Investigation of small mammal-borne viruses with zoonotic potential in South Africa

by
Ndapewa Laudika Ithete
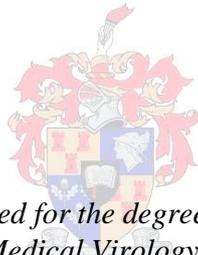
Dissertation presented for the degree of *Doctor of Philosophy (Medical Virology) in the*
Faculty of Medicine and Health Sciences at
Stellenbosch University

Supervisor: Prof Wolfgang Preiser

December 2013

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2013
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
'Eqr{tki j v'Í '4235'Uvgnngpdquej 'Wpkxgtukv{
Cnn'tki j vu'tgugtxgf '
"

Summary

The emergence and re-emergence of viral human pathogens from wildlife sources in the recent past has led to increased studies and surveillance of wildlife for potentially zoonotic agents in order to gain a better understanding of the pathogens, their sources as well as events that may lead to viral emergence. Of the >1407 known human pathogens, 13% are classified as emerging or re-emerging, and 58% as zoonotic; 37% of the (re-)emerging and 19% of the zoonotic pathogens are RNA viruses, accounting for the majority of recently emerged infectious diseases with a zoonotic origin, such as HIV, Ebola, Hendra, Nipah, Influenza and SARS.

This study focusses on potentially zoonotic viruses hosted by rodents (*Muridae family*), shrews (order previously known as Insectivora/Soricomorpha, now reclassified as Eulipotyphla) and bats (order Chiroptera). Rodents and bats represent the largest (~40%) and second largest (~25%) mammalian orders and both occur on every continent except Antarctica. Together, the three mammalian orders investigated represent the most relevant potential sources of new zoonoses.

In this study I investigated the occurrence of astroviruses, arenaviruses, coronaviruses and hantaviruses in South African small mammal species belonging to the orders mentioned above. These viruses have either been implicated in recent emerging zoonotic events or are considered to have the potential to cause cross-species transmissions resulting in a zoonotic event. In the first part of the study specimens collected from various bat, rodent and shrew species were screened for viral sequences by broadly reactive PCRs; positive samples were characterised by sequencing and sequence analysis. A separate part of the study focussed on hantavirus disease in humans: a seroprevalance survey was conducted to determine the presence of hantavirus antibodies in the local population. Additionally, acutely ill patients with potential hantavirus disease were tested in an attempt to identify possible acute infections and define clinical hantavirus disease in South Africa.

Screening of rodent and shrew specimens resulted in the identification of eight novel arenavirus sequences. Seven of the sequences are related to Merino Walk virus, a recently identified South African arenavirus, and the eighth sequence represents a novel lineage of Old World arenaviruses.

Screening of bat specimens resulted in the identification of highly diverse novel astrovirus and coronavirus sequences in various South African bat species, including the identification

of a viral sequence closely related to the recently emerged Middle East Respiratory Syndrome coronavirus.

While the study did not identify hantavirus infections in any of the acutely ill patients, it found seroprevalences similar to those observed in Europe and West Africa.

The results obtained highlight the importance of small mammals in the emergence of potential zoonoses and further reinforce the importance of viral surveillance of relevant wildlife species. Further in-depth studies of naturally infected reservoir host populations are required in order to gain a better understanding of virus-host dynamics and the events that lead to virus emergence.

Acknowledgments

"Yours, LORD, is the greatness and the power and the glory and the majesty and the splendour, for everything in heaven and earth is yours".1 Chronicles 29:11

Nature is relentless and unchangeable, and it is indifferent as to whether its hidden reasons and actions are understandable to man or not.

Galileo Galilei

Table of Contents

Abbreviations

AstV	Astrovirus
bp/kb	base pairs/kilobases
BLAST	Basic Local Alignment Sequence Tool
BtAstV	Bat Astrovirus
BtCoV	Bat Coronavirus
cDNA	complementary deoxyribonucleic acid
CoV	Coronavirus
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DOBV	Dobrava virus
EID	Emerging infectious diseases
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FAO	Food and Agriculture Organisation of the United Nations
GPC	Glycoprotein precursor
GSH	Groote Schuur Hospital
HCoV	Human coronavirus
HCPS/HPS	Hantavirus cardiopulmonary syndrome
HFRS	Haemorrhagic fever with renal syndrome
HTNV	Hantaan virus
ICTV	International Committee on Taxonomy of Viruses
IFA/IIFT	Immunofluorescence Assay/Indirect immunofluorescence test
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IUCN	International Union for the Conservation of Nature

JUNV	Junin virus
MEGA	Molecular Evolutionary Genetics Analysis
MERS-CoV	Middle East Respiratory Syndrome coronavirus
mRNA	messenger ribonucleic acid
MWV	Merino Walk virus
N/NP	nucleocapsid protein
NCBI	National Center for Biotechnology Information
NE	Nephropathia epidemica
NGS	Next-generation sequencing
NHLS	National Health Laboratory Services
nsp	Non-structural protein
ORF	Open reading frame
OUD	NHLS Oudtshoorn
PAA	NHLS Paarl
PCR	Polymerase chain reaction
PUUV	Puumala virus
RCF	Relative centrifugal force
RDA	Representational difference analysis
RNA	Ribonucleic acid
RdRp	RNA-dependent RNA polymerase
rN	recombinant nucleocapsid protein
RSA	Republic of South Africa
RT	Reverse transcription
SANGV	Sangassou virus
SARS-CoV	Severe Acute Respiratory Syndrome coronavirus
SARSr-CoV	Severe Acute Respiratory Syndrome-related coronavirus

SEOV	Seoul virus
sgRNA	subgenomic ribonucleic acid
SISPA	Sequence-independent single primer amplification
SNV	Sin Nombre virus
SSH	Suppression subtractive hybridisation
TEMED	N,N,N',N'-Tetramethylethylenediamine
TPMV	Thottapalayam virus
TRS	transcription regulatory sequence
TYG	Tygerberg Hospital/NHLS Tygerberg Chemical Pathology
VHF	Viral Haemorrhagic Fever
VRD	NHLS Vredendal
WB	Western Blot
WHO	World Health Organisation
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Figures

List of Tables

Chapter 1

1   Introduction and Literature Review

1.1   Emerging infectious diseases and zoonoses

Emerging infectious diseases are defined as infections newly appearing or those that have existed previously and are rapidly increasing in incidence or geographic range (Morse 1995; Brown, 2004; Hui et al, 2006). Emerging infectious diseases are categorised as follows: (1) resurgent or recurring old diseases as a result of mutation in the infectious agent, (2) human disease with epidemiological evidence of transmission into a newly susceptible population and (3) disease outbreak in the population caused by a zoonotic agent (Kellam 1999).

A review by Woolhouse and Gaunt 2007, reported that of the 1399 known human pathogens, 13% can be classified as emerging or re-emerging. Viruses make up 15% (208) of all known human pathogens, but 77% of these human-pathogenic viruses are emerging or re-emerging, and viruses account for 37% of (re-)emerging human pathogens and 19% of zoonotic pathogens (Taylor *et al.* 2001; Woolhouse and Gowtage-Sequeria, 2005). RNA viruses make up the majority of viral pathogens that have emerged in recent decades, with examples including Human immunodeficiency virus (HIV), Hendra virus, Nipah virus and Severe Acute Respiratory Syndrome virus (SARS-CoV). This might be due to the fact that RNA viruses have much higher mutation rates allowing for rapid selective adaptation and increasing the chances of successful cross-species transmission when compared to DNA viruses, bacteria and parasites (Holmes 2009).

The World Health Organisation (WHO) defines zoonosis as "any disease of infection caused by all types of agents transmissible from vertebrate animals to humans and vice versa" (http://www.who.int/topics/zoonoses/en/). Prominent examples of zoonotic viral diseases are rabies, Ebola disease, West Nile fever, Hendra and Nipah encephalitis and hantavirus diseases (Rabozzi *et al.* 2012).

Zoonoses are divided into two categories; diseases where animal-to-human transmission to humans is a rare occurrence and once it occurs the epidemic is maintained by ongoing human-to-human transmission as in the case of HIV, Ebola virus and SARS-CoV. The second category includes diseases where transmission, direct or vector-mediated, is the source of human infection, but subsequent human-to-human transmission is rare or non-

existent as observed for lyssa-, Nipah, arena- and hantavirus infections (Bengis *et al,* 2004; Leslie and McQuiston, 2007).

1.2   History of emerging infectious diseases in human beings

The history of emerging infectious diseases in human beings can be divided into four transitions: the first occurred with the establishment of human settlements and the domestication of livestock 5000-10 000 years ago, which led to the increased contact between humans and pathogens hosted by livestock and pests such as rodents. These pathogens are the progenitors to now established infections such as influenza, smallpox, measles, tuberculosis, malaria amongst others. The second historic transition occurred 1500-3000 years ago, as military and commercial contact increased between early Eurasian civilisations resulting in an exchange of pathogens (McMichael 2004).

The third transition was marked by European exploration and the subsequent trans-oceanic spread of lethal diseases into susceptible populations: Charles Darwin described disease in Australian Aborigines "Wherever the European has trod, death seems to pursue the aboriginal. We may look to the wide extent of the Americas, Polynesia, the Cape of Good Hope, and Australia, and we shall find the same result." and "It is certainly a fact, which cannot be controverted, that most of the diseases that have raged in the islands during my residence there, have been introduced by ships; and what renders this fact remarkable is that there might be no appearance of the disease among the crew of the ship which conveyed this destructive importation" (*from the Voyage of the Beagle, 1839*). During the age of discovery, 10-15 million deaths are estimated to have occurred among the natives in the Americas and the Pacific islands after the introduction of smallpox and measles by European explorers and traders (McMichael 2004; Cleveland *et al.* 2007).

We are currently experiencing the fourth historic transition characterised by globalisation, dramatically increasing urbanisation and population density, modern technology, and climate change (McMichael 2004).

Emergence of new diseases in human beings can be attributed to combined effects of various factors, such as globalisation, reservoir host ecology, virus-host dynamics, climate change and human-induced landscape change (Vandegrift *et al.* 2011).

1.3   Factors associated with cross-over transmission and viral emergence

The factors associated with emerging viral diseases are divided into three categories: viral factors, human factors, and improved diagnostic and epidemiological tools allowing for the detection of previously undetected infectious agents as well new infectious disease agents (Bengis *et al,* 2004; Hui 2006).

1.3.1   Viral factors

More RNA viruses are associated with emerging diseases compared to DNA viruses. The association of RNA viruses with emerging infections is attributed to their evolutionary rates. The evolution of RNA viruses is as a result of three mechanisms: point mutations, recombination and reassortment (Holmes and Drummond 2007).

a) Point mutations

RNA viruses have very large population sizes in a host organism in a magnitude of 10^{12} virus particles. A single virus can replicate to produce as many as 10^5 viral copies in 10 hours (Moya *et al.* 2004). In addition the viral RNA polymerases are highly error-prone and lack proofreading activity. This results in extremely high mutation rates (range 10^{-4} to 10^{-5}). The mutation rates for SARS-CoV following the outbreak in late 2002, was $8.26 \times 10{-}6$ per nucleotide per day (3 mutations per RNA genome per replication) (Hui 2006). The genome size for RNA viruses ranges from 3 to 30kb, and size is limited by high mutations rates: the generation of mutations beyond the error threshold would generate too many deleterious mutations resulting in decreased viral production and subsequent population extinction. However RNA viruses with mutation rates just close to the error threshold should theoretically be able to produce beneficial mutations required for adaptation in a new environment such as a new host (Moya *et al.* 2004; Holmes and Drummond 2007).

b) Recombination

Genetic variability in RNA genomes can also be achieved by recombination. Recombination occurs when two viruses co-infect the same cell and a hybrid molecule is produced through copy-choice replication used in the production of subgenomic RNA; RNA-dependent RNA polymerase (RdRp) moves from one template to the other (strand switching) (Holmes 2009). Although not a key evolutionary strategy but rather a sporadic event, recombination can promote emergence by improving viral fitness through creating advantageous genotypes and

removing deleterious mutations (Holmes and Drummond 2007). Phylogenetic analysis of the SARS-CoV suggests that the virus may have arisen from a recombination event between two bat coronaviruses between ORF1 and ORF2 (Hon *et al.* 2008).

c) Reassortment

Gene assortment occurs in RNA viruses with multi-segmented genomes such as influenza viruses. As with recombination, co-infection of a single host cell by two viruses is required for the exchange of genetic material which occurs during packaging of viral particles; segments of different ancestry are packaged together into one virus (Holmes 2009). Influenza viruses emerge as a result of reassortment; analysis of the pandemic A/Influenza/H1N1 virus which emerged in 2009 showed that the virus contained segments of avian, classical swine, Eurasian swine and human H3N2 virus strains (Dawood *et al.* 2009).

1.3.2 Environmental factors

a) El Niño southern oscillation

ENSO is an annual climate cycle on earth which begins in the tropical pacific ocean; it results in either warmer (El Niño) or colder (La Niña) sea surface temperatures in 2 to 7 year cycles. El Nino leads to an increase in rainfall on the western Coast of north and South America and decreased rainfall in Indonesia and the pacific (Dearing and Dizney 2010). The El Nino events of 1992 led to increased autumn, winter and spring rainfall in New Mexico and Utah resulting in enhanced spring primary production and thus increase in food sources for *Peromyscus maniculatus* (host of Sin Nombre virus) (Yates *et al.* 2002) and an increase in rodent population densities. Higher populations of *P.maniculatus* in turn lead increased utilisation of resources leading to increased contact with human beings (Klein and Calisher 2007) and increased transmission of Sin Nombre virus (SNV) to humans.

b) Climate change – temperature

The global average temperature has increased by 0.8°C between 1906 and 2005 (Hansen *et al.* 2006); the outbreak of West Nile virus in New York in 1999 is attributed to above-average temperatures (Patz *et al.* 2008). The mild northern hemisphere winter of 1998-99 allowed for increased survival of *Culex* mosquitoes during hibernation and decreased precipitation during the subsequent spring and

summer causing a decrease in mosquito predators (frogs, lacewings and ladybugs) (Hui 2006), together resulting in a higher mosquito vector population.

1.3.3 Anthropogenic factors

a) *Human demographics and behaviour*

The population of human beings has increased dramatically since the industrial revolution (Figure 1.1); large population sizes and densities favour the spread and maintenance of pathogens and disease. For instance, a population of at least 200 000 is required for measles virus to persist in the population (Hui 2006).
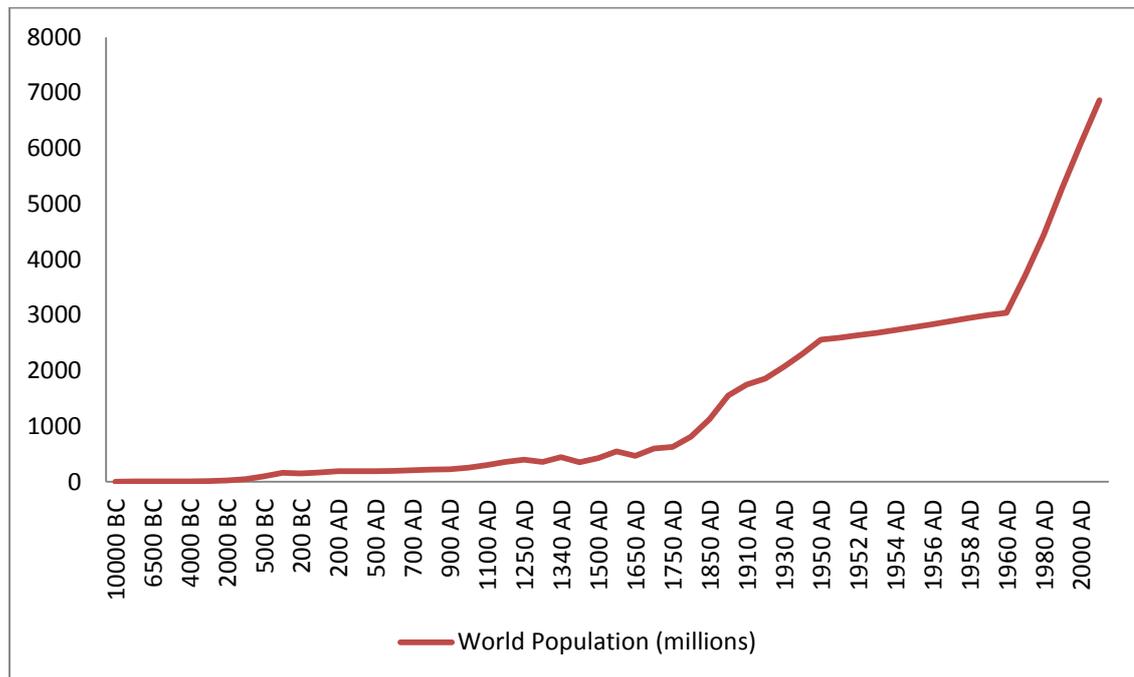
Figure 1.1 Growth of the World population. Graph illustrates growth from 10 000 BC to 2000 AD. A sharp increase commenced during the industrial revolution (~1760 AD to 1840 AD). Data obtained from the United States Census Bureau http://www.census.gov/population/international/data/worldpop/table_population.php

Urbanisation during which large populations move into cities with poor living conditions and poor hygiene in shanty towns means that exposure and spread of pathogens occurs more easily (Childs *et al.* 2007).

The increase in human population has also led to human encroachment into previously undisturbed habitats leading to deforestation and habitat fragmentation and increased human contact with infectious agents occurring in wildlife.

b) *Habitat destruction and fragmentation*

Destruction of forests to create agricultural land has led to the reduction of wildlife habitat; slash-and-burn deforestation in Malaysia resulted in the displacement of *Pteropus* bats forcing bats to forage on horticultural crops increasing the chances of bat contact with humans and livestock (Field *et al.* 2007). The bats were forced to roost in fruit orchards near which intensive pig farming was conducted. The Nipah virus (NiV) was transmitted from bats hanging in the fruit trees over the pig pens. The pigs acquired the infection (and acted as an amplifying host) and passed the infection on to the pig farmers (Field *et al.* 2007; Greger *et al.* 2007).

Deforestation and intensification of agricultural practices in South America has resulted in the increased contact between rodent hosts of arenaviruses and humans leading to the emergence of arenaviral haemorrhagic fevers in Argentina, Belize and Brazil (Greger *et al.* 2007). Argentine haemorrhagic fever (AHF) emerged after the pampas were cleared for the farming of maize; farming of maize led to an increase in the population of *Calomys musculinus*, the host of Junín virus (Enria *et al.* 1998).

c) *Consumption of bush meat*

As much as 3.4 million tons of bush meat are consumed in central Africa and up to 164000 tons are consumed in the amazon basin (Chomel *et al.* 2007). Hunting of peridomestic rodents is considered as a risk factor for Lassa infection. Consumption of rodents is common in Lassa endemic areas (ter Meulen *et al.* 1996).

Bats are hunted for food in Africa and south-east Asia (Mickleburgh *et al.* 2009); exposure to bat pathogens occurs through contact with bats through hunting, handling and consumption. Henipavirus-related sequences have been identified in *Eidolon helvum* in Ghana and Congo (Drexler *et al.* 2009; Weiss *et al.* 2012). Further studies are required to determine the risk of infection to high risk populations such as hunters and vendors (Weiss *et al.* 2012).

Consumption of non-human primates in central and west Africa is common place: as a source of food and as a source of income (Peeters *et al.* 2002). More than 60% of the population is exposed to blood and body fluids from non-human primates from hunting, butchering and petting in Cameroon. It is assumed that cross-species transmission of Simian immunodeficiency virus SIV from *Pan troglodytes* and *Cercocebus atys* led to the emergence of HIV-1 and HIV-2 in Central Africa during the 1940-50s (Sharp *et al.* 2001).

d) *Live animal markets or wet markets*

Animal markets are popular in both developing and developed countries providing freshly slaughtered or live animals for sale. In Africa and Asia, wildlife and exotic species are sold as well (Brown 2004). Animals are closely packed in cages, under poor hygienic conditions and often for prolonged periods; large quantities of animal excreta are shed and may contain high concentrations of zoonotic microbes (Woo *et al.* 2006). SARS-CoV emerged from the wet markets in the Guangdong province in Southern China: closely related viruses were isolated from palm civets (*Paguma larvata*) and raccoon dog (*Nyctereutes procyonoides*) in live markets (Woo *et al.* 2006). Li *et al* (2005) identified SARS-like CoVs in Chinese horse-shoe bats, it is hypothesised that the palm civets and racoon dogs were exposed infected bats and/or bat excreta while in captivity in the markets (Berger *et al.* 2004).

e) *Globalisation and travel*

Air travel has made it easier for infectious diseases to spread; an outbreak of monkeypox, a zoonotic virus from Central Africa, occurred in the United States after the importation of exotic African rodents, which then transmitted the virus to prairie dogs when caged together (Guarner *et al.* 2004). Prairie dogs (*Cynomys sp.*) are kept as pets which allowed for the opportunity to infect humans (Anderson *et al.* 2003).

