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Australian bat coronaviruses

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BSc

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Abstract

Coronaviruses were responsible for the global outbreak of severe acute respiratory syndrome (SARS) in 2003 and 2004, and the outbreak of Middle East respiratory syndrome (MERS) in 2012. Bats have since been identified as the natural hosts for a number of novel coronaviruses, including the likely ancestors to SARS and MERS coronaviruses. It is essential for Australia's biosecurity preparedness, and for broader understanding of this previously unknown group of viruses, that coronaviruses in bats in our region are identified, characterised and their ecology understood.

In Chapter 1, the relevant literature is reviewed, both in the context of my contribution to the Food and Agricultural Organisation of the United Nations publication 'Investigating the Role of Bats in Emerging Zoonoses', and additionally in updating subsequent research and emergence events.

Chapter 2 presents a novel peer reviewed and published methodology for collecting blood samples from small bats. This methodology was essential for the studies that followed.

Chapter 3 reports on the surveillance of 2,195 bats from Australia and neighbouring countries sampled between 1997 and 2009 for evidence of coronavirus infection. The study identified coronaviruses belonging to two genera (*Alpha*- and *Betacoronavirus*) in Australian bats, and serological evidence of infection of coronaviruses in bats from East Timor, Indonesia, Malaysia and Papua New Guinea. It also identified an interspecies transmission of a variant of the alphacoronavirus *Miniopterus bat coronavirus HKU8* from *Miniopterus spp* bats to bats of the genus *Rhinolophus*, supporting the hypothesis that bats from this genus are more likely to foster host shifts and pose a risk for the emergence of other bat coronaviruses. The study also elucidated the current diversity of coronaviruses in Queensland bats, and the findings are consistent with co-evolution with the occasional fostering of host shifts by bats of the genera *Hipposideridae* and *Rhinolophidae*. Further, they suggest that bat coronaviruses are as old as the most common bat ancestor - 65 million years.

Chapter 4 presents a longitudinal study of bats inhabiting an abandoned gold mine, which were sampled during spring, summer, autumn and winter between 2006 and 2008. The data and models from this study were used to develop a hypothesis of the infection dynamics of a novel *Alphacoronavirus* in *Miniopterus spp*. The hypothesis utilises a classical susceptible-infected-recovering (SIR) model, with individuals either susceptible to

infection, infected, or recovering from infection. An extension of the model considers pups that receive maternal antibody protection and tracks their progression through states of disease using a MSIR model, where a state of maternally derived immunity exists prior to becoming susceptible to infection. The findings suggested that bats have an anamnestic (immunological) memory which limits secondary coronavirus infections with a stronger and more rapid production of antibodies, compared to a primary infection.

In Chapter 5, a modified mark/recapture study on a maternal population of the Australian bat *Myotis macropus* identified that individual bats were infected with a novel unclassified putative *Alphacoronavirus* for up to 11 weeks. The observed pattern of infection supports not only a hypothesis of persistent coronavirus infection in bats, but also suggests that acute infection, and intermittent viral is possible.

The work in this thesis has made a major contribution to understanding the diversity and ecology of coronaviruses in bats. The findings have implications not only for Australia, where most of the studies were based, but also for the international community. The research highlighted the broad distribution of bat coronaviruses, both geographically and across bat species, demonstrated the risk of interspecies transmission, and modelled the infection dynamics of the viruses within individual bat species.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers associated with thesis

SMITH, C. S., DE JONG, C. E. & FIELD, H. E. 2010. Sampling small quantities of blood from microbats. *Acta Chiropterologica,* 12**,** 255-258.

Other peer-reviewed papers

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Contributor	Statement of contribution
Smith, C. S.	Wrote the paper (60%)
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Wang, L. F.	Wrote and edited paper (20%)

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Contributor	Statement of contribution
Smith, C. S.	Designed the experiment (60%) Performed the experiment (60%) Analysed the data (100%) Wrote the paper (60%)
De Jong, C. E.	Designed the experiment (20%) Performed the experiment (40%)

	Wrote and edited paper (20%)
Field, H. E.	Designed the experiment (20%) Wrote and edited paper (20%)

Contributions by others to the thesis

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This study was supported by the Australian Biosecurity Cooperative Research Centre for Emerging Infectious Diseases and partially by the Consortium for Conservation Medicine from the NSF/NIH Ecology of Infectious Diseases award (R01 TW05869) from the John E. Fogarty International Center and BQ, DAFF.

To my parents George and Gay, my children Duncan and Penelope and my darling wife Amanda, I thank you all for your love and support, and promise never to do it again.

Australian bat coronaviruses

Keywords

Australia, bat, coronavirus, epidemiology, identification, infection dynamics, MERS, molecular phylogenetics, phlebotomy, SARS

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Fields of Research (FoR) Classification

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Introduction

On the 21st February 2003 in the province of Guangdong (People's Republic of China), a person with flu like symptoms travelled to Hong Kong to visit family. Checking into their hotel they stayed only one night, on the ninth floor. The following morning the travellers' symptoms had not improved and they were admitted to hospital. Succumbing to disease, the traveller died the next day, from what was later diagnosed as severe acute respiratory syndrome or SARS. Prior to the travellers' death, ten other guests of the hotel, who were also checked in on the same day and resided on the same floor, were infected by the traveller. Epidemiological investigations later identified these ten guests as index patients for the subsequent outbreaks of SARS in China, Canada, Ireland, the United States of America, Germany, Singapore, Vietnam and Thailand. More incredibly one of the ten guests, who was admitted to a local hospital in Hong Kong, was directly linked to the infection of 99 health care workers, including 17 medical students, in that hospital (Centers for Disease Control and Prevention, 2003).

When the World Health Organisation declared the global outbreak over on the 5th July 2003, more than 8,000 cases with over 800 fatalities had been reported in 32 countries worldwide. The costs to the global economy was close to $US 40 billion, with the financial impact not due to the consequences of the disease itself but the impact of the disease on the behaviour of people within those economies. This containment of both microbial and economic pandemics is the reason for the importance of the global surveillance and monitoring of disease (Lee and McKibbin, 2004).

In March 2004, I and my colleagues commenced the field work that would later identify the natural reservoir host of a SARS-like coronavirus in bats (Li *et al.*, 2005). Upon my return to Australia, and given the importance of the global surveillance and monitoring of disease, I undertook this candidature in an attempt to identify any Australian bat coronavirus and elucidate their ecology. The first chapter of this thesis includes a literature review that was subsequently published as part of a FAO Animal Production and Health Manual in 2011 (Smith *et al.*, 2011b). To maintain its relevance, a brief review discussing coronaviruses in general and a selection of manuscripts published since 2011, has been included. At the time of my candidature, methodology available for sampling small quantities of blood from microbats was limited, and most were inappropriate or resulted in the animals death. Thus, the second chapter describes a technique for sampling small quantities of blood from

microbats which was published in 2010 (Smith *et al.*, 2010). Chapter 3 describes the Australian bat coronaviruses identified by myself, interspecies transmission of those coronaviruses, and also how they relate to other bat coronavirus identified worldwide. Following the identification of these coronaviruses, I planned two surveillance projects to study their ecology. The first, reported in Chapter 4, used a longitudinal survey (on a colony of bats infected with an *Alphacoronavirus*) to identify risk factors for infection and hypothesise a model for infection. The second, Chapter 5, utilised a modified mark/recapture method to observe natural infection in individuals and a general discussion presenting the final hypothesis is presented in Chapter 6.

Chapter 1 Literature review

At the time of publication, the FAO book chapter (included as Chapter 1 of this thesis) reported on the emergence and characterisation of bat coronaviruses from 17 studies (Smith *et al.*, 2011b). As of July 2013, the number of studies characterising bat coronaviruses had increased to 53 (Drexler *et al.*, 2014). Whilst the difference is substantial, many of the initial hypotheses discussed in the book chapter remain true, supported by these additional studies. The nomenclature for coronaviruses may have changed but the phylogeny of the groups remains the same (Gonzalez *et al.*, 2003, International Committee on Taxonomy of Viruses, 2009). In this review, I will generally discuss coronaviruses and a selection of manuscripts published since 2011 and more importantly, the emergence of another bat coronaviruses, Middle East respiratory syndrome (MERS-CoV), with fatal zoonotic consequences.

Coronavirus morphology and replication

Coronaviruses, of the order *Nidovirales*, family *Coronaviridae*, are the largest known non-segmented, single stranded, positive sense RNA viruses (27.6 to 32 kb), (Lai and

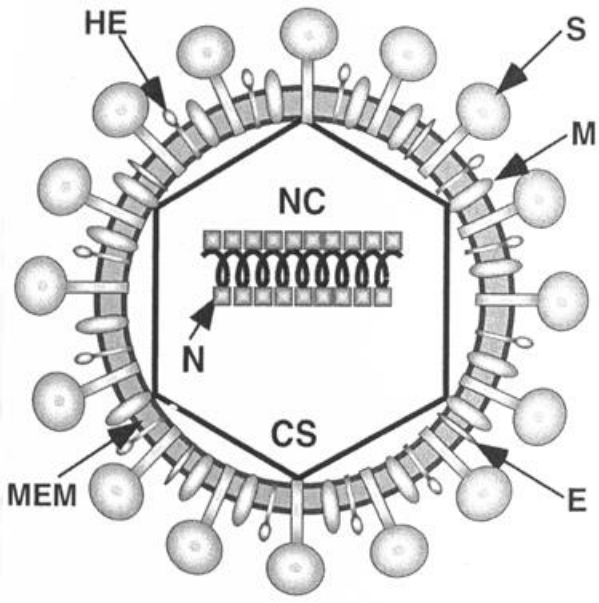


Figure 1. Schematic diagram of coronavirus morphology.

Lipid membrane (MEM), spike protein (S), small envelope protein (E), large membrane protein (M), nucleocapsid protein (N), hemagglutinin-esterase (HE), core shell (CS) and nucleocapsid (NC), (Spaan *et al.*, 2005).

Cavanagh, 1997, Spaan *et al.*, 2005). They can cause a range of syndromes including respiratory and gastroenteric disease in humans and respiratory, gastroenteric, neurological and hepatic disease in animals, often with significant economic consequences (Fraenkel-Conrat *et al.*, 1988, Lai and Cavanagh, 1997). Coronaviruses have large projections protruding from the envelope that are formed by trimers of the spike protein (Figure 1) and when viewed by electron microscopy (Figure 2), form the characteristic 'crown' that gave rise to the family's name. Coronaviruses have a diameter of 120-160 nm with an internal core shell 65 nm in diameter, protecting the nucleocapsid (Spaan *et al.*, 2005).

The lipid membrane envelope of coronaviruses, derived from the host cell, contains three proteins, the spike (S), small envelope (E), and membrane (M). The envelope of most

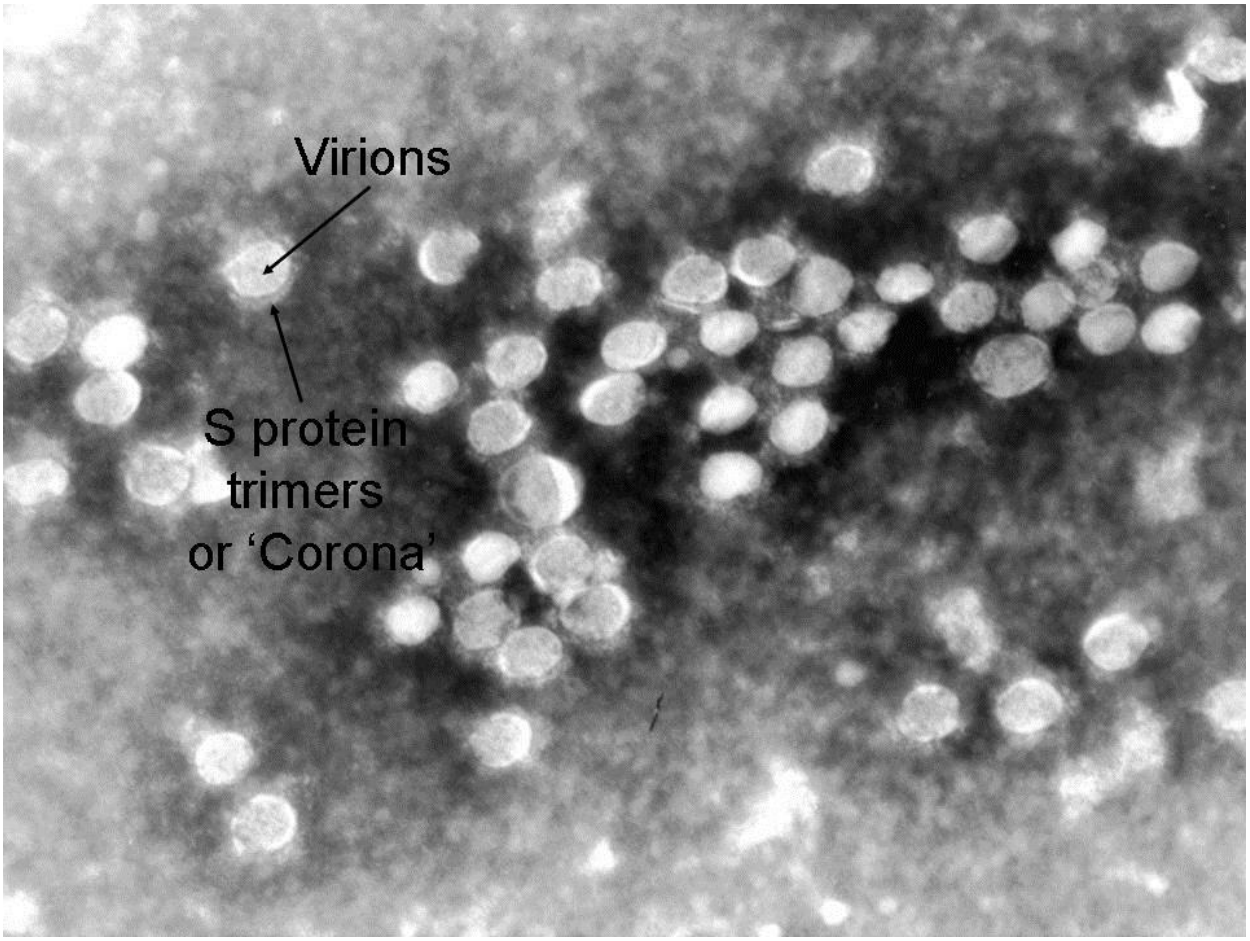


Figure 2. Electron micrograph of SARS coronavirus.

Electron micrograph of irradiated SARS coronavirus (H.sap/HKSAR/SARS-CoV/HKU-39849) showing the characteristic crown or 'Corona' that gave rise to the family's name. Micrograph: Howard Prior, Biosecurity Queensland, Department of Agriculture, Fisheries and Forestry.

Group 2 or *Betacoronaviruses* also contain a hemagglutinin-esterase (HE) protein. The S protein (1160-1452 aa, 180-220 kDa) has a highly exposed globular domain responsible for receptor binding, hemagglutination, membrane fusion and induction of neutralising antibodies. Immunisation with the spike protein alone can produce protection from challenge with some coronaviruses. The E protein contains 76-109 aa and has an apparent molecular mass of 9-12 kDa. The M protein (221-260 aa, 23-35 kDa) spans the envelope three to four times, it can induce interferon and together with the E protein, play an essential role in coronavirus virion assembly. The HE protein (65 kDa) found in the envelope of most *Betacoronaviruses* is apparently non-essential but has a receptor binding domain, hemagglutination activity and receptor destroying activities. The N protein (377 to 455 aa, 50-60 kDa) binds to the viral RNA and forms a helical nucleocapsid (Spaan *et al.*, 2005).

A large number of non-structural proteins are not incorporated into the virion, the largest of which are the replicase polyproteins. Approximately two thirds of the coronavirus genome (18 to 22 kb) contains two large open reading frames (ORF), designated ORF1a and 1b (Figure 3). Translation of ORF1a with a ribosome slip at the overlap of OFR1a and 1b yields replicase polyprotein 1a (450 kDa), whilst translation into ORF1b via a frame shift yields the replicase polyprotein 1ab. Both replicase polyproteins appear to be co- and post translationally processed, by viral proteases papain-like cysteine and 3CL proteinases, yielding 15-16 of mature replicase polyproteins, including the RNA-dependant RNA polymerase (RdRp) and an unknown number intermediate replicase polyproteins (Poon *et al.*, 2005, Spaan *et al.*, 2005). Downstream of ORF1b there are 3-13 additional ORFs that

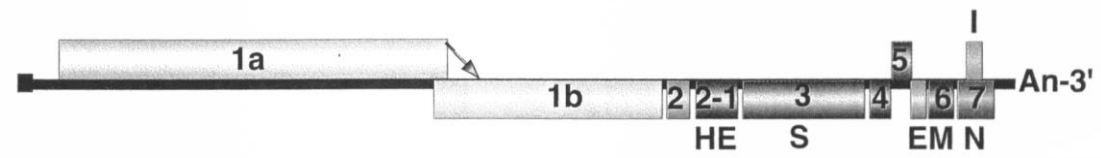


Figure 3. Representation of the coronavirus genome.

Representation of the genome of mouse hepatitis virus as a coronavirus genome example (Spaan *et al.*, 2005). Open reading frames (ORF) are represented by boxes. The proteins encoded by the ORFs are indicated; ORF1a encodes replicase polyprotein 1a and, together with ORF1b, replicase polyprotein 1ab. The 5' leader sequence is depicted by a small black box, hemagglutinin-esterase protein (HE), spike protein (S), small envelope protein (E), membrane protein (M), nucleocapsid protein (N), internal ORF (I) and poly(A) tail is indicated by An. No designated boxes are non-structural proteins and the arrow between ORF1a and 1b represents the ribosomal frame shifting site.

encode for structural and non-structural ‘accessory’ proteins, which at least in cell culture are largely non-essential (Spaan *et al.*, 2005).

Coronaviruses infect many mammals (Spaan *et al.*, 2005). Epithelial cells are the main sites of infection and induce respiratory or gastrointestinal disorders (Spaan *et al.*, 2005). Respiratory, faecal-oral and mechanical transmission are common but biological vectors are not known (Spaan *et al.*, 2005). Pigs, cats and domestic fowl may become persistently infected and shed virus from the enteric tract (Spaan *et al.*, 2005).

Using their S protein, coronaviruses will bind to surface molecules, including CEACAM1 glycoprotein, angiotensin converting enzyme 2 and aminopeptidase N, and when the HE protein is present can also bind to the N-acetyl neuraminic acid which serves as a co-receptor (Figure 4) (Crenim, 2008). Coronavirus replication proceeds through the translation of the full-length positive stranded genomic RNA in the cytoplasm of infected cells, the products of which are replicase polyproteins 1a and 1ab (Spaan *et al.*, 2005, Crenim, 2008). The replicase polyproteins then transcribe a full-length negative stranded RNA molecule from which 7 or more positive stranded nested subgenomic RNA molecules are transcribed, however, generally only the 5’-most ORF of the nested subgenomic RNA is translated (Figure 5) (Spaan *et al.*, 2005, Crenim, 2008). During transcription recombination can occur at a very high frequency and may allow coronaviruses to adapt to new hosts and ecological niches (Lau *et al.*, 2005, Spaan *et al.*, 2005, Woo *et al.*, 2006). The nucleocapsid is formed by the N protein binding to genomic RNA, and the M and E proteins which are expressed on the external surface of the endoplasmic reticulum and other Golgi membranes (Spaan *et al.*, 2005, Crenim, 2008). Virion assembly will continue with the nucleocapsid budding into the endoplasmic reticulum and being encased by its membrane (Spaan *et al.*, 2005, Crenim, 2008). The S and HE proteins, expressed on the internal surface of the endoplasmic reticulum, are not essential for virion assembly though the S protein is essential for infectivity (Spaan *et al.*, 2005). Assembled virions are transported by Golgi vesicles to the cell membrane and are exocytosed into the extracellular space (Crenim, 2008).

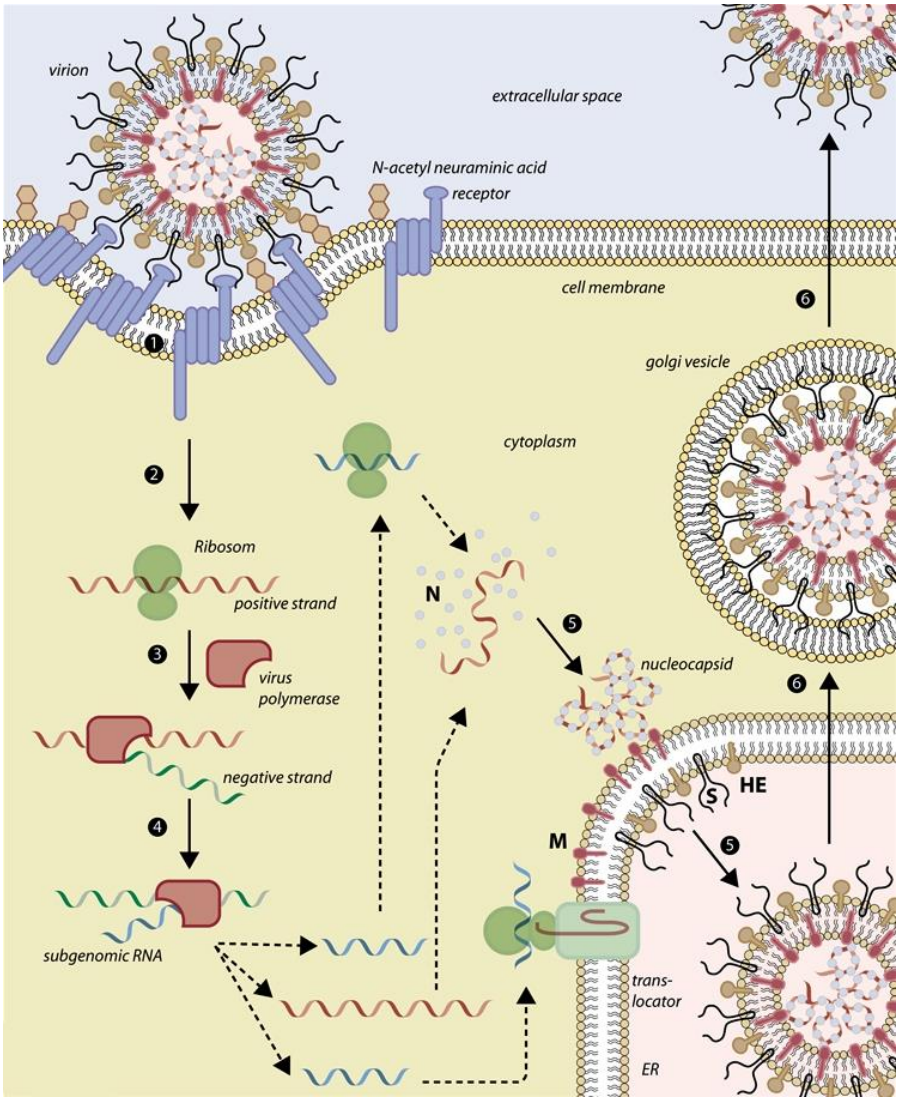


Figure 4. The coronavirus replication cycle.

