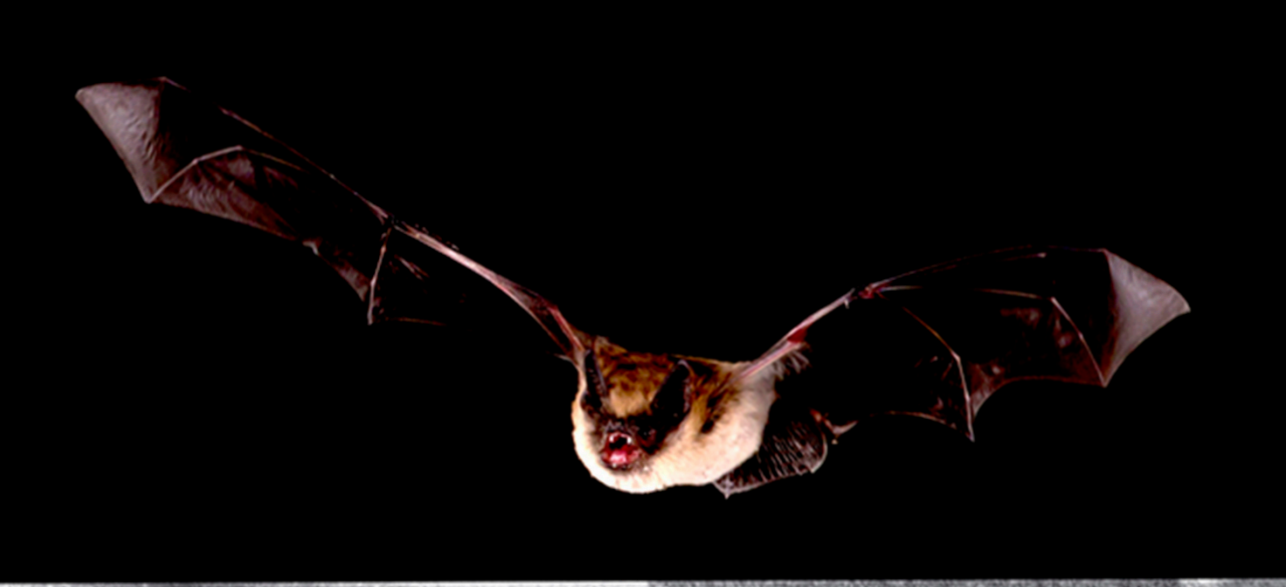

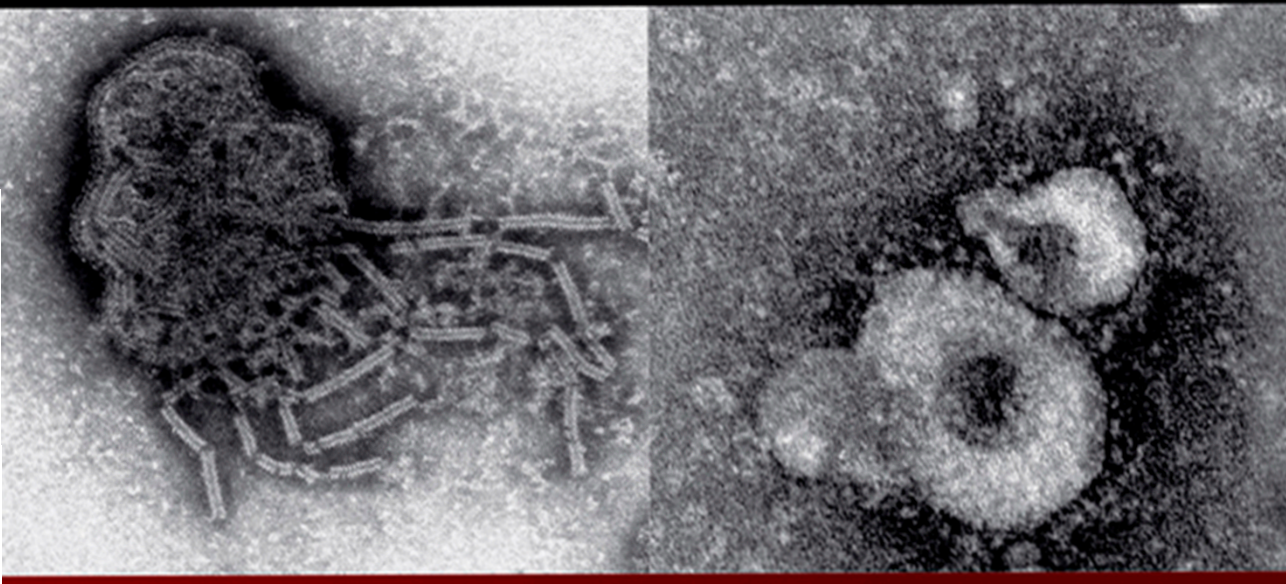

Edited by Lin-Fa Wang and Christopher Cowled

BATS AND VIRUSES

A New Frontier of Emerging Infectious Diseases

WILEY Blackwell

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PREFACE

The global burden of human suffering caused by infectious diseases has been lessened in the modern era by medical and technological advancements, yet the social costs of contemporary epidemics can still be devastating, and the economic costs can be orders of magnitude more severe than in the past. Modern communications have the ability to spread fear and panic to millions, while international air transport means the potential for infectious agents to move around the world within hours is a confronting reality. Globalization has connected countries and continents together in ways that make the impacts of emerging disease events in remote and distant places extend far beyond their geographic boundaries.

In the last two decades, some of the largest outbreaks of emerging infectious diseases, including the SARS virus outbreaks in 2003–2004, which caused an estimated more than $50 billion damage to the world economy, and the Ebola virus disease outbreaks in West Africa started from 2014, which has claimed more than 10 000 lives to date, have implicated bats as their primary source. It is now thought that 75% of all emerging human infectious diseases originate in other animals, and bats are being increasingly recognized as one of the most important reservoirs for emerging viruses. In addition to SARS and Ebola viruses, bats are implicated as the source of diverse human pathogens, including *Nipah virus*, *Hendra virus*, *Marburgvirus*, the newly emerged MERS virus, and more. As the only flying mammal on earth, the unique biological features of bats distinguish them from all other mammals. Recent studies suggest that bats' ability to live longer and harbor a large number of viruses without displaying clinical diseases may in fact be related to the adaptation to flight.

It is more than a century since an association was first recognized between bats and a zoonotic virus (rabies virus); however, this area of research has been neglected to a large degree, as reflected by the fact that only one dedicated book has ever been published on this important topic, and that was in 1974. It was in this context of rapid progress in bat and virus research and the lack of a dedicated book in this area for the last four decades, that we felt it timely to embark on the goal of publishing a dedicated volume summarizing the recent progress and state of play with regard to research into bats and their viruses. Our endeavor was greatly helped by the enthusiasm of the invited chapter authors, many of whom are recognized leaders in their fields. We would like to take this opportunity to formally thank all of the authors for their dedication and professionalism. We also wish to thank the staff at John Wiley & Sons, especially Mindy Okura-Marszycki and Stephanie Dollan, for strongly supporting our project from its inception through to final production.

In this volume, we have tried to put equal emphasis on both pathogen and host biology. While research in some areas, such as the physiology and biomechanics of bat

flight and echolocation, have a long history and are quite advanced, others, including bat genomics and immunology are still in their infancy and a lot more work needs to be done before one can present a complete picture. On the other hand, with recent advances in next generation sequencing, the characterization of bat viruses and/or viral genomic sequences have undergone exponential growth, as evident from the detailed descriptions of major bat-borne virus groups in the dedicated individual chapters. While such advances are exciting and represent great progress, many significant challenges remain, including but not limited to:

1. *Isolation of live virus from bat specimens*. With the rapid accumulation of viral sequences from metagenomics studies, the success rate of virus isolation still remains extremely low. The true association of some of these viral sequences with bats is yet to be proven, and it is not always clear what role bats play in the viral replication cycle. The need to understand the emergence of new human pathogens from wild reservoirs builds a strong case for the proper biological characterisation of both viruses and their natural hosts.
2. *The species-specific nature of the current bat research*. With over 1200 species of bats described, extreme caution needs to be placed on generalizing findings made from a limited number of bat species. It is important to recognize that it will be extremely difficult, if not impossible, to make any pan-bat claim from the current early studies on bats and their viruses.
3. *Cross-species comparative studies*. In addressing the question "Are bats special?" in their ability to coevolve with viruses, there is a need for in-depth comparative studies with other mammalian species, especially mouse and human. However, due to the lack of proper reagents (especially antibodies), cell lines, and bat colonies, the studies presented in this book can only be considered preliminary and much more work is required in future.
4. *Virus-centric focus*. Although there have been a few recent publications on bat-borne bacteria and parasites, the data are very limited in comparison to the large volume of virus-related publications. For this reason, we have limited the discussion of this book to viral pathogens; however we hope to include other pathogens in future edition(s).

Finally, as the field is moving forward very rapidly, we tried our best to capture the latest findings and knowledge at the time of publishing. However, with the book project spanning more than a year, it is inevitable that some of the most recent advances may have been overlooked or occurred too late for inclusion. With the pace of discovery accelerating, we look forward to a new era of research on bats and their viruses, which in 2015 looks promising indeed.

Lin-Fa Wang and Christopher Cowled
April 2015

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THE UNIQUENESS OF BATS

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1.1 INTRODUCTION

The aim of this chapter is to provide an overview of the distinctive features of bats, many of which are unique among mammals, and in particular to highlight features of their biology that may have some bearing on the high prevalence of viruses in this group (Luis *et al.*, 2013).

Bats are the only mammals with the capacity for powered flight. The associated skeletal adaptations of elongated forelimb bones were fully developed in the first fossil bat *Icaronycteris index* 50 million years ago, discovered in the Green River formation in Wyoming, USA (Jepsen, 1966, 1970). Also evident were auditory bullae at the base of the cranium, indicating the presence of large cochlea, associated with echolocation, which enabled bats to fly in darkness. Flight and echolocation allowed bats to occupy and eventually dominate the nocturnal aerial feeding niche where they are relatively free of competitors and predators. Among the exceptions are caprimulgid birds (nightjars and goatsuckers), an Old World bat hawk and a New World bat falcon. Owls cannot match the flight agility of bats, although they are opportunist predators at roosts (Fenton & Fleming, 1976).

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From the same Eocene fossil beds in Wyoming, Simmons *et al.* (2008) described *Onychonycteris finneyi,* of similar age to *Icaronycteris index* but more primitive, in that its limb bones are intermediate in proportion between terrestrial mammals and other Eocene bats. The cochlea is also smaller indicating that echolocation was either less well developed or absent, supporting the view that flight evolved before echolocation. However, in the absence of a more extensive fossil record, the evolutionary history of bats from a small terrestrial shrew-like early mammal of the Triassic to the fossil bats found 150 million years later is a matter of speculation. The most plausible hypothesis is that bats evolved from a species similar to modern day tree shrews, in which the limbs and digits became connected by folds of skin and the forelimbs became elongated to form an aerofoil (Smith, 1977; Hill & Smith, 1984). Jumping from branch to branch led to gliding and eventually to flapping flight.

With 1301–1331 species (January 2014, N.B Simmons, Pers. Comm.; Simmons, 2015) bats are the second largest order of mammals, and the number of species continues to rise as new ones are described or the taxonomy of particular genera is reviewed. Nevertheless, bats continue to account for about a fifth of all mammals because the same processes are happening in the largest order of mammals, the rodents, which has about twice as many species as bats. Bats are distributed throughout the world, with the exception of some isolated oceanic islands such as Tahiti and the polar regions, although they breed inside the Arctic Circle (Rydell, 1989). However, although there are twice as many bat species in the Old as in the New World, bats achieve their greatest species richness in South America (Hutson *et al.*, 2001; Jones *et al.*, 2009). Regardless of continent or scale, latitudinal gradients of richness are qualitatively similar and species number increases with decreasing latitude and decreases with increasing elevation (Willig *et al.*, 2003). Family-level species richness varies greatly, from one and two species in the Craseonycteridae and Myzopodidae respectively to more than 300 species in the Vespertilionidae (Simmons & Conway, 2003).

Historically, bats were divided into two suborders: the Megachiroptera consisted of a single family, the Pteropodidae – Old World vegetarians with large eyes, which do not echolocate; and the Microchiroptera with 16 families of echolocating and mainly insectivorous bats. Recent phylogenetic analysis has revised the classification of bats, and two new suborders have replaced the old: The Yinpterochiroptera consisting of the Pteropodidae and five other families grouped within the superfamily Rhinolophoidea; and the Yangochiroptera, with three superfamilies – the Emballonuroidea, the Vespertilionoidea and the Noctilionoidea, comprising a total of 13 families (Teeling *et al.*, 2005).

1.2 FLIGHT

The ability to glide by extending flaps of skin between the limbs has evolved several times among mammals, in the marsupial sugar gliders and flying phalangers of Australia and New Guinea, in placental mammals like flying squirrels and colugos of Asia, and anomalures of Africa. Bats are unique, however, in their capacity for powered flight. All forelimb bones in bats are elongated, although the ulna no longer extends to the wrist and is vestigial, so pronation and supination (turning about the elbow) is no longer possible nor desirable. Digit one – the thumb – is free and clawed and is important for climbing and grooming, and in some cases for aggression. Digits two and three are

generally close together and form the rigid leading edge of the aerofoil. Digits four and five support the wing membrane or patagium, which consists of a double layer of skin, well provided with elastic tissue so that it can change shape in flight but retracts and folds at rest. The hind limbs are also elongated, attached to the patagium and involved in steering. They are generally joined to the tail by the interfemoral membrane which is sometimes used in prey capture. The overall flexibility of the wing means that some bats have a unique flying attribute – the ability to carry out stall turns – to rotate through 90° in their own body length.

In contrast to birds, where most power for flight comes from two muscles – the pectoralis and the supracoracoideus working antagonistically – flight in bats is powered by nine pairs of muscles, mainly abductors and adductors, concentrated towards the midline (in contrast to the situation in terrestrial mammals where the flexors and extensors of the limbs power locomotion). In birds the muscles that elevate and depress the wings are on the ventral surface, whereas in bats the elevators are dorsal and the depressors are ventral. The keel on the sternum of bats is much less prominent than in birds. The muscles responsible for opening and closing the wing are also situated in the proximal parts of the forelimb and their power is transmitted by extended tendons. The wing opens and closes in one plane and twisting is eliminated. The clavicle braces the shoulder joint against the axial skeleton, in contrast to the situation in birds where the coracoid provides a more rigid brace.

Although the hind limbs may be elongated, the pelvic girdle is reduced compared with the pectoral girdle, and the diameter of the birth canal is reduced. However, across the pubic symphysis, the interpubic ligament joins the pubic bones ventrally, and can expand to increase the diameter of the birth canal from 2 to 35 mm in *Tadarida brasiliensis*. This expansion is under the influence of the hormone relaxin (Crelin, 1969). The hind limbs have become rotated by 90° in many bats, although they have retained the ability for terrestrial locomotion, sometimes impressively so (Lawrence, 1969; Riskin *et al.*, 2006). In some groups, however, such as horseshoe bats (family Rhinolophidae), the limbs have rotated though 180° and terrestrial locomotion is no longer possible so they can only hang or fly, although some species are able to land on the ground in pursuit of prey and lift off from a stationary position.

The overall shape of the wing is an important determinant of flight capability and foraging behaviour. Wing loading refers to the weight of the bat divided by the total area of the flight membrane, so that high wing loading occurs in a large bat with relatively small wings. Aspect ratio is the square of the wingspan divided by the wing area and is low in bats with short broad wings and high in bats with long narrow ones. A principal components analysis between increasing wing loading and aspect ratio reveals four broad flight capabilities (Norberg & Rayner, 1987):

1. Slow open-air flight, long distance migration.
2. Slow maneuverable flight in cluttered environments.
3. Fast flight in cluttered environments.
4. Fast open-air hawking, short range migration.

Of particular relevance in the context of disease transmission is the migratory ability of bats which will be considered later. In addition, O'Shea *et al.* (2014) have

hypothesized that bat flight provides the selection pressure for co-existence with viruses through a daily cycle of activity that elevates metabolism and body temperature analogous to the febrile response in other mammals.

1.3 ECHOLOCATION

Although the role of the ears in enabling bats to avoid obstacles in the dark was established in the late 18th century by Spallanzani and Jurine, it was not until the mid-20th century that the use of echolocation to catch insects was revealed by Griffin and colleagues (Griffin, 1958). Echolocation involves the analysis by an animal of the echoes of its own emitted sound to gain information about its environment. Echolocation is used by most bats to detect obstacles and prey. It has also evolved in some nocturnal cave-nesting birds like swiftlets (*Collocalia*) of the Old World tropics and the South American oilbird (*Steatornis*) and among mammals in toothed whales and in some insectivores (Sales & Pye, 1974). It has, however, been best studied in bats where it has reached extraordinary levels of sophistication (Fenton, 2013).

Most bats produce their echolocation calls in the larynx, which in bats is proportionally larger, and is tensioned by well-developed cricothyroid muscles. Lips, flaps of skin and noseleafs appear to act as acoustic lenses, focussing outgoing signals. The external ears are generally large and have a cartilaginous projection or tragus at their base which may limit the receptive field to an area 30–40° either side of the midline and this may in turn affect the directionality of incoming echoes (Altringham, 2011).

Bats use a variety of echolocation calls with different combinations of signal strength, signal duration and pattern of frequency change over time. As they approach targets, they produce shorter and shorter signals to ensure the outgoing pulses do not mask returning echoes.

Echolocation calls are generally beyond the range of human hearing (and so by definition are ultrasonic) and in the frequency range 20–120 kHz. They are also of high intensity and loud enough to be uncomfortable if we could hear them. To avoid deafening themselves by their own emitted sounds, most echolocating bats separate pulse and echo in time. Furthermore, they disconnect one of the middle ear bones, the stapes, from the oval window at the entrance to the cochlea, by contracting the stapedius muscle. When foraging, bats emit pulses of ultrasound and the pulse repetition rate increases when the echoes indicate the presence of a prey item, in some cases up to 200 Hz. The stapedius muscle can operate at this frequency, one of the highest recorded in mammals (Altringham, 2011).

Horseshoe bats (Rhinolophidae), leaf-nosed bats (Phyllostomidae), and three species of moustached bats (Mormoopidae), separate pulse and echo in frequency. They do so by exploiting Doppler shifts of their outgoing signals, which are dominated by a single frequency.

High frequency sounds attenuate rapidly in air so that a bat echolocating with signals that have most energy at 30 kHz is unlikely to detect insect-sized targets beyond 40 m. The frequency of sound is inversely proportional to its wavelength and so the higher the frequency the smaller the wavelength. The best sound for detecting an object is one with a wavelength similar in length to the object, so that bats feeding on small insects tend to use high frequency short wavelength calls.

Bats emit echolocation calls in pulses either at a constant frequency (CF) or frequency modulated (FM) or a mixture of the two. FM pulses are short, typically 2–5 ms long and sometimes less than 0.2 ms. If pulses were longer, the bat would be listening to the echo before it had finished emitting the pulse and neural mechanisms of echo interpretation require that to be avoided (Altringham, 2011). According to the autocorrelation function proposed by Simmons (1971), a bat behaves as if it stored the emitted pulse (in the inferior colliculus of the midbrain) and cross-correlates it with the returning echo (Altringham, 2011).

CF calls are typically 10–50 ms in duration and often have an FM component at the end. Many species with long CF components to their calls use an auditory processing system that is tolerant of pulse-echo overlap. This is possible because bats in the families Hipposideridae and Rhinolophidae have an acoustic fovea – a region of the cochlea that is extremely sensitive to the echo frequency of their calls – so that pulse and echo are separated in frequency rather than in time (Neuweiler, 1990; Altringham, 2011).

The Pteropodidae is the only family of bats not to have evolved laryngeal echolocation and relies on sight for orientation, together with olfaction for finding food. However, one genus within the Pteropodidae, *Rousettus*, has evolved a system of echolocation by rapid tongue clicking (Holland *et al.*, 2004), and as a result can roost deep in caves. Other members of the family may also roost in caves but only within sight of the entrance.

1.4 COMMUNICATION

In addition to its role in avoidance of obstacles and detection of prey, the sounds produced by bats are also important in communication between individuals (Altringham & Fenton, 2003). Although echolocation calls may have a communication function (Möhres, 1966; Barclay, 1982), social calls have been identified which often have a lower frequency than echolocation calls and may be audible to the human ear. Examples are distress calls (Russ *et al.*, 1998), mating calls (Lundberg & Gerrell, 1986), copulation calls (Thomas *et al.*, 1979), isolation calls when young are separated from their mothers (de Fanis & Jones 1995) and, together with their scent and the spatial memory of the mothers, enable young to be located among many millions on the wall of cave roosts (Balcombe & McCracken, 1992).

1.5 FORAGING, DIET, AND ECOSYSTEM SERVICES

Although bats have evolved a wide range of diets, they have retained a relatively simple digestive system and a relatively short intestinal passage time (Tedman & Hall, 1985). The majority of bat species are insectivorous, catching their prey in free flight or in various degrees of clutter, or gleaning, in which insects are taken from substrates such as leaves, bark or the ground. Such gleaners often have particularly acute hearing (Coles *et al.*, 1989) and can detect their prey by listening to the sounds of shimmering wings, or rustling, as the insect moves (Anderson & Racey, 1981; Swift & Racey, 2002). The majority of nocturnal moths, and also green lacewings, have evolved hearing organs that detect the bats' ultrasound and enable them to take avoiding action. Some arctiid moths

have evolved organs which generate sound that cause approaching bats to abandon their attack (Jones & Rydell, 2003).

Bats in the family Pteropodidae have large eyes and good night vision and feed on fruit, flowers, nectar, pollen, and leaves. Fruit contains little or no protein but bats can extract protein from pollen and leaves (Kunz & Ingalls, 1994; Long & Racey, 2007). The New World family Phyllostomidae includes many species with a diet of fruit, nectar and pollen which also catch insects to satisfy their protein requirements. The extent to which these bats rely on echolocation when feeding on plant products is unclear. However, some New World flowers have evolved nectar guides which reflect ultrasound to attract bats and encourage their role in pollination (von Helversen & von Helversen, 1999, 2003). Although the majority of bat species are insectivorous, some have become carnivorous, taking small mammals, reptiles and amphibians from the ground (Patterson *et al.*, 2003). Even more specialized are the piscivores, which echolocate ripples on the water surface before lowering their often enlarged feet into the water to gaff small fish (Schnitzler *et al.*, 1994). Vampire bats are unique as the only mammals to subsist entirely on a diet of blood. An anticoagulant in their saliva maintains blood flow once an incision has been made by sharp incisor teeth (Hawkey, 1966; Fernandez, 1999).

At least one species of vampire bat has an infrared detector on the nose leaf to assist in the localisation of prey, which in the case of the common vampire bat *Desmodus rotundus* is commonly cattle (Kürten *et al.*, 1984). Quadripedal locomotion is also well developed in vampires, which alight on the ground near their prey and are adept at avoiding their moving hoofs. They climb up the leg to the neck, make an incision and feed by lapping the blood. Before they can fly, they must lose weight and the kidney switches to water-eliminating mode. Once back in the roost, they must digest blood with no access to drinking water, so the kidney switches to a water conserving mode more efficient than that of some desert rodents (McFarland & Wimsatt, 1965). Cattle are not, however, debilitated by the loss of blood but by the diseases transmitted by the bats, the most serious of which is rabies (see Chapter 3).

As knowledge of bat diets becomes more detailed, so does awareness of the ecosystem services they provide (Boyles *et al.*, 2011; Kunz *et al.*, 2011). By analyzing feces, the proportion of insect pests in the diet can be determined and a monetary value placed on a colony of many millions of free-tailed bats *Tadarida brasiliensis* roosting in caves in Texas, not just in reduction of crops lost to pests but also in reducing the number of pesticide treatments the crop requires (Cleveland *et al.*, 2006). Similar ecosystem services are provided by wrinkle-lipped free-tailed bats, which act as a potential biological pest control agent through eating rice crops in Asia (Leelapaibul *et al.*, 2005). Wherever large colonies of bats roost, their guano is harvested as a fertilizer rich in nitrogen and phosphates. In many countries in the developing world, this is often the preferred fertilizer, because farmers consider that, unlike chemical fertilizers, it improves soil quality.

Fruit-eating bats disperse seeds over long distances and play an important role in forest regeneration (Lobova *et al.*, 2009; Fleming & Kress, 2013). Some plant-eating bats have become adapted to a diet of pollen and nectar and pollinate high value commercial crops such as durian in Asia (Bumrungsri *et al.*, 2009) or commonly used vegetables such as stink bean or petai (Bumrungsri *et al.*, 2008) as well as charismatic plants, such as baobabs in Africa and Madagascar (Baum, 1995; Andriafidison *et al.*, 2006).

1.6 HETEROTHERMY, DAILY TORPOR, AND HIBERNATION

The adaptation that has enabled insectivorous bats to colonize the north and south temperate zones is the capacity for heterothermy, in which body temperature is allowed to fall, sometimes close to ambient, from which it spontaneously rewarms. This is not an intermediate state between cold-blooded vertebrates or ectotherms and warm blooded vertebrates or endotherms but is a specialised form of homeothermy. Many temperate-zone bat species make use of daily torpor, allowing their body temperature to fall to save the energy increment that would be required to maintain a high constant body temperature or homoeothermic state. In late summer, bats begin to accumulate body fat and as aerial insect density and ambient temperatures decline in autumn, the periods of daily torpor become longer and then continuous for days or weeks as the bat is hibernating. Hibernation is interrupted by spontaneous arousals, the frequency of which is related to ambient conditions. At latitudes where ambient temperatures are below freezing for long periods, arousals are less frequent than at 53°N in the UK, where over a 3-year period, pipistrelle bats *Pipistrellus pipistrellus* flew in every winter month and on a third of all winter nights (Avery, 1985). Bats will often fly when the winter temperature rises above the threshold for insect flight and winter feeding is often recorded although increases in body weight have yet to be established (Ransome, 2008). There may be other drivers of winter arousals such as the need to urinate, to drink or to check on ambient conditions, and perhaps reposition within the hibernaculum. Arousal from deep hibernation, in which the bat's body temperature is close to ambient, is energetically expensive in the amount of fat metabolized. That explains the mass winter mortality, of over six million bats of several species in North America, attributed to the cold-adapted fungus *Pseudogymnoascus destructans*. This invades the skin of the muzzle, forearm, and wing membranes, penetrating the epidermis and dermis and causing the bat to arouse repeatedly during hibernation. More fat is metabolised in doing so than will last the bat for the duration of winter and the bat starves to death (Reeder & Moore, 2013).

Although many groups of mammals (such as some carnivores and Eulipotyphla) and some birds (such as humming birds) make use of torpor, bats have taken the adaptation to extremes during pregnancy and lactation. If pregnant bats experience periods of inclement weather, so that their insect food is no longer flying, they will become torpid and the development of the fetus will be slowed or halted. Conversely, if they experience high ambient temperatures and abundant food during pregnancy, foetal development will accelerate (Racey, 1973a; Racey & Swift, 1981). Among mammals in general, the gestation period is fixed by the foetal genotype and is resistant to alteration by environmental factors (Racey, 1981). The fact that foetal development in bats may be slowed, stopped or accelerated depending on ambient temperature and food supply is unique among mammals and may be related to the fact that they have one of the slowest recorded rates of foetal growth (Racey, 1973a, 1981).

The timing of the reproductive cycle of male bats of the temperate zone is also influenced by changes in ambient temperature and food supply. Premature arousal of captive pipistrelle bats *Pipistrellus pipistrellus* from hibernation with an abundant food supply results in the initiation of spermatogenesis. Conversely, spermatogenesis is delayed in captive noctule bats *Nyctalus noctula* in which hibernation is prolonged by several months (Racey, 1971).

1.7 REPRODUCTION

Bats have unique features in their reproduction, the most significant of which is delayed fertilization associated with prolonged storage of fertile spermatozoa. All bats of the temperate zone are seasonally monestrus, and have only one birth period a year. Births occur in midsummer, and after lactation and weaning, copulation begins, so that the majority of females entering hibernation are inseminated. Spermatozoa are stored in the oviducts, in the utero-tubal junction or in the uterus, depending on the species (Racey, 1979). In the latter case, vast numbers are stored so that the uterus more closely resembles the thin-walled spermatheca, or sperm storage sac of an insect, than a mammalian uterus (Racey, 1975). After arousal from hibernation in spring, a single large follicle, which has been overwintering in the ovary, ovulates and is fertilized by one of the stored sperm, and the remainder are expelled (Potts & Racey 1971; Wimsatt *et al*., 1966; Wimsatt, 1969). Isolation experiments have demonstrated that the sperm stored by female bats can retain their fertility for as long as seven months (Racey, 1973b, 1979). Although several vertebrate and invertebrate species exceed this, among mammals it is unique.

Sperm storage in females of the temperate zone is associated with a unique adaptation in males – extreme asynchrony between the endocrine and exocrine functions of the testis. Spermatogenesis is initiated on arousal from hibernation in spring and proceeds during summer so that sperm are released from the seminiferous tubules in July and August in the northern temperate zone and the tubules then regress, as in most seasonally breeding mammals (Racey & Tam, 1974). In bats, however, regression is complete, with the tubules in winter consisting of a single layer of spermatogonia and Sertoli cells. Copulation begins in September in the northern hemisphere (Racey, 1979) and in some bat species, continues throughout winter when males are observed in hibernacula copulating with torpid females (Wimsatt, 1945; Stebbings, 1965). It is also observed in spring (Aubert, 1963). When noctule bats (*Nyctalus noctula*) were deprived of the opportunity to copulate in autumn and introduced to females at intervals during winter they did so and females became pregnant (Racey, 1973b). This demonstrated that sperm stored for up to 7 months in the epididymis of males also retains its fertility and that the Leydig cells of the testis continue to secrete androgens during winter to maintain the integrity of the epididymis, the viability of spermatozoa and libido (Racey, 1974).

One of the advantages of prolonged sperm storage in temperate zone bats is that it avoids the necessity of finding mates and copulating when in poor body condition at the end of hibernation, and females can ovulate and pregnancy can proceed once body condition has improved (Potts & Racey, 1971). Another advantage may be that if ovulation occurs in response to increased temperature and food supply, then births will be synchronised, also at an optimal time. This may also explain the occurrence of sperm storage in some tropical bats (Racey, 1979; Racey & Entwistle, 2000).

In tropical latitudes, seasonal monestry also occurs but there is a wider range of reproductive cycles, some of which are polyestrus, so that some species have more than one young per year. Some reproductive cycles also incorporate delays in implantation and development (Racey & Entwistle, 2000).

1.8 LIFE HISTORY STRATEGIES

A striking feature of the life history characteristics of bats is the general consistency among different species, which may vary by three orders of magnitude in body mass, and occupy tropical and temperate latitudes with a wide range of diets and social systems varying from monogamous to highly polygynous (Barclay & Harder, 2003). Bats are generally monotocous and rarely have more than one young a year, after a long gestation followed by an extended lactation. The body mass of the young at birth is on average 23% that of the mother and 76% of her body mass at weaning (Barclay & Harder, 2003). Puberty seldom occurs in the year of birth but generally occurs in the following year, although in some species it may be delayed (Racey & Entwistle, 2000). A unique feature of bats, for mammals of their size, is their longevity, and Barclay and Harder (2003) give an average of 16.1 years. The average maximum recorded life span of a bat is 3.5 times that of a terrestrial placental mammal of a similar size and records of individuals surviving for more than 30 years now exist for five species (Wilkinson & South, 2002). Barclay & Harder (2003) hypothesize that low resource availability may limit reproductive output but that the overall consistency in life history characteristics reflects the evolutionary consequences of flight, which is generally associated with low extrinsic mortality (mortality associated with predators, competitors or disease) and may itself permit reduced reproductive effort per breeding event. However, such low extrinsic mortality has been challenged recently by the effects of white nose syndrome. Wilkinson and South (2002) found that life span significantly increases with hibernation and body mass and decreases with reproductive rate but is not influenced by diet or colony size. They suggested that hibernation may provide a natural example of caloric restriction which increases longevity in other mammals.

1.9 ROOSTING ECOLOGY

1.9.1 Caves

When they are not foraging, bats need shelter and are the only group of vertebrates to have successfully exploited caves as permanent daytime shelters (Kunz, 1982). In these they form some of the largest aggregations of vertebrates recorded, particularly for bats of the genus *Tadarida*, *w*ith estimates of up to 20 million *Tadarida brasiliensis* in single caves in the southern United States (Davis *et al.*, 1962; McCracken, 2003). Half a million *Hipposideros caffer* and up to 200 000 *Miniopterus schreibersii* were estimated to occupy single caves in Africa and Australia respectively (Brosset, 1966; Dwyer & Hamilton-Smith, 1965). Caves often contain several bat species and an estimated 800 000 individuals in a cave in Mexico comprised four species of mormoopid (Bateman & Vaughan, 1974) and three quarters of a million bats of three species roosted in Cucaracha cave, Puerto Rico (Rodriguez-Duran & Lewis, 1987). Although it is likely that these populations have declined in recent years (Furey & Racey, in press), the occurrence of such high densities of mammals in confined spaces is unique. Considerable effort has been expended in recent years attempting to refine methods of estimating the numbers involved (Hristov *et al.*, 2013).

The benefits of cave roosting include environmental temperatures that approach thermoneutrality, when bats do not expend energy maintaining their body temperature, thus reducing the energy costs of homeothermy. Disbenefits include a high incidence of ectoparasites (Marshall, 1982) and possibly also of disease and increased competition for food, although these remain to be rigorously established. In the temperate zone, some insectivorous bats also roost in rock crevices, such as *Eumops perotus* in the western United States (Vaughan, 1959), and in the tropics some frugivores (fruit eaters) are also crevice roosting, such as *Eidolon dupreanum* in Madagascar (Racey *et al.*, 2009) Several thousand *Eidolon helvum* also roost on cliffs at Wli Falls, Ghana (Ottou, 2011).

1.9.2 Trees

In areas devoid of caves, tree cavities may be the only available roosts and are used by species in several bat families in both temperate and tropical zones, although the size of such cavities limits the number of occupants. Even smaller numbers roost beneath exfoliating bark (Kunz & Lumsden, 2003). A few tropical bat species, mostly members of the Emballonuridae, roost in relatively exposed situations on the sides of tree boles (Kunz & Lumsden, 2003).

1.9.3 Houses

The low conductivity of wood and its suitability as a roosting substrate, especially for homoeothermic bats of the temperate zone, may explain why bats have followed it into buildings, although in terms of the evolutionary history of bats, this must be a relatively recent occurrence. Nevertheless, some bats, especially vespertilionids of the temperate zone, are now among the most synanthropic of all vertebrates. (Synanthropes are defined as animals which live near and benefit from humans and their dwellings.) These bats frequently occupy the roof spaces of houses, churches and other buildings as maternity roosts during the summer period of pregnancy, parturition and lactation. This may bring them into conflict with the human occupants of such buildings mainly because of the smell of urine and feces, and occasionally because of associated bed bugs (Cimicidae), which feed on the blood of bats and humans (Marshall, 1982). Transmission of disease from bats roosting in houses to human occupants is, however, seldom recorded and none of the five deaths from European bat lyssavirus recorded among the 590 million people in Greater Europe in the last 30 years was attributed to bats roosting in the victims' houses (Racey *et al.*, 2012).

1.9.4 Foliage

The largest bats, members of the family Pteropodidae found in the Old World tropics, roost in trees and are typically observed hanging from branches. Although the roosts of *Pteropus* were historically huge, extending over 13 square kilometres, with estimates of 30 million occupants (Ratclife, 1932), they are now much reduced in size and numbers as a result of loss of habitat, hunting for food (Mickleburgh *et al.*, 2009) or persecution by fruit farmers because of crop raiding (Furey & Racey, in press). Today, the largest recorded aggregation of fruit bats is found roosting in a small area of swamp forest in Kasanka National Park in northern Zambia where an estimated eight million

Eidolon helvum begin to arrive in October each year, to feed on wild fruit in miombo woodlands, increase in numbers to a peak in November and depart in late December (Racey, 2004). In the temperate zone, day roosting in foliage is confined to individuals or small family groups of the North American genus *Lasiurus* (Kunz & Lumsden, 2003).

Some foliage-roosting bats occupy unfurling leaves in both New and Old World habitats, and have specialised wrist and foot pads to enable them to cling to the smooth leaf surfaces (Kunz & Lumsden, 2003). Thus disc-winged bats of the genus *Thyroptera* occupy the unfurling leaves of *Heliconia* and *Calathea* in the New World (Findley & Wilson, 1974) and in Madagascar the endemic sucker-footed bat *Myzopoda aurita* roosts in the semi-unfurled central leaf of the Traveller's tree *Ravenala madagascariensis* (Ralisata *et al.*, 2010). All these roosts are highly ephemeral and the bats must find a new one when the leaf unfurls. More permanent than unfurling leaves are tents, made by 19 species in both Old and New Worlds, often by biting the leaf veins and ridges of a wide range of plant species (Kunz & Lumsden, 2003).

1.9.5 Roosts of other species

A few bat species roost in abandoned arboreal ant and termite nests which they are thought to excavate (Kunz & Lumsden, 2003). In Australia, the golden-tipped bat *Phoniscus papuensis* roosts in bird nests which it also modifies (Schulz, 2000).

1.9.6 Roost fidelity

Many bat species, especially insectivorous bats of the temperate zone, are characterized by high roost fidelity, as revealed by long-term banding studies for bats in buildings (Ransome, 2008) and caves (Gaisler *et al.*, 2003). The roosts of many fruit bats in the tropics, such as those of *Eidolon helvum* in African cities such as Accra, Dar-es-Salaam, and Kampala, and *Pteropus rufus* in Berenty National Park, Madagascar (Long & Racey, 2007) have existed for as long as local people can remember. However, in all these situations, the composition of the roosting groups is not constant and roosting groups may fragment and reform in what has been termed fission-fusion behaviour (Kerth & König, 1999).

1.10 MIGRATION

Less than 7% of bats are known or suspected migrants (Krauel & McCracken, 2013) compared with 40% of birds, but migrate for similar reasons – to experience more favorable climatic conditions and feeding opportunities. Some bat species that give birth and suckle their young in the higher latitudes of the temperate zone migrate nearly 2000 km to lower latitudes and hibernate where the climate is not so severe (Hutterer *et al.*, 2005). Of the approximately 45 bat species in Europe, only six are such long-distance migrants (Fleming & Eby, 2003). A dozen are regional migrants that may move several hundred kilometres, and the remainder are so-called stationary or sedentary species that move tens of kilometres between summer and winter roosts, but rarely disperse more than 100 km (Hutterer *et al.*, 2005). The cave-roosting bent-winged bat *Miniopterus schreibersii* is one of the best studied regional migrants and also the most widespread,

ranging from Europe through Africa to Australia. In Portugal it shows strong philopatry, or loyalty to maternity roosts following weaning. Mating occurs in hibernacula which are also the colony's maternity roosts (Rodrigues & Palmeirim, 2008). As a result of this strict philopatry to maternity roosts, all gene flow is male-induced during regional migrations (Rodrigues *et al.*, 2010).

Like birds, bats may be facultative or obligate migrants. One of the best examples of the latter is the tree-roosting hoary bat *Lasiurus cinereus* which migrates long distances in a north–south direction in North America. Individuals move through New Mexico in spring (Valdez & Cryan, 2009) and are found in Alberta, Canada in July (Baerwald & Barclay, 2011). Regional migrants show more varied directions of movement and may radiate from a common hibernaculum in a star-shaped pattern in spring.

Migratory patterns of the free-tailed bat *Tadarida brasiliensis* are more difficult to characterise, because they are panmictic (mate randomly) and populations show no genetic structure (Russell *et al.*, 2005). The species appears to include facultative, partial and long distance migrants (Krauel & McCracken, 2013). In the south-eastern United States, the bats appear to be sedentary and become torpid during cold spells in winter (Cockrum, 1969; La Val, 1973). In the mid-continent, a large part of the population migrates long distances between Mexico and the USA (Cockrum, 1969) and includes one of the longest recorded insectivorous bat migrations of 1840 km (Glass, 1982).

Less is known about migration in Old World fruit bats than for insectivorous species, although in Australia radio-tracking has revealed that nomadic populations of the grey-headed flying fox *Pteropus poliocephalus* track patchy resources and migrate hundreds of kilometres between successive pulses of *Eucalyptus* flowering (Fleming & Eby, 2003). The larger size of these bats means that recent advances in satellite tracking, particularly reduction in mass of the transmitter package, has allowed its deployment. It has been inferred for some time that the largest of the African pteropodids, the straw-coloured fruit bat *Eidolon helvum*, which is widely distributed across the central belt of the continent, migrated north and south seasonally in search of food (Kingdon, 1974). Four individuals were tracked up to 2518 km from Kasanka National Park in northern Zambia to the Democratic Republic of Congo over 149 days. They travelled in a north-westerly direction at an average speed of 90 km/day (Richter & Cumming, 2006). Satellite tracking has also revealed foraging flights of 130 km in two hours as well as inter-roost movements of *Pteropus poliocephalus* of several hundred kilometres, often between different countries of South East Asia (Epstein *et al.*, 2009). The longest recorded distance travelled for a pteropodid bat appears to be 3000 km for *Pteropus alecto* (Breed *et al.*, 2010).

1.11 CLIMATE CHANGE

Bats are important indicators of climate change (Jones *et al.*, 2009). The effects of changes in temperature and food supply on the initiation of pregnancy and spermatogenesis in bats has already been described and although the studies concerned were carried out mainly on captive bats, their effects on the gestation length of *Pipistrellus pipistrellus* were confirmed in the field (Racey & Swift, 1981). In *Rhinolphus ferrumequinum*, the timing of births in midsummer was significantly advanced in warmer springs, by 18 days, when spring temperatures were elevated by 2°C (Ransome & McOwat, 1994).

More extreme disruption was reported in the mouse-eared bat *Myotis myotis* in southern Spain, where pregnancies and births occurred in December, 6 months before the usual time for this species (Ibañez, 1977).

Climate change is likely to affect the energy budgets of hibernating bats whose fat reserves must last the winter, and this in turn is likely to affect their distribution, as they seek colder temperatures in which to maintain torpor (Humphries *et al.*, 2002). Changes in elevational distribution have been described for 24 bat species previously associated with lowlands in Costa Rica, which over a 27-year period moved higher up the mountains, an effect which was at least partly ascribed to climate change (La Val, 2004). A Mediterranean species, *Pipistrellus kuhlii*, has undergone a substantial northward range expansion over the last 15 years and is now found in parts of Central and Eastern Europe (Sachanowicz *et al.*, 2006), an effect which may also be explained, at least in part, by climate change. A similar western range expansion has been recorded for *Pipistrellus nathusii* (Lundy *et al.*, 2010).

The most extreme effects of climatic changes have been recorded in Australia, where severe drought and cold in 2006 caused the deaths of several hundred pups of the southern bent-winged bat *Miniopterus schreibersii bassani*. Such mortality is likely to have had a significant effect on the population which had already declined dramatically since the 1960s (Bourne & Hamilton-Smith, 2007). In contrast, extreme heat, with ambient temperatures in excess of 42°C, caused the deaths of over 3500 individuals in mixed species colonies of *Pteropus alecto* and *Pteropus poliocephalus* in January 2002 in northern New South Wales. This event led to the documentation of similar occurrences and it transpired that over 30 000 *Pteropus* spp., mainly *Pteropus poliocephalus*, had died during 19 similar temperature extremes (Welbergen *et al.*, 2008).

The increased incidence of extreme weather events is likely to have serious effects on populations of tree-roosting bats, particularly endemic species on tropical islands. The population of *Pteropus rodricensis* in the Mascarene Islands was halved by a single cyclone (Carroll, 1988) and in the Samoan Islands populations of *Pteropus samoensis* were reduced by about 90% by a cyclone in the early 1990s (Craig *et al.*, 1994).

More recently, statistical approaches have been used to predict the effect of different climate change scenarios on the distribution of bat species in Europe (Rebelo *et al.*, 2010) and SE Asia (Hughes *et al.*, 2012).

1.12 DISEASE-RELATED MORTALITY

In view of the prevalence of viruses in bats (Messenger *et al.*, 2002; Calisher *et al.*, 2006, 2008) it is surprising that there are so few recorded instances of virus-related mortality, in contrast to the devastating effects of the cold-adapted fungus *Pseudogymnoascus destructans*, known as white nose syndrome (Reeder & Moore, 2013). Even in this case, it is not the fungus itself which is the direct cause of mortality but starvation caused by frequent arousals from hibernation and consequent depletion of fat reserves (Reeder & Moore, 2013). Several thousand *Tadarida brasiliensis* died in Carlsbad cavern, New Mexico in 1955 and 1956, and the fact that half of the 20 individuals sampled were rabies-positive suggested that rabies was the overall cause of death (Burns *et al.*, 1956), although Constantine (1967) later implicated inclement weather conditions during migration in 1956. The only other mass mortality attributed to rabies

was several hundred *Epomops dobsoni* in southern Africa, of which 10–15% were found to be infected with Lagos bat virus (King *et al.*, 1994).

Pierson & Rainey (1992) described apparent epidemic disease in *Pteropus mariannus* in Micronesia in the 1930s, involving mass die-offs at the same time as measles affected the human population. An epidemic of unknown etiology was also suspected of reducing populations of *Pteropus tonganus* in Fiji during the 1940s. More recently, in 1985, many dead *Pteropus neohibernicus* were found on the Admiralty Islands (Flannery, 1989) and a similar incident involved *Pteropus rayneri* on the Solomon Islands.

Mass die-offs of *Miniopterus schreibersii* were reported in caves across southern France, extending into Spain and Portugal in 2002, which reduced the population by 60–65%. Although the cause was unclear, herpes virus was isolated from bat lungs. Other bat species roosting in the caves were apparently unaffected (Roué & Nemoz, 2004). Other than with white nose syndrome, the causal relationships of the other death events were never conclusively established.

1.13 CONSERVATION AND DISEASE SURVEILLANCE

About a quarter of all bat species are globally threatened (Mickleburgh *et al.*, 2002), mainly as a result of habitat fragmentation or loss, as well as loss of roosts. They generally have a negative public image that influences the response to outbreaks of disease and sometimes results in calls for culls, although recent studies have highlighted the need to avoid disturbances that may precipitate viral spillovers (Peel *et al.*, 2013). Loss of native fruits in the tropics often leads to increased dependency on farmed fruits, which brings bats into conflict with fruit farmers (Abdul-Aziz *et al.*, in press). In the Old World tropics, both insectivorous and frugivorous bats are hunted for food, generally unsustainably, so that their populations are becoming depleted (Mickleburgh *et al.*, 2009). Hunters and others handling dead bats may succumb to zoonotic disease such as Ebola (Leroy *et al.*, 2009). The identification of such diseases in bats, which has gathered momentum in recent years, has led to a global hunt for more bat viruses, driven by generous funding. A dichotomy has arisen in the approaches adopted. Most investigations have followed non-destructive blood-sampling protocols recommended by the Food and Agriculture Organization of the United Nations (2011). Others have involved killing large numbers of bats (Sasaki *et al*., 2012), some species of which are of conservation concern. Others have involved moving large numbers of bats across international boundaries with no acknowledged authorisation (He *et al*., 2013). Bat biologists and conservationists are keen to work with virologists and public health officials to protect the public and reduce the risks posed by zoonoses, but in a way that also minimises the impact on bat populations (Racey *et al.*, 2012) and follows widely accepted protocols for the use of animals in research, such as those of Sikes *et al.* (2011). Such an approach has proved successful in dealing with European bat lyssavirus and has been widely adopted by global organizations such as EcoHealth Alliance.

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2

VIRUSES IN BATS: A HISTORIC REVIEW

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2.1 INTRODUCTION

Until relatively recently, the history of the discovery of bat viruses was the history of the discovery of rabies virus. That was because of the justifiable emphasis on the importance of this virus (order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*), a pathogen of humans, livestock, companion animals, and other vertebrates. Because of its importance and the absence of an effective and accepted treatment for this horrifying disease, rabies continues to be of prime importance and the history of rabies research has been scientifically far reaching.

Rabies may be the oldest human infectious disease known. Its origin has been associated with wolves (*Canis lupus*), which are now domesticated as dogs (*Canis familiaris*). According to George Baer (1991), in 2300 BCE dog owners in the Babylonian city of Eshnunna were fined heavily for deaths caused by their dogs having bitten people. In 500 BCE Democritus, a Greek philosopher, wrote of a case of canine rabies (Gr. *lyssa*: frenzy, madness; Lyssa was the Greek goddess of rage, fury, and rabies, known for driving mad the dogs of the hunter Acteon, causing them to kill him); in 400 BCE Aristotle wrote that "Dogs suffer from the madness. This causes them to become very irritable and all animals they bite become diseased." The first century CE Roman writer

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Cardanus described the saliva from a rabid dog as a "*virus*", Latin for "poison". This mixture of nonsense and shrewd observation continued for centuries; however, the only early accepted treatment for rabies was devised by Aulus Cornelius Celsus, a second century Greek philosopher, who suggested cleaning and cauterizing wounds caused by dogs, a treatment used for two centuries.

As observations of rabies spread from the Middle East to Wales, Germany, Spain, Belgium, Austria, Turkey, Hungary, France, and finally throughout Europe, condemnations of both affected people and dogs, questioning of religions and of superstitions, and clinical observations were made. Then, in 1703, a Catholic priest in Mexico described a case of rabies, but his Spanish superiors castigated him for his report. Nonetheless, rabies was reported in dogs and pigs in Barbados in 1750 and in cattle and people bitten by dogs in the French West Indies in 1776–1789. In 1804 a German scientist, Georg Gottfried Zinke, demonstrated that rabies could be passed through saliva from rabid dogs, yet it was not until 1881 that Louis Pasteur and Emile Roux began searching for a cure for rabies. Roux devised a vaccine against rabies, one consisting of macerated spinal cord from laboratory animals with rabies. The success of this vaccine, applied by Pasteur to a young boy, Joseph Meister, who had been attacked by a rabid dog, has since become a hallmark of medicine and of infectious diseases. These treatments were not, however, the end of studies of rabies and they did not further our understanding of the epidemiology of rabies.

In the 18th century, belief in vampires, allegedly dead persons who left their graves and killed people and animals, had raised great concern in the Balkans and stimulated an extensive debate in Europe; this historic phenomenon still awaits a plausible and inclusive explanation. Rabies may have played a key role in the development of the vampire legend, perhaps even zombie legends, given the coincident times of the outbreaks and the striking similarities between the disease and the popular stories. Prior to the recognition of rabies as a disease, and certainly prior to knowledge regarding its etiology, there were European legends of bats as vampires and as a cause of madness.

Terms and phrases such as "going bats", "batty", "bats in one's belfry", all popular descriptions of mental instability, have been used vernacularly in the US since the turn of the last century. Indeed, Ambrose Bierce wrote, "He was especially charmed with the phrase 'bats in the belfry', and would indubitably substitute it for 'possessed of a devil', the Scriptural diagnosis of insanity." Perhaps there was an imagined association of the seemingly erratic behavior of bats and mental illness.

Logically and significantly, Juan Gomez-Alonso, a Spanish physician presenting a fascinating hypothesis in *Neurology* (Gomez-Alonso, 1998), suggested that vampire and werewolf legends may have originated with a rabies pandemic in eastern Europe from 1721 to 1728 and that bats were prominent, if peripheral, life forms in these folktales. Whatever the reasons for these stories, whether brought to the New World from Africa or because Trinidad and Tobago and Haiti were hotbeds of rabies and inferred associations of rabies and bats, the mythology and superstitions surrounding bats served as an impediment to enthusiastic research on bats and infectious diseases. To some extent, though considerably lessening, this remains true to this day.

Antonio Carini, an Italian physician, bacteriologist and professor and director of the Pasteur Institute of São Paulo, Brazil, presented his finding that rabies of herbivores could be transmitted by bats (Carini, 1911) and the same conclusion was made by Queiroz Lima in Brazil (Queiroz Lima, 1934) and Pawan in Trinidad (Pawan, 1936).

First to isolate rabies virus was Pawan in 1931 (Pawan, 1936), who also made the connection between fruit-eating bats and paralytic rabies (Pawan, 1948). These studies truly moved rabies virus and bat research forward, but it was not until relatively recently that other viruses and bats themselves came into prominence.

In 1903, Adelchi Negri, an Italian physician, had reported his observations of what came to be called "Negri bodies", eosinophilic inclusions found in the cytoplasm of nerve cells containing rabies virus (Negri, 1903). It was not until 1953 that the first US case of rabies in a bat was reported from Pennsylvania by Witte (1954). For many years thereafter, diagnostic techniques began to be improved, epidemiologic investigations expanded, specific monoclonal antibodies produced and applied, and investigations of rabies virus put on a molecular basis but the primary advance in rabies diagnosis was the development of an immunofluorescence test in the 1950s, used to detect rabies virus antigens (Goldwasser & Kissling, 1958).

2.2 KNOWLEDGE OF BATS, BACKGROUND

Bats themselves have been intensely studied for many decades but those studies rarely included viral diseases of the bats being studied, instead focusing on bat behavior, echolocation, feeding and migratory patterns, hibernation, and other features of their diverse biology. The reasons for this seem to be that certain bat populations are threatened by human activities and studies of bats are thought of as intrusive and possibly damaging to their populations. Chiroptologists are understandably protective of bats, but few have been informed about infectious diseases until recently. For the reasons mentioned already, and because they have for the most part simply, if astonishingly, been overlooked, bats had been essentially ignored as hosts of infectious agents; therefore, with few exceptions, they were not included in surveillance schemes. This has made for a woeful lack of basic information about these ecologically and economically important creatures, resulting in our unpreparedness for infectious disease threats, such as the recent disastrous US epizootic of white-nose syndrome caused by the fungus *Geomyces destructans* (Warnecke *et al*., 2013).

Many early virus isolations and/or detections of viral nucleic acids or proteins in bats occurred when arbovirologists accidentally captured bats in nets intended for capturing birds. Not wanting to pass up an opportunity to test whatever flew into their nets, these investigators sampled the one or few bats they had captured and tested them for the presence of viruses in their blood and brains. Other investigators, either searching in general for viruses of bats or coming across them in studies peripheral to studies of rabies virus, also detected hitherto unrecognized viruses of bats. Serological detection of bat antibodies to various viruses have also been reported, but since it was difficult to differentiate genuine virus-specific antibodies from cross-reactive antibodies to distantly related viruses, those findings will not be discussed here.

2.3 EARLY, SOMEWHAT RANDOM BAT VIRUS DISCOVERIES

While searching for rabies virus in a colony of bats in California in 1954, Harald Johnson netted Mexican free-tailed bats (*Tadarida brasiliensis mexicana*), from which he isolated a virus he named "bat salivary gland virus" (Johnson, 1962). Later renamed Rio

Bravo virus, after the name of the school where the bats had been trapped, this flavivirus (family *Flaviviridae*, genus *Flavivirus*) was later shown to occur in bats elsewhere in California, Texas, New Mexico, Sonora State Mexico, and Trinidad. This was the first non-rabies virus to be recognized as originating from bats. Other viruses were incidentally discovered in bats, mostly by arbovirologists using nets to capture birds for surveillance purposes but some by investigators simply isolating viruses from wildlife or interested in bats. For example, Boulger and Porterfield isolated Lagos bat virus in Nigeria in 1956 (Boulger & Porterfield, 1958) and in the same year Tacaribe virus was isolated from fruit bats in Trinidad (Downs *et al*., 1963) and Kern Canyon virus was isolated from insectivorous bats in California (Murphy & Fields, 1967). Although not published immediately, Pavri *et al*. (1971) isolated a paramyxovirus from a fruit bat in India in 1964 and, also in 1964, Anderson *et al*. isolated Mount Elgon bat virus (Anderson *et al*., 1969) from an insectivorous bat.

Still in the "general survey" phase of field virology, Indian investigators Rajagopalan and colleagues isolated Kyasanur Forest disease virus from an insectivorous bat (Rajagopalan *et al*., 1969) and the same group isolated West Nile virus from a fruit bat (Paul *et al*., 1970), both in 1969. In 1970, Venezuelan equine encephalitis epidemic subtype IAB was isolated from a vampire bat captured in southern Mexico (Correa-Giron *et al*., 1972) and Tignor *et al*. reported the first studies of Duvenhage virus, a lyssavirus isolated from a human with a rabies-like disease who had been scratched by an insectivorous bat in South Africa (Tignor *et al*., 1977). Continuous studies of zoonotic diseases in Africa and elsewhere, principally yellow fever, Lassa fever, and others led to the isolation of Ife virus (family *Reoviridae*, genus *Orbivirus*) from straw-colored fruit bats in 1971 (Kemp *et al*., 1988); Rio Bravo and Tamana bat virus (family *Flaviviridae*, genus *Flavivirus*) from insectivorous bats in Trinidad; Venezuelan equine encephalitis enzootic subtype IF was isolated in 1978 from a fruit bat in Brazil (Calisher *et al*., 1982); Sindbis virus was isolated in 1981 from pooled organs of round-leaf bats (*Hipposideros* spp.) in Zimbabwe (Blackburn *et al*., 1982); and Zhang *et al*. (1989) detected chikungunya virus from bats in China.

What can be made of all these viruses having been isolated from bats, mostly from frugivorous or insectivorous bats? First, most bats feed on either fruits of various sources or on insects, so that one would expect that if a virus was to be detected in a bat, it would be detected in either a fruit bat or an insectivorous bat. Second, bats are like the rest of us: from time to time they are exposed to blood-feeding arthropods, some of which are infected with viruses and can transmit them. During arbovirus epidemics arthropod populations usually are elevated from the norm and a given arthropod is more likely to transmit an arbovirus than is usually the case. Whether an arbovirus-infected bat or a bat infected with any other virus serves as a reservoir or as the principal reservoir must first be determined before its role in natural transmission cycles can be resolved.

Inconsistent results have been obtained from the few experimental infections of bats that have been conducted. As an example, Tacaribe virus, the only arenavirus that has been detected in bats, was shown to cause fatal infections in experimentally-infected Jamaican fruit bats (*Artibeus jamaicensis*), suggesting that this bat is not a natural reservoir host of Tacaribe virus (Cogswell-Hawkinson *et al*., 2012). Alternatively, Watanabe *et al*. (2010), obtained evidence that both an alphacoronavirus and a betacoronavirus occurred in bats in the Philippines. Experimentally infecting colonized Leschenault's rousette bats (*Rousettus leschenaulti*) with one of the Philippine betacoronaviruses from

a lesser dog-faced fruit bat (*Cynopterus brachyotis*), they observed virus replication but no clinical signs of illness. This result parallels other observations with wild-caught coronavirus-infected bats. Third, our knowledge of the prevalence of viruses in bats has been skewed by the amount of effort expended or lack thereof; until recently, the cause has been the effort directed at studies of humans, arthropods, ground-dwelling small mammals, birds, and just about everything except bats. That is changing rapidly and the application of modern techniques for detection of viral genomes has increased the possibilities substantially. For example, in the intense search for the reservoir host of the recently recognized Middle East respiratory syndrome coronavirus, Memish *et al*. (2013) detected a partial RNA sequence of a beta-coronavirus with 100% identity to virus from the human index case-patient. This nucleotide sequence was obtained from a fecal pellet from an Egyptian tomb bat captured in 2012.

2.4 MORE RECENT BAT VIRUS DISCOVERIES

2.4.1 Marburg and Ebola viruses (order *Mononegavirales*, family *Filoviridae*, genera *Ebolavirus* and *Marburgvirus*, respectively)

Filoviruses were discovered because they cause severe, often fatal, hemorrhagic diseases in humans and other primates. In the late summer of 1967 a hemorrhagic fever epidemic was observed in patients, mostly laboratory workers, in Germany and in Serbia, at the time part of Yugoslavia. Through disease investigations, it was soon shown that it was transmitted from African green monkeys (*Chlorocebus sabaeus*) consigned from Uganda to Europe, but the cause of the disease was unknown, other than that it was a hitherto unrecognized virus, which was named Marburg virus for the city in Germany where the disease was first recognized. Another infection with this virus occurred in a traveler in Africa in 1975 (Gear *et al*., 1975). However, although a great deal was learned about this virus from pathologic and laboratory studies, its epidemiology remained undetermined; nonetheless, bits and pieces of field evidence suggested that bats might be associated with Marburg virus. It was not until 1999 that Swanepoel *et al*. (2007) detected Marburg virus RNA in Egyptian rousettes (*Rousettus aegyptiacus*), eloquent horseshoe bats (*Rhinolophus eloquens*), and a greater long-fingered bat (*Miniopterus inflatus*) captured in the Democratic Republic of the Congo, and Towner *et al*. (2009) isolated genetically diverse Marburg viruses from Egyptian rousettes.

In 1976 a series of severe and often fatal hemorrhagic fevers occurred in southern Sudan. Almost immediately after those cases were recognized, a similar disease was observed in humans in Zaire, nearly 1000 km away. A virus, termed Ebola virus, named after a river near the epidemic site in Zaire, was isolated from patients and partially characterized (Johnson *et al*., 1977) but, as with Marburg virus, early intensive field studies did not reveal the source of the virus. Then, between 2001 and 2003, Leroy and colleagues collected small vertebrates at sites where non-human primates had died, at the border between Gabon and the Republic of the Congo. They detected RNA of an *Ebolavirus* in bats, the RNA sequences being quite similar to that of the ebolavirus isolated from humans during the 1976 outbreak in Zaire; the bats were hammer-headed fruit bat (*Hypsignathus monstrosus*), Franquet's epauletted fruit bat (*Epomops franqueti*),

and little collared fruit bat (*Myonycteris torquata*) This demonstrated an association of bats with Ebola Zaire virus, and confirmed their speculation that ebolaviruses circulate in the forests of central Africa (Leroy *et al*., 2005).

2.4.2 Hendra and Nipah viruses (order *Mononegavirales*, family *Paramyxoviridae*, genus *Henipavirus*), and other paramyxoviruses

In 1994, in Queensland, Australia, a horse died of undiagnosed cause and 8–11 days later depression, anorexia, fever, dyspnea, ataxia, tachycardia, tachypnea, and nasal discharge was reported to be occurring in 17 other horses from the same area; 14 of those horses died or were euthanized. Five and six days, respectively, after the death of the index horse, a stable hand and a horse trainer, both of whom had had close contact with the sick horse's mucous secretions, were diagnosed with influenza-like illnesses. The stable hand recovered but the trainer developed pneumonitis, respiratory failure, renal failure, and arterial thrombosis, and died from cardiac arrest seven days after admission to the hospital. A virus of the family *Paramyxoviridae* cultured from his kidney was shown to be identical to a virus isolated from the lungs of five affected horses. The two affected humans and horses had antibody to the virus and the disease was reproduced in healthy horses following challenge with spleen–lung homogenates from infected horses (Selvey *et al*., 1995). Scattered other cases caused by this virus were identified but evidence for its otherwise occurrence were not obtained from vertebrates or arthropods in the associated areas until flying foxes ("fruit bats", genus *Pteropus*) were tested.

The etiologic agent was eventually named Hendra virus. More than one-fifth of the flying foxes in eastern Australia were shown to have neutralizing antibody to Hendra virus as did bats of multiple species of flying foxes in New Guinea. In 1996 Hendra virus was isolated from a flying fox (Halpin *et al*., 2000). Epidemiologic evaluations suggested that horses become infected with Hendra virus via direct or indirect contact with infected flying foxes, and that humans become infected with this virus via direct contact with infected horses. Severe rabies-like disease in humans has led to additional studies of flying foxes, resulting in a greater understanding of the epidemiology and geographic distribution of Australian bat lyssavirus, a rhabdovirus, suggesting that studies of viruses in flying foxes in Australia and Asia might be more productive than had been realized (Fraser *et al*., 1996).

A second paramyxovirus detected in flying foxes is Menangle virus (genus *Rubulavirus*), responsible for a zoonotic disease affecting pigs and humans in New South Wales, Australia, in 1997. Antibodies capable of neutralizing Menangle virus, were detected in flying foxes, providing serologic evidence of a bat origin for this virus; the virus later was isolated from black flying foxes (*Pteropus alecto*) (Barr *et al*., 2012). Samples of bats in Indonesia later showed the presence of henipavirus and rubulavirus RNAs.

Then, in 1998, yet another paramyxovirus, this one named Nipah virus, was recognized as the etiologic agent of a deadly disease of humans and pigs in Malaysia and Singapore (Chua *et al*., 2000). By June 1999 more than 250 human encephalitis cases, including more than 100 fatalities were diagnosed in Malaysia, and another 11 cases, including one fatality, were diagnosed in Singapore. Initially misdiagnosed as an epizootic of Japanese encephalitis, precious time was lost in controlling this epizoodemic. Eventually, control efforts included culling of all pigs on affected farms, which was

extremely costly in terms of near collapse of the billion-dollar pig-farming industry, heightened animosity between communities, and administrative costs in Malaysia.

Nipah virus was shown to be closely related to Hendra virus of Australia (Chua *et al*., 2000) and, because of their large genomes, their limited homologies with other paramyxoviruses, and other unique characteristics, these two viruses were placed in a separate genus (*Henipavirus*) within the family *Paramyxoviridae*. In addition, because of the similarity of Nipah and Hendra viruses, flying foxes were suspected as being somehow involved in the epidemiology of Nipah virus. Neutralizing antibodies to this virus were demonstrated in pteropid bats of five species in Malaysia, suggesting widespread infection in bats there. Soon thereafter the virus was detected in urine from Malaysian island flying foxes (*Pteropus hypomelanus*) (Chua *et al*., 2002). Taken together, the epidemiologic portrait was that climatic and human-driven ecologic changes, along with locations of pig farms in orchards which are home to fruit bats, provided settings in which Nipah virus can switch species, from fruit bats to pigs to humans. Nipah virus has also been associated with fruit bats in Cambodia (Lyle's flying fox, *Pteropus lylei*) (Reynes *et al*., 2005) and Thailand (Wacharapluesadee *et al*., 2005), as well as in India, Bangladesh, and Indonesia.

During early 2001, an outbreak of febrile illness associated with altered sensorium was observed in Siliguri, West Bengal, India; laboratory investigations did not immediately identify an infectious agent. Nipah virus infection had not been previously detected in India but because Siliguri is near the border with Bangladesh, where outbreaks of Nipah virus infection had recently been described, samples obtained during the Siliguri outbreak were retrospectively analyzed for evidence of Nipah virus infection. Nipah virus-specific immunoglobulin M (IgM) and IgG antibodies were detected in 9 of 18 patients. reverse transcriptase–polymerase chain reaction assays detected Nipah virus RNA in urine samples from five patients. Sequence analysis confirmed that the Nipah virus from humans in Siliguri was more closely related to Nipah virus isolates from Bangladesh than to Nipah virus isolates from Malaysia (Chadha *et al*., 2006).

In contrast to transmission of Nipah virus from bats elsewhere, in Bangladesh transmission has been found to be via drinking the sap of date palms (*Phoenix dactylifera*) and via person-to-person route. Nipah virus RNAs detected in Bangladesh are variable in their sequence, suggesting multiple introductions via Indian flying foxes (*Pteropus giganteus*), which migrate over long distances and are found in the Maldives, India, Bangladesh, China, Nepal, Pakistan, and Sri Lanka.

Other than the fact that it is a henipavirus, little is yet known about Cedar virus, RNA of which was detected in fruit bat urine in Australia in 2009 (Marsh *et al*., 2012). Interestingly, challenge studies with Cedar virus in ferrets and guinea pigs, both susceptible to infection and disease with known henipaviruses, confirmed virus replication and production of neutralizing antibodies, but clinical disease was not observed. Also, the major genetic difference between Cedar virus and Hendra and Nipah viruses lies within the coding strategy of the P gene, known to play an important role in evading the host innate immune system. Preliminary studies indicated that Cedar virus infection of human cells induces a more robust interferon-β response than does Hendra virus. Cedar virus is one well worth studying with an aim to produce a human and livestock vaccine to accompany the existing vaccine for Hendra virus, released for use in 2012 (Middleton *et al.*, 2014).

Intriguing evidence for the presence of a henipavirus in Africa was presented by Hayman *et al*. (2008) who reported finding antibody to henipaviruses in straw-colored fruit bats (*Eidolon helvum*) in Ghana. As a follow-up, Drexler *et al*. (2009) detected henipaviral RNA in a straw-colored fruit bat.

Putting these pieces together, it is clear that information regarding the distribution and medical/veterinary importance of the henipaviruses is not nearly complete.

2.4.3 Coronaviruses (order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*)

The seminal discovery of severe acute respiratory syndrome (SARS), first in several hundred people in Guangdong Province, People's Republic of China in late 2002, then elsewhere in the world, moved bat virus recognition from serendipitous, fragmented, and local, to well-planned, methodical, and global. Carlos Urbani, an Italian physician working for the World Health Organization (WHO) in Hanoi, Vietnam, treated the first SARS cases there and notified the WHO of the severity of this disease. Unfortunately, Urbani contracted the disease and died of it in March 2003, thereafter the WHO put the entire world on alert. SARS cases were diagnosed in patients in Hong Kong, Vietnam, and Canada, including in healthcare workers and household members who had cared for patients with the disease. Many of the cases were traced through chains of transmission to a healthcare worker from Guangdong Province who had visited Hong Kong, where he was hospitalized with pneumonia and died. By late April 2003, more than 4000 SARS cases and 250 SARS-related deaths were reported to the WHO from more than 25 countries. Most of these cases occurred after exposure to SARS patients in healthcare or household settings. With an incubation period usually from 2 to 7 days, widespread transmission can occur quickly. Infection is usually characterized by fever, followed a few days later by a dry nonproductive cough and shortness of breath. Death from progressive respiratory failure occurs in 3% to nearly 10% of cases. Clearly, the rapid alert from the WHO likely saved thousands or many more lives.

The WHO coordinated a massive international collaborative effort that included clinical, epidemiologic, and laboratory investigations, and initiated efforts to control the spread of the disease. Attempts to identify the causative agent of the outbreak were successful during the third week of March 2003, when laboratories in the United States, Canada, Germany, and Hong Kong isolated a novel coronavirus (SARS-CoV) from SARS patients. Unlike other human coronaviruses, this one could be isolated in Vero cells. SARS-CoV RNA has frequently been detected in respiratory specimens, and convalescent-phase serum specimens from SARS patients contain antibodies that react with SARS-CoV, altogether providing evidence that is was a newly recognized virus and associated with the disease. The source of the virus in nature had not been determined at that time but knowing that it was a coronavirus made the search easier.

Coronaviruses comprise a diverse group of large, enveloped, positive-stranded RNA viruses that cause respiratory and enteric diseases in humans and other animals. Their genomes are the largest (about 30 000 nucleotides) of any known RNA virus. For the purpose of this chapter, suffice it to say that a great deal has been learned about coronaviruses; this has been nicely summarized by Ksiazek and colleagues and Rota and colleagues in their papers describing molecular and other characteristics and properties

of SARS-CoV and comparing its genome to the genomic sequences of other coronaviruses (Ksiazek *et al*., 2003; Rota *et al*., 2003).

In the aftermath of SARS, the many iterations of a likely natural history scenario (Himalayan palm civets; *Paguna larvata*, and a raccoon dog, *Nyctereutes procyonoides*, from live markets of wild animals in mainland China) were more confusing than helpful to our understanding of the origin and spread of the virus. Poon *et al*. searching for the SARS CoV in Hong Kong bats, came close to succeeding, being the first to detect a (Group 1, i.e., alphacoronavirus) coronavirus in bats (Poon *et al*., 2005); a retrospective study of samples collected for other purposes demonstrated the presence of alphacoronavirus RNA sequence in an Australian bat captured in 1996 (L. Poon, personal communication, 2013). Then Lau *et al*. (2005) and Li *et al*. (2005) reported their detections of (Group 2, i.e., betacoronaviruses) SARS CoV-like viruses in bats; subsequently, it became apparent that bats are a natural source of at least some of the numerous alphacoronaviruses and betacoronaviruses world-wide (Osborne *et al*., 2011). Partial descriptions of many hitherto unrecognized coronaviral sequences have since been published in the scientific literature. Without more biological and epidemiological information it is difficult to determine whether these represent newly recognized viruses, are closely or distantly related strains, or are more items to add to lists; these are not tabulated in Table 2.1.

Obviously, the occurrences of Marburg virus disease, ebolavirus disease, henipavirus disease, Nipah virus disease, and SARS have served to invigorate studies of bats as well as the discovery of the viruses that cause these diseases. When taken together with knowledge of other viruses which had been known for many years, this motivated the scientific community to look even deeper into the relationships of bats and infectious agents and to stimulate studies of the biology of the bats themselves.

2.4.4 Other viruses detected in bats

Now that bats have become favored targets for virus discovery efforts, a plethora of viruses and viral nucleic acid sequences have been recognized using such approaches as full genome sequencing, deep sequencing, ultra deep sequencing, and metagenomics. Essentially, every effort to detect coronaviruses and herpesviruses in bats now are successful to some degree. Indeed, in 2013 Hall *et al*. detected coronaviral RNA in guano samples from lesser short-tailed bats (*Mystacina tuberculata*) nesting on a remote island off the south coast of New Zealand, an island with no mammals other than these bats. Because of the isolation and history of this island, the absence of other mammals on the island, and the unique nucleotide sequence of this ostensible virus, it is assumed that this virus has a very ancient relationship with these primitive bats.

Bats have been shown to harbor sequences of hitherto unrecognized viruses of the families *Adenoviridae*, *Arenaviridae*, *Astroviridae*, *Bornaviridae*, *Bunyaviridae*, *Caliciviridae*, *Circoviridae*, *Coronaviridae*, *Dicistroviridae*, *Filoviridae*, *Flaviviridae*, *Hepadnaviridae*, *Hepeviridae*, *Herpesviridae*, *Nodaviridae*, *Orthomyxoviridae*, *Papillomaviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picobirnaviridae*, *Picornaviridae*, *Polyomaviridae*, *Poxviridae*, *Reoviridae*, *Retroviridae*, R*habdoviridae*, *Togaviridae*, and *Totiviridae*. Some of these sequences almost undoubtedly reflect the diets of the bats (Li *et al*., 2010) but increasing success has been due in large part to improved molecular techniques, the availability of cell lines from bat tissues (Crameri *et al*., 2009), and novel approaches (Chu *et al*., 2008; Baker *et al*., 2013; Hayward *et al*., 2013).

TABLE 2.1 List of viruses with bat origin by virus family and genus, bat genus, number of viruses, and year of isolation or detection

Virus family	Virus genus	Number of viruses isolated or detected	Associated bat genus[a]	Year of first isolation or detection[b]
Adenoviridae	*Mastadenovirus*	3	*Pteropus* *Pipistrellus* *Rousettus*	2007
Arenaviridae	*Arenavirus*	1	*Artibeus*	1956
Astroviridae	*Mamastrovirus*	numerous	numerous	2005
Bornaviridae	*Bornavirus*	1	*Pipistrellus*	2009
Bunyaviridae	*Orthobunyavirus*	5	*Molossus* *Artibeus* *Tadarida*	1965
	Hantavirus	9	*Eptesicus* *Anoura* *Diphylla* *Pipistrellus* *Rhinolophus* *Nycteris* *Neoromicia* *Hipposideros*	1989
	Phlebovirus	3	*Micropteropus* *Pipistrellus* *Rousettus*	1981
	Nairovirus	5	*Rousettus* *Myotis* *Scotophilus* *Nyctalus*	1968
Caliciviridae	*Sapovirus*	1	*Hipposideros*	2010
Circoviridae	*Circovirus*	numerous	numerous	2008
	Cyclovirus	1	*Tadarida*	2009
Coronaviridae	*Alphacoronavius*	numerous	*Miniopterus* *Hipposideros* *Mystacina*	2003
	Betacoronavirus	2	*Rhinolophus* *Neoromicia* *Taphozous*	2003
Dicistroviridae	undetermined	1	*Pipistrellus*	2009
Filoviridae	*Marburgvirus*	1	*Rousettus*	1999
	Ebolavirus	1	*Hypsignathus* *Epomops* *Myonycteris*	2001
	Cuevavirus	1	*Miniopterus*	2002
Flaviviridae	*Flavivirus*	18	*Tadarida* *Epomophorus* *Myotis* *Scotophilus*	1954

TABLE 2.1 (*Continued*)

Virus family	Virus genus	Number of viruses isolated or detected	Associated bat genus[a]	Year of first isolation or detection[b]
			Cynopterus *Nycteris* *Miniopterus* *Pteronotus* *Pipistrellus* *Rousettus* *Eptesicus*	
	Hepacivirus	3	*Hipposideros* *Otomops*	2010
	Pegivirus	4	*Pteropus* numerous others	2007
	Pestivirus	1	*Rhinolophus*	2010
Hepadnaviridae	*Orthohepadnavirus*	5	*Hipposideros* *Miniopterus* *Uroderma* *Rhinolophus*	1985
Hepeviridae	unnamed	1	*Myotis*	2008
Herpesviridae	*Simplexvirus*	1	*Lonchophylla*	1984
	unnamed	1	*Miniopterus* *Carollia*	1964
	Cytomegalovirus	1	*Myotis*	1995
	Percavirus *Rhadinovirus* *Macavirus*	numerous	numerous	
Nodaviridae	*Nodavirus*	1	*Eptesicus*	2010
Orthomyxoviridae	*Influenzavirus* A	1	*Sturnira*	2009
Papillomaviridae	*Omegapapillomavirus*	1	*Myotis*	2010
	unnamed	1	*Miniopterus*	2010
Paramyxoviridae	*Morbillivirus*	1	*Desmodus*	2008
	Henipavirus	4	*Pteropus* *Eidolon*	1994
	Rubulavirus	10	*Sturnira* *Eidolon* *Pteropus* *Rousettus* *Epomophorus*	1979
	Pneumovirus	1	*Eidolon*	2008
	unnamed	1	*Rousettus*	1964
Parvoviridae	*Dependovirus*	1	*Myotis*	2007
	Bocavirus	1	*Myotis*	2010
	undetermined	1	*Artibeus*	2005
	unnamed	1	*Eidolon*	2005
Picobirnaviridae	*Picobirnavirus*	1	*Pipistrellus*	2009
Picornaviridae	*Kobuvirus*	1	*Eidolon*	2008

(*Continued*)

TABLE 2.1 (*Continued*)

Virus family	Virus genus	Number of viruses isolated or detected	Associated bat genus[a]	Year of first isolation or detection[b]
	unnamed	7	unidentified *Miniopterus* *Ia* *Rhinolophus* *numerous others*	1982
Polyomaviridae	undetermined	1	*Myotis*	2007
Poxviridae	*Molluscipoxvirus*	1	*Eidolon*	2009
	Chiropoxvirus	1	*Eptesicus*	2009
Reoviridae	*Orbivirus*	3	*Syconycteris* *Eidolon* *Nycteris*	1965
	Orthoreovirus	4	*Pteropus* *Rousettus*	1973
	Rotavirus	3	*Eidolon* *Myotis* *Aselliscus*	2007
Retroviridae	*Betaretrovirus*	numerous	numerous	many
	Spumavirus	1	*Rhinolophus*	2010
	Gammaretrovirus	1	*Eptesicus*	2010
Rhabdoviridae	*Lyssavirus*	13	numerous	1931
	Vesiculovirus	1	*Eptesicus*	2008
	unassigned	5	*Myotis* *Hipposideros* *Tadarida* *Rhinolophus*	1956
Togaviridae	*Alphavirus*	6	unidentified *Hipposideros* *Rhinolophus* *Desmodus* *Carollia* *Uroderma*	1963
Totiviridae	*Totivirus*	1	unknown	2007

[a] Many of the viruses listed in this table were first isolated from sources other than bats. The hosts listed here are the bats from which these viruses were first obtained or otherwise detected.
[b] Indicates the year of first detection of the virus or its partial or complete genomic sequence.
Note: Certain of the viral nucleic acid sequences detected have been identified to virus family or to genus but not to species (virus), thus this is a provisional list.

It is noted, however, that the trendline of Figure 2.1 reflects specific occurrences in the history of virus discovery, from the detection of rabies virus (1930–1939) through the peak of arbovirus discovery (1950–1979) when the Rockefeller Foundation and the US Centers for Disease Control and Prevention emphasized this work. As funding for such efforts decreased, so did virus discovery, until about 2000, when hitherto unrecognized diseases appeared and their etiologic agents recognized. Now that techniques are

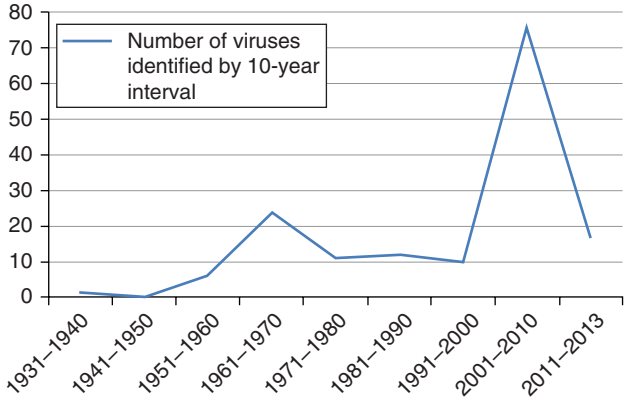

Figure 2.1 Trend of virus identification since 1930s. Number of viruses identified is plotted by 10-year intervals. The last interval (2011–2013) is not complete and hence does not reflect the real trend.

available for metagenomic analyses, the trendline is likely to rise again, and our ability to be more readily prepared for investigations, or even for predictions of future outbreaks remains to be seen; Rosenberg *et al*. (2013) have put this into a detailed context. One caution, however; because genomic sequences are not viruses, the finding of such sequences should not be taken as definitive proof of the presence of a particular virus (as for example in Table 2.1). Certainly, detection of such sequences can and should be taken as provisional or inferred evidence of the presence of a virus but further proof is necessary for more authoritative conclusions. Nonetheless, if viral genome sequences are all that are possible to obtain, such as when a virus cannot be cultured, then that will have to be taken as the best proof available.

Studies of bat retroviruses revealed that a diverse range of betaretroviruses have circulated in bats for most of their evolutionary history (Zhuo *et al*., 2013); kobuviruses (family *Picornaviridae*) have been detected in straw-colored fruit bats (Li *et al*., 2010); three sequences from various bats captured at various locations in Hong Kong represent three distinct virus species and may also represent members of a newly recognized genus of the family *Picornaviridae* (Lau *et al*., 2011); a rotavirus (family *Reoviridae*, genus *Rotavirus*) was detected in a straw-colored fruit bat captured in Kenya (Esona *et al*., 2010) and from a (possibly misidentified?) lesser horseshoe bat (*Rhinolophus hipposideros*) captured in China (He *et al*., 2013); Quan *et al*. (2013) have suggested that bats are a major natural reservoir for hepaciviruses and pegiviruses; and orthoreoviruses have been detected in bats in Australia (Nelson Bay, Pulau, Broome viruses) (Gard & Marshall, 1973; Pritchard *et al*., 2006; Thalmann *et al*., 2010, respectively) and China (Xi River virus) (Du *et al*., 2010). Another orthoreovirus, named Malaka virus, was isolated from a 39-year-old male in Malaysia (Chua *et al*., 2007). The patient had high fever and acute respiratory disease at the time of virus isolation. Two of his family members developed similar symptoms about a week later and had serological evidence of infection with the virus. Epidemiological tracing revealed that the family was exposed to a bat in the house a week before the first patient became ill. Genome sequence analysis indicated a close relationship between Melaka virus and Pulau orthoreovirus. It has

been assumed that Melaka virus is also a bat virus but it has not been detected in bats, so is not included in Table 2.1.

Unfortunately, in many laboratories virus isolation has now been supplanted by molecular detection of viral nucleic acids for virus discovery. In some ways this is limiting because we will not have the virus itself, only its genome (or partial genome) with which to conduct further work, such as biological characterization; a sequence is not a virus. Nonetheless, a complete RNA sequence is sufficient to reverse engineer a virus, to reconstruct a synthetic copy of that virus, such as has been done with RNA transcripts of a cDNA clone of hepatitis C virus (Kolykhalov *et al.*, 1997). Notwithstanding that relative shortcoming, rapid molecular detection of viral genomes has the advantages of specificity, of detecting viral genomes previously unrecognized, of simultaneous sampling of genomes from multiple viruses, of sampling over a wide range of materials and geographic areas, and of doing all this not only at continuing decreased cost but with increased safety for laboratory workers, who need not handle infectious samples.

Whether viruses or their genomes detected in bats indicate that bats are the natural reservoirs of viruses (for example, Quan *et al*., 2013) or whether they merely serve as just another vertebrate host, as accidental, unimportant hosts, must be answered for each virus discovered in them.

Finally, in an attempt to illustrate the diverse virus families or genera that different bats can harbor, a different format of virus list is presented in Table 2.2, focusing on the bat hosts, rather than the viruses.

2.5 SUMMARY

The prime difficulties in summarizing the available literature in Table 2.1 are that:

1. Many published papers fail to include either the species of bat from which a virus was identified, its year of collection, or both.
2. Some virus isolates were never fully identified, only detected, or identified only partially (to genus or family).
3. Certain papers describing endogenous or exogenous retrovirus sequences do not provide the species names of the bats from which they came or the years in which the bats were collected.
4. Some published "evidence" for the detection of a virus in a particular bat was simply the presence of antibody to that virus, or results of experimental infections, or based on other unwarranted assumptions.
5. Metagenomic studies of bat guano are generally useful as a screening tool, but only insofar as they indicate which bats frequent a particular site (usually a cave or under a tree) and which virus sequences (not viruses) have passed from the guts of some or all of those bats.
6. Some investigators use novel, non-standard nomenclature which cannot be comprehended by everyone else.
7. In the rush to publication, some few but popular journals do not provide adequate editorial assistance for authors who omit important historical information (exact location, date of collection, date of testing, and even name of the virus (who,

TABLE 2.2 List of bat genera (order Chiroptera) and viruses associated with them by family and genus

Bat host			Associated viruses	
Suborder	Family	Genus	Family	Genus
Yinpterochiroptera	Pteropodidae	*Pteropus*	*Adenoviridae*	*Adenovirus*
			Flaviviridae	*Pegivirus*
			Paramyxoviridae	*Henipavirus*
				Rubulavirus
			Reoviridae	*Orthoreovirus*
		Cynopterus	*Flaviviridae*	*Flavivirus*
		Eidolon	*Paramyxoviridae*	*Henipavirus*
				Rubulavirus
				Pneumovirus
			Parvoviridae	unnamed
			Picornaviridae	*Kobuvirus*
			Poxviridae	*Molluscipoxvirus*
			Reoviridae	*Orbivirus*
				Rotavirus
		Epomops	*Filoviridae*	*Ebolavirus*
		Epomophorus	*Flaviviridae*	*Flavivirus*
			Paramyxoviridae	*Rubula*
		Hypsignathus	*Filoviridae*	*Ebolavirus*
		Micropteropus	*Bunyaviridae*	*Phlebovirus*
		Myonycteris	*Filoviridae*	*Ebolavirus*
		Rousettus	*Adenoviridae*	*Mastadenovirus*
			Bunyaviridae	*Phlebovirus*
				Nairovirus
			Filoviridae	*Marburgvirus*
			Flaviviridae	*Flavivirus*
			Paramyxoviridae	*Rubulavirus*
				unnamed
			Reoviridae	*Orthoreovirus*
		Syconycteris	*Reoviridae*	*Orbivirus*
	Rhinolophidae	*Rhinolophus*	*Bunyaviridae*	*Hantavirus*
			Coronaviridae	*Betacoronavirus*
			Flaviviridae	*Flavivirus*
				Pestivirus
			Hepadnaviridae	*Orthohepadnavirus*
			Picornaviridae	unnamed
			Retroviridae	*Spumavirus*
			Rhabdoviridae	unassigned
			Togaviridae	*Alphavirus*
	Hipposideridae	*Hipposideros*	*Bunyaviridae*	*Hantavirus*
			Caliciviridae	*Sapovirus*
			Coronaviridae	*Alphacoronavirus*
			Flaviviridae	*Hepacivirus*
			Hepadnaviridae	*Orthohepadnavirus*
			Rhabdoviridae	unassigned
			Togaviridae	*Alphavirus*
		Aselliscus	*Reoviridae*	*Rotavirus*

(*Continued*)

TABLE 2.2 (*Continued*)

Bat host			Associated viruses	
Suborder	Family	Genus	Family	Genus
Yangochiroptera	Vespertilionidae	*Pipistrellus*	*Adenoviridae*	*Mastadenovirus*
			Bornaviridae	*Bornavirus*
			Bunyaviridae	*Hantavirus*
				Phlebovirus
			Dicistroviridae	undetermined
			Flaviviridae	*Flavivirus*
			Picobirnaviridae	*Picobirnavirus*
		Eptesicus	*Bunyaviridae*	*Hantavirus*
			Flaviviridae	*Flavivirus*
			Nodaviridae	*Nodavirus*
			Poxviridae	*Chiropoxvirus*
			Retroviridae	*Gammaretrovirus*
			Rhabdoviridae	*Vesiculovirus*
		Ia	*Picornaviridae*	unnamed
		Neoromicia	*Bunyaviridae*	*Hantavirus*
			Coronaviridae	*Betacoronavirus*
	Mormoopidae	*Pteronotus*	*Flaviviridae*	*Flavivirus*
		Myotis	*Bunyaviridae*	*Nairovirus*
			Flaviviridae	*Flavivirus*
			Hepeviridae	unnamed
			Herpesviridae	*Cytomegalovirus*
				Omegapapilloma-virus
			Papillomaviridae	*Dependovirus*
			Parvoviridae	*Bocavirus*
			Polyomaviridae	undetermined
			Reoviridae	*Rotavirus*
			Rhabdoviridae	unassigned
		Scotophilus	*Bunyaviridae*	*Nairovirus*
			Flaviviridae	*Flavivirus*
		Nyctalus	*Bunyaviridae*	*Nairovirus*
	Phylostomidae	*Artibeus*	*Arenaviridae*	*Arenavirus*
			Bunyaviridae	*Orthobunyavirus*
			Parvoviridae	undetermined
		Anoura	*Bunyaviridae*	*Hantavirus*
		Carollia	*Herpesviridae*	unnamed
			Togaviridae	*Alphavirus*
		Diphylla	*Bunyaviridae*	*Hantavirus*
		Desmodus	*Paramyxoviridae*	*Morbillivirus*
			Togaviridae	*Alphavirus*
		Lonchophylla	*Herpesviridae*	*Simplexvirus*
		Sturnira	*Orthomyxoviridae*	*Influenzavirus A*
			Paramyxoviridae	*Rubulavirus*
		Uroderma	*Hepadnaviridae*	*Orthohepadnavirus*
			Togaviridae	*Alphavirus*

TABLE 2.2 (*Continued*)

Bat host			Associated viruses	
Suborder	Family	Genus	Family	Genus
	Miniopteridae	*Miniopterus*	*Coronaviridae*	*Alphacoronavirus*
			Filoviridae	*Cuevavirus*
			Flavivirus	*Flavivirus*
			Hepadnaviridae	*Orthohepadnavirus*
			Herpesviridae	unnamed
			Papillomaviridae	unnamed
			Picornaviridae	unnamed
	Emballonuridae	*Taphozous*	*Coronaviridae*	*Betacoronavirus*
	Molossidae	*Molossus*	*Bunyaviridae*	*Orthobunyavirus*
		Tadaridae	*Bunyaviridae*	*Orthobunyavirus*
			Circoviridae	*Cyclovirus*
			Flaviviridae	*Flavivirus*
		Chaerephon	*Flaviviridae*	*Flavivirus*
			Rhabdoviridae	unassigned
		Otomops	*Flaviviridae*	*Hepacivirus*
	Mystacinidae	*Mystacina*	*Coronaviridae*	*Alphacoronavirus*
	Nycteridae	*Nycteris*	*Bunyaviridae*	*Hantavirus*
			Flaviviridae	*Flavivirus*
			Reoviridae	*Orbivirus*

other than the author and his mother, would remember the meaning of field/laboratory identifications such as "BaT123-V49C"?).

8. Many authors use the word "virus" when they mean "sequence". Many of these "data" were not included in Table 2.1. That table should not be considered final or up-to-date; while writing this chapter, the author became informed of numerous recent papers reporting the identifications of virus-specific nucleic acid sequences of multiple viral genomes. Previous reviews (Wang *et al*., 2011; Woo *et al*., 2006; Calisher *et al*., 2006) have been informative but studies of bat viruses are now so numerous and so productive that no review can be complete or totally inclusive. In order to try to pre-empt the emergence of bat-borne viruses, wide-scale surveillance efforts must and will be undertaken. Certainly, as additional studies of bats and their viruses are completed, it is likely that many more viruses, or their genomic sequences, will be detected in bats.

What began as an oddity became a trickle and is now a flood. Therefore, at this time little can be said in summary of these data. Considerable bias exists in tallying due to incidental, rather than designed captures of bats and the fortuity of isolating viruses from them. In addition, preferences for studying bats of particular species or types (insectivores, carnivores, frugivores) or ease of capture likely distort the apparent prevalence of viruses in the biome and do not help clarify which bats of the more than 1300 species are of greater or lesser importance as virus reservoirs and as transmitters of viruses. As we become more selective and sophisticated in searching for viruses or at

least for viral nucleic acids in bat tissues or body fluids, hundreds more viruses and viral sequences will undoubtedly be discovered and the true significance of bats in the natural transmission cycles of viruses and in causing diseases will become more clear-cut. Finally, it is important to recognize the difference between a virus and the sequence of a viral genome; a sequence is only one part of a virus, or, as Sir Peter Medawar aptly described it, "a virus is a piece of bad news wrapped up in protein."

Viruses of bats are highly diverse and their phylogeny might parallel the diversity of the bats themselves (Drexler *et al*., 2012a, b). Donaldson and colleagues studied the viromes (the sum total of all viruses found in members of a species) of bats of three species in Maryland, a mere 41 bats sampled on a single night and, after finding evidence of the presence of nucleic acid sequences of multiple viruses in fecal and oral swab samples, concluded that:

> *Given the depth of viral richness observed in bats, surprisingly little research has been conducted to determine which viruses are specific to bats, which viruses persistently infect different bat species, and which viruses are trafficked from one population to another with eventual dissemination to different species or reservoir hosts (Donaldson et al., 2010).*

Among many other such efforts, Wu and associates, studying bats in China, found evidence of the presence of viruses: in mammalian families (*Adenoviridae*, *Herpesviridae*, *Papillomaviridae*, *Retroviridae*, *Circoviridae*, *Rhabdoviridae*, *Astroviridae*, *Flaviridae*, *Coronaviridae*, *Picornaviridae*, and *Parvoviridae*); insect viruses, including those of the *Baculoviridae*, *Iflaviridae*, *Dicistroviridae*, *Tetraviridae*, and *Densoviridae*; fungal viruses, included those of the *Chrysoviridae*, *Hypoviridae*, *Partitiviridae*, and *Totiviridae*; and phages, including those of the *Caudovirales*, *Inoviridae*, and *Microviridae*, as well as unclassified phages (Wu *et al*., 2012). In addition to these viruses, probably associated with insects, plants, and bacterial flora related to the diet and habitats of bats, they identified complete or partial genome sequences of 13 novel mammalian viruses, including herpesviruses, papillomaviruses, a circovirus, a bocavirus, picornaviruses, a pestivirus, and a foamy virus. Further analyses indicated that these novel sequences showed little similarity with those of previously reported viruses (Wu *et al*., 2012). Dacheux *et al*. (2014) have reported nucleotide sequences of viruses of multiple families in tissues of bats in France. Their report included mention of nucleic acids of viruses of families infecting invertebrates, plants, fungi, protozoa, and bacteriophages, again intimating that perhaps not all viral nucleic acid sequences from bats represent definitive proof that such viruses were any more than "associated" with the bats in which they were detected.

Transmission between and among bats appears to be dependent on their colonial or individual features: ability to fly long distances, nutritional needs, peculiar immunologic characteristics, roosting behaviors, and perhaps even human impingement and global climate change. Suffice it to say, in brief, at this time it may be reasonably conjectured that many viruses, certain rhabdoviruses, herpesviruses, paramyxoviruses, filoviruses, and coronaviruses included, are primarily viruses of bats and that human, livestock, and wildlife infections with these viruses are coincidental and unfortunate. In order to obtain more definitive evidence that any viral nucleic acid sequence actually represents part of a complete virus, an infectious virus should be isolated and tested for biological characteristics using experimental infections of bats and other laboratory hosts.

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3

BAT LYSSAVIRUSES

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3.1 LYSSAVIRUS GENUS

Lyssaviruses constitute a genus (*Lyssavirus*) in the family *Rhabdoviridae* of enveloped single-stranded negative-sense RNA viruses. Rhabdoviruses have a monophyletic origin and a specific bullet-shaped virion morphology, which distinguish them from other taxa in the order *Mononegavirales*: the *Bornaviridae*, the *Filoviridae*, and the *Paramyxoviridae*. The majority of rhabdoviruses described to date (>200) are arthropod-borne (Dietzgen *et al*., 2011). However, lyssaviruses constitute an unusual exception. Lyssaviruses are transmitted directly between mammals, usually via a bite, causing the disease known as rabies; an acute progressive encephalitis, with nearly a 100% case fatality rate.

For decades in the early 20th century, it was believed that rabies virus was unique as the sole representative of its kind. However, discovery of several serologically and morphologically related viruses in Africa and Europe during the 1950–1960s resulted in the establishment of a group of 'rabies-related' viruses, also known as a 'rabies serogroup' (Shope *et al*., 1970). This group was supplemented further with other representatives, and the genus *Lyssavirus* was established under the auspices of the International Committee on the Taxonomy of Viruses (ICTV). The name of the genus originated from Greek mythology: Lyssa (Λυσσα) was a goddess, or spirit of rage, fury, raging madness,

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and frenzy (Theodorides, 1986). *Rabies virus* was established as the type species of the genus (Dietzgen *et al*., 2011).

The bullet-shaped lyssavirus virions are 130–250 nm in length and 60–100 nm in diameter. The lipid bilayer envelope is acquired from host cell membranes during budding. Protruding through the membranes glycoprotein (G) spikes are organized in trimers. The internal nucleocapsid (NC) core consists of a ribonucleoprotein (RNP) complex, comprising the genomic RNA, bound tightly to the nucleoprotein (N), together with an RNA-dependent RNA polymerase (L) and a phosphoprotein (P). The NC is active for transcription and replication; the RNP serves as a template processed by the L protein, which contains most enzymatic activities, and its cofactor, the P protein. The NC has a helical symmetry, about 700 × 20 nm in size. In the virion, the matrix protein (M) condenses the NC, interacts with the N-RNA complex and associates with the host-derived lipid bilayer containing the transmembrane G protein (Dietzgen *et al*., 2011).

