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FAO ANIMAL PRODUCTION AND HEALTH



manual

INVESTIGATING THE ROLE OF BATS IN EMERGING ZOONOSES

Balancing ecology, conservation and public health interest

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INVESTIGATING THE ROLE OF BATS IN EMERGING ZOONOSES

Balancing ecology, conservation and public health interest

Edited by
Scott H. Newman, Hume Field, Jon Epstein and Carol de Jong

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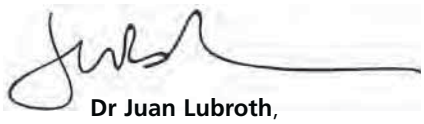
Foreword

With over 1,150 bat species worldwide – representing about twenty percent of the biodiversity of all mammalian species – they carry out important ecological and agricultural functions such pollination and dispersion of seeds. And while many tropical plant species depend entirely on bats for the distribution of their seeds, it is true that in the tropics bats can also be carriers of important diseases such as rabies, mokola, duvenhage, hendra or nipah viruses. These are the ones we know of today, but some 40 years ago, all we knew was about rabies. Is there more we should be doing?

This manual, "Investigating the role of bats in emerging zoonoses: Balancing ecology, conservation and public health interests" is an introduction to the complex issues associated with a One Health approach to understanding the biology and ecological importance of bats, and the drivers of zoonotic disease emergence from bats to people. As an introduction, this manual will provide a basis for understanding the need to balance natural resource management, disease surveillance, prevention and control.



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The authors would like to acknowledge all of the fieldworkers, researchers, biologists, medics, ecologists and veterinarians whose hard work contributed to the knowledge base presented in this manual. Special thanks to the staff of the Rabies Program CDC for their scientific expertise, dedicated service and international research collaboration.

The bat health and safety section has been developed by the Food and Agriculture Organization, the Australian Government Department of Agriculture, Fisheries and Forestry and the Australian Wildlife Health Network. Information is based upon guidance provided in Australian AUSVETPLANs, developed by government health and agriculture agencies and bat experts in Australia; guidance within Bat Manual chapters; and information provided in the Australian Immunisation Handbook 9th Edition 2008. Lyndel Post, Wildlife Health and Environment DAFF and Tiggy Grillo AWHN collated the information.

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Preface: Scope of this manual

Scott H. Newman [a]

This manual has been created for capacity development in countries interested in developing bat ecology, monitoring or disease surveillance programmes. It is intended for colleagues who have minimal knowledge about these topics and may be from public health, biology, wildlife, forestry, laboratory diagnostic, veterinary or agricultural professions. The manual contributes an important body of information about bat ecology, the ecological importance of bats, field techniques for studying bats and the most important infectious agents that are non-pathogenic to bats, but pose great risk to humans as zoonotic agents when they infect non-traditional host species. While bats may pose a risk to human health, it is important to realize that in most cases, zoonotic disease exposure from bats is a result of anthropogenic activities, and the ecological benefits of bats as pollinators or insect consumers far outweigh their zoonotic disease transmission potential. Therefore, understanding the ecology of the natural host of many potential zoonotic pathogens provides an opportunity for optimum management of the biological needs of bats and their habitats, ultimately ensuring the health of humans, livestock and wildlife species. This multisectoral approach, balancing the needs of people, wildlife, livestock and the environment, is part of a broader "One Health" approach, which is rooted in ideas that evolved more than 50 years ago.

The concept of addressing the connectivity between animal and human health is not new. In the 1960s, Calvin Schwabe, a veterinary epidemiologist and parasitologist in the United States, coined the expression "One Medicine" calling for a unified approach between veterinary and human medicine to combat zoonotic diseases – those diseases transmitted from animals to humans. The Manhattan Principles established in 2004 focus on the prevention of the emergence and re-emergence of diseases in the modern globalized world. More recently, a series of Ministerial conferences (Bamako, Mali 2006; New Delhi, India 2007; Sharm El Sheikh, Egypt 2008; Hanoi, Viet Nam 2010) resulting from the emergence and global spread of H5N1 highly pathogenic avian influenza (HPAI) have led international efforts towards addressing emerging infectious diseases at the animal-human-ecosystem interface, using the One Health approach and ensuring that health systems are capable of addressing high-impact disease threats that arise at the interface. It is recognized that to accomplish this requires inter-sectoral collaboration, timely and transparent communication, improved capacity, political commitment, regional and international cooperation, within the One Health framework.

Approximately 60 percent of emerging infectious diseases of humans are zoonotic and, since the 1940s, 70 percent originate from wildlife (often forest-dwelling) with wildlife-derived zoonotic diseases continuing to increase. These zoonotic pathogens have been identified in ungulates, carnivores, rodents, primates and non-mammal species, with important pathogens and diseases including HIV/AIDS, West Nile viruses, H5N1 HPAI, severe acute respiratory syndrome

[a] Food and Agriculture Organization of the United Nations

(SARS) and monkey pox. Most recently, bat-derived zoonotic pathogens such as Nipah and Hendra viruses, SARS-like coronaviruses, the Ebola and Marburg viruses, as well as various rabies-causing lyssaviruses, have gained notoriety as leading emerging diseases transmitted directly from bats to people, or via intermediate livestock and companion animal hosts, or fomites.

It has become clear that the emergence of infectious diseases, while complex in nature, is driven to some extent by ecosystem changes associated with growing global human population, increasing demand for animal protein by the growing middle-income class, more intensive farming systems, unsustainable natural resource consumption, biodiversity loss and habitat fragmentation, which lead to the loss of ecosystem services. Natural systems such as forests, grasslands, wetlands and oceans provide ecological services that all life depends on. Forests, for example, help purify air and water and mitigate greenhouse gas buildup in the atmosphere. Alteration in natural systems – whether in a rural, modified peri-urban or urban setting – results in decreased ecosystem services, leading to disease and increased health risks for all of the species in the ecosystem, including plants, wildlife, livestock and humans. Climate change and loss of ecosystem resilience, furthermore, are paving the road for the emergence of a series of new, multidimensional conservation and health challenges.

Approximately 70 percent of the 1.5 billion poorest people are dependent on livestock and natural resources. Poor sanitary and biosecurity conditions, in densely populated human-dominated, modified multi-species environments, provide opportunities for pathogens to transit more easily among potential host species. Subsistence bushmeat consumption, wildlife farming and trade bring people into contact with a great diversity of forest-dwelling species exposing people to novel pathogens. Intensive farming systems are also fertile breeding grounds for pathogens that can infect multiple hosts including livestock, wildlife and people.

In a globalized world where pathogens can travel the world in a day, emerging diseases, especially those affecting humans, livestock or wildlife, can have large negative socio-economic implications. Impacts can be severe for public health, livelihoods and food security, as well as for international trade and tourism. It is clear that the solution to the challenge of emerging infectious diseases relies on collaboration and integration of multiple disciplines and partners, including ministries of forestry and environment, agriculture and health. While more science is necessary to understand the complex relationships among disease emergence, transmission and ecological systems, science alone is not the solution. It is also essential to address the social and cultural dimensions of societies where issues concerning livestock, wildlife, humans and entire ecosystems intersect. Changes in thinking and behaviour must be encouraged, and future decision-making must be cognizant of the repercussions of poor natural resource management and their implications for civilization.

With the global response to H5N1 HPIA, reasonable capacity for regional disease surveillance, outbreak response, control and prevention in the agriculture sector has been established. However, there is still limited wildlife surveillance capacity, limited integration of wildlife expertise into epidemiological disease assessments, and often a lack of ecological information about wildlife host or transmission species. Furthermore, disease detection or emergence in people, livestock or wildlife rarely results in a multidisciplinary integrated response to determine drivers of emergence and to implement management actions. For field programmes targeting pandemic threats to be successful, they must take into account the broad range of stakeholder concerns, thus making animal disease prevention and control integral components of more general development activities.

Chapter 1
Emerging infectious diseases

Carol de Jong [a], *Hume Field* [a], *Scott H. Newman* [b] *and Jonathan H. Epstein* [c]

WILDLIFE AND EMERGING INFECTIOUS DISEASES

Although the current focus on emerging diseases in scientific literature and the popular press might suggest otherwise, novel diseases have occurred throughout history. By definition, every newly identified disease is novel. Today's endemic disease was yesterday's novel disease. This observation is not meant to invoke any complacency regarding the inevitability of disease emergence, nor to downplay the need for surveillance or discount the challenges associated with investigating and managing the outbreak of new diseases. Rather, it offers a window into the lessons of history.

Emerging infectious diseases (EIDs) are defined as infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range (Morens, Folkers and Fauci, 2004). Emerging infections have been a familiar threat since ancient times, with pandemics of cholera, influenza, smallpox and measles causing the deaths of millions of people worldwide. Since the 1940s, the incidence of EIDs has risen significantly and more than 300 infectious diseases have emerged (Jones *et al.,* 2008), most of which are viruses (Taylor, Latham and Woolhouse, 2001). More than 60 percent of EIDs are of zoonotic origin (Jones *et al.,* 2008), and in the last decade of the twentieth century zoonotic EIDs constituted 52 percent of all EID events (Taylor, Latham and Woolhouse, 2001).

Of all EIDs, zoonoses from wildlife represent the most significant, growing threat to global health. Among the zoonotic EIDs to emerge since the 1940s, the majority of EID events have originated in wildlife (71.8 percent) and their incidence has continued to increase (Jones *et al.,* 2008). Emerging zoonotic pathogens have been identified in ungulates, carnivores, rodents, primates, bats and other mammal and non-mammal species (Woolhouse and Gowtage-Sequeria, 2005). The best known EID of modern times, acquired immunodeficiency syndrome (AIDS), emerged from non-human primates around the early twentieth century (Worobey *et al.,* 2008). AIDS, which is caused by infection with one of two types of the human immunodeficiency virus (HIV), now threatens to surpass the Black Death of the fourteenth century and the 1918 to 1920 influenza pandemic, each of which killed 50 million people (Morens, Folkers and Fauci, 2004). Other recently emerged diseases, including Ebola virus, hantavirus, Nipah virus, West Nile virus, severe acute respiratory syndrome (SARS) coronavirus and highly pathogenic avian influenza (HPAI) virus, are examples of emerged or emerging zoonoses that have had (or threaten to have) a significant impact on human health.

[a] The State of Queensland, Department of Employment, Economic Development and Innovation (2011)

[b] Food and Agriculture Organization of the United Nations

[c] EcoHealth Alliance

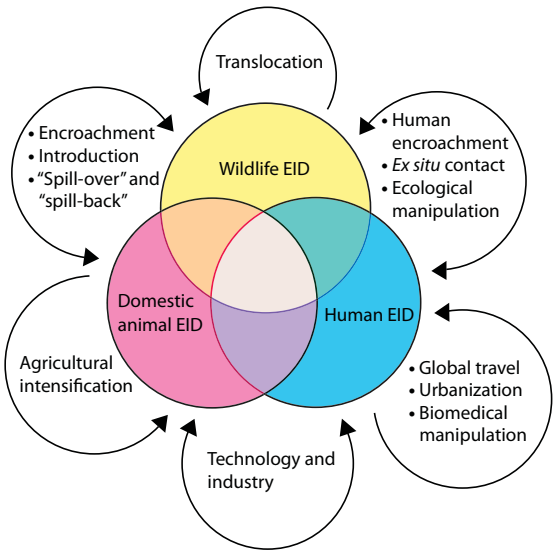
Understanding the factors that lead to pathogens jumping species or to increased contact among wildlife, livestock and humans is critical to understanding how diseases emerge from wildlife.

DRIVERS OF EMERGENCE

Wildlife populations constitute a large and often unknown reservoir of infectious agents (Chomel, Belotto and Meslin, 2007), playing a key role in emergence by providing a "zoonotic pool" from which previously unknown pathogens may emerge (Morse, 1995). The emergence of many zoonoses can be attributed to predisposing factors such as global travel, trade, agricultural expansion, deforestation and urbanization; such factors increase the interface and/or the rate of contact among human, domestic animal and wildlife populations, thereby creating increased opportunities for spill-over events to occur (Daszak, Cunningham and Hyatt, 2000; 2001). Lederberg, Shope and Oaks (1992) describe these changes as providing an "epidemiological bridge" that facilitates contact between the agent and the naive population. Daszak, Cunningham and Hyatt (2000) suggest that disease emergence from wildlife sources is primarily an ecological process, with emergence frequently resulting from a change in the ecology of the host or the agent or both. They suggest that most emerging diseases exist within a finely balanced host-agent continuum among wildlife, domestic animal and human populations. Any changes in the environment or host behaviour provide agents with favourable new ecological niches, allowing them to reach and adapt to new hosts and spread more easily between them (Morens, Folkers and Fauci, 2004).

FIGURE 1.1

The host-parasite continuum: most emerging diseases exist in a host-parasite continuum among wildlife, domestic animal and human populations



Source: Daszak, Cunningham and Hyatt, 2000

Pathogen adaptation and virulence are additional dynamics that have direct linkages to the ecological systems in which they occur. Regardless of whether the system is natural or agricultural, the key to pathogens' survival is their ability to adapt to the ever-changing environment. In natural systems, loss of biodiversity, changes in landscape ecology, climate change and other variables pose innate adaptation challenges for pathogens. In agricultural settings, farming modifications including intensification, changes in animal density or husbandry practices, use of pharmaceuticals and marketing create the adaptation challenges for pathogens. The pathogens that exist in wildlife or livestock hosts are therefore constantly challenged to adapt to new environmental circumstances for their survival, resulting in the emergence of "super pathogens" that can cross sectors such as the wildlife-livestock interface, and can ultimately infect humans when the opportunity arises.

Table 1.1 lists a range of drivers for the emergence of infectious disease identified by Daszak, Cunningham and Hyatt (2000), Morens, Folkers and Fauci (2004), Woolhouse and Gowtage-Sequeria (2005) and Chomel, Belotto and Meslin (2007). At the macro

TABLE 1.1
Drivers of emerging zoonoses

Human behaviour*
Cultural preference and celebrations
Food choices (bushmeat, live-animal markets, freshly killed)
Traditional medicine
Consumption instead of conservation
Ecotourism
Petting zoos
Exotic pet ownership
Modifications to natural habitats*
Communities and settlement encroaching on natural habitat
Development and construction
Water resource management (dams, redirecting river or ocean flow patterns)
Deforestation
Fragmentation of habitat
Loss of biodiversity and species
Waste and garbage management
Climate change
Changes in agricultural practices*
Expansion of livestock farming and encroachment
Intensification of production systems resulting in overcrowding, stress, and faster growing and input/output periods
More wastewater and faecal runoff into the environment
Farming of new species, including wildlife, without proper medical care, husbandry or biosecurity
Globalized international market chains

* The impacts are amplified by human demographics and socio-economic advancement from poverty towards middle income.

Source: Adapted from Chomel, Belotto and Meslin, 2007.

level, closer human contact with wildlife habitats, primarily caused by human population expansion into and modification of wildlife habitat, is considered a major driver in the emergence of zoonotic infections (Cunningham, 2005). At the microbial level, molecular changes may contribute to emergence, when mutation, recombination or reassortment occur or microbes switch from animal to human hosts (Morens, Folkers and Fauci, 2004).

IMPACT OF EIDs

EIDs are a significant threat to global public health, particularly considering that more than 25 percent of annual deaths worldwide are estimated to be directly related to infectious diseases (Morens, Folkers and Fauci, 2004). Economic losses associated with livestock morbidity and mortality threaten not only agricultural industries, but also wildlife-based economies such as wildlife tourism or the bushmeat trade (Chomel, Belotto and Meslin, 2007). Historically, wildlife diseases have been considered important only when agriculture or human health are threatened (Daszak, Cunningham and Hyatt, 2000). However EIDs are also a significant threat to species conservation and biodiversity. While wildlife species can be considered reservoirs of pathogens with the potential to infect humans and livestock, wildlife populations are themselves also threatened by introduced pathogens. Spill-over of infectious agents to wildlife populations is a particular threat to endangered species, where the presence of infected reservoir hosts can lower the pathogen's threshold density and lead to local population extinction (Daszak, Cunningham and Hyatt, 2000). For example, white nose syndrome, an emerging fungal pathogen of hibernating bats in northeastern North America first observed in 2006, has caused unprecedented bat mortality leading to losses of up to 95 percent in some hibernacula (Blehert *et al.,* 2009; Wibbelt *et al.,* 2010). Another (non-bat) example of the impact of EIDs on wildlife populations is high-pathogenicity avian influenza. While low pathogenic avian influenza was probably introduced from free-ranging waterfowl into poultry, the change from low to high pathogenicity occurred in poultry and spill-back into wildlife populations. This scenario has been responsible for a population-level impact on bar-headed geese (*Anser indicus),* as more than 6 000 individuals died during a single outbreak at Qinghai Lake in 2005 (Chen *et al.,* 2006; Zhou *et al.,* 2006).

BATS AND EIDs

In recent years, bats have been implicated in numerous EID events and are increasingly recognized as important reservoir hosts for viruses that can cross species barriers to infect humans and other domestic and wild mammals (Calisher *et al.,* 2006). Bats are second only to rodents in numbers of living genera and species, and are the largest order of mammals in overall abundance (Sulkin and Allen, 1974). They are unique in their vagility (potential for long-distance travel), and often aggregate in very large colonies (Turmelle and Olival, 2010). However, despite their abundance, relatively little is known about the species from which zoonotic viruses emerge to cause human disease (Calisher *et al.,* 2006). Much of the information gathered on the role of bats in the maintenance and spread of viruses has been from species of Microchiroptera (insectivorous bats), and there is relatively little information available for members of the suborder Megachiroptera (flying foxes and fruit bats) (Mackenzie, Field and Guyatt, 2003).

The role of bats in viral disease is well established (Sulkin and Allen, 1974), particularly their role as hosts for alpahviruses, flavirviruses, rhabdoviruses and arenaviruses (Mackenzie,

Field and Guyatt, 2003). Calisher *et al.* (2006) report on 66 viruses that have been isolated from or detected in bat tissues of 74 species (Table 1.2). Some viruses have been isolated from bats of only one species, and one from bats of 14 species. There are also many viral infections for which only serological evidence is available. Perhaps one of the highest-profile EID events in recent years – for which flying foxes have been identified as the natural host – is Nipah virus, which was identified as the cause of a major outbreak of disease in pigs and humans, resulting in 265 human cases of viral encephalitis (with a 38 percent mortality rate) and the eventual culling of 1.1 million pigs (Chua *et al.,* 2000). It is recognized that this catastrophic disease event was probably the result of several major ecological and environmental changes associated with deforestation and the expansion of non-industrial pig farming in association with the production of fruit-bearing trees. This combination created circumstances that led to the infection of pigs following indirect exposure to virus shed from fruit bats (Chomel, Belotto and Meslin*,* 2007). The highly infectious virus subsequently led to human cases (Daszak, Cunningham and Hyatt, 2001), most of which involved pig farmers or people associated with pig farming.

Bats possess certain characteristics that may maximize their effectiveness as reservoir hosts for viruses. Natural history features such as high species diversity, long life span, the capacity for long-distance dispersal, dense roosting aggregations (colony size), social behaviours and population structure, the use of torpor and hibernation, unique immunology and spatial population structure (Calisher *et al.,* 2006; Turmelle and Olival, 2009) have been suggested for the association of bats and EIDs. Evolutionary adaptations such as conserved

FIGURE 1.2
A property quarantined during an outbreak of Hendra virus in Queensland, Australia

HUME FIELD; © THE STATE OF QUEENSLAND (DEEDI)

FIGURE 1.3
Dense roosting behaviour of little bent-wing bats (*Miniopterus australis*) facilitating the transmission of virus among individuals

CAROL DE JONG; © THE STATE OF QUEENSLAND (DEEDI)

cellular receptors and pathways may also enhance the capacity for transmission of bat-associated viruses to other mammals (Calisher *et al.,* 2006).

So why are these diseases emerging now? When attempting to answer this question, it is appropriate to distinguish between emergence and detection. The identification of bat species as probable natural hosts of some recently described EIDs can be attributed

FIGURE 1.4
Typical roosting behaviour of flying foxes

DEBBIE MELVILLE; © THE STATE OF QUEENSLAND (DEEDI)

to the increased surveillance of bats after the initial discovery of antibodies to Hendra virus in Australian bats. However, targeted surveillance or improved diagnostic capabilities cannot explain the actual emergence of Nipah virus or SARS coronavirus, as evidenced by the consequent major disease outbreaks: even without targeted surveillance or improved diagnostic capabilities, the outbreaks would still have occurred (and possibly been identified), but their origins would have remained unknown. The absence of evidence of previous unidentified outbreaks from retrospective examination of historical case or necropsy data also supports emergence over detection. In addition, the temporal clustering of the identification of bat-associated agents (in Australia, new diseases associated with Hendra virus, Australian bat lyssavirus and Menangle virus occurred in the space of a few years) is consistent with a host-level effect. This contention is supported by earlier and concurrent identification of negative ecological impacts on (particularly pteropid) bat populations in Australia and Southeast Asia, manifested as declining populations and changed movement and foraging patterns. It was noted earlier that in addition to the presence of an agent, disease emergence requires an effective bridge from the natural host to a susceptible spill-over host (Lederberg, Shope and Oaks, 1992).

Such bridges result from anthropogenic or natural changes to the agent, the host or the environment. The available evidence suggests that Hendra and Nipah viruses are ancient viruses (Murray *et al.,* 1995; Gould, 1996) that are evolutionarily and immunologically well adapted to

their natural flying fox hosts, in whose populations they have long circulated. They remained primarily confined to these hosts until some change (or more probably sequence of changes) precipitated their emergence. As already mentioned, data on many fruit bat species suggest that populations in Australia and Southeast Asia are declining because of disruptions throughout their ranges. In Southeast Asia, anthropogenic activities (primarily habitat loss and hunting) have been identified as constituting the major threats. Deforestation for agricultural land, commercial logging or urban development is widespread, and results in loss or abandonment of roosting sites and loss of feeding habitats. Habitat loss from clearing is commonly exacerbated by tropical storms, as remnant forest is particularly prone to high wind damage. Hunting for consumption or crop protection, on both subsistence and commercial scales, results in the abandonment of roost and feeding sites (Mickleburgh, Hutson and Racey, 1992). In the scenario that emerges, flying fox populations are under stress, foraging and behavioural patterns are altered, niches expand, and livestock and humans come into closer contact. In Australia, this has been paralleled in recent decades by the geographic redistribution of flying fox camps into urban areas (L. Hall, personal communication), as the habitat loss and fragmentation associated with land-use change fundamentally alter the historical resource landscape.

MANAGEMENT STRATEGIES

The emergence and spread of infectious diseases in recent years has resulted in a major awakening of public health services. The involvement of veterinarians and other wildlife

FIGURE 1.5
Colony of more than 100 000 black flying foxes in an urbanized area of a coastal town in Queensland, Australia

CAROL DE JONG; © THE STATE OF QUEENSLAND (DEEDI)

specialists has highlighted the role that they can play in the surveillance, control and prevention of emerging zoonoses (Chomel, Belotto and Meslin, 2007). Current strategies for disease prevention and control in the spill-over host are directed towards minimizing direct or indirect contact with the natural host, improving farm-gate and on-farm biosecurity, and rapid disease detection and diagnosis. Additional strategies for Australian bat lyssavirus include the use of rabies vaccine for pre- and post-exposure prophylaxis in humans. Effective management strategies in the natural host are predicated on an understanding of the ecology of the disease in the natural host, and the identification and avoidance of factors putatively associated with

TABLE 1.2
Viruses isolated from naturally infected bats worldwide

Virus	Bat species (common name) [a]
Family *Rhabdoviridae*, genus *Lyssavirus*	
Rabies virus	Numerous bat species, essentially worldwide
Lagos bat virus	*Eidolon helvum* (African straw-coloured fruit bat), *Micropteropus pusillus* (Peters' lesser epauletted fruit bat), *Epomops dobsonii* (Dobson's epauletted fruit bat), *Nycteris gambiensis* (Gambian slit-faced bat), *Epomophorus wahlbergi* (Wahlberg's epauletted fruit bat)
Duvenhage virus	*Miniopterus* sp., *Nyctalus noctula* (noctule), *Vespertilio murinus* (parti-coloured bat), *Nycteris thebaica* (Egyptian slit-faced bat)
Australian bat lyssavirus	Megachiroptera (multiple *Pteropus* spp.), Microchiroptera sp. from Australia, *Saccolaimus flaviventris* (yellow-bellied pouched bat)
European bat lyssavirus 1	*Eptesicus serotinus* (serotine bat), *Rousettus aegyptiacus* (Egyptian rousette)
European bat lyssavirus 2	*Myotis myotis* (greater mouse-eared bat), *Myotis dasycneme* (pond bat), *Myotis nattereri* (Natterer's bat), *Miniopterus schreibersii* (Schreiber's bent-winged bat), *Rhinolophus ferrumequinum* (greater horseshoe bat), *Myotis daubentonii* (Daubenton's bat)
Aravan virus	*Myotis blythii* (lesser mouse-eared bat)
Khujand virus	*Myotis mystacinus* (whiskered bat)
Irkut virus	*Murina leucogaster* (greater tube-nosed bat)
West Caucasian bat virus	*Miniopterus schreibersii* (Schreiber's bent-winged bat)
Family *Rhabdoviridae*, genus unassigned	
Gossas virus	*Tadarida* sp.
Kern Canyon virus	*Myotis yumanensis* (Yuma bat)
Mount Elgon bat virus	*Rhinolophus eloquens* (eloquent horseshoe bat)
Oita 296 virus	*Rhinolophus cornutus* (little Japanese horseshoe bat)
Family *Orthomyxoviridae*, genus *Influenzavirus A*	
Influenza A virus	*Nyctalus noctula* (noctule)
Family *Paramyxoviridae*, genus *Henipavirus*	
Hendra virus	*Pteropus alecto* (black flying fox), *Pteropus poliocephalus* (grey-headed flying fox), *Pteropus scapulatus* (little red flying fox), *Pteropus conspicillatus* (spectacled flying fox)
Nipah virus	*Pteropus hypomelanus* (variable flying fox), *Pteropus vampyrus* (large flying fox), *Pteropus lyle* (Lyle's flying fox)

(Cont.)

TABLE 1.2 *(Cont.)*

Virus	Bat species (common name)
Family *Paramyxoviridae*, genus *Rubulavirus*	
Mapuera virus	*Sturnira lilium* (yellow epauletted bat)
Menangle virus	*Pteropus poliocephalus* (grey-headed flying fox)
Tioman virus	*Pteropus hypomelanus* (variable flying fox)
Family *Paramyxoviridae*, genus undetermined	
A parainfluenza virus	*Rousettus leschenaultii* (Leschenault's rousette)
Family *Coronaviridae*, SARS coronavirus	*Rhinolophus sinicus* (Chinese horseshoe bat), *Rhinolophus pearsonii* (Pearson's horseshoe bat), *Rhinolophus macrotis* (big-eared horseshoe bat), *Rhinolophus ferrumequinum* (greater horseshoe bat)
Family *Togaviridae*, genus *Alphavirus*	
Chikungunya virus[b]	*Scotophilus* sp., *Rousettus aegyptiacus* (Egyptian rousette), *Hipposideros caffer* (Sundevall's leaf-nosed bat), *Chaerephon pumilus* (little free-tailed bat)
Sindbis virus	*Rhinolophidae* sp., *Hipposideridae* sp.
Venezuelan equine encephalitis virus	*Desmodus rotundus* (vampire bat), *Uroderma bilobatum* (tent-making bat), *Artibeus phaeotis* (pygmy fruit-eating bat)
Family *Flaviviridae*, genus *Flavivirus*	
Bukalasa bat virus	*Chaerephon pumilus* (little free-tailed bat), *Tadarida condylura* (Angola free-tailed bat)
Carey Island virus	*Cynopterus brachiotis* (lesser short-nosed fruit bat), *Macroglossus minimus* (lesser long-tongued fruit 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* (Angola free-tailed bat)
Entebbe bat virus	*Chaerephon pumilus* (little free-tailed bat), *Mops condylurus* (Angola free-tailed bat)
Japanese encephalitis virus	*Hipposideros armiger terasensis* (great roundleaf bat, also known as Formosan leaf-nosed bat), *Miniopterus schreibersii* (Schreiber's long-fingered bat), *Rhinolophus cornutus* (little Japanese horseshoe bat)
Jugra virus	*Cynopterus brachiotis* (lesser short-nosed fruit bat)
Kyasanur Forest disease virus	*Rhinolophus rouxi* (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* (lesser dawn bat), *Cynopterus brachyotis* (lesser short-nosed fruit bat)
Rio Bravo virus	*Tadarida brasiliensis 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* (probably *Pipistrellus pipistrellus*, common pipistrelle)
Tamana bat virus	*Pteronotus parnellii* (Parnell's moustached bat)

(Cont.)

TABLE 1.2 *(Cont.)*

Virus	Bat species (common name)
Uganda S virus	*Rousettus* sp., *Tadarida* sp.
Yokose virus	Unidentified bat
Family *Bunyaviridae*, genus *Bunyavirus*	
Catu virus	*Molossus obscurus* (possibly *Molossus currentium*; Thomas's mastiff bat)
Guama virus	Unidentified bat
Nepuyo virus	*Artibeus jamaicensis* (Jamaican fruit-eating bat), *A. lituratus* (great fruit-eating bat)
Family *Bunyaviridae*, genus *Hantavirus*	
Hantaan virus	*Eptesicus serotinus* (serotine bat), *Rhinolophus ferrumequinum* (greater horseshoe bat)
Family *Bunyaviridae*, genus *Phlebovirus*	
Rift Valley fever virus	*Micropteropus pusillus* (Peter's pygmy epauletted fruit bat), *Hipposideros abae* (Aba leaf-nosed bat), *Miniopterus schreibersii* (Schreiber's long-fingered bat), *Hipposideros caffer* (Sundevall's leaf-nosed bat), *Epomops franqueti* (Franquet's epauletted bat), *Glauconycteris argentata* (common butterfly bat)
Toscana virus	*Pipistrellus kuhlii* (Kuhl's pipistrelle)
Family *Bunyaviridae*, genus unassigned	
Kaeng Khoi virus	*Chaerephon plicatus* (wrinkle-lipped free-tailed bat)
Bangui virus	*Scotophilus* sp., *Pipistrellus* sp., *Tadarida* sp.
Family *Reoviridae*, genus *Orbivirus*	
Ife virus	*Eidolon helvum* (straw-coloured fruit bat)
Japanaut virus	*Syconycteris australis* (southern blossom bat)
Fomede virus	*Nycteris nana* (dwarf slit-faced bat), *Nycteris gambiensis* (Gambian slit-faced bat)
Family *Reoviridae*, genus *Orthoreovirus*	
Nelson Bay virus	*Pteropus poliocephalus* (grey-headed flying fox)
Pulau virus	*Pteropus hypomelanus* (variable flying fox)
Broome virus	*Pteropus alecto* (black flying fox)
Family *Arenaviridae*, acaribe virus	
	Artibeus lituratus (great fruit-eating bat), *A. jamaicensis* (Jamaican fruit-eating bat)
Family *Herpesviridae*, genus unassigned	
Agua Preta virus	*Carollia subrufa* (grey short-tailed bat)
A cytomegalovirus	*Myotis lucifugus* (little brown bat)
Parixa virus	*Lonchophylla thomasi* (Thomas's nectar bat)
Family *Picornaviridae*, genus undetermined	
Juruaca virus	Unidentified bat

(Cont.)

TABLE 1.2 *(Cont.)*

Virus	Bat species (common name)
Unclassified	
Issyk-Kul (Keterah virus)[c]	*Nyctalus noctula* (noctule), *Eptesicus serotinus* (common serotine), *Pipistrellus pipistrellus* (common pipistrelle), *Myotis blythii* (lesser mouse-eared myotis), *Rhinolophus ferrumequinum* (greater horseshoe bat), *Scotophilus kuhlii* (lesser Asiatic yellow house bat), *Cynopterus brachyotis* (lesser short-nosed fruit bat), *Eonycteris spelaea* (lesser dawn bat), *Chaerephon plicatus* (wrinkle-lipped free-tailed bat), *Hipposideros diadema* (diadem leaf-nosed bat), *Taphozous melanopogon* (black-bearded tomb bat), *Rhinolophus lepidus* (Blyth's horseshoe bat), *Rhinolophus horsfeldi* (possibly *Megaderma spasma*, lesser false vampire bat)
Mojui dos Campos virus	Unidentified bat
Yogue virus	*Rousettus aegyptiacus* (Egyptian rousette)
Kasokero virus	*Rousettus aegyptiacus* (Egyptian rousette)

[a] Species names and common names are given according to Simmons, 2005 and other sources.
[b] Arthropod-borne viruses (arboviruses) isolated from or detected in bats were probably transmitted to them by arthropods, from another individual of the same bat species (reservoir host) or from another vertebrate reservoir host. With few exceptions, such as rabies virus, relatively little is known about the natural history of these viruses or about non-arthropod-transmitted viruses of bats.
[c] Issyk-Kul and Keterah viruses may be synonyms.

Source: Calisher *et al.*, 2006.

emergence, such as habitat loss, land-use change and demographic shifts. A possible future management strategy in reservoir populations is immunization using bait or plant-derived vaccinations.

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Chapter 2

Natural history, ecological and socio-economic value of bats

Tammy Mildenstein [a] *and Carol de Jong* [b]

Disease ecology, transmission and emergence are integrally linked to the host. It is therefore important to have a good understanding of host species, their natural history, and how they interact with humans. The following section provides a brief overview of bat taxonomy, distribution, biology and ecology, and a description of the importance of bats to humans. Given this manual's focus on sampling bats for tracking and understanding emerging infectious diseases (EIDs), this chapter highlights the aspects that are most important for an understanding of bats as pathogen reservoirs and transmitters. The references at the end of the chapter suggests further reading for more in-depth study.

TAXONOMY AND DISTRIBUTION

Bats (order Chiroptera) are a diverse group of mammals found on every major land mass except the polar regions and a few oceanic islands. There are 1 150 species of bats in the world (IUCN, 2010), meaning that one in five (21 percent) mammal species is a bat. Bats are distinguished from other mammals by their evolution of true flight, as opposed to the gliding capabilities of mammals in other orders.

Although the taxonomy of bats is currently being revised in the light of molecular phylogenetics, they have traditionally been split into two suborders – Microchiroptera and Megachiroptera – based largely on the evolution of echo-location (ability to navigate using the reflection of sound waves) (Koopman, 1993). The largest and most ecologically diverse of the two suborders is the Microchiroptera (Figure 2.1), the echo-locating bats, which include 963 species (IUCN, 2010). The Microchiroptera are widespread throughout the range of bats, with the greatest diversity occurring in the tropics (Findley and Wilson, 1983). They are primarily insectivorous, although some families have evolved more diverse diets.

The Megachiroptera (Figure 2.2) comprise 187 species of Old World bats (IUCN, 2010) which, with the exception of a single genus, do not echo-locate. As the name implies, Megachiroptera are, on average, larger in size than Microchiroptera, although considerable overlap exists: Megachiroptera weigh from 10 to 1 500 g, and Microchiroptera from 2 to 196 g (Mickleburgh, Hutson and Racey, 1992). Megachiroptera are commonly known as "fruit bats", because they eat fruit and nectar; fruit bats occur in the subtropical and tropical regions of the Old World, from the eastern Mediterranean and the Arabian Peninsula,

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across Africa to Asia, Australia and the islands in the Pacific (Rainey and Pierson, 1992). This designation is currently being re-evaluated, and new categories have been proposed, consistent with phylogenetic relationships.

NATURAL HISTORY

Given the global diversity and distribution of bats, it is not surprising that they have a vast natural history. Summaries of species-specific biology, ecology and conservation concerns are available from a number of sources (Mickleburgh, Hutson and Racey, 1992; Hutson, Mickleburgh and Racey, 2001; Kunz and Fenton, 2003; IUCN, 2010). This section provides a basic overview of the natural history of bats in general, emphasizing the areas most pertinent to understanding the diseases bats carry.

FIGURE 2.1
Example of a Microchiropteran: the little bent-wing bat (*Miniopterus australis*) weighs between 5 and 8 g

CAROL DE JONG; © THE STATE OF QUEENSLAND (DEEDI)

FIGURE 2.2
Example of a Megachiropteran: the Christmas Island flying fox (*Pteropus natalis*) is endemic to Christmas Island and weighs between 350 and 500 g

CAROL DE JONG; © THE STATE OF QUEENSLAND (DEEDI)

Roosting and aggregating behaviour

Given that bats spend roughly half their lives in the roosting environment, it is not surprising that roost sites play a large role in the biology and ecology of bats. Most bat species choose concealed roost sites, such as caves, mines (Figure 2.3), cavities or crevices in rocks and trees, under foliage, and in modified human-made structures (examples and references in Kunz and Pierson, 1994). Some colonially roosting megachiropteran species form conspicuous aggregations (also called "camps"), often using exposed tree branches (Figure 2.4) (examples in Mickleburgh, Hutson and Racey, 1992). Roost site occupation may be seasonal (e.g., during hibernation or maternity periods) or perennial, lasting year round in the same location for many years (Kunz and Pierson, 1994). In addition to their day-time roost locations, many bats also aggregate in night-time roosts, which are temporary and often close to feeding locations.

Bats are known for forming the largest aggregations of all mammals. Depending on the species, season, and location of the roost, colony sizes range from a few to millions of individuals. As with roost site occupancy, aggregating behaviour is a species-dependent trait, occurring seasonally (especially during hibernation and/or reproductive periods) in

FIGURE 2.3
A colony of little bent-wing bats (*Miniopterus australis*) roosting in a disused mine in Eastern Australia

CAROL DE JONG; © THE STATE OF QUEENSLAND (DEEDI)

some species and lasting year round in others. In particular, a few species of molossids and vespertilionids form the largest aggregations of Microchiroptera (McCracken and Gustin, 1991). More than one-third of megachiropteran species form colonies, with species from nine genera known to be strongly colonial: *Acerodon, Aproteles, Boneia, Dobsonia, Eidolon, Eonycteris, Notopteris, Pteropus* and *Rousettus* (Marshall, 1983; Pierson and Rainey, 1992).

Mating system and life history traits

Bat species exhibit a wide range of mating systems, from monogamy (uncommon in mammals), to lekking, to the promiscuous mating systems assumed in highly colonial species (Bradbury, 1977). The most prevalent mating system in bats is thought to be resource-defence polygyny, in which a male defends a harem of females for exclusive reproductive access (Kunz and Pierson, 1994). Most bat species are mono-oestrus, reproducing once per year, while some tropical species produce two or even three offspring each year (see examples in Kunz and Pierson, 1994). Whether mono-oestrus or polyoestrus, within most bat species a population's reproduction events are typically synchronized and defined by local seasons (Racey, 1982; Racey and Entwistle, 2000). Although there are solitary species of bats, in which a mother raises her young on her own, it is more typical for females to aggregate during the maternity season to give birth and raise their young.