The coronavirus replication cycle, full-length positive stranded genomic RNA (red), full-length negative stranded RNA (green), positive stranded nested subgenomic RNA (blue) (Crenim, 2008). (1-2) Using their S protein, coronaviruses will bind to surface molecules, including CEACAM1 glycoprotein, angiotensin converting enzyme 2 and aminopeptidase N and when the HE protein is present can also bind to the N-acetyl neuraminic acid which serves as a co-receptor. (3) Coronavirus replication proceeds through the translation of the full-length positive stranded genomic RNA in the cytoplasm of infected cells, the products of which are replicase polyproteins 1a and 1ab. (4) The replicase polyproteins then transcribe a full-length negative stranded RNA molecule from which 7 or more positive stranded nested subgenomic RNA molecules are transcribed. (5) The nucleocapsid is formed by the N protein binding to genomic RNA, and the M and E proteins which are expressed on the external surface of the endoplasmic reticulum and other Golgi membranes. Virion assembly will continue with the nucleocapsid budding into the endoplasmic reticulum and being encased by its membrane. (6) Assembled virions are transported by Golgi vesicles to the cell membrane and are exocytosed into the extracellular space.

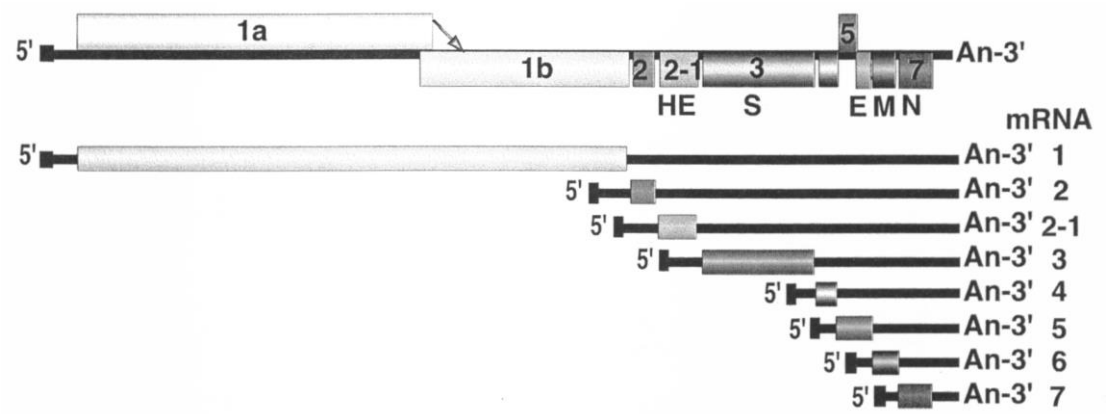


Figure 5. Coronavirus nested subgenomic RNA molecules.

Seven or more positive stranded nested subgenomic RNA molecules which are transcribed from a full-length negative stranded RNA molecule, generally only the 5'-most ORF of the nested subgenomic RNA is translated (Spaan *et al.*, 2005). The 5' leader sequence is depicted by a small black box, hemagglutinin-esterase protein (HE), spike protein (S), small envelope protein (E), membrane protein (M), nucleocapsid protein (N) and poly(A) tail is indicated by An. No designated boxes are non-structural proteins and the arrow between ORF1a and 1b represents the ribosomal frame shifting site.

Taxonomic classification

Prior to the global SARS pandemic only 12 species of coronaviruses had been recognised by the International Committee on Taxonomy of Viruses (2002). Historically, the genus *Coronavirus* (order *Nidovirales*, family *Coronaviridae*) were divided into three informal groups (1, 2 and 3) based on their antigenic and genotypic characteristics (Lai and Cavanagh, 1997). In 2003, it was proposed that the genera *Coronavirus* and *Torovirus* be redefined as two subfamilies within *Coronaviridae* and the three groups redefined as genera (Gonzalez *et al.*, 2003). However, it was not until 2009 that this proposal was ratified, with three genera *Alpha-*, *Beta-* and *Gammacoronavirus*, being named within the subfamily *Coronavirinae* (International Committee on Taxonomy of Viruses, 2009). A fourth genus, *Deltacoronavirus*, was added in 2011 (Figure 6) (International Committee on Taxonomy of Viruses, 2011).

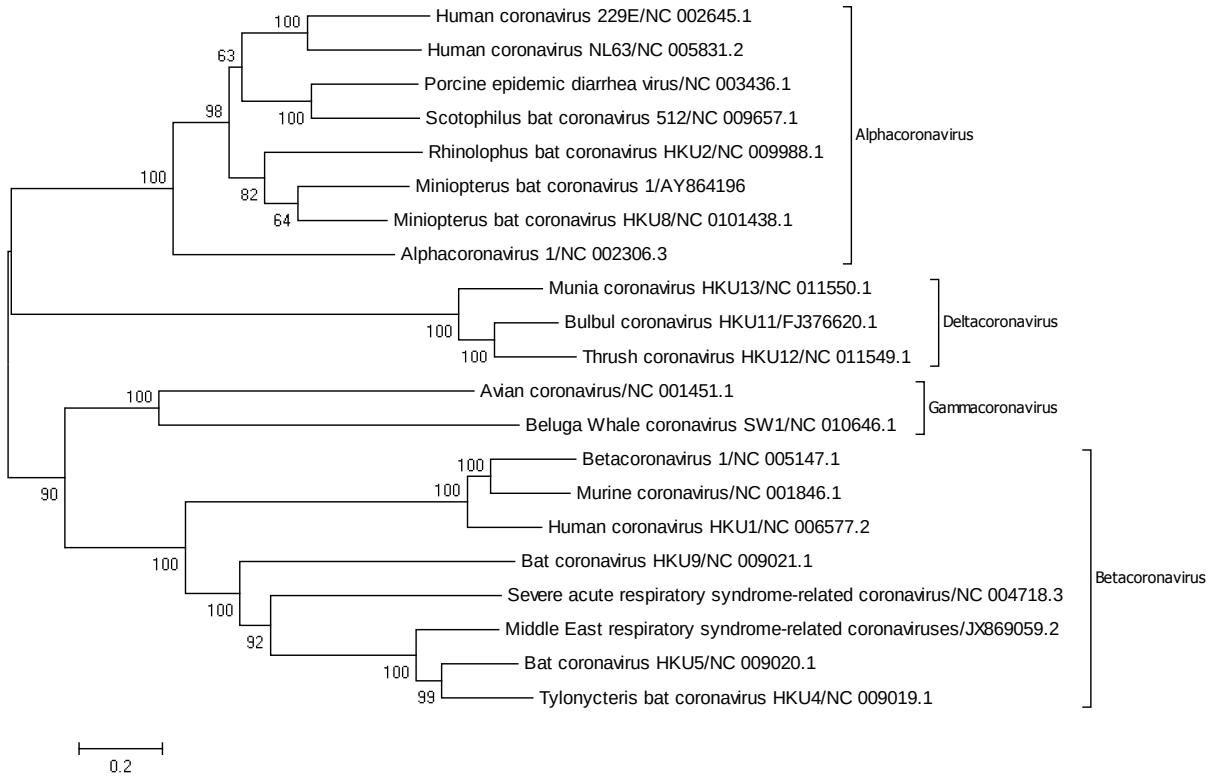


Figure 6. Nucleotide phylogenetic analysis of 21 reference coronaviruses representing each species and grouped by genus (complete genome sequence).

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (as in Chapter 3). The tree with the highest log likelihood (-575665.2715) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6851)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. There were a total of 34,919 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

The majority of detections of coronaviruses from bats, or other animals, have been from PCR targeting the RdRp (ORF1ab) gene. This PCR produces an amplicon size of only 440bp and prevents robust phylogenetic analysis. The difficulty in obtaining coronavirus isolates (due to the limited availability of appropriate cell lines (Crameri *et al.*, 2009)) from bats presents challenges for classifying these large and highly variable RNA viruses. To overcome these limitations, the ICTV proposed that comparison of coronaviruses using the pairwise amino acid difference of seven non-structual proteins would provide order.

Alternatively, pairwise amino acid distances of the RdRP-grouping units (816 nucleotides RdRp, nsp12) fragment.

Avian coronaviruses

Infectious bronchitis virus causes a highly contagious disease of chickens affecting the performance of both broilers and layers

Bovine coronavirus

Bovine coronavirus causes both respiratory and enteric disease, including calf diarrhoea, winter dysentery in adults and respiratory infections in cattle of all ages. Virus isolated from cattle with either enteric or respiratory disease are antigenically similar and studies suggest the antibodies to bovine coronavirus provide immunity (Weiss and Navas-Martin, 2005).

Feline coronavirus

Two variants of feline coronavirus (FCoV) are known, an avirulent form feline enteric coronavirus (FECV) commonly found in a carrier state in up to 90% of cats and the less common virulent form, feline infectious peritonitis virus (FIPV), which develops in 5% of cats infected with FECV. FIPV, which differs from FECV by only a single nucleotide polymorphism or deletion in the 3c gene, is selected during the persistent infection of predominantly intestinal epithelial cells (eneterocytes) and has the ability to replicate in macrophages leading to viremia and systemic spread of the virus, causing a severe and lethal disease (Hartmann, 2005, Weiss and Navas-Martin, 2005, Pedersen, 2009).

FECV is distributed worldwide and is endemic in multiple cat environments such as catteries, shelters and pet stores, where cats are regularly exposed oronasally to faeces (the major route of transmission) in litter trays shared with infected cats. It is relatively rare in free-roaming ownerless cats that do not use the same location to deposit their faeces. However, infection will spread rapidly amongst these free roaming ownerless cats if they are kept close together in a shelter. Most commonly, kittens are infected at 6-8 weeks of age once the maternal antibodies have waned and they are exposed to FECV. It has been shown that naturally infected cats shed FECV intermittently for periods up to 10 months but some become chronic shedders, doing so for years or a lifetime and provide a

constant source of infection to other cats. The viral load of FECV in faeces appears to decrease once the cat develops FIP (Hartmann, 2005, Weiss and Navas-Martin, 2005).

Whilst genetically distinguishable, FECV is closely related to transmissible gastroenteritis virus of pigs and canine coronavirus, and recombinants between these three viruses are known to occur (Pedersen, 2009)

Human coronaviruses

Prior to the emergence of SARS in 2003, two other coronaviruses, Human coronavirus 229E and OC43 (renamed Betacoronavirus 1), were both known to be etiological agents for disease in humans, both causing the common cold (Weiss and Navas-Martin, 2005, International Committee on Taxonomy of Viruses, 2012). Since then two other coronaviruses associated with respiratory disease in humans have also been identified; Human coronavirus HKU1 and NL63. Isolated from an elderly patient with pneumonia, HKU1 is difficult to propagate in cell culture and little is known of its biology. NL63 is an *Alphacoronavirus* isolated from a 7 month old child in the Netherlands suffering from bronchiolitis and conjunctivitis. It has subsequently been identified in other countries including Australia. NL63 is generally associated with infections of children but has also been detected in immunocompromised adults with respiratory tract infections (Weiss and Navas-Martin, 2005).

Murine coronaviruses

There are many variants of murine coronavirus (MHV). Commonly used laboratory variants provide animal models for encephalitis, hepatitis and demyelinating disease such as multiple sclerosis. Other variants cause enteric disease and are easily spread via the oral-faecal route (Weiss and Navas-Martin, 2005).

Porcine coronaviruses

Transmissible gastroenteritis virus (TGEV) is a major cause of viral enteritis and foetal diarrhoea in swine. The disease is most severe in neonates, infecting epithelial cells of the small intestine and leading to potential fatal gastroenteritis with significant economic losses. In adults, TGEV causes mild disease. An attenuated variant of TGEV, porcine respiratory virus (PRCoV), resulted from the deletion of up to 707 nucleotides in the 5’

region of the spike gene. This emergence of PRCoV from TGEV is an example of evolution with altered tissue tropism and virulence (Weiss and Navas-Martin, 2005).

Middle East respiratory syndrome coronavirus

In June 2013, a 60 year man was admitted to a hospital in Saudi Arabia with a seven day history of fever, cough, expectoration and shortness of breath. Findings from chest radiography were consistent with a lung infection and 11 days later the man died from progressive respiratory and renal failure. Subsequently, a novel coronavirus (Human coronavirus Erasmus Medical Centre, HCoV-EMC) isolated from the man's sputum was identified as the causative agent for his death, a constellation of symptoms now known as Middle East respiratory syndrome (MERS). Only three months later, a patient in a London hospital with reported travel to Saudi Arabia was reported to have been infected with the same virus, and cases continue to occur (Figure 7) (Zaki, 2013).

Characterisation of HCoV-EMC, now known as MERS-CoV, identified that its closest relatives were coronaviruses HKU4 and HKU5 isolated from bats in Hong Kong. It was hypothesised that the reservoir host for this new coronavirus could also be bats but molecular clock analysis had been unable to detect any direct ancestors. Anecdotal exposure histories suggested patients had been in contact with dromedary camels or goats (Reusken *et al.*, 2013, Zaki, 2013). Serological studies (which are best suited to screen animal populations for evidence of previous infection) later confirmed that dromedary camels from Omani and the Canary Islands (Spain) had specific antibodies against MERS-CoV spike protein (Reusken *et al.*, 2013). Soon after, MERS-CoV was identified in dromedary camels from a farm in Qatar linked to two human cases (Haagmans *et al.*, 2014). Subsequently, during the surveillance of bats in Saudi Arabia, a coronavirus which showed 100% nucleotide similarity to MERS-CoV was identified in a *Taphozous perforatus*. This discovery suggested that in addition to SARS, bats again might play a role in the infection of humans with coronaviruses (Ithete *et al.*, 2013, Memish *et al.*, 2013).

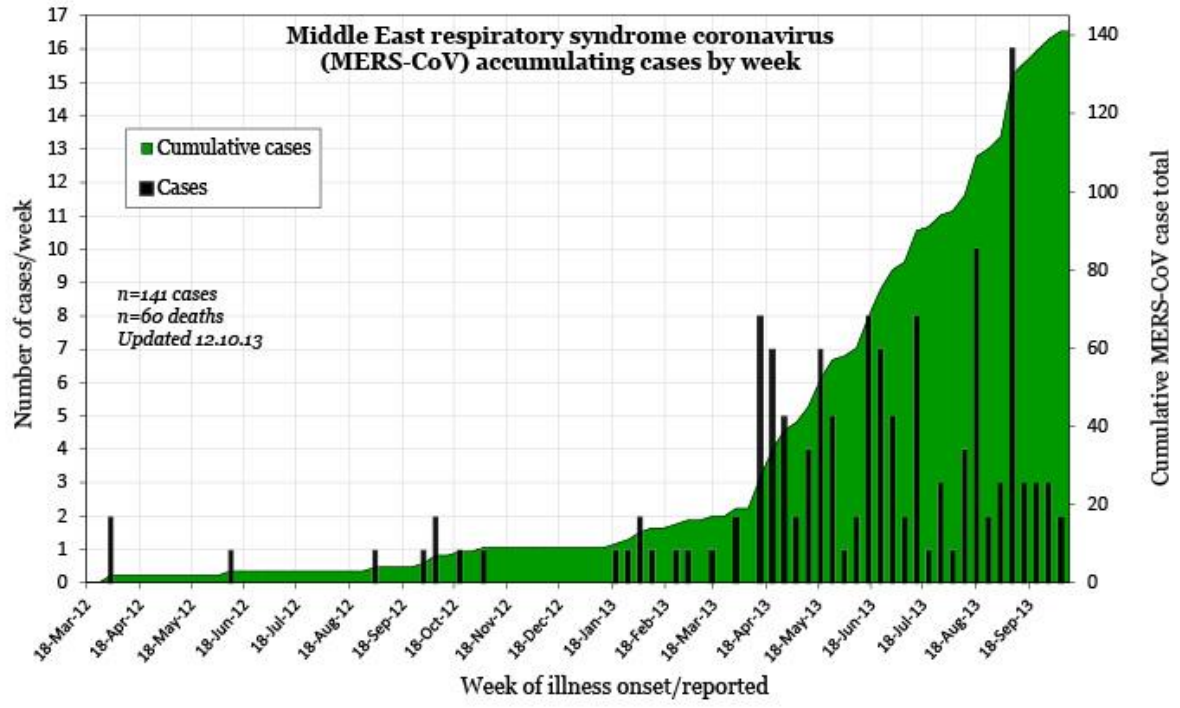


Figure 7. Weekly and cumulative cases of Middle East respiratory syndrome coronavirus (MERS-CoV).

(Mackay, 2013).

Gathering evidence

The global identification and characterisation of bat coronaviruses continues, clarifying the phylogeny between coronaviruses and highlighting the relevance of bats for their evolution (Quan *et al.*, 2010, Reusken *et al.*, 2010, Rihtaric *et al.*, 2010, Watanabe *et al.*, 2010, Smith *et al.*, 2011a, Lu and Liu, 2012, Shirato *et al.*, 2012, Tao *et al.*, 2012, Tsuda *et al.*, 2012, Anthony *et al.*, 2013, Corman *et al.*, 2013, Geldenhuys *et al.*, 2013, Goes *et al.*, 2013, Ithete *et al.*, 2013, Lelli *et al.*, 2013, Memish *et al.*, 2013, Drexler *et al.*, 2014). Additional studies discuss the ecology of the viruses and are discussed below (Lau *et al.*, 2010, Drexler *et al.*, 2011, Lau *et al.*, 2012).

Whilst interspecies transmission of coronaviruses is known to occur, they are poorly understood. Lau *et al.* (2012) identified the transmission of a novel bat coronavirus, HKU10, between bats from different suborders. Their data suggested an interspecies transmission of the coronavirus from *Rousettus leschenaultia* to *Hipposideros pomona*, circa 1959, with rapid evolution of the spike protein. In Chapter 3 of this thesis, I also

provide evidence that interspecies transmission was observed and supports the hypothesis that bats from the genus *Rhinolophus* may be more likely to foster host shifts than other species of bats, posing a risk for the emergence of other bat coronaviruses (Cui *et al.*, 2007).

Knowledge of the ecology of bat-borne viruses is lacking (Drexler *et al.*, 2011). Chapters 5 and 6 of this thesis attempt to address this lack of knowledge by investigating how coronaviruses are transmitted within a population of bats and maintained in individuals. Two recent studies also investigate the ecology of coronaviruses in bats (Lau *et al.*, 2010, Drexler *et al.*, 2011). Drexler *et al.* (2011) identified that there was strong and specific amplification of coronaviruses during the formation of a maternity colony of *Myotis myotis* and after parturition. It was hypothesised that the availability of susceptible bats during colony formation (mixing of infected and susceptible bats) and after parturition (the birth of susceptible pups) resulted in a viral epidemic that wanes as bats mount their own adaptive immunity. Lau *et al.* (2010) employed a mark-recapture study to identify the infectious period of coronaviruses in Chinese horseshoe bat (*Rhinolophus sinicus*). From 511 marked bats and 152 recapture events, they identified the longest shedding period was two weeks and viral clearance between two weeks and four months. From this, it was suggested that coronaviruses cause acute, self-limiting infection in horseshoe bats (Lau *et al.*, 2010).

In conjunction with the published book chapter, this brief review will serve to introduce the identification and ecology of bat coronaviruses.

BAT CORONAVIRUSES

Craig Smith [b], *Hume Field* [b] *and Lin-Fa Wang* [d]

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,

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

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)	
			serotinus	0 (1)	
		Ia	*io*	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).

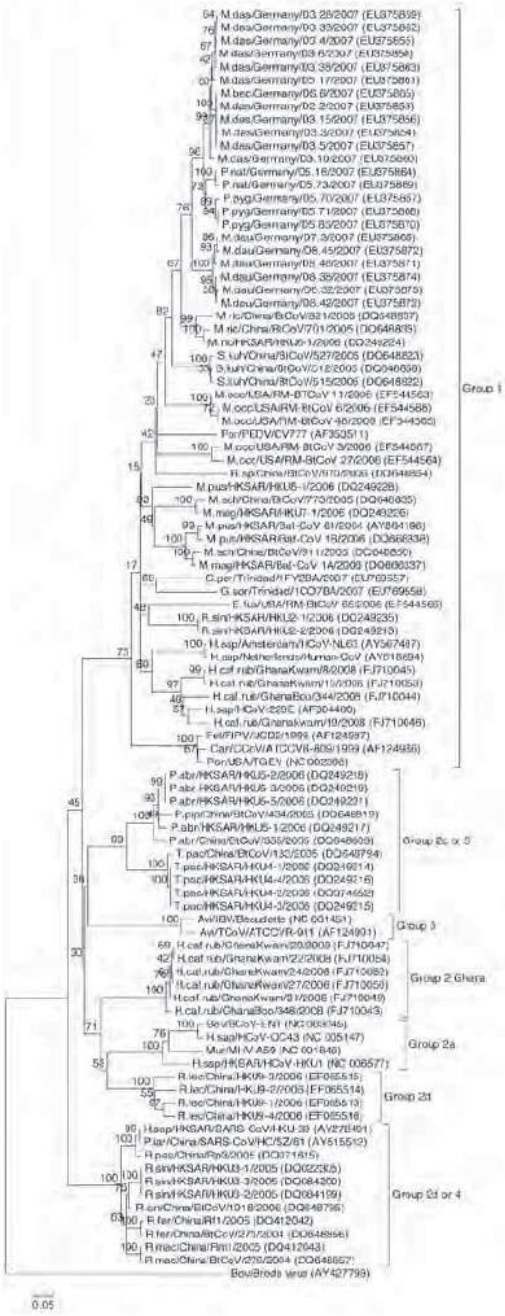
Li *et al.* (2005) found that SARS CoV and SARS-like CoVs share several unique open reading frames (ORFs) that are not found in any other CoVs, confirming an extremely close genetical relationship. Lau *et al.* (2005) concluded that SARS-like CoVs were an early split-off from other group 2 CoVs and should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

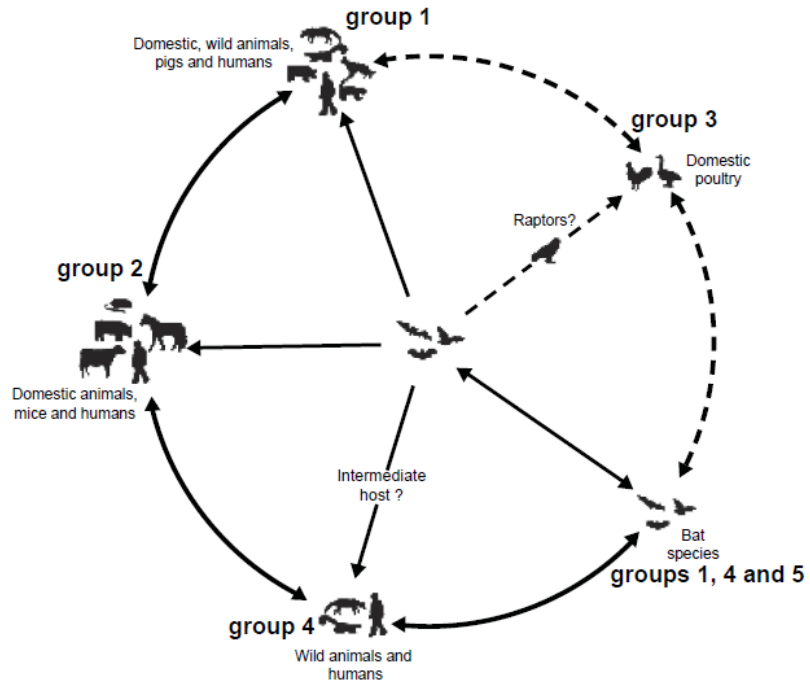
Recombination may allow adaptation to new hosts and ecological niches, and transmission of CoVs among bats, other wildlife, livestock, companion animals or humans (Lau *et al.,* 2005; Poon *et al.,* 2005; Tang *et al.,* 2006; Woo *et al.,* 2006; 2007). Chu *et al.* (2006) identified a group 1 bat CoV 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).