Viral proteins are multifunctional. The N is the major component of the NC and interacts actively with the RNA, L and P proteins. The P plays multiple roles during transcription and replication as a non-catalytic cofactor of the viral polymerase. It mediates the physical link and proper positioning of the L protein on the N-RNA template, and acts as a chaperone during synthesis of N, by forming N–P complexes that prevent N from self-aggregation and binding to cellular RNA (Dietzgen *et al*., 2011). The P protein was shown to have multiple binding sites for different viral or host proteins to allow their assembly into multimolecular complexes. The highly acidic N-terminal domain of the P protein has binding sites for the viral L and N proteins, host cell kinases and importins. The central domain has an L protein binding site and dimerization domain, and the C-terminal basic domain is involved in N-RNA binding. The P protein may also interact with the host's cellular transport systems, such as the dynein motor complex, nucleocytoplasmic transporters and microtubules to facilitate intracellular movement of viral components (Das *et al*., 2006; Albertini *et al*., 2008; Min *et al*., 2010). Furthermore, P interferes with the innate immune response by inhibiting different steps of the host cell interferon response (reviewed by Leyrat *et al*., 2011). The M, which is positioned in virions between the NC and viral envelope, binds the RNP and the cytoplasmic domain of the G, thereby facilitating maintenance of virion morphology and the budding process. In infected cells, the M appears to be involved in regulation of the genome RNA transcription. It also mediates such pathobiological effects as intracellular membrane redistribution and apoptosis. The G is assembled into trimers to form the virion surface spikes. As the only outer viral protein, the G interacts with host cell receptors, facilitating virus attachment and cellular entry. The G induces production of virus-neutralizing antibodies and elicits cell-mediated immune responses. The L has multiple domains and performs the functions required for genome transcription and replication, including RNA-dependent RNA polymerase, mRNA 5´ capping enzyme, cap methyltransferase, 3´ poly (A) polymerase and protein kinase activities.

The lyssavirus genome is represented by a non-segmented, linear, negative-sense single-stranded RNA, about 12 kb in length. It includes five major genes that are arranged in the order 3´-N-P-M-G-L-5´. Each of the individual genes is flanked by transcription initiation and termination/polyadenylation signals that are largely conserved among members of the same viral species. Transcription units are separated by short untranscribed intergenic regions. The ends of the genomic RNA, termed 3´ leader and 5´ trailer

sequences, exhibit terminal complementarity and contain promoter sequences that initiate replication of the genome and antigenome, respectively (Dietzgen *et al*., 2011).

Genetic distances between lyssavirus species are shorter than those in other rhabdovirus genera. At present, the genus includes 14 species and one putative member, known by a fragment of genome sequence only (Table 3.1, Figure 3.1). Demarcation criteria for lyssavirus species include:

1. Genetic distances, with the threshold of ~80–82% nucleotide identity for the complete N gene or ~80–81% nucleotide identity for the concatenated coding regions of N+P+M+G+L genes. Globally, all isolates belonging to the same species have higher identity values than the threshold, except the viruses currently included into the *Lagos bat virus* species. However, as these viruses are segregated into a monophyletic cluster in the majority of phylogenetic reconstructions, and in the absence of other demarcation characters, there is currently no plan to subdivide *Lagos bat virus* into several species, as has been proposed by several authors (Markotter *et al*., 2008; Delmas *et al*., 2008).
2. Topology and consistency of phylogenetic trees, obtained with various evolutionary models.
3. Antigenic patterns in reactions with anti-nucleocapsid monoclonal antibodies and serologic cross-reactivity using polyclonal antisera.
4. Whenever available, additional characteristics, such as ecological properties, host and geographic ranges, and pathological features are considered (Dietzgen *et al*., 2011).

Based on genetic distances and serologic cross-reactivity, the genus has been subdivided into two phylogroups (Badrane *et al*., 2001). The demarcation was based on phylogenetic relationships (~74% or more amino acid sequence identity within the G ectodomain between viruses of one phylogroup and less than ~64% sequence identity between viruses from different phylogroups); serologic cross-reactivity (cross-neutralization within phylogroups, but absence of cross-neutralization between members of different phylogroups); the presence of K/R333 in the G of Phylogroup I viruses and D333 in the G of Phylogroup II viruses, with implication for a reduced pathogenicity of the latter. In general, serologic cross-reactivity is somewhat correlated with genetic distances. Viruses that shared more than ~72% amino acid sequence identity within their G ectodomains cross-neutralized each other (Badrane *et al*., 2001).

Phylogroup I includes the species *Rabies virus* (RABV), *European bat lyssaviruses, type 1* (EBLV-1) and *type 2* (EBLV-2), *Duvenhage virus* (DUVV), *Australian bat lyssavirus* (ABLV), *Aravan virus* (ARAV), *Khujand virus* (KHUV), *Irkut virus* (IRKV), and *Bokeloh bat lyssavirus* (BBLV). Phylogroup II includes *Lagos bat virus* (LBV), *Mokola virus* (MOKV), and *Shimoni bat virus* (SHIBV). The remaining members of the genus, *West Caucasian bat virus* (WCBV), *Ikoma lyssavirus* (IKOV), and Lleida bat virus (LLEBV) cannot be included into either of these phylogroups. Although the latter three viruses are related phylogenetically (Figure 3.1), genetic distance between them are greater than the distances within Phylogroup I and Phylogroup II. No evidence for cross-neutralization between WCBV and IKOV were obtained (Horton *et al*., 2014). The same observation may be expected for LLEBV. Therefore, it is not possible to

TABLE 3.1 Viruses currently included in the genus *Lyssavirus*

Recognized and proposed species	Phylogroup	Natural host	Geographical range	Comments
Rabies virus; RABV (type species)	I	Bats (Chiroptera) of multiple species, terrestrial mammals (predominantly Carnivora)	Terrestrial mammals – worldwide (except Australia, Antarctica and several insular territories); bats – New World only	Responsible for the vast majority of human rabies cases in the world. All currently available human and veterinary vaccine strains originate from this species.
Australian bat lyssavirus; ABLV	I	Pteropodid bats (at least 4 species of *Pteropus* genus) and insectivorous bats (*Saccolaimus albiventris*)	Australia (perhaps, with several surrounding islands)	Given limited surveillance, host range among insectivorous bats may be greater. Three human cases documented.
European bat lyssavirus, type 1; EBLV-1	I	Insectivorous bats (predominantly *Eptesicus serotinus*)	The major part of Europe, from Spain to the Ukraine.	Given the limited surveillance in eastern Europe and Asia, may be distributed more broadly, along the reservoir species range. Spillover infections in wild and companion animals, as well as a human case have been documented.
European bat lyssavirus, type 2; EBLV-2	I	Insectivorous bats (predominantly *Myotis daubentonii* and *Myotis dasycneme*)	North-western Europe	Two human cases have been documented.

TABLE 3.1 (*Continued*)

Recognized and proposed species	Phylogroup	Natural host	Geographical range	Comments
Khujand virus; KHUV	I	Insectivorous bat *Myotis mystacinus*	Central Asia	Known by a single isolate. Given the limited surveillance in eastern Europe and Asia, may be distributed more broadly. No human cases have been documented.
Aravan virus; ARAV	I	Insectivorous bat *Myotis blythi*	Central Asia	Known by a single isolate. Given the limited surveillance in eastern Europe and Asia, may be distributed more broadly. No human cases have been documented.
Bokeloh bat lyssavirus; BBLV	I	Insectivorous bat *Myotis nattereri*	Europe	Known by 3 isolates. No human cases have been documented.
Irkut virus; IRKV	I	Insectivorous bat *Murina leucogaster*	Eastern Asia	Known by three isolates, from bats and from a human who developed rabies after bat bite.
Duvenhage virus; DUVV	I	Insectivorous bats	Sub-Saharan Africa	Known by 4 isolates, 3 of which came from humans, bitten by bats, and one from a bat, of the presumably *Miniopterus* species.

(*Continued*)

TABLE 3.1 (*Continued*)

Recognized and proposed species	Phylogroup	Natural host	Geographical range	Comments
Lagos bat virus; LBV	II	Pteropodid bats of several genera (*Eidolon helvum, Rousettus aegyptiacus, Epomophorus* spp. etc.)	Sub-Saharan Africa	Constitutes several lineages with long genetic distances. Potentially, in the future may be subdivided into 2–3 separate species. Spillover infections reported in wild and companion animals. No human cases documented to date.
Mokola virus; MOKV	II	Unknown	Sub-Saharan Africa	Twice isolated from shrews, once from a rodent. The majority of other isolates were obtained from companion animals, such as cats and dogs, as the result of spillover infections. Two human cases have been reported.
Shimoni bat virus; SHIBV	II	Insectivorous bat *Hipposideros commersoni* (*H. vittatus*)	Kenya	Known by a single isolate. Serologic surveys suggest that *Hipposideros commersoni* (*H. vittatus*) is the likely reservoir. No human cases have been documented.

TABLE 3.1 (*Continued*)

Recognized and proposed species	Phylogroup	Natural host	Geographical range	Comments
West Caucasian bat virus; WCBV	NA[a]	Insectivorous bats from genus *Miniopterus*	South-eastern Europe, probably Africa	Known by a single isolate from the Caucasia region. However, serologic surveys suggest that WCBV (or other serologically related virus) may be present in *Miniopterus* bats in Africa (Kenya). No human cases have been documented.
Ikoma lyssavirus; IKOV	NA	African civet (*Civettictis civetta*)	Tanzania	Known by a single isolate. The natural host is questionable. Given phylogenetic relatedness to the West Caucasian bat virus, the index case in African civet may result from a spillover infection of bat origin. No human cases have been documented.
Lleida bat lyssavirus; LLEBV	NA	Insectivorous bat *Miniopterus schreibersii*	Spain	Known by a single partial genome sequence with unsuccessful virus isolation efforts. No human cases have been documented.

[a]NA – neither a member of Phylogroup I nor II. Other Phylogroup not assigned.

assign these three viruses in a new Phylogroup III, based on the existing demarcation criteria. In general, the limited serologic cross-reactivity between lyssaviruses from different phylogroups is important from a public health and veterinary standpoint, as discussed below.

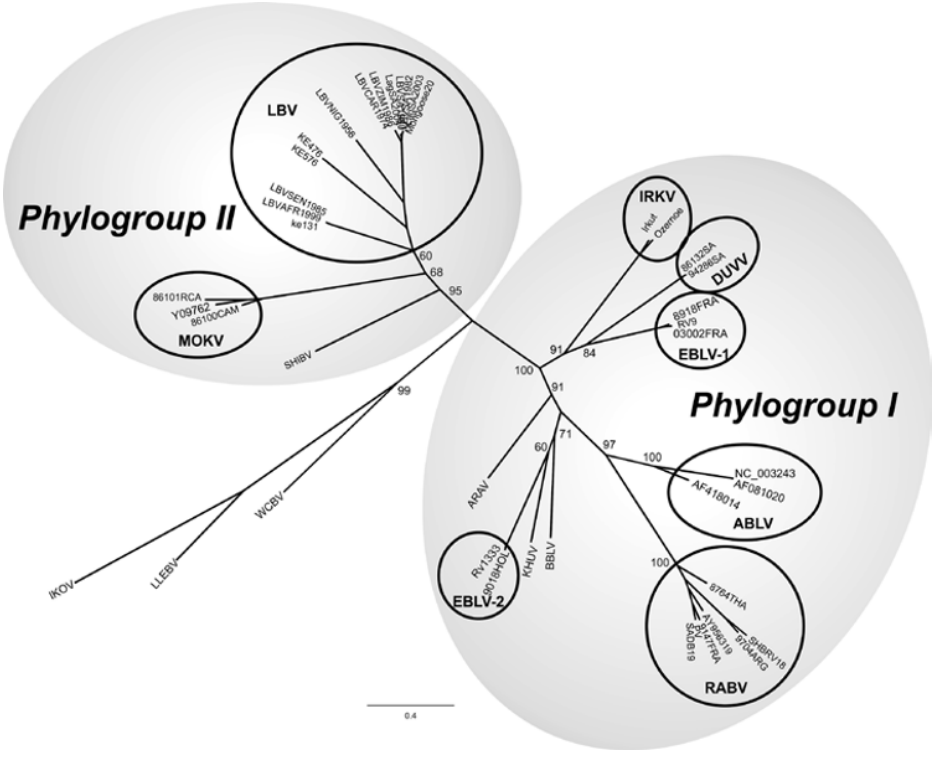

Figure 3.1 Phylogenetic tree of lyssaviruses based on nucleoprotein gene sequences. Branch lengths are drawn to scale, and bootstrap values (1000 replicates) are shown for key nodes. RABV, rabies virus; EBLV-1, European bat lyssavirus, type 1; EBLV-2, European bat lyssavirus, type 2; BBLV, Bokeloh bat lyssavirus; ARAV, Aravan virus; KHUV, Khujand virus; IRKV, Irkut virus; ABLV, Australian bat lyssavirus; LBV, Lagos bat virus; MOKV, Mokola virus; SHIBV, Shimoni bat virus; WCBV, West Caucasian bat virus; IKOV, Ikoma lyssavirus; LLEBV, Lleida bat lyssavirus.

3.2 PATHOBIOLOGY

All mammals are susceptible to lyssaviruses although bats and carnivores are the major natural reservoirs. It appears that bats are the primary reservoir hosts for lyssaviruses from at least 12 of the 14 identified species. In contrast, carnivores are reservoir hosts for RABV only (Rupprecht *et al*., 2011). Natural reservoirs for MOKV and IKOV are unknown as the majority of the available isolates originated from what are believed to be spillover infections in domestic animals (MOKV), as well as the single isolate of IKOV from an African civet (Marston *et al*., 2012). Serologic surveillance for MOKV is not reliable, as this virus demonstrates cross-reactivity with LBV, which is broadly circulating in African fruit bats (Kuzmin *et al*., 2008c; Dzikwi *et al*., 2010). No serologic surveillance data is available for IKOV to date.

Lyssaviruses are highly neurotropic. After delivery into a wound via a bite, lyssaviruses may undergo limited replication at the inoculation site, as was shown for skeletal

muscle cells (Tsiang, 1988). Thereafter (or immediately) the virions penetrate peripheral neurons, both sensory and motor fibers. The attachment to cell membrane receptors is mediated by the G spikes on the virion envelope. Several putative receptors were suggested for RABV attachment, including: nicotinic acetylcholine receptor, carbohydrate moieties, phospholipids, and gangliosides. Once bound, the virion enters the cell by endocytosis, and the endosomal vesicule is transported by retrograde axonal flow, dependent on the microtubule network, using dynein motors (Tordo *et al*., 2005). Following acidification, the viral membrane fuses with the endosome membrane, and the RNP is released into the cytoplasm, where transcription and replication occur. This process is mediated by the viral RNA-dependent RNA polymerase (including its cofactor, the P protein) whereas the RNP serves as a template and protects the RNA from the host nuclease activity. The translation is ensured by cellular machinery. The NC components accumulate as intracytoplasmic inclusions (historically known as Negri bodies) and presently serve as the major target for rabies diagnosis via antigen-detection methods. The G protein is delivered to the cytoplasmic membranes where, in association with the M protein, it mediates virion budding. The viral progeny are released at the synaptic junction and propagate up to the central nervous system (CNS) by using motor or sensory neurons and following neuronal connections. This activity avoids or limits surveillance by the host immune system, resulting in the absence of an innate and an early adaptive response. In addition, lyssavirus P protein selectively blocks interferon-signaling pathways in the infected cells via targeting STAT proteins (Wiltzer *et al*., 2012).

The duration of an asymptomatic incubation period is approximately 2 months on average (Tordo *et al*., 2005). However, it may be extremely variable, from a week to several months and even years (Boland *et al*., 2014). Being delivered to the CNS, the virus disseminates rapidly. Nearly all regions of the CNS may be affected, but the medulla oblongata is a principal area from which the virus is transported to the salivary glands along the innervation pathways (Figure 3.2). Virions released into the saliva provide transmission to other susceptible hosts via a bite. Virions appear in saliva usually during the clinical period of rabies but may be detected during the end of incubation period before obvious illness. At least for dogs, cats, and ferrets such virus excretion was documented for no longer than the last 10 days before advent of clinical signs (National Association of State Public Health Veterinarians, Inc., 2011). For bats and other wildlife, it may be longer, as detected in several experimental studies (Baer & Bales, 1967; Davis *et al*., 2012). Neuropathological changes observed in the infected brain are relatively mild histologically and include gliosis, slight neuronophagia, and perivascular infiltration with inflammatory cells, with rare involvement of meninges. Occasionally, more severe brain damage occurs, such as spongiform lesions, extensive neuronal degeneration, and widespread inflammation. Functional alteration of the CNS is much more significant than morphological representation. Apoptosis as a response to RABV infection is a prominent factor of neuron damage (Hemachudha *et al*., 2013). The reverse spread of the virus from the CNS to various tissues occurs during later stages of rabies via typical neuronal pathways, but viremia does not occur.

The clinical period is rapid and severe (1–10 days). A prodromal period is lacking specific clinical signs and is limited to fever, malaise, sometimes vague flu-like signs. Frequently, human patients feel paresthesia and tingling in the inoculation site. With the development of encephalitis, cerebral symptoms manifest. Two major clinical forms of

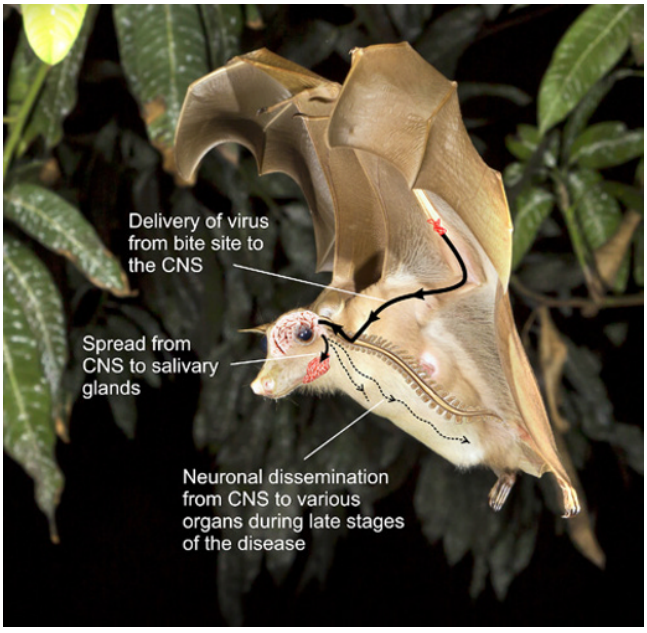

Figure 3.2 Schematic illustration of lyssavirus spread in an infected host. *(See insert for color representation of the figure).*

rabies have been described: furious and paralytic (although "mixed" forms are very common). When the disease is furious, animals or humans become agitated and aggressive. Insomnia, irritability, and anxiety are commonly observed. Other signs, such as pupillary dilation, altered phonation, aimless wandering and hyperactivity, drooling of saliva, and muscle tremors and seizures may be noted. Humans often develop hallucinations and delirium. Some symptoms, which have been considered as "classic"' but are only observed in 50% or less of human patients, are hydrophobia, aerophobia, photophobia, and phonophobia. Paralytic rabies is characterized by a greater prevalence of paresis and paralysis from the beginning of disease manifestations, whereas agitation and anxiety are moderate or absent. With the progression of CNS damage, sick animals or humans become comatose and die, usually due to respiratory failure or cardiac arrest (Hemachudha *et al*., 2013). Altered behavior of rabid animals and excretion of virus in saliva mediate transmission of the disease to other susceptible hosts.

The host immune system usually recognizes lyssavirus infection at a late stage, when the virus spreads from the CNS to peripheral organs. Given the short duration of the clinical period, immune responses cannot clear the virus easily. Indeed, various mammalian species exhibit different susceptibility to the variety of lyssaviruses, as at least in part resulting from apparent mutual adaptation of virus and its principal host. For example, canids are highly sensitive to homologous RABV variants, and develop a furious form of rabies with high titers of virus in salivary glands; this ensures transmission of the infection to a critical number of susceptible individuals before the death of the sick animal. Very low seroprevalence was demonstrated in natural populations of foxes, indicating that most events of their contact with RABV lead to a fatal infection

(Steck & Wandeler, 1980). In contrast, high seroprevalence was detected among bats (Steece and Altenbach, 1989; Kuzmin *et al*., 2008b, c; Dzikwi *et al*., 2010; Hayman *et al*., 2008). These gregarious mammals demonstrate moderate to low susceptibility to lyssaviruses. In most cases when bats encounter the virus, they develop an appropriate immune response and clear the virus before it reaches the CNS and causes clinical rabies. Therefore, a significant antibody prevalence in bat populations is attributed to abortive peripheral infection (frequently termed as "exposure") rather than to CNS infection (reviewed by Kuzmin & Rupprecht, 2007). Indeed, rare cases of survivorship after manifestation of clinical signs of rabies have been registered occasionally in different animal species (Hamir *et al*., 2011; Davis *et al*., 2013a). However, these sporadic events cannot be taken in support for a theory of lyssavirus persistence or a true 'carrier' state.

3.3 SURVEILLANCE AND DIAGNOSIS

Lyssavirus surveillance is targeted to obtain data on virus distribution, host range and circulation patterns from the field as opposed to experimental data generated in laboratory settings. The majority of findings relating to an understanding of bat rabies epidemiology and phylogeography, as described below in this chapter, were obtained via the establishment of a routine public health surveillance system in developed countries.

As lyssaviruses are neurotropic pathogens, CNS tissue is the only suitable target for accurate virus detection. Therefore, conclusive rabies diagnosis can be performed only post mortem. A combination of laboratory tests have been developed for antemortem diagnosis of rabies in humans (such as detection of viral antigens in the nerves surrounding hair follicles in skin, polymerase chain reaction (PCR)-amplification of viral RNA, isolation of infectious virus from saliva and skin, detection of specific antibodies in blood serum and cerebrospinal fluid, etc.), but none of these methods is sufficiently reliable when used alone. Antemortem methods are not used for rabies diagnosis in animals.

The existing routine surveillance network is based on the detection of rabies virus antigens and nucleic acids in animal CNS tissues. The majority of diagnostic samples originate from mammals (including bats) involved in exposure of humans or domestic animals, when a decision on post-exposure prophylaxis or quarantine must be made (National Association of State Public Health Veterinarians, Inc., 2011; World Health Organization, 2013). Although appropriate and economically feasible for such purposes, passive surveillance provides limited insight into host–pathogen ecology. Moreover, it is relatively efficient only in developed countries with well-established notification systems, effective delivery of biologics with cold chains, diagnostic capacity, inter-sectorial cooperation, and transparent communication networks.

Active rabies surveillance may utilize serologic tests, aiming to obtain indirect evidence of virus circulation via detection of specific antibodies. As discussed above, this approach is not reliable for the assessment of rabies virus circulation in most carnivores. However, it is useful for determination of herd immunity after implementation of oral rabies vaccination programs in carnivore populations (Rupprecht & Slate, 2012). Lyssavirus seroprevalence in bat populations is usually easy to demonstrate when appropriate methods are used (Trimarchi & Debbie, 1977; Steece and Altenbach, 1989; Brookes *et al*., 2005a; Kuzmin *et al*., 2008a; Dzikwi *et al*., 2010; Hayman *et al*., 2008).

Moreover, given the limited serologic cross-reactivity of certain lyssaviruses, comparative testing may provide inferences on the specific virus circulation in a given bat population (Kuzmin *et al*., 2008b,c; Kuzmin *et al*., 2011). From relatively large-bodied animals, serum samples may be obtained non-destructively, via peripheral venipuncture. However, it is technically difficult to obtain sufficient blood volumes from small insectivorous bats. In a majority of cases, serologic sampling of insectivorous bats is a part of a larger sampling aimed for broad pathogen discovery purposes (Wang *et al*., 2011), and this sampling is usually destructive.

Active lyssavirus surveillance of apparently healthy bats via collection of oral swabs for PCR amplification of viral RNA is usually not productive. Beyond rare exceptions (Echevarria *et al*., 2001; Serra-Cobo *et al*., 2002), testing of hundreds of oral swabs from bat populations produced negative results, even if the presence of lyssaviruses in these populations was demonstrated serologically and by sporadic isolation of the viruses from moribund and dead bats (Brookes *et al*., 2005a; Kuzmin *et al*., 2008c, 2010). This is likely caused by short and intermittent salivary excretion of lyssaviruses during the clinical course of rabies, or several days before the disease onset. Likewise, destructive sampling of apparently healthy bats for the detection of lyssaviruses in their CNS is not productive. The majority of such studies reported 0–0.5% of rabid bats, whereas this proportion was as high as 5–20% among moribund and dead bats (reviewed by Constantine, 1967a). In our experience, sampling CNS tissues and oral swabs from ~3000 apparently healthy bats collected in sub-Saharan Africa were negative for lyssaviruses. By comparison, 5 lyssavirus isolates were obtained from the brains of ~150 dead bats (found under their roosts), and in each case the virus was also detected in oral swab (Kuzmin *et al*., 2008c, 2010).

Indeed, CNS is the tissue of choice for rabies diagnosis. Historically, rabies diagnosis was based on the detection of Negri bodies in a Seller-stained CNS preparation. However, this method was not reliable, because similar "bodies" are present in other viral encephalitides (Tierkel & Atanasiu, 1996). The situation improved significantly with the advent of the fluorescent antibody test (FAT) in the late 1950s (Dean *et al*., 1996). In general, the method is based on detection of intracytoplasmic inclusions but with high specificity implied by the use of lyssavirus-specific antibodies, and sensitivity as of any fluorescent test compared to conventional histological testing. Several polyclonal and monoclonal antibody conjugates display broad pan-lyssavirus reactivity based on the conservation of viral NC, which predominates in the cytoplasmic inclusions (Horton *et al*., 2014). Other antigen-detection methods, such as the direct rapid immunohistochemical test (DRIT) also were shown to have high sensitivity and specificity and, unlike the FAT, do not require such expensive equipment as a fluorescent microscope (Dürr *et al*., 2008).

In contrast, methods based on the detection of nucleic acids (such as reverse transcription (RT)-PCR) at present are not recommended as primary tests for routine rabies diagnosis, due to their high sensitivity and possibility of cross-contamination, particularly in suboptimal laboratory settings (World Health Organization, 2013). However, RT-PCR is the method of choice for rabies diagnosis in non-CNS tissues or saliva, where presentation of lyssavirus antigens is limited and no cytoplasmic inclusions can be observed. Indeed, contamination may occur in such cases as well, even in the field (for example, during collection of oral swabs from bats and using only one pair of heavy-duty protective gloves for handling all animals). Therefore, sampling, testing

and interpretation of RT-PCR results should be done cautiously and compared to the results of other available diagnostic methods in toto.

The samples in which viral antigens or RNA cannot be easily detected require virus isolation in animal models (Koprowski, 1996) or cell cultures (Webster & Casey, 1996). Young (particularly suckling) mice are highly susceptible for intracranial lyssavirus inoculation, as well as neuronal cell cultures (such as the commonly used mouse neuroblastoma). In addition, several methods of enzyme-linked immunosorbent assay (ELISA) are available for lyssavirus antigen detection (Bourhy & Perrin, 1996). However, these did not find wide applicability, in part because of their complexity and limitations of sensitivity/specificity (compared to FAT and DRIT).

Lyssavirus-neutralizing antibodies in serum and cerebrospinal fluid were historically evaluated in a mouse model but at present are usually detected in cell culture via the rapid fluorescent focus inhibition test (RFFIT) or the fluorescent antibody virus-neutralization (FAVN) test (Smith *et al*., 1996; Cliquet *et al*., 1998). As suggested above, limited serologic cross-reactivity of lyssavirus G allows their differentiation in such virus-neutralizing tests, and is particularly useful for bat rabies surveillance (Kuzmin *et al*., 2008b, c; Kuzmin *et al*., 2011; Horton *et al*., 2014). Binding antibodies (targeting mainly the abundant viral NC) can be detected via an indirect FAT which is frequently used for antemortem testing of human samples. Due to the abundance of viral NC in infected cells, binding antibodies may be detected earlier than virus-neutralizing antibodies (Holzmann-Pazgal *et al*., 2009). Several modifications of the ELISA have been developed for antibody detection (Elmrgen & Wandeler, 1996). Several ELISA kits have been approved by Office International des Epizooties (World Organization for Animal Health) and are used broadly for detection of antibodies to RABV. However, no related work has been performed to date to assess the sensitivity and specificity of ELISA-based methods for the detection of antibodies against non-rabies lyssaviruses, and therefore their use in bat rabies surveillance is fairly limited.

3.4 GENERAL BIOLOGICAL CONSIDERATIONS ON BAT RABIES

Among zoonotic diseases increasingly recognized in bats, rabies is the best studied in part because of its long history of research (since the early 1900s) and the clear significance for agriculture and public health. Bats have been identified as principal reservoir hosts for such diverse lyssaviruses as LBV, DUVV, EBLV-1, EBLV-2, ABLV, IRKV, and BBLV. The RABV is maintained by bats in New World and by carnivores worldwide. Singular documentations of other lyssaviruses, such as ARAV, KHUV, WCBV, SHIBV, and LLEBV, do not allow conclusive inferences on principal reservoir hosts of these viruses, but they all were originally identified in bats. Phylogenetic placement within the genus also strongly suggests that these are bat lyssaviruses (Kuzmin *et al*., 2005, 2010, 2011; Aréchiga-Ceballos *et al*., 2013). Only MOKV and IKOV have not been found in bats to date, and reservoir hosts of these lyssaviruses remain elusive (Sabeta *et al*., 2007; Marston *et al*., 2012). IKOV clearly demonstrates close phylogenetic relatedness to WCBV and LLEBV, suggesting that it may be a bat-borne virus as well (Horton *et al*., 2014).

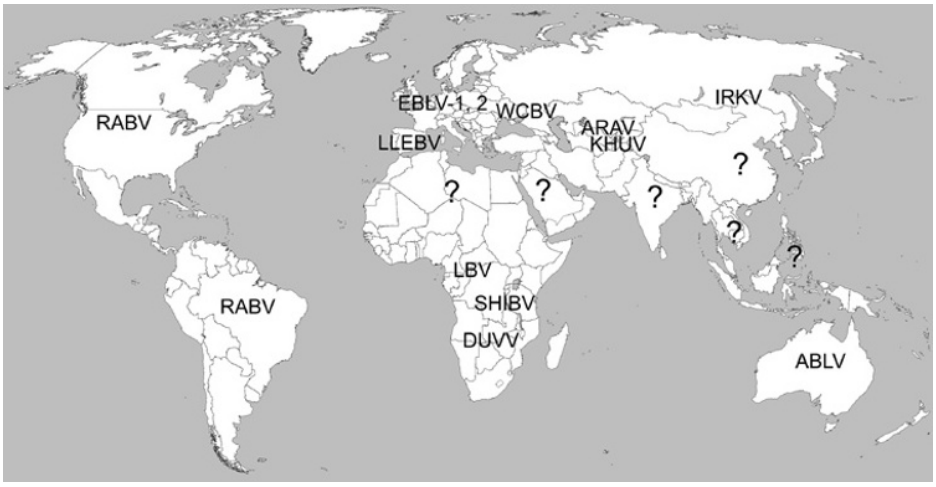

Figure 3.3 Global distribution of bat lyssaviruses. For virus abbreviations see text and Figure 3.1. Question marks indicate the territories with no isolates available for characterization.

Based on the variety of lyssaviruses identified in bats, these animals were proposed as being primarily affected by lyssavirus ancestors historically (Shope *et al*., 1970; Shope, 1982). Presumably, adaptation of the ancestral viruses to bats occurred somewhere in the Old World, perhaps in Africa or Eurasia, where the most divergent lyssaviruses are found today (Figure 3.3). Circulation of RABV among bats in the Old World was suggested repeatedly but has not been confirmed (Kuzmin *et al*., 2006a). By contrast, New World bats maintain circulation of RABV (although phylogenetic lineages of RABV associated with bats are different from the lineages circulating in carnivores), and it is unclear how New World bats acquired RABV in the absence of this virus in the Old World bats. A possibility exists that RABV circulates in Old World bats but has not been confirmed to date because of limited surveillance (Rupprecht *et al*., 2011). Alternatively, some ancient bat lyssaviruses and their hosts in the Old World may have gone extinct.

Several molecular clock estimates suggest that lyssaviruses are no more than 800 years old (Badrane & Tordo, 2001). Extant bat RABV appears no older than 300–500 years (Holmes *et al*., 2002; Hughes *et al*., 2005; Davis *et al*., 2006). However, lyssavirus genes are subjected to strong constraints applied by purifying selection (Holmes *et al*., 2002; Hughes *et al*., 2005; Davis *et al*., 2006). Such pressures can mask ancient origins of pathogens if the molecular clock estimations are based on recent sample sequences (Wertheim & Kosakovsky Pond, 2011). Alternative estimates suggest a greater age for American bat RABV lineages (Nadin-Davis *et al*., 2010; Kuzmina *et al*., 2013). It is possible that bat RABV was delivered to the New World prior to the Tertiary period (and perhaps, millions of years ago). Of course, such ancient viruses might be sufficiently different from contemporary RABV variants. Nevertheless, it is unlikely that these differences might be more significant than the differences between RABV and other lyssavirus species, as appears from phylogenetic reconstructions of the genus.

The ecological advantages of flight enhance the potential for invasion of new areas and rapid dispersal of pathogens by bats, much more readily than by terrestrial mammals. No geographic region, except Antarctica and a few very isolated islands, can be considered truly rabies-free based on the absence of the disease in carnivores. For example, ABLV was discovered in Australia in 1996, and three human cases have already been documented (Allworth *et al*., 1996; Hanna *et al*., 2000; ProMED-mail # 20130323.1600266). In the United Kingdom, EBLV-2 was identified in bats in 1996, and a human rabies case caused by this virus was diagnosed in 2002 (Fooks *et al*., 2003a). In the Irkutsk province of Eastern Siberia, which had been considered rabies-free for 35 years, IRKV was isolated from a bat in 2002 (Botvinkin *et al*., 2003), and a human case of bat origin was documented in 2007 in the Russian Far East (Leonova *et al*., 2009).

In general, patterns of lyssavirus circulation in bat populations are somewhat different from those in populations of carnivores (Table 3.2). Although it is well appreciated that in humans, carnivores, and laboratory rodents, rabies is almost invariably fatal, scientists still have no agreement on lyssavirus infection in bats. At least two major topics have been debated for decades, such as a possibility of a "carrier" state (e.g., prolonged virus excretion in saliva without clinical CNS infection) (Pawan *et al*., 1936; Sulkin *et al*., 1957; Sulkin, 1962; Echevarria *et al*., 2001; Serra-Cobo *et al*., 2002; Wellenberg *et al*., 2002; Aguilar-Setien *et al*., 2005; Vázquez-Morón *et al*., 2008), and non-bite virus transmission (Irons *et al*., 1957; Constantine, 1962). These topics are directly connected to the public health significance of bat rabies and are addressed below in detail.

In fact, bats are now the most prominent source of human rabies in the New World, Western Europe, and Australia, especially where the disease in carnivores has been

TABLE 3.2 Distinctive patterns of rabies in carnivore and bat populations

Facet	Carnivora	Chiroptera
Presence of the disease	Regional	Global
Lyssavirus species number	1 (given that the single available IKOV isolation was likely a spillover from a bat)	>12
Reservoir taxonomy	<12 genera	>12 genera
Susceptibility to homologous viruses	High	Moderate to low
Seroprevalence in natural populations	Usually <5% (in the absence of vaccination campaigns)	5–70% (directly proportional to the size and density of bat colonies)
Epizootic spread	5–40 km/year (in the absence of long-distant translocations by humans)	Correspond to migratory activity of reservoir species and may reach several hundred km/year
Public health burden (human cases per year)	>55,000 (over 90% caused by dog rabies)	<100
Control	Vaccination (parenteral and oral)	Limited options (historical population reduction for vampire bats only)

controlled. About 50% of the human cases caused by bat RABV variants, at least in North America, are "cryptic"; the patients could not recall a history of bat bite (Messenger *et al*., 2002; Blanton *et al*., 2009). Although it is generally thought that people may not pay attention to rather small lesions caused by bat bites and therefore do not remember the details of a bite (Messenger *et al*., 2002; Gibbons *et al*., 2002), the Advisory Committee on Immunization Practices (ACIP) recommends consideration of rabies prophylaxis in bat encounters in any case where a possibility of a bite cannot be reasonably ruled out, and a bat cannot be tested for rabies (Manning *et al*., 2008). Clearly, in the tropics where bats are most abundant and lyssaviruses are most diverse, the significance of bat rabies is much greater but is not addressed properly due to the lack of surveillance and limited diagnostic capacity, and is masked by the overarching problem of dog-mediated rabies.

3.5 GLOBAL DISTRIBUTION OF BAT LYSSAVIRUSES

3.5.1 The Americas

One of the major problems encountered by Europeans shortly after discovery of the Americas was vampire bat rabies. A paralytic disease in cattle, and sporadically in humans following vampire bat bites, was reported by the first Spanish colonists (reviewed by Baer, 1991). Hughes *et al*. (2005) suggested that the vampire bat RABV lineage is older than the RABV lineages associated with frugivorous and insectivorous bats in the Americas, and therefore may be their ancestor. However, this is questionable from the general phylogeny of American bat RABV lineages (Streiker *et al*., 2012b; Kuzmina *et al*., 2013). Considering that there are no vampire bats in the Old World where lyssaviruses hypothetically originated and where they circulate in frugivorous and insectivorous bats, there is no reason to expect that vampire bats were the first New World bats affected by rabies, unless we consider the supposition that bats acquired the virus from carnivores or other "terrestrial" mammals (Holmes *et al*., 2002), which does not sound a plausible hypothesis based on phylogeny of the extant RABV lineages, global animal ecology, and applied tenets of modern epizootiology.

Vampire bats are unique mammals that consume blood of other vertebrates as their only food. Three genera of vampire bats, belonging to the family Phyllostomidae, include the following species; the common vampire bat (*Desmodus rotundus*), which is the most abundant and prefers bovine blood; the hairy-legged vampire bat (*Diphylla ecaudata*), which is distributed mainly in cooler areas, up to the south of Texas historically, and prefers bovine and equine blood; and the white-winged vampire bat (*Diaemus youngi*), the rarest species found close to the equator, feeding mainly on birds (Nowak, 1999).

Vampire bat populations, which subsisted on blood of native animals prior to the arrival of European colonists, were significantly enlarged with the increasing numbers of prey, particularly livestock. The diagnosis of rabies in livestock was first made by the identification of Negri bodies in the brain of cattle during the outbreak of a previously undiagnosed disease in southern Brazil in 1911 (Carini, 1911). Rabies diagnosis in cattle as a consequence of virus transmission by vampire bats was also confirmed in Paraguay,

Argentina (reviewed by Baer, 1991), and in Trinidad, where rabies was detected not only in cattle but in several frugivorous and hematophagous bats (Pawan, 1936).

The disease caused by vampire bat RABV, in cattle, humans, and bats, is paralytic and virtually never furious. Such clinical manifestation may be dependent on some specific properties of the vampire bat RABV variant although no explanations from the standpoint of viral genome or pathobiological patterns of the infection have been proposed to date. Disease signs in livestock start with posterior incoordination, followed in later stages with anorexia, bellowing, weakness in the hind legs, and other signs of encephalitis. Finally, ascending paralysis, apnea, and death occur. Similar clinical signs and fatal outcomes are seen in humans. In a few documented cases, animals and humans recovered after a paralytic disease believed to be caused by contact with vampire bats. The majority of those cases were unavailable for laboratory confirmation (reviewed by Constantine, 1988). One of the examples was an outbreak of a paralytic disease among humans in Peru during 1996. At least nine deaths occurred, but also a few people recovered. Two fatal cases were confirmed to be rabies, but no samples were available from survivors (Warner *et al*., 1999). A recovery of a human from laboratory-confirmed rabies caused by the vampire bat RABV occurred in Brazil during 2008 (Filho, 2009). The case required implementation of intensive care and experimental rabies treatment.

The common vampire bat, *Desmodus rotundus,* became one of the most abundant bat species in tropical America due to an extensive food supply and ecological flexibility. According to a number of observations, rabies spreads through susceptible vampire bat populations as a "migratory epizootic". Some bats are killed by the disease; while others survive the exposure and develop immunity. After several years of reproduction, the bat population accumulates enough susceptible individuals to be vulnerable to another epizootic. In field studies, the virus prevalence among vampire bats varied from 0% in non-infected areas to as much as 14.3% in outbreak areas (Lord *et al*., 1975; Streicker *et al*., 2012a).

An idea that vampire bats may be asymptomatic rabies 'carriers', shedding the virus in their saliva for months or longer, was popular during initial studies of vampire bat rabies (Pawan, 1936). However, a significant limitation of the early studies was that the diagnosis was based on microscopic observation of intra-neuronal inclusion bodies, which are known to be unreliable for proper identification. As was suggested by Constantine (1988), early workers might have mistaken as rabies one or more other infectious agents that could persist in bat salivary glands. In several experimental studies, the disease in vampire bats was similar to rabies observed in other mammals (Moreno & Baer, 1980; Almeida *et al*., 2005a). The bats that developed signs of disease and excreted the virus via saliva soon died, whereas those that survived the inoculation without clinical signs never excreted the virus or had it in the brain as demonstrated upon euthanasia. Duration of the incubation period was dependent on the inoculation dose and varied from 5 to 57 days (usually 2–4 weeks), and duration of the clinical period was 1 to 28 days (usually no longer than 10 days). The virus was detected in bat saliva 0–8 days before the clinical onset. In a single case, when the duration of the clinic period was 28 days, virus appeared in saliva only during the last week before death. Hence, it is impossible to confirm that during the previous weeks the clinical signs in that bat were caused by rabies. In contrast, in an experimental study by Aguilar-Setien *et al*. (2005), vampire bats that succumbed to rabies did not excrete the virus in saliva, but virus was detected in oral swabs of three survivors that never developed apparent clinic signs of disease

during 2 years of observation, and no viral antigen or RNA was detected in their tissues upon euthanasia. No further corroborations of this observation were demonstrated.

Economic losses in livestock due to vampire bat rabies are tremendous. In the enzootic area from Mexico to Argentina, there is an at-risk population of more than 70 million head of cattle. The proportion of animals bitten by vampire bats may vary from 6% to 52% in a given herd, and bites may be multiple. As reviewed by Acha (1967) and Baer (1991), the estimated annual mortality of livestock due to vampire bat rabies in Latin America during the 1960s was more than 500 000 cases with an economic loss of about 50 million US$. During recent years, Latin American countries have reported reduced numbers of livestock rabies cases compared to previous years, 1869 to 3327 annually (Vigilancia epidemiologica de la rabia en las Americas, 2001–2003), but it is unclear whether this is the result of rabies control campaigns or an underestimation of the real situation. Understandably, data from rural localities is often missing or fragmented, and instead of individual cases, foci may be estimated.

Apparent spillovers of RABV from vampire bats into other potential vectors are uncommon. Bat species that share roosts with vampire bats appear to be infected most easily. Vampire bat RABV variants were diagnosed in frugivorous bats *Artibeus* spp. (Shoji *et al*., 2004). On several occasions, domestic cats infected with vampire bat RABV were secondary transmitters of the disease to humans. Of cattle slaughtered for human consumption in Mexico City, 40 of 1000 (4%) were found infected with RABV. No apparent human cases caused by the consumption of such carcasses were reported, but dogs, which ate meat from rabid livestock, developed a disease compatible with rabies after approximately one month (reviewed by Constantine, 1988).

At present, vampire bat distribution appears limited by the 10°C winter isotherm. However, fossil records indicate the presence of vampire bats in the western US (from west Texas to northern California), and in the eastern US (from Florida to West Virginia) between 30 000 and 5000 years ago (Ray *et al*., 1988). Climate change models predict that average temperatures are likely to increase by 1.7° to 2.8°C (3–5°F) along the Texas–Mexico border by 2080 and could rise as much as 3.9°C (7°F) in parts of Texas. Given this increase in average temperatures, winter minimums should also increase, opening new areas of northern Mexico and the southern United States to vampire bats (Mistry & Moreno-Valdez, 2010).

The first definitive case of rabies in an insectivorous bat, found outside the vampire bat distribution area, was documented during 1953 in a yellow bat (*Lasiurus intermedius*) in Florida (Sulkin & Greve, 1954). Other cases of insectivorous bat rabies were subsequently diagnosed in the US via enhanced surveillance. During 1957, bat rabies was reported in Canada. More recently, attention to bat rabies has increased since these animals have become the main source of indigenous human rabies in North America. About 1300–1600 rabid bats are documented in the US every year, which constitutes 21–24% of all animal rabies cases captured via the passive surveillance system. Bat rabies is reported from all 48 contiguous American states, and periodically from Alaska. Among bats reported as rabid during recent years: 46% were big brown bats (*Eptescicus fuscus*); 27% were Mexican free-tailed bats (*Tadarida brasiliensis*); 7% were hoary bats (*Lasiurus cinereus*); 6% were red bats (*Lasiurus borealis*); 3% were little brown bats (*Myotis lucifugus*); 2–3% were silver-haired bats (*Lasionycteris noctivagans*); and 1–2% were canyon bats (*Parastrellus hesperus*). Other bat species were

generally less than 1% of the registered cases (Blanton *et al*., 2010, 2012). Such distribution does not represent the true prevalence of rabies in different bats species, but rather the frequency of human contacts with certain species. For example, rabid bats are reported most frequently in the areas where they encounter humans often and diagnostic laboratories are available.

Distinct seasonal disease patterns were suggested for several bat species at moderate latitudes. Hoary bats (*Lasiurus cinereus*) and Mexican free-tailed bats (*Tadarida brasiliensis*) demonstrate two seasonal peaks of rabies prevalence. One peak occurs during April–June, after a period of high activity and interactions associated with the return from hibernation. A second peak, which is larger, appears during September–October. This is likely related to the increased mobility and contact rates of bats (with a significant proportion of young individuals never exposed to RABV) during their dispersion and migration towards overwintering locations. Since migratory bats are absent in their summer habitations for approximately 4–6 months, few if any rabies cases are registered in these species during this time. For non-migratory bats, such as *Eptesicus fuscus*, only one major peak is registered during the second half of the summer. This peak coincides with increased mobility of naïve young-of-the-year and the rearrangement of maternity colonies. Only occasionally, *Eptesicus fuscus* bats were found rabid in winter (Pybus, 1986).

During migratory stops, RABV is spread by bats along their geographic pathways. In the Americas, *Tadarida brasiliensis*, *Lasiurus* spp., and *Lasionycteris noctivagans* are the most important species in this respect. For example, a RABV isolated from a southern yellow bat (*Lasiurus ega*) in Paraguay demonstrated high genetic homology to RABV associated with hoary bats (*Lasiurus cinereus*) in North America (Sheeler-Gordon & Smith, 2001). As reviewed by Constantine (2003), hoary bats were sometimes found in the Galapagos Islands, and one was found in the Orkney Islands, north of Scotland. In fact, RABV associated with lasiurine bats form a monophyletic cluster, without respect to their geographic origin. This is in contrast to RABV lineages associated with *Tadarida brasiliensis*, *Eptesicus* spp., and *Myotis* spp. bats, which are different and geographically segregated (Oliveira *et al*., 2010).

As was revealed from the initial investigations, RABV isolates from various bat species differ in their pathobiological properties. For example, virus isolates from Mexican free-tailed bats demonstrated extremely short incubation periods in mice, whereas in the natural host the incubation periods could be as long as 181 days (Baer *et al*., 1980). Some bat viruses caused rabies in many carnivore species injected peripherally, whereas isolates from *Lasiurus borealis*, *Lasiurus cinereus*, *Lasionycteris noctivagans*, and *Eptesicus fuscus* injected via the same routes did not cause rabies in dogs, cats, foxes and skunks (Constantine, 1966a–c; Constantine & Woodall, 1966; Constantine *et al*., 1968).

All New World lyssaviruses belong to the rabies virus species. The bat-associated viral lineages belong to the 'indigenous American' group (Nadin-Davis, 2013), which also includes the raccoon, south-central skunk, several Mexican skunk RABV lineages, and is distinct from the RABV lineages associated with carnivores in the Old World, a part of which ('cosmopolitan' dog RABV) was delivered to the New World by European colonists and switched to several wildlife species such as Arctic, red, and gray foxes, coyotes, and northern populations of skunks (Figure 3.4). It appears that nearly each bat species harbors its own RABV lineage, and it was suggested that host phylogeny

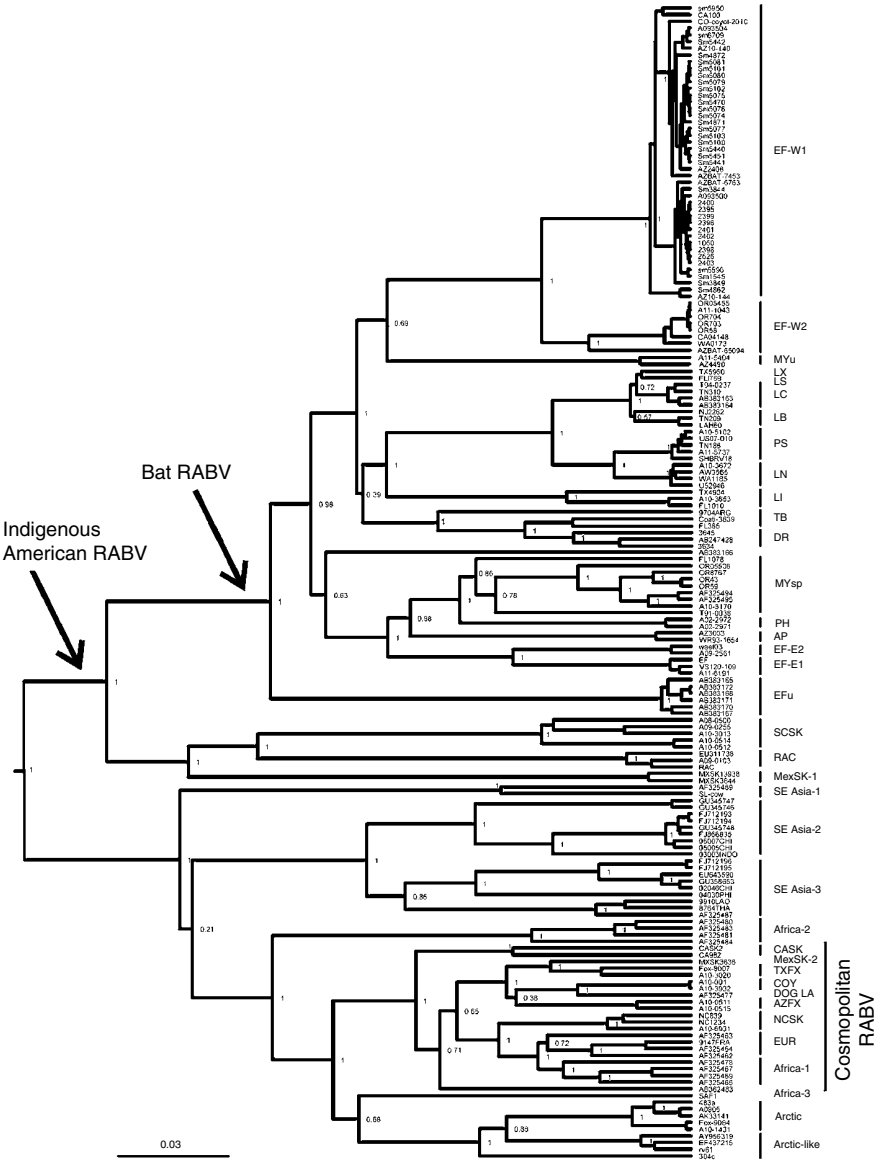

Figure 3.4 Bayesian tree of major RABV lineages based on the glycoprotein gene sequences. Lineage abbreviations: EF-W1 and EF-W2, *Eptesicus fuscus*, with predominantly western distribution; My, *Myotis yumanensis*; LX, *Lasiurus xanthinus*; LS, *Lasiurus seminolus*; LC, *Lasiurus cinereus*; LB, *Lasiurus borealis*; PS, *Perimyotis subflavus*; LN, *Lasionycteris noctivagans*; LI, *Lasiurus intermedius*; TB, *Tadarida brasiliensis*; DR, *Desmodus rotundus*; MYsp, *Myotis* spp; PH, *Parastrellus hesperus*; AP, *Antrozous pallidus*; EF-E1 and EF-E2, *Eptesicus fuscus*, with predominantly eastern and central distribution; EFu, *Eptesicus furinalis*; SCSK, south-central skunk; RAC, North American raccoon; MexSK-1, Mexican skunk, variant 1; SE Asia 1, 2 and 3 – diverse dog RABV lineages circulating in the South-East Asia; Africa-2, dog RABV lineage from the central and western Africa; CASK, California skunk; MexSK-2, Mexican skunk, variant 2; TXFX, Texas gray fox; COY, coyote; DOG LA, dog RABV from Latin America; AZFX, Arizona gray fox; NCSK, north-central skunk; EUR, fox viruses from moderate latitudes of Eurasia; Africa-1, dog RABV, broadly distributed in Africa; Africa-3, mongoose RABV from southern Africa; Arctic, Arctic RABV from Eurasia and North America; Arctic-like, Arctic-like RABV from southern and eastern Asia.

constrains the success of RABV transmission and establishment in a new host species (Streicker *et al*., 2010).

Furthermore, evolution rates in viral lineages associated with tropical bats are almost four times greater than evolutionary rates in lineages associated with bats in temperate latitudes. This may be a consequence of reduced relative seasonality in tropical bat activity and increased rates of virus transmission (Streicker *et al*., 2012a). In turn, modeling suggested that hibernation of bats is as an important factor for RABV perpetuation in populations of *Eptesicus fuscus* in moderate latitudes (George *et al*., 2011).

Because of their substantial public health significance (see Section 3.6), pathogenesis of bat rabies in the Americas (mainly in the US) has been studied more rigorously than in other parts of the world. Early studies were influenced by communications from Latin America, where vampire bats were believed to be asymptomatic "carriers" of rabies (Pawan, 1936). In a series of experiments aimed at investigating this concern, together with the mechanisms of RABV maintenance during hibernation (Sulkin *et al*., 1957, 1960, Sulkin, 1962), three significant findings were reported in the mid-20th century. The first finding indicated that it may be possible that viruses remain latent in a bat that was injected intramuscularly during simulated hibernation at low temperatures. When inoculated bats were returned to a warm environment and awakened, the viral infection was activated and clinical signs appeared after the same incubation period that was documented in the bats that were inoculated and maintained in an active state. A second finding suggested a role for interscapular brown adipose tissue (brown fat) as a depot for RABV storage during hibernation (Allen *et al*., 1964a, b). A third observation was the presence of RABV in salivary glands of bats in the absence of the virus in brain tissue, as shown via mouse inoculation (Sulkin *et al*., 1957, 1960). Unfortunately, some of these data appear inconsistent with regard to virus detection and subsequently may have led to misinterpretation. Although conservation of the virus during bat hibernation was confirmed (Sadler & Enright, 1959; Kuzmin *et al*., 1994; Kuzmin & Botvinkin, 1996), the general notion of salivary excretion in lieu of CNS replication and the particular function of brown fat during viral pathogenesis were not.

The finding of RABV in oral swabs and in salivary glands of bats in the absence of CNS infection is more complicated, as discussed above for vampire bat rabies (Aguilar-Setien *et al*., 2005). Several similar findings were reported for non-RABV lyssaviruses. Recently, one research group reported a recovery of two experimentally infected *Eptesicus fuscus* bats from a clinical neurologic disease that manifested 17 days post-inoculation with a homologous RABV isolate. Both bats improved after 4–5 days and recovered completely during following weeks. The virus was detected in oral swabs taken on day 17 post inoculation but not in the brains collected at euthanasia on day ~177 (Davis *et al*., 2013a).

Except for these two cases, rabies in *Eptesicus fuscus* bats inoculated with homologous or heterologous RABV variants was quite typical and fatal. Susceptibility to peripheral inoculation varied between18–80%, the duration of incubation periods was 13–140 days (one study describes an incubation period of at least 267 days in a naturally infected bat) (Davis *et al*., 2012), and the duration of clinical periods was 1–5 days. Virus appeared in oral swabs of bats 0–12 days before clinical onset, and was detected in salivary glands of 10–50% of rabid bats (always with the presence of the virus in the brain). No survivorship was reported, although due to the protocol requirements they all

were euthanized after onset of neurologic signs, and it was not possible to follow up the disease course (Jackson *et al*., 2008; Turmelle *et al*., 2010; Davis *et al*., 2013a, b).

In a study by Baer & Bales (1967), peripherally inoculated Mexican free-tailed bats had incubation periods of 24 to 125 days. Susceptibility was relatively low: the disease occurred in 14–33% of bats infected intramuscularly or subcutaneously and in 14% of bats infected intranasally. The virus could not be recovered from any organs in the absence of brain infection, and titers of the virus in extraneural tissues were low. No evidence of continuous virus excretion via saliva was noted. The longest period during which virus could be isolated from the saliva was 15 days before death (or 12 days before onset of the clinical signs).

Constantine (1967a) provided information on RABV distribution in tissues of 130 naturally infected Mexican free-tailed bats collected in Texas caves. The virus was detected as follows: brain 100%, salivary glands 79%, lungs 30%, and kidneys 12%. In further tests on 50 of those 130 bats, brown fat of two (4.0%) contained RABV, but all were negative for virus in liver, spleen, pectoral muscles, intestines, and fecal pellets. Later, the same author detected RABV in 5 of 15 (33%) impressions of nasal mucosa from naturally infected *Tadarida brasiliensis*. For two of those five, the virus was also isolated by mouse inoculation (Constantine, 1972).

These findings, coupled with reports about possible airborne human rabies exposure from bats in caves (Irons, 1957; Constantine, 1962), led to a series of transmission experiments performed by Constantine in caves inhabited by huge colonies of Mexican free-tailed bats. Several species of Carnivora, including foxes and coyotes, were housed in close-meshed cages in caves under the bat colony. After variable incubation periods, the animals developed furious rabies (Constantine, 1962, 1967b). In another experiment, RABV isolates from Mexican free-tailed bats and from salivary glands of a coyote, which had been infected in the bat cave, were further used for parenteral inoculation of carnivores, including those which could not be infected in the cave. Via this route, rabies developed in coyotes, foxes, dogs, raccoons and skunks which demonstrated a possibility to transmit the virus to carnivores (Constantine, 1966a–c).

Serologic responses of bats to RABV exposure and rabies seroprevalence of bats in natural habitats were addressed in several studies attempting to understand virus-host interactions and circulation patterns of RABV at a population level. Antibody prevalence was determined in populations of big brown bats (10%) and little brown bats (2%) in New York State (Trimarchi & Debbie, 1977), and was up to 80% in colonies of Mexican free-tailed bats (reviewed by Baer & Smith, 1991). For the latter species, it was suggested that seropositive females could protect their young by passive transfer of anti-RABV antibodies (Steece & Altenbach, 1989).

Such high seroprevalence rates suggest that the majority of RABV exposures in bats led to the development of a peripheral abortive infection with an appropriate immune response rather than to a productive CNS infection. Experimental studies demonstrated that singular exposures to RABV do not necessarily lead to the development of a detectable serologic response and, in turn, detectable virus-neutralizing antibodies do not necessarily protect animals from a chance to develop rabies after a new exposure to the virus (Turmelle *et al*., 2010; Davis *et al*., 2013a, b). In general, these results may be interpreted in the way that long-term repeated low-dose exposures to RABV along the life time of bats may confer reduced susceptibility of the animals to multiple exposures and explain the significant seroprevalence in bat populations (Turmelle *et al*., 2010).

Colonial bat species (e.g. *Tadarida brasiliensis, Eptesicus fuscus*, *Desmodus rotundus*) demonstrate limited susceptibility to indigenous RABV variants. The disease in individual bats is rarely furious, and predominant clinical signs include general exhaustion, weakness and paralysis. Colonial animals are in tight contact most of their lives. If they transmitted virus more efficiently and actively, the whole colony would die, eliminating the virus from circulation. To the contrary, solitary bat species frequently develop furious rabies and actively attack bats (or other animals) thereby facilitating virus transmission. Bell (1980) observed a *Lasiurus cinereus* bat, which successively attacked *Lasionycteris noctivagans*, *Tadarida brasiliensis* and *Eptesicus fuscus* bats during foraging, in which *Lasiurus cinereus* chased the victims, caught them and brought them to the ground. Later that night a *Lasiurus cinereus* was mist-netted approximately 50 m from the site. Presumably, it was the animal that was attacking the bats. It had fresh blood on its snout and around its head. However, no injuries were noted. Rabies diagnosis in this bat was confirmed in the laboratory.

Spillover infections and host shifts of bat RABV into carnivores are of significant importance because they help to understand the natural history and diversification of RABV, and because they raise public health concerns in areas where no "terrestrial" rabies is present. Bat viruses have been identified in cattle, cats, foxes and other mammals, however, very few events could be characterized as host shifts of bat RABV into carnivores. A limited rabies outbreak in red foxes (*Vulpes vulpes*) on Prince Edward Island (Canada), previously free of carnivore rabies, occurred during 1993. Monoclonal antibody typing of the viruses, isolated from rabid foxes, suggested their likely origin from mouse-eared bats, *Myotis lucifugus* or *Myotis septentrionalis* (Daoust *et al*., 1996). Another local outbreak, caused by a RABV variant associated with *Tadarida brasiliensis* bats, was documented in white-nosed coatis (*Nasua narica*) in Mexico (Aréchiga-Ceballos *et al*., 2010). At least three outbreaks in striped skunks (*Mephitis mephitis*) and one in gray foxes (*Urocyon cinereoargenteus*) caused by RABV variants associated with *Eptesicus fuscus* bats occurred in the Flagstaff area of Arizona during 2001–2009 (Leslie *et al*., 2006; Kuzmin *et al*., 2011). An outbreak in gray foxes caused by a RABV variant associated with *Myotis* bats was documented in Oregon during 2009, and another one caused by RABV variants associated with *Eptesicus fuscus* bats during 2010 (Kuzmin *et al*., 2011). A Mexican immigrant who died of rabies in the US in 2008 was infected by a RABV closely related to the variant encountered in *Tadarida brasiliensis* bats, but was exposed in Mexico to a carnivore, presumably a fox (Velasco-Villa *et al*., 2008). Repeated outbreaks in Arizona attracted significant attention, and extensive trap-vaccinate-release campaigns were implemented among skunks, along with oral vaccination of gray foxes. Other outbreaks were not managed but self-limiting. Presently, it remains unclear which conditions are needed for bat RABV to establish sustained circulation in carnivore populations.

3.5.2 Africa

In Africa, several species of non-RABV lyssaviruses have been identified in bats. Boulger & Porterfield (1958) isolated an agent from a pool of brains of straw-colored fruit bats (*Eidolon helvum*) at Lagos Island, Nigeria, during 1956. They named the agent "Lagos bat virus", and registered the agent as a possible arbovirus. The relatedness between LBV and RABV was not established for 14 years. Morphological investigations

undertaken during 1969–1970 demonstrated that LBV, together with the more recently isolated MOKV, were rhabdoviruses. Further studies revealed their antigenic relatedness to each other and to RABV, and the group of "rabies-related" viruses (further genus *Lyssavirus*) was established (Shope *et al*., 1970; Shope, 1982).

Thereafter, LBV was isolated several times, mainly from fruit bats across sub-Saharan Africa. Phylogenetic studies demonstrated that LBV is quite diverse genetically. Four lineages, A-D, were identified. Lineage A included the viruses found in *Eidolon helvum* bats in Senegal, Kenya, and an isolate imported to France with a presumable *Rousettus aegyptiacus* fruit bat imported from either Togo or Egypt. Lineage B included only the single initial isolate from Nigeria. Lineage C included the isolates obtained from epauletted fruit bats (*Epomophorus wahlbergi*, *Micropteropus pussilus*) from the Central African Republic and South Africa. Lineage D included three isolates obtained from *Rousettus aegyptiacus* bats in Kenya (Markotter *et al*., 2008; Kuzmin *et al*., 2008c; Kuzmin *et al*., 2010; our data, unpublished). Genetic distances between the isolates from lineage A and those from other lineages are greater than genetic distances within other lyssavirus species. Based on this, several authors suggested that lineage A may be potentially considered as a separate lyssavirus species (Delmas *et al*., 2008; Markotter *et al*., 2008). Conversely, LBV lineages demonstrate remarkable spatiotemporal stability. For example, the genome of the lineage A virus isolated in Kenya during 2007 was 99.8% identical to genome of the virus isolated in Senegal during 1985 (Kuzmin *et al*., 2008c). Similarly, genomes of the lineage C viruses from South Africa demonstrated very high levels of nucleotide identity over more than 25 years of sampling (Markotter *et al*., 2008).

Serologic studies identified a high seroprevalence of *Eidolon helvum* and *Rousettus aegyptiacus* fruit bats to LBV in such distant locations as Nigeria, Ghana, and Kenya. In different colonies, seroprevalence ranged from 19 to 67%, and was statistically greater in adult male bats (Dzikwi *et al*., 2010; Hayman *et al*., 2008, 2012; Kuzmin *et al*., 2008c). In contrast, no LBV-neutralizing antibodies were detected in the serum of insectivorous bats (Kuzmin *et al*., 2008c). These observations suggest that circulation patterns of LBV in the colonial African fruit bats are similar to the circulation patterns of RABV in colonial American bats.

The distribution of *Eidolon helvum* and *Rousettus aegyptiacus* fruit bats is not limited to sub-Saharan Africa. Both species are present in northern Africa and the Middle East. Moreover, a related bat species, *Rousettus leshenaulti*, is broadly distributed in southern Asia. There is a possibility that LBV, or perhaps other related lyssaviruses, circulate in fruit bats across these territories.

Not much is known on the pathobiology of LBV in the presumed natural hosts, fruit bats. Except the first finding in Nigeria where the virus was isolated from "apparently" healthy bats (recognizing they were shot at a distance in a tree roost), other isolates were obtained from the brains of moribund or dead bats. In carcasses of naturally infected bats the infectious virus or viral RNA was detected in various extraneural tissues, but the greatest titers (up to $10^{7.5}$ $MICLD_{50}$) were sometimes documented in salivary glands (our data, unpublished). In contrast, screening of ~1000 oral swabs from apparently healthy fruit bats from Kenya did not detect LBV (Kuzmin *et al*., 2008b, c). These findings imply that LBV causes a fatal disease in fruit bats, which most likely is transmitted via bites.

As a result of natural spillover infections, LBV was also isolated from an insectivorous slit-faced bat *Nycteris gambianus*, domestic cats and a dog, and a water mongoose (*Atilax paludinosus*). When LBV was detected in such naturally infected "terrestrial" mammals, these demonstrated clinical signs compatible with rabies (King & Crick, 1988; Mebatsion *et al*., 1992; Swanepoel, 1994; Markotter *et al*., 2006).

Initial studies using a mouse model suggested that LBV, similarly to MOKV, was not peripherally pathogenic to mice. This lack of pathogenicity was attributed to an amino acid substitution R/K333D in the viral G, and to the altered structure of the LC8 binding site of the P protein (Badrane *et al*., 2001; Mebatsion, 2001). The limitation of these first studies was that they were performed for only a few isolates of LBV and MOKV, with an unknown passage history. In other experiments by Markotter *et al*. (2009), at least three LBV isolates from lineages A and C demonstrated peripheral pathogenicity in a mouse model that was greater or equal to the peripheral pathogenicity of RABV. The same was shown later for isolates from lineage D (our data; unpublished). In general, the only isolate that consistently did not cause rabies in mice and hamsters via the peripheral route was a single available representative of lineage B (e.g., the first LBV isolate, described from Nigeria in 1956, and subjected to an unknown number of laboratory passages). In experimental settings, dogs did not present a productive infection after intramuscular administration of LBV (the only original isolate from Nigeria tested), even with doses $10^{6.5}$–$10^{7.5}$ $MICLD_{50}$. However, one of six monkeys inoculated intramuscularly with 10^{6} $MICLD_{50}$ of the same virus, developed bilateral paresis on day 22, but 'recovered' on day 86, and no virus was isolated from the animal at euthanasia on day 108 (Tignor *et al*., 1973).

Another African non-RABV lyssavirus, DUVV, was isolated during 1970 in South Africa from a human (for whom the virus was named) bitten on his lip by a bat while he was sleeping. According to the description given, the animal in question could have been a common bent-winged bat, such as *Miniopterus schreibersii*. The person did not receive medical treatment, and five weeks later developed rabies (Meredith *et al*., 1971). Further isolates of DUVV have also been identified in *Miniopterus* sp. in South Africa and in *Nycteris thebaica* in Zimbabwe (King & Crick, 1988). During recent years DUVV resulted in at least two more human deaths. One was reported from South Africa, and was similar to the index case (Paweska *et al*., 2006). The other occurred in Kenya, where a Dutch tourist in a field camp was attacked by a small bat, resulting in facial lacerations. Although the tourist was seeking medical service locally, rabies prophylaxis was not provided. Four weeks later, after returning home, she developed rabies (van Thiel *et al*., 2009). Despite fairly extensive surveillance attempts over more than 40 years, the reservoir Chiropteran host for DUVV remains elusive. Screening of ~3000 bat serum samples from Kenya identified only one specimen with a reasonably high antibody titer to DUVV. The sample was obtained from a *Miniopterus* sp. (our data, unpublished). Recent serologic studies in Swaziland suggest that *Nycteris thebaica* may be a regionally important host for DUVV (Markotter *et al*., 2013).

A lyssavirus that demonstrated genetic and serologic relatedness to LBV and MOKV but could not be included in either of these species was isolated from a dead insectivorous bat *Hipposideros commersoni* (in light of modern bat taxonomy it most likely was a *Hipposideros vittatus* (Simmons, 2005) in Kenya during 2009 and was

named SHIBV (Kuzmin *et al*., 2010). A rigorous quantitative comparison of antibody titers indicated that *Hipposideros commersoni/vittatus* bats are more likely to be reservoirs of this virus than fruit bats (Kuzmin *et al*., 2011).

The WCBV has never been documented in Africa to date, it is known only from a single isolate obtained from a *Miniopterus schreibersii* bat in the Caucasus Region (Botvinkin *et al*., 2003). However, significant apparent seroprevalence to this virus was detected in several species of *Miniopterus* bats collected in Kenya (Kuzmin *et al*., 2008b). Given the fact that WCBV does not cross-react serologically with other lyssaviruses (particularly, the same bat serum samples did not neutralize LBV, MOKV, RABV, and DUVV), this observation suggests that WCBV or some closely related virus, is present in Africa. Further, as *Miniopterus* bats are broadly distributed across sub-tropical and tropical areas of the Old World, they can maintain circulation of WCBV-related viruses quite widely. Recent detection of LLEBV (which is related to WCBV phylogenetically) in a *Miniopterus schreibersii* bat from Spain (Aréchiga-Ceballos *et al*., 2013) supports such an assumption. The isolation of another phylogenetically related lyssavirus, IKOV, occurred in an African civet (*Civettictis civetta*), that demonstrated clinical signs of furious rabies and attacked a person (Marston *et al*., 2012). This case occurred in Tanzania in an area controlled for carnivore rabies for a number of years. A possibility exists that the case in civet resulted from a spillover infection from a bat. Serum samples of Kenya bats that neutralized WCBV did not neutralize IKOV (Horton *et al*., 2014), and the reservoir host for IKOV remains to be determined.

3.5.3 Eurasia

Although the discovery of bat rabies in Germany occurred very shortly after the discovery of rabies in North American insectivorous bats (during 1954), not much attention was paid to this issue during the ensuing decades. Only 14 cases of bat rabies were diagnosed in different regions of Europe before 1985 (Schneider & Cox, 1994). These included the first case of human rabies after bat exposure in Europe, registered in the town of Voroshilovgrad (currently Lugansk) in the Ukraine during 1977 (Scherbak, 1982). Unfortunately, the virus was not stored for precise typing.

Further progress in European bat rabies investigations was associated with the development of monoclonal antibody technique (MAb) for lyssavirus typing during the 1970s (Wiktor & Koprowski, 1978). The typing of available European bat lyssavirus isolates with MAbs demonstrated that their antigenic patterns were similar to those of DUVV. For this reason, European bat lyssaviruses were designated initially into the same serotype 4 (and were referred to as DUVV-like). It was hypothesized that DUVV might have been delivered to Europe from Africa with migratory bats or via bats translocated by ships or other anthropogenic means (Schneider, 1982).

Surveillance and registration of rabid bats significantly increased in Europe since 1985, when two human cases of bat origin were documented in Russia and Finland (Lumio *et al*., 1986; Selimov *et al*., 1989). Additional studies with an extended panel of MAbs demonstrated that European bat lyssaviruses were similar, but not identical, to DUVV. Furthermore, they were different from each other (Dietzschold *et al*., 1988; King *et al*., 1990; Rupprecht *et al*., 1991). For a short period of time, the human isolate from Finland (also known as the 'Finman' virus), together with a few other isolates originating from *Myotis dasycneme* bats from the Netherlands, were segregated into a new

serotype 5; whereas other European bat isolates, obtained throughout Europe primarily from *Eptesicus serotinus* bats, and the human isolate from Russia (Yuli virus), were still considered as members of serotype 4 (DUVV-like). Often, they were also termed as "biotypes", to reflect their distinctions from the genuine African DUVV (King *et al*., 1990). The advent of gene sequence techniques for virus classification in the latter part of the 20th century led to the establishment of lyssavirus genotypes. The DUVV-like European viruses were segregated in genotype 5, and the "Finman-like" viruses were segregated into genotype 6 (Bourhy *et al*., 1992, 1993). At present, two species are established for these viruses by the ICTV. The *European bat lyssavirus, type 1* (EBLV-1) includes the isolates least different from DUVV and previously thought to be serotype 4 (genotype 5) members, whereas *European bat lyssavirus, type 2* (EBLV-2) includes the former members of serotype 5 (genotype 6) (Amengual *et al*., 1997; Fooks *et al*., 2003a). Extensive phylogenetic evaluation demonstrated that EBLV-1 can be subdivided into two lineages, "a" and "b". Furthermore, for EBLV-1a, a west-east distribution was suggested, whereas for EBLV-1b, a north-south distribution was suggested (Amengual *et al*., 1997). Given their extensive distribution throughout Europe, it should not be surprising to find EBLVs and their close relatives throughout the Mediterranean region, including North Africa and parts of Eurasia, considering routine exchanges of bat populations seasonally among these continents.

Comparatively, there is a remarkable difference between the number of bat species involved in RABV circulation in the Americas, and the depauperate taxa involved in EBLV circulation in Europe, as well as the difference between the phylogenetic diversity of RABV lineages encountered in American bats versus the genetic homogeneity of EBLVs. For example, about 95% of all EBLV-1 cases have been observed in serotine bats (*Eptesicus serotinus*) (Amengual *et al*., 1997; Schatz *et al*., 2012). A study of the subspecies *Eptesicus serotinus isabellinus* in southwestern Europe suggested that these bats may maintain circulation of EBLV-1 independently, and a phylogenetic lineage EBLV-1c was suggested for these viruses (Vázquez-Morón *et al*., 2008). To date, it appears that the overall EBLV-1 prevalence correlates with the abundance of serotine bats. In western and central Europe, the great majority of rabies cases were reported from their northern range, where the highest density of serotine bats was documented (Müller *et al*., 2007; Banyard *et al*., 2013). Antibodies to EBLV-1 were detected in *Eptesicus serotinus* in southern England, although no viral isolates were recovered (Harris *et al*., 2009). It is unclear how far the range of EBLV-1 is spread in the east. Surveillance in countries of Eastern Europe and Central Asia, where *Eptesicus serotinus* is present, is very limited. As there is evidence of genetic flow between *Eptesicus serotinus isabellinus* populations on both sides of the Strait of Gibraltar, it is possible that EBLV-1 is present in North Africa and beyond (Banyard *et al*., 2013). In addition, EBLV-1 was documented occasionally in *Nyctalus noctula* and *Vespertilio murinus* (Selimov *et al*., 1991), *Myotis myotis*, *Myotis dasycneme*, *Myotis daubentonii*, *Pipistrellus pipistrellus*, *Pipistrellus nathusii*, *Myotis natterreri*, *Rhinolophus ferrumequinum*, and *Miniopterus schreibersii* (Schneider & Cox, 1994; Serra-Cobo *et al*., 2002; Van der Poel *et al*., 2005). Antibodies to EBLV-1 were found additionally in *Tadarida teniotis* (Serra-Cobo *et al*., 2002). Other bat species may be important for the regional maintenance of EBLV-1, such as *Miniopterus schreibersii*, particularly when *Eptesicus serotinus* does not appear as an operative host in local dynamics (Pons-Salort *et al*., 2014).

EBLV-2 has been diagnosed infrequently in comparison to EBLV-1. This virus was isolated mainly from Daubenton's bats (*Myotis daubentonii*) and occasionally from pond bats (*Myotis dasycneme*). Presently, EBLV-2 is found in north-western Europe, including the Netherlands, Denmark, Switzerland, Germany, Finland, and the United Kingdom (Banyard *et al*., 2013). A singular records of EBLV-2 in *Nyctalus noctula* and *Vespertillio murinus* bats from the Ukraine (Selimov *et al*., 1991) was likely a mistake (Kuzmin *et al*., 2006a). It is unclear why the range of EBLV-2 does not correspond to the quite broad range of *Myotis daubentonii.* In addition to the index case in Finland, EBLV-2 claimed another human death in Scotland during 2002, in an unvaccinated bat rehabilitator who was subjected to bites from several bats (Fooks *et al*., 2003b).

Surveillance for bat rabies in Europe has been limited compared to that in the Americas because of existing conservational constraints (Banyard *et al*., 2013; Schatz *et al*., 2014). Active surveillance in Europe has been limited mostly to the screening of oral swabs for the presence of viral RNA, and to serological tests for antibody. During one survey in Spain, 15 of 71 oral swabs obtained from apparently healthy *Eptesicus serotinus* bats were reported positive for EBLV-1 RNA. Additionally, viral RNA was detected in 13 oral swabs, but only in 5 brains, of the 34 bats from which simultaneous testing of brains and oral swabs were available (Echevarria *et al*., 2001). In general, the authors detected viral RNA in 6.7% of harvested brains, but in 20% of harvested oral swabs. These findings suggested that bats might be "carriers" of EBLV-1 and shed virus in saliva without CNS infection. Further studies published by another Spanish team (Serra-Cobo *et al*., 2002) again reported the presence of EBLV-1 RNA in the esophagus–larynx–pharynx and lung of *Rhinolophus ferrumequinum* bats, with negative detection in brain. The same paper reported viral RNA in 3 of 27 blood pellet samples obtained from apparently healthy *Myotis myotis* bats. These bats were bled and released, therefore no further information on the presence of viral RNA and infectious virus in their tissues was available. EBLV-1 RNA was also detected in tissues of apparently healthy zoo bats, *Rousettus aegyptiacus*, which presumably acquired the infection from European insectivorous bats (Wellenberg *et al*., 2002).

A possibility of a 'carrier' state was suggested in one experimental study where American *Eptesicus fuscus* bats were inoculated with EBLV-1. One of the bats that survived inoculation without clinical signs and had no virus in CNS at euthanasia (day 67) had a positive oral swab on day 28 (Franka *et al*., 2008). However, other *Eptesicus fuscus* and *Eptesicus serotinus* bats inoculated with EBLV-1, and *Myotis daubentonii* inoculated with EBLV-2, did not show the presence of the viruses in oral swabs or salivary glands in absence of CNS infection. Susceptibility of bats to intramuscular and subcutaneous administration of the viruses was limited to 0–57%, and no bats developed rabies following oral and intranasal inoculation. Incubation periods in the peripherally infected bats were 7–67 days, and clinical periods were 1–8 days. Clinical signs included weight loss, weakness, ataxia, inability to fly, abnormal vocalization, and occasional aggression. The viruses were detected in bat brains, and less frequently in salivary glands, thyroid glands, lungs, kidneys, and other tissues, with the appearance of the viruses in oral swabs of bats 0–6 days before clinical onset (Franka *et al*., 2008; Freuling *et al*., 2009; Johnson *et al*., 2008; Kuzmin & Botvinkin, 1996). Experimental inoculation of fruit bats *Rousettus aegyptiacus* with two EBLV-1 isolates also demonstrated patterns of typical rabies rather than a carrier state. Of 22 bats inoculated, 7 (32%) succumbed to the disease. The virus was detected in the brain of each dying animal and in

the salivary glands of two of them. The tissues of all survivors were virus-negative (Van der Poel *et al*., 2000).

Additional surveys in Spanish bat populations demonstrated the presence of anti-EBLV-1 antibodies in 7.8% of serum samples. In some colonies, the prevalence was as high as 20–22% (Serra-Cobo *et al*., 2002). Screening of Scottish bats for antibodies to EBLV-2 demonstrated that 0.05 to 3.8% (95% confidence interval) of *Myotis daubentonii* bats were seropositive. However, in one location the prevalence was much higher, 16.3% (Brookes *et al*., 2005a; Harris *et al*., 2009). A very limited antibody response to EBLVs was registered in experimentally infected bats (Franka *et al*., 2008; Freuling *et al*., 2009; Johnson *et al*., 2008).

Another lyssavirus related to EBLV-2, BBLV, was discovered in Germany during 2010 (Freuling *et al*., 2011) and further isolated again in the same country and in France (Picard-Meyer *et al*., 2013). In all three instances, BBLV was found in moribund or dead Natterer's bats (*Myotis nattereri*) and is probably associated with this bat species. Given the phylogenetic relatedness of BBLV to EBLV-2, it is expected that serologic cross-reactivity is also high, and therefore it would be difficult to elucidate precise circulation patterns of BBLV from serologic surveillance. Preliminary studies have demonstrated the peripheral infectivity of BBLV for laboratory mice, and the reliable cross-reactive protection with rabies biologics against BBLV, as predicted from its relationship to other Phylogroup I viruses (Nolden *et al*., 2014).

A very divergent bat lyssavirus, WCBV, was isolated only once in the Caucasus Region from the brain of a mist-netted *Miniopterus schreibersii* bat during 2002 (Botvinkin *et al*., 2003). Phylogenetically, WCBV could not be included in either Phylogroup I or II (Kuzmin *et al*., 2005; Kuzmin *et al*., 2008d). Moreover, no serologic cross-reactivity was observed between WCBV and other lyssaviruses (Hanlon *et al*., 2005; Kuzmin *et al*., 2008b; Horton *et al*., 2010). Only one isolate of WCBV has been discovered to date, but serologic findings indicated that several species of *Miniopterus* bats from Kenya harbor WCBV-like neutralizing antibodies, which did not neutralize other tested lyssaviruses, including IKOV (Kuzmin *et al*., 2008b; Horton *et al*., 2014).

One important question addresses the pathogenicity of WCBV. As mentioned above, D_{333} in the G ectodomain of LBV and MOKV was a suggested reason for their reduced peripheral pathogenicity in mice (Badrane *et al*., 2001). The LC8 binding site of their P is also different from that of Phylogroup I lyssaviruses. The WCBV has E_{333} in the G ectodomain and the LC8 binding site of its P is different from both Phylogroup I and II lyssaviruses (Kuzmin *et al*., 2005). In our experiments, WCBV was nonpathogenic for 3-week-old mice and ferrets by intramuscular, subcutaneous, intraperitoneal and oral routes, even when doses of $10^{6.3}$ $MICLD_{50}$ were administered. However, 78% of Syrian hamsters, challenged intramuscularly with the same virus dose, developed typical rabies and succumbed. Big brown bats demonstrated limited susceptibility to the same dose of WCBV injected intramuscularly, and after their death, the virus was detected in their brain but not in extraneural tissues (Kuzmin *et al*., 2008a). Pathogenesis and circulation patterns of this virus should be studied more extensively.

Another divergent lyssavirus, LLEBV, was recently described from Spain. Viral RNA was detected in the brain of a *Miniopterus schreibersii* bat that was found moribund in a cave. The brain specimen was conserved inappropriately, and virus isolation was unsuccessful. Only a partial N gene fragment was PCR-amplified and sequenced (Aréchiga-Ceballos *et al*., 2013). Based on this sequence, LLEBV is most closely

related to WCBV and IKOV, however, it is still separated from these viruses by a long genetic distance. This finding supports the hypothesis that IKOV may also be a bat virus (likely associated with *Miniopterus* bats), and the case in the African civet probably resulted from a spillover infection (Marston *et al*., 2012; Horton *et al*., 2014). The extent to which *Miniopterus* bats harbor circulation of such lyssaviruses across their distribution range remains to be resolved.

Spillover of EBLV-1 into terrestrial animals has occurred infrequently, and no spillover information is available for other bat lyssaviruses encountered in Europe. Four EBLV-1 cases were registered in sheep from Denmark during 1998 and 2002 (Ronsholt, 2002), one case in a stone marten from Germany during 2001, and two cases in domestic cats in France during 2003 and 2007 (Dacheux *et al*., 2009). This scarcity may depend on a limited susceptibility of some terrestrial mammals to EBLVs. In one experiment, all ferrets inoculated intramuscularly with 10^6 foci-forming units (FFU) of EBLV-1 developed rabies, whereas, only 43% of ferrets inoculated with 10^4 FFU of EBLV-1 and none of those which received 10^4 FFU of EBLV-2 developed rabies (Vos *et al*., 2004a). Dogs were not susceptible to intramuscular inoculation with EBLV-1, whereas cats were, and they died of rabies within 15 days (Fekadu *et al*., 1988). Similarly, no virus was detected in inoculated foxes (Vos *et al*., 2004b). In another study, 14% of foxes developed rabies after intramuscular inoculation with 10^3 $MICLD_{50}$ of EBLV-1, but none of the foxes infected with the same dose of EBLV-2 (Cliquet *et al*., 2009). Sheep infected intramuscularly with EBLV-1 and EBLV-2 developed mild neurologic signs but recovered during the 94 days of observation, and their CNS tissues did not show the presence of viral antigens or nucleic acids. Therefore, the causative reason for their neurologic disorder cannot be explained by EBLV replication (Brookes *et al*., 2007).

In addition to the four human rabies cases of bat origin referred above, one other similar case was reported retrospectively from the town of Lugansk (formerly Voroshilovgrad; the place where a human case occurred in 1977). During 2002, a man died of rabies after a bite of a bat on his finger. The patient treated the bite wound himself, using an iodine solution, but did not seek rabies prophylaxis. No contacts with other mammals prior to disease were established (Botvinkin *et al*., 2006). Neither an antemortem nor postmortem virological investigation was performed. Thus, there are at least five documented human rabies cases of bat origin in Europe to date. EBLV-1 was the cause of one of them, EBLV-2 was the cause of two, and no agent identification was implemented for the other two cases.

Asia is the continent least explored for bat lyssaviruses. One historical record describes a probable "rabies virus" isolate from a bat in India and mentions a human rabies case after bat exposure (Pal *et al*., 1980), and one bat isolate in Thailand (Smith *et al*., 1968), both of Pteropodid bat origin. No rabies was found in 1013 bats examined in the Philippines (Beran *et al*., 1972) nor in 478 bats in Malaysia (Tan *et al*., 1969). Two human rabies cases after bat bites were suspected in northern China in 2002 and 2010; however, the diagnosis was based on clinical symptoms only, no virological assay was implemented, and postmortem samples were not stored (Tang *et al*., 2005; Liu *et al*., 2013).

Three isolates of RABV were reported from Siberian bats (Botvinkin, 1988; King *et al*., 1990; Botvinkin *et al*., 1992; Khozinski *et al*., 1991) but when they were genetically typed after a series of mouse passages, only laboratory strain CVS or Arctic RABV were identified (Kuzmin *et al*., 2006a). Therefore, it is not possible to evaluate

whether these isolates were mistaken initially or cross-contaminated during laboratory passages. Several RABV-positive bats were encountered in West Siberia in another study (Zaikovskaia *et al*., 2005). The authors collected 88 hibernating bats (mainly *Myotis daubentonii*). Brains of 24 (27.3%) were positive for lyssavirus antigens, and 17 (19.3%) were positive by RT-PCR. However, mouse isolation was inconsistent, and sequencing of the PCR products revealed a fox RABV variant which unlikely to occur in bats. A similar observation was recently reported from China (Wang *et al*., 2013). The authors collected 2969 bats of different species, and 85 (2.86%) of these were positive for RABV nucleic acid. In some locations, the infection prevalence was as high as 6.3–7.5%. Phylogenetic analysis of 10 positive samples demonstrated that all bat specimens were highly similar genetically to dog RABV that circulates in the same regions of China. Such unusual claims should be corroborated by additional studies, including field sampling and further laboratory survey to avoid a possibility of cross-contamination of the specimens.

Two distinct lyssaviruses were isolated from bats in Central Asia. One of them, ARAV, was obtained from the brain of a lesser mouse-eared bat (*Myotis blythii*) mist-netted in southern Kyrgyzstan in 1991 (Kuzmin *et al*., 1992). Another one, KHUV, was isolated in northern Tajikistan in 2001 from the brain of a whiskered bat (*Myotis mystacinus*) that landed on a building wall and allowed itself to be captured manually (Kuzmin *et al*., 2001). Phylogenetically, KHUV is closely related to EBLV-2 and BBLV, whereas ARAV is more distant, demonstrating moderate relatedness to the cluster of EBLV-2 and BBLV on one side, and to EBLV-1 on the other (Kuzmin *et al*., 2005, 2008c).

Experimental studies demonstrated low to moderate susceptibility of laboratory animals (such as mice, Syrian hamsters, and ferrets) and bats to peripheral inoculation of ARAV and KHUV. In one experiment, *Pipistrellus pipistrellus* bats were captured at the beginning of hibernation and transported to the laboratory in a cold environment. Half of the animals were awakened, inoculated intramuscularly with EBLV-1 and ARAV, and kept in an active stage at room temperature. Another half of the bats were inoculated and maintained in hibernation, at 2–5°C, during first 60 days post challenge. Thereafter, they were awakened and transferred to ambient room conditions as well. The incubation period in the latter group of bats after their awakening was the same as that in bats inoculated and maintained in an active state (Kuzmin *et al*., 1994; Kuzmin & Botvinkin, 1996). This demonstrates that viruses can be conserved in bats in an inactive state for at least 60 days of hibernation, and possibly longer. The duration of incubation periods in active bats was 14–67 days, and the duration of clinical periods was 1–13 days. Clinical signs were similar to those observed in bats infected with RABV and EBLVs. In some instances, signs of encephalitis, such as tonic–clonic convulsions, ascending paresis, and paralysis were observed. Biting behavior was observed repeatedly; however bats often were too weak, and could not actively chase and attack another bat. In other cases, hypersensitivity for high frequency sounds was reported. Finally, bats were seen often as emaciated and exhausted, unable to fly, without any specific signs of other brain dysfunction. They could only utter a prolonged loud vocalization and uncontrolled wing beats when disturbed. The viruses were detected in 100% of bat brains and in 33–100% of salivary glands, appearing in oral swabs 0–2 days before clinical onset (or 0–4 days before the death). The animals that survived inoculation never demonstrated signs of disease during the observation period, their oral swabs obtained in that period never

contained virus, nor did their brains and salivary glands harvested at the end of the experiment (Kuzmin & Botvinkin, 1996; Hughes *et al*., 2006).

Another bat lyssavirus, IRKV, was first isolated in 2002 in the town of Irkutsk (Eastern Siberia) from the brain of a greater tube-nosed bat (*Murina leucogaster*) that entered an apartment building, was captured, and died after several days of captivity with clinical signs of general weakness (Botvinkin *et al*., 2003). Phylogenetic analysis demonstrated that IRKV was related to the cluster of EBLV-1 and DUVV (Kuzmin *et al*., 2005, 2008c). In 2007, IRKV caused a human death in the Russian Far East. A girl was attacked by an unknown bat and bitten on her lip. She did not seek medical attention and developed rabies one month after the incident (Leonova *et al*., 2009). The obtained isolate (named Ozernoe) shared over 91% of nucleotide identity with the original IRKV isolate. During 2012, IRKV was isolated from an apparently healthy *Murina leucigaster* bat collected in the course of active surveillance in north-eastern China (Liu *et al*., 2013). Experimental studies demonstrated that susceptibility of rodents, ferrets and bats to IRKV was similar to their susceptibility to ARAV and KHUV, as described above. Incubation periods, clinical signs and distribution of IRKV in bat organs also followed the same patterns (Hughes *et al*., 2006).

As in other parts of the world, serologic surveys of Asian bats have provided some insights into lyssavirus circulation, even in the absence of viral isolates. Of 231 sera samples collected from the Philippines and screened against RABV and ABLV, 22 (9.5%) demonstrated neutralizing activity against ABLV, whereas no neutralization of RABV was detected (Arguin *et al*., 2002). At the time of that survey, no Asian bat lyssavirus isolates were available for comparative testing. Further studies were performed using ARAV, KHUV and IRKV for comparative tests *in vitro*, and presence of antibodies neutralizing these viruses was demonstrated in bat sera from Thailand (Lumlertdacha *et al*., 2005) and Bangladesh (Kuzmin *et al*., 2006b). In both of the latter studies, the greatest number of positive findings were made in pteropodid bats. In Thailand, 4.1% of tested *Pteropus lylei* bats were positive and in Bangladesh 2.4% of tested *Pteropus giganteus* bats were positive. In Cambodia, 14.7 to 16% of bat serum samples (both Mega- and Microchiroptera) were positive for antibodies against RABV, EBLV-1, ABLV and even LBV, although authors did not use Asian bat lyssaviruses for a comparison (Reynes *et al*., 2004). Similar serological studies conducted recently in Vietnam support the concept that bat lyssaviruses are widespread throughout Asia (Nguyen *et al*., 2014).

3.5.4 Australia

Prior to 1996, Australia had been considered free of rabies, except for a local rabies outbreak documented in dogs on the island of Tasmania during 1867 (Fraser *et al*., 1996). Following the discovery that flying foxes were a reservoir of Hendra virus, surveillance of these animals was increased, particularly in those who were found sick or injured. In 1996, a young female black flying fox (*Pteropus alecto*) was found under a fig tree, unable to fly, in Ballina, New South Wales. Tests for Hendra virus were negative, but evidence of severe nonsuppurative encephalitis was found in the brain. Typical inclusions of lyssavirus antigens were demonstrated through different brain areas. Another case was recognized retrospectively in a juvenile female of the same species from northern Queensland. This bat had been euthanized in 1995 with evidence of unusual aggressiveness. More isolates became available shortly thereafter via active targeted

surveillance. Gene sequences demonstrated that Australian isolates (originally named Ballina virus) were more related to classical RABV than to other lyssaviruses. Nevertheless, the amount of distinction suggested that a new genotype (further species), ABLV, should be established (Fraser *et al*., 1996; Hooper *et al*., 1997; Gould *et al*., 1998; Tordo *et al*., 2004).

There are four flying fox species in continental Australia: *Pteropus alecto*, *Pteropus poliocephalus*, *Pteropus scapulatus* and *Pteropus conspicillatus*, and ABLV was recognized in each of them, along the eastern coastal territory of the continent. Genetically, all of the pteropodid isolates were similar to each other, without any correlation to a particular host species or geographic location (Guyatt *et al*., 2003). Pteropodid bats roost in trees and form colonies ("camps") that frequently number in the thousands of animals belonging to one or several species. These colonies may fluctuate in size, depending on available food resource and season, and animals from one colony can move to another, especially during periods of migration. This dynamic 'fission–fusion' social structure has been invoked to explain the circulation of similar viruses in these animals (Hooper *et al*., 1997; Guyatt *et al*., 2003).

Several samples of ABLV were obtained from the insectivorous bat *Saccolaimus flaviventris*. The nucleotide sequences were segregated in another monophyletic clade, clearly distinguishable from the pteropodid clade (Gould *et al*., 2002). It remains to be clarified whether other species of Australian insectivorous bats participate in ABLV circulation, and if so, whether they maintain the same lyssavirus as *Saccolaimus flaviventris* or does additional variability occur.

In an experimental study, gray-headed flying foxes (*Pteropus polyocephalus*) demonstrated a moderate susceptibility (30%) to intramuscular inoculation with 10^5 $TCID_{50}$ of a homologous ABLV isolate. The incubation periods were 15–24 days. Clinical signs included general weakness, trembling, pareses and paralyses. The animals were euthanized on the onset of clinical signs, so the duration of clinical periods could not be assessed. The virus was detected in the brain of each clinical bat and at least in one saliva sample. Serologic response was not detected in the bats that developed rabies, but 70% of those bats that survived the challenge demonstrated various levels of ABLV-neutralizing antibodies in their sera during the following three months of observation (McColl *et al*., 2002). Dogs and cats inoculated intramuscularly with $10^{3.7}$–10^5 $TCID_{50}$ of ABLV demonstrated transient mild clinical signs, and no virus was detected in their tissues after euthanasia. Therefore, the observed clinical signs were unlikely to be caused by lyssavirus infection (McColl *et al*., 2007).

Spillover of ABLV into terrestrial mammals, (horses), has been documented only once in Queensland (ProMED-mail # 20130517.1720540). The horses developed clinical signs of encephalitis and were euthanized. Insectivorous bats were detected on that property in close proximity to the horses, and it is believed that bat bites caused the disease.

Three human cases of ABLV infection have been described to date. All were fatal, and clinical symptoms were compatible with rabies. The first one was reported very shortly after the virus was discovered in 1996. The patient was a woman, presumably infected by a *Sassolaimus flaviventris* bat in her care. The virus that was isolated was compatible with this bat species (Allworth *et al*., 1996; Gould *et al*., 2002). The second case occurred in another woman who developed rabies in 1998, approximately 27 months after presumable exposure from a bite by an unspecified flying fox. This isolate belonged to the pteropodid ABLV variant (Hanna *et al*., 2000; Warrilow *et al*.,

2002). The third case occurred during 2013 in a boy who developed rabies three weeks after a bat bite (Francis *et al*., 2014).

Little is known about ABLV circulation patterns. The virus was detected in 6% of sick, injured or orphan flying foxes submitted to diagnostic laboratories. In one study, 9.4% of flying foxes submitted to laboratories because they had bitten or scratched humans, or where testing was considered to be in the interest of public health, were ABLV-positive (Warrilow *et al*., 2003). Serological surveys of a mixture of sick and apparently healthy bats demonstrated that 16% of the bats were seropositive to ABLV (Hooper *et al*., 1997). It is interesting that the distribution range of *P. alecto* bats extends into Papua New Guinea and the eastern islands of Indonesia (Fraser *et al*., 1996). There is no reason to expect that distribution of ABLV is limited to continental Australia. For example, the presence of antibodies to this virus was demonstrated in 9.5% of bat serum samples collected in the Philippines (Arguin *et al*., 2002).