The outbreak of SARS-CoV in late 2002 exemplifies the importance of air travel in the spread of newly emerged pathogens: the disease was able to spread to 29 countries on five continents in a matter of months following the outbreak in late 2002 (http://www.who.int/csr/sars/country/2003_08_15/en/). Infection was spread by travellers on-board the flights as well as at destinations: forty flights were investigated for probable on-board transmission and five were associated with transmission including a flight from Hong Kong to Beijing during which 22 passengers may have contracted the infection (http://www.who.int/csr/don/2003_05_22/en/print.html).

1.4   Identification and characterisation of emerging viruses

Before the development of polymerase chain reaction (PCR), most viruses were discovered by virus isolation in cell cultures, by electron microscopy or by antigen detection methods such as enzyme immunoassays (EIA) and indirect immunofluorescence tests (IIFT) (Food and Agriculture Organisation of the United Nations (FAO) 2011).

Cell culture allows for the isolation of viral particles which can then be characterised and used in infectivity studies. An important limitation is that a permissive cell line is required for virus replication. Additionally, identification of virus replication normally depends on the observation of a cytopathic effect (CPE) and not all viruses cause CPE. Hantaviruses replication in Vero cells for instance does not produce a CPE so that infection has to be confirmed by immunofluorescence or reverse-transcription-PCR (RT-PCR) on ribonucleic acids (RNA) extracted from cell-culture supernatant (Kellam 1999). Another limitation of virus isolation is the need for biosafety (BSL) 3 or even 4 facilities when attempting to isolate highly pathogenic viruses such as viral haemorrhagic fever viruses (e.g. some arena-, hanta- and filoviruses). Nevertheless virus isolation may be useful for identification of new viruses (Wang and Daniels 2012).

Methods that aim to detect viral antigen or antibodies have been successful diagnosis of known infectious agents. Since an antigen or antibody is required for detection, these methods are limited to the detection of viruses for which antigens or monoclonal antibodies from antigenically related viruses are available (Richman 1993). EIA and immunoblots are useful for diagnosis of infection as they do not require whole viruses but instead make use of recombinant antigens. Recombinant proteins are produced by inserting PCR fragments of the required gene into an expression vector and culturing in suitable cells, e.g. *E.coli*, yeast or insect cells. Such approaches have been used to set up assays for the detection of antibodies against hantavirus, arenaviruses and henipaviruses. Antibody testing using recombinant antigens can be performed in BSL-2 laboratories, thus eliminating the need for higher BSL facilities which are expensive to maintain and also require specially trained personnel (Wang and Daniels 2012). Neutralisation test for highly pathogenic viruses such as hantavirus and arenavirus still need to be performed under BSL3 and BSL4 conditions since viruses are used in the assay. Neutralisation tests (plaque or focus reduction assays) are used measure virus neutralising antibodies in serial dilutions of patient samples such as serum. Neutralisation tests require a readily cultured cell line that permissive to the virus being investigated and requires as many as 6 days incubation are required to visualise results. Neutralisation assays are the gold standard for the detection of neutralising antibodies due to its high specificity and sensitivity (Mather *et al.* 2013).

Serological methods are especially useful when viral nucleic acids are absent (in cases when viraemia is transient) from the specimen (Lipkin and Firth 2013). Serological assays maybe used to determine the spread of an emergent disease even after infected individuals are past

the symptomatic stage of disease (Mather *et al.* 2013). It should be noted however, that if the infectious agent does not at least cross-react with the antigens used in the assay then false-negative results will be obtained. On the other hand, high cross-reactivity observed in EIA might lead to false positive results. It is therefore necessary to confirm results using a more specific assay for confirmation of the first test result (Kellam 1999). Electron microscopy (EM) was first developed in the 1930s; it became widely available in the 1960s and has been successfully used in the detection of human pathogenic viruses such as adeno-, entero- and paramyxoviruses during the 1970s and 80s (Roingeard 2008). EM can be used to identify viral particles in clinical samples such as tissue, faecal matter and serum, as well as *in vitro* cell cultures (Kellam 1999). The development of PCR and EIA assays during the 1990s led to a decline in the use of EM in routine diagnostics, but it is still useful in identification of novel viruses especially during outbreaks. The Hendra virus which caused an outbreak of severe respiratory disease in horses and human beings in Australia (1994) was identified as a mobillivirus by EM (Murray *et al.* 1995).

Molecular assays are highly favoured for virus identification due to their high sensitivity, ease of use and rapidity (results can be available within less than 24 hours). Methods such as conventional PCR, real-time PCR, nucleic acid sequence based amplification (NASBA) and loop mediated isothermal amplification (LAMP) have been used in routine diagnostics for identification and quantification of pathogens. These assays can also be used to identify novel viruses by designing degenerate primers targeting conserved regions of the viral genome (Kellam 1999; Beck *et al.* 2010; Delwart 2012) followed by sequencing and sequence analysis. This process is relatively easy due to genome databases such as Genbank (http://www.ncbi.nlm.nih.gov/genbank/) through which sequences of known viruses are readily available.

The main disadvantage of the abovementioned methods is the need for prior knowledge of viral sequences for primer design. Sequence-independent methods such as suppression subtractive hybridisation (SSH) and representational difference analysis (RDA) have been used in the identification of Human herpesvirus 8 (Mokili *et al.* 2012). Sequence-independent single primer amplification (SISPA) was developed to identify unknown viral nucleic acids and was first used to identify norovirus from faecal material. More recently it was, slightly modified, used to identify HCoV-NL63 (van der Hoek *et al.* 2004; Bexfeld and Kellam 2010). SISPA involves ligating adaptor molecules to fragmented viral DNA or copy DNA (cDNA) and amplifying the material using universal primers and finally Sanger sequencing to

identify the amplified fragments (Djikeng *et al.* 2008). More recently next-generation sequencing (NGS) methods and platforms have become available. NGS is theoretically able to detect any virus present in a sample (also referred to as the "virome"). Various platforms are available that vary in terms of biochemistries, sequencing protocols, throughput and sequence length, including Illumina sequencing systems (Illumina), 454 pyrosequencing platforms GS FLX+ and GS Junior (Roche), Ion Torrent and SOLiD (Life Technologies) (Barzon *et al.* 2013). The 454 GS FLX+ has been used in the identification and characterisation of novel viruses including two arenaviruses, Lujo virus and Merino Walk virus (Mokili *et al.* 2012).

1.5   Small mammals as hosts of emerging viruses

Zoonotic pathogens (58%) are responsible for the majority of pathogens causing infections in human beings (Taylor *et al.* 2001). The current hypothesis on virus-host relationships is that infection is subclinical or non-pathogenic, but that spill-over into a different animal species may result in severe disease. Such cross-species transmission is considered to be an accidental event which may occur when one species intrudes into the natural habitat of another species (Wong *et al.* 2007). Emerging pathogens originate from a wide variety of animals including non-human primates, carnivores, ungulates, rodents, bats, marine mammals and birds (Cleveland *et al.* 2007). In this study we focus on rodent- and bat-borne viruses: rodents and bats have the highest species diversity of all mammalian species (~40% and ~20% respectively) (Wong *et al.* 2007). Both orders of mammals are hypothesised to possess special characteristics that make them ideal hosts of emerging viruses (Luis *et al.* 2013).

1.5.1   Bats

Bats represent the second largest order of all mammalian species (with more than 1200 species) (Woo *et al.* 2006) and form the order Chiroptera, which was previously divided into Megachiroptera (mega bats) and Microchiroptera (micro bats) where Pteropid bats were classified as Megachiroptera based on their inability to echolocate. The remaining bat species are divided into 17 different families (Monadjem *et al.* 2010) as Microchiroptera. The proposed reclassification to the order places *Pteropodidae* and *Rhinolophidea* in the infraorder Yinpterochiroptera. The remaining superfamilies (*Emballonuroidea, Noctilionoidea* and *Vespertilionoidea*) previously classified under microchiroptera are now classified as Yangochiroptera (Miller-Butterworth *et al.* 2007). Bat species occur on every continent except the Polar Regions and few islands in Oceania (Calisher *et al.* 2006).

Bats have number important ecological roles; frugivorous bats act as pollinators and seed dispersers for more than 300 tropical plant species. Insectivorous bats play a role in controlling insect populations including crop pests which may be beneficial to farmers by minimising crop loss (Monadjem *et al.* 2010). Their guano is harvested for use as fertiliser and for the manufacture of antibiotics, gasohol and soaps (Calisher *et al.* 2006).

In the past two decades, bats have been increasingly implicated as the hosts of various zoonotic human pathogens including coronaviruses, filoviruses (Ebola and Marburg), lyssaviruses (Rabies) and paramyxoviruses (Hendra and Nipah) (Turmell and Olival 2009). Research interest in bats has especially increased in the aftermath of the SARS-CoV outbreak in late 2002. The implication of hosts in such relevant emerging infectious diseases has led researchers to hypothesise that bats possess certain characteristics that make them ideal viral reservoirs (Calisher *et al.* 2006; Wong *et al.* 2007). Bats are the only mammals that have flight ability which allows them to interact with other animals and carry viruses between different locations. They also congregate in colonies that contain between 10 and ≥500 000 individuals, and their longevity (10-35 years) provides opportunities for transmission of viruses (Chan *et al.* 2012).

A recently published study by Luis *et al* (2013) tested this hypothesis by analysing literature on bat and rodent viruses, testing for association between characteristics such as sympatry, torpor, population structure, longevity and zoonotic viral richness. Results showed that bats harbour more viruses per host species when compared to rodents; bat viruses had broader host range (4.51 bat species per virus). The effect of sympatry (defined as the occurrence of two or more species in the same geographical location without interbreeding [http://wordnetweb.princeton.edu]) was higher in bats than rodents and it also correlated positively with viral richness and this may be due to the fact that different bat species can roost together whereas different rodent species do not nest together. The study also found that torpor correlated negatively with viral richness and this may be due to decrease in contact between individuals and thus exposure to viruses during hibernation. Longevity higher body mass and smaller litter size correlated positively with viral richness and the authors speculate that this because viruses have a higher fitness in longer lived hosts because the host is infected for a longer period of time (Luis *et al.* 2013). Bats roost in natural habitats such as caves, rock crevices, and tree cavities. They also roost in man-made structures such as mine shafts, roofs, bridges and tombs (Wong *et al.* 2007; Chan *et al.* 2012). Human activities such as bushmeat hunting, encroachment, deforestation and agricultural intensification lead to loss

of roosting sites and feeding habitat (Field *et al.* 2009). Bats forced to forage and roost closer to human habitats thus increasing contact between bats and humans, and livestock or companion animals result in cross-species transmission that results in outbreaks (Leroy *et al.* 2009; Chan *et al.* 2012).

1.5.2   Rodents and insectivores

1.5.2.1   Rodents

The order Rodentia is the most species-rich mammalian order, accounting for 43% of the total number of mammalian species (≥2000 species). Rodents occur on every continent except Antarctica (Meerburg *et al*. 2009). Rodents often congregate together in large nests in urban, rural (peridomestic) and sylvan habitats (Wong *et al.* 2007). Rodents such as rats (*Rattus rattus, R. norvegicus*), mice (*Mus musculus*) and hamsters (*Mesocricetus auratus*) are used as animal models in the study of human diseases and as test subjects in the pharmaceutical industry in drug efficacy tests and they are also kept as household pets. At the other end of the spectrum rodents are often considered pests and are responsible for crop losses (especially cereals) and spoilage of food stores (Meerburg *et al.* 2009). They have also been implicated as reservoir hosts of zoonotic bacterial and viral pathogens such as *Leptospira spp., Yersinia pestis,* and viral haemorrhagic fever (VHF) viruses including arena- (Junin, Machupo, Lassa) and hantaviruses (Hantaan, Dobrava, Sin Nombre) (Davis *et al.* 2005). Transmission of pathogens between rodents and humans is affected by viral, environmental and anthropogenic factors (cf. section 1.3).

Most studies conducted on cross-species transmission are on *Calomys musculinus* (host of Junin virus), *Peromyscus maniculatus* (host of Sin Nombre virus) and *Clethrionomys glaroelus* (host of Puumala virus) (Mills and Childs 1998; Davis *et al.* 2005). A 3 year study on *C. musculinus* on the Argentine pampas showed the effect of climate change on the rodent population and subsequent transmission of Junin virus (JUNV) to humans. When the rodent population was low, human infection was also rare for two seasons. In the third year of the study, a mild winter allowed rodent reproduction through the winter and high overwinter survival. Reproduction during the spring led to an even higher increase in population and the high abundance of food sources during the following summer led to an epidemic of argentine haemorrhagic fever (Mills and Childs 1998).

A similar phenomenon has been observed for Puumala hantavirus in *C. glareolus*, where mild winter temperatures, higher precipitation with subsequently increased food sources and

resulting increase in rodent population lead to more cases of nephropathia epidemica (NE) (Escutenaire *et al.* 2002). The chances that an individual animal will transmit the virus is also dependent on the time elapsed since infection. It has been shown that even though Puumala virus (PUUV) shedding is chronic in *C. glareolus*, recently infected animals are more infectious (Davis *et al.* 2005). Newly infected individuals are introduced into the population every season in the form of young animals following the loss of protection offered by maternal antibodies. The prevalence of infection within a population increases with age and is generally higher in males. Transmission among males is believed to occur mainly during breeding season during their fights over territory and mates (Kuenzi *et al.* 2001). The prevalence of JUNV was higher in *C. musculinus* adult males with scars compared to those without scars (Mills and Childs 1998).

1.5.2.1   *Insectivores*

Shrews and moles were initially classified as members of the mammalian order *Soricomorpha/Insectivora* in the families *Soricidae* and *Talpidae* respectively. Recent re-classification has placed these two families in a new order *Eulipotyphla*, with hedgehogs forming the third family in the order, *Erinaceidae* (Douady *et al.* 2002; http://www.nhc.ed.ac.uk/index.php?page=24.134.166.495). The *Soricidae* family consists of more than 370 species and there are more than 40 species in the *Talpidae* family (http://www.departments.bucknell.edu/biology/resources/msw3/browse.asp?id=13700021). The majority of species are nocturnal and spend their days in underground nests or in forest undergrowth (Riber 2006). Their diet mainly consists of invertebrates such as insects, arachnids and earthworms. Unlike rodents, insectivores do not aggregate together in nests or colonies. The interest in insectivores as hosts of emerging zoonoses has been renewed since their implication as hosts of bornaviruses which cause progressive meningoencephalomyelitis in domestic animals such as horses and sheep. Evidence of bornavirus infection has also been detected in neuropsychiatric patients (Hilbe *et al.* 2006; Kinunen *et al.* 2013).

Thottapalayam virus was the first hantavirus isolated from an insectivore host *Suncus murinus* in India in 1964 (Song *et al.* 2007). The next shrew-borne hantavirus was identified in *Crocidura theresae* in Guinea, West Africa (Klempa *et al.* 2007) and since then numerous *Soricidae*-borne hantaviruses have been identified in North America, Europe and Asia (Kang et al. 2011a). *Talpidae*-borne hantaviruses have also been identified recently (Arai *et al.* 2008; Kang *et al.* 2011b). Generally insectivore-borne hantaviruses are not associated with

disease, but studies in Indonesia have shown evidence of Thottapalayam virus (TPMV) infection in patients with febrile disease (Okumura *et al.* 2007). As with bats, human activities such as deforestation and encroachment may force rodents and shrews to nest in peridomestic habitats (e.g. houses, barns and sheds), thus increasing contact with human beings and the chances for cross-species transmission. Peridomestic deer mice have higher prevalences of SNV and this may be due to a restriction in movement inside buildings resulting in increased concentration of infectious urine and faeces. The situation is exacerbated by the longer viral persistence of virus indoors compared to outdoors, where infectious particles disperse quickly or are destroyed by the sun's UV rays (Kuenzi *et al.* 2001).

1.6   Important examples for potentially emerging and zoonotic viruses

A literature survey by Luis *et al* (2013) on rodent and bat-borne emerging zoonoses confirms the conclusions made by Woolhouse and Gowtage-Sequeria (2005); statistical analysis showed that zoonotic viruses were more likely to be RNA viruses with multi-segmented genomes and replicating in the cytoplasm. Replication in the cytoplasm means that viruses bypass the need to interact with complex host cell replication machinery located in the nucleus (Luis *et al.* 2013). In this study we chose four RNA viruses implicated in emerging zoonoses.

1.7   Introduction to hantaviruses

Hantavirus disease was described thousands of years ago by Chinese physicians long before the identification of the etiological agents. But the disease gained prominence in recent history following the outbreak of what was termed Korean haemorrhagic fever disease in UN troops during the Korean War (1950-53) which was later renamed haemorrhagic fever with renal syndrome (HFRS) (Lee *et al.* 1978). A milder form of HFRS known as nephropathia epidemica (NE) was described in Scandinavia and Russia in the 1930s and 1940s (Klein and Calisher 2007). The hantavirus disease in the New World came to the forefront following an outbreak of disease then called Four Corners disease and later renamed hantavirus cardiopulmonary syndrome (HPS or HCPS) (Nichol *et al.* 1993).

The identification of Hantaan virus (HTNV) as the causative agent of HFRS and its reservoir host *Apodemus agrarius coreae* (Lee *et al.* 1978, 1981), led to the search for hantaviruses in rodents and subsequent discoveries of Puumala virus (PUUV) as the etiological agent of NE, Seoul virus in *Rattus norvegicus* and the first New World hantavirus to be identified,

14

Prospect Hill virus in *Microtus pennsylvanicus* (Lee *et al.* 1985). Studies following the identification of HTNV revealed that the virus belongs to the family *Bunyaviridae*, in a new genus *Hantavirus* (McCormick *et al.* 1982). TPMV was the first hantavirus identified in the 1960s and was previously unclassified but studies conducted since the establishment of the hantavirus genus classified TPMV as a hantavirus based on ultrastructures and genome sequence analysis (Song *et al.* 2007).

1.7.1 Molecular biology of hantaviruses

Hantavirus virions are spherical in shape and have a diameter of 80-120nm. They have an envelope consisting of two gylcoproteins, G_n and G_c embedded in a golgi membrane derived lipid bilayer. Within is a trisegmented genome with a small (S), medium (M) and large (L) segment (**Figure 1.2**). The genome segments are linear negative-sense single-stranded RNA molecules and flanked at the 5' and 3' end by highly conserved terminal complementary panhandles UAGUAGUAUUGC…and AUCAUCAUCUG… respectively (Hooper and Schmaljohn 2001). The S segment is 1.6 to 2kb long and encodes the nucleocapsid protein and non-structural proteins, New World viruses have an additional +1 overlapping reading frame (ORF) which codes for Non-structural proteins (NS). The M segment is ~3.6kb long and contains genes for glycoproteins G_n and G_c and the L segment is 6.5kb long and encodes for viral RNA-dependent RNA polymerase (RdRp) (King *et al.* 2012).