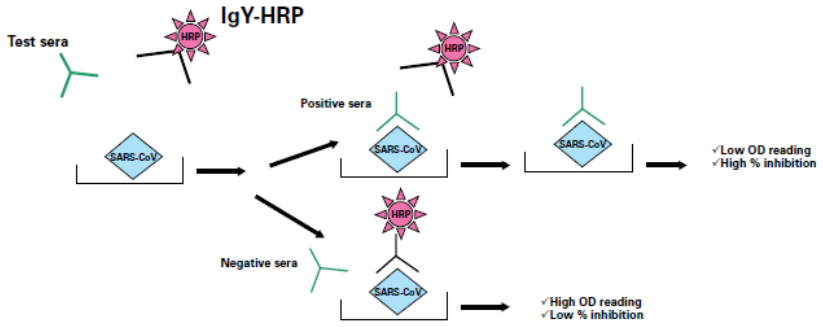
Quantitative real-time PCR: Quantitative real-time PCR targeting the polymerase and nucleocapsid genes have been developed by Ng *et al.* (2003).

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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Chapter 2 Sampling small quantities of blood from bats

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SHORT NOTES

Sampling small quantities of blood from microbats

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Key words: bats, bleeding, blood, mammals, plasma, sampling, serum

INTRODUCTION

Sampling blood from bats can be valuable for a range of studies including antibody detection for disease surveillance (Young *et al.*, 1996; Johara *et al.*, 2001; Li *et al.*, 2005), analysis of blood biochemistry (McLaughlin *et al.*, 2007) and populations genetics (Cardinal and Christidis, 2000; Appleton *et al.*, 2004). However, sampling sufficient volumes of blood, plasma or serum to satisfy a study's requirements from microbats can be challenging.

In the past, a range of techniques have been used including cardiac puncture (La Motte, 1958), bleeding from the orbital sinus (Baer, 1966), nicking a brachial or jugular vein with a scalpel (Baer and McLean, 1972) and puncture of the propatagial or uropatagial vein (Gustafson and Damassa, 1985; Entwistle *et al.*, 1994; Wimsatt *et al.*, 2005; Ellison *et al.*, 2006). Cardiac puncture yields good quantities of blood, however considerable mortality is often experienced (La Motte, 1958; Baer, 1966). Bleeding from the orbital sinus has commonly been used to sample bats, however yielding sufficient volumes of blood can sometimes be difficult (Baer and McLean, 1972) and Swann (1997) identified that the technique may have an adverse affect on the survival of some species of rodents. As such, cardiac puncture and orbital bleeding are no longer recommended as appropriate techniques for bleeding animals that are intended for release, however, cardiac puncture is still appropriate when exsanguination under anaesthesia is required (Morton *et al.*, 1993). Morton (1993) also recommended that a scalpel blade should not be used as it was imprecise and may lead to accidental mutilation of the animal, or operator if the animal was not adequately restrained. Several studies have described the sampling of blood via venipuncture using a heparinised haematocrit tube or glass micropipette and were able to yield sufficient volumes of blood (10–200 µl) to satisfy the study's requirements. They also identified that neither bleeding nor the use of anaesthesia had an effect on survival (Baer and McLean, 1972; Gustafson and Damassa, 1985; Wimsatt *et al.*, 2005; Ellison *et al.*, 2006). It is important that bleeding techniques are continually refined (Morton *et al.*, 1993) and so we describe a technique for sampling small quantities of blood from microbats and report the volumes taken from 1,129 bats.

MATERIALS AND METHODS

Bats were caught between 2006 and 2009 using a handnet or harptrap and placed individually into light-weight cloth bags (10 cm × 15 cm) secured with a drawstring (Hall, 1979). These cloth bags were then suspended from plastic tubing inside a polythene cooler using plastic clothes pegs (Hall, 1979). A thermometer and hygrometer were used to monitor the internal environment of the cooler so that it could be maintained at a temperature and humidity similar to that of the bats roost. The coolers' lid was left slightly ajar to allow adequate ventilation and to prevent excess humidity.

Morphometric measurements were taken from the bats before being bled. The bats' mass was measured to the nearest 0.5 g using a spring balance and its forearm length was measured to the nearest 0.1 mm using callipers. For bleeding, bats were manually restrained between the thumb and palm of the non-preferred hand. The bats' wing was extended until its fore and upper arm formed a 90° angle and then restrained between the fore and middle finger (Fig. 1A). The venipuncture site was prepared with a 70% ethanol swab and a sterile 25 g needle was used to puncture either the brachial (Fig. 1B) or the propatagial vein. Venous blood would then bead on the surface of the skin (Fig. 1C) and could be collected in 12 µl aliquots using a 20 µl micropipette and sterile tip (Fig. 1D). The first aliquot of blood

was added directly to 108 µl of phosphate buffered saline (PBS). Additional aliquots of blood were sampled and added to the same PBS until the maximum recommended blood volume was collected (less than 10% of the circulating blood volume or 6 µl/g of an animals mass, (Morton *et al.*, 1993). A clean cotton wool ball and pressure from the thumb were applied to the venipuncture site until bleeding ceased. Additional 108 µl aliquots of PBS were immediately added to the sampled blood to achieve a final dilution of 1:10 and mixed briefly using the pipette. Blood was centrifuged or allowed to settle overnight at 4ºC and the diluted plasma fraction removed for storage at -20ºC and later analysis. A volume of PBS equivalent to the plasma fraction was added to the remaining blood cells to maintain a 1:10 dilution and provide a haemostatic buffer. Alternatively, the sampled blood could be applied directly to filter paper (Ruangturakit *et al.*, 1994). A subset ($n = 89$) of the 1,129 bats that we bled had their blood sample observed for any evidence of clotting.

Field work was conducted with approval from: Animal Ethics, Queensland Primary Industries and Fisheries (QPIF), Department of Employment, Economic Development and Innovation (DEEDI); Environmental Protection Agency, Queensland Parks and Wildlife Service and the Northern Territory Parks and Wildlife Commission (NTPWC).

Results

We bled 1,129 individuals representing eight species of microbats (Table 1). On average we collected 4 µl of blood/g of the bats' mass (SD = 1.6, min–max = 0.1–12.0). Experienced operators could sample a bat in less than six minutes and for each 12 µl of blood sampled we were able to retrieve 100 µl of plasma diluted 1:10 in PBS. Partial clotting was observed in approximately 2% of samples ($n = 2$). All bats were released at their site of capture and observed flying back to or around the entrance of the roost; no deaths were recorded whilst bats were in our care.

Discussion

We have described a technique to sample up to 6 µl of blood/g from microbats. When removing this volume of blood from rats, K. J. Nahas, P. Provost, C. Sobry and Y. Rabemampianina

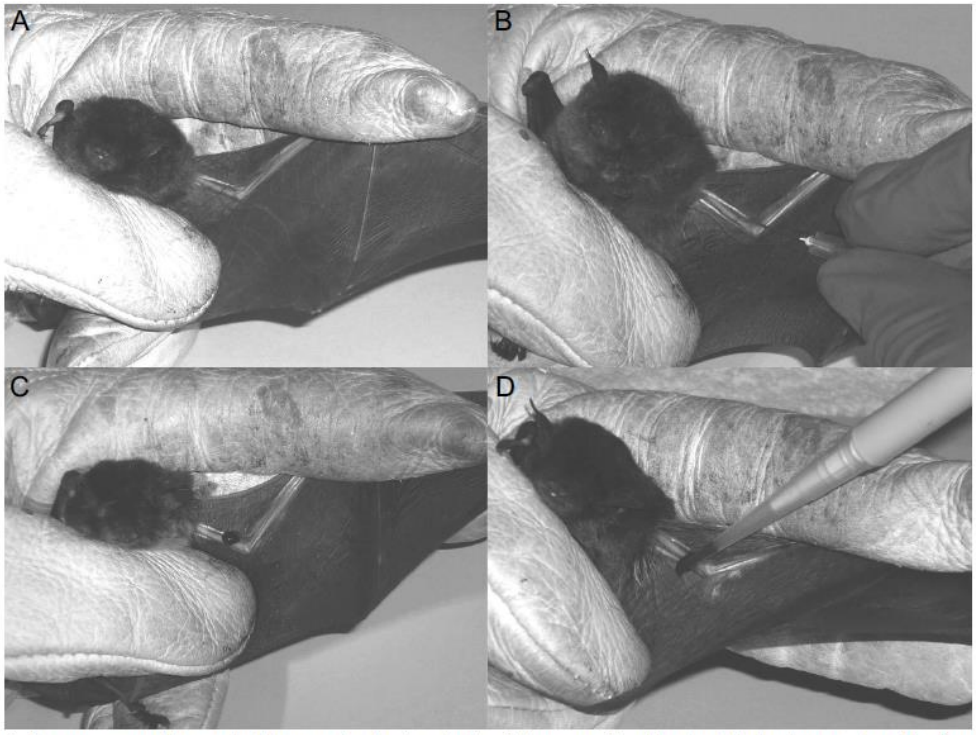

FIG. 1. Bats were manually restrained between the thumb and palm of the non-preferred hand and their wing extend until its fore and upper arm formed a 90° angle (A). The bleed site was prepared with a 70% ethanol swab and a 25 g needle was used to puncture either the brachial (B) or the propatagial vein. Venous blood would then bead on the surface of the skin (C) and could be sampled using a micropipette and sterile tip (D)

TABLE 1. Mean volume of blood/g of the bats' mass sampled from 1,129 bats representing eight species of microbats; $\bar{x} \pm$ SD (min–max)

Species	*n*	Blood volume (µl)	Mass (g)	Blood volume/Mass (µl/g)
Hipposideros ater	27	33 ± 9 (12–48)	6.1 ± 0.6 (5.0–7.0)	5.4 ± 1.5 (2.4–8.7)
Macroderma gigas	38	43 ± 18 (12–60)	104.6	0.4 ± 0.2 (0.1–0.6)
Miniopterus australis	180	37 ± 11 (12–60)	7.5 ± 0.8 (5.5–10.5)	5.0 ± 1.5 (1.1–9.2)
M. schreibersii	273	49 ± 14 (12–84)	14.2 ± 1.6 (10.0–18.0)	3.5 ± 1.0 (0.7–6.3)
Myotis adversus	31	51 ± 13 (12–60)	10.4 ± 1.2 (8.0–12.5)	4.9 ± 1.4 (1.0–7.5)
Rhinolophus megaphyllus	471	44 ± 12 (12–72)	11.2 ± 1.5 (8.0–15.5)	4.0 ± 1.2 (1.1–7.6)
Rhinonycteris aurantius	78	27 ± 10 (12–48)	8.2 ± 0.8 (6.5–10.5)	3.3 ± 1.2 (1.3–6.0)
Vespadelus troughtoni	31	33 ± 12 (12–72)	5.3 ± 0.6 (4.0–6.5)	6.1 ± 2.3 (2.0–12.0)

(unpublished data) identified that haematological parameters including red blood cell count, haemoglobin level, haematocrit, mean corpuscular volume and red cell distribution width all returned to normal within 14 days. We found that a 25 g needle was suitable for puncturing the brachial or propatagial vein of the insectivorous bats that we bled, however, a smaller 27 g needle may be preferred by the operator for puncturing other veins, including the interfemoral (Wimsatt *et al.*, 2005) or the brachial or propatagial vein of smaller insectivorous bats. On the rare occasion when the brachial artery, which lies adjacent, was accidently punctured instead of the vein, extraneous bleeding occurred (9.2 µl/g collected from a *M. australis* and 12 µl/g collected from a *V. troughtoni*). When this occurred, the beaded blood was immediately collected using a larger micropipette and a clean cotton wool ball and pressure from the thumb were applied to the puncture site until bleeding ceased. In these cases, with the immediate response to a punctured artery and even sometimes with a punctured vein, extraneous blood was lost onto the cotton wool. This loss was neither quantified nor included in the analysis. However, given that the mean volume of blood/g of the bats' mass sampled did not exceed 6 µl for this study there was often still a volume of blood available to be lost to the cotton wool. It is for this reason and for the benefit of the bats being sampled that we recommend aiming to collect less than 6 µl of blood/g of the bats' body mass.

We observed that experienced operators could sample a bat within six minutes. This included taking morphometric measurements, sampling blood, ensuring that bleeding had ceased, recording details and preparing equipment for the next bat to be sampled. Manual restraint and bleeding without anaesthesia simplifies fieldwork and does not effect the survival of bats (Entwistle *et al.*, 1994; Wimsatt *et al.*, 2005; Ellison *et al.*, 2006) and most small rodents (Swann *et al.*, 1997), since the associated stress of anaesthesia would probably be greater than the discomfort of venipuncture (Morton *et al.*, 1993). Also, by wearing leather and nitrile gloves, and by discarding used needles directly into a biohazard container after venipuncture, we found it a simple task to manually restrain bats without the need for anaesthesia whilst decreasing the risk of a bat bite or needle stick injury, as was also found by Ellison (2006).

Our technique of immediately diluting blood 1:10 in PBS allowed the retrieval of plasma without the need for anti-coagulants. For each 12 µl of blood sampled we were able to retrieve 100 µl of diluted plasma. This diluted plasma fraction was removed for storage at -20ºC where IgE antibodies are stable for at least 37 years (Henderson *et al.*, 1998). Alternatively, sampled blood could be applied to filter paper, where IgG antibodies are stable for at least five months (Ruangturakit *et al.*, 1994). Partial clotting was observed in approximately 2% of blood samples, but even with these clotted samples we were able to retrieve sufficient volumes of serum to satisfy the study's requirements. Antibody detection tests, such as an enzyme-linked immunosorbent assay (ELISA) require only a small volume of undiluted serum or plasma, approximately 2 µl, which is usually diluted 1:50 during the test methodology. To perform an ELISA using our diluted plasma we modified the ELISA methodology to account for the existing dilution.

No deaths were recorded whilst bats were in our care and upon release bats were observed flying back to or around the entrance of the roost. Whilst we are unable to comment on the long-term survival of these released bats, Entwistle (1994), Wimsatt (2005) and Ellison (2006) all reported that sampling blood from bats did not decrease their survival rate when compared to control groups that were also captured and handled but not bled. In an unrelated mark-recapture study in which we used our blood sampling technique (C. S., Smith, C. E. de Jong,

G. Crameri, J. MaEachern, M. Yu *et al*., unpublished data), we recaptured 42 of 52 *Myotis macropus*. This study did not have a control group and calculating survival rates was not possible, however, it was encouraging to observe the short-term (three months) survival of recaptured bats which we had sampled.

Acknowledgements

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Chapter 3 Identification and inter-species transmission of Australian bat coronaviruses: the precursors for emergence and indications of host taxonomy tropism suggesting co-evolution

Introduction

Coronaviruses were responsible for the global outbreak of severe acute respiratory syndrome (SARS) in 2003 and 2004, and the outbreak of Middle East respiratory syndrome (MERS) in 2013 (Drosten *et al.*, 2003, Zaki *et al.*, 2012). Bats have since been identified as the natural hosts for a number of novel coronaviruses, including the likely ancestors to SARS and MERS coronaviruses (Lau *et al.*, 2005, Li *et al.*, 2005, Memish *et al.*, 2013). Even before the identification MERS-like coronaviruses in bats, it was suspected that they could host a large diversity of novel coronaviruses (Woo *et al.*, 2006). The identification and characterisation of coronaviruses found in Australasian bats is essential to advance our understanding of this diversity and elaborate on the ecology and evolution of bat coronaviruses, and inform biosecurity preparedness.

Materials and methods

Sampling

A total of 2,195 bats from Australia and neighbouring countries were sampled between 1997 and 2009 for evidence of coronavirus infection (Figure 8). Bats were caught using harp traps (Figure 9), then individually housed in clean cloth bags and a polythene cooler until sampled (Figure 10). A single faecal pellet (collected directly from a defecating bat or from its clean calico bag) was placed into 1 ml of sucrose potassium glutamate albumin (SPGA) with added penicillin, streptomycin and fungizone. When no faecal pellet was obtained, the anus was swabbed. Insectivorous bats were bled as described by Smith *et al.* (2010) in Chapter 2 but briefly, a 25 g needle was used to puncture either the brachial or the propatagial vein. Venous blood would then bead on the surface of the skin and could be collected using a micropipette and sterile tip (Figure 11). Collected blood was diluted 1:10 in phosphate buffered saline to limit clotting. All bats were released at their point of capture within 6 hours. Twenty bats caught in central Queensland in an unrelated study in 1997, which had been euthanased and subsequently stored at -70ºC, were also sampled. These bats had a 2 mm^2 section of their intestine homogenised in 1 ml of SPGA.

Forty eight faecal samples collected from Taiwanese bats and civets were placed into 1 ml of AVL from the QIAamp® Viral RNA Mini Kit (QIAGEN) and stored at room temperature for 1 week until extracted. Additional serum samples collected from the previous surveillance of bats (East Timor, n=36; Indonesia, n=67; Malaysia, n=101 and Papua New Guinea, n=65) and subsequently stored at -20°C, were also tested for evidence of coronavirus infection.

Sampling was conducted with approval from the Department of Primary Industries and Fisheries, Queensland, Animal Ethics (SA 2006/06/117 and SA 2007/005/194), Environmental Protection Agency, Queensland Parks and Wildlife Service (WISP03887606 and WISP04906107).

Coronavirus detection and sequencing

Template RNA was extracted from 560 μl of SPGA using the QIAamp® Viral RNA Mini Kit (QIAGEN) following the manufacturer's instructions (QIAGEN, 2010). Reverse transcription followed by cDNA amplification using a polymerase chain reaction (RT-PCR) targeting a conserved region of the coronavirus RdRp gene, as described by Poon *et al.* (2005), was performed using the Superscript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen). Amplicons consistent with the expected length of 440 nucleotides were purified using the QIAquick® PCR Purification Kit (QIAGEN) as per the manufacturer's instructions (QIAGEN, 2008). Purified amplicons were directly sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as per the manufacturer's instructions (Applied Biosystems, 2002). The extension products were purified using the ethanol/EDTA precipitation method (Applied Biosystems, 2002) and analysed at the Griffith University DNA Sequencing Facility (Brisbane, Australia). Nucleotide sequence traces were edited using Sequence Scanner v1.0 (Applied Biosystems). The final consensus sequences, derived from sense and anti-sense primers, were deposited in GenBank under accessions numbers EU834950-EU834956. In an attempt to obtain additional sequence for phylogenetic analysis, ten pairs of additional primers targeting regions of the RdRp, nucleocapsid and spike genes were applied (Li *et al.*, 2005, Poon *et al.*, 2005, Chu *et al.*, 2006).

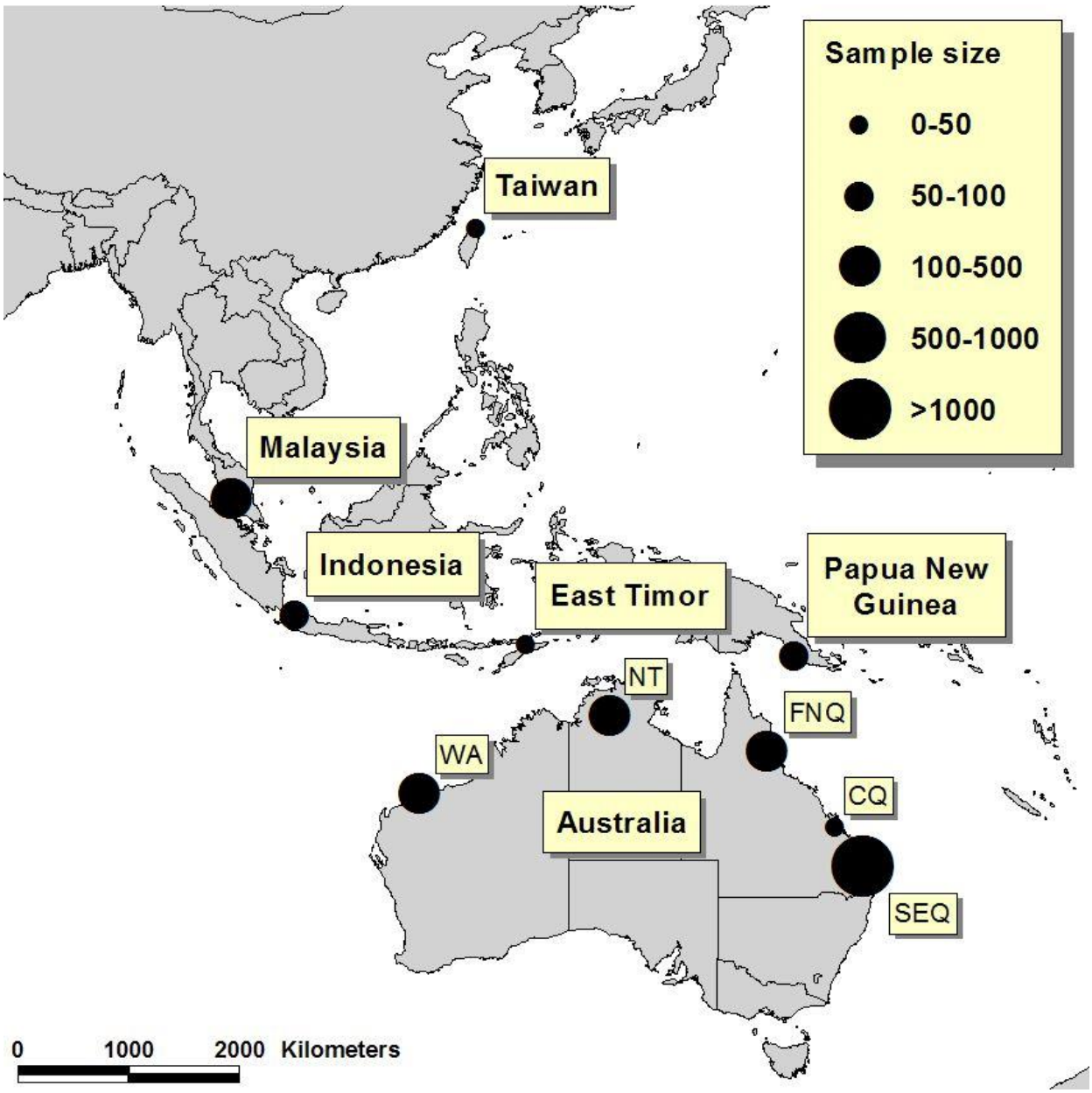

Figure 8. Sample locations for Australasian bat coronavirus surveillance.

Locations of 2,195 bats from Australia and neighbouring countries sampled between 1997 and 2009 for evidence of coronavirus infection. Australasian bats were sampled from south-east Queensland (SEQ, n=1162), central Queensland (CQ, n=42), far-north Queensland (FNQ, n=222), the Northern Territory (NT, n=333), Western Australia (WA, n=119) and Taiwan (n=48). Additionally, archived bat samples from East Timor (n=36), Indonesia (n=67), Malaysia (n=101) and Papua New Guinea (n=65) were also sampled.