Unlike other small mammals, bats tend to have slow foetal growth, low reproductive

FIGURE 2.4
A colony of up to 1 million little red flying foxes (*Pteropus scapulatus*) roosting in Melaleuca vegetation along a creek in southeast Queensland

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rates and long life spans (Racey, 1982; Kunz and Pierson, 1994; Racey and Entwistle, 2000). Bat pregnancies may last three to six months and are often variable (both among and within species) in response to environmental conditions (Racey, 1982; Racey and Entwistle, 2000). Bats typically have only one young per year, although some species (typically vespertilionids) may have twins (Racey and Entwistle, 2000). Female bats suckle their young longer relative to other mammalian species, waiting until the young are nearly as large as mature adults before weaning (Kunz and Stern, 1995). Life spans vary by species and the environment in which they live, but 15 years is not uncommon (Kunz and Pierson, 1994).

Feeding habits

The majority of bat species are insectivorous. These species represent every family in the Microchiroptera and are found throughout the global distribution of bats. Insectivores' diets range from general to highly specialized, preying on a diverse array of insects and arthropods, including moths (favoured by many species), spiders and small crustaceans (Kunz and Pierson, 1994 and citations therein).

About a quarter of all bats are frugivorous and/or nectivorous, meaning that they specialize on fruits and/or nectar and pollen from flowers. These bat species are found only in the subtropics and tropics and include all the Old World megachiropteran species and some subfamilies of the New World *Phyllostomidae*. Old World bats in this group may be

both frugivorous and nectivorous, but are usually identified as either fruit bats or blossom bats, depending on their feeding preference (Gould, 1977). Unlike most fruit bat species in the Old World, many of the frugivorous and nectivorous bat species in the New World have broad diets, which may include insects (von Helversen, 1993).

The small number of bat species that remain are either carnivorous, specializing on small vertebrates (e.g., birds, lizards, rodents, frogs), or sanguivorous, eating primarily blood from mammals and birds (examples in Kunz and Pierson, 1994).

Activity patterns

Bats are predominantly nocturnal, resting during the day and feeding at night, although some bat species are partially or completely diurnal (e.g., those on some islands; Kunz and Pierson, 1994). Most species of Microchiroptera and Megachiroptera depart from roost sites at early dusk to forage, and return to their day roosts by dawn (Kunz, 1982). The distance that bats travel during their foraging activities varies by species, habitat type, location, season, colony size and food availability. Microchiroptera have been tracked travelling 10 to 15 km from their day roost during foraging activities and may venture as far as 80 km (examples in Kunz and Pierson, 1994). Megachiropteran species have been known to travel as far as 87.5 km from their day roost for foraging (Epstein *et al.,* 2009). Female bats are likely to travel shorter foraging distances during lactation periods, as they are limited by the increased weight of carrying their young (especially some species of Megachiroptera) and/or the need to return to the roost to nurse young left behind.

In addition to their daily movements, some bat species are also known for long-term, long-distance migration (Bisson, Safi and Holland, 2009). In northern temperate regions, Microchiroptera forced to deal with cold seasons/winter, when food supplies are lacking, migrate south to less extreme winter climates (Strelkov, 1969). Nectivorous and frugivorous species in both the New and Old Worlds also move over very long distances to follow flowering and fruiting seasons, travelling as far as 2 000 km (Richter and Cumming, 2008). For example, seven radio-collared *Pteropus vampyrus* individuals in Southeast Asia were tracked moving hundreds of kilometres to roost sites in several different countries in the course of a year (Epstein *et al.,* 2009).

ECOSYSTEM ROLES

As the primary predators of nocturnal insects, insectivorous bats play a key role in regulating prey populations. Because these bats are highly mobile, they are also very effective in supporting their vegetative habitats, scattering nutrients across the landscape as they fly (Rainey *et al.,* 1992).

Fruit bats in the Old and New Worlds are ecologically important as seed dispersers and pollinators (see section on Positive roles of bats in human society). As they travel long distances during foraging, they distribute seeds and pollen across large areas, which is especially crucial to the regeneration of cleared areas (Fleming, 1988). In cases such as islands with few wildlife species, fruit bats are thought to play a "keystone" role in forest maintenance and community structure as the sole pollinators and seed dispersers of local plants (Rainey *et al.,* 1995).

SOCIO-ECONOMIC ROLES

Bats and humans have a long and complicated relationship. Many of the negative perceptions people associate with bats are based on myths, fears and misinformation. There are some valid cases where bats pose problems to humans, but humans also value bats in a number of ways. People benefit from bats both directly, for food and for support of their agricultural crops, and indirectly, for the ecological roles bats play in maintaining ecosystems and thus securing the beneficial services rendered by those ecosystems.

Negative roles of bats in human society

Fear and lack of knowledge about bats

Bats are disliked and feared in many regions of the world (e.g., North America) (Kellert, 1980), probably owing to a history of negative mythology and lack of understanding about bats. Some cultures maintain positive feelings about bats, such as in China, where they are symbols of happiness and longevity, and Poland, where they are believed to bring good luck. Elsewhere, bats are associated with death and darkness, and traditional stories have evolved in which bat-like characters play sinister roles causing harm to people. In Malaysia, for example, bats are considered dirty and are associated with evil spirits and vampires. Of the Malaysians interviewed, most (76 percent) had negative feelings towards bats, and half (49 percent) did not like bats at all. This matches the general lack of knowledge about basic bat natural history. Fewer than 5 percent of Malaysians interviewed knew that bats pollinated flowers, dispersed seeds, ate insect pests or produced guano that could be used as fertilizer (Kingston *et al.,* 2006).

Bats as orchard pests, intruders and disease vectors

Aside from folklore-inspired fears and misunderstanding due to lack of education, some concerns about bats are tied to reality. The most notable examples are among fruit farmers in the Old World, who continually face problems with fruit bats feeding on their fruit crops and aggregating in human-built structures, and the even more widespread fear of bats as disease vectors (see Chapter 5 for more information). Although these concerns are based in truth, fear and lack of education often lead to an exaggerated public response. In addition, people often fail to recognize the role that they themselves play in inviting conflict with bats. For example, in the Philippines, some fruit bat species forage on agricultural crops, but this may be because agricultural development has displaced their natural habitat. Studies have shown that bats prefer to forage in undisturbed natural forest, even when agricultural areas are available nearby (Mildenstein *et al.,* 2005). With an ever-increasing human population, people have fractured natural landscapes and moved into and around bat habitats, creating more opportunities for negative interactions. It follows that human-bat conflicts have become far more pronounced in recent times (Daszak, Cunningham and Hyatt, 2000).

Positive roles of bats in human society

Bats as food

An obvious direct benefit of bats to humans is as a food source. Although bat hunting is illegal throughout most of the Old World, people hunt bats for meat in much of

Asia, on islands in the Pacific and Indian Oceans, and in some parts of West Africa (Mickleburgh, Waylen and Racey, 2009; Figure 2.5). The hunted species are predominantly megachiropterans, but a few species of insectivorous bats are also hunted in Asia and Africa (Mickleburgh, Waylen and Racey, 2009). For some cultural groups, the hunting and eating of bats is linked to traditional customs and beliefs, such as indigenous tribes in the Philippines that believe bat meat has special medicinal properties (Mildenstein, 2002); other examples are in Swensson (2005) and Jenkins and Racey (2008). To other people, such as the Chamorros and Carolinians in the Mariana and Carolina Islands of The Federated States of Micronesia, fruit bats are a highly valued delicacy, traditionally eaten at celebrations. In many parts of Asia, however, bats are not a special food, and it is common for them to be hunted opportunistically as a novel supplemental food source (e.g., many bat hunters in the Philippines eat bats as a "finger food") (Mildenstein, 2002). Given the known levels of population decline in the Old World, fruit bat harvest is generally assumed to be unsustainable and a threat to the long-term persistence of Old World fruit bats (Mickleburgh, Waylen and Racey, 2009; IUCN, 2010).

Although Old World fruit bats were added to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendices in 1989 to stop international bat trade, a substantial amount of hunting still occurs for local sale. The cost of a bat seems to vary with the amount of effective law enforcement. A single large fruit bat can be sold for as little as USD 0.60 in the Philippines, where there is minimal implementation of bat

FIGURE 2.5
Flying foxes for sale in a community market in Manado, Indonesia

HUME FIELD; © THE STATE OF QUEENSLAND (DEEDI)

hunting regulation, or as much as USD 100 in the Mariana Islands, where poaching is punished far more strictly.

Guano

The faecal matter of insect-eating bats, called guano, is well-known around the world as a fertilizer for agricultural crops. Guano is regularly harvested on a small scale by local farmers for personal use. However, because guano is such an effective and valued resource, it is often harvested commercially at larger caves for international trade. A 2 lb (0.9 kg) bag of bat guano may sell for as much as USD 35 (prices vary according to the chemical content of the guano, and where and how it was harvested). As a large cave-dwelling bat population may deposit 85 to 100 tonnes of guano per year (Beck, 2010), the value of the guano added each year to the cave would as much as USD 3.5 million. It should be noted that while guano is a highly valued by-product of bat populations, guano mining is a major threat to bat populations around the world. Bat populations are very sensitive to disturbance, and cave ecosystems depend on guano as a source of nutrients.

Pest control

Unlike the previous two examples, some of the benefits that humans receive from bats do not affect the bats themselves. One of these is the control of insects. Insectivorous bats consume large volumes of insects (often as much as their own body weight each night), which translates into sizeable economic benefits, as many of the insects eaten are agricultural pests (Whitaker, 1993). The results of research in south-central Texas, United

FIGURE 2.6
Bags of bat guano harvested from a cave in the Philippines

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States of America concluded that the nearby population of Mexican free-tailed bats is so effective in insect control that it saves local farmers nearly USD 1.7 million a year in pesticide costs, which represents a quarter of the annual value of their crops (Cleveland *et al.,* 2006).

Pollinators/seed dispersers for economically important species

Old and New World fruit bats are important as pollinators and seed dispersers for many economically valuable plants, including those used as dyes, fibres, food, medicine, ornament and construction material (Fujita and Tuttle, 1991). A review in the Old World alone concluded that 289 species of plants rely to some degree on bats for pollination and/or seed dispersal, resulting in 448 economically valuable products (Fujita and Tuttle, 1991). Even more significant, some economically important plants depend almost exclusively on bats, including baobab in Africa and durian and petai in Southeast Asia (Kunz and Pierson, 1994). To give an idea of the value of bats' contribution to the production of these crops,

FIGURE 2.7
A good illustration of the importance of flying foxes as pollinators

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the international durian market was valued at USD 1.5 billion in 1998 (Lim, 1998). This is an even more significant sum in the less developed countries where durian is typically grown.

Ecosystem services

Humans rely on natural ecosystems for the services they provide (e.g., clean air and clean water), and bats play key roles in securing these important services by supporting the ecosystems that produce them. Bats maintain their habitats by regulating insect populations and cycling nutrients (insectivorous bats) and by pollinating flowers and dispersing seeds (nectivorous and frugivorous bats). Because bats are highly mobile, they perform these ecological roles across wide landscapes, supporting forest regeneration and maintenance at large scales. Subic Bay Forest Watershed Reserve in the Philippines is a good example of a bat-maintained forest that also provides ecosystem services for local industry. Subic Bay Freeport is the fastest growing industrial port in the Philippines, supporting USD 0.98 billion in international exports a year (Salonga, 2009). All the freshwater required by the freeport's businesses comes from the adjacent forest watershed reserve, which is host to one of the country's largest roosts of fruit bats. The bats in this roost forage throughout the watershed reserve but show a preference for the riparian corridors, which are the water storehouses of the forest (Mildenstein *et al.,* 2005).

Ecotourism

A final example of a socio-economic benefit of bats is the ecotourism industry. Worldwide, bat roosts are becoming an increasingly popular attraction for ecotourists (e.g., in Costa Rica, Lao People's Democratic Republic, Madagascar, the Philippines, and North America; examples in Pennisi, Holland and Stein, 2004). There are many success stories of communities overcoming their fears of local bat roosts and developing the roosts into tourist destinations. Perhaps the best known example is Congress Avenue Bridge in Austin, Texas, United States of America (Bat Conservation International, 2010). Under a bridge in the middle of this metropolis is a roost of 1.5 million Mexican free-tailed bats. The large roosting colony was originally slated for removal, but an education campaign led to its protection and popularization. Now, more than 140 000 visitors come to Austin each year to witness the evening emergence of this massive bat colony, bringing in USD 3.2 million directly and as much as USD 8 million indirectly in tourist revenue (Ryser and Popovici, 1999). The city also benefits from the estimated 15 tonnes of insects that the bats eat each night. As illustrated by Austin's Congress Avenue Bridge bat roost, ecotourism holds much promise for the future of bat conservation. The development of bat roosts for ecotourism encourages roost site protection and education and awareness about bats, while establishing a local economy tied directly to protecting the bats.

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Chapter 3

Bat population abundance assessment and monitoring

Tammy Mildenstein [a]

Surveys and monitoring are staple wildlife management tools commonly included in wildlife research and conservation programmes. Surveys and monitoring provide managers with important baseline information, methods for tracking wildlife characteristics over time, and feedback in an adaptive management framework. The terms "survey" and "monitoring" overlap in colloquial meaning and use. This chapter defines a "survey" as an assessment of a wildlife trait at a single point in time and "monitoring" as a series of surveys in which the trait is assessed repeatedly to track changes over time (following Morrison *et al.,* 2001).

In wildlife research, the number of parameters that can be surveyed and monitored is as large as the number of potential wildlife research questions. Common parameters include genetics, movement patterns, diet, habitat use, reproductive measures and survival. The trait most commonly monitored by wildlife managers is likely population abundance, both for practical reasons and to comply with goals set by management offices and conservation organizations. Population-level responses integrate meaningful impacts on individuals (e.g., behavioural, physiological and fitness impacts) into practical metrics (e.g., population size and trend) that can be used to track changes over time. As a result, natural resource management tends to focus on the population level (e.g., United States National Park Service; Wright, Dixon and Thompson, 1932; NPS, 2006), and standardized criteria for assessing conservation status are based on population metrics (e.g., Red List threat status assessment criteria; IUCN, 2010).

This manual includes a chapter on bat population survey and monitoring for several reasons. Understanding disease ecology depends on understanding the ecology of disease hosts, and veterinarians and biologists sampling bats in the wild have a unique opportunity to learn more about these potential host populations during their fieldwork. Using the simple population abundance estimation tools described in this chapter, researchers can gain reliable information on the size of the populations they have sampled. These numbers can then be used to gauge the sampling effort required and to determine the scope of inference and power of the disease study. Population abundance data that can be gathered during studies of disease prevalence may also play an important role in supporting bat conservation. Although bats are often conservation priorities (Mickleburgh, Hutson and Racey, 1992), many bat populations remain virtually unknown. Managers can use population size and distribution information to fill gaps in baseline knowledge, identify research priorities, and channel their limited resources to obtain the greatest conservation effect.

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This chapter provides an overview of population abundance assessment and is meant as an introduction to bat surveying and monitoring, rather than a comprehensive review. It outlines population census, sampling and indexing techniques, and describes how the resulting population size estimates could be incorporated into monitoring programmes to enhance the study of bats as hosts to infectious disease. The chapter discusses fundamental design considerations for monitoring programmes and provides references for more in-depth study and examples. It concludes with a description of how research teams can use the measurement of bat population abundance to refine disease sampling protocols, interpret the scope inference of a study, and evaluate the power of research results.

POPULATION ABUNDANCE ASSESSMENT

One of the most basic pieces of descriptive information about a wildlife population is its abundance, or size. Knowing a population's abundance gives managers a baseline for comparisons with other populations and with the same population over time, to evaluate the population's responses to environmental change, human disturbance and management activity. It also helps researchers to design effective sampling protocols and interpret study results.

A population's abundance can be counted directly in a census or estimated from partial counts and/or indices that are closely correlated with the population size. Selection of the most appropriate and effective method depends on the species and population of interest, where it is located, and survey constraints such as the resources and time available to the assessors. All population abundance assessment methods use a count divided by the fraction of the population that has been counted, or the detection rate. In a census, all individuals are counted, so the detection rate is 100 percent. When the population size is sampled or indexed, the extra step of dividing the count by the sampled fraction is needed to convert the measured population count or index into an estimate of abundance for the entire population.

Censusing bat colonies

A census, or complete count, is when all the individuals in a population are counted in a particular area at a particular time. By definition, a census therefore assumes that the detection rate is 100 percent and that no animals move into or out of the population during the counting (i.e., the population is closed) (Jarman, Smith and Southwell, 1996). Strictly speaking, these assumptions mean that it is often impossible to conduct a true census of a bat population. However, counting bats directly may still provide the best assessment of population abundance, especially when roosting colonies can be observed clearly and attention is given to making the detection rate as close to 100 percent as possible.

Counting at the roost site takes advantage of bats' natural aggregating behaviour as an opportunity to assess the population size when it is in one place. Census methods are considered most reliable in small colonies ($N < 1\ 000$) (Kunz *et al.,* 2009), but they have also been shown to be effective in larger colonies when suitable observation stations, trained observers and observation equipment are available (Mildenstein and Boland, 2010; Mildenstein, Stier and Cariño, 2002). Censusing is not feasible for solitary species, for species that roost in small groups in cavities or foliage, or for very large colonies that cannot be viewed in their entirety at the roost or during exit flights (Kunz, 2003).

Biologists have typically censused bat populations using three different methods at or around the roost site: direct roost counts, departure counts, and disturbance counts and disturbance counts (introduced in Racey, 1979). Direct roost counts are recommended when there are suitable vantage points from which the entire roosting population can be seen clearly. Departure counts offer a suitable alternative when a roost site cannot be reached for observation and are the most effective census technique for bats that depart from cavities (Kunz *et al.,* 2009). Disturbance counts are not usually recommended because of the unreliable data they produce and the risk of injury to the bats (Garnett, Whybird and Spencer, 1999). This section describes direct roost counts and departure counts, discusses the advantages and disadvantages, and provides recommendations for conducting each censusing technique.

Direct roost counts

If a bat colony can be observed at its roosting site, it is possible to assess abundance directly by counting all the individuals at the roost. This method can yield reliable abundance assessments for both microbats (Microchiroptera) (Tuttle, 2003; Hoying and Kunz, 1998; Kunz, 2003; Kunz and Reynolds, 2003) and megabats (Megachiroptera) (Eby *et al.,* 1999; Garnett, Whybird and Spencer, 1999; Utzurrum *et al.,* 2003) and is the preferred abundance assessment method when the population size, roost location and observation stations are optimal for visual counting.

The major drawback to this method is that it depends heavily on having suitable observation stations from which to count the roosting individuals. Observation locations are often limited, and sometimes the best available locations do not provide views of the entire roost, because of local topography and/or vegetation. Observation locations that are too close to roosting site may lead to disturbance and eventual flushing of the bats (Tuttle, 2003), and locations that are too far from the roost result in lower detection rates (Mildenstein and Boland, 2010). Even with optimal observation locations, detection rates can be reduced by poor lighting or weather conditions at the time of counting (Mildenstein and Boland, 2010). In addition, bats in dense aggregations may be missed when they are obscured by other bats, and/or be double-counted when they move around the roost during counting.

Because direct roost counts are sensitive to observation locations, ample time and resources should be allocated to reconnaissance prior to counting, to identify and develop observation locations that offer good views of the entire roost. The stations should be close enough for counting individual bats but far enough away and/or protected so that observers do not disturb the roosting bats. Distant observation locations are often preferable when they provide wider view sheds and more protection from sensitive colonies, and researchers have successfully used binoculars and spotting scopes to enhance their capability of seeing and counting bats (Figures 3.1 and 3.2). High-resolution photography has also proved useful, allowing researchers to zoom in on images of distant and/or densely clustered bat populations and use computer software tools to "mark" bats as they count them (Boland, 2009).

Departure counts

When it is not possible or not advisable to count bats at their roost site, the population may be counted as it leaves the roost, especially in the evening when the bats depart in search of food. Abundance assessments using this technique are also called "exit counts", "evening

FIGURE 3.1
A bat colony that is not sensitive to human presence allows counters to use close observation stations and count bats without the need for visual aids

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FIGURE 3.2
Population abundance assessment of a sensitive bat colony may require very distant observation stations and spotting scopes for censusing

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emergence counts" when used for cavity-dwelling bats, and "evening dispersal counts" or "fly-out counts" when used for tree roosting bats (especially Megachiroptera) (Kunz *et al.,* 2009). Whatever the name, the concept is the same, to count bats as they depart from their diurnal roost site to begin their evening foraging activities. Departure counts are censuses because bat populations aggregate in roosting colonies and depart as a group from the roost each evening to forage (Figure 3.3).

Counting bats as they depart from their roost is a suitable alternative to censusing colonies with inaccessible roost sites, and often provides the only effective population assessment method for bats that roost in cavities, such as mines, buildings, caves, rock crevices and tree cavities (Kunz *et al.,* 2009). For populations that can be counted at the roost, departure counts can be used in a double sampling framework to be compared with roost counts to estimate detection rates (Williams, Nichols and Conroy, 2002).

FIGURE 3.3
Mexican free-tailed bats (*Tadarida brasiliensis*) emerge from their cave roost in Texas. Observers counting this colony would likely count by units of 50 or more as the bats pass a landmark such as the tree in the foreground. Sensitive video cameras could also be used to record/count the stream of bats

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The major drawback of censusing using departure counts stems from the assumption of a 100 percent detection rate, which is often impossible for several reasons. Suitable observation stations that provide visual access to all departure flight paths are not always available, and the flight paths that the bats use vary, often depending on the seasonal availability of food and/or the current weather conditions (especially wind). Vegetation and topography along the flight paths may obscure visibility, and the diminishing light reduces detectability as the evening progresses. It is also common for many individuals to depart from the roost when it is too dark to see them, and some do not depart from the roost at all. Very large colonies may be difficult to count accurately if large numbers of departing bats are dispersed over a wide area or, conversely, if they are in densely populated flight streams. The reliability of count data is also very dependent on observers' training and experience (Mildenstein, in preparation; Mildenstein and Boland, 2010).

Departure counts can yield the most reliable data when conducted on small to medium-sized populations that depart in a concentrated group and are easily observed (Kunz, 2003; McCracken, 2003). Because departure counts depend on good observation stations, reconnaissance prior to counting is highly recommended for identifying the bats' departure routes and the best possible counting locations for observers. An optimal counting location is at some distance from the roost along a departure flight path, from which a distinct group or stream of departing individuals can be counted as they fly against the night sky (Figure 3.3). The number of observation locations needed depends on the particular colony and the departure characteristics of the bats. Counting teams typically develop a station for each of the flight paths used by bats leaving the roost. Wide flight paths are often split among several observers using topographic or vegetative landmarks to divide the sky. Observation against the night sky provides the greatest contrast at low light levels and increases the length of time that bats can be counted. When resources are available, count reliability can be improved with the use of light-gathering binoculars, night-vision goggles, thermal infrared imaging and/or video recorders with a night-vision or low light feature. (These technologies fall outside the scope of this overview, but see reviews and examples in Kunz *et al.,* 2009; Wescott and McKeown, 2004; Elliott *et al.,* 2006).

Estimating bat population abundance

The abundance of a bat population can also be estimated from any of several count statistics. In general, the estimation process involves counting a sampled portion of the population and dividing the count by the sampling fraction to extrapolate to the total population size ($\hat{N}$). This means dividing the count (C) by the portion of the total that was counted (ß), so that $\hat{N}$ = (number counted)/(fraction counted) = C/ß. The fraction counted can be defined by any sampling process, such as probability of observation (or detection rate), spatial sampling and capture rate (examples in Kunz *et al.,* 2009; Williams, Nichols and Conroy, 2002).

Estimating a population's abundance offers some noteworthy advantages over censusing. Unlike censusing, population estimation techniques do not depend on seeing all of the individuals of a population, so usually require less time and effort. In addition, count statistics can often be derived from sampling efforts that are already taking place (e.g., capturing bats for disease sampling). This section describes how to estimate population abundance using partial roost counts, capture/recapture data, and direct and indirect

indices of abundance, all of which complement the disease sampling protocols outlined in this manual.

An obvious disadvantage of population estimation is that the reliability of the population size estimate is only as good as the estimator. It is therefore very important to take time to measure the portion of the population that is being counted/sampled and to understand how that fraction relates to the rest of the population.

Partial counts of colonies

Researchers often know where a bat colony can be seen, either at the roost or when departing from a roost site, but they do not have the time or resources to develop optimal observation stations and to census the colony. Partial counts of the roosting colony can then be used to estimate total population size. As with censusing, partial counts assume that the population is closed during counting and that observers see and record all the individuals in the fraction of the population they intend to count. It is also assumed that the population is uniformly distributed across the sample area, so the size of the sampled fraction of the population can be extrapolated to represent the entire population.

Partial or incomplete counts can result from interference with the observation of an entire bat population, or from capture or observation techniques that sample only part of a bat population. As a simplified example, assume that 50 bats were counted at a roost site. Because of very tight clustering at the roost, it is estimated that only one fourth of the bats could be seen and counted. Using the count (C = 50) and the sampling fraction (ß = 0.25), the estimated total population size of bats at the roost would be $\hat{N} = C/ß = 50/0.25 =$ 200 bats. Similarly, if 30 bats were captured at a cave entrance and it is assumed that they represent only one tenth of the total number in the cave, the estimated total population size would be $\hat{N} = C/ß = 30/0.1 = 300$ bats.

The same concept can be applied to spatial sampling, where the number of individuals counted in a fraction of the total area is used to estimate the total population size. For example, assume that only a fraction (α) of a roosting colony can be counted, because the only available observation station provides a limited view of the colony. If 100 bats are counted from the observation station, and the station's view shed covers one fifth of a colony's total roosting area ($\alpha = 0.2$), the total population of the colony can be estimated by dividing the count at the observation station by the fraction of the total area that was counted ($\hat{N} = C/\alpha = 100/0.2 = 500$ bats).

For more complex population sampling, multiple estimators can be combined to estimate total population size from count data. Building on the two examples in the previous paragraphs, assume that at the observation station in the second example (the station from which one fifth of the roost site could be seen) the bats were also tightly clumped, as in the first example, such that only one fourth of the bats in view could be distinguished and counted. The estimation of the total population size at such a roost would have to incorporate both spatial sampling (from the observation station's partial view of the roost) and partial observability (from the dense clustering) to determine the fraction of the population that was counted. In this example, if 100 individuals were counted from the station, the estimated total population size would be $\hat{N} = C/ß\alpha = 100/(0.25)(0.2) = 2\ 000$ bats.

Using capture/recapture data to estimate population size

Population abundance can be estimated from wildlife capture data when recaptured individuals can be identified in subsequent capture sessions (described in Mills, 2007). This method uses the known number of individuals originally captured (or marked) and assumes that the proportion of recaptured individuals in a subsequent sample is the same as the proportion of the total population captured in the original sample.

Although abundance estimation using mark-recapture data can be complicated, the basic idea is similar to the partial count estimation method: the total population size is estimated by using a count divided by the fraction of the population that was sampled. In this case, the count is the total number of animals captured in the first capture session (M), and the sample fraction is the proportion of individuals captured in the second session that were recaptures. So, the estimated size of the population ($\hat{N}$) equals the number of individuals captured in the first session (M), divided by the fraction of the second set of captured individuals that was marked (m/n), or ($\hat{N}$) = M/(m/n) = M(n/m).

For example, assume that 40 bats were captured and marked in the first capture session (M = 40). In a second capture session, 50 bats were captured (n = 50), of which five were marked (m = 5). The estimated total abundance of the population being sampled would be $\hat{N}$ = M(n/m) = (40)(50/5) = 400 bats. This is just a simplified example. Abundance estimation equations usually include a correction factor and can be complicated by multiple capture sessions, and/or when captured individuals are tracked over time. See Mills (2007) and Williams, Nichol and Conroy (2002) for more details on calculating population estimates from mark-recapture data.

Mark-recapture methods are useful for estimating the population sizes of bats that roost alone or in small groups and of colonizing bats in inaccessible roosting locations. When bat capturing is already planned (e.g., to sample bat populations for disease), this population abundance estimation technique can be incorporated with very little effort. Reliable estimates using mark-recapture techniques are based on several assumptions. As in the other population estimation methods, it is assumed that the population is closed across capture sessions, although some estimation techniques are robust to open populations (Pollock, 1982). It is also assumed that all individuals have the same probability of being captured and the same survival rates and that detection rates are 100 percent (i.e., marks will not be lost or overlooked) (Kunz *et al.,* 2009). However, software programs have been developed that can tolerate violations to many of these assumptions (e.g., Program Mark).[1]

Indices of bat population abundance

An index is a measurable parameter that varies in proportion to the parameter of interest (Thompson, White and Gowan, 1998). Researchers may choose to measure an index rather than the population abundance, especially if it is more efficient or cost-effective to do so (Conroy, 1996). The reliability and usefulness of a population abundance index are based on how closely the index and the true abundance are correlated, which is often difficult to measure. In many cases, true population abundance cannot be measured to quantify its relationship with an index (often, this is why the index is being used in the first place). Even

[1] www.phidot.org/software/mark/background/

when the relationship has been established for a specific use, it is often not known how the index varies with species, population, gender, reproductive status, capture method, habitat type, season, weather conditions, etc. (Kunz *et al.,* 2009). In these cases, abundance indices can be used as a measure of "relative abundance" for comparison among populations and for tracking population changes over time, as long as special attention is given to standardizing the measurements taken.

Indices commonly used to assess bat population abundance include capture rates, direct observations (e.g., partial counts, bat activity) and indirect signs of bats (Kunz *et al.,* 2009). This section describes three population abundance indices that show the most promise for use during disease sampling fieldwork: capture rates, density measurements, and guano deposition. Although measuring the relationship between an index and the true population abundance is beyond the scope of disease studies, these indices may serve as measures of relative abundance that can be used for comparison over a long-term disease sampling programme.

Capture rate

In a study that involves capturing bats, the number of bats captured per unit of effort/time can index the abundance of the population being sampled (for a more detailed description see Kunz *et al.,* 2009). This capture index assumes that changes in capture rates reflect proportional changes in the abundance of the population being sampled (Conroy, 1996), and is usually expressed as the number of bats caught per net-hour, per night, per mist net night, or per net metre per night. The reliability of a capture index depends on standardized capture methods appropriate to the species being captured (Hodgkison *et al.,* 2004) and an understanding of capture variability among individuals within the target population.

Bat density

Bat density (in roosts or during foraging activity) can be measured through visual or acoustic observation, providing an index to abundance. Assuming uniform density, measurements of bat density in a known area can be used to estimate population abundance over a larger area (Conroy, 1996). Density measures can be made of bats at their roost (Figure 3.4) and typical colony density values have been established for a few species (e.g., 2000 bats/m^2 for *Miniopterus schreibersii*; Rodrigues and Palmeirim, 2008). This technique can also be especially helpful in assessing population abundance for species that roost solitarily or in small groups, and it may be incorporated into other ongoing fieldwork (Kunz *et al.,* 2009). Away from the roost, if researchers have the time to count the bats detected within a known area, the density of bat activity can also be a useful index to relative abundance (Hayes, 1999; Hayes, Ober and Sherwin, 2009), offering a point of comparison for subsequent sampling sessions. This technique is improved with bat species identification training and access to night-vision equipment and/or ultrasonic detectors (Parsons and Jones, 2000; Walsh and Catto, 2004).

Guano deposition

Estimates of bat faecal deposition (e.g., guano) have been used as an index to bat population size (Tuttle, 1979; 2003). This indirect index to population abundance assumes a consistent

FIGURE 3.4
The population abundance of the gray bat (*Myotis grisescens*) in a cave could be estimated by carefully counting the bats in a small area and multiplying the resulting bat density by the total bat-occupied area of the cave

relationship between the guano accumulation under a roosting colony and the number of bats present. Although regularly used in fieldwork, the effectiveness of guano deposition as an abundance index has not been thoroughly evaluated, because of the lack of data on seasonal changes in bats' foraging habits and roost use and lack of information on the biotic and abiotic factors that influence faecal accumulation and deposition measurement (Kunz *et al.,* 2009).

For frugivorous bats, the use of faeces as an index to population abundance remains unexplored. Faecal deposition rates of Old World fruit bats have not been studied, but fruit bat droppings can be collected under a roost and enumerated (Stier and Mildenstein, 2005), suggesting that this may provide a useful surrogate to the direct assessment of population abundance (Figure 3.5).

SURVEY AND MONITORING DESIGN CONSIDERATIONS

Bat population surveys and monitoring can provide important data to support bat research and conservation management. Monitoring programmes can use population abundance data to track changes over time in the size, roost site occupation, seasonal movement patterns, distribution and connectivity of bat populations. However, the use and application of population abundance assessments depend entirely on the quality of the data and how they were acquired. To detect changes in a population trait over time, measurements should reflect changes in the trait itself, rather than changes in the ways in which data

FIGURE 3.5

Faecal samples from large fruit bats (*Pteropus mariannus'* droppings shown here) can be collected opportunistically and non-invasively. Fruit bat biology suggests that each faecal sample collected under the roost in the morning represents one bat individual

Source: Stier and Mildenstein, 2005

were collected. It is therefore important that the survey design reflects the study goals, that the methodology is standardized and repeatable, and that the sources of variability in the data are well understood. The following should be considered when designing and implementing bat population assessments.

Define the research goals

Population abundance assessment is usually conducted for a specific reason. The first step of a population survey is establishing research and management goals and identifying the role that a population abundance estimate will have in reaching those goals (Hayes, Ober and Sherwin, 2009). Having a clear understanding of the reason for the population survey will guide assessors in choosing the appropriate assessment method, planning the assessment and evaluating the results.

Be prepared

Whether the goal is a one-time measure of population size to determine the sampling effort required or the establishment of a population abundance baseline for comparison with future assessments, it is critical that surveyors are well prepared for abundance estimation. Background literature should be gathered on the target species and population of interest,

and reconnaissance should be conducted at the study site to identify the location of the roost, suitable observation stations, and other important details about the terrain where the assessment will take place. Surveyors should be trained in population abundance assessment techniques and know the assumptions implicit to the methods they are using. Potential sampling errors can often be mitigated if the sources of error are identified at the fieldwork planning stage.

Standardize methods

For any survey technique, it is critical that assessors describe their methods clearly and standardize them across assessments for comparison among replicates and/or with future assessments. For example, many bat species exhibit synchronized seasonal birth pulses (Kunz and Pierson, 1994). Repeated counts of the same population must be made at the same time of year if population size comparisons are to be made across time. Observer training and the use of specified equipment and observation stations are also important in standardizing population assessment protocols.

Understand sources of variance

There are numerous sources of variance in population abundance data. Population assessment methods have inherent sources of error (e.g., due to observer differences, and the accessibility of observation locations), and the bat populations themselves are not static, exhibiting fluctuations in size (e.g., synchronized birth pulses) and temporal and spatial variability in foraging activity (Gannon, Sherwin and Haymond, 2003) and roosting (Sherwin, Gannon and Altenbach, 2003). It is important for the surveyors to understand their methodology and how to minimize variance in their data resulting from factors other than changes in the trait of concern. Multiple assessments should be made to reduce the effects of natural variance (Mills, 2007; Williams, Nichols and Conroy, 2002). Training improves observer quality (HaySmith *et al.,* 2009) and sampling variance decreases as the sampling fraction is increased: detectibility (ß) increases with increased effort, and spatial sampling fraction (α) is increased by increasing the amount of area that is counted (Williams, Nichols and Conroy, 2002).

Colony size does not necessarily equal population size

In addition to these general recommendations for conducting bat population assessments, readers should also be aware of two considerations specific to bat roost surveys. First, a bat colony is not always the same as a bat population, and it is important for researchers to understand the population structure of the bats they are studying. Although roosting colonies are often considered to be isolated populations, there are cases where individuals transfer regularly among colonies (Horn and Kunz, 2008). A census of the population must therefore include all interbreeding colonies, if it is to yield reliable population abundance data (Kunz *et al.,* 2009).

Similarly, many roosting colonies contain multiple species of bats, and the total colony size should not be assumed to be equally divided among co-roosting species (Figure 3.6). Species-specific population abundance assessment at the colony depends on how effectively observers can identify the co-roosting bat species. Although the total number of individuals

FIGURE 3.6
Example of a mixed-species roosting colony at Subic Bay, Philippines. Population abundance surveys coupled with species composition sampling revealed that *Pteropus vampyrus* individuals outnumbered *Acerodon jubatus* individuals in a ratio of 6:1

Source: Mildenstein, in preparation

can sometimes be counted directly at a roost site, it is unlikely that all individuals can be identified to species. The species composition at the roost site can be estimated by sampling areas within the roost and identifying the proportion of the sampled bats that belong to each species. Species-specific population abundance estimates can be made by multiplying the total colony count by the estimated species proportions.

Sensitivity of roost sites to disturbance

A second important survey consideration specific to bat population measurement is the long-term importance of roost sites to bats. Bat colonies can be extremely sensitive to disturbance at the roost, especially when hibernating (Tuttle, 1979; 2003) or when pups are present (Mann, Steidl and Dalton, 2002). Because many species show high fidelity to roost sites (Lewis, 1995), disturbance that leads to abandoning the roost can be devastating (Kunz *et al.,* 2009). Although wildlife research often aims to support conservation, some bat study activities have disturbed roosting colonies and resulted in population declines (examples in Kunz *et al.,* 2009). Therefore, veterinarians and biologists should plan their bat research activities with care to avoid disturbing the bats they hope to protect.

APPLICATION OF BAT POPULATION SURVEYS AND MONITORING TO THE STUDY OF BAT-BORNE DISEASES

Sampling design

Assessment of bat population abundance can enhance disease study in several ways. At the onset of a disease survey, knowing the size of the study population can help gauge the sampling effort required. Some sampling protocols specify a minimum portion of the target population that should be sampled. In these cases, researchers will need to know the total population size to determine how many bats they will need to capture and sample for the study. For example, a sampling protocol suggests that a minimum of 10 percent of the total bat population should be sampled; if researchers count 1 000 individuals at the roost, they will aim to capture 100 individuals (0.1 x 1 000) to follow the protocols for their study.

When the approximate rate of disease infection occurrence in a bat population is known, the size of the target population can be used to determine the sampling effort required for the study to be able to detect the disease. For example, if the rate of infection for a certain virus tends to be about 1 percent in bat populations, and the study population is assessed at 5 000 bats, only about 50 bats (0.01 x 5 000) in this population may test positive for infection. Assuming uniform capture probability, researchers would have to catch and test at least 100 bats to detect a single infected individual.