Viral RNA

Nucleocapsid protein

Ribonucleoprotein: Viral segments encpasidated by the nucleoprotein

RNA-dependent RNA polymerase

Spike Glycoproteins: G_n and G_c
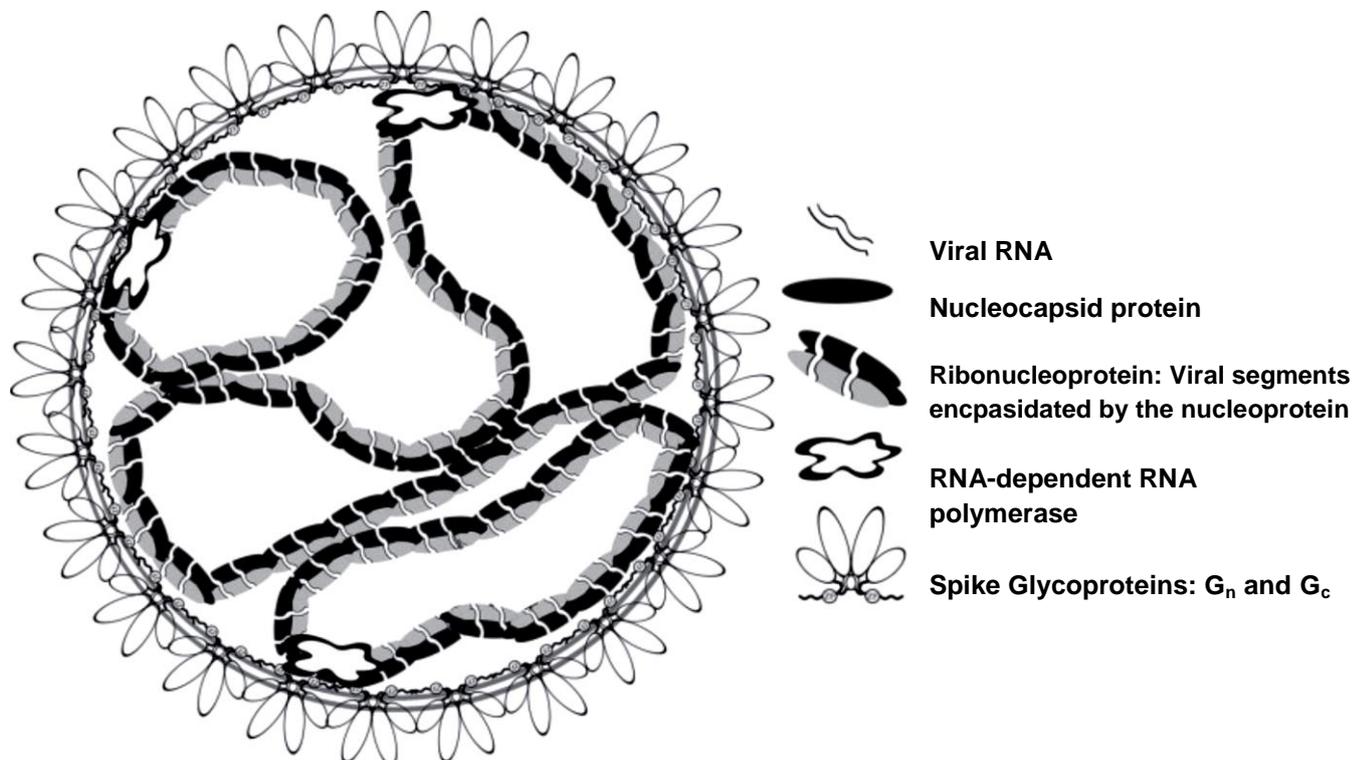
Figure 1.2 Schematic illustration of a hantavirus virion: the virus has a trisegmented genome; segments associate with the nucleoprotein (N) and are enclosed in lipid bilayer membrane which contains Gn and Gc assembled in Spike complex which protrudes from the surface of the membrane (Hepojoki *et al.* 2012). *Image used with permission from the Society for General Microbiology*.

Hantaviruses are able to infect endothelial, epithelial and immune cells (macrophages, dendritic cells and lymphocytes). The viruses enter the cells by attachment of G_n to a cell surface receptor, β1- and β3-integrin for non-pathogenic and pathogenic hantaviruses, respectively (Gavrilovskaya *et al.* 2002). Recent studies have found additional receptors involved in hantavirus cell entry; decay-accelerating factor (DAF/CD 55) was shown to act as a receptor in addition to β3-integrins for pathogenic Old-World viruses Dobrava and Puumala and β1-integrins in Tula virus (Krautkrämer and Zeier 2008). Klempa *et al* (2012a) showed that Sangassou virus makes use of β1-integrins and neither β3-integrin nor DAF/CD55 as observed for pathogenic Old World viruses.

Following attachment, cell entry occurs by receptor-mediated endocytosis involving clathrin-coated pit pathway, caveolae-pathway or macropinocytosis (Hooper and Schmaljohn 2001; Jin *et al.* 2002; Ramanathan and Jonsson 2008). The viruses then move to the endosome and lysosomes from which genome is released into the cytoplasm. Viral RdRp initiates transcription resulting in the production of S, M and L mRNA molecules. Translation then occurs at free ribosomes S and L transcripts, and M mRNA is translated at the rough endoplasmic reticulum (Jonsson *et al.* 2010). These initial stages result in the production of structural proteins, N, G_n and G_c, and more RdRp required for assembly and packaging of new virions. Viral RdRp switches to replication mode and genomic segments are produced and encapsidated by N to form ribonucleoproteins (RNPs). Mature RNPs are then transported to the Golgi apparatus where they interact with the Golgi membrane studded with newly translated Gn and Gc (Hussein *et al* 2011). The interactions of RNPs and glycoproteins lead to budding of virions from the Golgi apparatus but events are not clearly understood and there is an alternative hypothesis suggesting that New World hantavirus virions assemble and mature at the plasma membrane (Jonsson *et al* 2010).

1.7.2   Taxonomy and phylogeny of hantaviruses

The genus *Hantavirus* was established as part of the *Bunyaviridae* family in the 1980s following the identification of Hantaan virus (the prototype for the genus). Other genera in the family are *Nairovirus, Orthobunyavirus, Phlebovirus* and *Tospovirus* (King *et al.* 2012).

Current International Committee on Taxonomy of Viruses (ICTV) guidelines for the determination of species within the genus are as follows: (i) the hantavirus species is found in a unique ecological niche i.e in a different primary reservoir species or subspecies, (ii) it displays ≥7% amino acid sequence identity with other species in the genus when full S and M segment sequences are compared, (iii) it must show at least four-fold difference in a two-way cross-neutralisation tests and (iv) hantavirus species do not naturally form reassortant viruses with other species (Maes *et al.* 2009). There are exceptions to the second criterion as result of the occurrence of recent host-switching events (King *et al.* 2012) as described for SNV and New York virus, and for Dobrava and Saaremaa virus (Plyusnin *et al* 2001; Plyusnin *et al* 2002).

Hantaviruses are divided into New World and Old World viruses; and before the identification of novel hantaviruses in insectivores and more recently in bats, members of the genus were phylogenetically divided into two clades Old World and New World, with as TPMV an outlier. New World hantaviruses are hosted by rodents of the subfamilies *Sigmondontinae, Neotominae* and *Arvicolinae*, and Old World hantaviruses hosted by rodents in the *Murinae* and *Arvicolinae* subfamilies (Klein and Calisher 2007).

A recent study on hantaviruses in insectivores and bats conducted extensive phylogenetic analysis of long-standing members of the genus and the novel viruses recently identified (Figure 1.3). Phylogenetic inferences based on the S segment showed that hantaviruses are divided into four well supported phylogroups: Phylogroup I is composed of *Soricidae*-borne viruses Thottapalayam and Imjin and phylogroup II consists of bat-borne viruses and European *Talpidae*-borne viruses. The third phylogroup consists of *Soricidae* and *Talpidae* insectivore-borne as well as *Murid* rodent-borne hantaviruses. *Arvicolinae, Neotominae, Talpidae* and *Sigmondontinae* hosted viruses make up the fourth phylogroup, although these viruses do not form monophyletic clades as observed in the other phylogroups. Similar groupings were observed when the M segment was used for analysis, although phylogroup II was basal instead of group I in S segment analysis suggesting that reassortment may have occurred among phylogroup I and II viruses (Guo *et al.* 2013).

Analysis of the partial L segment which is the most abundantly available sequence for recently identified novel hantaviruses, yielded a different picture; phylogroup II bat-borne viruses are split and a fifth phylogroup emerges. Analysis provides evidence for multiple cross-species transmission events between insectivore and bat-borne viruses in the evolutionary history of the genus (Guo *et al* 2013).
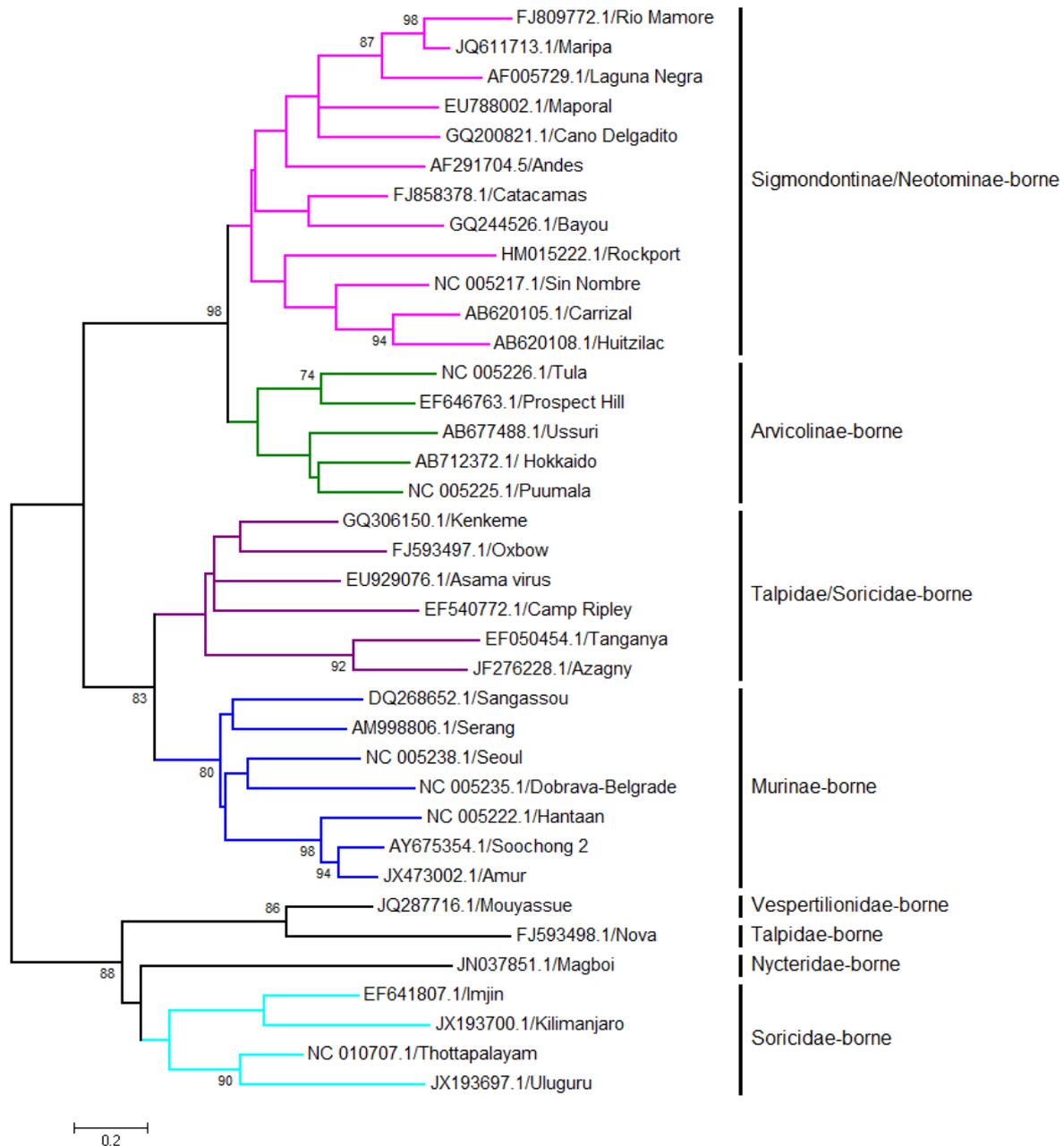
Figure 1.3 Evolutionary relationship between members of the *Hantavirus* genus. Maximum-likelihood inference based on the General time reversible model (GTR+I+G) performed in MEGA v5.05 on the partial *L* segment (corresponding to position 2956 to 3368 on the hantaan sequence NC005222): 1000 bootstrap replicates were used for inference to

obtain the consensus tree. Branches corresponding to partitions reproduced in less than 50% were collapsed. The percentage of replicate trees in which associated taxa clustered together in bootstrap analysis are shown at the branch nodes (values less than 70% are excluded from the tree). Virus names and NCBI Genbank numbers are indicated at the end of the branches. Families and subfamilies of the reservoir hosts are indicated to the right of the taxa (Guo *et al.* 2013).

1.7.3   Animal hosts of hantaviruses

Hantaviruses are unique in the Bunyaviridae family in that, unlike other members of the family, they are not transmitted by arthropods but instead are hosted by a variety of small mammals. Initially found hantaviruses were primarily associated with members of rodent families *Muridae* and *Cricetidae* with a solitary insectivore-borne virus, TPMV (Table 1.1). It was assumed that each hantavirus had co-evolved with a primary reservoir host (Klein and Calisher 2007). In recent years hantaviruses have been identified in a variety of insectivore and bat species (**Error! Not a valid bookmark self-reference.**, **Table 1.2** and **Table 1.3**); at least 24 novel hantavirus species have been identified in insectivores belonging to the order *Soricomorpha* and at least four bat hantaviruses (**Table 1.4**) have been identified in Brazil (de Araujo *et al.* 2012), China (Guo *et al.* 2013) and West Africa (Sumibcay *et al.* 2012; Weiss *et al.* 2012).

Table 1.1 List of hantaviruses approved by the ICTV; listed with their reservoir hosts (Information compiled using King *et al.* 2012 and Klein and Calisher 2007).

Species	Host(s)	Subfamily, Family	Country First Detected
New World Viruses			
Andes	*Oligoryzomys longicaudatus*	*Sigmodontinae, Cricetidae*	Argentina
Bayou	*Oryzomys palustris*	*Sigmodontinae, Cricetidae*	USA
Black Creek Canal	*Sigmodon hispidus*	*Sigmodontinae, Cricetidae*	USA
Caño Delgadito	*Sigmodon alstoni*	*Sigmodontinae, Cricetidae*	Venezuela
El Moro Canyon	*Reithrodontomys mexicanus*	*Neotominae, Cricetidae*	USA
Isla Vista	*Microtus californicus*	*Arvicolinae, Cricetidae*	USA
Laguna Negra	*Calomys laucha*	*Sigmodontinae, Cricetidae*	Paraguay
Muleshoe	*Sigmodon hispidus*	*Sigmodontinae, Cricetidae*	USA
New York	*Peromyscus leucopus*	*Neotominae, Cricetidae*	USA
Prospect Hill	*Microtus pennsylvanicus*	*Arvicolinae, Cricetidae*	USA
Rio Mamoré	*Oligoryzomys microtus*	*Sigmodontinae, Cricetidae*	Bolivia
Rio Segundo	*Reithrodontomys mexicanus*	*Neotominae, Cricetidae*	Costa Rica
Sin Nombre	*Peromyscus maniculatus*	*Neotominae, Cricetidae*	USA
Old World Viruses			

Dobrava	*Apodemus flavicollis*	*Murinae, Muridae*	Slovenia
Hantaan	*Apodemus agrarius coreae*	*Murinae, Muridae*	Korea
Khabarovsk	*Microtus maximowiczii, M. fortis*	Arvicolinae, Cricetidae	Russia
Puumala	*Myodes glareolus*	Arvicolinae, Cricetidae	Sweden
Saaremaa	*Apodemus agrarius agrarius*	*Murinae, Muridae*	Estonia
Seoul	*Rattus norvegicus*	*Murinae, Muridae*	Korea
Thailand	*Bandicota indica*	*Murinae, Muridae*	Thailand
Thottapalayam	*Suncus murinus*	*Soricinae ,Soricidae*	India
Topografov	*Lemmus sibiricus*	*Arvicolinae, Cricetidae*	Russia
Tula	*Microtus arvalis, M. rossiaemeridionalis*	*Arvicolinae, Cricetidae*	Russia

Table 1.2 New World hantaviruses not yet approved as members of the *Hantavirus* genus by the ICTV. Soricid-borne hantaviruses are highlighted in grey.

Virus	Host(s)	Subfamily, Family	Country First Detected
Araraquara	*Bolomys lasisuris*	*Sigmodontinae, Cricetidae*	Brazil
Bermejo	*Oligoryzomys chacoensis*	*Sigmodontinae, Cricetidae*	Argentina
Bloodland Lake	*Microtus ochrogaster*	*Arvicolinae, Cricetidae*	USA
Blue River	*Peromyscus leucopus*	*Neotominae, Cricetidae*	USA
Calabazo	*Zygodontomys brevicauda*	*Sigmodontinae, Cricetidae*	Panama
Choclo	*Oligoryzomys fulvescens*	*Sigmodontinae, Cricetidae*	Panama
Juquitiba	*Oligoryzomys nigripes*	*Sigmodontinae, Cricetidae*	Brazil
Lechiguanas	*Oligoryzomys flavescens*	*Sigmodontinae, Cricetidae*	Argentina
Limestone Canyon	*Peromyscus boylii*	*Neotominae, Cricetidae*	USA
Maciel	*Bolomys obscurus*	*Sigmodontinae, Cricetidae*	Argentina
Maporal	*Oecomys bicolor*	*Sigmodontinae, Cricetidae*	Venezuela
Monongahela	*Peromyscus maniculatus*	*Neotominae, Cricetidae*	USA
Orán	*Oligoryzomys longicaudatus*	*Sigmodontinae, Cricetidae*	Argentina
Pergmino	*Akodon azarae*	*Sigmodontinae, Cricetidae*	Argentina
Ash River	*Sorex cinereus*	*Soricinae, Soricidae*	USA
Camp Ripley	*Blarina brevicuda*	*Soricinae, Soricidae*	USA
Fox Creek	*Sorex palustris*	*Soricinae, Soricidae*	USA
Iamonia	*Blarina carolinensis*	*Soricinae, Soricidae*	USA
Jemez Springs	*Sorex monticolus*	*Soricinae, Soricidae*	USA
Powell Butte	*Sorex vagrans*	*Soricinae, Soricidae*	USA
Tualatin River	*Sorex trowbridgii*	*Soricinae, Soricidae*	USA
Castelo dos Sonhos	*unknown*		Brazil
Hu39694	*unknown*		Argentina

Table 1.3 Old World hantaviruses not yet approved as species in the *Hantavirus* genus.
Soricid-borne hantaviruses are highlighted in light grey and the talpid-borne virus in dark grey.

Virus	Host(s)	Subfamily, Family	Country First Detected
Amur/Soochong	*Apodemus peninsulae*	*Murinae, Muridae*	China
Da Bie Shan	*Niviventer confucianus*	*Murinae, Muridae*	China
Gou	*Rattus rattus*	*Murinae, Muridae*	China
Muju	*Myodes regulus*	*Arvicolinae, Cricetidae*	Korea
Sangassou	*Hylomyscus simus*	*Murinae, Muridae*	Guinea
Serang	*Rattus tanezumi*	*Murinae, Muridae*	Indonesia
Tigray	*Stenocephalemys albipes*	*Murinae, Muridae*	Ethiopia
Vladivostok	*Microtus fortis*	*Arvicolinae, Cricetidae*	Russia
Yuanjing	*Microtus fortis*	*Arvicolinae, Cricetidae*	China
Altai	*Sorex araneus*	*Soricinae, Soricidae*	Russia
Artybash	*Sorex spp*	*Soricinae, Soricidae*	Russia
Azagny	*Crocidura obscurior*	*Crocidurinae, Soricidae*	Cote d'Ivoire
Cao Bang	*Anourosorex squamipes*	*Soricinae, Soricidae*	Vietnam
Imjin	*Crocidura lasiura*	*Crocidurinae, Soricidae*	Japan
Kenkeme	*Sorex roboratus*	*Soricinae, Soricidae*	Russia
Lena River	*Sorex caecutiens*	*Soricinae, Soricidae*	Russia
Lianghe	*Anourosorex squamipes*	*Soricinae, Soricidae*	China
Tanganya	*Crocidura theresae*	*Crocidurinae, Soricidae*	Guinea
Yakeshi	*Sorex isodon*	*Soricinae, Soricidae*	China
Asama	*Urotrichus talpoides*	*Talpinae, Talpidae*	Japan

Table 1.4 Novel hantaviruses recently identified in bats

Virus	Host(s)	Family	Country First Detected
Huangpi	*Pipistrellus abramus*	*Vespertiliondae*	China
Longquan	*Rhinolophus affrinis, R. sinicus, R. monoceros*	*Rhinolophidae*	China
Xuan Son	*Hipposideros Pomona*	*Hipposideridae*	Vietnam
Magboi	*Nycteris hispida*	*Nycteridae*	Sierra Leone
Mouyassué	*Neoromicia nana*	*Vespertiliondae*	Cote d'Ivoire

The prototype of the genus, HTNV, was identified in the late 1970s in the striped field mouse, *Apodemus agrarius coreae,* whose range includes most of China, north and south Korea and eastern Russia (Lee *et al.* 1981). Members of the *Apodemus* genus occur across Eurasia into the United Kingdom (U.K.) and mainland Europe. These mice host a variety of

21

hantaviruses: *A. peninsulae* which occurs in China and Russia hosts variants of HTNV including Amur/Soochong virus, pathogenic to humans as well. In Europe the geographic range of *A. flavicollis* extends from the U.K. over most of central Europe and the Balkans into west Russia. *A. flavicollis* is host to Dobrava virus (DOBV) which is associated with severe haemorrhagic fever virus in central Europe and the Balkans. Another member of the *Apodemus* genus, *A. agrarius agrarius*, hosts Saaremaa virus, a variant of DOBV in countries of the former USSR. Another pathogenic variant of DOBV was more recently described in the *A. ponticus* in Russia (Klempa *et al.* 2008).