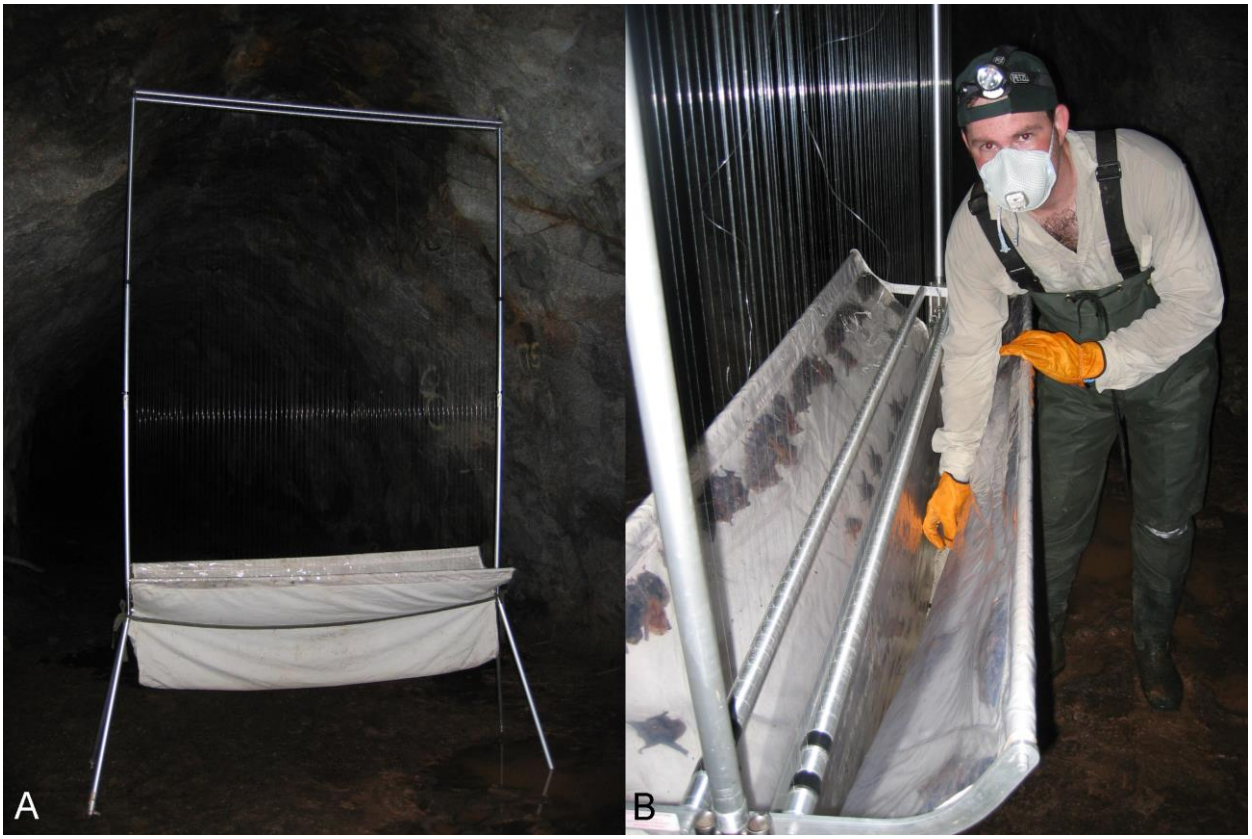

Figure 9. A collapsible bat trap.

The collapsible bat trap (A), commonly known as a harp trap was developed by Tidemann and Woodside (1978) based on the original designs of Constantine (1958) and Tuttle (1974). The trap is a common tool used for the capture of insectivorous bats and is best placed in the natural flight path of bats, including; roads, trails, streams and roost entrances. The trap is light and portable and can be set up in 5 minutes by a single person (Tidemann and Woodside, 1978). The author removing captured bats from the bag of a harp trap (B).

Coronavirus classification

Because of the difficulties in isolating bat coronaviruses, or the presence of faecal substances that often contribute to the inhibition of RT-PCR, obtaining a sequence from the seven genes in ORF1ab (as formally required for classification) is infrequent (Drexler *et al.*, 2010). The 440bp amplicon, derived from the universal coronavirus RT-PCR used in this and other ecological studies (Poon *et al.*, 2005), is often insufficient to obtain reliable resolution in phylogenetic analysis (Drexler *et al.*, 2010). To obtain a surrogate estimation of taxonomy, Drexler et al. (2010) overlapped and extended the sequencing of this 440bp universal amplicon downstream towards the 5' end of coronaviruses, producing a 816bp gene fragment which was used to calculate the distance for all available coronaviruses. This 816bp gene fragment or RdRp grouping unit (RGU) was then used as the basis for defining species separation in mammalian coronaviruses; i.e. >4.8% amino acid distance for *Alphacoronaviruses* and >6.3% amino acid distance for *Betacoronaviruses* (Drexler *et al.*, 2010). However, the field and lab work in this study preceded the publication of Drexler *et al.* (2010), and only 440bp were available for virus classification. Acknowledging this limitation, this study will utilise the concept of the RGU to calculate distance of coronaviruses, which is adequate for the primarily, disease ecology focus of the work.

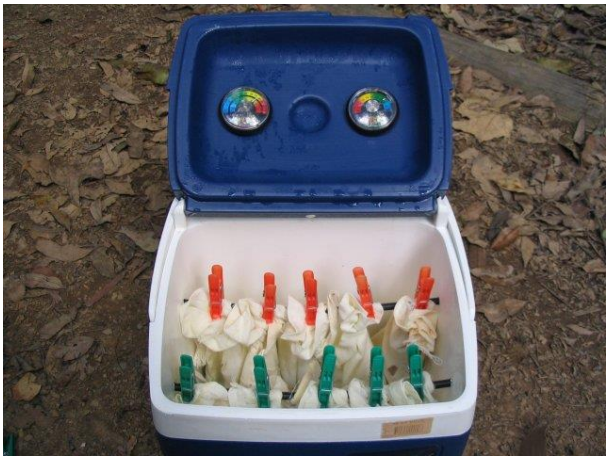

Figure 10. Polythene cooler used to house and transport bats.

Based on the design by Hall (1979), clean cloth bags contain an individual bat and are suspended from plastic tubing inside a polythene cooler using plastic clothes pegs, a thermometer and hygrometer were used to monitor the internal environment of the cooler so that it could be maintained at a temperature and humidity similar to that of the bats roost. The coolers' lid was left slightly ajar to allow adequate ventilation and to prevent excess humidity.

Molecular phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-16168.6385) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6965)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.0000% sites). The tree is drawn to scale, with branch

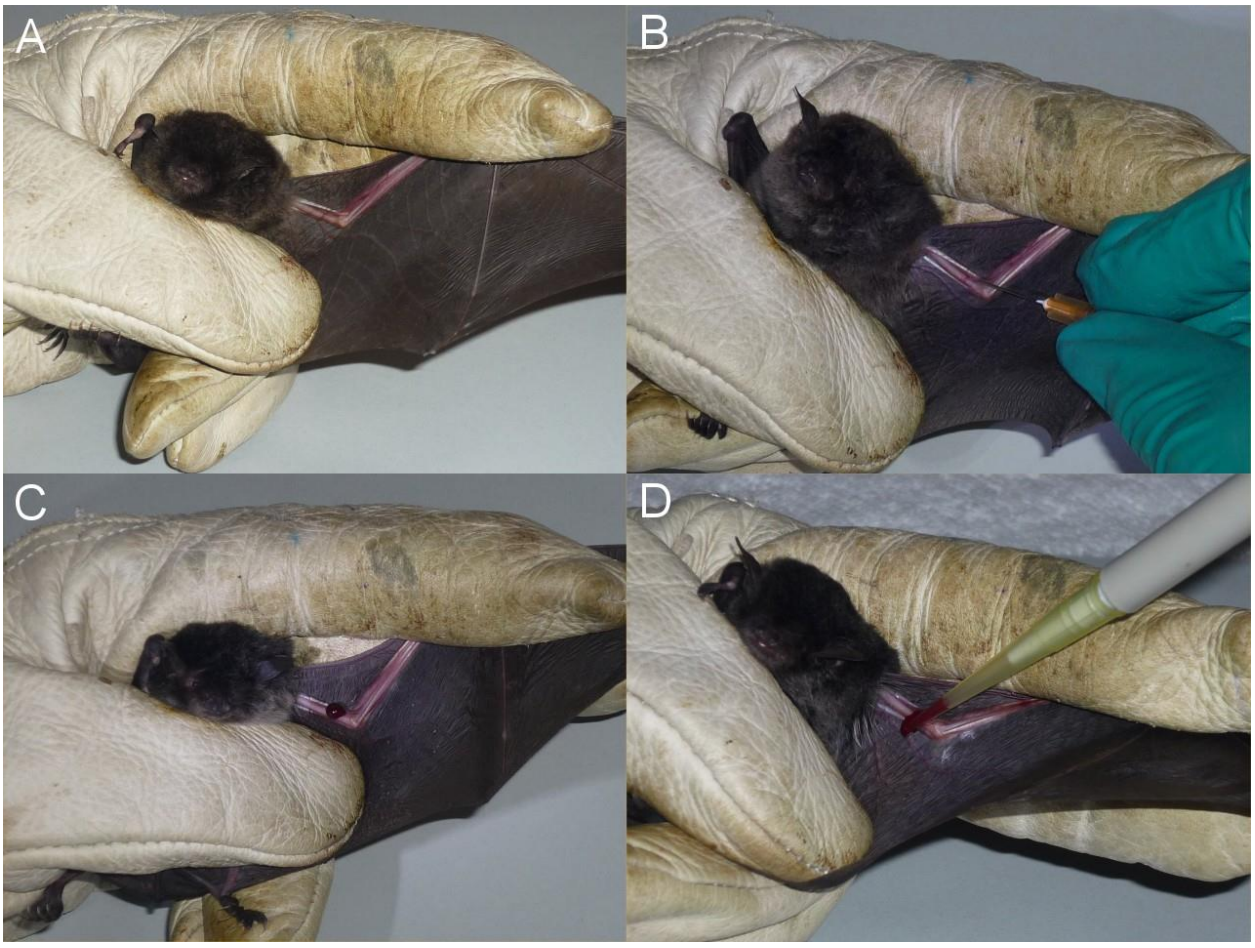

Figure 11. Sampling small quantities of blood from bats.

Bats were manually restrained between the thumb and palm of the non-preferred hand and their wing extend until its fore and upper arm formed a 90° angle (A). The bleed site was prepared with a 70% ethanol swab and a 25 g needle was used to puncture either the brachial (B) or the propatagial vein. Venous blood would then bead on the surface of the skin (C) and could be sampled using a micropipette and sterile tip (D). Colour plate from Smith *et al.* (2010).

lengths measured in the number of substitutions per site. The analysis involved 43 nucleotide sequences. There were a total of 878 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

Anti-coronavirus antibody detection

Anti-coronavirus antibodies were detected using a modified SARS coronavirus crude antigen ELISA developed by Yu et al. (2006). Whilst using the same antigen (gamma-irradiated SARS-CoV, grown in Vero E6 cells), the scarcity of the horseradish peroxidase (HRP) conjugated anti-coronavirus chicken antibodies (developed for the competitive ELISA) were replaced with HRP-conjugated Protein AG for the detection of bat anti-coronavirus antibodies bound directly to the antigen.

Tissue Tropism

To identify tissues tropism of Australian bat coronaviruses, a subset of 30 bats (*Miniopterus australis*, n=14; *M. schreibersii*, n=16) from south-east Queensland, had throat swabs and blood samples, in addition to the faecal samples or rectal swabs, tested for the presence of coronavirus RNA by RT-PCR, as above.

Results

Coronavirus identification

Sequencing of amplicons and subsequent phylogenetic analyses identified four coronaviruses in seven species of Australian bats. An *Alphacoronavirus* was identified in *M. australis* and *M. schreibersii* sampled between 2006 and 2008, from south-east and far-north Queensland and the Northern Territory (Figure 12 and Table 1). This coronavirus shares >99% RGU similarity with the ICTV reference virus *Miniopterus bat coronavirus HKU8* and based on classification of coronaviruses for this study should be considered a variant of that species. This variant of *Miniopterus bat coronavirus HKU8* was also identified in a single *Rhinolophus megaphyllus* from far-north Queensland and a single *M. australis* sampled in 1997 from central Queensland. *Miniopterus bat coronavirus HKU8* has also been identified in *Miniopterus spp* from Hong Kong and Bulgaria (Poon *et al.*, 2005).

A second *Alphacoronavirus* was identified in both *Myotis macropus* and *Vespadelus pumilus* from south-east Queensland (Figure 12 and Table 1). This *Alphacoronavirus*

shares only 89% RGU similarity to any other coronavirus and should be considered its own species. This putative species is most closely related to another putative species identified in *Pipistrellus kuhlii* from both Italy and Spain (Lelli *et al.*, 2013).

A *Betacoronavirus* identified in a single *Rhinonicteris aurantia* from the Northern Territory was most closely related to another *Betacoronavirus* identified in *Hipposideros caffer ruber* from Ghana. However, this relationship has a RGU similarity <81% and the two Betacoronaviruses should be considered individual putative species (Figure 12 and Table 1).

A second *Betacoronavirus* identified in *Pteropus alecto* should also be considered as a new putative species as it has <87% RGU similarity with its closest related coronavirus hosted in *Rousettus aegyptiacus* from Kenya and *Cynopterus brachyotis* from the Philippines (Figure 12 and Table 1).

Anti-coronavirus antibody detection

Anti-coronavirus antibodies were detected in all species of bats in which coronavirus RNA was detected (where serum or plasma was available for testing), except *R. aurantia* (n=105) (Table 1). Anti-coronavirus antibodies were also detected in an additional 18 species of bats from Australia, East Timor, Indonesia, Malaysia and Papua New Guinea (Table 1)

Tissue Tropism

Coronavirus RNA was detected in 11 faecal samples or rectal swabs from the subset of 30 bats that were sampled to identify tissue tropism. Of these 11 bats, coronaviruses RNA was detected in the throat swabs of only two bats. No coronaviruses was detected in any other throat swab or in any blood samples from the 30 bats.

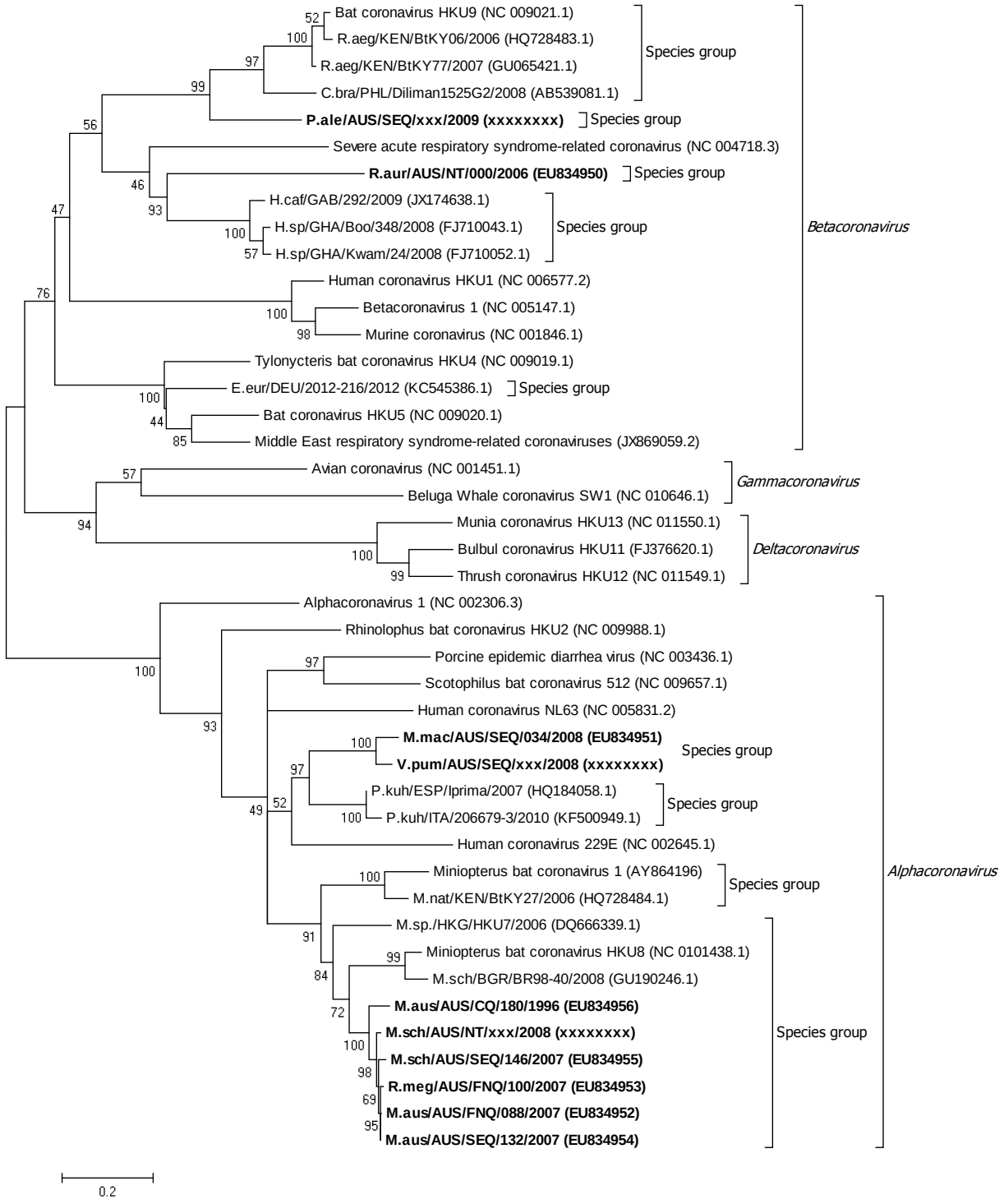

Figure 12. Nucleotide phylogenetic analysis of coronaviruses identified in Australian bats.

Coronaviruses identified in this study are in bold. Square brackets are used to identify species and genus groups. Coronavirus nomenclature: Host species/country of origin/laboratory identification/year collected (GenBank accession).

Figure 13. The demon of Bamford mine.

One hundred meters into the mines adit (horizontal shaft), a hand net is used to capture *Rhinolophus megaphyllus*. Deposits of copper (coloured blue) can be seen on the exposed rock.

Table 1. Surveillance for coronaviruses surveillance in bats in Australasia.

[1]Locations within Australia, central Queensland (CQ), far-north Queensland (FNQ), south-east Queensland (SEQ), Northern Territory (NT) and Western Australia (WA).

[2]Tested using universal coronavirus RT-PCR used in this and other ecological studies, (Poon *et al.*, 2005). No. detected (No. tested).

[3]Tested using SARS coronavirus crude antigen ELISA developed by Yu *et al.* (2006). No. detected (No. tested).

Suborder	Family	Genus	Species	Location[1]	Coronavirus RNA[2]	Coronavirus antibodies[3]
Pteropodiformes	*Hipposideridae*	*Hipposideros*	*ater*	Australia (FNQ)	0 (29)	0 (29)
				Australia (NT)	0 (27)	1 (4)
				Australia (WA)		0 (31)
			terasensis	Taiwan	0 (2)	
		Rhinonicteris	*aurantia*	Australia (NT)	1 (126)	0 (105)
	Megadermatidae	*Macroderma*	*gigas*	Australia (NT)	0 (57)	1 (63)
				Australia (WA)		17 (21)
	Pteropodidae	*Acerodon*	*celebensis*	Indonesia		0 (15)
		Cynopterus	*spp.*	Malaysia		11 (15)
		Dobsonia	*anderseni*	Papua New Guinea		1 (18)
			peronii	Indonesia		0 (1)
			praedatrix	Papua New Guinea		0 (21)
		Eonycteris	*spp.*	Malaysia		11 (12)
		Macroglossus	*minimus*	Papua New Guinea		0 (2)
			spp.	Indonesia		0 (3)

		Pteropus	*alecto*	Australia (SEQ)	4 (33)	9 (34)
				Indonesia		0 (36)
				Papua New Guinea		10 (11)
			capistratus	Papua New Guinea		0 (7)
			conspicillatus	Australia (FNQ)		6 (40)
			griseus	East Timor		0 (1)
			hypomelanus	Malaysia		0 (34)
			neohibernicus	Papua New Guinea		4 (6)
			poliocephalus	Australia (SEQ)	0 (27)	12 (73)
			scapulatus	Australia (NT)		3 (40)
			vampyrus	East Timor		4 (35)
				Malaysia		12 (32)
		Rousettus	*amplexicaudatus*	Indonesia	0 (6)	
			spp.	Indonesia		1 (6)
	Rhinolophidae	*Rhinolophus*	*megaphyllus*	Australia (FNQ)	1 (58)	5 (61)
				Australia (SEQ)	0 (448)	13 (399)
			monoceros	Taiwan	0 (41)	
Vespertilioniformes	*Emballonuridae*	*Saccolaimus*	*flaviventris*	Australia (WA)		0 (18)
		Taphozous	*spp.*	Australia (WA)		8 (38)
				Malaysia		1 (4)
	Miniopteridae	*Miniopterus*	*australis*	Australia (CQ)	1 (20)	15 (30)
				Australia (FNQ)	14 (30)	16 (30)
				Australia (SEQ)	38 (154)	80 (124)

				Australia (WA)		1 (1)
			schreibersii	Australia (NT)	6 (59)	26 (56)
				Australia (SEQ)	63 (238)	145 (211)
	Molossidae	*Chaerephon*	*jobensis*	Australia (WA)		2 (4)
		Mormopterus	*beccarii*	Australia (SEQ)	0 (3)	40 (41)
			norfolkensis	Australia (SEQ)	0 (1)	
	Vespertilionidae	*Chalinolobus*	*spp.*	Australia (WA)		2 (4)
		Myotis	*macropus*	Australia (FNQ)	0 (31)	18 (31)
				Australia (SEQ)	13 (64)	
		Nyctophilus	*bifax*	Australia (SEQ)	0 (6)	
			gouldi	Australia (SEQ)	0 (7)	
		Scotophilus	*spp.*	Malaysia		4 (4)
		Scotorepens	*greyii*	Australia (SEQ)	0 (1)	
			rueppellii	Australia (SEQ)	0 (1)	
			spp.	Australia (SEQ)		24 (24)
				Australia (WA)		0 (1)
		Vespadelus	*findlaysoni*	Australia (WA)		0 (1)
			pumilus	Australia (SEQ)	1 (4)	
			troughtoni	Australia (FNQ)	0 (31)	5 (31)
Feliformia	*Viverridae*	*Paguma*	*larvata*	Taiwan	0 (5)	

Discussion

Identification of coronavirus RNA and anti-coronavirus antibodies in Australasian bats

Whilst acknowledging that the 440bp amplicon derived from the universal coronavirus RT-PCR is often insufficient to obtain reliable resolution in phylogenetic analysis, this study used it to identify four coronaviruses (including three putative novel coronaviruses) in seven species of Australian bats, and detected anti-coronavirus antibodies in an additional 18 species from Australia, East Timor, Indonesia, Malaysia and Papua New Guinea. These identifications and detections support the hypothesis of Woo *et al.* (2006) that bats host a large diversity of novel coronaviruses, possibly due to their own diversity. It also demonstrates the ability for interspecies transmission or spillover of coronaviruses amongst bats, which advances our understanding of the ecology of bat coronaviruses and informs biosecurity preparedness.

Host tropism of bat coronaviruses

Bat coronaviruses have a narrow host range and are generally bat species or genus specific, independent of location (Poon *et al.*, 2004, Chu *et al.*, 2006, Tang *et al.*, 2006, Woo *et al.*, 2006, Gloza-Rausch *et al.*, 2008, Pfefferle *et al.*, 2009, Drexler *et al.*, 2014). Drexler *et al.* (2010) hypothesised that these virus-host associations or tropism could be used in a prospective manner to predict the geographic distribution of bat coronaviruses. Indeed, in support of this contention, Drexler *et al.* (2010) did identify the *Alphacoronaviruses Miniopterus bat coronavirus HKU8* (previously reported in *Miniopterus spp* from the People's Republic of China and Hong Kong Special Administrative Region) in *M. schreibersii* from Bulgaria, over 8,000 km away. The validity of this hypothesis was also confirmed by the current study's identification of *Miniopterus bat coronavirus HKU8* in *M. australis* and *M. schreibersii* from Australia, some 7,000 km from Hong Kong and almost 15,000 km from Bulgaria. Similarly, in support of this hypothesis and the host tropism of bat coronaviruses, is the identification of a novel putative *Betacoronavirus* (by this study) in *Rhinonicteris aurantia* from the Northern Territory, being most closely related to another putative *Betacoronavirus* identified by Pfefferle *et al.* (2009) in *H. caffer ruber* from Ghana, both coronaviruses are h osted by bats of the same family, *Hipposideridae*. Again, the putative *Betacoronavirus* identified in *P. alecto* from south-east Queensland, was most

closely related to the *Betacoronavirus Bat coronavirus HKU9*, identified in *Rousettus spp* from the People's Republic of China and Kenya, and from *Cynopterus brachyotis* from the Philippines, all of which belong to the family *Pteropodidae*. This relationship of related bat coronaviruses being hosted by bats of the same family has also been reported for coronaviruses that are hosted by *Vespertilionidae* (Cui *et al.*, 2007) and by *Rhinolophidae* (Lau *et al.*, 2005, Li *et al.*, 2005). With the reclassification of the taxonomy of bats using comparative-method and molecular studies (Hutcheon and Kirsch, 2006), the suborder *Pteropodiformes* now comprises, amongst others, bats from the families *Hipposideridae*, *Rhinolophidae* and *Pteropidae*. With the identification of *Betacoronaviruses* predominantly from bats of these families, the relationship of related bat coronaviruses being hosted by bats of the same species or genus can now be extended to bats of the same family or suborder; it also suggests that other *Betacoronaviruses* may be hosted by other *Pteropodiformes* (*Craseonycteridae*, *Megadermatidae* and *Rhinomatidae*).