Inference and power of disease study results

Knowing the abundance of the study population can also help to justify the scope of inference and evaluate the power of the study's results. The scope of inference of a study is the population to which the research results from the sampled population can be extended. As a simplistic example, if bats were randomly captured and sampled for disease at the mouth of a cave, the results of the research could be inferred to apply to the entire population within the cave. However, if only a certain segment of the bat population emerged from that particular cave entrance (e.g., just young males) the results of the study could only be applied to that segment (young males) of the population. Thus, knowledge of the population guides disease research design and helps provide a context for the results of disease study.

A study's power is its ability to detect an effect (Williams, Nichols and Conroy, 2002). In other words, the power of a disease surveillance study is the likelihood that, by design, it will discover disease in a population if the disease is there. Intuitively, a study's power is related directly to both the effect size (e.g., the prevalence of disease in a population) and the intensity of sampling effort (e.g., the fraction of the total population being sampled). Because the sampling fraction depends on the total population abundance, knowing the size of the study population is crucial to assessing the study's power. Conversely, if a surveillance protocol specifies a goal of a certain power for the study, and the approximate effect size is also known, the required sample size can be calculated using the population abundance (Mills, 2007; Williams, Nichols and Conroy, 2002).

Tracking changes over time

Bat population size provides a context for the results of disease research on bats. Routine population assessments should be included in any long-term study of bats as hosts to

disease to inform researchers and managers about the population trajectory of the hosts. Changes in or movements of bat populations are likely to have an impact on the diseases the bats carry. It is prudent for disease studies to include efforts for tracking the populations that are hosts to diseases of concern.

CONCLUSION

The scientific rigour and application of studies of disease in wild populations of bats can be strengthened when the size of the study population is known. In designing the disease investigation protocol, the size of the study population can help determine the number of individuals that must be sampled for disease. After the data have been collected, the population size can also offer a point of comparison for evaluating the study's scope of inference and power of detection. In longer-term disease monitoring programmes, tracking changes in disease presence can be informed by tracking changes in the host population, including fluctuations in bat population size and distribution. The size of a bat population can be censused by counting individuals directly at the roost or as they leave the roost, or estimated using partial counts, capture-recapture data or indices of abundance. Each of the population abundance assessment methods described here has advantages and disadvantages (Table 3.1), and most are simple and can be efficiently incorporated in disease study protocols.

TABLE 3.1
Bat population abundance assessment techniques: when used, advantages and disadvantages

Method type	Sampling strategy	When used	Advantages	Disadvantages
Census	Direct roost count	Roost site can be seen	Yields a very good count and other important roost data	High effort, depends on having good observation station(s)
Census	Departure count	Departure routes are known and can be seen	Simple and quick to perform	Depends on having observation stations and trained observers
Estimate	Spatial sampling	Part of roost or foraging area can be seen very clearly	Fairly simple and quick to perform	Depends heavily on the accuracy of estimated detection probability
Estimate	Capture-recapture sampling	Bats are being captured and recaptures can be identified	Can use capture sessions that are already planned; yields a robust estimate	High effort; many assumptions
Index	Capture rates	Bats are being captured	Does not require extra effort/ in-depth analysis	Assumes equal capture probabilities; may yield only relative abundance data
Index	Density	Bats observed at roosting or foraging sites	Low effort when incorporated in other field methods	Assumes uniform density; may yield only relative abundance data
Index	Faecal deposition	Roosting sites (especially) and/or foraging sites are accessible	Simple, non-invasive, easy to add to other field efforts	Requires research of the relationship between faecal deposition rates and population size

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Chapter 4

Disease surveillance in free-ranging bat populations: challenges and logistical considerations

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INTRODUCTION

Infectious diseases of wildlife are an increasingly significant cause of emerging human infections, leading to greater recognition of the importance of understanding the ecology of wildlife microbial agents (Daszak, Cunningham and Hyatt, 2000; 2001; Binder *et al.,* 1999; Daszak *et al.,* 2004). Wildlife disease surveillance is critically important for epidemiological investigations of the human and animal diseases that may be linked to free-ranging animal reservoirs, such as West Nile virus encephalitis, avian influenza, Ebola, Nipah and Hendra viruses, and hantavirus (Field *et al.,* 2001; Halpin *et al.,* 2007; Leroy *et al.,* 2005; 2004; Li *et al.,* 2005; Webster *et al.,* 1992; Eidson *et al.,* 2001; Peters, Simpson and Levy, 1999); for understanding infectious disease in human populations that may have historical or evolutionary relationships with animal agents, such as HIV/AIDs and malaria (Hahn *et al.,* 2000; Rich *et al.,* 2009); and for understanding the spectrum of microbial agents that naturally circulate in wildlife species and that may emerge in human or other animal populations (Jones *et al.,* 2008; Epstein *et al.,* 2010a).

Because of the complexity of wildlife-human or wildlife-livestock-human disease transmission, a multi-disciplinary scientific approach to studying disease dynamics and emergence is often necessary (Daszak *et al.,* 2004). Principles of human and veterinary medicine, epidemiology, ecology, microbiology and molecular biology are all important for understanding the ecology and emergence of infectious agents from wild animal reservoirs. When an aetiological agent in a disease outbreak has been identified as originating in wildlife, studying the ecology of the host and any potential vectors, including their interactions with people and/or domestic animals, is critical to assessing the risk of repeated spill-over.

Studies of infectious agents in free-ranging wildlife are replete with challenges. They often focus on identifying the natural reservoir of a target microbe. 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

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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. If a potential reservoir is identified, 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 presence of neutralizing antibodies against the target pathogen in serum can be used as evidence of a population's past exposure to the pathogen. For pathogens that cause acute infection in an animal, direct detection may be very difficult, but serology can offer an effective means of screening a population for exposure, assuming that antibodies persist over time. Population distribution and abundance are also key elements for the study of pathogens in wildlife.

There are significant challenges to obtaining any or all of this information from free-ranging wildlife. Statistical analyses may indicate the need to sample a certain number of individual animals to achieve statistical significance. Animals often live in difficult environmental conditions (remote, inaccessible, extreme in climate), or they may be solitary or scarce, making it difficult to achieve large numbers of samples. Wildlife study needs technical skill in locating and safely capturing and handling the target species, which may require specialized equipment, including tranquillizers or other anaesthetic techniques. Sampling wild animals can present physical risks to the scientist, such as being bitten, scratched, kicked or crushed. There are also risks to the animal, and animal welfare must be considered when designing wildlife studies that include capture, restraint and the collection of biological samples. In conclusion, safe and effective collection, storage and transportation of biological samples from field sites to the laboratory are absolutely critical

FIGURE 4.1
Sampling a ghost bat at a disused gold mine in a remote part of Northern Territory, Australia

AMANDA MCLAUGHLIN; © THE STATE OF QUEENSLAND (DEEDI)

to the successful detection of pathogens. Maintenance of a cold chain is one of the most important considerations when working with wildlife in remote settings (Figure 4.1).

SAMPLING BIASES

Several designs may be used for wildlife disease studies, including cross-sectional, longitudinal and experimental studies. Longitudinal studies are particularly useful for understanding temporal or seasonal patterns of infection in animal populations. Wildlife capture is often opportunistic, and the animals captured rarely represent a truly random sample. Factors associated with trap placement, including position, height and even time of capture, may select for certain individuals or even certain species. For instance, canopy nets or harp traps are often used to capture insectivorous bats, and it has been shown that different species forage at different heights within a tropical forest (Hodgkison *et al.,* 2004). The height of a canopy net will therefore influence which species is captured. Nets may also inherently select for particular individuals, such as slower or weaker animals that are unable to manoeuvre away from a net, or careless individuals that are less wary of obstacles. Pteropid fruit bats roost in trees, in colonies of structured and segregated populations based on age, sex and social dominance. A mist net placed in a location that is convenient for the scientist may therefore result in a biased selection based on age or sex. Recapture bias may also occur if traps are used in the same location over a long trapping period. Another challenge inherent to wildlife disease studies arises when trying to capture rare, solitary or nomadic animals, which may yield low sample numbers. Results may lack statistical robustness and be difficult to interpret. Biases are often unavoidable and must simply be acknowledged as limitations of the study.

Sick versus healthy animals

When the research objectives include detecting a pathogen that may occur at low incidence, it is sometimes advantageous to bias the study deliberately, by sampling animals that are more likely to be infected, such as sick or injured individuals. This only works if the microbe of interest causes detectable disease in its host. Rabies and other lyssaviruses, including Australian bat lyssavirus, can be shed asymptomatically by bats, but also cause neurological disease in infected bats. Studies of Australian bat lyssavirus targeting sick and injured bats have achieved higher detection rates than those that sample healthy populations (McCall *et al.,* 2000). Many zoonotic viruses with bat reservoir hosts, such as Hendra, Nipah and Marburg viruses do not appear to cause any clinical disease in infected bats (Halpin *et al.,* 2007; Li *et al.,* 2005), so when surveying bats for these agents or for other human or livestock pathogens that may be carried by bats, it is important to include apparently healthy bats in the study.

CAPTURE TECHNIQUES: SAFETY AND EFFICACY

Depending on the species of bat being studied, commonly used capture techniques include mist nets and harp traps. Mist nets can be used to capture large or small bats in open spaces near roost or feeding sites. The nets are typically attached to two poles either in a static assembly, where the net remains in a fixed position, or on a rope and pulley system, where the net can be elevated to capture the bats, and lowered to extract them once

FIGURE 4.2
A mist net erected between two masts on a rope and pulley system. The net can be elevated and lowered to capture and extract bats

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FIGURE 4.3
A canopy net can be employed where bats are roosting, foraging and flying at heights greater than the height of a mast

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caught (Figure 4.2). Canopy nets can be hung from tree branches at various elevations, for sampling bat species that forage at specific heights (Hodgkison *et al.,* 2004; Figure 4.3). Harp traps employ a system of two staggered rows of parallel nylon filaments strung within

FIGURE 4.4
A harp trap is used to capture echo-locating microchiropterans

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a rectangular aluminium frame. They are designed to capture echo-locating bats that might otherwise evade mist nets (Figure 4.4). Harp traps are most effective when placed at the openings of caves or in flyways that are heavily travelled by bats. An advantage of harp traps is that bats flying into the strings slide down the trap into a collecting bag (Figure 4.5). There is none of the entanglement that occurs with mist nets, and harp traps can capture a large number of bats in a short period. Although giant harp traps have been designed for the capture of large Pteropodid bats, mist nets are far more practical and more widely used. Regardless of which technique is used, it is important that field personnel monitor the nets or traps regularly to prevent bat injury or death, which can occur as a result of extensive struggling, entanglement, predation or exposure to the elements. It is recommended that mist nets are actively monitored throughout the trapping session and that bats are extracted from the net as soon as possible after capture.

Scientists capturing bats for disease surveillance must consider the safety of both the field personnel and the bats being sampled. Depending on the pathogen under study and the questions being asked, effective surveillance can often be achieved through non-lethal means that use safe and effective capture and release methods. However, in some instances, destructive sampling is necessary, such as with newly discovered host-pathogen relationships when the pathogenesis of an agent is unknown, such as Marburg virus in bats (Towner *et al.,* 2009), or when the pathogenesis is well described and virus can most reliably be detected in specific tissues, such as with lyssaviruses that can only reliably be detected in brain tissue from an infected animal using a direct fluorescence antibody test (Bourhy *et al.,* 1989). The conservation status of the target bat species should be considered when designing surveillance

FIGURE 4.5
A strategically placed harp trap in a disused mine in northern Australia has captured numerous eastern horseshoe bats (*Rhinolophus megaphyllus*)

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strategies, to minimize the impact on the population. Whether destructive or non-destructive sampling techniques are used, animal welfare should always be carefully considered when designing capture and sampling methods. The approval of an institutional animal care and use committee (IACUC) is necessary before beginning surveillance activity. In emergency situations such as outbreak investigations that require rapid action, IACUC review may not be possible, but humane sampling techniques should still be employed. Bats can be injured while they are entangled in a net or being removed from the net by a scientist, or during biological sample collection. There is also the potential for injury and exposure to zoonotic agents for the person handling the bats. Personal protective equipment (PPE) and immunization against rabies virus are important occupational safety measures when working with bats.

In some cases, particularly with large Pteropodid bats (e.g., *Pteropus* and *Aceradon* spp.) chemical restraint is appropriate to minimize stress and the risk of injury to both the bat and the handler. Several anaesthetic protocols have been described, including both injectable and inhalant anaesthetic agents (Heard, Beale and Owens, 1996; Jonsson *et al.,* 2004; Sohayati *et al.,* 2008; Heard, 2003). Anaesthesia should be administered by trained personnel – if possible, under the supervision of a veterinarian (Figure 4.6). Smaller bats

FIGURE 4.6
A field anaesthetic machine and anaesthetised black flying fox (*Pteropus alecto*)

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can be manually restrained during sample collection, and should be carefully monitored for signs of distress, including excessive vocalization, gaping or strained breathing. In the event of distress, it is recommended that the bat be placed in a cotton bag or pillow case, or a cage, and be allowed to recover before the sampling continues.

SAMPLING PROTOCOLS

Depending on the target pathogen, there are various strategies for sampling bats. Under non-destructive (non-lethal) sampling protocols, blood is collected. The volume collected is dictated by the animal's mass and total blood volume. Typically, less than 10 percent of total blood volume should be collected at one time (Morton *et al.,* 1993). Smith, de Jong and Field (2009) describe blood collection from small bats weighing less than 100 g. Briefly, blood is collected via the puncture of the brachial vein near the bat's elbow. The blood is allowed to pool on the surface of the vessel and is collected using a pipette. It is then diluted in phosphate buffered solution (PBS) at a fixed ratio (Figures 4.7 and 4.8). Dilute serum can then be separated and used in an enzyme linked immunosorbent assay (ELISA) or other serological assay. In larger bats, blood may be collected using standard phlebotomy techniques, either into a sterile collection tube or on to filter paper (Figures 4.9 and 4.10). For bats weighing more than 100 g, particularly large frugivorous bats, blood can most easily be collected from one of three veins: the cephalic ("propetagial") vein, the brachial vein, or the saphenous ("uropetagial") vein (Figure 4.11).

FIGURE 4.7
Bats are manually restrained between the thumb and palm of the non-preferred hand, with the wing extended until the fore- and upper arm form a 90° angle (A). The bleed site is prepared with a 70 percent ethanol swab, and a 25 g needle is used to puncture either the brachial (B) or the propatagial vein. Venous blood then beads on the surface of the skin (C) and can be sampled using a micropipette and sterile tip (D)

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FIGURE 4.8
A blood sample collected from a microbat is placed into a sterile 0.5 ml tube containing PBS

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FIGURE 4.9

A blood sample collected from a flying fox is placed into a sterile tube, which will be centrifuged and the serum fraction removed for serological studies

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FIGURE 4.10

A drop of blood can be collected on to filter paper, where antibodies for serological studies will be stable for at least five months without refrigeration

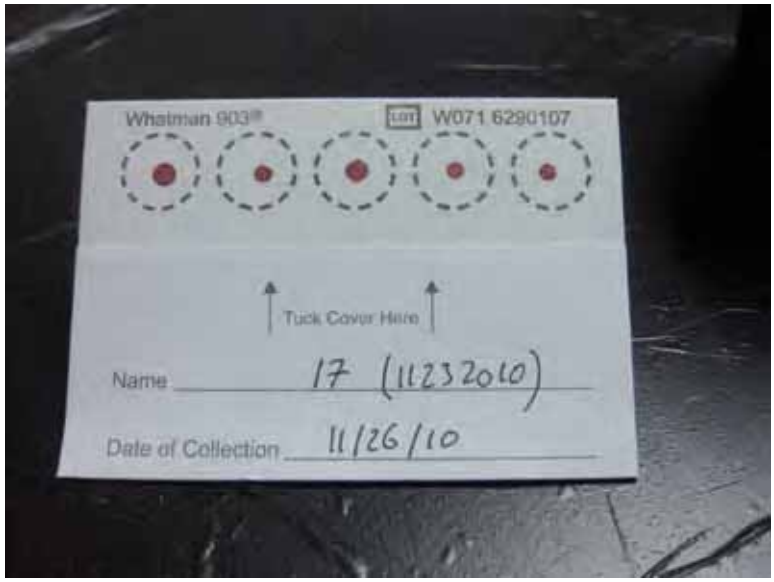

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FIGURE 4.11

a: Three easily accessible venipuncture sites on larger bats (*Pteropus vampyrus* shown): the cephalic, brachial and saphenous veins.

a)

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b: The cephalic vein is often the most accessible as it is highly visible when the bat's wing is extended. A small clip can be used to clamp the vessel while drawing blood.

b)

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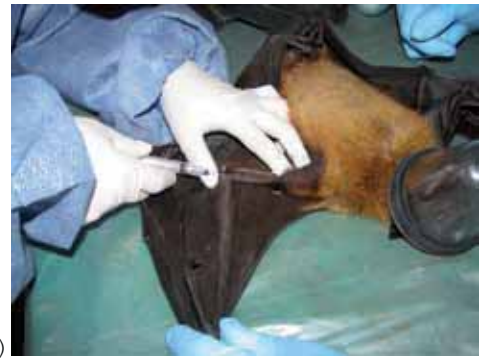
c: The brachial vein is best reached when the bat's arm is positioned with the elbow bent at 90°. Note that the brachial artery runs in close association with the vein. Care should be taken as the brachial vessels are at higher pressure than the cephalic or saphenous veins, and large hematomas may form if lacerated.

c)



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d: The saphenous vein is also easily accessible in large fruit bats, and is preferable if manual restraint is used without anesthesia.

d)

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FIGURE 4.12
A saliva sample collected from the throat of a spectacled flying fox (*Pteropus conspicillatus*) with a sterile polyester-tipped swab

FIGURE 4.13
An urogenital swab collected from the penis of a spectacled flying fox (*Pteropus conspicillatus*)

Saliva, urine and faeces may be collected to detect viral shedding. Typically, a sterile polyester-tipped swab can be used to collect samples from the oropharynx (Figure 4.12). Faeces and urine may be collected directly. Depending on the size of the bat, rectal or urogentital swabs may be used to collect faecal material or urine respectively (Figure 4.13). These samples should be placed in either a lysis buffer (e.g., tri-reagent or ribonucleic acid [RNA]) or a viral transport medium, and then frozen for transport. Samples collected for bacterial culture should be stored in the appropriate transport medium and kept cold (e.g., on ice) during transport.

TRANSPORT

Of paramount importance for sample quality is ensuring a sufficient cold chain from the field to the laboratory. Liquid nitrogen dewars allow samples to be frozen at ultra-low temperatures (-140 °C) and can be transported into the field. Vapour-cooled containers or "dry shippers" (Figure 4.14) have proved particularly useful in the field because they do not require nitrogen in its liquid form, which can spill; they remain cold for approximately two to three weeks without further addition of liquid nitrogen. In remote settings, it may not be possible to carry liquid nitrogen or nitrogen dry shippers. Wet ice can provide short-term cold storage, but samples collected for culture or molecular diagnostics should ideally be transferred to a freezer or liquid nitrogen within 12 to 24 hours of collection, to prevent degradation of virus or viral nucleic acid, which can decrease the likelihood of detection using isolation or molecular techniques.

DIAGNOSTIC ASSAYS/INTERPRETATION

Pathogen-specific diagnostic assays and their interpretation are discussed in chapters 5 and 6. In general, diagnostic assays must be interpreted with caution, particularly when testing for a newly discovered agent for which assays may not yet have been validated or optimized. The sensitivity and specificity of tests may vary. Serological assays, including ELISA and serum neutralization assays, can be subject to cross-reactivity of antibodies and antigen, depending on the test used. For example, serum neutralization assays for Nipah virus may cross-react with Hendra virus, or other unknown henipaviruses (Epstein *et al.,* 2008). In addition, interpretation of the presence of immunoglobulin G (IgG) antibodies via ELISA provides information about past infection or exposure only, and not current infection. Because the duration of IgG antibody persistence in wildlife species is usually unknown, serology must be interpreted with caution when trying to determine incidence of infection. Age-stratified analysis of serological test results has been used to narrow down the likely timing of infection by focusing on juvenile animals whose approximate age can be determined (Epstein *et al.,* 2010b; Plowright *et al.,* 2008.

PCR has become a widely used tool for the detection of pathogen nucleic acid in clinical samples. The advantages of molecular techniques such as PCR include their relatively high sensitivity and specificity, and the ability to work with non-infectious nucleic acid under lower biosafety laboratory conditions. PCR detection of agents that cause acute infection in the host, such as henipaviruses, is challenging because of the apparently short period of infection in chiropteran hosts (Middleton *et al.,* 2007; Williamson *et al.,* 2000). Thus the window of opportunity for detecting viral nucleic acid in excreta (when the animal is shedding virus) is relatively small. Studies that have successfully used PCR to detect henipavirus nucleic acid have focused on screening high volumes of pooled excreta, such as urine, rather than

FIGURE 4.14
A dry shipper is filled with liquid nitrogen prior to field work in remote parts of the Philippines

samples from individual bats, to increase the likelihood of detection (H. Field, unpublished; Epstein *et al.,* 2010b; Wacharapluesadee and Hemachudha, 2007; Wacharapluesadee *et al.,* 2009). These studies have provided information about population-level infection dynamics based on the timing of detections. When testing individual animals for the presence of viral nucleic acid, a quantitative PCR may help differentiate between a current infection (a high amount of nucleic acid) and the presence of remnant virus or nucleic acid from a prior infection, although no specific parameters have been established to make this distinction.

Viral isolation and bacterial culture remain the gold standard for determining whether an animal is infected with or shedding a viable pathogen. Unlike PCR, one of the major challenges in culturing emerging viruses such as Nipah, Hendra, Ebola and Marburg viruses is that many of them are classified as biosafety level-4 pathogens, which require the highest level of containment – something very few laboratories have. Another challenge to viral culture is the varying ease with which bat-borne viruses grow in standard animal-derived cell lines. Recently, diagnostic laboratories have developed bat cell lines, which may enhance the ability for bat viral pathogens to be cultured (Crameri *et al.,* 2009; Krahling *et al.,* 2010).

Ideally, bat surveillance strategies should include sample collection for multiple diagnostic modalities so that if a microbial agent of importance 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 preservative at ultra-cold temperatures (in liquid nitrogen or a -80 C° freezer) for viral culture.

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Chapter 5

Significant zoonotic diseases identified in bats

INTRODUCTION

Scott H. Newman [a]

In recent years, bats have gained significant notoriety for being implicated in numerous emerging infectious disease events, and their importance as reservoir hosts for viruses that can cross species barriers to infect humans and other domestic and wild mammals is increasingly recognized (Calisher *et al*., 2006). Historically, there has been a significant body of work on the role of Microchiroptera (insectivorous bats) as reservoirs of infectious agents, but relatively little available information on members of the suborder Megachiroptera (flying foxes and fruit bats) (Mackenzie, Field and Guyatt, 2003). Although the role of bats in harbouring viruses (alphaviruses, flaviviruses, rhabdoviruses and arenaviruses) is well established (Sulkin and Allen, 1974; Mackenzie, Field and Guyatt, 2003), there is increasing global interest in evaluating the broad range of potential infectious agents that bats harbour, with a particular focus on potential emerging pandemic threats. This concern may be somewhat exaggerated, as bats themselves do not represent the real threat to people as regards potential pathogens leading to large-scale zoonotic disease events. However, it is worth investigating the infectious agents harboured by bats, and integrating this information with an understanding of the risk of transmission from bats to people or livestock, which may serve as intermediate hosts and transmission vectors linking bats and people. This manual therefore elaborates on some of the most important families of viruses, including henipaviruses, lyssaviruses, bat coronaviruses and filoviruses.

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HENIPAVIRUSES

Hume Field [b] *and Jonathan H. Epstein* [c]

Introduction

Hendra and Nipah viruses are novel paramyxoviruses (genus *Henipavirus*) responsible for serious illness and death in livestock and humans. Hendra virus (HeV) was first described in September 1994 in an outbreak of acute respiratory disease in horses in the Brisbane suburb of Hendra, Australia (Selvey *et al.,* 1995). Twenty horses and two humans were infected, with one human and 13 horse fatalities. In 13 additional incidents to date, the case fatality rates have been approximately 75 percent in horses and 50 percent in humans. Wildlife surveillance identified fruit bats of the genus *Pteropus* (commonly known as "flying foxes") as the natural host of HeV, while human cases have been attributed to exposure to infected horses.

Nipah virus (NiP) was first described in 1999 from a major outbreak of disease in pigs and humans in peninsular Malaysia. More than 1 million pigs were culled to contain the outbreak. Of 265 reported human cases, 105 were fatal (Chua *et al.,* 2000). Direct contact with infected pigs was identified as the predominant mode of human infection. Fruit bats of the genus *Pteropus* were again identified as natural hosts (Johara *et al.,* 2001). Since 2001, seasonal outbreaks of NiV-associated disease in humans have been described in India and Bangladesh (Chadha *et al.,* 2006; Luby *et al.,* 2007). Several significant differences distinguish NiV infections in Bangladesh from those in Malaysia: mortality rates have been high, averaging 70 percent and exceeding 90 percent in some outbreaks; infection in humans has not necessarily associated with disease in pigs (or other livestock species); epidemiological evidence suggests direct bat-to-human infection via consumption of contaminated food; and there is evidence of horizontal human transmission (Luby *et al.,* 2009). Subsequent surveillance has identified evidence of henipavirus infection in *Pteropus* (and related) bat species across their global distribution (Halpin *et al.*, 2007). Recent reports suggest an even wider global occurrence of henipaviruses, with evidence of cross-neutralizing viruses in bats in Africa and China (Hayman *et al.*, 2008; Li *et al.,* 2008).

Hendra virus

History and impact

HeV causes sporadic disease in horses and humans. It has been reported only in Australia, where 14 incidents involving 48 confirmed or possible equine cases with a 75 percent case fatality rate, and seven confirmed human cases with a 50 percent case fatality rate were reported from 1994 to 30 June 2010. HeV was first described in 1994 in Brisbane, Australia after a sudden outbreak of an acute respiratory syndrome in thoroughbred horses in a training stable (Murray *et al.,* 1995a). This outbreak was characterized by severe respiratory signs and high mortality. The causal agent was initially unknown, but was quickly shown to be a previously undescribed virus of the family *Paramyxoviridae*. The virus was initially

[b] The State of Queensland (Australia), Biosecurity Queensland

[c] EcoHealth Alliance

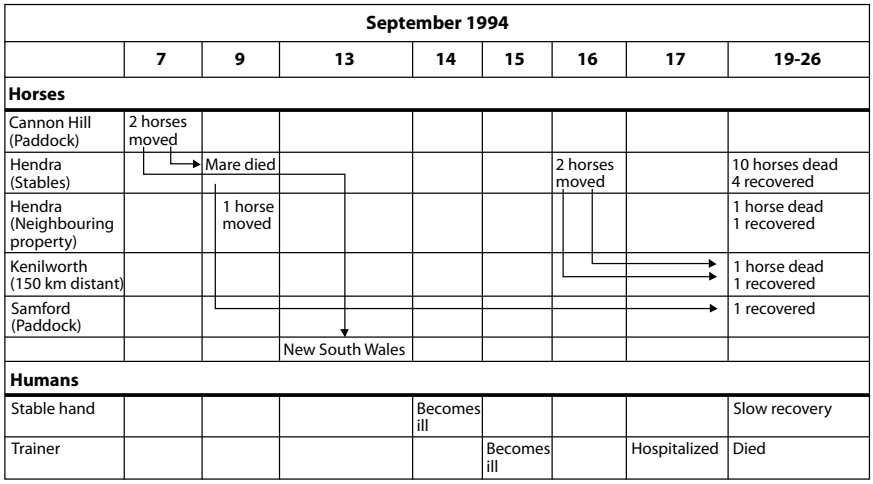
named equine morbillivirus (EMV), but was later renamed Hendra virus (after the Brisbane suburb where the outbreak occurred), and a new genus (*Henipavirus*) was ascribed.

Although HeV has a high case fatality rate in both horses and humans, it has not been highly infectious in these species to date. The putative index case in the first identified spill-over (Brisbane, 1994) was a heavily pregnant thoroughbred mare named Drama Series, at pasture in a Brisbane suburb. When observed to be ill (7 September 1994), she was moved to a training stable housing 23 other thoroughbreds in the Brisbane suburb of Hendra, where she died after a two-day illness. Twelve additional horses in the stable and an adjoining training stable died acutely over the following 14 days (Figure 5.1) (Murray *et al.,* 1995a). Clinical signs in this outbreak included fever, facial swelling, severe respiratory distress, ataxia and, terminally, copious frothy (sometimes blood-tinged) nasal discharge. There were four non-fatal cases, two of which retained mild neurological signs. A further three horses in the stable were subsequently found to have sero-converted without demonstrable clinical signs. All seven were subsequently euthanized (Baldock *et al.,* 1996; Douglas, Baldock and Black, 1997).

The trainer and a stable hand who were directly involved in nursing the index case became ill with a severe influenza-like illness within a week of contacting this horse. The trainer was hospitalized and subsequently died after respiratory and renal failure. Infection with HeV was demonstrated in both the trainer and the stable hand (Selvey *et al.,* 1995).

Comprehensive serological studies were an integral part of the early outbreak investigations. No evidence of HeV infection was found in 800 domestic animals surveyed on the case properties or on in-contact properties. These included 387 horses, 287 cattle, goats and pigs, 23 dogs, 64 cats and 39 poultry (Baldock, Douglas and Black, 1996; Rogers *et al.,* 1996). Particular effort was directed towards surveying the broader Queensland horse

FIGURE 5.1
Chronology of equine and human cases of disease attributed to HeV infection



Source: Murray *et al*., 1995b.

TABLE 5.1
Confirmed HeV incidents (as of 15 October 2010)

Location	Date
Mackay, Queensland	August 1994
Hendra, Queensland	September 1994
Cairns, Queensland	January 1999
Cairns, Queensland	October 2004
Townsville, Queensland	December 2004
Peachester, Queensland	June 2006
Murwillumbah, New South Wales	October 2006
Peachester, Queensland	June 2007
Cairns, Queensland	July 2007
Redlands, Queensland	June 2008
Proserpine, Queensland	July 2008
Cawarral, Queensland	August 2009
Bowen, Queensland	September 2009
Tewantin, Queensland	May 2010

population, with a further 2 024 horses from 166 properties covered by a structured survey (Ward *et al.,* 1996). With the exception of the seven horses that survived infection in the Hendra outbreak, none of the surveyed domestic animals showed serological evidence of exposure to HeV. The negative findings of the highly sensitive "gold standard" serum neutralization test (SNT) provided a level of confidence that HeV was not established in the Queensland horse population, and that in-contact domestic animals were not the source of infection. Baldock *et al.* (1996) also contended that the pattern of the Brisbane outbreak suggested that HeV infection was not highly contagious in horses, and probably required direct contact or mechanical transmission of infectious body fluids for natural transmission to occur. Subsequent experimental trials supported these field observations.

Epidemiology, pathogenesis and clinical presentation

This section draws heavily on the Queensland Government Biosecurity Queensland HeV Web site,[2] particularly the *Guidelines for veterinarians handling potential Hendra virus infection in horses* (version 4). It should be noted that epidemiological information about HeV is incomplete and is the subject of ongoing research.

The following are key features of HeV infection:

- There are no characteristic signs of infection in horses.
- Initial clinical signs in experimentally infected horses were an increase in body temperature and heart rate, and discomfort or restlessness expressed by weight shifting among both fore and hind limbs. This progressed to depression.

[2] www.dpi.qld.gov.au/4790_13371.htm

- As disease progresses, a predominance of neurological or respiratory signs tends to be seen.
- By the time a horse is showing clinical signs, virus is systemically widespread throughout the body and body fluids.
- Viral material could be detected in the nasal discharge of horses for four days prior to the onset of fever, and five days prior to the onset of clinical signs.
- Experimental studies in horses identified HeV in respiratory secretions, saliva and urine.
- In naturally infected horses, viral material has been detected in blood, nasal secretions and a wide range of body tissues.
- The incubation period in horses (time from exposure to appearance of first signs) is between five and 16 days. The course of illness for fatally infected horses is typically two days from first signs to death. The case fatality rate in horses is approximately 75 percent.
- Pathogenesis is associated with virus-induced damage to the vascular endothelium and subsequent vasculitis; the variable clinical presentation primarily observed reflects the first compromising endothelial damage sustained by the organ system(s).
- Horse-to-horse transmission has been more efficient in stabled than paddock situations.
- HeV may survive on fomites for variable periods, depending on climatic conditions (from several hours under hot and dry conditions to several days under cool and damp conditions) and infection may transfer to other horses via fomites contaminated by an infected horse.
- HeV uses a cell surface glycoprotein, ephrin B2, as a cell receptor. This receptor has a widespread cellular distribution, especially in vascular endothelial cells.
- There appears to be little genetic change in the virus structure from the original isolate in 1994.
- Experimentally infected horses did not transmit the virus to in-contact horses. Experimentally infected cats and guinea pigs were susceptible to HeV infection. In an experimental setting, a horse was infected following contact with the urine of an infected cat. Experimentally infected dogs, rabbits, chickens, rats and mice did not develop clinical disease, but some developed antibodies to HeV.
- Experimental studies in pigs produced a range of outcomes from no clinical disease to severe interstitial pneumonia.
- There is no evidence of natural infection in any non-equine domestic species in contact with naturally infected horses.
- Seven cases of human infection have been recorded. All cases had exposure either during autopsy of infected horses or from close contact with infected horses.

Disease ecology and natural reservoir

There is compelling evidence that flying foxes are the natural host of HeV:

- Neutralizing antibodies have been found in all four flying fox species on mainland Australia, across their geographic range, including in historic archived samples.
- Viral genome is frequently detected in the urine of free-living flying foxes; virus isolation has been successful on some occasions.
- Experimental infection in flying foxes does not cause clinical disease.

HeV has been identified in the birthing fluids, placental material and aborted pups of flying foxes. However the precise mode of bat-to-horse transmission has yet to be identified.

Epidemiologically, all HeV cases reported some level of flying fox activity in the vicinity, but not necessarily the presence of a roosting colony.

The presence of flying foxes' favoured food trees in the paddock of the primary cases in each incident is a consistent paddock-level risk factor. In addition, the majority of incidents coincide with flying foxes' gestation/early lactation period (August to October) (Field *et al.,* 2000), although more recent spill-overs (in May to July) have weakened this correlation. This seasonality in recent spill-over events strongly suggests a biological or ecological basis for spill-over from flying foxes to horses. However, the prevalence of infection in individual flying fox populations may vary from year to year, and a reliable method of predicting the high-risk period is not available.

HeV is present in flying fox populations in Australia and Papua New Guinea.

Index cases (the first confirmed cases) have typically been horses in paddocks or kept outside in areas that are attractive to flying foxes.

Ultrastructural, molecular and phylogenetic studies

Ultrastructural studies (Murray *et al.,* 1995b; Hyatt and Selleck, 1996) of the newly identified virus showed it to be pleiomorphic, ranging in size from 38 to more than 600 nm, and enveloped, with surface projections of 10 to 18 nm. Nucleocapsids were 18 nm wide and exhibited a herringbone pattern with 5 nm periodicity. These features indicated that the virus was a member of the family *Paramyxoviridae*, possibly genus *Paramyxovirus* or *Morbillivirus*. Antisera from a range of paramyxoviruses, morbilliviruses and pneumoviruses failed to neutralize the virus, although very weak immunofluorescent and protein immunoblot reactions to rinderpest antiserum were recorded (Murray *et al.,* 1995b). The virus did not exhibit detectable haemagglutination or neuraminidase activity. These features suggested the virus was a morbillivirus (Murray *et al.,* 1995b). Comparative sequence analyses by polymerase chain reaction (PCR) of a portion of the matrix protein supported this, with phylogenetic analysis indicating that the virus was distantly related to other known morbilliviruses (Murray *et al.,* 1995b). Hence the name equine morbillivirus (EMV) was tentatively ascribed to the virus. It was noted that the phylogenetic analysis suggested that the virus had not resulted from single- or multiple-point mutations from a closely related virus, and that emergence from a natural host was the most probable explanation of its origin (Murray *et al.,* 1995b).

Subsequent studies of the complete nucleotide sequence of the matrix (M) and fusion (F) proteins, and partial sequence information from the PV proteins, confirmed that a greater homology existed between EMV and known morbilliviruses than between EMV and other genera of the family *Paramyxoviridae* (Gould, 1996). Nevertheless, sequence comparisons revealed a large degree of divergence with other morbilliviruses, implying that an argument could be made for placing EMV in a new genus, and that additional sequence data were necessary to determine the precise position of EMV within the family.

Sequencing of the entire genome confirmed EMV as a member of the sub-family *Paramyxovirinae*, but identified differences that supported the creation of a new genus. These differences included a larger genome size, the replacement of a highly conserved

sequence in the L protein gene, different genome end sequences, and other sequence and molecular features (Wang *et al.,* 2000). The authors proposed *Henipavirus* as the new genus, with Hendra virus (see following) the type species and Nipah virus (see separate section) the second member. Concurrently, it was being argued that the name "equine morbillivirus" was inappropriate, as mounting evidence suggested that this was neither an equine virus nor a morbillivirus (Young *et al.,* 1997; Wang *et al.,* 1998). Thus the virus was renamed Hendra virus, after the location of the first known outbreak. The International Committee on Taxonomy of Viruses (ICTV) has formally recognized the genus *Henipavirus* and the name Hendra virus (ICTV, 2000).

Several other previously unknown members of the family *Paramyxoviridae* have been described in recent years. These include phocine distemper virus and cetacean morbillivirus (genus *Morbillivirus*), responsible for disease epidemics in marine mammals (Osterhaus *et al.,* 1990; Taubenberger *et al.*, 1996); Menangle virus (genus *Rubulavirus*), which caused severe reproductive disease in a commercial piggery in Australia in 1997 (Philbey *et al.,* 1998); NiV (genus *Henipavirus*), responsible for a major epidemic in pigs and humans in Malaysia in 1998 and 1999 (Chua *et al.,* 2000); Salem virus (unclassified), possibly associated with a disease outbreak in horses in New Hampshire and Massachusetts, United States of America in 1992 (Renshaw *et al.,* 2000); Tupaia paramyxovirus (unclassified), isolated from an apparently healthy tree shrew (*Tupaia belangeri*) in Thailand (Tidona *et al.,* 1999); Tioman virus (genus *Rubulavirus*) and Pulau virus (unclassified) isolated from flying foxes in Malaysia during attempts to isolate NiV (Chua *et al.,* 2001). Tioman and Menangle viruses are phylogenetically closely related. Tupaia and Salem viruses both share some sequence homology with HeV and NiV, but have features that preclude their inclusion as henipaviruses or morbilliviruses. Palau virus has subsequently been characterized as an orthoreovirus (Pritchard *et al.*, 2006).

