whittington, R.J., Taragel, C.A., Ottaway, S., Marsh, I., Seaman, J. and Fridriksdottir, V. (2000c) Molecular epidemiological confirmation of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. *Veterinary Microbiology* 79, 311–322.

29 Ruminant Aspects of Paratuberculosis Vaccination

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29.1 Introduction

The history of the use of vaccination for the control of paratuberculosis disease in ruminants dates back to 1926, when Vallee and Rinjard (1926) reported the use of live paratuberculosis bacilli adjuvanted in olive oil, paraffin and pumice powder. Subsequently, all paratuberculosis vaccines that have been used on a significant scale for the control of this disease have been whole bacilli from various species of *Mycobacterium avium* mixed with an oil adjuvant. While there have been differences between vaccines in the type of oil used, whether the bacilli were live or dead, and the strains of *M. avium* subsp. *paratuberculosis* (*MAP*) and *M. avium* employed, paratuberculosis vaccines have used the same basic formulation of whole bacilli in an oil adjuvant. Some of the differences in the whole-cell vaccines, such as the use of live bacilli versus killed bacilli, have not been sufficiently evaluated to determine whether or not they significantly affect the efficacy of paratuberculosis vaccines. What is apparent is that the immune responses to live *MAP* in an oil adjuvant are significantly different from those to the same organism in the absence of adjuvant (Begg and Griffin, 2005). Hence, considerable care should be exercised when comparing live *MAP* bacilli in oil vaccines with the live tuberculosis (TB) vaccine, *Mycobacterium bovis* BCG, which is used without an accompanying adjuvant.

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This review is a summary of the use of vaccines for the control of paratuberculosis. Most of the examples used in this review relate to whole *MAP* bacilli in oil vaccines and these provide a benchmark for the evaluation of the next generation of vaccines (see Huygen *et al.*, Chapter 30, this volume). As new-generation paratuberculosis vaccines will need to exceed the efficacy of *MAP* bacilli in oil vaccines to gain acceptance, in this regard, they will conceptually play a similar role to BCG, which serves as the reference point for new TB vaccines.

Over the years, there has been a very patchy uptake of vaccination for the control of paratuberculosis, which is a reflection of the significant deficiencies in the vaccines, as well as an unrealistic view of the cost-effectiveness of other control measures. What is apparent is that in some circumstances where other control measures have failed, current vaccines have proven to be cost-effective in reducing the production losses caused by *MAP*. For example, paratuberculosis was introduced into Iceland in 1933 with an importation of 20 Karakul sheep from Halle, Germany (Fridriksdottir *et al.*, 2000). This was followed by the observation of clinical cases of paratuberculosis in Icelandic sheep 5 years later (see Begg and Whittington, Chapter 15, this volume). Widespread vaccination of sheep in Iceland virtually eliminated clinical paratuberculosis, where prior to the vaccination programme the average annual mortality rate from *MAP* in adult sheep was 8–10%. Likewise, compulsory vaccination of goats in Norway was credited with the elimination of clinical paratuberculosis in that country (Saxegaard and Fodstad, 1985), and, more recently, vaccination has been used to help control clinical paratuberculosis in sheep in Australia (Reddacliff *et al.*, 2006).

In contrast to the usage of paratuberculosis vaccines in small ruminants, vaccination does not currently play a central role in any country for the control of this disease in cattle. Historically, extensive use of vaccination in cattle was reported to be effective in controlling clinical paratuberculosis in cattle in France (Vallee *et al.*, 1934), the Netherlands (Benedictus *et al.*, 2000) and the UK (Doyle, 1964). Reasons for discontinuing paratuberculosis vaccines in cattle included interference with skin testing for bovine TB, failure to prevent infection and the presence of large lesions at the inoculation site.

29.2 Characteristics of Vaccines Desired by Farmers/Veterinarians

The essential characteristic of a paratuberculosis vaccine is that it reduces the production losses caused by *MAP* (i.e. reduced disease without preventing infection). However, the ideal paratuberculosis vaccine might also prevent animals from becoming infected and/or serve as an immunotherapeutic when inoculated into already-infected animals (Table 29.1).

Table 29.1. Characteristics of whole-bacilli-in-oil vaccines, contrasted with a hypothetical, ideal *MAP* vaccine.

Vaccine characteristic	Whole-bacilli-in-oil vaccine	Ideal *MAP* vaccine
Prevent clinical disease in uninfected animals	Yes	Yes
Prevent clinical disease in already-infected animals	Yes, in early stages of infection	Yes
Prevent establishment of infection	No	Yes
Injection site lesions	Yes	No
Injury resulting from self-inoculation of vaccine	Yes	No
Cause false-positive responses for immune-based tests for bovine TB	Yes	No
Cause false-positive responses for immune-based tests for *MAP*	Yes	No

Furthermore, the ideal vaccine would have no adverse side effects, such as injection site lesions, false-positive reactions to immunodiagnostic assays for bovine TB and paratuberculosis or injury resulting from self-inoculation. Such an ideal vaccine would have the potential to eradicate *MAP* from a herd or flock. The current whole-bacilli-in-oil paratuberculosis vaccines fall considerably short of being ideal, but this set of desirable characteristics will be the ultimate goal of researchers developing the next generation of vaccines.

29.3 Measures of Protection

Measures of vaccine efficacy have been based on the following.

1. Clinical disease, including mortality and production parameters such as milk production, productive life, wool production and numbers of offspring raised. Positive cost–benefits of vaccination against paratuberculosis accrue from a reduction or elimination of clinical disease caused by *MAP*. A retrospective analysis of a vaccination trial in the Netherlands demonstrated a positive cost–benefit from vaccination against *MAP* of US$142 per cow arising from reduced clinical disease (van Schaik *et al.*, 1996). Modelling studies have been used to compare the relative cost–benefits of vaccination with other control measures. Groenendaal and Galligan (2003) modelled the economic consequences of control programmes for mid-sized dairy farms in the USA and found that vaccination but not test-and-cull strategies were economically attractive. While subclinical paratuberculosis can result in reduced productivity, these losses are minor compared with those accruing from clinical disease.

2. Faecal shedding is the most practical test for ante-mortem evaluation of the efficacy of paratuberculosis vaccines (Kalis *et al.*, 2001). The most valuable information comes not only from determining whether an animal is faecal-culture-positive but also by obtaining an estimate of the level of shedding (Eppleston *et al.*, 2005; Reddacliff *et al.*, 2006). Sheep with multi-bacillary paratuberculosis have been shown to excrete over 10^8 bacilli per g of faeces (Whittington *et al.*, 2000). This level of excretion is several orders of magnitude greater than that found in cattle with paratuberculosis (Jorgensen, 1982). The importance of the multi-bacillary animals, or supershedders as they are sometimes referred to, is that, although they are only a small proportion of the infected animals, they provide the overwhelming majority of environmental contamination in flocks and herds. Vaccination has been observed to delay the onset of faecal shedding in sheep (Reddacliff *et al.*, 2006), and ideally evaluation of a paratuberculosis vaccine should include faecal culturing on multiple occasions.

Post-mortem evaluation, including assessment of microscopic and macroscopic lesions and bacterial load. Ideally, a post-mortem evaluation of animals in paratuberculosis vaccine trials should not only determine whether an animal is infected but also provide an estimate of the level of infection and an assessment of the macro- and microscopic lesions (Hines *et al.*, 2007a). Quantitative bacteriology is a useful measure of protection for the evaluation of vaccines against chronic infections such as TB and paratuberculosis, where vaccination immunity is often not sufficient to completely prevent infection. Lesion-severity scoring systems have been developed for evaluating vaccines (Hines *et al.*, 2007b; Mackintosh *et al.*, 2008). The number of tissues examined from each animal will be determined by the number of animals in the vaccine trial and the time interval between challenge and the post-mortem examination. When the interval is as short as 50 days, up to 15 small intestinal tissues may need to be examined to reveal differences between vaccine groups (Uzonna *et al.*, 2003).

3. Immune correlates can be monitored. There are few studies of the protective immune response to *MAP*, in contrast to the vast amount of information of the immune response to other mycobacteria, especially members of the *Mycobacterium tuberculosis* complex. The expectation is that the protective immune response to *MAP* will be similar to that seen in TB. In both diseases, the causative

organism resides in macrophage and cellular immune responses are observed. While gamma interferon plays a central role in immunity, it has been shown that on its own it is not a reliable correlate of protection in TB (Wedlock *et al.*, 2007). While there are some imprecise immune correlates of vaccine protection against TB, there are no established surrogates that can be used as a substitute for bacteriological and/or histopathological assessment. For instance, although delayed-type hypersensitivity responses mediated by T lymphocytes are a prominent feature of mycobacterial infection (including BCG vaccinations), these responses do not reliably predict protection in mycobacterial diseases, such as TB or leprosy (Fine *et al.*, 1994). Whole mycobacterial bacilli-in-oil adjuvants are a potent stimulator of a range of immune responses, including adaptive immune responses and potentially innate immune activation. Wentink and colleagues (1993) observed that cattle inoculated with a *MAP*-in-oil vaccine that subsequently were skin-test-negative to johnin were more likely to have post-mortem evidence of paratuberculosis compared with animals that were skin-test-positive. Whether these findings point to aspects of the vaccination process or host-dependent resistance requires further study.

29.4 Assessing Vaccine Efficacy

Thorough evaluation of vaccines against paratuberculosis is technically challenging, time consuming and very expensive. Consequently, there are few reported studies where the efficacy of paratuberculosis vaccines has been well documented. In some studies, it has been difficult to distinguish possible vaccine effects from benefits obtained from the implementation of other management practices designed to reduce the exposure to *MAP*.

Vaccine efficacy can be assessed using experimental challenge models and field trials based on natural challenge. The advantages and disadvantages of the two approaches are summarized in Table 29.2. Vaccination in experimental challenge models provides a means for identifying the best candidates and these can subsequently be assessed in a field trial.

Table 29.2. Determination of vaccine efficacy by experimental challenge and field assessment.

	Vaccination/ experimental challenge	Field assessment
Challenge with *MAP*	High dose of organisms required. The challenge regime may not adequately replicate the conditions occurring with natural infection.	Natural challenge. The level of challenge may vary from herd to herd (flock to flock).
Numbers of animals required for vaccine assessment	Possible to assess vaccines using relatively small numbers of animals (e.g. 6–10 animals/ treatment group).	Much larger numbers of animals required. Often need to carry out the field assessment on multiple herds/flocks.
Measure of protection	Because small numbers of animals can be used, vaccine efficacy can be based on detailed post-mortem findings, such as bacteriological assessment of multiple tissues.	Because large numbers of animals are required, vaccine efficacy assessed on clinical disease and/or faecal shedding.
Duration of efficacy study	Can be relatively short (3–6 months) and based on detailed post-mortem findings.	Duration of study needs to be long enough to ensure there is time for the development of clinical disease (2–5 years).