Seoul virus (SEOV), first described in Korea, is hosted by the ubiquitously distributed *Rattus norvegicus* and has been identified in rats in Europe, the Americas and North Africa (Heyman *et al.* 2004). A related lineage, Gou virus (GOUV), is hosted by *Rattus rattus* in China and *Rattus tanezumi* hosts the Serang virus (SERV) in Indonesia and *Bandicota indica* the closely related Thailand virus (THAIV) (Pattamadilok *et al.* 2006).

Puumala virus (PUUV), which causes NE, a mild form of HFRS, is hosted by *Myodes glareolus* (formerly *Clethrionomys glareolus*), the bank vole, which occurs in most of Europe including Scandinavia, U.K., west Europe including north Spain and extends into Russia and north Turkey (Olsson *et al.* 2010).

In the New World hantaviruses are mainly hosted by rodents of the family *Cricetidae*, (Subfamilies: *Arvicolinae, Sigmondontinae* and *Neotominae)*. Sigmondontine and Arvicolinid rodents occur in South America and host a variety of human pathogenic viruses: in Brazil Araraquara (ARAV) and Juquitiba (JUQV) are hosted by *Oligoryzomys nigripes* and *Bolomys lasiurus* respectively. *O. nigripes* geographical range extends from Brazilian highlands into Paraguay, Uruguay and North-east Argentina, whereas *B. lasiurus* occurs in most of Brazil and into Bolivia and Paraguay. In Argentina a variety of *Oligoryzomys* species host pathogenic hantaviruses Andes (ANDV), Laguna Negra (LANV) and Bermejo (BERV) (Table 1.1 and 1.2); non-pathogenic viruses are hosted by *Akodon azarae* and *Bolomys obscurus*. Three more Sigmodontine-borne viruses are associated with disease in humans: Choclo (CHOV), Rio Mamoré (REMV), Lechiguanas (LECV) *Calomys laucha*, *O. microtus* and *O. fulvescens* in Paraguay, Bolivia and Panama (Klein and Calisher 2007). No human-pathogenic hantaviruses have been identified in Mexican rodents to date, despite the occurrence of disease-causing hantaviruses both north and south of Mexico (Jonsson *et al.* 2010).

In North America, hantaviruses are hosted by members of the *Sigmondontinae*, *Neotominae* and *Arvicolinae* subfamilies. *Peromyscus* species, *P. maniculatus* and *P. leucopus* host human pathogens SNV, Monongahela (MONV) and New York virus (NYV). Black Creek Canal (BCCV) and Bayou (BAYV), the only recognised Sigmodontine-borne hantaviruses in North America, are hosted by *Sigmodon hispidus* first identified in Florida, and *Oryzomys palustris* in Louisiana, which are also associated with disease in humans. None of the *Arvicolinae*-borne viruses identified in America, Asia and Europe are shown to cause disease in human beings. Prospect hill virus (PHV) was the first New World to be identified and it is hosted by *Microtus pennsylvanicus.* More arvicolind-borne viruses were identified in North America in *Microtus* species following the identification of SNV in the early 1990s and elsewhere in Asia and Europe viruses were identified *Microtus* species (**Table 1.3**) (Klein and Calisher 2007; Jonsson *et al.* 2010).

TPMV was the first shrew-borne hantavirus to be identified in the 1960s, and several more shrew-borne hantaviruses were found over the past decade; most of these viruses are hosted by members of the *Soricinae* and *Crocidurinae* subfamilies (Table 1.2 and 1.3).Other insectivores in which novel hantaviruses have been identified belong to the *Talpidae* family (Guo *et al.* 2013).

The first report of hantaviruses in bats was by Kim *et al* (1994); hantaviruses were isolated in *Eptesicus serotinus* and *Rhinolophus ferrum-equinum*. Hantaviruses have recently been identified in insectivorous bats in West Africa, Vietnam and China (**Table 1.4**). However, additional hantavirus sequences have been identified in Brazil in a vampire bat *Diphylla ecaudata* and a omnivorous bat *Anoura caudifer* (de Araujo *et al.* 2012).

In Africa, following the identification of Sangassou virus in *Hylomyscus simus* in Guinea (Klempa *et al.* 2006), numerous other viruses have been identified in other rodent and shrew species in other West African countries (Klempa *et al.* 2007, Weiss *et al.* 2011 and *Sumibcay et al. 2012*) and in East Africa as well (Meheretu *et al.* 2012). One study conducted by Lee *et al* (1999) surveyed South African rodents for hantavirus using serological techniques found evidence of infection in *Aethomys namaquensis* and *Tatera leucogaster*.

1.7.4 Hantavirus infection and disease

1.7.4.1 Infection in the reservoir host

Although hantaviruses are associated with a variety of small mammal species in Africa, America, Asia and Europe, human pathogenic viruses are all from the *Murinae* rodents in Europe and Asia (with the exception of PUUV which is hosted by *M. glareolus*). In the New World, pathogenic viruses are hosted by *Neotominae* and *Sigmondontinae* rodents.

Current knowledge of virus-host interaction of hantaviruses is predominantly based on studies performed on rodents and shrews to a lesser extent. Bat viruses have only been identified in the last two years and nothing is known about viral dynamics in naturally infected or lab infected study populations.

Studies in naturally infected rodent populations show that hantavirus infection in rodent hosts is asymptomatic and lifelong. Experimental infection of *Apodemus* with HTNV showed that animals had transient viraemia for up to 12 days and persistent viral shedding in the urine for a year after infection was observed. Viral antigen was detected in the lungs consistently but detected at certain times in other organs. The increase in viral shedding coincided with the spring and summer months, the breeding season (Meyer and Schmaljohn 2000).

 Studies in *C. glareolus* the host of PUUV, showed that vertical transmission from dam to progeny does not occur and horizontal transmission is the means by which the virus is transmitted between individuals in a colony. Studies *R. norvegicus* have also shown that adults have higher seroprevalence rates than juveniles. New-borns are protected from infection by maternal antibodies. The same phenomenon was observed for PUUV infection in *C. glareolus* (Kallio *et al.* 2010). Infection rates increased with age and coincide with the loss of protection provided by the maternal antibodies and changes in behaviour.

Studies have also shown that infection rates in adults are higher in males than females. Males shed virus via more routes than females in urine, faeces and saliva, and shed virus for longer periods of time as well (Escutenaire *et al.* 2002). Higher infection rates were also associated with males with the most wounds. The higher infection rates in breeding males are due to behaviour; fighting during breeding season and territory increases the risks of transmission through biting. Males also facilitate the spread of infectious excreta when marking their territory and to indicate social status during breeding season. Exploration of shelters and colony range by males puts them at a higher risk of infection than females (Escutenaire *et al.* 2002; Hinson *et al.* 2004).

1.7.4.2 Animal-to-human transmission and disease in human beings

Hantavirus infection in reservoir hosts is shown to be asymptomatic and sub-clinical, but the picture is different for hantavirus infection in human beings. Although hantaviruses have been identified in various shrew and bat species, disease in humans is only associated with rodent-borne hantaviruses (Jonsson *et al.* 2010).

Mechanisms for the rodent-to-human transmission are not well understood but evidence suggests that it is a combination of host, environmental and human factors. Environmental factors that influence host habitat such as rainfall, temperature, food availability may cause fluctuations in the host population density, host immunity and host behaviour resulting in the increased risk of transmission to human beings (Klein and Calisher 2007). For instance, the El Niño Southern Oscillation has been implicated in the outbreak of HCPS in the Four Corners region in the USA in 1993. El Niño events led to an increase in rainfall and winter temperatures, which in turn led to abundance in food sources for rodents and a subsequent increase in rodent population density ~2 years later (Dearing and Dizney 2010). The increase population also means higher infection rates and following the scarcity of food as a result of the population explosion, there is increased contact between humans and rodents as the rodents encroach on human settlements in search of food.

Studies performed in Germany and Sweden showed a positive relationship between increase in summer and autumn temperatures and the outbreak of NE. higher summer temperatures lead to increased mast seed production in beech and oak trees resulting in increased food source for *C. glareolus* the PUUV host. Additionally warmer autumn temperatures mean that the bank voles used fewer resources in order to maintain their high metabolic rate thus improving their chances of surviving the winter and thus a subsequent increase in population in the subsequent spring (Tersago *et al.* 2009; Schwarz *et al.* 2010).

Habitat encroachment and fragmentation by human activities (deforestation, farming and urbanisation) has been implicated as a factor for increase in transmission of hantavirus to human beings. Studies in Panama showed that hantavirus prevalence was higher in rodents in fragmented habitats and close to farm pasture which coincides with a loss in species diversity as well (Suzán *et al.* 2008). Destruction of host habitat also leads to increased prevalence of hosts in farming settings where hosts establish colonies in buildings such as barns and sheds thus increasing transmission of virus between individuals in a colony and transmission to humans (Dearing and Dizney 2010).

Transmission to humans occurs directly through biting or indirectly via inhalation of infected aerosolised particles from rodent excreta. Transmission occurs as result of rodent-human contact when rodents enter human dwellings or during human outdoor activities (Schwarz *et al.* 2010). People with outdoor occupations such as farmers and mammalogists are at risk of being exposed to aerosolised rodent excreta and subsequent infection (Klein and Calisher 2007). Working in a space without adequate ventilation such as when cleaning out rodent infested sheds and barns results exposure to virus (Dearing and Dizney 2010).

There are two diseases associated hantavirus infection in human beings; haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and in the Americas hantavirus infection results in hantavirus cardiopulmonary syndrome (HCPS).

1.7.4.3 HFRS

HFRS gained prominence when it was detected in United Nations troops during the Korean War (1950-53). The disease was then described as Korean haemorrhagic fever (Lee *et al.* 1978). Since then outbreaks of HFRS occurred in China, Russia and central Europe and the Balkans, and there are approximately 150 000 cases of HFRS reported every year. The majority of illnesses are attributed to SEOV and HTNV infections in Asia, the number of hospitalised cases in China ranges from 50 000 to 100 000 annually, and the mortality rate ranges from 0.2% for PUUV infection to 15% for DOBV infection (Fulhorst *et al.* 2011).

HFRS incubation period is approximately three weeks (range 1-8 weeks). Classical disease involves five clinical stages; febrile, hypotensive, oliguric, polyuric and convalescence (Jonsson *et al.* 2010). The febrile phase is characterised by flu-like nonspecific symptoms such as fever, backache, abdominal pain, myalgia and malaise and lasts for 3 to 5 days. Patients may present with photophobia, pharynx enathema, conjunctival haemorrhage and transient visual impairment as well (Krüger *et al.* 2001). Between 11 and 40% of patients' progress to the hypotensive or shock phase which is characterised by nausea and vomiting, thirst, restlessness, vascular leakage, abdominal pain, and tachycardia and a third of the patients develop shock and mental confusion. This phase lasts for 3 to 6 days and is followed by the oliguric phase during which interstitial haemorrhage and interstitial infiltrates result in acute renal failure. 30% of the patients present with cerebral, conjunctival and gastrointestinal haemorrhages (Muranyi *et al.* 2005). Patients also experience pain in the abdomen and back, as well as reduced urinary output (Hammerbeck *et al.* 2009). The oliguric phase lasts for up to 16 days and 50% of disease-related fatalities occur due to renal insufficiency, shock and

haemorrhage (Jonsson *et al.* 2010). The polyuric and diuretic phase signals the start of recovery with polyuria greater than 3L to 6L/day resulting in fluid and electrolyte shifts (Hammerbeck *et al.* 2009; Fulhorst *et al.* 2011). The convalescent phase during which full recovery occurs ranges from 3 weeks to 6 months (Muranyi *et al.* 2005).

Nepthropathia epidemica (NE) is a milder variant of HFRS; it was first described in the 1930s in Scandinavia and its causative agent PUUV was identified in the 1980s (Schmaljohn *et al.* 1985). The disease occurs mostly in Scandinavia and Central Europe and more than 90% of the cases are asymptomatic or subclinical (Settergren *et al.* 2000). The disease is characterised by sudden onset of fever, headache, and abdominal pain and clinical presentation is similar to classical HFRS; patients present with conjunctival haemorrhages, palatine petechiae and truncal petechial rash, and neurologic manifestations such as seizures and bladder paralysis is observed in 1% of the patients. In severe cases of NE, fatality rates range from 0.1 to 1.0% (Muranyi *et al.* 2005).

1.7.4.4 HCPS

Hantavirus cardiopulmonary syndrome (HCPS) was discovered in 1993 following an outbreak of disease caused by SNV hosted by *Peromyscus maniculatus* in the Four Corners Region in the United States (Nichol *et al.* 1993). A few years later an outbreak of HCPS occurred in Argentina caused by the Andes virus hosted by *Oligoryzomus longicaudatus* (Simpson *et al.* 2010).

HCPS consists of four clinical phases: prodrome, cardiopulmonary phase, diuresis and convalescence and incubation period ranges from 9 to 33 days (Jonsson *et al.* 2010). Symptoms during the prodromal phase are similar to those observed in HFRS febrile phase (Simpson *et al.* 2010) and it lasts for 3 to 6 days. The second phase includes the onset of respiratory symptoms such as a non-productive cough and dyspnea; patients deteriorate rapidly, and respiratory failure occurs as a result of noncardiac pulmonary oedema and hypotension within 4 to 24 hours (Hammerbeck *et al.* 2009). Death occurs after 2-10 days of disease onset with fatalities ranging between 30 and 50% (Jonsson *et al.* 2010). Patients that survive the cardiopulmonary enter the diuretic/convalescent phase during which rapid clearing of pulmonary oedema and diuresis occurs as well as the resolution of fever and shock. Convalescence takes up to 3 months (Fulhorst *et al.* 2011).

In general, person-to-person transmission of hantavirus does not occur but it has been reported for the 2002 ANDV outbreak in Argentina (Martinez *et al.* 2005).

Numerous studies have been performed to determine the occurrence of hantavirus disease in Africa. The first studies were conducted in the 1980s in Central (Coulaud *et al.* 1987; Gonzalez *et al.* 1989), East (Salah *et al.* 1988; Rodier *et al.* 1993) and West Africa (Gonzalez *et al.* 1984; Tomori *et al.* 1986) serological evidence of infection was reported but no virus was isolated from any of the study subjects. In the late 1990s Lee *et al* (1999) conducted a seroprevalence study in South Africa; sera from the general population as well as patients suffering from renal dysfunction were tested for hantavirus antibodies, and a seroprevalence of ranging from 0.57% in the rural Eastern Cape province, to 4.2% in rural the Western Cape province was reported. More recently, Klempa *et al* reported on studies in Guinea conducted in areas near where the Sangassou host was identified (Klempa *et al.* 2010) and in other provinces in Guinea (Klempa *et al.* 2012). The population-based survey reported a seroprevalence of 1.2% and specific neutralising antibodies were identified in sera from two serosurvey samples and two patients with fever of unknown origin (Klempa *et al.* 2010). In the subsequent study (Klempa *et al.* 2012) sera was collected from patients who'd experienced high fever in the 3 months prior to sample collection and a seroprevalence of 1.2% was reported as well (Klempa *et al.* 2010).

1.7.5   Identification and diagnosis of hantaviruses infection

HFRS and HCPS disease result in IgG and IgM antibody response against the nucleocapsid (N) protein and serological assays are most commonly used to confirm infection (Jonsson *et al.* 2010). N protein is the most immunodominant antigen (Yamada *et al.* 1995), indirect immunofluorescence test (IIFT) was the first serologic test to be used in the diagnosis of HFRS in Europe and Asia. Slides with infected virus are required for IIFT each microscopic slide must consist of 50% infected cells. IIFT is highly specific but it is labour-intensive and it requires virus isolation, which is difficult as BSL-3 facilities are required (de Figueiredo *et al.* 2009). Alternative assays that do not require infectious virus make use of recombinant N protein instead: various groups have established ELISA and Western Blot assays using recombinant N protein as antigen, and commercially developed assays have also been developed. The serotyping of hantavirus infection may be achieved by focus reduction neutralisation test (FRNT). FRNT is the gold standard for the identification and confirmation of hantavirus infection but it requires BSL-3 facilities for virus isolation and preparation of plates for sample testing (Jonsson *et al.* 2010).

Molecular technique assays such as reverse-transcription PCR and real-time PCR assays have been established for the detection of hantavirus RNA. Assays for the identification of unknown viruses are based consensus or degenerate primers targeting a conserved region of the viral genome (Lednicky 2003). Pan-PCR assays designed for the detection known and unknown members of the genus target conserved regions in the RNA-dependent RNA polymerase gene on the L segment (Klempa *et al.* 2006; Arai *et al.* 2008; Kang *et al.* 2009). Species-specific assays are setup in areas where the hantavirus species causing disease in the population is known (Garin *et al.* 2001; Kramski *et al.* 2007).

1.7.6   Prevention and treatment of hantavirus disease

Treatment for hantavirus is mostly supportive, patients with severe disease are placed in intensive care units; patients with HFRS usually require haemodialysis and where HCPS patients may require mechanical ventilation and oxygenation (Bi *et al*. 2008).

Ribavirin, a broad spectrum antiviral has been shown to have beneficial effects if administered early during the course of disease. Clinical trials in China showed that the drug was effective in reducing patient mortality. The drug was also able to decrease risk of transition in to the oliguric phase and the occurrence of haemorrhages (Krüger *et al.* 2001).

Hantavirus vaccines currently available are only approved for use in China and South Korea. The vaccines are based on inactivated HTNV and SEOV; in Korea Hantavax consists of inactivated HTNV cultured in suckling mouse brain; two basic doses and a booster are required (Bi *et al.* 2008). There are four vaccines available in China and these vaccines are part of the expanded immunisation programme and therefore available for free to persons living in HFRS endemic regions (Zhang *et al.* 2010). The vaccines are derived from inactivated cell culture derived HTNV and SEOV, a bivalent vaccine containing both viruses is also available (Bi *et al.* 2008).

There are no vaccines available against DOBV and PUUV disease in Europe nor are there vaccines available against hantavirus disease in the Americas. Most efforts for developing vaccines in Europe and America are aimed vaccines that eliminate the need to culture high virus titres (which is highly hazardous and requires BSL-3 containment).

Candidate vaccines suggested so far are vector-based: a recombinant vaccinia virus (VACV)-vectored vaccine expressing full length M and S segments has gone as far as phase II trials. No adverse effects were observed in the subjects but only 26% of volunteers developed neutralising antibodies (Schmaljohn *et al.* 2009). Another option for a molecular vaccine is

gene gun-delivered DNA plasmid. This offers an advantage over vector-based vaccines because they do not consist of proteins and pre-existing vector immunity is not hindrance. Recently a phase I study was published in which HTNV and PUUV M-segments were cloned into a vector and administered to human subjects intradermally. About 30% of the subjects developed high titres of neutralising antibodies and cross-neutralisation ability was generally low. More investigations are required to determine viability of DNA vaccines (Boudreau *et al.* 2012).

The most effective means of controlling hantavirus infection in humans is to limit exposure to infectious rodents and their excreta. In China public awareness campaigns and control of rat populations in residential areas have been used and there has been a decrease in HFRS incidence (Zhang *et al.* 2010). Campaigns in the USA are conducted by the Centers for Disease Control and Prevention (CDC) to educate the public on how to minimise the risk of hantavirus infection (http://www.cdc.gov/hantavirus/hps/prevention.html).

1.8   Introduction to arenaviruses

Arenaviruses belong to the single genus *Arenavirus* in the family *Arenaviridae*. The genus is named for the sandy-like appearance of the viral particles when viewed by electron microscopy (arena, Latin, meaning sand) (Emonet *et al.* 2009). Lymphocytic choriomeningitis virus (LCMV), the prototype virus of the family, was discovered by three separate research groups in the 1930s: Armstrong and Lille in 1934, Traub in the same year, and Rivers and Scott in 1935. Subsequently, Tacaribe, Junin and Machupo viruses were identified in Central and South America in the 1950s and 1960s. The first African arenavirus, Lassa was discovered in 1969, in Nigeria (Frame *et al.* 1970). Arenaviruses are hosted by members of the mammalian order *Rodentia* with the exception of Tacaribe, which is hosted by *Artebeus* bats (Downs *et al.* 1963), in which they cause persistent unapparent infection with prolonged or lifelong vireamia (Enria *et al.* 2011). Of the known arenavirus species nine are associated with human disease: LCMV with acute central nervous system disease and congenital disease and Guanarito, Junin, Lujo, Lassa, Machupo, Sabiá and Whitewater Arroyo with severe haemorrhagic fever (HF) (Gonzalez *et al.* 2007).