3.6 PUBLIC HEALTH AND VETERINARY SIGNIFICANCE OF BAT RABIES

Over 99% of human rabies cases globally are caused by dog-mediated disease (Lembo *et al*., 2011). Nevertheless, bat rabies constitutes a complex transdisciplinary problem, which becomes particularly prominent after elimination of the infection in dog populations. One of the challenges is associated with lyssavirus diversity. Genetic distances among lyssaviruses correlate with antigenic distances. Those viruses that share over 74% amino acid sequence within the G ectodomain sufficiently cross-neutralize each other (Badrane *et al*., 2001). All commercially available rabies biologics are based on several well-studied strains of RABV. They are efficacious against Phylogroup I lyssaviruses (Figure 3.1) but do not elicit sufficient protection against Phylogroup II lyssaviruses, WCBV, and IKOV (Hooper *et al*., 1997; Badrane *et al*., 2001; Brookes *et al*., 2005b; Hanlon *et al*., 2005; Horton *et al*., 2014). By inference, it is unlikely that these biologics will protect against such divergent lyssavirus as LLEBV.

To overcome this problem, several different vaccine approaches have been investigated. The first potential MOKV vaccine (which was also aimed to protect against LBV as these viruses within Phylogroup II cross-react serologically (Badrane *et al*., 2001), a recombinant baculovirus expressing MOKV G elicited protection against the homologous virus but not against a heterologous challenge with RABV (Tordo *et al*., 1993). Several vectors and promoters were implemented in DNA vaccines against MOKV, which expressed either the G, N, or both genes. Neither vaccine was fully protective in a single immunization, although booster doses increased their efficacy significantly, except for the vaccine based on the N gene (Nel *et al*., 2003).

Other studies explored DNA vaccines that expressed MOKV and RABV chimeric G genes (Bahloul *et al*., 1998; Jallet *et al*., 1999). The utility of this strategy was in the observation that lyssavirus G can be divided into two parts, separated by a flexible bridge. Each of these parts contains one of the two important antigenic domains in the elicitation of a protective immune response, the antigenic sites II (NH_2 part) and III (COOH part). Such chimeric vaccines induced virus-neutralizing antibodies against RABV, EBLV-1, EBLV-2, MOKV, and LBV, for example against a variety of Phylogroup I and II viruses available at the time. The only virus that for some reason was neutralized

weakly was DUVV (Bahloul *et al*., 1998; Jallet *et al*., 1999). Indeed, one would not expect that these vaccines confer protection against WCBV, IKOV, and LLEBV, as discovered later.

Experimental vaccinia viruses encoding G genes of RABV, MOKV and WCBV, either singly or in dual combinations, were constructed in another study (Weyer *et al*., 2008). Constructs expressing a single G gene protected animals against challenge with homologous virus. Similarly, recombinants expressing G genes from two lyssaviruses induced protection against both homologous viruses, but no significant cross-reactivity was observed (e.g., the RABV+MOKV vaccine elicited protection against RABV, MOKV, and LBV but did not protect from WCBV, whereas the RABV+WCBV vaccine protected mice from RABV and WCBV only).

The increasingly recognized antigenic variability within the *Lyssavirus* genus may be a problem in such vaccine development endeavors. For example, if one viral G can protect against the cross-reacting MOKV and LBV, another G is needed for protection against WCBV, and likely different G proteins will be needed for protection against IKOV and LLEBV. Obviously, there are limitations to the number of G genes inserted into a viral genome. However, insertion of at least two additional G genes in the RABV genome was successful (Faber *et al*., 2009). Construction of three chimeric G genes, and a replacement of the original G gene with these three constructs in a lyssavirus backbone, may potentially cover all antigenic variety of lyssaviruses detected to date. However, it is unclear whether all the inserted G proteins will be expressed efficiently and represented equally on the virion surface. Likely, larger DNA-viruses should be considered for creation of such a recombinant "pan-lyssavirus" vaccine. Unfortunately, at least two major obstacles lie ahead. Considering the lag from creation of new concepts to experimental development and clinical trials, many years will likely elapse between today's ideas and tomorrow's practices. The second obstacle is related to demands of the global market. As long as non-Phylogroup I lyssaviruses are not recognized as a significant veterinary or public health threat, it is unlikely that pharmaceutical companies will be sufficiently interested to invest funds in creation of biologics dedicated solely for the needs of developing countries where these viruses circulate.

Despite the fact that rabies biologics provide complete protection against Phylogroup I lyssaviruses, people still die of rabies caused by these variants. Vampire bat rabies is the major public health threat in Latin America. Such factors as anthropogenic environmental modifications, growing livestock populations, and climate change, bring vampire bats in contact with domestic animals and humans more frequently. A significant increase of vampire bat attacks was reported in 1996 from the Amazonian region of Brazil (Schneider *et al*., 1996). Of 129 people interviewed, 23% had been bitten by vampire bats during the previous year, with an average of 2.8 bites per attacked person. It appears that bats did not maintain RABV at that time, because neither human nor animal rabies was reported. However, the situation changed dramatically at the beginning of 2004, when at least 22 human cases occurred. Among 250 persons interviewed in the state of Para, Brazil, 140 had been bitten by bats during the previous year (ProMED-mail, archive # 20040520.1349, 20040527.1428), and each sixth or seventh such bite caused rabies. Between December of 2006 and February of 2007, an outbreak involving 527 persons bitten by vampire bats claimed at least 23 deaths in southeastern Peru (Salmón-Mulanovich *et al*., 2007). In 2009, 19 cases of human rabies transmitted by vampire bats were reported from five outbreaks in the Amazon region (Canahuiri, 2009;

Canahuiri & Vargas, 2009). Brazil and Peru consistently report the greatest number of human rabies cases associated with vampire bat bites, although under-reporting is still significant given the lack of communications from remote Amazon regions (Schneider *et al*., 2009). Not all bites of rabid vampire bats cause the disease in humans, likely depending on virus dose and site of the bite, as RABV-neutralizing antibodies were detected in 11% of human serum samples collected in two communities of the Amazon region (Gilbert *et al*., 2012).

Human rabies of nonhematophagous bat origin has been a significant public health concern in North America. In South America it may be masked by the problem of vampire bat rabies, and at earlier times due to uncontrolled dog rabies. The suggestions of airborne exposure of two humans after a visit to Frio cave in Texas, inhabited by millions of Mexican free-tailed bats (Irons, 1957; Constantine, 1962), was considered unreliable. The victims had often visited caves, and could have forgotten incidents of bat bites. Interviews with relatives or friends of other "cryptic" rabies patients usually helped to identify some kind of contact with a bat, but there often occurred several weeks or months before the onset of symptoms of the disease (Gibbons *et al*., 2002; Messenger *et al*., 2002). Still, among 39 human rabies cases registered in the US during 2000–2011 (Blanton *et al*., 2010, 2011, 2012), 27 were caused by bat RABV variants (including four cases of transplantation of organs and vessels from a donor infected with a Mexican free-tailed bat RABV variant). Among the remaining 12 cases, only 1 was caused by an indigenous raccoon RABV variant whereas the other 11 were acquired from dog exposures abroad. A history of a bite or other direct physical contact with a bat was recovered for 16 patients. Other patients or their relatives either could not recall any incidents of bat exposure, or mentioned that bats were seen in the houses several months before disease onset. Six human deaths were caused by RABV variants associated with Mexican free-tailed bats (*Tadarida brasiliensis*), five by a variant associated with silver-haired bat (*Nasionycteris noctivagans*), five by a variant associated with tricolored bat (*Perimyotis subflavus*), and at least one by a variant associated with a *Myotis* sp. One case occurred after a bite from a vampire bat during a previous stay in Mexico.

One patient from Texas survived a neurologic disease presumably diagnosed as rabies, based on the presence of RABV-binding antibodies in the serum and CSF. This patient had a history of visitation to a bat cave approximately one month before the disease onset, where several disturbed bats flew into her face. She was admitted repeatedly to several hospitals with recurring mild neurologic symptoms (Holzmann-Pazgal *et al*., 2009). This patient never required intensive care, which was in contrast to other known cases of human survival from rabies, including a case that occurred in Wisconsin during 2004, and led to the development of the Milwaukee protocol for rabies treatment (Willoughby *et al*., 2005). No viral isolates or RNA were identified in any survivor from the disease acquired from non-hematophagous bats. The diagnosis in every case was based on exposure history, a compatible incubation period, clinical signs, and a RABV serologic response. Therefore, it is unknown which RABV variants caused rabies in these survivors.

The viruses associated with silver-haired (*Lasionycteris noctivagans*) and tricolored (*Perimyotis subflavus*) bats are segregated into two phylogenetically related lineages and were previously termed as one viral variant, the silver-haired bat rabies virus (SHBRV). As inferred from a molecular clock estimate, their divergence might have occurred ~50 years ago (Kuzmina *et al*., 2013). In fact, neither silver-haired nor

tricolored bats are involved frequently in human encounters. The prevalence of SHBRV among the RABV variants causing human rabies cases over the variants associated with common dwelling-roosting bats appears significant (Messenger *et al*., 2002). Investigations into SHBRV implied that this virus may have enhanced pathogenicity. For example, the SHBRV was better adapted to fibroblasts (BHK-21) and epithelial cells (MA-104) compared with a coyote RABV (Morimoto *et al*., 1996). This trait appeared to correlate with an ability to be more effective in replicating in the dermis at the inoculation site. However, since other bat RABV variants were not tested in this regard, and related *in vivo* experiments have not been reported to date, it is not clear whether the reported findings are specific to SHBRV.

Some bites, especially if they were inflicted by small bat species, may be ignored because they were not recognized as dangerous by the patient. The ACIP has introduced an improved guide for human rabies post-exposure prophylaxis (Manning *et al*., 2008). According to this document, rabies vaccination should be considered if a bat is found indoors, and humans in the same room are unaware that a bite or direct contact might have occurred (e.g., a sleeping person awakens to find a bat in the room, or a bat is found in the room with a previously unattended child, mentally disabled or intoxicated person).

The number of human rabies cases attributed to bat exposure in the Old World is limited. Notably, they were all caused by Phylogroup I lyssaviruses, and obviously could be prevented by routinely administered post-exposure rabies prophylaxis. The real number of human cases may be under-reported, and non-Phylogroup I lyssaviruses can also be pathogenic for humans even if no such reports are available to date from the areas with poor surveillance systems and limited laboratory capacity.

Attempts to control bat-mediated rabies have been implemented consistently for the disease in vampire bats only. Approaches include: prophylactic vaccination of livestock, post- and pre-exposure vaccination of humans, and selective population reduction of vampire bats. One can estimate the benefits obtained with vaccination of livestock, making a comparison of the vaccination cost versus economic losses caused by rabies epizootics in naïve herds. Post-exposure rabies prophylaxis of humans is problematic in remote localities in Latin America due to the necessity for maintaining the cold chain for biologics, transportation limitations, and associated costs. Pre-exposure vaccination should be administered to persons of high exposure risk and ideally should be included in childhood vaccination programs in remote areas.

Methods of vampire bat population reduction include use of anticoagulants (such as warfarin), which are deadly for vampire bats. The warfarin jelly is applied on the backs of captured bats, and the animals are released. In the roost, during self- and allogrooming, other vampire bats consume the jelly on those that are treated (up to 20 or more vampire bats may be killed for each one treated). They also smear the jelly on the roost walls, excrete warfarin with urine and feces, and finally the whole roost becomes contaminated and deadly for vampire bats (and other species). Another approach includes application of warfarin to the fresh vampire bat bite wounds on cattle, because bats often return to bites made the previous night. This strategy may be quite practical for farms with a limited number of livestock, but not for large herds. Warfarin that is injected intramuscularly into cattle will circulate in the blood during the next 3–4 days, killing vampire bats which may feed on the animal. This approach is preferred by many ranchers, but is dangerous for calves and should never be used for horses (Greenhall, 1993).

However, a recent study demonstrated that culling campaigns fail to reduce rabies in vampire bat populations (as shown by seroprevalence dynamics) and are perhaps counterproductive for disease control owing to the targeted removal of adults, but potentially greater importance of naive juvenile and sub-adult bats for RABV transmission (Streicker *et al*., 2012b).

Experiments with oral vaccination demonstrated that 12.5–50% of vampire bats develop antibody responses and survive further RABV challenge (Aguilar Setien *et al*., 1998; Almeida *et al*., 2005b). Potentially, oral rabies vaccines could be applied as a jelly on bat backs, similarly to anticoagulants, so that the animals consume it during self- and allogrooming.

Population reduction approaches cannot be implemented for non-hematophagous bats: first, because they would be ineffective, and second, because many bat species are vulnerable and are legally protected. Educational campaigns targeting health professionals and different demographic groups of the general public should be implemented routinely (Racey *et al*., 2013). Examples can be found at the websites of the Global Alliance for Rabies Control (http://rabiescontrol.net), Bat Conservation International (http://www.batcon.org), Bat Conservation Trust (http://www.bats.org.uk), Centers for Disease Control and Prevention (http://www.cdc.gov), and other organizations. In fact, the message is reasonably simple; to increase public awareness, to avoid physical contact with bats, to administer pre-exposure rabies prophylaxis to all persons (and animals) of high exposure risk, and to administer post-exposure rabies prophylaxis in every case of bat exposure (including such circumstances where a bite cannot be ruled out as described in the ACIP recommendations) if the biting animal cannot be tested for rabies. Contacts with sick and injured bats are most dangerous, as such animals have been found to have a significantly higher probability to be rabid compared to randomly collected bats (McCall *et al*., 2000). Eviction of bats from houses and public buildings may lead to dispersal of infected individuals over greater territories and mixing with other colonies, thereby facilitating further rabies spread (Streicker *et al*., 2013). Preventing bats from access to human dwellings is a more plausible strategy to avoid exposure.

3.7 CONCLUSIONS

Clearly, bats play a key role in lyssavirus evolution, distribution and ecology. In contrast to the well-studied carnivore rabies, many aspects of bat rabies remain elusive particularly from the standpoint of the commonly accepted hypothesis that lyssaviruses originated in bats in the Old World. For example, why are non-RABV lyssaviruses dominant in the Old World, but are not present in the New World? How did New World bats acquire RABV in the apparent absence of this virus in Old World bats? What are the biological underpinnings in the New World that supported the radiation of multiple bat species maintenance and circulation of distinct RABV lineages, whereas by comparison in the Old World, only a few bat species maintain circulation of genetically homogeneous lyssaviruses, such as EBLV-1, EBLV-2, and BBLV? How can mechanisms of bat innate and adaptive immunity be exploited to develop better interventions against rabies? Besides migration, torpor, and hibernation, what other physiological or population-level processes permit viral perpetuation in space and time? What is the role

of airborne infection in gregarious bat colonies and will conventional biologics prevent fatal aerosol exposures? Can a present day understanding of bat rabies allow for predictive modeling of lyssavirus spillover and adaptation to carnivore hosts?

The general body of knowledge to date indicates that bats infected with lyssaviruses develop fatal rabies similar to other mammals. However, several contradictive reports suggest the existence of a "carrier" state. This possibility must be ruled out promptly as it may significantly influence public health policies with regard to rabies prophylaxis. At present, the absence of rabies virus in the brain of a biting bat is sufficient for discontinuing rabies prophylaxis, and no human rabies cases have resulted from this practice to date.

The non-Phylogroup I lyssaviruses are detected increasingly often in the Old World and deserve special attention, because conventional rabies biologics do not elicit protection against these agents. Hence, there is a critical need to establish host range, circulation patterns, and pathobiological properties of such viruses in bats, and to develop new biologics capable of offering suitable protection.

Strategies to manage rabies in bat populations remain to be developed. The existing approaches for control of the disease in vampire bats are not very successful or sustainable, and require re-evaluation. The same approaches cannot be implemented for non-hematophagous bats, which require invention of new methodologies. Differential education campaigns targeting the general public and health care professionals, all levels of rabies prophylaxis (including pre- and post-exposure prophylaxis), and avoidance of direct contact with bats (without destruction of their habitats) must be rigorously implemented at present.

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4

BAT PARAMYXOVIRUSES

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4.1 INTRODUCTION TO THE PARAMYXOVIRIDAE

Bats have been implicated as an important source of new and emerging paramyxoviruses. The family *Paramyxoviridae* is divided into two subfamilies, the *Paramyxovirinae* (paramyxoviruses) and *Pneumovirinae* (pneumoviruses). The paramyxoviruses are currently grouped into seven genera: *Morbillivirus*, *Respirovirus*, *Rubulavirus*, *Avulavirus*, *Henipavirus*, *Aquaparamyxovirus* and *Ferlavirus*, while the pneumoviruses are grouped into two genera: *Pneumovirus* and *Metapneumovirus* (International Committee on Taxonomy of Viruses, 2013). All viruses isolated from or detected in bats to date, belong to the subfamily *Paramyxovirinae*, with the exception of the partial pneumovirus sequences identified by Drexler *et al.* (2012). In this context, the discussion of pneumovirus will be limited to the introduction section of this chapter. Within this chapter the term "paramyxovirus" will be used to describe members of the subfamily *Paramyxovirinae* whereas "pneumoviruses" will be used to describe members of the subfamily *Pneumovirinae*.

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4.1.1 Virus structure

Paramyxovirus virions are generally spherical, but can also be pleomorphic (Figure 4.1). Particles range from 40–540 nm in diameter (Loney *et al.*, 2009; Terrier *et al.*, 2009) and filamentous forms can be longer (Liljeroos *et al.*, 2013). Virions are composed of two structural components; the helical ribonucleoprotein (RNP) core

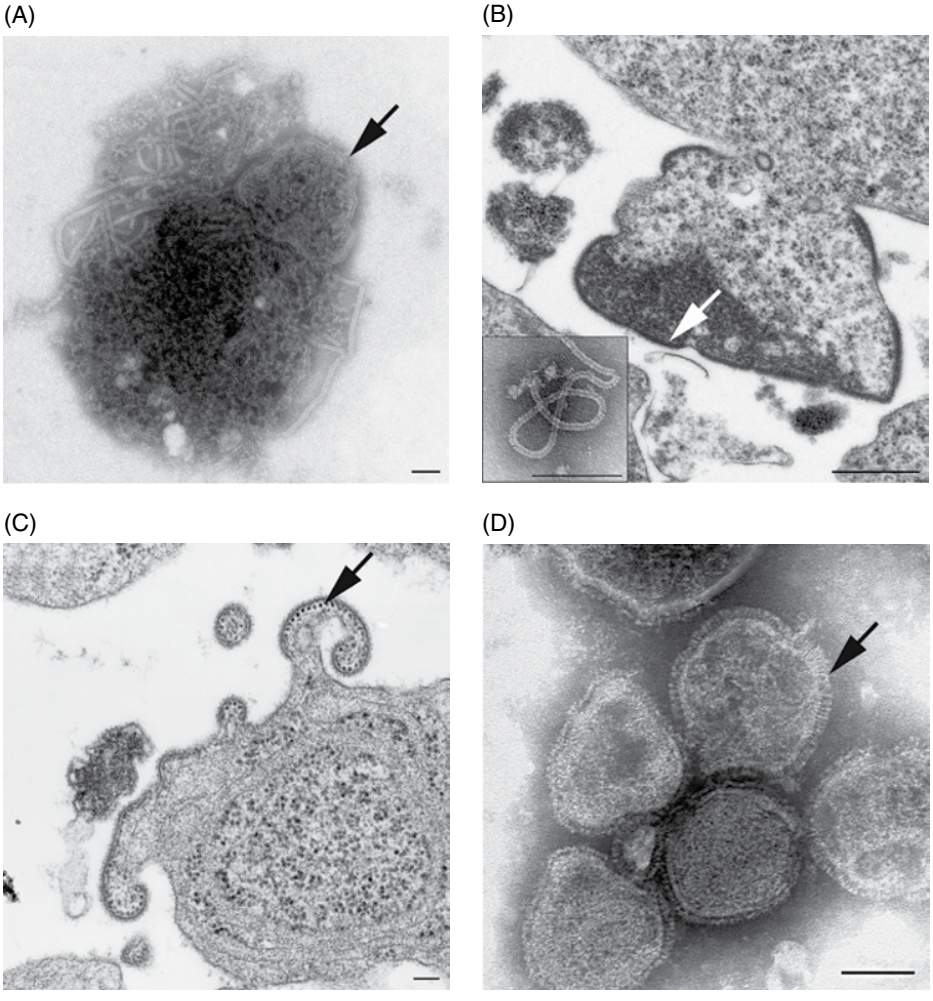

Figure 4.1 Transmission electron micrographs of viruses within the subfamily *Paramyxovirinae*. (A) Hendra virus, negatively stained. Arrow indicates virus particle, which is adjacent to extensive amounts of nucleocapsid released from that particle. (B) Cedar virus budding from the plasma membrane of a host cell. White arrow indicates underlying nucleocapsid in thin section EM. (B, inset) Cedar virus nucleocapsid displaying typical herringbone morphology. (C) Tioman virus budding from the plasma membrane of a host cell. Arrow indicates underlying nucleocapsid in thin section EM. (D) Menangle virus, negatively stained. Arrow indicates glycoprotein fringe. Scale bars represent 100 nm in all images except (B), which is 500 nm.

and the lipoprotein bilayer membrane, acquired from the host cell, which constitutes the envelope. The negative-sense, non-segmented, single-stranded (NNS) genomic RNA is always tightly encased by nucleocapsid proteins in a left-handed coil with distinct 'herringbone' morphology (Figure 4.1B, inset). The nucleoprotein (N), in conjunction with the phosphoprotein (P) and large (L) protein, together form a complex that has RNA-dependent RNA polymerase activity. In pneumoviruses an additional protein, transcription processivity factor M2-1, is associated with the polymerase complex and is essential for replication (Collins & Karron, 2013). Projecting 8–12 nm from the lipid bilayer of the viral envelope are two to four different glycoproteins which can be readily observed by electron microscopy (EM; Figure 4.1D) (Lamb & Parks, 2013). All paramyxoviruses possess attachment (HN, H or G) proteins and fusion (F) proteins that are activated by proteolytic cleavage (Conzelmann 1998; Collins & Karron, 2013; Lamb & Parks, 2013). The pneumoviruses only contain G attachment proteins, which do not possess any sequence or structural homology to their paramyxoviruses counterparts, along with an F fusion glycoprotein. A small hydrophobic (SH) protein is present in all members of the *Pneumovirinae* (Collins & Karron, 2013), some rubulaviruses (Hiebert *et al.*, 1985; Elango *et al.*, 1989), and the unclassified rodent paramyxoviruses J virus (JPV) and Beilong virus (BeiPV) (Jack *et al.*, 2005, 2008; Li *et al.*, 2006). JPV and BeiPV also contain a transmembrane (TM) protein (Jack *et al.*, 2005, 2008; Li *et al.*, 2006). The matrix (M) protein is the most abundant protein in the virion, and is basic and slightly hydrophobic. The M protein plays a key role in viral morphogenesis as glycoproteins are anchored in the gaps between the M proteins (Battisti *et al.*, 2012). The M protein also interacts with the lipid bilayer and nucleocapsids (Lamb & Parks, 2013) to initiate virus assembly and budding (Harrison *et al.*, 2010).

4.1.2 Genome organization

4.1.2.1 Paramyxoviruses All paramyxoviruses contain a NNS RNA genome ranging from 15 178 (Porcine rubulavirus) to 19 212 (BeiPV) nucleotides in length. Paramyxovirus genomes contain six to eight genes, flanked by a conserved 3′ leader (55 nucleotides for all members of the subfamily) and a 5′ trailer (50–161 nucleotides) extragenic regions (Lamb & Parks, 2013). The term "gene", when used in reference to paramyxoviruses, refers to the sequence encoding a single mRNA, even if more than one open reading frame (ORF) is present or more than one protein is encoded. The N, P, M, F, attachment (HN/H/G), and L genes are present in all paramyxoviruses and the order is conserved (Figure 4.2). In addition, some paramyxovirus species possess additional genes, such as the SH gene. Genes are flanked at both 5′ and 3′ ends of the ORFs by untranslated regions (UTRs) and separated by intergenic regions. The 5′ UTR contains a gene start (GS) sequence utilized for transcription initiation and the 3′ UTR contains a gene end (GE) region responsible for transcription termination. The GE sequence contains a stretch of four to seven uridine residues that act as a template for polyadenylation of the mRNA. The intergenic region is three nucleotides in length for viruses in the genera *Morbillivirus*, *Respirovirus*, and *Henipavirus*, but can vary in length from 1–124 nucleotides for viruses in the genera *Rubulavirus* and *Avulavirus* (Lamb & Parks, 2007; Lau *et al.*, 2010).

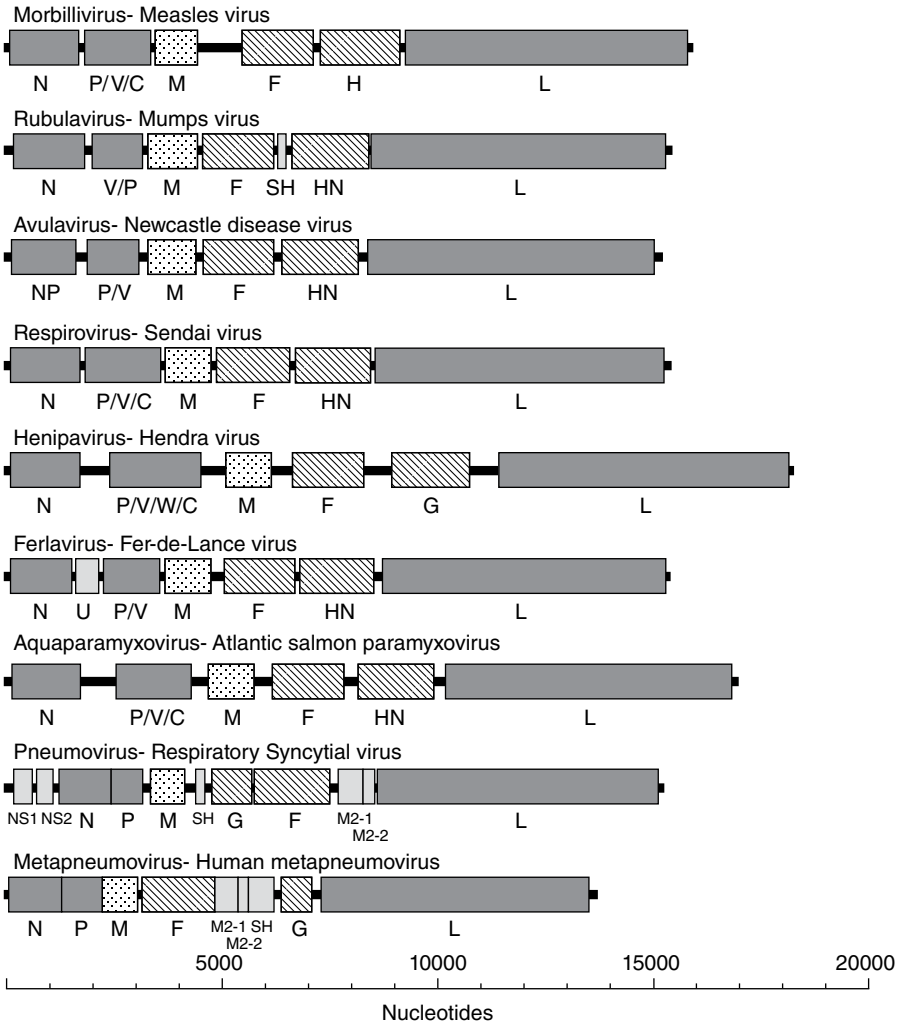

Figure 4.2 Representative genomes of paramyxoviruses and pneumoviruses from each genus. The genomes, coding regions and untranslated regions are drawn to scale. The conserved genes present in all genomes are indicated as follows: dark shaded = RNA polymerase and nucleocapsid genes (N, P and L); slanted = envelope membrane protein genes (F and attachment protein); dotted = matrix protein (M). The light shaded boxes indicate genes which are not commonly shared amongst members of the family. The scale at the bottom represents genome size in nucleotides.

4.1.2.2 Pneumoviruses Pneumoviruses contain NNS RNA genomes with lengths ranging from 13 335 (human metapneumovirus) to 15 222 (human respiratory syncytial virus) nucleotides. The genomes of the *Pneumovirinae* contain 8–10 genes, although the genomic organization is not identical between the *Pneumovirus* and *Metapneumovirus* genera. Pneumoviruses contain 10 genes; non-structural (NS) protein 1, NS2, N, P, M1, SH, G, F, M2, and L. The metapneumoviruses lack the non-structural proteins NS1 and NS2, and contain eight genes (Figure 4.2). Genomes are flanked by a 3' leader (41–44 nucleotides)

and a 5' trailer (91–155 nucleotides) extragenic regions (Collins & Karron, 2013; Decaro *et al.*, 2014). Poorly conserved intergenic regions of variable lengths (1–190) separate the gene boundaries of the pneumoviruses (Collins & Karron, 2013). A unique feature of the genus *Pneumovirus* is the overlapping M2/L gene, which is not observed in any other paramyxovirus (Collins *et al.*, 1987).

4.1.3 Paramyxovirus replication

The paramyxovirus life cycle begins with virus adsorption and entry into a host cell. Entry into cells requires the fusion of the virion envelope with the host cell membrane. Nearly all paramyxoviruses that have been examined to date require both the membrane-anchored attachment and F glycoproteins for efficient fusion to occur. Membrane fusion is triggered at the cell surface in a receptor-dependent, pH-independent manner (Lamb & Jardetzky, 2007; Lamb & Parks, 2013). The attachment protein recognizes cell surface receptors, and these receptors vary amongst different paramyxoviruses. Paramyxoviruses in the genera *Rubulavirus*, *Respirovirus* and *Avulavirus* encode an attachment (HN) protein that both binds to and cleaves sialic acid receptors, promoting virus attachment during entry and budding after infection, respectively (Jardetzky & Lamb, 2014). The morbilliviruses, including measles virus (MeV) and canine distemper virus (CDV), have an H attachment glycoprotein, which possesses only hemagglutinating activity and does not bind to sialic acid receptors. Signaling lymphocyte activation molecule (SLAM; CD150) is the cellular receptor for the morbilliviruses (Hsu *et al.*, 2001; Tatsuo *et al.*, 2000, 2001). Nectin-4 is a cellular receptor used by the morbilliviruses for entry specifically into epithelial cells (Muhlebach *et al.*, 2011; Noyce *et al.*, 2011, 2013; Noyce & Richardson, 2012; Pratakpiriya *et al.*, 2012). Vaccine and laboratory adapted strains of MeV utilize CD46 as a receptor (Dorig *et al.*, 1993; Naniche *et al.*, 1993). The henipaviruses use ephrin-B2 as the major entry receptor (Bonaparte *et al.*, 2005; Negrete *et al.*, 2005; Marsh *et al.*, 2012). Ephrin-B3 was identified as a second entry receptor for Nipah virus (NiV) and Hendra virus (HeV) (Negrete *et al.*, 2006; Bishop *et al.*, 2007), but not for the newest member of the genus, CedPV (Marsh *et al*., 2012).

In contrast to the paramyxoviruses, efficient fusion for the pneumoviruses does not require interaction with the homologous attachment protein. As the *Pneumovirinae* G proteins are not structurally related to the *Paramyxovirinae* HN/H/G proteins and are also not required for F activation, the entry mechanisms appear to differ substantially between the viral subfamilies (Jardetzky & Lamb, 2014). The pneumovirus G protein participates in attachment by binding glycosaminoglycans, which are long unbranched chains of repeating disaccharide subunits on the outer surface of the cell (Collins & Karron, 2013; Hallak *et al.*, 2000). Endocytosis has been implicated as a possible route of respiratory syncytial virus (RSV) entry (Kolokoltsov *et al.*, 2007; Krzyzaniak *et al.*, 2013). Nucleolin was recently implicated as an RSV receptor (Tayyari *et al.*, 2011), but it appears that this protein interacts with the F protein rather than the G attachment protein (Mastrangelo & Hegele, 2013).

Following virion attachment to a permissive and receptor-bearing host cell, viral F proteins are triggered, resulting in the fusion of virion membranes with target cell membranes via a process that is driven by the refolding of F proteins from initial metastable states into more stable hairpin structures (Russell & Luque, 2006). Fusion of the virion and plasma membranes results in delivery of the viral nucleocapsid into the cytoplasm, where all aspects of replication take place. Only virions that contain a functional viral RNA-dependant RNA

polymerase (vRdRp) can initiate infection and this polymerase complex acts as both a transcriptase and a replicase. The helical, RNase-resistant RNP is the template for all RNA synthesis and the RNA genome is neither capped nor polyadenylated.

All paramyxovirus RNA synthesis begins with a single entry of the vRdRp at the 3´ end of the genome. Primary transcription occurs with negative-sense viral genomic RNA within RNPs serving as template for the production of mRNAs. Initiation of mRNA synthesis is dependent on the distance between the genome 3´ end and GS signal (Cordey & Roux, 2006). The strength of the replication promoter is important in determining initiation of mRNA synthesis (Vulliemoz *et al.*, 2005). A scanning model predicts that ribosomes initiate translation at the first AUG codon that is in favourable context according to the "Kozak" sequence 5´-GCCRCCAUGG-3´ (Kozak, 1986). Monocistronic mRNA produced by the vRdRp contains a 5´ cap (Abraham *et al.*, 1975) and is polyadenylated by a stuttering mechanism that reiteratively copies a short run of four to seven uridines, leading to the termination and release of the mRNA. After releasing the mRNA, the vRdRp remains attached to the genome template before reinitiating transcription at the downstream gene. Most viruses in the *Paramyxovirinae*, but none in the *Pneumovirinae*, engage in RNA editing by adding non-templated G residue(s) during transcription, resulting in reading frame of different mRNAs to alternatively encode the P or V proteins (Kolakofsky *et al.*, 2005). Transcription for all mRNA starts at the GS signal. Notably, the GS signal for the L gene of the pneumoviruses is located within the upstream M2 gene, thus the two genes overlap by 68 nucleotides (Collins *et al.*, 1987). Overlapping genes do not exist in any other members of the *Paramyxoviridae* characterized to date. It has been suggested that in order to transcribe the L gene, the vRdRp utilizes retrograde scanning, reading the transcript in both directions (Fearns & Collins, 1999).

Transcription follows the "stop–start" model first described for vesicular stomatitis virus (VSV) (Blumberg *et al.*, 1981). In this model transcription is sequential, where the vRdRp moves along the RNA template, stopping and starting at gene junctions, generating individual mRNAs. This stop-start mechanism of transcription is not perfect and failure of vRdRp to reinitiate transcription at a downstream site results in a gradient of mRNA production inversely proportional to the distance from the 3´ end of the genome (Cattaneo *et al.*, 1987; Lamb & Parks, 2013), and this transcription gradient is maintained throughout infection (Plumet *et al.*, 2005).

Most paramyxoviruses circumvent the interferon (IFN) response by interaction and interference with the cellular pathways involved in the host innate immune response. Virus infected cells may be triggered to secrete type I IFNs, antimicrobial peptides, cytokines, chemokines, and other metabolites which act to coordinate the production of an antiviral state, induce complex processes relating to inflammation and facilitate the development of adaptive immunity (as reviewed in Kumar *et al.*, 2011). The P gene products from viruses within the subfamily *Paramyxovirinae* have been demonstrated to inhibit both dsRNA signaling (Naniche *et al.*, 2000; He *et al.*, 2002; Komatsu *et al.*, 2002; Poole *et al.*, 2002) and IFN signaling (Horvath, 2004; Rodriguez & Horvath, 2004; Conzelmann, 2005). The ability of viruses to inhibit IFN pathways is considered to be important determinants of virulence and host range. As the levels of newly synthesized viral proteins build up, the viral polymerase enters a replication mode. The switch is triggered by levels of the nucleocapsid protein. Viral genomes are no longer transcribed, but rather are replicated in a two-step process that involves first the production of positive-sense antigenomes from genomic templates, and subsequently the production of negative-sense genomes from antigenomic templates (Lamb & Parks, 2013). The

antigenome serves solely as a replication intermediate for genome synthesis. During genome replication, all gene junction signals and editing sites are ignored by the vRdRp. Paramyxovirus RNA is required to be bound by a helical nucleocapsid in order to be infectious. For efficient replication, paramyxovirus genomic RNA must contain a total number of nucleotides that is a multiple of six, dubbed the 'rule of six' (Calain & Roux, 1993). The RNA polymerase initiates more efficiently when *cis*-acting promoter sequences are found in the correct context with relation to N subunits, determined by the length of the entire genome (Egelman *et al.*, 1989; Hausmann *et al.*, 1996). Association of N monomers with hexameric genomic nucleotide sequences begins with the first nucleotide at the 5′ end of the genome and continues until the genome is precisely covered by N subunits to the 3′ end. Although the paramyxoviruses strictly obey the "rule of 6", no such requirement exists for the pneumoviruses. Although paramyxovirus genomes are non-segmented, not all budding particles receive only a single copy of genomic RNA. Some virions containing multiple genome copies are released (Rager *et al.*, 2002; Loney *et al.*, 2009). Apart from incorporation into new virions during the budding process, progeny negative-sense genomes can serve as templates to produce additional antigenomes, or they enter into a secondary transcription phase to synthesize more viral mRNA.

To complete the infectious cycle, newly synthesized viral proteins and RNPs assemble together at plasma membranes of the host cell in preparation for particle budding and release of progeny virions. Prior to localization, nucleocapsids assemble in the cytoplasm. The position of M proteins underneath the cellular plasma membrane allows for the interaction with both RNPs and the cytoplasmic tails of viral glycoproteins (Takimoto & Portner, 2004; Harrison *et al.*, 2010).

Polarized epithelial cells that line body surfaces possess apical and basolateral surfaces, and budding of paramyxoviruses only occurs from the apical surface (Blau & Compans, 1995; Roberts *et al.*, 1995; Bose *et al.*, 2001; Zhang *et al.*, 2002). Polarized budding has important consequences for pathogenesis, as budding from the apical surface favours restriction of infection to the epithelial layer, while budding from the basolateral surface allows development of a systemic infection.

4.2 BATS AS A MAJOR SOURCE OF NEW PARAMYXOVIRUSES

4.2.1 Sampling methods

Paramyxoviruses have been isolated or detected in bat tissues, serum, urine, saliva, uterine fluid and foetuses (Wang *et al.*, 2013). In the wild, bats are trapped, sampled, and either released or euthanized for further processing. Mist nets are typically used by bat biologists to capture wild bats. Mist nets are usually made of nylon mesh suspended between two poles, and when properly deployed are virtually invisible. There are several disadvantages to using mist nets. They are very time consuming to set up. Animals caught in the net can become entangled, so the net must be checked often and the animal removed promptly. Disentangling an animal from a mist net can be difficult, stressing the animals in the process and must be done carefully by trained personnel.

Traditional approaches of collecting specimens for virus isolation from fruit bats involve mist-netting or shooting, both of which are labour intensive, and the latter requires killing of the animals (Johara *et al.*, 2001). During the search for the natural

reservoir of NiV in 1999, a novel method was developed to collect urine samples from bats (Chua, 2003b). An urgent need to identify the natural reservoir of NiV was required to obtain a better understanding of the mode of spillover into livestock. A preliminary survey was conducted with regard to the roosting behaviour of the fruit bats and then pooled urine samples were collected. In the afternoon prior to the day of collection, the exact spots where the urine and feces of fruit bats were expected to be deposited, as indicated by the presence of previous droppings on the ground, were carefully delineated. In the early morning of the following day, prior to the fruit bats returning to roost, clean translucent plastic sheets were placed at the marked spots directly under the roosting areas. Sterile cotton swabs were used to soak up the urine as soon as it landed on the plastic sheet and immediately placed into viral transport media (Chua, 2003b). As this procedure is minimally invasive, the collection process can be repeated. This is particularly important if excretion of infectious agents from the host is intermittent and periodic in nature. One of the key benefits of this technique is that it supplants the need to kill animals, which is of paramount importance from welfare and conservation perspectives. In addition to the isolation of NiV, this particular urine collection study also led to the isolation of two other previously unknown infectious agents from fruit bats, Tioman virus (Chua *et al.*, 2001) and Pulau virus (Pritchard *et al.*, 2006). Since its first successful use, this urine-based paramyxovirus isolation has been further optimized and applied to the successful isolation of multiple paramyxoviruses in Australia (Marsh *et al.*, 2012; Barr *et al.*, 2014)

4.2.2 Methodologies utilized in the detection and characterization of paramyxoviruses

4.2.2.1 Virus isolation Although molecular characterization is possible without ever isolating the live virus, virus isolation remains critical for further investigation beyond initial sequence characterization, such as pathogenesis studies. Cell culture systems are routinely used to isolate paramyxoviruses from biological or environmental samples. Different cell lines, such as Vero (African Green monkey kidney) (Chua *et al.*, 2001; Aljofan *et al.*, 2009; Kurth *et al.*, 2012; Marsh *et al.*, 2012; Wilkinson *et al.*, 2012), PK15 (pig kidney) (Moreno-Lopez *et al.*, 1986), RK13 (rabbit kidney) (Halpin *et al.*, 2000; Sasaki *et al.*, 2012) and PaKi (bat kidney) (Crameri *et al.*, 2009; Barr *et al.*, 2012; Marsh *et al.*, 2012) are commonly used for virus isolation from various bat samples. Virus replication, hence success of isolation, is cell line dependant. With the increasing frequency of bat-borne viruses crossing the species barrier and causing severe disease in humans and other animals, there is an urgent need for the establishment of robust cell lines from various bat species to facilitate virus isolation and basic research. Although bat cell lines are available, they differ in their value for comprehensive studies depending on their susceptibility to infection with paramyxoviruses (Crameri *et al.*, 2009; Hoffmann *et al.*, 2013). Virus susceptibility of a host species and its derived cell lines do not always match, or the cell line may change over time. For example, human HeLa USU cells were not susceptible to either HeV or NiV infection, due to the lack of expression of ephrin-B2 (Bonaparte *et al.*, 2005). Another problem typically encountered during virus isolation attempts is a lack of cytopathic effect (CPE). Cells are observed daily for toxicity, contamination or CPE, but CPE may not always be present (Barr *et al.*, 2012; Lau *et al.*, 2010). This can be overcome by monitoring virus growth by alternative molecular methods such as polymerase chain reaction (PCR).

4.2.2.2 ***Serology*** Diagnosis of infection is ideally performed by detection of viral RNA in an acute-phase blood or serum sample, but serological testing still remains the gold standard to confirm whether a human or other animal has been exposed to a pathogen. Serological diagnosis relies on detection of paramyxovirus specific immunoglobulin M (IgM) and IgG in enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA). Exposure may be ascertained by monitoring seroconversion from negative to positive virus-specific IgM antibody status, or by demonstration of an increase in IgG antibody titer in paired (acute and convalescent) serum specimens. Small changes in viral genetic and protein sequences will be largely tolerated by most serological tests; therefore tests based on one virus are usually capable of detecting antibodies to a related virus. While this complicates the diagnosis of a specific agent, it can also sometimes be an advantage; for example, serum cross-reactivity played a major role in the rapid establishment of NiV diagnosis/surveillance capability in Malaysia using the HeV antibody ELISA (Harcourt *et al.*, 2000; Marsh *et al.*, 2012; Wang & Daniels, 2012). Antigenic cross-reactivity has been used to prove the relatedness between other viruses, such as the bat and pig strains of MenPV (Barr *et al.*, 2012). Serology that is reliant on antigenic cross-reactivity needs to be interpreted carefully. During the characterization of CedPV, Vero cells infected with CedPV were able to be stained with anti-HeV antibodies by immunohistochemistry (IHC). However, HeV- or NiV-neutralizing antibodies were unable to neutralize CedPV infection, demonstrating that CedPV and the other henipaviruses share cross-reactive antigenic regions but not cross-neutralizing epitopes (Marsh *et al.*, 2012).

Confirmation of infection should be made by serum neutralization test (SNT), which is one of the most specific serological methods. Virus neutralization tests are usually based on CPE or the plaque-reduction neutralization test. Antibodies in serum bind to viral proteins and prevent the virus from attaching and entering host cells, hence reducing the viral titer.

Several bat paramyxoviruses are zoonotic, and the henipaviruses require biosafety level 4 (BSL-4) containment. In order to negate the requirement for BSL-4 containment, which is not widely available, pseudotyped virus particles provide an alternative diagnostic method that can be performed at BSL-2 conditions. VSV pseudotype particles displaying NiV F and G were used as a substitute for NiV virions (Kaku *et al.*, 2009; Tamin *et al.*, 2009). Multiplexed microsphere assays for henipaviruses have also been used as a surrogate for virus neutralization (Bossart *et al.*, 2007).

4.2.2.3 PCR Detection and identification of paramyxoviruses is routinely attempted using cell culture, EM, antigen detection assays, serologic assays, and nucleotide sequence-based assays such as PCR, with limitations in each system. Genome, antigen, and antibody-based assays are usually too specific to detect novel viruses. Cell-culture isolation will only allow successful detection of viruses that are capable of replicating in the culture system used and will require further characterization. EM requires a fairly high titer of virus for visualization and also requires further characterization. To increase the ability to detect novel viruses, broadly reactive PCR assays were developed (Tong *et al.*, 2008). The primers for these PCR assays were developed from highly conserved regions of the genome with the aim of detecting both known and novel paramyxoviruses. To maintain the relative specificity while increasing the breadth of detection, two strategies are combined in these assays, i.e., the use of consensus-degenerate hybrid oligonucleotide primers and the employment of semi-nested

PCRs. To achieve less degeneracy and greater sensitivity, the *Paramyxovirinae* was further divided into two subgroups of genera based on RNA polymerase gene relatedness, the *Morbillivirus-Respirovirus-Henipavirus* subgroup and the *Rubulavirus-Avulavirus* subgroup (Tong *et al.*, 2008). Consensus primers based on these groupings have been successfully used in the identification and characterization of CedPV (Marsh *et al.*, 2012), AchPV 1 and 2 (Baker *et al.*, 2013), several henipaviruses in African bats (Drexler *et al.*, 2009; Weiss *et al.*, 2012) and unclassified paramyxoviruses in Indonesia (Sasaki *et al.*, 2012), Europe (Drexler *et al.*, 2012; Kurth *et al.*, 2012), Africa (Baker *et al.*, 2012; Drexler *et al.*, 2012) and South America (Drexler *et al.*, 2012). Another PCR assay has been described that detects all genera of the *Paramyxoviridae* using a single set of primers, without the requirement of semi-nested or nested PCR (van Boheemen *et al.*, 2012).

4.2.2.4 Next-generation sequencing When the broadly active PCR assays described previously are to be used as a pathogen discovery tool, success depends on knowing, or at least suspecting, some information about the infectious agent. Pan-microbial DNA microarrays such as the ViroChip (Chen *et al.*, 2011), GreeneChip (Palacios *et al.*, 2007) and Lawrence Livermore Microbial Detection Array (Gardner *et al.*, 2010) are other sequence-based assays. Although useful for the detection for a wide spectrum of pathogens, microarrays are still limited by the genome sequence data available at the time of design. Next-generation sequencing (NGS), otherwise known as massively parallel or deep sequencing, involves the analysis millions of sequences derived from nucleic acids unbiasedly amplified (Liu *et al.*, 2012; Radford *et al.*, 2012). Third generation sequencing is a relatively new platform where PCR is not required before sequencing (Schadt *et al.*, 2010). NGS functions independently of the need for *a priori* knowledge about the target sequence, as opposed to conventional PCR, but the properties of the assay that promote detection through random amplification lead to higher concentrations of the more abundant transcripts, such as host genomic material. Targeted enrichment of viral genomes over host genetic material can therefore greatly enhance the discovery rate of novel viruses (Gnirke *et al.*, 2009; Depledge *et al.*, 2011; Oude Munnink *et al.*, 2013; Cotten *et al.*, 2014).

A recent example of the utilization of NGS in the context of virus discovery and disease investigation occurred in 2012, when a severe disease affected a wildlife biologist shortly after her return from rural Africa to the United States. After several known suspect pathogens were ruled out as the cause of her illness, a combination of NGS and metagenomic analysis identified a novel paramyxovirus, Sosuga virus, related to rubula-like viruses isolated from fruit bats (Albarino *et al.*, 2014). Following initial discovery by NGS, the virus genome was completely characterized by use of standard sequencing techniques.

Like PCR, NGS can enable detection of the presence of a virus, but the presence of viral nucleic acid does not prove that the virus is the causative agent of a disease. Proof of disease causation can only be established via traditional methods that depend on the availability of a live virus isolate.

4.2.2.5 Electron microscopy Paramyxoviruses have a typical morphology that is readily recognized by EM (see Figure 4.1). EM plays a crucial role in the early preliminary identification of the causative agent of an outbreak. Rapid identification of the

virus can subsequently determine the appropriate effective control measures to halt the outbreak. When clinical data is inconclusive, a range of molecular and biochemical assays can be used to identify associated infectious agents. However, if the agent is novel, there is a high probability that these assays will be ineffective. EM is not restricted by the existence of predefined probes (antibodies, antigens, nucleic acids). EM has been instrumental in the identification and characterization of most of the bat paramyxoviruses where virus isolation has been successful (Sundqvist *et al.*, 1990; Henderson *et al.*, 1995; Hyatt & Selleck, 1996; Chua *et al.*, 2001, 2007; Yaiw *et al.*, 2008b; Albarino *et al.*, 2014). EM will remain an important frontline method for rapid virus identification/exclusion investigation of any outbreak of new and unusual cases of illness with suspected infectious etiology.

4.3 KNOWN BAT PARAMYXOVIRUSES

Several bat paramyxoviruses have been discovered and characterized, although not all viruses can be classified within the current genera (Figure 4.3). It is expected that the number of bat-borne viruses within *Paramyxoviridae* will continue to expand in the future.

4.3.1 Hendra virus (HeV)

HeV (Figure 4.1A) was first identified in 1994 as the causative agent of an outbreak of acute respiratory disease in horses and two humans in Hendra, a suburb of Brisbane, Australia. This virus was initially named equine morbillivirus, but following the isolation and characterization of HeV, the genome size and lack of conservation with other known paramyxoviruses lead to the proposal of a new virus genus within the *Paramyxovirinae* (Wang *et al.*, 2000). Several years later, the identification of NiV and demonstration of its close similarity to HeV lead to the creation of the genus *Henipavirus* (Eaton *et al.*, 2007).

Following the identification of HeV in 1994, 55 outbreaks have been reported in Australia as of 1 September 2014, with 90 individual horses. As a result of these outbreaks 88 individual horses have either died or been euthanized. In horses, HeV causes a severe, often fatal, febrile illness associated with respiratory and neurologic signs. HeV in both naturally and experimentally infected horses is a rapidly progressing disease, with death occurring usually within 48 hours after the onset of clinical signs. The incubation period for horses is believed to range from 5–10 days following infection (Murray *et al.*, 1995; Williamson *et al.*, 1998; Marsh *et al.*, 2011).

A total of seven human HeV infections have occurred to date, with four succumbing to their infections. All human infections have resulted from close physical contact with infected horses. Symptoms have varied between patients, with an estimated incubation period of 7–10 days. Initial disease signs are influenza-like, then progress to a fulminating encephalitis with multi-organ failure (Selvey *et al.*, 1995; Playford *et al.*, 2010). In one case, late onset encephalitis was observed 13 months after an unrecognized exposure via infected horses. After the initial exposure, the individual suffered from an influenza-like illness and aseptic meningitis (Baldock *et al.*, 1996; O'Sullivan *et al.*, 1997).

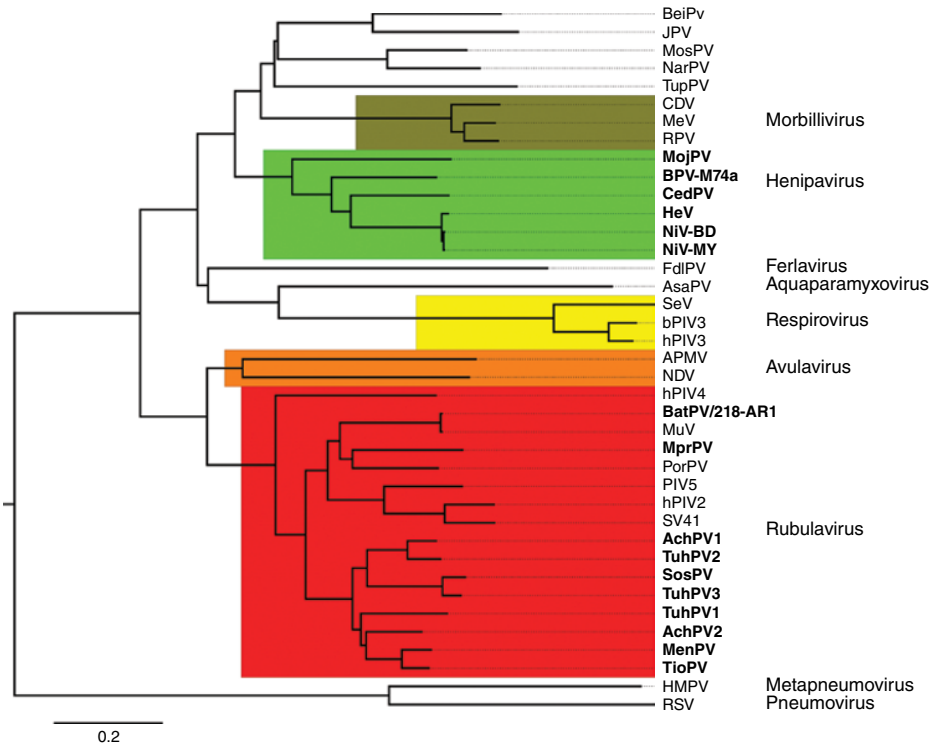

Figure 4.3 Phylogenetic tree based on the N protein sequences of selected paramyxoviruses. Virus name (abbreviation) and GenBank accession numbers are as follows: Achimota virus 1 (AchPV1) JX051319; Achimota virus 2 (AchPV2) JX051320; Atlantic salmon paramyxovirus (AsaPV) EU156171; Avian paramyxovirus 6 (APMV6) AY029299; Bat paramyxovirus/Epo spe/218-AR1/DRC/2009 (BatPV/218-AR1) HQ660095; Bat paramyxovirus/Eid hel/GH-M74a/GHA/2009 (BatPV-M47a) HQ660129; Beilong virus (BeiPV) DQ100461; Bovine parainfluenza virus 3 (bPIV3) AF178654; CDV AF014953; Cedar virus (CedPV) JQ001776; Fer-de-lance virus (FdlPV) AY141760; Hendra virus (HeV) AF017149; Human metapneumovirus (HMPV) AY297749; Human parainfluenza virus 2 (hPIV2) AF533010; Human parainfluenza virus 3 (hPIV3) Z11575; Human parainfluenza virus 4a (hPIV4a) AB543336; J virus (JPV) AY900001; Menangle virus (MenPV) AF326114; Measles virus (MeV) AB016162; Mojiang virus (MojPV) KF278639; Mossman virus (MosPV) AY286409; Mapeura virus (MprPV) EF095490; Mumps virus (MuV) AB000388; Nariva virus (NarPV) FJ362497; Newcastle disease virus (NDV) AF077761; Nipah virus, Bangladesh strain (NiV-BD) AY988601; Nipah virus, Malaysian strain (NiV-MY) AJ627196; Parainfluenza virus 5 (PIV5) AF052755; Porcine rubulavirus (PorPV) BK005918; RSV U39661; Rinderpest virus (RPV) Z30697; Sendai virus (SeV) M19661; Simian virus 41 (SV41) X64275; Sosuga virus (SosPV) KF774436; Tioman virus (TioPV) AF298895; Tuhoko virus 1 (TuhPV1) GU128080; Tuhoko virus 2 (TuhPV2) GU128081; Tuhoko virus 3 (TuhPV3) GU128082; Tupaia paramyxovirus (TupPV) AF079780. *(See insert for color representation of the figure).*

In recent years HeV has also been reported to naturally infect dogs. On two separate occasions dogs have tested positive to infection, with these dogs being present on properties with known HeV infected horses. In 2011, a dog tested seropositive to HeV on a property where three horses were infected with HeV, and in 2013, another dog tested positive to HeV by PCR.

Very early after the initial discovery of HeV, the natural reservoir was identified as flying foxes or fruit bats, with seroprevalence varying from 10–50% in different colonies (Young *et al.*, 1996; Halpin *et al.*, 2000). HeV has also been isolated on several occasions from three different species of Australian pteropid bats: *Pteropus alecto*, *P. poliocephalus* and P. conspicillatus (Halpin *et al.*, 2000; Smith *et al.*, 2011).

The most promising approach to prevent HeV disease in Australia is a recently licenced equine vaccine, Equivac®HeV. This vaccine is a recombinant form of the HeV G glycoprotein. *In vivo* experiments in horses showed seroconversion following vaccination and prevented disease following exposure to an otherwise lethal HeV challenge (Middleton *et al.*, 2014). In addition, vaccination prevented viral shedding and replication of HeV in horse tissues.

4.3.2 Nipah virus (NiV)

NiV first emerged in Malaysia in 1998 (Chua *et al.*, 1999, 2000). Initially, the virus caused mild respiratory and neurological disease in swine and was associated with an acute and frequently fatal febrile encephalitic disease in humans (Chua, 2003a). Initially, the disease outbreak was attributed to Japanese encephalitis virus, but laboratory analysis subsequently lead to the identification of a novel paramyxovirus related to HeV. By April 1999, 265 cases of febrile encephalitis had been reported in Malaysia with 105 deaths. NiV also spread to neighbouring Singapore via infected pigs being transported to an abattoir. This resulted in infection of 11 abattoir workers, with 1 fatality (Paton *et al.*, 1999; Chua *et al.*, 2000). Cessation of pig movement, culling of infected animals (over 1 million pigs), and ongoing post-outbreak serological surveillance to support quarantine measures resulted in effective control of the outbreak, with no further cases of either human or porcine infection identified to date in Malaysia (reviewed by Chua, 2010).

Since the 1998–1999 outbreak, Malaysia has reported no further cases of NiV infection, however, the virus continues to spill over and cause disease in other countries. Since 2001, human cases of NiV have occurred almost annually in Bangladesh and sporadically in neighbouring India. Case fatality rates for these later outbreaks were higher than those observed in the Malaysian outbreak (Luby *et al.*, 2009b).

The incubation period for NiV infection is similar to HeV and has been estimated at 1–2 weeks. The initial symptoms of NiV are non-specific and include headaches, fever, dizziness, and muscle pain. As the disease progresses, neurological symptoms become the dominant feature and, depending on the strain, respiratory involvement to various degrees. Respiratory involvement has been a more predominant feature of NiV infection in Bangladesh and India, occurring in approximately 60–75% of patients in Bangladesh (Chong *et al.*, 2008; Hossain *et al.*, 2008), and 51% of patients during the 2001 outbreak in India (Chadha *et al.*, 2006). Most deaths following NiV infection are due to encephalitis and severe CNS dysfunction (Goh *et al.*, 2000).

The most significant difference seen with NiV in Bangladesh and India compared with Malaysia was the transmission route. Human infections in Malaysia were almost all associated with contact with infected pigs (Tan *et al.*, 1999). In contrast, in Bangladesh, no intermediate animal host has been identified, and human-to-human transmission has been observed (Chadha *et al.*, 2006; Luby *et al.*, 2009a). Mortality rates of outbreaks in India and Bangladesh (43–100%) have also been higher than the 38.5% rate reported for Malaysia (Chua, 2003a; Chong *et al.*, 2008).

Based on the phylogenetic similarity between NiV and HeV, the initial search for a wildlife reservoir for NiV focused on bats. Investigation of bat colonies across peninsular Malaysia revealed evidence of NiV-neutralizing antibodies in both of the local pteropid species; the Island flying fox, *Pteropus hypomelanus*, and the Malaysian flying fox, *P. vampyrus*, as well as two non-pteropid fruit bats and an insectivorous bat species (Yob *et al.*, 2001; Shirai *et al.*, 2007). In 2002, NiV was also isolated from urine and a swab of partially eaten fruit collected from *P. hypomelanus* on Tioman Island, off the coast of peninsular Malaysia (Chua *et al.*, 2002).

Following the first recorded outbreaks of human NiV infection in India and Bangladesh in 2001, *P. giganteus*, a flying fox found across the Indian subcontinent, was suspected as the reservoir host for NiV in these countries. In 2003 in Naogaon, Bangladesh, the site of a human NiV outbreak two years earlier, antibodies against NiV were detected in *P. giganteus* (Hsu *et al.*, 2004). Serological evidence of NiV infection was also detected in *P. giganteus* sampled during an outbreak of human disease in Goalanda, Bangladesh, in 2004 (ICDDR, 2004). Screening of a single colony of *P. giganteus* in northern India, following the 2001 outbreak of human disease, demonstrated neutralizing antibodies to NiV in over 50% of serum samples tested (Epstein *et al.*, 2008). To date, no NiV isolate has been reported from bats in either Bangladesh or India.

4.3.3 Menangle virus (MenPV)

MenPV (Figure 4.1D) is a zoonotic paramyxovirus, first identified in a disease outbreak in pigs in 1997 at a piggery in New South Wales, Australia (Chant *et al.*, 1998; Philbey *et al.*, 2008). MenPV has been tentatively classified in the genus *Rubulavirus*. In this outbreak, pigs suffered a reproductive disease, with disease signs including increased fetal abnormalities and stillborn piglets. Virus was isolated from the lung, brain and heart of stillborn piglets using BHK21 cells. No disease was observed in postnatal animals, but high-titer neutralizing antibodies were found in adult pigs at two piggeries associated with the outbreak (Philbey *et al.*, 2008). The virus was also shown to be zoonotic, with two piggery workers with high-level exposure developing a serious influenza-type illness and rash during the outbreak. These individuals also developed neutralizing antibodies to MenPV (Chant *et al.*, 1998).

Bats were hypothesized to be the source of the MenPV outbreak. Gray-headed flying foxes (*Pteropus poliocephalus*) and little red flying foxes (*Pteropus scapulatus*) roosting near the piggery were investigated for their involvement. Using serology, MenPV-neutralizing antibodies were detected in gray-headed flying foxes, black flying foxes (*Pteropus alecto*) and spectacled flying foxes (*Pteropus conspicillatus*), but not in little red flying foxes in either pre- or post-outbreak serum samples (Philbey *et al.*, 2008). Other species were investigated, including rodents, birds, cattle, sheep, cats and a dog, and all were found to be negative.

In 2009, MenPV was isolated from a bat roost at Cedar Grove, South East Queensland, Australia (Barr *et al.*, 2012). Black flying foxes were the predominant species in this colony at the time of sampling. The virus isolated was sequenced and demonstrated a 94% nucleotide sequence identity to the virus isolated from pigs in 1997, with amino acid sequence identities of greater than 96% for all genes. This provided strong evidence supporting the original hypothesis that the outbreak of MenPV

infection in pigs and humans in 1997 was probably the result of a spillover from bats roosting near the piggery.

4.3.4 Cedar virus (CedPV)

CedPV (Figure 4.1B) is a novel paramyxovirus first isolated from bats at Cedar Grove, South East Queensland, Australia (Marsh *et al.*, 2012). This virus shares significant features with the known henipaviruses, NiV and HeV. The genome size (18 162 nucleotides) and organization is very similar to HeV and NiV, with the genome being most similar to HeV. The amino acid sequence identity for individual proteins compared to HeV ranges from 25–60% identity. Interestingly, CedPV also uses ephrin-B2 as a functional cellular receptor for entry during infection, in common with both HeV and NiV; however, CedPV cannot utilize ephrin-B3, which is also used as a receptor by the other two henipaviruses.

Unlike HeV and NiV, for which antisera raised against either one can cross neutralize the other, CedPV neutralizing sera is unable to neutralize either HeV or NiV, and vice versa. Despite this lack of cross neutralization, the nucleocapsid protein displays antigenic cross-reactivity with henipaviruses, as demonstrated by two-way staining utilizing antisera generated against nucleocapsids of CedPV and HeV.

Two striking differences were observed between CedPV and HeV/NiV. First, CedPV does not cause disease in animal models of infection. Preliminary challenge studies with CedPV in ferrets and guinea pigs, animal models both susceptible to infection and disease with the other known henipaviruses, did not result in the development of clinical signs or pathology following challenge with an equivalent dose of virus. A serial sacrifice experiment was able to confirm virus replication in ferrets, with virus replication detected by both real-time PCR and immunohistochemical staining of N antigen in tissues. Ferrets and guinea pigs also produced neutralizing antibodies following challenge.

The second notable difference between CedPV and the other henipaviruses is the lack of editing of P gene transcripts to produce a V or W protein. Paramyxoviruses edit the P gene mRNA transcript by inserting additional non-templated G residues at a site known as the editing site, resulting in proteins with identical N-terminal regions but different C terminal sequences. The additional viral proteins are called V and W proteins. For many of the paramyxoviruses, these V and W proteins have been demonstrated experimentally to antagonize the host innate immune responses, such as inhibiting the expression of IFN. The RNA editing site has the sequence AAAAGGG, and is absolutely conserved in all known HeV and NiV isolates sequenced to date, yet is absent from the CedPV P gene. Sequencing of P gene mRNA from CedPV infected cells, both by Sanger sequencing (Marsh *et al.*, 2012) and Illumina sequencing (G. Marsh, unpublished data) has failed to detect any sign of RNA editing for this gene. Therefore, CedPV is the first paramyxoviruses demonstrated to lack this editing function.

In order to better understand the pathogenicity differences between CedPV and the other henipaviruses, the ability of CedPV to inhibit the IFN pathways in human cells was investigated. HeV and NiV infection of human cells has previously been demonstrated to inhibit the expression of type I IFN (Virtue *et al.*, 2011). Following infection of HeLa cells with CedPV, significant upregulation of the IFNβ gene was observed, suggesting that CedPV lacks the ability to antagonize this pathway (Marsh *et al.*, 2012), but the role that this plays in terms of pathogenicity requires further investigation.

4.3.5 Mapuera virus (MprPV)

MprPV was isolated from the salivary glands of a healthy fruit bat (*Sturnira lilium*) captured in a tropical rain forest in Brazil in 1979. This virus was initially characterized morphologically as a paramyxovirus by electron microscopy (Zeller *et al.*, 1989). N gene sequences placed MprPV within the *Rubulavirus* genus (Henderson *et al.*, 1995). This virus has never been associated with disease in any human or animal, however experimental intracranial infections of mice were shown to be fatal (Zeller *et al.*, 1989).

4.3.6 Porcine rubulavirus (PorPV)

PorPV infection or "blue eye" disease was an emerging disease first identified in La Piedad, Michoacan, Mexico in 1980 (Stephan *et al.*, 1988). The causative agent of this disease was previously known as La-Piedad-Michoacan paramyxovirus (LPMV), with disease being characterized by encephalitis and respiratory disease in piglets, reproductive failure in adult pigs, and occasional corneal opacity in all ages (Moreno-Lopez *et al.*, 1986; Stephan *et al.*, 1988). The natural reservoir of this virus is not known, however due to its close genetic relationship with MprPV (Wang *et al*., 2007) and preliminary serological surveillance data (Salas-Rojas *et al*., 2004), it has been suggested that bats are likely reservoirs of this virus.

4.3.7 Tioman virus (TioPV)

TioPV (Figure 4.1C) was isolated from urine of the island flying fox (*Pteropus hypomelanus*) collected from Tioman Island off the eastern coast of peninsular Malaysia, while investigating the reservoir of NiV (Chua *et al.*, 2001). This virus is closely related to MenPV and has also been tentatively classified in the genus *Rubulavirus*. A sero-survey of inhabitants of Tioman Island (169 individuals) demonstrated five individuals (1.8%) had neutralizing antibodies to TioPV, suggesting previous infection of the island population with TioPV or a similar virus (Yaiw *et al.*, 2007).

Due to its close relationship with MenPV, an experimental challenge of pigs was performed (Yaiw *et al.*, 2008a). Pigs were challenged with TioPV either oronasally or subcutaneously, with some pigs developing fever, but no other clinical signs. All pigs seroconverted, producing neutralizing antibodies to TioPV. Virus could be detected in a range of tissues, particularly the tonsillar epithelium, and could be re-isolated from oral swabs. This suggested that pigs could become naturally infected with TioPV and could facilitate virus transmission to humans following contact with oral secretions.

4.3.8 Achimota viruses (AchPV)

AchPV1 and 2 are two different paramyxoviruses, which have been isolated from urine samples collected from *Eidolon helvum* bats roosting in Accra, Ghana (Baker *et al.*, 2013). These two viruses, although not closely related, cluster with the other bat-borne rubula-like viruses. Both of these viruses were able to infect Vero and PaKi cells; however, they produced different CPE and AchPV2 grew to a higher titer. No serological cross-reactivity of the AchPVs was observed with other known paramyxoviruses, as sera raised against other closely and more divergent viruses was unable to neutralize

either AchPV1 or 2. Interestingly, human sera collected in Ghana and Tanzania from both healthy and febrile humans was able to neutralize AchPV2. This suggests that AchPV2 has zoonotic potential, with either AchPV2 or a closely related virus having infected humans in the past. Whether these viruses can cause disease in humans or animals is currently unknown.

4.3.9 Tukoko viruses (ThkPV)

TuhPV 1, 2, and 3 are novel paramyxoviruses that have been detected and sequenced from *Rousettus leschenaultii* in China (Lau *et al.*, 2010). Although full genome sequences were able to be obtained from bats, these viruses were unable to be cultured in the laboratory. These sequences are closely related to the other bat-borne rubula-like viruses, MenPV and TioPV. Unfortunately, without live viruses, the potential of these viruses to cause disease in humans and animals cannot be ascertained.

4.3.10 Sosuga virus (SosPV)

In 2012, a wildlife biologist returned to the US and developed a severe acute febrile illness after spending 6 weeks in South Sudan and Uganda collecting bats and rodents from remote rural areas for ecological research (Albarino *et al.*, 2014). During the field trip, whilst wearing different levels of personal protective equipment (PPE), the biologist manipulated animals in traps and mist nets, performed dissections, collected blood and tissues, and visited caves with large bat populations. Symptoms upon hospital admission included fever, malaise, headache, generalized myalgia and arthralgia, neck stiffness, a metallic taste, sore throat, and a maculopapular rash that was present later in the infection. The biologist was discharged from hospital after 2 weeks, but considerable sequelae (myalgia, arthralgia, headache, malaise, and fatigue) persisted for several months (Albarino *et al.*, 2014).

NGS was performed on RNA extracted from blood and serum samples of the patient and metagenomic analysis revealed a novel paramyxovirus (Albarino *et al.*, 2014) most closely related to ThkPV-3, a rubula-like virus isolated from *Rousettus leschenaultii* fruit bats in southern China (Lau *et al.*, 2010). The novel paramyxovirus was provisionally named Sosuga virus in recognition of its probable geographic origin (South Sudan, Uganda). The complete genome of SosPV has been sequenced and is 15 480 nucleotides in length, conforming to the 'rule of 6' (Calain & Roux, 1993). The genome organization is typical of the rubulaviruses, and phylogenetic analysis clearly demonstrated that SosPV clusters with other bat-borne rubula-like viruses (Albarino *et al.*, 2014). Virus isolation was unsuccessful when patient blood was used to inoculate Vero-E6, Vero-SLAM, and H292 cells, but virus was detected following intracranial inoculation of suckling mice (Albarino *et al.*, 2014). The exact source SosPV remains unknown, but sequence similarity with other bat-derived rubula-like viruses, in addition to the circumstances surrounding the biologist's illness is highly suggestive of a bat origin for this new paramyxovirus.

4.3.11 Other paramyxoviruses

Globally, many groups are investigating bats as a natural reservoir of paramyxoviruses by collecting bat urine samples and testing for the presence of virus by PCR using

universal primers (Tong *et al.*, 2008). Using this approach many novel paramyxoviruses have been detected and reported from around the world, including Ghana, Zambia, Democratic Republic of Congo, Gabon, Central African Republic, Comoros, Mauritius, Madagascar, Germany, Bulgaria/Romania, Thailand, Indonesia, Costa Rica, Panama, and Brazil (Drexler *et al.*, 2009 2012; Baker *et al.*, 2012; Chintapitasakul *et al.*, 2012; Kurth *et al.*, 2012; Sasaki *et al.*, 2012; Wilkinson *et al.*, 2012; Muleya *et al.*, 2014). These viruses belong to the *Henipavirus*, *Morbillivirus*, *Rubulavirus*, and *Pneumovirus* genera. This suggests that many other novel paramyxoviruses are present in bats and we have only detected and/or isolated a small percentage of the diversity in bats. Future virus discovery projects will certainly result in the isolation of additional novel paramyxoviruses.

Mojiang virus (MojPV) is a novel henipa-like virus detected in rats (*Rattus flavipectus*) in Yunnan Province, China in 2012 (Wu *et al.*, 2014). This virus was detected in rectal swabs collected from rats in a cave. Six months previous to this, three humans visited this cave and developed a severe pneumonia. Although isolated from rats, microbats (*Rhinolophus ferrumequinum*) also reside in this cave, so it is possible that this detection in rats may represent a spillover event from bats. Full genome sequence analysis of MojPV demonstrated that the closest relatives are the henipaviruses, with the genome length (18 404 nucleotides) and gene order consistent with that observed for HeV and NiV. Amino acid sequence identity ranged from 38–63% compared to HeV and NiV. If rats are the natural reservoir of this virus it would suggest that henipaviruses have a broader natural reservoir range than just bats. The discovery of MojPV, a rodent virus with close genetic relatedness to a bat henipa-like viruses, warrants further surveillance to locate this or other closely related viruses in bats co-localized with the rodent population in China.

4.4 RISKS, CONTROL, AND PREVENTION

4.4.1 Risk of spillover

An understanding of the epidemiology of bat paramyxoviruses is essential for determining the risk factors associated with the transmission of virus from the reservoir host to either an intermediate host or directly to humans. Some potential routes for zoonotic pathogen spillover from bats to humans are via urine, feces, saliva, and during hunting and preparation of bat meat for food. Viruses of the family *Paramyxoviridae* detected in bat species have been identified in fruit bats across Africa, Australia, South America, Asia, Madagascar, and Europe (Chua *et al.*, 2002a, b; Halpin *et al.*, 2000, 2011; Wong *et al.*, 2007; Hayman *et al.*, 2008; Drexler *et al.*, 2009; Lau *et al.*, 2010) and bats frequently exist in close proximity to large human settlements and farming areas. Knowledge of virus diversity and distribution among fruit bats in different regions is required to determine factors triggering spillover events and routes of transmission to and among human populations.

For example, to reduce the risk of NiV infection in pigs, good farm management is essential, with regular monitoring of herd health and early recognition of disease syndromes, clearly defined protocols for introducing new stock, and an ongoing disease surveillance program (Field *et al.*, 2004). Fruit trees or orchards can be removed from

the immediate vicinity of pig sheds, wire screening of open-sided pig sheds to prevent fruit bat access, and ensuring that roof run-off does not enter pig pens are additional measures that may be taken to prevent the interaction of bats and pigs (Mackenzie *et al.*, 2003; Field *et al.*, 2004). In the case of HeV, a high level of awareness and preparedness should be maintained by veterinarians and horse handlers when attending to horses with respiratory or neurological disease. Until HeV is diagnostically excluded, extreme care should be exercised and nasal secretions, saliva and urine should be treated as potentially infectious. Rapid laboratory testing capability is also an important aspect of disease control and management.

The destruction of native forest habitats by humans either to clear land for agriculture or to harvest timber has greatly increased the risk of fruit bats encountering spillover hosts as they seek new roosts and alternative food sources, and thus also increased the risk of emergence of epizootic disease (Halpin *et al.*, 2007).

4.4.2 Reservoir host management

Reservoir host management requires an understanding of the host behaviour and ecology. Although bats are the reservoirs for several characterized paramyxoviruses, there are currently no active bat vaccination programs in place to prevent circulation and spread of these viruses. HeV is currently the only bat-borne paramyxovirus for which a vaccine exists, but given the many difficulties and unique challenges posed by the management of viral zoonoses in wildlife populations, it is unlikely that vaccination of bats will become a viable control measure in the near future. Population reduction as a control strategy is generally considered undesirable and inappropriate, as the culling methods used are expensive, ineffective or inhumane, and animal species may become endangered in their natural environment. Population reduction activities may also cause stress to the bats, which in turn may result in increased viral shedding, thus increasing spillover potential.

4.4.3 Vaccines

Vaccines against human measles and mumps paramyxoviruses have been available since the 1960s. In addition, several vaccine candidates for human parainfluenza virus and RSV are currently in various stages of clinical trial. Effective veterinary vaccines are available to protect pets and livestock against CDV and Newcastle disease virus. Currently, the only vaccine that exists for a bat paramyxovirus is Equivac®HeV (Zoetis, Parkville, VIC., Australia), the HeV vaccine approved for use in horses. Equivac®HeV was launched in November 2012 and is the first vaccine licenced and commercially deployed against a BSL-4 agent. The HeV subunit vaccine consists of a recombinant soluble and oligomeric form of the G glycoprotein (Bossart *et al.*, 2005). Vaccine efficacy in immunized horses was assessed against the clinical, virologic, and pathologic features of HeV infection (Marsh *et al.*, 2011; Middleton *et al.*, 2014). The formal launch of Equivac®HeV represents the culmination of many years of research where multiple studies with the vaccine were conducted in animal infection models. Studies using NiV in cats (Mungall *et al*., 2006; McEachern *et al*., 2008) and monkeys (Bossart *et al*., 2012) and HeV in ferrets (Pallister *et al*., 2011) and nonhuman primates (Mire *et al*., 2014) provided strong evidence that the HeV glycoprotein subunit-based vaccine could prevent

not only disease but also infection in animals exposed to otherwise lethal doses of either HeV or NiV. The HeV vaccine has the potential for breaking the chain of HeV transmission from bats to horses to humans, thereby protecting both horse and human health.

4.5 CONCLUSIONS

Since the discovery of HeV in fruit bats in Australia, bats have been investigated as a source of novel paramyxoviruses. In the past 20 years, many new viruses have emerged from bats, with several being associated with disease outbreaks in both humans and animals. The past several years has seen a significant increase in the detection and isolation of bat-borne paramyxoviruses, with more than 20 viruses isolated and many others detected via sequence information.

Based on past experience, paramyxoviruses will remain high on the list as a virus family to be investigated during outbreaks of new disease, particularly if bat involvement is suspected. As technologies like NGS are further developed and refined, we will continue to see an increase in full genome sequences of bat-borne paramyxoviruses. One of the challenges in characterizing viruses detected directly from bats without any association with disease will be determining the level of containment necessary for safe handling of the virus. Risk assessments are essential to estimate the potential of any novel virus to cause pandemic disease of humans or animals. Just how many paramyxoviruses are harboured by bats remains to be seen and this exciting area of research will no doubt continue to yield interesting results in the future.

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5

BAT CORONAVIRUSES

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5.1 INTRODUCTION

Bats have been documented as natural hosts of human diseases since the beginning of the 20th century (Courter, 1954). In the last 40 years, several emerging human viral infections have been linked to bats, including lyssavirus (Samaratunga *et al.*, 1998; Stantic-Pavlinic, 2005), Ebola virus (Leroy *et al.*, 2005), Hendra virus (Halpin *et al.*, 1999), and Nipah virus (Chua *et al.*, 2002). Coronaviruses were not detected in bats until 2005, just after the outbreak of severe acute respiratory syndrome (SARS) (Lau *et al.*, 2005; Li F *et al.*, 2005b; Poon *et al.*, 2005). Uncovering the origin of the SARS coronavirus led to the discovery of coronaviruses in bats. Since then a variety of coronaviruses have been identified in more than 100 bat species distributed throughout Asia, Australia, Europe, Africa, and America (Shi, 2013; Chen *et al.*, 2014) (Table 5.1). Fifty-nine full-length genomes of bat coronaviruses have been sequenced to date, and partial coronavirus genomic sequences deposited in GenBank have exceeded 600 (Chen *et al.*, 2014).

Coronaviruses belong to the subfamily *Coronavirinae*, family *Coronaviridae*, a monophyletic cluster in the order *Nidovirales* (de Groot *et al.*, 2012). The coronavirus genome has a positive-sense single-stranded RNA of 26–32 kb in size, the largest

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TABLE 5.1 Coronaviruses detected in bats

Bat family and species	Bat CoV species	Country	Genomic sequence	Reference
Vespertilionidae				
Eptesicus fuscus	Unclassified alphacoronavirus	USA	Partial	Donaldson *et al.*, 2010; Huynh *et al.*, 2012; Osborne *et al.*, 2011
Eptesicus isabellinus	Unclassified betacoronavirus	Spain	Partial	Falcon *et al.*, 2011
Eptesicus serotinus	Unclassified betacoronavirus	Italy	Partial	De Benedictis *et al.*, 2014
Eptesicus spp.	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Hypsugo savii	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011
	Unclassified betacoronavirus	Italy	Partial	Lelli *et al.*, 2013
Miniopterus africanus	Unclassified coronavirus	Kenya	Partial	Tong *et al.*, 2009
Miniopterus australis	Unclassified alphacoronavirus	Australia	Partial	unpublished
Miniopterus fuliginosus	Unclassified alphacoronavirus	Japan	Partial	Shirato *et al.*, 2012
Miniopterus inflatus	Unclassified alphacoronavirus	Kenya	Partial	Tao *et al.*, 2012; Tong *et al.*, 2009
Miniopterus magnater	Alphacoronavirus 1A	China	Full length	Chu *et al.*, 2008
	Alphacoronavirus HKU7	China	Partial	Chu *et al.*, 2006; Woo *et al.*, 2006
	Alphacoronavirus HKU8	China	Full length	Chu *et al.*, 2008; Chu *et al.*, 2006
Miniopterus natalensis	Unclassified alphacoronavirus	Kenya	Partial	Tong *et al.*, 2009
Miniopterus pusillus	Alphacoronavirus 1B	China	Full length	Chu *et al.*, 2008; Chu *et al.*, 2006; Poon *et al.*, 2005
	Alphacoronavirus HKU8	China	Partial	Woo *et al.*, 2006
Miniopterus schreibersii	Unclassified alphacoronavirus	Spain,	Partial	Falcon *et al.*, 2011
	Alpacoronavirus 1B	China	Partial	Tang *et al.*, 2006; Vijaykrishna *et al.*, 2007
	Unclassified alphacoronavirus	Bulgaria	Partial	Drexler *et al.*, 2010
	Unclassified alphacoronavirus	Australia	Partial	unpublished
Miniopterus spp.	Unclassified coronavirus	Kenya	Partial	unpublished
	Unclassified coronavirus	South Africa	Partial	Geldenhuys *et al.*, 2013
Myotis bechsteini	Unclassified alphacoronavirus	Germany	Partial	Gloza-Rausch *et al.*, 2008
Myotis blythii	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011

Myotis dasycneme	Unclassified alphacoronavirus	Germany	Partial	Gloza-Rausch *et al.*, 2008
	Unclassified betacoronavirus	Netherlands	Partial	Reusken *et al.*, 2010
Myotis daubentonii	Unclassified alphacoronavirus	Germany	Partial	Gloza-Rausch *et al.*, 2008
	Unclassified alphacoronavirus	United Kingdom	Partial	August *et al.*, 2012
	Unclassified betacoronavirus	The Netherlands	Partial	Reusken *et al.*, 2010
	Unclassified coronavirus	China	Partial	He *et al.*, 2014
	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011
	Unclassified coronavirus	Germany	Partial	Drexler *et al.*, 2010
Myotis davidii	Unclassified coronavirus	China	Partial	He *et al.*, 2014
Myotis lucifugus	Unclassified alphacoronavirus	USA	Full length	unpublished
	Unclassified alphacoronavirus	USA	Partial	Dominguez *et al.*, 2007
Myotis macropus	Unclassified alphacoronavirus	Australia	Partial	unpublished
Myotis myotis	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011
	Unclassified coronavirus	Germany	Partial	Drexler *et al.*, 2011
Myotis nattereri	Unclassified alphacoronavirus	United Kingdom	Partial	August *et al.*, 2012
Myotis ricketti	Alphacoronavirus HKU6	China	Partial	Woo *et al.*, 2006
Myotis rufus	Unclassified alphacoronavirus	Brazil	Partial	Goes *et al.*, 2013
Myotis velifer	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Myotis volans	Unclassified alphacoronavirus	USA	Partial	Osborne *et al.*, 2011
Neoromicia cf. zuluensi	Unclassified betacoronavirus	South Africa	Partial	Ithete *et al.*, 2013
Neoromicia spp.	Unclassified coronavirus	South Africa	Partial	Geldenhuys *et al.*, 2013
Nyctalus lasiopterus	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011
Nyctalus leisleri	Unclassified alphacoronavirus	Bulgaria	Partial	Drexler *et al.*, 2010
Nyctalus noctula	Unclassified betacoronavirus	Italy	Partial	Lelli *et al.*, 2013
	Unclassified betacoronavirus	Netherlands	Partial	Reusken *et al.*, 2010
Pipistrellus abramus	Betacoronavirus HKU5	China	Full length	Woo *et al.*, 2007
	Betacoronavirus HKU5	China	Partial	Lau *et al.*, 2013; Woo *et al.*, 2006
Pipistrellus kuhlii	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011; Lelli *et al.*, 2013
	Unclassified alphacoronavirus	Saudi Arabia	Partial	Memish *et al.*, 2013
	Unclassified betacoronavirus	Italy	Partial	Lelli *et al.*, 2013

(*Continued*)

TABLE 5.1. (*Continued*)

Bat family and species	Bat CoV species	Country	Genomic sequence	Reference
Pipistrellus nathusii	Unclassified betacoronavirus	Romania	Partial	Annan *et al.*, 2013
	Unclassified betacoronavirus	Ukraine	Partial	Annan *et al.*, 2013
	Unclassified alphacoronavirus	Germany	Partial	Gloza-Rausch *et al.*, 2008
Pipistrellus pipistrellus	Unclassified betacoronavirus	Netherlands	Partial	Reusken *et al.*, 2010
Pipistrellus pygmaeus	Unclassified betacoronavirus	Romania	Partial	Annan *et al.*, 2013
	Unclassified alphacoronavirus	Germany	Partial	Gloza-Rausch *et al.*, 2008
Pipistrellus spp.	Unclassified alphacoronavirus	Spain	Partial	Falcon *et al.*, 2011
Pipistrellus subflavus	Unclassified alphacoronavirus	USA	Partial	Huynh *et al.*, 2012
Scotoecus spp.	Unclassified coronavirus	Kenya	Partial	Tong *et al.*, 2009
Scotophilus kuhlii	Unclassified alphacoronavirus	Philippines	Partial	Watanabe *et al.*, 2010
	Bat alphacoronavirus 512	China	Full length	Tang *et al.*, 2006
Tylonycteris pachypus	Betacoronavirus HKU4	China	Full length genome	Lau *et al.*, 2013; Tang *et al.*, 2006; Woo *et al.*, 2007
Vespertilio superans	Unclassified bat betacov/SC2013	China	Full length	Yang *et al.*, 2014
Rhinolophidae				
Rhinolophus affinis	Bat SARS-related coronavirus	China	Full length	He *et al.*, 2014
Rhinolophus blasii	Bat SARS-related coronavirus	Bulgaria	Full length	Drexler *et al.*, 2010
	Unclassified alphacoronavirus	Bulgaria	Partial	Drexler *et al.*, 2010
Rhinolophus cornutus	Unclassified betacoronavirus	Japan	Partial	unpublished
Rhinolophus euryale	Alphacoronavirus 1B	Bulgaria	Partial	Drexler *et al.*, 2010
	Bat SARS-related coronavirus		Partial	Drexler *et al.*, 2010
Rhinolophus ferrumequinum	Unclassified alphacoronavirus	Bulgaria	Partial	Drexler *et al.*, 2010
	Bat SARS-related coronavirus	Bulgaria	Partial	Drexler *et al.*, 2010
	Bat SARS-related coronavirus	China	Full length	Li W *et al.*, 2005a; Tang *et al.*, 2006
	Unclassified alphacoronavirus	China	Partial	Tang *et al.*, 2006; Vijaykrishna *et al.*, 2007
	Unclassified alphacoronavirus	China	Partial	He *et al.*, 2014
Rhinolophus hipposideros	Unclassified coronavirus	Slovenia	Partial	Rihtaric *et al.*, 2010
	Unclassified betacoronavirus	Italy	Partial	Lelli *et al.*, 2013
	Unclassified alphacoronavirus	China	Partial	He *et al.*, 2014

Rhinolophus landeri	Unclassified coronavirus	Kenya	Partial	unpublished
Rhinolophus macrotis	Bat SARS-related coronavirus	China	Full length	Li *et al.*, 2005; Tang *et al.*, 2006
Rhinolophus megaphyllus	Unclassified alphacoronavirus	Australia	Partial	unpublished
Rhinolophus mehelyi	Bat SARS-related coronavirus	Bulgaria	Partial	Drexler *et al.*, 2010
Rhinolophus pearsonii	Bat SARS-related coronavirus	China	Full length	Li *et al.*, 2005
Rhinolophus pusillus	Bat SARS-related coronavirus	China	Full length genome	Yang *et al.*, 2013
Rhinolophus sinicus	Bat SARS-related coronavirus	China	Full length	Ge *et al.*, 2013; Lau *et al.*, 2005; Li *et al.*, 2005; Ren *et al.*, 2006; Yuan *et al.*, 2010
	Alphacoronavirus HKU2	China	Full length	Lau *et al.*, 2007; Woo *et al.*, 2006
Rhinolophus spp.	Unclassified coronavirus	Kenya	Partial	unpublished
	Unclassified coronavirus	Rwanda	Partial	unpublished
Rhinonicteris aurantia	Unclassified betacoronavirus	Australia	Partial	unpublished
Rhinopomatidae				
Rhinopoma hardwickei	Unclassified alphacoronavirus	Saudi Arabia	Partial	Memish *et al.*, 2013
Hipposideridae				
Hipposideros armiger	Unclassified alphacoronavirus	Thailand	Partial	unpublished
	Betacoronavirus HKU10	China	Partial	Ge *et al.*, 2012
Hipposideros caffer	Unclassified betacoronavirus	Gabon	Partial	unpublished
Hipposideros commersoni	Unclassified coronavirus	Kenya	Partial	Tong *et al.*, 2009
	Unclassified betacoronavirus	Nigeria	Partial	Quan *et al.*, 2010
Hipposideros diadema	Unclassified alphacoronavirus	Philippines	Partial	Tsuda *et al.*, 2012
Hipposideros larvatus	Unclassified coronavirus	Thailand	Partial	unpublished
Hipposideros pomona	Alphacoronavirus HKU10	China	Full length	Lau *et al.*, 2012
Hipposideros spp.	Unclassified alphacoronavirus	Ghana	Partial	Pfefferle *et al.*, 2009
Pteropodidae				
Cynopterus brachyotis	Unclassified betacoronavirus	Philippines	Partial	Watanabe *et al.*, 2010
Epomophorus labiatus	Unclassified coronavirus	Kenya	Partial	unpublished
Eidolon helvum	Unclassified betacoronavirus	Kenya	Partial	Tao *et al.*, 2012; Tong *et al.*, 2009
Micropteropus pusillus	Unclassified coronavirus	Central African Republic	Partial	unpublished

(*Continued*)

TABLE 5.1. (*Continued*)

Bat family and species	Bat CoV species	Country	Genomic sequence	Reference
Ptenochirus jagori	Unclassified betacoronavirus	Philippines	Partial	Tsuda *et al.*, 2012
Pteropus giganteus	Unclassified beta- and gammacoronavirus	Bangladesh	Partial	Anthony *et al.*, 2013a
Rousettus aegyptiacus	Unclassified coronavirus	Kenya	Partial	Tong *et al.*, 2009
Rousettus leschenaultii	Betacoronavirus HKU9	China	Full length	Lau *et al.*, 2010; Woo *et al.*, 2007
	Alphacoronavirus HKU10	China	Full length	Lau *et al.*, 2012
Phyllostomidae				
Anoura geoffroyi	Unclassified coronavirus	CostaRica	Partial	Corman *et al.*, 2013
Artibeus jamaicensis	Unclassified coronavirus	Panama	Partial	Corman *et al.*, 2013
	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Artibeus lituratus	Unclassified coronavirus	Panama	Partial	Corman *et al.*, 2013
	Unclassified betacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Artibeus phaeotis	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Carollia brevicauda	Unclassified coronavirus	Brazil	Partial	Corman *et al.*, 2013
Carollia perspicillata	Unclassified alphacoronavirus	Trinidad	Partial	Carrington *et al.*, 2008
	Unclassified betacoronavirus	CostaRica	Partial	Corman *et al.*, 2013
	Unclassified coronavirus	Brazil	Partial	Corman *et al.*, 2013
	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Carollia sowelli	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Glossophaga soricina	Unclassified alphacoronavirus	Trinidad	Partial	Carrington *et al.*, 2008
Lonchorhina aurita	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Phyllostomus discolor	Unclassified coronavirus	Panama	Partial	Corman *et al.*, 2013
Molossidae				
Chaerephon plicata	Bat SARS-related coronavirus	China	Full length	Yang *et al.*, 2013
Chaerephon pumilus	Unclassified alphacoronavirus	Kenya	Partial	Tong *et al.*, 2009
Chaerephon spp.	Unclassified alphacoronavirus	Kenya	Partial	Tong *et al.*, 2009
Molossus currentium	Unclassified coronavirus	Brazil	Partial	Corman *et al.*, 2013

Molossus rufus	Unclassified coronavirus	Brazil	Partial	Corman *et al.*, 2013
Mops midas	Unclassified coronavirus	South Africa	Partial	Geldenhuys *et al.*, 2013
Nyctinomops laticaudatus	Unclassified betacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Otomops martiensseni	Unclassified coronavirus	Kenya	Partial	Tong *et al.*, 2009
Tadarida brasiliensis	Unclassified alphacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Nycteridae				
Nycteris	Unclassified betacoronavirus	Ghana	Partial	Annan *et al.*, 2013
Emballonuridae				
Taphozous perforatus	Unclassified betacoronavirus	Saudi Arabia	Partial	Memish *et al.*, 2013
Mormoopidae				
Pteronotus davyi	Unclassified betacoronavirus	Mexico	Partial	Goes *et al.*, 2013
Pteronotus parnellii	Unclassified betacoronavirus	CostaRica	Partial	Corman *et al.*, 2013
	Unclassified betacoronavirus	Mexico	Partial	Anthony *et al.*, 2013b
Megadermatidae				
Cardioderma cor	Unclassified alphacoronavirus	Kenya	Partial	Tong *et al.*, 2009
Mystacinidae				
Mystacina tuberculata	Unclassified alphacoronavirus	New Zealand	Partial	Hall *et al.*, 2014

genome among the RNA viruses. The virions are 120–160 nm in diameter, spherical, and enveloped. The name of coronavirus was inspired by the equidistribution of the spike glycoproteins on the virion surface when viewed under the electron microscope giving the viral particle the appearance of the solar corona (Lai *et al.*, 2007). In the latest release of Virus Taxonomy by the International Committee on Virus Taxonomy (ICTV) in 2013, the *Coronavirinae* subfamily has four defined genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and the new genus *Deltacoronavirus* (de Groot *et al.*, 2012; ICTV, 2013). Among the classified coronavirus species, 15 belong to *Alphacoronavirus* and *Betacoronavirus* and mainly infect mammals, including humans, pigs, cats, and bats; two belong to *Gammacoronavirus* and only infect birds; while two belong to *Deltacoronavirus* and infect marine mammals. Remarkably, among the 15 classified *Alphacoronavirus* and *Betacoronavirus* species, 8 are from bats (Table 5.2). It has been suggested that bats are ideal hosts for both alphacoronaviruses and betacoronaviruses and may play an important role in the ecology and evolution of coronaviruses (Woo *et al.*, 2012).

In this chapter we provide an overview of the discovery and history of bat coronaviruses with a focus on SARS-CoV and the Middle East respiratory syndrome coronavirus (MERS-CoV), both of which resulted in pandemics. We then turn to the genetic diversity of bat coronaviruses and discuss the taxonomic position and potential risk these coronaviruses pose to humans and other animals.

5.2 HUMAN DISEASES RELATED TO BAT CORONAVIRUSES

Coronaviruses usually cause mild respiratory symptoms in humans. Prior to the outbreak of SARS only two coronaviruses were known to infect humans: hCoV-229E and hCoV-OC43 (Vetterlein & Hesse, 1965; McIntosh *et al.*, 1967). Since then, four additional coronaviruses have been discovered in human patients: SARS-CoV (Falsey & Walsh, 2003), human coronavirus hCoV-NL63 (van der Hoek *et al.*, 2004), human coronavirus hCoV-HKU1 (Woo *et al.*, 2005), and MERS-CoV (Zaki *et al.*, 2012). SARS-CoV and MERS-CoV are highly pathogenic in humans and cause severe acute respiratory distress, with a high rate of mortality. Remarkably, both viruses are believed to have originated from bats.

5.2.1 SARS

5.2.1.1 SARS and SARS-CoV SARS, also known as infectious atypical pneumonia, is a novel emerging infectious disease that caused the first global pandemic of the 21st century (Zhong *et al.*, 2003). In November 2002, the first case of SARS was recorded in Foshan city, Guangdong Province, China (Chinese SARS Molecular Epidemiology Consortium, 2004; Song *et al.*, 2005). The disease spread rapidly to Beijing, Shanxi, Hong Kong, and other Provinces and regions across China. By July 2003, SARS had spread to 28 countries throughout the globe. According to the World Health Organization (WHO) there were 8096 reported cases and 774 deaths (WHO, 2004a). In that same year a novel coronavirus called SARS-CoV was isolated and identified to be responsible for the pandemic (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003).