29.5 Synergistic Effects of Vaccination and Management Procedures

There are reports that, in a few cattle herds, cases of clinical paratuberculosis continued among vaccinated stock (Spears, 1959). In a retrospective study of vaccinated herds in the UK, Wilesmith (1982) reported that clinical cases of paratuberculosis occurred in 9.7% (17) of herds after 10 years of vaccination use. In contrast, 37.7% (66) of the study herds became free of clinical disease in the first year following vaccination, and by 6 years after the start of vaccination 83% (146) of the herds had obtained freedom from clinical disease. The reasons for the failure of vaccination are unknown but could include: very heavy challenge with *MAP*; low standards of husbandry, hygiene or nutrition; and misuse of the vaccine. The provision of piped water supplies (as opposed to water from ponds and ditches) and the culling of offspring of clinical cases were found to be associated with a reduced time taken for the extinction of clinical disease (Wilesmith, 1982). Modelling studies by Groenendaal and Galligan (2003) predict that improved calf-hygiene strategies are critically important in every paratuberculosis control programme, including vaccination.

29.6 Vaccination Schedules

The schedule of vaccination will depend on the disease process of paratuberculosis, systems used for managing the hosts and the characteristics of the vaccine. There is a significant, albeit imperfect, body of evidence to show that the greater the age of animals when first exposed to infection with *MAP*, the less likely they are to develop clinical disease (Hines *et al.*, 2007a). Exposure to *MAP* can occur immediately after birth, before it is practically possible to vaccinate animals. A further challenge for controlling paratuberculosis by vaccination is the occurrence of intrauterine infection, documented in cattle (Whittington and Windsor, 2009), sheep (Lambeth *et al.*, 2004) and farmed deer (Thompson *et al.*, 2007). While the clinical significance of *in utero* infection with *MAP* is unknown, it highlights a possible situation whereby the ability for vaccination to protect animals from paratuberculosis is compromised. Whether vaccination of the dam can be used to prevent the spread of *MAP* to the fetus is unknown. For vaccination to be successful in controlling paratuberculosis, it must protect young animals, especially in the first year of life. While there is limited evidence as to the protective immune responses needed against paratuberculosis, it would appear that these will predominantly be cell-mediated. In contrast, antibody responses are very unlikely to play a significant role in immune protection against paratuberculosis. Thus, passive antibody from the mother in colostrum will not protect her offspring and is unlikely to be a determining factor when developing a vaccination schedule for paratuberculosis.

29.7 Duration of Immunity and Revaccination

There are no robust data on the duration of immunity generated by paratuberculosis vaccines. Difficulties arise in trying to assess this because a significant reduction in vaccine immunity may be offset by animals becoming naturally more resistant as they become older. Revaccination of cattle was extensively practised in France, with the recommendation that it be carried out when the vaccination nodule regressed, at 12–18 months of age (Vallee *et al.*, 1934). In the early investigations of paratuberculosis vaccines in cattle in the UK, revaccination was often carried out, usually once, but two to three times in some herds (Doyle, 1964). The benefits of revaccination have not been well documented and experimental investigations indicate that it may enhance the development of clinical disease. Stuart (1965) observed significant protection against clinical paratuberculosis in calves vaccinated once at 1 week old but not in another group which were vaccinated at 1 week of age and revaccinated 1, 2.5 and 4 years later. Comparable experiments carried out in sheep with revaccination 11 months after the initial vaccination did not increase the immunity to

experimental challenge and may have led to a deleterious outcome (Gilmour and Angus, 1973). Current recommendations for whole-bacilli-in-oil paratuberculosis vaccines are that they are only used once.

29.8 Therapeutic Effects of Vaccination

There is both field and experimental evidence to show that the whole-bacilli-in-oil vaccines induce some protection against the development of clinical disease when administered to animals likely to have already been exposed to *MAP*. The initial vaccination trials carried out on Icelandic sheep showed a significant reduction in clinical disease when animals were vaccinated at 8–10 months of age (Sigurdsson, 1952). Subsequently, vaccination was successfully carried out on a large scale in Iceland on 4–6-month-old lambs, an age when at least some of them would have already been infected with *MAP* (Fridriksdottir *et al.*, 2000). In an experimental challenge trial, Gwozdz and colleagues (2000) observed a reduced mycobacterial burden in sheep vaccinated with a whole-bacilli-in-oil vaccine 2 weeks after oral challenge with *MAP*. The mechanism of the therapeutic effects of paratuberculosis vaccination is unknown and could be due to stimulation of both innate and adaptive immune responses. These observations of a therapeutic effect of paratuberculosis vaccination are in contrast to the lack of any effect of BCG on TB. A major concern has been raised that post-exposure vaccines for TB may have serious adverse effects by producing an inappropriate immune response, such as the 'Koch reaction', with necrosis and bacterial dissemination (Orme, 2006). There is no evidence to indicate whether or not a similar phenomenon will be observed with paratuberculosis vaccines.

29.9 Host Differences and *MAP* Subtypes

There are some differences between hosts in their susceptibility to paratuberculosis, which may affect the ability of vaccines to protect against this disease. Outbreaks of fatal para tuberculosis occur in farmed deer that are 8–15 months old, which is significantly earlier than that observed in cattle and sheep (Mackintosh *et al.*, 2004). Whether or not there is a relationship between the susceptibility of a host to *MAP* and the effectiveness of vaccination is unknown. While there has been a suggestion that whole-bacilli-in-oil vaccines are more efficacious in small ruminants than in cattle, there is limited evidence to support this view. If such a difference does exist it may relate to the distribution of subtypes of *MAP* in different hosts. There are two major subtypes of *MAP*, one of which predominates in cattle and the other in sheep (Collins *et al.*, 1990). The phenotypic importance of these strains of *MAP* is discussed elsewhere (see Stevenson, Chapter 12, this volume).

29.10 Interference with Ante-mortem Tests for Bovine TB

MAP shares many antigens in common with other mycobacterial species, including *M. bovis,* the cause of bovine TB. This is evidenced by cattle and farmed deer vaccinated with whole-cell-in-oil vaccines against *MAP* that have positive responses on skin testing to both avian and bovine tuberculins (Hebert *et al.*, 1959; Mackintosh *et al.*, 2005). Vaccine-induced tuberculin reactivity can persist for long periods, with positive caudal-fold skin tests to bovine tuberculin recorded in 45% of 68 calves that were inoculated 2 years previously at 2–4 weeks of age with a live *MAP* bacilli-in-oil vaccine (Milestone, 1989). In animals inoculated with whole *MAP* bacilli vaccines, the response to the avian tuberculin is normally greater than that to the bovine tuberculin, and use of a comparative skin test will identify most paratuberculosis-vaccinated cattle that do not have bovine TB as having non-specific skin-test responses. In contrast, caudal-fold skin testing using only bovine tuberculin will result in large numbers of vaccinated animals giving false-positive reactions. Currently antibody tests are routinely used in farmed deer, but not cattle, for the diagnosis of bovine TB.

Studies by Mackintosh and colleagues (2008) clearly showed that vaccination with bacilli-in-oil vaccines significantly interferes with the performance of the serological test currently being used in New Zealand for the diagnosis of bovine TB in farmed deer.

In most countries, control of bovine TB has taken priority over paratuberculosis. Consequently, the use of paratuberculosis vaccination in cattle has been restricted and tightly regulated in those countries with compulsory bovine TB eradication programmes. The use of live *MAP* vaccines is contraindicated in cattle and farmed deer herds that are infected with *M. bovis*. A limited amount of experimental evidence indicates that it is difficult to detect *M. bovis* infection in cattle and farmed deer in vaccinated herds (Ritchie *et al.*, 1952; Inglis and Weipers, 1963; C.G. Mackintosh, personal communication). The apparently successful *MAP* vaccination programme in France was abandoned because it interfered with the eradication of bovine TB (Vallee *et al.*, 1934).

29.11 Interference with Immune-based Tests for Paratuberculosis

Inoculation with whole *MAP* bacilli-in-oil vaccines induces an immune response that will result in positive reactions being recorded in immune-based tests for paratuberculosis (Muskens *et al.*, 2002). Cattle have been shown to respond positively in one or more routine diagnostic tests for paratuberculosis for many years after vaccination (Muskens *et al.*, 2002). Consequently, the currently available paratuberculosis vaccines preclude control programmes using the combined approach of vaccination and the identification of the more heavily infected animals with immune-based tests. Furthermore, immune-based tests cannot be used to determine the *MAP* infection status of animals that farmers may wish to move out of infected herds or flocks. Given that vaccination does not prevent infection with *MAP*, vaccinated animals should not be moved to uninfected flocks or herds.

29.12 Conclusions

There are still no ideal, cost-effective methods for the control of paratuberculosis. Eradication of *MAP* from flocks and herds using a test-and-slaughter approach is extremely difficult with the currently available diagnostic tests, and even if achievable will take many years. Furthermore, control schemes for paratuberculosis based on this approach are unlikely to be cost-effective, especially in the absence of financial support from the state. While the only available paratuberculosis vaccines are those based on whole *MAP* bacilli in oil, their use is likely to be predominantly in small ruminants. If new vaccines can be developed to overcome at least some of the deficiencies of the whole *MAP* bacilli-in-oil formulations, vaccination has the potential to be an attractive, cost-effective method for the control of paratuberculosis in all hosts, including cattle.

References

Begg, D.J. and Griffin, J.F. (2005) Vaccination of sheep against *M. paratuberculosis*: immune parameters and protective efficacy. *Vaccine* 23, 4999–5008.

Benedictus, G., Verhoeff, J., Schukken, Y.H. and Hesselink, J.W. (2000) Dutch paratuberculosis programme history, principles and development. *Veterinary Microbiology* 77, 399–413.

Collins, D.M., Gabric, D.M. and de Lisle, G.W. (1990) Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. *Journal of Clinical Microbiology* 28, 1591–1596.

Doyle, T.M. (1964) Vaccination against Johne's disease. *Veterinary Record* 76, 73–77.

Eppleston, J., Reddacliff, L., Windsor, P., Links, I. and Whittington, R. (2005) Preliminary observations on the prevalence of sheep shedding *Mycobacterium avium* subsp. *paratuberculosis* after 3 years of a vaccination programme for ovine Johne's disease. *Australian Veterinary Journal* 83, 637–638.

Fine, P.E., Sterne, J.A., Ponnighaus, J.M. and Rees, R.J. (1994) Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity. *Lancet* 344, 1245–1249.

Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S. and Gudmundsdottir, K.B. (2000) Paratuberculosis in Iceland: epidemiology and control measures,

past and present. *Veterinary Microbiology* 77, 263–267.

Gilmour, N.J.L. and Angus, K.W. (1973) Effect of revaccination on *Mycobacterium johnei* infection in sheep. *Journal of Comparative Pathology* 83, 437–445.

Groenendaal, H. and Galligan, D.T. (2003) Economic consequences of control programs for paratuberculosis in midsize dairy farms in the United States. *Journal of the American Veterinary Medical Association* 223, 1757–1763.