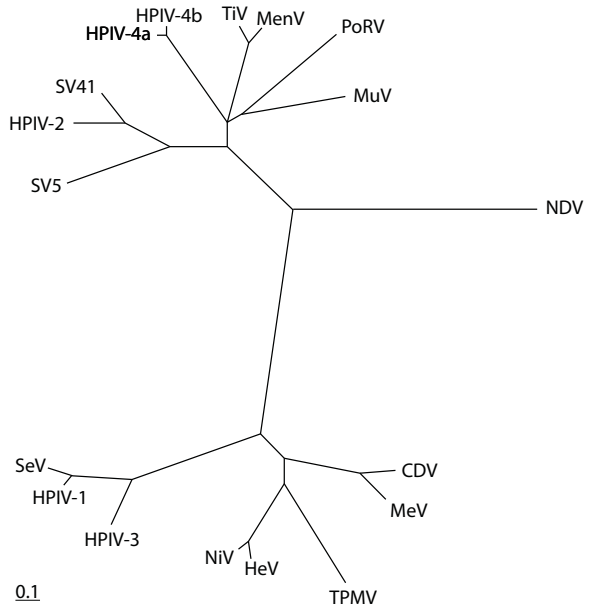
There are two reported isolations of paramyxoviruses from bats prior to the description of HeV in flying foxes in 1996: a sub-type of parainfluenza virus type 2 from the fruit bat species *Rousettus leschenaulti* in India (Pavri, Singh and Hollinger, 1971); and Mapuera virus from another fruit bat species, *Sturnira lilium*, in Brazil (Henderson *et al.,* 1995). Both of these belong to the genus *Rubulavirus,* but are unrelated to Menangle and Tioman viruses. However Mapuera virus is closely related to porcine rubulavirus (formerly La Piedad paramyxovirus), a novel paramyxovirus that caused serious disease in pigs in Mexico (Moreno-Lopez *et al.,* 1986). Figure 5.2 presents a phylogenetic representation of the family *Paramyxoviridae*.

Nipah virus

History and impact

NiV is the second novel paramyxovirus linked to flying foxes. It is zoonotic, has a wide host range, and is phylogenetically close to HeV. Unlike HeV in horses, NiV is highly infectious in pigs, with a high proportion of infected pigs being sub-clinically infected (Nor, Gan and Ong, 2000). The case fatality rate in humans is high, as with HeV. NiV was first described following a major outbreak of disease in pigs and humans in peninsular Malaysia between September 1998 and April 1999, resulting in the death of 105 humans and the culling of more than 1 million pigs (Chua *et al.,* 1999a; Nor, Gan and Ong, 2000). Initially attributed

FIGURE 5.2
A phylogenetic representation of the family *Paramyxoviridae*



A phylogenetic tree based on the deduced amino acid sequences of the matrix protein of members of the family *Paramyxoviridae*. Branch lengths represent relative evolutionary distances.
CDV = canine distemper virus.
HPIV = human parainfluenza virus.
MenV = Menangle virus.
MeV = measles virus.
MuV = mumps virus.
NDV = Newcastle disease virus.
NiV = Nipah virus.
PoRV = porcine rubulavirus.
SeV = Sendai virus.
SV = simian virus.
TiV = Tioman virus.
TPMV = Tupaia paramyxovirus.

Source: Chua *et al.*, 2002.

to Japanese encephalitis virus, the primary disease aetiology was subsequently shown to be a previously undescribed virus of the family *Paramyxoviridae*. Preliminary characterization of an isolate at the United States Centers for Disease Control and Prevention (CDC) at Fort Collins and Atlanta showed that the new virus, subsequently named Nipah virus, had ultrastructural, antigenic, serologic and molecular similarities to HeV (CDC, 1999). Retrospective investigations suggest that NiV has been responsible for disease in pigs in peninsular Malaysia since late 1996, but the disease was not recognized as a new syndrome because the clinical signs were not markedly different from those of several endemic diseases, and because morbidity and mortality were not remarkable (Aziz *et al.,* 1999).

Following the Malaysian outbreak and its subsequent characterization, NiV has been recognized as the aetiologic agent in more than ten outbreaks of encephalitis in Bangladesh

and West Bengal, India since 2001. Outbreaks in Bangladesh have shown strong spatial and temporal clustering, with cases occurring in the western half of the country (the two Indian outbreaks occurred in West Bengal, near the Bangladesh border) and all cases occurring between November and April (Luby *et al.,* 2007). NiV encephalitis outbreaks in Bangladesh have also included chains of human-to-human transmission, a marked distinction from the Malaysian outbreak (Gurley *et al.,* 2007; Luby, Gurley and Hossain, 2009). To date, there have been more than 200 cases of NiV encephalitis in Bangladesh, with an average mortality rate of 71 percent, although the case fatality rate in some outbreaks exceeded 90 percent (Luby *et al.,* 2007).

Epidemiology, pathogenesis and clinical presentation

Malaysia: The epidemic primarily affected pig and human populations, although horses, dogs and cats were also infected. The disease in pigs was highly contagious, and clinical disease was characterized by acute fever with respiratory and/or neurological involvement. Incubation was estimated to be seven to 14 days. Crude case fatality rate was low (< 5 percent), and a notably large proportion of infected pigs were asymptomatic (Nor, Gan and Ong, 2000). The clinical course appeared to vary with age. Sows primarily presented with neurological disease, and sows and boars sometimes died peracutely. In weaners and porkers a respiratory syndrome predominated, frequently accompanied by a harsh non-productive (loud barking) cough. It is unclear whether respiratory and neurological symptoms observed in suckling piglets were directly attributable to infection. Epidemiological evidence suggests that the movement of pigs was the primary means of spread among farms and regions (Nor, Gan and Ong, 2000). The primary mode of transmission on pig farms was believed to be via the respiratory route; later laboratory evidence provided support for this contention.

The predominant clinical syndrome in humans was encephalitic rather than respiratory, with clinical signs including fever, headache, myalgia, drowsiness and disorientation, sometimes proceeding to coma within 48 hours (Chua *et al.,* 1999a; Goh *et al.,* 2000). The majority of human cases had a history of direct contact with live pigs. Most were adult male Chinese pig farmers (Chua *et al.,* 1999a; Parashar *et al.,* 2000).

Evidence of infection has also been found in dogs, cats and horses (Chua *et al.,* 1999b; Nor, Gan and Ong, 2000; Mills *et al.,* 2009). The initially high prevalence of infection in dogs in the endemic area during and immediately following the removal of pigs suggests that dogs readily acquired infection from infected pigs. The far lower antibody prevalence and restriction of infection to within 5 km of the endemic area suggests that NiV did not spread horizontally within dog populations (Field *et al.,* 2001). The risk of natural infection in cats directly from pteropid bats appears to be low (Epstein *et al.,* 2006).

Bangladesh: Serological evidence suggests that *Pteropus giganteus*, the sole pteropid species on the Indian subcontinent, is a natural reservoir for NiV in Bangladesh and India (Hsu *et al.,* 2004; Epstein *et al.,* 2008). These bats are abundant and widely distributed across the subcontinent (Bates and Harrison, 1997). Systematic sero-epidemiological surveillance of *P. giganteus* has shown widespread evidence for infection throughout Bangladesh (Epstein *et*

al., 2010). There has been epidemiological evidence to suggest that cases in Bangladesh have been associated with contact with sick livestock, and direct bat-to-human transmission also appears to occur through the ingestion of date-palm sap, presumably contaminated with bat excreta (Luby *et al.,* 2009).

Disease ecology and natural reservoir

Malaysian bats became a surveillance priority in determining the origins of the virus because laboratory evidence suggested a close relationship between HeV and NiV, and because flying foxes had been shown to be a putative natural host of HeV. Neutralizing antibodies to NiV were found in 21 of 324 bats, from five of 14 species surveyed. These were the megachiropteran species *Pteropus vampyrus* (five of 29 surveyed), *P. hypomelanus* (11 of 35), *Cynopterus brachyotis* (two of 56), and *Eonycteris spelaea* (two of 38), and the microchiropteran species *Scotophilus kuhli* (one of 33) (Johara *et al.,* 2001). Subsequently, NiV was isolated from urine collected from a sero-positive colony of *P. hypomelanus* on Tioman Island (Chua *et al.,* 2001) and *P. vampyrus* in Perak state. By the time NiV had been identified as the causative agent in human outbreaks of encephalitis in Bangladesh, the link between henipaviruses and flying foxes had been discovered. Bats, including 50 *Pteropus giganteus,* were screened for antibodies to NiV during an outbreak investigation in Meherpur and Nagaon districts in 2003. Two of 25 *P. giganteus* tested in Nagaon were positive for antibodies against NiV on a capture enzyme linked immunosorbent assay (ELISA) (Hsu *et al.*, 2004). In 2003, a colony of *P. giganteus* in northern India, approximately 1 200 km from the border with Bangladesh in an area not known to have had any human NiV infections, was screened for NiV antibodies. Some 42 percent (n = 80) were found to be sero-positive by serum-neutralization assay, further substantiating the hypothesis that pteropid bats were likely to be a natural reservoir for henipaviruses throughout their range (Epstein *et al.,* 2008). Current research focuses on understanding the temporal and spatial dynamics of NiV in *P. giganteus* in Bangladesh by conducting longitudinal studies at single colonies over several years, to determine patterns of viral shedding. Early sero-epidemiological results indicate that NiV circulates widely in flying foxes throughout Bangladesh (Homaira *et al.,* 2007; Epstein *et al.,* 2010), despite the spatial clustering of human cases, suggesting that human demography and behaviour may play a significant role in zoonotic transmission.

Ultrastructural, molecular and phylogenetic studies

Initial electron microscopic studies showed that the ultrastructure of NiV was consistent with that of viruses of the family *Paramyxoviridae*, and immunofluorescence tests of infected cells suggested a virus related to HeV. Preliminary nucleotide sequencing also indicated that NiV was related to HeV (CDC, 1999). Virus particles were pleiomorphic, ranging from 120 to 500 nm, and enveloped, with surface projections measuring 10 nm. Typical herringbone nucleocapsid structures were seen, approximately 1.67 μm in length and 21 nm wide. NiV-infected cells reacted strongly with HeV antiserum, but not with antisera for other paramyxoviruses, including measles virus, respiratory syncytial virus and parainfluenza 1 and 3. No reactivity was seen with other viruses, including herpes virus, enteroviruses and Japanese encephalitis virus (Chua *et al.,* 2000). Cross-neutralization studies have shown at least a fourfold difference in neutralizing antibodies between HeV and NiV (Chua *et al.,* 2000; Johara *et al.,* 2001).

Later, more extensive nucleotide sequence studies found that the nucleo (N) protein, phosphor (P) protein and matrix (M) gene of NiV shared a 70 to 78 percent nucleotide homology with those of HeV, supporting the findings of others (see section on the History and impact of NiV) that HeV and NiV are phylogenetically closer to each other than to any other viruses in the subfamily *Paramyxovirinae* (Chua *et al.,* 2000; Harcourt *et al.,* 2000).

Molecular characterization of full genomic sequences of NiV from humans in Malaysia and Bangladesh have shown that there is an overall nucleotide homology of 91.8 percent between the strains, with the majority of nucleotide variation occurring in the P gene. Amino acid homology is predicted to be higher (> 92 percent), particularly in the coding regions (Harcourt *et al.,* 2005). In Thailand, many sequences from the NiV L gene have been obtained from pooled urine samples collected underneath large colonies of *Pteropus lylei* (Wacharaplusadee *et al.,* 2005; 2009). Phylogenetic analysis shows considerable strain variation within a colony at the same point in time and over time. Because so few NiV isolates have been obtained from bats, few comparative data are available from the whole genome. However, a recent isolate from *P. vampyrus* in Malaysia has found significant variation from the *P. hypomelanus* isolate from Tioman Island.

Diagnostics

This section draws primarily on the paper of Daniels, Ksiazek and Eaton (2001). It describes diagnostic methods for detecting virus, virus antigen or virus nucleotide sequence (evidence of current infection) and antibody (evidence of past infection). It also makes reference to appropriate diagnostic samples, gold standard tests and test limitations, and to test interpretation in bats.

Henipaviruses are classified internationally as biosafety level (BSL) 4 agents. This classification has diagnostic ramifications for tests that necessarily involve live virus (virus isolation beyond primary diagnosis and serum neutralization tests [SNTs]), and such work should be carried out only under physical containment (PC) level-4 conditions after appropriate training.

Virus isolation: Daniels, Ksiazek and Eaton (2001) comment that HeV grows well in Vero cells from a range of tissue specimens including brain, lung, kidney and spleen. Cytopathic effect (CPE) usually develops within three days, initially manifested by the formation of syncytia containing 20 or more nuclei, and subsequently by punctate holes in the cell monolayer. Virus isolates can be specifically identified by immuno-staining, neutralization with specific antiserum, PCR and electron microscopy. Virus isolation is an important diagnostic tool, and where appropriate protocols and training are in place, Daniels, Ksiazek and Eaton (2001) suggest that primary virus isolation (for diagnostic purposes) can be performed in PC3 laboratories. They caution that any cultures developing characteristic CPE should be forwarded to a PC4 laboratory for further work.

Immuno-histochemistry: In contrast to virus isolation, immuno-histochemistry (IHC) is performed on formalin-fixed tissues, so the same biosafety constraints do not apply. Daniels, Ksiazek and Eaton (2001) comment that IHC is a useful and safe technique that can detect virus antigen in a range of tissues. They recommend the submission of a range of tissues (including spleen), noting that virus may clear from lung tissue early in an infection. They also

note that because IHC uses formalin-fixed tissues, the technique is useful for retrospective investigations on archived materials. The availability of a range of mono - and polyclonal - antisera means that test sensitivity and specificity can be tailored to testing objectives.

Electron microscopy: Negative-contrast electron microscopy (EM) and immuno-electron microscopy were an integral part of the initial HeV diagnostic effort (Murray *et al.,* 1995a). Daniels, Ksiazek and Eaton (2001) recognize the value of both in providing rapid and valuable information on virus structure and antigenic reactivity during primary virus isolation, and see these and other EM techniques as complementing other diagnostic methodologies.

PCR and sequencing: PCR is a powerful molecular technique able to amplify segments of virus genes. The diagnostic PCR in routine use at the Australian Animal Health Laboratory (AAHL) employs a set of nested primers to amplify segments of the HeV matrix gene that codes for the relatively conserved matrix protein (Daniels, Ksiazek and Eaton, 2001). The ability to select primer sets for various genes means that test sensitivity and specificity can be tailored to testing objectives. The technique can be used as a primary diagnostic tool to detect virus sequences in fresh or formalin-fixed tissue, and fixed or cerebrospinal fluid, or as an adjunct to virus isolation for rapidly characterizing virus isolates. The inherent high sensitivity of PCR associated with the amplification process creates an ever-present risk of laboratory contamination and subsequent false positive test results. This issue can largely be addressed by appropriate laboratory design, personnel training and internal and external quality control programmes.

Sero-neutralization tests: The SNT is regarded as the gold standard serologic test for HeV. Sera are incubated with live virus in microtitre plates to which Vero cells are added. Initial serum dilutions of 1:2 or 1:5 can be used. Cultures are read at three days, and the sera that block CPE are regarded as positive (Daniels, Ksiazek and Eaton, 2001). The use of live virus and the attendant biosafety issues mean that SNTs should be performed in only PC4 facilities.

ELISA tests: ELISA tests provide a rapid, inexpensive and safe means of conducting serologic investigations. AAHL initially developed an indirect ELISA for the detection of immunoglobulin G (IgG) antibodies to HeV in horses. Subsequently, a range of refinements has been explored to reduce non-specific reactions and improve test specificity relative to the SNT. A competitive ELISA format using monoclonal antibodies is now being developed. The incorporation of A protein and G protein conjugates rather that anti-species conjugates has broadened the application of the indirect ELISA beyond horses (Daniels, Ksiazek and Eaton, 2001). This ELISA has been used to screen *Pteropus giganteus* sera from India, and compares favourably with the SNT, with 91 percent sensitivity and 75 percent specificity (Epstein *et al.,* 2008). However, there are a number of shortcomings of the current ELISA in relation to HeV surveillance in wildlife populations.

Multiplexed micosphere assays: Bossart *et al.* (2007) developed two multiplexed microsphere assays that utilize recombinant soluble attachment glycoproteins (sGs). The antibody detection assay can detect and differentiate between anti-HeV and anti-

NiV antibodies; the receptor inhibition assay, which incorporates the cellular receptor, recombinant ephrin-B2sGHeV- and sGNiV-coupled beads, offering enhanced specificity. Both assays offer improved performance compared with available ELISAs, and the receptor inhibition assay is effectively a surrogate virus-neutralization test. Both require small volumes of sera, can be done quickly, and do not require high biocontainment.

Test limitations for wildlife species

Serologic tests specifically developed for diseases of wildlife are limited; serologic tests used in wildlife studies are commonly transposed from those for domestic species. The validity of such tests and the interpretation of their results can therefore be problematic. Gardner, Hietala and Boyce (1996) raise two fundamental points about the transposition of serologic tests to wildlife species. First they note that many tests have not been adequately evaluated in the domestic species for which they were developed, so data on inherent test sensitivity and specificity are lacking. Second, they argue that even if the test has been validated in domestic species, test characteristics should not be assumed to be the same in wildlife species, given the possible differences in pathogen strains, host responses and exposure to cross-reacting infections in wildlife species.

However, the authors make several positive observations. First, while species-specific reagents are rarely available for wildlife species, this potential major impediment has been largely overcome by the development of protein A and/or G complexes, which are used as alternatives to secondary antibody in these tests. Second, although test sensitivity and specificity can be difficult and expensive to establish, relative values can be obtained through comparison with known positive and negative samples. Third, as ELISA sensitivity and specificity are directly influenced by the cut-off value, it may be appropriate to present results as a receiver operating characteristic (ROC) curve, or report them using several cut-off points. Fourth, ELISAs using monoclonal antibodies can reduce cross-reactivity between antigenically related agents, thus improving specificity. Fifth, the positive predictive value of a test can be improved by testing high-risk groups, or retesting "positive" samples and using series interpretation of the results.

Conclusion

The available evidence suggests that Hendra and Nipah are ancient viruses, well adapted to their natural hosts, in whose populations they have long circulated. Current evidence points to multiple chiropteran host species, although the degree to which detections in non-pteropid species (except in Africa) are the result of incidental transmission is not clear. Further studies are needed to elucidate how widely henipaviruses circulate, both geographically and taxonomically. It appears that a wide range of mammalian hosts are susceptible to henipaviruses owing to their reliance on the ephrin B2 receptors for cell entry. Ephrin B2 receptors are highly conserved across mammalian taxa and found in many different tissue types, allowing viral entry via both respiratory and gastrointestinal routes. The close phylogenetic relationship between Hendra and Nipah viruses is consistent with a common progenitor virus.

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LYSSAVIRUSES

Janine Barrett [b]

Introduction

Lyssaviruses (family *Rhabdoviridae*, genus *Lyssavirus*) are the aetiological agents for rabies – an acute, progressive viral encephalitis. With the notable exception of fewer than ten recent cases, rabies is fatal in humans. Lyssaviruses occur on all continents apart from Antarctica.

Lyssaviruses are membrane-bound, bullet-shaped virions, with a genome consisting of single-stranded, negative-sense ribonucleic acid (RNA). The approximately 12 000 base-pair genome encodes five monocistronic genes: a nucleoprotein (N protein), a phosporotein (P protein), a matrix (M) protein, a glycoprotein (G protein) and a RNA-dependent RNA (RdRP) polymerase (L). Together with the RNA, the N, P and L proteins form an inner ribonucleoprotein core, or nucleocapsid. The outer G protein forms spikes that project through the virion membrane. The M protein links the inner nucleocapsid and the outer membrane-bound G protein (Finke and Conzelmann, 2005).

Lacking relevant proof-reading ability during replication, most viral variation arises via the survival of mutations, known as genetic drift. Lyssaviruses are relatively fragile and do not persist in the environment.

Currently, 12 putative viral species or genotypes reside in the genus *Lyssavirus* (Table 5.2). These have been grouped into phylogroups based on pathogenicity and immunogenicity (Badrane *et al.,* 2001; Kuzmin *et al.,* 2010)

The major host reservoirs are members of the orders Carnivora (e.g., dogs, foxes, mongoose, raccoons, skunks, etc.) and Chiroptera (bats). There are very limited numbers of isolates for some lyssaviruses. Mokola may be maintained among insectivores such as shrews (order Soricomorpha). Bats are considered the major hosts for all lyssaviruses except Mokola, and perpetuate a wide diversity of viral antigenic variants in those genotypes that have been adequately studied. Genotype 1 rabies virus is the type species of the genus, and the most significant lyssavirus in terms of public health and veterinary impacts.

Vampire bat-variant rabies virus in Latin America causes a significant number of human deaths each year, and there are very rare cases due to other bat lyssavirus genotypes elsewhere (Table 5.2).

History and impact

Compared with the long history of rabies virus in carnivores and humans, recognition of bat lyssaviruses is comparatively recent and – with the notable exception of rabies virus in Latin American vampire bats, particularly *Desmondus rotundus* – bat lyssaviruses rarely cause rabies in humans and other animals. Allusions to bat-borne rabies first appeared in the 1500s in a description by Spanish conquistadores in Latin America of human and livestock deaths following bat bites, but the association was not confirmed until 1921 by Haupt dn Rehaaq in Brazil (Kuzmin and Rupprecht, 2007; Freuling *et al.,* 2009b).

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[b] The State of Queensland, Department of Employment, Economic Development and Innovation (2011)

FIGURE 5.3
Structural diagram of a lyssavirus

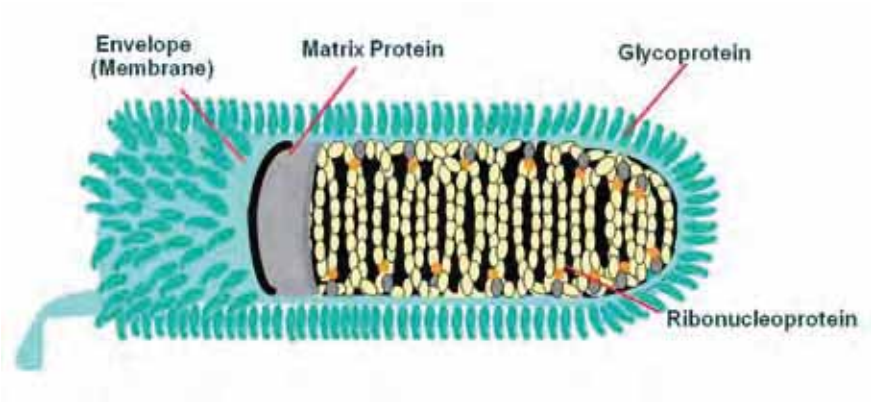

FIGURE 5.4
Electronmicrograph of bullet-shaped Lagos bat virus particles budding from an intracellular inclusion body

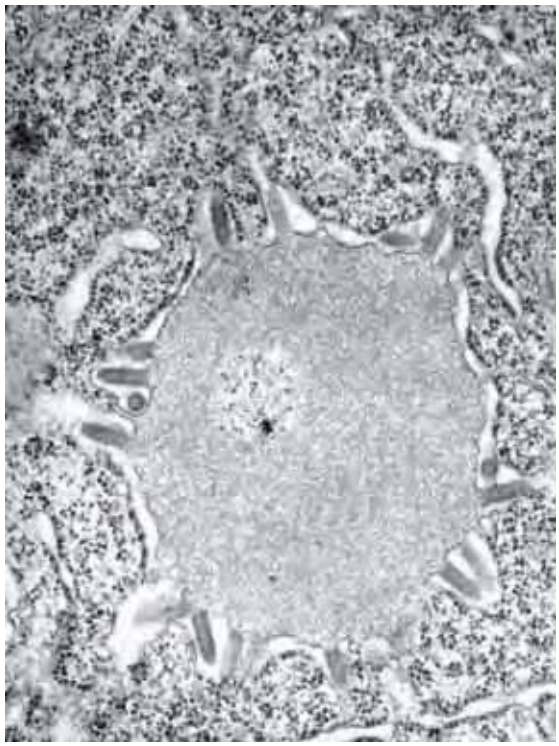

TABLE 5.2
Summary of the lyssaviruses

Phylogroup	Genotype	Lyssavirus	Maintenance hosts	Location	Number of human cases
I	1	Rabies virus	Canivora and multiple species of insectivorous and haematophagus bats	Carnivores on all continents except Australia and Antartica Insectivorous and haematophagus bats in the Americas	> 55 000/year attributed to carnivore-variants 100s/year (estimated) due to vampire bat rabies 1-4/year attributed to other bat rabies variants
II	2	Lagos bat virus	Unconfirmed – a few isolates from Megachiroptera (*Epomophos wahlbergi* – Wahleberg's epauletted fruit bat) and Microchiroptera	Africa	None reported to date
II	3	Mokola	Unconfirmed – a few isolates from shrews No bat isolates	Africa	2 (both in 1970)
I	4	Duvenhage virus	Unconfirmed – a few isolates from insectivorous bats (*Miniopterus* spp. and *Nycteris thebaica)*	Africa	3 (1970, 2006 and 2007)
I	5	European bat lyssavirus-1 (EBLV-1)	Insectivorous bats – typically *Eptesicus serotinus* (serotine bat)	Europe	2 (1977 and 1985)[b]
I	6	European bat lyssavirus-2 (EBLV-2)	Insectivorous bats – typically *Myotis daubentonii* (Daubenton's bat)	Western Europe	2 (1985 and 2002)[b]
I	7	Australian bat lyssavirus	Flying foxes (*Pteropus* sp.) and at least one species of insectivorous bat (*Saccolaimus flaviventris* – yellow-bellied sheath-tail bat)	Australia	2 (1996 and 1998)
I		Aravan virus[a]	Unconfirmed – single isolate from *Myotis blythii* (lesser mouse-eared bat) 1991	Central Asia (Kyrgystan)	None reported to date
I		Khujand virus[a]	Unconfirmed – single isolate from *Myotis mystacinus* (whiskered bat) 2001	Central Asia (Tajikistan)	None reported to date
I		Irkut virus	Unconfirmed – single isolate from *Murina lecuogaster* (greater tube-nosed bat) 2002	Eastern Siberia	1 (2007)
III		West Caucasian bat virus[a]	Unconfirmed – single isolate from *Miniopterus schreibersii* (Schreiber's bent-winged bat) 2002	Western Caucasus mountains	None reported to date

(Cont.)

TABLE 5.2 *(Cont.)*

Phylogroup	Genotype	Lyssavirus	Maintenance hosts	Location	Number of human cases
II		Shimoni[a]	Unconfirmed – single isolate from *Hipposideross commersoni* (Commerson's leaf-nosed bat) 2009	Africa	None reported to date

[a] Single isolates only.

[b] There are accounts of a few (< 5) human rabies deaths following bat bites from which no virus has been retained for genotyping, but that are considered likely to be EBLV-1, EBLV-2, West Caucasian bat virus or other yet to be identified lyssaviruses.

Of the bat lyssaviruses, rabies virus in vampire bats has the most significant impact on human and veterinary health, causing a significant but poorly documented number of human deaths, along with between 100 000 and 500 000 cattle deaths, at a cost of more than USD 42 million a year (Greenhall and Schmidt, 1988).

Reports of human deaths in the United States of America following bites by insectivorous bats in the 1950s led to the recognition of bat rabies among insectivorous bats in North America (Kuzmin and Rupprecht, 2007; Constantine, 2009). Bat-associated rabies in the United States causes about one to four human deaths each year and rare disease occurrences in other animals.

Surveys of African mammals led to the recognition of Lagos bat virus (1956), Mokola virus (1968) and Shimoni bat virus (2009) (Kuzmin *et al.,* 2010). Duvenhage virus was first detected in a human case (1970). African bat lyssaviruses appear to be rare causes of disease in domestic animals or humans. However, carnivore-variant rabies is relatively common in Africa, differentiation of bat lyssaviruses from carnivore rabies virus requires genotype-specific tests, and there is little systematic surveillance in Africa. The actual impact of bat lyssaviruses in Africa is therefore likely to be masked by the far greater impact of carnivore-variant rabies virus (Struthers, 1991).

Reports of rabies in insectivorous bats of Europe between 1954 and 1984 led to the recognition of two bat lyssaviruses: European bat lyssavirus-1 (EBLV-1), and European bat lyssavirus-2 (EBLV-2). EBLVs are very rare causes of disease in humans (the last was in 2002) and other animals (Freuling *et al.,* 2009b).

Australia was believed to be free of all lyssaviruses until the recognition of Australian bat lyssavirus (ABLV) in *Pteropus* sp. and one species of insectivorous bat in 1996. There have been two human deaths attributed to bites from bats, the last in 1998, and no detection in animals other than bats (Barrett, 2004).

The diagnosis of rabies, without further characterization of the virus, in pteropid bats in India (1980) and Thailand (1967) means it is likely that ABLV or other lyssavirus(es) are present in Megachiroptera and other species throughout Asia.

A series of bat surveys between 1990 and 2002 led to the discovery of single isolates of Aravan virus, Khujand virus, Irkut virus and West Caucasian bat virus in insectivorous bats of Central Asia and Eastern Europe.

Despite the low case rate outside Latin America, the cost of managing potential exposure to bat lyssaviruses through contact with bats has a significant impact on public health. Bat lyssaviruses appear to have no significant impact on bat populations, particularly compared with the devastating impact of the recently emerged fungal white-nose syndrome (Frick *et al.,* 2010).

Epidemiology and disease ecology

Lyssaviruses have been detected in most of the bat species that have been appropriately surveyed, and should be presumed to be present in all bat species globally. Many countries have little to no surveillance of bats, and epidemiological and disease ecology data are very limited for the African and Eurasian lyssaviruses. The absence of isolates from bats in India and Southeast Asia probably reflects a lack of surveillance rather than an absence of lyssaviruses.

Within each bat lyssavirus genotype for which there are adequate isolates there are bat species-specific virus variants or biotypes that are maintained by particular bat species within geographically discrete areas. In contrast to dog-variant rabies virus, which is present on all continents except Australia and Antarctica, the distribution of each genotype of bat lyssavirus appears to be limited to specific bat species within a single continent (Table 5.2). Most of the data on bat lyssaviruses relate to genotype 1 bat rabies virus, EBLV-1 and the pteropid-variant of ABLV, and are presumed to be biased by human factors that influence the likelihood of a bat being submitted to diagnosis.

The considerable differences between the ecology of bats and that of carnivores need to be considered when extrapolating from the larger body of knowledge about carnivore rabies epidemiology. Lyssaviruses are found across diverse bat species, whether they are insectivorous, frugivorous or haematophagous; hibernate and/or migrate or do not; are solitary or roost in colonies of a few individuals to millions of bats of single or mixed species; and (in contrast to most carnivores) bear young (usually one at a time) once or twice per year. The extent to which these factors influence and interact in the disease ecology of bats is unclear and may be specific to the relationship between each bat species and each virus variant.

Transmission is rarely observed among bats. Presumably, as for carnivore-variant rabies, transmission between bats is by transfer of infectious saliva through biting and licking. Aerosol transmission in caves may play a role in bat rabies epidemiology, particularly among bats that roost in caves in large, high-density colonies (Constantine, 2009). Transmission between solitary bat species and arboreal flying foxes in Australia presumably depends on direct bite contact.

When adequately sampled, bats from nearly all of the more than 40 bat species present in the United States of America are found to be infected with many distinct variants of rabies virus (Streicker *et al.,* 2010). Enzootic cases occur in every state except Hawaii (Blanton, Palmer and Rupprecht, 2010). Most isolations of rabies virus in bats of the Americas come from relatively few species of the genera *Desmodus, Eptesicus, Lasionycteris, Lasiurus, Myotis* and *Tadarida*. Paradoxically, among the bats submitted to diagnostic testing at state laboratories, studies have shown higher infection rates in solitary bats and those living in small groups than in colonial species (Blanton, Palmer and Rupprecht, 2010). The occurrence of bat rabies in the Americas is comparatively high, with 1 625 laboratory-diagnosed cases

among the 27 915 bats examined during 2009 in the United States alone (Blanton, Palmer and Rupprecht, 2010).

In Europe, a total of 889 lyssavirus-infected bats had been detected by 2010 (Freuling and Muller, 2010), with isolates from only ten of the 45 known bat species (Freuling *et al.,* 2009b). All European bat cases have been due to either EBLV-1 or EBLV-2. The majority of characterized cases are of EBLV-1 in the serotine bat (*Eptesicus serotinus).* EBLV-1 occurs as two sub-lineages, 1a in many countries of Western and Central Europe, and 1b in the serotine bat and *E. isabellinus* (recently identified as a separate species) in the Iberian Peninsula and France. EBLV-2 is isolated only sporadically, and only from *Myotis daubentonii* and *M. dascyneme* (Freuling *et al.,* 2009b). European insectivorous bats roost in large colonies, some migrate over large distances, and roost sites may contain colonies of different species, allowing horizontal spread within and among species. Whether detection of EBLV in species other than *Eptesicus* and *Myotis* reflects maintenance within primary hosts or simply spill-over to these other species, or why the numbers of species involved and individual cases detected are considerably lower than in the Americas are unclear.

In Australia, only 187 cases of ABLV have been diagnosed in bats (Australian Wildlife Health Network, 2010), which probably reflects the relatively low surveillance pressure in Australia compared with North America and Europe. ABLV occurs as two variants (Guyatt *et al.,* 2003; Barrett, 2004). The Pteropid-variant has been isolated from all four of the common *Pteropus* spp. on mainland Australia: little red flying fox (*P. scapulatus*), black flying fox (*P. alecto*), grey-headed flying fox (*P. poliocephalus*) and spectacled flying fox (*P. conspicillatus*). Flying foxes roost in trees, in large camps of thousands to hundreds of thousands. Camps often contain one or more species, and black, grey-headed and little red flying foxes migrate for distances ranging from hundreds to thousands of kilometres in search of fruit, blossoms and leaves. It remains unclear whether all four species act as maintenance hosts or one or more species maintain the virus with frequent spill-over to other flying foxes. Surveillance of healthy wild-caught individuals suggests that the prevalence of ABLV in the general bat population is less than 1 percent (McCall *et al.,* 2000). Analysis of

FIGURE 5.5
Serotine bats (*Eptesicus serotinus*) – primary maintenance host of EBLV-1

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prevalence and risk factors in flying foxes submitted to lyssavirus testing indicates that three factors – species, age and health status – are associated with the proportion of infection. In particular, 20 to 30 percent of flying foxes showing signs of central nervous system (CNS) disease were ABLV-positive, compared with fewer than 2 percent of sick injured or orphaned bats without CNS signs (Barrett, 2004).

Surveillance of insectivorous bats in Australia has been limited, and the yellow bellied sheath-tailed bat (*Saccolaimus flaviventrus*) variant of ABLV is the only virus identified in any of the more than 60 insectivorous bat species in Australia. These bats are solitary, roost in tree hollows and are rarely encountered, and little is known about the epidemiology of this distinct variant.

Clinical disease due to lyssavirus infection is believed to be fatal in all mammals. The moderate to high sero-prevalence in healthy bats around the globe suggests either clinical recovery or subclinical resolution of infection (so-called "abortive" infection), where an early adequate immune response clears infection prior to the onset of clinical signs. There is no recognized carrier state for lyssavirus infection. An apparently normal animal may excrete virus for several days prior to developing clinical signs.

Human, livestock, wildlife and environmental pespectives

The disease ecology of bat lyssaviruses in humans and other animals reflects the interactions of many factors that influence contact between bats and other species, including those that influence bat, human and other animal behaviour and the effect of lyssavirus encephalitis on bat behaviour.

On very rare occasions bat lyssaviruses are transmitted to humans via organ transplants or laboratory exposure. Experimental evidence has demonstrated the potential for aerosol transmission within bat caves (Constantine, 2009). Secondary transmission via an infected animal other than a maintenance host species is well documented for carnivore-variant rabies (e.g., dog-variant rabies virus transmitted to humans via cats), but appears to be less

FIGURE 5.6
Little red flying foxes (*Pteropus scapulatus*) – one of four host species for the pteropid-variant of ABLV

FIGURE 5.7
Spectacled flying foxes (*Pteropus conspicillatus*) – one of four host species for the pteropid-variant of ABLV

common for bat lyssaviruses. Transmission of bat rabies virus from affected cats to humans has been documented (Badilla *et al.,* 2003).

Vampire bat rabies: Vampire bats' feeding on the blood of mammals results in a unique epidemiology and disease ecology for vampire bat-variant rabies transmission. The vampire bat population of Latin America is believed to have increased substantially following European introduction of large prey, notably livestock such as cattle (Hughes, Orciari and Rupprecht, 2005; Kuzmin and Rupprecht, 2007). Vampire bats feed during the night and can feed repeatedly on sleeping people and other animals without waking them. Blood feeding leads to high levels of bat-bite exposure and fatal disease in humans and livestock, particularly cattle. Given the remoteness of localities in regions such as Amazonia, the regularity of prior outbreaks, the increased environmental intrusion for mining and timber resources, the rapid conversion of rain forest to livestock and agricultural pursuits, and the lack of adequate laboratory-based surveillance, more than 500 human cases a year may occur in the Americas related to bat lyssaviruses, primarily owing to highly adaptive populations of vampire bats (Schneider *et al.,* 2009).

Vampires are not cold-tolerant, and the distribution of vampire bats in Latin America is partially limited by minimum temperatures (Greenhall and Schmidt, 1988). Higher global temperatures arising from climate change will increase the potential range of vampire bats and the distribution and impact of bat rabies (Hughes, Orciari and Rupprecht, 2005).

Other bat lyssaviruses: In contrast to haematophagus bats, transmission to humans by frugivourous (megachiropteran) or insectivorous (microchiropteran) bats is rare, but has

FIGURE 5.8
Vampire bat (*Desmondus rotundus*) captured in a mist net

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emerged as the prominent source of human rabies in areas where carnivore-variant rabies has been controlled. There are typically one to two human fatalities a year due to bat rabies in the United States of America, and as few as ten human cases of EBLV-1, EBLV-2, ABLV, Duvenhage or Mokola have been reported since their discovery.