TABLE 5.2 Classified species and prototype strains in coronavirus genera

Species	GenBank accession No. of representative strains	Host	Reference
Genus *Alphacoronavirus*			
Alphacoronavirus 1			
Canine coronavirus	GQ477367	Dog	Herrewegh *et al.*, 1998
Feline coronavirus type I	EU186072	Cat	Tekes *et al.*, 2008
Feline coronavirus type II	AY994055	Cat	Barker *et al.*, 2013
Porcine respiratory coronavirus	DQ811787	Pig	Zhang *et al.*, 2007b
Transmissible gastroenteritis virus	AJ271965	Pig	Almazan *et al.*, 2000
Human coronavirus 229E	NC_002645	Human	Thiel *et al.*, 2001
Human coronavirus NL63	NC_005831	Human	van der Hoek *et al.*, 2004
Miniopterus bat coronavirus 1			
Miniopterus bat coronavirus 1A	NC_010437	Bat	Chu *et al.*, 2008
Miniopterus bat coronavirus 1B	NC_010436	Bat	Chu *et al.*, 2008
Miniopterus bat coronavirus HKU8	NC_010438	Bat	Chu *et al.*, 2008
Porcine epidemic diarrhea virus	NC_003436	Pig	Bridgen *et al.*, 1998
Rhinolophus bat coronavirus HKU2	NC_009988	Bat	Lau *et al.*, 2007
Scotophilus bat coronavirus 512	NC_009657	Bat	Tang *et al.*, 2006
Genus *Betacoronavirus*			
Betacoronavirus 1			
Bovine coronavirus	NC_003045	Cattle	Chouljenko *et al.*, 2001
Canine respiratory coronavirus	JX860640	Dog	Lim *et al.*, 2013
Equine coronavirus	NC_010327	Horse	Zhang *et al.*, 2007a
Humancoronavirus OC43	NC_005147	Human	Vijgen *et al.*, 2005
Dromedary camel coronavirus HKU23	KF906251	Camel	Woo *et al.*, 2014
Murine coronavirus			
Murine hepatitis virus	NC_001846	Mouse	Leparc-Goffart *et al.*, 1997
Rat coronavirus	NC_012936	Rat	Stephensen *et al.*, 1999
Human coronavirus HKU1	NC_006577	Human	Woo *et al.*, 2005
Pipistrellus bat coronavirus HKU5	NC_009020	Bat	Woo *et al.*, 2007
Rousettus bat coronavirus HKU9	NC_009021	Bat	Woo *et al.*, 2007
Tylonycteris bat coronavirus HKU4	NC_009019	Bat	Woo *et al.*, 2007
Severe acute respiratory syndrome-related coronavirus			
SARS-related human coronavirus	NC_004718	Human	Marra *et al.*, 2003
SARS-related palm civet coronavirusSZ3	AY304486	Civet	Guan *et al.*, 2003
SARS-related Rhinolophus bat coronavirus Rp3	DQ412042	Bat	Li W *et al.*, 2005a

(*Continued*)

TABLE 5.2 (*Continued*)

Species	GenBank accession No. of representative strains	Host	Reference
Genus *Deltacoronavirus*			
Bulbul coronavirus HKU11	FJ376620	Avian	Woo *et al.*, 2009
Thrush coronavirus HKU12	NC_011549	Avian	Woo *et al.*, 2009
Munia coronavirus HKU13	NC_011550	Avian	Woo *et al.*, 2009
Genus *Gammacoronavirus*			
Avian coronavirus			
Duck coronavirus	JF705860	Avian	Chen *et al.*, 2013
Infectious bronchitis virus	AY646283	Avian	Binns *et al.*, 1986
Turkey coronavirus	EU022526	Avian	Cao *et al.*, 2008
Beluga Whale coronavirus SW1	NC_010646	Whale	Mihindukulasuriya *et al.*, 2008

The overall genomic organization of SARS-CoV is similar to that of other coronaviruses. Six open reading frames (ORFs) are conserved throughout the *Coronavirnae* and arranged in the 5´ to 3´ direction: ORFs 1a and 1b, together comprising the *replicase* genes and taking approximately two-thirds of the genome, and the ORFs encoding for the structural proteins including the spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N). Between ORF1b and the structural protein genes, or within the N gene, there are eight auxiliary genes: ORF3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b which are specific to SARS-CoV (Chim *et al.*, 2003; Rota *et al.*, 2003; Ruan *et al.*, 2003; Snijder *et al.*, 2003) (Figure 5.1). By phylogenetic analysis using the conserved polymerase genes, SARS-CoV forms a distinct group within the genus *Betacoronavirus*. These distinct genomic characteristics classify SARS-CoV as a novel coronavirus species (Gorbalenya *et al.*, 2004).

5.2.1.2 Animal origins of SARS-CoV Epidemiological studies indicated that all initial SARS cases had recently had contact with animals. The search for the animal origin of SARS-CoV was first conducted in 2003 in a live animal market in Shenzhen, Guangdong Province. Serological evidence of SARS-CoV was discovered in masked palm civets (*Paguma larvata*), raccoon dogs (*Nyctereutes procyonoides*), and Chinese ferret badgers (*Melogale moschata*) (Guan *et al.*, 2003). Two full-length genomic sequences, SZ3 and SZ16, were identified from nasal swabs from masked palm civets. The genomes of SZ3 and SZ16 showed 99.8% nucleotide (nt) pairwise identities to that of human SARS-CoV strain Tor2. But a 29 nt insertion was found in ORF8 in the genome of SZ3 and SZ16, which forms a complete ORF8 instead of two split ORFs as in the human SARS-CoV. This insertion was also found in a few of the early human SARS isolates, but was completely deleted in most of the isolates of the early phase patients and all isolates of the middle and late phase patients. This breakthrough initially connected SARS-CoV to the masked palm civet (Guan *et al.*, 2003).

In succession, different research teams investigated the antibodies of SARS-CoV in both wild and domestic animal traders, market managers, and food traders in markets in

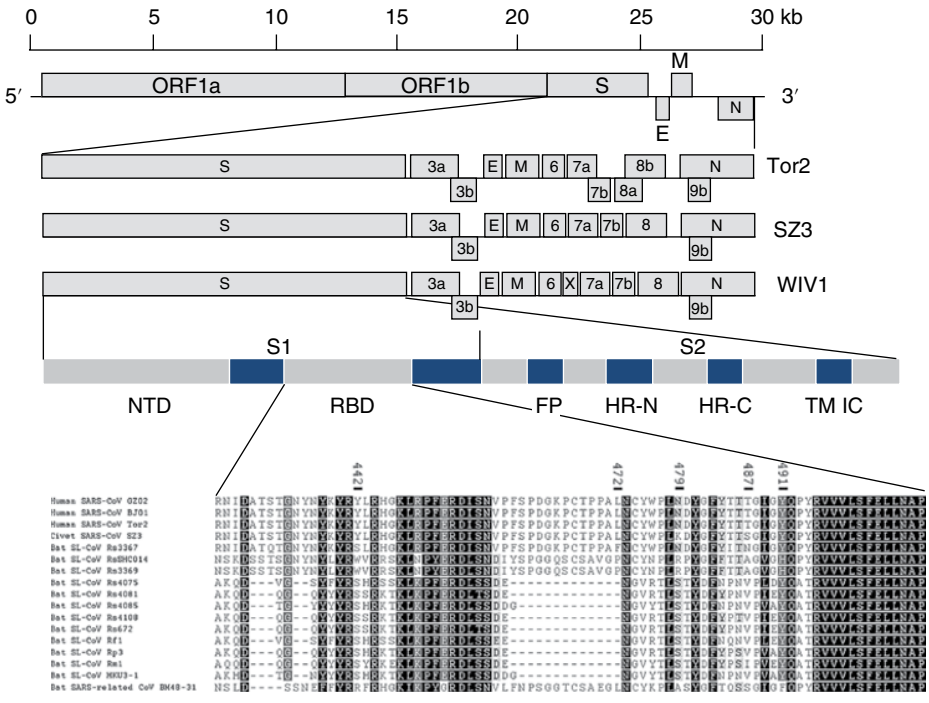

Figure 5.1 Comparison of genome organization and RBD sequences of SARS-CoVs and SL-CoVs, Genes are identified by their transcript names (1a, 1b, 3a, 3b, 6, X, 7, 7a, 7b, 8, and 9b), or the abbreviated name of their protein product (S, spike; E, envelope; M, membrane; N, nucleocapsid). Partial sequences (426–507 aa) of the RBD domain containing the key receptor binding sites (indicated by amino acid numbers) are shown. NTD, N-terminal domain; RBD, receptor binding domain; HR-N, heptad-repeat N-terminal domain; HR-C, heptad-repeat C-terminal domain; TM, transmembrane domain; IC, intracellular tail. Literature references are described in the text. GenBank accession numbers: WIV1 (bat SARS-like CoV WIV1), KF367457; Tor2 (human SARS-CoV Tor2), JX163924; SZ3 (civet SARS-CoV SZ3), AY304486; Rs3367, KC881006; RsSCH014, KC881005; Rs3369, KC880986; Rs4075, KC880993; Rs4081, KC880999; Rs4085, KC880992; Rs4108, KC881001; Rp3, DQ071615; Rs672, FJ588686; Rf1, DQ412042; Rm1, DQ412043; HKU3, DQ022305; BM48–31, NC_014470.

Shenzhen and Guangzhou. The data showed that the prevalence of antibodies in traders who dealt with masked palm civets could be as high as 72.7%, which was significantly higher than that in other human populations (Guan *et al.*, 2003; Xu *et al.*, 2004a; Xu *et al.*, 2004b). These results suggested that SARS-CoV infection was due to direct exposure to wild animals (especially masked palm civets) (Xu *et al.*, 2004a, b).

During the second stage of the SARS outbreak from 16 December 2012 to 8 January 2013, four patients in Guangzhou were diagnosed with SARS-CoV infection (Liang *et al.*, 2004). All four patients had had direct or indirect contact with wild animals or house rats before the onset of clinical symptoms. Consistently, SARS-CoV was detected by reverse transcription–polymerase chain reaction (RT-PCR) in palm civets and raccoon dogs from markets and restaurants in Guangzhou. The full-length genomic

sequences of SARS-CoV from two patients and the palm civets had 99.89% similarity (Chinese SARS Molecular Epidemiology Consortium, 2004; Kan *et al.*, 2005; Song *et al.*, 2005). These results again suggested that civets were the origin of human SARS-CoV during the 2003–2004 outbreak.

To further confirm civets as natural or reservoir hosts of SARS-CoV, Kan *et al.* (2005) investigated >1000 farmed civets in 12 Provinces in China in 2004. In contrast with the market civets in Guangdong Province during the SARS outbreak, all of the farmed civets tested negative for SARS-CoV by RT-PCR assay (Kan *et al.*, 2005). An investigation conducted in wild civets collected in Hong Kong confirmed that they were not infected by SARS-CoV (Poon *et al.*, 2005). At the same time, Wu *et al.* (2005) performed an experimental infection in civets using two human isolates of SARS-CoV, BJ01 (with the 29 nt deletion) and GZ01 (without the 29 nt deletion). All 10 inoculated civets displayed clinical symptoms such as fever, lethargy, and loss of aggressiveness (Tu *et al.*, 2004; Wu *et al.*, 2005). This demonstrated that civets were just as susceptible to SARS as humans. These findings suggested that civets may not be natural reservoir hosts of SARS-CoV after all, but rather were intermediate hosts that had facilitated transmission from its natural reservoir into humans.

5.2.1.3 Bat SARS-like CoV In 2005 two independent teams in China reported their discovery of coronaviruses closely related to human SARS-CoV in horseshoe bats. Both teams found serological and genomic evidence of SARS-like coronavirus (SL-CoV) in bat samples collected from Guangdong, Guangxi, Hubei, Tianjin, and Hong Kong. All bat SL-CoV positive samples came from *Rhinolophus* bats in the family Rhinolophidae, including *R. sinicus*, *R. pusillus*, *R. macrotis*, and *R. ferrumequinum* (Lau *et al.*, 2005; Li W *et al.*, 2005a; Ren *et al.*, 2006; Yuan *et al.*, 2010). Full-length genomic sequences of four bat SL-CoV isolates (HKU3, Rp3, Rf1, and Rm1) were identified from bat fecal samples. The genomic sequence comparison revealed that these bat SL-CoVs shared identical genomic organization and had 87–92% nt identities to human or civet SARS-CoVs. Like the coronaviruses in civets or early phase patients, bat SL-CoVs were shown to have a 29 nt insertion in ORF8. Except for the spike protein, ORF3, and ORF8, all bat SL-CoV proteins shared high amino acid sequence identities of 93–100% with their homologs in human or civet SARS-CoVs (Lau *et al.*, 2005; Li W *et al.*, 2005a; Ren *et al.*, 2006).

Subsequent investigations demonstrated that SL-CoVs were not only present throughout China, but also in Europe and Africa (Drexler *et al.*, 2010; Lau *et al.*, 2010a; Yuan *et al.*, 2010). In Europe, a high prevalence of SL-CoVs was detected in *Rhinolophus* bat species in Bulgaria, Slovenia, and Italy. These European SL-CoVs were genetically distinct from those from China. The Slovenian strains shared 85% nucleotide identity and 95.6% amino acid identity to the Chinese strain Rp3, while the Bulgarian strain BM48–31 was shown to be highly divergent from Chinese SL-CoVs in proteins encoded by ORF3b and ORF6, and lack the coding capacity for ORF8 (Drexler *et al.*, 2010; Rihtaric *et al.*, 2010; Balboni *et al.*, 2011). In Africa, betacoronaviruses related to SARS-CoV were detected in both *Hipposideros* and *Chaerephon* bats from Ghana, Kenya, and Nigeria. Compared to *Rhinolphus* SL-CoVs from Eurasia, the betacoronaviruses from African non-*Rhinolophus* bat species were much more phylogenetically distant to SARS-CoV. For example, Zaria bat coronavirus from *Hipposideros commersoni* in Nigeria possesses three overlapping ORFs between the M and N genes and two conserved stem-loop II motifs (Pfefferle *et al.*, 2009; Tong *et al.*, 2009; Quan *et al.*, 2010).

Prior to 2013, all discoveries of SL-CoVs were based on genomic evidence. No SL-CoV had been successfully isolated *in vitro*. In 2013 a breakthrough was achieved that provided the strongest evidence to date of the origin of SARS-CoV. At a single bat colony in Yunnan Province, Ge *et al*. (2013) conducted a 12-month longitudinal survey (April 2011–September 2012) of SL-CoVs in a colony of *Rhinolophus sinicus* bats. Both a high prevalence and high genetic diversity of SL-CoVs were observed. Analysis of the S protein sequences indicated the presence of seven different strains of SL-CoVs existing in the bat colony including two newly detected strains and five other strains similar to Rs672, HKU3–1, Rp3, Rf1, and Rm1, respectively (Ge *et al.*, 2013). The full-length genomes of the two novel strains (SL-CoV RsSHC014 and Rs3367) were determined by sequencing. The overall nucleotide sequence identity between SL-CoV RsSHC014, Rs3367 and human SARS-CoV genomes (Tor2 strain) was 95%, much higher than previously observed for bat SL-CoVs in China (88–92%) or Europe (76%). Higher sequence identities were also observed at the protein level between these new SL-CoVs and SARS-CoVs, particularly on the S proteins in which no deletions were observed (Figure 5.1). Most importantly, a live SL-CoV (SL-CoV WIV1) was isolated from bat fecal samples. Furthermore, SL-CoV WIV1 was demonstrated to use the same receptor as the SARS-CoV for cell entry. Serum-neutralization assays, using nine convalescent sera from human SARS patients, showed that seven of these were able to completely neutralize 100 $TCID_{50}$ (tissue culture infectious dose 50) of WIV1 at dilutions between 1:10 to 1:40, further confirming the close relationship between WIV1 and SARS-CoV. These data provided the strongest evidence to date that Chinese horseshoe bats are the natural reservoir of SARS-CoV.

5.2.1.4 Mechanisms of interspecies transmission of SARS-CoV Understanding the mechanism of how SARS-CoV was transmitted to humans is a big concern. The S protein of coronaviruses is responsible for receptor binding, fusion, and determining viral host tropism (Lai *et al.*, 2007). The S protein is a membrane-bound trimer and contains two subunits; receptor-binding subunit S1 and membrane-fusion subunit S2 (Figure 5.1). The S2 subunits from *Alphacoronvirus* and *Betacoronavirus* share sequence and structural homology and also contain homologous heptad-repeat segments that fold into a conserved trimer-of-hairpins structure essential for membrane fusion (Zheng *et al.*, 2006). The S1 subunits from *Alphacoronvirus* and *Betacoronavirus* have no obvious sequence homology. The S1 regions contain receptor-binding domains (RBD) that are sufficient for high-affinity binding to a viral host receptor (Gallagher & Buchmeier, 2001).

A metallopeptidase, angiotensin-converting enzyme 2 (ACE2) was identified as the functional receptor of the SARS-CoV (Li *et al.*, 2003). Detailed peptide mapping revealed that a fragment of 193 aa (aa 318–510) in the S protein was sufficient to bind human ACE2 (Wong *et al.*, 2004).

Based on the epidemiologic data, Li *et al*. (2005b) investigated the molecular mechanism of interspecies transmission of SARS-CoV from non-human animals to humans by using two human SARS-CoV strains isolated from the 2002–2003 (Tor2) and 2003–2004 (GZ03) SARS outbreaks, and one strain isolated from palm civets (SZ3). They found that all three S proteins bound to and utilized palm civet ACE2 efficiently, but GZ03 and SZ3 S proteins utilized human ACE2 markedly less efficiently than did the Tor2 S protein. Binding assays combining the various point mutations indicated that the

difference in binding efficiency was caused by the alteration of S protein residues 479 and 487, and the adaptation of the S protein to human ACE2 is facilitated by alteration of residue 479 to asparagine and of 487 to threonine (Li W *et al.*, 2005b; Qu *et al.*, 2005). In the second SARS outbreak (2003–2004), the individuals infected by GZ03 appeared to have less severe symptoms and no secondary transmission was observed, all of which may have been due to fewer mutations of key residues in the S proteins of GZ03 (Li W *et al.*, 2005b). The structure of SARS-CoV S protein RBD (aa 306–527) complexed with human receptor ACE2 (aa 19–615) further confirmed the above results (Li F *et al.*, 2005). In addition, Li (2008) resolved the complexed structures of RBDs from various human and civet SARS-CoV strains with a chimeric ACE2 bearing the critical N-terminal helix from civet and the remaining peptidase domain from human. The results showed that the major species barriers are determined by interactions between four ACE2 residues (residues 31, 35, 38, and 353) and two RBD residues (residues 479 and 487) (Li, 2008).

For bat SL-CoVs, Ren *et al.* (2008) analyzed the receptor usage of one SL-CoV strain (Rp3) by combining a human immunodeficiency virus-based pseudovirus system with cell lines expressing ACE2 molecules of humans, civets, or horseshoe bats (Ren *et al.*, 2008). The results demonstrated that the previously discovered SL-CoV strain Rp3 could not use ACE2 as receptor. However, the chimeric Rp3-S protein with a minimal insert region (human SARS-CoV S amino acids 310–518) gained the ability to enter cells via human ACE2 (Ren *et al.*, 2008). This result was further confirmed by a recombinant bat SL-CoV based on the HKU3 genome backbone carrying the RBD of the human SARS-CoV S in ACE2 humanized mice (Becker *et al.*, 2008). These results indicate that very few evolutionary changes (likely recombination events) may be required to confer bat SL-CoV with the ability to infect humans.

Receptor analysis of the recent SL-CoV isolate WIV1 revealed another possible route of coronavirus transmission to humans (Ge *et al*., 2013). It was demonstrated that WIV1 replicates efficiently in HeLa cells expressing ACE2 from humans, civets, or Chinese horseshoe bats and that it can grow in African green monkey kidney cells (Vero E6), human alveolar basal epithelial cells (A549), pig kidney 15 cells (PK-15), and *Rhinolophus sinicus* kidney cells (RSKT). WIV1 has high similarity in the RBD (96% identity) with human SARS-CoV. Of the two critical residues described above, residue 479 is identical (asparagine) between WIV1 and human SARS-CoV. Instead of threonine at residue 487 for SARS-CoV, WIV1 has asparagine. The ability of SL-CoV WIV1 to use the human ACE2 receptor suggests that direct bat to human infection is a plausible scenario for some bat SL-CoVs.

5.2.2 Middle East respiratory syndrome (MERS)

5.2.2.1 MERS and MERS-CoV The first human MERS case was reported on 13 June 2012 in Jeddah, Saudi Arabia. Shortly thereafter a novel coronavirus HCoV-EMC/2012 now known as MERS-CoV was isolated from a patient (Bermingham *et al.*, 2012; Zaki *et al.*, 2012; de Groot *et al.*, 2013). By 23 February 2015 a total of 1026 laboratory-confirmed cases of MERS-CoV infection, including at least 376 related deaths (WHO 2015) have been reported – about 37% mortality. Since its first documented case in Saudi Arabia, MERS-CoV infection has been reported throughout the Middle East: United Arab Emirates, Qatar, Oman, Jordan, Kuwait, Yemen, Lebanon, and Iran. Spread by travellers, the infection was reported globally in the United

Kingdom, France, Tunisia, Italy, Malaysia, Philippines, Greece, Egypt, United States, Netherlands, and Algeria. Current epidemiological data show that MERS-CoV exhibits limited human-to-human transmission (Health Protection Agency UK Novel Coronavirus Investigation team, 2013; Mailles *et al.*, 2013). Phylogenetic analysis of the *replicase* gene of coronaviruses with completely sequenced genomes showed that MERS-CoV is most closely related to two Chinese bat coronavirus species in the genus *Betacoronavirus*: *Tylonycteris bat coronavirus HKU4* (BtCoV-HKU4) and *Pipistrellus bat coronavirus HKU5* (BtCoV-HKU5) (Woo *et al.*, 2007). MERS-CoV was found to have 75% and 77% amino acid sequence identity, respectively, with BtCoV-HKU4 and BtCoV-HKU5, based on conserved *replicase* genes. According to the classification criteria of the ICTV, MERS-CoV represents a novel coronavirus species (van Boheemen *et al.*, 2012). Sequence comparison revealed that similar viral sequences to MERS-CoV have been detected in *Pipistrellus pipistrellus* in the Netherlands in 2008 and *Eptesicus isabellinus* in Spain in 2011 prior to the outbreak of MERS (Falcon *et al.*, 2011; Reusken *et al.*, 2010). MERS-CoV, HKU4, HKU5, and several similar coronaviruses from Europe cluster together as a new group within the *Betacoronavirus* genus.

Based on full-length genomic sequences, MERS-CoVs isolated from patients are phylogenetically classified into two lineages: A and B. The viral genomes detected in the earliest cases in humans (MERS-CoV and Jordan-N3/2012) fall into lineage A and are genetically distinct from the later cases (England-Qatar/2012, England2-HPA, and others) that fall into lineage B. The accumulation of genetic diversity, including changes in the S protein, suggests that the natural reservoirs of MERS-CoV are geographically widespread (Cotten *et al.*, 2013).

5.2.2.2 Animal origins of MERS-CoV Because of the phylogenetic similarities between MERS-CoV and bat coronaviruses, the search for the animal reservoir of MERS-CoV was initially focused on bats. A betacoronavirus closely related to MERS-CoV was detected in *Nycteris* bats in Ghana with a prevalence of 24.9% and clustered as a basal sister clade with MERS-CoV, HKU4, and HKU5. *Pipistrellus* bats in Romania and Ukraine were found to harbor coronaviruses which share higher homologs with MERS-CoV than HKU4 and HKU5, around 87.1 to 88.1% nucleotide identity and 98.3% amino acid identity based on a 904-bp fragment of the RdRp gene (Annan *et al.*, 2013). Another betacoronavirus was reported in *Eptesicus serotinus* from northern Italy and this strain (ITA26/384/2012) shared 96.9% amino acid identity with MERS-CoV in an 816-bp RdRp fragment (De Benedictis *et al.*, 2014). In the Western Hemisphere a MERS-related coronavirus was discovered in a *Nyctinomops laticaudatus* bat from Mexico. Its partial non-structural protein 14 (nsp14) sequence had 85.7% nucleotide identity and 95.5% amino acid identity with that of MERS-CoV (Anthony *et al.*, 2013b). Nevertheless, none of the bat betacoronaviruses mentioned above is likely to be a direct ancestor of MERS-CoV (Lau *et al.*, 2013).

Available epidemiology data indicate that some MERS patients had a history of exposure to dromedary camels and goats (Albarrak *et al.*, 2012; Buchholz *et al.*, 2013). Serological evidence against the MERS-CoV S protein revealed that Omani camels – but not European sheep, goats, cattle, and other camelids – had a high prevalence of neutralizing antibodies against MERS-CoV (Hemida *et al.*, 2013; Reusken *et al.*, 2013a, b; Alagaili *et al.*, 2014; Alexandersen *et al.*, 2014). A retrospective search for MERS-CoV antibodies indicated that the virus could be traced to as early as 1992 in the Middle East (Alagaili *et al.*, 2014; Alexandersen *et al.*, 2014; Meyer *et al.*, 2014).

Genomic sequences and viral isolation further confirmed the origin of MERS-CoV in Camels. Haagmans *et al.* (2014) first detected MERS-CoV RNA from 3 out of 14 dromedary camels from a farm in Qatar linked to two human cases in October 2013 (Haagmans *et al.*, 2014). Chu *et al.* (2014) identified MERS-CoV from nasal swab specimens in 4 out of 110 apparently healthy dromedaries in Egypt and obtained a near-full-length genome sequence of camel MERS-CoV (NRCE-HKU205), which had an overall nucleotide similarity of 99.2–99.5% to the human isolate. Unlike human MERS-CoVs, NRCE-HKU205 has 12 aa differences (residues 23, 26, 194, 434, 666, 696, 756, 886, 888, 918, 1158, and 1333) in the S protein (Chu *et al.*, 2014). In June 2014 direct evidence was provided for camel-to-human transmission of MERS-CoV when a previously healthy 43-year-old Saudi man developed respiratory symptoms after caring for ill camels, several of which had been exhibiting nasal discharge (Azhar *et al.*, 2014; Memish *et al.*, 2014). MERS-CoVs isolated from the nasal swabs of this patient and from one of the camels were almost identical (Azhar *et al.*, 2014). Phylogenetic analysis of MERS-CoV genomes obtained from human cases and camels suggests that multiple zoonotic spill-over events have occurred since the beginning of the MERS-CoV epidemic (Cotten *et al.*, 2013, 2014; Alagaili *et al.*, 2014; Briese *et al.*, 2014; Kupferschmidt, 2014). These data further suggest that MERS-CoVs have been circulating in dromedary camels for at least two decades if not longer and can be transmitted from camels to humans through close contact.

5.2.2.3 Mechanisms of interspecies transmission of MERS-CoV Viral receptor analysis is important in understanding the interspecies transmission of MERS-CoV and is helpful for antiviral drug screening and vaccine development. Soon after the MERS outbreak, a cellular molecule, dipeptidyl peptidase 4 (DPP4, also known as CD26) was identified as a functional receptor for MERS-CoV (Raj *et al.*, 2013). DPP4 is relatively conserved among mammalian species, and most cell lines derived from human, bat, non-human primate or swine were found to be susceptible to MERS-CoV infection. However, cell lines originating from mice, hamsters, dogs, and cats were not susceptible (Chan *et al.*, 2013; Raj *et al.*, 2013). DPP4 from camel, goat, cow, and sheep can be also recognized by MERS-CoV and can support MERS-CoV replication (Barlan *et al.*, 2014; van Doremalen *et al.*, 2014). These findings suggest that the MERS-CoV receptor DPP4 plays a pivotal role in the observed species tropism of MERS-CoV and may be a restriction factor for interspecies transmission of MERS-CoV.

5.3 GENETIC DIVERSITY OF BAT CORONAVIRUSES

5.3.1 Alphacoronaviruses

Alphacoronaviruses infect various mammalian species including humans, pigs, cats, and bats (Pedersen *et al.*, 1984; Kusanagi *et al.*, 1992; van der Hoek *et al.*, 2004; Chu *et al.*, 2008). Among the eight currently established species within the genus *Alphacoronavirus*, four were identified in Chinese insectivorous bats: *Miniopterus bat coronavirus 1* (1A and 1B), *Miniopterus bat coronavirus HKU8*, *Scotophilus bat coronavirus 512*, and *Rhinolophus bat coronavirus HKU2* (Tang *et al.*, 2006; Lau *et al.*, 2007; Chu *et al.*, 2008) (Table 5.1 and Figure 5.2).

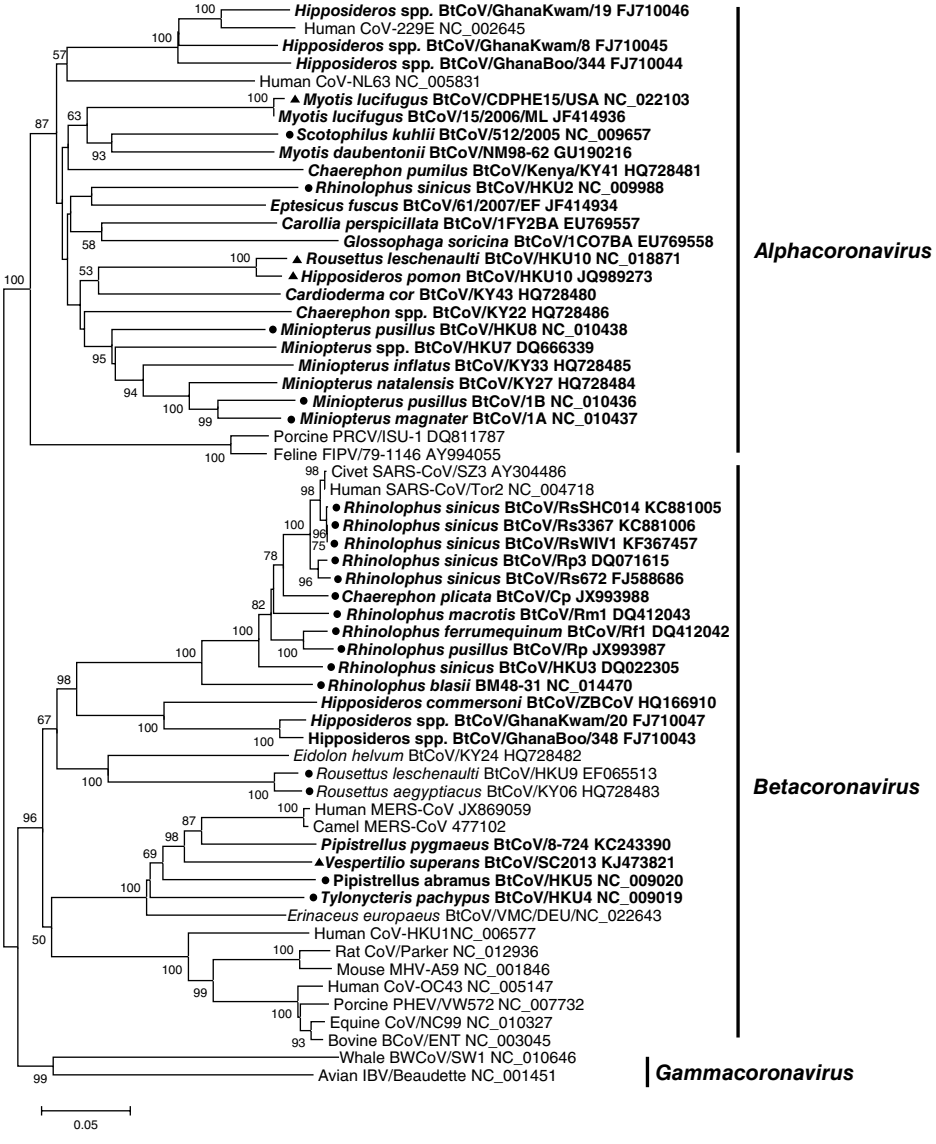

Figure 5.2 Phylogenetic analysis of coronaviruses derived from bats and other species. The phylogenetic tree was constructed based on partial RdRp sequences (816 nt). The available RdRp sequences (≥816 nt) were extracted from GenBank and aligned using ClustalW. The alignment was used for tree construction by the neighbor-joining method using MEGA (Version 5.1). Numbers above branches indicate bootstrap values calculated from 1000 bootstrap replicates (values ≥50 are shown). All the coronaviruses derived from bats are drawn in bold. The bat coronaviruses were named following bat species, plus BtCoV, strain name, and GenBank accession number. Classified bat coronavirus species are indicated by filled circles. Unclassified bat coronaviruses with full-length genomes are indicated by filled triangles.

The first bat alphacoronavirus, designated *Miniopterus bat coronavirus 1* (BtCoV 1), was reported from three different *Miniopterus* bat species in Hong Kong (Poon *et al.*, 2005). The high prevalence rate (63%) of this virus in *Miniopterus pusillus* suggests that it might be a commonly circulating coronavirus in this species in Hong Kong. In two subsequent studies targeting bats in Hong Kong, four distinct alphacoronaviruses including BtCoV 1A, BtCoV 1B, BtCoV HKU7, and BtCoV HKU8 were found in *Miniopterus* bats. These viruses are closely related genetically and are derived from a common ancestor (Chu *et al.*, 2006; Woo *et al.*, 2006). BtCoV 1A and 1B are two very close – but distinct – lineages divided from the previously reported BtCoV 1 and they have apparent host restriction to *Miniopterus magnater* and *M. pusillus*, respectively. Moreover, coinfections of BtCoV 1B and HKU8 were commonly observed among *M. pusillus* (Chu *et al.*, 2008). With the availability of full genome sequences, BtCoV 1A and 1B have been assigned to the same species within the genus *Alphacoronavirus*, known as *Miniopterus bat coronavirus 1*. BtCoV HKU8 represents another species. The co-presence of genetically diverse but related alphacoronaviruses in *Miniopterus* bats in a small geographical region suggests that alphacoronaviruses have coevolved in this genus for a long time (Chu *et al.*, 2006).

Besides *Miniopterus*, bats of other genera in China have also been demonstrated to harbor alphacoronaviruses. Genetically divergent alphacoronaviruses were found in *Myotis*, *Scotophilus*, and *Rhinolophus* bats from different locations in China. Phylogenetic analysis has revealed host species specificity among these bat coronaviruses (Tang *et al.*, 2006; Woo *et al.*, 2006; He *et al.*, 2014). Interestingly, an alphacoronavirus identified in *Rhinolophus sinicus* from Hong Kong (BtCoV HKU2) possesses a unique genetic feature compared to all other alphacoronaviruses; its spike protein contains a short peptide homologous to a corresponding peptide within the RBD of SARS-CoV S protein. This suggests that the spike protein of BtCoV HKU2 could have been acquired from SARS-CoV via recombination (Lau *et al.*, 2007). Another alphacoronavirus (BtCoV HKU10) was detected in insectivorous bats (*Hipposideros pomona*) from Hong Kong and fruigivorus bats (*Rousettus leschenaultii*) from Guangdong and potentially represents a novel alphacoronavirus species. The genome sequences of *Hipposideros* CoV HKU10 and *Rousettus* CoV HKU10 share high similarity except in the S gene. Evidence was found for a recent transmission of BtCoV HKU10 from *R. leschenaultii* to *H. pomona* and it is the first evidence for interspecies transmission of coronavirus between different suborders of bats (Lau *et al.*, 2012)

In addition to China, detection of alphacoronaviruses in bats has been reported in many other countries throughout the globe. The coronaviruses detected in *Miniopterus fuliginosus* from Japan show a close relationship to BtCoV HKU7 from *M. magnater* in Hong Kong (Shirato *et al.*, 2012). In the Philippines two alphacoronaviruses were found in *Scotophilus khulii* and *Hipposideros diadema*, respectively, and they share the highest nucleotide sequence identity of 95% and 80% respectively to the strains previously described in China in the partial RdRp gene (Tsuda *et al.*, 2012; Watanabe *et al.*, 2010). In Europe a number of alphacoronaviruses with a wide diversity and distribution were reported from multiple bat species in Spain and Germany, including *Myotis* sp., *Pipistrellus* sp., and *Nyctalus lasiopterus*. Some European bat alphacoronaviruses are related to those found in Asia while others are distinct and clustered in new monophyletic clades (Gloza-Rausch *et al.*, 2008; Drexler *et al.*, 2011; Falcon *et al.*, 2011). A great diversity of bat alphacoronavirus is also present in Africa. Three different coronaviruses

BtKY22, BtKY41, and BtKY43 were identified from *Chaerephon* and *Cardioderma* bats in Kenya. Genomic characterization suggests they are members of the genus *Alphacoronavirus*, but they are phylogenetically distant from any other bat coronaviruses and likely to represent three novel species. Additionally, viruses belonging to the established species *Miniopterus bat coronavirus 1* were detected in Kenyan bats as well (Tao *et al.*, 2012). In North America where bat species different from the Eastern Hemisphere are distributed, three clusters of alphacoronaviruses were found in *Eptesicus fuscus* and *Myotis occultus* inhabiting the Rocky Mountain region and exhibit significant dissimilarity with the alphacoronaviruses of Asian bats in the highly conserved RdRp region (Dominguez *et al.*, 2007). More recently a novel alphacoronavirus was discovered in guano of lesser short tailed bats (*Mystacina tuberculata*) on a remote offshore island in New Zealand with 80% nucleotide identity to BtCoV HKU8. Interestingly, despite the geographic and evolutionary isolation of the host species, this virus has not diverged significantly from other alphacoronaviruses (Hall *et al.*, 2014). Moreover, although most studies suggest host species restriction of bat alphacoronavirus, different bat species from the same colony have been found to harbor alphacoronaviruses of the same genetic lineage, which indicates a great complexity of the ecology of this viral genus in bats (Tang *et al.*, 2006; Falcon *et al.*, 2011).

Finally, there have been two reports of bat alphacoronaviruses closely related to human pathogenic coronaviruses. BtCoV Hipposideros/GhanaKwam/19/2008 was detected in *Hipposideros caffer ruber* in Ghana. Its RdRp fragment shares 92% nucleotide sequence identity with Human coronavirus 229E and they are predicted to share a most recent common ancestor only 200 years ago (Pfefferle *et al.*, 2009). Another bat coronavirus derived from the North American tricolored bat (*Perimyotis subflavus*) was predicted to share common ancestry with Human coronavirus strain NL63. Their most recent common ancestor was calculated to have occurred approximately 563–822 years ago (Huynh *et al.*, 2012).

In summary, alphacoronaviruses infect a wide range of different species and exhibit remarkably high genetic diversity in bats. Natural infection of different bat species with different alphacoronaviruses is globally present and bats are suggested to be an ancestral source of this coronavirus genus. More alphacoronaviruses have yet to be discovered in bats elsewhere in the near future.

5.3.2 Betacoronaviruses

Compared with bat alphacoronaviruses, bat betacoronaviruses have been identified from fewer host species and show less genetic diversity (He *et al.*, 2014). Bat betacoronaviruses are distributed among three of the four betacoronavirus lineages. *Betacoronavirus* group B contains diverse SARS-like bat coronaviruses while group C betacoronaviruses include diverse MERS-related bat coronaviruses. These viruses have already been discussed above.

The other bat-associated betacoronavirus species, *Rousettus bat coronavirus HKU9*, is currently the sole species belonging to *Betacoronavirus* group D. BtCoV HKU9 was first discovered in *R. leschenaultii* bats in Guangdong Province in China. Complete genome sequencing of four BtHKU9 strains revealed a marked nucleotide and amino acid sequence polymorphism among isolates (Woo *et al.*, 2012). Interestingly, the same bat could be coinfected with two or three distinct genotypes of

BtCoV HKU9. The presence of diverse genotypes of BtCoV HKU9 in *R. leschenaultii* bats is likely due to a combination of mutation and recombination that may have been facilitated by dense roosting behavior and long range foraging of this particular bat species (Lau *et al.*, 2010b). Additionally, BtCoV HKU9 strains were detected in *Hipposideros* sp. samples collected in Yunnan Province (Ge *et al.*, 2012). The sequence variation between different strains is consistent with what was found in *R. leschenaultii* in Guangdong and further demonstrates the genetic diversity of BtCoV HKU9 in bat populations.

Although they are not as abundant or diverse as bat alphacoronaviruses, studies of the distribution, genetic diversity, and evolution of bat betacoronaviruses are of special importance, since many pose potential threats to human health. It is highly likely that additional betacoronaviruses will be identified in bats. The huge diversity of alphacoronaviruses and betacoronaviruses in bats supports the hypothesis that bats are ideal hosts for these viruses and fuel the evolution and dissemination of these two genera (Woo *et al.*, 2012).

5.3.3 Gammacoronaviruses

Currently, the sole recorded bat gamacoronavirus (PgCoV-4) was found in one Indian bat (*Pteropus giganteus*) in Bangladesh (Anthony *et al.*, 2013a). The sequence of a partial RdRp fragment (294 nt) of PgCoV-4 is close to avian infectious bronchitis virus (IBV) with 92% nt identity and 98% aa identity, respectively. Phylogenetically, PgCoV-4 closely clusters with IBV and falls into the genus *Gammacoronavirus*.

5.3.4 Classification of coronaviruses

According to the ICTV criteria for classification of coronaviruses, only viruses with complete genome sequences are considered for taxonomy. This standard compares the pair-wise evolutionary distances using coronavirus family-wide conserved domains in the replicase polyprotein pp1ab, which consists of seven peptide subunits: nsp3, nsp5, nsp12 (RdRp), nsp13, nsp14, nsp15, and nsp16. Viruses sharing less than 90% sequence identity in the conserved replicase domains with any other established member of the family may be considered representatives of a new species. Viruses sharing less than 46% sequence identity in the aforementioned conserved replicase domains with any other established members of the family may be considered representatives of a new genus (de Groot *et al.*, 2012). Under these criteria, 59 bat coronaviruses with full-length genome sequences were divided into eight established species, including *Miniopterus bat coronavirus 1* (2 genomes, 1A and 1B), *Rhinolophus bat coronavirus HKU2* (4 genomes), *Miniopterus bat coronavirus HKU8* (one genome), *Scotophilus bat coronavirus 512* (one genome), *Tylonycteris bat coronavirus HKU4* (5 genomes), *Pipistrellus bat coronavirus HKU5* (4 genomes), *Rousettus bat coronavirus HKU9* (8 genomes), and *Severe acute respiratory syndrome-related coronavirus* (25 genomes, HKU3, Rp3, WIV1, and etc), and 2 unassigned species including *Bat coronavirus HKU10* (8 genomes) and *Bat coronavirus CDPHE15/USA/2006* (one genome) (Table 5.1). For investigation of bat coronaviruses, most research is based on PCR assays targeting the conserved RdRp fragment. Usually, the PCR amplicon sizes range from 121 nt to 440 nt

(Tong *et al.*, 2009; Anthony *et al.*, 2013b; Memish *et al.*, 2013). Based on these RdRp fragments, these viruses may be roughly assigned to a new species or a new genus. Complementary to this, Drexler *et al*. (2010) developed RdRp-based grouping units (RGU) for coronaviruses by comparing amino acid identities translated from an 816-bp fragment of the RdRp. Criteria for defining separate RGU in mammalian CoV were greater than 4.8% amino acid distance for alphacoronaviruses and greater than 6.3% distance for betacoronaviruses. Recently, human MERS-CoV and *Pipistrellus* bat HKU5 were found to have only 5.1% difference in the RGU motif. Yet, MERS-CoV and HKU5 are clearly two distinct species. For accommodating these situations, Drexler *et al*. (2014) revised the RGU threshold for betacoronaviruses to 5.1%. In addition to the above full-length or partial genomic sequences, there are hundreds of additional partial RdRp sequences deposited in GenBank. These sequences are shorter than 816 nt and remain as yet unclassified.

5.4 CONCLUSIONS

A number of conclusions can be drawn based on our current knowledge of bat coronaviruses:

1. Bats carry a great genetic diversity of coronaviruses and are probably the natural and ancestral reservoirs of alphacoronaviruses and betacoronaviruses. Some of these viruses have evolved to infect other species.
2. Diverse SL-CoVs are circulating in horseshoe bats and one of them (the prototype of SARS-CoV) was transmitted to civets through S gene mutations, resulting in the 2002–2003 SARS pandemic. Some bat SL-CoVs such as WIV1 can use the cellular ACE2 receptors of humans, civets, and bats, suggesting the potential for direct transmission from bats to other animals including humans.
3. Bats are likely natural reservoirs of MERS-CoV or an ancestral MERS-like CoV. Future investigation of MERS-CoV and its origins should focus on bats of the following families: Molossidae, Vespertilionidae, Nycteridae, and Emballonuridae.
4. From 55 published articles on bat coronaviruses at the time of preparation for this book chapter, more than 102 bat species from around the world have been shown to carry coronaviruses (Chen *et al.*, 2014). Currently, eight bat coronaviruses have been classified as species, but more than one hundred bat coronaviruses (or strains) have not yet been classified. However, as there are more than 1200 bat species in the world, large numbers of new bat coronaviruses likely await discovery.
5. Bat coronaviruses have not been fully discovered due to the diversity of bats across the globe. Through the lesson of SARS and MERS, the transmission of animal coronaviruses to humans can be expected to continue. To address public health concerns regarding coronaviruses, strategies should be properly developed for rapid diagnosis and evaluation of the ability for cross-species transmission of animal, and particularly bat, coronaviruses.

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6

BAT FILOVIRUSES

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6.1 INTRODUCTION

Marburgvirus (MARV) and *Ebolavirus*, the only two genera in the family *Filoviridae* of the order *Mononegavirales*, are enveloped viruses with non-segmented, negative-sense, single-stranded RNA genomes (Sanchez *et al*., 2007; Easton & Pringle, 2012). These viruses are the causative agents of rapidly progressive hemorrhagic fevers, with high mortality rates among humans and non-human primates in Africa (Leroy *et al*., 2004).

To date, seven species of filoviruses have been identified and classified (Kuhn *et al*., 2013). Specifically, the genus *Ebolavirus* is composed of five recognized species: *Tai Forest ebolavirus* (TAFV), *Reston ebolavirus* (RESTV), *Sudan ebolavirus* (SUDV), *Ebola virus* (EBOV) (formely Zaïre ebolavirus:ZEBOV), and *Bundibugyo ebolavirus* (BDBV). The genus *Marburgvirus* consists of only one species, *Marburg margburgvirus*

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(formerly referred to as *Lake Victoria marburgvirus*) (Kuhn *et al*., 2011; Adams *et al*., 2012), which includes two strains characterized by approximately 20% genetic divergence: *Marburgvirus* (MARV) and *Ravn virus* (RAVV). Additionally, a tentative genus "*Cuevavirus*", composed of a single species called *Lloviu virus* (LLOV) has been proposed (Kuhn *et al*., 2010).

Since their discovery 45 years ago, filoviruses have caused only a small number of documented outbreaks in humans, yet have gained a severe reputation due to their extreme virulence and high mortality rate in humans. In this chapter, we present an overview of current knowledge on the epidemiology and ecology of bat filoviruses and finally describe briefly the different techniques currently used to identify and characterize these viruses.

6.2 *MARBURGVIRUS* OUTBREAKS

The first recognized filovirus, MARV, was discovered in 1967 after the accidental infection of laboratory workers who were working in close contact with green monkeys (*Cercopithecus aethiops*) imported from Entebbe (Uganda) to facilities in Frankfurt, Germany (six cases, two fatalities), Marburg, Germany (24 cases, 5 fatalities), and Belgrade in the former Yugoslavia (two cases, zero fatalities) (Stile *et al*., 1968). Between the initial discovery and the late 1990s, sporadic cases were reported in South Africa (Johannesburg) in 1975 (three cases, one fatality) and Kenya (Nairobi) in 1980 (two cases, one fatality) and 1987 (one case, one fatality) (Gear *et al*., 1975; Smith *et al*., 1982; Johnson *et al*., 1996). In addition, two laboratory infections were reported in Russia in 1988 and 1990, one of which had a fatal outcome (Kuhn, 2008; Nikiforov *et al*., 1994). Thereafter, MARV caused two major outbreaks in Democratic Republic of Congo (DRC) in 1998–2000 and then in Angola in 2004–2005. A large outbreak occurred in the neighboring rural locations of Durba and Watsa (eastern DRC) between 1998 and 2000, affecting 154 people and causing 128 fatalities (Bausch *et al*., 2006). Most patients worked in gold mining in Goroumbwa cave. The Durba and Watsa outbreaks were characterized by the circulation of several strains, highlighting multiple independent introductions from an unknown natural reservoir (Bausch *et al*., 2003, 2006; Colebunders *et al*., 2007). The largest recorded MARV outbreak to date took place in Angola in 2004–2005, causing 252 cases and 227 fatalities (Towner *et al*., 2006). Between 2007 and 2008, three small outbreaks occurred in western Uganda, in Kamwenge and Ibanda districts, among gold miners in Kitaka cave (Adjemian *et al*., 2011), with a case fatality rate (CFR) of 25%. More recently, outbreaks of Marburg hemorrhagic fever (MHF) were reported in early September and in November 2012 in Ibanda and Kabale districts, and 14 cases including 7 fatalities were registered (CFR of 50%) (Mbonye *et al.,* 2012). Finally, the two most recent human cases reported to date affected two tourists, an American and a Dutch, who visited Python Cave in the Maramagambo forest in Uganda, known to harbor large bat colonies (Timen *et al*., 2009).

With a total number of 462 cases and 375 fatalities (CFR 81%), MARV represents a significant threat to global public health (Slenczka & Klenk, 2007).

6.3 *EBOLAVIRUS* OUTBREAKS

6.3.1 *Ebolavirus* and *Sudan ebolavirus*

Ebolavirus first emerged in the form of two almost simultaneous outbreaks in Sudan and the DRC in 1976. Each outbreak was caused by a distinct species of *Ebolavirus*, namely *Sudan Ebola virus* (SUDV) and *Ebolavirus* (EBOV) (Smith, 1978; Johnson, 1978).

In Sudan, the outbreak was centered in the cities of Nzara and Maridi and caused 152 fatalities from 284 cases. In DRC, the epicenter of the outbreak was Yambuku. This outbreak resulted in 280 fatalities from 318 registered cases. Later in 1977, one fatal case was reported to Tandala, DRC. The analysis resulted in the isolation of EBOV (Heymann *et al*., 1980). Another outbreak caused by SUDV happened in 1979, once again in Nzara and Maridi, with 34 cases and 22 fatalities (Baron *et al*., 1983). Thereafter, EBOV re-emerged in 1995 and a large outbreak occurred in and around Kikwit in the south of DRC. This outbreak caused 255 fatalities among 315 cases (Khan *et al*., 1999).

Three other outbreaks from EBOV occurred in northeast Gabon between 1994 and 1997 (Amblard *et al*., 1997; Gorges-Courbot *et al*., 1997a, b; Georges *et al*., 1999), causing a total of 143 cases including 97 fatalities. The period 2000–2008 was marked by repeated outbreaks of EBOV and the resurgence of SUDV. Between 2001 and 2005, Gabon and Republic of Congo (RC) have been affected by five outbreaks of EBOV (Leroy *et al*., 2002, 2004; Pourrut *et al*., 2005; Formenty *et al*., 2006) leading to 392 cases and 339 fatalities. Twelve years after the Kikwit Ebola outbreak in 1995, an Ebola haemorrhagic fever (EHF) outbreak in the Occidental Kasaï province of DRC between May and November 2007, led to more than 260 cases and caused 186 fatalities (CFR 71,5%) (Leroy *et al*., 2009).

The most recent outbreak in Guinea began in February of 2014 and has become the largest Ebola outbreak in history, with cases still occurring at the time of writing. Since the beginning, new cases and deaths attributable to Ebola virus disease continue to be reported by the Ministries of Health in Six West African countries: Guinea, Liberia, Sierra Leone, Nigeria, Senegal and more recently Mali. According to the latest World Health Organization (WHO) updates, the cumulative number of cases attributed to Ebola virus disease in the six countries stands at 25 207, including 10 459 fatalities (Guinea: 3492 cases and 2314 fatalities, Liberia: 9712 cases and 4332 fatalities, Sierra Leone: 11 974 cases and 3799 fatalities, Nigeria: 20 cases and 8 fatalities, Senegal: 1, nonfatal case, Mali: 8 cases and 6 fatalities). The Ebola outbreak in Guinea was likely caused by an EBOV lineage that has spread from Central Africa into Guinea and West Africa in recent decades, and does not represent the emergence of a new divergent and endemic virus (Dudas & Rambaut, 2014).

Seven years after the outbreak of EBOV in the Occidental Kasaï province of DRC in 2007, EBOV reemerged in August 2014 in the DRC in the province of Equateur. This outbreak is ongoing and separate from the West African outbreak (the viral strain being closely related to the strain responsible for the outbreak of Ebola in Kikwit in 1995), and has to date caused 62 cases and 35 deaths according to the WHO.

Four outbreaks of SUDV have occurred over the past 12 years. Indeed, SEBOV re-emerged between August 2000 and January 2001 in Uganda, affecting mainly the

town and district of Gulu. This outbreak caused 425 cases and 173 deaths (Okware *et al*., 2002; Lamunu *et al*., 2004). In 2004 an outbreak in the Yambio district of Sudan led to 17 cases and 7 deaths (Towner *et al*., 2004; World Health Organization, 2004; Onyango *et al*., 2007). In May 2011, a case of EHF was reported in a 12-year-old girl from Luweero district, central Uganda (Shoemaker *et al*., 2012). In 2012, two outbreaks of SUDV were reported in Uganda; first, in July-August 2012 in the Kibaale district, 24 cases and 17 fatalities were reported; while in November 2012, in the Luweero and Kampala districts, 10 cases and 5 fatalities were reported (World Health Organization, 2012).

6.3.2 *Tai Forest* and *Bundibugyo ebolaviruses*

Twenty-five years after the initial outbreak of SUDV in 1979, Ebola virus disease reappeared in November 1994. This new outbreak was characterized by the emergence of a new species, *Tai Forest ebolavirus* (TAFV) which caused a single case in a Swiss ethologist who fell ill a few days after performing a necropsy on a chimpanzee (*Pan troglodytes*) found dead in the Tai National Park, south-western Ivory Coast (Le Guenno *et al*., 1995, 1999; Formenty *et al*., 1999). Moreover, the outbreak of 2007–2008 in Uganda (Bundibugyo district) highlighted yet another new species of *Ebolavirus*, named *Bundibugyo ebolavirus* (BDBV). This outbreak caused 116 cases and 39 fatalities (CFR 34%) (Wamala *et al*., 2010). This strain re-emerged again in August 2012, from Isiro and Viadana in Orientale Province, eastern DRC, causing 52 cases and 25 fatalities.

6.3.3 *Reston ebolavirus*

An additional species of Ebolavirus, *Reston ebolavirus* (RESTV) was first isolated in 1989 from *Cynomolgus* monkeys (*Macaca fascicularis*) imported from the Philippines and housed in a quarantine facility in Reston, Virginia (United States). These monkeys developed hemorrhagic disease associated with high mortality. Thereafter, subsequent detections in captive *Cynomolgus* monkeys have been reported in Texas in the United States, Italy and the Philippines (Jahrling *et al*., 1990; Dalgard *et al*., 1992; Hayes *et al*., 1992; Rollin *et al*., 1999). Although no disease was observed in humans during these episodes, several animal care staff in the United States and the Philippines showed seroconversion (Miranda *et al*., 1991; Miranda *et al*., 1999). These findings are supported by the detection of IgG antibodies to RESTV in pig farm workers and pig slaughterhouse workers thought to have come in contact with sick pigs in the Philippines in 2009 (Miranda & Miranda, 2011), suggesting a possible pig-to-human transmission.

6.4 FILOVIRUSES IN YINPTEROCHIROPTERAN BATS

For more than 20 years following the initial outbreaks, efforts to identify natural reservoir hosts and/or vectors of filoviruses were unsuccessful (Breman *et al*., 1999; Leirs *et al*., 1999; Monath, 1999; Reiter *et al*., 1999). Rodents (Morvan *et al*., 1999) and bats (Arata & Johnson, 1978) had long been considered potential reservoir hosts, however firm evidence was sorely lacking. In the mid 1990s, experimental studies on plants and animals in Africa resulted in the infection of both insectivorous and frugivorous bats by

EBOV (Swanepoel *et al*., 1996). Finally, in 2005 the first conclusive evidence of the presence of filoviruses in wild bats was obtained (Leroy *et al*., 2005). Several species of African, Asian, and European bats, including representatives from both major bat lineages, have now been identified as probable reservoir hosts for filoviruses (Table 6.1) (Leroy *et al*., 2005; Pourrut *et al*., 2009; Towner *et al*., 2007; Maganga *et al*., 2011; Negredo *et al*., 2011).

6.4.1 Ebolaviruses

The first evidence of the presence of EBOV among naturally infected fruit bats was provided by the detection of viral RNA and antibodies in three species of fruit bats collected in Gabon and RC (*Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata*) (Leroy *et al*., 2005). Subsequently, in other studies, EBOV-specific immunoglobulin G (IgG) were found in *Micropteropus pusillus* (4/197), *Myonycteris torquata* (19/573), *Epomops franqueti* (36/805), *Hypsignathus monstrosus* (9/125), and *Rousettus aegyptiacus* (24/307) (Pourrut *et al*., 2007, 2009), and in one *Eidolon helvum* species captured in Accra in 2008 (Hayman *et al*., 2010). Recently, anti-EBOV antibodies were once again detected in sera from *Epomops franqueti* and *Hypsignathus monstrosus* in addition to *Epomophorus gambianus* species in Ghana (Hayman *et al*., 2012). Finally, Olival *et al*. (2013) detected anti-EBOV antibodies and EBOV antigen in *Rousettus leschenaultii* (5/141) captured in Bangladesh in 2010–2011.

Miranda and Miranda (2011) argued that the detection of RESTV in monkeys and its isolation in pigs in the Philippines (Barrette *et al*., 2009) signifies the ability of RESTV to infect multiple species. Indeed, although viral RNA of RESTV has never been detected in bats, serological evidence of infection in bats has been provided. Taniguchi *et al*. (2011) detected RESTV-reactive antibodies in *Rousettus amplexicaudatus* bats captured in 2008–2009 in the Philippines, at the sites near areas where *Cynomolgus* monkeys and swine have been found infected. In addition, in their study, Hayman *et al*. (2012) found serological markers of RESTV in *Epomophorus gambianus*, *Epomops franqueti*, and *Hypsignathus monstrosus* in Ghana. Finally, Yuan *et al*. (2012) found serological evidence of RESTV infection in several species of bats in China: *Cynopterus sphinx* (2/2), *Hipposideros pomona* (3/39), *Hipposideros* spp., *Rhinilophus affinis* (1/69), and *Rousettus leschenaultii* (11/126).

Moreover, anti-ebolavirus antibodies have been found in *Nannonycteris veldkampii* (1/4) in Ghana (Hayman *et al*., 2012). In this same study, a case of seropositivity with both RESTV and EBOV was reported in one individual of the *E. gambianus* species.

6.4.2 *Marburgvirus*

Based on the discovery of EBOV in fruit bats in Gabon and RC (Leroy *et al*., 2005), and on epidemiologic linkage of MHF cases to a gold mine containing sizeable numbers of bats during a large MHF outbreak in Durba, DRC in 2000 (Bausch *et al*., 2003, 2006), Towner *et al*. (2007) screened a wide variety of bats species and reported MARV infection in the Egyptian fruit bat (*Rousettus aegyptiacus*) by the presence of virus-specific RNA and antibody. Thereafter, MARV-specific RNA and antibodies were detected once again in *Rousettus aegyptiacus* bats in DRC and Kenya, but also in an insectivorous bat *Rhinolophus eloquens* in DRC (Swanepoel *et al*., 2007; Kuzmin *et al*., 2010). Finally,

TABLE 6.1 Bat genus and species found naturally infected by filoviruses (PCR and/or serology)

Bat suborder	Bat species	Detection method	Filovirus species	Country	References
Yinpterochitoptera	*Rousettus aegyptiacus*	Serology, PCR, Isolation	*Marburg marburgvirus*	Gabon, DRC, Kenya, Uganda	Towner *et al*., 2007
	Rhinolophus eloquens				Swanepoel *et al*., 2007
	Hipposideros spp.	Serology, PCR			Kuzmin *et al*., 2010
	Hypsignathus monstrosus	Serology			Towner *et al*., 2009
	Epomops franqueti	Serology			Maganga *et al*., 2011
	Micropteropus pusillus	Serology			
	Eidolon helvum	Serology	*Zaire ebolavirus*	Gabon, RC, Ghana, Bangladesh	Leroy *et al*., 2005
	Epomophorus gambianus	Serology			Pourrut *et al*., 2007
	Epomops franqueti	Serology, PCR			Pourrut *et al*., 2009
	Hypsignathus monstrosus	Serology, PCR			Hayman *et al*., 2010
	Micropteropus pusillus	Serology			Hayman *et al*., 2012
	Myonycteris torquata	Serology, PCR			Olival *et al*., 2013
	Rousettus aegyptiacus	Serology			
	Rousettus leschenaultii	Serology			
	Cynopterus sphinx	Serology	*Reston ebolavirus*	Philippines, Ghana, China	Taniguchi *et al*., 2011
	Epomophorus gambianus	Serology			Hayman *et al*., 2012
	Epomops franqueti	Serology			Yuan *et al*., 2012
	Hipposideros Pomona	Serology			
	Hipposideros spp.	Serology			
	Hypsignathus monstrosus	Serology			
	Rhinolophus affinis	Serology			
	Rousettus amplexicaudatus	Serology			
	Rousettus leschenaultia	Serology			
Yangochiroptera	*Miniopterus inflatus*	Serology, PCR	*Marburg marburgvirus*	DRC	Swanepoel *et al*., 2007
	Mops condylurus	Serology	*Zaire ebolavirus*	Gabon	Pourrut *et al*.,2009
	Miniopterus schreibersii	Serology	*Reston ebolavirus*	China	Yuan *et al*., 2012
	Pipistrellus pipistrellus	Serology			
	Myotis ricketti	Serology			
	Myotis spp.	Serology			
	Scotophilus kuhli	Serology			
	Miniopterus schreibersii	PCR, HTS	*Lloviu Cuevavirus*	Spain	Negredo *et al.,* 2011

MARV was isolated from *R. aegyptiacus* bat tissues in Uganda (Towner *et al*., 2009). In this same study, MARV-specific antibodies were detected in bat sera. Finally, MARV IgG was found in bats (*Epomops franqueti*, *Hypsignathus monstrosus*, *Micropteropus pusillus*) captured in Gabon and RC between 2003 and 2008 (Pourrut *et al.,* 2009). Maganga *et al*. (2011) demonstrated that MARV is enzootic in *R. aegyptiacus* bat species in Gabon. Taken together, these findings highlighted the role of the common Egyptian fruit bat as a reservoir species for MARV. Moreover, in Uganda, Towner *et al*. (2009) detected MARV RNA in only 1 (0.2%) of 609 insectivorous bats (*Hipposideros* spp.) suggesting that infection in this species represented spillover from *R. aegyptiacus* bats.

6.5 FILOVIRUSES IN YANGOCHIROPTERA BATS

Filovirus infections have been also detected from insectivorous bats in Africa, Asia and Europe.

6.5.1 *Ebolaviruses*

Ebolavirus-specific RNA and antibodies have been detected in insectivorous bats in Africa. Indeed, EBOV-specific IgG was found in microchiropteran bats (including *Mops condylurus* among other bats species) in Gabon (Pourrut *et al*., 2009). In addition, Yuan *et al*. (2012) provided serological evidence of REBOV infection in Vespertilionidae bats in China: *Miniopterus schreibersii* (2/23), *Pipistrellus pipistrellus* (4/35), *Myotis ricketti* (4/83), *Myotis spp.* (3/118), and *Scotophilus kuhli* (1/25).

6.5.2 *Marburgvirus*

In DRC, MARV RNA was detected in one (3%) of 33 insectivorous bats (*Miniopterus inflatus*), caught in the entrances of Goroumbwa Mine (Swanepoel *et al*., 2007). No anti-MARV antibodies were detected in this individual. *Miniopterus inflatus* could be a reservoir of MARV even if the low rate of infection of this virus in this bat species might result of either: (i) The small number of individuals screened for MARV, as very few studies have investigated MARV in this species (Swanepoel *et al*., 2007; Maganga *et al*., 2011); or (ii) spillover from circulation of virus in *R. aegyptiacus* bats as these two species are known to live in close proximity in caves in some parts of Africa (DRC or Gabon for example).

6.5.3 *Cuevavirus*

The detection of nucleotide sequences and specific antibodies against filoviruses (MARV and EBOV) in bat species of the genus *Miniopterus* in DRC and China would suggest that bats in this genus could host viruses of the family *Filoviridae* (Swanepoel *et al*., 2007; Yuan *et al*., 2012). This hypothesis seems to be strengthened by the recent discovery of a new filovirus in a bat belonging to the genus *Miniopterus*. Indeed, nucleotide sequences of a novel filovirus, named *Lloviu virus* (LLOV), were detected in extracts from lung, liver, rectal swabs, or spleen of five dead *M. schreibersii* among 34

bat carcasses (25 *M. schreibersii* and 9 *Myotis myotis*) collected from Cueva del Lloviu (Lloviu cave) in Northern Spain in 2002 (Negredo *et al*., 2011).

6.6 ECOLOGICAL AND EPIDEMIOLOGICAL PATTERNS IN BATS

In this section we document geographic, ecological and epidemiological characteristics of filoviruses associated bats to understand their occurrence in their natural hosts. Since the more general discussion of bat biological features in relation to their reservoir host role will be discussed in Chapters 10 and 11, we will concentrate our discussion on the more filovirus-specific aspects.

6.6.1 An extended natural geographic distribution

The geographic distribution of filovirus disease spreads generally across the humid African forest. Peterson *et al*. (2004) predicted that filoviruses would occur across the humid rain forests of central and western Africa (for EHF) and the drier and more open areas of central and eastern Africa (for Marburg HF). Indeed, between 2005 and 2008, MARV- and EBOV-infected bats were found in six countries located in humid and drier areas of central, eastern Africa and western Africa (Gabon, RC, DRC, Uganda, Kenya, and Ghana) (Leroy *et al*., 2005; Pourrut *et al*., 2007, 2009; Swanepoel *et al*., 2007; Towner *et al*., 2009; Hayman *et al*., 2010, 2012; Kuzmin *et al*., 2010; Maganga *et al*., 2011; Hayman *et al*., 2012). While Marburg virus appears to be confined to bats in parts of Africa, since the early 2010s we seem to be seeing an extension of the range of the Ebolaviruses in Asia, with the serological evidence of bats infected with Ebola Reston, in the Philippines (Taniguchi *et al*., 2011, and Ebolavirus, in Bangladesh and China (Yuan *et al*., 2012; Olival *et al*., 2013) (Figure 6.1). These findings are consistent with the ecological niche models for EHFs, which had identified broader potential distributional areas in Southeast Asia, suggesting that similar ecological conditions to those identified in Africa exist in the Philippines, for example (Petersen *et al*., 2004).

The recent discovery of cave bats infected by *Lloviu virus* in Europe (Negredo *et al.,* 2011) is intriguing since it is completely outside of the previously described range for filoviruses, i.e. Africa or Asia (Petersen *et al*., 2004). The findings thus extend the natural geographical range of bat filoviruses (Figure 6.1).

6.6.2 Bats as drivers of filoviruses emergence and spillover?

6.6.2.1 Filovirus transmission between bats As for lyssaviruses (Banyard *et al*., 2010) and coronaviruses (Poon *et al*., 2005; Chu *et al*., 2008; Lau *et al*., 2012), it has been shown that frequent interspecies transmissions of filoviruses are possible between conspecific bats living in the same roosting sites. Indeed, evidence of interspecies transmission has been established for MARV. MARV was detected in two different species of bats, *Rousettus aegyptiacus* and *Hipposideros* sp. bats living in Kitaka cave in Uganda (Towner *et al*., 2009) and also in two other bat species, *Miniopterus inflatus* and *Rousettus aegyptiacus*, caught in Goroumbwa mine in the DRC (Swanepoel *et al*., 2007). These bat species are known to live in close proximity suggesting that transmission could occur by direct contact through biting and scratching during bat-bat

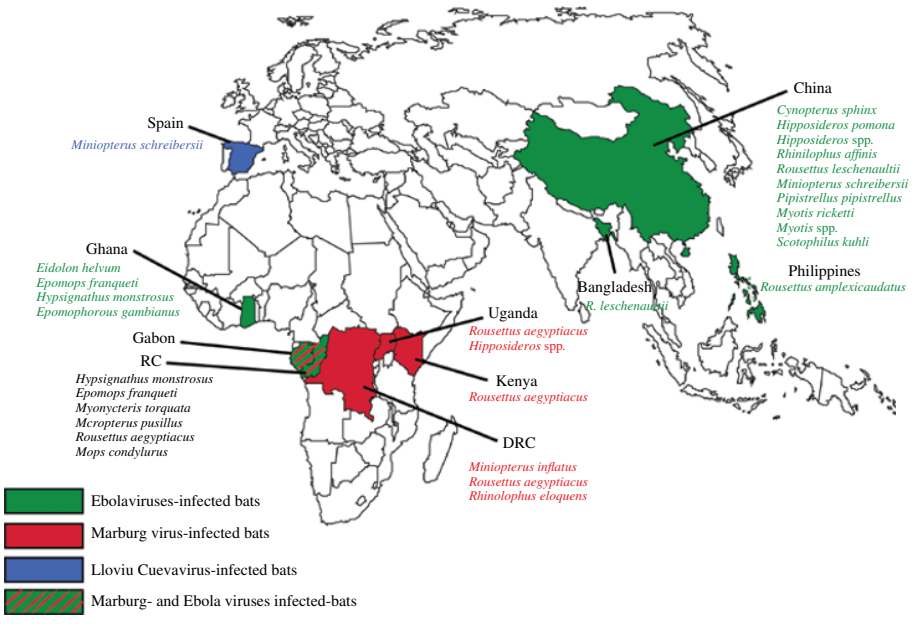

Figure 6.1 Geographic locations of bat species filoviruses-infected in the world. *(See insert for color representation of the figure).*

aggression. Otherwise, vertical transmission of MARV infection has been suspected by Towner *et al*. (2009) in *R. aegyptiacus* species, with the detection of MARV RNA in 10.3 % of juveniles tested (8/78). However, no clear evidence of this transmission pathway was found. Sexual transmission of filoviruses among bats has also been suggested together with the previous discovery of EBOV in reproductive tissue of infected humans (Rodriguez *et al.*, 1999; Rowe *et al*., 1999), the observation of active Marburg virus transmission via semen (Martini & Schmidt, 1968), and more recently the discovery of one bat with MARV-positive reproductive tissue (uterus/ovary) (Amman *et al*., 2012).

6.6.2.2 Filovirus outbreaks linked to bat exposure Numerous human outbreaks of MHF were linked to visitation in caves or mines usually inhabited with colonies of bats (Bausch *et al*., 2006). First, in 1975 MHF was developed in one of two Australian tourists who slept in rooms inhabited by insectivorous bats at two locations in Zimbabwe and had also visited the Chinhoyi caves, which are occupied by bats. Subsequently, a nursing sister who had cared for both patients also developed the disease (Conrad *et al*., 1978; Towner *et al*., 2009). In 1980 (Smith *et al*., 1982) and 1987 (Johnson *et al*., 1996), MHF was diagnosed in two patients who visited Kitum Cave on the Kenyan side of Mount Elgon, inhabited by bats. Surprisingly, although this cave and others are often frequently visited by tourists and local people, and although MARV RNA was detected in *R. aegyptiacus* bats tissues collected at Kitum Cave in July 2007 (Kuzmin *et al*., 2010), no further cases of MHF linked to this location have been reported. In 1998–2000, a small outbreak of MHF in Durba village, DRC, affected gold miners in Goroumbwa Mine where large colonies of bats roosted. This outbreak consisted of repeated occurrences of short transmission chains arising in workers

(Bausch *et al*., 2006). In 2007, in Uganda, the investigation of an MHF outbreak in miners in Kitaka cave where large numbers of insectivorous and fruit bats were present revealed that MARV strains identified in bats were identical to those detected in the miners (Towner *et al*., 2009). Finally, the two most recent human cases of MARV reported to date affecting two tourists, an American and a Dutch, who visited the Python Cave near Queen Elizabeth National Park in Uganda which is known to harbor large *R. aegyptiacus* colonies, reinforced the risk of filovirus transmission to humans who visit caves inhabited by bats (Timen *et al*., 2009).

Although no definitive proof that humans can be directly infected by bats was found, Arata & Johnson (1978) suspected the first recognized outbreak of EHF linked to bat exposure in six patients who worked in a room where bats roosted in a cotton factory in Sudan in 1976, where a large outbreak of Ebola virus occurred the same year. In addition, Leroy *et al*. (2009) showed that the outbreak in Luebo (DRC) in 2007 was linked to human exposure to the putative fruit bat reservoir during a massive arrival of migratory fruit bats to this region, strongly suggesting that humans can be directly infected by bats.

It has been speculated that the absence of extensive MARV transmission to humans may result from: (i) The small number of putative reservoirs infected at any one time; or (ii) the possibility, however slight, of rare mutations in filoviruses allowing for successful transmission from a reservoir to humans or non-human primates (Monath, 1999).

The direct transmission of filoviruses from bats to humans remains to be clearly established as well as patterns of its transmission. However, Leroy *et al*. (2009) suggested that Ebolavirus can be contracted by humans through direct exposure to blood of infected bats. Kuzmin *et al*. (2011) argued that bats could represent a spillover infection from some other source and that the identity of strains isolated from both bats and humans does not necessarily mean that humans acquired the virus from bats. We cannot exclude the possibility that both bats and humans could be independently and simultaneously infected from some other source in mines and caves.

In conclusion, although bat–human transmission has not been conclusively proven, the potential risks of filovirus spillover from bat populations should be taken into account to prevent and control the emergence and reemergence of these devastating infectious diseases.

6.6.2.3 Seasonality of filovirus infection in bats Filoviruses, as with henipaviruses and lyssaviruses, have extremely low detection frequencies, thus causing viruses to be encountered too rarely to enable the characterization of virus over time (Lehle *et al*., 2007; Pourrut *et al*., 2007; Wacharapluesadee *et al*., 2010; Turmelle *et al*., 2010). However, in the case of filoviruses, birthing and breeding seasons would be important factors involved in the dynamics of bat filoviruses infection. Indeed, Amman *et al.* (2012), argued that birthing would be linked to increased infection prevalence and ultimately spillovers for Marburg virus in bats. Amman *et al*. (2012) showed that old juveniles of the bat species *R. aegyptiacus* were most likely to be actively infected with Marburg virus. Otherwise, in the study of Pourrut *et al*. (2009), the authors showed that pregnant female bats were statistically more likely to be infected by Ebolavirus than nonpregnant females. Moreover, Plowright *et al*. (2008) found that pregnant and lactating females of the species *Pteropus scapulatus* were characterized by a greater risk of Hendra virus infection than other individuals. According to these authors, pregnancy

would contribute to altered immune responses in favor of viral replication in the organs or even the appearance of the virus in the bloodstream. These findings suggest that these seasonal periods might represent periods of heightened risk for humans due to potentially increased viral shedding.

6.6.3 Uncertainty surrounding the identification of the *Lloviu virus* reservoir

An animal reservoir can be defined as a taxon that continuously harbors higher genetic virus diversity without injury to itself and serves as a source of infection for other susceptible host species. Natural and experimental infections of bats by filoviruses do not produce any disease (Swanepoel *et al*., 1996), even if some viruses appear to cause clinical disease in wild-living bats, including lyssaviruses (McColl *et al*., 2002; Banyard *et al*., 2010). Experimental infections *in vivo* and *in vitro* have shown that Ebola virus can persist as an asymptomatic or subclinical infection in a reservoir species such as bats, with few or no transmission events, and could be activated sporadically under certain conditions of immunodeficiency (Gupta *et al*., 2004; Strong *et al*., 2008). These conditions might include pregnancy and other stresses (Strong *et al*., 2008), periods of switching foods types (Groseth *et al*., 2007) or other infections.

First, it has to be recognized that LLOV was found in the dead *M. schreibersii* bats, but not in healthy *M. schreibersii* or in bats of other species sharing the same caves (such as *M. myotis*). Second, these findings suggest that LLOV is the first filovirus that causes disease in bats, which questions the role of this species as a natural reservoir host. It is possible that *M. schreibersii* represents a spillover host and the natural reservoir could be a bat of an unknown species or another non-bat animal host. Further investigation is needed to fully understand the epidemiology and ecology of this new filovirus.

6.7 BAT FILOVIRUS CHARACTERIZATION

The identification of geographic ranges of circulation for the various filoviruses has for a long time been based solely on the observation of outbreak events. However, in order to better understand the transmission of this virus, it seems indispensable to look at its circulation in the natural environment, and specifically in animal reservoirs. For all the techniques described below, blood and tissue samples for suspected filovirus infections must be handled at biosafety level 4 (BSL-4), both in the field (Figure 6.2) and in the laboratory.

6.7.1 Filovirus isolation

Following the initial *Ebolavirus* outbreak in 1976, research of filoviruses in animal reservoirs was principally based on virus isolation. The identification of filoviruses by virus isolation is time consuming yet cost-effective, and must be conducted in a BSL-4 laboratory. Indeed, it requires several days for tissue culture and complementary tests for species characterization. Virus isolation is more reliable from blood or liver samples than from mucosal swabs or other fluids. Even though filoviruses grow well in a large variety of cell lines, the most commonly used cell lines for virus

Figure 6.2 Biosafety in the field during the collection of samples from bats potential filoviruses reservoirs. *(See insert for color representation of the figure).*

isolation and propagation of filoviruses are Vero or Vero E6 cells (*Cercopithecus aethiops*, African green monkey kidney).

6.7.2 Filovirus RNA detection

Currently, the most widely applied test is based on the detection of viral RNA in tissues and fluids by reverse transcription–polymerase chain reaction (RT-PCR) assay. This technique has been adapted to the field and for animal screening (Towner *et al*., 2004; Pourrut *et al*., 2009). Initially, conventional RT-PCR assays required confirmation by sequencing of PCR amplicons. Nowadays, real time RT-PCR assays have been implemented for filovirus detection and species identification (Drosten *et al*., 2002; Gibb *et al*., 2001; Huang *et al*., 2012), and are superior due to their higher sensitivity and rapid acquisition of results. Typically, these molecular assays target the nucleoprotein gene (Ogawa *et al*., 2011; Huang *et al*., 2012).

6.7.3 Filovirus antigen detection

The detection of viral antigen by enzyme-linked immunosorbent assay (ELISA) is still used as a diagnostic assay for EBOV and MARV infections. This test is reasonably sensitive, highly specific and can be easily conducted without extensive specialized equipment. Quantification by immunocapture ELISA technology has been tested and applied on sera collected from several animal species (including bats) to test for the presence of anti-EBOV IgG (Morvan *et al*., 1999; Leroy *et al*., 2005).

6.7.4 Whole genome amplification

The *Ebolavirus* and *Marburgvirus* genomes are about 19 000 nucleotides long and are transcribed into eight major subgenomic mRNAs. These mRNAs encode seven structural proteins: nucleoprotein (NP), virion protein 35 (VP35), VP40, glycoprotein (GP), VP30, VP24, and RNA-dependent RNA polymerase (L), as well as two nonstructural

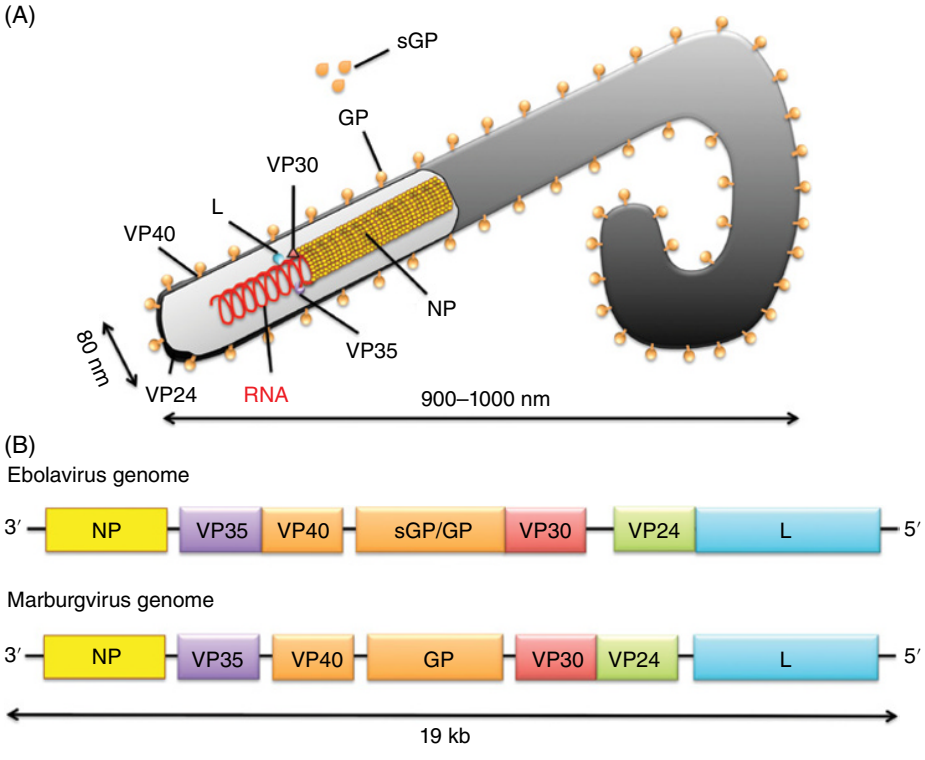

Figure 6.3 (A) A schematic illustration of a filovirus particle is presented. Four proteins are involved in the formation of the ribonucleoprotein complex: polymerase or large protein (L), nucleoprotein (NP), virion structural protein 30 (VP30), VP35. The glycoprotein (GP) is a type I trans membrane protein and is anchored with the carboxy-terminal part in the virion membrane. The soluble GP (sGP) is a non-structural glycoprotein secreted from infected cells and is only secreted by ebolaviruses. VP40 and VP24 are membrane-associated proteins. (B) Schematic representation of Ebolavirus (EBOV) and Marburgvirus (MARV) genomes. *(See insert for color representation of the figure).*

proteins, soluble glycoprotein (sGP) and small soluble glycoprotein (ssGP) (Figure 6.3). After viral RNA extraction and RT-PCR, whole genome sequencing is currently obtained through primer-walking PCR and traditional Sanger sequencing as previously described (Towner *et al.*, 2006).

6.8 CONCLUSIONS

Throughout this chapter we have highlighted the role of potential reservoir host that bats may play in the epidemiology of filoviruses in parts of Africa, and now in Asia and Europe. Knowledge of reservoir species is needed to understand and prevent viral emergence in human and animal populations. To this end, efforts must focus on the development or use of techniques that are increasingly sensitive (high-throughput sequencing for example) to detect filoviruses within their hosts and to further investigate the genome of the filoviruses identified.

The knowledge of ecological determinants that would promote infection of these animals by filoviruses is an important issue in order to understand and predict disease emergence at local and regional, spatial and temporal scales. The current outbreak of EHF affecting West Africa threatens to spread more broadly and encourages us to increase our surveillance efforts, for example through the creation of sub-regional programs that foster collaboration between different research teams in different countries.

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7

BATS AND REVERSE TRANSCRIBING RNA AND DNA VIRUSES

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7.1 INTRODUCTION TO REVERSE TRANSCRIBING RNA AND DNA VIRUSES

7.1.1 Retroviruses

The first descriptions of an association between retroviruses and bats were *in vitro* studies with the bat lung cell line, TB1 Lu (NBL-12), derived from a female bat. These cells were used to detect and propagate bovine leukemia virus (Mihailescu *et al.*, 1980; Patrascu, 1988), and to demonstrate the presence of retroviruses in human breast cancer

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tissue (Ilyin & Morozov, 1980). These early studies with Type C and Type D retroviruses that are now recognized as gammaretroviruses and betaretroviruses respectively, provided evidence that bats might be able to support the replication of reverse transcribing viruses, at least at the cellular level.

Retroviruses belong to the *Retroviridae* family and comprise a large and diverse range of pathogenic and non-pathogenic viruses. Retroviral particles are enveloped with a diameter of 100–150 nm and harbor two copies of a single-stranded positive-sense genomic RNA of 7–12 kb (Vogt, 1997). The hallmark of retroviruses is their unique capacity to convert their single-stranded RNA genome into a dsDNA form in a process called reverse transcription, which occurs in the host cell cytoplasm and is catalyzed by the virion-encoded reverse transcriptase enzyme (Telesnitsky & Goff, 1997). The linear dsDNA reverse transcription product, which forms part of the preintegration complex, enters the nucleus and is inserted permanently into the host DNA chromosome by the viral integrase to form proviral DNA that acts as a template for viral gene expression (Brown, 1997).

Retroviruses have either simple or complex genomes such as gammaretroviruses and lentiviruses, respectively. All retroviral genomes contain the canonical *gag*, *pro*, *pol*, and *env* genes flanked by long-terminal repeat (LTR) sequences in the provirus (Vogt, 1997) (refer to Table 7.1 for a full list of the abbreviations and symbols used in this chapter). The *gag* gene encodes the Gag (group-specific antigen) polyprotein that is proteolytically cleaved into the internal viral structural proteins: matrix, capsid and nucleocapsid. The *pro* gene encodes the viral protease, *pol* encodes reverse transcriptase and integrase enzymes, and *env* the surface (SU) and transmembrane (TM) envelope glycoproteins required for viral attachment and entry (Vogt, 1997). During virus maturation the viral protease cleaves the proteins encoded by *gag*, *pol*, and in some cases *env*, to generate an infectious virus. In addition to the canonical genes, complex retroviruses carry additional genes such as the human immunodeficiency virus type 1 (HIV-1) *tat* and *rev* that are critical for virus replication and *vif*, *vpr*, *vpu*, and *nef*, important for viral pathogenesis. An overview of the retroviral life-cycle is described in Vogt (1997).

Retroviruses are classified into two subfamilies. The *Orthoretrovirinae* subfamily contains the genera *Alpharetrovirus, Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus*, and *Lentivirus*. The *Spumaretrovirinae* subfamily comprises the single genus, *Spumavirus*. Retroviruses have been discovered in a broad range of vertebrates including mammals, reptiles, birds and amphibians and can cause leukaemia, lymphoma and immunodeficiency in some species (Rosenberg & Jolicoeur, 1997). Retroviruses that can be transmitted horizontally from one individual to another are known as "exogenous retroviruses" (XRVs). However, as discussed below, retroviruses can also be inherited vertically from parent to offspring and are referred to as "endogenous retroviruses" (ERVs). A few endogenous retroviruses are also able to produce infectious retroviruses (Denner, 2010; Weiss, 2013). However, on the whole, endogenous retroviruses are defective and represent fossil records of extinct viruses. Recent studies reveal the presence of endogenous gammaretroviruses and betaretroviruses in bats of different suborders demonstrating that bats have been infected with retroviruses historically. However, while some bat ERVs have been found to express viral mRNAs *in vivo*, exogenous bat retroviruses remain to be discovered (Cui *et al.*, 2012a, b; Hayward *et al.*, 2013b; Zhuo *et al.*, 2013).

TABLE 7.1 Abbreviations used in this chapter

Abbreviation	Definition
BaEV	baboon endogenous virus strain M7
βERV	endogenous betaretrovirus
βXRV	exogenous betaretrovirus
C	core protein
cccDNA	covalently closed circular DNA
Env	envelope
EqERV	equine endogenous retrovirus
ERV	endogenous retrovirus
EsRV	*Eptesicus sortinus* retrovirus
eSRV	simian endogenous retrovirus
FeLV	feline leukemia virus
F-MuLV/FMLV	Friend murine leukemia virus
Gag	group-specific antigen
GaLV	gibbon ape leukemia virus
gENTV	enzootic nasal tumor virus
HBHBV	horseshoe bat hepatitis B virus
HBV	hepatitis B virus
HBx	X protein
HERV-K	human endogenous retrovirus-K
HERV-K113	human endogenous retrovirus-K113
HERV-K115	human endogenous retrovirus-K115
HERV-K-TR	human endogenous retrovirus-K-TR
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HMTV	human mammary tumor virus
JSRV	Jaagsietke sheep retrovirus
KoRV	koala retrovirus
LINEs	long interspersed nuclear elements
LTR	long-terminal repeat
M1RV	*Megaderma lyra* retrovirus
MDEV	*Mus dunni* endogenous retrovirus
MIERV-βC	*Myotis lucifigus* endogenous retrovirus betaretrovirus C
MLV	murine leukemia virus
MMTV	mouse mammary tumor virus
M-CRV/MCRV	murine type C retrovirus
M-MuLV/MMLV	Moloney murine leukemia virus
MPMV	Mason–Pfizer monkey virus
MrRV	*Myotis ricketti* retrovirus
oENTV	ovine enzootic nasal tumor virus
OOEV	*Orcinus* orca endogenous retrovirus
ORF	open reading frame
P	polymerase
PaERV-βA	*Pteropus alecto* endogenous retrovirus betaretrovirus A
PaRV	*Pteropus alecto* retrovirus
PERV	porcine endogenous retrovirus

(*Continued*)

TABLE 7.1 (*Continued*)

Abbreviation	Definition
PERV-A	porcine endogenous retrovirus type A
PERV-B	porcine endogenous retrovirus type B
PERV-C	porcine endogenous retrovirus type C
Pol	polymerase
preXMRV-1/2	pre-xenotropic murine leukemia virus-related virus 1 and 2
Pro	protease
PvERV	*Pteropus vampyrus* endogenous retrovirus
RMLV	Rauscher murine leukemia virus
RaFV-1	*Rhinolophus affinis* foamy virus type 1
RaRV	*Rhinolophus affinis* retrovirus
RBHBV	roundleaf bat hepatitis B virus
RC-DNA	relaxed circular DNA
RD114	RD114 feline endogenous retrovirus
RpeRV	*Rhinolophus pearsoni* retrovirus
R-MuLV	Rauscher murine leukemia virus
REV	reticuloendotheliosis virus
RfRV	*Rhinolophus ferrumequinum* retrovirus
RfEnv01	*Rhinolophus ferrumequinum* Envelope 01
R1RV	*Rousettus leschenaultii* retrovirus
RpuRV	*Rhinolophus pusillus* retrovirus
RmRV	*Rhinolophus megaphyllus* retrovirus
S	surface protein
SFV	simian foamy virus
SINEs	short interspersed nuclear elements
SIV	simian immunodeficiency virus
SMR	squirrel monkey retrovirus
SRV1	simian retrovirus 1
SRV4	simian retrovirus 4
SU	surface
TE	transposable element
TBHBV	tent-making hepatitis B virus
TM	transmembrane
TvERV	Common brushtail possum endogenous retrovirus
XMRV	xenotropic murine leukemia virus-related virus
XRV	exogeneous retrovirus

7.1.2 Hepadnaviruses

Hepatitis B virus (HBV) is the prototype species of the family *Hepadnaviridae*, which is comprised of two genera: *Orthohepadnavirus* and *Avihepadnavirus*, associated with mammals and birds, respectively. Members of the *Orthohepadnavirus* genus are found in humans, great apes, woolly monkeys, woodchucks, and squirrels while avian hepadnaviruses infect duck, heron, goose, stork, and crane (Simmonds, 2001). Hepadnaviruses infect hepatocytes, causing a transient and chronic infection of the liver. Hepadnavirus infections are usually restricted to the species from which the virus was isolated or to closely related species (Seeger & Mason, 2000). HBV is a major human pathogen infecting

more than 40% of the global population and resulting in an estimated 240 million chronic carriers in 2005 (Ott *et al.*, 2012) with 10–25% of chronic carriers developing either fatal liver cancer or cirrhosis (Seeger & Mason, 2000).

Hepadnaviruses are DNA viruses, in contrast to retroviruses that have an RNA genome, although they share a common evolutionary origin (Miller & Robinson, 1986). The distinguishing feature of hepadnavirus replication is protein-primed reverse transcription, which differs mechanistically from retroviruses (Summers & Mason, 1982). Hepadnaviral particles are enveloped and contain an inner icosahedral core encasing a partially double-stranded relaxed circular DNA (RC-DNA) genome of approximately 3.2 kb (Beck & Nassal, 2007). The genome encodes four overlapping open reading frames (ORFs) for the viral core protein (C), surface protein (S), polymerase (P), and X protein (HBx) (Beck & Nassal, 2007).

Following viral entry, the RC-DNA is converted into covalently closed circular DNA (cccDNA) in the nucleus of the host cell. Thus, unlike retroviruses that insert their DNA genome into the host cell chromosome, the hepadnaviral DNA usually remains episomal during its productive life cycle. However, cccDNA is known to integrate into the hepatocyte genome and this is associated with the development of hepatocellular carcinoma (Bonilla Guerrero & Roberts, 2005). The cccDNA acts as a template for the synthesis of full length and subgenomic viral RNAs by the host RNA polymerase II. The larger, pregenomic RNA (pgRNA) is selectively packaged into capsids along with the viral P protein, and a reverse transcriptase that converts the RNA template into partially double-stranded RC-DNA in the host cell cytoplasm. Thus hepadnaviral reverse transcription occurs in the producer cell in contrast to retroviruses where it takes place in the target cell. The fate of the nucleocapsids containing mature RC-DNA is either intracellular amplification of cccDNA or acquisition of an outer envelope by budding into the endoplasmic reticulum and release from the cells as progeny virions. The biology and life-cycle of HBV has been extensively reviewed (Nassal & Schaller, 1996; Seeger & Mason, 2000; Beck & Nassal, 2007)

Findings from the study of endogenized HBV sequences present in host genomes suggest that birds are the likely ancestral hosts of hepadnaviruses (Cui & Holmes, 2012; Liu *et al.*, 2012; Suh *et al.*, 2013). These viruses infiltrated their avian hosts at least 19 million years ago (Gilbert & Feschotte, 2010). In contrast, hepadnavirus fossils have not yet been found in mammalian genomes, suggesting these viruses only recently entered these hosts (Gilbert & Feschotte, 2010; Suh *et al.*, 2013), which is supported by the recent discovery of pathogenic bat hepadnaviruses that are serologically related to human HBV (Drexler *et al.*, 2013).

7.2 ENDOGENOUS RETROVIRUSES IN BATS

7.2.1 Endogenous retroviruses: A transposable element subclass

ERVs belong to the general class of transposable elements (TEs) that are present in all mammalian genomes including those of bats (Cui *et al.*, 2012a, b; Hayward *et al.*, 2013a, b; Zhang *et al.*, 2013; Zhuo *et al.*, 2013). Unlike XRVs, the entire ERV life-cycle can occur within the confines of the cell, culminating in copying of their DNA from one genomic location to another. There are two classes of retroelements (also known as

retrotransposons or class I transposons) and these are LTR retrotransposons and non-LTR retrotransposons with both classes reported in the genomes of *Pteropus alecto* and *Myotis* spp. (Hayward *et al.*, 2013b; Zhang *et al.*, 2013; Zhuo *et al.*, 2013). ERVs are LTR-containing retrotransposons that replicate their DNA by reverse transcription of an RNA intermediate, catalysed by reverse transcriptase (Boeke *et al.*, 1985; Garfinkel *et al.*, 1985; Boeke & Stoye, 1997; Symer & Boeke, 2010; Finnegan, 2012). This replication mechanism is distinct to DNA transposons (class II transposons) that amplify their genomic DNA by a cut and paste mechanism in the absence of an RNA intermediate (Kurth & Bannert, 2010) and several DNA transposon families are present in the genome of *M. lucifigus* (Pritham & Feschotte, 2007; Ray *et al.*, 2007, 2008).

ERVs have much in common with retroviruses and can possess *gag*, *pro*, *pol*, and *env* genes, flanked by a pair of LTRs (Symer & Boeke, 2010; Stoye, 2012). They replicate as either transposable elements or XRVs, depending on the integrity of their *env* genes. ERVs lacking *env* genes tend to proliferate rapidly within host genomes highlighting the efficiency of ERV intracellular amplification (Magiorkinis *et al.*, 2012). Non-LTR retrotransposons, so named for their lack of terminal repeats shared by retroviruses, include the mammalian short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) and are present in vast copy numbers within vertebrate genomes (Smit, 1996; Stoye, 2012). LINEs (but not SINEs) encode reverse transcriptases, which are then exploited by both LINEs and SINEs for retrotransposition (Kurth & Bannert, 2010; Symer & Boeke, 2010). Both LINEs and SINES have been reported in the genomes of *P. alecto* and *M. davidii* (Zhang *et al.*, 2013).

7.2.2 Endogenous retroviruses originate from exogenous retroviruses

XRVs spread throughout the animal kingdom, typically by integration of their proviral DNA into the nuclear genome of somatic cells followed by virion production and horizontal transmission to successive hosts. Occasionally XRVs become integrated into their host's germline cells, leading to Mendelian inheritance of the retrovirus. Such vertically transmitted ERVs initially maintain the structure of the original XRV (Weiss, 2006), and like their somatic cell-integrated exogenous progenitors, are capable of producing infectious virions and horizontal transmission. However, the eventual fate of many ERVs is genetic fossilization as a consequence of facing selective pressure from the host. Over millions of years ERV genomes accumulate random mutations including deletions and insertions that usually put an end to their capacity for producing functional gene products and infectious virions.

7.2.3 Endogenous retrovirus nomenclature

ERVs are described in the literature by a complicated nomenclature. The current taxonomic scheme for XRVs divides them into seven genera and endogenized members of these genera may be referred to using these names. However, an alternative scheme originally based on the ERVs present in the human genome, is also regularly used. This scheme divides ERVs into three broad classes (Class I, II and III). Class I ERVs phylogenetically cluster alongside the *Gammaretrovirus* and *Epsilonretrovirus* genera, Class II ERVs alongside the *Betaretrovirus, Deltaretrovirus*, *Alpharetrovirus*, *and Lentivirus*

genera, and Class III ERVs alongside the *Spumavirus* genus (Gifford & Tristem, 2003). It is worth noting that ERVs representing all exogenous retroviral genera with the exception of the *Deltaretrovirus* genus have been described in the literature (Jern & Coffin, 2008). Neither scheme comprehensively encompasses all known ERVs and the taxonomy and nomenclature of XRVs and ERVs has been reviewed by Jern *et al.* (2005).