Gwozdz, J.M., Thompson, K.G., Manktelow, B.W., Murray, A. and West, D.M. (2000) Vaccination against paratuberculosis of lambs already infected experimentally with *Mycobacterium avium* subsp. *paratuberculosis*. *Australian Veterinary Journal* 78, 560–566.

Hebert, C.N., Doyle, T.M. and Paterson, A.B. (1959) Tuberculin sensitivity in attested cattle vaccinated against Johne's disease. *Veterinary Record* 71, 108–111.

Hines, M.E., Stabel, J.R., Sweeney, R.W., Griffin, F., Talaat, A.M., Bakker, D., Benedictus, G., Davis, W.C., de Lisle, G.W., Gardner, I.A., Juste, R.A., Kapur, V., Koets, A., McNair, J., Pruit, G. and Whitlock, R.H. (2007a) Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Veterinary Microbiology* 122, 197–222.

Hines, M.E., Stiver, S., Giri, D., Whittington, L., Watson, C., Johnson, J., Musgrove, J., Pence, M., Hurley, D., Baldwin, C., Gardner, I.A. and Aly, S. (2007b) Efficacy of spheroplastic and cell-wall component vaccines for *Mycobacterium avium* subsp. *paratuberculosis* in experimentally-challenged baby goats. *Veterinary Microbiology* 120, 261–283.

Inglis, J.S.S. and Weipers, M. (1963) The effect of Johne's vaccination on the efficacy of the single intradermal comparative tuberculin test. *British Veterinary Journal* 119, 426–429.

Jorgensen, J.B. (1982) An improved method for culture of *Mycobacterium paratuberculosis*. *Acta Veterinarinia Scandanavia* 23, 325–335.

Kalis, C.H., Hesselink, J.W., Barkema, H.W. and Collins, M.T. (2001) Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. *American Journal of Veterinary Research* 62, 270–274.

Lambeth, C., Reddacliff, L.A., Windsor, P., Abbott, K.A., McGregor, H. and Whittington, R.J. (2004) Intrauterine and transmammary transmission of *Mycobacterium avium* subsp. *paratuberculosis* in sheep. *Australian Veterinary Journal* 82, 504–508.

Mackintosh, C.G., de Lisle, G.W., Collins, D.M. and Griffin, J.F. (2004) Mycobacterial diseases of deer. *New Zealand Veterinary Journal* 52, 163–174.

Mackintosh, C.G., Labes, R.E. and Griffin, J.F. (2005) The effect of Johne's vaccination on tuberculin testing in farmed red deer (*Cervus elaphus*). *New Zealand Veterinary Journal* 53, 216–222.

Mackintosh, C.G., Labes, R.E., Thompson, B.R., Clark, R.G., de Lisle, G.W., Johnstone, P.D. and Griffin, J.F.T. (2008) Efficacy, immune responses and side-effects of vaccines against Johne's disease in young red deer (*Cervus elaphus*) experimentally challenged with *Mycobacterium avium* subsp. *paratuberculosis*. *New Zealand Veterinary Journal* 56, 1–9.

Milestone, B.A. (1989) Vaccination of cattle against Johne's disease – further observations. *Surveillance* 16(4), 22–23.

Muskens, J., van Zijderveld, F., Eger, A. and Bakker, D. (2002) Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. *Veterinary Microbiology* 86, 269–278.

Orme, I. (2006) Safety issues regarding new vaccines for tuberculosis, with an emphasis on post-exposure vaccination. *Tuberculosis* 86, 68–73.

Reddacliff, L., Eppleston, J., Windsor, P., Whittington, R. and Jones, S. (2006) Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Veterinary Microbiology* 115, 77–90.

Ritchie, J.N., Robertson, A. and Muir, R.O. (1952) Response to the single intradermal comparative test in animals sensitized by injection with *M. johnei* and by the bovine type of *M. tuberculosis*. *State Veterinary Journal, London* 7, 28–37.

Saxegaard, F. and Fodstad, F.H. (1985) Control of paratuberculosis (Johne's disease) in goats by vaccination. *Veterinary Record* 116, 439–441.

Sigurdsson, B. (1952) Vaccination against paratuberculosis (Johne's disease). *Journal of Immunology* 68, 559–565.

Spears, H.N., III (1959) Vaccination against Johne's disease: the results of a field trial experiment. *Veterinary Record* 71, 1154–1156.

Stuart, P. (1965) Vaccination against Johne's disease in cattle exposed to experimental infection. *British Veterinary Journal* 121, 289–318.

Thompson, B.R., Clark, R.G. and Mackintosh, C.G. (2007) Intra-uterine transmission of *Mycobacterium avium* subsp. *paratuberculosis* in subclinically affected deer (*Cervus elaphus*). *New Zealand Veterinary Journal* 55, 308–313.

Uzonna, J.E., Chilton, P., Whitlock, R.H., Habecker, P.L., Scott, P. and Sweeney, R.W. (2003) Efficacy of commercial and field-strain *Mycobacterium*

paratuberculosis vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* 21, 3101–3109.

Vallee, H. and Rinjard, P. (1926) Etudes sur l'enterite paratuberculeuse des bovides (note préliminaire). *Revue Générale de Médécine Vétérinaire* 35, 1–9.

Vallee, H., Rinjard, P. and Vallee, M. (1934) Sur la prémonition de l'entérite paratuberculose des bovides. *Revue Générale de Médécine Vétérinaire* 43, 777–779.

van Schaik, G., Kalis, C.H., Benedictus, G., Dijkhuizen, A.A. and Huire, R.B. (1996) Cost–benefit analysis of vaccination against paratuberculosis in dairy cattle. *Veterinary Record* 139, 624–627.

Wedlock, D.N., Denis, M., Vordermeier, H.M., Hewinson, R.G. and Buddle, B.M. (2007) Vaccination of cattle with Danish and Pasteur strains of *Mycobacterium bovis* BCG induce different levels of IFN gamma post-vaccination, but induce similar levels of protection against bovine tuberculosis. *Veterinary Immunology and Immunopathology* 118, 50–58.

Wentink, G.H., Bongers, J.H., Vos, J.H. and Zeeuwen, A.A.P.A. (1993) Relationship between negative skin test with Johnin after vaccination and post mortem findings. *Veterinary Record* 132, 38–39.

Wilesmith, J.W. (1982) Johne's disease: a retrospective study of vaccinated herds in Great Britain. *British Veterinary Journal* 138, 321–331.

Whittington, R.J. and Windsor, P.A. (2009) *In utero* infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. *The Veterinary Journal* 179, 60–69.

Whittington, R.J., Reddacliff, L.A., Marsh, I., McAllister, S. and Saunders, V. (2000) Temporal patterns and quantification of excretion of *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Australian Veterinary Journal* 78, 34–37.

30 Development of New Paratuberculosis Vaccines

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30.1 Introduction

Infection of ruminants, particularly cattle, by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is common in most countries and leads to sufficient clinical paratuberculosis to result in significant economic losses (Ott *et al.*, 1999; DEFRA, 2008). Most national control programmes for paratuberculosis place priority on the reduction of clinical disease, because eradication of infection remains unrealistic. This is because the insensitivity of diagnostic tests (Barrington *et al.*, 2003) enables a proportion of subclinical animals to remain as a continuing source of herd infection (Tiwari *et al.*, 2006; Nielsen and Toft, 2008). Against this background, the implementation of large-scale vaccination programmes appears an attractive alternative. However, the provision of an effective *MAP* vaccine has been frustratingly elusive. Despite 80 years of vaccine development, no formulation has yet been found that can protect all animals against disease, let alone prevent shedding from subclinical animals or provide total protection from infection. Current vaccines often also cause large granulomas at the inoculation site (Windsor and Eppleston, 2006) and induce cross-reactivity to tuberculin screening tests, which provides a significant disincentive towards their usage, particularly in cattle (Rosseels and Huygen, 2008). These issues are discussed in more detail elsewhere (see de Lisle, Chapter 29, this volume).

The principal reasons behind these failures are still far from clear but stem from the

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exquisite ability of *MAP* to avoid immune recognition, establish long-term intracellular persistence and dysregulate immune responses. It is probable that factors inherent within vaccine formulations have reduced appropriate induction of the early innate and adaptive cellular mechanisms essential to clear *MAP* effectively during primary infection, especially in particularly susceptible animals such as neonates (Larsen *et al.*, 1975). This lack of clearance allows development of long-term *MAP* persistence in the host and sometimes induces suppressive immune responses that lead to clinical disease. Suppression can also interfere with expansion of vaccine-primed, long-term protective mechanisms, which, although sufficient to contain clinical manifestations in some animals, remain ineffective against intestinal infection and shedding (Coussens, 2004). Factors attributable to these failures include the use of vaccine strains that are not *MAP* (Uzonna *et al.*, 2003); the inability to present immunodominant, *MAP*-specific antigens; the use of insufficiently modified *MAP* vaccine strains that allow mycobacterial mechanisms that subvert immune responses to remain active; the use of vaccines that induce major humoral responses that are not protective; and the use of modes of antigen delivery that fail to promote correct Th1 imprinting and immune memory. Some of these issues are discussed in more detail elsewhere (see Stabel, Chapter 21, this volume).

The general international trend in vaccine development has progressed from live attenuated strains to killed strains and then to subunit delivery. In the case of paratuberculosis, the first classical vaccination strategy using live *MAP* strains appeared promising (Vallée *et al.*, 1934), although accurate estimates of initial efficacy are difficult to establish from published data (Sigurdsson, 1956). These vaccine strains have now been lost, but it is likely that they were not markedly attenuated (Doyle, 1964) but instead relied upon subcutaneous compartmentalization of the inoculum in an oil and pumice-based adjuvant to optimize antigen presentation, inhibit growth and prevent dissemination. Subsequently, a series of live strains of UK origin were similarly used from the 1940s to 1980s. These were reportedly attenuated by serial passage on solid media (although the number of subcultures was never recorded) and were assessed for virulence by oral administration to a variety of hosts (Doyle, 1964; Stuart, 1965). While the details of these validations were sparse and did not include long-term follow-up, these vaccines were used widely and relatively successfully (Wilesmith, 1982). A mixture of the UK strains 316 and 2e was administered live in an oil-based emulsion to Icelandic lambs in the 1960s (Sigurdsson, 1960), reducing mortality from 11 to 0.8%, and to Norwegian goats in the 1960s and 1970s, reducing infection rates from 53 to 1% (Saxegaard and Fodstad, 1985). Both of these strains were derived from early 1960 stocks and were shown recently to contain genomic deletions and phenotypic characteristics associated with attenuation (T.H. Bull, 2009, unpublished results). In contrast, it is not clear if another of these UK strains, designated 316F, was ever attenuated. It originated from a 1970s subculture of vaccine stocks held at Weybridge VLA, UK and was subsequently used as the seed for various vaccine formulations. Strain 316F was used in the UK until 1978 and in the live commercial vaccine (Neoparasec) used in France and Hungary in the 1980s (Argent, 1991; Kormendy, 1994) and in New Zealand up until 2002 (Begg and Griffin, 2005). A recent subculture of 316F, originally obtained from Weybridge VLA, UK in 2001, has been shown to be as effective as the virulent reference strain K-10 in producing disseminated infection in BALB/c mice. Genomic studies of 316F and 316v, a similar Weybridge strain used for *MAP* ELISA testing in Australia from 1986 (Milner *et al.*, 1987), show them to have the same gene complement as the virulent reference strain K-10. Whether 316F can cause disease in ruminants remains to be tested.