From 1946 to 2009, at least 57 of 250 cases of human rabies in the United States of America were attributed to bat rabies variants (Messenger *et al.,* 2003; Blanton, Palmer and Rupprecht*,* 2010). Of the bat-variant cases, the majority (at least 30) were associated with a single variant, known as the Ln/Ps variant, found among silver-haired (*Lasionycteris noctivagans*) and eastern tricoloured (*Perimyotis subflavus*) bats.

Of the remaining bat-variant cases, at least 13 were attributed to a rabies virus variant associated with Mexican free-tailed bats (*Tadarida brasiliensis*), two to a variant associated with big brown bats (*Eptesicus fuscus*), and two to a variant associated with *Myotis* spp. The variants associated with six cases prior to 1970 and four cases from 2004 to 2009 remain unknown.

Intriguingly, the silver-haired and eastern tricoloured bat species associated with the variant causing most human cases in the United States of America are far less common near human dwellings than are more colonial bat species, such as *Eptesicus* or *Myotis*. The epidemiological basis for this remains unclear. In addition, in many human cases a bite or other exposure to a bat may not initially be reported, although subsequent investigation usually reveals that the infected person has had contact with or proximity to a bat. Most microchiropteran bats have small but extremely sharp teeth, and blood or a wound does not have to be evident for a bite to transmit infection. The histories of human bat rabies cases in the United States suggest that people may not seek medical care following bat contact because they do not realize the risks, do not appreciate the significance of a comparatively small wound, or are unaware that exposure has occurred.

Many European bats lead generally secluded lives, and human contact with sick or injured bats is uncommon. The relatively low rate of human cases in Europe may be

FIGURE 5.9
Eastern tricoloured bat (*Perimyptis subflavus*) – one of two species associated with the variant of bat rabies most frequently isolated from human rabies in the United States of America

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because the bats that people most frequently encounter, such as the common pipistrelle (*Pipistrellus pipistrellus*), are rarely associated with EBLV (Freuling *et al.,* 2009a). Education and vaccination of bat rehabilitation workers can minimize the risk to public health.

In Australia, people most frequently come into contact with bats when they attempt to rescue sick, injured or orphaned flying foxes. Loss of habitat through agriculture and urbanization has contributed to the urbanization of the four common species of flying foxes. They are frequently injured or trapped on powerlines, barbed wire and fruit tree netting, and are comparatively large (300 to 900 g) and obvious, bringing them into contact with humans. As in Europe, Australians have less frequent contact with microchiropoterans, and contact with the solitary yellow-bellied sheath-tailed bat (*Saccolaimus flavientris*), which is the only known microbat host for ABLV, is very rare.

Of the two human cases of ABLV to date, one involved an unvaccinated bat rehabilitator bitten by a rescued microchiropteran (*S. flavientris*), and the other a woman who was bitten when she tried to remove a flying fox that had flown, unprovoked, on to the back of a child

at night. Both bats displayed uncharacteristic aggression, which is an uncommon (occurring in about 20 percent of cases) clinical sign of ABLV but should be considered as a high risk factor for ABLV transmission (Barrett, 2004).

The vast majority of lyssavirus infections in livestock are the result of spill-over of carnivore rabies variants. Bat lyssavirus spill-over is most frequent with genotype 1 bat rabies viruses in the Americas, resulting in dead-end spill-over infections of cattle, cats, foxes and other mammals (Kuzmin and Rupprecht, 2007). The factors driving emergence of a variant associated with big brown (*Eptesicus fuscus*) and Myotis bats as a self-sustaining epizootic in skunks in Arizona, United States of America are unclear (Leslie *et al.,* 2006).

Spill-over of EBLV-1 is rare, with detections in sheep, a stone marten and two cats (Freuling *et al.,* 2009b). Spill-overs of Lagos bat and Mokola viruses have been detected in cats, dogs and wild carnivores (Kuzmin and Rupprecht, 2007; Markotter *et al.,* 2008). There have been no detections in animals other than bats of ABLV, Duvenhage, EBLV-2 or those lyssavirus genotypes for which only single isolations have been made, although their similarity to bat rabies and EBLV-1 suggests that exposure may lead to undetected clinical disease in other mammals on rare occasions.

The evolution of lyssaviruses in bats or carnivores has been the subject of considerable debate (Holmes *et al.,* 2002; Davis *et al.,* 2005; Hughes, Orciari and Rupprecht, 2005; Davis, Bourhy and Holmes, 2006; Hughes, 2008). It remains unclear whether lyssavirus genotypes evolved first in bats or carnivores. It as been proposed that genotype 1 rabies virus was introduced to American bats by terrestrial animals during European colonization.

Control and prevention

Vaccination programmes to control lyssavirus infection in maintenance host species are the most effective means of controlling spill-over infection to humans and other animals. Animal control, parental and oral vaccination programmes have been successful in eradicating or controlling carnivore rabies in dogs and wildlife, including foxes, raccoons and skunks (Rupprecht *et al.,* 2008). Vaccination of free-living bats is not feasible, and in the absence of effective control strategies for eradicating lyssaviruses in bats, it is necessary to manage spill-over of bat lyssaviruses to humans and other animals.

The impact of vampire bat rabies on livestock can be minimized by the prophylactic vaccination of cattle. The unique blood-feeding behaviour of vampire bats, and their sensitivity to anticoagulants also allow the local suppression of vampire bat populations by poisoning. Anticoagulant gel can be applied to the coats of Judas bats and spread through the colony through communal grooming, or anticoagulant can be administered topically to recent wounds or intramuscularly to cattle, to be transferred to vampire bats during blood feeding (Kuzmin and Rupprecht, 2007). Culling of other bats is inappropriate owing to the significant role they play in the environment. In Europe and Australia, the value of bats is recognized by their legal protection and conservation. Due to the rare incidence of other bat lyssaviruses in other animals, pre-exposure vaccination specifically to provide protection against them is not justified, except for in rare or valuable individuals.

The World Health Organization (WHO) and the United States' Advisory Committee on Immunization Practices publish regularly updated guidelines and recommendations on the use of vaccines for management of human rabies (WHO, 2007; Manning *et al.,*

2008; Rupprecht *et al.,* 2010). All current commercial human and animal vaccines used for lyssavirus prophylaxis were developed for the prevention of genotype 1 rabies virus. Rabies vaccines and immune globulin have been shown to provide adequate cross-protection against phylogroup 1 lyssaviruses (EBLV, ABLV and Duvenhage) but not the more divergent phylogroup 2 and 3 viruses, particularly Lagos bat, Mokola, West Caucasian and Shimoni bat viruses (Brookes *et al.,*2005; Hanlon *et al.,* 2005; Kuzmin *et al.,* 2010). Poor cross-protection against the divergent viruses is supported by cases of Lagos bat and Mokola virus infection in rabies-vaccinated cats and dogs. Recommendations for the use of rabies vaccines to provide cross-protection against ABLV in humans are included in *The Australian immunisation handbook* (Australian Government, 2008).

Prevention of bat lyssavirus spill-over in humans is based on the management of any bat bite (penetration of the skin by teeth) or non-bite exposure, defined as contamination of open wounds, abrasions (including scratches) or mucous membranes with saliva or other potentially infectious material (e.g., neural tissue). Key elements of the published recommendations include:

- limiting contact with bats through education programmes and exclusion housing or netting to prevent contact with bats during the night;
- avoiding bat bites and scratches through use of appropriate bat handling techniques and protective equipment, including puncture-resistant gloves;
- pre-exposure vaccination if continuous, frequent or infrequent contact with bats is anticipated, particularly for veterinary staff, cavers, bat ecologists, laboratory workers and others who work with bats or lyssaviruses;
- prompt and thorough cleansing of bites and other wounds associated with bats;
- post-exposure vaccination for bites or other potential exposure to bat lyssavirus, to provide active (rabies vaccines) and passive (rabies immunoglobulin) immunity, as recommended by WHO or relevant national guidelines.

Pre-exposure prophylaxis does not eliminate the need for risk management following a bite or non-bite exposure to a lyssavirus, but it simplifies post-exposure treatment by eliminating the need for human rabies-immune globulin and decreasing the number of post-exposure vaccine doses to two injections. Pre-exposure prophylaxis reduces the risk to individuals where medical attention or rabies biologics are unavailable, or when post-exposure vaccination is delayed or not sought after unapparent exposure. People at continuous or frequent risk of exposure should ensure they maintain an adequate immune response by monitoring their serum titres of rabies virus-neutralizing antibodies and getting booster vaccinations according to international or relevant national recommendations (WHO, 2007; Australian Government, 2008; Manning *et al.,* 2008; Rupprecht *et al.,* 2010).

Pathogenesis and clinical presentation

Virus introduced through the skin or mucous membranes may undergo replication in local tissues, or enter the nerve process directly and ascend to the nerve cell bodies of ganglia, the spinal cord or brain stem. Virus replicates in the nerve cell cytoplasm, forming accumulations of viral protein that may become large enough to be visualized by histology or immuno-histochemistry (IHC), and rapidly disseminates through the nervous system and to non-neural tissue via nerve processes (Gosztonyi, 1994).

By the time clinical signs develop, virus is widespread in neuronal and non-neural tissues. Spread to the salivary glands and excretion in saliva enables further transmission.

The incubation period in humans is typically one to three months, but may vary from less than one week to more than a year (WHO, 2007), and a similar range of incubation times from weeks to months have been documented in bats (Field, McCall and Barrett, 1999; Constantine, 2009). The location of viral sequestration and the factors responsible for the delayed progression of long incubation periods are unknown. The precise mechanisms producing nervous system dysfunction and death are complex. Disruption of cellular functions, loss of homeostasis and inflammation usually culminate in multi-system organ failure and death.

Classically, clinical rabies in humans and dogs is described as "furious" (encephalitic) or "dumb" (paralytic). However, the clinical presentations of all genotypes of lyssavirus in all species span a broad spectrum of signs reflecting abnormal function of the central, peripheral and autonomic nervous systems. Infections with different lyssaviruses are not clinically distinguishable.

Clinical signs in bats reflect alterations in behaviour and motor function and rapidly progress to death within hours or days. In Microchiroptera and flying foxes, signs include unusual aggression or tolerance of people, fighting with other bats, being active and away from roosts and camps during the day, biting and vocalizing, and being grounded and unable to fly (Barrett, 2004; Constantine, 2009). Flying foxes infected with ABLV are frequently found on the ground, hanging inappropriately low in trees or on human-made structures away from their camps (e.g., in backyards). In most cases, the clinical signs are dominated by paresis or paralysis. A minority of cases (19 of 74, 19 percent) showed signs of overt aggression, including flying out of trees in unprovoked attacks, and repeated attempts to bite (Barrett, 2004).

As natural infections in bats are rarely observed, and testing for lyssavirus is usually post-mortem, diagnosis of lyssavirus in bats is biased towards cases with fatal outcomes. The full scope of the natural history of lyssavirus infection in bats is probably not known. The high – typically 5 to 20 percent – sero-prevalence of lyssavirus antibodies in bat populations (Dzikwi *et al.,* no date; Lumlertdacha *et al.,* 2005; Kuzmin *et al.,* 2008; Vazquez-Moron *et al.,* 2008) and experimental inoculations of EBLV (Vos *et al.,* 2004; Brookes *et al.,* 2007) and ABLV (McColl *et al.,* 2007) suggest that these viruses may be less virulent than genotype 1 rabies virus.

Diagnostics

Definitive diagnosis of lyssavirus infection is based on viral isolation or detection of viral antigens, antibodies or viral genome. Lyssavirus identification to the level of specific variants requires antigenic typing with a panel of monoclonal antibodies, or molecular characterization. Detection of lyssavirus antibodies indicates current infection, past exposure or rabies vaccination.

Primary identification of a lyssavirus infection is typically made by direct examination of brain impressions and demonstration of viral inclusions (antigens). Virus may also be isolated in the brains of inoculated animals or cell cultures. Electron microscopy may be used to determine a morphologic identification of lyssaviruses by examination of the virion ultrastructure in cell cultures or tissues. Standard protocols for laboratory diagnosis of rabies

are published by the World Organisation for Animal Health (OIE) and the World Health Organization (WHO).

Appropriate samples

Definitive diagnosis of lyssavirus infection requires examination of fresh or 10 percent-buffered formalin-fixed tissues.

Primary diagnosis should be performed using fresh CNS samples, particularly the brain stem, whenever possible. These samples are usually only available post-mortem. Tests are available for formalin-fixed tissues, but fixed samples are not recommended for primary diagnosis unless fresh brain tissues are unavailable owing to longer tissue processing and testing times.

Removal of CNS tissues should be performed by trained, rabies-vaccinated personnel, using appropriate personal protective equipment (PPE; gowns or lab coats with sleeves, double-latex or heavy rubber gloves, face shields). Use of a biosafety cabinet is recommended.

When CNS tissues are not available, non-CNS tissues or saliva may be tested, but test sensitivity is likely to be reduced by the relatively low concentration of virus in non-CNS tissues and fluids, increasing the potential for false negative results.

Serum and cerebrospinal fluid may be tested for the presence of anti-lyssavirus antibodies, indicating an immune response to current or prior infection. Sero-conversion may be late or absent in clinical cases.

All unfixed samples should be shipped on dry ice (or icepacks for same-day delivery) to the diagnostic laboratory, by the most expedient method.

Unfixed tissue samples may be stored for up to 48 hours at 4 °C, or for up to four weeks at -20 °C. Unfixed tissues stored for longer periods should be kept at -80 °C. Serum and cerebrospinal fluid samples for serologic testing should be stored at -20 °C or below. Saliva or other biological fluid samples should be stored at -80 °C or below.

Haemolysis of red cells compromises the test reliability of serum samples and can be minimized if whole blood is centrifuged and serum separated prior to transport. Whole blood should not be shipped in the same container as samples on dry ice, because there is a risk of freezing and haemolysis, regardless of packing insulation. Whole-blood samples should be centrifuged and serum removed prior to freezing.

Formalin-fixed tissues should remain in the fixative for a minimum of 24 to 48 hours at room temperature. Thereafter, formalin-fixed brain tissues should be stored in 70-percent ethanol at room temperature for long-term storage, and never frozen.

Paraffin-blocked tissues and tissue sections (slides) should be stored at ambient temperatures and not frozen.

Available tests

A range of tests for lyssavirus provide different degrees of specificity for the particular genotype or variant. For routine primary lyssavirus diagnosis, antigen detection tests, such as the direct fluorescent antibody test, are highly sensitive and broadly reactive, and are less expensive and less time-consuming than other methods.

Viral protein detection: Lyssavirus diagnosis is most efficiently based on the detection of abundant intracellular collections of viral proteins (antigen) that form viral inclusions. Direct

detection methods can be used to detect viral antigens and histopathological changes and to observe virion morphology.

All virus antigen tests require thorough tissue sampling (including complete cross-sectioning of the brain stem and cerebellum) to ensure that samples include adequate quantities of viral product. These methods provide rapid diagnosis within minutes or hours without the need for amplification as required for genome or live virus detection:

1. antigen detection:
   a. direct fluorescent antibody (DFA) test and formalin-fixed DFA;
   b. immuno-histochemistry (IHC) – direct rapid IHC test (DRIT) or routine IHC tests;
   c. antigenic typing;
2. histology;
3. electron microscopy.

The standard test for virus antigen detection in CNS tissues is the DFA test on fresh brain impressions (Centres for Disease Control and Prevention; WHO). This test is easy to perform, highly specific and approximately 100 percent sensitive for most genotypes, and can be completed within three to four hours. The DFA conjugates consist of lyssavirus-specific fluorescein-labelled antibodies that are visible with a fluorescent microscope. The conjugates may be hyper-immune polyclonal or monoclonal antibodies directed against one or more highly conserved rabies or variant-specific epitopes. Morphologically fluorescein-labelled intracellular inclusions occur as fluorescent large or small (oval or round) inclusions, dust-like particles or strands.

The DRIT is an alternative antigen test that uses a cocktail of purified biotinylated anti-rabies virus nucleocapsid monoclonal antibodies to detect antigen. The test has demonstrated preliminary sensitivity and specificity equal to that of the DFA test in detecting rabies virus antigens (Lembo *et al.,* 2006). Advantages of this procedure include the rapidity of the test protocol (one hour for completion), the fixation of brain touch impression slides in formalin (inactivating rabies virus), and the minimal equipment requirements (ambient incubation temperatures, standard light microscope).

Antigenic typing in indirect fluorescent antibody tests is an inexpensive, rapid and easily performed method of determining virus variants in a few hours. Panels of murine anti-rabies virus nucleoprotein monoclonal antibodies (MABs) are used to distinguish virus variants by the different reaction patterns. Tests are performed on acetone-fixed brain impression slides or virus-infected cell culture slides. Amplification of virus by inoculating cell cultures or animals may be required if the primary test material contains low levels of antigen. If antigenic typing results are inconclusive, additional testing can be performed at reference laboratories, which have a more extensive panel of MABs and resources for sequence analysis.

Routine formalin-fixed CNS tissue samples cannot be tested by the standard DFA or DRIT tests because the chemical cross-linking of proteins that occurs during the fixation process alters virus antigen structure and availability. Formalin-fixed tissue samples that have been processed, embedded in paraffin and sectioned may be tested by a formalin-fixed DFA (FFDFA) or an IHC test.

The FFDFA uses fluorescein-labelled conjugates in modifications of the standard DFA that include proteinase K digestion to disassociate chemical bonds and expose rabies virus epitopes (Whitfield *et al.,* 2001). The FFDFA requires a high affinity and a high-titred

commercial source of polyclonal anti-rabies virus conjugate, and may require working dilutions of rabies virus conjugate that are five to ten times more concentrated, to detect antigen in formalin-fixed tissues.

IHC tests are a standard light microscope alternative to the FFDFA for formalin-fixed tissues and paraffin-embedded sections (Hamir *et al.,* 1996). Like the FFDFA, the IHC protocol includes enzyme digestion (pronase instead of proteinase K) to disassociate cross-linking of protein bonds. Lyssavirus antigen will appear as large or small (oval or round) inclusions or dust-like particles in the colour of the selected chromagen within the cytoplasm of infected neurons against a light-blue background of the haemotoxylin-stained tissue. Advantages of the IHC test over the FFDFA include the ability to test for virus antigen and other aetiologies simultaneously, by including antibodies to other agents; the ability to examine the histopathology of tissues; and the ability to observe slides with a standard light microscope. Disadvantages are the time required for the procedure – approximately six hours – and the number of test components to optimize.

Routine histological stains (e.g., haemotoxylin and eosin) may detect abnormal histopathology consistent with encephalomyelitis. More specialized stains (e.g., Sellers) can be used to observe typical viral eosinophilic intracytoplasmic inclusions, such as classical Negri bodies within neurons. Although of historical importance, these older diagnostic methods lack both the specificity and the sensitivity of modern antigenic or genomic tests.

Direct observation of virions by electron microscopy allows the examination of virus ultrastructure, shape and size (Gosztonyi, 1994). This technique provides supportive evidence of a rhabdovirus infection, but is less sensitive, not specific for the lyssavirus genus, genotype or variant, and too costly and time-consuming for routine diagnosis.

Genome detection:

1. reverse transcriptase polymerase chain reaction (RT-PCR);
2. RT-PCR with sequencing and phylogenetic characterization;
3. real-time PCR.

Molecular methods amplify and detect viral RNA using RT-PCR (Coertse *et al.,* 2010). These are the most sensitive and specific tests for virus diagnosis, but the reliability of RT-PCR depends on a number of variables: the sample; the methods for RNA extraction and RT-PCR; selection of appropriate primers for amplification; the quality of reagents; individual technical expertise; avoidance of contamination; interpretation of the results; and the confirmatory methods.

Molecular techniques are most useful when genotype- or variant-specific molecular typing is required, to confirm a positive diagnosis from other methods, and to provide maximum sensitivity when fresh CNS tissues are not available, particularly if the only available samples are non-CNS tissues that have low quantities of antigen. Sequencing and phylogenetic analysis provide a very high degree of resolution of the virus variant.

Real-time PCR tests are highly sensitive, exquisitely specific, very rapid and less susceptible to contamination, but may fail to detect virus if there is sequence variation. These tests are most useful when the likely genotypes and variants are known and limited, so that a small panel of primers and probes can detect all anticipated variants, as is the case in Australia. Elsewhere, the diversity among viral variants and the lack of non-degenerate universal

primers have limited the use of real-time PCR. A real-time RT-PCR for primary diagnosis developed with degenerate primers and probes is reported to have sufficient sensitivity to detect all major rabies virus variants (genotype 1), including Aravan and Khujand viruses. The future incorporation of real-time techniques that detect all lyssaviruses, including the most divergent Mokola, Lagos bat, West Caucasian and Shimoni viruses, will allow for more rapid diagnosis, less risk of cross-contamination, and test automation.

Live virus detection:

1. *in vivo* isolation (e.g., mouse inoculation test);
2. cell culture isolation.

Isolation methods amplify infectious virus in samples, and may be applied as an alternate confirmatory test to the standard DFA test. Classical methods include *in vivo* isolation in animals (usually intra-cerebral inoculation of suckling mice) and *in vitro* virus isolation in cell cultures. The identification of virus amplified by *in vivo* inoculation and cell cultures should be confirmed by antigenic or molecular methods.

For most routine diagnostic needs, inoculation of cell cultures, such as mouse neuroblastoma cells, provides the same sensitivity as animal inoculation, but with quicker results and without the ethical issues or maintenance involved in the use of laboratory animals. Propagation, amplification and quantification of virus and antibodies with cell cultures are also used to produce vaccines, determine the safety of vaccine lots, and study the pathogenesis of rabies virus in particular cells.

Antibody detection: Genotype 1 rabies virus neutralization tests are the standard tests for detecting antibodies to the rabies G protein, particularly for determining if rabies vaccination has produced adequate titres of rabies virus-neutralizing antibody. These tests are also used to detect cross-reactive immune responses to other lyssaviruses. A lack of validated serological tests for other bat lyssavirus genotypes hampers the sensitivity and interpretation of serological surveys of bats outside the Americas (Barrett, 2004; Freuling *et al.,* 2009b).

The rapid fluorescent focus inhibition test (RFFIT), fluorescent antibody virus neutralization (FAVN) test and the older mouse neutralization test (MNT) are highly specific tests that measure the ability of rabies virus antibodies in serum or cerebrospinal fluid samples to neutralize a known standard challenge virus dose. The RFFIT and FAVN both exhibit the same sensitivity and specificity in determining rabies virus-neutralizing antibodies, and test results are comparable and equivalent when converted to IU/ml (Briggs *et al.,* 1998). The RFFIT is the gold standard for determining whether or not vaccination has produced adequate titres of rabies virus-neutralizing antibody.

Indirect fluorescent antibody (IFA) tests are sensitive methods for detecting specific rabies virus IgM and IgG antibodies. Unlike the neutralization tests, the IFA test detects antibodies to rabies virus proteins other than G, and predominantly to the ribonucleoprotein. The endpoint antibody titre is the last dilution demonstrating specific fluorescence. The IFA titres cannot be interpreted as neutralizing antibody levels because the test is not G protein-specific.

ELISA methods have also been used for wildlife, but may lack sensitivity or give inconsistent results (Knoop *et al.,* 2010).

Test limitations

Test limitations arise from issues related to sample selection and storage, test performance quality, test sensitivity and specificity, delay before a diagnosis can be made, and the expertise, infrastructure and equipment required to perform the test reliably (Fooks *et al.,* 2009). The validity of different laboratory diagnostic tests depends on appropriate storage for the sample and test type. Test performance quality can be assured through quality control, laboratory accreditation and staff proficiency programmes. There is need to identify which tests can provide reliable results in the required time from the available samples, staff and equipment.

Published standard procedures for DFA, DRIT, RT-PCR and isolation methods maximize sensitivity by testing the CNS tissues most likely to be positive in rabid animals (brain stem and cerebellum). Diagnostic sensitivity and specificity can be enhanced by testing in parallel or series, usually by using broadly reactive antibody conjugates (e.g., DFA) in combination with genotype- or variant-specific antigenic or genomic assays (e.g., real-time PCR) to confirm and characterize further any positive results. Nucleotide sequence analysis, rather than antigenic typing, may be required to differentiate some variants in rabid bats.

Problems of cross-contamination can be avoided by processing necropsy samples separately, using separate containers for fixation and washing in DFA tests, and using different laboratory areas for processing samples for RT-PCR. There are no universal RT-PCR primers for all lyssaviruses, so multiple broadly reactive or degenerate primers are needed to rule out lyssavirus infection.

Most commercial diagnostic test reagents (e.g., antibody conjugates) have been developed for the diagnosis of genotype 1 rabies virus variants. These reagents cross-react very well with most genotypes, including Australian and European bat lyssaviruses, but the ability of a particular product to detect the more divergent lyssaviruses should be confirmed. The ability of reagents to detect novel bat lyssavirus will need to be established as further genotypes are discovered.

Test interpretation

The limitations, accuracy, specificity and sensitivity of each diagnostic test should be understood before interpreting test results. Consideration should be given to factors that could have led to false positive or negative results, and – where reasonable – test results should be confirmed by a combination of antigenic and genomic tests or via a reference laboratory.

Depending on the specificity of the test, a positive result is indicative of rhabdovirus infection (electron microscopy) or lyssavirus infection (broadly reactive antigenic tests, non-specific genomic tests, histopathology, *in vivo* or cell culture isolation), or can provide resolution down to the level of the specific genotype or variant (antigenic profiles, genomic sequencing and phylogenetic analysis, or real-time RT-PCR). Care must be taken not to over-interpret less specific results.

Samples that produce weak, non-specific reactions or unusual or inconclusive results should be confirmed by alternative testing methods or submission to a reference laboratory.

Diagnosis to the level of virus variant is very useful in a range of circumstances, including when determining the virus variants in human cases with unclear or unknown virus exposure

histories; discovering the emergence of new viruses; monitoring the epidemiologic spread or re-emergence of virus in defined geographical areas; detecting spill-over or host-switching of variants from the predominant host species to another species; and monitoring the success of rabies vaccination programmes.

Genetic typing is becoming routine as more laboratories are able to extract RNA, perform RT-PCR tests, and sequence viruses. The N protein gene has been the one most frequently utilized in molecular epidemiology studies. Older studies focused their analyses on short sequences of fewer than 400 nucleotides. Improving technologies have expanded the focus from single gene sequences to whole viral genomes. Genbank now holds thousands of N gene sequences (complete and partial) for comparisons, and lyssavirus researchers are focusing on the G, P and L genes. These data may assist in understanding specific gene functions in host species, viral pathogenesis, replication and virion formation.

Serology is useful for studying population levels of exposures and infection dynamics. Interpretation of bat lyssavirus serology is complicated by its reliance on detecting cross-reactions in rabies virus tests, and the use of modified genotype-specific assays that have not been standardized or validated. Post-exposure serology in a clinically normal subject is of no value for predicting whether or not clinical disease will develop for risk evaluations.

Conclusions

- Species-specific lyssavirus variants should be presumed to be present in all bat species globally.
- Enhanced surveillance and applied research are needed for a better understanding of the epidemiology, disease ecology, pathogenesis and immunobiology of lyssaviruses in bats.
- Pre- and post-exposure prophylaxis should be implemented in accordance with WHO/OIE or relevant national guidelines as part of any programme that includes direct contact with bats, whether or not a bat lyssavirus has been reported in the area or species.
- New biologics are needed for the development of a pan-lyssavirus vaccine and prophylactic control.

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BAT CORONAVIRUSES

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Introduction

The sudden emergence of severe acute respiratory syndrome (SARS) in late 2002 and its rapid global spread brought the concept and consequences of infectious disease emergence into sharp public focus. The early epidemiological clues to a wildlife origin and the subsequent detection of SARS coronavirus (CoV) in civets (*Paguma larvata*) in wet markets in southern China underlined the increasingly evident association between wildlife and emerging zoonoses. However, although it was acknowledged that the human outbreak likely originated from contact with infected market animals, it was not clear that these species were the natural reservoir of the virus. The wildlife trade in southern China is dynamic and opportunistic, and it was hypothesized that infection spilled from a less frequently traded natural reservoir to civets and other immunologically naïve species at some point in the wildlife supply chain, leading to a cycle of infection in the Pearl Delta wet markets of Guangdong, and from there to humans. A team of scientists from China, Australia and the United States of America spent two years searching for the SARS virus reservoir in nature, taking a targeted approach to the surveillance of wildlife species in southern China, and using both serologic and molecular detection methods. In bats, they identified a cluster of SARS-like CoVs from which (phylogenetic analyses indicate) the SARS CoV emerged.

An understanding of the dynamics of infection in both the natural system and wildlife markets is essential for managing the risk of future SARS outbreaks. The SARS case study offers an insight into the drivers for and complexity of disease emergence from wildlife.

CoVs (order Nidovirales, family *Coronaviridae*) cause a range of disease syndromes, including respiratory and gastroenteric disease in humans, and respiratory, gastroenteric, neurological and hepatic disease in animals, often with significant public health and economic consequences (Fraenkel-Conrat, Kimball and Levy, 1988; Lai and Cavanagh, 1997). CoVs have historically been divided into three groups (groups 1, 2 and 3) based on their antigenic and genotypic characteristics (Lai and Cavanagh, 1997). Group 2 CoVs include the SARS CoV, the aetiological agent responsible for the global outbreak of SARS. Post-SARS, bats have been identified as a natural reservoir of multiple novel group 1 and 2 CoVs, including SARS-like CoVs, the likely ancestors of SARS CoV (Lau *et al.,* 2005; Li *et al.,* 2005).

This chapter draws heavily on the unsubmitted Ph.D. thesis of Smith (unpublished).

History and impact

SARS was first reported in February 2003 in China. When the World Health Organization (WHO) declared the outbreak over on 5 July 2003, more than 8 000 cases (more than 800 fatal) had been reported in 32 countries worldwide. Knowledge of the origin of emerging agents and an understanding of the factors associated with emergence are fundamental to managing the risk of subsequent spill-overs and associated disease outbreaks. With SARS,

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a succession of phylogenetic and epidemiological findings suggested that the outbreak had a wildlife origin and originated in "wet markets" in southern China. Wildlife markets are complex and dynamic places, with a random mix of farmed and wild-caught wildlife housed, sold and slaughtered side-by-side. A WHO mission to China in August 2003 developed a causal model with interacting natural, market, human and peri-human animal components. This model was a useful tool not only for conceptualizing the likely complexity of the system, but also for identifying possible transmission control points. For example, regulation (or elimination) of the trade in wild-caught wildlife might control transmission to market and farm populations, and thus to humans; elimination of infection in the farmed wildlife population and ongoing monitoring might control transmission within this group, and thus to wildlife markets and humans.

Identifying the factors associated with the emergence of SARS requires an understanding of the ecology of infection both in the natural reservoir and in secondary market reservoir species. Thus, a necessary extension of understanding the ecology of the reservoir is an understanding of the trade and the social and cultural context of wildlife consumption. It is known that a wholesale and retail structure for the wildlife trade exists in southern China, with multiple wholesalers providing multiple retailers at the city level. It is also known that some wildlife are farmed and some wild-caught. However, what about the marketing structure? Do some dealers buy and sell from both sources? How much farm-to-farm trading occurs? Do farms periodically augment their stock from the wild?

The wildlife trade is driven by a complex mix of economic, social and cultural factors. The demand for and consumption of wildlife in southern China have increased in recent years, purportedly owing to improved economic conditions. Increases in legal and illegal wildlife trade have paralleled this growth in demand, with animals reportedly channelled from many and various locations in Southeast Asia. A rich cultural heritage underlies wildlife consumption in China. Different species and dishes are favoured for a range of social, business and health reasons. For example, the masked palm civet (*Paguma larvata*), the putative source of the human SARS outbreak, was historically eaten in winter when fresh fruit was often unavailable. People believed that eating the animal (known colloquially as the "fruit fox" or "flower fox" because of its dietary preferences) provided the same health benefits as eating fruit. In the markets, wild-caught civets still attract a price premium, because people believe they are more health-giving (and taste better) than their grain-fed farmed counterparts.

Although Guan *et al.* (2003) identified SARS CoV in *P. lavarta* and other species in wet markets in mainland China, other studies (Tu *et al.*, 2004) suggested these species were not the natural reservoir of the virus.

At the time of writing, 109 species of bats, representing 11 families and 44 genera, have been surveyed for CoVs (Table 5.3) (Lau *et al.,* 2005; Li *et al.,* 2005; Poon *et al.,* 2005; Chu *et al.,* 2006; Tang *et al.,* 2006; Woo *et al.,* 2006; Dominguez *et al.,* 2007; Lau *et al.,* 2007; Muller *et al.,* 2007; Woo *et al.,* 2007; Brandao *et al.,* 2008; Carrington *et al.,* 2008; Gloza-Rausch *et al.,* 2008; Misra *et al.,* 2009; Pfefferle *et al.,* 2009; Tong *et al.,* 2009; Reusken *et al.,* 2010). CoVs were detected in 36 species, and anti-CoV antibodies in a further seven species (Tables 5.3 and 5.4). Because of the low concentration of ribonucleic acid (RNA) in bat samples, generation of long sequences from novel bat CoVs is difficult and technically demanding (Pfefferle *et al.,* 2009).

TABLE 5.3
Global surveillance for CoVs and anti-CoV antibodies in bats

Suborder	Family	Genus	Species	PCR	Serology
Pteropodiformes	*Hipposideridae*	*Hipposideros*	*abae*	0 (16)	
			armiger	0 (113)[5]	0 (12)
			caffer		0 (14)[2]
			caffer ruber	12 (59)	
			commersoni	1 (10)	0 (16)
			larvatus	0 (2)	
			pomona	0 (23)[2]	
			pratti	0 (9)	
			ruber	0 (6)	
	Megadermatidae	*Cardioderma*	*cor*	1 (13)	
	Pteropodidae	*Casinycteris*	*argynnis*		0 (3)
		Cynopterus	*sphinx*	0 (50)[3]	0 (17)[SNT]
		Eidolon	*helvum*	6 (222)[2]	0 (6)
		Epomophorus	*gambianus*		0 (10)
			wahlbergi	0 (3)	0 (2)
		Epomops	*franqueti*		0 (5)
		Hypsignathus	*monstrosus*		1 (11)
		Lissonycteris	*angolensis*	0 (10)	1 (18)
		Myonycteris	*torquata*		1 (7)
		Rousettus	*aegyptiacus*	55 (630)[4]	28 (171)[2]
			leschenaulti	0 (2)	2 (184)[SNT]
	Rhinolophidae	*Aselliscus*	*stoliczkanus*	0 (7)	
		Coelops	*frithi*	0 (6)	
			larvatus	0 (3)	
		Rhinolophus	*macrotis*	1 (38)	
			affinis	0 (96)[5]	0 (2)
			darlingi		0 (1)
			ferrumequinum	5 (49)[2]	0 (4)[SNT]
			fumigatus		1 (204)
			landeri		0 (2)
			luctus	0 (4)	
			macrotis	1 (8)	5 (7) [SNT]
			malayanus	0 (15)	
			osgoodi	0 (2)[2]	
			pearsoni	4 (78)[2]	13 (46) [SNT]
			pusillus	0 (135)[4]	2 (6) [SNT]
			rex	0 (2)	
			rouxi	0 (6)	
			sinicus	120 (719)[6]	31 (37)

(Cont.)

TABLE 5.3 *(Cont.)*

Suborder	Family	Genus	Species	PCR	Serology
			sp.	0 (7)	
			thomasi	0 (12)	
Vespertilioniformes	*Emballonuridae*	*Coleura*	*afra*	0 (35)[2]	
		Taphozous	*hildegardeae*	0 (3)	
			mauritianus		0 (1)
			spp.	0 (8)[2]	
	Miniopteridae	*Miniopterus*	*africanus*	1 (8)	
			inflatus	7 (12)	1 (34)
			magnater	18 (218)[5]	0 (23)
			minor	1 (16)	
			natalensis	1 (7)	
			pusillus	22 (103)[5]	0 (24)
			schreibersii	18 (140)[3]	0 (1)
	Molossidae	*Chaerephon*	*pumilus*	2 (7)	0 (54)[2]
			sp.	7 (38)	
		Molossus	*major*	0 (25)	
		Mops	*condylurus*		14 (115)
			midas		0 (15)
		Otomops	*martinsseni*	2 (19)	
		Tadarida	*brasiliensis*	0 (1)	
	Mormoopidae	*Mormoops*	sp.	0 (1)	
		Pteronotus	*pamelli*	0 (31)	
	Noctilionidae	*Noctilio*	*leporinus*	0 (6)	
	Nycteridae	*Nycteris*	*argae*		0 (1)
			hispida	0 (1)	
			thebaica		0 (6)
	Phyllostomidae	*Carollia*	*perspicillata*	1 (5)	
		Desmodus	*rotundus*	1 (17)[2]	
		Glossophaga	*soricina*	1 (21)	
		Phyllostomus	*hastatus*	0 (11)	
	Vespertilionidae	*Barbastella*	*leucomelas*	0 (1)	
		Eptesicus	*fuscus*	1 (25)	
		Ia	*serotinus* *io*	0 (1) 0 (8)	
		Glauconycteris	*beatrix*	0 (1)	
		Lasionycteris	*noctivagans*	0 (2)	
		Murina	*leucogaster*	0 (5)	
		Myotis	*altarium*	0 (1)	0 (1) [SNT]
			bechsteinii	1 (13)	
			bocagei		0 (1)

(Cont.)

TABLE 5.3 *(Cont.)*

Suborder	Family	Genus	Species	PCR	Serology
			brandtii	0 (4)	
			chinensis	0 (14)[3]	0 (3)
			ciliolbrum	0 (1)	
			dasycneme	37 (172)	
			daubentonii	16 (141)[2]	
			emarginatus *evotis*	0 (6) 0 (4)	
			lucifugus	3 (31)	
			myotis	0 (4)	
			mystacinus	0 (4)	
			nattereri *occultus*	0 (2) 5 (16)	
			ricketti	14 (105)[6]	0 (2)
			sp.	0 (80)	
			volans	0 (6)	
		Neoromicia	*tenuipinnis*	0 (4)	
		Nyctalus	*aviator*	0 (6)	
			noctula	5 (43)[4]	0 (2)
			plancyi	0 (1)	0 (1) [SNT]
		Pipistrellus	*abramus*	18 (58)[3]	
			capensis		0 (1)
			deserti	0 (1)	
			nanulus	0 (6)	
			nathusii	2 (30)	
			pipistrellus	8 (35)	
			pygmaeus	3 (57)	
			sp.	0 (1)	
		Plecotus *Scotomanes*	*auritus* *ornatus*	0 (7) 0 (1)	
		Scotophilus	*borbonicus*		0 (1)
			dinganii		0 (5)
			kuhlii	5 (43)	
		Tylonycteris	*pachypus*	6 (35)[2]	

[n] Combined results from multiple ([n]) studies.