7.2.4 Role of transposable elements and endogenous retroviruses in disease and host evolution

TEs including ERVs can be neutral, deleterious, or beneficial to the host and have been major drivers of genomic and biological diversity in vertebrates over millions of years (Bohne *et al.*, 2008; Stoye, 2012). Although TEs have been regarded in the past as harmful genetic elements in the host genome, recent findings suggest that they can have an overall beneficial role through gene regulation and exaptation where they are co-opted by the ancestral host to perform essential physiological roles (Kurth & Bannert, 2010; Rebollo *et al.*, 2012). ERVs have been implicated in host diseases such as cancer through insertional mutagenesis (Ruprecht *et al.*, 2008), and have had a positive role in shaping the transcriptional regulation network of the p53 human tumor suppressor protein (Wang *et al.*, 2007). A notable example of beneficial ERVs or their genes is *syncytin*, which has a fundamental role in the placentation of mammals. *Syncytin* evolved by exaptation of an envelope protein of retroviral origin by ancestral hosts and was domesticated in different mammalian species, including humans (Lavialle *et al.*, 2013). Another example is the *Fv1* gene, which is homologous to the *gag* gene of a class III ERV, encoding a host intracellular restriction factor that protects mice against infection by murine leukemia virus (MLV) (Best *et al.*, 1996). The co-opted *Fv1* virus resistance gene demonstrates strong positive selection in members of the *Mus* genera suggesting an antiviral role throughout *Mus* evolution (Yan *et al.*, 2009; Yap *et al.*, 2014). Thus co-opted ERV genes tend to maintain their intact forms in the host genome and are expressed as host genes.

TEs comprise approximately 50% of the mammalian genome (Zamudio & Bourc'his, 2010) while it is estimated that LTR-retrotransposons (that include ERVs) represent 8% and 10% of human and mouse genomes, respectively (Lander *et al.*, 2001; Waterston *et al.*, 2002). Intriguingly, bats have the smallest genome size among mammals (Smith & Gregory, 2009; Zhang *et al.*, 2013). In accordance with their short genome length (~2 gigabases), bat LTR-retrotransposons are also amongst the lowest found in mammals with regard to both copy number ($3.9–4.5 \times 10^5$) and genomic composition (4.8–5.5%) (Zhang *et al.*, 2013). Intact ERVs have been reported in humans, mice, cats, koalas, pigs, and bats (Baillie *et al.*, 2004; Tarlinton *et al.*, 2006; Lee & Bieniasz, 2007; Cui *et al.*, 2012a, b; Stoye, 2012; Hayward *et al.*, 2013b) and some of them are active and infectious such as ERVs in mice, koalas, and pigs (Patience *et al.*, 1997; Tarlinton *et al.*, 2006; Stoye, 2012). There is molecular evidence that ERVs in bats may express infectious virus (Hayward *et al.*, 2013b). In contrast, extinction of LINE-1 retroelement activity occurred early in the ancestry of the family Pteropodidae (Cantrell *et al.*, 2008).

Class II DNA transposons, which make up 3% of the human genome (Lander *et al.*, 2001), are of interest, since the prevailing view was that they have been extinct in mammals for at least 37 million years (Lander *et al.*, 2001; Waterston *et al.*, 2002; Pace & Feschotte, 2007). Opposing this view, an active DNA transposon, *piggyBat*, has been

identified in the genome of the little brown bat (*Myotis lucifugus*) (Pritham & Feschotte, 2007; Mitra *et al.*, 2013), suggesting the tolerance of DNA transposon invasion in vespertilionid bats (Ray *et al.*, 2007, 2008). The recent and likely ongoing waves of DNA transposition in *Myotis* bats represent an extraordinary opportunity to study how this activity shapes the genome and the evolution of new bat species (Mitra *et al.*, 2013).

7.2.5 Endogenous retroviruses as fossil records of ancient exogenous retroviruses

ERVs, as non-replicating fossils, serve as historical records of extinct infectious XRV progenitors. By analyzing the phylogenetic relationships between these fossilized ERVs we can learn much about the shared history of bats and retroviruses throughout the course of their co-evolution. ERVs are seen throughout the entire metazoan animal kingdom (Gifford & Tristem, 2003), which mirrors the broad host spectrum of retroviruses. Recently, by screening a few bat genomes and transcriptomes, we and others have found that bats have a variety of ERVs in their genomes, some of which are still transcriptionally active (Cui *et al.*, 2012a, b; Hayward *et al.*, 2013b; Zhuo *et al.*, 2013). As large-scale genome sequencing comes of age, comparative genomics becomes the most powerful tool for studying ERVs. With more bat genomes becoming available (see Chapter 13), researchers are likely to establish a better understanding of retroviral evolutionary history and diversity. For example, the screening of 60 vertebrate genomes uncovered a remarkable depth in gammaretroviral diversity, inferring that the diversity of exogenous gammaretroviruses is underestimated, and that rats may have acted as overlooked facilitators in the global spread of mammalian gammaretroviruses (Hayward *et al.*, 2013a).

7.3 GAMMARETROVIRUSES IN BATS OF DIFFERENT SUBORDERS

7.3.1 Gammaretroviruses: host range and diseases

Gammaretroviruses belong to a major genus in the family *Retroviridae*, *Gammaretrovirus* and cluster within Class I ERVs in phylogeny (Jern *et al.*, 2005). Gammaretroviruses generally do not undergo interclass transmission and to date, no member of this genus has been shown to infect and cause disease in humans. In this regard the xenotropic murine leukemia virus-related virus (XMRV) that contaminated reagents and cell lines was incorrectly associated with human prostate cancer and chronic fatigue syndrome (Rezaei *et al.*, 2013; Bhardwaj & Coffin, 2014). Regardless, gammaretroviruses are well recognised because of certain viruses within this genus that have been extensively studied. For example, MLV and feline leukemia virus (FeLV), are linked with disease such as malignancies, immunodeficiencies, and neurologic disorders in their hosts (Rosenberg & Jolicoeur, 1997). KoRV, which is associated with neoplasia in koalas (Hanger *et al.*, 2000), is especially intriguing since this recently integrated ERV is transitioning from an exogenous to an endogenous form in real time (Tarlinton *et al.*, 2006). The porcine endogenous retrovirus (PERV) has received attention in the context of xenotransplantation of porcine tissues, which may offer a solution to the shortage of human donor organs. Pigs are natural hosts for PERVs, so possible cross-species transmission of PERV to human recipients has been a major concern (Paradis *et al.*, 1999).

7.3.2 Discovery of gammaretroviruses in bats

In 2012, we reported the discovery of an intact endogenous gammaretrovirus in the greater horseshoe bat (*Rhinolophus ferrumequinum*), named RfRV (Cui *et al.*, 2012b) (Figure 7.1A). Using *de novo* transcriptome sequencing, we found a complete bat retrovirus present in the brain tissue of the greater horseshoe bat (Cui *et al.*, 2012b). The RfRV genome is 8356 nt in length and has the typical genomic structure of a simple retrovirus (Figure 7.1A). However, RfRV also has several atypical features including a premature stop codon near the 3´ end of *pol*, a predicted ORF containing a partial integrase coding sequence, and two AUG initiation codons for *env*, together indicating that it is a defective virus. Our investigation of the phylogenetic position of RfRV demonstrated that it is a gammaretrovirus and that it is basal to the phylogeny of all extant mammalian gammaretroviruses that were examined (Figure 7.2). However, a subsequent pan-phylogenomic analysis, which included ERVs, as well as XRVs from a wider range

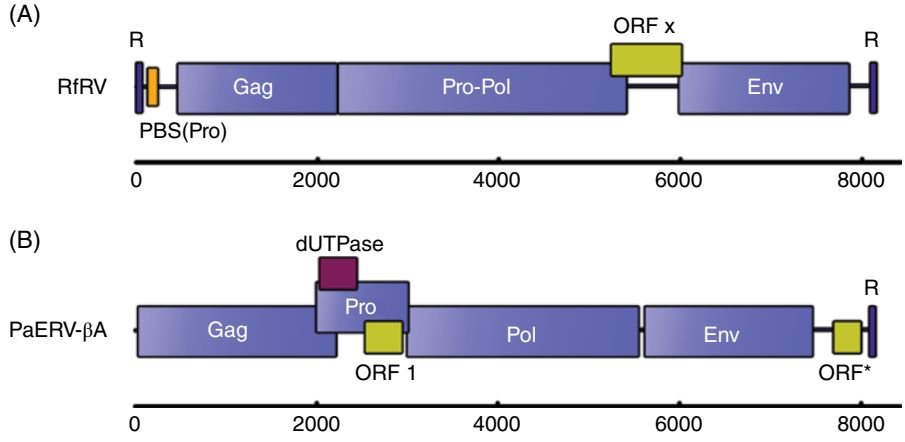

Figure 7.1 Full length retroviral transcripts from bats of different suborders. (A) Structure of the *Rhinolophus ferrumequinum* retrovirus (RfRV) genomic transcript. The 3´ end of RfRV *pol* gene is truncated by a premature stop codon and a new open reading frame (ORF), ORF x, partially encoding the integrase protein, overlaps with *pol* and *env*. The complete RfRV genome is 8356 nucleotides (nt), comprising the genes *gag* (nucleotide positions 621 to 2348), *pol* (nt 2349 to 5594), *env* (nt 5990 to 7867), and direct repeats (R) at both ends. A proline tRNA primer-binding site, PBS (Pro), is present at nt 152 to 173. (B) Structure of the ***P**teropus **a**lecto* **e**ndogenous **r**etrovirus – **beta**retrovirus **A** (PaERV-βA) genomic transcript. Two contigs were identified in the *P. alecto* Illumina-sequenced transcriptome that overlapped by 3,152 nt with 100% sequence identity which were used to assemble the PaERV-βA genomic transcript. Shown are the retroviral genes: *gag*, *pro*, *pol*, and *env*, which have been rendered defective by random mutation since integration, and the betaretroviral dUTPase domain in *pro*, two unique ORFs, and the 3´ terminal repeat region. ORF* does not appear to be genuine, but rather has arisen as a result of an insertion mutation that has disrupted a stop codon. Figure 7.1B is reproduced from (Hayward *et al.*, 2013b) by the publication authors through the Creative Commons Attributions License 4.0 (http://creativecommons.org/licenses/by/4.0/) agreement with BioMed Central. *(See insert for color representation of the figure).*

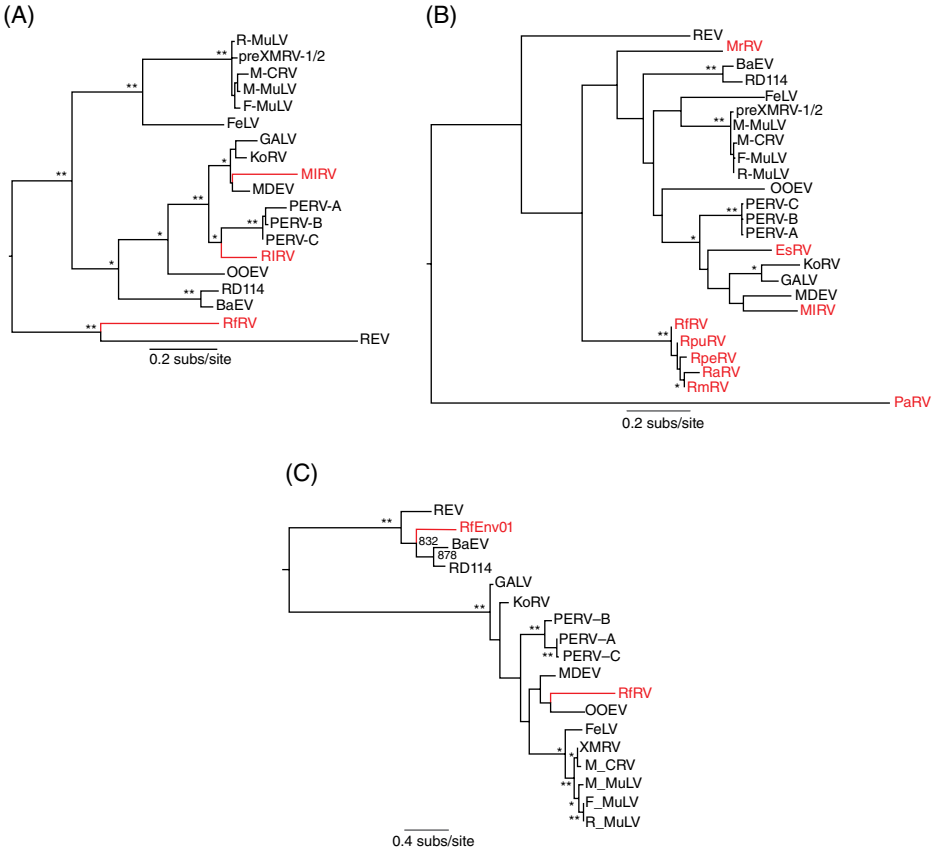

Figure 7.2 Phylogenetic analysis of bat gammaretroviruses and non-bat extant gammaretroviruses. Maximum likelihood phylogenetic trees of (A) Gag, (B) Pol, and (C) Env. Bars represent amino acid substitutions per site and the trees are midpoint rooted for clarity only. Only bootstrap values higher than 70% are shown, such as two stars denoting values ranging from 90% to 100% and one star 70% to 90%. Detailed information regarding sequences can be found in (Cui *et al.*, 2012a, b) and the Accession number for the *Eptesicus serotinus* bat retrovirus (EsRV) is AGN73375. *(See insert for color representation of the figure).*

of host taxa suggests that RfRV is positioned within the gamma-ERV clade and that some of the other bat ERVs and the non-bat ERVs (e.g., pig and tenrec) show more ancient features by being positioned deeper in the phylogenetic tree (Hayward *et al.*, 2013a). These results suggest that the origin RfRV is complicated and that more than one host species may be involved.

Interestingly, the avian reticuloendotheliosis virus (REV), recently shown to have a mammalian origin and introduced into birds in the mid-20th century through accidental human intervention (Niewiadomska & Gifford, 2013), is also basal in our phylogenetic analysis (Cui *et al.*, 2012b). To expand our understanding of basal bat gammaretroviruses, we screened 10 more bat species sampled in both China and Australia. We verified

that six bat species, the least horseshoe bat (*Rhinolophus pusillus*), Pearson's horseshoe bat (*R. pearsoni*), smaller horseshoe bat (*R. megaphyllus*), intermediate horseshoe bat (*R. affinis*), Rickett's big-footed bat (*Myotis ricketti*), and black flying fox (*P. alecto*), also harbor similar viruses (named RpuRV, RepRV, RmRV, RaRV, MrRV, and PaRV, respectively) that are actively transcribed (Cui *et al.*, 2012b). Phylogenetic analysis shows that all these bat viruses are consistently basal to extant mammalian gammaretroviruses and that PaRV is located even deeper than avian REV (Figure 7.2). These data and those from other studies showing that REV is located within a clade of hedgehog and bat ERVs (Hayward *et al.*, 2013a) and the fact that REV-related ERVs are present in Malagasy carnivores and Australian monotremes (Niewiadomska & Gifford, 2013) suggest that bats might be a mammalian REV host and a possible vector responsible for cross-species transmission to mammals located in geographically distinct regions.

A statistical test for codivergence between gammaretroviruses and their bat hosts shows weak support across the data set, which could suggest episodes of coevolution during the history of the virus spread (Cui *et al.*, 2012b). Later, we reported that two other bats, Leschenault's rousette (*Rousettus leschenaultii*) and the greater false vampire bat (*Megaderma lyra*), also harbor the gammaretroviruses R1RV and M1RV, respectively (Cui *et al.*, 2012a). Both R1RV and M1RV are defective due to truncations in *pol*, but are actively transcribed. These bat retroviruses are distinct from RfRV (Cui *et al.*, 2012a) and are embedded within the diversity of mammalian gammaretroviruses (Figure 7.2). In this regard, RlRV has a close relationship with PERVs, and M1RV, KoRV, gibbon ape leukemia virus (GaLV) and *Mus dunni endogenous virus* (MDEV) and falls into a monophylogenetic cluster (Figure 7.2A). However, due to clear genetic divergence between bat and non-bat viruses, some intermediate retroviruses (and probably different hosts) are likely to exist. Regardless, these observations provide evidence for potential cross-species transmission of retroviruses between bats and non-bat mammals.

Due to the nature of retroviral endogenization, large numbers of ERVs are expected in bat genomes. Our genomic mining of the little brown bat (*Myotis lucifugus*) and large flying fox (*Pteropus vampyrus*) revealed diversified ERVs in these genomes, with the time of integration widely ranging from 2.4 to 64.6 million years ago (Cui *et al.*, 2012a). Another in-depth mining of the little brown bat genome discovered six groups of Class I ERVs, suggesting our current knowledge of the circulation of bat retroviruses could be underestimated (Zhuo *et al.*, 2013). The observations that bats harbor phylogenetically distinct viruses indicate that they have played important roles in the diversification of mammalian gammaretroviruses.

7.4 BETARETROVIRUSES IN BATS OF DIFFERENT SUBORDERS

7.4.1 Betaretroviruses: host range and diseases

The known XRVs of the genus *Betaretrovirus* (βXRVs) are hosted by a diverse range of mammals, having been identified in primates, sheep, goats, and rodents (Graff *et al.*, 1949; Sonigo *et al.*, 1986; De las Heras *et al.*, 1991; York *et al.*, 1992). In contrast to gammaretroviruses, betaretroviruses demonstrate a greater capacity to jump between diverse species (Baillie *et al.*, 2004; Hayward *et al.*, 2013b). The βXRVs have long been

associated with malignancies in their hosts. The Jaagsiekte sheep retrovirus (JSRV) causes pulmonary carcinoma in sheep, the Mason–Pfizer monkey virus (MPMV) causes immunosuppression and wasting in rhesus monkeys, and the mouse mammary tumor virus (MMTV), as its name suggests, is responsible for breast cancer in mice (Sonigo *et al.*, 1986; Wootton *et al.*, 2005; Cadieux *et al.*, 2009).

Endogenous betaretroviruses (βERVs) are Class II ERVs and have been identified in more mammalian hosts than are known to be infected by extant βXRVs. This group includes the human endogenous retrovirus-K (HERV-K) group reviewed by Mayer & Meese (2002). βERVs may be ubiquitously distributed among mammals, having been reported in primates, horses, lemurs, rodents, bears, and bats (Baillie & Wilkins, 2001; Baillie *et al.*, 2004; van der Kuyl, 2011; Hayward *et al.*, 2013b; Mayer *et al.*, 2013; Zhuo *et al.*, 2013).

7.4.2 Betaretroviruses in bat transcriptomes and genomes

Recent work by our group, which identified the presence of βERVs in bats, reported the expression of betaretroviral transcripts encoding all of the major betaretroviral gene products in *P. alecto*, the smaller horseshoe bat (*R. megaphyllus*), and the greater horseshoe bat (*R. ferrumequinum*) (Hayward *et al.*, 2013b). Importantly, the expression of a full genomic betaretroviral transcript, named *Pteropus alecto* endogenous retrovirus – betaretrovirus A (PaERV-βA), was described in the *P. alecto* transcriptome. However, it was determined that this genomic transcript was produced by a fossilized βERV rather than an actively replicating βXRV since the protein coding domains contained frameshift errors and premature stop codons that rendered them defective (Figure 7.1B), and because the PaERV-βA transcript was very similar to the PvERV-βK endogenous retrovirus present in the *P. vampyrus* genome demonstrating 94% nucleotide identity (Hayward *et al.*, 2013b).

Analyses of the *P. vampyrus* and *M. lucifugus* genomes unveiled a greater diversity of integrated βERVs within the *Betaretrovirus* genus than has previously been recognized (Hayward *et al.*, 2013b; Zhuo *et al.*, 2013). These surveys of the βERVs led to some intriguing implications about the evolution of the *Betaretrovirus* genus and the role played by bat hosts. Phylogenetic analyses show that bat βERVs are broadly spread across the entire breadth of the betaretroviral phylogeny (Figure 7.3) in a similar manner to that previously described for the βERVs of rodents (Baillie *et al.*, 2004). These phylogenies hint toward a possible role for bats and rodents as primary reservoirs of βXRVs with occasional cross-species transmission into other mammalian hosts, including humans.

The genomic analysis of *M. lucifugus* uncovered one intact βERV named MIERV-βC (Hayward *et al.*, 2013b). While it remains possible that this βERV is still capable of producing infectious virus it should be noted viral integration is estimated to have occurred 4.2 million years ago and nucleotide polymorphisms are likely to exist within the protein coding domains which may yet render the viral gene products non-functional.

7.4.3 Extensive diversity among bat betaretroviruses

Such is the diversity of bat βERVs that we were compelled to propose the existence of eight distinct sub-groups (Groups I–VIII) of the genus *Betaretrovirus*, all but one of which are represented by βERVs in bats (Figure 7.4) (Hayward *et al.*, 2013b).

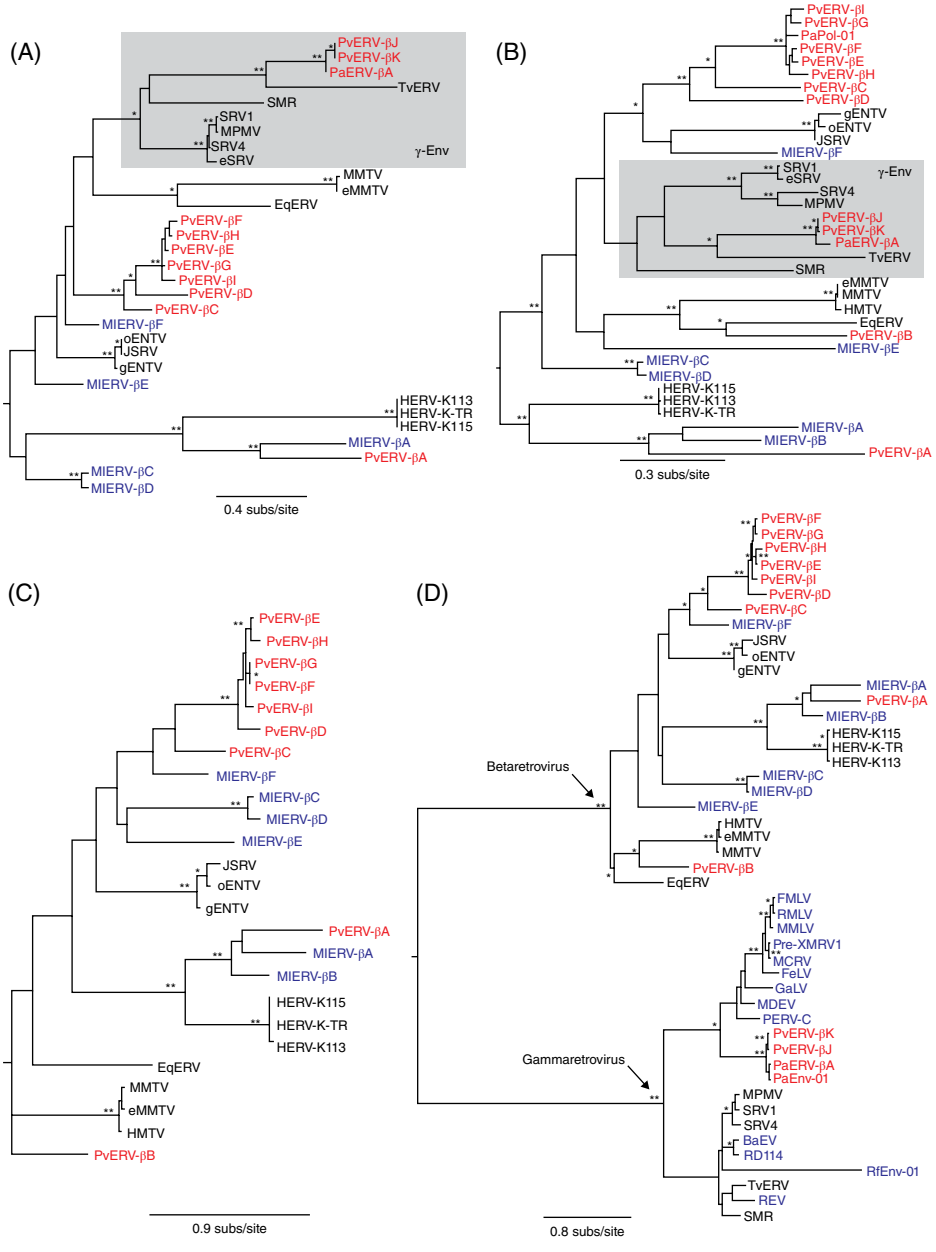

Figure 7.3 Phylogenetic relationships of bat and non-bat extant betaretroviruses with comparison to gammaretroviral Env sequences. Maximum likelihood phylogenetic trees of betaretroviral (A) Gag, (B) Pol, and (C) Env amino acid sequences and (D) betaretroviral compared to gammaretroviral Env amino acid sequences. Bootstrap values <70% are not shown, and branch lengths are draw to scale of amino acid substitutions per site. Bootstrap values are denoted as **>90%; *>70%; and <90%. The trees are midpoint rooted for purposes of clarity only. βERV proteins of *P. vampyrus* and *P. alecto* are highlighted in red text. βERVs of *M. lucifigus* and *R. ferrumequinum* are highlighted in blue text. Non-bat betareroviruses and gammaretroviruses are in black and teal text, respectively. The clades within (A) Gag and (B) Pol trees, highlighted with a grey rectangle (ϒ-Env), contain betaretroviruses whose Env sequence is not sufficiently closely related to the Env of other betaretroviruses to be included in the Env tree. Their phylogenetic relationship to gammaretroviruses is revealed in the (D) Env tree. Detailed information regarding sequences can be found in (Hayward *et al.*, 2013b). Figure is reproduced from (Hayward *et al.*, 2013b) with copyright permissions as described in the legend of Figure 7.1. *(See insert for color representation of the figure).*

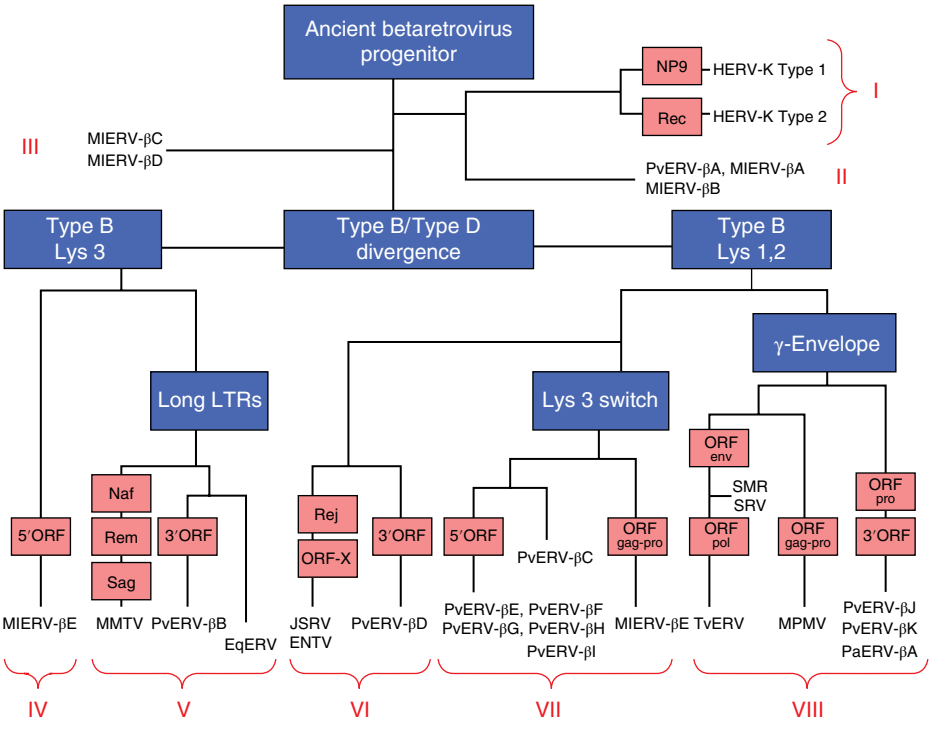

Figure 7.4 A proposed series of events leading to the current diversity in the genus *Betaretrovirus*. The proposed series of evolutionary events leading to eight distinct sub-groups of betaretroviruses based on a combination of the phylogenetic analyses of Gag, Pol, and Env protein sequences, and the genomic features and organizations of individual betaretroviruses. Abbreviations are defined in the legend of Table 7.1. Figure is reproduced from (Hayward *et al.*, 2013b) with copyright permissions as described in the legend of Figure 7.1. *(See insert for color representation of the figure).*

Betaretroviruses are recognized as a heterogeneous genus. Prior to the current taxonomic scheme for XRVs, complex "Type B" and simple "Type D" retroviruses were described as distinct groups differing in terms of genetic structure, morphological characteristics, and nuclear mRNA export mechanisms (Vogt, 1997). These two groups were later formally merged into the genus *Betaretrovirus* (Vogt, 1997). It is now clear that the divisions between various βXRVs run deeper than the Type B and Type D divide. Research over the last decade has revealed that betaretroviruses are more complex than previously recognized, with accessory genes being uncovered in the Type D JSRV (Hofacre *et al.*, 2009; Nitta *et al.*, 2009), and the revelation that both Type B and Type D betaretroviruses make use of two, possibly redundant, nuclear mRNA export strategies (Mertz *et al.*, 2005; Nitta *et al.*, 2009; Boeras *et al.*, 2012). Our analysis of bat βERVs revealed that, in addition to the broad phylogenetic diversity of betaretroviruses, additional significant evolutionary differences exist among betaretroviral subgroups.

An analysis of the nuclear mRNA export strategies of bat βERVs revealed that members of groups I to VII make use of an accessory export protein (Hayward *et al.*,

2013b). Phylogenetic analysis of the envelope proteins revealed that group VIII viruses emerged though a single recombination event between a betaretrovirus and a gammaretrovirus, in which the betaretrovirus replaced its *env* gene with the gammaretroviral equivalent. Amazingly, this event resulted in the newly emerged group VIII lineage of betaretroviruses evolving from a seemingly complex retrovirus into a simple retrovirus, as the accessory export protein was encoded within its betaretroviral *env* gene and was lost in the exchange (Hayward *et al.*, 2013b). This expanded previous studies regarding the nature of the Type D *env* gene, which had concluded that a recombination event gave rise to the Type D lineage (Sommerfelt & Weiss, 1990; Sonigo *et al.*, 1986). Rather, we revealed that the Type D lineage, that includes JSRV, which does not possess a gammaretroviral *env* gene, had emerged prior to the recombination event as a result of the switch to the use of an alternative tRNA-primer binding site (Hayward *et al.*, 2013b).

Bat βERVs were found to possess a surprisingly large number of additional ORFs (Hayward *et al.*, 2013b). Even though the analysis restricted reporting of unique ORFs to those spanning at least 100 codons, and many retroviral accessory proteins use ORFs smaller than this (Bannert *et al.*, 2010), we identified 10 unique ORFs in bat βERVs within groups IV through VIII (Hayward *et al.*, 2013b). While we cannot yet know with any certainty that these ORFs are indeed protein coding, much less speculate on their function, they hint at the possibility of a new level of previously unrecognized complexity within the genus *Betaretrovirus*.

Estimations of the time since integration of the original βXRVs into bat genomes reveal that betaretroviruses have been infecting bats for a significant portion of their evolutionary history, with integration times spanning as far back as ~36 million years ago (mya) until at least as recently as ~3 mya (Hayward *et al.*, 2013b; Zhuo *et al.*, 2013). Given this long and likely uninterrupted history of betaretroviral infection of bats it will not be surprising if one or more bat βXRVs are soon discovered.

7.5 PATHOGENIC HEPADNAVIRUSES RELATED TO HBV IN BATS

Until recently there appeared to be no known zoonotic reservoir for human HBV. However, a survey of 54 bat species for the presence of hepadnaviral DNA revealed ten out of 3080 specimens that were positive in three bat species (Drexler *et al.*, 2013). These are the tent-making bat (*Uroderma Bilobatum)*, a New World bat from Panama, and two Old World bats, the roundleaf bat (*Hipposideros cf. rube)* and the halcyon horseshoe bat (*Rhinolophus alcyone*), both from Gabon (Drexler *et al.*, 2013). Based on the convention for hepadnaviruses, the three unique hepadnavirus species were named the roundleaf bat HBV (RBHBV), the horseshoe bat HBV (HBHBV), and the tent-making bat HBV (TBHBV) and have genomes of 3368 nt, 3377 nt and 3149 nt, respectively (Drexler *et al.*, 2013). The nucleotide sequences of RBHBV, HBHBV, and TBHBV vary by at least 35% compared to any known hepadnaviruses (Drexler *et al.*, 2013). They all contain four ORFs corresponding to the S, P, C, and HBx found in hepadnaviruses (Drexler *et al.*, 2013). The genome organization of these bat viruses, together with their genome lengths and phylogenetic relatedness places them in the *Orthohepadnavirus* genus (Drexler *et al.*, 2013). In this regard, the Old and New World bat viruses form two monophyletic clades with all primate hepadnaviruses and the TBHBV New World bat

hepadnavirus forms a sister clade with the primate viruses (Drexler *et al.*, 2013). A hepadnavirus identified in the long-fingered bat (*Miniopterus fuliginosus*) from Myanmar (He *et al.*, 2013a) forms a sister clade with the African bat viruses RBHBV and HBHBV (Drexler *et al.*, 2013).

Full-length molecular clones of RBHBV, HBHBV, and TBHBV were resurrected from bat tissues and the Old World bat viruses were transfected into hepatocytes to evaluate the seroreactivity of bat sera to the expressed viral antigens (Drexler *et al.*, 2013). Antibody detection rates were as high as 18.4% in hipposiderid bats (9 of 49 animals) and 6.3% in rhinolophid bats (1 of 16 animals) with titres ranging from 1:100 to 1:1,600 (Drexler *et al.*, 2013). These bat viruses are antigenically related to human HBV, with the New World bat demonstrating particularly close serological relatedness to primate hepadnaviruses consistent with their phylogeny (Drexler *et al.*, 2013).

Notably, bat hepadnaviruses are capable of infecting human hepatocytes using the human HBV receptor SLC10A1 and, typical of hepatitis viruses, target the liver of infected bats (Drexler *et al.*, 2013). Bat hepadnaviruses are inhibited by the reverse transcriptase inhibitor, entecavir, approved for HBV treatment (Drexler *et al.*, 2013). While the presence of a bat reservoir for hepadnaviruses and the ability of these viruses to infect human cells suggest their zoonotic potential, a major caveat of these studies is that their replication in a primate species needs to be demonstrated. Nevertheless, the inability of high titer anti-HBV sera from vaccinated individuals to neutralize the New World bat hepadnavirus, TBHBV, may present a challenge for vaccination strategies to eradicate HBV from humans.

7.6 BAT METAGENOMICS STUDIES

Metagenomics studies of viral genetic material present in bat tissue, bodily fluids, and feces have aimed to define viral diversity and to detect potential zoonotic viruses circulating in bats. These studies were made possible due to the advent of next generation sequencing (Delwart, 2007), which, unlike polymerase chain reaction (PCR), does not require pre-existing knowledge of the target viral sequence. Several studies have elucidated viral genetic material associated with insectivorous and frugivorous bats from distinct geographical regions including USA, China, Myanmar, Africa, and France (Donaldson *et al.*, 2010; Li *et al.*, 2010; Ge *et al.*, 2012; Wu *et al.*, 2012; Baker *et al.*, 2013a; He *et al.*, 2013b; Dacheux *et al.*, 2014). These studies detected RNA and DNA viruses enriched from feces, oral, urine, and various tissue samples where nucleic acid was amplified by sequence-independent PCR and sequenced using next-generation sequencing platforms. The bat material contained unknown viruses and sequences related to known viruses that infect plants, fungi, and insects. Most of the sequences associated with bat material are in fact from bacteria and the host while others are of cryptic origin; for example the insect viruses are likely derived from the diet of bats. Notably, bat viral sequences related to known viruses tend to be highly divergent at the nucleotide level often requiring translation into protein for taxonomic classification. Several studies have revealed viral sequences from the *Retroviridae* family (Ge *et al.*, 2012; Wu *et al.*, 2012; Baker *et al.*, 2013a; He *et al.*, 2013b; Dacheux *et al.*, 2014). These were detected in bat fecal samples (Ge *et al.*, 2012), pharyngeal and anal swabs (Wu *et al.*, 2012), lung and other tissue (Baker *et al.*, 2013a; He *et al.*, 2013b; Dacheux

et al., 2014). However, none of these retroviral sequences are full-length and the possibility that they are from endogenous retroviral DNA or transcribed RNA cannot be excluded. In fact, due to the nature of the short viral sequences it would be difficult to definitively classify bat retroviruses using phylogenetic methods. Furthermore, verification of next generation sequencing requires PCR amplification and sequencing from the original sample. To date, there are no studies that have conclusively demonstrated the presence of an exogenous retrovirus in bats. In contrast, metagenomics analysis of tissue samples, which included liver, from Myanmar reported the first hepadnavirus sequence that comprised a large proportion of the mammalian viral sequences analysed (He *et al.*, 2013b) that has been confirmed by full genome sequencing and serological studies in bats (Drexler *et al.*, 2013).

The first metagenomics study that reported retroviral sequences was performed on fecal samples from insectivirorous bats in China (Ge *et al.*, 2012). These included sequences related to Moloney murine leukemia virus (M-MuLV) and XMRV. However, given that taxonomic analysis was performed on reads of only 35 nucleotides (nt) and that XMRV is a known laboratory contaminant (Rezaei *et al.*, 2013; Bhardwaj & Coffin, 2014), the confidence that these sequences represent real bat retroviruses is low. A subsequent metagenomics analysis of RNA and DNA viruses present in 11 insectivorous bat species from six provinces in China reported the discovery of a bat spumavirus (RaFV-1) in the intermediate horseshoe bat (*Rhinolophus affinis*) (Wu *et al.*, 2012). The partial RaFV-1 genome sequence of 2856 nt covers the encoded C-terminus of Pol and the N-terminus of Env with 52–59 and 36–38% amino acid similarity to other foamy viruses. The presence of RaFV-1 was verified by PCR and re-sequencing. Phylogenetic analysis of RaFV-1 shows that it clusters with members of the spumavirinae subfamily including human foamy virus. Analysis of mammalian viruses present in the lung of the straw-coloured fruit bat (*Eidolon helvum*) from Africa revealed that 21% of viral sequences were related to retroviruses (Baker *et al.*, 2013b). These sequences were derived from *gag*, *pol*, and *env* with the majority related to gammaretroviruses and betaretroviruses. The longest ORF represented a partial gammaretroviral polymerase protein sequence (th_NODE_62045) that was phylogenetically distinct from avian and mammalian gammaretroviruses. This sequence, which was not confirmed by PCR amplification, contained many stop codons highly indicative of an endogenous retrovirus. A metagenomic study of insectivorous bats from Myanmar and China remarkably revealed the presence of 23 contigs (of average size 114 bp) with similarity to deltaviruses in addition to gammaretroviral, betaretroviral, and spumaviruses (He *et al.*, 2013b). The sampled tissue was pooled from the laryngopharynx, trachea, lung, heart, spleen, stomach, gut, kidney, and bladder. However, whether these represent ERV or XRVs is uncertain, and endogenous deltaviruses have never been reported. A gammaretrovirus was identified in the lung tissue of the serotine bat (*Eptesicus serotinus*), a bat with anthropophilic behavior that was sick but negative for rabies (Dacheux *et al.*, 2014). Several contigs and individual reads with high-scoring segment pairs were discovered related to gammaretroviral *pol* and *env* with a 155 amino acid fragment of Pol, verified by PCR. Phylogenetic analysis of this Pol fragment showed that it was distinct from other bat gammaretroviruses M1RV, MrRV, RpuRV, RaRV, RmRV, and PaPV. Given that it is a partial sequence, the possibility that it represents an ERV cannot be ruled out. A survey of viral sequences in feces from bat species in China reported that retroviral sequences were the most frequently identified, particularly in bats belonging

the family *Hipposideridae* that are considered primarily insectivorous (Yuan *et al.*, 2014). The sequences were mainly related to the *Gammaretrovirus* and *Betaretrovirus* genera, in addition to sequences that could not be classified. The majority of translated contigs contained stop codons suggesting a probable ERV origin (Yuan *et al.*, 2014). Thus, while metagenomics studies in bats have identified sequences corresponding to several retroviral genera, notably gammaretroviruses, betaretroviruses, and spumaretroviruses, they are all from partial sequences and their verification as XRVs as well as their phylogenetic positions remains to be determined.

7.7 BATS AS POTENTIAL RESERVOIRS FOR RETROVIRAL AND HEPADNAVIRAL ZOONOSES

Bats have several properties that make them ideal viral reservoirs. They roost in large populations facilitating spread amongst bats, they are flying mammals enabling geographical spread, they have a long life-span providing greater opportunity for establishment of chronic–persistent infections, and they hibernate (an immunosuppressive state that could delay viral clearance) (Wang *et al.*, 2011). The uniqueness of bats with regard to rodents as a viral reservoir has been questioned; however bats harbor more zoonotic viruses per species and have greater capacity for interspecies transmission than their rodent counterparts (Luis *et al.*, 2013). See more discussion on this topic in Chapter 11.

While XRVs that circulate in bats are yet to be identified, the presence of a large diversity of ERVs in bats suggests their likely existence (Cui *et al.*, 2012a, b; Hayward *et al.*, 2013b; Zhuo *et al.*, 2013). It is well established that retroviruses can cross the species barrier from animals to humans, where in many cases the animals are nonhuman primates. The most notable retroviral zoonosis is HIV, a lentivirus that causes acquired immune deficiency syndrome (AIDS), with an estimated 78 million individuals infected globally since 1981 (UNAIDS, 2013). Retroviruses such as HIV require cutaneous or mucous membrane exposure to blood and/or bodily fluids for transmission. In this regard, HIV type 1 (HIV-1) and HIV type 2 (HIV-2) are thought to have entered the human population through multiple spillover events of simian immunodeficiency viruses (SIV) that naturally infect African primates hunted for bushmeat (Hahn *et al.*, 2000; Peeters *et al.*, 2002; Aghokeng *et al.*, 2010).

Cross-species transmission from non-human primates to humans of simian foamy virus (SFV), a spumavirus, and simian T-lymphotropic viruses, which are deltaretroviruses, have been reported through close contact such as bites and scratches (Betsem *et al.*, 2011) and through consumption of bushmeat (Wolfe *et al.*, 2005). There is also some evidence of cross-species transmission of betaretroviruses between humans and other animal species. Recent research has implicated the betaretroviruses JSRV (found in sheep) and MMTV (found in mice) in zoonotic transmission to humans with associated diseases of the lungs, breast tissue, and kidneys. However, these associations remain controversial and are still under active investigation (Lawson *et al.*, 2010; Mason, 2011; Selmi, 2011; Linnerth-Petrik *et al.*, 2014; Wang *et al.*, 2014). In contrast to retroviruses, the zoonotic potential of bat hepadnaviruses appears to be more tangible given the identification of bat orthohepadnaviruses that are serologically related to human HBV (Drexler *et al.*, 2013).

Similar to non-human primates, bats are also hunted as bushmeat and thus the potential for transmission of either exogenous retroviruses or hepadnaviruses directly to humans is possible. A global survey of the use of bats as bushmeat report a high level of activity throughout Asia, the Pacific Islands, some Western Indian Ocean islands, Sub-Saharan Africa, and South America (Mickleburgh *et al.*, 2009). The scale of hunting and bushmeat consumption of the fruit bat, *Eidolon helvum*, in Ghana, West Africa, is reported to be greater than previously appreciated. This is because fruit bats do not follow the conventional commodity chain for bushmeat sold in specialized bushmeat markets and in restaurants and instead are sold in marketplaces or kept by hunters for personal consumption (Kamins *et al.*, 2011). The bat taxa, *Urodemera* spp., *Hipposideros* spp., *Rhinolophus* spp., and *Miniopterus* spp. associated with the presence of bat hepadnaviruses are hunted, traded, and consumed in Cambodia, Myanmar, Papua New Guinea, Thailand, Vietnam, Bangladesh, China, Equatorial Guinea, Guinea, and South Africa (Mickleburgh *et al.*, 2009). *Rhinolophus* spp., *Myotis* spp., *Pteropus* spp., *Rousettus* sp., and *Megarderma* spp. associated with the presence of endogenous gammaretroviruses and *Pteropus* spp. and *Rhinolophus* spp. associated with endogenous betaretroviruses have been reported to be hunted in all of the abovementioned regions except South Africa and Sub-Saharan Africa (Mickleburgh *et al.*, 2009). Thus reports of bushmeat consumption intersect with bats carrying hepadnaviruses or potentially carrying retroviruses.

7.8 CONCLUSIONS

Mining of the bat genome, transcriptome and metagenome confirms the presence of retroviral sequences in bats. The fossil record of endogenous retroviruses suggests that ancient bats or their common ancestors have been infected with retroviruses in the past and that these viruses include orthologs of currently circulating gammaretroviruses and betaretroviruses. While sequences from other retroviral genera have been reported in metagenomic studies of bat material including deltaviruses, which would be notable given that endogenous deltaretroviruses have not been reported in vertebrates, the short sequences obtained from these metagenomic studies make these findings inconclusive. Mining the bat genome for ERVs has uncovered a greater diversity than observed with currently circulating (extant) retroviral counterparts and the identification of Class I ERVs that remain to be classified. The study of βERVs, in addition to highlighting their large diversity, also suggests that they have a greater propensity compared to gammaretroviruses to cross the species barrier and are more likely to spillover to humans. Geographic and systematic large-scale screening studies of active bat retroviruses are anticipated in the future to elucidate the detailed viral transmission routes between bats and other species. The discovery of a hepadnavirus in bats that is serologically related to a known human pathogen exemplifies bats as a rich source of potential viral pathogens. The practice of hunting bats for bushmeat appears to be common and spans many countries providing an opportunity for the spread of retroviruses and hepadnaviruses. Bats comprise more than one fifth of all mammalian species with the most recent common ancestor traced back to 64 million years ago (Teeling *et al.*, 2005). With such a deep ancient root as well as species richness, a great diversity of ERVs as well as XRVs is anticipated to be discovered in future studies.

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8

BAT REOVIRUSES

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8.1 INTRODUCTION

8.1.1 Background

During the past two decades researchers have increasingly recognized bats as potential reservoir hosts for emerging human-pathogenic viruses. Besides the highly pathogenic bat viruses belonging to the families *Filoviridae*, *Coronaviridae*, and *Paramyxoviridae*, members of the family *Reoviridae* were also isolated from bats and suspected to cause diseases in humans. It is assumed that reoviruses have frequently crossed the species barrier between bats and humans in Australasia, starting with the first isolation of a bat reovirus in Australia and continuing with the isolation of bat reoviruses from patients in Southeast Asia. Until recently the phenomenon of bat reovirus emergence was thought to be restricted to members of the species *Nelson Bay orthoreovirus* and the geographical region of Australasia; however, very recently, it has become apparent that the same situation might also be present in Europe. Many reoviruses have already been associated with bats, and in this chapter we will briefly summarize the bat and bat-related reoviruses that we know so far.

8.1.2 Reovirus taxonomy and disease epidemiology

The name reovirus is an abbreviation for **r**espiratory, **e**nteric **o**rphan virus. This reflects the virus's ability to infect the respiratory and enteric tract of humans, while at the time of its first description in 1959 it was not yet associated with a clinical disease in humans

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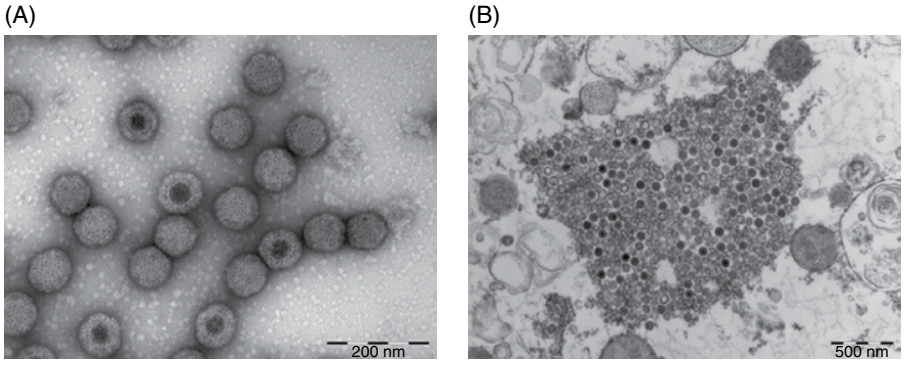

Figure 8.1 Electron micrographs of orthoreoviruses. (A) Negative staining of cell culture supernatant from Vero E6 cells infected with Mammalian orthoreovirus strain T3/Bat/Germany/342/08. Approximate diameter = 70 nm, typical double-layered capsid structure. Bar indicates 200 nm. (B) Ultrathin sectioning of Vero E6 cell culture infected with Mammalian orthoreovirus strain T3/Bat/Germany/342/08. Inclusion body, bar indicates 500 nm.

(orphan). Since then, however, many reoviruses have been isolated and identified as etiologic agents of disease in humans. All members of the family *Reoviridae* share distinct characteristics: An icosahedral, non-enveloped particle structure of about 60–80 nm in diameter and a typical double- to triple-layered capsid structure with an outer and inner protein shell (Figure 8.1) (Büchen-Osmond, 2003). Within the inner capsid, all reoviruses have a segmented, double-stranded RNA genome; nevertheless, the number of segments varies depending on the genera from 10 (e.g., genus *Orthoreovirus*) to 12 (e.g., genus *Coltivirus*) (Day, 2009).

The family *Reoviridae* is divided into the subfamilies *Sedoreoviridae* and *Spinareoviridae* (King *et al.*, 2011). The subfamily *Sedoreoviridae* comprises six genera, one of which contains highly virulent strains in humans (genus *Rotavirus*; type species: Rotavirus A) and one in animals (genus *Orbivirus*; type species: Bluetongue virus) (King *et al.*, 2011). As estimated by the WHO in 2008, about 450 000 children worldwide younger than 5 years of age die every year from a rotavirus infection. The low-income countries are predominantly affected by the severe diarrheal disease caused by rotaviruses, although it is preventable by vaccination (World Health Organization, 2014). Out of six known serotypes the most predominant is Rotavirus A, transmissible via the fecal–oral and respiratory routes of infection. The animal-pathogenic Bluetongue virus (genus *Orbivirus*) is an arbovirus transmitted by mites to animals (livestock animals and wild ruminants) in Australia, Asia, the Middle East, Africa, Europe, and the USA. Infections with Bluetongue virus are often subclinical in animals. However, outbreaks of severe Bluetongue disease occur on a regular basis all over the world. The pathology of severe Bluetongue cases in animals is similar to the pathology of hemorrhagic fever viruses in humans (Maclachlan, 2011).

The subfamily *Spinareoviridae* consist of nine distinct genera, containing the human-pathogenic genera *Coltivirus* (type species: Colorado tick fever virus) and *Orthoreovirus* (type species: mammalian orthoreovirus, MRV) (King *et al.*, 2011).

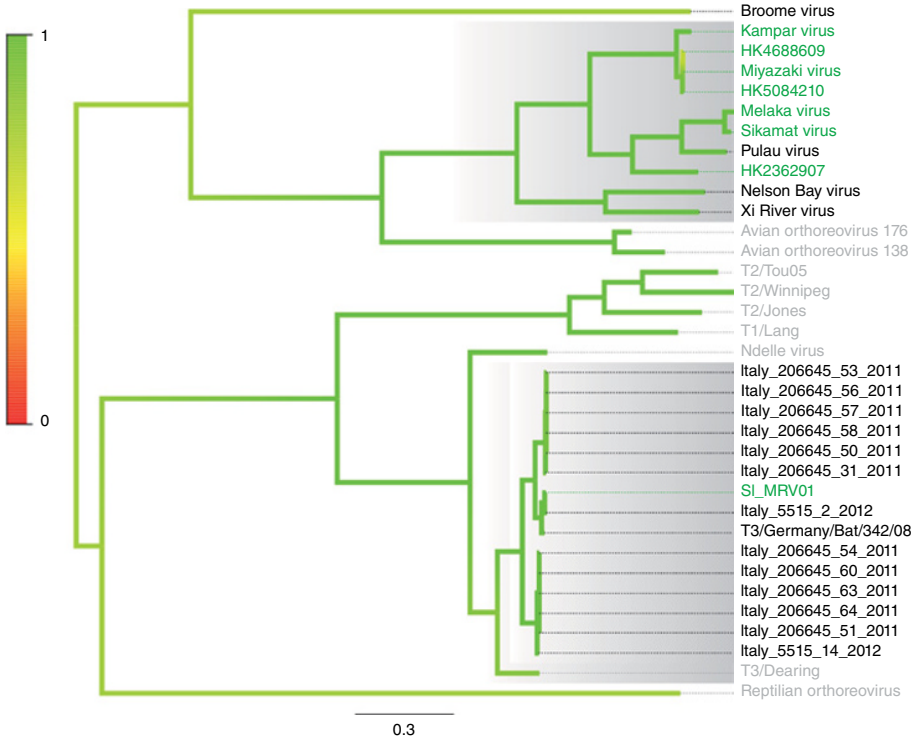

Figure 8.2 Phylogeny of orthoreoviruses. Posterior probabilities are given by color code represented by colored left-hand scale bar. Taxa are written in black (isolated from bats), green (isolated from humans), or gray (taxa provided for tree construction). The genus *Nelson Bay orthoreovirus* is depicted by the upper gray-shaded area and viruses of type 3 (T3) genus *Mammalian orthoreovirus* in the lower gray-shaded area. For Bayesian calculation of phylogenetic tree MrBayes v3.1.2 was used (Huelsenbeck *et al*., 2001), following a first model selection by using jModelTest (Posada, 2008), and model GTR+ G (gamma distribution) was selected for the alignment. The calculation parameters were as follows: number of runs: four, number of generations: 10 000 000 (partial S1 segment; 1369 nt), sample frequency: 100 and burn in: 25%. The results were finally visualized by the FigTree v1.2.1 program (http://tree.bio.ed.ac.uk/), a graphical viewer of phylogenetic trees. The scale-bar at the bottom represents the evolutionary distance of nt substitutions per position. The calculations were unrooted, but for visualization mid-point root was applied. *(See insert for color representation of the figure)*.

Colorado tick fever virus is transmitted to humans by ticks that are abundant in the Rocky Mountains in the USA and Canada. The associated disease may be so severe as to require hospitalization, and in rare cases hemorrhagic courses have also been observed (Calisher, 1994). The genus *Orthoreovirus* is divided into five distinct species: *Avian orthoreovirus*, *Baboon orthoreovirus*, *Reptilian orthoreovirus*, *Nelson Bay orthoreovirus* (also referred to as *Pteropine orthoreovirus*), and *Mammalian orthoreovirus* (King *et al.*, 2011) (Figure 8.2). Two of these five species are associated with bats: *Mammalian orthoreovirus* and *Nelson Bay orthoreovirus*.

8.2 ORTHOREOVIRUSES OF BATS AND HUMANS

In 1964, Stanley *et al.* examined the ecology and epidemiology of reoviruses in animals to prove the hypothesis of man being the major source of reoviruses (Stanley *et al.*, 1964). However, they found antibodies to MRV type 1 in sera from Western Australian bats. In several consecutive studies reoviruses of different species have been detected and isolated from bats and humans all over the world (Figure 8.3). The following sections summarize the detection of reoviruses in bats and their possible zoonotic potential. An additional overview is given in Table 8.1.

8.2.1 *Nelson Bay orthoreovirus*

Nelson Bay orthoreoviruses are sometimes also referred to as *Pteropine orthoreoviruses*, as all bat orthoreoviruses clustering in this species have been isolated from fruit bats of the family Pteropodidae. But more recently it has become evident that the species *Nelson Bay orthoreovirus* contains orthoreoviruses from two different hosts: bats and humans.

8.2.1.1 Isolated from bats The species *Nelson Bay orthoreovirus* is named after the type species Nelson Bay virus (NBV), first isolated in 1970 from the blood of a flying fox (*Pteropus poliocephalus*) in New South Wales, Australia (Gard & Compans, 1970). NBV was isolated during an epidemiologic investigation of arboviruses in the Nelson Bay area and constitutes the first reovirus isolated from a bat. Moreover, NBV was the first mammalian reovirus known to be capable of inducing syncytia formation in cell culture and animals (Wilcox & Compans, 1982). In addition to viruses of the

Figure 8.3 Map of reoviruses associated with bats worldwide. The dots indicate the sample origin of the virus isolates described. The color code indicates if viruses were isolated from humans or bats and whether these isolates cluster within the species *Nelson Bay orthoreovirus* or *Mammalian orthoreovirus*. *(See insert for color representation of the figure).*

TABLE 8.1 Orthoreoviruses isolated from bats and bat-related orthoreoviruses isolated from humans

Strain	Year	Host	Origin	Sequences available	Cluster	Reference
Nelson Bay	1970	*Pteropus* (Megachiroptera)	Australia	Genome	Pteropine ORV	Gard & Compans, 1970
Pulau	1999	*Pteropus* (Megachiroptera)	Malaysia	S1,S2,S3,S4, genome	Pteropine ORV	Pritchard *et al*., 2006; Voon *et al*., 2011
Broome	2002	*Pteropus* (Megachiroptera)	Australia	Genome	Own cluster	Thalmann *et al*., 2010
Melaka	2006	Human (Bat associated)	Malaysia	S1,S2,S3,S4, genome	Pteropine ORV	Chua *et al*., 2007; Voon *et al*., 2011
Kampar	2006	Human (Bat associated)	Malaysia	S1,S2,S3,S4, genome	Pteropine ORV	Chua *et al*., 2008; Voon *et al*., 2011
HK23629/07	2007	Human (Bat associated)	Hong Kong/ Indonesia	S1,S2,S3,S4	Pteropine ORV	Cheng *et al*., 2009; Wong *et al*., 2012
Miyazaki-Bali/2007	2007	Human (Bat related)	Japan/Indonesia	S1,S2,S3,S4	Pteropine ORV	Yamanaka *et al*., 2014
HK46886/09	2009	Human (Bat associated)	Hong Kong/ Indonesia	S1,S2,S3,S4	Pteropine ORV	Wong *et al*., 2012
HK50842/10	2010	Human (Bat related)	Hong Kong/ Indonesia	S1,S2,S3,S4	Pteropine ORV	Wong *et al*., 2012
Xi River	2010	*Rousettus* (Megachiroptera)	China	S1,S3	Pteropine ORV	Du *et al*., 2010
Sikamat	2010	Human (Bat associated)	Malaysia	S1,S2,S3,S4	Pteropine ORV	Chua *et al*., 2011
T3/Bat/Germany/342/08	2010	Vespertilionid (Microchiroptera)	Germany	Genome	Mammalian ORV	Kohl *et al*., 2012
Bat MRV019/09	2011	Vespertilionid (Microchiroptera)	Germany	partial L1	Mammalian ORV	Kohl *et al*., 2012
Bat MRV021/09	2011	Vespertilionid (Microchiroptera)	Germany	partial L1	Mammalian ORV	Kohl *et al*., 2012
T3/Bat/Italy/130366/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al*., 2013
T3/Bat/Italy/155012/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al*., 2013
T3/Bat/Italy/191797/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1	Mammalian ORV	Lelli *et al*., 2013
T3/Bat/ Italy/206645–31/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al*., 2013
T3/Bat/ Italy/206645–50/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial S1	Mammalian ORV	Lelli *et al*., 2013

(*Continued*)

TABLE 8.1 (*Continued*)

Strain	Year	Host	Origin	Sequences available	Cluster	Reference
T3/Bat/ Italy/206645–51/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–53/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–54/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–56/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–57/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–58/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–60/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–63/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/206645–64/2011	2011	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/Italy/5515–1/2012	2012	Vespertilionid (Microchiroptera)	Italy	partial L1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/Italy/5515–2/2012	2012	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/Italy/5515–3/2012	2012	Vespertilionid (Microchiroptera)	Italy	partial L1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/Italy/5515–4/2012	2012	Vespertilionid (Microchiroptera)	Italy	partial L1	Mammalian ORV	Lelli *et al.*, 2013
T3/Bat/ Italy/5515–14/2012	2012	Vespertilionid (Microchiroptera)	Italy	partial L1, S1	Mammalian ORV	Lelli *et al.*, 2013
Cangyuan	2012	*Pteropus* (Megachiroptera)	China	partial L-, M-, S-segments	Pteropine ORV	NCBI AccNo. KC994903–912 (unpublished)
SI-MRV01	2012	Human (bat related)	Slovenia	Genome	Mammalian ORV	Steyer *et al.*, 2013

species NBV, avian orthoreoviruses and baboon orthoreovirus are the only fusogenic reoviruses known so far, representing three of the five subspecies of orthoreoviruses.

Between 1998 and 1999 a severe outbreak of Nipah virus took place in Malaysia (see Chapter 4). Subsequently, animals on Tioman Island were investigated to determine the natural reservoir host species. A novel reovirus, Pulau virus (PuV), was coincidently isolated from bats (*Pteropus hypomelanus*) during the investigation, along with Nipah and Tioman paramyxoviruses (Pritchard *et al.*, 2006). Phylogenetic analysis found PuV to be closely related to NBV, and assigned as a new strain of the *Nelson Bay orthoreovirus* species. PuV induces syncytial formation in cell culture and shows cross-neutralization with NBV.

Broome virus (BroV) was isolated in 2002 from organ tissues of a flying fox (*Pteropus scapulatus*) found sick in Broome, Western Australia (Thalmann *et al.*, 2010). The bat showed neurologic symptoms suggesting an Australian bat lyssavirus (ABLV) infection. Tests confirmed the ABLV infection in the bat's brain and salivary glands. In addition, the remaining internal organs were pooled and inoculated onto cell cultures, resulting in the coincidental isolation of a novel reovirus, BroV. Phylogenetic analysis reveals significant divergence between BroV and the existing members of the *Nelson Bay orthoreovirus* species, suggesting it should possibly be considered a sixth distinct species within the genus *Orthoreovirus* (Figure 8.2).

The isolation of Xi River virus was reported in 2010 (Du *et al.*, 2010). Bats (*Rousettus leschenaultii*) were trapped in the vicinity of the Xi River in Guangdong Province, China. Cell cultures were inoculated with lung tissues of these bats. Sequence analysis proved Xi River virus to be an additional member of the species *Nelson Bay orthoreovirus*. Sequences of yet another *Nelson Bay orthoreovirus*, Cangyuan virus, also isolated from *Rousettus leschenaultii*, are available in NCBI Genbank (unpublished, refer to Table 8.1).

8.2.1.2 Isolated from humans The first bat-related orthoreovirus was isolated from a human in March 2006. Throat swabs from a 39-year-old male exhibiting respiratory symptoms, high fever, cough, and severe sore throat were inoculated onto cell cultures for virus isolation (Chua *et al.*, 2007). The virus was named Melaka virus (MelV) after the Malaysian city where it was first isolated. Two of the patient's children developed high fever and lethargy a week after the onset of the father's symptoms, and he reported that a bat had strayed into his house 7 days before he became ill. In follow-up serological testing, the patient, his pregnant wife, and the two children were positive for neutralizing antibodies to MelV, suggesting a possible human-to-human transmission. MelV was further characterized and phylogenetically identified as a member of the species *Nelson Bay orthoreovirus*. MelV shows cross-neutralization with PuV and vice versa, but does not display any neutralizing effects with MRV sera. A further cross-neutralization study with 109 sera from volunteers, collected during the search for Nipah virus on Tioman island (the same search that yielded PuV), revealed a MelV seroprevalence of 13%. MelV was the first virus identified within the species *Nelson Bay orthoreovirus* capable of infecting humans.

Five months later, in August 2006, a 54-year-old patient from Kampar, Malaysia presented with high fever, respiratory symptoms, and vomiting (Chua *et al*., 2008). The local doctor sent a throat swab from the patient to the National Public Health Laboratory where another novel reovirus was isolated from the sample. This virus was

named Kampar virus (KamV) and is phylogenetically closely related to MelV. As in the case of MelV, human-to-human transmission was suspected. Both the patient's wife and the local doctor had cross-neutralizing sera in follow-up tests. No direct contact with bats was reported, but the patient's house was surrounded by fruit trees frequently visited by bats.

During the years 2007, 2009, and 2010, three novel orthoreoviruses were isolated in Hong Kong from patients with symptoms of influenza-like illness (Cheng *et al*., 2009; Wong *et al*., 2012). All three patients (51-year-old male, 26-year-old female, and 29-year-old female) reported to have been traveling in Indonesia shortly prior to onset of symptoms. Further characterization of the novel strains HK23629/07, HK46886/09, and HK50842/10 showed their relation to the *Nelson Bay orthoreovirus* species. Only in the case from 2009 (strain HK46886/09) a previous contact with bats was reported. The woman had visited a bat cave in Bali during her stay in Indonesia.

In November 2007, a Japanese traveler (38-year-old male) developed high fever, joint pain, sore throat, and cough 11 days after returning to Japan from Bali, Indonesia (Yamanaka *et al*., 2014). A throat swab was taken and sent to the Miyazaki Prefectural Institute for virus isolation. After successful isolation the strain was named Miyazaki-Bali/2007. Forty-six serum samples were obtained from the patient's family members and caretakers, but none of these tested positive for Miyazaki-Bali/2007 antibodies. Although no serologic comparison was performed with other strains of the species *Nelson Bay orthoreovirus*, phylogenetic analysis proved its clustering with this species.

In March 2010 a 46-year-old male patient from Sikamat, Malaysia, presented with influenza-like illness with high fever, severe sore throat, and prostrating myalgia (Chua *et al*., 2011). A throat swab was taken and a novel reovirus, Sikamat virus, was isolated. Like the other isolates described earlier, Sikamat virus is another relative of the viruses within the species *Nelson Bay orthoreovirus*, which is also underlined by cross-neutralization with the other strains. The patient and his family spent their weekends in their village house which was surrounded by fruit trees inhabited by fruit bats, but no direct contact was reported. The patient's wife and one of his children had positive immunoglobulin M (IgM) and IgG antibody results for Sikamat virus.

8.2.2 Other bat-related orthoreoviruses

Mammalian orthoreoviruses (MRV) are highly divergent and opportunistic and have been demonstrated to be found in a variety of mammals including humans. MRV is associated with either unapparent or rather mild courses of disease of the respiratory or gastro-intestinal tract. Rarely, more virulent and also neurotropic strains have been reported (Hermann *et al*., 2004; Ouattara *et al*., 2011; Tyler *et al*., 2004).

8.2.2.1 Isolated from bats The first bat MRV strains T3/Bat/Germany/342/08, Bat MRV 019/09, and Bat MRV 021/09 were isolated in 2010 and 2011 from Bavarian bats (*Plecotus auritus*, *Myotis mystacinus*, *Pipistrellus pipistrellus*, and *Pipistrellus nathusii*) in Germany (Kohl *et al*., 2012). Strain T3/Bat/Germany/342/08 was found to be closely related to strain T3D/04, which was obtained from a dog pup suffering on hemorrhagic enteritis from a co-infection with parvovirus (Decaro *et al*., 2005). This is remarkable as the donor bat of strain T3/Bat/Germany/342/08 also showed hemorrhagic

enteritis. In the same year 19 novel MRV isolates were reported from various Italian bats (Table 8.1) (Lelli *et al*., 2013). Taken together, the 3 German and 19 Italian isolates constitute the first description of orthoreoviruses in bats outside of Australia and Asia, and their first detection in microbats. In contrast, all reoviruses of the species *Nelson Bay orthoreovirus* were isolated from either humans or fruit bats.

8.2.2.2 Isolated from humans In 2013 a 17-month-old child was hospitalized with acute gastroenteritis and a 5-day history of diarrhea at UMC Ljubljana, Slovenia (Steyer *et al*., 2013). A novel orthoreovirus was isolated from stool samples and named SI-MRV01. Phylogenetic analysis showed that strain SI-MRV01 is very closely related to strain T3/Bat/Germany/342/08. Epidemiological investigation showed no evidence of human-to-human transmission as none of the family members reported similar signs of disease. No contact with bats was reported, but the child had had close contact with the grandparents' dog.

8.3 BAT ORBIVIRUSES

In 1971 and 1974, bats (*Eidolon helvum*) were captured in Nigeria, Cameroon, and the Central African Republic, and eight similar virus strains were isolated and named Ife virus, after the city of Ife, Nigeria (Kemp *et al*., 1988). While no sequence data has yet been obtained, Ife virus was classified as the first bat orbivirus according to growth characteristics, serology, and electron microscopy. Another bat orbivirus, Japanaut virus, was reported in 1975 (Schnagl & Holmes, 1975). Japanaut virus was isolated from the blood of a Blossom bat (*Syconycteris crassa*) in the Sepik district of Papua New Guinea. Similar to the case of Ife virus, no sequence data of Japanaut virus is currently available, and the virus was taxonomically placed according to growth characteristics and electron microscopy. The case is similar for Fomédé virus which was repeatedly isolated from Nycteridae bats (*Nycteris gambiensis* and *Nycteris nana*) in Guinea (Boiro *et al*., 1986; Butenko, 1996; Konstantinov *et al*., 2006) before it was identified as an orbivirus by electron microscopy (Zeller *et al*., 1989).

8.4 BAT ROTAVIRUSES

During a field survey in 2007, fecal swabs from *Eidolon helvum* bats were collected in Vihiga and Maseno, Kenya (Esona *et al*., 2010). Subsequent polymerase chain reaction (PCR) screening revealed the first known bat rotavirus A strain (Bat/KE4852/07) in four individual bats. The authors found possible genomic reassortment between the described strain and human rotavirus A strains. A metagenomic study published recently described the sequence of yet another bat rotavirus A strain from France that is rather distantly related to known rotavirus A strains (bat rotavirus A b8) (Dacheux *et al*., 2014). In 2011 the first Asian bat rotavirus A strain (RVA/Bat-tc/MSLH14/2012/G3P) was isolated in Yunnan province, China, from *Rhinolophus hipposideros* bats (He *et al*., 2013). The strain was found to be related to feline and canine rotavirus strains. Sequence data of yet another, unpublished bat rotavirus strain (RVA/Bat/MYAS33/2013NSP5) is available in GenBank (Table 8.2).

TABLE 8.2 Reoviruses from the genus *Orthoreovirus* detected in bats

Strain	Year	Host	Origin	Data type	Sequences available	Cluster	Reference
Japanaut virus	1974	*Syconycteris* (Megachiroptera)	Papua New Guinea	Isolate	n/a	Orbivirus	Schnagl & Holmes, 1975
Fomédé virus	1978	Nycteridae (Microchiroptera)	Guinea	Isolate	n/a	Orbivirus	Boiro *et al.*, 1986
Ife virus	1988	*Eidolon* (Megachiroptera)	Nigeria, Cameroon, Central African Republic	8 isolates	n/a	Orbivirus	Kemp *et al.*, 1988
Bat/KE4852/07	2007	*Eidolon* (Megachiroptera)	Kenia	Fecal swabs	Partial genome	Rotavirus	Esona *et al.*, 2010
Bat rotavirus strain b8	2009	Vespertilionid (Microchiroptera)	France	Metagenome	Partial genome	Rotavirus	Dacheux *et al.*, 2014
RVA/Bat-tc/MSLH14/2012/G3P	2011	Vespertilionid (Microchiroptera)	China	Metagenome, isolate	Genome	Rotavirus	He *et al.*, 2013
RVA/Bat/MYAS33/2013NSP5	2013	n/a	China	n/a	Partial genome	Rotavirus	NCBI AccNo KF64918*6–1*88 (unpublished)

8.5 ZOONOTIC POTENTIAL OF BAT REOVIRUSES

The history of Pteropine orthoreoviruses (species *Nelson Bay orthoreovirus*) impressively demonstrates the ongoing occurrence of bat-related virus outbreaks in humans; however, none of the viruses were isolated simultaneously from both bats and humans, and thus the suspected inter-species transmission has yet to be proven. When looking at the phylogenetic relationship of the viruses within the *Nelson Bay orthoreovirus* species, no clear cluster differentiation between bat-borne and human-isolated strains is visible (Figure 8.2). One may expect these viruses to cluster in groups based on their host, but instead they appear randomly intermixed, providing further evidence of possible transmission from bats to humans. Viruses from the species *Nelson Bay orthoreovirus* have been isolated from pteropid bats, which are also reservoir hosts for zoonotic viruses such as Hendra virus and Nipah virus, which are capable of inducing severe and lethal courses of infection in humans (Clayton *et al*., 2012; Smith & Wang, 2013). Ecological factors underlying the emergence of zoonotic paramyxoviruses such as Hendra and Nipah have been identified (Chapter 10). As *Nelson Bay orthoreovirus* and other zoonotic strains have emerged concurrently in the same host species, and the same geographical region (Figure 8.3), we must consider the possibility that the same or similar ecological factors are driving the emergence of bat reoviruses.

The detection of mammalian orthoreoviruses in European bats was first described in 2012 (Kohl *et al*., 2012; Lelli *et al*., 2013). As mentioned, they display opportunism and are capable of infecting a range of different host species. These newly described mammalian orthoreoviruses cluster together in phylogenetic analyses (Figure 8.2) reminiscent of the species *Nelson Bay orthoreovirus.* The only human-isolated, bat-related mammalian orthoreovirus (SI-MRV01) is clearly more closely related to the bat isolates than to any known human isolate (Steyer *et al*., 2013). Considering the probable bat-to-human transmission events that have occurred in Southeast Asia, we may be seeing a very similar situation amongst European strains. Surveys on European bat reoviruses are fairly rare, and, to our knowledge, no consecutive surveys on bat reovirus infections in humans have been performed. A broad serological survey on these viruses in European bat species known to host these viruses, and potentially susceptible human populations, might enlighten the zoonotic potential of these viruses in Europe. All species of European bats have been described to co-roost in human dwellings. We need to clarify this situation, since the possible threat to human health remains uncertain.

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9

OTHER BAT-BORNE VIRUSES

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9.1 INTRODUCTION

Bats were first recognized as having an impact on human health in the 1920s, with the finding that bats have the ability to carry and transmit rabies virus (family *Rhabdoviridae*, genus *Lyssavirus)* to humans (Calisher *et al.*, 2006). Notably, it is now understood that all but one of the known lyssaviruses are identified as having bat reservoirs (Rupprecht *et al.*, 2011). Fear of rabies virus infection has kept people away from bats, but with increasing urbanization, movement of people and animals, and encroachment into undeveloped wildlife habitats, humans are increasingly coming into contact with wildlife, including bats, that they otherwise would not be in contact with (Jones *et al.*, 2008).

Aside from being a natural reservoir of rabies virus, bats have recently gained attention as potential hosts of new and emerging infectious diseases due to the recent outbreaks/emergences of paramyxoviruses (Hendra and Nipah) (Murray *et al.*, 1995; Selvey *et al.*, 1995; Chua *et al.*, 2000, 2002; Yob *et al.*, 2001; Field *et al.*, 2011; Smith *et al.*, 2011), Ebola virus (Leroy *et al.*, 2005, 2009), and coronaviruses (severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS))

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(Guan *et al.*, 2003; Li *et al.*, 2005; Tong *et al.*, 2009; Graham *et al.*, 2013; Memish *et al.*, 2013). In these outbreaks, bats were implicated as the source of viruses that directly or through an intermediate host led to human infections (Smith & Wang, 2013). The SARS-CoV outbreak in 2002/2003 with its global spread and approximately 8000 cases over 6 months highlighted the potential of bats to be a source of novel human pathogens (Graham *et al.*, 2013).

With the availability of sensitive molecular tools including metagenomics/next-generation sequencing and generic viral taxa-group polymerase chain reaction (PCR) assays, it has recently been possible to detect a wide range of viruses in bats, (Donaldson *et al.*, 2010; Li L *et al.*, 2010; Ge *et al.*, 2012; Tse *et al.*, 2012b; He *et al.*, 2013; Coffey *et al.*, 2014; Dacheux *et al.*, 2014), though little is known of the biology of these infections. None of these recently discovered novel viruses first identified in bats have yet been shown to be a significant public health concern; nevertheless, the potential for bat viruses to emerge as important pathogens should not be underestimated. In this chapter we survey bat-borne viruses other than those discussed in other chapters. The viral families discussed herein are listed in Table 9.1 along with the bat species identified as harboring the virus.

9.2 RNA VIRUSES

9.2.1 Influenza viruses

Influenza viruses belong to the family *Orthomyxoviridae*, and are grouped in three genera: *Influenzavirus A*, *B*, and *C*. Virus particles are enveloped and are comprised of eight negative-sense, single-stranded RNA segments (Palese &Shaw, 2007). Influenza A viruses are isolated from many animal species including humans, pigs, dogs, horses, mink, felids, marine mammals, and a wide range of birds (Webster *et al.*, 1992; Fouchier *et al.*, 2007) and their zoonotic spread to humans occurs and presents a public health threat. Influenza B and C viruses are predominantly human pathogens and have been sporadically isolated from seals and pigs, but their zoonotic spread is not known to be important to human disease (Guo *et al.*, 1983; Osterhaus *et al.*, 2000). In contrast, zoonotic transmission is important to human influenza A virus infection. Influenza A viruses are continually undergoing molecular changes through mutations (antigenic drift), reassortment (antigenic shift), and in rare instances, recombination, causing yearly epidemics and sometimes pandemics (Palese, 2004; Lozano *et al.*, 2012; Fineberg, 2014; Wang *et al.*, 2014). Birds are considered the primary natural reservoir for influenza A viruses and 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes of influenza virus A have been detected in many different combinations in wild birds and poultry throughout the world (Alexander, 2000; Munster & Fouchier, 2009). Influenza A was recently detected in fruit bats from Guatemala (*Sturnira lilium*) and Peru (*Artibeus planirostris*) with low frequencies, 0.9% and 0.8%, respectively (Tong *et al.*, 2012, 2013). High rates of immunoglobulin G (IgG) positivity against recombinant bat-derived HA and/or NA in both Guatemalan bats (38%) and Peruvian bats (50%) (Tong *et al.*, 2013) suggest influenza infection in New World bats is common. Phylogenetically, the influenza viruses detected in bats are quite distinct from all known influenza viruses but are most closely related to influenza A viruses (Figure 9.1).

TABLE 9.1 Viruses identified from bats, worldwide

Virus	Bat species (common name)
Family *Orthomyxoviridae*, genus *Influenzavirus A*	
Influenza A virus	*Sturnira lilium* (little yellow-shouldered bat), *Artibeus planirostris* (flat-faced fruit-eating bat)
Family *Togaviridae*, genus *Alphavirus*	
Chikungunya virus	*Scotophilus* sp., *Rousettus aegyptiacus* (Egyptian fruit bat), *Rousettus leschenaulti* (Leschenault's rousette), *Hipposideros caffer* (Sundevall's roundleaf bat), *Chaerephon pumilus* (little free-tailed bat)
Sindbis virus	*Rhinolophus* sp., *Hipposideros* sp.
Venezuelan equine encephalitis virus	*Desmodus rotundus* (common vampire bat), *Uroderma bilobatum* (tent-making bat), *Artibeus phaeotis* (pygmy fruit-eating bat), *Artibeus turpis* (teapa fruit-eating bat), *Carollia perspicillata* (Seba's short-tailed bat)
Eastern equine encephalitis virus	*Myotis* sp., *Eptesicus* sp.
Western equine encephalitis virus	*Eptesicus* sp.
Family *Bunyaviridae*, genus *Nairovirus*	
Ahun virus	*Myotis mystacinus* (whiskered bat), *Pipistrellus pipistrellus* (common pipistrelle)
Issyk-Kul virus	*Nyctalus noctula* (common noctule)
Family *Bunyaviridae*, genus *Phlebovirus*	
Rift Valley fever virus	*Micropteropus pusillus* (Peter's dwarf epauletted fruit bat), *Hipposideros abae* (Aba roundleaf bat), *Miniopterus schreibersii* (common bent-wing bat), *Hipposideros caffer* (Sundevall's roundleaf bat), *Epomops franqueti* (Franquet's epauletted bat), *Glauconycteris argentata* (silvered bat)
Toscana virus	*Pipistrellus kuhlii* (Kuhl's pipistrelle)
Malsoor virus	*Rousettus* sp.
Family *Bunyaviridae*, genus *Hantavirus*	
Hantaan virus	*Epteiscus serotinus* (serotine bat), *Rhinolophus ferrumequinum* (greater horseshoe bat)
Mouyassué virus	*Neoromicia nanus* (banana pipistelle)
Magboi virus	*Nycteris hispida* (hairy slit-faced bat)
Xuan Son virus	*Hipposideros pomona* (Pomona roundleaf bat)
Huangpi virus	*Pipistrellus abramus* (Japanese house bat)

(*Continued*)

TABLE 9.1 (*Continued*)

Virus	Bat species (common name)
Longquan virus	*Rhinolophus affinis* (intermediate horseshoe bat), *Rhinolophus sinicus* (Chinese rufous horseshoe bat), *Rhinolophus monoceros* (Formosan lesser horseshoe bat)
Family *Bunyaviridae*, genus *Orthobunyavirus*	
Kaeng Khoi virus	*Chaerephon plicatus* (wrinkle-lipped free-tailed bat), *Taphozous theobaldi* (Theobald's tomb bat)
Catu virus	*Molossus obscurus* (Thomas' mastiff bat)
Guama virus	Unidentified bat
Nepuyo virus	*Artibeus jamaicensis* (Jamaican fruit bat), *Artibeus lituratus* (great fruit-eating bat)
Mojui dos Campos virus	Unidentified bat
Bangui virus	*Tadarida* sp., *Pipistrellus* sp., *Scotophilus* sp.
Family *Flaviviridae*, genus *Flavivirus*	
Bukalasa bat virus	*Chaerephon pumilus* (little free-tailed bat), *Tadarida condylura* (Angolan free-tailed bat)
Carey Island virus	*Cynopterus brachyotis* (lesser short-nosed fruit bat), *Macroglossus minimus* (long-tongued nectar bat)
Central European encephalitis virus	Unidentified bat
Dakar bat virus	*Chaerephon pumilus* (little free-tailed bat), *Taphozous perforatus* (Egyptian tomb bat), *Scotophilus* sp., *Mops condylurus* (Angolan free-tailed bat)
Entebbe bat virus	*Chaerephon pumilus* (little free-tailed bat), *Mops condylurus* (Angolan free-tailed bat)
Sepik virus	Unidentified bat
Japanese encephalitis virus	*Hipposideros armiger terasensis* (great roundleaf bat), *Miniopterus schreibersii* (common bent-wing bat), *Rhinolophus cornutus* (little Japanese horseshoe bat)
Dengue virus	*Myotis nigricans* (black myotis), *Pteronotus parnelli* (Parnell's mustached bat), *Natalus stramineus* (Mexican funnel-eared bat), *Artibeus jamaicansis* (Jamaican fruit bat), *Carollia brevicauda* (silky short-tailed bat)
West Nile virus	*Eptesicus fuscus* (big brown bat), *Tadarida brasiliensis* (Mexican free-tailed bat), *Myotis lucifugus* (little brown bat), *Myotis septentrionalis* (Northern long-eared myotis), *Rousettus leschenaulti* (Leschenault's rousette)

TABLE 9.1 (*Continued*)

Virus	Bat species (common name)
Jugra virus	*Cynopterus brachyotis* (lesser short-nosed fruit bat)
Kyasanur Forest disease virus	*Rhinolophus rouxii* (rufous horseshoe bat), *Cynopterus sphinx* (greater short-nosed fruit bat)
Montana myotis leucoencephalitis virus	*Myotis lucifugus* (little brown bat)
Phnom-Penh bat virus	*Eonycteris spelaea* (cave nectar bat), *Cynopterus brachyotis* (greater short-nosed fruit bat)
Rio Bravo virus	*Tadarida braziliensis mexicana* (Mexican free-tailed bat), *Eptesicus fuscus* (big brown bat)
St. Louis encephalitis virus	*Tadarida brasiliensis mexicana* (Mexican free-tailed bat)
Saboya virus	*Nycteris gambiensis* (Gambian slit-faced bat)
Sokuluk virus	*Vespertilio pipistrellus* (common pipistrelle)
Tamana bat virus	*Pteronotus parnellii* (Parnell's mustached bat)
Uganda S virus	*Rousettus* sp., *Tadarida* sp.
Yokose virus	Unidentified bat
Family *Arenaviridae*, genus *Arenavirus*,	
Tacaribe virus (TCRV)	*Artibeus lituratus* (great fruit-eating bat), *Artibeus jamaicensis* (Jamaican fruit bat), *Desmodus rotundus* (common vampire bat), *Sturnira lilium* (little yellow-shouldered bat), *Platyrrhinus helleri* (Heller's broad-nosed bat)
Family *Picornaviridae*, genus *Kobuvirus*	
Bat kobuvirus	Unidentified bat
Family *Picornaviridae*, genus *Mischivirus*	
Miniopterus schreibersii picornavirus 1	*Miniopterus schreibersii* (common bent-wing bat)
Family *Picornaviridae*, genus unassigned	
Bat picornavirus 1	*Miniopterus pusillus* (small bent-wing bat), *Miniopterus schreibersii* (common bent-wing bat)
Bat picornavirus 2	*Miniopterus magnate* (Western bent-wing bat)
Bat picornavirus 3	*Hipposideros armiger* (great roundleaf bat), *Rhinolophus sinicus* (Chinese rufous horseshoe bat)
Ia io picornavirus 1	*Ia io* (great evening bat)
Rhinolophus affinis picornavirus 1	*Rhinolophus affinis* (intermediate horseshoe bat)

(*Continued*)

TABLE 9.1 (*Continued*)

Virus	Bat species (common name)
Family *Astroviridae*, genus *Mamastrovirus*	
Unassigned species	*Hipposideros armiger* (great roundleaf bat), *Hipposideros larvatus* (intermediate roundleaf bat), *Hipposideros pomona* (Pomona roundleaf bat), *Ia io* (great evening bat), *Miniopterus magnater* (Western bent-wing bat) , *Miniopterus pusillus* (small bent-wing bat), *Miniopterus schreibersii* (common bent-wing bat), *Myotis chinensis* (large myotis), *Myotis myotis* (greater mouse-eared bat), *Myotis ricketti* (Rickett's big-footed bat), *Pipistrellus abramus* (Japanese house bat), *Rhinolophus ferrumequinum* (greater horseshoe bat), *Rhinolophus pearsonii* (Pearson's horseshoe bat), *Rhinolophus rouxii* (rufous horseshoe bat), *Rousettus leschenaultii* (Leschenault's rousette), *Scotophilus kuhlii* (lesser Asiatic yellow bat), *Taphozous melanopogon* (black-bearded tomb bat), *Tylonycteris robustula* (greater bamboo bat)
Family *Caliciviridae*, genus *Sapovirus*	
Bat sapovirus	*Hipposideros pomona* (Pomona roundleaf bat)
Family *Adenoviridae*, genus *Mastadenovirus*	
Ryukyu virus 1	*Pteropus dasymallus yayeyamae* (Ryukyu flying fox)
Bat adenovirus 2	*Pipistrellus pipistrellus* (common pipistrelle)
Bat adenovirus 3	*Myotis ricketti* (Rickett's big-footed bat)
Unassigned species	*Nyctalus noctula* (common noctule), *Rhinolophus ferrumequinum* (greater horseshoe bat), *Myotis sp.*, *Scotophilus kuhlii* (lesser Asiatic yellow bat), *Desmodus rotundus* (common vampire bat), *Eidolon helvum* (straw-coloured fruit bat), *Antrozous pallidus* (pallid bat), *Tadarida brasiliensis* (Mexican free-tailed bat), *Chaerephon* sp., *Otomops martienssi*
Family *Herpesviridae*, subfamily *Alphaherpesvirinae*	
Unassigned species	*Eidolon dupreanum* (Madagascan fruit bat), *Eidolon helvum* (straw-coloured fruit bat), *Pteropus lylei* (Lyle's flying fox), *Lonchophylla thomasi* (Thomas' nectar bat)

TABLE 9.1 (*Continued*)

Virus	Bat species (common name)
Family *Herpesviridae*, subfamily *Betaherpesvirinae*	
Bat betaherpesvirus 1	*Myotis nattereri* (Natterer's bat), *Pipistrellus pipistrellus* (common pipistrelle)
Bat betaherpesvirus 2	*Miniopterus fuliginosus* (Eastern bent-wing bat)
Rhinolophus ferrumequinu betaherpesvirus 1	*Rhinolophus ferrumequinum* (greater horseshoe bat)
Tylonycteris robustula betaherpesvirus 1	*Tylonycteris robustula* (greater bamboo bat)
Miniopterus schreibersii herpesvirus	*Miniopterus schreibersii* (common bent-wing bat)
Unassigned species	*Rousettus aegyptiacus* (Egyptian fruit bat), *Eptesicus fuscus* (big brown bat)
Family *Herpesviridae*, subfamily *Gammaherpesvirinae*	
Bat gammaherpesvirus 1	*Eptesicus serotinus* (serotine bat), *Myotis nattereri* (Natterer's bat), *Pipistrellus nathusii* (Nathusius' pipistrelle), *Pipistrellus pipistrellus* (common pipistrelle)
Bat gammaherpesvirus 2	*Myotis nattereri* (Natterer's bat), *Myotis myotis* (greater mouse-eared bat)
Bat gammaherpesvirus 3	*Nyctalus noctula* (common noctule), *Myotis nattereri* (Natterer's bat), *Myotis myotis* (greater mouse-eared bat)
Bat gammaherpesvirus 4	*Nyctalus noctula* (commone noctule), *Myotis nattereri* (Natterer's bat)
Bat gammaherpesvirus 5	*Pipistrellus nathusii* (Nathusius' pipistrelle)
Bat gammaherpesvirus 6	*Pipistrellus pipistrellus* (common pipistrelle)
Bat gammaherpesvirus 7	*Pipistrellus pipistrellus* (common pipistrelle)
Myotis ricketti gammaherpesvirus 1	*Myotis ricketti* (Rickett's big-footed bat)
Myotis ricketti gammaherpesvirus 2	*Myotis ricketti* (Rickett's big-footed bat)
Unassigned species	*Rousettus aegyptiacus* (Egyptian fruit bat), *Eptesicus serotinus* (serotine bat), *Hipposideros diadema* (diadem leaf-nosed bat)
Family *Herpesviridae*, unassigned subfamily	
Agua Preta virus	*Carollia subrufa* (gray short-tailed bat)
A cytomegalovirus	*Myotis lucifugus* (little brown bat)
Family *Poxviridae*, subfamily *Chordopoxvirina*e	
WA2011	*Eptesicus fuscus* (big brown bat)
Unassigned species	*Eidolon helvum* (straw-coloured fruit bat), *Miniopterus schreibersii* (common bent-wing bat)

(*Continued*)

TABLE 9.1 (*Continued*)

Virus	Bat species (common name)
Family *Polyomaviridae*, genus *Orthopolyomavirus*	
Bat polyomavirus	*Myotis lucifugus* (little brown bat)
Bat polyomavirus 2a	*Desmodus rotundus* (common vampire bat)
Bat polyomavirus 2b	*Pteronotus parnellii* (Parnell's mustached bat)
Bat polyomavirus 2c	*Artibeus planirostris* (flat-faced fruit-eating bat)
Bat polyomavirus 3a	*Artibeus planirostris* (flat-faced fruit-eating bat), *Sturnira lilium* (little yellow-shouldered bat)
Bat polyomavirus 3b	*Molossus molossus* (velvety free-tailed bat)
Bat polyomavirus 4a	*Artibeus planirostris* (flat-faced fruit-eating bat)
Bat polyomavirus 4b	*Carollia perspicillata* (Seba's short-tailed bat)
Chaerephon polyomavirus 1	*Chaerephon sp.*
Otomops polyomavirus 1	*Otomops martiensseni* (large-eared free-tailed bat)
Otomops polyomavirus 2	*Otomops martiensseni* (large-eared free-tailed bat)
Eidolon polyomavirus 1	*Eidolon helvum* (straw-coloured fruit bat)
Cardioderma polyomavirus	*Cardioderma cor* (heart-nosed bat)
Miniopterus polyomavirus	*Miniopterus inflatus* (greater long-fingered bat)
Pteronotus polyomavirus	*Pteronotus davyi* (Davy's naked-backed bat)
Unassigned species	*Rousettus aegyptiacus* (Egyptian fruit bat)
Family *Parvoviridae*, genus *Bocavirus*	
Myotis myotis bocavirus 1	*Myotis myotis* (greater mouse-eared bat)
Family *Parvoviridae*, genus *Dependovirus*	
Unassigned species	*Antrozous pallius* (pallid bat), *Myotis daubentonii* (Daubenton's bat), *Myotis ricketti* (Rickett's big-footed bat), *Rhinolophus sinicus* (Chinese rufous horseshoe bat), *Rhinolophus affinis* (intermediate horseshoe bat), *Rhinolophus pearsonii* (Pearson's horseshoe bat), *Rhinolophus macrotis* (big-eared horseshoe bat) , *Hipposideros armiger* (great roundleaf bat), *Hipposideros larvatus* (intermediate roundleaf bat), *Scotophilus kuhlii* (lesser Asiatic yellow bat), *Miniopterus schreibersii* (common bent-wing bat), unidentified bat
Family *Parvoviridae*, unclassified genus	
Eidolon helvum bat parvovirus 1	*Eidolon helvum* (straw-coloured fruit bat)
Artibeus jamaicensis bat parvovirus 1	*Artibeus jamaicensis* (Jamaican fruit bat)
Family *Papillomaviridae*	
Rousettus aegyptiacus papillomavirus 1	*Rousettus aegyptiacus* (Egyptian fruit bat)
Miniopterus schreibersii papillomavirus 1	*Miniopterus schreibersii* (common bent-wing bat)

TABLE 9.1 (*Continued*)

Virus	Bat species (common name)
Miniopterus schreibersii papillomavirus 2	*Miniopterus schreibersii* (common bent-wing bat)
Myotis ricketti papillomavirus 1	*Myotis ricketti* (Rickett's big-footed bat)
Eidolon helvum papillomavirus 1	*Eidolon helvum* (straw-coloured fruit bat)
Pteropus giganteus papillomavirus 1	*Pteropus giganteus* (Indian flying fox)
Epteiscus serotinus papillomavirus 1	*Epteiscus serotinus* (serotine bat)
Epteiscus serotinus papillomavirus 2	*Epteiscus serotinus* (serotine bat)
Epteiscus serotinus papillomavirus 3	*Epteiscus serotinus* (serotine bat)
Rhinolophus ferrumequinum papillomavirus 1	*Rhinolophus ferrumequinum* (greater horseshoe bat)
Unassigned species	*Eidolon helvum* (straw-coloured fruit bat), unidentified bat

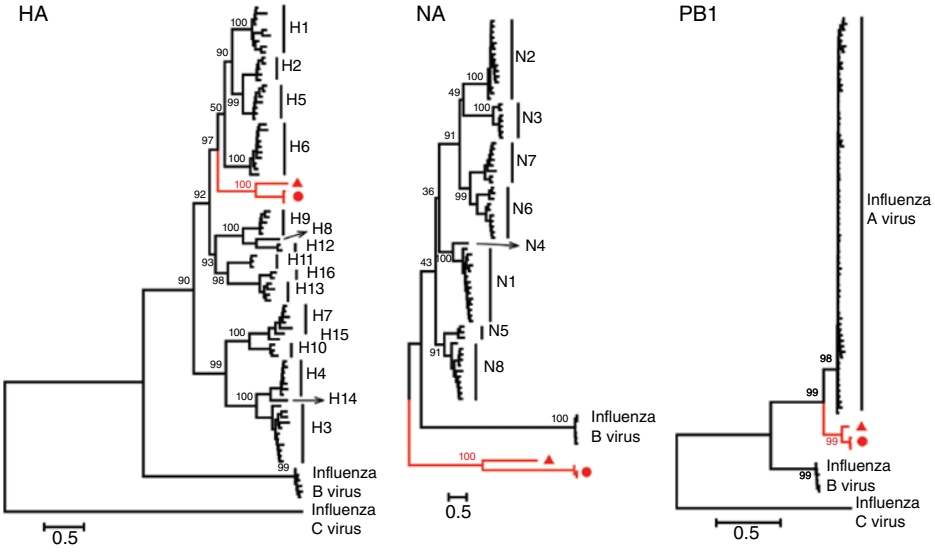

Figure 9.1 Evolution of influenza virus in New World bats. Phylogenetic relationships of influenza A viruses sampled from bats (red branches) and other animals ('non-bat', black branches) based on the amino acid sequences of three representative gene segments (HA, NA, and PB1). *(See insert for color representation of the figure).*

Structural features of the genomes of these bat influenza viruses are also suggestive of a type A influenza virus. The three bat influenza viruses identified in Guatemala were nearly identical to each other (96–99% nucleotide identity) and have been classified as a novel influenza A virus species, H17N10 (Tong *et al.*, 2012). An additional influenza virus was identified in a Peruvian fruit bat that is phylogenetically distinct from the ones identified in Guatemala, representing a second novel subtype, H18N11

(Tong *et al.*, 2013). The sequence differences among these viruses in some gene segments is greater than all other mammalian and avian species combined, suggesting a potentially important, likely ancient, reservoir for diversity of influenza viruses (Tong *et al.*, 2013).

Notably, the HA and NA-like (NAL) molecules of the newly detected bat influenza viruses do not appear to have sialic acid binding activities which fits with the high degree of divergence observed at the sequence level (Shi *et al.*, 2010; Li *et al.*, 2012; Zhu *et al.*, 2012, 2013; Sun *et al.*, 2013; Wu *et al.*, 2014). Crystal structures of the newly identified H17, H18, N10, and N11 have been obtained and help provide some additional insight. Structurally, both H17 and H18 are similar to other influenza A HAs (Gamblin & Skehel, 2010; Sun *et al.*, 2013; Tong *et al.*, 2013; Zhu *et al.*, 2013). However, H17 and H18 lack many of the known conserved residues in the receptor binding sequence (RBS) (Tong *et al.*, 2013; Zhu *et al.*, 2013). Both H17 and H18 lack the ability to bind a variety of sialic acids and glycans, suggesting that these bat influenza viruses utilize a unique protein receptor (Tong *et al.*, 2012, 2013; Sun *et al.*, 2013; Zhu *et al.*, 2013). The newly identified NAL molecules, N10 and N11, share very little sequence identity to other influenza NA genes, but are structurally similar. Both N10 and N11 NALs do contain calcium which is found in all known influenza A and B NA active sites for stabilization (Li *et al.*, 2012; Zhu *et al.*, 2012; Tong *et al.*, 2013). Very few of the amino acid residues within the putative neuraminidase active site conserved among other influenza viruses are conserved in N10 and N11 and the putative active site is much larger than other known neuraminidases (Li *et al.*, 2012; Tong *et al.*, 2013; Zhu *et al.*, 2012). Taken together, these results indicate that sialic acid is neither a receptor for virus attachment nor a substrate for virus release, suggesting a novel mechanism of bat influenza A virus attachment and activation of membrane fusion for entry into host cells (Tong *et al.*, 2013).