Concerns with the use of live strains on grounds of health and safety, short shelf life and potential spread to the environment led to the introduction of heat-killed, whole-cell vaccines (Emery and Whittington, 2004). Strains used for these formulations have included the 316F strain grown in various types of liquid cultures given at different concentrations (Emery and Whittington, 2004), virulent *MAP* bison strains (Singh *et al.*, 2007) and *MAP* cell-wall-deficient preparations

(Hines *et al.*, 2007a). One commercial killed 316F vaccine (Gudair) has been trialled extensively and registered in a national control programme (Reddacliff *et al.*, 2006). Yet, even with a recent improvement of adjuvant and delivery (Silirum), killed whole-cell vaccines are unable to eliminate *MAP* infection from herds (Garrido *et al.*, 2007).

Subunit vaccines that incorporate immunodominant *MAP* proteins have been developed more recently and are discussed in detail in this chapter. Their use aims to avoid the induction of suppressive immune responses by whole-cell vaccine components and circumvent problems of cross-reactions with tuberculin in cattle. The recent availability of genomic (Wu *et al.*, 2007; Paustian *et al.*, 2008; Castellanos *et al.*, 2009) and proteomic (Leroy *et al.*, 2007; Hughes *et al.*, 2008) arrays have provided tools for identifying potential immunodominant targets, many of which are currently under investigation. More research in this area is undoubtedly required, as the longevity and type of immunological memory that these vaccines may induce are poorly understood and so far none of these subunit vaccines has been shown to be significantly better than killed vaccines. The notable recent success using whole-cell BCG, an attenuated *Mycobacterium bovis* strain, to prime with directed subunit boosting against *Mycobacterium tuberculosis* challenge (Hawkridge *et al.*, 2008) suggests that combinations of whole-cell and subunit strategies may also be applicable for paratuberculosis. New live attenuated strains of the *M. tuberculosis* complex produced by molecular genetic techniques are being investigated as improved replacements for BCG (Hoft *et al.*, 2008), and similar approaches are being investigated for producing new attenuated strains of *MAP* as candidate vaccines.

The complex balance of immunological responses required to achieve effective immunity from any *MAP* vaccine over long periods is particularly challenging. Humoral and mucosal immunity may have some impact, but the priming and maintenance of appropriate Th1 responses is crucial (Coussens, 2004; Vordermeier *et al.*, 2006). Differential responses to vaccination between neonates and adults suggest immunological maturity and sensitization from encounters with other mycobacteria may be important contributory factors and that vaccination approaches may need to be tailored to these variations in status. The size of *MAP* reservoirs also suggests that exposure to *MAP* is often almost inevitable and that even eradication programmes that include vaccination may not be successful unless the vaccine used also has a therapeutic effect. Current vaccines are aimed principally at prophylactic protection, although there is evidence that some delivery systems are suitable for treatment of infected animals (Bull *et al.*, 2007). The possibility that chronic *MAP* infection in humans may be involved in the development of Crohn's disease raises the possibility that a vaccine adapted for human use could provide an alternative direction for treatment, and such a vaccine would need to act therapeutically. However, the current goal in vaccine development is firmly focused on providing better protection of ruminants against disease and elimination of shedding from subclinically infected animals.

30.2 Live Vaccines

30.2.1 Attenuated strains of mycobacteria

As discussed in the introduction, the first vaccines were delivered live in aggressive adjuvants such as mineral oil or pumice, and because of this they stimulated a wide range of immunological responses, in a similar way to the dead whole vaccines that have replaced them. A recent attempt to induce protection using a non-adjuvanted live strain of *MAP* was unsuccessful (Begg and Griffin, 2005). This is contrary to the situation pertaining to the use of BCG in protecting against tuberculosis, where live BCG without an adjuvant gives very much better protection than dead BCG administered in a mineral oil adjuvant (Griffin *et al.*, 1999). One possible reason for the lack of success of non-adjuvanted live *MAP* vaccines is that the vaccine was not delivered in the most appropriate way, and if it had been delivered in a mild adjuvant such as a lipid matrix, as was done very recently, it

would give protection (Griffin *et al.*, 2009). Another possibility is that *MAP* strain 316F is so attenuated that by itself it does not survive in the host for long enough to stimulate a good immunological response. This situation applies in the case of tuberculosis vaccination, where *M. bovis* BCG is known to replicate for some time in a host and engenders a good protective response against tuberculosis (Andersen and Doherty, 2005), whereas strains of *M. bovis* that are too attenuated do not (Collins *et al.*, 2002).

The first slow-growing mycobacteria for which molecular genetic techniques were developed were strains of the *M. tuberculosis* complex, and many different approaches have now been used to produce attenuated live strains of these organisms with potential as candidate vaccines. These were produced by random mutagenesis, which was followed by a wide range of selection methods aimed at identifying a small number of mutants, which were subsequently tested for attenuation in animal models, and also by directed mutagenesis, where allelic exchange techniques are used to inactivate chosen genes (Collins, 2000). Additional genes to enhance immunogenicity have also been incorporated into the already attenuated *M. bovis* BCG, the world's most used vaccine (Andersen and Doherty, 2005), and the first of these vaccines are already in early human trials (Ly and McMurray, 2008). Similar approaches for producing attenuated strains of *MAP* have been achieved more recently (see Chacon and Barletta, Chapter 9, this volume).

The definition of virulence factors may provide targets for vaccine development. For this purpose, Cavaignac *et al.* (2000) produced a library of insertional mutants of *MAP* and screened them *in vitro* for properties such as auxotrophy that might correlate with attenuation. One of these auxotrophs, with an interruption in a hypothetical protein gene, has recently been found to be attenuated in both mice and goats (D.M. Collins, 2009, unpublished results). Instead of screening transposon mutants by laboratory methods, Shin *et al.* (2006) produced a library of insertional mutants of *MAP*, and used a high-throughput sequencing protocol to identify 288 disrupted genes. From 11 mutants selected for virulence testing in a mouse model, seven had deficient *in vivo* growth characteristics. Recent screening of *MAP* transposon mutants in cattle macrophages has also been used to identify potential virulence genes (Scandurra *et al.*, 2009).

In the first study reported of allelic exchange in *MAP*, three separate genes were successfully inactivated (K.T. Park *et al.*, 2008). Two of these genes were chosen because their homologues had already been shown to be important for virulence in the *M. tuberculosis* complex. The same rationale has also been used to choose several other genes that have very recently been inactivated in *MAP* (D.M. Collins, 2009, unpublished results). The concept of employing BCG as a vaccine vector to deliver *MAP* antigens in the context of a live mycobacterial strain is also being investigated. BCG itself appears to give some protection in mice against infection with *MAP* (Heinzmann *et al.*, 2008; Roupie *et al.*, 2008a), and this protection increased when BCG expressed a group of genes from an operon in a putative pathogenicity island in *MAP* (Heinzmann *et al.*, 2008).

Development of new live *MAP* strains as vaccines is still in the early stages and several important issues will need to be considered, similar to some of those pertaining to live tuberculosis vaccines (Kamath *et al.*, 2005), before the commercial stage is reached. Despite regulatory issues outlined in Section 30.5, and potential interference with diagnostic tests, the use of well-characterized *MAP* mutants as vaccines remains attractive, as they would be cheaper than subunit-based vaccines and might offer better protection.

30.2.2 Live vectors expressing subunits

The delivery of pathogen-specific subunit antigens by recombinant live heterologous vectors including attenuated bacteria and replication-deficient viruses is an alternative vaccination strategy already being trialled for use in both animal and human diseases (Xing *et al.*, 2005; Bejon *et al.*, 2007; Weyer *et al.*, 2007; Hawkridge *et al.*, 2008). The rationale for this approach relies on the properties of vectors that cause transient intracellular infection but

not disease and which naturally direct antigen processing towards cell-mediated responses. As this type of immune emphasis is thought to be essential for any successful *MAP* vaccine, live vector delivery systems may provide an attractive alternative to recombinant subunit preparations, which have the additional complication of requiring non-specific adjuvants or cytokine therapy to drive cell-mediated bias. Optimal live vector delivery regimens can require multiple vaccinations, but, because complete cycles of intracellular replication are not required for successful antigen delivery and presentation, repeated exposure to the same vector can potentially be used (Gabitzsch *et al.*, 2009). Vector combinations have included naked DNA and bacterial or viral priming, followed in each case by a viral vector boost. BCG used as a bacterial prime with the recombinant modified vaccinia Ankara virus (rMVA) as a boost provides some protection against *M. tuberculosis/M. bovis* infections in both humans and cattle (Vordermeier *et al.*, 2006; Tchilian *et al.*, 2009), suggesting that this type of approach may also be suitable for *MAP* vaccination. Since recombinant BCG for the delivery of *MAP* antigens would produce problems with cross-reactivity to tuberculin testing in cattle (Hope and Villarreal-Ramos, 2008), alternative priming vectors, such as attenuated *MAP* strains or recombinant human adenovirus, simian adenovirus or fowlpox, may be more appropriate for bovine vaccination against *MAP*. This field of research is still in its infancy, but initial studies in mice using a combination of *MAP* antigens delivered by replication-deficient human adenovirus serotype 5 priming and rMVA boosting have shown some promise in both therapeutic and prophylactic regimens against *MAP* challenge (Bull *et al.*, 2007).

30.3 Subunit-based Vaccines

30.3.1 Introduction

The identification of immunodominant protein antigens inducing strong Th1-type immune responses during the first asymptomatic stage of the disease and the demonstration of their protective potential in experimental infection models (mouse and target species) is central to the development of subunit-based vaccines. If effective immunization of animals with recombinant proteins in adjuvant or with DNA vaccines encoding immunogenic antigens (or a combination of DNA and protein) can be achieved, this would overcome the interference issues linked to whole-cell-based vaccines. The entire genome sequence of the K-10 strain of *MAP* has recently become available (see Bannantine *et al.*, Chapter 10, this volume) and provides a tool for the identification of *MAP* antigens useful for more effective immunoprophylaxis (Li *et al.*, 2005).

30.3.2 Immunodominant Th1 antigens identified

SOD (superoxide dismutase, MAP2121c) is a 23 kD exported protein of virulent mycobacteria that is considered a virulence factor, as it interferes with macrophage bactericidal properties and has anti-apoptotic properties (Hinchey *et al.*, 2007). Vaccination of mice with recombinant *MAP* SOD protein was reported to induce a mixed Th1/Th2 response (IFN-γ, IL-6, TNF-α), significant antibody production and a delayed-type hypersensitivity reaction (Mullerad *et al.*, 2002a). In cattle, SOD is a strong stimulus for γδ T cells, thought to be important in the early stages of infection and in granuloma formation (Shin *et al.*, 2005).