Interspecies transmission of an Australian bat coronavirus: the precursor for emergence

Despite our intensive surveillance (n=506) of the Australian bat *R. megaphyllus*, from the genus that hosts SARS-like coronaviruses in China (Lau *et al.*, 2005, Li *et al.*, 2005), coronavirus RNA was only detected in one bat (Figure 12 and Table 1). This coronavirus was identified as a variant of the *Alphacoronavirus Miniopterus bat coronavirus HKU8* and was identical to the variant identified in *M. australis* at the same roost. This identification is strongly suggestive that the moment of interspecies transmission or spill-over of an *Alphacoronavirus* from *M. australis* to *R. megaphyllus* was observed. Whilst environmental contamination of the samples cannot be excluded, interspecies transmission, or spill-over, and host shifting (defined as interspecies transmission followed by establishment and long-term persistence in the new host species) has been suggested as an explanation for the relatedness of bat coronaviruses identified in different species of bats, and as a driver for their evolution through adaption within the new host species (Poon *et al.*, 2005, Wang *et al.*, 2006, Cui *et al.*, 2007, Vijaykrishna *et al.*, 2007, Chu *et al.*, 2008, Gloza-Rausch *et al.*, 2008, Pfefferle *et al.*, 2009). Emergence of zoonotic viruses from a wildlife reservoir host requires four events; (1) interspecies contact, (2) interspecies transmission of the virus (or spill-over), (3) establishment and long-term persistence in the new host (or host shift), and (4) virus adaptation within the new host (Wang *et al.*, 2006, Cui *et al.*, 2007). This study identified two of the four events that are required for the successful emergence

of an Australian bat coronavirus; (1) there was opportunity for interspecies contact between *M. australis* and *R. megaphyllus* at the same location (Figure 14), and, (2) inter-species transmission of an *Alphacoronavirus* from *M. australis* to *R. megaphyllus* was observed. However, neither (3) establishment or long-term persistence of the virus in the new host, or (4) virus adaptation in the new host were identified. Bats from the genus *Rhinolophus* may be more likely to foster host shifts than other species of bats and pose a risk for the emergence of other bat coronaviruses (Cui *et al.*, 2007). This study identified the interspecies transmission of a variant of *Miniopterus bat coronavirus HKU8* which supports this contention. Additionally, the findings support the hypothesis that the presence of bats from the genus *Rhinolophus* is a risk for the emergence of both SARS-like and other bat coronaviruses (Cui *et al.*, 2007), and could indicate that we have detected the precursors required for the emergence of an Australian bat coronavirus. However, the lack of evidence for the establishment of this coronaviruses in the genus *Rhinolophus* suggests a low likelihood of emergence at this time.

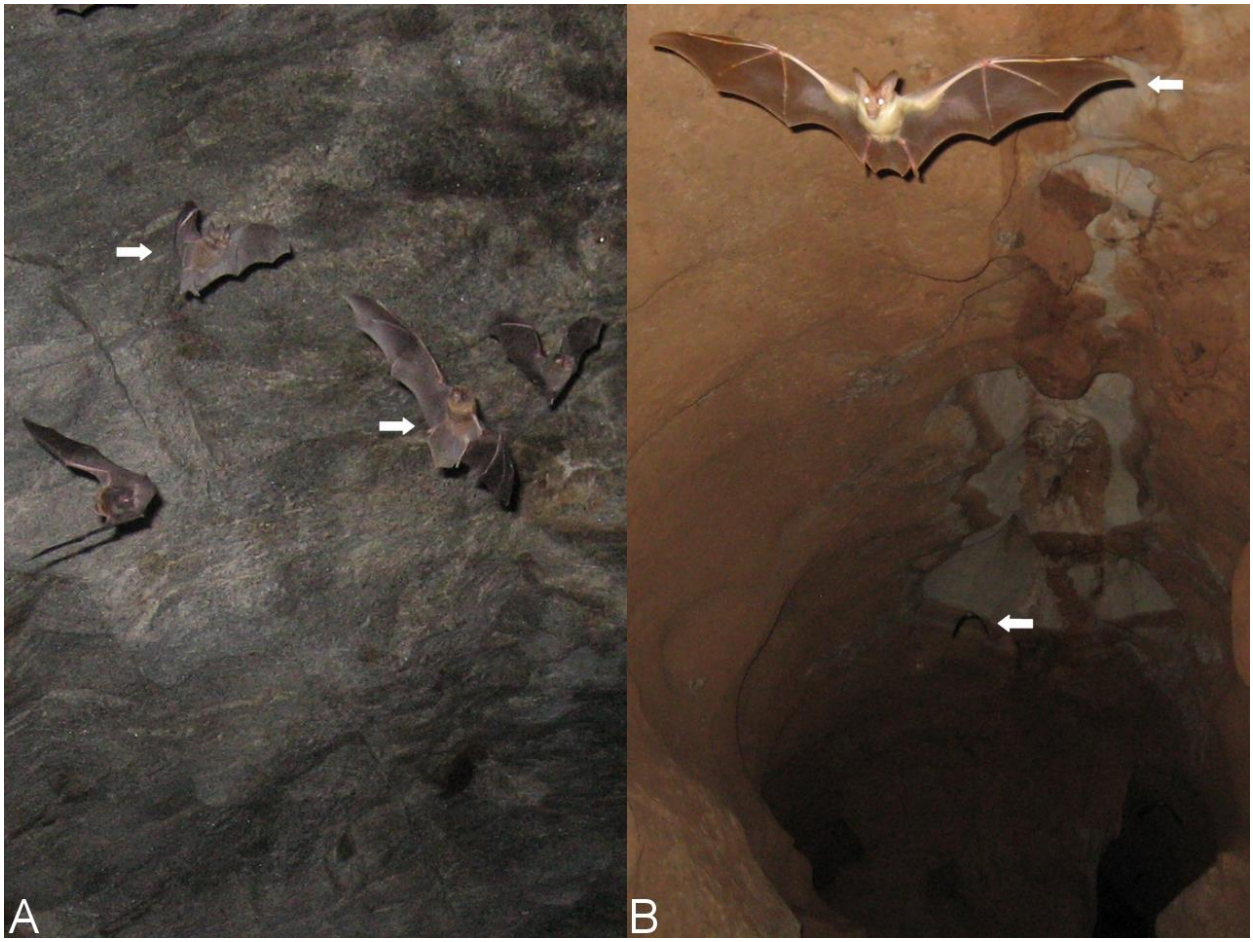

Figure 14. Interspecies contact of Australian bats.

The presence of Australian bats utilising the same roosts and flyways illustrates the potential for interspecies contact, the first event required for the emergence of zoonotic viruses (Wang *et al.*, 2006). Panel A: *Rhinolophus megaphyllus* (left arrow) and *Miniopterus spp* (right arrow) from far north Queensland; Panel B: *Macroderma gigas* (top arrow) and *Rhinonicteris aurantia* (bottom arrow) from the Northern Territory.

Coronavirus evolution

Given this general host tropism for bat coronaviruses, two methods of evolution have been proposed to explain coronavirus diversity in bats and other species (Cui *et al.*, 2007, Vijaykrishna *et al.*, 2007). Divergent evolution requires the inter-species transmission of a common ancestor bat coronavirus between related species of bats and subsequent adaption and establishment in the new host would result in families and suborders of bats having related coronaviruses, whilst transmission between unrelated species of bats or other species would result in a more divergent coronaviruses (Wang *et al.*, 2006, Lau *et al.*, 2012). However, to account for the identification of related coronaviruses in related

species of bats in different locations throughout the world, divergent evolution would require the global distribution of each newly diverged coronavirus, a process that may be possible given some bats' ability for range movement (Breed *et al.*, 2010), but not all. An alternative explanation for the diversity of coronaviruses is co-evolution of bats and coronaviruses (Cui *et al.*, 2007), whereby the divergence of each bat species was mirrored by the divergence of the coronavirus it hosted. This method of evolution would account for the diversity, relatedness and global distribution of bat coronaviruses but would require that bat coronaviruses are as old as the most common bat ancestor, 65 million years (Churchill, 2008). However, co-evolution alone does not explain the presence of different coronavirus genera in the same species or genus, i.e. *Hipposideridae* and *Rhinolophidae* hosting both *Alpha* and *Betacoronaviruses* (Woo *et al.*, 2006) which would require some host shifting, an ability previously reported in *Rhinolophidae* (Cui *et al.*, 2007). The most plausible scenario is that the current diversity of coronaviruses in bats was the result of co-evolution with the occasional fostering of host shifts by *Hipposideridae* and *Rhinolophidae*.

A *Betacoronavirus* in flying foxes: implications for bush meat?

A *Betacoronavirus* was identified in *P. alecto* from south-east Queensland. Whilst other coronaviruses have been identified in bats from the family *Pteropodidae* (Woo *et al.*, 2007, Tong *et al.*, 2009), this is the first identification of a coronavirus in a flying fox (genus *Pteropus*). Also, the detection of anti-coronavirus antibodies in *P. alecto*, *P. conspicillatus*, *P. neohibernicus*, *P. poliocephalus*, *P. scapulatus*, and *P. vampyrus* from far north and south-east Queensland, the Northern Territory, East Timor, Malaysia and Papua New Guinea suggests that coronaviruses are widely distributed amongst species of this genus and their distribution.

Flying foxes are commonly hunted and are an important source of bush meat in many countries throughout their distribution (Epstein *et al.*, 2009). The presence of flying foxes in live animal markets (where they are sold for human consumption) creates a scenario similar to that found in the People's Republic of China where bats from the genus *Rhinolophus* are also sold for human consumption and are thought to have been responsible for the spill-over of SARS into civets (Guan *et al.*, 2003, Tu *et al.*, 2004). When assessing the risk of the emergence of other bat coronaviruses, the presence of flying foxes in live animal markets should be considered a factor as they could provide an alternate route for emergence.

Genetic instability of a *Betacoronavirus*

A *Betacoronaviorus* was identified in *R. aurantia* from the Northern Territory. This coronavirus is unique in that it had an inserted codon in the RNA-dependant RNA polymerase gene. This codon (GCT) is inserted at nucleotide position 423 of the PCR amplicon or at nucleotide position 15,632 when compared with the complete genome sequence of SARS coronavirus (HKU-39849, Genbank accession AY278491.2, data not shown). Whilst the function of this inserted codon (if any) is unknown, it illustrates the variety of mechanisms (including insertions, deletions, mutations and recombination) that coronaviruses use to maintain their genetic instability, and as a result generate diversity (Lai and Cavanagh, 1997). This diversity provides variants with evolutionary advantages, including the adaptation to a new host or greater pathogenicity (Lai and Cavanagh, 1997).

Anti-coronavirus antibody detection

Anti-coronavirus antibodies were detected in all species of bats in which coronavirus RNA was detected (where serum or plasma was available for testing), except *R. aurantia*. Of the 126 *R. aurantia* surveyed, coronavirus RNA was detected in only 1 bat. This sample size was sufficient to detected coronavirus or anti-coronavirus antibodies at a prevalence of 2% (Cannon and Roe, 1982), and suggests that either infection occurs at this low level and we were unable to detect antibodies using the SARS crude antigen ELISA, or that *R. aurantia* also has the ability to foster host-shifts of coronaviruses from other species (Cui *et al.*, 2007) and this host-shifting is a rare event. For the latter scenario to occur, the coronaviruses detected in *R. aurantia* would need to have been transmitted from a species of bat with which interspecies contact was possible (Wang *et al.*, 2006). In the Northern Territory, *R. aurantia* was caught roosting with both *H. ater* and *Macroderma gigas* (Figure 14). Further surveillance of both Australian *H. ater* and *M. gigas* is necessary to identify the coronaviruses hosted by these species and determine if they are the same or closely related to that identified in *R. Aurantia*. If so, it would be another example of interspecies transmission of coronaviruses in Australian bats. Also of interest, are *Scotorepens* whose prevalence of anti-coronavirus antibody prevalence was 100% (n=24), which strongly suggests a high rate of coronavirus infection.

Tissue tropism

The majority of coronaviruses previously reported in bats were detected in faecal samples or rectal swabs 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). From the subset of 30 bats that were sampled to identify tissue tropism in Australian bat coronaviruses, coronavirus RNA was detected in faecal samples or rectal swabs of 11 bats. Of these 11 bats, coronaviruses RNA was detected in the throat swab of only two bats and was not detected in any blood samples. Whilst this sample size limits statistical analysis, it suggests that bat coronaviruses will only be detected in throat swabs secondary to detection in faecal samples or rectal swabs, confirming a predominantly enteric tropism of bat coronaviruses. It also indicates that blood samples are not useful for the detection of bat coronaviruses.

Co-habitation of civets (*Paguma larvata*) and bats (*Rhinolophus monoceros*)

Whilst no evidence of coronavirus infection was detected in either the civets (*Paguma larvata*) or bats (*R. monoceros*) from Taiwan (Table 1), both were found co-habiting the same cave. This scenario illustrates the potential for interspecies contact between bats of the genus known to host SARS-like coronavirus (Lau *et al.*, 2005, Li *et al.*, 2005), and a non-bat species considered to be the origin of the SARS outbreak in humans (Guan *et al.*, 2003, Tu *et al.*, 2004). This observation suggests a potential alternate route for the emergence of SARS-like coronaviruses other than the live animal markets of the People's Republic of China, as this cave was also frequented by humans for the purpose of mining guano (pers. comm. Chao-Lung).

Conclusion

This study identified coronaviruses in Australian bats and evidence of infection of coronaviruses in bats from East Timor, Indonesia, Malaysia and Papua New Guinea. It also identified an interspecies transmission of an Australian bat coronavirus, supporting the hypothesis that the presence of bats from the genus *Rhinolophus* is a risk for the emergence of both SARS-like and other bat coronaviruses (Cui *et al.*, 2007). Whilst the precursors required for the emergence of an Australian bat coronavirus were detected, there appears to be a low risk of the emergence at this time. The study extended the known relationship of related bat coronaviruses being hosted by bats of the same species or genus to bats of the same family or suborder. It also elucidated the current diversity of coronaviruses in bats suggesting that it is the result of co-evolution with the occasional fostering of host shifts by *Hipposideridae* and *Rhinolophidae*, and that bat coronaviruses are as old as the most common bat ancestor, 65 million years (Churchill, 2008).

These findings advance our understanding of the diversity of coronaviruses in bats. This diversity, the global distribution of bats and the propensity of coronaviruses to successfully cross species barriers suggests SARS-like coronaviruses may not be the only example of a bat coronavirus being the cause of future disease outbreaks.

Chapter 4 *Alphacoronavirus* infection dynamics in a population of *Miniopterus spp.*

Introduction

Relatively little is known about the ecology and infection dynamics of coronaviruses in wild animals (Poon, Chu et al. 2005) and whilst many surveys have been conducted to identify coronaviruses in bats, few have reported more than descriptive statistics (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, Tong *et al.*, 2009). However, some putative risk factors for the infection of bats with coronaviruses (assumed through detections of genomic material by RT-PCR) have been reported and most appear to be associated with maternal colonies. Sub-adults, lactating females, and more generally, any female bat associated with maternal colonies, and even the formation of the maternal colony itself, have all been reported as risk factors for infection (Gloza-Rausch *et al.*, 2008, Pfefferle *et al.*, 2009, Drexler *et al.*, 2011). These risk factors and peaks of infection, characterised by increased virus concentration and prevalence, are hypothesised to be due to the formation of a colony of sufficient size and density as to allow attainment of a critical basic reproductive rate in susceptible bats and also due to a new wave of susceptible bats within the colony - juveniles who have lost their perinatal protection but not yet mounted their own adaptive immunity (Gloza-Rausch *et al.*, 2008, Drexler *et al.*, 2011). It was also suggested by Gloza-Rausch *et al.* (2008) that the lower detection rates of coronavirus in adult bats

Figure 15. An abandoned gold mine in south-east Queensland, Australia.

With a drive length of 60m, this mine was abandoned in the 1920's and is now inhabited by bats.

Figure 16. Bats roosting at the mines entrance.

For public safety, the mine is barred but it still allows access by bats which can often be seen roosting near the entrance.

could be due to partial immune protection from previous infection earlier in life, as with other bovine, murine and porcine coronaviruses (Weiss and Navas-Martin, 2005).

Studies of bat adaptive immunity have provided evidence for both the antibody and cell-mediated (innate) immunity in bats (Barrett, 2004, Field, 2005, Plowright *et al.*, 2008, Breed *et al.*, 2011, Baker *et al.*, 2013, Epstein *et al.*, 2013, Baker *et al.*, 2014). Although bats appear to share most features of the immune system with other mammals, qualitative and quantitative differences in immune responses have been reported. These differences may allow the asymptomatic nature of viral infections in bats (Baker *et al.*, 2013).

The ability for antibodies to provide protection from infection is an important feature of the immune system (Baker *et al.*, 2013). Not only have neutralising antibodies in bats been shown to confer protection but it has also been demonstrated that maternal immunity is passed from dams to pups, with the duration of maternal immunity lasting up to eight months (Field, 2005, Plowright *et al.*, 2008, Breed *et al.*, 2011, Baker *et al.*, 2013, Epstein *et al.*, 2013, Baker *et al.*, 2014). Using my technique described in Chapter 2 (Smith *et al.*, 2010), this study endeavoured to elucidate the immunological response by bats to an *Alphacoronavirus* infection and identify any other risk factors that may contribute to the dynamics of their infection.

Materials and methods

Sampling

An abandoned gold mine in south-east Queensland, Australia (Figure 15 & 16), was selected for this study due to its inhabitance by three species of bats, *Miniopterus australis*, *M. schreibersii* (Figure 19) and *Rhinolophus megaphyllus* (Figure 18), and also due to the previous detection, in bats from this mine, of a variant of the *Alphacoronavirus, Miniopterus bat coronavirus HKU8* (Chapter 3). Approximately 180 bats (30 *M. australis*,

30 *M. schreibersii* and 60 *R. megaphyllus*) were sampled once each season over a period of two years, between 2006 and 2008. A collapsible bat trap (Figure 9 & 17), placed at the entrance of the mine, caught bats as they returned to roost each morning after a nights foraging. Bats were then individually housed in clean cloth bags and a polythene cooler until sampled (Figure 10). A single faecal pellet (collected directly from a defecating bat or from its clean cloth bag) was placed into 1 ml of sucrose potassium glutamate albumin (SPGA) with added penicillin, streptomycin and fungizone. When no faecal pellet was obtained, the anus was swabbed and the swab placed into 1 ml SPGA. Bats were manually restrained and bled as described in Chapter 2 (Smith *et al.*, 2010). Briefly, a 25 g needle was used to puncture either the brachial or the propatagial vein. Venous blood would then bead on the surface of the skin and could be collected using a micropipette and sterile tip (Figure 11). Collected blood was diluted 1:10 in phosphate buffered saline to limit clotting. Bats were sexed based on the presence of external genitalia; male bats have an obvious penis (Churchill, 2008). Female bats were assigned to one of two age classes (Churchill, 2008);

- Adult: bats that are in reproductive condition (pregnant) or have reproduced in previous years (developed teats)
- Sub-adult: bats that are adult size but have not yet reached sexual maturity (not pregnant and minute teats)

Male bats can reportedly be aged more subjectively, based on knobbly wing joints indicating immature cartilaginous epiphyises in the forearm long bones (Churchill, 2008). I initially attempted this approach, but subsequently abandoned it because of concerns of mis-classification, and so all males bats were placed in the age class Male.

All bats were then temporarily marked with a non-toxic pen inside their ear (Figure 20), to prevent recapture and sampling of the same bat within a season, and released at the entrance of the mine within 6 hours.

Sampling was conducted with approval from the Department of Primary Industries and Fisheries, Queensland, Animal Ethics (SA 2006/06/117 and SA 2007/005/194), Environmental Protection Agency, Queensland Parks and Wildlife Service (WISP03887606 and WISP04906107).

Figure 17. Preparing the collapsible bat trap

Scientist Carol de Jong, prepares the collapsible bat trap for placement at the mines entrance.

Coronavirus detection and sequencing

Template RNA was extracted from 560 μl of SPGA using the QIAamp® Viral RNA Mini Kit (QIAGEN) following the manufacturer's instructions (QIAGEN, 2010). Reverse transcription followed by cDNA amplification using a polymerase chain reaction (RT-PCR) targeting a conserved region of the coronavirus RNA-dependent RNA polymerase gene, as described by Poon et al. (2005), was performed using the Superscript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen).

Anti-coronavirus antibody detection

Anti-coronavirus antibodies were detected using a modified SARS coronavirus crude antigen ELISA developed by Yu et al. (2006). Whilst using the same antigen (gamma-irradiated SARS-CoV, grown in Vero E6 cells), the scarcity of the horseradish peroxidase (HRP) conjugated anti-coronavirus chicken antibodies (developed for the competitive ELISA) were replaced with HRP-conjugated Protein AG for the detection of bat anti-coronavirus antibodies bound directly to the antigen.

Descriptive statistics

Figure 18. *Rhinolophus megaphyllus*.

One of the three species of bats that inhabit the mine, *Rhinolophus megaphyllus*, more commonly known as the Eastern horseshoe bat, named after its large elaborate noseleaf that assist with echolocation.

Descriptive statistics, including mean prevalence and the calculations of 95% confidence intervals for binomial populations (Wilson 1927) were calculated in Excel®.

Determining risk factors through multivariable analysis

To prevent bias of the regression coefficients and allow valid interpretation of multivariable analysis, the datasets were edited as suggested by Peduzzi *et al.* (1996) and Pedhazur (1997), in that;

1. The number of bats per cohort (observations) must be greater than 10
2. There must be at least one test detection (event) per cohort
3. The number of events must not equal the number of observations

Modelling of binomial proportions (logistic regression, GenStat® 11th Edition) was used to identify risk factors associated with a particular outcome (detection of coronavirus by RT-PCR or anti-coronavirus antibodies by ELISA). The general strategy for building a logistic regression model was as suggested by Hill and Ward (2008):

1. “Perform univariable logistic regression to identify potential risk factors, also known as the unadjusted odds. For each variable, note the change in deviance to the model and the p-value associated with this change. (Note: The ‘total’ deviance measures the difference between the observed data and what is predicted by the model containing only the intercept, the ‘residual’ deviance measures the difference between the observed data and what is predicted by the model that includes a variable. The difference between these two deviances follows a Chi-square (χ^2) distribution with the number of degrees of freedom in the model. Variables whose deviance p-value is <0.25 should be considered for inclusion in the model, variables with a deviance p-value >0.25 are unlikely to be risk factors for the outcome but should be considered as potential confounders.”