To date, although attempts to culture bat influenza viruses have been unsuccessful both in a variety of cell culture systems and chicken embryos, which are routinely used for influenza virus culture, viral transcription from reporter minigenomes is functional in human and primate cells (Tong *et al.*, 2012, 2013). A recent study found that a recombinant virus with six of the eight bat influenza virus A/little yellow-shouldered bat/Guatemala/164/2009 genes and the remaining two chimeric genes encoding the hemagglutinin and neuraminidase proteins from A/SC35M (seal influenza A) was able to replicate well in mammalian cells and mice, but poorly in avian cells and chicken embryos, and was unable to reassort with other influenza A viruses (Juozapaitis *et al.*, 2014). A more recent study suggests that the bat-influenza virus is unlikely to reassort with an influenza A virus or influenza B virus and spread to other species even if they were to infect the same host cell (Zhou *et al.* 2014).

In addition avian and swine influenza A viruses have recently been grown in bat cell lines derived from: *Rousettus aegyptiacus*, *Hypsignathus monstrosus*, *Epomops buettikoferi*, *Rhinolophus alcyone*, *Carollia perspicillata*, and *Tadarida brasiliensis*, indicating a possibility that bats could serve as vessels for influenza virus reassortment (Hoffmann *et al.*, 2013). Since the detection of novel influenza A viruses in New World fruit bats is quite recent, additional detection, serology and biological characterization studies are needed to assess the prevalence and diversity of influenza A viruses present in bats, and their potential to cross species barriers and emerge in new hosts.

9.2.2 Alphaviruses

Alphavirus is a genus in the family *Togaviridae*. Alphaviruses have spherical, enveloped particles (70 nm diameter) with a positive sense, single-stranded RNA genome ranging in length between 11 000 and 12 000 nucleotides. Alphaviruses are an important cause of arthropod-borne encephalitis in humans and also infect various other vertebrates such as rodents, fish, birds, and larger mammals such as horses, as well as invertebrates (Griffin, 2007; Knipe & Howley, 2013). A few alphaviruses causing human disease have been identified in bats, including Venezuela, eastern and western equine encephalitis viruses (VEEV, EEEV, and WEEV, respectively) from various bat species in multiple regions of America (Price, 1978a; McLean *et al.*, 1979; Ubico & McLean, 1995), Sindbis virus from *Rhinolophidae* and *Hipposideridae* bats (Blackburn *et al.*, 1982; Calisher *et al.*, 2006) and Chikungunya virus from *Rousettus leschenaulti*, *R. aegyptiacus*, *Hipposideros caffer*, *Scotophilus* sp., and *Chaerephon pumilus* bats (Calisher *et al.*, 2006). Notably, antibodies to VEEV were detected more frequently in bats that roosted in close proximity to humans and livestock (Blackburn *et al.*, 1982). Sindbis virus can infect and cause neurological symptoms in cave bats (*Myotis lucifugus*) under laboratory conditions (Brueckner *et al.*, 1956; Thompson *et al.*, 2014). These data may suggest a possible role of bats in the enzootic maintenance and spread of these important zoonotic alphaviruses. However, further study is needed to clarify the role of bats as amplification/reservoir hosts for these alphaviruses.

9.2.3 Bunyaviruses

Viruses from the family *Bunyaviridae* form enveloped, spherical virions (about 80–120 nm in diameter) and have a negative-sense, single-stranded, tripartite segmented RNA genome. The *Bunyaviridae* is a large family comprising over 300 individual virus species grouped into five genera: *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (Schmaljohn & Nichol, 2007). With the exception of *Hantavirus*, a genus of viruses which is transmitted by rodents, other members of the family *Bunyaviridae* are transmitted via arthropods and infect a variety of vertebrate or plant hosts. Bats are also known to harbor members of *Nairovirus*, *Phlebovirus*, *Hantavirus*, and *Orthobunyavirus.*

9.2.3.1 Nairoviruses The Ahun virus was identified in two different insectivorous bat species, *Myotis mystacinus* and *Pipistrellus pipistrellus*, collected from two different geographic locations in close proximity to humans in France. Phylogenetic analysis demonstrated that the Ahun virus has diverged considerably from the other known viruses in the genus *Nairovirus* (Dacheux *et al.*, 2014). Since nairoviruses are predominantly tick-borne and bats are frequently parasitized by ticks, this virus is likely transmitted by ticks and could potentially infect other mammalian hosts. The Issyk-Kul virus (ISKV) is another distinct nairovirus isolated from both insectivorous bats (*Nyctalus noctula*) and ticks in Kirghizia (Lvov *et al.*, 1973a).

9.2.3.2 Phlebovirus Rift Valley fever (RVF) virus was isolated from Peter's epauletted fruit bat (*Micropteropus pusillus*) and the aba roundleaf bat (*Hipposideros abae* sp.) in the Republic of Guinea (Boiro *et al.*, 1987). Furthermore, *Miniopterus schreibersii* and *Eptesicus capensis* are known to be susceptible to experimentally induced RVF

virus infection. Though both RVF virus antigen and specific antibodies to RVF virus were identified among wild bats, none of the bats developed signs of clinical illness. It is possible that these species of bat play a role in RVF virus transmission between mosquitoes and other domestic and wild mammals (Boiro *et al.*, 1987; Oelofsen & Van der Ryst, 1999). Malsoor virus is a bat phlebovirus isolated from *Rousettus* sp. bats from Mahabaleshwar, Maharashtra State, India. Phylogenetic analysis showed closer clustering of Malsoor virus with Heartland virus and severe fever with thrombocytopenia syndrome virus (SFTS), both of which have caused severe human diseases elsewhere (Mourya *et al.*, 2014). Toscana virus (TOSV) is an arthropod-borne virus mainly identified from *Phlebotomus* species, being a major cause of meningitis and encephalitis in Mediterranean countries, and has also been isolated once from a bat in areas where *Phlebotomus perniciosus* and *P. perfiliewi* were present (Verani *et al.*, 1988). However no hemagglutination-inhibiting antibodies were found in sera from these bats and the role of bats in the maintenance of the transmission cycle of TOSV remains unclear.

9.2.3.3 Hantavirus Hantaviruses are etiological agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans (Schmaljohn *et al.*, 2007; Zeier *et al.*, 2005). Rodents have long been recognized as natural reservoirs for hantaviruses, but more recently the host range has been expanded by the discovery that shrews, moles and bats can also be infected and might serve as additional reservoirs (Kim *et al.*, 1994). Hantaan virus and Puumala virus were both isolated in rodents and cause severe human diseases in Korea, however both are also prevalent in rhinolophus bats, which are suspected as a natural reservoir of hantaviruses in Korea (Lee, 1998). Several other identified bat hantaviruses include Mouyassué virus (MOUV) from banana pipistrelle bats (*Neoromicia nanus*) captured near Mouyassué village in Cote d'Ivoire, West Africa (Sumibcay *et al.*, 2012), Magboi virus (MGBV) from hairy split-faced bats (*Nycteris hispida*) found near the Magboi River in Sierra Leone (Weiss *et al.*, 2012), Xuan Son virus (XSV) in Pomona round leaf bats (*Hipposideros pomona*) from Vietnam (Arai *et al.*, 2013), Huangpi virus (HUPV) in the Japanese house bat (*Pipistrellus abramus*), and Longquan virus (LQUV) in rhinolophus bats (*R. affinis*, *R. sinicus*, *R. monoceros*) in China (Guo *et al.*, 2013). In the phylogenetic tree, MOUV, XSV, MGBV, HUPV, and LQUV are highly divergent and basal to all other rodent- and soricomorph-borne hantaviruses, with the exception of Nova virus and Altai virus in insectivore species (shrews and moles) (Weiss *et al.*, 2012; Guo *et al.*, 2013). These bat hantaviruses tend to form monophyletic groups but with great diversity (Guo *et al.*, 2013). Because the phylogenetic trees of the hantaviruses do not always match those of their mammalian hosts, it is likely that cross species infection and co-divergence have contributed to hantavirus evolution (Kang *et al.*, 2009; Ramsden *et al.*, 2009; Guo *et al.*, 2013). Overall, it appears that bats are likely to be important natural reservoir hosts of some hantaviruses, although more evidence is required to elucidate such associations.

9.2.3.4 Orthobunyavirus The Kaeng Khoi (KK) virus was repeatedly isolated from *Chaerephon plicatus* and *Taphozous theobaldi* bats from Thailand in 1969, 1970, and 1971 (Williams *et al.*, 1976; Neill, 1985) and 30 years later its minor variant named as Cambodian bat virus was identified from dead *Chaerephon plicatus* bats in Kampot, Cambodia (Osborne *et al.*, 2003). The low nucleotide variation among KK viruses has

been observed over 30 years and across a broad geographical area, similar to that seen for other members of the *Orthobunyavirus* genus, suggests these viruses are genetically relatively stable in nature, possibly related to their need to grow and compete in both invertebrate and vertebrate hosts (Brockus & Grimstad, 2001; Osborne *et al.*, 2003). In the phylogenetic tree, the KK virus forms a unique group within the genus *Orthobunyavirus* which is consistent with serological cross reactivity studies. Genetically, the closest match is with members of the California encephalitis serogroup (<60% nucleotide identity). KK virus-neutralizing antibodies were detected in bat guano workers in a bat cave in Thailand (Neill, 1985) and KK virus was also isolated from bat bugs (Cimicidae*)* in the same cave, suggesting that Cimicidae bugs might be vectors of KK virus transmission (Williams *et al.*, 1976). Several other orthobunyaviruses, like Catu virus and Guama virus from Guama serogroup, Nepuyo virus from Group C serogroup, Mojui dos Campos virus and Bangui virus have also been isolated from wild caught bats (Berge, 1975; Miura & Kitaoka, 1977; Calisher *et al.*, 2006). Mojui dos Campos virus and Bangui virus remain ungrouped, since they do not share antigenic cross activity with other orthobunyaviruses.

9.2.4 Flaviviruses

Flavivirus is a genus of viruses in the family *Flaviviridae*. Its members share several common features: enveloped and spherical virion that is 40–65 nm in diameter, a nucleocapsid in icosahedral-like symmetry and positive-sense, single-stranded RNA genome of approximately 10 000–11 000 bases (Lindenbach, 2007). Most of these viruses are arthropod-borne viruses (arboviruses), which are transmitted to host animals by arthropod vectors such as mosquitoes and ticks. Hence, flaviviruses can be divided into three groups: mosquito-borne, tick-borne, and unknown vector groups. The known bat flaviviruses are mainly in the unknown vector group but also include several in the mosquito-borne and tick-borne groups. About 60% of flaviviruses are known to be etiological agents of human diseases, of which dengue virus types 1–4 (DENV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis viruses (TBEV) are recognized important human pathogens with significant public health and economic impacts. The role of bats in the transmission and ecology of DENV, WNV, SLEV, YFV, TBEV and other known human flaviviruses is not well understood, but several studies have demonstrated that bats of some species are susceptible to infection by these viruses and some of them have been detected genetically and/or serologically from bats of various species in multiple locations (Sulkin *et al.*, 1966; Paul *et al.*, 1970; Miura & Kitaoka, 1977; Platt *et al.*, 2000; de Thoisy *et al.*, 2009; Jeffrey Root, 2013). For example, DENV with evidence of nucleic acid, antibody or both was repeatedly identified from bats in various regions of America where all four serotypes (DENV-1, DENV-2, DENV-3, DENV-4) are present and DENV disease is endemic (Platt *et al.*, 2000; Aguilar-Setien *et al.*, 2008; de Thoisy *et al.*, 2009; Machain-Williams *et al.*, 2013). WNV was isolated in fruit bats from Southern India in 1968 and was found to be genetically similar to a human patient strain isolated in 1967 in the same region (Paul *et al.*, 1970). Serological screening also suggested that WNV occurred in bats in the Yucatan Peninsula of Mexico (Machain-Williams *et al.*, 2013) and in North America (Pilipski *et al.*, 2004; Bunde *et al.*, 2006; Jeffrey Root, 2013). SLEV was isolated from Mexican free-tailed bats (*Tadarida b.*

mexicana) collected during an epidemic of St. Louis encephalitis in Houston, Texas in 1964 (Phillips & Melnick, 1965). Later serologic data from a field survey suggested SLEV also occurs in bats in the Yucatan Peninsula of Mexico (Machain-Williams *et al.*, 2013) and in brown bats in Ohio, USA (Herbold *et al.*, 1983), which were indeed shown to be susceptible to experimental SLEV infection (Sulkin *et al.*, 1964, 1966). JEV can infect humans and a variety of vertebrate animals. Carried by mosquitoes, wild pigs and water birds are considered the natural reservoirs of JEV (Mackenzie *et al.*, 2004). The role of bats in JEV epidemiology has been explored in multiple locations since 1963 when bats were demonstrated susceptible to JEV infection (Sulkin *et al.*, 1970). JEV viruses and/or serum antibody against JEV have been identified from bats in multiple regions in Asia where outbreaks of Japanese encephalitis constantly occur (Sulkin *et al.*, 1970; Miura *et al.*, 1970; Sulkin & Allen, 1974; Banerjee *et al.*, 1988; Mackenzie *et al.*, 2004; Cui *et al.*, 2008; Wang *et al.*, 2009; Zhang *et al.*, 2009; Liu *et al.*, 2013). No conclusive evidence of YFV exists yet in wild bats; however, bats can be infected with YFV following ingestion of infected mosquitoes and low prevalence of antibody in bats to YFV was reported previously, which could be due to serological cross reactivity (Sulkin, 1962; Simpson & O'Sullivan, 1968). However, Sepik virus (SEPV), Entebbe bat virus (ENTV), Sokuluk virus (SOKV), and Yokose virus (YOKV) are genetically and antigenically related to YFV and have been identified in bats from Papua New Guinea, Africa, central Asia, and far east Asia, respectively (Lvov *et al.*, 1973b; Boiro *et al.*, 1987; Kuno *et al.*, 1998; Tajima *et al.*, 2005; Kuno & Chang, 2006; Watanabe *et al.*, 2010b). Unlike the mosquito-borne flaviviruses YFV, JEV, SLEV, Jugra virus and SEPV, some bat flaviviruses like Dakar bat virus, Bukalasa bat virus, Carey Island virus, Entebbe bat, Phnom-Penh bat virus, Rio Bravo bat virus, Montana Myotis leukoencephalitis virus (MML), YOKV, Tamana bat virus (TABV), and SOKV have been identified only from bats and not from any arthropod vector (Lvov *et al.*, 1973b; Tajima *et al.*, 2005; Calisher *et al.*, 2006; Kuno & Chang, 2006). ENTV, YOKV, and SOKV in the mosquito-borne group replicate in mosquito cells and further study of potential arthropod vectors may identify vectors for these viruses in the future (Kuno & Chang, 2006). In the past, taxonomic classification of the over 80 members of the genus *Flavivirus* was mainly based on antigenic cross-reactivity in neutralization, complement fixation and hemagglutination tests. More recently, phylogenetic analysis based on genetic relatedness has shown that mode of transmission correlates strongly with phylogeny with tick-borne, mosquito-borne, and unknown vector viruses representing major splits. The relative positions of the mosquito-borne, tick-borne and unknown vector clades, however, differ depending on which gene is analyzed (*NS3*, *NS5*, or full genome) (Kuno *et al.*, 1998; Billoir *et al.*, 2000; Cook & Holmes, 2006; Kuno & Chang, 2006; Volkova *et al.*, 2012). The bat flaviviruses in the mosquito-borne clade are with groups of JEV, DENV, WNV, SLEV, YFV-like, such as SEPV, ENTV, SOKV, YOKV, Jugra virus, and Uganda S virus, respectively. Of the mosquito-borne clade, vectors for ENTV, SOKV, and YOKV are unknown, suggesting an unrecognized vector or loss of vector-borne transmission (Cook & Holmes, 2006; Kuno & Chang, 2006). The bat flaviviruses within the unknown vector clade have only been isolated from bats and are grouped into five different subclades (Rio Bravo, MML, Bukalasa, Dakar, Phnom Penh), with TABV being highly divergent from the others and listed as an unclassified flavivirus although it shares many characteristics with the flaviviruses (Cook & Holmes, 2006; de Lamballerie *et al.*, 2002). It has been suggested that flaviviruses of unknown vectors

including the bat- and insect-specific flaviviruses are more ancient and diverged before the vector-borne viruses (Billoir *et al.*, 2000; de Lamballerie *et al.*, 2002; Cook & Holmes, 2006; Cook *et al.*, 2012; Volkova *et al.*, 2012).

9.2.5 Arenaviruses

The family *Arenaviridae* is comprised of bisegmented, negative-sense RNA viruses known for causing lymphocytic choriomeningitis and hemorrhagic fevers in humans. Members of *Arenaviridae* are mainly associated with rodents, while Tacaribe virus (TCRV) poses an exception to this rule and is presently the only case of natural arenavirus infection in bats. TCRV was first isolated from six great fruit-eating bats (*Artibeus lituratus*) and five Jamaican fruit bats (*A. jamaicensis*) collected in the 1950s near Port-of-Spain, Trinidad (Downs *et al.*, 1963). Although no isolations or virus sequences have been obtained since then, TCRV positive sera were later found in *A. jamaicensis*, *A. lituratus*, the common vampire bat (*Desmodus rotundus*), the little yellow-shouldered bat (*Sturnira lilium*), and Heller's broad nosed bat (*Platyrrhinus helleri*) from Trinidad (Price, 1978b), as well as in a great fruit-eating bat from Guatemala (Ubico & McLean, 1995).

Currently, arenaviruses can be divided into two serogroups; the LCMV-Lassa virus (Old World) complex and the TCRV (New World) complex. TCRV is closely related to viruses that are highly pathogenic to humans, including Junin, Machupo, Guanarito, and Sabia viruses (Bowen *et al.*, 1996). Despite this, TCRV itself is not a recognized human pathogen. The only known TCRV infection was laboratory acquired and had flu-like symptoms (Cogswell-Hawkinson *et al.*, 2012). The true reservoir host for TCRV is still unclear at this stage. On the one hand, TCRV has only been detected and isolated from *Artibeus* sp. bats and the search for other potential hosts, especially small rodents, has been unsuccessful (Downs *et al.*, 1963), favoring the hypothesis that *Artibeus* bats are its natural host. On the other hand, Jamaican fruit bats have been experimentally infected with TCRV but to low titer, short duration, at low inoculum and with a high fatality rate at high inoculum, and transmission between infected and uninfected bats was not demonstrated (Price, 1978b). This is in contrast to arenaviruses in rodents, which establish persistent infections with no significant pathology. These observations suggest that Jamaican fruit bats are not likely to be a natural reservoir for TCRV (Price, 1978b).

9.2.6 Picornaviruses

The family *Picornaviridae* is comprised of small, positive-sense RNA viruses that infect a wide range of vertebrates. It is a large family with more than 12 established genera and an increasing number of unclassified and highly diverse species. Within the family tree, bat picornaviruses can be classified under four major lineages (Figure 9.2). The first picornavirus discovered in bats was a kobuvirus. It was recovered from a metagenomics study of a bat guano collected from various roost sites at Point Reyes National Seashore of North California (Li L *et al.*, 2010). Later, using degenerative primer sets targeting conserved regions in the 3D gene, three groups of sapelovirus-related picornaviruses were discovered from alimentary specimens from bats from rural areas of Hong Kong (Lau *et al.*, 2011), i.e. bats from the *Hipposideros*, *Miniopterus*, *Pipistrellus*, and *Rousettus* genera, with detection rates of 0.3% ~ 3.2%. In a third study, diverse viruses

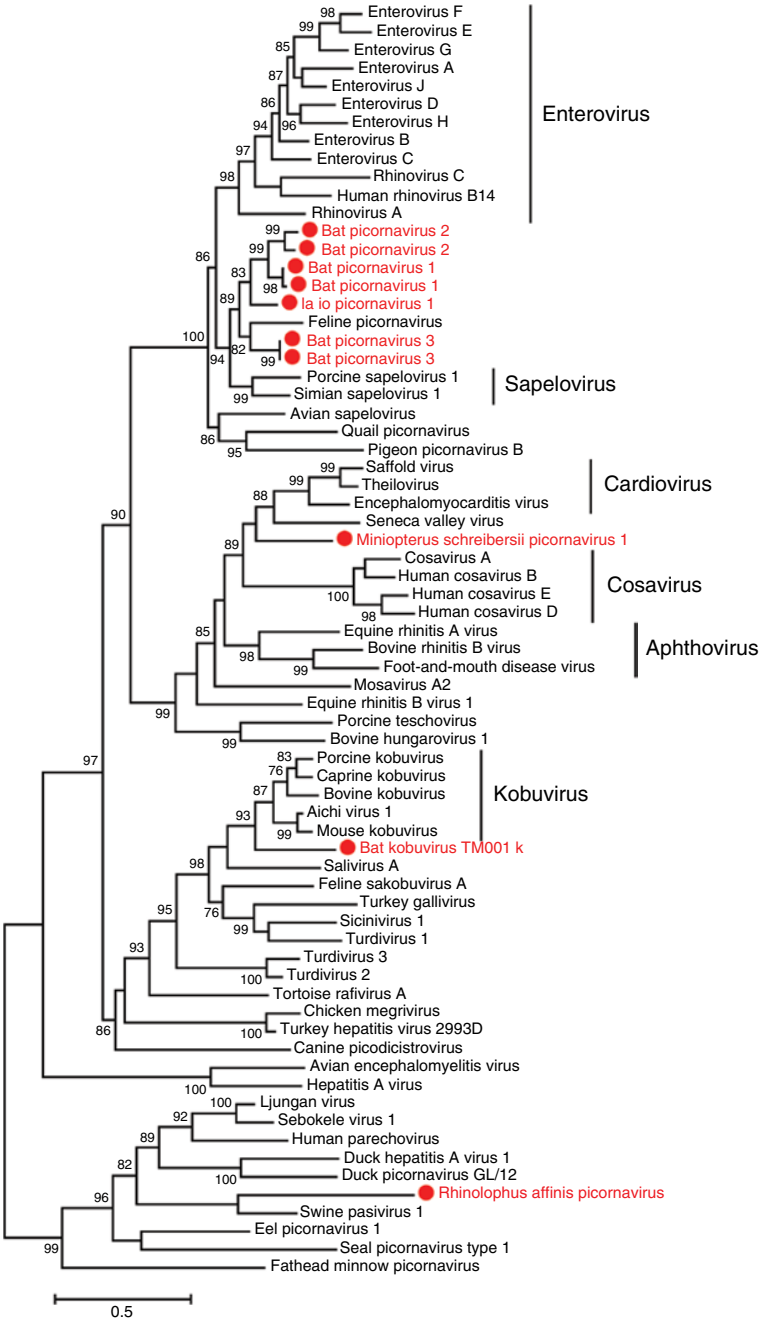

Figure 9.2 The phylogenetic positions of bat picornaviruses within the context of the entire family *Picornaviridae*. The tree is reconstructed using maximum likelihood methods implemented in PhyML program and is mid-point rooted. The data set is comprised of partial and complete polyprotein sequences of bat picornavirus as well as those of the type species within the family. The bat sequences are indicated in red. The names of the species and genera are labeled to the right of the tree. *(See insert for color representation of the figure).*

within the family *Picornaviridae* were discovered by deep sequencing of bat pharyngeal and anal swab samples (Wu *et al.*, 2012). The viruses were recovered from three bat species; the common bent-wing bat (*Miniopterus schreibersii*), great evening bat (*Ia io*), and the intermediate horseshoe bat (*Rhinolophus affinis*), collected from Yunnan, Guizhou, and Hainan provinces of China, respectively (Wu *et al.*, 2012). In total, there are at least seven types (species) of bat picornaviruses discovered to date.

The largest lineage, which contains bat picornavirus 1–3 (Lau *et al.*, 2011) and *Ia io* picornavirus 1 (Wu *et al.*, 2012), is related to the genera *Sapelovirus* and *Enterovirus*, although substantial differences between the genome structures are observed among these viruses (Lau *et al.*, 2011). Furthermore, bat viruses within this lineage are not monophyletic: Bat picornavirus 3 is actually more closely related to feline picornavirus (Lau *et al.*, 2012) than to the other bat picornaviruses within this cluster, which implies more complex host dynamics involving multiple mammalian species. Indeed, the lack of correlation between host specificity and phylogenetic clustering further indicates the presence of active cross-species transmission among these bat picornaviruses (Lau *et al.*, 2011). The remaining bat picornaviruses, Miniopterus schreibersii-1, Bat kobuvirus, and Rhinolophus affinis-1, belong to distinct phylogroups in the phylogeny (Figure 9.2). Each of them is represented by a single virus and all three are distantly related to the other known picornaviruses, namely, genera *Cardiovirus* and *Senecavirus*, genus *Kobuvirus*, and the as-yet unclassified Swine pasivirus 1, respectively. There is little information on the biology of infection and disease for these bat viruses. One study of captured bats positive for Bat picornavirus 1–3 showed no disease associated with infection (Lau *et al.*, 2011). Compared to coronaviruses and astroviruses, the detection rate for bat picornaviruses is very low. It is likely that more picornaviruses will be detected in future studies, however, care must be taken in interpreting the results of guano metagenomics because viruses identified could come from their diet and not from infection (Donaldson *et al.*, 2010; Li L *et al.*, 2010; Ge *et al.*, 2012; Wu *et al.*, 2012).

9.2.7 Astroviruses

Astroviruses are known for causing enteric infections in infants and young children. The family *Astroviridae* is comprised of two genera, *Mamastroviruses* (MAstVs) and *Avastroviruses* (AAstVs), which infect mammalian and avian hosts, respectively. Since 2008, there has been a proliferation of bat-borne astrovirus detections through active surveillance of viruses in bats in Hong Kong (Chu *et al.*, 2008), mainland China (Zhu *et al.*, 2009; Xiao *et al.*, 2011), and Germany (Drexler *et al.*, 2011). These detections have led to a great expansion of the known diversity within MAstVs. Metagenomics studies of bat fecal samples have detected much of this diversity (Li L *et al.*, 2010; Wu *et al.*, 2012). Remarkably, within guano samples collected at bat roost sites, Li *et al.* (2010) were able to recover a highly divergent astrovirus-like sequence (677 bp) which occupied a basal position between MAstVs and AAstVs, although further verifications of both sequence and host are certainly required. Like bat coronaviruses, astroviruses were recovered from both fecal and oral samples in a number of bat genera, namely, *Miniopterus*, *Myotis*, *Hipposideros*, *Rhinolophus*, *Pipistrellus*, *Scotophilus*, and *Taphozous* (Chu *et al.*, 2008; Zhu *et al.*, 2009; Drexler *et al.*, 2011; Xiao *et al.*, 2011). Importantly, the prevalence rates for astroviruses in bats are generally very high. The total rate in rectal samples reached 46% and 44.8% in studies by Chu *et al.* (2008) and

Zhu *et al.* (2009), respectively. Interestingly, the prevalence rates showed significant seasonal (Drexler *et al.*, 2011) and geographic (Xiao *et al.*, 2011) variation such that infection dynamics of astroviruses in bats may resemble those of human and domestic animals. There is no evidence for disease associated with these infections in bats (Drexler *et al.*, 2011).

In the phylogeny, bat astroviruses take up a substantial part of the diversity within MAstVs and have a paraphyletic relationship where some of the viruses cluster with other members of MAstV (Chu *et al.*, 2008; Zhu *et al.*, 2009). Based on the RNA-dependent RNA polymerase (RdRp) gene phylogenetic tree, bat astroviruses form two distinct clades; one is "bat-only", and one that clusters with human/mink/ovine/porcine astroviruses. This topology implies independent host switching events between bat and non-bat hosts (Zhu *et al.*, 2009). Within the bat astroviruses, the phylogeny shows various degrees of correlation with host specificity (Zhu *et al.*, 2009), which may reflect distinct host spectrums of these viruses.

9.2.8 Caliciviruses

Caliciviruses can be divided into five genera: *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus*, and *Nebovirus*. The genera *Norovirus* and *Sapovirus* are associated with enteric disease in humans. Bat calicivirus was first identified using targeted reverse transcription (RT)-PCR screening, which involved 728 rectal swabs from 14 bat species captured in rural areas of Hong Kong (Tse *et al.*, 2012a). Virus was only recovered from *Hipposideros pomona* samples with low prevalence rate (1.56%). To date, this virus remains the sole representative of caliciviruses in bats.

The bat calicivirus isolate was assigned as a divergent member of the genus *Sapovirus*, based on genomic structure, sequence similarity, and phylogenetic analyses of the major conserved proteins (ORF1 precursor polyprotein and VP1) (Tse *et al.*, 2012a). While sharing elements of commonality with sapoviruses, the bat virus has several important and unique features that distinguish it from the rest of genus, including a highly divergent VP2, a different reading frame, a high G+C content, and low CpG suppression (Tse *et al.*, 2012a). Interestingly, the on-site examination of the captured bats showed no obvious signs of enteric disease in infected animals (Tse *et al.*, 2012a). There is insufficient data to determine whether bats are an important reservoir species for sapoviruses.

9.3 DNA VIRUSES

9.3.1 Adenoviruses

The *Adenoviridae* family of viruses is characterized by having linear, double-stranded DNA genomes 26–48 kilobases in length. *Adenoviridae* can be classified into five genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus*. All human adenoviruses are within the genus *Mastadenovirus*, which also contains viruses that infect other mammals. Adenoviruses are associated with a variety of illnesses in humans including respiratory disease, conjunctivitis, and gastroenteritis (Berk, 2007). The first bat adenovirus was identified while attempting to

establish a bat spleen cell line from *Pteropus dasymallus yayeyamae*. Cytopathic effect was seen at an early passage, indicating the presence of a virus (Maeda *et al.*, 2008). Subsequently, adenoviruses have been described in bats from guano samples (Drexler *et al.*, 2011; Li L *et al.*, 2010; Li Y *et al.*, 2010b), oral and rectal swabs (Wu *et al.*, 2012; Baker *et al.*, 2013), urine (Baker *et al.*, 2013), and organs from healthy and moribund/dead animals (Maeda *et al.*, 2008; Sonntag *et al.*, 2009; Kohl *et al.*, 2012; Raut *et al.*, 2012; Lima *et al.*, 2013). In addition, bat adenoviruses identified from these studies have a wide geographic distribution and host range, which includes *Pteropus dasymallus yayeyamae*, *Pipistrellus pipistrellus*, *Nyctalus noctula*, *Rhinolophus ferrumequinum*, *Myotis* sp., *Scotophilus kuhlii*, *Desmodus rotundus*, *Eidolon helvum*, *Antrozous pallidus*, and *Tadarida brasiliensis* (Maeda *et al.*, 2008; Sonntag *et al.*, 2009; Li L *et al.*, 2010; Li Y *et al.*, 2010b; Drexler *et al.*, 2011; Raut *et al.*, 2012; Wu *et al.*, 2012; Lima *et al.*, 2013; Baker *et al.*, 2013). Adenoviral DNA has also been detected in *Chaerephon* sp. and *Otomops martienssi* bats from Kenya (Conrardy *et al.*, 2014). Based on phylogenetic analysis of a nearly complete (BtAdV-TJM) and complete (BtAdV2) bat adenovirus genome sequences, they are most closely related to canine adenoviruses and tree shrew adenovirus (Kohl *et al.*, 2012; Li Y *et al.*, 2010b). There is strong support based on multiple phylogenetic analyses that bat adenoviruses 2 and 3 share a distant common ancestor with canine adenovirus (Kohl *et al.*, 2012). Based on currently available data, adenoviruses appear to be fairly diverse and prevalent within bat populations (Li Y *et al.*, 2010b). Interestingly, several studies have demonstrated the presence of adenoviruses in bats without obvious detriment to the host. In particular, histopathological examination of infected tissues suggests that infection causes little or no disease (Maeda *et al.*, 2008; Kohl *et al.*, 2012; Raut *et al.*, 2012; Lima *et al.*, 2013). Transcriptional analysis of a bat adenovirus-infected bat cell line revealed differential expression of host immune response genes, which may be responsible for lessening the consequences of infection (Wu *et al.*, 2013).

9.3.2 Herpesviruses

The family *Herpesviridae* is delineated by having large, double-stranded DNA genomes of roughly 120–240 kilobases. Herpesviruses are classified into three subfamilies *Alphaherpesvirinae* (αHV), *Betaherpesvirinae* (βHV), and *Gammaherpesvirinae* (γHV) (Pellett & Roizman, 2007), and human pathogens are present in all three subfamilies. A common characteristic of herpesviruses is the ability to establish a lifelong persistent infection within the host. Herpesviruses of all three subfamilies have been detected in bats from North America, South America, Europe, Africa, and Asia. Specifically, the viruses have been found in both healthy and moribund/dead bats from organ tissue (spleen, lungs) (Wibbelt *et al.*, 2007; Molnar *et al.*, 2008; Watanabe *et al.*, 2009, 2010a; Janoska *et al.*, 2011; Zhang *et al.*, 2012; Dacheux *et al.*, 2014), and throat swabs/saliva and rectal swabs (Donaldson *et al.*, 2010; Razafindratsimandresy *et al.*, 2009; Wu *et al.*, 2012). Due to the large size of herpesvirus genomes, only one complete bat herpesvirus genome (MsHV, a βHV) has been published (Zhang *et al.*, 2012). In phylogenetic analyses, bat herpesviruses within all three subfamilies (alpha-, beta-, and gamma-) tend to cluster with other bat herpesviruses within the same subfamily. They are distantly related to some human herpesviruses along with some non-human primate herpesviruses (Razafindratsimandresy *et al.*, 2009; Janoska *et al.*, 2011; Wu *et al.*, 2012; Zhang

et al., 2012). Several additional bat herpesviruses were identified in metagenomic studies but the sequence data is insufficient for robust phylogenetic analysis (Donaldson *et al.*, 2010; Baker *et al.*, 2013; Dacheux *et al.*, 2014). The biology of bat herpesvirus is not well understood. Analysis of a full-length bat betaherpesvirus genome, MsHV, demonstrated the presence of multiple genes associated with putative host immune evasion (Zhang *et al.*, 2012). In humans, herpesvirus infection of an immune-competent host is generally mild and/or asymptomatic, depending on the herpesvirus, and persists for the lifetime of the host. Further studies will need to be carried out to understand herpesvirus infections of bats.

9.3.3 Poxviruses

The *Poxviridae* family is comprised of viruses with large double-stranded DNA genomes (130–300 kilobases) that, uniquely, replicate in the cytoplasm rather than the nucleus. *Poxviridae* is divided into two subfamilies: *Chordopoxvirinae* (vertebrate hosts) and *Entomopoxvirinae* (insect hosts). Within *Chordopoxvirinae* are diverse viruses with a broad host range, which includes several important human pathogens such as variola virus (causative agent of smallpox) and molluscum contagiosum virus (MCV) (Moss, 2007). Furthermore, poxviruses from other vertebrates can cross the species barrier and infect humans as well (i.e. cowpox and monkeypox viruses (Shchelkunov, 2013)). To date, only a limited number of poxviruses have been identified in bats and none have been fully sequenced. The first bat poxvirus was identified from synovial tissue in a symptomatic *Eptesicus fuscus* bat in the United States and is most closely related to Cotia virus, an unclassified murine poxvirus in *Chordopoxvirinae*. The bat that carried this virus had infected synovial tissue with visibly swollen joints consistent with arthritis (Emerson *et al.*, 2013). A similar disease syndrome can be found in human smallpox infections, which are characterized by osteomyelitis with arthritis, but the number of cases are very limited (Eeckels *et al.*, 1964). Subsequently, potential poxvirus genome fragments were discovered in a metagenomics study on throat swabs of *Eidolon helvum*. The sequences recovered are related to molluscum contagiosum virus (MCV), a human poxvirus that has no known animal reservoir. The result was further confirmed by PCR examination on additional throat swabs from *Eidolon helvum* bats (5 of 40 samples positive) (Baker *et al.*, 2013). A third bat poxvirus was identified in a cutaneous lesion on a *Miniopterus schreibersii bassanii* bat from South Australia. The identification was based on electron microscopy only and no genetic characterization of the virus was performed (McLelland *et al.*, 2013).

9.3.4 Polyomaviruses

Polyomaviruses are small, encapsidated, double-stranded DNA viruses that infect mammals as well as birds. Their ~5 kilobase genome is packaged into a 45 nm particle. In humans, infection typically occurs in early childhood and is persistent throughout life without pathogenic consequences. Polyomaviruses can cause disease in immunocompromised individuals and distinct types of human polyomaviruses are continually being discovered (Imperiale, 2007). The first bat polyomavirus was discovered in Canadian *Myotis* sp. bats in 2009 (Misra *et al.*, 2009). Since then, several additional bat polyomaviruses have been characterized by molecular approaches, including metagenomics

approaches (Fagrouch *et al.*, 2012; Baker *et al.*, 2013; Tao *et al.*, 2013). Bat polyomaviruses have been found in bats from South America and Africa with prevalence rates ranging between 11–40%, indicating a global distribution (Fagrouch *et al.*, 2012; Tao *et al.*, 2013). Bats harbor an extensive diversity of polyomaviruses, which are distantly related to primate polyomaviruses, rodent polyomaviruses, sea lion polyomavirus, and bovine polyomavirus (Misra *et al.*, 2009; Tao *et al.*, 2013). Bat polyomaviruses have been identified in tissues, oral, and rectal swabs and do not appear to be associated with any morbidity (Misra *et al.*, 2009; Fagrouch *et al.*, 2012; Tao *et al.*, 2013).

9.3.5 Parvoviruses

The *Parvoviridae* family of viruses is distinguished by single-stranded DNA genomes approximately 5 kb in length. The *Parvovirinae* subfamily, which infects vertebrates, is divided into five genera: *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus*, and *Parvovirus.* A unique genus of *Parvoviridae* is the *Dependovirus* genus, consisting of viruses that usually require coinfection with a helper virus (such as an adenovirus) to replicate (Berns & Parrish, 2007). Several viruses within the *Parvoviridae* family are important human and animal pathogens. In a metagenomics study characterizing the virome of bat guano, a parvovirus-like sequence was identified in guano from a bat roost near San Saba, Texas (Li L *et al.*, 2010). Based on limited sequence analysis, the parvovirus-like sequences were distantly related to known parvoviruses. Since the guano was collected non-invasively the species of bat that harbored the parvovirus-like sequence was not identified but was likely one of the following species: *Tadarida brasiliensis*, *Myotis velifer*, *Nycticeus humeralis*, or *Perimyotis subflavus.* In the same study, an adeno-associated virus was identified from guano of a California bat belonging to one of the following species: *Antrozous pallidus*, *Myotis* spp., *Tadarida brasiliensis* (Li L *et al.*, 2010). A second study using serum samples identified two bat parvoviruses, one in *Eidolon helvum* and a second in *Artibeus jamaicensis* (Canuti *et al.*, 2011). While detections in bat guano do not distinguish between infection and ingested virus, the detection of viruses in serum indicate that these bats were actively infected. Recently, a metagenomics study identified a bat bocavirus from oral/rectal swabs of *Myotis myotis* from China (Wu *et al.*, 2012). Several adeno-associated viruses (AAV, members of the *Dependovirus* genus) have been identified in a number of Chinese bats (22.4% prevalence) (Li Y *et al.*, 2010a; Ge *et al.*, 2012;). The bat AAVs were identified from fecal swabs from 10 different insectivorous bat species (*Rhinolophus affinis*, *Rhinolophus sinicus*, *Rhinolophus pearsoni*, *Rhinolophus macrotis*, *Hipposideros armiger*, *Hipposideros larvatus*, *Myotis daubentoni*, *Myotis ricketti*, *Scotophilus kuhlii*, *Miniopterus schreibersii*).

The bat parvoviruses are related to several different genera within *Parvovirinae*. The bat bocavirus from China is most closely related to bovine parvovirus-1 by analysis of the NS1 protein and the VP1 protein (Wu *et al.*, 2012). Limited sequences from a metagenomics study detected sequences that matched with members of the genera *Parvovirus*, *Erythrovirus*, and *Bocavirus*, but additional studies are needed to determine whether these sequences represent true bat parvoviruses (Ge *et al.*, 2012). There have been numerous AAV sequences identified in bats at robust prevalence levels, and sequence has been obtained for the replication-related protein and capsid protein open reading frames for one bat AAV. Phylogenetic analysis showed that the bat AAV was

most closely related to a porcine AAV and is distantly related to primate AAVs (Li Y *et al.*, 2010a; Ge *et al.*, 2012).

9.3.6 Papillomaviruses

Members of the family *Papillomaviridae* contain ~8 kilobase circular, double-stranded DNA genomes and are non-enveloped. *Papillomaviridae* has large genetic diversity with species/types that infect humans, non-human primates, a large diversity of other mammals, birds, and reptiles (Bernard *et al.*, 2010). Human papillomaviruses (HPV) are important human pathogens, and include types or strains that are associated with cancers in humans (Howley & Lowy, 2007). The first report of a bat papillomavirus was in 2006 in a *Rousettus aegyptiacus* bat that had basosquamous carcinoma (McKnight *et al.*, 2006; Rector *et al.*, 2006). Since that study, papillomaviruses have been identified in bats from a number of anatomical sites including rectal swabs (Tse *et al.*, 2012b), hair follicles/bulbs (Garcia-Perez *et al.*, 2013), and oral swabs, urine, and lung tissue (Baker *et al.*, 2013; Garcia-Perez *et al.*, 2014). Tse and colleagues identified a unique bat papillomavirus from a rectal swab taken from a *Miniopterus schreibersii*; however further sampling by either rectal swabs (95 bats), or an additional 419 samples from various anatomical sites failed to detect any additional cases (Tse *et al.*, 2012b). The low prevalence rate may be due to the specimen type. Indeed, a 2014 study found Spanish bats harboring papillomaviruses in oropharyngeal swabs with a prevalence rate of 21%. Four distinct bat papillomaviruses were detected in *Eptesicus serotinus* and one was detected in *Rhinolophus ferrumequinum* (Garcia-Perez *et al.*, 2014). Additionally, potentially unique papillomavirus sequences have been identified by a metagenomics approach in *Eidolon helvum*, in which sequences were primarily identified from throat swabs, but also in lungs and urine to a lesser extent (Baker *et al.*, 2013). Finding papillomaviruses in bats in mucosal areas (rectal/oral swabs, hair follicles/bulbs) is compatible with the known mucosal epithelium tropism of human papillomaviruses.

The fully sequenced bat papillomaviruses show distinct phylogenetic relationships to other papillomaviruses with at least five unique bat papillomavirus lineages present (Garcia-Perez *et al.*, 2013). Bat papillomavirus lineages are interspersed throughout the *Papillomaviridae* phylogenetic tree, indicating multiple evolutionary events that led to papillomavirus infection of bats (Garcia-Perez *et al.*, 2014). Most of the bat papillomaviruses have been identified in healthy bats; however the fact that the first bat papillomavirus was identified in a basosquamous carcinoma in a *Rousettus aegyptiacus* bat (McKnight *et al.*, 2006; Rector *et al.*, 2006), indicates the pathogenic potential of at least one bat papillomavirus.

9.4 CONCLUSIONS

In summary, bats naturally harbor viruses from many different families. Bats are increasingly recognized as reservoirs for many viruses that can infect humans, domestic animals and other wildlife (Calisher *et al.*, 2006). But for many other viruses detected in bats, the role of bats as either amplification or reservoir hosts is unknown. The high population density, ability to migrate over long distances, species diversity, and various other features of their physiology and ecology contribute to the diversity of viruses

found to date and likely many new viruses to be found in the future, including some with risk of transmission to humans (Calisher *et al.*, 2006). With the ever expanding list of novel bat viruses comes the challenge of determining which viruses to prioritize for further study. This challenge is compounded by the inability to culture and characterize the biological features of some of these viruses. The development of additional and diverse bat cell lines and better tools to study disease and immune responses in bats will help address these challenges. Further study of the virology, ecology, and epidemiology of virus infections of bats will provide valuable general insights into the origin and spread of emerging and re-emerging zoonotic viruses.

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10

ANTHROPOGENIC EPIDEMICS: THE ECOLOGY OF BAT-BORNE VIRUSES AND OUR ROLE IN THEIR EMERGENCE

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10.1 INTRODUCTION

The majority of emerging zoonoses are driven by human activities. An unknown diversity of viruses, bacteria, and other microbial flora exist within wild animal populations, just as with humans. Many of these microbes exist benignly within their particular host species, while others may act as pathogens – causing disease either in the natural host or when transmitted to an immunologically naïve host. Most cases of consequent disease outbreaks in wildlife that result from such microbial transmission go unnoticed, primarily due to a lack of surveillance for wildlife disease. However, when wildlife viruses make their way into livestock and/or human populations and cause detectable disease, they are more likely to get noticed, and trigger a more intensive investigation. Zoonotic transmission is the exchange of pathogens between wildlife and humans, and can be bidirectional (Epstein & Price, 2009). Viral spillover likely happens more frequently than is identified; zoonotic disease emergence typically follows human activities that alter the environment, such as urbanization, agricultural expansion, and deforestation, creating circumstances that increase contact with wild

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animal populations, and the opportunity for wildlife viruses to make the jump into new hosts (Daszak *et al.*, 2001; Patz *et al.*, 2004; Wolfe *et al.*, 2007). In Africa and China, the hunting and wildlife trade (which involves either killing and butchering animals *in situ* or in central markets after they have mixed with other species) are primary examples of activities that put those who handle these animals and come in contact with their bodily fluids at increased risk of exposure to any potentially pathogenic microbes that the animal may be excreting. Hunting of nonhuman primates in central Africa facilitated human immunodeficiency virus (HIV) emergence (Hahn *et al.*, 2000), and similarly, contact with nonhuman primates and other animals has been associated with outbreaks of Ebola virus in central Africa, and wildlife contact is presumed to be the cause of the current 2014–2015 outbreak of Ebola virus Zaire in West Africa (Baize *et al.,* 2014; Olival & Hayman, 2014).

There are multiple examples of zoonotic disease outbreaks that have been associated with anthropogenic changes to the environment, many of which have involved bats and their viruses. Ebola virus, severe acute respiratory syndrome coronavirus (SARS-CoV), Nipah and Hendra viruses – all are carried by bat reservoirs, and each has spilled over into animals and people, causing significant disease, and in the case of SARS, the first pandemic of the 21st century. In this chapter, we discuss these viruses and the ecological drivers of their emergence in some detail, and highlight how understanding the ecology of both host and pathogen is vital to mitigating outbreaks and potential global pandemics.

10.2 THE BAT–HUMAN AND BAT–LIVESTOCK INTERFACE: THE IMPORTANCE OF DISEASE ECOLOGY

Understanding the drivers of zoonotic disease emergence is critical to mitigating spillover, and so too is understanding the ecology of natural reservoirs. In order to truly understand how these viruses operate in nature, and how likely they are to jump from their wildlife hosts into livestock or humans, it is necessary to establish a broad framework to study the dynamics of the virus in its host, and the way that host interacts with its environment, conspecifics, and other potential animal hosts, including humans (Wood *et al.*, 2012). Which host species is a reservoir for the pathogen of interest? How abundant is this species? What is the level of contact with humans, and via which activities that would allow pathogens to spill over from wildlife into livestock or humans? There is substantial evidence that anthropogenic activities drive disease emergence from animal reservoirs. These activities include agricultural expansion or intensification, deforestation, hunting, wildlife trade, and global travel (Daszak & Cunningham, 2003; Patz *et al.*, 2004; Jones *et al.*, 2008). When a zoonotic pathogen is able to circulate within a human population, social dynamics facilitate larger outbreaks and global travel allows for local epidemics to become pandemics. Understanding the ecology of host species is fundamental to understanding how host and pathogen interact at a subpopulation and population level, and provides insight to factors that influence infection and transmission dynamics within and between species.

There are more than 1200 species of bats in the world, forming the order Chiroptera, with two sub-orders: Yinpteropchiroptera and Yangochiroptera (Teeling *et al.*, 2005). This makes bats the second most speciose taxonomic group of mammals after rodents, representing 20% of mammalian diversity (Teeling *et al.*, 2005). Bats are present on every continent and in every environment in which humans occur (Teeling *et al.*, 2005). Further, bats

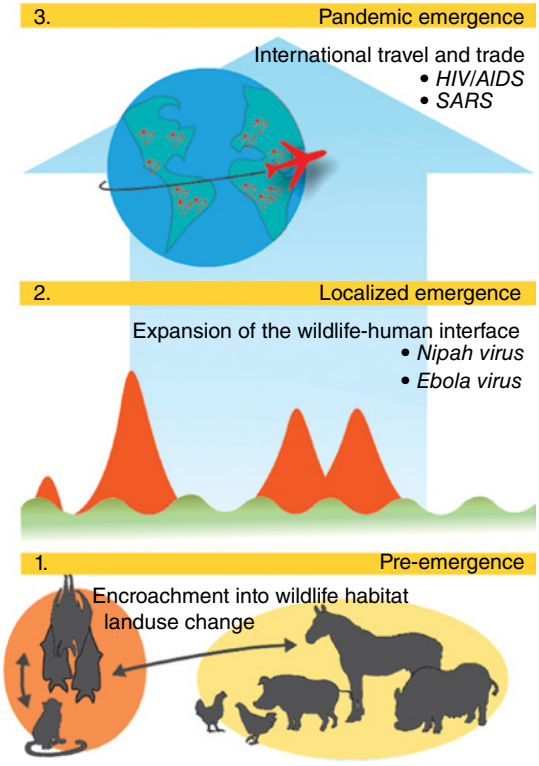

Figure 10.1 Schematic illustration of three stages of zoonotic pathogen emergence from a wildlife reservoir. Step 1 represents the human–wildlife interface. Activities that expand this interface and promote contact with wildlife (e.g., bats) provide opportunities for viruses which may constitute normal flora in their animal reservoirs, to jump into people either directly or via livestock. Occasionally spillover results in a localized disease outbreak in people, which either fades out or persists (step 2). Global travel allows infected individuals to introduce the pathogen to new regions, potentially leading to pandemic spread (step 3). Interventions at step 1 are necessary to minimize the health and economic impacts of disease emergence (reprinted from Morse *et al.*, 2012). *(See insert for color representation of the figure).*

successfully exploit human dwellings, constructs, and food resources, and thus contact between humans, domestic animals and bats, occurs in multiple ways, both directly and indirectly. The association between bats and zoonotic viruses is reviewed extensively in Chapter 2, here we highlight some of the anthropogenic drivers that have facilitated contact between bats, people and livestock, and the emergence of significant zoonotic pathogens.

Bats typically avoid contact with people. The majority of bats are insectivores that hunt moths, beetles, mosquitoes, and other insects. Insectivorous bats frequently live in caves in large colonies, though many live in trees, rocky crags, abandoned buildings, and occasionally, in inhabited buildings. Frugivorous bats, another large and diverse group of bats, are typically colonial and roost in trees, often near or within human settlements in rural or urban environments (Nowak, 1994; Epstein *et al.*, 2009; Hahn *et al.*, 2014a, b). They forage for fruit or flowers from a large variety of plants, including

BOX 10.1 Examples of anthropogenic activities that led to viral spillover

The majority of emerging zoonoses originate in wildlife, and human activities which increase contact among wildlife, livestock and people, are the major drivers of spillover (Daszak & Cunningham, 2003). Figure 10.1 illustrates a three-stage process of disease emergence which begins with the human-wildlife interface. The emergence of Nipah virus in Malaysia in 1998 was due to the expansion and intensification of pig farming, with larger pig farms and the high throughput of pigs facilitating the maintenance of the virus (Pulliam *et al.*, 2012). The presence of fruit orchards, with trees overhanging the pig pens, allowed fruit debris from feeding flying foxes to drop into pig pens, and putatively facilitate transmission from bats to pigs (Chua *et al.*, 2002a). In Bangladesh, cultivated date palm sap, a delicacy enjoyed by generations of Bangladeshi people, and which the resident pteropid species *Pteropus giganteus* has learned to exploit, constitutes the major route of zoonotic transmission for Nipah virus (Luby *et al.*, 2006, 2007).

commercially cultivated fruit trees. Most contact between bats and people is incidental. For example, bats that take up residence inside an attic may find their way into living areas, where their presence precipitates attempts to catch and remove them. In the context of disease transmission, bite or scratch contact is the primary mechanism by which bat pathogens such as rabies and other bat lyssaviruses directly infect humans (McCall *et al.*, 2000; De Serres *et al.*, 2008).

However, indirect contact with bat excreta is the most common route of exposure to bat viruses for people (Wood *et al.*, 2012). Nipah virus, Hendra virus, Ebola virus and Marburg virus are each associated with fruit bat reservoirs, and exposure to virus via bat excreta has been hypothesized as the main route of spillover from bats to humans (see Box 10.1) or other animals (Leroy *et al.*, 2005; Luby *et al.*, 2006; Field *et al.*, 2007; Towner *et al.*, 2009; Halpin *et al.*, 2011).

There is also substantial evidence that bats are reservoirs of filoviruses, including Ebola viruses and Marburg virus, as well as yet uncharacterized filoviruses (Hayman *et al.*, 2012; Leroy *et al.*, 2005; Olival *et al.*, 2013; Towner *et al.*, 2009). Ebola virus in the Congo, which causes a fatal hemorrhagic fever in both nonhuman primates and people, has been transmitted to humans via the consumption of infected animals – carcasses in many cases, as part of traditional hunting practices (Leroy *et al.*, 2004). In December, 2013, an outbreak of Ebola Zaire virus, of unprecedented magnitude in West Africa, began in Guéckedou, Guinea, following a single introduction from an unknown animal reservoir into the human population (Gire *et al.*, 2014). Human social dynamics, rather than repeated introductions from an animal reservoir, have been responsible for the rapid and uncontrolled spread of Ebola virus disease through Guinea, Sierra Leone, and Liberia, tragically underscoring the role of human–wildlife interaction in the emergence of novel epidemics and pandemics.

SARS CoV, which represents the first global pandemic of the 21st century, emerged from bats through the live animal markets of southern China in 2003 (Li *et al.*, 2005). The close caging of various mammalian species including bats, and the general lack of effective biosecurity practices in handling and butchering animals in live animal markets facilitated the infection of multiple species, including civets, raccoon dogs, and ferret badgers, all of which were initially suspected as being the primary source of the virus in early investigations (Guan *et al.*, 2003). The identification of SARS-like coronaviruses in bats, and the recent discovery of a strain capable of directly infecting people

(Ge *et al.*, 2013), illustrates the importance of identifying wildlife reservoirs for zoonotic viruses. Without this information, depopulation of a presumptive intermediate host, as was the case with civets, would potentially serve as short-term solution, but would be insufficient to prevent reintroduction and potential reemergence.

10.3 APPROACHES TO UNDERSTANDING THE ECOLOGY OF BAT-BORNE VIRUSES

Studies of infectious agents in free-ranging bats, as with any group of free-living wild animals, are replete with challenges. Foremost, such populations are uncontrolled and often undefined, which leads to a host of logistical and study design limitations, namely, difficulties in obtaining representative samples, recapturing individuals, and tracking their infection status through time, physical risks associated with handling wildlife and the hazards of wildlife capture, and uncertainties around what constitutes a "population" to name a few. Epidemiological studies often focus on identifying the natural reservoir of a target pathogen or suspected pathogen. There are varying viewpoints on how to define a wildlife reservoir (Haydon *et al.*, 2002), although most conceptual frameworks include the persistence of the pathogen within the species, the pathogen's ability to replicate within an individual of the species and be transmitted, and a requirement that individuals of the species be present within an ecosystem where transmission is occurring or has occurred. Many bats, such as *Pteropus vampyrus* in Malaysia, live in remote or poorly accessible habitats. *Pteropus vampyrus*, the largest of the bat species with a body mass averaging 800 g, roost in treetops and have been subject to decades of intensive hunting with firearms in Malaysia (Gumal, 2004; Mickleburg *et al.*, 1992; Epstein *et al.*, 2009). Some of the larger colonies can be found deep inside mangrove swamps that have no access from land and require small boats to approach via tidal channels. The height of the trees in which they roost, and the twice daily alternating tidal inundation and soft, exposed mud flats beneath, makes capture and sampling very difficult. Other bat species, such as *Miniopterus scherbersii*, the presumptive host of *Ebolavirus Reston* in the Philippines, may roost in large caverns, making the placement of harp traps or nets difficult. Techniques for capture and sampling of bats have been thoroughly reviewed elsewhere (Food and Agriculture Organization of the United Nations, 2011), and as such will not be detailed here.

Assuming one is able to catch and sample enough individuals to answer an epidemiological question with statistical robustness; ensuring appropriate sample handling and transport to a laboratory for diagnostic testing are important considerations. An effective cold chain is essential for optimizing the value of hard-earned samples, particularly in the tropics where ambient temperatures can quickly render biological samples (molecular or viral culture) nonviable. Ideally, liquid nitrogen or dry ice should be brought to the field to allow for the immediate freezing of samples at ultracold temperatures.

An important consideration with specific pathogens and bats, as with any large, diverse taxonomic group, is to differentiate between a species which carries a pathogen, and the entire taxon of bats. The focus at the species level and not the entire Order Chiroptera is significant when it comes to ascertaining the prevalence, maintenance, and transmission dynamics of pathogens among bats. Once a bat species is identified as a

potential reservoir for a pathogen of interest, the prevalence of the pathogen can be determined either through direct detection using culture and isolation or by molecular techniques such as polymerase chain reaction (PCR). The type of biological sample collected will influence the ability to detect a specific virus or other agent, and consideration must be given to the study design and whether sampling will be destructive (animals killed to collect samples from internal organs and other tissues) or nondestructive.

There are important considerations when deciding on a sampling approach, including the scientific questions that the study is intended to answer: for example, what viruses are these bats shedding versus what viruses do they carry? What is the prevalence of a given virus? This typically requires individual sampling (Figure 10.2A), whereas an effort to obtain viral isolates or genetic sequence would benefit from sampling as much of the population as possible, and pooled urine or faeces may be a more effective sampling technique (Figure 10.2B). One should consider the conservation status of the target species and the practicality of catching individual animals as opposed to collecting guano or urine droplets from beneath a colony. The latter is often a good strategy for maximizing sample collection with minimal sampling effort, though if resources allow, a combined approach provides maximum information.

Pathogens that cause acute infection and a short clinical course, and are present at a low prevalence within a population can be difficult to detect by virus isolation or PCR in individual animals without a very large sample size. Serology can offer an effective means of screening a population for past exposure or infection, assuming that antibodies persist over time. There are a variety of well-established platforms that can be used to detect antibodies to a specific agent, including: enzyme-linked immunosorbent assays (ELISAs), immunofluorescent antibody test (IFAT), Western blot, and viral neutralization tests (VNTs). While the first three measure the presence of antibodies, but not their ability to destroy a virus, the latter, the VNT, is a functional assay which measures the serum's ability to bind to and neutralize active virus *in vitro*. VNTs also provide titers, which is a measure of how dilute serum can become while still effectively stopping virus. One advantage of ELISAs over VNTs is that they do not require the use of live viral cultures and thus can be performed with minimal biosafety conditions. Serology can be used to inform dynamic models, such as the ***S***usceptible-***I***nfectious-***R***ecovered (SIR) models, that are used to explain infection patterns over time. Population distribution and abundance are also key elements for the study of pathogens in bats.

10.3.1 Observational study design

There are several types of observational study designs that can be used to understand the epidemiology of viruses and other pathogens in bats. Cross-sectional studies examine the proportion of individuals that are infected at the time of sampling (e.g., 20 bats infected out of 100 sampled = 20% prevalence). This type of study provides a snapshot of the temporal dynamics within a population. A cross-sectional study can be highly informative in terms of establishing disease prevalence, age or sex biases, and clinical impact of infection (by collecting data on the physical status of each animal sampled). A longitudinal study provides more information on the temporal dynamics of a pathogen and is generally a series of cross-sectional samples collected at regular time intervals. In human longitudinal studies, it is often possible to follow individuals and their infection status over time. Unfortunately, with bats, as with most wild animals that live in large

(A)

(B)

Figure 10.2 When designing a disease ecology study it is important to determine whether (A) individual-level sampling or (B) population-level sampling (e.g., pooled urine collection), is sufficient to test hypotheses or provide epidemiological data. A combined approach may provide optimal data. Photo credits: (A) M. Hillyard, (B) J. Mencher, copyright 2014, EcoHealth Alliance. *(See insert for color representation of the figure)*.

populations, it is very difficult to reliably recapture individuals, although it may happen on occasion. Thus, longitudinal studies of free-ranging bats have typically relied on each sample being representative of the population as a whole, with data describing trends in infection status over time *within the population* rather than within individuals. Rahman *et al.* (2013) conducted a 36-month longitudinal study of Nipah virus seroprevalence in a population of *P. hypomelanus* on Tioman Island in Malaysia. The authors reported significant differences in seroprevalence over time, which may have been due to a viral outbreak within the population, or transition of juvenile bats into adults over the course of the study (Figure 10.3).

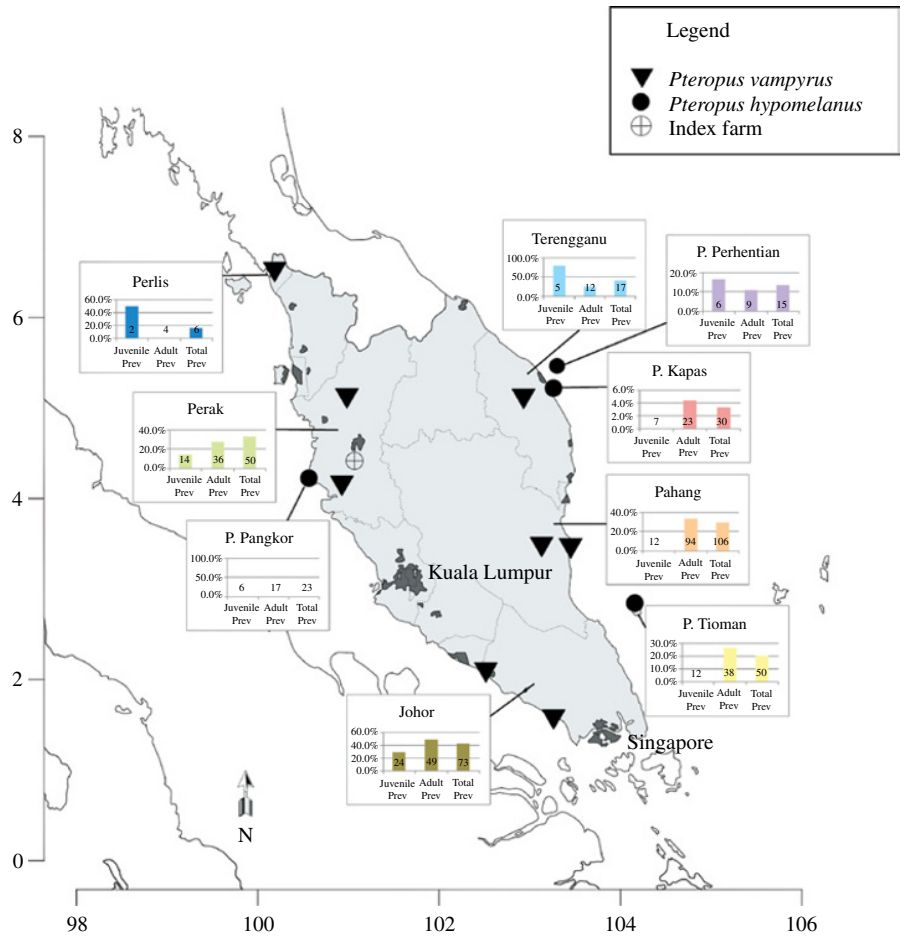

Figure 10.3 Seroprevalence of Nipah virus in *Pteropus* spp. in peninsular Malaysia. A country-wide survey was conducted to determine the spatial distribution of Nipah virus and to characterize the risk factors involved in bat infections (reprinted from Rahman *et al.*, 2013). *(See insert for color representation of the figure).*

Once a bat species is identified as a potential reservoir for a pathogen of interest, the prevalence of the pathogen can be determined by direct detection using culture and isolation or by molecular techniques, such as PCR. The type of biological sample collected will influence one's ability to detect a specific virus or other agent. Ideally, bat surveillance strategies should include sample collection for multiple diagnostic modalities, so that if a microbial agent of import or interest is detected by one test, then another confirmatory test may be conducted. Standard strategies used by the authors include the collection of duplicate samples, with one set preserved in a lysis buffer for molecular diagnostic screening, and a second set preserved either in a viral transport medium (when viral detection is the objective of the study) or frozen without any preservative at ultra-cold temperatures (in liquid nitrogen or a –80°C freezer) for viral culture.

When designing a study, one must determine whether it will be sufficient to detect the pathogen in excreta (saliva, urine, or feces) as routes of excretion, or whether it is

necessary to look at organ tissues to detect the pathogen and/or to better understand which tissues the pathogen may infect. This question will influence whether sampling will be destructive or non-destructive as discussed above. There are arguments for and against each approach, and one should approach study design with consideration of the conservation status of the target species, the impact that destructive sampling may have on the population and with a general philosophy of maximizing information while minimizing impact on the animal.

Serology can be used to assess changes in a population's overall susceptibility to infection (seroprevalence) over time by providing a series of snapshots at given time intervals. Longitudinal studies of anti-Hendra virus antibodies in *P. scapulatus* and anti-NiV antibodies in *P. hypomelanus* were used to test hypotheses about seasonality of henipaviruses in order to assess whether certain times of year had higher risk of spillover (Plowright *et al.*, 2008; Rahman *et al.*, 2013). Serological data is commonly used as a proxy for actual infection when studying viruses associated with acute infectious periods that make detection of infected individuals difficult. Population distribution and abundance are also key elements for the study of pathogens in bats.

10.3.2 Mathematical models

Dynamic models have become increasingly integrated into epidemiologic studies of pathogens in host populations. Models provide an analytical platform that allows the testing of hypotheses regarding the impacts of host biology on: the dynamics of a pathogen within a specific population; the impact of interventions such as vaccination; or to identify the impact of population size or demography on disease dynamics. Mathematical models have been used to describe a variety of zoonotic viruses in wildlife hosts, including hantavirus, Nipah virus, Ebola virus, and Hendra virus (reviewed in Allen *et al.*, 2012). A common type of dynamic model used in epidemiology is called a compartmental model, as it describes sections or "compartments" of a population in terms of their state of infection. These are well described elsewhere, but briefly, SIR models use a series of differential equations to describe a proportion of a population that occupies one of three potential states of infection: "S" = a proportion of a population that is *susceptible* to infection by a pathogen (not yet exposed); "I" = the proportion that is *infectious* (infected and able to transmit to susceptible hosts); and "R" = the proportion that has been infected and is now *removed* (immune or dead) from further infection (this also includes individuals who were resistant or otherwise immune to infection) (Figure 10.4). SIR models can incorporate

$$\frac{dS}{dt} = -\beta \frac{S}{N} I,$$

$$\frac{dI}{dt} = \beta \frac{S}{N} I - \gamma I - \alpha I = I\left(\beta \frac{S}{N} - \gamma - \alpha\right),$$

$$\frac{dR}{dt} = \gamma I$$

Figure 10.4 A standard SIR model, where β = the transmission rate for a pathogen within the population; γ = the recovery rate from infection; and α = is the mortality due to infection. d*S*/d*t* is the change in the proportion of the population that is susceptible over time; d*I*/d*t* is the change in the infected proportion of the population over time, and d*R*/d*T* is the change in the proportion of recovered individuals in the population over time. N = the total population size.

biological parameters to increase their realism and improve their predictive value, such as birth rate, baseline mortality rate, immigration/emigration, duration of infection, and age categories. Some models add an additional state: "Exposed," to signify the proportion of the population that has been exposed to a pathogen but it not yet infectious, thus creating an S-E-I-R, model. These models are useful for exploring seasonal dynamics or other temporal patterns of infection and can be used to help predict times of peak infection. This is particularly relevant to understanding risk of spillover to other animal or human hosts.

One caveat to interpreting dynamic models is that they are limited in their accuracy by the data that is available. Longitudinal data sets are particularly useful for feeding and validating models. A model can be parameterized either with theoretical or observed values, and the parameters can be each be adjusted in order to assess their individual impact on the dynamics. However, a model is only as good as the data it uses. Models of human diseases, such as measles, are quite robust since there is an abundance of disease and demographic data available from human populations (Bjornstad *et al.*, 2002; Keeling & Grenfell, 2002). However, when it comes to wildlife diseases, data is understandably much harder to acquire, and while dynamic disease models have been used to examine wildlife disease, they must be interpreted carefully based on the assumptions made about host life history traits or disease impacts given limited data.

There are significant challenges to obtaining biological and life history data from free-ranging bats. Additionally, there is a relatively little known about how viral pathogens are maintained in bat populations. Rabies virus in *Eptesicus fuscus*, the Little Brown bat of North America, is an example of a bat host and associated virus that have been well studied and for which disease models have been developed (Georgea *et al.*, 2011; O'Shea *et al.*, 2011). *Pteropus alecto* and *P. poliocephalus*, two of the four Australian flying fox species that have been associated with Hendra virus, have had reasonably good ecological and physiological studies conducted on individuals both from wild populations as well as in captivity (Hall & Richards, 2000). These studies have included radio and satellite telemetry to measure local and long-range movements, which are critical for understanding how populations are connected; this in turn provides insight and real-world data to help parameterize infectious disease models that test hypotheses about how Hendra virus may persist within the species over time (Eby, 1991; Eby *et al.*, 1999; Tidemann *et al.*, 1999; Plowright *et al.*, 2011; Smith *et al.*, 2011; Tidemann & Nelson, 2011). For many other chiropteran species such as *Rousettus aegyptiacus*, *Hypsignathus monstrosus*, *Rhinolophus ferrumequinum*, or *Pteropus* spp., that have been associated with Marburg virus, Ebola virus, SARS-CoV, or Nipah virus, respectively, far less is known about long-term infection dynamics (Leroy *et al.*, 2005; Li *et al.*, 2005; Towner *et al.*, 2009; Halpin *et al.*, 2011). A major challenge to acquiring long-term disease data for bats is obtaining sufficient long-term funding; however, there have been a few longitudinal studies focusing on emerging zoonotic viruses such as Marburg virus, Hendra virus, bat coronaviruses, and Nipah virus, which have provided important insights into the dynamics of these zoonotic pathogens in nature. These are reviewed in detail in other chapters of this book.

10.3.3 Outbreak response and long-term ecological study

Two distinct scenarios involving bat-borne zoonoses require the implementation of the epidemiological principles described above: an outbreak investigation, and the

long-term ecological study of the dynamics of a virus and its natural host, which includes transmission to humans or other animals. To illustrate both, we describe the initial outbreak investigations of Nipah virus and a multi-year follow-up study, as well as our investigations of Reston Ebola virus (REBOV) in the Philippines.

10.3.3.1 Outbreak response: Nipah virus, Malaysia 1998–1999 Disease emergence usually occurs without any pre-warning. The detection of a novel disease and the success of any outbreak response depends on the resources available, the systems in place, and the individuals involved. The emergence of Nipah virus in Malaysia in 1998 is an excellent illustration of the complexities and challenges in such a scenario.

Initially, the connection between human cases and disease in pigs was not made, as the sporadic human cases presented to multiple hospitals over space and time, constraining the ability to identify a cluster of cases (CDC, 1999). When the epidemiological association between porcine and human cases was made (as a consequence of both informal and formal contact between animal and human health authorities), the initial thinking (confounded by ambiguous diagnostics) was that this was an aberrant Japanese encephalitis virus (JEV) outbreak (Chua *et al.*, 2000; Nor *et al.*, 2000b); understandable when the existing paradigm was that disease in pigs and associated humans indicates JEV, notwithstanding epidemiological consistencies in this instance. Thus initially, the outbreak response focused on JEV vaccination of pigs and mosquito vector control, which, because of the contrasting disease ecology, was totally ineffective in containing the spread of the novel Nipah virus. It was only when a skeptical Malaysian scientist excluded known arboviruses and sought international collaboration that the novel Hendra-like virus was identified (Chua *et al.*, 2000). The Malaysian government then moved quickly to assemble a multidisciplinary outbreak response team of Malaysian and international scientists. This team sought first to contain the outbreak in pigs and humans, and second to identify the origins of the virus. Thus the first priority was to confirm that pigs were the source of human infection, and "hot" pig farms were targeted for immediate investigation; active porcine cases were assessed and necropsied under elevated biosecurity protocols, and a range of tissue samples forwarded to the Australian Animal Health Laboratory (AAHL) for virus isolation at Biosafety Level 4 (BSL4) (Daniels *et al.*, 2001). When cell culture confirmed that the porcine isolates were identical to the human isolates, the response effort broadened to understanding the epidemiology and characteristics of pig to pig and pig to human transmission, to containing the outbreak in pigs, and to investigating the origins of the virus. The Malaysia Department of Veterinary Services declared Nipah virus as a notifiable disease and declared disease control and eradication areas. A cross-departmental taskforce made decisions on the evacuation of farming areas, the destruction of pigs and pig farms, and the payment of compensation. When the outbreak was under control, national testing and surveillance programs were implemented, and established that Nipah virus infection was not endemic in the Malaysian pig population (Nor *et al.*, 2000a).

10.3.3.2 Ecological investigation: why did Nipah virus emerge? Earlier research on the origins of Hendra virus in Australia (Young *et al.*, 1996) was fundamental to quickly identifying pteropid bats (flying foxes) as the likely natural reservoirs of Nipah virus. Antibodies to a Hendra-like virus were identified in both species of pteropid bat present in Malaysia, and the virus itself was isolated from *Pteropus hypomelanus*,

the Variable flying fox, on Tioman Island (Johara *et al.*, 2001; Chua *et al.*, 2002b). However, *P. hypomelanus* is restricted to islands, and is not found near the index farm. Was Nipah virus also circulating broadly in *Pteropus vampyrus*, which is found across mainland peninsular Malaysia (and in Thailand and Indonesia), and why had Nipah first emerged in Ipoh in the north of Malaysia, when there were many other pig farms in overlapping with flying fox habitat? A large-scale ecological study was designed to test the hypothesis that both *P. vampyrus* and *P. hypomelanus* were natural reservoirs for Nipah virus, and that the size and structure of the pig farm was a factor in Nipah virus emergence.

The investigation was composed of a multiyear, multifaceted study of Nipah virus in bats as well as an analysis of disease data from the index farm using SIR models. A spatial study was conducted to determine the spatial distribution of Nipah virus in bats across Malaysia and a longitudinal study of a single population of *P. hypomelanus* on Tioman Island was used to identify seasonal trends in viral dynamics within bats. Satellite telemetry was used to observe local and long-range movements of *Pteropus vampyrus* in order to better understand Nipah virus data from the bat studies. Lastly, experimental infection studies under BSL4 conditions were conducted (at AAHL) to examine the pathophysiology of Nipah virus in *Pteropus vampyrus.*

Results from the country-wide survey of bats indicated that Nipah virus did indeed circulate amongst both species of pteropid bat, as IgG antibodies against Nipah virus were detected in nearly every colony sampled (Figure 10.3). Viral RNA was not detected in any of the urine, rectal, or oral swabs collected, nor was virus isolated, which suggested that Nipah virus prevalence was low and a relatively rare infection in bats. The serological data from the longitudinal study did not indicate a seasonal pattern of infection; however, a spike in seroprevalence amongst juvenile bats suggested that an outbreak had occurred within the study population during the three-year period of study. Given that this was the same population from which Chua had originally isolated Nipah virus years earlier (Johara *et al.*, 2001; Chua *et al.*, 2002b), it appeared that Nipah virus was maintained within the population - probably by the pulse of juveniles born each year which would increase the susceptible proportion of the population. The satellite telemetry study (Epstein *et al*., 2009) substantially improved our understanding of host ecology. Although the cost of the satellite transmitters limited the overall number of bats that could be collared, the data showed that *P. vampyrus* were highly mobile, flying hundreds of kilometers, and across international boundaries over the 9-month period of data collection (Figure 10.5).

This supported the hypothesis that Nipah virus could be maintained at low incidence (<1%) if colonies were connected via migratory individuals (Epstein *et al.*, 2009; Rahman *et al.*, 2013). Further, the telemetry results demonstrated that the home range of *P. vampyrus* included Indonesia and Thailand, reinforcing the notion that Nipah virus was likely enzootic throughout their entire range, rather than just within Malaysia.

So, the ecological study of Nipah virus in bats suggested that this was a virus that circulated widely in both species of *Pteropus* bats; however, the question remained as to why Nipah virus emerged on the farm in Ipoh as opposed to any other pig farm in Malaysia. One of the striking facts about the index farm was that it was by far the largest pig farm in Malaysia, with more than 30 000 head of pigs and was an industrialized structure, meaning that pigs were segregated by stage of production, and that there was a particularly high throughput of individuals both coming into the farm (via birth) and leaving the farm (to market). Fortunately, the farm kept good digital records of pig

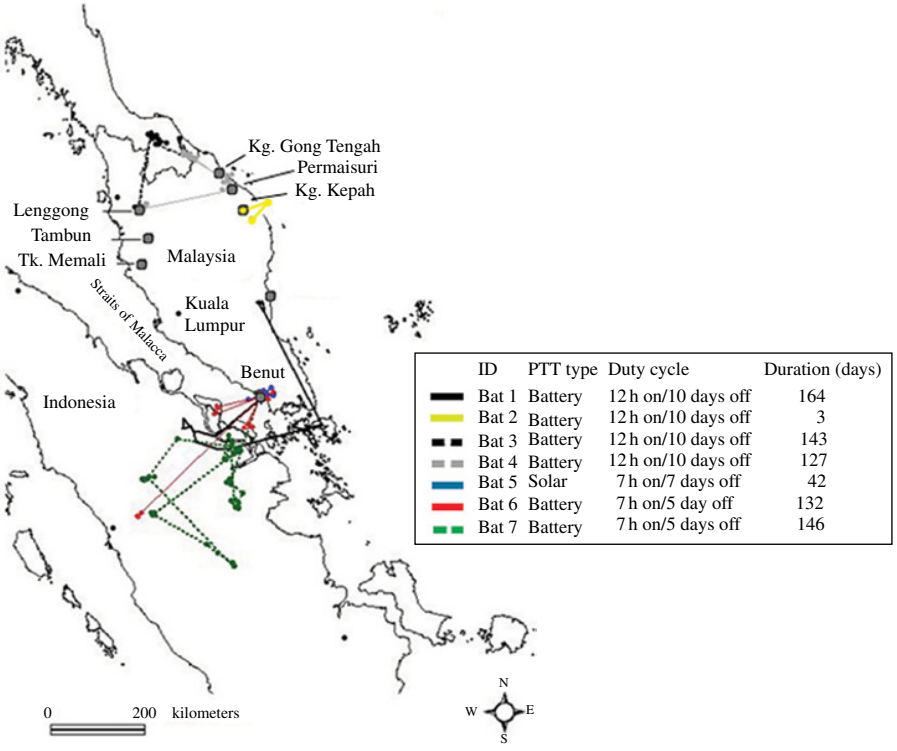

Figure 10.5 Satellite telemetry data showing *Pteropus vampyrus* flight paths and roost locations. The data were used to look at mobility and connectivity among bat colonies and illustrate that flying foxes are highly mobile and utilize habitat in Malaysia, Indonesia and Thailand (reprinted from Epstein *et al.*, 2009). *(See insert for color representation of the figure).*

morbidity and mortality, including the mortality rates associated with Nipah virus (Nor *et al.*, 2000a) as well as birth rates and numbers of each age group, all of which would be used to parameterize a dynamic disease model. A Susceptible-Exposed-Infected-Recovered model that included a component of the Recovered fraction that had maternal antibodies (i.e., the SEIRA model), was developed and used to simulate outbreaks of Nipah virus. It had been hypothesized that the proximity of mango and other fruit trees to the pig enclosures led to the spillover of Nipah virus from bats by way of dropped fruit laden with saliva, or excreta falling into the pig enclosures (Chua *et al.*, 2002a). The analysis of outputs from the SEIRA model revealed some critically important factors that allowed Nipah virus to emerge on this farm and create a long-term sustained outbreak, such that pigs and people would be infected over more than a year: (1) the large herd size of the farm was necessary to support a sustained Nipah outbreak in pigs; (2) the rapid turnover of pigs created a steady supply of susceptible individuals to help sustain Nipah virus in the population; (3) multiple introductions of Nipah virus from bats, rather than a single event, would be necessary to create a sustained outbreak in a pig population (which was actually observed) and the initial introduction of Nipah virus from bats primed the population for a longer-term outbreak (Pulliam *et al.*, 2012). In fact, the heterogeneous mixture of immune and susceptible pigs in the large herd created

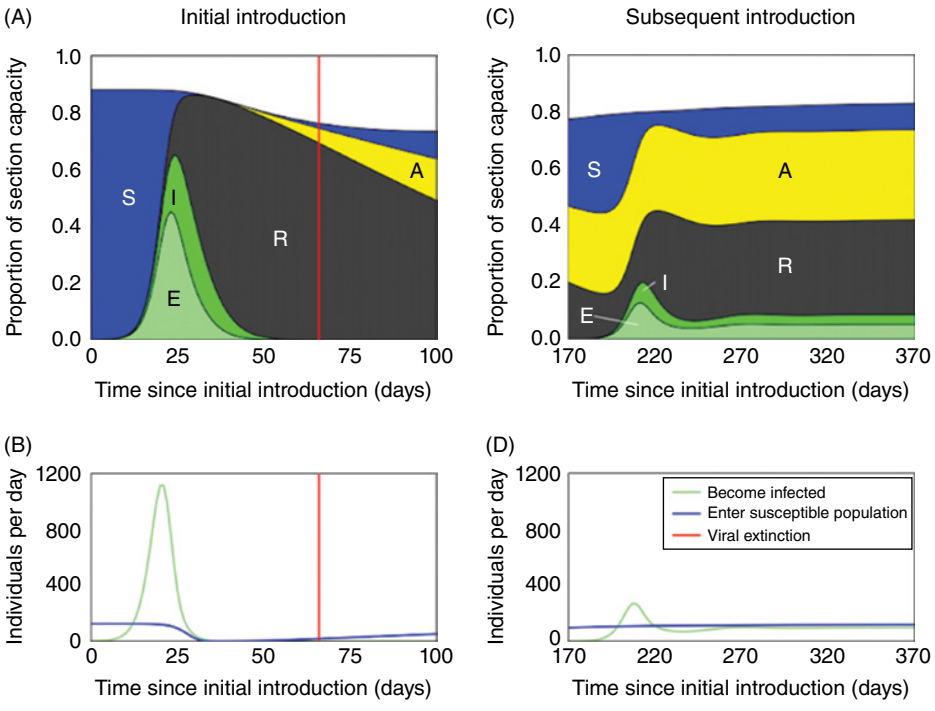

Figure 10.6 Deterministic model results illustrating Nipah virus dynamics in the growing section of the index farm. Individuals are characterized as belonging to one of five states: susceptible (S), immune – maternal antibodies (A), immune – recovered from infection (R), exposed (E), and infectious (I). The top panels illustrate the infection/immunity profile of the growing section following (A) initial introduction of the virus and (B) subsequent introduction. The qualitative difference in infection dynamics results primarily from the prevalence of maternal antibodies in the young pig population. (C, D) Following the initial introduction of the virus (C), the rate of replenishment of the susceptible population in the growing section (solid blue line) declines, as many individuals are immune, having been infected while in the breeding sections. The rate at which individuals are infected (green line) declines in consequence. When the virus is reintroduced (D), many individuals entering the growing section have maternal antibodies. Loss of maternal antibodies after entry into the growing section provides a source of susceptibles independent of the presence of infection (blue line), allowing the virus to persist (reprinted from Pulliam *et al.*, 2012). *(See insert for color representation of the figure).*

an environment where Nipah virus could circulate at a low level, but persistently over the course of more than a year (Figure 10.6). The model suggested that had the farm been smaller, as most other farms were, the result of a Nipah virus introduction from bats would have resulted in a rapid, intense outbreak of Nipah virus that would have run its course in just a few days or weeks, rather than be sustained over a year. Without the high rate of introduction of susceptible pigs, the surviving herds would have remained largely resistant to infection.

The combination of studying the ecology of Nipah virus in bats and modeling the outbreak in pigs provided important insights into why Nipah virus emerged on the index

farm in Malaysia. The field studies confirmed that pteropid bats were the natural reservoir for Nipah virus in Malaysia (this was further supported by the experimental infections and subsequent viral isolation from naturally infected wild-caught bats (Rahman *et al.*, 2010, 2013; Halpin *et al.*, 2011) and while the opportunity for viral spillover from bats to pigs likely existed in other locations and at other times, the size and structure of the pig farm in Ipoh was critical to sustaining a Nipah virus outbreak in pigs and subsequently people. This work also provides an important example of one of the major anthropogenic drivers of disease emergence: agricultural intensification.

10.3.3.3 Reston ebolavirus The emergence of REBOV illustrates a somewhat novel anthropogenic driver for emergence. Ebolaviruses were first described in 1976, associated with highly lethal outbreaks of human hemorrhagic fever in central and western Africa (Bres, 1978). REBOV was first described in 1989, associated with an outbreak of fatal hemorrhagic disease in Philippines-sourced cynomolgus monkeys (*Macaca fascicularis*) in a primate facility in Reston, Virginia, USA (Geisbert *et al.*, 1992). While monkeys were clinically and fatally infected, infected facility workers exhibited no symptoms, in stark contrast to other known ebolavirus infections in humans. Similar scenarios were subsequently seen again in primate facilities in the USA and in a facility in Italy (Rollin *et al.*, 1999; Perini, 2000). Surveillance of licenced export primate facilities in the Philippines found endemic infection in one of five facilities. Depopulation of this facility and revised monitoring and export protocols saw export re-established without subsequent incident (Miranda *et al.*, 1999). No conclusions were reached about the origin of infection in the monkeys. No further detections of REBOV were forthcoming for nearly two decades, until in December 2008 REBOV was detected coincident with an outbreak of severe porcine reproductive and respiratory syndrome (PRRS) in commercial pig farms in the Philippines (Barrette *et al.*, 2009). PRRS is caused by porcine reproductive and respiratory syndrome virus, a member of the genus *Arterivirus*. Screening of associated pig farm workers also revealed evidence of REBOV infection, but all remained healthy and none had symptoms or history of attributable clinical disease. The Philippines government requested assistance from international agencies to assess the implications for the pig industry and for human health, and to identify the origins of the virus. Investigations of the latter were informed by previous studies elsewhere, which had shown that bats were reservoirs of Ebola viruses (Leroy *et al.*, 2005; Olival & Hayman, 2014), and revealed evidence of infection in a suite of Philippine bat taxa (Taniguchi *et al.*, 2011; Jayme *et al.*, pesonal communication) suggesting they are the likely reservoir of REBOV in nature.

10.4 ANTHROPOGENIC ACTIVITIES DRIVE ZOONOTIC DISEASE EMERGENCE FROM BATS

10.4.1 Agricultural expansion/intensification: Nipah virus

The majority of emerging zoonotic viruses can be traced back to wildlife, yet there is an increasing body of scientific evidence to suggest that people, not bats, have by and large been the instigators of these spillover events (Jones *et al.*, 2008). Activities that alter our environment and increase our opportunities for direct or indirect contact with wildlife,

Figure 10.7 *Pteropus giganteus*, found in the Indian subcontinent, is one of the largest bat species. Photo by J. Epstein, EcoHealth Alliance copyright 2014. *(See insert for color representation of the figure).*

such as expansion of intensive farming, wildlife trade, urbanization, and global travel, have led to an increase in viral spillover, and the emergence of some significant zoonotic viruses – some of which have become global pandemics.

Perhaps one of the most illustrative examples of a manmade epidemic is the Nipah virus outbreak in Malaysia, described above. Interestingly, there was only the single epidemic in Malaysia, which concluded in 1998. However, additional outbreaks were subsequently recognized in South Asia, prompting a second long-term ecological study in order to understand what appeared to be a very different situation.

In 2001, Nipah virus was first recognized as the cause of fatal encephalitis in Bangladesh and India (though the Siliguri outbreak would be retrospectively diagnosed years later), and outbreaks recurred in Bangladesh on a near annual basis (Chadha *et al.*, 2006; Luby *et al.*, 2007). *Pteropus giganteus* is the sole member of the genus extant in South Asia, and it is abundant throughout India and Bangladesh (Bates & Harrison, 1997). This was the presumptive reservoir, and early investigations showed that antibodies to Nipah virus were indeed present in *P. giganteus* in India and Bangladesh (Figure 10.7) (Hsu *et al.*, 2004; Epstein *et al.*, 2008). Additional studies of Nipah virus in bats have detected viral RNA and anti-Nipah immunoglobulin G (IgG) antibodies in multiple locations in Bangladesh and India, confirming this species as a natural reservoir (Epstein *et al.*, 2012; Yadav *et al.*, 2012).

In marked contrast to Malaysia, it appeared that there was direct bat-to-human transmission in Bangladesh via a food-borne route (Luby *et al.*, 2006). Date palm sap is harvested during the winter months (November–April) and is collected by shaving the bark off a portion of the trunk of a date palm tree (*Phoenix sylvestris*) and letting the sap run down the shaved part into a clay collecting pot, which is hung from the tree. The pots are collected from the trees early in the morning by *gachis* who sell the sap door to door while it is fresh. As it turned out, the date palm sap is an attractant to frugivorous bats – namely *P. giganteus* and smaller species from the same family, *Cynopterus sphinx* and *Rousettus*

Figure 10.8 A date palm sap collector (*gachi*) demonstrates using a bamboo skirt to cover his collection pot as a means to prevent bats from contaminating sap with excreta. Photo by J. Epstein, copyright 2014, EcoHealth Alliance. *(See insert for color representation of the figure).*

leschenaultii (Khan *et al.*, 2011). Epidemiological evidence has linked date palm sap consumption to several outbreaks in Bangladesh (Luby *et al.*, 2009). In this context, the cultivation of date palm sap (an anthropogenic activity) provided bats with access to a food resource they otherwise would not have had, which incidentally allowed for the contamination of the sap with saliva or other excreta containing Nipah virus. What makes Nipah virus of particular concern is that it has demonstrated the ability to be transmitted from person to person, and has been associated with a mean case fatality rate of about 75% (Gurley *et al.*, 2007; Luby *et al.*, 2007; Homaira *et al.*, 2010). The predictable and recurring spillover of Nipah virus from bats to people, coupled with an ability to spread (albeit to a very limited extent) among people, and the fact that this is happening in one of the most populous places on Earth, brings attention to the pandemic potential of this virus, and an urgent need to limit the opportunities for spillover, by reducing the interface between bats and people (Luby, 2013). One of the ways that this is being done is through the introduction of bamboo skirts which wrap around the palm tree trunk and cover the clay pot as well as the shaved area, thereby preventing bats from accessing the sap stream and the pot (Figure 10.8) (Khan

et al., 2009, 2012). Studies are underway to assess the efficacy of this intervention, as well as the cultural beliefs and practices that may influence the use of bamboo skirts (Khan *et al.*, 2009; Nahar *et al.*, 2013).

10.4.2 Urbanization: Hendra virus

Five years prior to the discovery of Nipah virus in Malaysia, an outbreak of severe acute respiratory disease occurred in horses in a racing stable in Hendra, a suburb of Brisbane in the eastern Australian state of Queensland. Fourteen horses were affected with respiratory and neurological signs, and the horse's trainer became sick and died after being exposed to the horses (Selvey *et al.*, 1995). The cause of the outbreak was a novel paramyxovirus, initially characterized as most similar to those within the genus *Morbillivirus*, which includes canine distemper virus. Initially called equine morbillivirus, Hendra virus, as it was subsequently called, was traced back to pteropid bats (of which there are four species in Australia) as the natural reservoir. Since 1994, Hendra virus has cause numerous outbreaks in horses across Queensland and the adjoining state of New South Wales, and evidence of infection has been detected in each of the four species of flying fox present within this range (Field *et al.*, 2011). While the definitive mode of transmission between bats and horses is still uncertain, the most biologically plausible and parsimonious hypothesis is that infected bats feeding or roosting in trees within horse paddocks contaminate the area beneath, and horses are exposed either by direct contamination or by ingesting contaminated feed or water (Field *et al.*, 2010, 2011). Infected horses are then able to transmit the virus to other horses, and to humans (Field *et al.*, 2007). Outbreaks in horses are sporadic, though since 2006, there has been a marked increase in the frequency and number of equine cases identified. In 2011, an unprecedented number of cases were detected, concurrent with an increased frequency and level of detection in flying foxes under ongoing disease surveillance (Mahalingam *et al.*, 2012). It remains unclear what drives outbreaks in bats that would lead to increased spillover; however, recent research suggests that two species (*Pteropus alecto* and *P. conspicillatus*) play a primary reservoir role (Smith *et al.*, 2014).

Epidemiological and broader phylogenetic evidence suggests that Hendra virus recently emerged within Australia rather than being introduced from offshore (see Box 10.2). It is probable that sporadic cases have occurred historically, but were not identified, and that improved detection and reporting is responsible for the apparent increase in frequency of cases.

Pteropid bats are highly mobile and display nomadic and migratory behavior (Eby, 1991; Plowright *et al.*, 2011; Smith *et al.*, 2011; Tidemann & Nelson, 2011), and migration of infected individuals has been postulated as the main means of allowing Hendra virus persistence via a meta-population structure (Plowright *et al.*, 2011). The hypothesis was that urbanization played a role in attracting flying foxes to utilize humanized environments, which provided protected food and roosting resources, and resulted in reduced migratory movements. Plowright *et al.* (2011) modelled Hendra virus infection dynamics over time, hypothesizing decreased connectivity among populations and found that outbreaks within urban bat populations increased in amplitude coinciding with seasonal birthing (Plowright *et al.*, 2011). More recent telemetry studies demonstrate a high level of connectivity between flying fox populations at local, regional and

BOX 10.2 The long-term relationship between bats and their viruses

Paramyxoviruses, coronaviruses, and lyssaviruses are believed to have coevolved over millions of years along with their chiropteran hosts (Wertheim *et al.*, 2013), and this may be the case with many of the zoonotic viruses that originate in bats. This long-term relationship between host and virus may explain why bats experience only mild pathology and may appear clinically normal while infected, compared to the severe disease these viruses cause in other mammalian hosts. The increase in zoonotic pathogen spillover from bats and other hosts in recent history points to the significance of environmental or ecological changes that create new opportunities for these viruses to jump into other hosts (Daszak & Cunningham, 2003; Patz *et al.*, 2004; Jones *et al.*, 2008).

inter-regional levels (Edson *et al*., personal communication), prompting consideration of alternative hypotheses of transmission and infection dynamics in the natural reservoir. When Hendra virus recrudescence was modeled in flying foxes by Wang *et al*. (2013), patterns of long-term persistence emerged and were indistinguishable from infection dynamics that included immigration into a population. In support of this model, Nipah virus recrudescence was observed in a study of wild-caught *Pteropus vampyrus* in Malaysia where a Nipah virus-negative bat, sequestered from other bats, seroconverted and then began shedding virus which was then detected by viral isolation (Rahman *et al.*, 2011).

If indeed suburban and urban environments in Queensland, which include horse farms, are a driving factor in Hendra virus spillover, then the interface where Hendra virus is transmitted from bat to horse becomes a crucial piece of the puzzle, and the route of transmission must be blocked, which may involve excluding horses from beneath trees that may host flying foxes. Another intervention that has recently been developed is a Hendra virus vaccine, which is commercially available for horses, and could create an effective barrier to spillover that would significantly reduce the risk of human infection, as well as horse infection. The challenge, of course, is whether there is enough public interest in using the vaccine, or modifying the horse's environment to reduce the risk of infection (Kung *et al.*, 2013). To date there has been modest uptake of the vaccine, with the majority of the horse population estimated to be unvaccinated. The importance of understanding not just the science of disease management, but the drivers of human behavior and decision-making is further illustrated by the findings of a recent survey of horse owners in Australia, wherein a substantial proportion of respondents indicated that they had not implemented recommended exposure risk mitigation measures even though they were aware of them. As with Nipah virus, interventions must be practical in order to achieve compliance from the general public. The interface between horses and flying foxes is the result of human alteration of the environment (e.g., placing horse paddocks in flying fox habitat, or developing urban centers that provide preferable habitat for bats); and the responsibility rests on public health officials, veterinarians, policy makers, and ultimately horse owners to reduce the risk of spillover by reducing opportunities for horses to be exposed to bat excreta and Hendra virus.

In Australia, there is clear evidence of an expanded bat–human and parallel bat–livestock interface in recent decades. There are multiple components to this: first, increased human population; second, loss of habitat associated with anthropogenic land-use; third, demographic shifts to a periurban "tree change" lifestyle. People and

flying-foxes have similar biogeographic preferences, both clustering along the coast, and particularly around the mouths of coastal rivers. Thus, while the overall increase in human population size has been moderate, the increases in human population density have been much greater. Conversely, while the total flying fox population has significantly decreased over the decades, the flying-fox population density has been less affected. Add to this the highly mobile and nomadic nature of flying foxes, and there remains the ability for large numbers and high density of flying foxes to occur periodically at individual roost locations. The number and nature of flying fox roost locations has also changed – historically there were fewer, but larger and more permanently occupied roosts, whereas now there are more roosts that are smaller and intermittently occupied. In addition, there are now more roosts in urban locations, and consequently a greater public profile and frequency of interaction with people. With this increased overlap in human and bat habitat, the possibility of increased zoonotic disease transmission exists, and there is ongoing public debate about the effective management of these ecologically important animals, such that the public health risks (and public perception of these risks) are mitigated while the conservation of bats as an important wildlife resource are also considered. This debate gets at the heart of the very dilemma that populations around the world will inevitably grapple with as humans further encroach on wildlife habitat: how to reconcile conservation of natural resources with public health and economic development. It is becoming increasingly clear from scientific study of disease emergence that human activities are precipitating increased contact with wildlife, and driving pathogen spillover, making it necessary to better understand these underlying processes that lead to zoonotic disease outbreaks and modify our behavior if we wish to reduce the risk of outbreak.