Another antigen that might be involved in the innate immune response to *MAP* is MPP14, a 14 kD secreted *MAP* protein that can induce strong IFN-γ responses in both experimentally infected and uninfected calves. However, these responses may interfere with diagnostic testing using the IFN-γ test (Olsen and Storset, 2001).

The three members of the Ag85 complex, Ag85A (MAP1609c), Ag85B (MAP0126) and Ag85C (MAP3531c), are highly conserved proteins with mycolyl-transferase activity, which are present in all mycobacterial species and abundantly secreted in mycobacterial culture filtrate. The antigens from *M. tuberculosis* are

among the most promising vaccine candidates for human tuberculosis and are currently being tested in clinical trials both as a fusion protein (Ag85B–ESAT-6) and as a recombinant modified vaccinia Ankara virus encoding Ag85A in a BCG prime–MVA–Ag85A boost protocol (Ly and McMurray, 2008). In the case of *MAP*, strong T-cell responses (proliferation, IL-2 and IFN-γ) can be detected against Ag85A and Ag85B and to a lesser extent Ag85C in low- and medium-shedder animals but not in culture-negative cows, whereas IL-4 levels are very low (Shin *et al.*, 2005). *MAP* Ag85 antigens have also been reported as immunodominant in experimentally infected cattle and mice (Mullerad *et al.*, 2002b; Rosseels *et al.*, 2006a) and also when delivered to mice as DNA (Rosseels *et al.*, 2002).

Heat-shock proteins Hsp65 (GroEL) and Hsp70 (DnaK) can also induce specific immune responses in *MAP*-infected and *MAP*-vaccinated cattle. As with PPD responses, the *MAP* Hsp70-specific, cell-mediated immune responses decrease upon progression to the clinical stage of the disease. Hsp65 induces less prominent responses compared with Hsp70 but shows a similar pattern relative to disease stages (Koets *et al.*, 1999).

P22 (22 kD) is an exported *MAP* protein belonging to the LppX/LprAFG family of putative mycobacterial lipoproteins. IFN-γ responses against this protein were detected in sheep vaccinated with the live attenuated Neoparasec vaccine, and antibodies were detected by Western blot analysis in 10 out of 11 vaccinated sheep, in 2/2 clinically affected cows and in 11/13 subclinically infected cows (Dupont *et al.*, 2005). The P22 protein induces good IFN-γ and antibody responses in sheep when administered as recombinant protein in a water-in-oil emulsion (Rigden *et al.*, 2006). Another lipoprotein, the 19 kD MAP0261c, has been reported to stimulate strong humoral but weak IFN-γ production in infected cattle (Huntley *et al.*, 2005a).

The alkyl hydroperoxide reductases AhpC and AhpD are constitutively expressed by *MAP in vitro*, and homologous antigens can be detected in *M. tuberculosis* during exposure to oxidative stress but not in *M. avium* subsp. *avium*. In goats experimentally infected with *MAP*, antibodies against AhpC but not against AhpD could be detected and both these antigens elicited a strong IFN-γ response (Olsen *et al.*, 2000).

Two *MAP* proteins belonging to the PPE family, MAP1518 and MAP3184, elicit significant IFN-γ levels in macrophages of experimentally infected Holstein calves (Nagata *et al.*, 2005). These may have vaccine potential, as PE/PPE proteins are implicated as virulence factors in *M. tuberculosis* and a number of PE/PPE proteins of *M. tuberculosis* are promising tuberculosis vaccine candidates (Skeiky *et al.*, 2004; Romano *et al.*, 2008).

Further investigation of *MAP* proteins involved in host interactions during the early and asymptomatic stages of *MAP* infection is required. Recently, Wu *et al.* (2007) reported on the so-called stressome of *MAP*, characterized by gene expression profiling of *MAP* exposed to different stress conditions or shed in cow faeces. They identified a novel set of putative virulence genes, but to what extent these are also involved in early and asymptomatic cellular immune control remains to be determined. In this context, it is important to note that similar stress-induced proteins of *M. tuberculosis* are strongly recognized by T cells from healthy, PPD-positive donors (Leyten *et al.*, 2006) and also during persistent *M. tuberculosis* infection in mice (Roupie *et al.*, 2007).

30.3.3 Protein subunit candidates

Chen *et al.* (2008) immunized C57BL/6 mice with a recombinant 74F polyprotein, generated by the sequential linkage of three *MAP* ORFs, in monophosphoryl lipid A (MPL) (Table 30.1). There were antigen-specific IFN-γ responses and reductions in bacterial loads in liver, spleen and lymph nodes of vaccinated animals at week 12 post-challenge, but the differences from control animals were not so clear-cut at other times.

Koets *et al.* (2006) reported vaccination experiments with recombinant *MAP* Hsp70 protein mixed with dimethyl dioctadecyl

Table 30.1. Summary of protein and DNA vaccine candidates tested in animals.

Vaccine type	Other name(s)	Size kDa	Function	Tested species	Challenge strain	Dose and route of infection	Comments	Reference
Protein vaccines								
MAP3527–MAP1519 fusion protein: 17.6 kDa C-terminal fragment of MAP3527–MAP1519–14.6 kDa C-terminal of MAP3527	MAP 74F	74	MAP3527 (PepA) is a 34 kDa trypsin-like serine protease, containing C-terminal PDZ domain. MAP1519 is a hypothetical protein belonging to the PPE protein family.	C57BL/6 mice	*MAP* strain 66115-98 (from an infected cow)	10^9 CFU intraperitoneally 3 weeks after the last immunization	Immunized subcutaneously twice, 3 weeks apart, with 50 µg/animal of fusion protein in MPL. Control group received MPL alone. Reduction in bacterial loads in liver (12 weeks), spleen (8–16 weeks) and lymph nodes (12 weeks).	Chen *et al.*, 2008
MAP3840	Hsp70, dnaK	70	Chaperonin	40 female calves	*MAP* from infected cow	At least 2×10^4 CFU, orally; nine gavages over 21-day period	Immunized subcutaneously twice, 308 days apart, with 200 µg recombinant Hsp70 in DDA. Significantly reduced shedding of *MAP* in the faeces during 2 years following experimental challenge.	Koets *et al.*, 2006
MAP0216 MAP1609c MAP3531c MAP0187c	Ag85AA g85BAg 85C SOD	32 30 32 23	Antigen 85complex (Ag85) includes Ag85A, Ag85B and Ag85C, which are mycolyl-transferases involved in cell wall synthesis. SOD is superoxide dismutase.	24 calves	*MAP* strain 66115-98	1×10^7 CFU orally for 7 consecutive days, 4 weeks after the last immunization	Immunized subcutaneously with mix of 100 µg of each protein in MPL or intramuscularly with MPL + 100 µg bovine IL-12 DNA. Some protection induced but no significant differences between any vaccinated groups.	Kathaperumal *et al.*, 2008
DNA vaccines								
Mix of 26 antigens as pDNA; MAP numbers 0448, 1301, 1308, 2491,			The 26 genes present in more than one protective clone array are indicated. These genes coded for transport/binding, proline-rich	BALB/c mice	*MAP* strain 6112	1×10^8 CFU intraperitoneally 2 weeks after the last immunization	Immunized with 2 µg of pDNA pool (by gene gun) and boosted 3 weeks later. Identification of the protective	Huntley *et al.*, 2005b

(*Continued*)

Table 30.1. *continued*

Vaccine type	Other name(s)	Size kDa	Function	Tested species	Challenge strain	Dose and route of infection	Comments	Reference
3498c, 1239c, 1493c, 1912, 2239, 3049c, 3131, 3171c, 0047c, 1003c, 2191, 2192, 3737, 1796c, 1871c, 2171c, 2174c, 2175c, 2230c, 2604c, 3742, 3764c			antigens (PPE family), membrane proteins, macrophage cell entry proteins and mycobactin/ polyketide synthases				antigens in the DNA pool was not performed.	
Mix of pDNA encoding MAP0216 MAP1609c MAP3531c MAP0187c MAP2121c	Mix of Ag85A Ag85B Ag85C SOD MMP-1	 32 30 32 23 35	Antigen 85complex (Ag85) includes Ag85A, Ag85B and Ag85C, which are mycolyl-transferases involved in cell wall synthesis. SOD is superoxide dismutase. MMP-1 is major membrane protein of unknown function.	C57BL/6 mice	*MAP* isolate from an infected cow	1×10^9 CFU intravenously 3 weeks after the last immunization	Immunized intramuscularly with 50 µg of each DNA three times at 3-week intervals. A significant reduction in the bacterial burden in the spleen and liver compared with controls was found. Relative severity of the liver and spleen histopathology paralleled the *MAP* culture results.	S.-U. Park *et al*., 2008
MAP0586c (pDNA)		33	Transglycosylase	BALB/c and C57BL/6 mice	*MAP* ATCC 19698	2×10^6 CFU intravenously 6 weeks after the last immunization	Immunized intramuscularly with 100 µg DNA four times at 3-week intervals or three times with DNA and boosted with 50 µg of recombinant protein. Both DNA and DNA/protein combinations effectively reduced the number of bacteria in spleen, as determined by luminometry and CFU plating. DNA/protein combination was as effective as *M. bovis* BCG vaccine ($P > 0.05$) 8 weeks after challenge.	Roupie *et al*., 2008a
MAP3936 (pDNA) BCG3866c MAV0214	Hsp65 Ag85A Ag85A	65 32 32	GroEL-like type I chaperonin.	Lambs	*MAP* from a patient with Crohn's disease	2×10^9 CFU orally 3 months after the last vaccination	Immunized intramuscularly three times at 20-day intervals. Histopathology of post-mortem tissue sections revealed absence of lesions or bacteria in the groups vaccinated with the three DNA vaccine constructs.	Sechi *et al*., 2006

ammonium bromide (DDA) adjuvant in 40 female calves (Table 30.1). This vaccine significantly reduced shedding of bacteria in faeces during the 2 years following experimental challenge with an admittedly low dose (2×10^4 CFU). The vaccine enabled the serological differentiation of vaccinated from infected animals, as infection induced only weak Hsp70-specific antibodies. The interference of this vaccine with tuberculin skin testing has yet to be evaluated.

More recently, 5–10-day-old Holstein–Friesian calves were vaccinated with a mix of four recombinant proteins (Ag85A, Ag85B, Ag85C and SOD) administered subcutaneously in MPL or MPL+IL-12 adjuvant (Kathaperumal *et al.*, 2008) (Table 30.1). Vaccination induced strong antibody responses to all four proteins. Antigen-specific IL-2, IFN-γ and TNF-α were detected in vaccinated animals, but only after an oral challenge with 10^7 CFU of *MAP* strain 66115-98 given for 7 consecutive days. Although the 16 weeks between challenge and sacrifice was too short (and the infecting dose probably too low) to evaluate vaccine efficacy, culture results indicated a protective trend, with *MAP* isolated from 7/18 vaccinated calves and from 6/8 control animals.