Figure 19. Roosting *Miniopterus spp.*

Commonly known as bentwing bats, *Miniopterus spp*. roost densely together, possibly facilitating the transmission of coronaviruses.

2. “Use the univariable model with the lowest deviance p-value as the foundation. One at a time, add the remaining variables, whose deviance p-value <0.25, and note the change in deviance to the model and the p-value associated with this change. The added variable with the lowest deviance p-value (now significant at a p-value <0.05) can be added to the model.”

Figure 20. A bat marked with a non-toxic pen inside its ear.

This bat was identified as being a recapture (from sampling a few days prior) by the temporary mark from a non-toxic pen inside its ear.

3. "Using the above two-variable model, continue adding, one at a time, the remaining variables. As before, note the change in deviance to the model and the p-value associated with this change. Continue adding variables to the model until they no longer significantly improve the fit (the p-value associated with the change of any added variable is no longer <0.05)."

4. "Check for interaction. Using the multivariable model, add, one at a time, all possible two way interactions of the risk factors. Note the change in deviance to the model and the p-value associated with this change. If more than one interaction term improves the models fit, use the multivariable model and the best fitting interaction to determine whether any additional interaction terms further improve the model."

5. "Check for confounding. Using the multivariable model with any interactions, add, one at a time any potential confounders. If the addition of a potential confounder changes the odds ratio associated with any risk factor by >10%, then that variable is a confounder."

6. "Assess the overall adequacy of the model. As previously stated, deviance follows an approximate Chi-square distribution, if the model fits well, residual deviance (the difference between the observed data and what is predicted by the final multivariable model with any interactions and confounders) will not be statistically significant."

Referents were manually selected but were generally those with the greatest observations to minimise aliasing categories in the logistic regression model.

Results

Bats from the mine were sampled each season over a two year period between 2006 and 2008. Using the previously outlined methodology to edit data, the following cohorts were

removed each of the datasets (RT-PCR and ELISA) before analysis and are shown in Appendix 1 & 2

For the RT-PCR dataset of 518 results; the following cohorts were removed before analysis;

- Species *R. megaphyllus* removed, no RT-PCR detections (392 results remaining)
- Unknown Sex removed (391 results remaining)
- Unknown Age removed (381 results remaining in final dataset, Table 8)

For the ELISA dataset of 457 results, the following cohorts were removed before analysis;

- Species *R. megaphyllus* removed, as above, no RT-PCR detections (335 results remaining)
- Unknown Sex removed (334 results remaining, Table 9)

Descriptive statistics, model building strategies, multivariable analysis and model predictions for the detection of coronavirus by RT-PCR (n=381) and anti-coronavirus antibodies by ELISA (n=334) are presented below.

Table 2. Descriptive statistics for the detection of coronavirus RNA by RT-PCR.

Variable	Category	Detected (Total)	Prevalence (95% CI)
Season			
	Spring	44 (158)	28 (21-35)
	Summer	16 (49)	33 (21-47)
	Autumn	25 (95)	26 (19-36)
	Winter	16 (79)	20 (13-30)
Species			
	Miniopterus australis	38 (154)	25 (19-32)
	Miniopterus schreibersii	63 (227)	28 (22-34)
Sex			
	Male	52 (189)	28 (22-34)
	Female	49 (192)	26 (20-32)
Age			
	Male	52 (189)	28 (22-34)
	Female sub-adult	29 (95)	31 (22-40)
	Female adult	20 (97)	21 (14-30)

Table 3. Model building strategy for the multivariable analysis of the detection of coronavirus RNA by RT-PCR

Variable	Residual deviance	P
Season	437.9	0.434
Species	440.2	0.503
Sex	440.5	0.660
Age	438.0	0.261
Season+Species+Season*Species	437.0	0.815
Season+Sex+Season*Sex	434.4	0.506
Season+Age+Season*Age	429.9	0.458

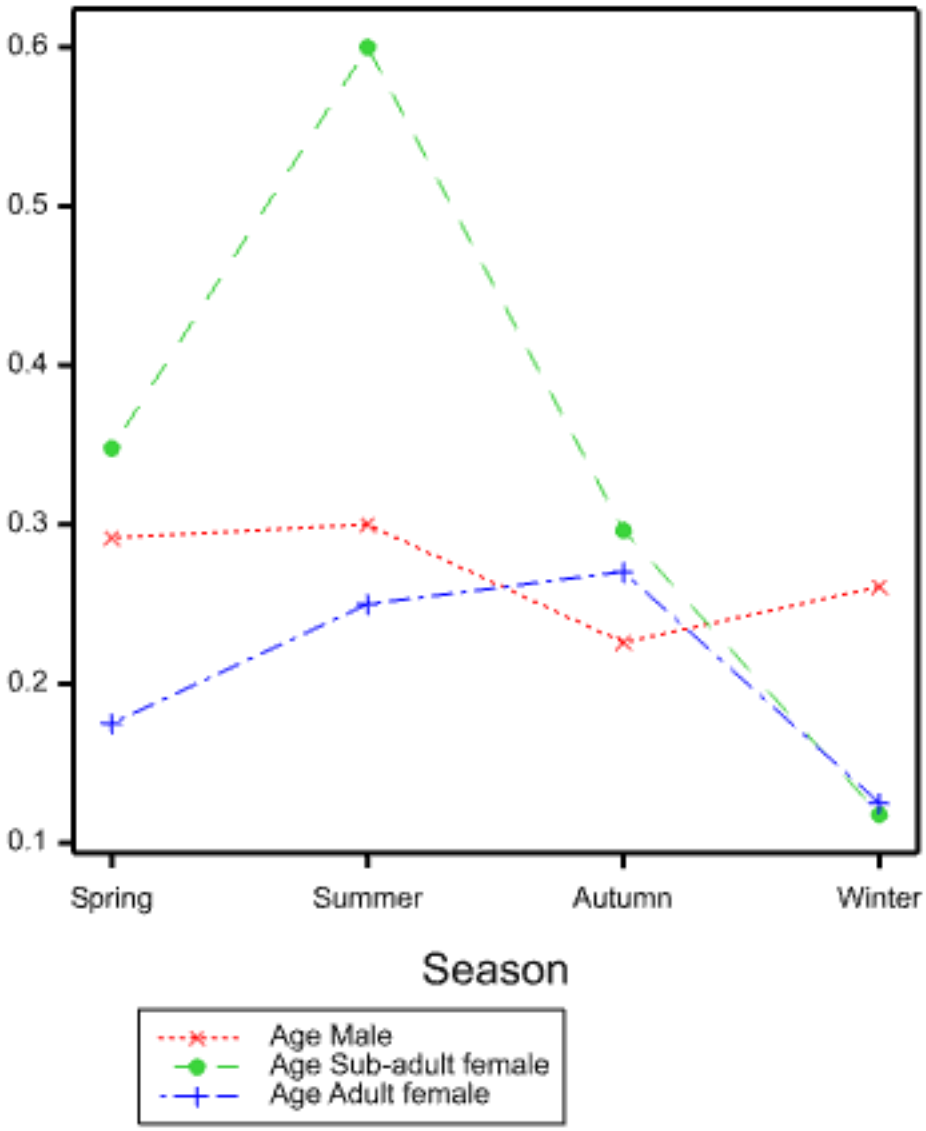

Figure 21. Multivariable model for the seasonal prediction of the detection of coronavirus by RT-PCR in *Miniopterus spp*.

The final model suggests an increase in the prevalence of coronavirus RNA (likely due to infection) rate in sub-adult females over spring and summer, during the formation of maternal colonies.

Table 4. Descriptive statistics for the detection of anti-coronavirus antibodies by ELISA.

Variable	Category	Detected (Total)	Prevalence (95% CI)
Season			
	Spring	92 (125)	74 (65-81)
	Summer	36 (39)	92 (80-97)
	Autumn	64 (94)	68 (58-77)
	Winter	33 (76)	43 (33-55)
Species			
	Miniopterus australis	80 (124)	65 (56-72)
	Miniopterus schreibersii	145 (210)	69 (62-75)
Sex			
	Male	105 (166)	63 (56-70)
	Female	120 (168)	71 (64-78)
Age			
	Male	105 (166)	63 (56-70)
	Female sub-adult	45 (79)	57 (46-67)
	Female adult	75 (89)	84 (75-90)

Table 5. Model building strategy for the multivariable analysis of detection of anti-coronavirus antibodies by ELISA.

Variable	Residual deviance	P
Season	387.2	<0.001
Species	421.2	0.395
Sex	419.3	0.111
Age	403.8	<0.001
Season+Species	387.2	0.981
Season+Sex	382.1	0.023
Season+Age	365.8	<0.001
Season+Age+Species	365.6	0.652
Season+Age+Sex	-	-
Season+Age+Season*Age	352.1	0.034

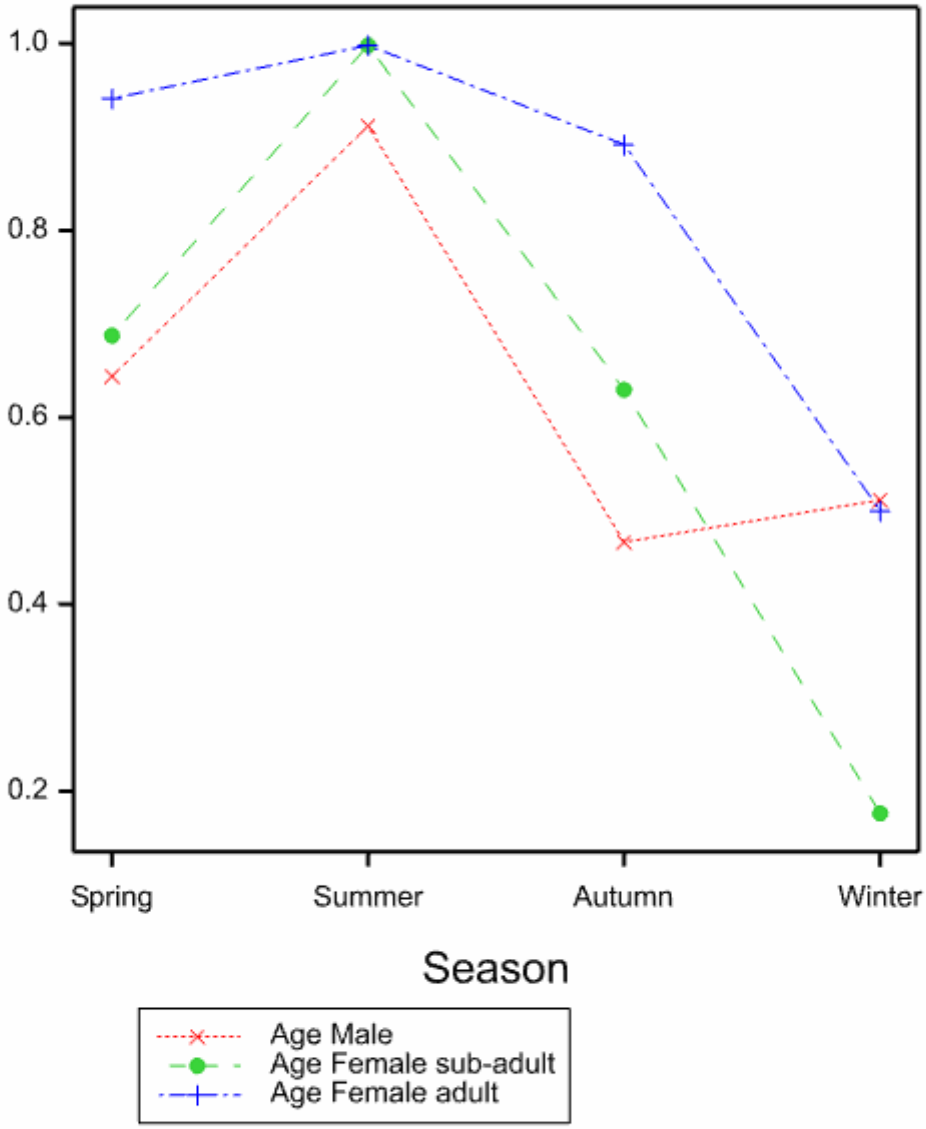

Figure 22. Multivariable model for the seasonal prediction of the detection of anti-coronavirus antibodies by ELISA in *Miniopterus spp*.

The final model suggests an increase in the prevalence of antibodies (possibly in response to a recent infection) in all cohorts over spring and summer, during the formation of maternal colonies.

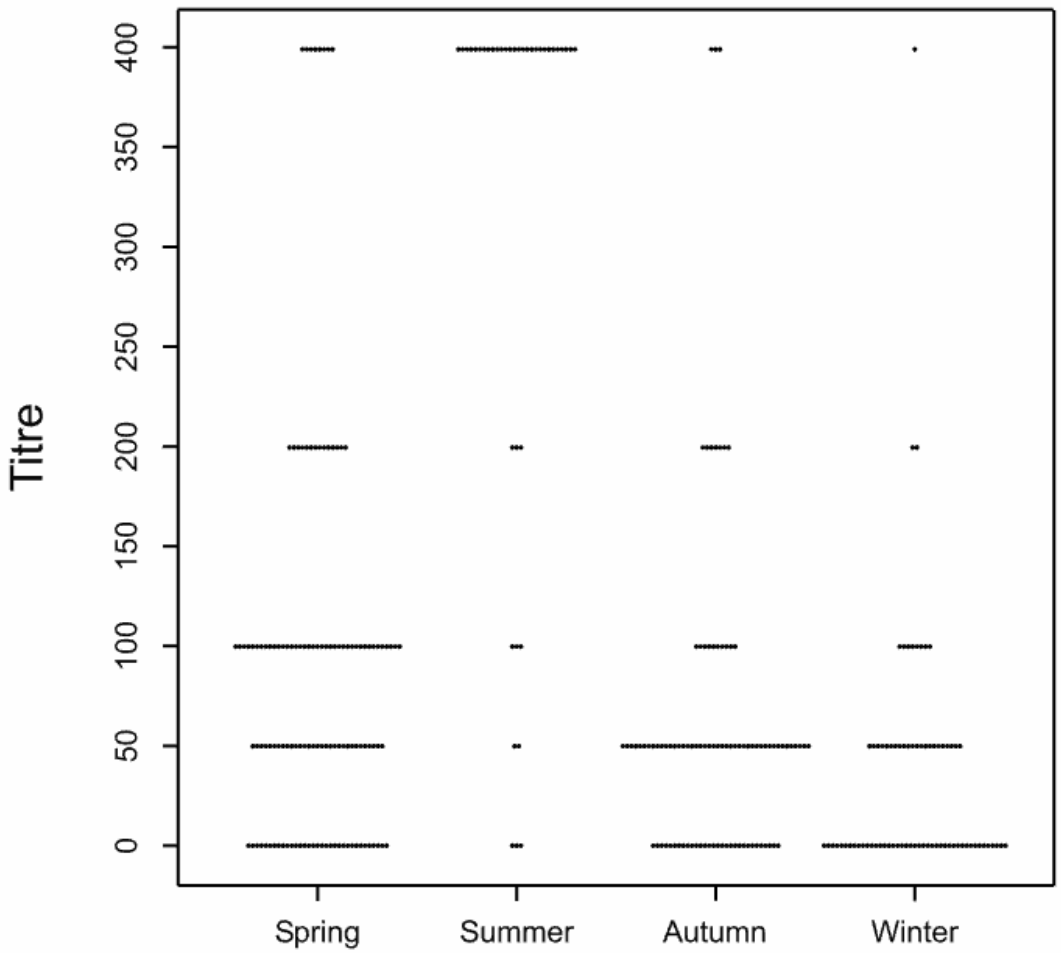

Figure 23. Seasonal variation in anti-coronavirus antibody titres in *Miniopterus spp*.

A dot histogram illustrates the increased anti-coronavirus antibody titre in summer suggesting an immunological response to a recent infection.

Discussion

Minopterus spp.* and *Miniopterus bat coronavirus HKU8

Miniopterus spp., specifically *Miniopterus schreibersii*, have the widest natural distribution of any bat species, extending from Europe, to southern Africa, to south-east Asia and Australia, and across to Japan, New Guinea and the Solomon Island (Churchill, 2008).

Throughout its range, this genus has been found to be infected with the alphacoronaviruses, *Miniopterus bat coronavirus 1* and *Miniopterus bat coronavirus HKU8* whilst displaying no signs of disease (Poon *et al.*, 2005, Chu *et al.*, 2006, Tang *et al.*, 2006, Woo *et al.*, 2006, Muller *et al.*, 2007, Woo *et al.*, 2007, Tong *et al.*, 2009). These unique attributes of a highly prevalent coronavirus, in a common bat, easily captured using recognised techniques, made it an excellent host to study. Also, with my technique for sampling small quantities of blood from bats, (Smith *et al.*, 2010), it provided a unique opportunity to study the immunological response by bats to coronavirus infection.

Modelling the infection of an *Alphacoronavirus* in *Miniopterus spp.*

My predictive modelling for the detection of coronavirus RNA (which is defined as excretion from an infected individual), suggests a pronounced increase in the viral prevalence of infected sub-adult females during spring and summer (Figure 21). Whilst not statistically significant, the putative identification of this risk factor for infection (sub-adult bats) was previously suggested by Drexler *et al.* (2011) and Gloza-Rausch *et al.* (2008) and supports the model's ability to predict the patterns of infection of coronavirus in *Miniopterus spp*. Also predicted by this model was a subtle increase in the prevalence of infection in adult females, also during spring, summer and autumn. In south-east Queensland's spring and summer, *Miniopterus spp* will form maternal colonies and give birth to pups (Churchill, 2008). Thus, my predictive model now appears to capture other previously reported factors for an increased rate of coronavirus infection - formation of maternal colonies and the ongoing lactation of adult females (Gloza-Rausch *et al.*, 2008, Drexler *et al.*, 2011). Whilst comment on the dynamics of infection of males is not possible (due to possible confounding of mixed ages), it is interesting to note that prevalence remains relatively stable and does not decrease in winter as does the rate of infection for both sub-adult and adult females.

As with the model for the detection of coronavirus RNA, the model for the detection of anti-coronavirus antibodies, also predicted an increased prevalence during summer (Figure 22). However, this model predicted not only a dramatic increase in the prevalence of anti-coronavirus antibodies in sub-adult females, but also in adult females and males. These antibodies then appeared to wane over the coming seasons, with males and adult females dropping to a seroprevalence of approximately 50% and sub-adult females down past 20%. In support of this model, is the titre of anti-coronavirus antibodies, for each season

(Figure 23). The measurements indicate that the median titre of 0 in winter and 1:50 in both spring and autumn, were in direct contrast to a median titre of 1:400 in summer.

Caveats for interpretation

There are several caveats for interpretation of this study's results. Whilst a valid and significant model for the detection of anti-coronavirus antibodies was built, the same was not the case for the detection of viral genome by RT-PCR, as all variables were forced into this model, with the model that produced the lowest deviance being selected for interpretation (season and age, Table 3). However, this is the same model that produced a statistically significant model for the detection of anti-coronavirus antibodies (Table 5). This consistency of variables between models, and the previous identification of these variables as risk factors for the detection of coronavirus, provides confidence for its use in modelling the prediction of coronavirus prevalence in *Miniopterus spp.* (Gloza-Rausch *et al.*, 2008, Drexler *et al.*, 2011). It should also be noted, that any observational or predicted differences between sub-adult and adult female bats could also be true for sub-adult and adult male bats, however, the inability to accurately age male bats will seriously confound this cohort's results. An effort was made to age bats using other morphological measurements (weight and forearm length), but with no significant difference identified between sub-adult and adult female bats (not shown), this strategy was abandoned. Any future study elaborating on this study's predictions will require an accurate ageing methodology for male bats.

A hypothesis of the infection dynamics of an Alphacoronavirus in *Miniopterus spp*.

By themselves, each of these models and the antibody titre measurements provided valuable information on the ecology of a virus in a population, but together, this information can be used to form a hypothesis of the infection dynamics in that population. Below (Figure 24), is an attempt to describe that hypothesis. Due to possible confounding of males by age, this hypothesis is presented and argued from the female population of bats, where accurate aging was possible.

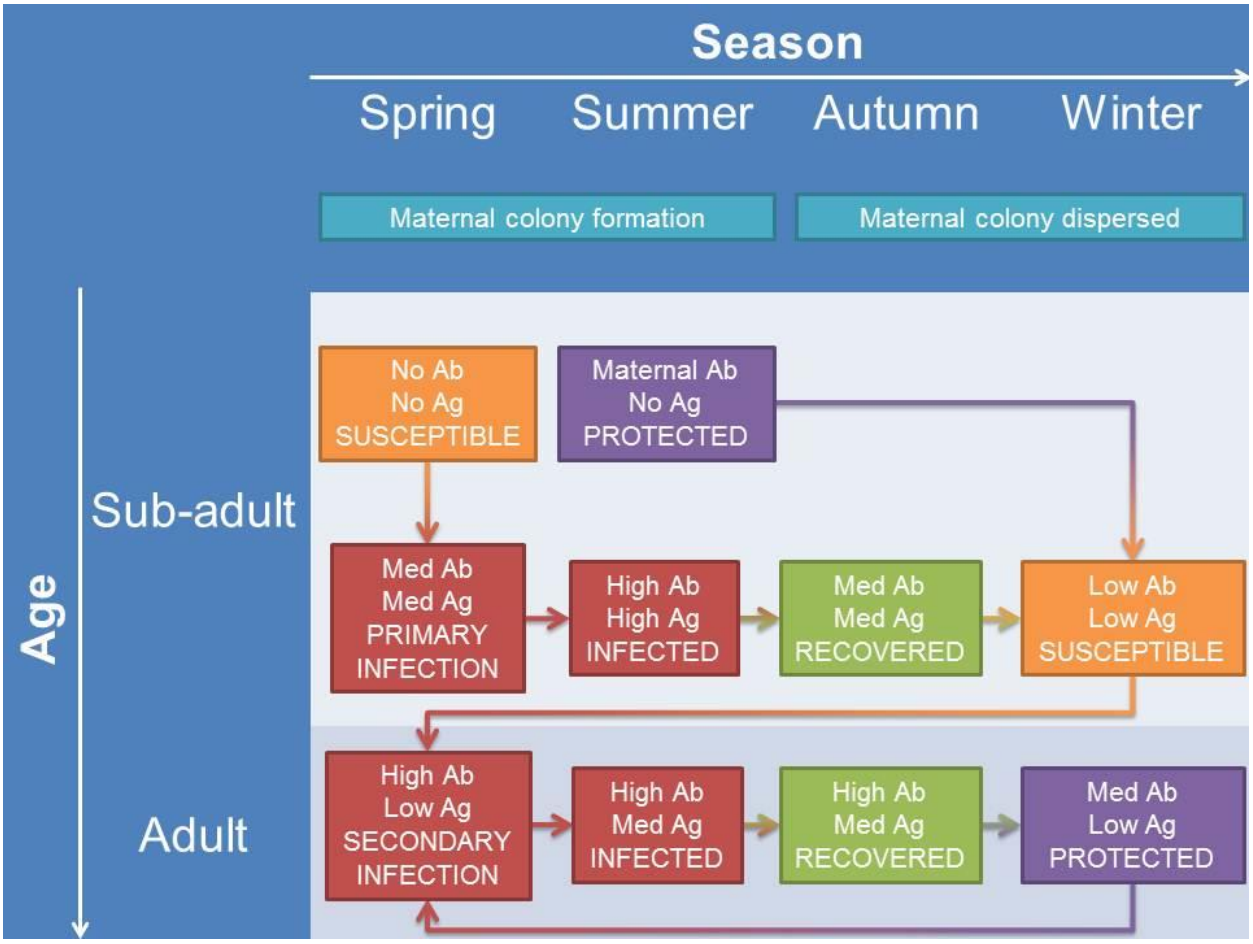

Figure 24. Hypothesis of the infection dynamics of an *Alphacoronavirus* in *Miniopterus spp.*

1. Spring (Year 1): Juvenile female bats (*Miniopterus spp.*) born within the confines of a maternal colony have not received adequate protection from maternal antibodies (passed across the placenta and additionally through colostrum from their mother). Susceptible, these bats succumb to their first (primary) infection by coronavirus but initiate an immunological response, including the production of anti-coronavirus antibodies. Alternatively, some bats are protected by maternal antibodies and remain so until winter, at which time the maternal antibodies have waned sufficiently to result in that cohort being susceptible to infection (Field, 2005, Plowright *et al.*, 2008, Epstein *et al.*, 2013).
2. Summer (Year 1): As more susceptible sub-adults become infected, both the viral and serological prevalence for this cohort increases.
3. Autumn (Year 1): Eventually, with the dispersal of the maternal colony and the sub-adults immunological response having conquered the infection, the viral prevalence of this cohort begins to decrease.