10.4.3 Wildlife trade: SARS-CoV

SARS was first reported in February 2003 in China. When the World Health Organization declared the outbreak over on 5 July 2003, more than 8000 cases (over 800 fatal) had been reported in 32 countries worldwide. The economic cost has been estimated to be more than US$50 billion (Lee & McKibbin, 2004). The outbreak occurred at a time in history when knowledge of the origin of an emerging agent and an understanding of the factors associated with emergence, were recognized as being fundamental to controlling an outbreak, and to managing the risk of subsequent spillovers and associated disease outbreaks. To this end, a succession of phylogenetic and epidemiological findings suggested that SARS had a wildlife origin, and that "wet markets" in southern China were the origin of the outbreak (Guan *et al.*, 2003; Ksiazek *et al.*, 2003; Xu *et al.*, 2004). Subsequently, two groups independently identified SARS-like coronaviruses in species of bats in China (Lau *et al.*, 2005; Li *et al.*, 2005). Li *et al*. (2005) found compelling serological and molecular evidence of a cluster of SARS-like coronaviruses in several species of free-living horseshoe bats (*Rhinolophus* spp.) in southern China. They contended that the virus responsible for the SARS outbreak in humans in 2003 emerged from this cluster of viruses, and that bats are the origin of the SARS coronavirus. Cui *et al*. (2007) suggested that *Rhinolophus* species were more likely to foster host shifts of coronaviruses than other bat species, and that this propensity, when combined with the potential for close contact between bats, civets and humans in the wildlife trade in southern China, supported SARS-like coronaviruses as being the source of the SARS

Figure 10.9 Masked palm civets (*Paguma lavarta*) traded in wet markets in China were a putative intermediate host of SARS and the immediate source of SARS infection in humans. Photo by H. E. Field, 2014. *(See insert for color representation of the figure)*.

coronavirus. This argument was consistent with molecular analyses by Vijaykrishna *et al*. (2007), which indicated a recent host shift of coronaviruses from bats to palm civets or other animals, and subsequently humans. The recent findings of Ge *et al.* (2013) – which included the isolation of a virus that is the closest match, genetically, to SARS-CoV to date, and which uses the angiotensin-converting enzyme-2 receptor to enter to human lung tissue cells – confirm Rhinolophidae as the natural host of SARS coronavirus and strongly support the hypothesis that direct transmission between bats and humans is possible.

The approach of Mills and Childs (1998) to the investigation of infectious diseases in wildlife populations highlights the fundamental need to understand the ecology of the disease in order to achieve effective control or prevention. However, the emergence of SARS suggests that the fusion of ecological factors with cultural and economic factors can greatly complicate the investigation of emerging infectious disease from wildlife. Identifying the drivers for the emergence of SARS requires not only an understanding of the ecology of infection in the natural reservoir (and *a priori*, the identification of the natural reservoir), but also an understanding of the ecology of infection in the secondary market reservoir species (especially masked palm civets, Figure 10.9) that were the immediate source of human infection. Thus, a necessary extension of understanding the ecology of disease in the reservoir (and fundamentally the ecology of the reservoir), is an understanding of the wildlife trade, and of the social and cultural context of wildlife consumption. A rich cultural heritage underlies wildlife consumption in China. Many people, particularly in southern China still seek *ye wei* – the wild taste – and believe that it endows added social status, prosperity, and health benefits derived from the traits of the animal, or from specific parts of the animal.

Increasing affluence in parts of China, associated with a burgeoning economy, appears to have triggered an increased demand for wildlife, and fostered an increased wildlife trade (both legal and illegal) in China and neighboring countries to satisfy

Figure 10.10 Poor biosecurity measures and close human–animal contact in wet markets in southern China promoted cross-species transmission of novel viruses. Photo by H. E. Field, 2014. *(See insert for color representation of the figure).*

this demand. The dynamic demographic shifts in China (as people move from country to city and from region to region to pursue economic opportunities) add additional complexity. For example, people moving from southern China to Beijing take with them their demand for *ye wei*, and so restaurants and the supporting wildlife trade move with them. As China's human resources become internationally mobile, it is likely that supply will continue to follow demand. Detailed records of numbers, species, origin, destination, temporal patterns of supply and demand of wildlife seem to be unavailable, but what do we know? We know that a wholesale and retail structure exists in the wildlife trade in southern China, with multiple wholesalers providing multiple retailers at a city level. We know that some wildlife are farmed and some wild-caught. What about the marketing structure? Are there dealers who buy and sell from both sources? How much farm-to-farm trading occurs? Do farms periodically augment their stock from the wild? To answer these questions, it is necessary to understand what drives the wildlife trade – a complex mix of economic, social, and cultural factors. This complexity is illustrated by the anecdote that in southern China, wild-caught civets attract a price premium, because people believe they are more health-giving (and taste better) than their grain-fed farmed counterparts.

What is the role of the wet markets in facilitating disease transmission? Typical trading and husbandry practices in wildlife markets throughout China and Asia show little or no awareness of biosecurity. There appears to be no attempt to quarantine animals from different sources, cages are often crowded and heavily contaminated with feces and spoiled food, dead animals are often observed in cages or in the market walkways, and different species typically juxtapose or share cages (see Figure 10.10 for example).

10.4.4 Bushmeat hunting: Ebola virus

Despite the fact that we have been aware of Ebola virus since 1976, little was known about its reservoir until 2005, when antibodies against Ebola virus and viral nucleic acid were found in several species of bat in Central Africa, most frequently in three frugivorous species: *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycterus torquata* (Leroy *et al.*, 2005). Ebola outbreaks in the Republic of Congo and Gabon from 2000–2003 were associated with mortality events in gorillas and chimpanzees that preceded the human outbreaks (Leroy *et al.*, 2002, 2004; Walsh *et al.*, 2003). While it remains uncertain exactly how gorillas and chimpanzees are infected, the practice of hunting and butchering great apes, and collecting and consuming carcasses from the forest, facilitates the zoonotic transmission of Ebola virus (Leroy *et al.*, 2002, 2004).

Filoviruses are reviewed in depth in Chapter 6, and the discussion here is limited to bushmeat hunting as another example of human behavior that expands the wildlife–human interface and promotes contact. Hunting wildlife for sustenance and as a commodity is commonplace across Central and West Africa (Wilkie & Carpenter, 1999; Kamins *et al.*, 2011). There have been more than 20 outbreaks of Ebola virus since its discovery in 1976; however, the majority of them (prior to 2014) have been small, localized outbreaks affecting fewer than 1000 people (Olival & Hayman, 2014). The fact that there have been relatively few Ebola virus outbreaks over the past 40 years is surprising, given the opportunity for spillover through ongoing bushmeat hunting and the relatively high (20%) prevalence of viral RNA detected in bats (Leroy *et al.*, 2005). However, most Ebola outbreaks have been tied to contact with wildlife other than bats. In fact, there is no specific evidence of direct bat-to-human infection with Ebola virus, though in an outbreak in Luebo, DRC in 2007, there was evidence that fruit bats (*H. monstrosus* and *E. franqueti*) had aggregated near a village that hunted them at the time that the index case become infected (Leroy *et al.*, 2009). Cases of Marburg virus have more often been linked to exposure to bats (*Rousettus aegyptiacus*), as detailed in Chapter 6.

Fortunately, the majority of Ebola virus outbreaks have occurred in relatively isolated communities where movement of infected individuals and contact with other people was fairly limited. By contrast, the 2014 Ebola outbreak in West Africa highlights the catastrophic consequences that can follow a single spillover event given the right circumstances: an outbreak in a populous region, where social customs promote close contact with the sick or dead (e.g., in funeral rituals); where fear and a lack of understanding of infectious disease and mistrust of government promotes transboundary movement to seek medical care or to evade public health authorities; and where there is high connectivity with urban centers (Baize *et al.*, 2014; Wesolowski *et al.*, 2014). Guéckedou, in Guinea, ground zero for the 2014 outbreak, is connected to the capital, Conakry by a highway, and also is situated on the border with Sierra Leone and Liberia. Conakry became the first urban center to experience an Ebola outbreak. It is evident that public health systems in Guinea, Sierra Leone, and Liberia were inadequately prepared to handle an outbreak of Ebola virus disease, and this situation has contributed to this being the largest outbreak in history, with more than 10,000 cases identified and the number rapidly increasing (CDC, 2014).

Bats are regularly hunted and consumed in West Africa, and though it is currently unknown whether the index case had contact with a bat or some other animal (Baize *et al.*, 2014), understanding which species of bat may carry Ebola in this region and

intensive educational campaigns to change the way people handle bats are necessary to reduce the risk of future outbreaks not only in the countries presently experiencing Ebola virus disease, but in other countries that have Ebola reservoir species that are hunted and eaten.

10.5 OUTBREAK MITIGATION: MANAGING THE INTERFACE

It seems that the more scientists look for viruses in bats, the more they find, and evidence is mounting to suggest that not only do bats carry significant zoonotic viruses, but that they may have been the original reservoir for what are now considered human pathogens, including measles and hepatitis C virus (Drexler *et al.*, 2012; Epstein *et al.*, 2010; Quan *et al.*, 2013). Two new strains of influenza virus have recently been found in bats, suggesting that influenza A viruses may have originated in bats as well (Tong *et al.*, 2012, 2013).

In Australia, the increasing urban presence of flying foxes has meant increased public demand for flying-fox management. Large numbers of flying foxes in an urban environment can undoubtedly cause noise, soiling, and consequent loss of social amenity for people living in the immediate vicinity, and threaten economic loss for local businesses. This situation places increasing pressure on local authorities to remove flying fox colonies. Historically, this was often done by the local gun club indiscriminately shooting up the colony, which had major animal welfare and ecological consequences, and was of variable success in relocating the colony. Nonetheless, there are still periodic calls for culling. Flying foxes are regarded by some as vermin and their urban presence provokes strong negative emotions in these individuals. A larger section of the community supports dispersal of colonies by nonlethal means; however, any attempt to disperse a colony is fraught with uncertainty as to the outcome. There are numerous examples of colonies moving a short distance to an even more problematic location, or to colonies fragmenting and occupying multiple locations. More broadly, there is increasing awareness that dispersing colonies is simply moving the "problem" to another community, but this is juxtaposed with a desire/demand for an immediate "solution" from those negatively affected for the animals (Kung *et al.*, personal communication).

In Uganda, a mass culling of *R. aegyptiacus* occurred in 2008 at Kitanga mine following several cases of Marburg virus hemorrhagic fever (Amman *et al.*, 2014). The cave had been completely depopulated. In 2012, an outbreak of Marburg virus occurred in a nearby town. Affecting 15 people, this was the largest outbreak of Marburg virus to date. Bats were observed back in Kitanga mine, though at a much reduced abundance. Studies of 400 bats showed that Marburg virus RNA was present in 13.3% of the bats. In 2007–2008, a longitudinal study of bats in this location found the prevalence in this population to be significantly lower at 5.1% (Amman *et al.*, 2014). The authors of the study concluded that the mine was repopulated by bats susceptible to Marburg virus, and that subsequent introductions of virus led to a larger proportion of infected individuals, which thereby increased the risk of spillover to a local human population (Amman *et al.*, 2014).

This is an example of the type of unintended consequence that can occur by displacing or removing a local bat population. Additionally, flying foxes provide valuable ecosystem services such as pollination and seed dispersal, and extermination or even

disruption of wild populations may be detrimental to forest ecosystems. It is far more pragmatic, ecologically sound, and cost effective to focus on changing human behaviours that lead to disease outbreaks than to attempt to exterminate wild bat populations. The processes that cause outbreaks are well understood, and *simple*, effective solutions that modify high risk behaviors (e.g., using bamboo skirts to cover date palm sap pots to protect sap from bat excreta), are the best way to prevent future disease outbreaks.

10.6 CONCLUSIONS

The emergence of zoonotic viruses that cause high mortality rates in humans and livestock, and their association with bats, has led to unprecedented levels of research on the viruses and their chiropteran hosts. However, there is still much to learn regarding the ecology of viruses such as Ebola virus, Middle East respiratory syndrome coronavirus, and henipaviruses, as well as the increasing numbers of novel viruses that are identified. Important questions remain as to how to relate viral genotype to phenotype and clinical outcomes in people. An oft-asked question is whether bats are unique in their ability to carry viruses with little or no clinical impact. Little is understood about the bat immune system and how it responds to viral infection, though important research is underway to elucidate immunological mechanisms in bats (see Chapter 14). Much work is needed to understand these fundamental aspects of bat biology and physiology, as well as viral genetics and the ecology of the zoonotic viruses described in this chapter. Identifying predictable patterns in bat infection as well as ecological processes that create contact between people and bats, will allow us to develop predictive models for spillover, and reduce the risk of human outbreaks.

For those who wish to undertake field studies in bats, obtaining viral isolates that allow for whole-genome analyses and experimental studies will be critically important for understanding both the spectrum of strain diversity within a viral genus and the functional differences that result from these genetic differences. Sample collections from wild bats should focus on maximizing information about tissue tropism and routes of viral excretion, though care should always be taken to perform humane, ethical animal capture and sampling techniques, and sampling strategies should be based on knowledge of abundance and conservation status of the bat species being studied. Viruses should not be studied in isolation and gathering careful biological and ecological information about the bats being studied as well as the way in which they relate to local human and livestock populations is critically important for identifying strategies for reducing the risk of viral spillover. Multidisciplinary teams are best equipped to conduct comprehensive studies of the ecology of zoonotic viruses in bats and the human activities that promote viral spillover, bearing in mind that anthropogenic activities such as deforestation, agricultural intensification, hunting and wildlife trade are major drivers of zoonotic disease emergence. These activities are likely to continue to intensify as human populations grow and demand for natural resources increases; therefore it is incumbent upon us, the scientific community, continue to broaden our understanding of how zoonotic viruses operate in their hosts and identify strategies that protect both human health and ecologically important bat species. Balancing anthropogenic environmental change with ecosystem protection may ultimately reduce the risk of disease emergence by limiting our contact with bats and other wildlife.

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11

ARE BATS REALLY "SPECIAL" AS VIRAL RESERVOIRS? WHAT WE KNOW AND NEED TO KNOW

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11.1 INTRODUCTION

The notion that bats are "special" or "unique" as reservoir hosts for viral pathogens has recently become in vogue in both the media and scientific literature (Dobson, 2005; Wang *et al.*, 2011; Olival *et al.*, 2012; Luis *et al.*, 2013; O'Shea *et al.*, 2014). This was initially based on the relatively recent finding that a number of high-profile zoonotic viruses (such as severe acute respiratory syndrome coronavirus (SARS-CoV), Ebola and Marburg viruses, and Hendra and Nipah viruses) have bat origins. It has been further developed through the hypothesis that the life history traits of bats compared to other mammals may make them unique and exceptional hosts for viruses (Luis *et al.*, 2013; O'Shea *et al.*, 2014), and with the finding that they harbor more viruses than some other groups of mammals (Luis *et al.*, 2013). However, claims of bats being "special" as viral reservoirs are often made in isolation, without a proper comparative approach that includes data from a wide array of animal taxonomic groups. In this chapter, we extend our previously published preliminary analysis of this issue (Olival *et al.*, 2012), and take a critical and objective look at what may, or may not, make bats "special" as disease reservoirs.

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11.2 WHAT FACTORS MAY MAKE A HOST TAXON "SPECIAL" AS A VIRAL RESERVOIR?

In order to make claims that some host taxonomic groups, such as those within a specific mammalian Order, are "special" or "unique" as compared to other taxonomic groups, it is first necessary to define what we mean by "special". There are six criteria that can be used to test whether a given group of mammals differs from others in terms of their importance or significance as disease reservoirs. We use the term taxonomic group to generally refer to any hierarchical taxonomic level which can be compared to others in the same level, for example a genus as compared to other host genera, or, as often used here, an Order compared to other Orders in the same Class (Mammalia).

1. Greater number of pathogens (greater viral richness) than other taxonomic groups.
2. Higher proportion of zoonotic diseases than other taxonomic groups.
3. Unique set of pathogens as compared to other taxonomic groups.
4. Ecological, behavioral, or life-history traits that differentially favor pathogen diversity as compared to other taxonomic groups.
5. Ecological, behavioral, or life-history traits that differentially favor interspecies transmission or spillover as compared to other taxonomic groups.
6. Unique immune system traits or responses that result in more frequent asymptomatic infection as compared to other taxonomic groups.

11.3 FACTORS THAT MAY CONFOUND INVESTIGATIONS OF WHETHER OR NOT A TAXONOMIC GROUP IS "SPECIAL"

The ability to determine whether a host is "special", based on the above six criteria, is highly dependent on the availability of unbiased and comprehensive datasets for comparable taxonomic groups. With a complete and unbiased dataset, simple statistical approaches can be used to test hypotheses related to the six criteria listed previously (Hypothesis 1: Bats harbor a greater number of viruses than other taxonomic groups). However, the availability of unbiased data is rare, and therefore methods to differentiate certain taxonomic groups over others may be confounded by sampling bias (as in, not distributing pathogen discovery research equally) and other factors that affect probability of detection (for instance, lack of clinical signs or lack of immunological reagents). We address specific research limitations and causes for bias in the comparative analyses next.

11.3.1 Research bias towards certain hosts and pathogens

We examined the number of virus-related research studies published per year for each major mammalian order, through a keyword search of ISI Web of Science including order name, common name, and virus keywords, such as "((chiroptera OR bat*) AND (virus* OR viral))." The number of bat virus studies has grown dramatically, especially in the last 10 years, as compared to some other groups of mammals (Figure 11.1). Other orders, including primates and rodents, have an order of magnitude greater number of

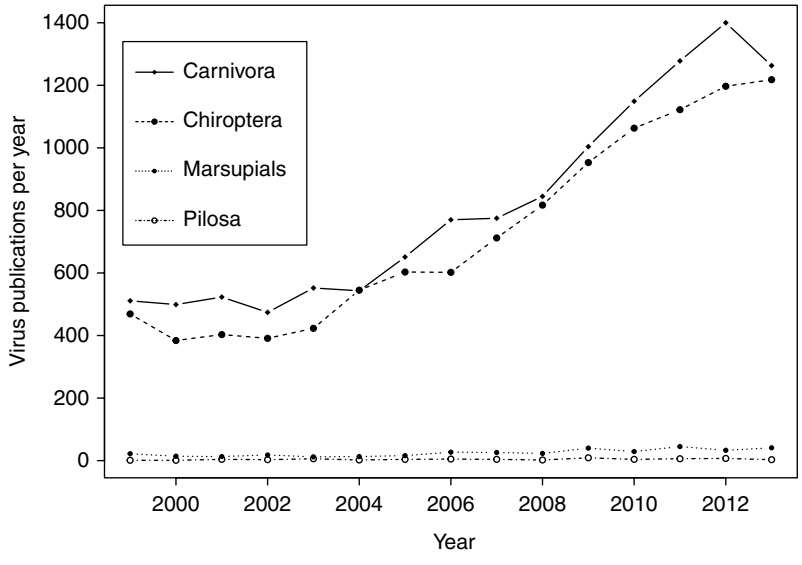

Figure 11.1 Number of virus research papers published per year from 1999–2013 for bats, carnivores, marsupials, and sloths.

studies published than bats; for example, there were 23 012 virus studies on rodents and 70 900 studies on primates in 2013.

However, the bias toward a very large number of studies in rodents and primates may be due to studies using experimental animal models, including *Rattus rattus* and macaques – common viral infection lab models – and may not reflect surveillance in wild species. We find that the number of virus studies published per year for the Order Carnivora is roughly equivalent to the number of papers published on bats – although this too could be biased higher due to lab or domestic animals such as using ferrets for influenza studies, and dogs and cats. Other, less speciose orders like Pilosa (sloths and anteaters), Didelphimorphia (opossums), and Diprotodontia (kangaroos, wallabies, etc.) (Note: 'Marsupials' combines these two orders in Figure 11.1), have been subject to very little virus research ('Marsupials' 1999–2013, range 12–45 studies per year; Pilosa, range 1–9 studies). Neither of these "control taxonomic groups" has seen the dramatic increase in viral research in recent years that bats, carnivores, rodents, and primates all have. This begs the rhetorical question: Are sloths special? Or any other understudied order of mammals? We know that, overall, Pilosa is a much less diverse order of mammals, but on a per species basis do they harbor more viruses, more unique viruses, or more zoonotic viruses but we just do not know it yet because we have not looked?

11.3.2 Lack of thorough disease ecology studies

One criterion for defining whether a given taxonomic group is "special" would be to assess whether or not humans share some unique ecological interface or likelihood of contact with that group which differs from other groups. For example, bats, due to the synanthropic roosting behavior of many species, have been implicated as being more

likely to come into contact with humans than many other groups of mammals, and overall in Australasia make up a higher proportion of species that use human-modified habitat (McFarlane *et al.*, 2012). While this is true for some species of bat that are adaptable to human dwellings, many species of bat are also obligate forest-dwelling species that need pristine forest for survival. For example, over 125 species of bats, including many obligate forest interior species, have been described from one rich rainforest community in Malaysia (Kingston *et al.*, 2006). Furthermore, while some bat species are capable of roosting and foraging in close proximity to humans, several species of rodents are also tolerant of disturbed habitat and act as common as "peri-domestic" species around the world. In ranking mammalian orders by emerging infectious disease host status and use of human modified habitat, McFarlane *et al.* (2012) found that rodents, primates, and carnivores all had higher odds ratios than bats.

While these literature review studies are important, in order to properly assess levels of wildlife contact with humans, thorough ecological studies and properly designed studies for data collection are needed. Several detailed disease ecology studies of bat–human ecological interaction have been published over the last decade, but these remain limited to a few species. This includes the use of telemetry to identify overlap and habitat use in flying foxes in order to understand the ecology of henipaviruses (Epstein *et al.*, 2009; Newman *et al.*, 2011; Smith *et al.*, 2011) and Reston Ebola virus (de Jong *et al.*, 2013). Geographical information systems (GIS)-based approaches to estimate likelihood of contact and spillover have also been used to estimate risk of Nipah virus spillover at a coarse scale (Hahn *et al.*, 2014a, b). Camera traps have been used to understand specific bat foraging ecology and have led to implementation of a low-tech barrier solution to reduce the risk of Nipah virus transmission via bat contact (Khan *et al.*, 2011). Most recently, under the USAID PREDICT project, we have begun to systematically quantify human contact with bat, primate, and rodent wildlife species across tropical land-use gradients on three continents (in the Brazilian Amazon, Borneo rainforest, and Ugandan Bwindi Rainforest). This project, named Deep Forest, uses a combination of ecological surveys using standardized trapping at each field site and standardized behavioral questionnaires administered to people living around each field site and those who interact with wildlife. Ultimately, these studies are relatively few, and are often preliminary in nature, so it is not yet possible to deduce how frequently people make contact with bats, under what circumstances they do so, and whether this is increasing over time so that it could contribute to increased viral emergence from bats.

11.3.3 The ability to measure immune responses and detect illness in hosts

Reservoirs of zoonotic viruses are typically able to support replication of the viral agent with only minor clinical signs of infection. Some authors have suggested that another criteria defining bats as "special" disease reservoirs is their ability to become infected with diverse viral pathogens, but show no clinical signs. Unfortunately, very few studies have been conducted to test this phenomenon in bats, largely because such trials are difficult to conduct in the field, and usually involve experimental infections of previously uninfected hosts. Clearly, the absence of prior infection cannot be determined with any level of confidence in wild-caught animals. Very few laboratory colonies of bats have been set up, and little work has so far been conducted to test this hypothesis. Experimental

infection studies of *Pteropus* sp. bats with henipaviruses (Halpin *et al.*, 2011; Middleton *et al.*, 2007), recent work on infection in *Rousettus* sp. bats with Marburg virus (Towner, 2013), and other studies have increased our understanding of bat immune response, but are insufficient to fully understand the diversity of host–pathogen interactions involving bats. The results of these studies do not support the claim that bats show no clinical signs when challenged with virus. Rather, these studies are inconclusive to date in this subject, suggesting that they may not be unusually refractory to viral infection and may be similar to other reservoir host–pathogen relationships that have coexisted for long periods of time. One definition of a reservoir host is a species that has coevolved with a given pathogen, and is immunologically conditioned to its infection (Ashford, 2003; Haydon *et al.*, 2002; Olival *et al.*, 2012). For example, rodents have co-evolved with dozens of different Hantaviruses, with most viral strains and species being highly species-specific and seeming to cause only mild pathology in their natural host species. Experimental research on various rodent models has shown that rodents have a similar ability to become infected, shed virus, but show little to no clinical signs when infected with Hantaviruses (Schountz & Prescott, 2014). Does this make rodents special because they do not get sick from Hantaviruses, but other hosts (i.e., humans) will show pathology and die from these viruses? This indeed leads us to a critical and still unanswered scientific question, whether we are talking about bats, rodents, or some other group of mammals: why do some individuals/genotypes/species get sick while others do not? And what can we learn about this process for the benefit of humans? It seems the uniqueness of the bat immune response remains an open question, and we should be reminded that a broader comparative approach is needed.

Another limitation in assessing clinical signs and determining if bats get "sick" or not from viral infection is a lack of bat-specific reagents for immunological studies (See Chapter 14 for further discussion) and of captive bat colonies for experimental work. Recently established captive colonies, such as *Rousettus aegyptiacus* at the Centers for Disease Control (CDC) in the US, are beginning to make experimental work more accessible and these efforts are shedding light on filovirus infections in bats (Olival & Hayman, 2014; Towner, 2013).

Finally, there has been little work so far on defining clinical signs in bats, or the clinical progression of illnesses. Do bats develop the same clinical features as other mammalian hosts? Should we expect elevated temperatures? What are the baseline physiological data we are measuring against? With the exception of a very few bat species kept in captivity (for instance, at Lubee Bat Conservancy and other zoos around the world), we have no idea as to what the physiological parameters for a "healthy bat", such as temperature or blood cell count, should be. Levinson *et al.* (2013) conducted a literature review to assess if viral discovery efforts targeted clinically "sick" wildlife, and if this was more effective than viral discovery in seemingly healthy wildlife hosts. They first found that only about half of all studies reported the health condition of the host animals. They argue that this can be improved with the inclusion of wildlife veterinarians in viral discovery efforts – and the general move toward more interdisciplinary "One Health" research teams. Second, they found for those studies that did report the health condition of animals, that there was no significant effect on the number of viruses found per host species in those animals that were reported as sick vs. those that were not. Interestingly, they did find that the proportion of hosts that were symptomatic differed by host order. Overall, bats were reported as being asymptomatic more often than

other host groups (Levinson *et al.*, 2013). However, is this due to the fact that sick, wild bats are difficult to detect in the field, or is it a real physiological or immunological effect that was captured in the reported studies? These questions remain unanswered, but it is likely a combination of both factors. More systematic approaches to assessing health status in wildlife are needed to explore this further, rather than reliance on disparate studies published over the years.

11.4 VIRAL DIVERSITY IN BATS COMPARED TO OTHER MAMMALIAN HOSTS

In order to assess whether bats harbor a disproportionate number of viruses compared to other groups of mammals, and whether they harbor a disproportionate number of zoonotic viruses, we updated our literature review and data extraction process that included all literature describing mammalian viruses and their hosts published over the previous 50 years (Olival *et al.*, 2012).

11.4.1 Do bats harbor a disproportionate number of viruses?

In a two order comparison of bats and rodents, Luis *et al.* (2013) found that overall bats harbored 2.71 total viruses per species, and rodents harbored 2.48. While their study did use robust modeling approaches including phylogenetic correction, these values of mean number of viruses per host cannot be taken to suggest that bats really do harbor more virus than other mammals. While accessible, these summary statistics do not properly account for research bias, and further include only comparison of two mammalian orders. To address this latter issue, we used our database of mammalian viruses to examine viral richness across all mammalian orders for which we had data. In Figure 11.2 we summarize the known viral richness for the six most speciose orders. In this broad data set, although mean values will obviously differ between orders, these differences in viral richness across orders are not statistically significant. Thus, using a very large comparative dataset including 593 unique viruses and 768 mammal species from 13 different orders, bats do not stand out as having a significantly greater per-species viral diversity than other mammalian orders.

11.4.2 Do bats harbor a disproportionate number of zoonoses?

In their analysis, Luis *et al.* (2013) also found that bats harbored slightly more zoonotic viruses on a per species basis, 1.79 mean zoonotic viruses per bat species, and 1.48 per rodent species. We also addressed this hypothesis using our large comparative data set to include more than the two orders they looked at. In Figure 11.3 we show the proportion of zoonotic viruses on a per-species basis for each of 13 mammalian orders. As is evident, there is a paucity of data for several orders, including Cingulata, Pilosa, Didelphimorphia, and Eulipotyphyla, even though the proportion of zoonotic viruses for each of these orders is close to 100%. This finding clearly reflects the role of research bias (which we did not correct for here). It is not necessarily the case that these hosts harbor only viruses that can infect humans, but rather that we are biased in our available assays and surveillance priorities towards viruses that infect humans. No doubt each of

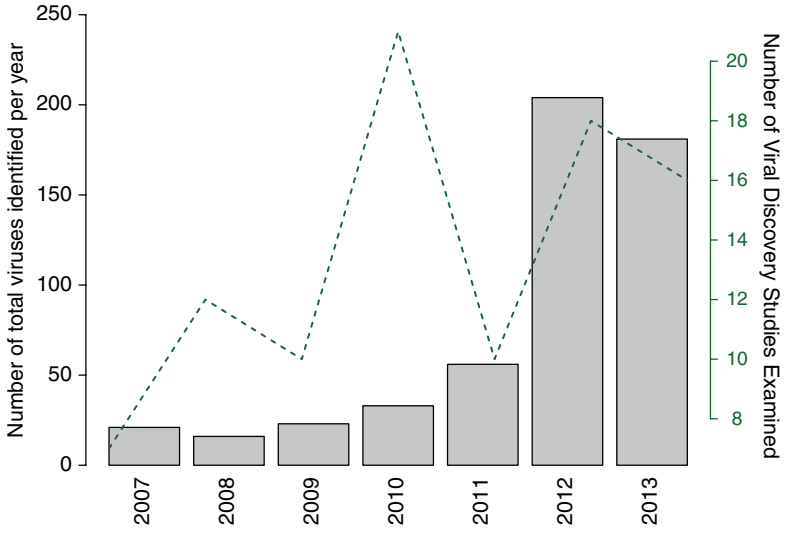

Figure 11.2 Viral discovery publications per year for bats from the past 7 years, and total number of viruses found in those studies (dashed line). Data from Weekley & Olival (in preparation).

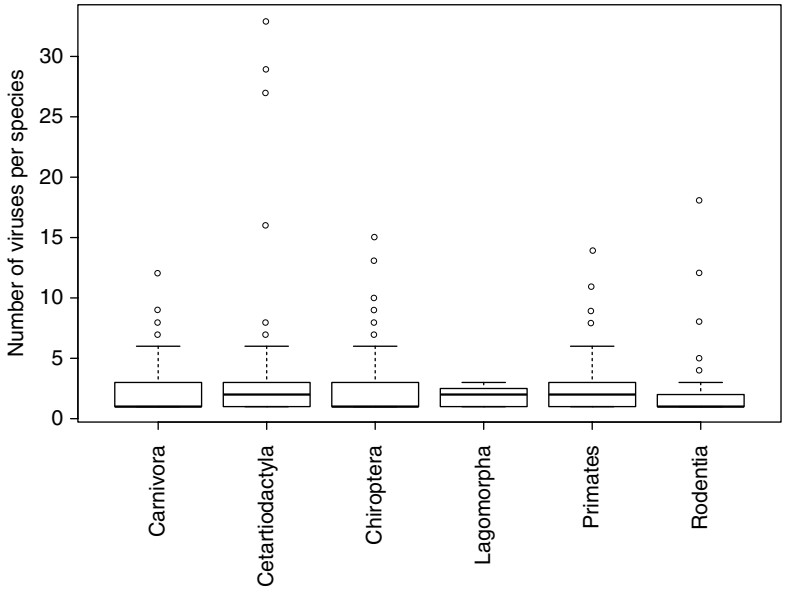

Figure 11.3 Boxplot showing total viral richness, species level data aggregated by order, for over 700 species of mammals across six orders with the most data available. Data updated from Olival *et al.* (2012).

these orders harbors a unique pool of viruses, but to date we have lacked the tools and motivation to describe this viral diversity. For the orders that do have significant data available, we find that bats, rodents, and primates all have a significantly higher proportion of zoonoses than other orders, but the differences between them are not significant (Figure 11.3). Again, our findings do not suggest that bats are more special than either rodents or primates in terms of their ability to harbor zoonotic viruses.

11.4.3 Focused literature review of bat viral discovery efforts from the past 7 years

We conducted a detailed quantitative review of the bat viral discovery literature published between 2007 and 2013, to identify specific variables to better target future viral discovery efforts in bats (Weekley & Olival, unpublished data, in preparation). In this review, data were extracted from 94 bat virus studies, including host species identification, sample types, virus detection methods, and viral identification to at least the family level, and the number of total and novel viruses found per study.

We found that the number of studies have increased over this 7-year period, as have the number of bat species sampled per study, the number of novel and total viruses found by year of publication, and increased reliance on molecular detection methods. Figure 11.4 shows the total number of viruses identified in these studies per year from 2007–2013, and the number of viral discovery studies investigated (dashed line). There is an increasing trend of more studies yielding more total viruses in bats; however this relationship is not significant for several reasons. For example, in 2010 there were a large number of studies published, but most of these were describing single viruses from single host species. Research efforts have shifted to larger discovery studies involving multiple bat host species for a given viral family (Drexler *et al.*, 2012; Anthony *et al.*, 2013b; Quan *et al.*, 2013) and multiple viral families for a given host species (Anthony *et al.*, 2013a). This has resulted in a large increase in viral detection and discovery in bats, which does not relate linearly to research effort as measured by the number of publications per year. This is important, as it may skew findings if using linear methods to correct for research effort, and reflects the surge in viruses discovered in bats over the last two years.

Across the 94 bat studies examined, over 60 000 samples were taken from over 44 000 individual bats comprising 17 families, 110 genera, and 340 species, with 24 viral families identified. The majority of viruses described were found in only four host families (Vespertilionidae, Pteropodidae, Rhinolophidae, and Hipposideridae), with mean viral prevalence highest in feces and tissue samples. These results have important implications for how future viral discovery studies in bats are designed, how samples are collected, what samples should be screened for different viral families, and whether lethal sampling is effective (Weekley & Olival, unpublished data).

11.5 LIFE HISTORY TRAITS: ARE BATS UNIQUE?

Do bats have unique life history and ecological characteristics that make them better positioned to harbor and transmit viruses to humans than other mammals? First, it is important to recognize that bats are an extremely diverse group and have a wide range of life-history traits, morphologies, and ecologies (Nowak, 1999). Thus there are two

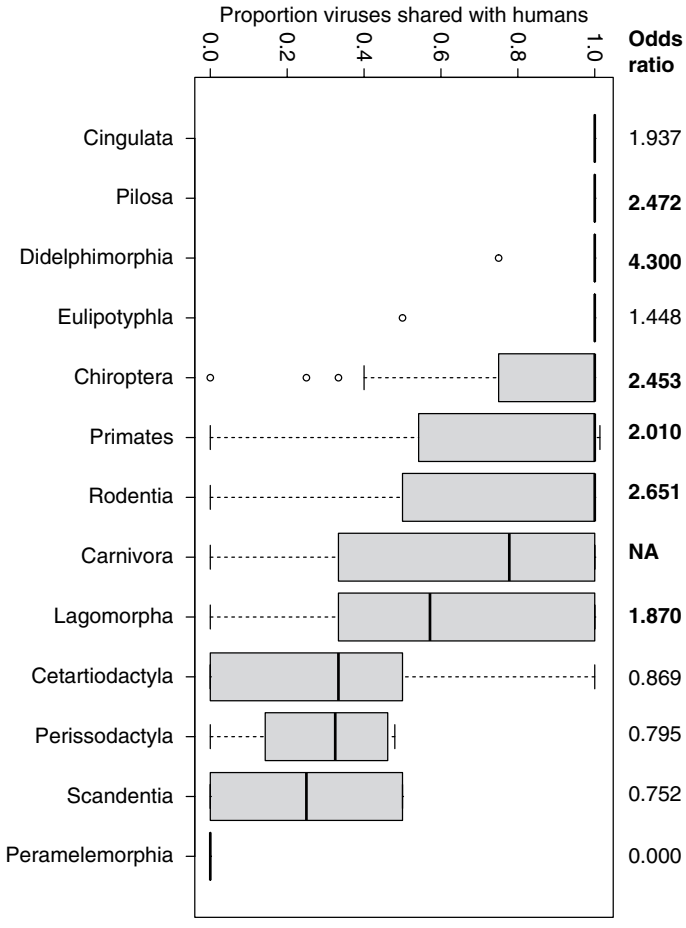

Figure 11.4 Boxplots based on species-level data of proportion of viruses for each species in an Order that are zoonotic. Black bar is median value, box shows interquartile range of proportion of zoonotic viruses across the order. Odds ratio calculated relative to default Order in analysis, Carnivora. Note: while Cingulata, Pilosa, Didelphimorphia, and Eulipotyphla have the highest overall proportion of zoonotic viruses per species, they are also the most data deficient Orders and are highly skewed.

types of comparisons in question. First, there is the *intra*-order comparison; that is, are there specific species of bats that harbor more viruses or more zoonoses than other bat species? Second, the *inter*-order comparison; do bats harbor more viruses because of life history traits that are distinct from all other groups of mammals? Several studies have addressed the former question previously using data acquired from literature reviews, and factors like geographic range area and shape, population structure, sympatric overlap with other hosts, and the use of torpor were each found to be significant in various studies (Turmelle & Olival, 2009; Olival *et al.*, 2012; Luis *et al.*, 2013; Gay *et al.*, 2014). In this chapter, we will take a closer look at the latter question: do bats differ in their life history traits in comparison to other groups of mammals?

At this large, comparative scale, the first thing that comes to mind that sets bats apart from all other mammals is their ability to fly. Bats are the only mammals capable of self-powered flight. Most bats (excluding the family Pteropodidae) have also evolved echolocation as a sensory trait, which they share with marine mammals through convergent evolution. O'Shea *et al.* (2014) proposed an interesting hypothesis that bats' ability to fly may have conferred some unique selective force for dealing with viral infection due to the metabolic rates and elevated body temperatures that are a by-product of the physiologically demanding act of flight. Essentially, these daily, elevated body temperatures may mimic a regular febrile response to limit infections. This hypothesis needs to be tested, but of all the life history traits, self-powered flight is certainly one that sets bats apart from all other mammals. To see whether, across all ~5000 mammalian species, we could identify other life-history traits uniquely different in bats, we analysed species level life-history data from the PanTHERIA database (Jones *et al.*, 2009). Figure 11.5 shows boxplots of species-level data aggregated by mammalian order for life-history traits that have been previously been suggested as significant in influencing viral diversity. Of the six traits examined, the only trait for which bats seem to stand out (though influenced strongly by a series of outlier species), is population group size. The *y*-axis of Figure 11.5 for Population Group Size was truncated to a maximum group size of 5000 individuals to make the data more visible across orders, but there are a handful of

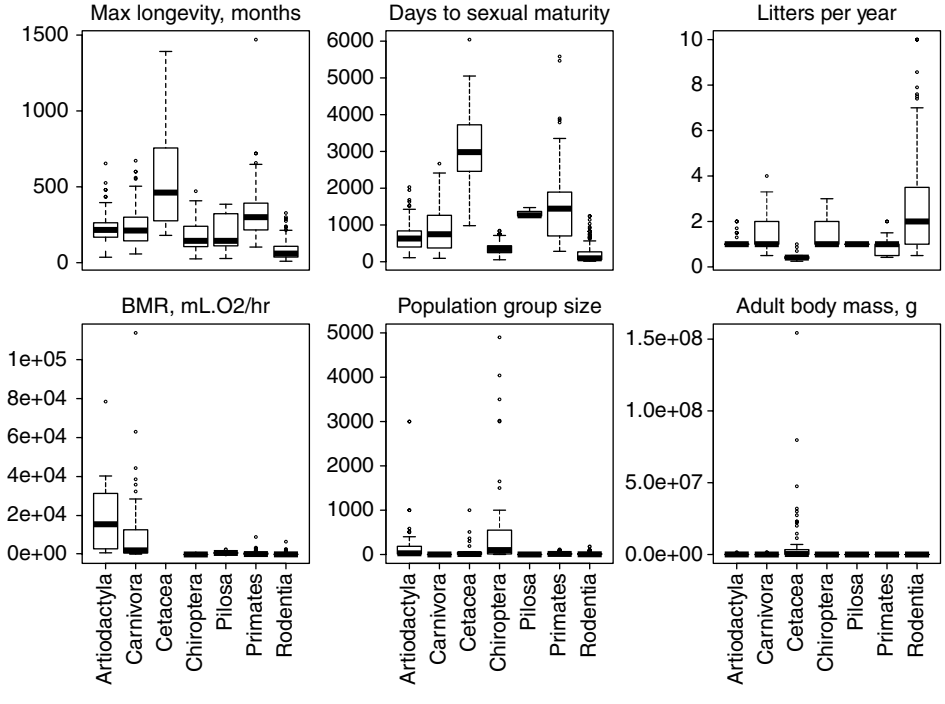

Figure 11.5 Boxplots of life-history trait data from PanTHERIA species-level database, grouped by Order for seven mammalian orders. Bats are not significantly differentiated from other mammals for any of these traits that have been previously suggested to influence viral diversity.

bat species that aggregate in population sizes much larger than this. For example, the Brazilian free-tailed bat and Straw-colored fruit bat are two outliers not shown on the figure, which both have population sizes in the millions. However, while these outliers are often cited as examples of bats having much larger population sizes than all other mammals, the totality of the species-level data shows that there is no statistically significant difference between orders for this trait. In summary, of the life history traits we examined, none seem to clearly set bats apart as unique from other mammals when viewed aggregated across species. It should be noted, however, that we did not examine some traits that have been suggested as being unique in bats because we did not have good comparative data across the groups (for example maximum heart rate or maximum oxygen consumption rate). More comparative research and species-specific data are needed to see how different bats are as a whole for other traits such as these.

11.6 DISTRIBUTION AND DIVERSITY OF BAT VIRUSES, AND WAYS TO TARGET FUTURE DISCOVERY EFFORTS

Whether or not bats are "special", we know they harbor some viral pathogens of serious consequence to human health. Several zoonotic viruses such as Ebola, Marburg, Nipah, Hendra, Middle East respiratory syndrome, and SARS-like coronaviruses, have bats as the most likely natural reservoirs (Calisher *et al.*, 2006; Ge *et al.*, 2013; Memish *et al.*, 2013; Olival *et al.*, 2012; Olival & Hayman, 2014). Due to the emergence of these high consequence pathogens in human populations, there has been a growing interest in analytical approaches that may begin to forecast bat-borne disease outbreaks and spillover events to other hosts. Can we predict the next bat-borne zoonoses? Can we allocate our surveillance resources to the geographic localities and species most likely to harbor the next big emerging infectious disease?

Brierely *et al.* (personal communication) laid out a framework to identify the drivers of zoonotic bat-borne virus emergence. They divided the processes of disease emergence into principle components and examined drivers at each stage: drivers of pathogen richness, drivers of transmission opportunity and drivers of infection success. This overall structure allows for different aspects of the emergence process to be tested against various potential "drivers", or causes of virus sharing, using spatial analyses that account for research bias. In this analysis, host diversity and climatic variability seem to drive pathogen richness; while human population density, bushmeat hunting and livestock production are significant drivers of transmission opportunity between bats and humans. Mapping the outputs of these spatial models for each stage of the emergence process separately can help to identify high priority locations for pathogen discovery in ways that may not overlap with those for public health interventions.

Others have focused on modeling which host species are most likely to harbor more viruses/zoonoses. These studies have given us clues to life history traits that may help predict viral richness within, but not between, different mammalian groups (Altizer *et al.*, 2003; Turmelle & Olival, 2009; Cooper *et al.*, 2012; Luis *et al.*, 2013; Gay *et al.*, 2014). Within bats, results from these studies suggest that species that are more frequently studied (research bias), with more fragmented range area, more structured populations, smaller litter size, larger body mass, and greater longevity and litters per year all may be more likely to harbor zoonoses and virus diversity in general. Species that are sympatric

with a large number of other species are also more likely to harbor a greater diversity of viruses (Luis *et al.*, 2013). Targeting bat species with these traits for viral discovery may prove particularly fruitful, as compared to randomly selecting species for surveillance.

Far less research has been conducted on targeting surveillance at key transmission and ecological interfaces, even for known zoonoses. For example, despite several bat species being identified as the origin of filoviruses, only a handful of studies have investigated the ecology of these bats with respect to viral shedding or high risk contact with people (for a review see Olival & Hayman, 2014). The exception seems to be the henipaviruses, largely due to the high number of outbreaks of Nipah virus in Bangladesh and Hendra virus in Australia identified recently. It is increasingly apparent that spillover of Nipah virus in Bangladesh is largely through the consumption of date palm sap contaminated by bats feeding from sap collection jars (Rahman *et al.*, 2012). In Australia, emergence of Hendra virus has occurred repeatedly via bat to horse viral spillover, followed by human contact with sick horses (Plowright *et al.*, 2011). It is, however, unclear whether these risk factors are responsible for spillover in other countries or are risk factors for other viruses carried by bats. Perhaps one of the challenges to progress is that ecological studies are complex, expensive and require multi-year surveillance to obtain the power necessary to test hypotheses. For example, it took over 5 years of research to identify the most likely underlying driver of Nipah virus emergence in Malaysia (Pulliam *et al.*, 2012). This included field capture and sampling of *Pteropus* bats, satellite telemetry, analysis of climate trends, veterinary epidemiology of pig farms, and complex mathematical modeling of viral dynamics. To understand the broader context of human–bat interactions, and their consequences for viral spillover will require similar long-term studies at geographically disparate sites, and the sampling of multiple species of bats representing the phylogenetic diversity of the Order Chiroptera.

11.7 SUMMARY AND FUTURE RESEARCH

The review presented here, and our literature and data analyses do not give a clear indication of whether bats are "special" for zoonotic viruses – that is, they do not definitively demonstrate that bats do, or do not, harbor a greater diversity of zoonotic or potentially zoonotic viruses than other taxonomic groups or have a greater propensity for spillover of zoonotic viruses. However, it does appear that bats, along with rodents and primates, not only make up the vast majority of mammal species in the world (~70%), but also seem to harbor a high proportion of zoonotic viruses. On the other hand, it is also clear that other mammalian taxonomic groups have had very little viral discovery research conducted on them, and with more dedicated research these groups may also turn out to be important for zoonoses. Our review does suggest that there are major gaps to be addressed before this hypothesis can be tested, particularly in the clinical signs that bats exhibit when infected as "natural" reservoirs, and in the mechanisms by which bat life history traits do or do not make them more likely to be reservoirs. Given the new analyses reported here, and the substantial gaps in other studies, we conclude that the evidence does not yet support the hypothesis that bats are "special" in their relationship with viruses. That said, our analyses provide substantial support that bats rank highly, along with primates, rodents, and potentially other mammalian groups, as hosts for a significant number of known and as-yet undiscovered zoonotic viruses,

many of which are, or may be significant public health threats. Like primates and rodents, bats may not be particularly "special" reservoirs, but simply reservoirs, and therefore should continue to be the focus of viral research and discovery.

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12

ANIMAL MODELS OF RECENTLY EMERGED BAT-BORNE VIRUSES

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12.1 INTRODUCTION

Animal infection models have long provided a surrogate means of studying the pathogenesis of human infectious diseases; different animal models may be used to reflect different disease aspects and understanding key elements of the human infection is necessary to optimize studies in animals. More recently it has become possible to design certain animal models using knock-in, knock-out, and humanized mice – immunodeficient mice into which human hematopoietic stem cells are injected to create mice carrying functioning human cells, tissues, and organs (Nomura *et al.*, 2008).

In addition to pathogenesis studies, such models allow assessment of candidate vaccines and therapeutics including preliminary estimation of dose rates and regimes. Animal models that are fit-for-purpose will replicate the virus of interest, preferably inducing clinical disease and pathology that closely mimic the human illness after exposure via known or plausible natural routes. The outcomes of exposure in the model must also be reproducible and well characterized. In the case of BSL-3 (Biosafety Level-3) or BSL-4 pathogens, the "Animal Efficacy Rule" introduced by the US Food and Drug Administration (FDA) in 2002 recognizes evaluation of countermeasures in two different animal models that appropriately reproduce the disease seen in humans.

Bats and Viruses: A New Frontier of Emerging Infectious Diseases, First Edition.
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In this chapter we discuss various animal infection models as they relate to recently emerged zoonotic bat-borne viruses: coronaviruses (severe acute respiratory syndrome (SARS)), filoviruses (Ebola and Marburg), and paramyxoviruses (Hendra and Nipah). Within each group of viruses, we briefly discuss the human clinical disease, then review four different types of animal model: (i) small laboratory mammals which are often used because of their size, ease of handling, availability of immunological reagents, facility for host adaptation of virus via multiple passages, and capability for genetic manipulation; (ii) non-human primates that are employed as human surrogates; (iii) spillover hosts that may play key roles in the impact of an infection on the community; and (iv) reservoir hosts for what they reveal about virus-host co-evolution.

12.2 SARS CORONAVIRUS

Until recently the only coronaviruses known to cause disease in humans were those associated with the common cold. In 2003 severe acute respiratory syndrome coronavirus (SARS-CoV) emerged and spread rapidly around the globe resulting in over 700 deaths with an overall mortality rate of 9.6% (Zhao *et al.*, 2014). Cynomolgus macaques were used to fulfill Koch's postulates and established SARS-CoV as the causative agent of the new disease syndrome (Fouchier *et al.*, 2003; Kuiken *et al.*, 2003). Since then, an array of different animals have been used as models of SARS-CoV infection although their disease is less severe and the course is shorter than in humans; no single model replicates all aspects of human disease. In addition to SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) is also a bat-borne virus of interest, but no animal models currently exist for this virus.

12.2.1 Human disease

SARS primarily affects the human respiratory tract. The incubation period of 2–10 days is characterized by fever, headache, and myalgia, which progresses to cough, shortness of breath and rapid breathing, pneumonia, pleurisy, and diarrhea (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003). During the course of infection, pulmonary alveoli and bronchioles become inflamed and necrotic, resulting in pulmonary hemorrhage, congestion, edema, and hyaline membrane formation (Ding *et al.*, 2003; Nicholls *et al.*, 2003b; Tse *et al.*, 2004). Deregulation of proinflammatory cytokine production is thought to be responsible for the severe lung damage, manifested as diffuse alveolar damage (DAD), which is the major pathology associated with the infection (Nicholls *et al.*, 2003a). Necrosis and/or inflammation of varying severity may also be seen in many other organs (reviewed in Gu & Korteweg, 2007). Age was found to be a predictor of disease severity and mortality with an increased mortality rate of 38–50% among the elderly (Chan *et al.*, 2003a, b).

12.2.2 Small animal models

Mice, hamsters, and ferrets are commonly used as experimental models for SARS-CoV infection. Inbred mice (BALB/c, C57BL/6, 129S) replicate the virus to moderate levels in nasal turbinates and lung but the course of infection is very short – virus is cleared by

5–7 days post infection – and there is no clinical disease (Glass *et al.*, 2004; Subbarao *et al.*, 2004). However, like in humans, age affects the course of the disease and aged BALB/c mice develop clinical signs including weight loss, ruffled fur, and dehydration (Roberts *et al.*, 2005a; Rockx *et al.*, 2009). Viral replication in the respiratory tract leads to DAD, pneumonitis, and hyaline membrane formation so the model can be used for pathogenesis studies and the evaluation of therapeutics (van den Brand *et al.*, 2014).

Serial passage of SARS-CoV (Urbani) in mice produced a strain, MA15, which replicated rapidly in young BALB/c mice to produce clinical signs including pneumonitis (Roberts *et al.*, 2007). Virus grew to very high titers in the lungs, causing damage to bronchiolar and alveolar epithelial cells and viral RNA was detected in a range of tissues. The disease course in these mice was very short, with death occurring 3–5 days after intranasal inoculation, and some aspects of the human disease such as DAD and edema did not develop. Although MA15 does not replicate all the features of SARS-CoV infection in humans it nonetheless provides a model for the investigation of SARS-CoV pathogenesis in young immunocompetent mice.

Some workers have elected to modify the host through genetic engineering. Transgenic mice expressing the human version of the SARS-CoV receptor angiotensin-converting enzyme-2 (ACE2) developed a lethal infection after inoculation with a human strain of the virus, displaying clinical signs such as weight loss, high virus titers in lung and brain, and upregulation of proinflammatory cytokines (McCray *et al.*, 2007; Tseng *et al.*, 2007). A STAT1 (signal transducer and activator of transcription-1)-deficient mouse model not only demonstrated the importance of interferon in SARS-CoV clearance, but also produced a model in which infection was prolonged with interstitial pneumonia and extrapulmonary spread (Hogan *et al.*, 2004).

Together, the various mouse models have been widely used to assess vaccines and therapeutics including DNA and recombinant vaccines (Bisht *et al.*, 2004; Yang *et al.*, 2004) and monoclonal antibodies (Greenough *et al.*, 2005), and also to show that mice re-exposed to SARS-CoV showed no sign of the (antibody-dependent) disease induction found in cats upon re-infection with the feline infectious peritonitis coronavirus (Subbarao *et al.*, 2004).

The Golden Syrian hamster produces a model where SARS-CoV consistently replicates to high levels in the upper and lower respiratory tract and the course of infection is comparatively long. There are no overt clinical signs but a later study showed a decreased ability to use a treadmill (Roberts *et al.*, 2008). Pneumonitis and pulmonary consolidation were present with evidence of spread to the liver and spleen (Roberts *et al.*, 2005b). Disease severity has been increased in an immunosuppressed hamster model that displays a longer disease course with higher mortality, expanded tissue tropism, and increased viral pathology including in the lung (Schaecher *et al.*, 2008). The hamster has been used to evaluate therapies for SARS-CoV infection (Roberts *et al.*, 2006), to assess the immunogenicity of the spike protein of SARS-CoV, and to measure the capacity of antibodies against this protein to mediate antibody dependent enhancement of disease (Kam *et al.*, 2007).

There are conflicting reports on the outcome of SARS-CoV exposure in ferrets. Several studies have reported clinical disease in ferrets (Martina *et al.*, 2003; ter Meulen *et al.*, 2004; Chu *et al.*, 2008; See *et al.*, 2008), while Weingartl *et al.* (2004a) reported subclinical infection. Where observed, ferrets developed clinical signs similar to those seen in humans, such as sneezing, nasal discharge and fever. Lesions in the respiratory

tract are also similar to those seen in humans, African green monkeys (AGMs), and macaques, with severe DAD and alveolar edema but no hyaline membranes or syncytia; lesions also occurred in the lung, spleen, liver, and lymph nodes (van den Brand *et al.*, 2008, 2014). The ferret model has now been successfully used to assess a number of vaccines (Darnell *et al.*, 2007; See *et al.*, 2008) and therapeutics (ter Meulen *et al.*, 2004) and to investigate the host response to SARS-CoV infection (Danesh *et al.*, 2011). One disadvantage of the ferret model has been a lack of specific immunological reagents, although this is slowly being remedied (Danesh *et al.*, 2008; Ochi *et al.*, 2008).

12.2.3 Nonhuman primates

The pathology seen in fatal human SARS is best replicated in cynomolgus macaques and AGMs, although even in these animals the disease is less severe than in humans with variable virus replication and reduced mortality. Initial studies in cynomolgus macaques (Fouchier *et al.*, 2003; Kuiken *et al.*, 2003) described lethargy, skin rash, and respiratory distress with interstitial pneumonia, with replication in the tissues. However, a later study comparing SARS-CoV infection in AGMs, rhesus, and cynomolgus macaques reported no clinical signs of disease in any of these nonhuman primates (NHPs) although all replicated the virus in both the upper and lower respiratory tract (McAuliffe *et al.*, 2004). Age is a factor in the disease seen in cynomolgus macaques (Smits *et al.*, 2010), with disease severity increasing with age. Many other factors may affect the outcome of SARS studies in NHPs, including virus strain and dose (Subbarao & Roberts, 2006); although the variation seen in animal models may simply reflect the variation seen in the human infection (van den Brand *et al.*, 2014). Nevertheless, the larger group sizes required to take account of this variability can be problematic in NHPs. In spite of this, NHPs have been used to evaluate a range of vaccines and therapeutics from recombinant vaccines to siRNA therapies, (reviewed in Nagata *et al.*, 2010).

12.2.4 Spillover hosts

SARS-like coronaviruses were first identified in Himalayan palm civets and a raccoon dog taken from a live animal market in Guangdong in China (Guan *et al.*, 2003; Webster, 2004). Experimental inoculation of civets with two human isolates of SARS-CoV resulted in lethargy, fever, and leukopenia, as well as histopathology indicating pneumonia (Wu *et al.*, 2005). While there is evidence of SARS-CoV infection in other animals including foxes, cats and rats (Wang *et al.*, 2004), pigs and chickens (Weingartl *et al.*, 2004b; Chen *et al.*, 2005) there are few experimental studies in these animals. A single study showed that cats could be infected with SARS-CoV and developed lesions in the respiratory tract (Martina *et al.*, 2003). In contrast, SARS-CoV failed to replicate in chickens, turkeys, geese, ducks and quail (Swayne *et al.*, 2004) and no clinical signs were observed in young pigs and chickens (Weingartl *et al.*, 2004b).

12.2.5 Reservoir host

In 2005 *Rhinolophus* bats were identified as the reservoir of SARS-like CoVs based on serology and sequencing of viral genomes (Lau *et al.*, 2005; Li *et al.*, 2005). Then in 2013 two coronaviruses closely related to human SARS-CoV were isolated from

horseshoe bats in China, which were able to use the same ACE2 receptor for entry as for SARS-CoV (Ge *et al.*, 2013). These findings suggested that bats were likely the reservoir host of the SARS-CoV responsible for the major outbreaks in 2002–2003 and that there are many other SARS-like CoVs circulating in this group of bats. The latter point is supported by further serological surveys indicating high seroprevalence and wide distribution of seropositive bats (summarized in Wang *et al.*, 2006) and phylogenetic analyses of a large number of bat CoVs (Tang *et al.*, 2006; Vijaykrishna *et al.*, 2007; Yang *et al.*, 2013). However, there is very little data on experimental infection of bats with these viruses. Leschenault's rousette bats were fed intestine samples from a lesser dog-faced fruit bat (*Cynopterus brachyotis)* in the Philippines, which was positive by polymerase chain reaction (PCR) for a group 2 CoV. Although there was evidence of replication based on PCR detection of viral RNA in the intestine, no clinical signs were observed (Watanabe *et al.*, 2010).

In another study examining the effect of natural infection in bats, SARS-like CoVs were detected in the alimentary canal of 9.3% of 1401 Chinese horseshoe bats sampled over 4 years. Bats carrying the virus showed no signs of disease but were observed to have lower body weights (Lau *et al.*, 2010).

12.3 FILOVIRUSES

The first documented filovirus outbreak in humans took place in Germany and Yugoslavia in 1967 and the causative agent was named *Marburgvirus* (MARV). Nine years later a second filovirus, *Ebolavirus* (EBOV) emerged in Africa and both viruses have re-emerged sporadically since then. For a long time NHPs were the only animal models in which wild type viruses produced signs resembling the human disease. Studies in mice, guinea pigs, and hamsters could only be carried out with virus that had been adapted to the host. More recently, a marmoset model has shown promise as a small NHP model that replicates the unadapted virus to produce disease similar to that seen in humans.

12.3.1 Human disease

MARV and EBOV infections in humans are clinically indistinguishable. The incubation period ranges from 4–21 days and initial symptoms are influenza-like with fever, myalgia, headache, and lethargy followed by nausea and vomiting. A maculopapular rash may also occur (Martini, 1971). The disease then progresses to involve the respiratory, neurological, urinary, and vascular systems. Disseminated intravascular coagulation does not occur with certainty in humans (reviewed in Sanchez *et al.*, 2007). Death occurs from shock and multiple organ failure (reviewed in Feldmann & Geisbert, 2011). The infection is frequently lethal in humans and outbreak fatality rates have ranged from 65–90%. Mortality has been attributed to production of proinflammatory cytokines and suppression of both innate and adaptive immunity, the latter resulting from massive apoptosis of T lymphocytes (Wauquier *et al.*, 2010). The paucity of human samples means that animal models are essential, not only for assessment of vaccines and therapeutics, but also to enhance understanding of the disease in humans.

12.3.2 Small animal models

Guinea pigs and mice have traditionally been the small animal infection models for EBOV and MARV, but both rely on the use of virus strains adapted by serial passage**.** Adult immunocompetent mice are resistant to infection with wild type EBOV and MARV, attributed to a strong type I interferon response (Bray, 2001), while guinea pigs infected with wild type EBOV or MARV only develop a transient fever (Simpson *et al.*, 1968). EBOV was adapted to produce disease in adult mice by serial passage in suckling mice (Bray *et al.*, 1998); and mice infected with the adapted virus display clinical signs including ruffled fur, lethargy, and weight loss and die 5–7 days postinfection. Necrosis occurs in the liver and spleen together with lymphocyte apoptosis characteristic of the infection in humans and NHPs. Intravascular coagulation, a defining characteristic of the disease in NHPs, does not occur consistently in mice (Bray *et al.*, 2001; Gibb *et al.*, 2001).

A lethal model of MARV in immunocompetent mice was not developed until 2009 and necessitated viral passage through severe combined immunodeficient (SCID) mice and then immunocompetent BALB/c mice (Warfield *et al.*, 2009). The resulting virus induced fatal disease in BALB/c mice within 5–10 days with many of the characteristics of the infection in NHPs, including high levels of viremia, lymphopenia, coagulopathy, and liver damage.

Filoviruses were also adapted to guinea pigs by serial passage (Ryabchikova *et al.*, 1996; Connolly *et al.*, 1999), inducing fever and dehydration, followed by death 7–9 days post infection. Virus replicated to high titers in spleen, liver, adrenal gland and lung. Lymphopenia and necrosis of the liver and spleen were observed. Guinea pigs also developed coagulation abnormalities although not to the extent seen in NHPs (Connolly *et al.*, 1999; Reed & Mohamadzadeh, 2007). Bystander lymphocyte apoptosis, important in mice and NHPs, has not been observed in guinea pigs (Bray *et al.*, 1998, 2001).

Evaluation of vaccines and therapeutics in mice and guinea pigs is not necessarily predictive of their effects in NHPs. A comparative study (Wahl-Jensen *et al.*, 2012) indicated that of five vaccines providing some protection to rodents, three offered no protection in NHPs. Similarly, of three postexposure therapeutics successfully tested in rodent models, only one was found to be effective in NHPs.

More recently, mouse-adapted EBOV has been used to create a lethal Syrian hamster model (Ebihara *et al.*, 2013) that replicates aspects of the disease seen in humans and NHPs. Hamsters develop clinical signs with ruffled fur and lethargy followed by death at 4–5 days post infection. Importantly hamsters also develop coagulopathy that is very similar to that seen in macaques infected with wild type EBOV (Ebihara *et al.*, 2011; Geisbert *et al.*, 2003a). Necrosis and apoptosis were noted mainly in lymphoid organs and the liver. A strong type I interferon response, thought to be the reason that adult mice were resistant to wild type EBOV (Bray, 2001), was not found in the early stage of infection with the adapted virus. The utility of this model is increasing with the ongoing development of reagents and sequencing of the Syrian hamster genome.

12.3.3 Nonhuman primates

Primates are the only animals yet identified in which wild type EBOV and MARV produce lethal infection, strongly resembling human disease (Bray & Geisbert, 2005; Sanchez *et al.*, 2007). As a result, infection with EBOV and MARV has been extensively

investigated in NHPs and is better understood in these than in any other species including humans. Studies have been carried out in baboons (Perry *et al.*, 2012) and African green monkeys (Davis *et al.*, 1997) but the following descriptions relate to infection in macaques. Infection progresses similarly for either virus and results in fever and viremia with clinical signs including anorexia, severe weight loss and dehydration followed by maculopapular rash, diarrhea and death 5–8 days postinfection. These filoviruses initially infect monocytes, macrophages, and dendritic cells and spread systemically, causing splenitis and hepatitis. Spread to regional lymph nodes via the lymphatic system results in extensive lymphocyte apoptosis, interfering with both innate and adaptive immune responses. Infected macrophages release proinflammatory cytokines, which trigger coagulation abnormalities leading to hemorrhage, thrombosis, and death (Geisbert *et al.*, 2003b, 2007; Fritz *et al.*, 2008; Hensley *et al.*, 2011). Minor differences from the human infection include shorter incubation period in macaques and uniform lethality, which has been shown using a number of different routes of infection and with very small doses of EBOV (Johnson *et al.*, 1995; Jaax *et al.*, 1996). Numerous vaccines have been evaluated in macaques, (reviewed in Geisbert *et al.*, 2010a) as well as various postexposure treatments, including treatments for coagulopathy and sepsis, interfering RNAs to suppress viral replication, and monoclonal antibodies (reviewed in Wong *et al.*, 2014).

Finally, a small animal NHP model has recently been developed using marmosets. Marmosets infected with wild type EBOV or MARV developed a systemic lethal disease with several features of the human infection including weight loss, fever, lymphoid necrosis (and hepatic necrosis as well in the case of EBOV) (Carrion *et al.*, 2011). Importantly, marmosets also develop disseminated intravascular coagulation. A small NHP model is particularly useful for agents such as EBOV and MARV that must be used under high containment, and marmosets have been used to model other hemorrhagic diseases such as Lassa hemorrhagic fever and Argentine hemorrhagic fever (Samoilovich *et al.*, 1984).

12.3.4 Spillover hosts

Filovirus disease in experimentally infected NHPs has been well documented but NHPs are also thought to be a spillover host for EBOV and MARV – possibly via the consumption of fruit contaminated by infected fruit bats. Natural infection of NHPs is suggested by evidence such as EBOV outbreaks in humans that are coincident with decimation of NHP populations and the linking of human EBOV outbreaks to handling of dead animals by hunters or villagers (Bermejo *et al.*, 2006; Leroy *et al.*, 2004). The source of the original MARV virus outbreak in Germany was African green monkeys (*Cercopithecus aethiops*) imported from Uganda (Smith *et al.*, 1967).

Reston EBOV (REBOV) is an EBOV isolate that causes fatal disease in macaques but asymptomatic infection in humans (Hayes *et al.*, 1992; Miranda *et al.*, 1999). Isolation of REBOV from farmed pigs in the Philippines (Barrette *et al.*, 2009) and detection of antibody to REBOV in six people with exposure to pigs or pig products suggested for the first time that pigs may be a source of filovirus transmission to humans. Experimental inoculation showed that REBOV did not cause clinical disease in pigs (Marsh *et al.*, 2011b) although the virus replicated to high levels in lung and lymphoid tissue with shedding from the nasopharynx. Sequence variation among

different REBOV isolates suggested that REBOV may have spilled over from an unknown host into monkeys and pigs (Barrette *et al.*, 2009).

12.3.5 Reservoir host

Current studies suggest that fruit bats are the reservoir host for EBOV and MARV. Very early studies of EBOV replication in species spanning a number of classes showed that two fruit bat species were the only ones to replicate the virus to high titre, despite an absence of observed clinical signs (Swanepoel *et al.*, 1996). In 2005, over 1000 small animals including bats, birds and other vertebrates were tested for evidence of EBOV infection in an area where an EBOV outbreak in human and apes had recently occurred and EBOV RNA was detected in three species of fruit bats – *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquate.* Despite this, no virus was isolated and the bats did not appear to be ill (Leroy *et al.*, 2005). Investigation of an EBOV outbreak in the Democratic Republic of Congo in 2007 linked the outbreak to a massive bat migration and a patient identified as the most likely initial human victim, who had bought bats to eat from a local hunter (Leroy *et al.*, 2009).

Unlike EBOV, MARV has been isolated from wild-caught fruit bats. The bats, which displayed no signs of illness, were caught in a cave in Uganda where miners had contracted MARV (Towner *et al.*, 2009). Evidence of MARV infection in bats has been reported in fruit bats at other sites, and also in insectivorous bats (Swanepoel *et al.*, 2007; Towner *et al.*, 2007).

The apparent asymptomatic replication of EBOV and MARV in bats suggests a long period of co-evolution between virus and host, characteristic of a virus in its natural reservoir. However, the reservoir hosts for EBOV and MARV have not been definitively determined – and an EBOV-like virus has been linked with mortality in insectivorous bats in Spain (Negredo *et al.*, 2011).

12.4 PARAMYXOVIRUSES

The henipaviruses, Hendra virus (HeV) and Nipah virus (NiV), have both emerged in the last 20 years to cause repeated disease outbreaks in humans and other animals. In contrast to other paramyxoviruses, a wide range of species are readily infected by henipaviruses. As a result, development of animal models has been relatively straightforward with no adaptation of virus or engineering of susceptible hosts required.

12.4.1 Human disease

HeV infection in humans is similar to infection caused by NiV. An incubation period ranging from 5–14 days is followed by an influenza-like illness, which can include fever, headache, myalgia, and lethargy. At this point patients may either recover or progress to pneumonia and/or encephalitis with a high case fatality rate. Relapsing or late-onset encephalitis after apparent recovery from acute infection is a feature of both infections. There is evidence that viremia occurs after primary virus replication in respiratory and lymphoid tissues (Chua *et al.*, 2001; Wong *et al.*, 2002c; Pallister *et al.*, 2011a). Viral tropism for endothelial cells then leads to infection of blood vessels

resulting in widespread vasculitis with syncytia formation in affected tissues and thrombosis, ischemia, and microinfarction in most major organs (Wong *et al.*, 2002b). Pulmonary edema occurs, with alveolar hemorrhage and pneumonia (OSullivan *et al.*, 1997; Wong *et al.*, 2009). Viral tropism for neurons means the central nervous system (CNS) is particularly severely affected and parenchymal necrosis is characteristic of infections at this site (Wong *et al.*, 2002a).

12.4.2 Small animal models

A range of small animal models have been developed for the henipaviruses, including cats, ferrets, guinea pigs, hamsters, and mice. Cats infected with henipaviruses develop fever, depression, and elevated rates of respiration (Westbury *et al.*, 1995, 1996). Infection of endothelial cells leads to widespread systemic vasculitis and virus can be isolated from lung, spleen, brain, kidney, and urine (Westbury *et al.*, 1995), as well as placenta and placental fluids (Mungall *et al.*, 2007). The disease in cats is largely pulmonary with thrombosis, fibrinoid necrosis, and endothelial syncytia observed in the lungs (Hooper *et al.*, 1997b; Middleton *et al.*, 2002; Williamson *et al.*, 1998). Cats do not develop the neurological signs seen in humans and other animal models, although meningitis still occurs. Nonetheless, henipavirus infection in the cat model is reproducible and has been successfully used to assess potential vaccine candidates (Mungall *et al.*, 2006; McEachern *et al.*, 2008).

Unlike cats, ferrets reproduce both the respiratory and the neurological aspects of henipavirus disease seen in humans, making them a particularly suitable model for henipavirus studies (Bossart *et al.*, 2009). Fever is the first clinical sign seen in ferrets infected with HeV or NiV, followed by lethargy and neurological signs such as tremors, hind limb paresis, myoclonus, and depression (Bossart *et al.*, 2009; Pallister *et al.*, 2011a, b). Histologically, there is widespread systemic vasculitis affecting major organ systems. As in humans the organ systems most affected are the lungs, with necrotizing alveolitis and syncytia in alveolar epithelium, together with the central nervous system where brain parenchyma including neurons become infected. In addition to replicating human henipavirus disease very closely, henipavirus infection in ferrets is lethal over a wide range of doses, providing a consistent outcome that facilitates their use in evaluation of vaccines and therapeutics (Bossart *et al.*, 2009; Pallister *et al.*, 2009, 2011b). Comparison of transmission routes of NiV Bangladesh and NiV Malaysia in ferrets has shown that higher levels of oral shedding of the Bangladesh strain might be a factor in human to human transmission of this particular virus (Clayton *et al.*, 2012).

Clinical signs of NiV or HeV infection in guinea pigs are variable, ranging from no clinical signs at all to death, and the outcome of infection with a given dose of HeV or NiV can vary within and between experiments (reviewed in Geisbert *et al.*, 2012). Widespread vasculitis occurred in HeV infected guinea pigs (Hooper *et al.*, 1997b; Torres-Velez *et al.*, 2008) and the animals developed encephalitis but not pulmonary edema (Williamson *et al.*, 2001). Gross histological lesions are similar in both infections. The variability in the outcome of infection means that guinea pigs have not been used for the evaluation of vaccines and therapeutics, however the guinea pig model has been successfully used as a virulence control in experiments to ensure the integrity of the virus inoculum (Middleton *et al.*, 2007) and it has been suggested that

replication in the bladder and placenta might make it a useful model for transmission studies (Williamson *et al.*, 2001).

Hamsters infected with HeV or NiV reproduce the disease seen in humans, with the development of both respiratory and neurological signs (Guillaume *et al.*, 2009; Wong *et al.*, 2003) although variable outcomes have been reported in NiV infected hamsters (summarized in Geisbert *et al.*, 2012). These differences were shown to depend on the dose and route of inoculation with low doses leading to the development of neurological signs and high doses to the development of respiratory signs (Rockx *et al.*, 2011). In contrast, hamsters infected with HeV developed both respiratory and neurological signs as seen in acute human infection and appear to become more resistant to HeV infection with age (Guillaume *et al.*, 2006, 2009).

Until recently, attempts to infect mice with both HeV and NiV had been unsuccessful (Westbury *et al.*, 1995; Wong *et al.*, 2003) and mice were thought to be resistant to infection. However, mice proved to be susceptible to henipavirus infection when Dups *et al.* (2012) showed that aged, but not juvenile, C57BL/6 mice could be infected with a high dose of HeV delivered intranasally and developed signs of ataxia, muscle tremors, and hypersensitivity. All mice developed encephalitis, but inflammatory lesions and HeV antigen were confined to the brain, and in particular to afferent olfactory pathways. A transient respiratory infection was cleared early in the infection and systemic vasculitis characteristic of the infection in other animals and humans did not develop. Similar findings were reported in BALB/c mice. These features of HeV infection in the aged mouse make it an ideal model for studying infection of the brain by the olfactory route (Dups *et al.*, 2012). They may also be important to gaining a greater understanding of recrudescence using the wide array of tools available for mice, including transgenic mice. In a transgenic mouse model lacking the receptor for type I interferon, mice developed fatal encephalitis following either HeV or NiV infection (Dhondt *et al.*, 2012). Like aged mice, these mice had vasculitis and parenchymal inflammation in the brain but did not develop a respiratory infection.

12.4.3 Nonhuman primates

NHP models for the henipaviruses have been established in squirrel monkeys and AGMs. Infection with a high dose of NiV in squirrel monkeys produced nonuniform results with approximately 50% of the infected animals showing neurological and respiratory symptoms (Marianneau *et al.*, 2010). As a result this model has not been widely used in the evaluation of therapeutics. In contrast, infection with HeV and NiV was uniformly lethal in AGMs (Geisbert *et al.*, 2010b; Rockx *et al.*, 2010), with NiV doses as low as 2×10^4 plaque-forming units (pfu) producing a systemic infection with respiratory and neurological disease and the involvement of multiple organs. The most common lesion observed is systemic vasculitis, with vascular lesions in the brain and respiratory epithelium, and the most severely affected organ is the lung with congestion, hemorrhage, and pulmonary edema. Neurological signs included severe depression and a reduced ability to move, and henipavirus antigen was detected in endothelial cells in the brain as well as in neurons. Syncytial cells, considered the hallmark of paramyxovirus infection, were prominent in numerous tissues. AGMs have now been used to evaluate vaccines and therapeutics including ribavirin (Rockx *et al.*, 2010), a human monoclonal antibody (Bossart *et al.*, 2011; Geisbert *et al.*, 2014) and a subunit vaccine (Bossart *et al.*, 2012; Mire *et al.*, 2014).

12.4.4 Spillover hosts

Spillover hosts play a role in the transmission of both HeV and NiV to humans. All known human infections with HeV have so far occurred only after exposure to infected horses. In contrast, during the initial outbreak of NiV in humans in Malaysia/Singapore in 1998–1999, the virus was spread to humans via infected pigs. Both horses and pigs act as amplifying hosts, in which the viruses replicate to high levels.

As in humans, HeV infection in horses causes vascular disease with fever and neurological and respiratory signs. Early clinical signs of depression and increased respiratory rate progress to frothy nasal discharge, and further neurological signs such as ataxia, head pressing, and myoclonic twitches (Murray *et al.*, 1995a; Hooper *et al.*, 1997a; Williamson *et al.*, 1998; Marsh *et al.*, 2011a). Systemic vasculitis occurs with the involvement of multiple organs and syncytial cells can be seen in the endothelial cells in blood vessels in lungs, kidney, and lymph nodes. Major pathology occurs in the lung with congestion, pulmonary edema (Murray *et al.*, 1995b), and hemorrhage (Marsh *et al.*, 2011a).

Most reports of henipavirus infection in pigs involve NiV with one report only of HeV infection in Landrace and Gottingen minipigs (Li *et al.*, 2010), where respiratory and transient neurological signs were observed. NiV infection in pigs is largely asymptomatic. Fever is a common symptom, but there is also an age-related effect, with respiratory signs principally seen in younger pigs and neurological signs in older pigs. The infection is characterized by systemic vasculitis, alveolitis, and meningitis (Mohd Nor *et al.*, 2000; Middleton *et al.*, 2002) and experimental infection suggested that infection of the CNS in pigs could occur via the olfactory route (Weingartl *et al.*, 2005). A recombinant canarypox vaccine has been assessed in pigs and was shown to prevent shedding and reduce NiV replication (Weingartl *et al.*, 2006).

During the Malaysian outbreak increased numbers of dying dogs were noted on farms where NiV outbreaks occurred (Parashar *et al.*, 2000) and serological studies showed that dogs were commonly infected (Field *et al.*, 2001; Mills *et al.*, 2009). Two dogs living near a pig farm died shortly before their owner contracted a NiV infection (Tan *et al.*, 1999). HeV infection has also been recorded in 2 dogs in Australia on properties where horses had been infected with HeV. The infection in dogs was asymptomatic (Promed, 2011). The role of dogs in onward transmission of henipavirus infection to humans is not known.

12.4.5 Reservoir host

Viral infection of a reservoir host may not lead to clinical disease, and so far this has been the case for fruit bats infected with henipaviruses. Infection of *Pteropus alecto*, *P. poliocephalus*, or *P. vampyrus* resulted in inconsistent seroconversion without clinical disease or gross pathology (Williamson *et al.*, 1998, 2000; Middleton *et al.*, 2007; Halpin *et al.*, 2011). Isolation of HeV from the urine and fetal tissues of wild-caught bats (Halpin *et al.*, 2011) and the isolation of NiV from the urine of experimentally infected pteropid bats (Middleton *et al.*, 2007) suggest possible transmission routes within natural populations. However, the lack of generation of high-titer isolates, combined with the serological observations, suggest that current assumptions about host/pathogen relationships that center on such laboratory markers may not be so applicable to coevolved pathogens in reservoir hosts.

12.5 CONCLUSIONS

Experimental studies using live animals exposed to emerging zoonotic diseases are conducted within a complex decision-making framework that incorporates consideration of biocontainment, occupational safety, science quality, and harm minimization to animals. As described above, additional complexity arises from the variation in patterns of expression of infection within and between animal species. In the case of newly emerged viruses, it is also currently not feasible to predict with accuracy the outcome of exposure of species not identified as part of the original field event; even NHP are not reliable surrogates for human infection and disease. So, while there is probably general agreement that species with the appropriate biological characteristics should be selected for use as an infection model in research studies, it may be less clear how such criteria are agreed upon. For example, species selection may be driven by constraints of infrastructure and limitations of staff expertise and reagent availability as well as by scientific questions pertaining to the pathogenesis of acute disease and its control; mechanisms of development of chronic, persistent or recrudescent disease and their prevention; or the dynamics of pathogen maintenance in reservoir hosts to assist in management of transmission risk. Data generated from properly designed animal infection experiments always contribute to the overall host–pathogen story, but the fitness-for-purpose of the model should always be assessed in the context of specific scientific objectives. In particular, translation of research findings from animal studies to humans continues to present a major challenge, and will remain a fertile area of investigation for many years to come.

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13

BAT GENOMICS

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13.1 INTRODUCTION

The order Chiroptera has many unique biological adaptations that have made it attractive for genome sequencing and comparative genomics. Specialized traits, including flight, echolocation, hibernation/torpor, longevity, and antiviral immunity have all been investigated in bats using genomics methods. Genome sequencing studies have also shed light on the phylogenetic placement of bats and on the evolutionary constraints that have acted on the pan-mammalian genome.

Transcriptomics has also been employed to illuminate biological traits such as hibernation and echolocation. In the context of genomics, transcriptome information can provide valuable support for gene/transcript annotation models, generally in combination with homology and *de novo* gene prediction. In the absence of genome information, *de novo* transcriptome assemblies can provide a catalog of expressed transcripts specific to certain tissues or conditions. Here, we review the major bat genome projects and discuss some of the limitations of the resulting data. We also review those studies that have employed transcriptomics to elucidate molecular aspects of unique bat traits.

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13.2 GENOMICS

Sequencing of the human genome remains one of the greatest scientific achievements of humankind. The Human Genome Project, an extensive international collaboration, took over 10 years to complete. Since the publication of the human genome in 2001 (Lander *et al.*, 2001), many other mammalian genomes have been sequenced, including mouse (Waterston *et al.*, 2002), rat (Gibbs *et al.*, 2004), dog (Lindblad-Toh *et al.*, 2005), and cow (Elsik *et al.*, 2009). Significant technological advancements, namely the application of next generation sequencing (NGS), have revolutionized mammalian genome sequencing. NGS has drastically reduced the cost and time required for sequencing genomes, so it is now feasible to sequence the genomes of non-model organisms, including wildlife species. These improvements, however, have not eliminated all the difficulties associated with sequencing projects. It is important to note that mammalian genomes typically contain a high proportion of repetitive sequences (Lander *et al.*, 2001; Cordaux & Batzer, 2009). This repetitive content can cause significant problems for genome assembly, particular when only short read technologies are used.

13.2.1 The era of bat genomics

In 2011 an ambitious project known as the 29 Mammals Project published draft genome assemblies for 29 mammalian species, including the little brown bat (*Myotis lucifugus*) and the large flying fox (*Pteropus vampyrus*) (Lindblad-Toh *et al.*, 2011). The project aimed to identify constrained elements in the human genome based on the evolutionary constraint across eutherian mammals. This was achieved by creating low to medium coverage genome assembles for 24 mammalian species, along with higher coverage assemblies for human, chimpanzee, mouse, dog, and opossum. The 29 species were chosen to generate maximum novel branch length while representing the four major mammalian clades. Comparative genomics revealed the existence of over 3.5 million constrained elements across the species, accounting for approximately 4% of the human genome. Such elements included previously undetected exons, RNA structural families and regulatory factors such as promoters. While this project did not examine either of the bat genomes in detail, the contribution that this project made to bat biology should not be underestimated. Researchers now had access to genomic resources for two of the major Chiroptera suborders. The era of bat genomics was born.

Since the release of the *M. lucifugus* and *P. vampyrus* genomes in 2011, a further seven bat genomes have been sequenced and published (Table 13.1). The seven genomes were all published in 2013 and represent diverse taxa, including members of both the Yinpterochiroptera and Yangochiroptera suborders. While the motivation for each genome project varied, the methodologies used were highly similar. With the exception of the original *M. lucifugus* and *P. vampyrus*, all other bat genomes have been sequenced and assembled entirely from Illumina short-read data. In contrast, *M. lucifugus* and *P. vampyrus* genomes were sequenced using bacterial artificial chromosome (BAC) or fosmid libraries with Sanger sequencing technology. Consequently, the coverage of the *M. lucifugus* and *P. vampyrus* genomes is lower compared to the seven species that were sequenced entirely by NGS (Table 13.1).

Genome annotation has been undertaken using a variety of strategies. For the *P. alecto* and *M. davidii* genomes, genes were predicted using a combination of homology-based prediction, *de novo* prediction and transcriptome data (Zhang *et al.*, 2013). Similarly, *M. brandtii* genes were annotated using a combination of homology-based prediction and transcriptome sequence information (Seim *et al.*, 2013). The *M. lucifugus* and *P. vampyrus* genomes, however, were annotated through the Ensembl pipeline, based on similarity to the human genome (Lindblad-Toh *et al.*, 2011). Homology-based gene prediction was also used to annotate the *Rhinolphus ferrumequinum*, *Megaderma lyra*, *Pteronotous parnellii*, and *Eidolon helvum* genomes. In each case, the number of predicted genes has been similar to that reported for other mammals (Elsik *et al.*, 2009; Gibbs *et al.*, 2004; Lander *et al.*, 2001; Lindblad-Toh *et al.*, 2005; Waterston *et al.*, 2002). The total number of predicted genes varied from ~17 000 genes in *P. vampyrus* to ~26 000 genes in *M. brandtii*; however, these may be outliers, as the remaining seven bat gene sets contained between 19 728 and 21 705 genes, respectively (Table 13.1).

13.2.2 Phylogenomics

Genome sequencing studies have also contributed to the ongoing debate regarding the phylogenetic relationship of bats to other mammals. Bats reside within the superorder Laurasiatheria (Murphy *et al.*, 2001; Springer *et al.*, 2004). However, their placement within this clade remains unclear. A number of hypotheses for the interordinal relationships of Laurasiatheria have been proposed (Zhou *et al.*, 2012). Two prevailing theories suggest that bats may form a sister clade to Fereuungulata (Cetartiodactyla + Perissodactyla + Carnivora + Pholidota) (Murphy *et al.*, 2001, 2007; Zhou *et al.*, 2012) or alternatively, may reside within Pegasoferae (Chiroptera + Perissodactyla + Carnivora + Pholidota) (Nishihara *et al.*, 2006). Phylogenomics analysis based on 2654 single-copy orthologous genes from *M. brandtii* suggest this species diverged from the *Equus* (horse) lineage ~ 82 million years ago (mya) (Seim *et al.*, 2013). This proposition is consistent with phylogenomics analysis of the related species *M. davidii* and *P. alecto*. Indeed, based on 2497 single copy orthologous genes these species also appeared to diverge from a common ancestor with horse ~ 88 mya (Zhang *et al.*, 2013). While these studies support the theory of bats as a member of Pegasoferae (Chiroptera + Perissodactyla + Carnivora + Pholidota) (Nishihara *et al.*, 2006), phylogenomics analysis of six additional bat species observed conflicting findings. Indeed, maximum likelihood reconstructions based on 2320 coding DNA sequences from *M. lucifugus*, *P. vampyrus*, *E. helvum*, *R. ferrumequinum*, *M. lyra*, and *P. parnellii* found that bats form a sister group within the clade of ungulates, cetaceans, and carnivores (Fereuungulata) (Tsagkogeorga *et al.*, 2013). This study also found strong statistical evidence to support the suborder classification of Yinpterochiroptera and Yangochiroptera.

13.2.3 Immunity

Emerging infectious diseases pose a significant threat to the world's human population. It is now recognized that many emerging and re-emerging human infectious diseases are derived from wildlife. In a systematic review, Luis *et al.* (2013) demonstrated that

TABLE 13.1 Genome statistics for nine published bat genomes based on GenBank entries

Species	*Pteropus alecto*	*Myotis davidii*	*Myotis brandtii*	*Rhinolophus ferrumequinum*	*Megaderma lyra*	*Eidolon helvum*	*Pteronotus parnellii*	*Myotis lucifugus*	*Pteropus vampyrus*
Common name	Black flying fox	David's Myotis	Brandt's bat	Greater horseshoe bat	Greater false vampire bat	Straw-colored fruit bat	Parnell's mustached bat	Little brown bat	Large flying fox
Suborder	Yinpterochiroptera	Yangochiroptera	Yangochiroptera	Yinpterochiroptera	Yinpterochiroptera	Yinpterochiroptera	Yangochiroptera	Yangochiroptera	Yinpterochiroptera
Family	Pteropodidae	Vespertillonidae	Vespertillonidae	Rhinolophidae	Megadermatidae	Pteropodidae	Mormoopidae	Vespertillonidae	Pteropodidae
Sequencing platform	Illumina HiSeq 2000	Illumina HiSeq 2000	Illumina HiSeq 2000	Illumina HiSeq 2000	Illumina HiSeq 2000	Illumina HiSeq 2000	Illumina HiSeq 2000	Sanger	Sanger
Sequencing Library†	Mate-pair/short PE	Mate-pair/short PE	Mate-pair/short PE	Short PE	Short PE	Short PE	Short PE	BAC	40 kb Fosmids and 4kb PE
Coverage (×)	110	110	120	17	18	18	17	7	2.89
Total sequence length	1 985 958 707	2 059 799 708	2 107 242 811	1 926 439 238	1 735 931 796	1 837 754 460	1 960 317 893	2 034 575 300	1 996 076 410
Scaffold number/ N50	65 598/15 954 802	101 769/3 454 484	169 750/3 225 832	160 500/21 151	192872/16 881	133538/27 684	177401/22 675	11654/4 293 315	96944/124 060
Contig number/ N50	170 164/31 841	325 280/15 182	325414/23 289	290 685/11 659	518 327/7 043	288446/12 668	443121/9 502	72785/64 330	388808/8 527
Annotated genes	21 392	21 705	25 918	20 424	20 043	20 455	20 357	16 990	19 728
GenBank assembly	GCA_000325575.1	GCA_000327345.1	GCA_000412655.1	GCA_000465495.1	GCA_000465345.1	GCA_000465285.1	GCA_000465405.1	GCA_000147115.1	GCA_000151845.1
Reference	Zhang *et al.*, 2013	Zhang *et al.*, 2013	Seim *et al.*, 2013	Parker *et al.*, 2013	Parker *et al.*, 2013	Parker *et al.*, 2013	Parker *et al.*. 2013	Lindblad-Toh *et al.*, 2011	Lindblad-Toh *et al.*, 2011

*Includes mitochondrial DNA.

†PE; paired-end. Mate-pair PE libraries contain ≥2 kb insert length. Short PE libraries contain <800 bp inserts.

bats harbor proportionally more zoonotic viruses than any other mammalian order, including rodents. Bats are natural reservoir hosts for many highly pathogenic viruses including Hendra and Nipah paramyxoviruses, severe acute respiratory syndrome (SARS)-like and Middle East respiratory syndrome (MERS) coronaviruses, rabies and other lyssaviruses and Ebola and Marburg filoviruses. With the notable exception of rabies viruses, bats appear to harbor many viruses asymptomatically. Consequently, the mechanisms by which bats may coexist with highly pathogenic viruses has become an intriguing question and has prompted the emergence of bat immunology as a significant field of contemporary research. Comparative studies between resistant and susceptible hosts may yield novel insights into the molecular mechanisms of antiviral immunity.

Considering the ancient lineage of Chiroptera, bats have undoubtedly coevolved with viruses over millions of years. Consequently, we would expect signatures of coevolution to be visible at the host–pathogen interface, that is, the innate immune system. Zhang *et al.* (2013) investigated signatures of positive selection within genes of the immune system for both *P. alecto* and *M. davidii* compared to their orthologs in seven other mammalian species. Evidence of positive selection was observed in genes spanning a diverse range of immunological functions, including interferon (*IFNG*), interferon receptors (*IFNAR1*), interferon-stimulated genes (*ISG15*), interleukins (*IL18*), and toll-like receptors (TLR7). Accelerated evolution of innate immune genes may be a direct consequence of prolonged viral exposure. This evolutionary adaptation may contribute to bats ability to harbor viruses with few signs of disease.

13.2.4 Gene family expansion

Comparative genome analysis of *M. brandtii* with other mammals also demonstrated significant changes in the immunological gene repertoire. Expansion of leukocyte receptor complex (LRC) gene families (including the leukocyte immunoglobulin-like receptors; LILRs) was observed in both *M. brandtii* and *M. davidii* (Seim *et al.*, 2013; Zhang *et al.*, 2013). Considering the important immunological role of LRC members, expansion of these gene families within the *Myotis* lineage may have considerable immunological consequences. However, unlike the *Myotis* lineage, no expansion of these gene families was observed in the Pteropid bat, *P. alecto* (Zhang *et al.*, 2013). A number of other examples of gene family expansion within the *Myotis* lineage have been reported, including duplication of *FBXO31* (involved in ubiquitination) and *RNASE4* (digestive enzyme) (Seim *et al.*, 2013; Zhang *et al.*, 2013). Extensive gene duplication within the *Myotis* genome may be a consequence of DNA transposon activity. Indeed, class II transposable elements such as members of the *Helitron* family are believed to have significantly shaped the genome content of the vesper bat lineage (Pritham & Feschotte, 2007).

13.2.5 Longevity

Bats have remarkably long lifespans given their body size. *M. brandtii* has the longest recorded lifespan of all bats (exceeding 40 years) and an adult body weight of only 4 to 8 g. This disparity between bats' body weight and longevity has been investigated

through comparative genomics. One study found that the long-lived bats *M. brandtii*, *M. lucifiugus*, *Eptesicus fuscus*, and *Tadarida bradsiliensis* shared unique amino acid substitutions/deletions in the growth hormone receptor (GHR) and insulin-like growth factor 1 receptor (IGF1R) (Seim *et al.*, 2013). The authors hypothesize that mutations in GHR/IGF1R genes combined with specialized adaptations such as hibernation and low reproductive rate are responsible for the exceptional lifespan of *M. brandtii.*

13.2.6 Hibernation

Prior to the availability of whole genome sequences, the molecular control of hibernation was investigated largely on a gene-by-gene basis (Chen *et al.*, 2008; Eddy & Storey, 2003, 2004, 2007). However genome sequencing of hibernating bats has allowed investigators to examine this adaption on a more global scale. Seim *et al.* (2013) examined changes in gene expression in the liver of *M. brandtii* after 6 months of hibernation. Gene ontology (GO) enrichment analysis demonstrated a down-regulation of genes associated with protein synthesis, glycolysis, splicing, and mitochondrial respiration. Upregulated genes were involved in digestion and peptidase activity, including lipid metabolism (Seim *et al.*, 2013). It is generally accepted that during hibernation there is a shift from carbohydrate-based metabolism to lipid metabolism (Lyman & Chatfield, 1955). Enzymes responsible for lipid metabolism, such as pancreatic triacylglycerol lipase, have been found to be more abundant during hibernation in the thirteen-lined ground squirrel (Squire & Andrews, 2003; Squire *et al.*, 2003). Interrogation of the *M. davidii* genome revealed that a related lipase known as bile salt stimulated lipase (BSSL or CEL) had expanded to six copies (Zhang *et al.*, 2013). This gene was also upregulated in the liver of hibernating *M. brandtii* (Seim *et al.*, 2013). This gene was not expanded in the non-hibernating bat *P. alecto* (Zhang *et al.*, 2013). Together these studies suggest BSSL/CEL may have an important role the hibernation process of *Myotis* bats, particularly in regard to lipid metabolism.

13.2.7 Echolocation and convergent evolution

Echolocation remains one of the most remarkable adaptations observed in bats, a phenomenon that is shared by the odontocetes (toothed whales and dolphins). It is now understood that echolocating bats do not form a single phylogenetic group. In fact, some echolocating bats are more closely related to non-echolocating Old World fruit bats than they are to other echolocating bats (Tsagkogeorga *et al.*, 2013). This finding suggests that echolocation has either evolved at least twice in the Chiroptera lineage, or if it evolved only once, that it was then subsequently lost from the main lineage of Old World fruit bats. To investigate the evolution of echolocation, Parker *et al.* (2013) undertook a genome-wide analysis of sequence convergence across 22 mammalian species, including four echolocating bats (*M. lucifugus*, *R. ferrumequinum*, *M. lyra*, and *P. parnellii*) and two nonecholocating bats (*P. vampyrus*, *E. helvum*). A total of 2326 orthologous coding sequences were included, of which ~200 genes showed evidence of convergence. Many of these genes were related to hearing and deafness (Parker *et al.*, 2013). Unique amino acid changes shared amongst echolocating mammals were also reported by Seim *et al.* (2013), some of which are known to be expressed in the inner ear. Positive selection of

known echolocation related genes such as *SLC25A5* (Prestin) (Li *et al.*, 2010) and *TMC1* (Davies *et al.,* 2012*)* have also been validated through genome sequencing (Zhang *et al.*, 2013).

13.2.8 Genomic adaptations associated with flight

Bats are the only mammals to have evolved true flight. Studies of this adaptation have focused on genetic changes in genes associated with energy metabolism and its byproducts. Using the public genomic resources, evidence of positive selection was detected in the mitochondrial and nuclear encoded oxidative phosphorylation genes of *M. lucifugus* and *P. vampyrus* (Shen *et al.*, 2010). The authors further investigated 77 oxidative phosphorylation genes from *Rousettus leschenaultia*, *Cynopterus sphinx*, *Miniopterus fuliginosus*, and *Scotophilus kuhlii* and found that genes involved in energy metabolism have evolved under adaptive evolution within the common ancestral bat lineage. Using a similar approach, Zhang *et al*. (2013) found evidence of positive selection of genes involved in the DNA damage response/DNA repair pathway within the ancestral bat lineage. The authors proposed that the accelerated evolution of these genes may be directly related to minimizing or repairing damage caused by the deleterious byproducts of increased metabolism, namely reactive oxygen species (Zhang *et al.*, 2013).

More wide-ranging genomic changes have also been associated with the evolution of flight. All bats sequenced to date have an estimated genome size of ~2 Gb (Table 13.1). This is consistent with previous observations that bats have smaller genomes compared to other mammals (Burton *et al.*, 1989; Smith & Gregory, 2009). The smaller genome size of bats is thought to be related to the reduced cell size and the increased metabolic demands of flight. Like bats, birds also have smaller genome sizes compared to mammals (Hughes & Hughes, 1995), suggesting that reduced genome size may be a common adaptation among flying vertebrates.

13.2.9 Limitations of genome sequencing

Like all genomes, the nine available bat genomes undoubtedly contain errors and gaps. In most cases the bat genomes have been assembled entirely from Illumina NGS data. This has the advantage in that high read depth is obtained. Indeed, the *P. alecto*, *M. davidii*, and *M. brandtii* assembled genomes have average read depths of over 100× (Table 13.1). High coverage, combined with high accuracy of Illumina sequencing, means sequence mutations such as single nucleotide polymorphisms can be identified with high confidence. The trade off, however, is that short read data generally produces highly fragmented genome assemblies. This is particularly evident around repetitive sequences and regions with high GC content. Indeed all of the publicly available bat genomes remain fragmented into thousands of contigs/scaffolds (Table 13.1).

Additional technologies and/or computational approaches can assist to resolve the problem of repetitive regions. When a BAC or fosmid library is available, long read technologies such as Roche 454, or even Sanger sequencing, can be used to improve draft genome assemblies. While this approach has been used successfully within our laboratory to resolve difficult regions of the *P. alecto* genome, it is laborious and unfeasible on a large scale. Physical mapping technologies such as optical mapping have been

used to improve the genome assemblies of other mammalian species and thus may prove useful for improving the published bat genomes. By way of example, the genome of the domestic goat (*Capra hircus*) was significantly improved by combining optical mapping with Illumina short read data, resulting in super-scaffolds with an N_{50} length five times longer than achieved using fosmid end-sequencing of the assembled Illumina reads (Dong *et al.*, 2013b). Long-read single-molecule sequencing, such as the Pacific Biosciences RS II system (PacBio), is also gaining popularity. This platform generates read-lengths of greater than 10 kb, which in most cases should span considerable regions of repetitive sequences. Reads can be assembled either *de novo* or as hybrid assemblies with Illumina short-read data. This approach has been used to improve genome assemblies for a number of species including *Drosophila* sp. (English *et al.*, 2012), chimpanzee (Huddleston *et al.*, 2014), and human.