30.3.4 DNA vaccines

DNA vaccines are very effective (particularly in small rodents) in inducing humoral and cellular immune responses needed for protection against intracellular mycobacterial pathogens (Huygen, 2005, 2006) and are already available for some veterinary viral diseases (Meeusen *et al.*, 2007). These vaccines do not require a cold chain and are very stable. Experimentally, large numbers of DNA vaccine candidates can be easily produced, as purification of the protein antigens is not required.

Using expression library immunization, Huntley *et al.* (2005b) reported on the protective potential of a plasmid mix encoding 26 *MAP* antigens that conferred significant protection on BALB/c mice against intraperitoneal challenge with 10^8 CFU of *MAP* (Table 30.1). Genes in the protective mix encoded transport/binding, membrane and virulence proteins and mycobactin/polyketide synthases, but further analysis of the respective antigens has not been performed to our knowledge.

The DNA vaccine potential of two proteins, MAP0586c and MAP4308c, previously identified by postgenomic and immunoproteomic analysis of the *MAP* secretome as novel serodiagnostic antigens has been recently evaluated (Leroy *et al.*, 2007). Immunization of mice with plasmid DNA encoding MAP0586c and MAP4308c induced strong Th1-type immune responses, whereas only DNA encoding MAP4308c stimulated antibody responses. *MAP*-infected BALB/c mice also generated strong MAP0586c-specific T-cell responses and could be partially protected against infection following DNA vaccination, indicating that this putative transglycosylase warrants further investigation (Roupie *et al.*, 2008a) (Table 30.1).

S.-U. Park *et al.* (2008) reported recently on immunization of C57BL/6 mice with a mix of five plasmids encoding Ag85A, Ag85B, Ag85C, SOD and a 35 kD protein, MAP2121c (Table 30.1). Mice were vaccinated three times and challenged by an intraperitoneal injection of 10^9 CFU of *MAP* 3 weeks after the second booster. This resulted in a significant reduction in the bacterial load in spleen and liver of vaccinated mice. Unfortunately, these results cannot be easily compared with a previous study by the same group using protein immunization (Chen *et al.*, 2008) as the counts are analysed differently.

Potency of DNA vaccines in larger animals and humans has usually been found to be considerably lower than in small rodents, but a recent study in sheep provided sufficiently encouraging results to continue optimizing this form of vaccination for paratuberculosis. DNA vaccines encoding Ag85A from *M. bovis* BCG and from *M. avium* subsp. *avium* and Hsp65 from *MAP* were evaluated in groups of five lambs each (Sechi *et al.*, 2006) (Table 30.1). Lambs were vaccinated intramuscularly three times (0, 20 and 40 days) from 5 months of age and challenged with 2×10^9 *MAP*, 3 months after the last vaccination. Histopathology of

post-mortem tissues after 1 year revealed no lesions in the three DNA-vaccinated groups, but lesions were readily observed in the control group.

30.4 Assessment of Vaccine Efficacy in Different Animal Models

Ruminant models are certainly required for the final testing of vaccines (see Hines, Chapter 19, this volume), and a few subunit candidates have already been tested in cattle and sheep (Sechi *et al.*, 2006; Kathaperumal *et al.*, 2008). However, testing of vaccine candidates in ruminants is very expensive, and mouse models are being used for initial testing (see Talaat, Chapter 20, this volume). Although the mouse is not a target species for Johne's disease, it is useful for testing vaccine candidates, owing to the wide range of immunological tools, the various genetic backgrounds of inbred strains and the low cost of purchase and maintenance (Hines *et al.*, 2007b). Protective efficacy in mice is generally demonstrated by comparing bacterial replication in naive versus vaccinated animals. Replication in liver and spleen is usually monitored by CFU plating but can also be monitored by luminometry using luminescent *MAP* transformed with a bacterial luciferase (Rosseels *et al.*, 2006b). Luminescent *MAP* has been used for testing whole-cell-based and subunit *MAP* vaccines in mice (Rosseels *et al.*, 2006b; Roupie *et al.*, 2008a) and also for investigating the role of the *Slc11a1* gene in innate resistance and susceptibility to *MAP* (Roupie *et al.*, 2008b).

30.5 Vaccine Regulatory and Production Issues

The successful introduction of a new *MAP* vaccine into the market will involve not only provision of clear and repeatable experimentation results demonstrating significant efficacy but also the need to establish regulated manufacturing processes that are capable of distributing a consistently safe and effective product. National Regulation Authorities (NRAs) are responsible for control of these processes. Their recommendations and licence requirements must be taken into account, even at the initial vaccine design stage, to stand any chance of obtaining market authorization, and this inevitably results in vaccine development being a long, complex and costly undertaking. NRAs were initially instigated to standardize methods in the light of tragic vaccine contamination incidents such as smallpox in 1901 (Lilienfeld, 2008), BCG in 1931 (Calmette, 1931) and polio in 1955 (Nathanson and Langmuir, 1995), and are now in place in all developed countries, as well as other countries who are licensing vaccine products as a pre-qualification for sale to United Nations agencies (Milstein and Belgharbi, 2004). However, despite many years of WHO guidelines (WHO, 1992), there is no international agreement on market authorization and this can lead to new vaccines being only regionally available.

International cooperation is also hindered by significant complexities in the way regulations are administered between countries. Usually two or three different agencies are involved in each country, and the agencies in different countries have different combinations of responsibilities and are governed by very different legislation. Controlled storage of master seed stocks of a stringently defined vaccine strain is mandatory in all countries to ensure consistent quality. Standard testing for strain purity and contamination that conforms to a country's good manufacturing practice (GMP) regulations is also required. In the European Union, for example, GMP is required for the 'active' antigen alone and for the finished vaccine product containing the 'active' antigen in combination with delivery organisms and/or adjuvant preparations (Heldens *et al.*, 2008). Regular testing of manufactured vaccine batches is needed throughout the designated shelf life to determine the degree of safety, potency and efficacy to the target species from single, repeated and up to tenfold overdoses. This includes a recommendation for an *in vivo* test to predict clinical efficacy, although this has not been possible in some cases and has never been applied to previous *MAP* vaccines. It remains to be shown that any such test would be applicable

to measure the activity of a new *MAP* vaccine, and demonstrations of immunological activity as a measure of potency may be more practical. Exceptionally, for some recombinant proteins and polysaccharide vaccines, physico-chemical testing that correlates well with biological effectiveness is an acceptable and preferred replacement (Milstein, 2004). This may also be applicable to certain *MAP* subunit vaccines.

Many countries require vaccines that comprise or derive from live genetically modified organisms (GMOs) to have additional assessments, particularly with reference to virulence and biosafety. In the European Union, for example, important factors include: zero transfer of genetic material from the vaccine into the host chromosome (EMeA, 2004); a minimum requirement to show genetic stability and an absence of reversion to virulence in a GMO during at least five consecutive passages within a designated test animal species (EMeA, 2007); no significant increase in capacity for survival or shedding over the parent wild-type organisms in either the environment or susceptible hosts; and an optimum route of administration, particularly relevant to orally administered vaccines.

The requirement for complete regulatory compliance has been escalated by the expectation of zero risk demanded from the public, and this has led to introduction of larger clinical trials and widening of the safety margins for GMO release. This has meant that older vaccines registered 10 years ago would not necessarily be successful in the current regulatory climate. As of 2008, for example, consent for GMOs that contain antibiotic markers will not be given. This may affect vaccines which use plasmids expressing immunologically active agents delivered via bacterial vectors such as BCG. Resistance to heavy metals may be acceptable, provided that this phenotype does not confer a fitness advantage on the GMO (Favre and Viret, 2006).

The current indication that live attenuated vaccine strains or replication-deficient delivery vectors may provide modes for improved vaccine efficacy has presented a new wave of applications for trials of GMOs in animals and humans. Concerns for the safe release of GMOs has prompted the need for NRAs to provide precise definitions of what constitutes a GMO whilst still allowing dynamic review. However, there is no universally accepted definition of a GMO, so, for example, *Salmonella typhi* Ty21a, which is an attenuated strain isolated after chemical and UV mutagenesis, is not a GMO in the European Community but is in Canada. Of additional interest, however, and a point that is still not clearly defined in the law of some countries, is the status of the recipient of a GMO or DNA vaccine and whether the actual act of vaccination demands that the recipient be reclassified as a GMO in itself. As an additional control on GMO release, some countries have also introduced the concept of a 'new organism'. New Zealand regards all organisms that were not present in the country immediately before 1998 or subsequently eradicated as 'new' and thus subject to special conditional release application (SCNZ, 2003). While none of the new generation of paratuberculosis vaccines has yet reached these regulatory hurdles, the hurdles will certainly ensure that it is many years before an entirely new *MAP* vaccine is licensed for use.

References

Andersen, P. and Doherty, T.M. (2005) The success and failure of BCG – implications for a novel tuberculosis vaccine. *Nature Reviews Microbiology* 3, 656–662.

Argent, G. (1991) Efficiency of vaccination and other control measures estimated by faecal shedding in a regional control program. In: Chiodini, R.J. and Kreeger, J. (eds) *Proceedings of the 3rd International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, pp. 495–498.

Barrington, G.M., Gay, J.M., Eriks, I.S., Davis, W.C., Evermann, J.F., Emerson, C., O'Rourke, J.L., Hamilton, M.J. and Bradway, D.S. (2003) Temporal patterns of diagnostic results in serial samples from cattle with advanced paratuberculosis infections. *Journal of Veterinary Diagnostic Investigation* 15, 195–200.

Begg, D.J. and Griffin, J.F. (2005) Vaccination of sheep against *M. paratuberculosis*: immune parameters and protective efficacy. *Vaccine* 23, 4999–5008.

Bejon, P., Ogada, E., Mwangi, T., Milligan, P., Lang, T., Fegan, G., Gilbert, S.C., Peshu, N., Marsh, K. and Hill, A.V. (2007) Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS ONE* 15, e707.

Bull, T.J., Gilbert, S.C., Sridhar, S., Linedale, R., Dierkes, N., Sidi-Boumedine, K. and Hermon-Taylor, J. (2007) A novel multi-antigen virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis*. *PLoS ONE* 2, e1229.

Calmette, A. (1931) Epilogue de la catastrophe de Lübeck. *Presse Médicale* 2, 17.

Castellanos, E., Aranaz, A., Gould, K.A., Linedale, R., Stevenson, K., Alvarez, J., Dominguez, L., de Juan, L., Hinds, J. and Bull, T.J. (2009) Discovery of stable and variable differences in the *Mycobacterium avium* subsp. *paratuberculosis* type I, II, and III genomes by pan-genome microarray analysis. *Applied and Environmental Microbiology* 75, 676–686.