1.8.1   Molecular biology of arenaviruses

The virions are spherical to pleomorphic in shape, and have a diameter ranging from 110 to 130nm (King *et al.* 2012). The virion envelope is derived from the host cell and it is embedded with club-shaped projections composed of GP1 and GP2. Within the virus the two

RNA segments interact with the nucleocapsid protein to form ribonucleoprotein (Figure 1.4) (Howard 2008; Enria *et al.* 2011).
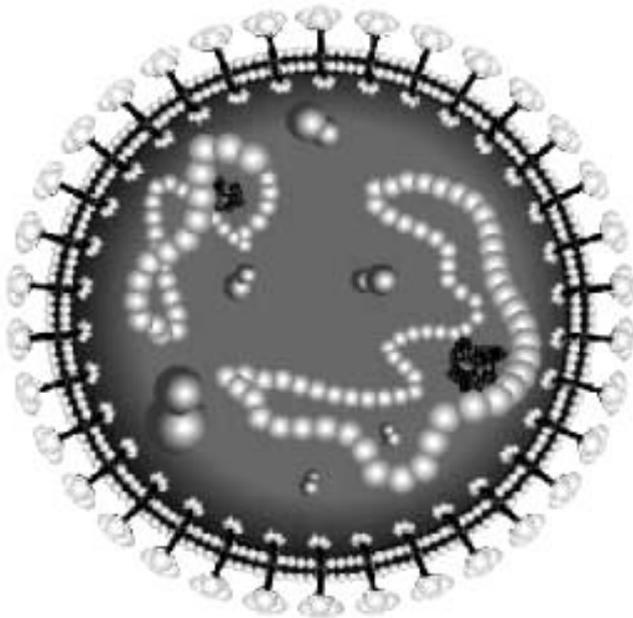
Figure 1.4 Illustration of the arenavirus virion. The host-derived envelope is lipid bilayer which contains the glycoproteins GP1 and Gp2. The L and S RNA segments interact with the nucleocapsid protein to form a ribonucleoprotein. Host cell derived ribosomes contained within the virion contribute to the sandy-like appearance that is observed with electron microscopy (Source: Rodas and Salvato 2006)

The arenavirus genome is bi-segmented and classified as negative sense although the 5' end of the large (~7.3kb) and small (~3.5kb) segment are positive sense, this genome arrangement is referred to as ambisense and it has been observed in some bunyaviruses as well (King *et al.* 2012). The large (L) segment codes for the viral RNA-dependent RNA polymerase (RdRp) and the zinc-binding protein (Z) and the short (S) segment codes for the envelope glycoprotein (GPC) as well as the nucleocapsid protein (NP) (Gonzalez *et al.* 2007). Both segments contain conserved reverse-complementary non-coding 19-nucleotide sequences at 5'and 3' termini (King *et al.* 2012), it is speculated that these regions serve as binding sites for L protein (RdRp) (Enria *et al*. 2011). The genes on each segment are separated by a non-coding intergenic region (IGR) which serves as a bona fide transcription- termination signal (Figure 1.5) (Lopez and Franze-Fernandez 2007).
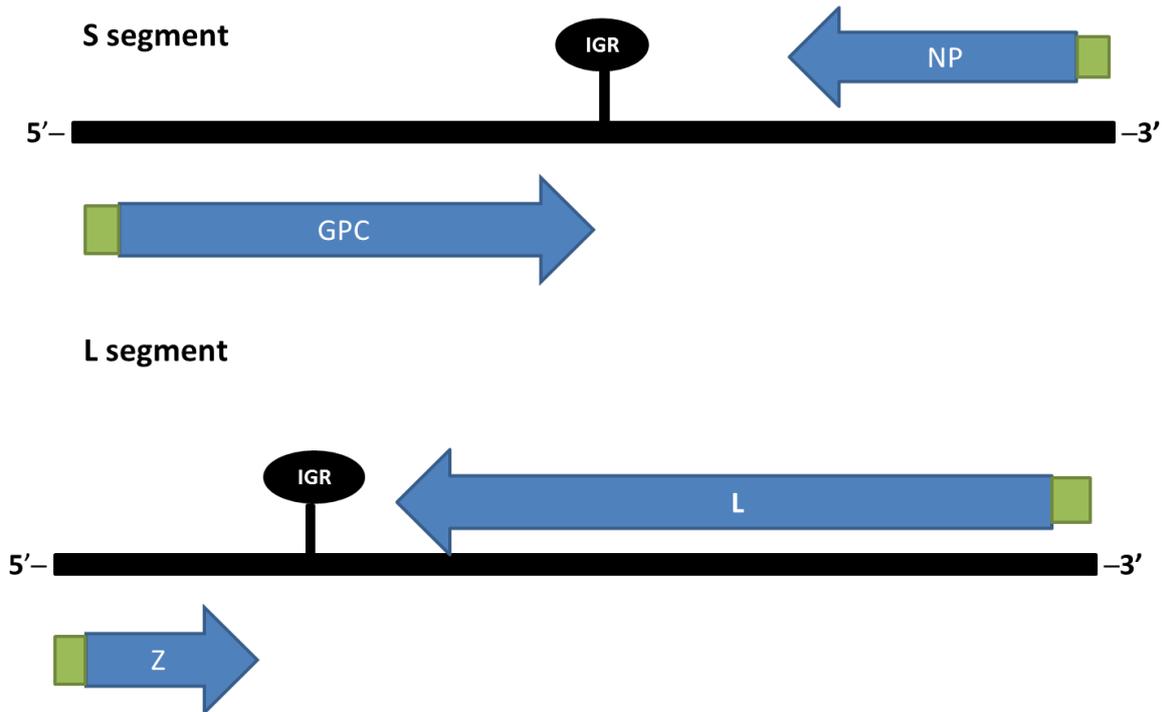
Figure 1.5 Arena virus genome organisation. The genome is composed of two single-stranded segments; the S segment is ~3.5kb long and contains the genes for glycoprotein and the nucleocapsid proteins. The L segment codes for Z ring finger matrix protein and the L RNA-dependent RNA polymerase. The arenaviruses uses an ambisense coding strategy to synthesise the two polyproteins from the genes (blue) that are separated by non-coding intergenic region (IGR) and flanked by complementary non-coding pan-handle sequences (green) at the 5' and 3' end of the segments (Modified from Meyer *et al.* 2002; Moraz *et al.* 2011).

Arenavirus cell entry is facilitated by the attachment of GP1 to at least one cellular receptor. Old World arenaviruses and clade C New World viruses enter the cell by binding to the α-dystroglycan. Clade B New World viruses (Guanarito, Junin, Machupo and Sabia) bind to human transferrin receptor-1. Following attachment to a cell surface protein, virus enters the cell by direct fusion of the viral envelope to the host cell membrane; alternatively the virus can enter by receptor-mediated endocytosis in clathrin- and non-clathrin coated vesicles (Kunz, Borrow, and Oldstone 2002). GP-2 mediates fusion of the vesicle to a low-acid endosome resulting in the release of the nucleocapsid into the cytoplasm (Enria *et al*. 2011).

Arenavirus life cycle occurs in the cytoplasm of the host cell and produced virions exit the cell by budding from the cell membrane within 16-24 hours of infection (in most cases infection is non-cytolytic) (Meyer, de la Torre and Southern 2002). The before replication can take place the NP and L genes are transcribed and translated, the L protein and NP facilitate the synthesis of complementary-sense RNA, which in turn is used as a transcription

template for the synthesis of GPC and Z mRNA as well as full-length negative-sense RNA (Enria *et al.* 2011; *Fenner's Veterinary Virology* 2011). L protein facilitates transcription of viral mRNA by cleaving caps and up to 7 bases from cellular mRNA (cap snatching), which then serve as primers to initiate transcription of the viral mRNA (Garcin and Kolakofsky 1992). The processing of GPC is not fully understood, following translation the signal peptide from the N-terminus is cleaved and the GPC is glycosylated and transported from the endoplasmic reticulum to the Golgi apparatus where GPC is cleaved into GP-1 and GP-2, and transported to the cell membrane where virion budding occurs. The Z protein serves a role in the synthesis of vRNA and mRNA, it also serves as matrix protein interacting with NP, GP-1 and GP-2 in particle assembly and budding (Enria *et al.* 2011). The process by which arenavirus particles are assembled has not been fully elucidated; newly synthesised genomic RNA molecules, NP, L interact with the nucleocapsid to form the ribonucleoprotein intracellularly, the ribonucleoproteins are then packed into virions at the cell membrane along with host-cell ribosomes (Buchmeier 2002). Budding from the cell membrane lead to the maturation of the virions, it is the last step of the life cycle (*Fenner's Veterinary Virology* 2011).

1.8.2   Taxonomy and phylogeny of arenaviruses

The *Arenaviridae* family contains 24 species that have been approved by the International Committee on Virus Taxonomy (ICTV) and 8 viruses awaiting approval (Emonet *et al,* 2009; King *et al.* 2012). Species demarcation criteria have been established for the family by the ICTV and a virus may be classified as a species within the family if:

a. It is associated with a specific host or groups of species
b. Its presence is confirmed in a defined geographical area
c. it is the etiological agent (or not) of human disease
d. antigenic cross-reactivity differs significantly, including the lack of cross-neutralisation where applicable
e. there is significant amino acid sequence difference from other species in the genus

Species definition of viruses is polythetic; therefore new isolates are placed in existing species if they share 4 or 5 common features and classified as a tentative species if 2 differences are observed with any other arenavirus (Clegg 2002; Emonet *et al.* 2009). Using the above criteria to demarcate can prove to be difficult, as in the case of Chapare, Dandenong, Lujo and Sabia which have been isolated from human beings but no rodent

reservoir host has been identified as yet. Another difficulty is the availability of BSL-4 facilities for the determination of antigenic cross-reactivity of a new isolate. These difficulties have led to the increased reliance on genetic criteria (virus definition is based on a statistical difference in the sequences of the new isolate and other members of an existing species) to define species. The use of genetic criteria to define virus species is not without its problems; which part(s) of the genome do we use for analysis and what is the degree of diversity required to classify two viruses as members of different species (Emonet *et al.* 2009).

There is no set genetic criterion for defining species in the genus. Bowen *et al* (2000) proposed 12% amino acid sequence difference (uncorrected p-distance) in full-length NP for defining arenavirus species. This benchmark was suggested based on the fact that 12% is the highest intra-species amino acid difference reported (between Lassa virus strains, LP and GA391) and 13% the lowest inter-species difference (between Junin and Machupo) reported. There are no set cut-off values for analysis using GPC or L for use in species definition.

Arenaviruses are divided into two lineages: Old World (LCMV-Lassa sero-complex) and New World viruses (Tacaribe sero-complex) based on genetic (**Figure 1.6**), serologic and geographical data. The New World viruses are further subdivided into 4 clades: Clade A consists of Allpahuayo, Flexal, Parana, Pichinde and Pirital. Clade B consists of haemorrhagic fever viruses Chapare, Guanarito, Junin, Machupo and Sabia, along with Tacaribe and Amapari. Clade C has only two viruses Latino and Oliveiros. The fourth clade, A/rec consists of Bear Canyon, Catarina, Skinner Tank, Tamiami and Whitewater Arroyo (Briese *et al.* 2009; Emonet *et al.* 2009; Enria *et al.* 2011). It is postulated that A/rec clade viruses are the result of an ancient recombination event between a clade A and clade B virus; phylogenetic analysis shows that the North American viruses cluster with clade A viruses when the *N* gene is used for analysis and cluster with clade B when *GPC* gene is used (Charrel *et al.* 2001; Charrel *et al.* 2008).
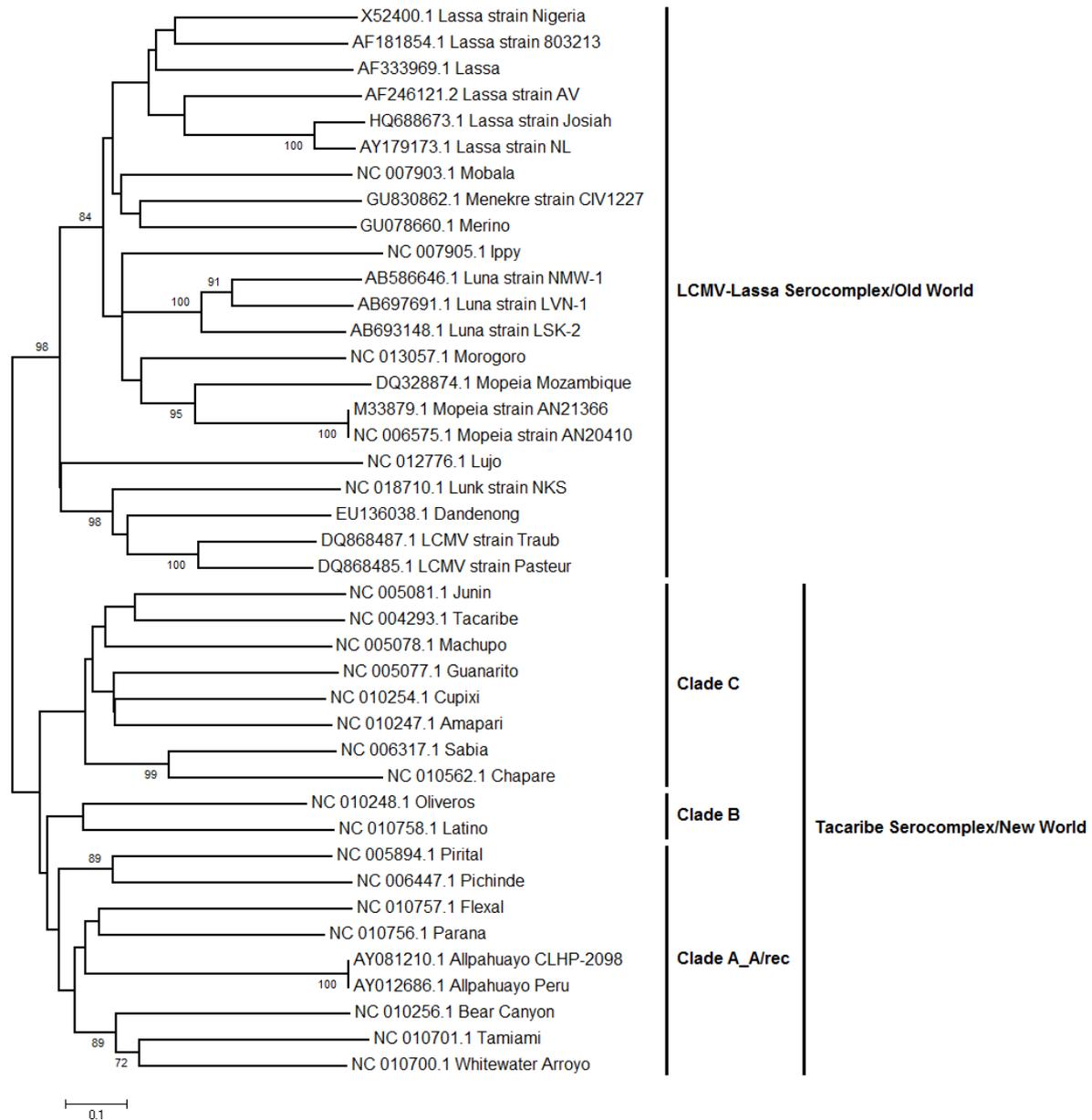
Figure 1.6 Evolutionary relationship of taxa in the *Arenaviridae* family inferred using the *N* gene

The evolutionary history was inferred in MEGA v5.1 using the Neighbour-Joining method and the bootstrap consensus tree inferred from 1000 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrap replicate values are displayed at the branch nodes and all values lower than 70% were removed from the tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. A 301 amino acid sequence fragment of the N gene (corresponding to position 1445 to 3144 bp on Lujo virus S segment NC_012776.1) was used in the analysis.

In the Old World lineage, Lujo is the closest to the New World virus followed by LCMV (Briese *et al.* 2009). Phylogenetic analysis by Emonet *et al* (2006) using GPC, NP, L and Z

35

genes showed that the Old World viruses form a monophyletic clade and LCMV roots the other members of the complex. Analysis also showed that Mopeia and Mobala share a common ancestor (Emonet *et al.* 2006) along with the lately discovered Luna virus (Ishii *et al.* 2011) and Morogoro virus (Günther *et al.* 2009). The Gbagroube virus recently identified in *Mus setulosus* is the closest relative to Lassa virus, and analysis shows that the two virus lineages diverged 1000 years ago (Coulibaly-N'Golo *et al.* 2011). The Menekre virus in the serocomplex is undefined; the virus clusters with Ippy in *GPC* gene analysis, unique lineage in *N* gene analysis and clusters with Mobala and Mopeia in *L* segment analysis.

1.8.3   Animal hosts of arenaviruses

Arenaviruses are hosted by rodents in the *Muridae* and *Cricetidae* families; Old World viruses are hosted by members of the *Murinae* subfamily in the *Muridae* family and New World viruses are hosted by members of the *Neotominae* (in North America) and *Sigmodontinae* (South America) subfamily in the *Cricetidae* family (Enria *et al.* 2011). The only exception is Tacaribe which is hosted by *Artibeus* bats in Trinidad (**Table 1.5** and

Table 1.6). Murine rodents are the ecological equivalents of the Sigmodontine rodents in the Americas and there are more than 300 genera and more than 1300 species in the *Muridae* family; they make up for 40% of all mammalian species.

It is now widely accepted that diversity observed in arenaviruses is the result of co-speciation/divergence between arenaviruses and *Muridae* rodents (Gonzalez *et al*. 2007). Co-speciation occurs when two species closely associated, in this case virus and rodent host speciate or evolve in parallel. The (rodent radiation) hypothesis put forward suggests that: a common rodent was infected with an ancestral arenavirus 35 million years ago, the holoarctic cricetids spread into the Americas North to South evolving into *Neotominae* and *Sigmodontinae* subfamilies. The cricetids migrated to Asia as well, continuing on to Europe and Africa and evolved into the *Muridae* family (Gonzalez *et al.* 2007; Zapata and Salvato 2013). Another hypothesis suggests that it is a combination of co-speciation and virus transfer events among rodent species that responsible the co-divergence inconsistencies observed for some members of the arenavirus family (Gonzalez *et al.* 2007). Analysis on New World viruses by Irwin *et al* (2012) suggests that clade A, B and C viruses mainly evolved by host switching events and geographic proximity of their hosts, and not through co-divergence with their respective hosts. This hypothesis is supported by the fact that some viruses are hosted by two or more hosts as in the case of Bear Canyon virus (BCNV) by *Neotoma macrotis* and *Peromyscus californicus*, Cajimat *et al* (2007) hypothesise that cross-species jump occurred where BCNV crossed from *N. macrotis* to *P. californicus*. The occurrence of Pirital and Guanarito virus in one species, *Zygodontomys brevicuda* also supports the hypothesis by Irwin *et a*l. Assessment of Old World viruses by Hugot *et al* (2001) and Coulibaly-N'Golo *et al* (2011), suggests that they may have co-evolved with their host, but host-switching and extinction events may have eliminated clear evidence of co-evolution.

Infection of the natural host species usually results in a mild or inapparent long-term/life-long infection. Virus is maintained within a host population horizontally: An infected animal sheds viruses in urine and faeces which is then aerosolized and inhaled by the animals in the colony as a result of biting and scratching (especially in adult males). Vertical transmission from dam to offspring may also occur (Enria *et al.* 2011). Infection in the host is chronic, sporadic vireamia occurs along with viruria; virus shedding may be long-term or life-long. The nature of infection depends on factors such as sex, age, genetic composition, immunological resistance and history of infection. For instance neonate mice infected usually become chronic carriers and shed virus in urine for their entire life-span (Gonzalez *et al.* 2007).

Table 1.5 Old World arenaviruses include the ubiquitously distributed LCMV and viruses from Africa; with their rodent hosts and the countries in which they were identified. Confirmed human pathogenic viruses are marked with an asterix.