4. Winter (Year 1): The serological prevalence for this cohort has also been decreasing for some time now, as antibodies to the primary infection wane and maternal antibodies are lost. All sub-adult bats are now again, susceptible to infection.
5. Spring (Year 2): Last year's sub-adult bats are now one year old and aged as adult. Returning to the maternal colony, they are again exposed to the coronavirus resulting in a secondary infection (for bats who have only just lost their maternal antibodies, this will be their primary infection).
6. Summer (Year 2): This secondary infection is similar to the first in that there is an immunological response, however, this response is dramatically different in that there is a stronger and more rapid production of antibodies and an apparent quashing of infection (suggested by low viral prevalence).
7. Autumn (Year 2): Even after dispersal of the maternal colony and having recovered from the infection, the prevalence of antibodies remains high in adult females.
8. Winter (Year 2): This high serological prevalence continues into winter, and unlike sub-adults, adults now have a protective component against future coronavirus infection.

This ability for an immunological system to recognise a virus, or other antigen, from a previous infection is an important immunological asset, it allows the rapid production of antibodies that appear to control infection. This anamnestic or immunological memory response by bats to coronaviruses is not unique, other studies have suggested that long-term repeated infection of bats with rabies virus may confer significant immunological memory and reduced susceptibility to infection (O'Shea *et al.*, 2014). It also suggests that if bats have this immunological memory and are not actively producing antibodies at the time of sampling, then cross-sectional surveys underestimate the amount of exposure to an antigen (Turmelle *et al.*, 2010).

Conclusion

The data and models from this study were used to develop a hypothesis of the infection dynamics of an *Alphacoronavirus* in *Miniopterus spp*. The hypothesis is similar to the classical SIR model, where individuals are either susceptible to infection, infected, or recovering from that infection. Field (2005) used SIR models to describe the infection dynamics of Hendra virus in flying-foxes, and determined population size, infection and recovery rates were all key parameters. There is also an elaboration of the model were if a

pup has received protection from maternal antibodies, their progression through states of disease could be tracked using the MSIR model, where a state of maternally derived immunity exists before becoming susceptible to infection. The study also suggested that bats have an anamnestic or immunological memory which may limit secondary coronavirus infections with a stronger and more rapid production of antibodies, compared to a primary infection.

Chapter 5 Maintenance of a coronavirus infection in a population of Australian bats (*Myotis macropus*) by persistent infection of individuals

Introduction

In spite of the potential for serious consequences of virus epidemics emerging from bats, knowledge is currently lacking on their ecology. For example, it is still unknown how these viruses, with human pathogenic potential, are maintained, amplified or controlled in bats (Drexler *et al.*, 2011). Drexler *et al.* (2011) identified two peaks of amplification of coronaviruses, characterised by increased virus concentration and increased detection rates, upon the formation of a colony of *Myotis myotis* in Germany and following parturition. It was hypothesised that the initial peak was probably due to the formation of a colony of sufficient size and density to allow the establishment of a critical basic reproductive rate in susceptible bats. The second peak, after parturition, was associated with a new wave of susceptible bats, newborn pups who had lost their perinatal protection but not yet mounted their own adaptive immunity (Drexler *et al.*, 2011). In another attempt to better define the epidemiology of coronaviruses, Lau *et al.* (2010) marked 511 Chinese horseshoe bats (*Rhinolophus spp*) from 11 sites and recaptured 113 (22%). From this study it was estimated that viral clearance occurred between 2 weeks and 4 months after infection and suggested that coronaviruses in Chinese horseshoe bats caused an acute self-limiting infection associated with weight loss. It was also identified that the peak activity for coronaviruses was during spring, soon after hibernation, and that mating and feeding activity may have facilitated the spread of the virus within and between roosts. In Chapter 4 of this thesis, it was identified that throughout a two year study, a population of Australian bats (*Miniopterus australis* and *M. schreibersii*) was constantly infected with a variant of the *Alphacoronavirus* (*Miniopterus bat coronavirus HKU8*) at a prevalence of at least 17%. In an attempt to identify the ways in which coronaviruses are maintained at a relatively high viral prevalence, we conducted a mark-recapture study on another population of Australian bats (*Myotis macropus*) which was infected with a putative novel *Alphacoronavirus*.

M. macropus is primarily a costal species, with its distribution extending from the Kimberley in northern Western Australia, around to Victoria and South Australia. This bat

can be distinguished from all other bats in the *Vespertilionidae* family by its disproportionately large feet. *M. macropus* rakes these large feet over the water's surface and catches small fish, prawns and aquatic insects. These bats also forage on flying insects, including moths, beetles and spiders. They generally roost near water in caves, trees hollows and under bridges in small groups (less than 15), but colonies of several hundred are known. The number of litters a female will produce each year varies with latitude. In Victoria (lowest latitude of its distribution), a female will have only one pregnancy with a single young born in November or December. In northern New South Wales (lower-middle latitude) two litters of single young are produced in October and January. The first ovulation occurs in August and the second occurs soon after birth of the first litter. Both pregnancies last 12 weeks and females continue to lactate with the first young in the second pregnancy. Lactations lasts eight weeks and mother and pup roost and forage together for another 3 - 4 four weeks. Only dominant males who have an estalished territory mate, defending a harem of 1 - 12 females from other males. In northern Queensland (higher latitude), females have three pregnancies per year (Churchill, 2008).

Methods

Sampling

A colony of *M. macropus* (Figure 25), in which we had identified a putative novel *Alphacoronavirus* (Chapter 3), roosted in the lifting holes of a bridge in south-east Queensland (Figure 26). Eight sampling events commenced on the 13th January 2009 and continued weekly over two months until the 2nd March. A ninth and final sampling event occurred one month later, 31st March 2009. During the first 4 sampling events bats were marked with implantable radio frequency identification transponders, more commonly known as 'microchips', subcutaneously on the dorsum as described by Wimsatt *et al.* (2005) (Figure 27). During a sampling event when a bat was marked or recaptured, a single faecal pellet (collected directly from a defecating bat or from its clean calico bag) was placed into 1 ml of sucrose potassium glutamate albumin (SPGA) with added penicillin, streptomycin and fungizone. When no faecal pellet was obtained, the anus was swabbed and the swab placed into 1 ml SPGA, as above. Pregnancy status of female bats was determined by palpation.

Sampling was conducted with approval from the Department of Primary Industries and Fisheries, Queensland, Animal Ethics (SA 2006/06/117 and SA 2007/005/194), Environmental Protection Agency, Queensland Parks and Wildlife Service (WISP03887606 and WISP04906107).

Coronavirus detection and sequencing

Template RNA was extracted from 560 μl of SPGA using the QIAamp® Viral RNA Mini Kit following the manufacturer's instructions (QIAGEN, 2010). Reverse transcription followed by cDNA amplification using a polymerase chain reaction (RT-PCR) targeting a conserved region of the coronavirus RNA-dependent RNA polymerase gene, as described by Poon et al. (2005), was performed using the Superscript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen). Amplicons consistent with the expected length of 440 nucleotides were purified using the QIAquick® PCR Purification Kit as per the manufacturer's instructions (QIAGEN, 2008). Purified amplicons were directly sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit as per the manufacturer's instructions (Applied Biosystems, 2002), the extension products were purified using the ethanol/EDTA precipitation method (Applied Biosystems, 2002) and analysed at the Griffith University DNA Sequencing Facility (Brisbane, Australia). Nucleotide sequence traces were edited using Sequence Scanner v1.0 (Applied Biosystems). The final consensus sequence were derived from sense and anti-sense primers and a reference sequence (*M.mac/AUS/SEQ/034/2008*) deposited in GenBank under accession EU834951.

Figure 25. A female *Myotis macropus* (Bat 22) and her 2 week old pup.

This female had an implantable radio frequency identification transponder, more commonly known as a 'microchip', subcutaneously implanted on the dorsum during Week 2 of the mark-recapture study, when she was identified (by palpation of the abdomen) as being pregnant. She was recaptured on Week 4 and was again identified as being pregnant, on Week 5 she had given birth and the pup was attached. On Week 7 the pup was still attached and they were both photographed. When recaptured on Week 12 the pup was no longer attached and was assumed to have weaned, roosting separately with the other weaned pups that were observed in the colony. Photograph courtesy of Steve Parish.

Statistical analysis

Binomial confidence intervals (95%) for a proportion (or prevalence) were calculated using Wilson (1927). To ascertain whether bats with multiple detections (Bats 1-7) were being reinfected on a regular basis, we assumed that a detection was evidence of a reinfection and tested the null hypothesis that the rate of infection in these bats was the same as those with single detections (Bats 8-23) using a chi-square test of association with a Yates value corrected for continuity (www.vassarstats.net). In an attempt to identify risk factors that may be used to differentiate recaptured bats with multiple detections and recaptured

bats with single detections, modelling of binomial proportions (logistic regression, GenStat Fifteenth Edition, VSN International Ltd) was employed.

Figure 26. *Myotis macropus* roosting in the lifting holes of a bridge in south-east Queensland.

Removal of bats from these relatively shallow holes provided a successful capture rate.

Results

Sampling

Fifty two bats were marked during the first 4 sampling events (weeks 1-4). Forty two (81%) of the marked bats were recaptured on subsequent sampling events (weeks 2-8 and 12) and often they were recaptured more than once (Table 6). Recaptured bats were sampled on each occasion. The reproductive status of the 16 adult females captured in the study was assessed (Table 7). Females were observed to be pregnant between weeks 1-5 (13th January-9th February), have dependant young between weeks 3-5 (27th January – 9th February) and lactating between weeks 3-12 (27th January – 31st March).

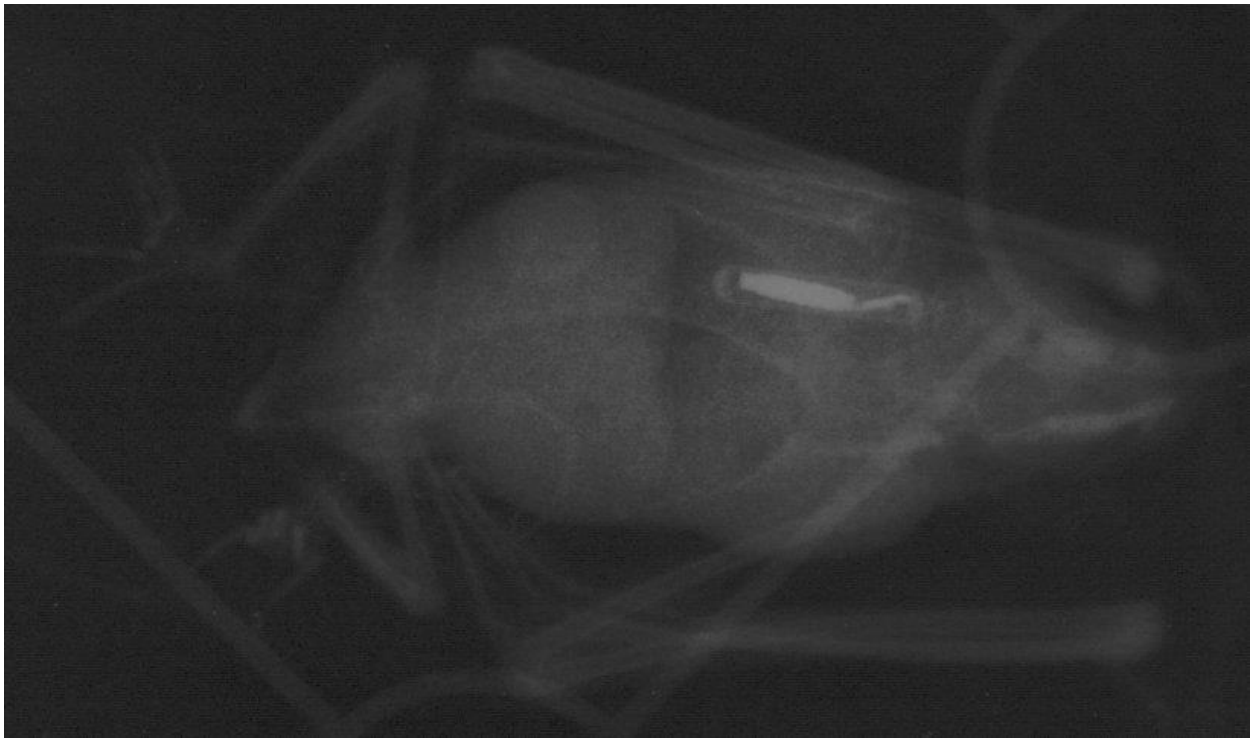

Figure 27. A radiograph of a male *Myotis macropus*.

A radiograph of a male *Myotis macropus* with an implantable radio frequency identification transponder, more commonly known as a 'microchip', subcutaneously implanted on the dorsum. Radiograph courtesy of Kenilworth Veterinary Clinic.

Coronavirus detection and sequencing

There were multiple detections of coronavirus RNA in seven of the recaptured bats (17%, Bats 1-7), single detections of coronavirus in 16 (38%, Bats 8-23) and coronavirus was not detected in 19 (45%, Bats 24-42). The seven recaptured bats which had multiple detections of coronavirus had coronavirus detected over periods of 1, 8 (n=2), 9, 10 (n=2) and 11 weeks, a mean of 8 weeks. Sequencing of the purified amplicons and subsequent phylogenetic analysis identified three genotypes (A, B and C) of a putative novel *Alphacoronavirus* infecting the population. Lack of complete sequence precluded classification as described in Chapter 3. Of the ten bats that were not re-captured, five were coronavirus-positive and five were coronavirus-negative (Table 2).

Statistical analysis

The prevalence of coronavirus RNA in 52 *Myotis macropus* from this study is presented in Figure 28. Assuming that a detection was evidence of a reinfection, the null hypothesis

that the rate of infection in bats with multiple detections (Bats 1-7) was the same as those with single detections (Bats 8-23) was rejected (χ^2=11.2, d.f.=1, p=0.0019). Modelling of binomial proportions (logistic regression) did not identify any correlations between recaptured bats with multiple detections and recaptured bats with single detections, and age (χ^2=2.05, d.f.=2, p=0.359) or sex (χ^2=0.76, d.f.=1, p=0.383).

Table 6. Detection of a putative novel *Alphacoronaviruses* in a 52 *Myotis macropus* from a mark-recapture study conducted over 3 months.

[A, B and C]Coronavirus genotypes

					Week								
Recaptured	**Coronavirus RNA**	**Bat**	**Sex**	**Age**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**12**
Recaptured													
	Multiple Detections												
		1	Male	Unknown	+[C]	-						+[C]	+[C]
		2	Female	Adult	-	+[A]	+[A]				+[A]		+[C]
		3	Female	Sub-adult		+[A]	+[A]		-				
		4	Female	Sub-adult		+[B]					-	-	+[A]
		5	Male	Unknown			+[A]	+[A]	-		-		+[A]
		6	Male	Unknown				+[C]					+[C]
		7	Male	Unknown				+[A]					+[B]
	Single Detection												
		8	Female	Sub-adult	+							-	
		9	Male	Unknown		+	-					-	
		10	Male	Unknown		+	-				-		
		11	Male	Unknown		+	-	-			-	-	
		12	Female	Sub-adult		+	-		-				
		13	Female	Sub-adult		+						-	-
		14	Male	Unknown			+	-	-		-	-	
		15	Male	Unknown			+		-				
		16	Male	Unknown	-		+						
		17	Female	Adult		-	+						
		18	Female	Adult				-	+				-
		19	Female	Adult	-			-					+
		20	Female	Adult		-	-						+
		21	Female	Adult		-	-	-					+
		22	Female	Adult		-		-	-				+
		23	Female	Adult			-	-	-				+
	Not Detected												
		24	Female	Adult	-		-					-	-
		25	Female	Adult	-	-	-				-		
		26	Female	Sub-adult	-	-							

		27	Male	Unknown	-	-						
		28	Female	Adult	-					-		-
		29	Male	Unknown	-			-		-		
		30	Male	Unknown	-						-	
		31	Female	Sub-adult	-				-	-		
		32	Female	Adult		-			-			-
		33	Female	Adult		-	-					-
		34	Female	Sub-adult		-	-					-
		35	Male	Unknown			-			-		-
		36	Female	Adult			-			-		-
		37	Female	Adult			-		-	-		
		38	Female	Adult			-					-
		39	Female	Sub-adult				-			-	-
		40	Male	Unknown				-			-	-
		41	Male	Unknown				-			-	-
		42	Male	Unknown				-				-
Not Recaptured												
	Single Detection											
		43	Male	Unknown		+						
		44	Male	Unknown		+						
		45	Female	Sub-adult			+					
		46	Female	Sub-adult			+					
		47	Male	Unknown				+				
	Not Detected											
		48	Male	Unknown	-							
		49	Female	Adult		-						
		50	Male	Unknown		-						
		51	Male	Unknown			-					
		52	Female	Sub-adult				-				

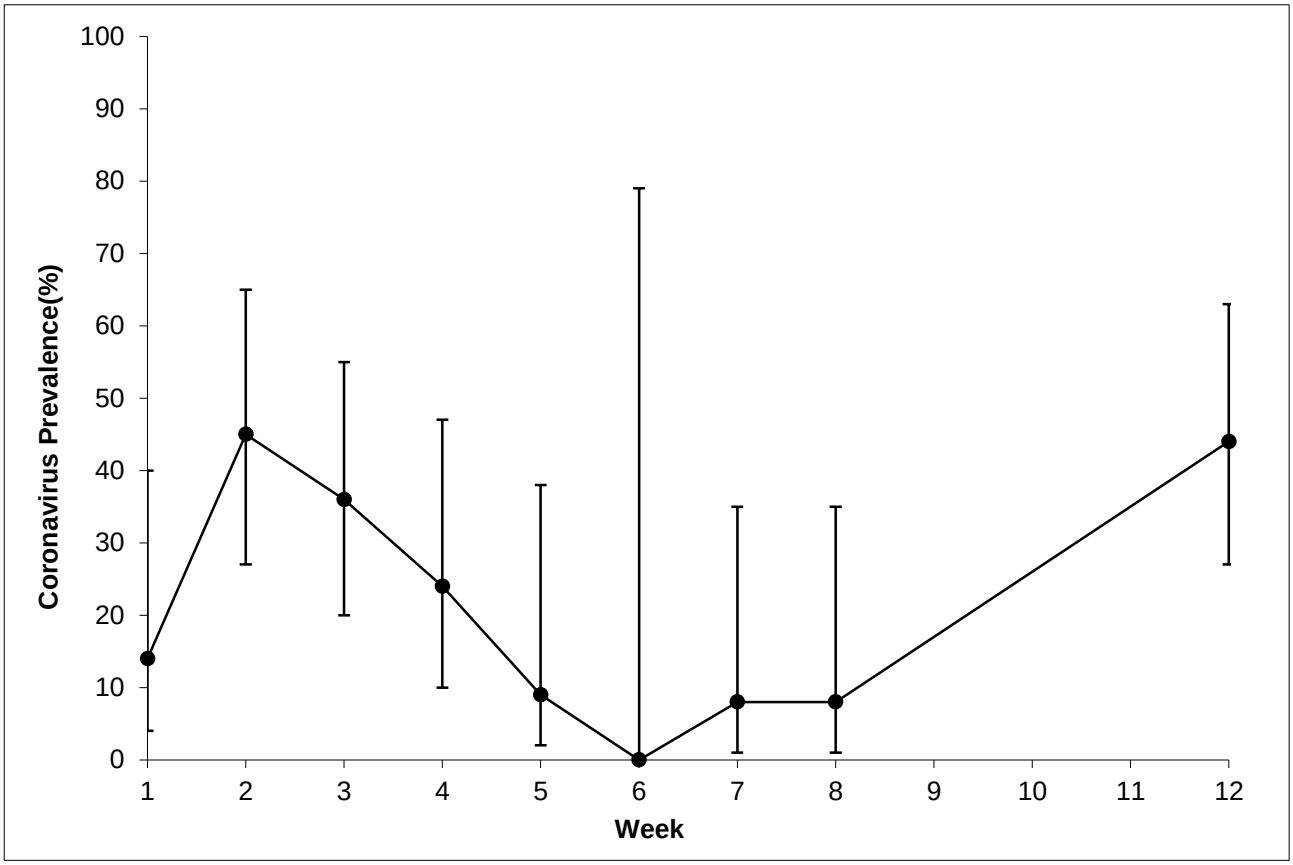

Figure 28. Prevalence of a putative novel *Alphacoronaviruses* in a 52 *Myotis macropus* from a mark-recapture study conducted over 3 months.

Error bars indicate 95%CI (Wilson, 1927)

Table 7. Reproductive status of the adult females *Myotis macropus* captured in this study.

When captured, a three letter coding system was used to describe the reproductive status. Pregnancy (P), dependant young (D) and lactating (L) are recorded in that order if observed. If not observed, a dash is recorded as a placeholder, i.e. a pregnant female who has no dependant young and is not lactating will be represented by P--. Whilst a female who has given birth (no longer pregnant) and now has dependant young and is lactating will be represented by -DL.

	Week								
Bat	**13th Jan**	**21st Jan**	**27th Jan**	**3rd Feb**	**9th Feb**	**15th Feb**	**23rd Feb**	**2nd Mar**	**31st Mar**
2	P--	P--	P--				--L		--L
24	P--		---						--L
28	P--						--L		---
25	P--	P--	P--				--L		
33		P--	P--						---
17		P--		P--	-DL				--L
14		P--	P--						
32		P--			P-L				---
49		P--							
15		P--	P--	---					---
11		P--	P--						---
38			P--						---
37			P--		P-L		--L		
36			---						---
20			PDL		P--				---
23				P--	P-L				---
P	←				→				
D			←		→				
L			←						→

Discussion

Persistent or long-term infection

This study identified that Australian bats (*Myotis macropus*) were infected with a putative novel *Alphacoronavirus* over periods of up to 11 weeks. This period of infection in the colony is consistent with that observed by Lau *et al.* (2010) of between 2 weeks and 4 months. However, whereas Lau *et al.* (2010) suggested that SARSr-Rh-BatCoV caused an

acute, self-limiting infection in individual Chinese horseshoe bats, it appears that our virus is capable of a persistent or long-term infection of bats for almost 3 months. Persistent infection has previously been suggested as playing a role in the maintenance of coronaviruses in populations of bats, as it does for other coronavirus, including feline coronaviruses were it has been shown that naturally infected cats shed FECV intermittently for periods up to 10 months but some (~15%) become chronic shedders, doing so for years or a lifetime (Addie *et al.*, 1995, Hartmann, 2005, Weiss and Navas-Martin, 2005, Chu *et al.*, 2006, Tang *et al.*, 2006). This study is unique in that it identified a pattern of infection in individual bats, not populations of bats, that supports the hypothesis for persistent infection.