Cavaignac, S.M., White, S.J., de Lisle, G.W. and Collins, D.M. (2000) Construction and screening of *Mycobacterium paratuberculosis* insertional mutant libraries. *Archives of Microbiology* 173, 229–231.

Chen, L.-H., Kathaperumal, K., Huang, C.-J., McDonough, S.P., Stehman, S., Akey, B., Huntley, J.F., Bannantine, J.P., Chang, C.-F. and Chang, Y.-F. (2008) Immune responses in mice to *Mycobacterium avium* subsp. *paratuberculosis* following vaccination with a novel 74F recombinant polyprotein. *Vaccine* 26, 1253–1262.

Collins, D.M. (2000) New tuberculosis vaccines based on attenuated strains of the *Mycobacterium tuberculosis* complex. *Immunology and Cell Biology* 78, 342–348.

Collins, D.M., Wilson, T., Campbell, S., Buddle, B.M., Wards, B.J., Hotter, G. and de Lisle, G.W. (2002) Production of avirulent mutants of *Mycobacterium bovis* with vaccine properties by the use of illegitimate recombination and screening of stationary phase cultures. *Microbiology* 148, 3019–3027.

Coussens, P.M. (2004) Model for immune responses to *Mycobacterium avium* subspecies *paratuberculosis* in cattle. *Infection and Immunity* 72, 3089–3096.

DEFRA (2008) Veterinary surveillance: disease profiles. Available at: www.defra.gov.uk/animalh/diseases/vetsurveillance/profiles/spjohnes.pdf (accessed 16 January 2009).

Doyle, T.M. (1964) Strains of *Mycobacterium johnei* used for the preparation of vaccine. *State Veterinary Journal* 19–20, 154–155.

Dupont, C., Thompson, K., Heuer, C., Gicquel, B. and Murray, A. (2005) Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Medical Microbiology* 54, 1083–1092.

EMeA (2004) Committee for Medicinal Products for Veterinary Use: EMEA/CVMP/004/04-FINAL: Guideline on live recombinant vector vaccines for veterinary use. Available at: http://www.emea.europa.eu/pdfs/vet/iwp/000404en.pdf (accessed 16 January 2009).

EMeA (2007) VICH Topic VL41. Guideline on target animal safety: examination of live veterinary vaccines in target animals for absence of reversion to virulence. Available at: http://www.emea.europa.eu/pdfs/vet/vich/105204enfin.pdf. (accessed 16 January 2009).

Emery, D.L. and Whittington, R.J. (2004) An evaluation of mycophage therapy, chemotherapy and vaccination for control of *Mycobacterium avium* subsp. *paratuberculosis* infection. *Veterinary Microbiology* 104, 143–155.

Favre, D. and Viret, J.-F. (2006) Biosafety evaluation of recombinant live oral bacterial vaccines in the context of European regulation. *Vaccine* 24, 3856–3864.

Gabitzsch, E.S., Xu, Y., Yoshida, L.H., Balint, J., Gayle, R.B., Amalfitano, A. and Jones, F.R. (2009) A preliminary and comparative evaluation of a novel Ad5 [E1-, E2b-] recombinant-based vaccine used to induce cell mediated immune responses. *Immunology Letters* 122, 44–51.

Garrido, J.M., Geijo, M.V., Molina, E., Sevilla, I., Plazaola, J.M., Puentes, E. and Juste, R.A. (2007) Preliminary evaluation of a field trial on the use of vaccination in dairy cattle farms with paratuberculosis. In: Nielsen, S.S. (ed.) *Proceedings of the 9th International Colloquium on Paratuberculosis*. International Society of Paratuberculosis, Madison, Wisconsin, www.paratuberculosis.org/pubs/proc9/section5.htm (accessed 16 January 2009).

Griffin, J.F., Mackintosh, C.G., Slobbe, L., Thomson, A.J. and Buchan, G.S. (1999) Vaccine protocols to optimise the protective efficacy of BCG. *Tubercle and Lung Disease* 79, 135–143.

Griffin, J.F.T., Hughes, A.D., Liggett, S., Farquhar, P.A., Mackintosh, C.G. and Bakker, D. (2009) Efficacy of novel lipid-formulated whole bacterial cell vaccines against *Mycobacterium avium* subsp. *paratuberculosis* in sheep. *Vaccine* 27, 911–918.

Hawkridge, T., Scriba, T.J., Gelderbloem, S., Smit, E., Tameris, M., Moyo, S., Lang, T., Veldsman, A., Hatherill, M., Merwe, L., Fletcher, H.A.,

Mahomed, H., Hill, A.V., Hanekom, W.A., Hussey, G.D. and McShane, H. (2008) Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa. *Journal of Infectious Diseases* 198, 544–552.

Heinzmann, J., Wilkens, M., Dohmann, K. and Gerlach, G.F. (2008) *Mycobacterium avium* subsp. *paratuberculosis*-specific *mpt* operon expressed in *M. bovis* BCG as vaccine candidate. *Veterinary Microbiology* 130, 330–337.

Heldens, J.G., Patel, J.R., Chanter, N., Ten Thij, G.J., Gravendijck, M., Schijns, V.E., Langen, A. and Schetters, T.P. (2008) Veterinary vaccine development from an industrial perspective. *The Veterinary Journal* 178, 7–20.

Hinchey, J., Lee, S., Jeon, B.Y., Basaraba, R.J., Venkataswamy, M.M., Chen, B., Chan, J., Braunstein, M., Orme, I.M., Derrick, S.C., Morris, S.L., Jacobs, W.R., Jr. and Porcelli, S.A. (2007) Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. *Journal of Clinical Investigation* 117, 2279–2288.

Hines, M.E., Stiver, S., Giri, D., Whittington, L., Watson, C., Johnson, J., Musgrove, J., Pence, M., Hurley, D., Baldwin, C., Gardner, I.A. and Aly, S. (2007a) Efficacy of spheroplastic and cell-wall competent vaccines for *Mycobacterium avium* subsp. *paratuberculosis* in experimentally-challenged baby goats. *Veterinary Microbiology* 120, 261–283.

Hines, M.E., Stabel, J.R., Sweeney, R.W., Griffin, F., Talaat, A.M., Bakker, D., Benedictus, G., Davis, W.C., de Lisle, G.W., Gardner, I.A., Juste, R.A., Kapur, V., Koets, A., McNair, J., Pruitt, G. and Whitlock, R.H. (2007b) Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Veterinary Microbiology* 122, 197–222.

Hoft, D.F., Blazevic, A., Abate, G., Hanekom, W.A., Kaplan, G., Soler, J.H., Weichold, F., Geiter, L., Sadoff, J.C. and Horwitz, M.A. (2008) A new recombinant bacille Calmette–Guérin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *Journal of Infectious Diseases* 198, 1491–1501.

Hope, J.C. and Villarreal-Ramos, B. (2008) Bovine TB and the development of new vaccines. *Comparative Immunology, Microbiology and Infection Diseases* 31, 77–100.

Hughes, V., Bannantine, J.P., Denham, S., Smith, S., Garcia-Sanchez, A., Sales, J., Paustian, M.L., McLean, K. and Stevenson, K. (2008) Proteome-determined *Mycobacterium avium* subspecies *paratuberculosis*-specific proteins: immunogenicity in ovine paratuberculosis. *Clinical and Vaccine Immunology* 15, 1824–1833.

Huntley, J.F., Stabel, J.R. and Bannantine, J.P. (2005a) Immunoreactivity of the *Mycobacterium avium* subsp. *paratuberculosis* 19-kDa lipoprotein. *BMC Microbiology* 5, 3.

Huntley, J.F., Stabel, J.R., Paustian, M.L., Reinhardt, T.A. and Bannantine, J.P. (2005b) Expression library immunization confers protection against *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infection and Immunity* 73, 6877–6884.

Huygen, K. (2005) Plasmid DNA vaccination. *Microbes and Infection* 7, 932–938.

Huygen, K. (2006) DNA vaccines against mycobacterial diseases. *Future Microbiology* 1, 63–73.

Kamath, A.T., Fruth, U., Brennan, M.J., Dobbelaer, R., Hubrechts, P., Ho, M.M., Mayner, R.E., Thole, J., Walker, K.B., Liu, M., Lambert, P.H, AERAS Global TB Vaccine Foundation and World Health Organization (2005) New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. *Vaccine* 23, 3753–3761.

Kathaperumal, K., Park, S.-U., McDonough, S., Stehman, S., Akey, B., Huntley, J., Wong, S., Chang, C.-F. and Chang, Y.-F. (2008) Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine* 26, 1652–1663.

Koets, A.P., Rutten, V.P., Hoek, A., Bakker, D., van Zijderveld, F., Muller, K.E. and van Eden, W. (1999) Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis. *Veterinary Immunology and Immunopathology* 70, 105–115.

Koets, A., Hoek, A., Langelaar, M., Overdijk, M., Santema, W., Franken, P., Eden, W. and Rutten, V. (2006) Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. *Vaccine* 24, 2550–2559.

Kormendy, B. (1994) The effect of vaccination on the prevalence of paratuberculosis in large dairy herds. *Veterinary Microbiology* 41, 117–125.

Larsen, A.B., Merkal, R.S. and Cutlip, R.C. (1975) Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *American Journal of Veterinary Research* 36, 255–257.

Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K. and Wattiez, R. (2007) Antigen discovery: a

postgenomic approach to paratuberculosis diagnosis. *Proteomics* 7, 1164–1176.

Leyten, E.M., Lin, M.Y., Franken, K.L., Friggen, A.H., Prins, C., van Meijgaarden, K.E., Voskuil, M.I., Weldingh, K., Andersen, P., Schoolnik, G.K., Arend, S.M., Ottenhoff, T.H. and Klein, M.R. (2006) Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes and Infection* 8, 2052–2060.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S. and Kapur, V. (2005) The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proceedings of the National Academy of Sciences of the USA* 102, 12344–12349.

Lilienfeld, D.E. (2008) The first pharmacoepidemiologic investigations: national drug safety policy in the United States, 1901–1902. *Perspectives in Biology and Medicine* 51, 188–198.

Ly, L.H. and McMurray, D.N. (2008) Tuberculosis: vaccines in the pipeline. *Expert Review of Vaccines* 7, 635–650.

Meeusen, E.N., Walker, J., Peters, A., Pastoret, P.P. and Jungersen, G. (2007) Current status of veterinary vaccines. *Clinical Microbiology Reviews* 20, 489–510.

Milner, A.R., Lepper, A.W., Symonds, W.N. and Gruner, E. (1987) Analysis by ELISA and Western blotting of antibody reactivities in cattle infected with *Mycobacterium paratuberculosis* after absorption of serum with *M. phlei*. *Research in Veterinary Science* 42, 140–144.

Milstein, J.J. (2004) Regulation of vaccines: strengthening the science base. *Public Health Policy* 25, 173–189.

Milstein, J.J. and Belgharbi, L. (2004) Regulatory pathways for vaccines for developing countries *Bulletin of WHO* 82, 128–133.