Virus	Host(s)	Countries
Gbagroube	*Mus (Nannomys) setulosus*	Cote d'Ivoire
Ippy	*Arvicanthus spp*	Central African Republic
Kodoko	*Mus Nannomys minutoides*	Guinea
Lassa*	*Mastomys spp*	Nigeria, Ghana, Sierra Leone, Cameroun, Guinea, Mali, Cote d'Ivoire
LCMV*	*Mus musculus*	Worldwide distribution
Lujo*	*unknown*	South Africa, imported from Zambia
Luna	*Mastomys natalensis*	Zambia
Lunk	*Mus minutoides*	Zambia
Menekre	*Hylomyscus sp*	Cote d'Ivoire
Merino Walk	*Myotomys unisulcatus*	South Africa
Mobala	*Praomys jacksoni*	Central African Republic
Mopeia	*Mastomys natalensis*	Mozambique, Zimbabwe
Morogoro	*Mastomys natalensis*	Tanzania

Table 1.6 New World arenaviruses with rodent and bat host species and country in which they were identified; organised according to clade. Confirmed human pathogenic viruses are marked with an asterix

Virus	Host(s)	Countries
Clade A		
Allpahuayo	*Oecomys bicolor*	Peru
Flexal*	*Neocomys spp*	Brazil
Paraná	*Oryzomys buccinatus*	Paraguay
Pichinde	*Oryzomys albigularis*	Columbia
Pirital	*Sigmodon alstoni*	Venezuela
Clade A/B rec		
Bear Canyon	*Peromyscus californicus*	USA
Big Bushy Tank	*Neotoma albigula*	USA
Catarina	*Neotoma micropus*	USA
Skinner Tank	*Neotoma mexicana*	USA
Tamiami	*Sigmodon hispidus*	USA
Tonto Creek	*Neotoma albigula*	USA
Whitewater Arroyo	*Neotoma micropus*	USA
Clade B		
Amapari	*Oryzomys gaedi, Neocomys guianae*	Brazil
Chapare*	*Unknown*	Bolivia
Cupixi	*Oryzomys capito*	Brazil
Guanarito*	*Zygodontomys brevicuda*	Venezuela
Junín*	*Calomys musculinus, Calomys laucha*	Argentina
Machupo*	*Calomys callosus*	Bolivia
Sabiá*	*Unknown*	Brazil
Tacaribe	*Artibeus literatus*	Trinidad
Clade C		
Latino	*Calomys callosus*	Bolivia
Oliveros	*Bolomys obscurus*	Argentina

Rodents live in densely populated colonies and have a longevity that is sufficient to act as reservoir hosts to parasites during any season (Salazaar-Bravo, Ruedas and Yates 2002).

1.8.4   Epidemiology of arenaviruses

The actual mode of transmission of arenaviruses from rodents to humans is not known (Enria *et al.* 2011). It postulated the infection probably occurs through the inhalation of aerosolised particles and or contact with from urine and faeces from an infected animal. Other suggested mode of transmission is direct contact with rodent blood or through biting (Charrel and de

Lamballerie 2003). Infection from coming into contact with food that is contaminated with rodent excreta has also been hypothesised for Bolivian haemorrhagic fever (HF) and Lassa fever (ter Meulen *et al.* 1996; Gonzalez *et al.* 2007). In West Africa, ter Meulen *et al.* (1996) found that higher seroprevalence of Lassa was found in populations in which more than 90% of the population consumed rodents.

Factors driving the emergence of disease outbreaks in humans are not well understood. However, the risk of human infections is increased by rodents that live near human dwellings whether it is in towns or villages. In the case of Bolivian and Venezuelan HF, encroachment into forests to clear land for agricultural purposes lead to increased contact between humans and the reservoir hosts of the Machupo and Guanarito viruses (Enria *et al.* 2011).

Argentine HF, which is caused by Junin virus infection, is endemic to the humid pampas of eastern Argentina. The outbreak in the 1950s is associated with deforestation and intensive farming in the area, which resulted in the infection of mostly male agricultural workers from transmission that occurred from the inhalation of infectious aerosols that were formed as the rodents and excreta where caught and shredded in the mechanical harvesters during the harvesting of corn (Maiztegui *et al.* 1975). The geographic range of Argentine HF is much smaller than that of its principal host, *Calomys musculinus*; it is distributed throughout central and north-eastern Argentina (Enria *et al.* 2011). The majority of human infections are as a result of contact with infectious rodent excreta but evidence of person-to-person transmission has been documented for the outbreak that occurred in 1996 (Padula *et al.* 1998).

Lassa fever is endemic to West Africa and cases have been described from Nigeria, Guinea, Liberia and Sierra Leone as well some neighbouring countries such as Burkina Faso and Ivory Coast (Enria *et al.* 2011). The majority of Lassa cases are acquired by local residents are attributed to the capture and handling of rodents for dietary consumption as well as the contamination of food stores with rodent excreta during harvest season. Human-to-human transmission has also been observed among patients in hospital (ter Meulen *et al.* 1996). The Lassa endemic regions are popular with tourists from abroad and there have been reports on imported Lassa infection in Germany, Netherlands, England, Japan and the United States of America (Gonzalez *et al.* 2007).

The other African arenavirus for which pathogenicity in humans is confirmed is the Lujo virus; it caused severe disease in patients from Zambia and, was imported into South Africa

resulting in a nosocomial outbreak, in late 2008. The reservoir host of Lujo has not been identified as of yet (Paweska *et al.* 2009).

LCMV is the only arenavirus with worldwide distribution as its hosts *Mus domesticus* and *Mus musculus* have a worldwide geographical range although cases have only been reported in the Americas and Europe. The manner in which LCMV is transmitted to humans is not known, but infection occurs mostly in young adults. Although experimental evidence suggests that infection is via inhalation of infected aerosols, direct contact with rodents, and rodent bites (Gonzalez *et al.* 2007).

1.8.5   Arenavirus disease in humans

Not all arenaviruses cause disease in human beings; nine arenaviruses are associated with disease in human beings. Among these, five viruses cause HF disease in the Americas and three in Africa while LCMV is responsible for human infection worldwide (Ogbu *et al.* 2007; Paweska *et al.* 2009; Charrel and de Lambarellie 2010).

1.8.5.1 Human infections in the New World

The first recognised American arenavirus is the Junin virus, which was identified as the causative agent of Argentine haemorrhagic fever after the first outbreak in the 1950s. The incubation period before the onset of illness, ranges from 4-21 days and at onset patients present with chills, malaise, myalgia, headache and slight increase in body temperature (to 38-39°C). Multi-system involvement follows several days later and patients experience gastrointestinal distress with abdominal pain, nausea and vomiting, diarrhoea, as well as neurological symptoms such as dizziness, disorientation, and tremor of the tongue and arms by the end of first week. Patients also experience backache, photophobia and flushing over the face, neck and chest area (Peters 2002; Enria *et al.* 2011).

In the second week, 70 to 80% of patients begin to improve; the remaining 20 to 30% progress to severe haemorrhagic and neurologic manifestations, which begin with confusion, intention tremors and increased irritability and progress to delirium, convulsions and coma. Shock and bacterial infection resulting in pneumonia, septicaemia and thrush are also observed in the patients and usually lead to further complications in the patient. By the third week of illness patients begin to improve and a long convalescence period follows (Enria *et al.* 1998).

 The clinical manifestations of Bolivian and Venezuelan HF are similar to those observed for Argentine HF, the mortality rate for all three HFs ranges between 15 and 30%. Nosocomial outbreaks have been reported for Bolivian HF (Gonzalez *et al.* 2007) and patients with Venezuelan HF presented with a sore throat as one of the initial symptoms and hearing loss during convalescence (Enria *et al.* 2011). There are only three recorded cases for Brazilian HF which is caused by the Sabia virus. Of the three cases, two were laboratory cases and one natural infection. The clinical manifestations are similar to those observed for other South American HFs; however liver necrosis and jaundice were observed in the one natural infection case (Gonzalez *et al.* 2007; Enria *et al.* 2011).

Whitewater Arroyo virus (WWAV) was identified in *Neotoma albigula* in the 1990s in New Mexico, but the only recorded cases of human disease where reported between June 1999 and May 2000. Three females in California presented with fever, headache and myalgia and later developed respiratory distress and haemorrhagic manifestations. Two of the patients also suffered from liver failure. The clinical course of disease was similar to that observed for South American HFs and infection with WWAV was confirmed by PCR (CDC 2000, Derlet and Richards 2000).

1.8.5.2 Human infections in the Old World

There are thirteen arenaviruses that have been identified in Africa to date; six have been approved by the ICTV as species and 7 are yet to be approved. Only two of the African viruses are associated with human disease, Lassa and Lujo (King *et al.* 2012; Coulibaly-N'Golo *et al.* 2011). Lassa fever is named for the town where it was identified in American missionaries working in northern Nigeria who presented with febrile disease during the first epidemic (McCormick and Fisher-Hoch 2002). The incubation before onset of illness is between 3 and 21 days and onset patients present with generalised weakness, fever, malaise and headache (Enria *et al.* 2011). Fifty percent of the patients also present with joint and lumbar pain, nausea and vomiting, diarrhoea along with abdominal pain, and 60% develop a non-productive cough (McCormick and Fisher-Hoch 2002). Within a few days, patients progress to retrosternal chest pain, pharyngitis and conjunctivitis, and small number of patients present with facial and neck swelling, subconjunctival haemorrhage and gastrointestinal and vaginal bleeding. Elevated respiratory rates are observed in most patients. Hypotension, tachycardia and encephalopathic and neurologic manifestations are present in the more severely ill patients (Enria *et al.* 2011). At day 10 patients that survive defervesce by then the patient is in recovery with the exception of sensorineural hearing loss in 30% of

recovering patients. Hearing recovers in three to four months after infection in half the patients which becomes permanent if it does not resolve after one year (McCormick and Fisher-Hoch 2002). Patients with severe illness rapidly progress to shock, delirium, seizures, coma and eventually death (Enria *et al.* 2011).

Serologic evidence of Lassa infection has been reported in Senegal, Guinea, Liberia and Cameroon. Disease cases have been reported in Liberia, Sierra Leone, Guinea, Ghana, Senegal, Gambia and Mali, with 15 to 20% mortality (Gonzalez *et al.* 2007).

Lujo virus caused a nosocomial outbreak involving five patients, one of whom survived. The index patient was from an agricultural farm outside of Lusaka (Zambia) she initially presented with headache and malaise and later presented with diarrhoea and vomiting, fever, chest pain and sore throat. A few days later she developed a rash on most of her body, myalgia and facial swelling. After being admitted to hospital in Johannesburg, she presented with cerebral oedema, acute respiratory distress, renal dysfunction, and lack of pupillary and corneal reflexes. A physician and paramedic were potentially exposed during the flight to South Africa and the paramedic presented with symptoms of illness 9 days later, once the link between the two patients was established, patient 2 was diagnosed with haemorrhagic fever. Tracing of contacts for the patients was the instituted; 2 nurses and a cleaner at the hospital succumbed to the illness. Specimens were obtained from patients 2 and 3 and screened for known VHF: PCR for Old World arenavirus confirmed the presence of arenavirus and subsequent sequencing analysis and phylogenetic analysis confirmed that the causative agent was a novel arenavirus (Paweska *et al.* 2009).

1.8.5.3 Lymphocytic choriomeningitis

LCMV infections may occur worldwide wherever; its hosts *Mus musculus* and *Mus domesticus* are present. Human infections are most prevalent during the autumn when rodent population is at its highest and the rodents move into barns and homes to obtain shelter against the cold weather. Infections and outbreaks have been attributed to contact with wild and urban rodents, infected laboratory mice and hamsters as well as pet hamsters (Gonzalez *et al.* 2007; *Fenner's Veterinary Virology* 2011).

Onset of LMCV disease is characterised by fever, malaise, myalgia, weakness, and gastrointestinal symptoms with a cough or sore throat. Testicular pain, parotid pain and inflammation may also occur in the patients. This phase may last for a few days or for as long as 2-3 weeks (Gonzalez *et al.* 2007; Enria *et al.* 2011). In rare cases patients also present with

a rash, arthralgia and arthritis and in even rarer cases myocarditis might occur. Febrile prodrome occurs in less than 10% of the cases during an outbreak and it presents with a brief defervescence, followed by aseptic meningitis and patients experience severe headaches. In cases where disease is severe, patients develop encephalitis and most patients make full recovery (Buchmeier, de la Torre and Peters 2006).

LCMV is also recognised as an important congenital disease. Most cases of symptoms of disease are mild but in rare occurrences neurologic manifestations include myelitis, paralysis, hydrocephalus (Buchmeier, de la Torre and Peters 2006).

1.8.6   Diagnosis and detection of arenavirus infection

Arenavirus detection is performed by virus isolation, antigen-capture ELISA, neutralisation test and PCR. Haemorrhagic fever-causing arenaviruses are classified as BSL-4 pathogens so handling of human and rodent specimens needs to be handled in the appropriate facilities. Viruses can be isolated from blood or serum obtained during the acute febrile phase (McCormick and Fisher-Hoch 2002; Buchmeier *et al.* 2007); Arenaviruses can grow on Vero E6 and BHK-21 cells as well primate and rodent fibroblast cells, and can be isolated from urine, throat swabs, blood and tissue even necropsy tissue with the exception of brain. Rodent urine, blood, throat swabs and tissue are also suitable for virus isolation. A cytopathic effect is not always observed and virus replication in culture needs to be confirmed by detection of viral antigens by IIFT and immunoperoxidase assay (Howard 2008). Viral antigen can be detected in blood, serum and tissue homogenate using antigen-capture ELISA assays. This is especially useful when viral nucleic acids are undetectable and it is too early to detect antibodies against developed against the virus in the patients. Rodent blood, urine and throat swabs are suitable for the detection of viral antigens (Buchmeier *et al.* 2007).

Because of the safety concerns in virus isolation alternative methods for diagnosis are required. Serological techniques are used for routine diagnostics: IIFT and ELISA are used to detect virus-specific antibodies in patient sera. Antibodies are detectable from the late acute phase and into early convalescence (McCormick and Fisher-Hoch 2002). Serological assays based on recombinant antigens have been developed; this eliminates the need to work with infectious material (Fukushi *et al.* 2012). Recombinant assays developed using Lassa recombinant NP and GP2 antigen are able to detect other Old World viruses and weak cross-reactivity has been observed for some New World viruses (Branco *et al.* 2008). Likewise for assays developed using Junin recombinant NP antigens, a strong cross-reactivity is observed

for New World viruses and weak cross-reactivity was observed against LCMV (Machado *et al.* 2010).

Molecular techniques such as have become highly favourable because their rapidity. Virus isolation can take as long as 7-10 days to develop CPE and viral antibodies are only detected from the late acute phase onwards. The high sensitivity and the ability to detect replicating virus early during the infection allows for appropriate treatment (McCormick and Fisher-Hoch 2002). PCR assays based on primers that target a conserved region either on the S and L segment; assays have been established to detect all members of the family or targeting either serocomplex (Lozano *et al.* 1997, Vieth *et al.* 2007). The Lujo virus was detected in patient sera using Pan-Old World PCR assays with primers designed to detect conserved regions in the *GPC* and *NP* genes on the S segment for all Old World arenaviruses (Paweska *et al.* 2009). Real-time PCR assays have also been developed and result can be obtained within 24 hours leading to a quick diagnosis and timely treatment (Grajkowska *et al.* 2009).

1.8.7   Prevention and treatment of arenavirus infection in humans

To prevent arenavirus disease rodent-to-human, human-to-humans and infectious sample to laboratory personnel transmission must be interrupted. Rodent control has been used to reduce cases of Bolivian HF. *Calomys callosus* were trapped and removed from the towns in the Bent district where Bolivian HF is endemic. Infections still occur outside the towns in the rural areas where trapping of rodents is impractical (Mercado *et al.* 1975; Enria *et al.* 2011). Rodent control interventions have not been successful in preventing or reducing the incidence of Argentine HF as exposure to Junin virus occurs in rural areas and during the harvesting season (Gonzalez *et al.* 2007). A collaboration between the governments of Argentina and the United States led to the production of a live attenuated vaccine, named Candid#1 and its efficacy was proved in a double-blinded trial where 15 000 agricultural workers at risk of exposure were enrolled. The vaccine was shown to have 84% efficacy and since been used to vaccinate high risk populations, thus reducing the morbidity and mortality from Argentine HF. Candid#1 might be effective in providing protection against Bolivian HF (caused by the Machupo virus) but it does not provide any cross-protection against Guanarito and Sabia viruses (Enria *et al.* 2011).

There is vaccine available against Lassa virus, but there are feasible candidates that have been tested in hamsters and non-human primates. Guinea pigs and non-human primates immunised with a vaccinia-vectored Lassa GP gene had partial protection from disease and

vireamia. Another promising candidate is the Yellow Fever 17D-based (YF17D) vectors expressing Lassa truncated GPC, protected 80% of the guinea pigs against fatal Lassa fever proof-of-concept experiments (Bredenbeek *et al.* 2006). Since YF17D is one of the most safe and efficacious with a favourable benefit-risk profile and is cost-effective, the development of a yellow fever/ Lassa fever chimera vaccine would be an ideal solution in a region where both Yellow fever and Lassa fever are endemic. The genetic YF17D backbone has been used in the development of vaccines against Japanese encephalitis (JE), Dengue and west Nile virus, which are currently in phase II-III clinical testing. A chimera JE vaccine (IMOJEV®) is the first YF17D-vectored vaccine to be licensed for use in the general population in Australia and Thailand (Carrion *et al.* 2012).

1.8.8   Cross-species transmission and zoonoses

Although reassortment of arenaviruses has been shown experimentally, it is currently understood that reassortment does not play a role in the evolution of arenaviruses (Gonzalez *et al.* 2007). Intra-segmental recombination has been documented for North American arenaviruses Whitewater Arroyo, Tamiami and Bear Canyon. Analysis of viral sequences shows that these viruses emerged from recombination between lineage A and B viruses; Charrel *et al* (2001) showed that N gene is derived from lineage A viruses and GPC is derived from lineage B viruses. Intra-segmental recombination most likely occurred during co-infection of a rodent by a lineage A and lineage B viruses (Charrel *et al.* 2002). The occurrence of two or more rodents each hosting a different virus in the same geographic area has been documented in South America. *Z. brevicuda* and *S. alstoni* the hosts of Guanarito and Pirital virus are sympatric; it is therefore plausible that cross-species transmission can occur between sympatric rodent species (Fulhorst *et al.* 1999; Gonzalez *et al.* 2007).

1.9   Introduction to coronaviruses

Coronaviruses (CoV) are members of the *Coronavirinae* subfamily, together with the *Torovirinae* forming the family *Coronaviridae (*King *et al.* 2012*). Coronaviridae* are members of the order *Nidovirales* along with *Ateriviridae* and *Roniviridae* (Cavanagh and Britton 2008). *Coronaviridae* have the largest RNA genome known so far, which ranges in size from 27 to 32kb (Baker 2008). The first CoV isolated, in the 1930s, was the avian infectious bronchitis virus (IBV)

Until the outbreak of SARS coronavirus in the Guangdong province of China in late 2002, CoV infections in humans were associated with 25% of upper respiratory infection

(Cavanagh 2004). The first human CoV were identified in the 1960s (Tyrell and Bynoe 1965); CoV 229E in Chicago medical students (Hamre and Procknow 1966), soon after the OC43 was isolated on human embryonic tracheal organ cultures inoculated with material from adults suffering from upper respiratory disease (McIntosh *et al.* 1967). CoV cause respiratory and enteric disease in chickens, cattle, pigs, cats and dogs, and disease can be fatal in young animals (Baker 2008).

1.9.1 Molecular biology of coronaviruses

Coronaviruses are spherical, with a diameter range of 120-160nm, particles have petal-shaped "spikes" that are 20nm long in the membrane (Figure 1.7). Virions have a lipid membrane with protein spike proteins and integral membrane protein (Peiris and Poon 2009). The RNA molecule interacts with the nucleocapsid protein to form the nucleoprotein (Cavanagh and Britton 2008). The coronavirus genome is a positive-sense, single-stranded molecule that is capped at the 5'end and polyadenylated at the 3'end.
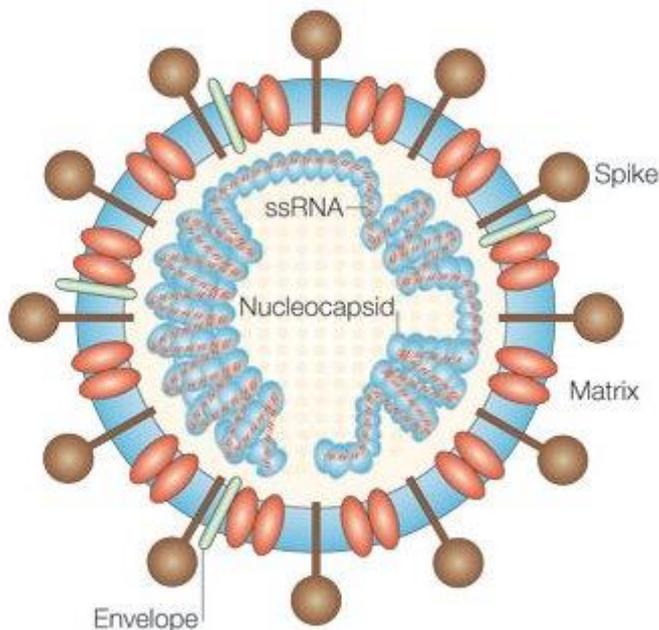
Figure 1.7 Schematic representation of the SARS-CoV virion. Host cell derived membrane contains spike proteins (S) and glycoprotein (GP1 and 2), as well as an integrated membrane protein. Within the virion, the nucleocapsid protein interacts with the positive-sense single-stranded RNA molecule (Perlman and Dandekar 2005). *Used with permission from Nature Publishing Group*.