[SNT] Confirmatory serological results. Indirect immunofluorescence test, serum neutralization test (SNT) or western blot results are not included unless they were used as the primary test for anti-CoV antibody detection.

Sources: Smith, unpublished. Combined results for the detection of CoV by polymerase chain reaction (PCR) in faeces or anal swabs, and detection of anti-CoV antibodies by enzyme linked immunosorbent assay (ELISA) from 17 studies (Lau et al., 2005; Li *et al*., 2005; Poon *et al*., 2005; Chu *et al*., 2006; Tang *et al*., 2006; Woo *et al*., 2006; Dominguez *et al*., 2007; Lau *et al*., 2007; Muller *et al*., 2007; Woo *et al*., 2007; Brandao *et al*., 2008; Carrington *et al*., 2008; Gloza-Rausch *et al*., 2008; Misra *et al*., 2009; Pfefferle *et al*., 2009; Tong *et al*., 2009; Reusken *et al*., 2010).

TABLE 5.4
Global surveillance for CoVs in bats

Authors	Location	Species	Name[6]	Group	GenBank Accession[7]
Poon *et al.* (2005)	China, Hong Kong SAR[1]	*Miniopterus pusillus*	M.pus/HKSAR/Bat-CoV 61/2004	1	AY864196
Lau *et al.* (2005)	China, Hong Kong SAR[1]	*Rhinolophus sinicus*	R.sin/HKSAR/HKU3-1/2005	2b[4]	DQ022305
			R.sin/HKSAR/HKU3-2/2005	2b	DQ084199
			R.sin/HKSAR/HKU3-3/2005	2b	DQ084200
Li *et al.* (2005)	China	*Rhinolophus ferrumequinum*	R.fer/China/Rf1/2005	2b	DQ412042
		Rhinolophus macrotis	R.mac/China/Rm1/2005	2b	DQ412043
		Rhinolophus pearsoni	R.pea/China/Rp3/2005	2b	DQ071615
Tang *et al.* (2006)	China	*Myotis ricketti*	M.ric/China/BtCoV/701/2005	1	DQ648833
			M.ric/China/BtCoV/821/2005	1	DQ648837
		Miniopterus schreibersii	M.sch/China/BtCoV/773/2005	1	DQ648835
			M.sch/China/BtCoV/911/2005	1	DQ648850
		Pipistrellus abramus	P.abr/China/BtCoV/355/2005	2c[5]	DQ648809
		Pipistrellus pipistrellus	P.pip/China/BtCoV/434/2005	2c	DQ648819
		Rhinolophus ferrumequinum	R.fer/China/BtCoV/273/2004	2b	DQ648856
		Rhinolophus macrotis	R.mac/China/BtCoV/279/2004	2b	DQ648857
		Rhinolophus sinicus	R.sin/China/BtCoV/1018/2006	2b	DQ648795
		Rhinolophus sp.	R.sp/China/BtCoV/970/2006	1	DQ648854
		Scotophilus kuhlii	S.kuh/China/BtCoV/512/2005	1	DQ648858
			S.kuh/China/BtCoV/515/2005	1	DQ648822
			S.kuh/China/BtCoV/527/2005	1	DQ648823
		Tylonycteris pachypus	T.pac/China/BtCoV/133/2005	2c	DQ648794
Woo *et al.* (2006)	China, Hong Kong SAR[1]	*Miniopterus magnater*	M.mag/HKSAR/HKU7-1/2006	1	DQ249226
		Miniopterus pusillus	M.pus/HKSAR/HKU8-1/2006	1	DQ249228
		Myotis ricketti	M.ric/HKSAR/HKU6-1/2006	1	DQ249224
		Pipistrellus abramus	P.abr/HKSAR/HKU5-1/2006	2c	DQ249217
			P.abr/HKSAR/HKU5-2/2006	2c	DQ249218
			P.abr/HKSAR/HKU5-3/2006	2c	DQ249219
			P.abr/HKSAR/HKU5-5/2006	2c	DQ249221
		Rhinolophus sinicus	R.sin/HKSAR/HKU2-1/2006	1	DQ249235
			R.sin/HKSAR/HKU2-2/2006	1	DQ249213
		Tylonycteris pachypus	T.pac/HKSAR/HKU4-1/2006	2c	DQ249214
			T.pac/HKSAR/HKU4-2/2006	2c	DQ074652

(Cont.)

TABLE 5.4 *(Cont.)*

Authors	Location	Species	Name[6]	Group	GenBank Accession[7]
			T.pac/HKSAR/HKU4-3/2006	2c	DQ249215
			T.pac/HKSAR/HKU4-4/2006	2c	DQ249216
Chu *et al.* (2006)	China, Hong Kong SAR[1]	*Miniopterus magnater*	M.mag/HKSAR/Bat-CoV 1A/2006	1	DQ666337
		Miniopterus pusillus	M.pus/HKSAR/Bat-CoV 1B/2006	1	DQ666338
Woo *et al.* (2007)	China	*Rousettus lechenaulti*	R.lec/China/HKU9-1/2006	2d	EF065513
			R.lec/China/HKU9-2/2006	2d	EF065514
			R.lec/China/HKU9-3/2006	2d	EF065515
			R.lec/China/HKU9-4/2006	2d	EF065516
Dominguez *et al.* (2007)	United States of America	*Eptesicus fuscus*	E.fus/USA/RM-BtCoV 65/2006	1	EF544566
		Myotis occultus	M.occ/USA/RM-BtCoV 3/2006	1	EF544567
			M.occ/USA/RM-BtCoV 6/2006	1	EF544568
			M.occ/USA/RM-BtCoV 11/2006	1	EF544563
			M.occ/USA/RM-BtCoV 27/2006	1	EF544564
			M.occ/USA/RM-BtCoV 48/2006	1	EF544565
Gloza-Rausch *et al.* (2008)	Germany	*Myotis bechsteinii*	M.bec/Germany/D6.6/2007	1	EU375865
		Myotis dasycneme	M.das/Germany/D2.2/2007	1	EU375853
			M.das/Germany/D3.3/2007	1	EU375854
			M.das/Germany/D3.4/2007	1	EU375855
			M.das/Germany/D3.5/2007	1	EU375857
			M.das/Germany/D3.6/2007	1	EU375858
			M.das/Germany/D3.10/2007	1	EU375860
			M.das/Germany/D3.15/2007	1	EU375856
			M.das/Germany/D5.17/2007	1	EU375861
			M.das/Germany/D3.28/2007	1	EU375859
			M.das/Germany/D3.33/2007	1	EU375862
			M.das/Germany/D3.38/2007	1	EU375863
		Myotis daubentonii	M.dau/Germany/D7.3/2007	1	EU375866
			M.dau/Germany/D8.32/2007	1	EU375875
			M.dau/Germany/D8.38/2007	1	EU375874
			M.dau/Germany/D8.42/2007	1	EU375873
			M.dau/Germany/D8.45/2007	1	EU375872
			M.dau/Germany/D8.46/2007	1	EU375871
		Pipistrellus nathusii	P.nat/Germany/D5.16/2007	1	EU375864
			P.nat/Germany/D5.73/2007	1	EU375869
		Pipistrellus pygmaeus	P.pyg/Germany/D5.70/2007	1	EU375867
			P.pyg/Germany/D5.71/2007	1	EU375868
			P.pyg/Gremany/D5.85/2007	1	EU375870

(Cont.)

TABLE 5.4 *(Cont.)*

Authors	Location	Species	Name[6]	Group	GenBank Accession[7]
Brandao *et al.* (2008)[2]	Brazil	*Desmodus rotundus*	D.rot/Brazil/Bat CoV DR/2007	2	EU236685
Carrington *et al.* (2008)	Trinidad	*Carollia perspicillata*	C.per/Trinidad/1FY2B/2007	1	EU769557
		Glossophaga soricine	G.sor/Trindad/1CO7B/2007	1	EU769558
Tong *et al.* (2009)[3]	Kenya	*Cardioderma cor*	C.cor/Kenya/BtKY03/2006	1	GQ920802
		Chaerephon pumila	C.pum/Kenya/BtKY40/2006	1	GQ920836
			C.pum/Kenya/BtKY41/2006	1	GQ920837
		Chaerephon sp.	C.sp/Kenya/BtKY14/2006	1	GQ920813
			C.sp/Kenya/BtKY15/2006	2	GQ920814
			C.sp/Kenya/BtKY17/2006	1	GQ920815
			C.sp/Kenya/BtKY21/2006	2	GQ920819
			C.sp/Kenya/BtKY22/2006	1	GQ920820
			C.sp/Kenya/BtKY39/2006	1	GQ920835
		Eidolon helvum	E.hel/Kenya/BtKY18/2006	2	GQ920816
			E.hel/Kenya/BtKY19/2006	2	GQ920817
			E.hel/Kenya/BtKY20/2006	2	GQ920818
			E.hel/Kenya/BtKY23/2006	2	GQ920821
			E.hel/Kenya/BtKY24/2006	2	GQ920822
		Hipposideros commersoni	H.com/Kenya/BtKY07/2006	2	GQ920806
		Miniopterus africanus	M.afr/Kenya/BtKY42/2006	1	GQ920838
		Miniopterus inflatus	M.inf/Kenya/BtKY30/2006	1	GQ920829
			M.inf/Kenya/BtKY31/2006	1	GQ920830
			M.inf/Kenya/BtKY31/2006	1	GQ920831
			M.inf/Kenya/BtKY33/2006	1	GQ920832
			M.inf/Kenya/BtKY34/2006	1	GQ920833
			M.inf/Kenya/BtKY35/2006	1	GQ920827
			M.inf/Kenya/BtKY36/2006	1	GQ920828
			M.inf/Kenya/BtKY37/2006	1	GQ920834
		Miniopterus natalensis	M.nat/Kenya/BtKY27/2006	1	GQ920824
		Otomops martiensseni	O.mar/Kenya/BtKY02/2006	1	GQ920801
		Rousettus aegyptiacus	R.aeg/Kenya/BtKY05/2006	2	GQ920804
			R.aeg/Kenya/BtKY06/2006	2	GQ920805
			R.aeg/Kenya/BtKY08/2006	2	GQ920807
			R.aeg/Kenya/BtKY09/2006	2	GQ920808
			R.aeg/Kenya/BtKY10/2006	2	GQ920809
			R.aeg/Kenya/BtKY11/2006	2	GQ920810

(Cont.)

TABLE 5.4 *(Cont.)*

Authors	Location	Species	Name[6]	Group	GenBank Accession[7]
			R.aeg/Kenya/BtKY12/2006	1	GQ920811
			R.aeg/Kenya/BtKY13/2006	1	GQ920812
			R.aeg/Kenya/BtKY25/2006	2	GQ920823
			R.aeg/Kenya/BtKY28/2006	1	GQ920825
			R.aeg/Kenya/BtKY29/2006	1	GQ920826
		Scotoecus sp.	S.sp/Kenya/BtKY04/2006	1	GQ920803
Pfefferle *et al.* (2009)	Ghana	*Hipposideros caffer ruber*	H.caf.rub/GhanaBoo/8/2008	1	FJ710045
			H.caf.rub /GhanaBoo/10/2008	1	FJ710053
			H.caf.rub /GhanaBoo/19/2008	1	FJ710046
			H.caf.rub /GhanaBoo/20/2008	2 Ghana	FJ710047
			H.caf.rub /GhanaBoo/22/2008	2 Ghana	FJ710054
			H.caf.rub /GhanaBoo/24/2008	2 Ghana	FJ710052
			H.caf.rub /GhanaBoo/27/2008	2 Ghana	FJ710050
			H.caf.rub /GhanaBoo/31/2008	2 Ghana	FJ710049
			H.caf.rub /GhanaBoo/344/2008	1	FJ710044
			H.caf.rub /GhanaBoo/348/2008	2 Ghana	FJ710043
Reusken *et al.* (2010)	Netherlands		N.noc/VM182/2007/NLD	1	GQ2599960
			N.noc/VM176/2007/NLD	1	GQ2599961
			N.noc/VM366/2008/NLD	1	GQ2599962
			N.noc/VM199/2007/NLD	1	GQ2599963
			P.pipi/NLD/VM312/2008	1	GQ2599964
			M. das/NLD/VM3/2007	1	GQ2599965
			M. das/NLD/VM34/2006	1	GQ2599966
			M. das/NLD//VM84/2007	1	GQ2599967
			M. das/NLD/VM105/2006	1	GQ2599968
			M. das/NLD/VM62/2007	1	GQ2599969
			M. das/NLD/VM73/2007	1	GQ2599970
			M. dau/NLD/VM222/2007	1	GQ2599971
			M.dau/NLD/VM303/2008	1	GQ2599972
			M. dau/NLD/VM361/2008	1	GQ2599973
			M. das/NLD/VM7/2007	1	GQ2599974
			M. das/NLD/VM284/2008	1	GQ2599975
			M. das/NLD/VM2/2007	1	GQ2599976
			P. pipi/NLD/VM314/2008	2c	GQ2599977

[1] SAR = Special Administrative Region.

[2] 136 nucleotide sequence of the conserved region of ORF1b (RNA-dependent RNA polymerase[RdRP]) only, identified to group level only, excluded from further phylogentical analysis.

[3] 121 nucleotide sequence of the conserved region of ORF1b (RdRP) only, identified to group level only, excluded from further phylogentical analysis.

[4] Putative group 2b (proposed group 4 by some authors).

[5] Putative group 2c (proposed group 5 by some authors).

[6] Coronavirus nomenclature: host species/country of origin/laboratory identification/year collected.

[7] GenBank accession for the conserved region of ORF1b (RdRP) or the entire genome sequence from which the conserved region was trimmed.

Sources: Smith, unpublished. Combined results for the detection of CoVs by PCR in faeces or anal swabs (Lau *et al.,* 2005; Li *et al.,* 2005; Poon *et al.,* 2005; Chu *et al.,* 2006; Tang *et al.,* 2006; Woo *et al.,* 2006; Dominguez *et al.,* 2007; Woo *et al.,* 2007; Brandao *et al.,* 2008; Carrington *et al.,* 2008; Gloza-Rausch *et al.,* 2008; Pfefferle *et al.,* 2009; Tong *et al.,* 2009; Reusken *et al.,* 2010).

Group 1 bat coronaviruses

Multiple authors (Poon *et al.*, 2005; Tang *et al.*, 2006; Woo *et al.*, 2006; Chu *et al.*, 2006; Dominguez *et al.*, 2007; Gloza-Rausch *et al.*, 2008; Carrington *et al.*, 2008: Tong *et al.*, 2009; Misra *et al.*, 2009; Pfefferle *et al.*, 2009) identified group 1 CoVs in bats from a range of genera (*Cardioderma, Carollia, Chaerephon, Eidolon, Eptesicus, Glossophaga, Hipposideros, Miniopterus, Myotis, Otomops, Pipistrellus, Rhinolophus, Rousettus, Scotoecus, Scotophilus* and *Tylonycteris*) (Tables 5.3 and 5.4).

Group 1 bat CoVs have nucleotide sequence similarity (of 54 to 75 percent) to non-bat group 1 CoVs. They are highly divergent and related to CoVs previously identified from domestic animals (Figure 5.10; Poon *et al.,* 2005; Tang *et al.,* 2006). Pfefferle *et al.* (2009) identified a group 1 bat CoV in *Hipposideros caffer ruber* that shared 92 percent sequence similarity to the human CoV (hCoV)-229E. Group 1 bat CoVs have lower nucleotide sequence similarity to other CoVs from groups 2 and 3 (22 to 74 percent) and are distinguished from these groups by the addition of 14 amino acids in the spike (S) protein (Poon *et al.,* 2005; Tang *et al.,* 2006).

Group 2b (proposed group 4 by some authors) bat coronaviruses

Lau *et al.* (2005), Li *et al.* (2005) and Tang *et al.*(2006) identified SARS-like CoVs in bats from the genus *Rhinolophus* (*R. ferrumequinum, R. macrotis, R. pearsoni, R. sinicus*). SARS-like CoVs identified in these bats had 88 to 94 percent nucleotide sequence similarity to SARS CoVs identified in humans and masked palm civets (*Paguma larvata)* (Lau *et al.,* 2005; Li *et al.,* 2005). Li *et al.* (2005) compared the replicase polyprotein (RdRP), small envelope, membrane and nucleocapsid proteins with the transcription regulatory sequences (required for subgenomic RNA transcription) of SARS CoV and SARS-like CoVs, and identified high similarity (96 to 100 percent). However, the spike protein had only 64 to 80 percent similarity, and although anti-SARS-like CoV antibodies had a level of cross-reactivity among all SARS-like CoVs, they failed to neutralize SARS CoV (Li *et al.,* 2005; Tang *et al.,* 2006). This suggests that the direct progenitor of the SARS CoV detected in *P. lavarta* has yet to be identified (Tang *et al.,* 2006).

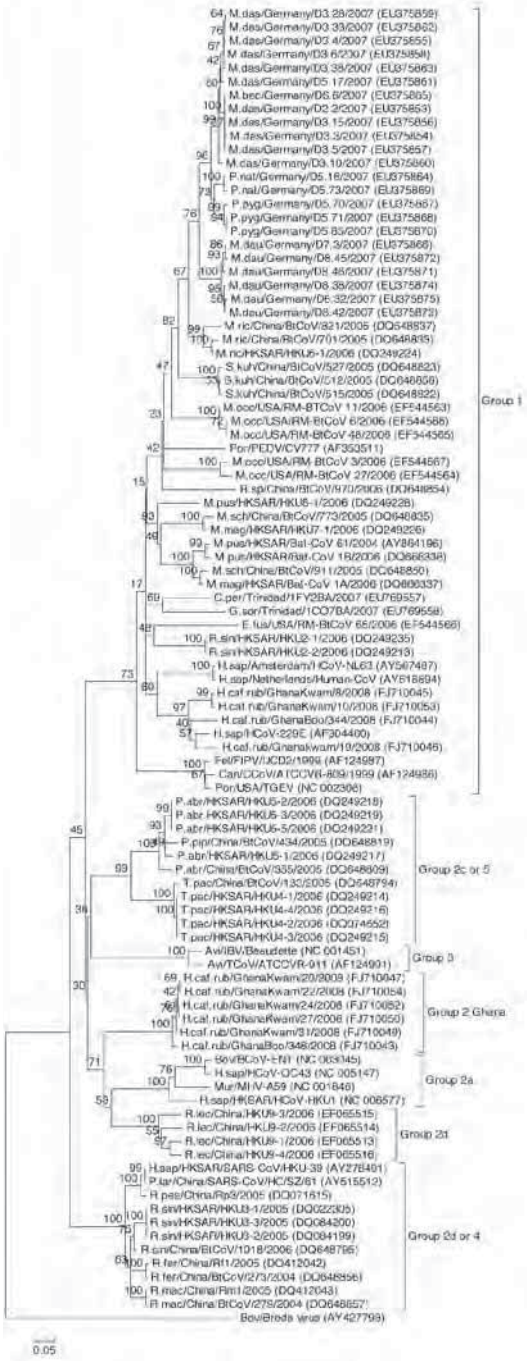
Li *et al.* (2005) found that SARS CoV and SARS-like CoVs share several unique open reading frames (ORFs) that are not found in any other CoVs, confirming an extremely close genetical relationship. Lau *et al.* (2005) concluded that SARS-like CoVs were an early split-off from other group 2 CoVs and should form the new putative group 2b, while Tang *et al.* (2006) named the putative group 4.

Muller *et al.* (2007) detected anti-SARS-like CoV antibodies in African bats and suggested that they could host group 2b CoVs. Tong *et al.* (2009) identified a bat CoV in *Chaerophon* spp., which was phylogenetically related to other SARS-like CoVs, but this analysis was conducted on only a 121 nucleotide sequence derived from the RdRP gene.

Group 2c (proposed group 5 by some authors) bat coronaviruses

Woo *et al.* (2006) identified two different CoVs, each in a different genus of bat (*Pipistrellus* and *Tylonycteris*). As these formed distinct phylogenetic groups, but were closely related to other group 2 CoVs, it was postulated that they should constitute a new subgroup, group 2c (called group 5 by some authors) (Woo *et al.,* 2007). Woo *et al.* (2006) also identified the

FIGURE 5.10
Phylogenetic reconstruction of the relationships among 96 CoVs using a 443 nucleotide-conserved region of ORF1b (RdRP)



presence of a quasi-species with two peaks (T and C) consistently observed at nucleotide position 1279 of the RdRP gene in ORF1b of HKU5-1.

Group 2d bat coronaviruses

Woo *et al.* (2007) identified bat CoV HKU9 in *Rousettus lechenaulti* from China, Hong Kong SAR and proposed the novel subgroup group 2d.

Group 2 coronaviruses

Although Tong *et al.*, (2009) conducted analysis on only a 121 nucleotide sequence derived from the RdRP gene, Group 2 CoVs were identified in bats from the genera *Chaerophon, Hipposideros* and *Rousettus*. It is suggested that the bat CoVs identified in *Rousettus* are similar to the bat CoV HKU9, identified in *R. lechenaulti* from China, Hong Kong SAR and are likely to be genetically related to other group 2d bat CoVs (Tong *et al.,* 2009). Brandao *et al.* (2008) also identified a group 2 bat CoV in *Desmodus rotundus,* but having analysed only a 136 nucleotide sequence were unable to specify which sub-group of group 2. Pfefferle *et al.* (2009) identified group 2 bat CoVs in *Hipposideros caffer rubber,* which reliably formed a new sub-group sharing a common ancestor with group 2b SARS-like CoVs identified in bats.

The reconstruction shown in figure 5.10 was generated using a maximum composite likelihood neighbour-joining methodology, bootstrapped with 1 000 replicates and pair-wise deletions (Smith, unpublished). The numbers at the nodes indicate the percentage of bootstrap trees containing this node. Coronavirus nomenclature: host species/country of origin/laboratory identification/year collected (GenBank accession).

Epidemiology and disease ecology

Gloza-Rausch *et al.* (2008) identified that young age and lactation were significantly correlated with the detection of bat CoVs, but that sex and pregnancy were not, and suggested that bat CoVs could maintain themselves through infection of immunologically naive young, rather than circulating in a population throughout the year. However Chu *et al.* (2006), Tang *et al.* (2006) and Dominguez *et al.* (2007) suggested that a high viral prevalence of CoVs in bats at different locations throughout the year, and an absence of unusual mortality or illness imply that CoVs establish persistent or long-term infection in bats, a characteristic that has been detected in pigs, cats, dogs and cattle.

Poon *et al.* (2005), Chu *et al.* (2006), Woo *et al.* (2006), Tang *et al.* (2006), Gloza-Rausch *et al.* (2008) and Pfefferle *et al.* (2009) found that bat CoVs have a narrow host range and are bat genus/species-specific. Poon *et al.* (2005) identified the same CoV in three species of *Miniopterus* (*M. magnater, M. pusillus* and *M. schreibersii*) but did not detect any CoV in *Myotis chinensis* or *Myotis ricketti*, which frequently co-habit with *Miniopterus pusillus*, concluding that this CoV has a narrow host range. Chu *et al.* (2006) later confirmed this narrow host range, identifying that the group 1 bat CoV bat CoV 1A was exclusively identified in *Miniopterus magnater* while the similar bat CoV 1B was exclusively identified in *M. pusillus*. Tang *et al.* (2006) found that two species of bat (*Miniopterus schreibersii* and *Myotis ricketti*) from the same cave in Guangxi, mainland China each had a different group 1 bat CoV. Woo *et al.* (2007) also identified host tropism, concluding that the group 2c bat

CoVs HKU4 and HKU5 and the Group 2d bat CoV HKU9 were each limited to an individual species (*Tylonycteris pachypus, Pipistrellus abramus* and *Rousettus lechenaulti* respectively).

Lau *et al.* (2005), Woo *et al.* (2006) and Tang *et al.* (2006) also found that one genus/species of bat may host different CoVs, including ones from different groups. Woo *et al.* (2006) identified both group 1 bat CoVs (HKU2) and group 2b SARS-like CoVs (HKU3 and BtCoV/1018) in *Rhinolophus sinicus,* and Tang *et al.* (2006) identified group 1 (BtCov/970/06), group 2b (BtCoV/273/04) and group 2d (BtCoV/355/05) CoVs in *R. ferrumequinum*. These findings suggest that genetically divergent bat CoVs are commonly present in and specific to different bat species (Tang *et al.,* 2006).

Woo *et al.* (2006) and Tang *et al.* (2006) postulated that the diversity of CoVs in bats could be related to bats' unique properties. The diversity of bat species (bats account for 980 of the world's 4 800 recorded mammalian species) potentially provides a large number of different cell types to host different CoVs (Woo *et al.,* 2006). Their ability to fly provides great mobility and allows the possible exchange of viruses with other bat populations or other mammals (Tang *et al.,* 2006; Woo *et al.,* 2006). The roosting of large numbers of bats together also facilitates the exchange of viruses among individual bats (Tang *et al.,* 2006; Woo *et al.,* 2006). However, this diversity could also be attributable to the high mutation rates of CoVs and RNA viruses in general and to the higher chance of recombination of CoVs owing to their unique replication mechanism (Woo *et al.,* 2007). This diversity of CoVs in bats suggests that bats play an important role in the ecology and evolution of CoVs and implies that there are probably a great number of CoVs yet to be identified in bats and other animals (Lau *et al.,* 2007; Woo *et al.,* 2007).

CoVs in bats have a stable genetic population, suggesting that they are endemic, although the epidemic-like growth in all other animals indicates repeated inter-species transmissions and occasional establishment (Vijaykrishna *et al.,* 2007). Together with the positive selection pressure observed in SARS CoV identified in masked palm civets and humans, these findings support the hypothesis that SARS CoV diverged from closely related SARS-like CoVs in bats in 1986, 17 years before the SARS outbreak, and resided in an unknown intermediate host until it was introduced into the masked palm civet and human populations (Vijaykrishna *et al.,* 2007).

Poon *et al.* (2005) found that the viral sequence of CoVs identified in three species of *Miniopterus* (*M. magnater, M. pusillus* and *M. schreibersii*) were highly similar, implying that frequent interspecies transmission occurred. As the majority of *M. pusillus* were infected with this CoV (63 percent, n = 19), the authors concluded that it was likely they were the major reservoir host. Chu *et al.* (2008) also suggested interspecies transmission of bat CoVs; the bat CoVs HKU7 and HKU8 identified at relatively low rates in the genus *Miniopterus* showed a close genetic relationship to the bat CoV Shandong/977/2006 identified in *Rhinolophus ferrumequinum*. Gloza-Rausch *et al.* (2008) also suggested that the bat CoV identified in *Myotis bechsteinii* (BtCoV/M.bec/Germany/6.6/2004), which is closely related to the bat CoVs identified in *M. dascycneme*, could have been the result of interspecies transmission. Pfefferle *et al.* (2009) also identified a group 1 bat CoV in *Hipposideros caffer ruber*, which shared 92 percent sequence similarity to the human CoV hCoV 229E. The authors suggested that this was the result of interspecies transmission 208 to 322 years ago, but postulated that direct transmission from bats to humans would have been difficult

owing to the small viral load normally detected in bat faeces. These findings suggest that some bat CoVs have the ability for interspecies transmission, which is relevant to the genesis of SARS CoV in masked palm civets and humans (Chu *et al.,* 2008).

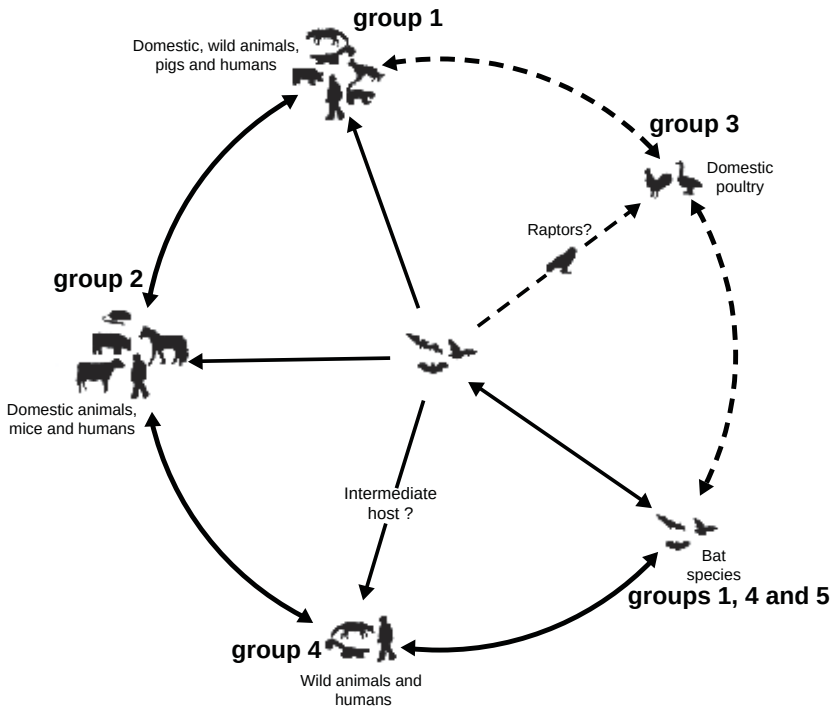
Recombination may allow adaptation to new hosts and ecological niches, and transmission of CoVs among bats, other wildlife, livestock, companion animals or humans (Lau *et al.,* 2005; Poon *et al.,* 2005; Tang *et al.,* 2006; Woo *et al.,* 2006; 2007). Chu *et al.* (2006) identified a group 1 bat CoV in *Miniopterus magnater,* which fell into lineage 1B in RdRP nucleotide sequence analysis but clustered with lineage 1A when the nucleo (N) gene was used for analysis. Chu *et al.* (2006) suggested that a recombination of lineages 1A and 1B may have occurred and that there was ample opportunity for co-infections and recombination of bat CoVs. Chu *et al.* (2008) later confirmed co-infection of bat CoVs by identifying both bat CoV 1B and HKU8 in *Miniopterus pusillus*, suggesting that this could provide opportunities for recombination of bat CoVs. In addition, a 14 amino acid conserved region found in the S protein of all group 1 CoVs is deleted from a group 1 bat CoV (HKU2), SARS and SARS-like CoVs (Lau *et al.,* 2007). So although HKU2 is a group 1 CoV, Lau *et al.* (2007) conclude that it appears to have acquired its S protein through a recombination event with SARS or a SARS-like CoV from group 2b, or that HKU2, SARS and SARS-like CoVs had a common ancestor. Woo *et al.* (2007) identified the non-structural proteins 7a and 7b in the group 2d bat CoV HKU9, previously only recognized in feline infectious peritonitis virus (FIPV), a group 1 CoV. These two genes identified in HKU9 were shown to be under high selective pressure, which may have been due to recent acquisition by combination (Woo *et al.,* 2007). Although this is further evidence of recombination, such recombination would have required infection of an individual animal (bat or cat) with both HKU9 and FIPV, which would have required an inter-species transmission event.

CoVs identified in bats have great genetic diversity and are older than any CoVs previously identified in other animals, suggesting that bats are likely to be the natural reservoir host for all known CoVs, including human cold CoVs (Figure 5.11; Vijaykrishna *et al.,* 2007).

Similarities among bat CoVs, SARS-like CoVs and SARS CoV suggest a common ancestor, while differences in the nucleotide sequence of the S protein distinguish between SARS-like CoVs in bats and SARS CoV in humans and masked palm civets (Lau *et al.,* 2005; Ren *et al.,* 2006). A 29 nucleotide region present in ORF8 of SARS-like CoVs identified in bats, SARS CoV identified in masked palm civets and SARS CoV identified in human cases from the early phase of the SARS outbreak were deleted from the SARS CoV identified in human cases from the middle to late phases of the outbreak, indicating the evolution of an increasingly pathogenic CoV responsible for the SARS outbreak (Lau *et al.,* 2005; Li *et al.,* 2005). Ren *et al.* (2006) also found that in spite of the evidence for strong positive selection of SARS CoV, indicating a recent interspecies transmission, SARS-like CoVs in bats did not demonstrate this positive selection and had evolved independently within bats for a relatively long time.

Woo *et al.* (2007) identified two closely related group 2c CoVs (HKU4 and HKU5, from *Tylonycteris pachypus* and *Pipistrellus abramus* respectively) and speculated that they originated from a common ancestor, diverging into two different CoVs through adaptation in different hosts and ecological niches.

FIGURE 5.11
Postulated evolution of CoVs



All animal and human CoVs (groups 1, 2, 4 and 5, also known as groups 1, 2a, 2b and 2c) evolved from the interspecies transmission of CoVs from bats (solid lines). Interspecies transmission and evolution of group 3 CoVs in poultry possibly resulted via an intermediary host such as a raptor (dashed lines) preying on bats and poultry.

Source: Vijaykrishna *et al*., 2007.

Pathogenesis and clinical presentation

SARS patients presented with symptoms after a mean incubation period of six to seven days (ranging from one to 20 days) (Chan-Yeung and Xu, 2003; Huo *et al.,* 2003; Tsang *et al.,* 2003; Wu *et al.,* 2003). The first symptom in 85 to 100 percent of patients was a fever (> 38 °C) for a mean duration of nine days (Booth *et al.,* 2003; Tsang *et al.,* 2003; Wu *et al.,* 2003; Liu *et al.,* 2004; Muller *et al.,* 2006). Other symptoms included fatigue (in 7 to 94 percent of patients), a non-productive cough (63 to 86 percent), sputum production (67 percent), chills and rigors (8 to 56 percent), headache (11 to 37 percent), general malaise (a general feeling of illness, 36 percent), myalgia (muscle pain or tenderness, 18 to 49 percent), dyspnoea (difficulty in breathing, 42 to 80 percent), sore throat (10 percent), vomiting and neck pain (Booth *et al.,* 2003; Huo *et al.,* 2003; Rainer *et al.,* 2003; Tsang *et al.,* 2003; Wu *et al.,* 2003; Xiao *et al.,* 2003; Babyn *et al.,* 2004; Liu *et al.,* 2004; Wong *et al.,* 2004). Diarrhoea was reported in 10 to 66 percent of patients and rhinorrhoea in 2 to 23 percent, but these were not predictors of SARS (Booth *et al.,* 2003; Babyn *et al.,* 2004; Liu *et al.,* 2004; Wong *et al.,* 2004; Muller *et al.,* 2006).

Laboratory findings included leucopenia (low white blood cell count, in 33 to 68 percent of patients), lymphopenia (low lymphocyte count, 53 to 95 percent), thrombocytopenia (low platelet count, 28 to 40 percent), hypocalcaemia (60 percent), hypoxaemia (low concentration of oxygen in arterial blood), elevated levels of lactate dehydrogenase (indicating anaerobic respiration, 58 to 88 percent) and aspartate aminotransferase or alanine aminotransferase (indicating hepatic cellular damage, 27 to 62 percent) (Booth *et al.,* 2003; Huo *et al.,* 2003; Tsang *et al.,* 2003; Wu *et al.,* 2003; Liu *et al.,* 2004; Wong *et al.,* 2004; Muller *et al.,* 2006). Levels of creatine kinase (indicating muscle damage) were reported as high by Liu *et al.* (2004) (at 18 to 32 percent) but were found to be normal by Tsang *et al.* (2003). Abnormal chest radiographs were noted in 61 to 80 percent of patients (Huo *et al.,* 2003; Zhao *et al.,* 2003; Babyn *et al.,* 2004; Paul *et al.,* 2004). Abnormalities included small or large, single or multifocal patchy shadows or opacities (23 to 60 percent), which appeared after two to five days, and ground-glass-like opacification or consolidation (31 to 45 percent), which appeared after six to 19 days (Lu *et al.,* 2003; Zhao *et al.,* 2003; Babyn *et al.,* 2004; Guo *et al.,* 2004; Paul *et al.,* 2004).

Diagnostics

The majority of CoVs identified in bats were identified from faecal material, indicating a predominantly enteric tropism (Lau *et al.,* 2005; Poon *et al.,* 2005; Chu *et al.,* 2006; Tang *et al.,* 2006; Dominguez *et al.,* 2007; Lau *et al.,* 2007). CoVs were also detected in oral swabs, but not in blood or serum (Lau *et al.,* 2005; Li *et al.,* 2005; Poon *et al.,* 2005; Chu *et al.,* 2006; Tang *et al.,* 2006; Woo *et al.,* 2006; Dominguez *et al.,* 2007; Lau *et al.,* 2007; Muller *et al.,* 2007; Woo *et al.,* 2007; Pfefferle *et al.,* 2009).

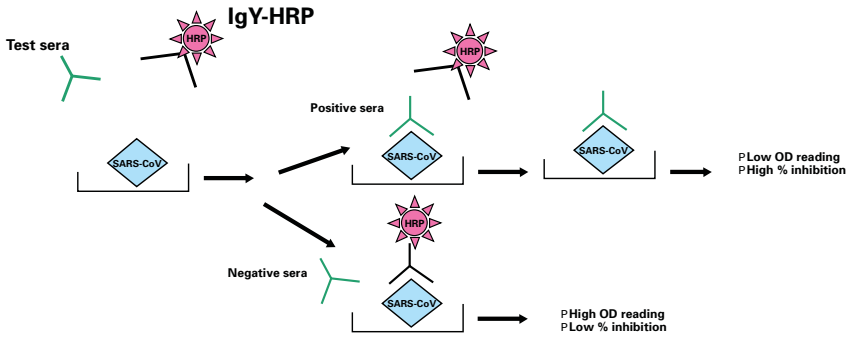
Quantitative real-time PCR: Quantitative real-time PCR targeting the polymerase and nucleocapsid genes have been developed by Ng *et al.* (2003).

Reverse transcriptase PCR (RT-PCR): Reverse transcription followed by complementary deoxyribonucleic acid (cDNA) amplification using a RT-PCR targeting a conserved region of the polymerase gene is described by Poon *et al. (*2005). Amplicons consistent with the expected length of 440 nucleotides can be sequenced and phylogenetically compared with other known CoVs.

Competition ELISA: Yu *et al.* (2006) mapped the immunodominant regions of both N and S proteins using a panel of SARS CoV sera generated in different animal species. Recombinant proteins corresponding to the immunodominant regions of the N and S proteins were used to produce chicken polyclonal antibodies for development of a competition ELISA. To simplify the procedure, horseradish peroxidase (HRP)-conjugated chicken antibodies were developed so that the detection of anti-SARS CoV antibodies could be achieved in a single incubation step (Figure 5.12).