The apparent discrepancy between an acute infection observed by Lau *et al.* (2010) and a persistent infection interpreted from this study's results requires clarification. It is possible that the discrepancy is real and there are true variations in patterns of infection for different species of coronaviruses and bats, or it could be that the limited rate of recapture of infected bats in the study by Lau *et al.* (2010) precluded an accurate interpretation of infection. Whilst a significant marking effort of 511 bats was made by Lau *et al.* (2010), only 113 (22%) bats were recaptured and coronavirus was only ever detected in 63 of the 511 bats (12%), limiting the number of bats from which interpretations could be made. Of these 63 bats, shedding of coronavirus was detected in only one bat on more than one occasion (two weeks apart) and ten bats which were detected shedding coronavirus at one sampling event were not detected shedding when recaptured (between 4 and 16 months later), providing an interpretation of an infectious period of between 2 weeks and 4 months. Conversely, whilst only employing 52 marked bats, our study had a viral prevalence of 54% (28 bats) and a recapture rate of 81% (42 bats). The weekly sampling events and the affinity of bats for the lifting holes in which they roosted, provided a unique opportunity to frequently recapture marked individuals that were shedding coronavirus. This increased probability of recapture of bats shedding coronavirus allowed interpretation of the pattern of infection at a resolution not previously studied. Thus, the current study is possibly more accurate than that of Lau *et al.* (2010), and the suggestion of persistent infection of coronaviruses in bats is likely to be sound.

Why not reinfection?

Sequencing of the purified amplicons from recaptured bats with multiple detections of coronavirus (Bats 1-7) and subsequent phylogenetic analysis identified three genotypes of

the putative novel *Alphacoronavirus*. These genotypes differed by eight single nucleotide polymorphisms, from a possible 440 nucleotides, and all were degenerate (translating only one phenotype). This suggests that the different genotypes are likely members of the viral quasispecies infecting the host, since all members of a quasispecies are likely to be present in all infected hosts it is unlikely that these genotypes can be used to determine reinfection.

To further investigate the possibility of reinfection, the study tested the null hypothesis that the rate of infection in bats with multiple detections (Bats 1-7) was the same as those with single detections (Bats 8-23). To accomplish this, each detection of the putative novel *Alphacoronavirus* in bats with multiple detections (Bats 1-7) was assumed to be a reinfection. The null hypothesis was rejected, indicating that the rate of infection in bats with multiple detections was not the same as that of bats with single detections. Hypotheses to explain this scenario include;

(1) Bats 1-7 were persistently infected and were responsible for the acute, self-limiting infection of Bats 8-23

(2) Bats 1-7 had their health or immunity compromised and were susceptible to re-infection at a rate greater than Bats 8-23

(3) All bats were persistently infected but Bats 8-23 were intermittently shedding when captured

Poor health or compromised immunity

Previous studies have suggested that poor health or compromised immunity, associated with pregnancy and lactation, are risk factors for increased seroprevalence of viruses in bats (Plowright *et al.*, 2008, Breed *et al.*, 2011). Similarly, a correlation between the detection of coronaviruses in female bats associated with maternity colonies has also been established (Gloza-Rausch *et al.*, 2008, Pfefferle *et al.*, 2009). The colony used in this study had been selected for its ease of access and the unique roosting behaviour of bats in the bridges lifting holes, providing a successful recapture rate. It was opportunistically and irregularly sampled over the previous year, with a coronavirus RNA detection prevalence of between 30% (19-45%, 95%CI) one year prior to the commencement of the mark-recapture study, and 0% (0-15%, 95%CI) three months prior. It was only during the first sampling event that the majority of female adults (88%) were identified as being pregnant and that the study site was considered a maternity colony. In agreement with

Gloza-Rausch *et al.* (2008), Pfefferle *et al.* (2009) and Drexler *et al.* (2011), it appears that the site has an increased prevalence of coronavirus when used as a maternity colony (during the mark-recapture study and exactly one year prior), as opposed to other times (three months prior) when no coronavirus was detected and no pregnant females were observed. However, these correlations do not extend to recaptured bats with multiple detections (Bats 1-7), with modelling of binomial proportions (logistic regression) not identifying any correlation with age (χ^2=2.05, d.f.=2, p=0.359) or sex (χ^2=0.76, d.f.=1, p=0.383). With no correlation with age or sex and using these same variables as markers for pregnancy and lactation (adult females), there are no indications that recaptured bats with multiple detections of coronavirus (Bats 1-7) are so because of poor health or compromised immunity, associated with pregnancy and lactation.

Acute, self-limiting infection or intermittent shedding?

A SARS coronavirus crude antigen ELISA developed by Yu *et al.* (2006) and used effectively in Chapter 3, was not successful in detecting antibodies in these bats. It appears that either the test was not suitable for detection of antibodies against the novel *Alphacoronavirus* present in this colony or that antibodies were not raised against the infection. The limited availability of diagnostic tools for the detection of bat coronaviruses precluded further serological analysis and differentiation between an acute, self-limiting infection (in which a rising antibody titre would be expected) and long-term infection with intermittent shedding (in which a relatively stable antibody titre would be expected). Similarly, the lower sensitivity of a traditional gel based PCR (as compared to quantitative real time PCR), the presence of inhibitory factors in the faecal pellets and anal swabs collected for testing, and variations of viral shedding in individuals precludes determination if recaptured bats that were virus-negative on re-capture had an acute infection or were intermittently shedding.

Susceptible bats through migration or birth

Migration of bats has previously been shown to play a role in the maintenance of viruses; immigration allows the maintenance of an infection through newly introduced susceptible individuals (Drexler *et al.*, 2011, Plowright *et al.*, 2011). However, the population of bats used in this study appeared relatively closed with the population size remaining between 72 and 101 bats (data not shown) and apparent high fidelity to the roost site (assumed from the high recapture rate of marked bats, 81%). It is therefore unlikely that immigration

of susceptible bats was responsible for the maintenance of the *Alphacoronavirus* in this relatively small and closed population. Throughout a three year study, Drexler *et al.* (2011) observed that strong and specific amplification of RNA viruses, including coronaviruses, occurred upon colony formation and following parturition. It was suggested that the initial peak, upon colony formation, was due to the massing of enough susceptible bats to reach a critical basic rate of viral reproduction and that the second amplification peak was associated with the establishment of susceptible subpopulation of newborn pups losing their perinatal immunity. Interestingly, two apparent peaks of infection (not statistically significant) were also observed during the current three month study of a maternal colony. Whilst bats occupied this colony irregularly throughout the year, it was upon the formation of the maternity colony that the first peak was observed (Figure 28), coinciding with the observations of Drexler *et al.* (2011). The second peak followed two months later, as it did for Drexler *et al.* (2011), but cannot be attributed to the maternal antibody loss in the subpopulation of newborn pups in this study, as none were sampled. Indeed, the second peak resulted from detections of coronavirus RNA in almost all the bats with multiple detections (Bats 1-2, 4-7) and a number of single detections in adult females (Bats 19-23), some of whom had been pregnant and lactating. This second peak is more suggestive of infection of a cohort (adult females) from persistently infected bats or the synchronised intermittent shedding of the same cohort who may now have poor health or compromised immunity after weaning a pup.

Conclusion

This study identified that Australian bats (*Myotis macropus*) were infected with a novel putative *Alphacoronavirus* over periods of up to 11 weeks. The pattern of infection observed supports not only the hypothesis for persistent infection of coronaviruses in bats but also suggests an acute infection or intermittent viral shedding in others.

Chapter 6 General discussion

A defining event

The global SARS outbreak in 2003 was a defining event in emerging infectious diseases (EIDs) awareness. Prior to SARS, the perception in 'developed' countries was that EIDs were confined to 'under-developed' countries; a reflection of inadequate socio-economic circumstances, of limited public health resources, and a consequence of entrenched cultural practices. While elements of these factors undoubtedly underpin disease emergence, this perception is naïve in that it ignores the exponential expansion of global connectivity (predominantly by air travel) in recent decades. SARS, and more recently the emergence of MERS in Saudi Arabia and Ebola in Africa, demonstrated that disease emergence in a remote region or area threatens countries and people around the globe.

As a consequence of my earlier role in a multi-institutional, multi-disciplinary international team that identified *Rhinolophus* bat species as the putative natural reservoir of a SARS-like coronavirus in China (Li *et al.*, 2005), the initial focus of this thesis was to identify any SARS-like coronaviruses in Australian bats. Reassuringly for Australia's public health and biosecurity imperatives, this research found no evidence of SARS-like coronaviruses in Australian bats. However, clear evidence of other bat coronaviruses was found and their discovery redirected the research focus to elucidate their diversity and relatedness to identified bat coronaviruses worldwide, the process of evolution that they had undergone, and an understanding of their dynamics of infection and maintenance in host populations.

In Chapter 1, the current literature on bat coronaviruses was reviewed. My initial research was at the forefront of this area of research, and I was invited to contribute to a chapter in the Food and Agricultural Organisation (FAO) of the United Nations in their publication "Investigating the role of bats in emerging zoonoses: Balancing ecology, conservation and public health interests" (Newman *et al.*, 2011). Because of the novelty and impact of SARS a wave of global research paralleled mine requiring particular update of the literature review over the course of the thesis. Chapter 2 described a novel technique that I developed and published to collect blood samples from very small bats. This technique represents a major methodological advance in the surveillance of bats for EIDs, and has been widely cited (Racey *et al.*, 2011, Anthony *et al.*, 2013, Olival *et al.*, 2013, Larison *et al.*, 2014, Olival and Hayman, 2014, Sheta *et al.*, 2014, Olival *et al.*, 2015).

Australian bat coronavirus infection dynamics

The data and models from Chapter 4 support a hypothesis regarding the infection dynamics of a novel putative *Alphacoronavirus* in *Miniopterus spp*. The hypothesis is that the formation of a maternal colony and ongoing lactation are risk factors for infection (as previously identified by Drexler *et al.* (2011) and Gloza-Rausch *et al.* (2008)), and that a susceptible-infected-recovering (SIR) model, or a maternal-SIR for sub-adults with protective maternal antibodies, could describe an individual bat's state of infection, and that bats have an immunological memory which may limit secondary coronavirus infections, with a stronger and more rapid production of antibodies. Chapter 5 identified that individual *Myotis macropus* were infected with a novel putative *Alphacoronavirus* over periods of up to 11 weeks, this observed pattern of infection supports the hypothesis of persistent infection of coronaviruses in some individual bats. Patterns of infection in other individuals are suggestive of intermittent viral shedding (of persistently infected bats) but could also be interpreted as an acute infection (lack of antibody detection in this species precluded distinguishing between the two). While taking care to avoid over-interpretation, Chapter 5 suggests that another paradigm could be added to the hypothesis of the infection dynamics for bat coronaviruses from Chapter 4 - that of a carrier state, where some infected bats become chronic shedders. This carrier state (Figure 29) could potentially then be a source of infection to a colony, maternal or otherwise. Potentially, a carrier status could be responsible for both primary and secondary infections of other bats, either alternating between being a carrier and being infected (having a secondary infection), or just being a carrier. Persistent infection has previously been suggested as playing a role in the maintenance of coronaviruses in populations of bats, as it does for other coronaviruses, including feline coronaviruses. Naturally infected cats shed FECV intermittently for periods up to 10 months, but some (~15%) become chronic shedders, doing so for years or a lifetime (Addie *et al.*, 1995, Hartmann, 2005, Weiss and Navas-Martin, 2005, Chu *et al.*, 2006, Tang *et al.*, 2006).

This hypothesis warrants further investigation, including the production of statistically significant models from surveillance data. This was not possible within the logistical and funding constraints of this thesis, but with additional surveillance from the same or similar sites, increased sample sizes, and appropriate tools to age male bats, this hypothesis could be thoroughly tested. A mark-recapture study conducted over an entire year would allow an understanding of infection dynamics outside of parturition and birthing.

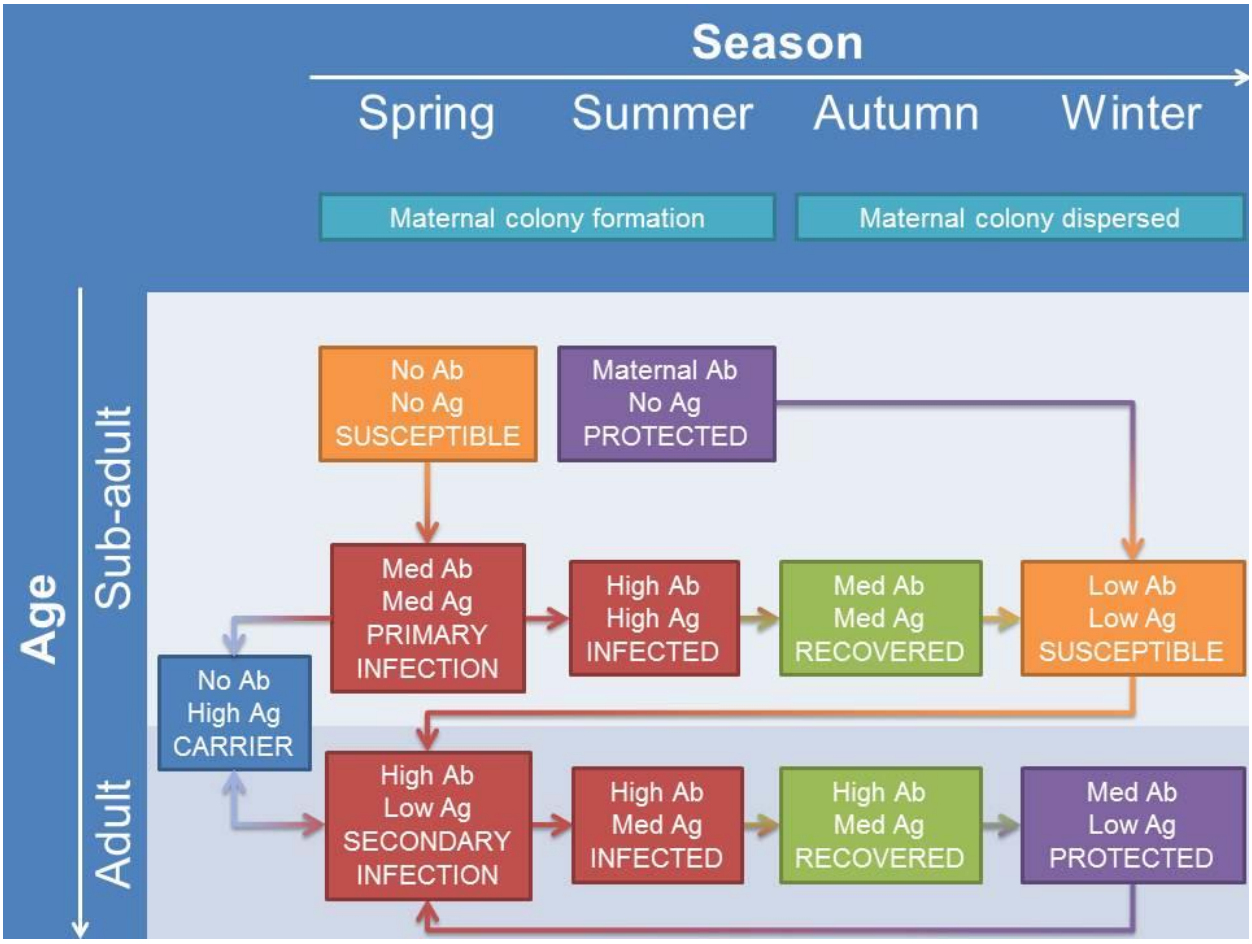

Figure 29. A revised hypothesis for the infection dynamics of coronaviruses in bats.

When discussing the infection dynamics of bat coronaviruses it would be remiss to ignore the unique biology of these, the only mammals with the ability for true sustained flight. Flight has previously been linked with viral infection dynamics, O'Shea *et al.* (2014) suggested that elevated metabolism and body temperature generated during daily cycles of flight was analogous to a febrile response in other mammals and on an evolutionary scale produced a diversity of viruses more tolerant of the fever response. Also, it has been suggested that reactive oxygen species (a by-product of metabolism) placed positive selective pressure on a high proportion of the genes in the DNA damage checkpoint. These flight induced adaptions may have had inadvertent effects on bat immune function and life expectancy (Zhang *et al.*, 2013).

By themselves these adaptations in response to the evolution of flight could have an effect on viral infection dynamics, but the product of flight itself (general frequent and long distance movement (Roberts *et al.*, 2012)) would also surely have some selective pressure on viruses hosted by bats. For example, in Chapters 4 and 5 increased prevalence of

coronavirus was associated with the formation of maternal colonies as did Drexler *et al.* (2011) and Gloza-Rausch *et al.* (2008). Whilst it is reasonable to assume that this increased viral prevalence is the result of the congregation of susceptible bats, conversely, a survival strategy is required for the coronaviruses during periods of its host's dispersal (when flight has afforded the bats the ability to separate over large distances). Could it also be that whilst bats have adapted to the evolution of flight by controlling the damage of DNA and effects of viral infection, viruses have also evolved with the product of flight to survive periods of time when susceptible hosts are sparse? Is this the difference that fundamentally drives different transmission dynamics of coronaviruses in bat populations and requires a persistent infection for bat coronaviruses to endure?

Continued surveillance

Collectively, this thesis provides evidence of a diversity of coronaviruses (belonging to both *Alpha* and *Betacoronavirus* genera) in bats throughout Australasia. It demonstrates firstly that coronaviruses are not recent introductions to Australian bats, and secondly supports a hypothesis of an ancient, complex and adaptive evolutionary association. More specifically, it supported hypotheses that bats from the genus *Rhinolophus* may be more likely to foster host shifts than other species of bats, and their presence increases the risk of emergence of both SARS-like and other bat coronaviruses. Further, it extended the known relationship of bat coronaviruses hosted by bats of the same species or genus to bats of the same family or suborder. It also indicated that the current diversity of coronaviruses in bats is the result of co-evolution with the occasional fostering of host shifts by *Hipposideridae* and *Rhinolophidae*, and that bat coronaviruses are likely to be as old as the most common bat ancestor - 65 million years.

Following on from the above, while the lack of detection of SARS coronaviruses in this study provides preliminary evidence of the lack of occurrence in Australian bat populations, it would be inappropriate to over-interpret the absence of evidence. Indeed, the detection of a broadly clustering SARS-like *Betacoronavirus* in *Rhinonicteris* (from the Northern Territory) warrants urgent follow-up. More broadly, additional and targeted surveillance of putative higher risk host species is required to confirm or refute the preliminary findings and hypotheses of this thesis. A complementary and parallel research approach could be to screen potentially susceptible close contact non-bat populations for evidence of spillover. This was initially a part of the PhD research plan however limited resources precluded its implementation. Structured surveillance of demonstrated

coronavirus susceptible species such as rodents (Wang *et al.*, 2015) or other native mammal populations in the immediate vicinity of identified infected bat populations would confirm or refute spillover potential. The co-habitation of bats and civet cats in caves in China (Chapter 3) appears to provide opportunity for the spillover of coronaviruses from their natural reservoir host to an amplifying host; however, surveillance of wild civet cats shows an absence of infection in the natural population. Are the dense and diverse population of animals in Chinese wet markets a requirement for spillover or does it also occur in nature, generally resulting in the death of a solitary dead-end host? If death is the result, then rural areas in countries like Australia will largely protect it from EIDs, as dead-end hosts are unlikely to have contact with other humans or livestock. However, encroachment of humans into native areas and fragmentation of remnant areas decrease this isolation and leave us vulnerable to EIDs, coronavirus, Ebola, Hendra and Nipah virus are all the result of human encroachment into native areas, increasing contact with wildlife and promoting spillover of EIDs.

Notwithstanding this project's research outputs, it is evident that coronavirus surveillance in Australian bats is incomplete and that a wider spectrum of bat species needs to be investigated. A timely example of this is the recent identification of MERS-like coronaviruses in bats from the genus *Taphozous spp* in Saudia Arabia. Whilst no suitable samples (faeces or anal swabs) were available from Australian *Taphozous* for coronavirus detection or identification, anti-coronavirus antibodies were detected in over 20% of *Taphozous* serum samples collected for this thesis in Australia. If a general rule of species tropism for bat coronaviruses (discussed in Chapter 3) is applied to these findings, it is suggestive of a MERS-like coronavirus circulating in Australian bats, and with Queensland's substantial camel export industry, requires immediate attention. A high prevalence of anti-coronavirus antibodies were also detected in *Mormopterus becarii* and *Scotorepen spp* and indicates that likely not all Australian bat coronaviruses were identified in this study.

When first drafted in 2011, this thesis included the paragraph, "*These findings advance our understanding of the diversity of coronaviruses in bats. This diversity, the global distribution of bats and the propensity of coronaviruses to successfully cross species barriers suggests SARS-like coronaviruses may not be the only example of a bat coronavirus being the cause of future disease outbreaks*." With the emergence of MERS in September 2012, it took only a year to validate these words, providing an enduring

reminder that as we travel through our lives, altering the environment in which we live, we facilitate contact between species that have never met and potentially provide opportunities for the spillover of viruses that are still unnamed.

Appendices

Table 8. RT-PCR dataset collected each season over two years between 2006-2008.

Season	Species	Sex	Age	Detected (Total)
Spring				
	M. australis			
		Female		
			Adult	2 (7)
			Sub-adult	2 (16)
		Male		
			Unknown	9 (33)
	M. schreibersii			
		Female		
			Adult	5 (33)
			Sub-adult	14 (30)
		Male		
			Unknown	12 (39)
Summer				
	M. australis			
		Female		
			Adult	1 (3)
			Sub-adult	1(2)
		Male		
			Unknown	4 (13)
	M. schreibersii			
		Female		
			Adult	1 (1)
			Sub-adult	2 (3)
		Male		
			Unknown	8 (27)
Autumn				
	M. australis			
		Female		
			Adult	5 (19)
			Sub-adult	3 (8)
		Male		
			Male	4 (18)
	M. schreibersii			
		Female		
			Adult	5 (18)
			Sub-adult	5 (19)
		Male		
			Unknown	3 (13)
Winter				
	M. australis			
		Female		
			Adult	0 (4)

			Sub-adult	1 (10)
		Male		
			Unknown	6 (21)
	M. schreibersii			
		Female		
			Adult	2 (12)
			Sub-adult	1 (7)
		Male		
			Unknown	6 (25)
Total				**101 (381)**

Table 9. ELISA dataset collected each season over two years between 2006-2008.

Season	Species	Sex	Age	Detected (Total)
Spring				
	M. australis			
		Female		
			Adult	3 (4)
			Sub-adult	5 (8)
		Male		
			Unknown	14 (22)
	M. schreibersii			
		Female		
			Adult	29 (30)
			Sub-adult	17 (24)
		Male		
			Unknown	37
Summer				
	M. australis			
		Female		
			Adult	1 (1)
		Male		
			Unknown	7 (9)
	M. Schreibersii			
		Female		
			Adult	1 (1)
			Sub-adult	3 (3)
		Male		
			Unknown	24 (25)
Autumn				
	M. australis			
		Female		
			Adult	16 (19)
			Sub-adult	5 (8)
		Male		
			Unknown	11 (18)
	M. schreibersii			

		Female		
			Adult	17(18)
			Sub-adult	12 (19)
		Male		
			Adult	3 (12)
Winter				
	M. australis			
		Female		
			Adult	2 (4)
			Sub-adult	3 (10)
		Male		
			Unknown	13 (21)
	M. schreibersii			
		Female		
			Adult	6 (12)
			Sub-adult	0 (7)
		Male		
			Unknown	9 (22)
Total				**(201) 334**

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