CoV virions attach to the host cell through the interactions of the spike protein with specific cell surface receptors. The N-terminal end of spike mediates attachment to the receptor and the C-terminal end facilitates the fusion of the viral membrane with that of the host cell

(Belouzard *et al.* 2012). The genomic RNA is released into the cytoplasm and the entire replication cycle takes place in the cytoplasm where genomic, negative-sense RNA intermediates and subgenomic RNA molecules are synthesised (Cavanagh and Britton 2008).

The 5' end of the genomic mRNA consists of open reading frames 1a and b (ORF1ab), which occupies two-thirds, of the genome (~21kb). ORF1ab is preceded by an untranslated (UTR) leader sequence and a transcriptional regulatory sequence (TRS); ORF1ab is followed by ORF2 which encodes structural proteins which occur in the following order: spike-envelope-membrane-nucleocapsid, with the exception of clade 2a betacoronaviruses which contain haemagluttinin esterase gene before the spike encoding region. In between the structural protein genes, are accessory proteins and TRSs and the 3'end of the genome is polyadenylated (Figure 1.8) (Baker 2008, Woo *et al.* 2012b).

Alphacoronavirus

Former Clade 1a

5'- | ORF1a | ORF1b | S | 3 | mp | E | M | N | 7 | -AAA(n)-3'

Former Clade 1b

5'- | ORF1a | ORF1b | S | mp | E | M | N | 7 | -AAA(n)-3'

Betacoronavirus

Former Clade 2a/HKU1, BCoV

5'- ▮ | ORF1a | | ORF1b | HE | S | 4 | 5 | M | 6 | 7 | N | 7 | -AAA$_{(n)}$-**3'**

Former Clade 2b/HKU3_SARS-CoV

5'- ▮ | ORF1a | | ORF1b | S | 3 | E | M | 6 | 7 | 8 | N | 9 | -AAA$_{(n)}$-**3'**

Former Clade 2c/HKU4, HKU5

5'- ▮ | ORF1a | | ORF1b | S | 3 | E | M | N | -AAA$_{(n)}$-**3'**

Former Clade 2d /HKU9

5'- ▮ | ORF1a | | ORF1b | S | 3 | E | M | N | 7 | -AAA$_{(n)}$-**3'**

Gammacoronavirus

IBV

5'- ▮ | ORF1a | | ORF1b | S | 3 | E | M | 6 | N | -AAA$_{(n)}$-**3'**
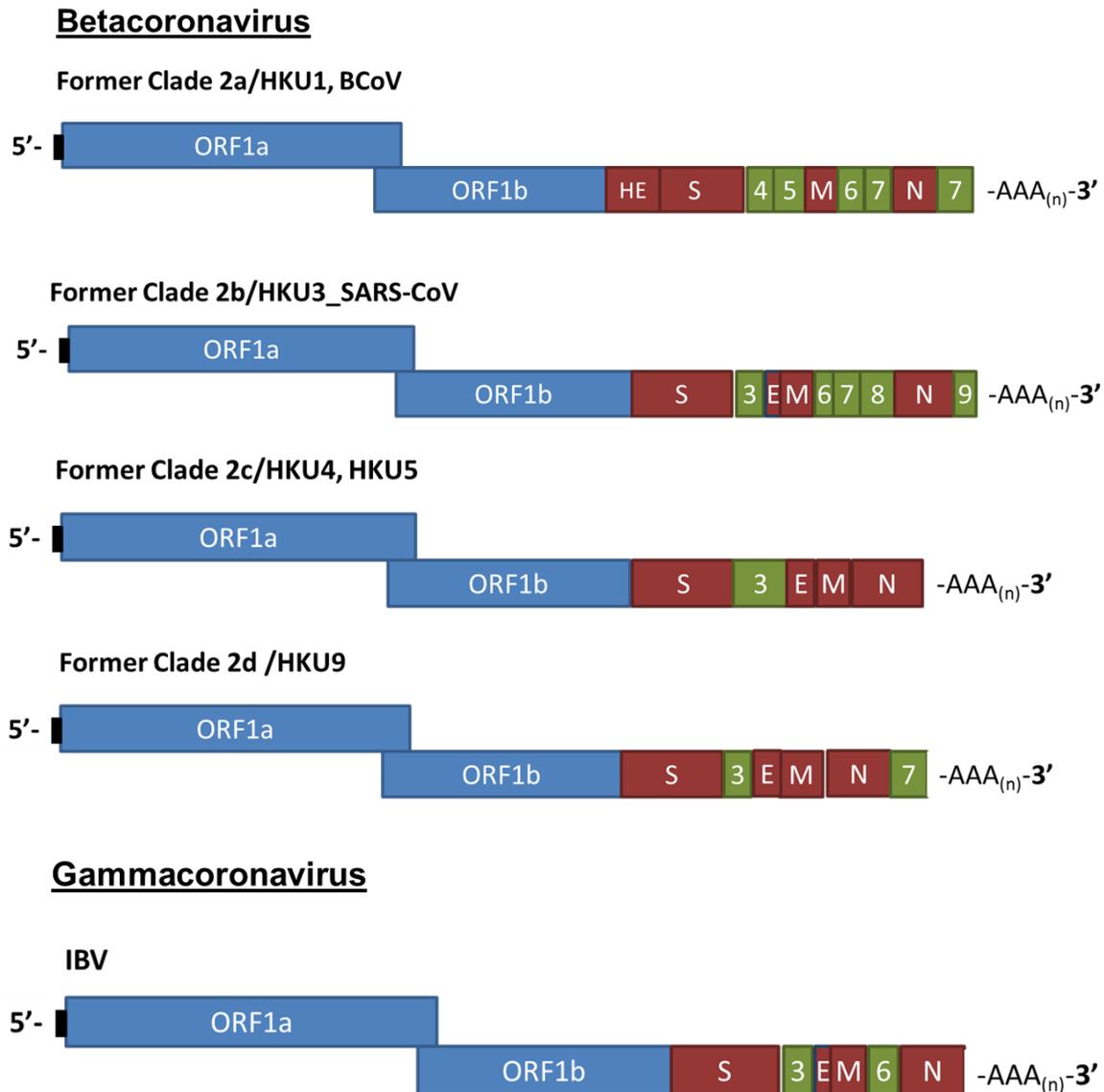
Figure 1.8 Schematic diagram of genome organisation within the recognised genera in the subfamily *Coronavirinae*. Approximately two-thirds of the genome is occupied by ORF1ab (blue) which is preceded by an untranslated leader sequence (5'UTR) (black rectangle). ORF1ab is followed by ORFs encoding the structural proteins (maroon) interspersed by accessory protein genes (green) which are named according to the ORF in which they are encoded (Modified from Baker 2008 and King *et al.* 2012).

ORF1ab is translated by ribosomes into polyprotein 1a and -1 ribosomal frameshift occurs to produce polyprotein 1ab (pp1ab), which is subsequently cleaved into 15 or 16 non-structural proteins (nsp1-16) by papain-like proteinases and the main proteinase (M^{Pro} or 3CL^{Pro}) and RNA-dependent RNA polymerase (Table 1.7) which are required for the first steps of virus replication (van Boheeman *et al.* 2012).

Table 1.7 Non-structural proteins encoded in ORF1ab, cleaved from the replicase

polyprotein 1ab (Modified from King *et al.* 2012 and Baker 2008).

Non-structural	Name	Assigned Function(s)
1		Interferon antagonist, host cell mRNA degradation, translation inhibtion, cell cycle arrest
2	Unknown	Interacts with replicase/transcriptase complex
3	Papain-like proteinase PL1^{pro}	polyprotein processing
	Papain-like proteinase PL2^{pro}	polyprotein processing, deubiquitinating enzyme
	ADP-ribose-1" phosphatase	RNA binding
4	Unknown	possibly associated with formation of double-membrane formation
5	Main proteinase (M^{pro})	polyprotein processing
6	Unknown	possibly associated with formation of double-membrane formation
7	—	single-stranded RNA binding
8	Noncanonical "secondary " RdRp	forms hexadecameric supercomplex with nsp7
9	—	single-stranded RNA binding, associates with replicase/transcript
10	Dodecameric zinc finger protein	associates with replicase/transcriptase complex, stimulates methyltransferase activity in nsp16
11	Unknown	Unknown
12	RNA-dependent RNA polymerase	formation of subgenomic and genomic RNA
13	Helicase, RNA 5'triphosphotase	
14	3'→5' exoribonuclease	Required for RdRp fidelity
	Guanine-N7-methyltransferase	Involved in RNA cap formation
15	Hexameric uridylate-specific endoribonuclease	
16	Ribose-2'-O-methyltransferase	Involved in RNA cap formation

The rest of the genome extending towards the 3'end encodes for structural proteins envelope
(E), membrane (M), nucleocapsid (N) and spike (S); are translated from subgenomic (sg)
mRNA molecules (

Table 1.8). Accessory proteins specific to coronavirus lineages are interspersed between the structural proteins (Figure 1.8).

Table 1.8 Structural proteins encoded by ORFs at the 3'end of the coronavirus genome

Protein	Size (aa)	Function
Spike	1128-472	mediates receptor binding and fusion between viral and host cell membrane Determines host tropism
Envelope	74-109	plays role in virion assembly and morphogenesis (it is also a virulence factor for SARS-CoV
Membrane	218-263	plays a role in virion assembly and packaging viral genome into virions
Nucleocapsid	349-470	plays a role in RNA synthesis and translation

Subgenomic RNAs contain a common short 5' leader sequence (identical to the 5'end of the genomic mRNA) and 3'end transcription unit (containing one or more ORFs). Subgenomic RNA is formed by continuous synthesis using negative-sense RNA as a template. The negative sense RNA used as a template for sgRNA is synthesised using the discontinuous RNA synthesis: negative-sense RNA synthesis is initiated at the 3'end of the genome and terminates once a TRS has been copied. This negative-sense strand is then translocated to the 5'end of the genome where it fuses as a result of the complementary base pairing between the copied TRS and the genomic TRS downstream of the 5'end leader sequence. Negative-sense strand continues until the 5'end producing a chimeric negative-sense sg RNA molecule which in turn serves as a template for the synthesis of sg mRNA molecules used in the translation of the structural and accessory proteins in the 3'end of the genome (Baker 2008; King *et al.* 2012).

Spike and membrane protein (Hemagluttinin esterase in clade 2a betacoronaviruses) are synthesised at the endoplasmic reticulum, both S subunits and the N-terminus of M are glycosylated. Virus assembly mostly occurs in the Golgi apparatus, it also occurs in the endoplasmic reticulum-Golgi intermediate and in the endoplasmic reticulum (Cavanagh and Britton 2008). The complete virions then bud off from the ER and Golgi apparatus and are transported to the cell surface by secretory vesicles and released from the cell by budding from the host cell membrane (Peiris and Poon 2009).

1.9.2   Phylogeny and taxonomy of coronaviruses

The international committee on virus taxonomy (ICTV) recognises 3 genera and within the subfamily *Coronavirinae* (Figure 1.9): the genus *Alphacoronavirus* consists of canine Alphacoronavirus 1, respiratory human CoVs NL63 and 229E, Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), feline CoV, and bat CoVs identified in *Miniopterus*, *Rhinolophus* and *Scotophilus* bat species (King *et al.* 2012).

The betacoronavirus genus is subdivided into 4 monophyletic clades, labelled A to D. Clade A consists of bovine CoV, murine hepatitis virus (MHV) and human CoVs HKU1 and OC43. Members of clade B are human SARS-CoV, SARS-related CoVs isolated from palm-civets and Chinese ferret badgers and HKU3 isolated from *Rhinolophus* bats. Clade D consists only of bat viruses HKU9 isolated from *Rossettus* bats, and up until 2012 Clade C included bat viruses only. Genetic and phylogenetic analysis of the Middle East respiratory syndrome coronavirus (MERS-CoV) responsible for severe respiratory disease in the Middle East showed that it is related to HKU4 and HKU5 bats isolated from *Pipistrellus* and *Tylonycteris* bats (Van Boheeman *et al.* 2012, Woo *et al.* 2012b and Annan *et al.* 2013).

The Gammacoronavirus genus is composed mainly of avian CoV such as Infectious bronchitis virus and Turkey coronavirus. This genus contains only one mammalian virus, the Beluga whale coronavirus SW1. A fourth *Deltacoronavirus* genus has been proposed (but not yet approved) and it contains avian CoV; Munia, Bulbul and Thrush, and closely related viruses identified in the Asian leopard cat and Chinese ferret badger (Woo *et al.* 2012a; King *et al.* 2012).
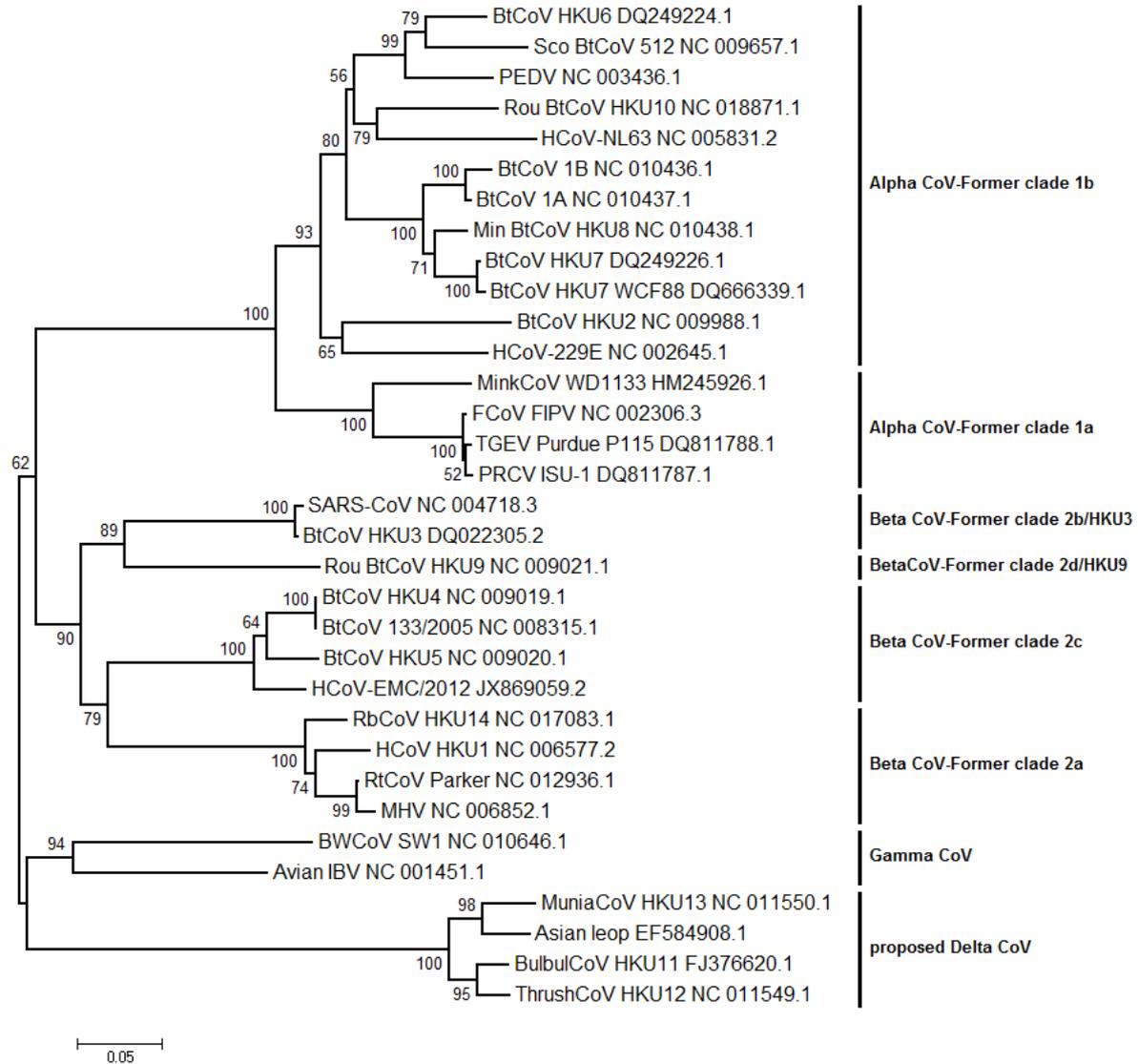
Figure 1.9 Phylogenetic relationship between member taxa of the subfamily *Coronavirinae*. Phylogenetic analysis was performed using coronavirus sequences downloaded from Genbank in MEGA v5.05 on a partial *RdRp* ~1400bp fragment (corresponding to positions 12897-14296bp in the Mink CoV genome HM245926.1), amino acid sequence alignment was performed using the Blosum model and an unrooted neighbour-joining tree was constructed using the percentage distance model. The relationship between taxa was confirmed by 1000 bootstrap replicates (Tamura *et al.* 2011).

Members of the *Coronaviridae* family are assigned to a genus and species based on rooted phylogeny and pair-wise evolutionary distance based on family-wide conserved domains in in ORF1ab; nsp 5, 12, 13, 14, 15 and 16 (Table 1.7). Newly identified viruses that do not cluster with current genera may be assigned to a new genus (King *et al.* 2012).

1.9.3   Coronavirus infection and disease

CoV target epithelial cells and therefore mostly associated with respiratory and gastrointestinal infection (King *et al.* 2012), and are transmitted via respiratory aerosols and the faecal-oral route. CoVs do not necessarily cause disease at the sites of infection, for instance infectious bronchitis virus (IBV) causes respiratory disease as well as kidney disease and damage to gonads in infected birds. Infection and disease is not necessarily species specific; both canine and feline CoVs are able to cause disease in pigs. Experimental infection of turkeys with bovine CoV causes gastroenteritis in the infected birds (Cavanagh and Britton 2008).

1.9.3.1   Disease in Animals

There are four animal coronaviruses associated with disease in the *alphacoronavirus* genus: transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus, TGEV causes the most severe disease in young piglets with vomiting, watery diarrhoea, weight loss and dehydration. Infection in adult swine is usually asymptomatic but in some outbreaks mortality may be high (*Fenner's Veterinary Virology 2011*). Porcine respiratory virus is a respiratory variant of TGEV, a 620 to 680 base pair deletion at the 5'end of the spike gene resulted in the loss of enteric tropism. Porcine respiratory virus causes mild respiratory disease in piglets and symptoms may include dyspnea, polypnea and anorexia. Disease is more severe when co-infection with other respiratory pathogens occurs. The third swine alphacoronavirus, porcine epidemic diarrhoea virus (PEDV), causes watery diarrhoea in pig but disease manifestations are less severe in 4-5 week old piglets present with diarrhoea and vomiting. PEDV was first described in Belguim in 1978, following an outbreak of disease swine breeding farms. Since then disease has been described in central and eastern Europe, and Asia (Song and Park 2012).

Feline infectious peritonitis virus (FIPV) is a systemic and often lethal disease in domestic and wild cats of the *Felidae* family; the disease was first described in domestic cats in the 1960s. Disease occurs in cats of all ages and infected animals are present with malaise, chronic fever and anorexia, as well as ocular and neurological symptoms. Canine coronaviruses cause a variety of disease; some strains cause gastroenteritis with symptoms similar to those observed in other animals, other strains are associated with respiratory disease and pantropic canine CoV cause severe systemic disease characterised by anorexia, diarrhoea, depression, vomiting, ataxia and seizures (*Fenner's Veterinary Virology 2011*).

Betacoronavirus genus includes bovine coronavirus (BCoV) which cause three types of syndromes in cattle, calf diarrhoea, and winter dysentery in adult cattle and respiratory disease in cattle of all ages. BCoVs were first reported as a cause of diarrhoea in claves in the USA in the 1970s and have since been reported in Europe and Asia (Decaro *et al.* 2008). Infected animals present with bloody diarrhoea, dehydration and respiratory manifestations (Fulton *et al.* 2012). Bovine-like coronaviruses have been isolated from dogs, humans, domestic ruminants and wild ruminants such as deer and giraffe (*Fenner's Veterinary Virology* 2011).

Canine respiratory virus (CRCoV) causes mild respiratory disease and is causative agent of canine infectious respiratory disease along with adenoviruses, parainfluenza virus, herpesvirus, reovirus and influenza viruses. CRCoV is most closely related to HCoV-OC43 and BCoV (Decaro *et al.* 2007).