Genome annotation remains a significant computational challenge, particularly in genomes with either low coverage or highly fragmented assemblies. Annotation strategies based only on homology preclude the discovery of novel genes and may bias the annotation towards less diverged loci. It should be acknowledged that genome annotation will never be perfect and that continued refinement and improvements are essential. The incorporation of RNA-Seq data and mass spectrometry based proteomics data in gene prediction pipelines will improve annotations in the future.

13.3 TRANSCRIPTOMICS AND MicroRNAs

Transcriptome sequencing has provided functional insights into some of the most important biological traits of bats, including immunity (Papenfuss *et al.*, 2012; Shaw *et al.*, 2012), echolocation (Dong *et al.*, 2013a), wing formation (Wang *et al.*, 2010), and antihemostatic properties of vampire bat saliva (Francischetti *et al.*, 2013).

13.3.1 Cataloging immune genes

By targeting specific tissues and cells, the immune gene repertoires of both the Jamaican fruit bat (*Artibeus jamaicensis*) and the Australian black flying fox (*P. alecto*) were successfully sequenced and annotated (Papenfuss *et al.*, 2012; Shaw *et al.*, 2012). As known viral reservoirs, the rapid discovery of immune relevant genes through transcriptome sequencing has enabled the host anti-viral response to be evaluated. *A. jamaicensis* immune relevant genes were identified through transcriptome sequencing of lung, spleen, kidney, and poly-IC stimulated primary kidney cells (Shaw *et al.*, 2012). Over 300 000 transcripts were assembled *de novo*, of which ~19% could be annotated by BLASTX to the NCBI non-redundant (nr) database. GO analysis was used to identify a total of 466 immune-related genes. A similar approach was utilized by Papenfuss *et al.* (2012), who sequenced transcripts derived from thymus, mitogen-stimulated spleen cells, white blood cells, lymph node, and bone marrow of *P. alecto*. Approximately 300 000 transcripts were assembled with ~51% showing homology to proteins in the NCBI nr database. Functional classification showed that 3.5% of transcripts (approximately 500) were immune relevant and encompassed many innate and adaptive immunological pathways. This study represented the first attempt to characterize the immune gene repertoire of a bat on a global scale. Perhaps one of the more surprising findings of this study was the

absence of natural killer (NK) receptors within the *P. alecto* transcriptome (Papenfuss *et al.*, 2012). Contraction of this gene family was later confirmed at the genome level within the *P. alecto* and *M. davidii* genomes (Zhang *et al.*, 2013). Furthermore, the NK receptor repertoire also appeared to be reduced in *A. jamaicensis* (Shaw *et al.*, 2012). Together these findings suggest bats may employ a novel class of NK receptors yet to be described.

13.3.2 Functional genomics of echolocation

Transcriptomics has also been used to investigate the molecular mechanisms controlling echolocation. In this study, the transcriptome of the inner ear was sequenced and compared between an echolocating species (Rickett's big-footed bat; *Myotis ricketti*) and a nonecholocating species (Greater short-nosed fruit bat; *Cynopterus sphinx*). Transcriptomes were assembled *de novo* for each species, and transcript expression levels determined by mapping sequence reads back to the *de novo* transcriptome. GO enrichment analysis of genes upregulated in the *M. ricketti* inner ear revealed an overrepresentation of terms related to auditory processes and ear development (Dong *et al.*, 2013a). One gene of particular note was *TMC1*, which encodes a transmembrane protein with ~57× higher transcript abundance in the inner ear of *M. ricketti* compared to *C. sphinx*. This gene was shown to evolve under positive selection in *M. lucifugus* (Davies *et al.*, 2012) and *M. davidii* (Zhang *et al.*, 2013).

13.3.3 MicroRNA discovery

Increased recognition of the importance of microRNAs in regulating eukaryotic gene expression has led to the identification and characterization of many bat microRNAs. Using NGS several groups have catalogued novel bat microRNAs across a diverse range of taxa including *A. jamaicensis* (Shaw *et al.*, 2012), *P. alecto* (Cowled *et al.,* 2014), and *E. fuscus* (Platt *et al.*, 2014). Indeed, 399 microRNAs were identified in the *P. alecto* genome, including over 100 that appeared unique amongst vertebrates (Cowled *et al.,* 2014). MicroRNAs have also been annotated on the *M. lucifugus* and *P. vampyrus* genome through the Ensembl annotation pipeline. The functional role of microRNAs in hibernation has been reported previously. Increased expression of eight microRNAs in the brain of *M. lucifugus* was found to be associated with hibernation (Biggar & Storey, 2014).

13.3.4 Bat specific gene discovery through transcriptomics

The identification of novel bat-specific genes through either transcriptome or genome sequencing projects is of great interest. As described above for *A. jamaicensis* and *P. alecto*, only a fraction of the *de novo* assembled transcripts show homology to known protein sequences. While erroneous transcripts may account for many of these sequences, others may represent truly novel genus-specific or species-specific genes. Increased confidence can be obtained by mapping the *de novo* assembled transcripts back to a reference genome as reported by Papenfuss *et al*. (2012). Novel bat-specific or species-specific genes may also be identified through genome projects, particularly when transcriptome sequencing is integrated into the genome annotation pipeline. However, sequence data alone is only the first step in novel gene discovery. Functional characterization of putative novel transcripts is essential.

13.4 CONCLUSIONS

There is no doubt that bat genomics has come a long way in a short period of time. The remarkable taxonomic diversity and specialized adaptations of bats have made them excellent candidates for whole genome sequencing and comparative genomics, and these projects have provided significant insight into traits such as flight, echolocation, hibernation, antiviral immunity, and longevity. Bat genomics has also contributed to resolving the phylogenetic placement of bats within the broader mammalian clade. The sequencing and annotation of nine bat genomes represents a significant resource for the scientific community, and while only a small number of transcriptome studies have been performed on bats to date, this will undoubtedly increase in the future.

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14

BAT IMMUNOLOGY

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14.1 INTRODUCTION

Despite the diversity of viruses carried by bats and the possibility that they may carry persistent viral infections, evidence from experimentally and naturally infected bats have demonstrated that they rarely display clinical or pathological signs of disease (Sulkin *et al*., 1966; Swanepoel *et al*., 1996; Williamson *et al*., 1998, 2000; Leroy *et al*., 2005, 2009; Middleton *et al*., 2007; Towner *et al*., 2009). There are also few reports of mass die-offs among bat populations and viral infections have not been reported as a major cause of bat deaths. The fungus that causes white nose syndrome (WNS) among North American microbats is the only pathogen that has been reported to cause mass mortalities among some bat populations (Blehert *et al.*, 2009). The long-term co-evolutionary history of bats and viruses has likely resulted in the establishment of a state of equilibrium, allowing both the viruses and their host to coexist in a disease-free state typical of reservoir hosts. It is possible that this evolutionary trade-off has beneficial consequences for the host, for example by conferring protection against other pathogens or even against predators (Wang *et al*., 2011).

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Despite the importance of bats as natural host reservoirs and the array of unique characteristics shared by this group of mammals, until relatively recently, bats have been among the least studied groups of mammals. As the only flying mammal, the evolution of flight has been hypothesized to have provided strong selective pressure on the immune system of bats and on the viruses themselves due to fluctuations in metabolic rate and body temperature (Zhang *et al.*, 2013; O'Shea *et al.*, 2014). Genetic changes coincident with adaptation to flight have been identified in the whole genomes of divergent bat species providing strong support for coevolution of the bat immune system with viruses (Zhang *et al.*, 2013). Higher body temperatures have also been hypothesized to have provided bats with enhanced immune responses and may help to explain why co-evolved bat viruses thrive in the cooler body temperatures of spillover hosts (O'Shea *et al.*, 2014). However, the nature of the immune response of bats to viruses is poorly understood and has significant potential to provide insights that may assist in developing novel strategies to redirect the immune response or treat viral diseases in other mammals. There has been a recent resurgence of interest in the area of bat immunology, in part due to the emergence and re-emergence of a number of viruses that have been linked to bats. Advances in technology including the availability of whole genome data and deep sequencing methods have greatly facilitated investigations into the immune system of bats, and there is now a need for the development of methods for evaluating the immune responses of bats and the development of antibody reagents. A recent review of bat antiviral immunity represented the first review of bat immunology published in almost four decades (Baker *et al.*, 2013). Progress in the area of bat immunology is rapidly increasing as new groups enter the field and technology provides opportunities for more rapid discovery. The purpose of this chapter is to provide an update on the current state of knowledge on bat immunology focusing on our understanding of antiviral immunity in bats.

14.2 IMMUNE TISSUES AND CELLS

Bats have bone marrow, thymus, spleen, and lymph nodes, similar to other mammals. However, no reports have described the histological organization and development of lymphoid tissue in bats and few bat specific reagents exist to identify different subpopulations of cells. Despite this, broad subsets of cells have been described based on morphological and physiochemical characteristics, demonstrating the presence of similar populations of cells in bats to other mammals. Morphological characteristics of cells using basic hematology and histology has confirmed the presence of lymphocytes, neutrophils, eosinophils, basophils, and macrophages in the Brazilian free-tailed bat, *Tadarida brasilensis* (Turmelle *et al.*, 2010a). All other reports of bat immune cell populations have focused on the Indian flying fox, *Pteropus giganteus*. Sarkar & Chakravarty (1991) identified macrophages, T-, and B-cell populations based on cellular adherence and scanning electron microscopy. This work confirmed that the ratio of macrophages:B cells:T cells of 1:2:9 is similar to mice, which had a ratio of 1:1:8. However, the size of cell populations identified in *P. giganteus* appeared to be smaller compared to other species with T cells ranging from 6–7 μm, B cells from 7–9 μm, and macrophages were 4–5 μm. Further studies to determine the size of different immune cell populations in a variety of bat species should be performed to confirm these

observations and determine their implications. Cells resembling follicular dendritic cells (FDCs) have also been described in *P. giganteus* (Sarkar & Chakravarty, 1991). FDCs are capable of capturing and retaining antigen in the form of immune complexes that can persist for months or even years and are important for the induction and maintenance of memory immune responses (Mandels *et al.*, 1980; Tew *et al.*, 1980). Evidence for the ability of some viruses to retain infectivity when complexed within human or mouse FDCs has been demonstrated (Keele *et al.*, 2008). However, whether FDCs play a role in the persistence of viral infections in bats awaits further investigation. Although natural killer (NK) cells have not been identified in bats, two major classes of NK cell receptors; killer inhibitory receptors (KIRs) and Ly49-like receptors are absent from transcriptome and genome datasets examined despite the identification of other known NK cell co-receptors (Papenfuss *et al.*, 2012; Zhang *et al.*, 2013). All other mammals examined to date have expanded either the KIR or Ly49 family of receptors (Kelley *et al.*, 2005). The characterization of bat NK cells is likely to provide some interesting insights into the control of viruses and tumors in bats.

14.3 INNATE IMMUNITY

One hypothesis for the ability of bats to remain asymptomatic to viral infection is that they are able to control viral replication very early in the immune response through innate antiviral mechanisms. Recent advances in the area of innate immunity, in part facilitated by the availability of whole genome data, have begun to provide insights into unique adaptations in the innate immune system of bats.

14.3.1 Pattern recognition receptors (PRRs)

PRRs are proteins predominately expressed by cells of the innate immune system to identify evolutionarily conserved pathogen-associated molecular patterns (PAMPs) associated with viruses, bacteria, fungi, and parasites. PRRs include Toll-like receptors (TLRs), RIG-like receptors (RLRs), and nonobese diabetic (NOD)-like receptors (NLRs), which provide the first line of host defense against infection (Bowie & Unterholzner, 2008). Here we describe the recent advances in the identification and understanding of PRRs in bats.

TLRs have been characterized from two fruit bats, *Pteropus alecto* and *Rousettus leschenaultii*. This work has confirmed the presence of transcripts corresponding to TLRs 1–10 and TLR13 in *P. alecto,* and TLRs 3, 7, and 9 in *R. leschenaultii*, indicating that bats are capable of recognizing a range of pathogens (Iha *et al.*, 2010; Cowled *et al.*, 2011; Papenfuss *et al.*, 2012). The TLRs responsible for viral nucleic acid sensing are TLRs 3, 7, 8, and 9 all of which appear to be conserved in bats, consistent with bats being capable of viral recognition similar to other species. The mRNA expression pattern of TLRs in *P. alecto* tissues suggests that they are predominantly expressed by professional immune cells, similar to other mammals (Cowled *et al.*, 2011). Recent genomic analysis revealed that TLR7 has undergone faster evolution in at least two bats, *P. alecto* and *Myotis davidii* (Zhang *et al.*, 2013). While the function of TLR7 in bats remains to be investigated, the coevolution of bats with viruses may have resulted in changes in TLR7 that may influence single-stranded RNA (ssRNA) recognition.

Genomic analysis has also shown that TLR13 is present in both *P. alecto* and *M. davidii* but among other mammals it has only been identified in rodents (Zhang *et al.*, 2013). Although its ligand is still unknown, knockdown of TLR13 in mice results in greater susceptibility to vesicular stomatitis virus (VSV), indicating its importance in viral recognition (Shi *et al.*, 2011). TLR13 in *P. alecto* contains stop codons within its open reading frame (ORF) and may represent a transcribed pseudogene, while *M. davidii* TLR13 contains an intact ORF which is potentially functional. The presence of TLR13 may confer upon bats additional viral sensing capability, and the transcription of a TLR13 pseudogene in *P. alecto* may indicate that it has only recently undergone inactivation (Cowled *et al.*, 2011).

RLRs include retinoic acid inducible gene I (RIG-I, also known as DDX58), melanoma differentiation associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RLRs are widely expressed in the cytoplasm of most cells, and recognize cyptoplasmic viral RNA (Yoneyama & Fujita, 2009). *P. alecto* contains all three RLRs, which share similarities in their predicted domain architecture and tissue expression patterns to their counterparts in humans and other mammals. Furthermore, stimulation of bat kidney cells with synthetic dsRNA (poly I:C) induces rapid upregulation of all three helicases, which is similar to other mammals (Cowled *et al.*, 2012; Papenfuss *et al.*, 2012). Analysis of bat transcriptome data has also identified members of the RLR pathway in *A. jamaicensis* (Shaw *et al.*, 2012). Interestingly, genomic analysis has indicated that RIG-I has undergone faster evolution in bats, which may in turn alter its functionality (Zhang *et al.*, 2013). However, no functional studies have been performed to examine the nature of bat RLRs to date.

NLRs are a large family of intracellular PRRs that regulate innate immunity in response to recognition of various PAMPs from bacteria, viruses and stressed or damaged cells (Martinon *et al.*, 2009). Activation of NLRs induces the production of inflammatory cytokines or activates the inflammasome complex. Although no studies have examined NLRs in detail in bats, two NLR family members – NLR family, pyrin domain containing 3 (NLRP3) and NLR family CARD domain containing 5 (NLRP5) – were identified in transcriptome data from *P. alecto* (Papenfuss *et al.*, 2012). NLRP5 has been proposed to function as a positive or negative regulator of the antiviral immune response and NLRP3 activates caspase-1 in the inflammasome which in turn cleaves interleukin (IL)-1b and IL-18 into active mature peptides (Schroder & Tschopp, 2010). The inflammasome can be activated in a similar manner by the non-NLR protein, absent in melanoma 2 (AIM2). Genomic analysis revealed that the AIM2 locus is absent from both *P. alecto* and *M. davidii* and NLRP3 has undergone positive selection (Zhang *et al.*, 2013). These changes have the potential to significantly impair the formation and/or function of inflammasomes in bats and dampen the inflammatory response against viruses.

14.3.2 Interferon (IFN) family members

The IFN system provides the first line of defense against viral infection in vertebrates. There are three types of IFNs, designated type I, II, and III, which differ in their amino acid sequences and the receptor complex they signal through. Of the three types, type I and type III IFN are induced directly in response to viral infection and are key cytokines capable of inducing an 'antiviral state' in infected and neighboring cells. This section will focus on type I and III IFNs in innate antiviral defense in bats.

Type I IFNs are a multigene family that includes IFNα and IFNβ, which have demonstrated antiviral activity, and other less well-defined IFNs, including IFNω, IFNε, IFNκ and IFNτ. In humans, IFNα consists of 13 genes while IFNβ, IFNω, IFNε, and IFNκ are each encoded by a single locus. Type I IFNs have been described in three species of fruit bats: *R. aegyptiacus*, the Malaysian flying fox, *P. vampyrus* and the Greenish naked-backed fruit bat, *Dobsonia viridis* and from two microbats, the little brown bat, *M. lucifugus* and the Serotine bat, *E. serotinus* (Omatsu *et al.*, 2008; He G. *et al.*, 2010; Kepler *et al.*, 2010; He X. *et al.*, 2014). He G. *et al.* (2010) described the cloning of eight IFNα subtypes and one pseudogene from *D. viridis* and provided evidence for positive selection in driving the evolution of the bat IFNα gene family. IFNω and IFNκ cDNAs have also been cloned from *E. serotinus* (He X. *et al.*, 2014). Using statistical methods for the assembly of genes from unassembled genome trace archives, Kepler *et al.* (2010) inferred that there were seven IFNα genes in *P. vampyrus* but only one IFNα pseudogene in *M. lucifugus*. Interestingly, IFNω and IFNδ genes appear to have expanded in both bats, with 28 IFNω and 14 IFNδ genes identified in *P. vampyrus* and 25 IFNω and 19 IFNδ in *M. lucifugus*. The expansion of IFNω is not unique to bats as there are 24 IFNω genes in the bovine type I IFN locus and 13 in feline (Yang *et al.*, 2007; Walker & Roberts, 2009). However, the simultaneous contraction of IFNα and expansion of IFNω has not been observed in other species. As only low coverage bat genomes have been used to identify IFNs, the exact numbers of type I IFN gene family members is yet to be confirmed. Antiviral activity of bat type I IFNs has only been examined for IFNω and IFNκ in *E. serotinus*. Both IFNω and IFNκ display antiviral activity against bat Lyssaviruses with evidence that the antiviral activity of IFNκ is weaker compared to IFNω (He X. *et al.*, 2014). Although IFNδ is known predominantly for its function in reproduction, evidence for high antiviral activity has been demonstrated in porcine cells (Lefevre *et al.*, 1998; Cochet *et al.*, 2009). The large size of the IFNδ family in bats suggests that similar to IFNω, it may be important in host defense in bats and IFNω and IFNδ may compensate for the contracted IFNα family.

Type III IFNs are also induced directly in response to viral infection and use a similar sensing pathway to type I IFN but signal through a different IFN receptor complex. In humans, three type III IFNs have been identified (IFNλ1, IFNλ2, and IFNλ3) and two (IFNλ2 and IFNλ3) exist in mice. Using the Ensembl database, Fox *et al.* (2009) identified five IFNλ loci in the *M. lucifugus* genome but only one contained a full-length ORF (Fox *et al.*, 2009). In pteropid bats, three IFNλ loci were identified in the genome of *P. vampyrus* using the Ensembl database, two of which (IFNλ1 and IFNλ2) were cloned from *P. alecto* cDNA (Zhou *et al.*, 2011b). Overall, the type III IFN family appears conserved with other species in terms of sequence and number of loci.

14.3.3 Production of IFNs by bat cells

The secretion of IFN by bat cells has been examined in response to stimulation with viruses and synthetic TLR ligands including poly I:C and lipopolysaccharide (LPS), demonstrating that IFN production pathways are functional in bat cells (Stewart *et al.*, 1969b; Crameri *et al.*, 2009; Kepler *et al.*, 2010; Zhou *et al.*, 2011b). The earliest evidence for IFN-like activity in stimulated bat cells was from a study by Stewart *et al.* (1969b), which demonstrated antiviral activity of supernatant from poly I:C stimulated embryo tissue cultures from the Mexican free tailed bat, *Tadarida brasiliensis* (Stewart

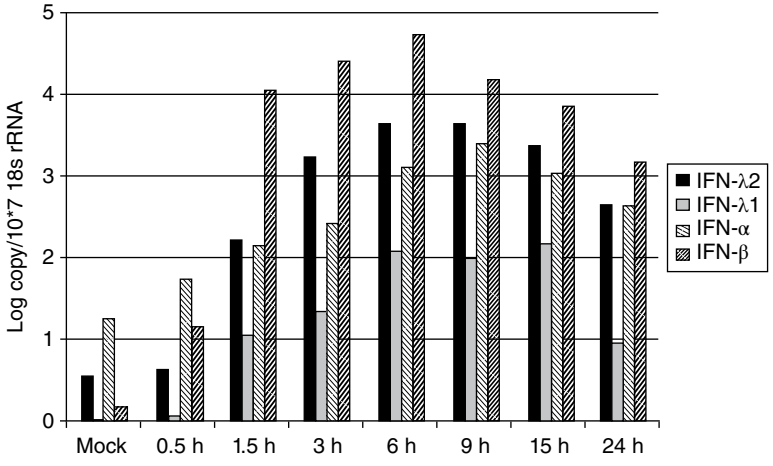

Figure 14.1 Production time course of bat type I and III IFNs on poly I:C transfection in the PaLuT02 bat lung cell line (Crameri *et al*., 2009). Cells were stimulated with poly I:C and collected at the indicated time points. IFN mRNA was measured by qRT-PCR. The data were normalized against the housekeeping gene 18s rRNA. Source: Zhou *et al*., 2011. Copyright 2011. The American Association of Immunologists, Inc.

et al., 1969a). Kepler *et al.* (2010) also demonstrated that poly I:C and LPS stimulated *P. vampyrus* peripheral blood mononuclear cells (PBMCs) produced high levels of IFNβ mRNA. IFNβ production peaked at 2 h following LPS or poly I:C treatment, with a 20–50-fold increase in IFNβ mRNA expression, followed by a gradual decrease to near baseline by 24 hours. Similar expression patterns for IFNα and IFNβ upon poly I:C stimulation were also reported in a *R. aegyptiacus* bat lung cell line, although IFNα showed a delayed response compared to IFNβ (Omatsu *et al*., 2008). In cloned *P. alecto* lung cells, poly I:C treatment induced both type I and type III IFNs as early as 0.5h, peaking at 6h followed by a decrease at 24h (Zhou *et al.*, 2011b). In addition, the two *P. alecto* type III IFNs are differentially induced relative to each other and to type I IFN after stimulation with poly I:C (Figure 14.1). Overall, these studies demonstrate the importance of type I IFNs in early antiviral defense.

Similar results have been observed in experimentally infected bat cells. Infection of *P. alecto* splenocytes with the bat paramyxovirus Tioman virus resulted in the down-regulation of type I IFNs and the upregulation of type III IFNs indicating that type III IFNs may play an important role in the ability of bats to coexist with viruses (Zhou *et al.*, 2011b). A similar pattern of type I IFN-independent IFNλ induction has only been reported in one other study, in which Hantaan virus was used to examine the IFN response of human epithelial cells (Stoltz & Klingström, 2010). In contrast, henipavirus infection antagonized type I and type III IFN production and signaling in *P. alecto* cells but only IFN production in human cells (Virtue *et al.*, 2011a, b). The difference in the behavior of bat IFNs upon Tioman and henipavirus infection may reflect different IFN production mechanisms in splenocytes, which are professional immune cells and cloned bat cells which are predominantly fibroblast-like cells (Crameri *et al.*, 2009) Similar to the responses of other mammalian cells, bat cells showed a delay in the production of IFNβ following VSV infection with a peak level of IFNβ by 8h and remaining almost

unchanged up to 24h (Kepler *et al.*, 2010). Since the majority of *in vitro* experimental infections have been performed using nonimmune bat cells, it will be necessary to examine IFN expression patterns in the immune cells upon viral infection.

IFN production is controlled by transcription factors that bind to the IFN promoter region and induce transcription of IFN genes. Few studies have examined the IFN production pathway in bat cells or the signaling molecules involved in IFN production. In humans and other species, transcription factor binding sites for IFN regulatory factors (IRFs) and nuclear factor kappa B (NF-κB) are located in type I and III IFN promoters (Osterlund *et al.*, 2007). The IRF family consists of nine members (IRF1 to IRF9), which share functional and structural characteristics. However, only IRF1, IRF3, IRF5, and IRF7 positively regulate type I and III IFN transcription. Nuclear factor (NF)-κB is a protein complex that includes five members: NF-κB1, NF-κB2, RelA, RelB, and cRel (Randall & Goodbourn, 2008). In a recent study, Zhou *et al.* (2014) identified all of the IRF family members in the *P. alecto* genome and reported that the *P. alecto* IFNβ promoter region contains typical IRF3 and IRF7 binding sites. He X. *et al.* (2014) also identified IRF and NF-κB binding sites in the IFNω and IFNκ promoters of *E. serotinus*. These studies provide the first evidence that bat IFN is likely induced though similar mechanisms to that of humans and other mammals. Furthermore, *P. alecto* IRF7 appears to have a broad expression pattern across all tissues (Zhou *et al.*, 2014), which contrasts to its restricted expression by immune cells in humans (Honda *et al.*, 2006). The unusual expression pattern of bat IRF7 might contribute to the ability of bats to coexist with viruses with a much broader distribution, providing bats with the ability to activate the IFN response in a wider subset of tissues and cells. Genome analysis of *P. alecto* and *M. davidii* has also demonstrated positive selection on the cRel gene, identifying amino acid changes that could potentially affect IκB (inhibitor of NF-κB) binding (Zhang *et al.*, 2013).

14.3.4 IFN receptors and downstream signaling molecules

Type I IFNs act through a heterodimeric receptor comprised of IFNαR1 and IFNαR2, which appears to be expressed ubiquitously in humans and other mammals (Randall & Goodbourn, 2008). In contrast, type III IFNs bind to a receptor complex including IFNλR1 (also called IL28Ra) and IL10R2 to elicit equivalent antiviral responses to type I IFNs. Unlike IFNαR, IFNλR1 has a limited tissue distribution pattern and is expressed predominantly by epithelial cells thus restricting the functionality of type III IFNs (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003; Sommereyns *et al.*, 2008). Only the *P. alecto* type III receptor complex (IFNλR1 and IL10R2) has been characterized in bats, and IFNλR1 appears to be a functional receptor (Zhou *et al.*, 2011a). The bat IFNλR complex has a wide tissue distribution and at the cellular level, both epithelial and immune cells are responsive to IFNλ treatment, which is consistent with a more important role of type III IFNs in antiviral immunity in bats (Zhou *et al.*, 2011a). This result is consistent with differences in the type III IFN response described above. Although no studies have examined bat type I IFN receptors, genome analysis has demonstrated that the IFNAR1 gene has undergone positive selection in *M. davidii* but not in *P. alecto*. However, whether this change has functional consequences for the IFNR remains to be examined.

After binding to their receptors, both type I and III IFNs activate the JAK-STAT (Janus/just another kinase-signal transducers and activators of transcription) pathway. The henipaviruses, HeV and Nipah virus (NiV) encode V proteins that bind to STAT1

and STAT2 proteins of host cells to block IFN responses. NiV blocks IFN signaling in cells from a variety of species including bat cells (Tb1-Lu from *T. brasiliensis*), consistent with similar mechanisms of IFN signaling in bats to that of other mammals. However, a mutant version of the NiV V gene which contains a single amino acid mutation in the STAT1/2 binding region retained some residual activity only in the bat cells. This result may reflect a difference in STAT signaling pathway in bats which may contribute to their ability to coexist with viruses (Hagmaier *et al.*, 2006). Stimulation of *R. aegyptiacus* cells using human IFNα resulted in the phosphorylation and translocation of bat STAT1 into the nucleus consistent with its activation in a similar manner to other species. Furthermore, inhibition of nuclear translocation of bat STAT1 was also observed in IFN-stimulated bat cells infected with rabies virus (Brzozka *et al.*, 2006; Fujii *et al.*, 2010). Overall, from the limited evidence collected to date, the IFN signaling pathway in bat cells appears to behave similarly to that of other mammals.

14.3.5 Interferon stimulated genes (ISGs)

Treatment of cells with type I and type III IFNs upregulates the expression of thousands of ISGs which in combination specify the antiviral state of infected and neighboring cells (Randall & Goodbourn, 2008). It is reasonable to hypothesize that the ability of bats to control viruses may be due to the presence of a special subset of ISGs which can broadly limit viral replication. Several ISGs corresponding to those of other mammals have been identified in different species of bats. Zhou *et al.* (2013) described three bat ISGs in *P. alecto*; protein kinase R (PKR, or eIF2aK2), orthomyxovirus-resistant gene 1 (Mx1 GTPase), and 2–5-oligoadenylate synthetase 1 (OAS1). These ISGs represent major antiviral pathways and are among the most extensively studied of the ISGs. Papenfuss *et al.* (2012) also identified a number of ISGs in *P. alecto* transcriptome data including Mx1, Mx2, OAS1, OAS2, OAS3, OAS-like (OASL), PKR, RNaseL, and ISG15. All of the bat ISGs examined to date appear to be conserved in sequence compared to other mammals with the exception of ISG15, which has undergone positive selection in *P. alecto* (Zhang *et al.*, 2013). In mice, ISG15 improves the efficiency of the IFN response (Zhou *et al*., 2007). Whether it has a role in the efficiency of the antiviral response of bats remains to be investigated.

The induction pattern of ISGs in bat cells by IFNs or poly I:C also appears to be similar to other species. In *P. alecto* kidney cells, OAS1, PKR and Mx1 are upregulated by treatment with recombinant bat IFNβ and IFNλ2 and ISG56 and RIG-I are induced by recombinant bat IFNλ2 (Zhou *et al.*, 2011b, 2013). Virtue *et al.* (2011a) showed that pteropid bat lung cell lines also produce ISG54 and ISG56 following treatment with universal type I IFN which is an IFNα hybrid constructed from recombinant human IFNα/δ. In the Serotine bat, *E. serotinus* cells, treatment with recombinant bat IFNω and IFNκ induced ISG56, Mx1 and IFIT3. Using poly I:C stimulation, which induces ISGs through PRRs, the genes RIG-I, MDA5, LGP2 and IRF7 were upregulated in bat cells indicating that these genes are also ISGs in *P. alecto* (Cowled *et al.*, 2012; Zhou *et al.*, 2014). Similarly the induction of OAS2 has been detected in *P. vampyrus* PBMCs following stimulation with poly I:C (Kepler *et al.*, 2010).

The ISG response has been examined in several *in vitro* viral infection experiments in bat cells. The bat-borne virus, Pteropine orthoreovirus NB (formerly known as Nelson Bay virus) induced Mx1, OAS1 and PKR in *P. alecto* cells and OAS2 was

upregulated in VSV infected PBMCs from *P. vampyrus* (Kepler *et al.*, 2010; Zhou *et al.*, 2013). Antiviral activity of recombinant bat IFNλ on Pulau virus infected *P. alecto* cells was accompanied by upregulation of ISG56 and RIG-I, consistent with ISG56 and RIG-I playing a role in restricting viral replication (Zhou *et al.*, 2011b). Similarly, the control of lyssavirus replication by IFNω in *E. serotinus* cells coincided with the induction of ISG56, Mx1 and IFIT3 (He X. *et al*., 2014). ISGs are also targeted by viruses as a mechanism of antagonizing the host immune response. For example, bat origin O'nyong-nyong virus infection ablates the expression of IFN stimulated genes p56 and MxA in African fruit bat *Eidolon helvum* cells and HeV blocks ISG54 and ISG56 expression in *P. alecto* cells (Biesold *et al.*, 2011; Virtue *et al.*, 2011a). In mouse and *Tadarida brasiliensis* cells, inhibition of the activity of both PKR and initiation factor 2a (EIF2a) has been demonstrated to play a role in the reactivation of Ebola virus infection in persistently infected mouse cells. *In vivo*, Ebola virus can also be evoked from mice 7 days after infection by inhibition of PKR and EIF2a, providing a potential spillover mechanism from bats to other susceptible species (Strong *et al.*, 2008).

14.3.6 MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules that are essential regulators of expression of eukaryotic genes, including elements required for viral replication. Only limited information exists on bat miRNAs, most of which focuses on regulation of hibernation, muscle development, focal adhesion and axon guidance in *M. lucifugus* (Kornfeld *et al.*, 2012; Maistrovski *et al.*, 2012; Biggar & Storey, 2014). The use of miRNAs as markers for WNS has also been explored in *M. lucifugus* (Iwanowicz *et al.*, 2013). Next generation sequencing has resulted in the identification of miRNAs in two species of bats. Shaw *et al*. (2012) identified 42 miRNAs in *A. jamaicensis* from transcriptome sequence data. Similarly, Cowled *et al.* (2014) identified 399 miRNAs in *P. alecto*, of which a sizeable proportion were unique amongst vertebrates. Analyses of predicted gene targets suggest potential roles for bat miRNAs in mediating virus-host interactions. Although further validation of the targets will be necessary, these results fill a major gap in bat genome research and may shed light on the ability of bats to harbor deadly viruses.

14.4 ADAPTIVE IMMUNITY

Some of the earliest studies of bat immunology examined antibody and cell-mediated responses in bats and provided evidence for qualitative and quantitative differences in adaptive immune responses and in the generation and maintenance of immunological memory. However, no work has been performed to understand how these differences relate to the ability of bats to control viral replication. Recently, there has been a resurgence of work concerning adaptive immunity, including the elucidation of the duration of maternal immunity and the first description of a bat MHC region (Epstein *et al.*, 2013; Baker *et al.*, 2014; Ng, 2014). This section will provide an overview of antibody and cell-mediated immune responses of bats, focusing on the nature of the adaptive immune response to viral infections.

14.4.1 Immunoglobulins

Bat immunoglobulin (Ig) genetics, antibody isotypes and antibody responses to model antigens have recently been reviewed by Butler *et al*. (2014) and therefore will only be briefly described here. Megabats and microbats have IgM, IgG, IgA, and IgE subtypes and both κ and λ light chains but within Chiroptera, IgD appears to be present only in microbats (McMurray *et al.*, 1982; Chakraborty & Chakravarty, 1984; Butler *et al.*, 2011; Papenfuss *et al.*, 2012; Wynne *et al.*, 2013). The antigen-binding variable (V) region repertoire of the antibody heavy chain in both megabats and microbats appears to be highly diverse and early indications are consistent with the possibility that bats may rely more on combinatorial diversity rather than somatic mutation (Baker *et al.*, 2010; Bratsch *et al.*, 2011; Seim *et al.*, 2013). This may indicate that the specificity of the antibody repertoire is hardwired into bats as a consequence of their long coevolutionary history with viruses. The extent of somatic mutation of bat antibodies and the specificity and avidity of Igs to viruses warrants further investigation.

14.4.2 Antibody mediated immune responses to experimental viral infections

Unlike conventional laboratory animals, no "clean" captive colonies of bats exist and experimental infections rely on the use of wild caught individuals, which represent a mixed population of unknown age, susceptibility, and prior viral exposure. The interpretation of antibody responses in bats is therefore extremely challenging. Bats are capable of mounting an antibody response to both viruses and model antigens (Hatten *et al.*, 1968, 1970; Chakraborty & Chakravarty, 1984; Halpin *et al.*, 2000; Lau *et al.*, 2005; Leroy *et al.*, 2005; Wellehan Jr *et al.*, 2009). Neutralizing antibodies to viruses including HeV, Ebola, and SARS-like CoV have also been detected in wild caught bats demonstrating that they are capable of mounting an antibody response (Halpin *et al.*, 2000; Lau *et al.*, 2005; Leroy *et al.*, 2005). The appearance of antibodies in bats appears to follow the same succession as that of other mammals with the early appearance of IgM, followed by IgG. However, there are some differences in the time course, quantity and duration of the antibody response and questions exist over the protective nature of antibodies in bats (Hatten *et al.*, 1968; McMurray *et al.*, 1982; Chakraborty & Chakravarty, 1984; Davis *et al.*, 2007; Wellehan Jr *et al.*, 2009; Turmelle *et al.*, 2010b).

A number of viruses have been used to experimentally infect bats, including rabies, Marburg, HeV, NiV, and Japanese B encephalitis virus (JEV) (Williamson *et al.*, 1998; Williamson *et al.*, 2000; Almeida *et al.*, 2005; Davis *et al.*, 2007; Middleton *et al.*, 2007; Turmelle *et al.*, 2010b; Halpin *et al.*, 2011; Paweska *et al.*, 2012). Rabies is the only virus known to cause clinical signs of disease in bats as a result of both experimental and natural infections. However, the development of disease is inconsistent and the factors responsible for the difference in disease outcome between individuals remain unknown. Furthermore, the ability of bats to control the replication of other viruses that are highly pathogenic to other mammals but in some instances succumb to rabies is not understood. To date, antibody responses are the only immune parameter measured in experimentally infected bats and evidence from these studies support the possibility that antibodies are unlikely to play a key role in the control of viral infections.

Rabies has been the most common virus used in experimental infections of bats with reports describing inoculation of a variety of species of bats with rabies variants. Aerosolized exposure is believed to be one mechanism for the transmission of rabies between individual bats (Constantine *et al.*, 1972). Wild caught *E. fuscus* and *T. brasiliensis* bats survive aerosolized rabies virus exposure and generate a neutralizing antibody response. However, this response was not always protective against subsequent intramuscular challenge with an amnestic response detected in 21 of the 24 challenged bats and the development of clinical rabies in ten animals (Davis *et al.*, 2007). Several studies have demonstrated that bats that are vaccinated or with prior antigen exposure are capable of clearing viral infection even in the absence of detectible neutralizing antibody (Seymour *et al.*, 1978; Sétien *et al.*, 1998; Aguilar-Setien *et al.*, 2002; Turmelle *et al.*, 2010b). Of 16 big brown bats (*E. fuscus*) that survived primary and secondary rabies virus challenge, 15 survived a tertiary challenge despite seroconversion being detected in only four of the surviving bats (Turmelle *et al.*, 2010b). Similarly, Almeida *et al*. (2005) described the intramuscular infection of 40 vampire bats (*Desmodus rotundus*) with rabies virus, of which 30 bats survived, but resistance was observed in bats that developed low or undetectable antibody and bats with high antibody titers. These studies provide support for the development of a protective response following repeated virus exposure, but the failure to detect an antibody response in many animals that survived infection may indicate that the nature of protective immunity in bats differs from other mammals.

Unlike rabies virus infections, experimental infections performed using HeV, NiV, Marburg, and JEV fail to result in clinical or pathological signs of disease in any species of bat but similar to rabies infection, the role of the antibody response in providing protection remains unclear and many animals survive infection in the absence of an antibody response. The henipaviruses, HeV and NiV have been used in a number of experimental infections of pteropid bat species to understand the nature of viral infection in the natural reservoir of these viruses. NiV infection of *P. poliocephalus* bats by subcutaneous injection resulted in the production of neutralizing antibody in all 11 individuals tested but in a separate study, only four of eight *P. vampyrus* bats that were infected by the intranasal/oral route produced a neutralizing antibody response (Halpin *et al.*, 2011; Middleton *et al.*, 2007). Both subcutaneous and intraoral/nasal routes of infection have also been used for HeV inoculation of pteropid bats. Neutralizing antibody responses were detected in 10 out of 20 *P. alecto* bats inoculated oral-nasally with HeV (Halpin *et al.*, 2011). Similarly, in *P. poliocephalus* bats infected with HeV, neutralizing antibodies were detected in two of four bats inoculated by subcutaneous injection and three of the four bats inoculated by the intranasal/oral route with none of the bats displaying clinical signs of disease (Williamson *et al.*, 1998). A second study of *P. poliocephalus* in late gestation infected subcutaneously with HeV, detected neutralizing antibodies in all four bats and no abnormalities were observed in the fetuses or adults post mortem (Williamson *et al.*, 2000). In other mammals, pregnancy results in a bias in the immune response towards humoral immunity and away from cell mediated immunity which could be harmful to the foetus (Szekeres-Bartho, 2002). Whether the nature of the maternal immune response facilitates greater production of an antibody response in infected bats during pregnancy remains to be investigated.

Similar results have been observed in bats infected with JEV and Marburg with differences also detected in the quantity of antibody produced. In big brown bats (*E. fuscus*) experimentally infected with JEV, not all bats develop a neutralizing antibody response

following subcutaneous JEV infection and the quantity of antibody produced appeared to be lower than that of other species. Studies have also failed to detect evidence of complement fixation or hemagglutination by JEV antigen (Leonard *et al.*, 1968; Sulkin *et al.*, 1966). This result may be a technical artefact or reflect differences in the reactivity of bat antibodies with complement, rather than the failure of these animals to produce an IgG response. In bats inoculated by the intraperitoneal and subcutaneous route with Marburg virus, all bats seroconverted but neutralizing antibody titers were low and not detected in all animals (Paweska *et al.*, 2012). Clearly, additional work is needed to understand the nature of the antibody response in bats, but the studies performed to date support the hypothesis that other innate or adaptive immune factors may play a more central role in viral clearance in bats.

Although individual variation makes it difficult to derive conclusions on the role of antibodies in bats, these differences are not surprising considering that wild caught individuals were used in each experiment. Gilbert *et al.* (2013) recently highlighted the difficulties associated with the interpretation of serology from wild-caught individuals for which the history of viral infection is unknown. These concerns are also valid for the interpretation of results from experimentally infected wild-caught individuals. An additional consideration is that all of the experimental infections described above have used bat-borne viruses, often performed on the natural reservoir species. Although it would be useful to determine whether a difference in the immune response occurs if bats are inoculated with a non-bat-borne virus, identifying a non-bat-borne virus is challenging and would additionally require bat cells to express the correct receptor for infection.

14.4.3 Maternally derived antibody protection

The waning of maternal antibody protection in juvenile bats has been implicated in increases in viral prevalence and spillover events from bats. Plowright *et al.* (2008) used modeling to demonstrate a correlation between waning maternal immunity and the peak annual spillover hazard for HeV. Maternal antibody dynamics have also been studied in captive bat populations in two separate studies which each examined changes in anti-henipavirus antibody concentrations. In the first, the serum antibody concentrations of dam–pup pairs was monitored in two experimental populations; wild-caught *P. alecto* naturally infected with HeV and captive *P. hypomelanus* vaccinated with canine distemper virus antigen. This study confirmed the transfer of antibodies from the dam to pup demonstrating a direct correlation between the serostatus of dams and pups and determined the duration of transferred immunity to be between 7.5–8.5 months (Epstein *et al.*, 2013). A correlation in henipavirus antibody serostatus of 13 *E. helvum* dam–pup pairs was also reported, with a gradual decline to undetectable levels in the pups by 4–12 months after birth. Seroconversion of young bats following the decline of maternal antibodies occurred between 6 and 12 months of age demonstrating the protective effect of maternally derived antibodies and the susceptibility of juveniles following the decay of maternal antibody (Baker *et al.*, 2014). The decline of maternal antibody in both studies is consistent with the presence of a susceptible group of juveniles in the population as early as four months postpartum for *E. helvum* and 7.5–8.5 months postpartum for the pteropid bats. This timeframe is consistent with a spike in viral infection in susceptible animals and/or spillover events to other susceptible species (Plowright *et al.*, 2011).

Rabies-specific antibodies in wild-caught adult and juvenile *T. brasiliensis* have also been examined from late pregnancy through to weaning. The percentage of wild caught

suckling bats with rabies antibodies approximated that of adult females consistent with the presence of maternally derived antibody. Furthermore, IgM antibodies (which do not cross the placenta or gut epithelium) were detected in suckling bats indicating that *in utero* infection may occur. In other cases, an increase in the levels of rabies specific IgM antibodies occurred in juveniles towards the end of lactation consistent with susceptibility early in life after the decline of maternal antibodies (Steece & Altenbach, 1989).

14.4.4 T-cell-mediated immune responses

Cell-mediated immune (CMI) responses are controlled by CD8 cytotoxic and CD4 helper T lymphocyte populations and result in the killing of virus infected cells or activation of the antibody and cytokine response. Although the populations of T cells in bats have not been characterized to date, transcriptome data has provided the first indication that the receptors and co-receptors present on T cells in other species are conserved in bats (Papenfuss *et al.*, 2012; Shaw *et al.*, 2012). The CD4 co-receptor has also been characterized in *R. aegyptiacus* (Omatsu *et al.*, 2006). Although no studies have examined the CMI responses of bats to viral infections, the generation of an IFNγ reagent for pteropid bats has been described and will assist in future studies to examine CMI in bats. IFNγ is produced by bat cells stimulated with mitogens such as PHA and ConA and recombinant bat IFNγ has antiviral activity against Semliki Forest virus and HeV *in vitro* (Janardhana *et al.*, 2012). At least *in vitro*, IFNγ appears to have similar activity to IFNγ from other mammals, consistent with its role in the CMI response.

A number of studies have described the *in vitro* responses of pteropid bats and microbats to T cell mitogens and mixed lymphocyte responses in pteropid bats (McMurray & Thomas, 1979; Chakraborty & Chakravarty, 1983; Chakravarty & Paul, 1987; Paul & Chakravarty, 1987). These studies have all reported delayed responses compared with those of conventional laboratory animals. The presence of suppresser T cells (now called regulatory T cells) was implicated in the delay in mitogenic responses of B cells in bats (Chakravarty & Paul, 1987). Whether these cells are involved in the delay in T cell mediated immune responses observed in bats remains to be determined, as does the correlation between mitogen stimulated responses and the role of T cells in a viral infection.

A number of studies have also performed experiments to try to understand the CMI response *in vivo* and have provided support for the generation of a CMI response in bats. However, once again, information is only available on the immune response of bats to model antigens. As described above, although a number of experimental infections on bats have been performed, no information was collected on CMI responses, due partly to the lack of reagents for identifying cell types in bats. *In vivo* CMI responses in bats have only been measured using a delayed-type hypersensitivity (DTH) tests using PHA skin tests or skin sensitivity to 2–4 dinitrofluorobenzene (DNFB) (Christe *et al.*, 2000; Allen *et al.*, 2009; Turmelle *et al.*, 2010a). Responses to DTH tests have been variable with only three of 12 *P. giganteus* responding to DNFB indicating that bats may not be as sensitive to this treatment as other species (Chakraborty & Chakravarty, 1983). Individual variation in the responses of *T. brasiliensis* to PHA injection has also been observed (Turmelle *et al.*, 2010a). Differences in PHA skin tests and subcutaneous PHA injection have been reported in *T. brasiliensis* and *M. myotis*, respectively, and may be related to environmental and physiological factors including roost ecology and pregnancy (Christe *et al.*, 2000; Allen *et al.*, 2009). These results again demonstrate the

difficulty of working with outbred wild-caught individuals where individual variation is inevitable and highlight the importance of taking environmental and physiological factors into consideration in the interpretation of immune responses.

14.4.5 The major histocompatibility complex (MHC)

The MHC is among the most gene-dense and polymorphic regions in mammalian genomes and plays an important role in resistance to infectious diseases, reproductive success, autoimmunity and transplantation. The MHC region of *P. alecto* was recently mapped providing the first glimpse into the content and organization of the MHC in bats (Ng, 2014). A partial MHC class I (MHC-I) region, and complete class II and class III regions were identified in the recently completed genome of *P. alecto* (Zhang *et al.*, 2013). All three bat MHC regions were highly contracted and although there was a high level of synteny with other species, the bat MHC also contained some unusual features compared to other mammals. In particular, recent data (Ng, 2014) indicate that the *P. alecto* MHC region is unique in that class I genes have only duplicated within one of the three class I duplication blocks identified in other mammals (Kulski *et al.*, 2002). Furthermore, the MHC class I genes contain unique insertions within their peptide binding groove which may play a role in their ability to bind and present a higher diversity of peptide antigen. Twelve MHC class II genes, with orthology to class II genes from other mammals, were also identified in the bat genome, including one class II gene that was located outside of the class II region (Ng, 2014).

Only limited work has been performed to examine the polymorphism of MHC genes in bats and has focused entirely on MHC class II genes of microbats using the class II DR beta (DRB) locus which is the most extensively studied MHC locus in mammals. Extreme differences in DRB polymorphism have been observed between two Mexican verpertilionid bat species with extensive polymorphism in *Myotis velifer* compared to extremely limited polymorphism in *Myotis vivesi* (Richman *et al.*, 2010). Population sizes have been suggested to account for the observed difference in MHC polymorphism between the two species. DRB diversity has also been examined in the bulldog bat, *Noctilio albiventris* which displayed MHC diversity within the range observed in other mammals and the sac winged bat, *Sacopteryx bilineata* which appeared to have low diversity (Mayer & Brunner, 2007; Schad *et al.*, 2011). Evidence for pathogen driven positive selection with the identification of unexpected homozygosity for a common allele was observed for a population of *S. bilineata* (Mayer & Brunner, 2007). DRB intron sequences from three species of bat (*R. aegyptiacus*, *C. perspicillata*, and *Phyllostomus discolor*) have also been used to infer phylogenetic relationships and to demonstrate the monophyly of Chiroptera (Kupfermann *et al.*, 1999). Overall, studies of DRB polymorphism in bats provide evidence for the influence of population size and pathogen pressure on the diversification of class II genes. The variation in polymorphism observed between bats may influence the ability of different populations of bats to respond to infections.

14.4.6 Cytokines

Cytokines and their receptors are among the most divergent and rapidly evolving genes known (Bird *et al.*, 2002). Many of the reagents available for human and mouse cytokines

do not cross-react with those from other species and the characterization of cytokines and their receptors has been problematic in more divergent species. However, several bat cytokine genes involved in the adaptive immune response have now been described, providing the first step in examining the cytokine profile of bats during infection. Genes encoding IL-2, IL-4, IL-6, IL-10, IL-12p40, and tumor necrosis factor (TNF) have been cloned from *R. leschenaultii* cDNA (Iha *et al*., 2010). Partial cDNAs for IL-10, IL-32a, TNF, and granulocyte colony-stimulating factor have been cloned from Seba's fruit bat (*C. perspicillata*) (Cogswell-Hawkinson *et al*., 2011). Type II transmembrane proteins belonging to the TNF family, APRIL (A proliferation-inducing ligand) and BAFF (B-cell activating factor) have been identified in *Verspertilio superans*. APRIL and BAFF play a role in B-cell survival, Ig secretion, isotype switching, and T-cell independent antibody responses and appeared to be highly conserved in both sequence and functional activity in *V. superans* (You *et al.*, 2012a, b). Similar to other mammals, the presence of a single IFNγ has been confirmed using whole genome data from *P. vampyrus* and *M. lucifugus* (Kepler *et al.*, 2010). The availability of bat genome and transcriptome datasets has also provided sequence data for a variety of cytokine genes from bats (Papenfuss *et al.*, 2012; Shaw *et al.*, 2012; Zhang *et al.*, 2013). This information should facilitate the characterization of bat cytokines to identify those for which cross-reactive antibodies from other species may be useful and provide information for the development of bat specific antibody reagents.

14.5 CONCLUSIONS

Although bats have traditionally been an understudied group of mammals, the emergence and re-emergence of viruses from bats has highlighted the need to understand how these reservoir hosts interact with and control viral infections. As discussed above, bats appear to share many of the immunological features of other animals, including the presence of immune cell populations and evidence for the conservation of immune genes across a variety of immune functions and pathways. However, evidence from work performed on both the innate and adaptive immune responses of bats is consistent with functional differences in the activation of the immune system. Studying wildlife presents significant challenges but advances in technology including new molecular techniques, proteomics, metabolomics, transcriptomics, and genomics are transforming the way we study non-traditional model organisms. The key feature of these new technologies is that they are species independent but they do require significant computational and bioinformatics time and resources to analyze the data generated. As discussed in Chapter 13, whole genome and RNA-Seq information is rapidly becoming available from a variety of bat species and has already provided clues to the nature of the immune response of bats. Further uses of RNA-Seq should include the dissection of the immune response in infected and uninfected bat tissues and cells to identify the pathways responsible for immune control under different conditions. Knockdown of immune genes using siRNAs is now performed on a routine basis in a variety of species, including bats, and will be useful for understanding the role of specific immune genes and pathways in response to viruses.

Based on the evidence collected to date, the area of innate immunity appears to be highly promising and additional work to dissect the various pathways of innate immune signaling will be required in the future. The work described above highlights several

recent discoveries in the area of innate immunity, including evidence for the loss of several innate immune gene loci in bats and functional differences in IFN production and signaling. Although cell lines have now been derived from a variety of bat tissues, additional immune cell lines will be critical in understanding the nature of innate immunity bats. Bats rarely display pathology in response to viral infections, the exception being the response of some individuals to rabies virus. Much of the pathology associated with infection in other species is caused by the over-activation of the immune system, particularly proinflammatory cytokine responses. Understanding how the inflammatory pathways are controlled in bats may provide us with insights into how the immune system of humans and other species can be redirected to prevent overactivation and pathology.

Although some work has been performed on cell-mediated immunity to model antigens, no studies have examined the cell-mediated immune response of bats to viral infection and the activation of different subsets of cells, including T- and B-cell subsets. Although some reagents generated for cells from other species may cross react with bat cells, the challenge in this area will be the development of bat-specific reagents for the identification of bat cells and the cytokines they produce.

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SPECIES INDEX

Viral Species Index

Bats and Viruses: A New Frontier of Emerging Infectious Diseases, First Edition.
Edited by Lin-Fa Wang and Christopher Cowled.

Bat Species Index (by common name)

Bat Species Index (by scientific name)

SUBJECT INDEX

Bats and Viruses: A New Frontier of Emerging Infectious Diseases, First Edition.
Edited by Lin-Fa Wang and Christopher Cowled.

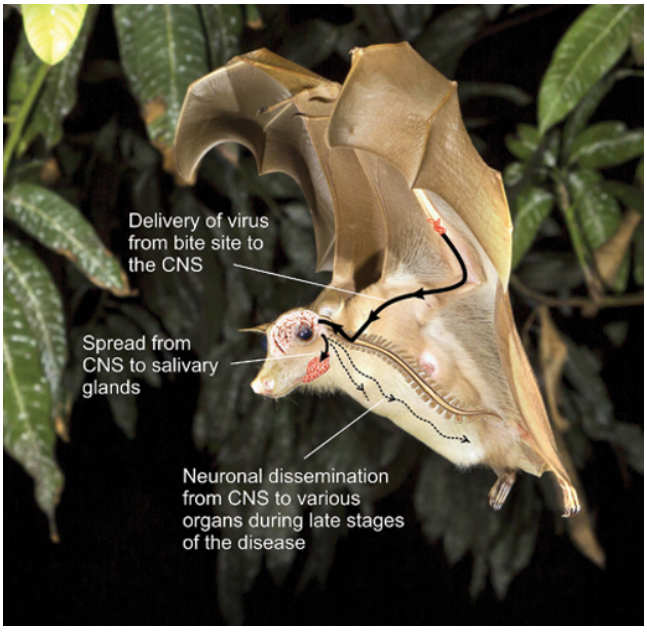

Figure 3.2 Schematic illustration of lyssavirus spread in an infected host.

Bats and Viruses: A New Frontier of Emerging Infectious Diseases, First Edition.
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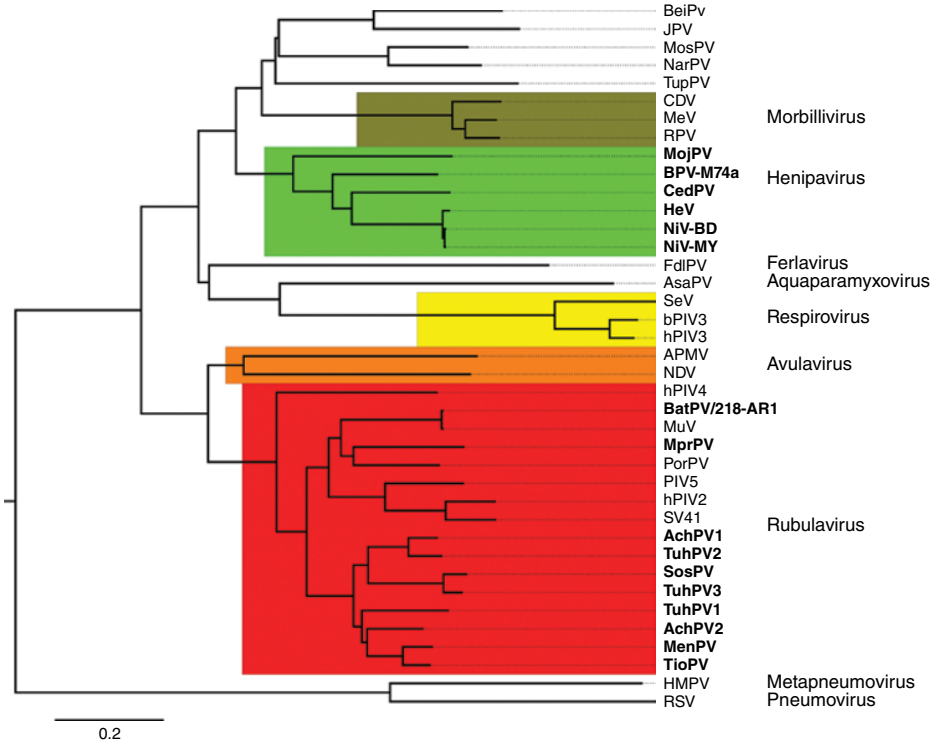

Figure 4.3 Phylogenetic tree based on the N protein sequences of selected paramyxoviruses. Virus name (abbreviation) and GenBank accession numbers are as follows: Achimota virus 1 (AchPV1) JX051319; Achimota virus 2 (AchPV2) JX051320; Atlantic salmon paramyxovirus (AsaPV) EU156171; Avian paramyxovirus 6 (APMV6) AY029299; Bat paramyxovirus/Epo spe/218-AR1/DRC/2009 (BatPV/218-AR1) HQ660095; Bat paramyxovirus/Eid hel/GH-M74a/GHA/2009 (BatPV-M47a) HQ660129; Beilong virus (BeiPV) DQ100461; Bovine parainfluenza virus 3 (bPIV3) AF178654; CDV AF014953; Cedar virus (CedPV) JQ001776; Fer-de-lance virus (FdlPV) AY141760; Hendra virus (HeV) AF017149; Human metapneumovirus (HMPV) AY297749; Human parainfluenza virus 2 (hPIV2) AF533010; Human parainfluenza virus 3 (hPIV3) Z11575; Human parainfluenza virus 4a (hPIV4a) AB543336; J virus (JPV) AY900001; Menangle virus (MenPV) AF326114; Measles virus (MeV) AB016162; Mojiang virus (MojPV) KF278639; Mossman virus (MosPV) AY286409; Mapeura virus (MprPV) EF095490; Mumps virus (MuV) AB000388; Nariva virus (NarPV) FJ362497; Newcastle disease virus (NDV) AF077761; Nipah virus, Bangladesh strain (NiV-BD) AY988601; Nipah virus, Malaysian strain (NiV-MY) AJ627196; Parainfluenza virus 5 (PIV5) AF052755; Porcine rubulavirus (PorPV) BK005918; RSV U39661; Rinderpest virus (RPV) Z30697; Sendai virus (SeV) M19661; Simian virus 41 (SV41) X64275; Sosuga virus (SosPV) KF774436; Tioman virus (TioPV) AF298895; Tuhoko virus 1 (TuhPV1) GU128080; Tuhoko virus 2 (TuhPV2) GU128081; Tuhoko virus 3 (TuhPV3) GU128082; Tupaia paramyxovirus (TupPV) AF079780.

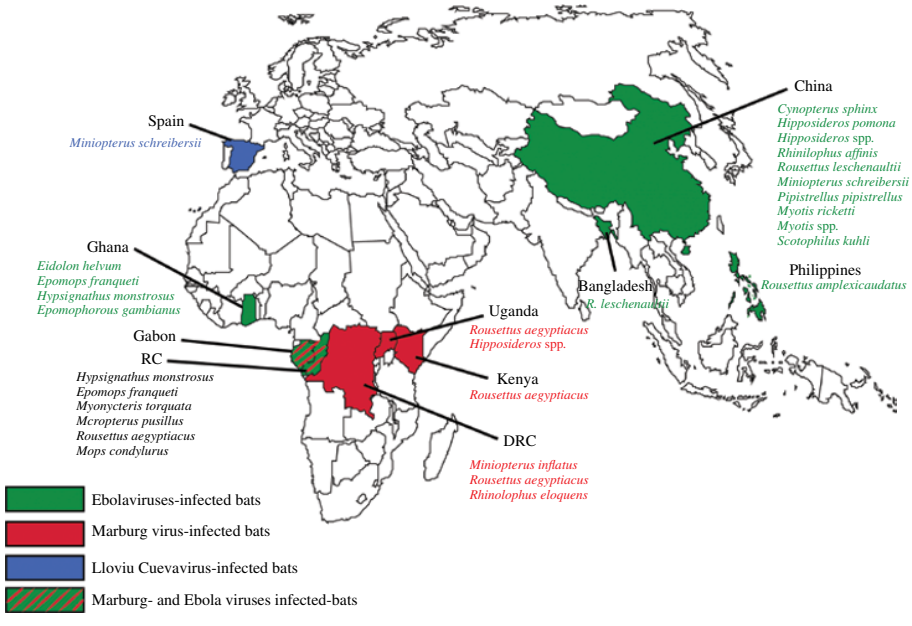

Figure 6.1 Geographic locations of bat species filoviruses-infected in the world.

Figure 6.2 Biosafety in the field during the collection of samples from bats potential filoviruses reservoirs

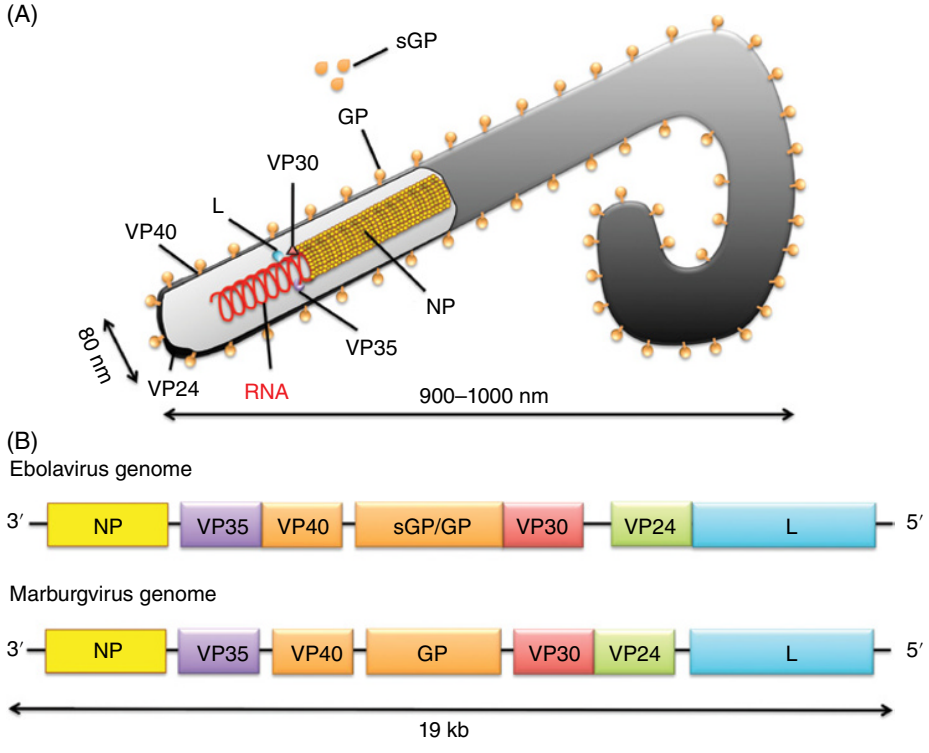

Figure 6.3 (A) A schematic illustration of a filovirus particle is presented. Four proteins are involved in the formation of the ribonucleoprotein complex: polymerase or large protein (L), nucleoprotein (NP), virion structural protein 30 (VP30), VP35. The glycoprotein (GP) is a type I trans membrane protein and is anchored with the carboxy-terminal part in the virion membrane. The soluble GP (sGP) is a non-structural glycoprotein secreted from infected cells and is only secreted by ebolaviruses. VP40 and VP24 are membrane-associated proteins. (B) Schematic representation of Ebolavirus (EBOV) and Marburgvirus (MARV) genomes.

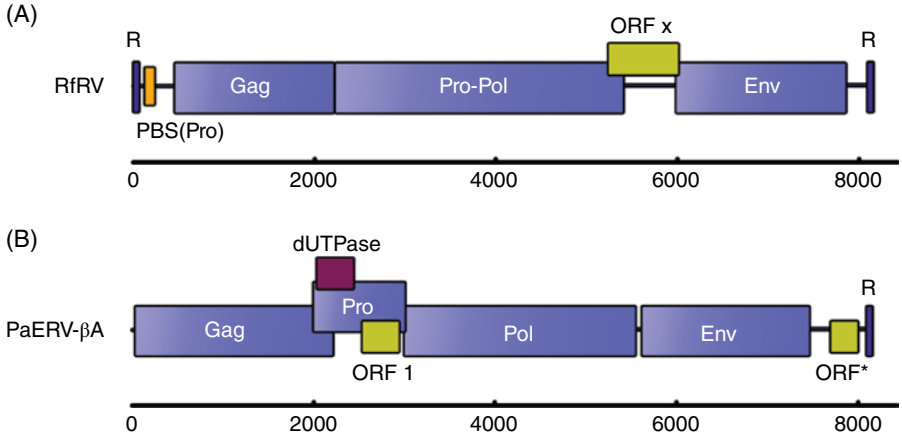

Figure 7.1 Full length retroviral transcripts from bats of different suborders. (A) Structure of the *Rhinolophus ferrumequinum* retrovirus (RfRV) genomic transcript. The 3′ end of RfRV *pol* gene is truncated by a premature stop codon and a new open reading frame (ORF), ORF x, partially encoding the integrase protein, overlaps with *pol* and *env*. The complete RfRV genome is 8356 nucleotides (nt), comprising the genes *gag* (nucleotide positions 621 to 2348), *pol* (nt 2349 to 5594), *env* (nt 5990 to 7867), and direct repeats (R) at both ends. A proline tRNA primer-binding site, PBS (Pro), is present at nt 152 to 173. (B) Structure of the ***P**teropus **a**lecto* **e**ndogenous **r**etrovirus – **beta**retrovirus **A** (PaERV-βA) genomic transcript. Two contigs were identified in the *P. alecto* Illumina-sequenced transcriptome that overlapped by 3,152 nt with 100% sequence identity which were used to assemble the PaERV-βA genomic transcript. Shown are the retroviral genes: *gag*, *pro*, *pol*, and *env*, which have been rendered defective by random mutation since integration, and the betaretroviral dUTPase domain in *pro*, two unique ORFs, and the 3′ terminal repeat region. ORF* does not appear to be genuine, but rather has arisen as a result of an insertion mutation that has disrupted a stop codon. Figure 7.1B is reproduced from (Hayward *et al.*, 2013b) by the publication authors through the Creative Commons Attributions License 4.0 (http://creativecommons.org/licenses/by/4.0/) agreement with BioMed Central.

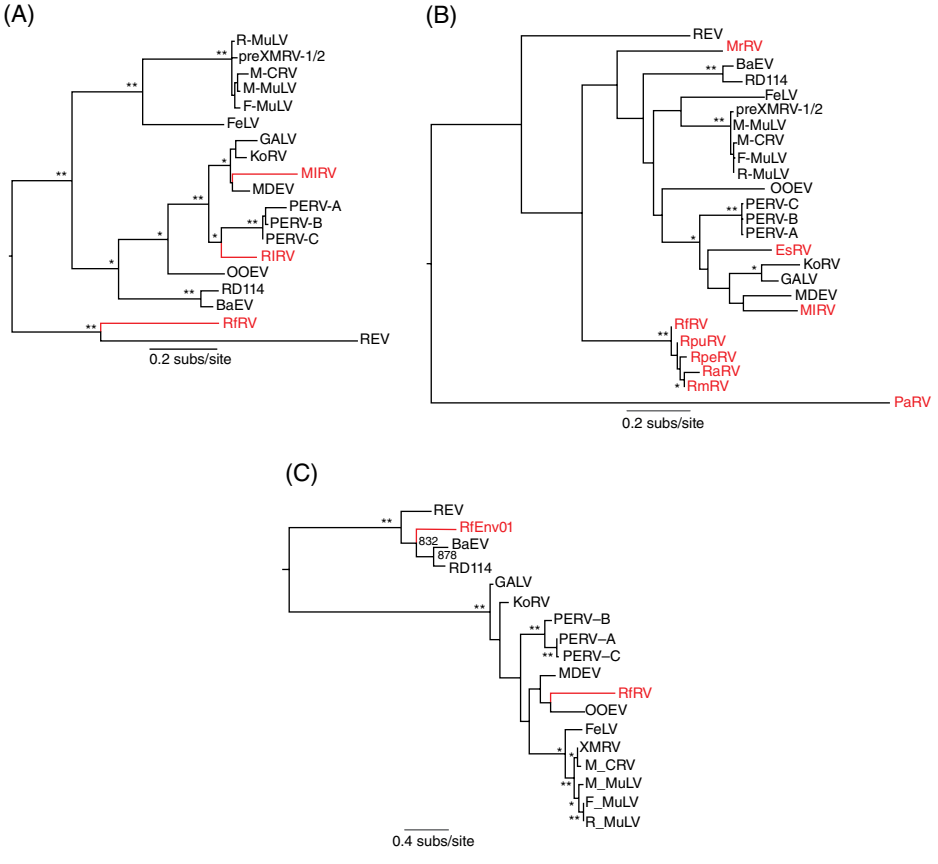

Figure 7.2 Phylogenetic analysis of bat gammaretroviruses and non-bat extant gammaretroviruses. Maximum likelihood phylogenetic trees of (A) Gag, (B) Pol, and (C) Env. Bars represent amino acid substitutions per site and the trees are midpoint rooted for clarity only. Only bootstrap values higher than 70% are shown, such as two stars denoting values ranging from 90% to 100% and one star 70% to 90%. Detailed information regarding sequences can be found in (Cui *et al.*, 2012a, b) and the Accession number for the *Eptesicus serotinus* bat retrovirus (EsRV) is AGN73375.

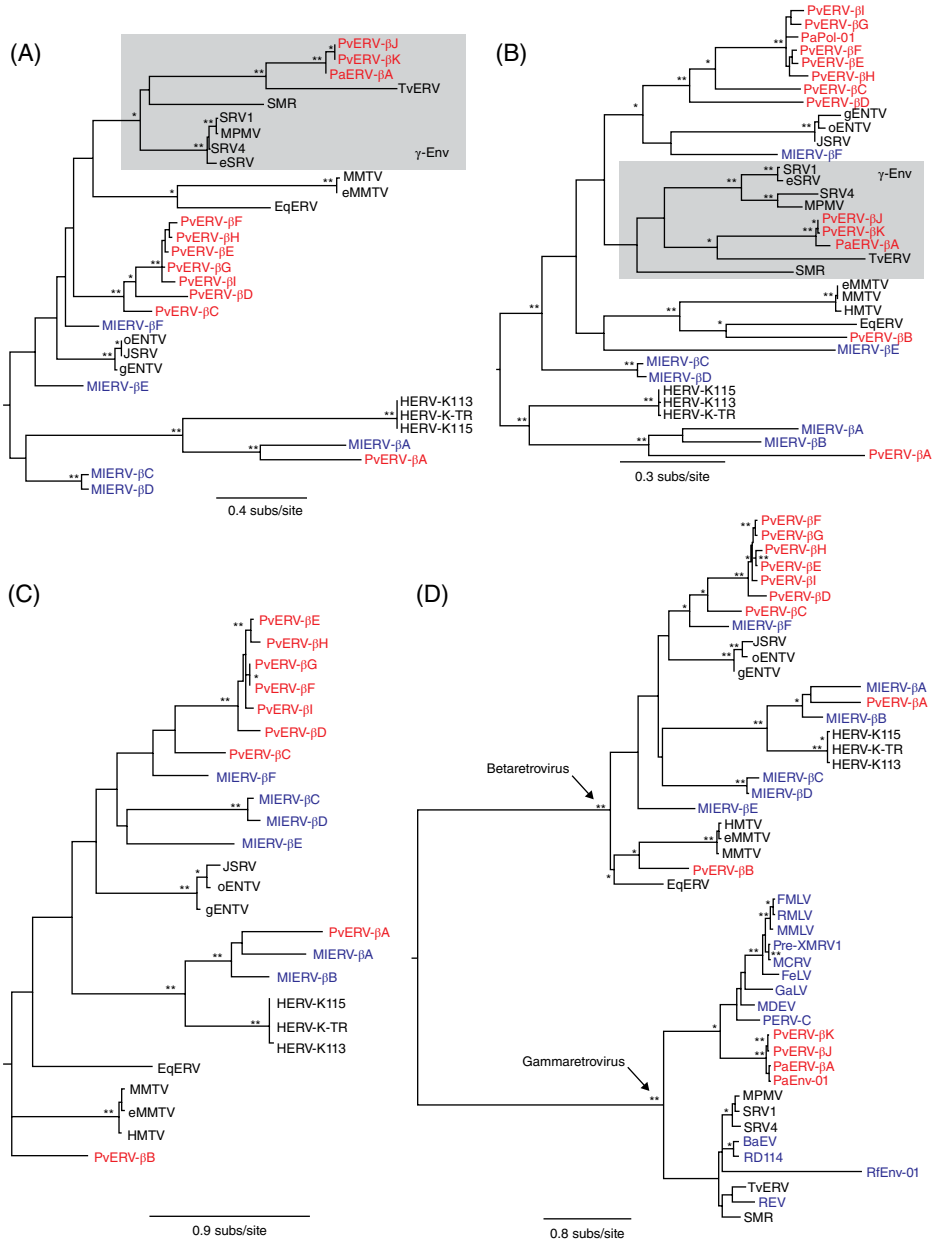

Figure 7.3 Phylogenetic relationships of bat and non-bat extant betaretroviruses with comparison to gammaretroviral Env sequences. Maximum likelihood phylogenetic trees of betaretroviral (A) Gag, (B) Pol, and (C) Env amino acid sequences and (D) betaretroviral compared to gammaretroviral Env amino acid sequences. Bootstrap values <70% are not shown, and branch lengths are draw to scale of amino acid substitutions per site. Bootstrap values are denoted as **>90%; *>70%; and <90%. The trees are midpoint rooted for purposes of clarity only. βERV proteins of *P. vampyrus* and *P. alecto* are highlighted in red text. βERVs of *M. lucifigus* and *R. ferrumequinum* are highlighted in blue text. Non-bat betareroviruses and gammaretroviruses are in black and teal text, respectively. The clades within (A) Gag and (B) Pol trees, highlighted with a grey rectangle (ϒ-Env), contain betaretroviruses whose Env sequence is not sufficiently closely related to the Env of other betaretroviruses to be included in the Env tree. Their phylogenetic relationship to gammaretroviruses is revealed in the (D) Env tree. Detailed information regarding sequences can be found in (Hayward *et al.*, 2013b). Figure is reproduced from (Hayward *et al.*, 2013b) with copyright permissions as described in the legend of Figure 7.1.

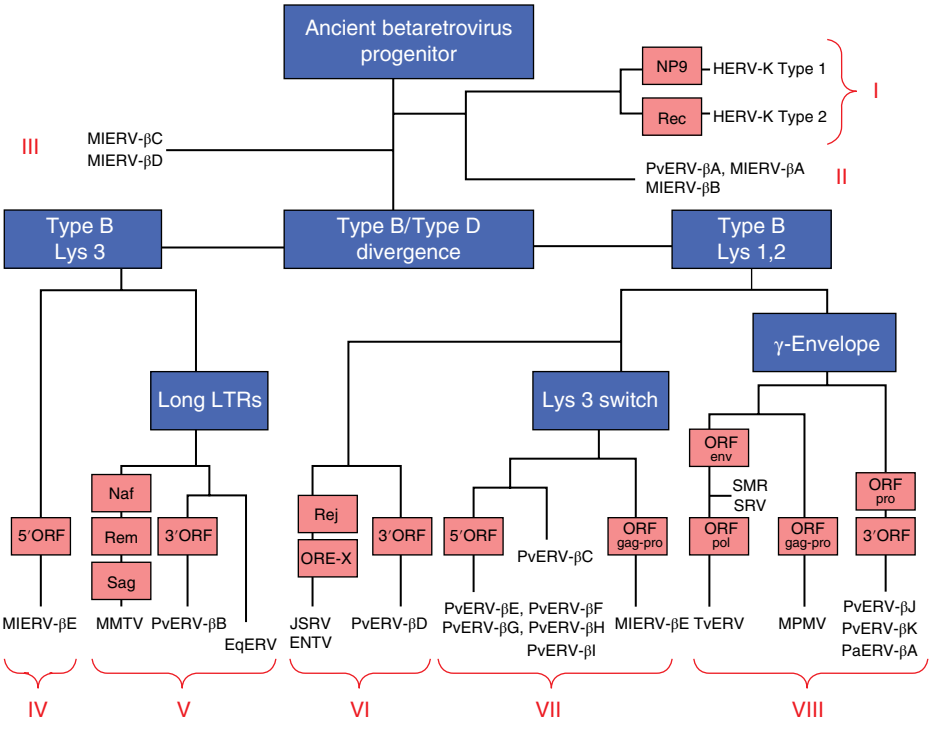

Figure 7.4 A proposed series of events leading to the current diversity in the genus *Betaretrovirus.* The proposed series of evolutionary events leading to eight distinct sub-groups of betaretroviruses based on a combination of the phylogenetic analyses of Gag, Pol, and Env protein sequences, and the genomic features and organizations of individual betaretroviruses. Abbreviations are defined in the legend of Figure 7.3. Figure is reproduced from (Hayward *et al.*, 2013b) with copyright permissions as described in the legend of Figure 7.1.

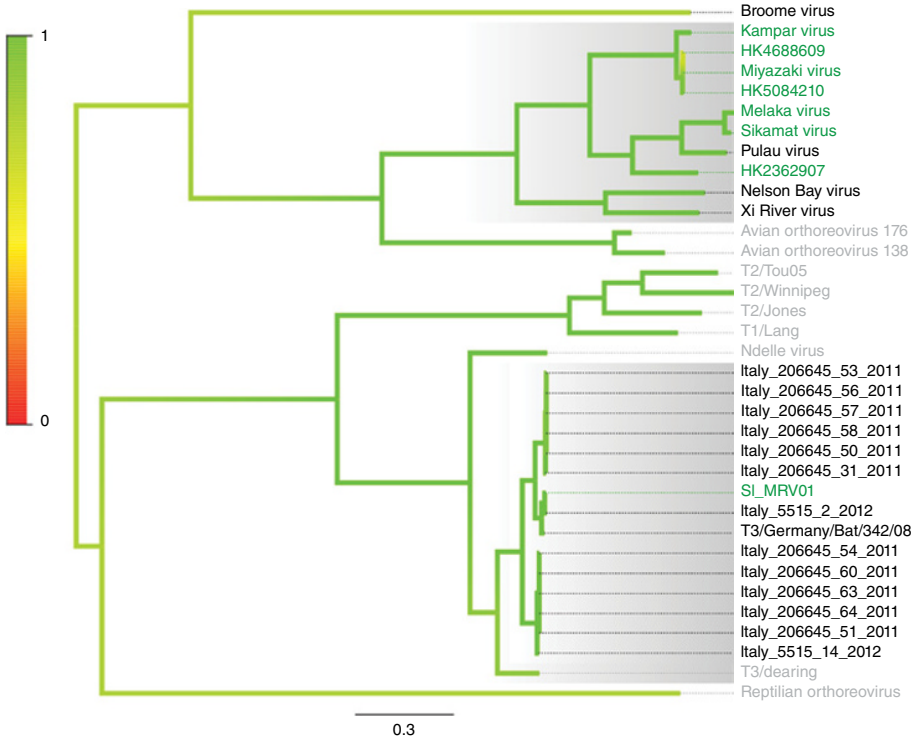

Figure 8.2 Phylogeny of orthoreoviruses. Posterior probabilities are given by color code represented by colored left-hand scale bar. Taxa are written in black (isolated from bats), green (isolated from humans), or gray (taxa provided for tree construction). The genus *Nelson Bay orthoreovirus* is depicted by the upper gray-shaded area and viruses of type 3 (T3) genus *Mammalian orthoreovirus* in the lower gray-shaded area. For Bayesian calculation of phylogenetic tree MrBayes v3.1.2 was used (Huelsenbeck et al., 2001), following a first model selection by using jModelTest (Posada, 2008), and model GTR+ G (gamma distribution) was selected for the alignment. The calculation parameters were as follows: number of runs: four, number of generations: 10 000 000 (partial S1 segment; 1369 nt), sample frequency: 100 and burn in: 25%. The results were finally visualized by the FigTree v1.2.1 program (http://tree.bio.ed.ac.uk/), a graphical viewer of phylogenetic trees. The scale-bar at the bottom represents the evolutionary distance of nt substitutions per position. The calculations were unrooted, but for visualization mid-point root was applied.

Figure 8.3 Map of reoviruses associated with bats worldwide. The dots indicate the sample origin of the virus isolates described. The color code indicates if viruses were isolated from humans or bats and whether these isolates cluster within the species *Nelson Bay orthoreovirus* or *Mammalian orthoreovirus*.

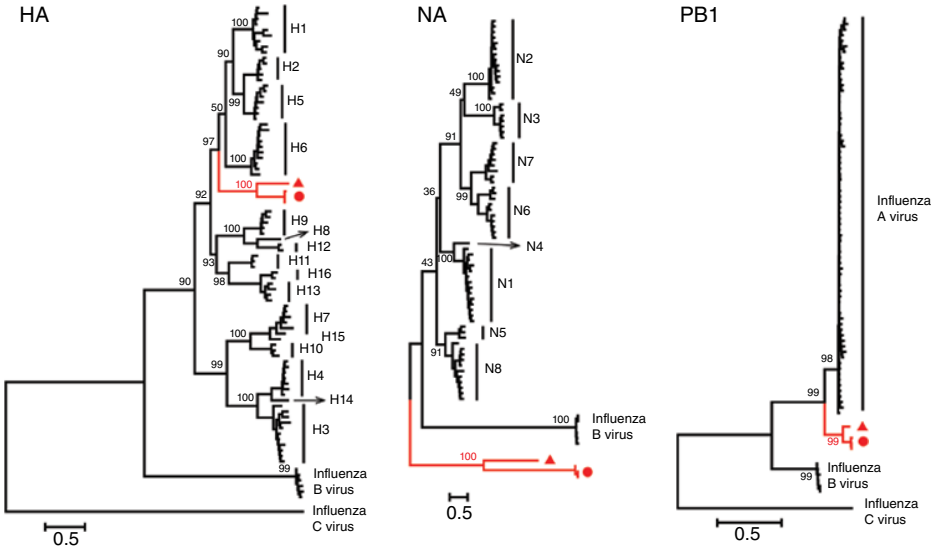

Figure 9.1 Evolution of influenza virus in New World bats. Phylogenetic relationships of influenza A viruses sampled from bats (red branches) and other animals ('non-bat', black branches) based on the amino acid sequences of three representative gene segments (HA, NA, and PB1).

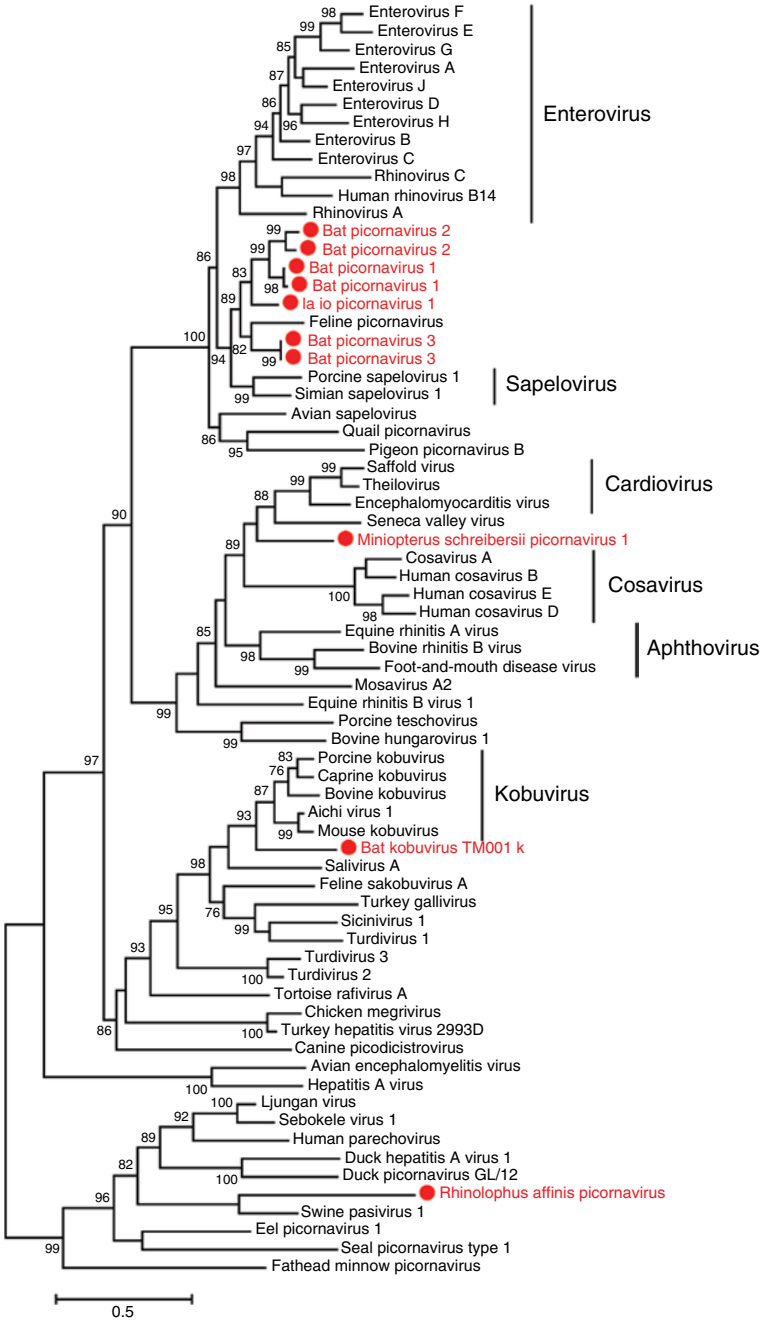

Figure 9.2 The phylogenetic positions of bat picornaviruses within the context of the entire family *Picornaviridae*. The tree is reconstructed using maximum likelihood methods implemented in PhyML program and is mid-point rooted. The data set is comprised of partial and complete polyprotein sequences of bat picornavirus as well as those of the type species within the family. The bat sequences are indicated in red. The names of the species and genera are labeled to the right of the tree.

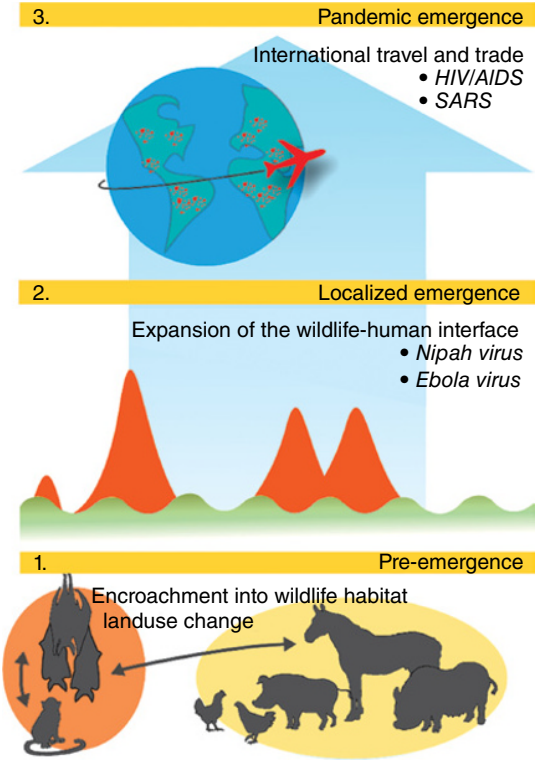

Figure 10.1 Schematic illustration of three stages of zoonotic pathogen emergence from a wildlife reservoir. Step 1 represents the human–wildlife interface. Activities that expand this interface and promote contact with wildlife (e.g., bats) provide opportunities for viruses which may constitute normal flora in their animal reservoirs, to jump into people either directly or via livestock. Occasionally spillover results in a localized disease outbreak in people, which either fades out or persists (step 2). Global travel allows infected individuals to introduce the pathogen to new regions, potentially leading to pandemic spread (step 3). Interventions at step 1 are necessary to minimize the health and economic impacts of disease emergence (reprinted from Morse *et al.*, 2012).

(A)

(B)

Figure 10.2 When designing a disease ecology study it is important to determine whether (a) individual-level sampling or (b) population-level sampling (e.g., pooled urine collection), is sufficient to test hypotheses or provide epidemiological data. A combined approach may provide optimal data. Photo credits: (a) M. Hillyard, (b) J. Mencher, copyright 2014, EcoHealth Alliance.

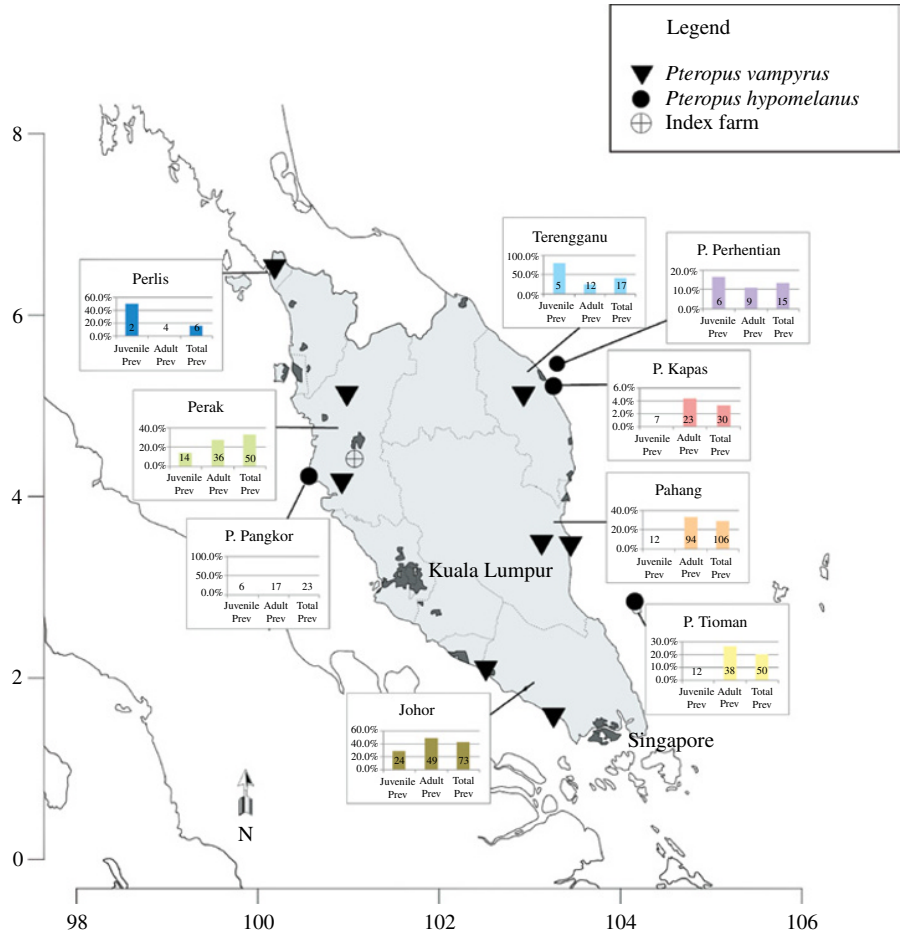

Figure 10.3 Seroprevalence of Nipah virus in *Pteropus* spp. in peninsular Malaysia. A country-wide survey was conducted to determine the spatial distribution of Nipah virus and to characterize the risk factors involved in bat infections (reprinted from Rahman *et al.*, 2013).

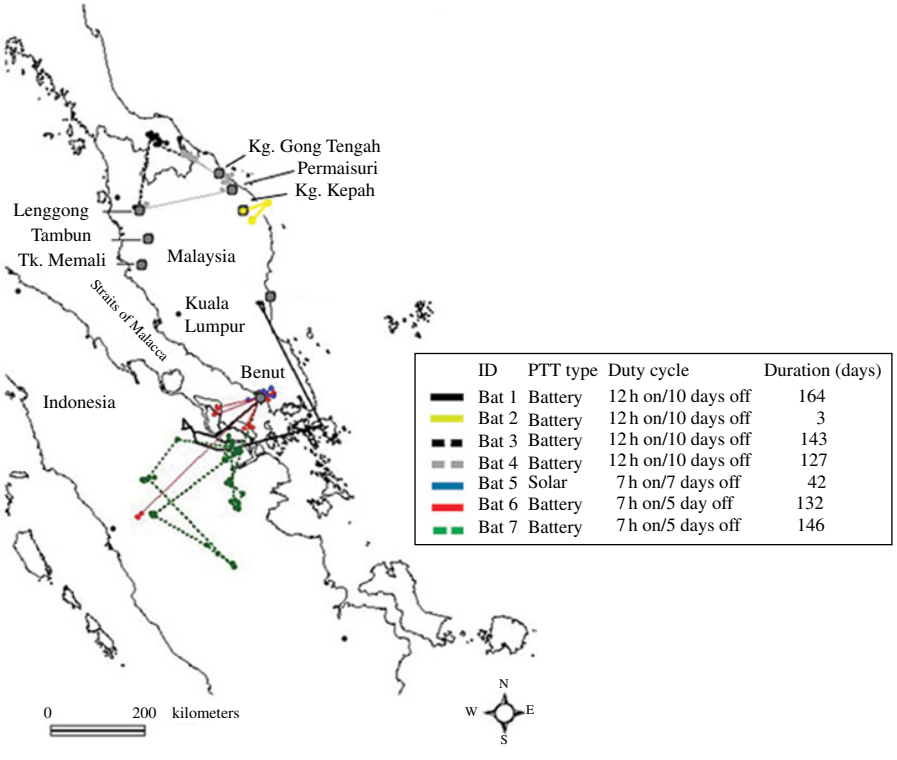

Figure 10.5 Satellite telemetry data showing *Pteropus vampyrus* flight paths and roost locations. The data were used to look at mobility and connectivity among bat colonies and illustrate that flying foxes are highly mobile and utilize habitat in Malaysia, Indonesia and Thailand (reprinted from Epstein *et al.*, 2009).

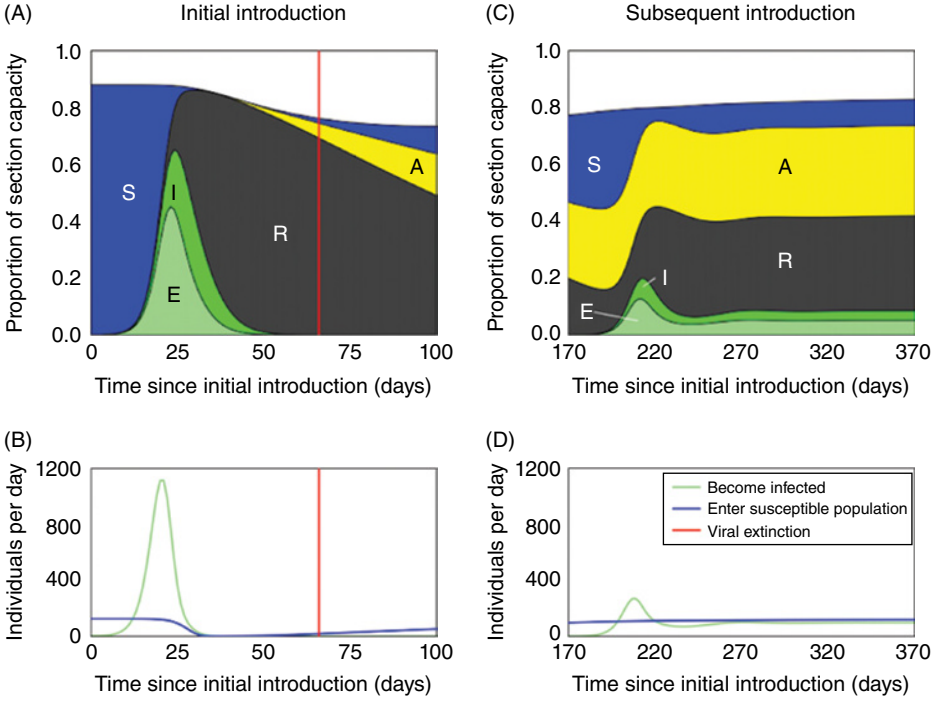

Figure 10.6 Deterministic model results illustrating Nipah virus dynamics in the growing section of the index farm. Individuals are characterized as belonging to one of five states: susceptible (S), immune – maternal antibodies (A), immune – recovered from infection (R), exposed (E), and infectious (I). The top panels illustrate the infection/immunity profile of the growing section following (A) initial introduction of the virus and (B) subsequent introduction. The qualitative difference in infection dynamics results primarily from the prevalence of maternal antibodies in the young pig population. (C, D) Following the initial introduction of the virus (C), the rate of replenishment of the susceptible population in the growing section (solid blue line) declines, as many individuals are immune, having been infected while in the breeding sections. The rate at which individuals are infected (green line) declines in consequence. When the virus is reintroduced (D), many individuals entering the growing section have maternal antibodies. Loss of maternal antibodies after entry into the growing section provides a source of susceptibles independent of the presence of infection (blue line), allowing the virus to persist (reprinted from Pulliam *et al.*, 2012).

Figure 10.7 *Pteropus giganteus*, found in the Indian subcontinent, is one of the largest bat species. Photo by J. Epstein, EcoHealth Alliance copyright 2014.

Figure 10.8 A date palm sap collector (*gachi*) demonstrates using a bamboo skirt to cover his collection pot as a means to prevent bats from contaminating sap with excreta. Photo by J. Epstein, copyright 2014, EcoHealth Alliance.

Figure 10.9 Masked palm civets (*Paguma lavarta*) traded in wet markets in China were a putative intermediate host of SARS and the immediate source of SARS infection in humans. Photo by H. E. Field, 2014.

Figure 10.10 Poor biosecurity measures and close human–animal contact in wet markets in southern China promoted cross-species transmission of novel viruses. Photo by H. E. Field, 2014.

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