Virus isolation: Attempts to isolate bat CoVs using African green monkey kidney (Vero E6), C6/36, Caso-2, colorectal adenocarcinoma (HRT-18G), foetal rhesus kidney (FRhK 4), human hepatoma (Huh-7 and Huh-7.5), human lung fibroblast (MRC-5), Madin-Darbyin canine kidney, rhesus monkey kidney (LLC-Mk2) and TB 1 LU cells, chicken embryonated eggs and primary bat kidney epithelial and lung fibroblast cells were unsuccessful (Lau *et al.,* 2005; Li *et al.,* 2005; Poon *et al.,* 2005; Chu *et al.,* 2006; Woo *et al.,* 2006; Lau *et al.,* 2007).

FIGURE 5.12
Competition ELISA procedure



HRP-conjugated chicken antibodies were developed so that the detection of anti-SARS CoV antibodies could be achieved in a single incubation step.

Source: Yu *et al.*, 2006.

Given the narrow host range of bat CoVs (Poon *et al.,* 2005; Chu *et al.,* 2006; Tang *et al.,* 2006; Woo *et al.,* 2006; Gloza-Rausch *et al.,* 2008; Pfefferle *et al.,* 2009), it is not surprising that all attempts to isolate them have been unsuccessful. However, with the development of bat cell lines (Crameri *et al.,* 2009), future attempts may be more successful.

Conclusion

The significance of cultural and economic drivers for disease emergence is being increasingly recognised. Parallels between the wet markets and SARS in China, and the bush meat trade and HIV-like viruses in Africa are evident. The need for a combination of "hard" and "soft" sciences and a "big-picture" view is increasingly evident. Continued surveillance will advance understanding of the diversity of CoVs in bats. This diversity, the global distribution of bats, and CoVs' propensity to cross species barriers successfully suggest that SARS-like CoVs may not be the only example of bat CoVs causing disease outbreaks.

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FILOVIRUSES

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Introduction

Filoviruses, which consist of Marburg and Ebola viruses, have collectively captured the public's imagination, as evidenced by sensationalistic non-fiction books and many press reports during viral haemorrhagic fever (VHF) outbreaks. Since the discoveries of Marburg virus in 1967 (Figure 5.13) and Ebola virus in 1976 (Figure 5.14), these viruses have caused more than 2 600 human infections, primarily in Africa, with an average case fatality of 72 percent. Although the disease burden caused by filoviruses is only a small fraction of those caused by more common diseases such as malaria and HIV, the combination of high case fatalities, rapid course of disease, human-to-human (contact) transmission, and general lack of available vaccines and therapies has placed filoviruses among the pathogens that immediately elicit fear among local communities and government agencies. History has shown that in rural African settings, filovirus outbreaks can be large (> 150 cases), necessitating rapid responses by the international community to help implement detection, case management and epidemiologic surveillance, usually for a duration of several months.

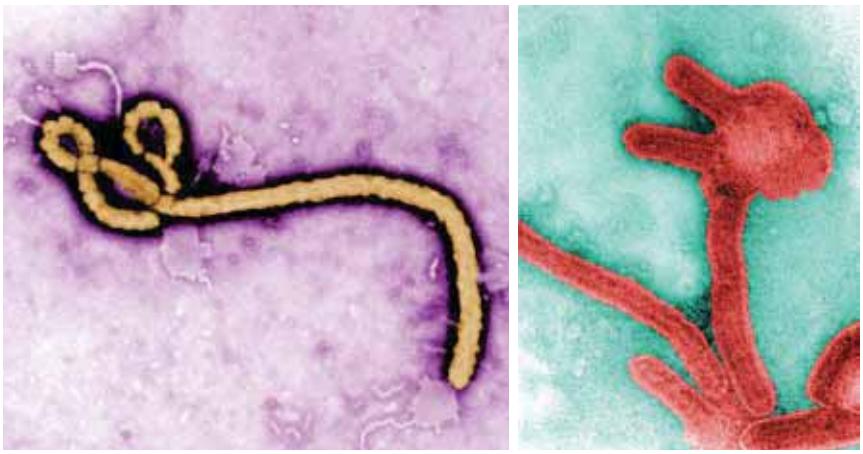
Currently there are five known species of Ebola virus (*Zaire*, *Sudan*, *Reston*, *Côte d'Ivoire* and *Bundibugyo ebolaviruses*) and one species of Marburg virus (*Lake Victoria marburgvirus*), each with clear differences in their respective pathogenicities in human and non-human primates (NHPs) (Hartman, Towner and Nichol, 2010; Towner *et al.,* 2008). Within the last dozen years, episodes of filovirus VHFs within the human population have been occurring with increasing frequency, now averaging one epidemic every one to two years (Table 5.5). These facts emphasize the paramount importance of investigations to identify the natural reservoirs of filoviruses, the results of which are implicating bats as natural hosts.

History: aetiology and linkage to natural host

Although the natural reservoirs for filoviruses have not been identified definitively, the cumulative evidence now shows that bats can be a source of infectious virus. The observations that first implicated bats where made when Marburg virus-infected monkeys consigned from Uganda to Europe in 1967 caused the first recognized outbreaks of Marburg haemorrhagic fever (MHF). The monkeys were caught or temporarily maintained on the shores and islands of Lake Victoria, and were all from areas where fruit bats were prevalent (Smith, 1982). In 1975, the second known outbreak of MHF occurred when travellers became infected and, seeking medical treatment, transmitted the virus to a health care worker in South Africa. In the previous two weeks, the travellers had slept in rooms containing insectivorous bats at two locations in Zimbabwe and had visited the Chinhoyi Caves (formerly the Sinoia Caves) where bats may also have been present (Conrad *et al.,* 1978). The following year, Ebola haemorrhagic fever (EHF) emerged for the first time in two simultaneous but geographically distinct outbreaks in Nzara, southern Sudan and Yambuku,

[e] Centers for Disease Control and Prevention

FIGURE 5.13/ 5.14
Electron micrograph



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Democratic Republic of the Congo (DRC, formerly Zaire). In the Sudan, the first six patients worked in a room of a cotton factory where bats were roosting (Arata and Johnson, 1978). In 1980 and 1987, two patients who developed MHF in Kenya had both visited Kitum Cave – which is known to harbour large colonies of bats – shortly before becoming ill (Smith *et al.,* 1982; Johnson *et al.,* 1996). In 1994, chimpanzees that developed EHF in Côte d'Ivoire had been observed feeding in a wild fig tree alongside fruit bats for two weeks before developing the disease; they then infected the investigating veterinarian (Formenty *et al.,* 1999a).

Reston ebolavirus, which has not been shown to be pathogenic in humans, was repeatedly imported via virus-infected monkeys consigned to the United States of America and Europe from the Philippines. On each occasion, the shipment originated from a single primate export facility located on the grounds of a former fruit orchard known to be frequented by fruit bats (Miranda *et al.,* 1999). In 1996, fruit bats were experimentally infected and shown to be capable of supporting Ebola virus replication for three weeks without developing overt disease (Swanepoel *et al.,* 1996). In 1998 to 2000, the longest known MHF outbreak on record occurred in Durba village in northeastern DRC, and consisted of multiple short transmission chains among workers in the Gorumbwa mine where vast numbers of bats roosted. Studies later found that multiple genetic lineages of virus circulated during this outbreak (Bausch *et al.,* 2006), some of which were detected in Egyptian rousette bats (*Rousettus aegyptiacus*) and two species of insectivorous bats that inhabited the mine, but no live virus was isolated (Swanepoel *et al.,* 2007). Retrospective investigations of hospital records found evidence of MHF in Durba six years earlier, but the outbreak went largely unrecognized. Ultimately, the mine flooded and the outbreak ceased.

In 2002, Ebola virus ribonucleic acid (RNA) was detected in three forest-dwelling species of fruit bats in the Gabon during an investigation that followed repeated outbreaks of EHF (Leroy *et al.,* 2005). In 2005, Marburg virus RNA was detected in *R. aegyptiacus* bats, also

in the Gabon, but in the absence of a corresponding outbreak of disease (Towner *et al.,* 2007b). In both investigations, efforts to isolate live virus from bats again proved fruitless. In 2007, a watershed year for filovirus emergence, an outbreak of EHF appeared in Luebo, DRC, to which bats were potentially linked (Leroy *et al.,* 2009). In the months prior to this, MHF resurged again, in miners working in the Kitaka mine in southwest Uganda (Ibanda district). This mine was found to be colonized by more than 100 000 *R. aegyptiacus* bats, from which Marburg virus was finally isolated multiple times over an eight-month period, and the genetic sequences closely matched those from the infected miners (Towner *et al.,* 2009). Within the following year, the same bat species was again associated with MHF when tourists from the United States and the Netherlands separately became ill after visiting a large colony of *R. aegyptiacus* at the nearby Python Cave in Queen Elizabeth National Park (Timen *et al.,* 2009). Also in 2007, investigations of a newly identified species of Ebola virus in Bundibugyo, Uganda (Towner *et al.,* 2008), revealed unconfirmed stories of children interacting with a bat among villagers known to consume bats occasionally. In 2008, *Reston ebolavirus* was found circulating for the first time among swine in the Philippines (Barrette *et al.,* 2009), where intensive pig farming operations were open-air and often in close proximity to fruit trees known to be seasonally frequented by bats.

These data provide a recurrent link, albeit circumstantial at times, between bats and filoviruses. The evidence linking *R. aegyptiacus* to outbreaks of MHF is convincing, given the repeated isolations of genetically diverse Marburg viruses similar to those found in humans. Scientists must continue their efforts to identify natural filovirus hosts, particularly for Ebola viruses, so as to determine the complex cycle of virus maintenance in nature. Such studies will be greatly enhanced through experimental infections of candidate reservoir species under controlled conditions.

Epidemiology and disease ecology in humans, livestock and wildlife: drivers of emergence and spill-over

Outbreaks of EHF and MHF are sporadic, often interspersed by years or even decades of no apparent disease activity. Epidemiologic investigations of filovirus outbreaks are usually difficult because the index case(s) is (are) often long-deceased, unknown or otherwise inaccessible. Nevertheless, genetic investigations can reveal insights into the transmission patterns and spread of filoviruses among human populations. Such studies have found many filovirus outbreaks to fall within two general categories: those that result from a single introduction into the human population; and those that result from multiple introductions.

Outbreaks shown to be single introductions followed by human-to-human transmission were the 1995 outbreak of EHF (*Zaire ebolavirus*) in Kikwit, DRC, the 2000 outbreak of EHF (*Sudan ebolavirus*) in Gulu, Uganda, and the 2005 outbreak of MHF in Uige, Angola (Table 5.5; Rodriguez *et al.,* 1999; Towner *et al.,* 2004; 2006). It is worth noting that these three outbreaks are among the largest ever recorded. Genetic sequencing of isolates from each of them found very few, if any, nucleotide differences between human infections occurring at the beginning and end of the outbreak, or between fatal and non-fatal cases. In some instances, identical full-length virus genome sequences (19114 nucleotides) were found in patients infected six weeks apart (Towner *et al.,* 2006). The interpretation that the Marburg virus outbreak in Angola (Uige) resulted from a single event stems from the

expectation that little or no virus evolution occurs during the repeated chains of human-to-human transmission over the course of a relatively short outbreak (< one year). Outbreaks such as these are often hospital-(Kikwit) (Khan *et al.,* 1999) or clinic-based (Uige) and may be explosive, resulting in many human cases over a short period.

Examples of outbreaks resulting from multiple spill-over events are the MHF outbreaks in Durba, DRC (Bausch *et al.,* 2006) and Ibanda, Uganda (Towner *et al.,* 2009), and the cluster of EHF (*Zaire ebolavirus*) outbreaks in Gabon from 2001 to 2003 (Table 5.5; Leroy *et al.,* 2004). Furthermore, the investigation of Ebola virus in Philippine swine revealed three distinct *Reston ebolavirus* lineages circulating among multiple pig farm operations (Barrette *et al.,* 2009). Viral genetic diversity accumulates while these viruses are maintained in nature. Repeated virus introductions into human and swine populations were evident in these instances through the detection of a diverse array of virus genetic variants circulating in the populations during the outbreak. More than 80 percent of the cases in Durba could be epidemiologically linked to subterranean mining activity or direct contact. Miners remained underground for days at a time, presumably exposing themselves to a natural source (Bausch *et al.,* 2003), which is now believed to include *Rousettus aegyptiacus* and perhaps other species of bats resident in the mine (Swanepoel *et al.,* 2007).

Studies conducted later in Uganda, in which Marburg virus was isolated from *R. aegyptiacus*, found no evidence of vertical transmission of virus among bats. Data suggest that juveniles may be exposed to virus at a particular stage of their development, possibly determined by factors such as waning maternal immunity (Towner *et al.,* 2009). Correlations between overt disease and Marburg virus infection in bats could not be determined, despite the detection of virus antigen in liver and spleen through immuno-histochemical staining. Moreover, all bats captured appeared to be active, healthy and capable of fulfilling their ecological function (Towner *et al.,* 2009).

The zoonotic sources of the virus that initiated the human EHF outbreaks in Gabon were of a different aetiology and coincided with declines in Central African wildlife (Leroy *et al.,* 2004). Genetically distinct virus chains of human-to-human transmission were linked to direct contact with infected NHPs (and one duiker) scavenged by villagers for consumption. Leroy *et al.* (2005) reported evidence of Ebola virus infection (virus antibody and RNA) in the fruit bats *Hypsignathus monstrosus*, *Epomops franquetti* and *Myonycteris torquata*. The genetic sequences of the virus RNA in the bats matched Ebola virus sequences from human isolates obtained during the same outbreak periods. Although not definitively shown, the assumption is that these bats, perhaps through some bodily fluids, transmit the virus to NHPs, which then serve as secondary amplification hosts. There is also a suggestion of seasonality in EHF outbreaks in this area. Primate mortalities have been reported to appear often at the end of dry seasons when food resources are more scarce, perhaps forcing a spatio-temporal clustering of frugivorous animals (Gonzalez, Pourrut and Leroy, 2007). Investigations to define natural Ebola transmission dynamics have been difficult and confounded by the inability to detect Ebola virus RNA reliably in these chiropteran species in the Gabon. Factors that contribute to difficulties in detection include sequence diversity, low viral loads, and the need to sample hundreds of specimens from any one species if only a small fraction is actively infected. The isolation of Marburg virus was therefore aided by the limited number of species found in subterranean environments, compared with those found in the surrounding forest, and the enhanced capability to capture large numbers of these species due to their restricted routes of escape.

Clinical presentation and pathogenesis

In humans, the most pathogenic filoviruses are *Zaire ebolavirus* and *Lake Victoria marburgvirus*, each capable of causing case fatalities ranging from 80 to 90 percent in rural African populations. Somewhat less pathogenic are *Sudan* and *Bundibugyo ebolaviruses*, causing case fatalities of 53 and 40 percent respectively (Macneil *et al.,* 2010), followed by *Reston ebolavirus,* which has not been demonstrated to cause any disease in humans. *Côte d'Ivoire ebolavirus* has caused only a single non-fatal case of EHF (Formenty *et al.,* 1999b). Human disease caused by filoviruses tends to manifest abruptly with non-specific symptoms such as fever, chills, myalgia and general malaise (Colebunders *et al.,* 2007). Additional symptoms may include lethargy, nausea, vomiting, abdominal pain, anorexia, diarrhoea, coughing, headache and hypotension (Hartman, Towner and Nichol, 2010). Despite reports in the popular literature, overt haemorrhagic symptoms such as rash, easy bruising, frequent nosebleeds, bleeding from venipuncture sites and bleeding from mucosal sites do not always occur, even in patients infected with more pathogenic filovirus species (Colebunders *et al.,* 2007). When present, these haemorrhagic symptoms generally develop late in the course of infection, during times of peak illness. The incubation period (asymptomatic period) ranges from two to 21 days (Sanchez, Geisbert and Feldmann, 2007), and in fatal cases, the mean time from symptom onset to death is eight to nine days, with patients often dying before the development of a humoral immune response (Ksiazek *et al.,* 1999; Baize *et al.,* 1999). The levels of viral genomic RNA in patient blood are 100 to 1 000 times higher in fatal than non-fatal cases (Towner *et al.,* 2004), and the progression of disease is more rapid. Where infections prove fatal, death is generally imminent shortly after the onset of coma, multi-organ failure and shock (Sanchez, Geisbert and Feldmann, 2007; Bwaka *et al.,* 1999); autopsies reveal extensive necrosis in a variety of organs, including liver, spleen, kidney, thymus, lymph nodes and reproductive organs (Zaki and Goldsmith, 1999).

For individuals who survive, convalescence can be prolonged, characterized by myalgia, arthralgia, muscle weakness, hepatitis, ocular disease, myelitis, hearing loss and even psychosis (Hartman, Towner and Nichol, 2010). Virus can be isolated up to 80 days after the onset of symptoms, in immunologically protected sites of the body, particularly in semen (Smith *et al.,* 1982; Rodriguez *et al.,* 1999; Emond *et al.,* 1977; Rowe *et al.,* 1999).

Filoviruses infect a wide variety of cell types and likely use one or more ubiquitously expressed proteins, such as lectins, to mediate cell entry (Simmons *et al.,* 2003). The cause of severe disease is likely to be a combination of host immune suppression, rapid viral replication and, ultimately, vascular dysfunction. Multiple studies have shown that the propensity for filoviruses to infect macrophages and dendritic cells, particularly in the early phases of infection, may be a root cause of increasing disease severity (Bray and Geisbert, 2005; Geisbert *et al.,* 2003a; 2003b). By infecting these central immune cells, the virus gains early entry into the lymph system, and ultimately enters blood circulation, thereby providing ready access to downstream target organs such as the liver and spleen. Once there, infection foci become established and release chemotactic factors that recruit more macrophages. Infected macrophages express tissue factor (TF) on the surface of the cells (Geisbert *et al.,* 2003a) and secrete high levels of inflammatory cytokines, which may result in uncontrolled inflammation (Simmons *et al.,* 2003; Gupta *et al.,* 2001). TF initiates the coagulation cascade, and when uncontrolled, leads to micro-thrombosis and disseminated intravascular coagulation (DIC) (Ruf, 2004). Micro-thrombosis associated with DIC leads in

turn to restricted blood supplies and multiple organ failure. Hallmarks of advanced disease include elevated liver enzymes, hepatocellular necrosis, and dysfunction of coagulation pathways and vascular systems (Zaki and Goldsmith, 1999).

Besides macrophages, dendritic cells are also early targets that when infected lead to immune suppression and disregulation. Infected dendritic cells fail to mature correctly, thereby abrogating their ability to provide co-stimulation of T cells (Bosio *et al.,* 2003; Mahanty *et al.,* 2003). Lymphocyte populations also decline rapidly, not by direct infection, but more likely due to bystander apoptosis (Baize *et al.,* 1999; Geisbert *et al.,* 2000). Filoviruses also actively inhibit the type I interferon system through a number of well-documented mechanisms involving the VP35 and VP24 proteins (Harcourt, Sanchez and Offermann, 1999; Hartman *et al.,* 2008; Hartman, Towner and Nichol, 2004; Reid *et al.,* 2006). The interferon response is a critical means by which host immune systems gain early control of virus replication; by impairing this central innate cellular mechanism, filoviruses can replicate unabated during the crucial early stages of infection.

Diagnostics

Samples suspected to contain filoviruses, especially those from infected patients, should be handled minimally and with extreme caution; any manipulations outside the field setting should be carried out in a class II biosafety cabinet within biosafety level (BSL) 4 containment. In a hospital setting, personal protective equipment (PPE) should include barrier gowns, two

FIGURE 5.15
Investigators donning full PPE for entering a subterranean mine in Uganda and capturing *R. aegyptiacus* bats infected with Marburg virus

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pairs of gloves, rubber boots, eye protection or face shield, and respiratory protection. When sampling wildlife in the field, additional measures may be necessary, including the use of bite-resistant gloves, insect repellent, hooded Tyvek coveralls, and head protection if working in confined spaces such as caves or mines (Figures 5.15 and 5.16). Positive air-purifying respirator units are particularly useful in meeting many PPE needs with a single piece of comfortable equipment. Whether in a field, hospital or laboratory setting, copious amounts of disinfecting agents such as chlorine bleach or Amphyl (hospital-grade Lysol) should be used to disinfect working surfaces and non-disposable equipment.

TABLE 5.5
Filovirus outbreaks in humans

	Date	Location	Cases	Fatality (%)
Zaire ebolavirus	1976	Zaire (now DRC)	318	88
	1977	Zaire (now DRC)	1	100
	1994	Gabon	49	65
	1995	DRC	315	88
	1996 (spring)	Gabon	37	57
	1996 (autumn)[a]	Gabon	60	75
	2001-2002	Gabon, Rep. of Congo	123	79
	2003 (spring)	Rep. of Congo	143	90
	2003 (autumn)	Rep. of Congo	35	83
	2005	Rep. of Congo	12	75
	2007	DRC	264	71
	2008	DRC	32	47
Sudan ebolavirus	1976	Sudan	284	53
	1979	Sudan	34	65
	2000	Uganda	425	53
	2004	Sudan	17	42
Côte d'Ivoire ebolavirus	1994	Côte d'Ivoire	1	0
Reston ebolavirus	1989-1990	United States of America	4	0
	1992	Italy	0	0
	1996	United States of America	0	0
	2008	Philippines	6	0
Bundibugyo ebolavirus	2007-2008	Uganda	131	40
Marburgvirus	1967	Germany, (Former) Yugoslavia, via Uganda	31	23
	1975[a]	Zimbabwe, South Africa	3	75
	1980	Kenya	2	50
	1987	Kenya	1	100
	1998-2000	DRC	154	83
	2005	Angola	252	90
	2007[b]	Uganda	4	25
	2007[c]	Uganda	1	0
	2008[c]	Uganda	1	100

[a] Subsequent transmission to a health care worker in South Africa.
[b] Linked to Kitaka mine, Ibanda, Uganda.
[c] Linked to Python Cave, Queen Elizabeth National Park, Uganda.

FIGURE 5.16
Field dissection of a bat potentially infected with Marburg virus

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For human samples, laboratory testing for acute case diagnosis includes virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA)-based antigen capture. Evidence of infection in the recent or distant past can usually be demonstrated using ELISA-based immunoglobin (Ig) M and IgG detection assays on serum from convalescent patients (Ksiazek *et al.,* 1999). For deceased patients, acute infection can often be detected by specific immuno-histochemical staining of tissues, including skin (Zaki *et al.,* 1999), but antigen detection, virus isolation and RT-PCR also work well, provided post-mortem blood can be drawn. Blood and serum are the preferred samples for diagnostic testing. However, if no other methods are available, recent data indicate that high levels of viral nucleic acid can be found in oral and nasal swabs from patients in the end stage of disease (Towner *et al.,* 2006; Formenty *et al.,* 2006; Grolla *et al.,* 2005). Antigen-capture ELISAs are broadly reactive and capable of detecting all known species of filoviruses, making them a critical component of any diagnostic testing regimen (Towner *et al.,* 2008). RT-PCR can be more sensitive and has the advantage of being used on inactivated material, but the method can be susceptible to virus genetic sequence diversity. Quantitative (Taqman) RT-PCR assays are ideal for detecting a dynamic range of viral loads, including trace quantities of viral nucleic acids. The method easily lends itself to high-throughput processing and is often preferred over standard (or nested) RT-PCR, which is more susceptible to false positives (Towner *et al.,* 2007a). In side-by-side sensitivity comparisons, quantitative (Q)-RT-PCR is on a par with nested RT-PCR, and both methods are more sensitive than virus isolation. A significant disadvantage with Q-RT-PCR is that it does not easily produce sequence information for downstream phylogenetic applications. A combination of ELISAs for viral antigen, IgG and IgM detection, along with virus isolation

and RT-PCR have proved to be a highly effective regimen for diagnosing suspected EHF and MHF patient samples from all stages of disease.

For wildlife, particularly bats, liver/spleen and blood (serum) have been the tissues of choice for filovirus screening. Virus has been found in other tissues, but the same animals were also positive by testing liver/spleen. For testing deceased animals such as NHPs that are in various stages of decomposition, bone marrow and skin may be the only options (Gonzalez, Pourrut and Leroy, 2007). In pigs infected with *Reston ebolavirus*, high viral loads were also seen in lung and lymph nodes (Barrette *et al.,* 2009). For wildlife screening, Q-RT-PCR and virus isolation are the diagnostic methods of choice for detecting active infections, while screening for virus-specific IgG is most useful for identifying animal populations with histories of exposures to filovirus infections.

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Chapter 6
Virus discovery

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INTRODUCTION

Among mammals, bats (order Chiroptera) are second only to rodents in number of species (more than 1 000) and abundance. Bats have been shown to be the reservoir hosts of a number of emerging viruses responsible for severe human and livestock disease outbreaks, including rabies, henipavirus and filovirus infections, as well as severe acute respiratory syndrome (SARS) (Calisher *et al.,* 2006; Woo *et al.,* 2006). A paralytic disease in cattle and, sporadically, in humans after vampire bat bites was reported from the time of the first Spanish colonists in Latin America. The diagnosis of rabies was first confirmed by the identification of Negri bodies in the brains of cattle during an outbreak in Brazil in 1911 (Carini, 1911). Vampire bats probably maintained rabies virus circulation for a long time prior to Europeans' discovery of America, and the association between vampire bites and the disease was understood by indigenous people, who cauterized or washed the bites to prevent the disease (Constantine, 1988). During the following century, especially in the last 15 to 20 years, increasing numbers of zoonotic pathogens have been identified in bats, leading to a surge of research interest and activities examining bats' role as an important reservoir host of zoonotic viruses.

It is now commonly accepted that viruses in bats are of high prevalence and genetic diversity, at least those in the families *Rhabdoviridae*, *Coronaviridae, Astrovirdae, Paramyoxviridae, Filoviridae, Reoviridae, Adenoviridae* and *Herpesviridae*. It is also observed that some of these viruses, which could be highly virulent in other mammalian hosts, seem to be relatively harmless in bats. The question remains whether these observations are related to fundamental differences in bats' innate ability to control virus infection, or are simply a reflection of sampling bias or the increased intensity of international surveillance in hunting for novel bat viruses. However, in spite of this uncertainty, it is clear that bats are an important source of zoonotic viruses and that there is potential for more bat-borne viruses to emerge and infect human and other animals. In this context, it is essential to develop a better understanding of bat virus diversity and ecology and of the factors important for virus spill-over from bats into other animals. Active surveillance and discovery of new bat viruses will form an important part of international efforts to improve the prevention and control of potential future outbreaks caused by bat-borne viruses.

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FIGURE 6.1
CDC investigators collecting *Rousettus aegyptiacus* bats in Kitum Cave (western Kenya), during surveillance for emerging zoonotic pathogens

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HISTORY OF VIRUS DISCOVERY IN BATS

Apart from rabies investigations, described in a separate section in Chapter 5 of this manual, the major wave of discovery of viruses in bats occurred between 1930 and 1970, following intensive efforts by government and private institutions to conduct field surveillance for viruses in vertebrates and invertebrates worldwide. Although the major aim was to survey arthropod-borne viruses, many so-called "orphan viruses" (i.e., without known disease association at the time of discovery) were also identified. More than 500 viruses were collected, and the list was published in the *International catalogue of arboviruses including certain other viruses* (Calisher *et al.,* 2006; Karabatsos, 1985).

In Australia, a wave of discovery began in 1994, when Hendra virus was discovered in Queensland following the death of more than ten horses and one human (Murray *et al.,* 1995). Fruit bats of the genus *Pteropus* were identified as the reservoir of Hendra virus (Halpin *et al.,* 2000; Young *et al.,* 1996; see also separate section in Chapter 5 for more details). The association of bats as the natural reservoir of Hendra virus was demonstrated after an extensive surveillance study of more than 40 different animal species in Queensland, Australia (Young *et al.,* 1996). This important discovery laid the foundation for the subsequent discovery of Nipah virus in human and pig specimens in Southeast Asia (Chua *et al.,* 2000; Epstein *et al.,* 2008; Montgomery *et al.,* 2008) and the eventual confirmation of flying fox as its natural reservoir (Chua *et al.,* 2002; Yob *et al.,* 2001). Surprisingly, recent serological and molecular studies suggest that a divergent henipavirus is present in western Africa – outside *Pteropus* bats' distribution range – in African straw-coloured fruit bats (*Eidolon helvum*) (Drexler *et al.,* 2009; Hayman *et al.,* 2008).

In contrast to henipaviruses, which were discovered after they caused outbreaks of disease, Tioman and Pulau viruses were first discovered as orphan viruses, but later facilitated the investigation of disease outbreaks caused by closely related viruses. Tioman virus, a novel paramyxovirus in the genus *Rubulavirus* (Chua *et al.,* 2001), and Pulau virus, a novel reovirus in the genus *Orthoreovirus* (Pritchard *et al.,* 2006), were discovered accidently during the search for Nipah virus in bat urine samples (Chua *et al.,* 2002; Yob *et al.,* 2001). The direct isolation of Tioman virus provided crucial supportive evidence for the bat origin of Menangle virus, a zoonotic paramyxovirus responsible for disease outbreaks in pigs and humans in Australia (Chant *et al.,* 1998).

The knowledge and reagent gathered from Pulau virus played a pivotal role in the rapid identification of Melaka virus, a zoonotic reovirus responsible for outbreaks of acute respiratory and enteric diseases in humans (Chua *et al.,* 2007). Molecular studies indicated that Melaka virus is highly related to the bat orthoreovirus, Pulau virus. This, together with epidemiological tracing studies, quickly identified bats as the origin of this novel zoonotic virus (Chua *et al.,* 2007). It is interesting to note that these viruses are also closely related to another orphan virus, Nelson Bay virus, which was isolated in the 1970s as part of arbovirus surveillance (Gard and Marshall, 1973). Since the discovery of Melaka virus, two additional related viruses were shown to be able to spill over and cause disease in humans. A Melaka-like virus, Kampar virus, was isolated from a throat swab of a male patient in Kampar, Perak, Malaysia who was suffering from high fever, acute respiratory disease and vomiting at the time of virus isolation (Chua *et al.,* 2008). Serological studies indicated that Kampar virus was transmitted from the index case to at least one other individual and caused respiratory disease in the contact case. Another related virus was isolated in China, Hong Kong Special Administrative Region (SAR) from a human respiratory patient who had had potential exposure to bats in Bali (Cheng *et al.,* 2009). Similar viruses are expected to be widely present in bats of different species at different geographic locations. This was confirmed by the recent isolation of the Xi River virus in Chinese *Rousettus* bats (Du *et al.,* 2010). Although it is not known whether all these related viruses are able to infect and cause disease in humans, it can be speculated that there have been undetected human infections from this group of viruses.

The SARS virus was responsible for the first serious and widespread zoonotic disease outbreak of the twenty-first century, with a huge global impact on health, travel and the economy (Peiris, Guan and Yuen, 2004). During the peak of the outbreak in 2003, viruses closely related to the human SARS coronavirus were isolated from civets and raccoon dogs (Guan *et al.,* 2003), and viral genomic materials were detected in cats, pigs and other animals (Wang *et al.,* 2006). However, further surveillance indicated that none of these animals were the natural reservoir of the virus. As demonstrated by a combination of serological and molecular methods, horseshoe bats in the genus *Rhinolophus* carry a group of coronaviruses that are closely related to the outbreak strains (Lau *et al.,* 2005; Li *et al.,* 2005). These SARS-like coronaviruses have an almost identical genome organization and a very similar sequence to the SARS virus, with the exception of the S protein, which is responsible for binding to the receptor on susceptible cell surface. It has not yet been proved whether any of the bat SARS-like coronaviruses will be able to adapt to infection in mammals other than bats and, considering the divergence of the S protein, whether intermediate evolutionary and adaptive chains exist between the human SARS coronavirus and bat SARS-like coronaviruses.

Filoviruses such as Marburg and Ebola viruses cause severe haemorrhagic fever with high fatality case rates in humans. They are also easily transmitted among humans, and several significant outbreaks have been reported from sub-Saharan Africa (Leroy *et al.,* 2005; Swanepoel *et al.,* 2007; Towner *et al.,* 2009). The index cases of Marburg infection occurred during 1967 among laboratory workers in Germany and the former Yugoslavia, who had handled tissues and blood of African non-human primates (Martini, 1969). However, the natural reservoirs of filoviruses were unknown for many years, in spite of significant international efforts: these viruses were identified only in moribund humans and apes. The situation changed between 2001 and 2005, when the ribonucleic acid (RNA) of Ebola virus was detected in tree-roosting fruit bats from the Gabon, although no direct link between human disease and bat exposure could be established at that time (Leroy *et al.,* 2005). More recently, an epidemiologic investigation putatively linked the index case of Ebola outbreak in the Democratic Republic of the Congo (DRC) in 2007 to contact with freshly killed fruit bats that were migrating in mass close to the outbreak villages and represented an important food source for local people (Leroy *et al.,* 2009).

Retrospective analysis demonstrated that the majority of human cases of Marburg virus infection could be linked to visits to caves and mines. Surveillance of a variety of animals in Durba mine (DRC) during the Marburg outbreak, demonstrated the presence of Marburg virus RNA in insectivorous bats from *Rhinolophus* and *Miniopterus* genera and in Egyptian fruit bats (*Rousettus aegyptiacus*), but not in animals from any of the other vertebrate or invertebrate groups investigated (Swanepoel *et al.*, 2007). Marburg virus RNA detected in *R. aegyptiacus* was also documented in the Gabon, Uganda and Kenya, while its detection in other bat species was only occasional. The infectious virus was isolated from *R. aegyptiacus* with high RNA load in Uganda (Kuzmin *et al.,*2010; Towner *et al.,* 2009; 2007). Gene sequences of Marburg virus strains detected in bats were identical to those detected in humans.

However, the ecology of filoviruses is still unknown. Reports on the sero-prevalence of bats are non-conclusive (Leroy *et al.,* 2005; Swanepoel *et al.,* 2007; Towner *et al.,* 2009). It is still unclear whether bats are the principal reservoir hosts of filoviruses, or represent spill-over infection from some other source. The identification of gene sequences from bat and human isolates does not necessarily mean that humans were infected from bats. Bats and humans could be independently and simultaneously infected from some other source in the mines and caves.

During the last 15 years or so there has been a dramatic increase in international attention to newly emerged or discovered bat viruses. This brief review of the recent history highlights how bat viruses have been discovered in very different scenarios, from the accidental discovery of orphan viruses to the confirmation of bat origins for known viruses, using targeted surveillance. It is anticipated that the advance of modern molecular tools and increased scientific activities in this field will uncover many more new bat viruses in the near future.

MOLECULAR APPROACHES TO VIRUS DISCOVERY IN BATS

Most viruses have been discovered routinely by cell culture isolation, electron microscopy, antigen detection assays (immunofluorescence assays or enzyme immunoassays [EIAs]), serologic assays and genome-based assays, such as polymerase chain reaction (PCR) assays.

Each method has limitations for systematic virus discovery. Cell culture isolation allows the detection of only those viruses that grow and replicate in the culture system used, and requires further characterization, usually by antigen- or genome-based assays. Electron microscopy is a relatively insensitive method for virus detection, requires a fairly high titre of virus for visualization, and also requires further characterization. Traditional antigen- and antibody-based assays rely on sera from previously infected hosts – which can be difficult to obtain – and usually detect a known virus that is suspected to be present in a biological sample, so are too specific to detect novel viruses. Genome-based PCR assays by single plex run in parallel, or multi-plex in conventional or real-time PCR can be used to identify or exclude immediately the presence of a known viral pathogen suspected to be present in a biological sample. PCR is very sensitive and specific, especially when coupled with sequencing of PCR products. However, its reliance on specific primers complementary to the pathogen genome sequence makes conventional PCR analysis unsuitable for screening of biological samples for the presence of unknown viruses.

Consensus-degenerate PCR

PCR primers are designed by using available sequences to identify the most conserved regions of the viral genome – usually the polymerase gene – among the family, subfamily or genus and then using degenerate primers, inosine residues and the consensus-degenerate hybrid oligonucleotide primer (CODEHOP) technique (Rose *et al.,* 1998) to develop primers that in theory should amplify all known or new members of a viral family, subfamily or genus. PCR methods using family, subfamily or genus consensus-degenerate primers have been used very successfully to identify and characterize a number of novel human viruses of known viral families, including coronaviruses (Sampath *et al.,* 2005; Ksiazek *et al.,* 2003; van der Hoek *et al.,* 2004), hepatitis G virus (Simons *et al.,* 1995), Sin Nombre virus (Nichol *et al.,* 1993), human retrovirus 5 (Griffiths *et al.,* 1997), and novel animal viruses such as macaque gamma-herpesvirus, bats herpesviruses (Wibbelt *et al.,* 2007), bat coronavirus, bat henipavirus (Drexler *et al.,* 2009), bat polyomavirus (Misra *et al.,* 2009), bat coronavirus (Lau *et al.,* 2005; Li *et al.,* 2005; Poon *et al.,* 2005; Tong *et al.,* 2009) and bat adenoviruses (Maeda *et al.,* 2008). However it may not be able to detect viruses of novel family for which no *a priori* sequence data exist. Its general and comprehensive approach needs to be automated in an innovative high-throughput system, so that a collection of all different family-restricted consensus-degenerate primers are compiled for systemic broad-range virus discovery.

Microarrays

A microarray is essentially hundreds or thousands of individual desoxyribonucleic acid (DNA) probes immobilized at defined locations on a solid support such as nylon membranes or glass slides. Nucleic acid hybridization is the central event in microarray technology. Two types of DNA microarray are currently used for virus identification: those using short oligonucleotide probes that are sensitive to single-base mismatches, which are used for genotyping and resequencing of known viruses such as the influenza chip (Kessler *et al.,* 2004; Sengupta *et al.,* 2003); and those based on using long oligonucleotide generic probes (60 or 70 mers) that tolerate sequence mismatches – a significant advantage over PCR-based methods –

which are used for identifying both previously recognized viruses and new or variant viruses, such as the Virochip (Wang *et al.,* 2002). Viral microarrays have been used successfully to detect novel human viruses (Kistler *et al.,* 2007; Wang *et al.,* 2003). Microarrays require sufficient sequence similarities between the virus and array oligonucleotides for hybridization to occur, making the detection of highly divergent novel viruses problematic.