Mullerad, J., Hovav, A.H., Fishman, Y., Barletta, R.G. and Bercovier, H. (2002a) Antigenicity of *Mycobacterium paratuberculosis* superoxide dismutase in mice. *FEMS Immunology and Medical Microbiology* 34, 81–88.

Mullerad, J., Michal, I., Fishman, Y., Hovav, A.H., Barletta, R.G. and Bercovier, H. (2002b) The immunogenicity of *Mycobacterium paratuberculosis* 85B antigen. *Medical Microbiology and Immunology* 190, 179–187.

Nagata, R., Muneta, Y., Yoshihara, K., Yokomizo, Y. and Mori, Y. (2005) Expression cloning of gamma interferon-inducing antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 73, 3778–3782.

Nathanson, N. and Langmuir, A.D. (1995) The Cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. *American Journal of Epidemiology* 142, 109–140.

Nielsen, S.S. and Toft, N. (2008) Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques *Veterinary Microbiology* 129, 217–235.

Olsen, I. and Storset, A.K. (2001) Innate IFN-gamma production in cattle in response to MPP14, a secreted protein from *Mycobacterium avium* subsp. *paratuberculosis*. *Scandinavian Journal of Immunology* 54, 306–313.

Olsen, I., Reitan, L.J., Holstad, G. and Wiker, H.G. (2000) Alkyl hydroperoxide reductases C and D are major antigens constitutively expressed by *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 68, 801–808.

Ott, S.L., Wells, S.J. and Wagner, B.A. (1999) Herd-level economic losses associated with Johne's disease on US dairy operations. *Preventive Veterinary Medicine* 40, 179–192.

Park, K.T., Dahl, J.L., Bannantine, J.P., Barletta, R.G., Ahn, J., Allen, A.J., Hamilton, M.J. and Davis, W.C. (2008) Demonstration of allelic exchange in the slow-growing bacterium *Mycobacterium avium* subsp. *paratuberculosis*, and generation of mutants with deletions at the *pknG*, *relA*, and *lsr2* loci. *Applied and Environmental Microbiology* 74, 1687–1695.

Park, S.-U., Kathaperumal, K., McDonough, S., Akey, B., Huntley, J., Bannantine, J.P. and Chang, Y.-F. (2008) Immunization with a DNA vaccine cocktail induces a Th1 response and protects mice against *Mycobacterium avium* subsp. *paratuberculosis* challenge. *Vaccine* 26, 4329–4337.

Paustian, M.L., Zhu, X., Sreevatsan, S., Robbe-Austerman, S., Kapur, V. and Bannantine, J.P. (2008) Comparative genomic analysis of *Mycobacterium avium* subspecies obtained from multiple host species. *BMC Genomics* 9, e135.

Reddacliff, L., Eppleston, J., Windsor, P., Whittington, R. and Jones, S. (2006) Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Veterinary Microbiology* 115, 77–90.

Rigden, R.C., Jandhyala, D.M., Dupont, C., Crosbie-Caird, D., Lopez-Villalobos, N., Maeda, N., Gicquel, B. and Murray, A. (2006) Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *Journal of Medical Microbiology* 55, 1735–1740.

Romano, M., Rindi, L., Korf, H., Bonanni, D., Adnet, P.-Y., Jurion, F., Garzelli, C. and Huygen, K. (2008) Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). *Vaccine* 26, 6053–6063.

Rosseels, V. and Huygen, K. (2008) Vaccination against paratuberculosis. *Expert Review of Vaccines* 7, 817–832.

Rosseels, V., Scanlan, V., Vanonckelen, A., Jurion, F., Palfliet, K., Marché, S., Godfroid, J., Walravens, K. and Huygen, K. (2002) Development of a plasmid DNA based *M. paratuberculosis* vaccine encoding immunodominant T cell antigens identified in mycobacterial culture filtrate. In: Juste, A.R., Geijo, M.V. and Garrido, J.M. (eds) *Proceedings of the Seventh International Colloquium on Paratuberculosis*. International Association for Paratuberculosis, Madison, Wisconsin, pp. 108–113.

Rosseels, V., Marché, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K. and Huygen, K. (2006a) Members of the 30- to 32-kDa mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infection and Immunity* 74, 202–212.

Rosseels, V., Roupie, V., Zinniel, D.K., Barletta, R.G. and Huygen, K. (2006b) Development of luminescent *Mycobacterium avium* subsp. *paratuberculosis* for rapid screening of vaccine candidates in mice. *Infection and Immunity* 74, 3684–3686.

Roupie, V., Romano, M., Zhang, L., Korf, H., Lin, M.Y., Franken, K.L., Ottenhoff, T.H., Klein, M.R. and Huygen, K. (2007) Immunogenicity of eight dormancy (DosR) regulon encoded proteins of *Mycobacterium tuberculosis* in DNA vaccinated and tuberculosis-infected mice. *Infection and Immunity* 75, 941–949.

Roupie, V., Leroy, B., Rosseels, V., Piersoel, V., Noël-Georis, I., Romano, M., Govaerts, M., Letesson, J.J., Wattiez, R. and Huygen, K. (2008a) Immunogenicity and protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c of *Mycobacterium avium* subsp. *paratuberculosis* secretome. *Vaccine* 26, 4783–4794.

Roupie, V., Rosseels, V., Piersoel, V., Zinniel, D.K., Barletta, R.G. and Huygen, K. (2008b) Genetic resistance of mice to *Mycobacterium paratuberculosis* is influenced by Slc11a1 at the early but not at the late stage of infection. *Infection and Immunity* 76, 2099–2105.

Saxegaard, F. and Fodstad, F.H. (1985) Control of paratuberculosis (Johne's disease) in goats by vaccination. *Veterinary Record* 116, 439–441.

Scandurra, G.M., Young, M., de Lisle, G.W. and Collins, D.M. (2009) A bovine macrophage screening system for identifying attenuated transposon mutants of *Mycobacterium avium* subsp. *paratuberculosis* with vaccine potential. *Journal of Microbiological Methods* 77, 58–62.

SCNZ (2003) Sustainability Council of New Zealand. Hazardous Substance and New Organisms Act 2003. Available at: http://www.sustainabilitynz.org/docs/HSNOAct_ProposedAmendments.pdf (accessed 16 January 2009).

Sechi, L., Mara, A.L., Cappai, P., Frothingham, R., Ortu, S., Leoni, A., Ahmed, N. and Zanetti, S. (2006) Immunization with DNA vaccines encoding different mycobacterial antigens elicits a Th1 type immune response in lambs and protects against *Mycobacterium avium* subsp. *paratuberculosis* infection. *Vaccine* 24, 229–235.

Shin, S.J., Chang, C.F., Chang, C.D., McDonough, S.P., Thompson, B., Yoo, H.S. and Chang, Y.F. (2005) *In vitro* cellular immune responses to recombinant antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* 73, 5074–5085.

Shin, S.J., Wu, C.W., Steinberg, H. and Talaat, A.M. (2006) Identification of novel virulence determinants in *Mycobacterium paratuberculosis* by screening a library of insertional mutants. *Infection and Immunity* 74, 3825–3833.

Sigurdsson, B. (1956) Immunological problems in paratuberculosis. *Bacteriological Reviews* 20, 1–13.

Sigurdsson, B. (1960) A killed vaccine against paratuberculosis (Johne's disease) in sheep. *American Journal of Veterinary Research* 21, 54–67.

Singh, S.V., Singh, P.K., Singh, A.V., Sohal, J.S., Gupta, V.K. and Vihan, V.S. (2007) Comparative efficacy of an indigenous 'inactivated vaccine' using highly pathogenic field strain of *Mycobacterium avium* subspecies *paratuberculosis* 'bison type' with a commercial vaccine for the control of Capri-paratuberculosis in India. *Vaccine* 25, 7102–7110.

Skeiky, Y.A., Alderson, M.R., Ovendale, P.J., Guderian, J.A., Brandt, L., Dillon, D.C., Campos-Neto, A., Lobet, Y., Dalemans, W., Orme, I.M. and Reed, S.G. (2004) Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. *Journal of Immunology* 172, 7618–7628.

Stuart, P. (1965) Vaccination against Johne's disease in cattle exposed to experimental infection. *British Veterinary Journal* 121, 289–318.

Tchilian, E.Z., Desel, C., Forbes, E.K., Bandermann, S., Sander, C.R., Hill, A.V., McShane, H. and Kaufmann, S.H. (2009) Immunogenicity and protective efficacy of prime–boost regimens with recombinant *ΔureC hly+ Mycobacterium bovis* BCG and modified Vaccinia Virus Ankara expressing *M. tuberculosis* antigen 85A against murine tuberculosis. *Infection and Immunity* 77, 622–631.

Tiwari, A., Vanleeuwen, J.A., McKenna, S.L., Keefe, G.P. and Barkema, H.W. (2006) Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Canadian Veterinary Journal* 47, 874–882.

Uzonna, J.E., Chilton, P., Whitlock, R.H., Habecker, P.L., Scott, P. and Sweeney, R.W. (2003) Efficacy of commercial and field-strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* 21, 3101–3109.

Vallée, H., Rinjard, P. and Vallée, M. (1934) Sur la préimmunisation de l'entérite paratuberculeuse des bovidés. *Revue Générale de Médécine Vétérinaire* 43, 777–779.

Vordermeier, H.M., Huygen, K., Singh, M., Hewinson, R.G. and Xing, Z. (2006) Immune responses induced in cattle by vaccination with a recombinant adenovirus expressing mycobacterial antigen 85A and *Mycobacterium bovis* BCG. *Infection and Immunity* 74, 1416–1418.

Weyer, J., Rupprecht, C.E., Mans, J., Viljoen, G.J. and Nel, L.H. (2007) Generation and evaluation of a recombinant modified vaccinia virus Ankara vaccine for rabies. *Vaccine* 25, 4213–4222.

WHO (1992) Expert committee on biological standardisation. 42nd report guidelines for national authorities on quality assurance for biological products. In: *Technical Report Series* 822, Annex 2.

Wilesmith, J.W. (1982) Johne's disease: a retrospective study of vaccinated herds in Great Britain. *British Veterinary Journal* 138, 321–331.

Windsor, P.A. and Eppleston, J. (2006) Lesions in sheep following administration of a vaccine of a Freund's complete adjuvant nature used in the control of ovine paratuberculosis. *New Zealand Veterinary Journal* 54, 237–241.

Wu, C.W., Schmoller, S.K., Shin, S.J. and Talaat, A.M. (2007) Defining the stressome of *Mycobacterium avium* subsp. *paratuberculosis in vitro* and in naturally infected cows. *Journal of Bacteriology* 189, 7877–7886.

Xing, Z., Santosuosso, M., McCormick, S., Yang, T.C., Millar, J., Hitt, M., Wan, Y., Bramson, J. and Vordermeier, H.M. (2005) Recent advances in the development of adenovirus- and poxvirus-vectored tuberculosis vaccines. *Current Gene Therapy* 5, 485–492.

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