Random PCR and shotgun sequencing

Arbitrarily primed PCR, representational difference amplification (RDA) and sequence-independent single-primer amplification (SISPA) of nuclease-protected viral particles followed by low-scale shotgun Sanger sequencing are strategies for amplifying genetic sequences with PCR without prior knowledge of the precise sequences and for detecting viruses through their protein sequence homologies with known viruses (Allander *et al.,* 2001; Lisitsyn and Wigler, 1993; van den Hoogen *et al.,* 2001). They rely on the degenerate binding of arbitrarily chosen primers to sample multiple complementary DNA (cDNA) or genomic DNA species during PCR, and yield viral amplicon "fingerprints", which are then shotgun-cloned and sequenced for viral identification. SISPA involves the directional ligation of a linker/adaptor oligonucleotide on to both ends of a target population of either DNA or double-strand cDNA (after reverse transcription from RNA). These technologies were used successfully to identify human meta-pneumovirus (van den Hoogen *et al.,* 2001), novel human and bovine parvoviruses, polyomaviruses (Allander *et al.,* 2001; Jones *et al.,* 2005; Gaynor *et al.,* 2007) and anelloviruses (Linnen *et al.,* 1996). However, they are technically challenging and of limited utility in rapidly detecting and identifying unknown viruses because they are complicated multi-step procedures, often require the infection of cultured cells, and often involve genomic sequence comparison between two related samples, uninfected negative control and infected sample containing virus.

High-throughput sequencing

The parallel, high-throughput capability offered by next-generation sequencing technology, including 454 sequencing and other sequencing platforms, makes them attractive for studies of microbial diversity-metagenomic sequencing. In theory, such metagenomic sequencing permits the identification of all nucleic acid sequences in a sample, provides the opportunity for considering a broader spectrum of organism, and is therefore ideal for application in novel virus discovery, although challenges remain owing to the high costs, risks of missing the less frequent viruses in a sample, and need for sophisticated analytical tools to obtain accurate microbial identification for hundreds or thousands of species in a reasonable time and at a reasonable cost. The availability of high-throughput sequencing has led to the discovery of a number of novel viruses (Finkbeiner *et al.,* 2009; Epstein *et al.,* 2010; Briese *et al.,* 2009). However, most high-throughput sequencing has been restricted to large genome centres, as it is not yet economically or logistically feasible for individual laboratories to use metagomic sequencing as a regular pathogen discovery approach.

Each of the technologies discussed in this section has strengths and weaknesses in terms of sensitivity, specificity, complexity, throughput and cost. The selection of approaches to virus detection and discovery in bats should allow for the systematic detection of a potentially unlimited range of viruses in the most cost-effective but comprehensive manner.

IMPROVEMENT OF VIRUS ISOLATION FROM BATS

Despite the rapid increase in the detection of novel viral sequences from bat specimens, there has been very limited success in the isolation of live bat viruses. This is best demonstrated by the total failure of many international groups' attempts to isolate coronavirus from bats, with more than 100 different bat coronavirus sequences detected by PCR surveillance in Asia, Africa, Europe, the Americas and Australia (see separate section on coronaviruses in Chapter 5 for more details).

Successful virus isolation will depend on many factors, each of which may play a different role in the isolation of a specific virus. The following are general considerations to be borne in mind when designing a specific virus isolation project.

Cell lines

It is self-evident that any successful isolation of live virus depends on the use of susceptible cell lines. The challenge for virus discovery is that any given cell's susceptibility to a new virus will be unknown. It is generally believed that cells from animals genetically related to the target hosts will have a high chance of being susceptible to viruses of the target hosts, as has been elegantly demonstrated by a recent publication studying host phylogeny constraints to the cross-species transmission of rabies virus in bats (Streickner *et al.,* 2010). This makes it important to develop appropriate bat cell lines from target hosts, to increase the chance of virus isolation from bats. Recently, two groups have successfully established stable cell lines from two different fruit bat species, *Rousettus aegyptiacus* (Jordan *et al.,* 2009) and *Pteropus alecto* (Crameri *et al.,* 2009).

Sampling strategy

This is especially important when trying to isolate viruses of certain known families. For example, for the isolation of henipaviruses or other paramyxoviruses it is best to take urine samples, which is a non-invasive and convenient method (Chua *et al.,* 2002). Faecal swabs are a better specimen when coronaviruses are the target (Lau *et al.,* 2005; Li *et al.,* 2005). If it is permitted to sacrifice bats for virus isolation, the strategic selection of organs will be important for the successful isolation of viruses of known tropism, such as brain tissue for lyssaviruses (Kuzmin *et al.,* 2008), and spleen and liver for filoviruses (Towner *et al.,* 2009; 2007).

Monitoring virus isolation

Although most viruses cause cytopathic effect (CPE), this may not be the case for an unknown virus in a novel bat cell line. If the aim is to capture all the live viruses from an isolation attempt, it might be necessary to rely on modern molecular techniques (e.g., microarray and next-generation sequencing) to monitor the virus isolation process. This will be very expensive, but the cost can be reduced by pooling samples, such as all the supernatant samples from the third passage of cells showing no obvious CPE.

Bat immunity

Receptor specificity has long been considered the main barrier for the cross-species transmission of viruses, and the innate immunity of host cells is increasingly recognized as another important barrier (Streicker *et al.,* 2010). Currently, there is very little published

knowledge on the innate immune system of any bat species, although progress is being made (Cowled *et al.,* 2010; Iha *et al.,* 2010; 2009). Once there is better understanding of this area, it will be possible to design and engineer bat cells that favour virus growth, as has been done for other mammalian cell systems (Young *et al.,* 2003).

Implications for human health

Because of the abundance of bats, particularly in the tropics, the bat-human interface is the important niche for pathogen spill-over and emergence. If contact with bats is the proven or suspected cause of a disease in humans, targeted surveillance must be initiated in local bat populations. Alternatively, a broad sampling approach may be implemented for pathogen discovery purposes. The surveillance activity must be approved by the relevant institutional animal care and use committee and the local or international authorities responsible for bat population management and conservation.

For pathogens that cause acute fatal disease in bats (such as lyssaviruses), sampling of sick and dead bats is far more valuable than collection of apparently healthy bats (Kuzmin *et al.,* 2008). For other infections with unknown ecology that may persist in bats, sampling of apparently healthy animals is recommended. Non-destructive sampling (e.g., collection of oral and faecal swabs, and peripheral bleeding) is feasible for relatively large-bodied bats (over 40 g), although the limitations of such sampling are obvious when body tissues are essential for virological testing (e.g., brain for lyssaviruses, liver and spleen for filoviruses). In cases of non-targeted broad surveillance, efforts must be made to create a representative panel of various bat species from different roosts.

During sampling, precautions against rabies (described in the section on Lyssaviruses in Chapter 5 of this manual) must be considered for each bat species at any geographic location around the world. The precautions for other pathogens vary, depending on the type of roost, the character of potential exposure, the bat species and the geographic location. Minimum personal protective equipment (PPE) such as latex (or preferably puncture-resistent nitrile gloves) and leather gloves may be used when sampling is conducted outside bat roosts (e.g., mist netting in an open area) in the Americas. Additional precautions should be considered when large aggregations of bats are investigated in Africa, owing to potential exposure to filoviruses, or Southeast Asia and Australia, owing to potential exposure to henipaviruses. PPE in such cases should include coveralls, gloves and respiratory protection with minimum P2 (N95) masks, or if available and preferred, powered air-purifying respirators, which must be rigorously disinfected after use. Significant PPE must also be used for handling and sampling bat species that are confirmed or suspected reservoirs of filoviruses and henipaviruses, as transmission mechanisms for these infections are currently unknown. All field and laboratory equipment, holding bags and other objects that may be contaminated during bat sampling must be disinfected after use, or disposed of.

Specific pre- and post-exposure prophylaxis is currently available only for rabies (caused by phylogroup 1 lyssaviruses, but not phylogroup 2 lyssaviruses or West Caucasian bat virus). For several other infections, experimental vaccines are under construction or laboratory trials are being made, but no prophylaxis is commercially available. The effectiveness of anti-viral medication for these pathogens is also unknown. In case of exposure, rigorous cleaning of the skin or wound with water and soap is mandatory, followed by disinfection with available

FIGURE 6.2
CDC investigators sampling collected bats in the field (Kenya), during surveillance for emerging zoonotic pathogens

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chemicals. Quarantine may be considered for encounters with highly contagious infections that can be transmitted among humans (such as filoviruses and Nipah virus).

CONCLUDING REMARKS

As for any other discovery process, no method for discovery of bat viruses will work in all scenarios. When convenient and possible, the use of a combination of different methods will undoubtedly increase the chance of success. It is conceivable that in the foreseeable future, molecular approaches will continue to lead the way in virus discovery, but technological advances in other areas, such as high-density epitope/peptide arrays and purpose-built engineered cell lines, are likely to complement molecular techniques, to achieve improved outcomes in virus detection and discovery. It should be noted that a newly discovered virus will not necessarily be pathogenic, even if it was discovered during a disease investigation. Establishing the causal relationship between a virus and a disease is a complicated process, which is beyond the scope of this chapter but has been covered by Lipkin (2010).

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Chapter 7

The use of telemetry to understand bat movement and ecology

Jonathan H. Epstein [a] *and Scott H. Newman* [b]

Migratory animals present specific management and conservation challenges. These include the need for large protected areas, such as for ungulates in Africa or North America (Berger, 1991; Thirgood *et al.,* 2004); poor knowledge of movement patterns and habitat use, such as for sea turtles (Lopez-Mendilaharsu *et al.,* 2005); different pressures on habitat in disparate regions within a species' home range, such as for neotropical migrant songbirds (Robbins *et al.,* 1989; Roca *et al.,* 1996); and inconsistent protection laws, particularly if a species' home range crosses national boundaries. In addition to conservation and management issues, epidemiological studies of wild animal hosts may be greatly informed by an understanding of the host's home range, foraging behaviour, daily habitat use or movements, and long-range seasonal movements. Movement data can help define a population; locate points of contact with other host species, including conspecifics and other species, including humans; and help define the geographic range of a pathogen, which may not be discernable through visual observation, colour marking or other monitoring techniques.

TRACKING TECHNOLOGY

Approaches to the local and long-range tracking of wildlife include direct marking, population genetics, isotope signatures and telemetry (Olival and Higuchi, 2006). Direct marking, or banding, has been widely used to track birds (Berthold and Terrill, 1991) and bats (Fleming and Eby, 2003). Movement can also be measured indirectly, including through population genetics. Desoxyribonucleic acid (DNA)-based methods have been used to describe the historical connectivity between populations (Paetkau *et al.,* 2004). Stable isotopes found in the tissues of migrant individuals are also used for the indirect inference of movement patterns, but this method lacks geographic resolution and is of questionable accuracy (Kelly and Finch, 1998; Rocque *et al.,* 2006). Although useful, none of these methods describe the specific paths of animal movements.

Telemetry has been used to track movements in a wide variety of animal species, from sea turtles to elephants to migratory birds (Galanti *et al.,* 2000; Bentivegna, 2002; Kenow *et al.,* 2002; Haines *et al.,* 2003). Animal tracking using telemetry has been critically important

[a] EcoHealth Alliance

[b] Food and Agriculture Organization of the United Nations

for understanding the movement ecology and population dynamics of these animals, and the resulting data have been used in management and conservation efforts. More recently, telemetry has been used in epidemiological studies, where understanding of the local and long-range migratory movements of the host species associated with pathogens has improved understanding of how pathogens may circulate within populations, how they can be transmitted over geographic ranges, and the seasonality or timing of pathogen dispersal (Breed *et al.,* 2010; Epstein *et al.,* 2009; Bourouiba *et al.,* 2010).

In the past, simple very high frequency (VHF) radio transmitters with accompanying power supply and antenna were attached externally to the animal or implanted. Recent technological advances have resulted in the development of Platform Transmitter Terminal (PTT) and Global Positioning System (GPS) transmitters with capabilities far beyond those of conventional VHF radio transmitters. Although modern PTT and GPS transmitters follow the same basic operating principles as VHF radio transmitters (emission of an electromagnetic signal at a specified frequency, which is detected by receivers tuned to the frequency), they use orbiting satellites to receive and relay transmitter signals. Thus, VHF, PTT and GPS transmitters have very different characteristics, rendering them suitable for very different species and studies (Table 7.1).

Although radio telemetry has long been the standard technology used to collect data on local or long-range migratory movements among a wide variety of species, including some bats, the mass of the transmitters has limited the size of the animals to which they can be applied (Wikelski *et al.,* 2007). Studies estimating the impact of added weight on a bat's ability to manoeuvre in flight led to the recommendation that a transmitter weighs less than 5 percent of the bat's body mass (Aldridge and Brigham, 1988), which is also the rule of thumb for

TABLE 7.1
Characteristics of radio transmitters used in bat telemetry studies

	Radio transmitter type		
	VHF	**Satellite (PTT)**	**Satellite (GPS)**
Transmitter weight	< 1 to 12 g	12-18 g	9-60 g
Species weight	> 20 g	> 500 g	> 450 g
Minimum cost	USD 100/each	USD 3 200/each	USD 3 800/each
Attachment	Collar	Collar	Collar
Power source	Battery	Battery or solar	Battery or solar
Duration	Days to months*	Months to years	Months to years
Range	0.1-100+ km*	Unlimited	Unlimited
Tracking	Manual	Satellite	Satellite
Tracking interval	Continuous*	4 hours	Continuous
Accuracy	± 5 m- km*	±100-200 m	±10-20 m
Frequency	VHF	UHF	UHF

* Depends on the size of the transmitter, use of aircraft for relocating animals (range 100+ km) and triangulation with resighting (accuracy 5 m).

Source: Adapted from FAO, 2007: Table 7.1

avian telemetry studies. Thus telemetry studies in small bats have been limited by the size of available devices. To date, studies using satellite PTTs and GPS data loggers have been limited to larger, frugivorous bats such as *Pteropus* or *Eidolon* species (Tidemann and Nelson, 2004; Richter and Cumming, 2008; Epstein *et al.,* 2009). As VHF transmitters have become smaller, now weighing as little as 0.37 g, small insectivorous bats can also be studied (Monadjem *et al.,* 2010).This section presents a review of VHF, PTT satellite and GPS satellite telemetry technologies for studying bat movements, and compares the advantages and disadvantages of each.

Radio telemetry

VHF radio transmitters are the most commonly used technology for tracking animals and have been in use for decades (Gillespie, 2001). Transmitters depend on a signal-emitting device (the radio transmitter), which is attached to the target animal, usually via a collar or harness (Figure 7.1a and 7.1b), although it is sometimes glued directly on to the animal's body. Transmitters vary in size and can be as small as a fraction of a gram. The transmitter emits a pulsating signal, which is received by a VHF antenna connected to a receiver that emits an audible tone. Radio transmitters vary in range, but typically signals can be detected over about 1 or 2 km, as long as they are unobstructed (e.g., with clear lines of sight between the transmitter and antennae). Signals can be heard for longer distances when animals are tracked from higher elevations, depending on the length of the antenna, location of the animal, size of the transmitter and other environmental characteristics that may attenuate the signal. The frequency of the tone emitted by the receiver changes according to the distance between the transmitter and the antenna. Antennae can be hand-held or mounted on a vehicle, boat or aeroplane (Michener and Walcott, 1966). Each radio telemetry unit can be set to emit signals of a specific frequency, and receivers can switch among frequencies so that a scientist can track multiple animals at the same time. Radio telemetry is very useful for locating animals to a particular location, such as a tree or burrow, as long as the animals are not out of the transmitter's range.

There have been several VHF telemetry studies of bats (Fleming and Eby, 2003). Some of the most extensive data sets on local and long-range movements of bats come from Australian studies describing the migratory movements and foraging behaviour of Old World fruit bats of the genus *Pteropus* (Eby, 1991; Palmer and Woinarski, 1999; Tidemann *et al.,* 1999; Palmer, Price and Bach, 2000; Markus and Hall, 2004). *Pteropus* species are gregarious, forming roosting colonies of varying sizes, depending on the habitat (Pierson and Rainey, 1992; Hall and Richards, 2000; Kunz and Jones, 2000). Australian *Pteropus* spp. are migratory (Fleming and Eby, 2003), but detailed movement data for other species are scant. Radiotelemetry studies of Australian *Pteropus* species show that individuals may fly up to 50 km each night to forage (Palmer, Price and Bach, 2000). Australian flying foxes occupy large home ranges, and are generally seasonally nomadic, flying hundreds of kilometres per week as part of their normal movement patterns, often in response to local food availability (Nelson, 1965; Eby 1991; Tidemann *et al.,* 1999; Hall and Richards, 2000; Kunz and Jones, 2000; Palmer, Price and Bach, 2000; Markus and Hall, 2004).

PTT satellite telemetry

Because of their adult size, Pteropodid bats, particularly those of the genera *Pteropus* and

FIGURE 7.1a

Pteropus vampyrus being fitted with a satellite collar while anaesthetized: note the finger under the collar used to test the collar's fit to allow for swallowing and unrestricted head movement

FIGURE 7.1b

Testing collar fit on _P. giganteus_. Once the collar is attached, the anaesthetized bat is held by the feet. In this position the collar can be checked for appropriate fit such that it is loose enough not to be constrictive and tight enough that it cannot be removed by sliding it over the bat's head

Eidolon have been the focus of bat PTT satellite telemetry studies. PTTs range from 5 to 22 g in weight, depending on whether they are solar- (the lightest) or battery-powered. There have been few satellite telemetry studies of bats, but PTT satellite telemetry has been used to describe the foraging and migratory movements of *Pteropus* and *Eidolon* species because of their conservation status and importance as reservoirs for emerging infectious diseases (Tidemann and Nelson, 2004; Richter and Cumming, 2008; Epstein *et al.,* 2009). The largest study to date was of seven healthy (body mass greater than 700 g) adult male *Pteropus vampyrus* collared with either a 20 g battery-powered PTT or a 12 g solar-powered PTT (from Microwave Telemetry, Maryland, United States of America) in peninsular Malaysia (Epstein *et al.,* 2009).

Additional sensors can be used in conjunction with standard satellite tracking PTTs. Integrated sensors can be used to corroborate location findings, such as sea-surface temperature for albatrosses (Shaffer *et al.,* 2005); collect behavioural information, such as dive depth and duration for sea turtles (Yasuda and Arai, 2005); or transmit data when otherwise not possible, such as through pop-up satellite archival tags for pelagic fish (Block *et al.,* 1998). The integration of GPS into satellite PTTs (see following section) has significantly improved the accuracy and spatial resolution of animal tracking studies (to within tens of metres), and will certainly become more widely used as sensors become smaller and lighter (Hulbert, 2001).

Data processing

PTT satellite locations are obtained using the Argos Service (Collecte Localisation Satellites [CLS], Ramonville Saint-Agne, France), which categorizes location errors into seven classes from smallest to greatest – 3, 2, 1, 0, A, B and Z (CLS, 2008) – and delivers data electronically to the user's e-mail address or a Web-accessible site. Movement and migration tracks are reconstructed using the best location data, with established error rates (classes of 3, 2, 1 and 0), which are subjectively evaluated using ecological knowledge about the study species. Classes A, B and Z data are often dismissed, as there are no upper limits to their error margins (CLS, 2008). Movement track and home range location data are imported into ArcView GIS 3.2 (ESRI, United States of America), and detailed tracks are constructed using Argos-tools extension (CLS, Largo, Maryland, United States of America). Home range analysis can be performed by using multiple techniques and combining data from multiple individuals (Figure 7.2; Rodgers and Carr, 2001; Seaman and Powell, 1996). Temporal, spatial, seasonal and statistical analyses are also possible, including evaluation of the potential role of bats in transmitting diseases to other locations or species. However, the greatest limitation is that the accuracy of PTT transmitters does not exceed +/- 100 m.

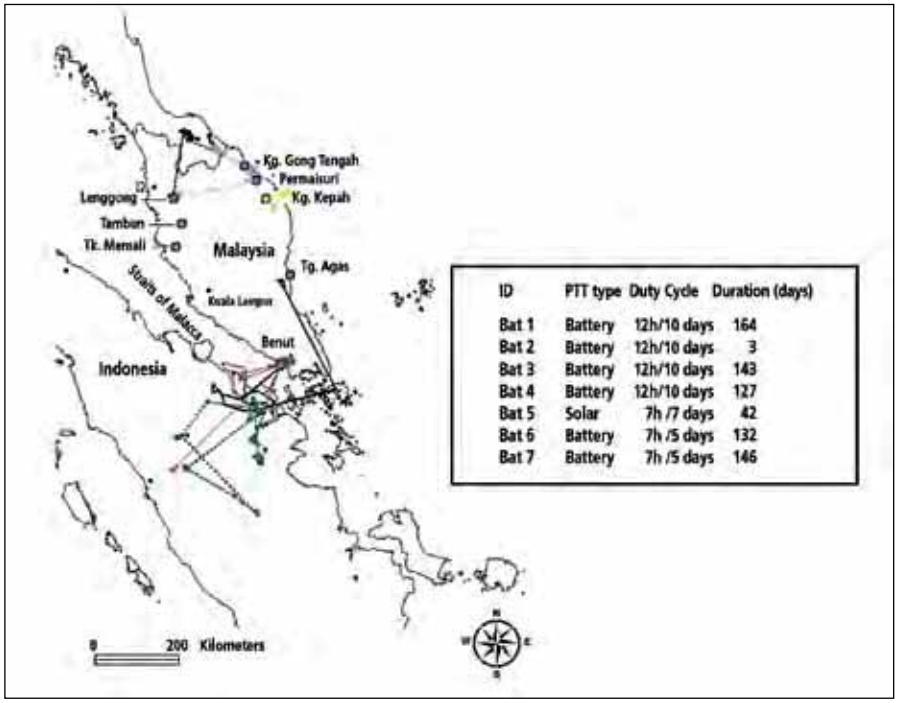
GPS satellite telemetry and GPS data loggers

Tomkiewicz *et al.* (2010) provide a thorough review of GPS animal tracking systems. Briefly, GPS tracking devices utilize GPS technology to obtain three-dimensional location data (latitude, longitude and altitude) from equipped animals. Data loggers use GPS technology and store locations on an internal memory requiring data to be remotely downloaded using either Bluetooth or GSM/GPRS networks, an antenna and receiver. This usually requires that the receiver and datalogger are within approximately 500 m although it is possible to recover data from longer distances if the receiver is located at a higher altitude than the

animal wearing the logger. In contrast, highly accurate data from GPS satellite transmitters (PTTs) are transmitted via ARGOS satellite. GPS devices (data loggers or PTTs) are far more accurate than standard satellite PTTs, and can record an animal's position to within +/- 20 m – far exceeding the accuracy of satellite telemetry. The position accuracy of GPS data loggers depends on similar factors to the accuracy of satellite telemetry: amount of forest or vegetation cover obstructing satellite reads; position and integrity of the antenna on the device; and battery power. In addition to location data, GPS technology can also collect animal position and environmental data, which can provide highly detailed descriptions of activity. GPS data loggers have been used in a few studies of *Rousettus aegyptiacus* bats in Israel (IBCRS, Prague abstract; Nathan *et al.*, 2010) and of *Eidolon helvum* in Africa (Dechman *et al.,* 2010). As with satellite PTTs, a duty cycle pre-programmed into each

FIGURE 7.2

Satellite telemetry study of seven adult male *P. vampyrus* bats in Peninsular Malaysia. Bats typically flew hundreds of kilometres over the duration of the study. Four bats captured and released at a large permanent colony in southwestern Peninsular Malaysia spent significant amounts of time roosting and foraging in Sumatra, Indonesia as well as Malaysia. Two bats (Bat 3 and 4) captured and released at the same colony flew in different directions following release, then reunited in southern Thailand approximately four months later. These studies illustrate the high mobility and multinational habitat use of this species, as well as the potential for connectivity among spatially distant colonies



Source: Epstein, 2009: No. 18.

transmitter determines the longevity of the device battery and the frequency or timing of the equipment for collecting geographic locations. Compared with satellite PTTs, GPS-quality data provide a better opportunity for evaluating exact habitat use and the interface or contact between bats and other species, including livestock and people.

DISCUSSION

Telemetry studies have proved to be invaluable for studying the ecology of bats, including their foraging and migratory movements, daily activity patterns and behaviour. More recently, telemetry has become an important tool for epidemiologists' understanding of the host range and movement of chiropteran and other bat species that can be reservoirs or transmission vectors for a variety of pathogens. When considering the use of telemetry devices on bats, the following should be taken into consideration:

- What hypothesis will be tested?
- What device(s) is (are) appropriate for testing the hypothesis?
- How many individual animals must be included in the study to test the hypothesis adequately?
- Animal welfare: Satellite and GPS telemetry studies in large fruit bats are relatively new, and an optimal collar that does not pose a risk to bats' health or affect their behaviour has not yet been developed. Scientists should consult the device's manufacturer and carefully consider the impact that any attached device may have on the welfare of the study animal. All study protocols using telemetry devices should be reviewed by an institutional animal care and use or equivalent committee, which must include a bat telemetry expert to ensure the ethically appropriate use of the equipment. The following are important welfare considerations:
  - Is the mass of the device < 5 percent of the animal's body mass?
  - What attachment apparatus will be used – a collar, harness or adhesive?
  - Where on the bat's body will the apparatus be attached?
  - Will the device fall off, or is it permanent?
  - Will the device remain on the animal long enough to obtain adequate data?
  - What is the conservation status of the target species?

A few additional considerations should be taken into account when using telemetry with bats rather than other animals. Solar-powered devices require exposure to sunlight, and bats' colonial nature and often tightly packed roosting behaviour may obscure solar cells on a device, preventing adequate recharging during the day. Bats often groom each other or fight, making antennae vulnerable to chewing or other damage that may destroy transmitter functionality. Some bat species roost in trees over water; if transmitters fall off they may not be recoverable. Inaccessible roosts or migratory bats may make data retrieval from GPS transmitters difficult, and species behaviour should be carefully considered when selecting the technology to use.

Each technology and attachment technique has advantages and disadvantages, and scientists should carefully consider the questions they wish to answer through telemetry. Table 7.2 summarizes the advantages and disadvantages of each technology.

TABLE 7.2
Advantages and disadvantages of telemetry technologies for use on bats

Technology	Advantages	Disadvantages
VHF/radio transmitter	Inexpensive, accurate, many manufacturers and suppliers Ideal for animals with small home ranges Mass suitable for wide range of species	Labour-intensive Limited range of receiver Long-range movement requires vehicle Animals may move out of range and be lost to the study
Satellite PTT	Can track animals anywhere in the world Data retrieval via Internet The least labour-intensive option Solar-powered transmitters can transmit for years Ideal for long-range movements	Relatively poor accuracy Not good for fine-resolution behavioural data Accuracy depends on available satellites and cover The most expensive option Data retrieval also expensive Cost limits study size Mass limits species size Few manufacturers and only one data vendor (Argos)
GPS data loggers and PTTs	Accurate location data Can record many locations and store on device Power-efficient Can record three-dimensional location and behavioural data Data easily importable into a Geographic Information System (GIS) Useful for high-resolution local data, e.g., foraging behaviour, and for high-frequency location recording to describe behaviour	Data retrieval requires either retrieving the device or being within range for downloading data via wireless – if animal leaves range data are lost Moderately expensive Mass limits species size Accuracy depends on satellite visibility/ cover

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Chapter 8

The global context of ensuring the health of people, wildlife, livestock and the environment

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At the global level, health care for people and animals, including wildlife, needs to move away from its current approach of hopping from one pandemic risk to the next, to adopt a more holistic view, based on an understanding of the drivers of disease emergence and the preventive measures that secure the health of people, livestock, wildlife and the environment. This approach will require further education about the interdependency of human, animal, wildlife and ecosystem health, accompanied by political, national and financial commitment to shift priority towards measures that prevent disease transmission across sectors and borders.

One of the greatest challenges associated with such an effort is the ability to balance the needs of people, wildlife and domestic animals in the face of limited natural resources and increasing global population, resource consumption and demand for livestock-based protein. Education can help this process, but behaviour must also be modified, and neither scientific knowledge nor education alone will lead to changes in human behaviour and decision-making at the global level. It is widely acknowledged that human behaviour underpins emerging infectious disease (EID) events and that multiple interrelated global factors drive these processes. There is need to demonstrate how individuals' decisions and people's lives in urban, suburban and rural areas depend on ecological health and ecosystem services. Change will require the integration of scientific research with educational outreach and consideration of cultural dimensions and local priorities.

Health management requires the renovation of current approaches, to place multiple disease concerns and impacts against the backdrop of sustainable agriculture, natural resource management and socio-economic development. The main elements of this collaboration entail broadening the approaches to health across relevant disciplines, by promoting horizontal and cross-sectoral collaboration to address current emerging zoonotic disease and veterinary public health challenges more sustainably and adequately. To be successful, pandemic threat programmes have to consider a wider range of stakeholder concerns. Livestock are often a central focus of zoonotic disease control, largely owing to domestic animals' economic importance, but also because more is understood about livestock diseases and their impact on human health. However, the majority of emerging

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zoonoses originate in wild animal species, about whose health very little is known. Animal disease prevention and control efforts, for both domestic animals and wildlife, will have to become integral components of more general development activities. Hence, there is a need to enhance animal health services, starting in the developing countries, and to define disease control and prevention in the context of natural resource management (i.e., the preservation of ecosystems), sustainable agriculture and rural development.

Bats (order Chiroptera) have recently been associated with an increasing number of viral pathogens that have caused significant human and livestock morbidity and mortality (Calisher *et al.,* 2006). This has led to increased research efforts regarding bats and their pathogens and, in the context of emerging pandemic threats, has raised the question as to whether bats are unique among wildlife in their capacity to act as reservoirs for zoonotic pathogens. Aspects of bats' natural history and ecology make them unique. They are the second most diverse group of mammals on earth (after rodents), with more than 1 150 species (IUCN, 2010). One in five mammal species is a bat and bats are found on every major land mass except the polar regions and a few oceanic islands. They are the only true flying mammals, and many species are migratory, flying hundreds or thousands of kilometres in the course of their seasonal movements. Collectively, bats are responsible for the consumption of millions of insects and the pollination of thousands of plants, flowers and fruit trees, and play a major role in seed dispersal in the forests and other habitats they occupy. In some forest habitats, they are a "keystone" species for maintaining ecosystems and are crucial to the regeneration of cleared forest areas. Bats' foraging nature makes them likely to combat vector-borne disease such as malaria, encephalitis and West Nile viruses, although this is difficult to quantify. Bats also provide protection against certain pest species that routinely affect agricultural crops – generating economic savings of billions of United States dollars on alternative pest control measures. Bats are invaluable for ensuring the food security of people who rely on fruit trees, and also provide food security to wildlife species that rely on vegetation, fruit and flowers.

From a pathogen host perspective, the sheer diversity of bat species makes it unsurprising that bats are the reservoir of many potentially infectious agents. Viruses such as Ebola virus, rabies, Nipah virus and severe acute respiratory syndrome (SARS)-like coronaviruses circulate in wild bat populations (Johara *et al.,* 2001; Rupprecht, Hanlon and Hemachudha, 2002; Leroy *et al.,* 2005; Li *et al.,* 2005; Calisher *et al.,* 2006). These pathogens have caused substantial disease in people and other animal species, but typically – with the exception of rabies virus – they appear not to cause any disease for their bat hosts. They have most likely co-evolved with bats to reach a point where bats can asymptomatically shed virus. The impact on wildlife, domestic animal and human hosts has been severe, and is associated with high levels of morbidity and mortality, as well as significant economic costs.

However, it is important to remember that people are responsible for changing landscapes, deforestation, encroachment and other activities that create opportunities for pathogens to jump host species and cause illness or death in other species. People can also serve as the solution, by making more responsible land-use planning choices, avoiding encroachment and minimizing risky behaviours or situations that lead to exposure to potential wildlife pathogens. From a scientific perspective, improved bat surveillance and diagnostic testing have also allowed the discovery of potential pathogens in bat species.

Although these "novel" infectious agents have likely been in bats for many years, it is only recently that scientists have searched for, and have tests that can identify, these pathogens. From a biological perspective, nothing has changed in the relationship between the bat and the infectious agent for which it is the reservoir.

It is therefore essential that the scientific community maintain both a public health and a conservation perspective, and prevent the vilification of bats for maintaining pathogens. This critical message should be conveyed to all sectors of society. The destruction of bats and their habitats represents a far greater risk to human health than the existence of pathogens carried by bats. The more that is understood about the ecology of hosts and their pathogens, and about the human activities that facilitate zoonotic pathogen transmission, the more effectively the risk of outbreaks of emerging pathogens can be reduced by altering behaviour.

This manual provides an introduction to the ecology of zoonotic pathogens carried by bats and the techniques that are used all over the world to study them. It aims to promote the responsible and sensible study of bats and the microbes they carry, and to reinforce the ecological importance of protecting this massively diverse and abundant group of animals.

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Chapter 9
Safe handling of bats

RISKS OF HANDLING BATS

The sampling of any wild animals can present physical risks to the handler, including the potential for injury through being bitten, scratched, kicked or crushed (see Chapter 4, p. 49). All bats should be considered to pose a risk for zoonotic infection of people, most frequently through bites, scratches, and contact with faeces, urine and saliva. In particular, there is a risk of exposure to some significant zoonotic agents for any person handling bats in either the field or the laboratory, entering bat colonies or working with laboratory samples obtained from bats.

WORKING SAFELY

Any person exposed to or working with bats should be immunized against rabies virus and should use personal protective equipment (PPE). These important occupational safety measures should be part of normal occupational health and safety procedures whenever bats or bat samples are handled:

- All personnel must be appropriately trained prior to handling bats or sampling bat populations or individual animals.
- All personnel should avoid bites and scratches from bats by using proper PPE.
- All field and laboratory staff should be trained in the correct use of PPE and the decontamination of reusable equipment.
- Before engaging in at-risk activities, recently vaccinated people should ensure that they have a protective vaccination titre.

Contamination with aerosols, saliva, urine and faeces is highly possible, so all the personnel who handle animals and/or animal parts must take all necessary precautions. These include the use of gloves, masks and eye protection.

Specific precautions against the risk of certain pathogens vary, depending on the type of bat roost, the nature of the potential exposure, the bat species and the geographic location:

- Minimum PPE such as latex and leather gloves should be used when sampling is conducted outside bat roosts (e.g. mist netting in an open area).
- Maximum precautions must be used for handling and sampling bat species that are confirmed or suspected reservoirs of filoviruses and henipaviruses, as the transmission mechanisms for these infections are currently unknown. PPE in such cases should include coveralls, gloves, eye protection, and respiratory protection using at a minimum, P2 (N95) masks, with powered air-purifying respirators (PAPR) available for those personnel that chose to use them. If PAPRs are used, they must be rigorously disinfected after use.

All field and laboratory equipment, holding bags and other objects that may be contaminated during bat sampling must be decontaminated and disinfected after use, or disposed of.

Key health and safety procedures include (Animal Health Australia, 2011):

- avoiding bat bites and scratches, through the use of appropriate bat handling techniques and protective equipment, including puncture-resistant gloves;
- pre-exposure vaccination, if continuous, frequent or infrequent contact with bats is anticipated, particularly for veterinary staff, cavers, bat ecologists, biologists, laboratory workers and others who work with bats or lyssaviruses;
- immediate and thorough cleansing of bites and other wounds associated with bats;
- post-exposure vaccination for bites or other potential exposure to bat lyssaviruses, to provide active (rabies vaccines) and passive (rabies immunoglobulin) immunity, as recommended by World Health Organization (WHO) or relevant national public health guidelines.

FIRST AID AND MEDICAL ASSESSMENT[3]

It is essential that whenever a possible exposure to any lyssaviruses or other zoonotic agent occurs (i.e. a bite, a scratch, a splash on to mucous membranes, or aerosol exposure in the field or laboratory), first aid is commenced as soon as possible to remove the virus from exposed tissue. Medical advice should always be sought without delay, irrespective of vaccination status, as post-exposure prophylaxis may be needed.

Proper cleansing of any wounds, abrasions and splashes is an important first aid measure in preventing rabies in people. If a person is bitten or scratched, or his/her mucous membranes (i.e. eyes, nose, mouth or existing wounds) are splashed with any bodily fluids from the animal, the affected area should be immediately and thoroughly washed with soap and water for at least 15 minutes. Scrubbing should be avoided, as this may cause abrasions that could facilitate entry of the virus into the wound. A virucidal antiseptic, such as povidone iodine, iodine tincture, aqueous iodine solution or alcohol (i.e. ethanol), may be applied to wounds after washing. After cleaning, medical advice should be sought immediately, irrespective of rabies vaccination status, as a booster dose(s) may be necessary.

Medical assessment and monitoring of people encountering highly contagious pathogens should be carried out, and quarantine may need to be considered.

Specific lyssavirus vaccination information

Prevention of spill-over of bat lyssaviruses to humans is based on the management of any bat bite (penetration of the skin by teeth) or any non-bite exposure, which is defined as contamination of open wounds or mucous membranes with saliva or other potentially infectious material (e.g., neural tissue), or contamination of abrasions (including scratches).

Pre-exposure prophylaxis does not eliminate the need for risk management following a bite or non-bite exposure to a lyssavirus, but it simplifies post-exposure treatment by eliminating the need for human rabies-immune globulin and decreasing the number of post-exposure vaccine doses to two injections. Pre-exposure prophylaxis reduces the risk to individuals where medical attention and rabies biologics are unavailable, or when post-exposure vaccination is delayed or not sought after unapparent exposure (see Chapter 5, p. 91).

[3] This section is based on Animal Health Australia, 2011.

People at continuous or frequent risk of exposure should ensure that they maintain an adequate immune response by monitoring their serum titres of rabies virus-neutralizing antibodies and getting booster vaccinations according to international or relevant national recommendations (WHO, 2007; Government of Australia, 2008; Manning *et al.,* 2008; Rupprecht *et al.,* 2010).

***Note**:* Specific pre- and post-exposure prophylaxis is currently available for only rabies (caused by phylogroup 1 lyssaviruses), but not for other phylogroup lyssaviruses. However it is considered likely that the use of rabies vaccine will mitigate the risk of infection with some other phylogroup lyssaviruses, such as Australian bat lyssavirus. For several other infections, experimental vaccines are under construction or laboratory trials in progress, but no prophylaxis is commercially available. The effectiveness of anti-viral medication for these pathogens is also unknown. People handling bats should ensure that they are aware of current technical advances in both vaccines and anti-viral medications for bat viruses with zoonotic potential (see Chapter 6, p. 144).

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Capacity development is one of the pillars through which the Food and Agriculture Organisation of the United Nations supports member countries. This manual serves as a resource for better understanding the ecology of bats, their natural history, their role in providing ecosystem services, techniques used for monitoring populations, and for the detection, identification and monitoring of viruses naturally circulating in bats and that can have significant implication if they are transmitted to people either through direct contact, or indirectly, through livestock. This manual will engage professionals from multiple disciplines ranging from public health and veterinary medicine to natural resource managers and biologists, but most importantly, highlights the need to understand the anthropogenic drivers resulting in disease transmission from bats to people.

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