Porcine haemagluttinating encephalomyelitis virus (HEV) was first isolated from the brains from suckling piglets with encephalomyelitis in Canada in the late 1950s. HEV infection is endemic in major pig-raising countries in Asia, Europe and North America (*Fenner's Veterinary Virology 2011*). HEV disease is infrequent and the virus primarily affects piglets under the age of <3 weeks. Infected animals exhibit anorexia, depression dehydration, vomiting and wasting disease, and 50% of the infected piglets presented with non-suppurative encephalomyelitis (Quiroga *et al.* 2008).

Mouse hepatitis virus (MHV) (Mouse coronavirus) is a collection of strains that cause a variety of diseases such as respiratory, gastrointestinal, neurologic disease or in combination with hepatic disease. Neurotropic strains of MHV have the ability to cause acute encephalitis with or without chronic demyelination; this makes MHV infection in small rodents is ideal as disease models for multiple sclerosis (Bender and Weiss 2010; Cowley and Weiss 2010). Strain JHM was first isolated in 1949 from the brain of a paralysed mouse and A59 was isolated in 1961 by another group from a mouse with leukaemia. These two strains are used to study coronavirus-induced central nervous system disease and are therefore important in the study human CNS diseases such as multiple sclerosis (Bender and Weiss 2010).Sialoacryadenitis virus consists of a variety of strains as observed for MHV. It is highly contagious in naïve rat populations and virus can cause disease in rats of all ages but it is most severe in young rats. Symptoms include nasal and ocular discharge, photophobia, cervical swelling and dyspnea (*Fenner's Veterinary Virology* 2011).

Equine coronavirus (ECoV) was first isolated from a faecal matter of a foal with diarrhoea in 1999 in the USA (Guy *et al.* 2000) and it clusters together with HCoV-OC43 and BCoV phylogenetically, however very little is known about the clinical significance of the virus (Zhang *et al.* 2007).

Two avian coronavirus species are recognised in the *gammacoronavirus* genus; Turkey CoV was first identified in turkeys in the USA in 1951. It is associated with enteric disease and occurs throughout the world (*Fenner's Veterinary Virology 2011*). Infectious bronchitis virus (IBV) is the prototype of the family and is hosted by *Gallus gallus* (chicken). It causes the respiratory disease but it replicates in non-respiratory epithelial cells causing disease in kidneys and gonads. Infected birds, especially chicks present with nasal discharge, râles, watery eyes and lethargy. IBV infection in young chicks may result in death but death mostly occurs following a secondary bacterial infection and older birds present with a less severe form (Cavanagh 2007).

1.9.3.2 *Infection and disease in humans*

The first known HCoVs 229E and OC43, isolated in the 1960s are associated with respiratory and enteric disease in infants, immunocompromised individuals and the elderly. Infection usually occurs in the upper respiratory tract and is associated with 25% of common cold cases. Illness usually presents with symptoms such as nasal discharge, sore throat, malaise and headache, and lasts for an average of 6/7 days (Cavanagh 2007).

In November 2002, an outbreak of severe acute respiratory disease occurred in the Guangdong province in Southern China. The causative agent of the disease was a novel coronavirus, SARS-CoV. Patients presented with high fever, dyspnoea, headache, dry cough, malaise and hypoxemia (Dorsten *et al.* 2003; Ksiazek *et al.* 2003). The incubation period from point of infection to onset of disease ranges from 3 to 5 days (in some cases up to 10 days). Virus is transmitted from person-to-person via respiratory aerosols generated by coughing, sneezing, or through contact with contaminated environmental surfaces and fomites. Most of patients had mild to intermediate illness which resolved in with bed rest and oxygen supplementation. Up to 36% of the patients required intensive care, and up to 26% of the patients progressed to acute respiratory distress and required invasive ventilatory support (Ho, 2011). A total of 8422 cases of SARS-CoV infection and 916 fatalities were recorded (10% fatality rate) (http://www.who.int/csr/sars/country/2003_08_15/en/index.html).

Following the SARS outbreak, there was a renewed interest in HCoVs and two novel coronaviruses associated with mild upper respiratory disease have been identified so far. HCoV-NL63 was isolated from a 7-month old infant with bronchiolitis, and the same virus was isolated from an 8-month old infant with pneumonia in the Netherlands in 2004 (Fouchier *et al.* 2004; Wevers and van der Hoek, 2009). Global distribution has been proved as the virus has been identified in countries on every continent; the virus has been detected in 1-9.3% of the respiratory tract samples tested. Patients infected with NL-63 presented with fever, cough, sore throat and rhinitis (Fielding 2011).

The second coronavirus is HKU1, a betacoronavirus first isolated from two adult patients in Hong Kong (Woo *et al.* 2005). The clinical picture presented in patients is similar to that observed for NL63 as well as OC43 and 22E. The virus has been identified in patients with mild respiratory illness in Australia (Sloots *et al.* 2006), France (Vabret *et al.* 2006), USA (Esper *et al.* 2006), South Africa (Smuts and Hardie 2006) and Brazil and most recently in Korea (Lee *et al.* 2013). Both HKU1 and NL63 are associated with common colds, and are associated with more severe disease in young children, immunocompromised adults and the elderly (Wevers and van der Hoek 2009). There is evidence that suggests that HKU1 is associated with gastrointestinal disease (Vabret *et al.* 2006).

A sixth human coronavirus was reported in September 2012. The virus was isolated from a patient who presented with unexplained severe acute respiratory disease; the patient had travelled to the Saudi Arabia and Qatar. PCR, Sanger sequencing and subsequent phylogenetic analysis showed that the sequence was a novel betacoronavirus belonging to lineage 2c with bat viruses HKU4 and HKU5 (Bermingham *et al.* 2012; Woo *et al.* 2012a). This virus was named Middle East Respiratory Syndrome (MERS-CoV), infection is severe community-acquired pneumonia with possible renal failure; patients presented with arthralgia, cough, myalgia (Chan *et al.* 2012). Up until 4 October 2013, a total of 136 cases of laboratory-confirmed infection and 58 deaths have been confirmed (http://www.who.int/csr/don/2013_10_04/en/index.html).

1.9.4   Identification and diagnosis of coronavirus infection

Before the SARS-CoV outbreak, HCoV (OC43 and 229E) infections were associated with the common cold which is not considered serious illness. Detection of infection was primarily by antigen-capture ELISA on respiratory samples. Coronaviruses have been isolated from various cell lines; 229E was isolated from embryonic lung fibroblasts, SARS-CoV from Vero

and rhesus kidney cells, NL63 can be cultured on tertiary monkey kidney cells and HKU1 was isolated from hepatocarcinoma cell lines HuH7 (Peiris and Poon 2009).

More recently, the most common method for detection of infection is PCR. Assays based on primers that target conserved regions of the genome especially the *RdRp* gene have been established and especially useful in the detection of novel viruses in the event of outbreaks: MERS-CoV was identified using such an assay (Zaki *et al.* 2012). More specific assays have been developed to identify new cases during an outbreak (Poon *et al.* 2003; Corman *et al.* 2012).

Serological assays such as EIA, IIFT and neutralization tests have been used in the detection of viral antigen and/or antibodies. Commercial and in-house IIFT assays were developed for the serodiagnosis of SARS-CoV (Müller *et al.* 2007; Chan *et al.* 2004). Antibody ELISAs based on recombinant N antigen eliminate the need to work in BSL-3 with infectious material, however they have a high sensitivity because of the high cross-reactivity between coronaviruses which may result in false positive results. ELISA results should therefore be confirmed with a more specific assay such as Western Blot using recombinant spike antigen (Woo *et al.* 2004). Although electron microscopy is no longer used in routine diagnostics, it can be useful in the identification of newly emerged viruses. EM can be used view infected patient specimens such as tissue or cell culture isolates. SARS-CoV was identified in patient bronchoalveolar lavage specimen using EM (Ksiazek *et al.* 2003).

1.9.5   Cross-species transmission and zoonotic potential of coronaviruses

The unique mechanism (discontinuous transcription) by which CoVs and other nidoviruses replicate during which random template (strand) switching occurs (see section 1.1.1) means that CoVs have a high homologous recombination frequency as high as 25% in the event of co-infection (Woo *et al.* 2009). Their RNA-dependent RNA polymerase has an infidelity resulting in high mutation rates in the order of one mutation per 10^3 to 10^4 nucleotides (Woo *et al.* 2009; Woo *et al.* 2012a). Thirdly, CoVs have possess the largest known RNA genome (range ~25 – 32kb), which gives the virus extra flexibility to accommodate and modify genes (Woo *et al.* 2009). These features allow for the emergence of novel viral variants and species that are capable of crossing the species the barrier and infecting non-reservoir hosts.

There is evidence for multiple incidents of cross species transmission of CoVs between animals, from animals-to-humans, and from humans to humans (Bolles *et al.* 2011). The most well-known example is SARS-CoV which emerged in the live animal markets of Guangdong

province (China). The current hypothesis is that, SARS-CoV originated in *Rhinolophus spp* bats, was transmitted to an intermediate host(s), the palm civet (*Paguma larvata*) and racoon dog (*Nyctereutes procyonoides*), and finally human beings (Guan *et al.* 2003; Li *et al.* 2005).

Bovine coronavirus has been implicated as the ancestor to HCoV-OC43; the viruses share high antigenic and genetic similarities. Full genome amino acid sequence comparison between a laboratory strain of HCoV-OC43 and BCoV Mebus strain showed sequence identity ranging between 93% and 98% in the various structural and non-structural gene ORFs used for analysis. Molecular clock analysis suggests that the cross-species jump of BCoV into human beings occurred in 1890 resulting in the emergence of HCoV-OC43 (Vijgen *et al.* 2005). More recently HEC4408 was isolated from a child with acute diarrhoea (in 1998) and sequence analysis showed that the isolate is a BCoV (Vijgen *et al.* 2005).

In 2009, Pfefferle *et al.* reported on close relatives of HCoV-229E identified in *Hipposideros spp* in Ghana. Sequence analysis of an 817bp *RdRp* gene fragment, showed that BtCoV/Hip sp/GhanaKwam/19/2008 and HCoV-229E have a 91% nucleotide identity. Molecular dating analysis was performed and results showed that the two viral sequences diversified in 1803. This evidence suggests that HCoV-229E may have emerged from a bat coronavirus ~210 years ago (Pfefferle *et al*. 2009; Huynh *et al.* 2012). Huynh et al (2012) described a novel bat coronavirus in big brown bats (*Eptesicus fuscus*) that is closely related to HCoV-NL63 and phylogenetic analysis shows that the NL63 may have originated from bats in between 1190 and 1449 AD.

A reverse zoonosis event is also postulated to have occurred; with spill-over infection from humans into pigs which resulted in the emergence of porcine epidemic diarrhoea coronavirus (PEDV) (Bolles *et al.* 2011) but more analysis is required to solidify this hypothesis.

Experimental evidence provided by three different groups' shows that two type II feline coronavirus (FCoV) strains arose from independent recombination events between type I FCoV and canine coronavirus (CCoV).

1.9.6   Identification of novel coronaviruses in Africa

Since the outbreak of SARS-CoV and the establishment of *Rhinolophus* bats as the source of the cross-species transmission, a number of studies have been conducted investigating the presence of coronaviruses in African bat species. Tong *et al* (2009) reported 41 novel CoV sequences in Kenyan bats; the majority of viruses clustered with known lineages and four sequences identified represent novel coronavirus lineages. Studies conducted in Ghana and

Nigeria, have identified viruses distantly related to SARS-CoV as well as an ancestor to HCoV-229E (Pfefferle *et al.* 2009; Quan *et al.* 2010; Annan *et al.* 2013). There are two studies in the literature on South African bat coronaviruses, Müller *et al* (2007) conducted a serological survey on bat specimens from South Africa and the Democratic Republic of the Congo and found antibody evidence of infection. A more recent study by (Geldenhuys *et al.* 2013) identified novel coronavirus sequences in three South African bat genera: *Miniopterus*, *Neoromicia* and *Mops* trapped in three provinces, Gauteng, NorthWest and Limpopo.

In our study we aimed to identify and characterise novel coronavirus sequences in South African bat species trapped at locations in the Western Cape, KwaZulu Natal and Limpopo province.

1.10 Introduction to astroviruses

Astroviruses were first identified in children suffering from diarrhoea by electron microscopy in 1975. Astroviruses have been implicated as causative agents of enteric disease in young children and there are studies that suggest that astroviruses the second most common agent (after rotaviruses) responsible for gastroenteritis in children (Madeley and Cosgrove 1975; de Benedictis *et al*. 2011). Astroviruses have been isolated from faecal matter of a variety of mammalian (humans, cats, dogs, deer, mice, pigs, sheep and mink) and avian species (chickens, guinea fowls and turkeys). Infection in mammals is associated with gastroenteritis in young mammals; in birds it is associated with fatal hepatitis in ducks and interstitial nephritis in chickens (King *et al.* 2012). Despite the worldwide distribution of infection and endemic nature, very little is understood about their evolution (Strain *et al.* 2008).

1.10.1 Characteristics of astroviruses

Astroviruses are non-enveloped viruses with a diameter that ranges from 28 to 30 nm, the virions are spherical in shape and five or six pointed star is visible on the surface of ~10% virions (Figure 1.10) (King *et al.* 2012).
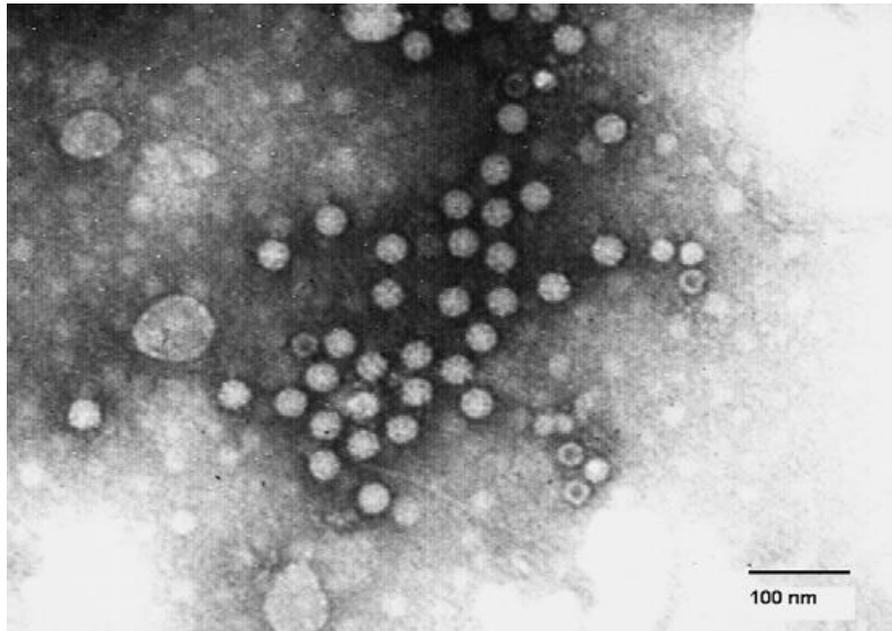
Figure 1.10 Electron microscope image (36 000 Kx) of astrovirus from a faecal sample obtained from diarrhoeic puppy. Star-like appearance of astrovirus is readily observed (Toffan *et al.* 2009). (*Image used with permission from Elsevier B.V*).

The genome is composed of a single-stranded RNA molecule that is 6.8 to 7.9 kb long and contains an non-translated region (NTR) at the 5' end, which is followed by three open reading frames; ORF1a, ORF1b and ORF2 (Figure 1.11). The ORFs are followed by an NTR at the 3' end and a polyadenylated-tail (de Benedictis *et al.* 2011). The virion RNA molecule is infectious and serves as mRNA template for translation of non-structural polyproteins, nsp1a and nsp1ab (King *et al.* 2012).
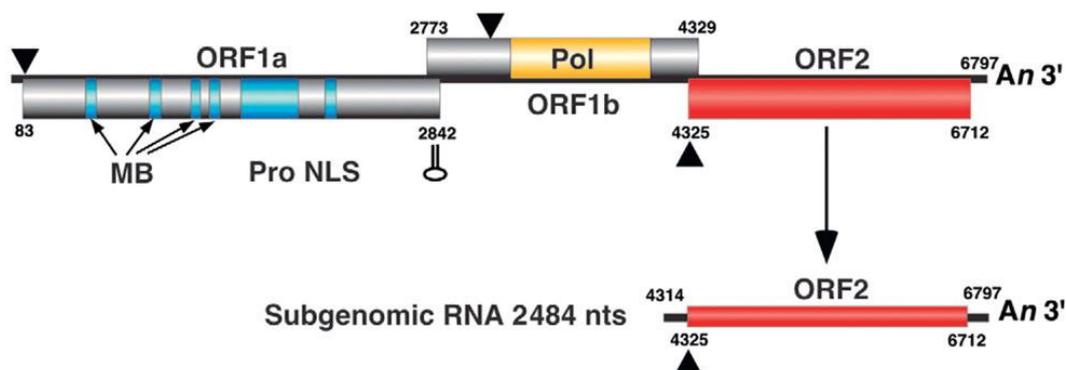
Figure 1.11 Genome organisation of human astrovirus 1: Two-thirds of the genome is occupied by ORF1ab; ORF1a codes for the serine protease (Pro) and 1b codes for RNA-dependent RNA polymerase (Pol). ORF2 occupies the 3'end of the genome and encodes for structural proteins (Ball *et al.* 2005) (*Image used with permission from Elsevier B.V.*).

There's very little information available on the astrovirus attachment and entry into the cell, and the receptor for virus entry is not known. Human astroviruses (HAstV 1-8) can infect Caco2 cell line but another human colon adenocarcinoma cell line (HCT-15) only supports HAstV 1 infection suggesting that multiple receptors are involved in virus attachment and entry (Brinker *et al.* 2000; de Benedictis *et al.* 2011). Work by Donelli *et al* (1992) suggests that receptor-mediated endocytosis is a possible mechanism for astrovirus entry into cells.

The mechanism by which the RNA molecule is released into the cytoplasm is not fully understood. Virus replication occurs in the cytoplasm and once the RNA molecule is released the ORF1ab is translated by host cell machinery into ~110kDa polypeptide which is then cleaved by both cellular and viral proteases into five different peptides (de Benedictis *et al.* 2011). The polypeptide contains several conserved motifs such as transmembrane domains, nuclear localisation sequence and serine protease (Figure 1.1). The polypeptide is subsequently cleaved by cellular and viral proteases into a minimum of five peptides whose functions is yet to be determined. Transmembrane domains assist with replication at the endoplasmic reticulum (ER) membrane (Moser and Schultz-Cherry 2005). NSP1a/4 plays a role in viral replication, it co-localises with viral RNA at the ER. ORFa and ORFb overlap by 70 nucleotides and a frame-shift into the -1 frame results in the translation of ORFb into an ORF1a/b fusion peptide which is subsequently, cleaved releasing the RNA-dependent RNA polymerase (RdRp) (Figure 1.11) (Méndez *et al.* 2013).

RdRp expression leads to the production of minus-strand viral RNA strand, which subsequently results in the generation of multiple copies of positive-sense strands and polyadenylated subgenomic RNA (sgRNA) of ORF2 containing 5' and 3' end UTRs, ORF2 is consists of two coding regions; the first region is highly conserved and encodes for one polyprotein component as well as VP34 which plays a role in virion assembly. The 3'end of ORF2 is highly variable and contains the genes for the remaining polyprotein capsid components, and VP25 and VP27 components of the spikes (Ulloa and Gutiérrez 2010). ORF2 is translated into an 87kDa structural protein which is then cleaved by cellular proteases in order to obtain the individual structural proteins (de Benedictis *et al.* 2011). The individual capsid protein components assemble spontaneously to from icosahedral structures and positive-sense RNA genomes are packaged into the structures by interacting with the first 70 amino acids of the capsid protein. Immature virions are released from the cell by cell lysis; the capsid proteins are further processed by trypsin-like enzymes producing mature infectious viruses (*Fenner's Veterinary Virology* 2011).

63

1.10.2 Phylogeny and taxonomy of astroviruses

Analysis conducted by Lukashov and Goudsmit (2002) led to the division of astroviruses into 2 genera; *Mammastrovirus* which includes all viruses identified in mammals and *Avastrovirus* genus is comprised of astroviruses identified in birds (Figure 1.12).

Astroviruses are classified based on species of origin which does not necessarily correspond to phylogenetic relationships. Species demarcation criteria for *Astroviridae* are currently being redefined (King *et al.* 2012). The Astrovirus study group has submitted a proposal for the genetic classification of astroviruses using phylogenetic analysis of the full amino acid sequence ORF2 (which codes for the capsid polyprotein). The group proposed that the mean amino acid p-distance between members of the same species is between 0.006 and 0.312, and the difference between two different species ranges from 0.350 to 0.750 (Bosch *et al.* 2010).
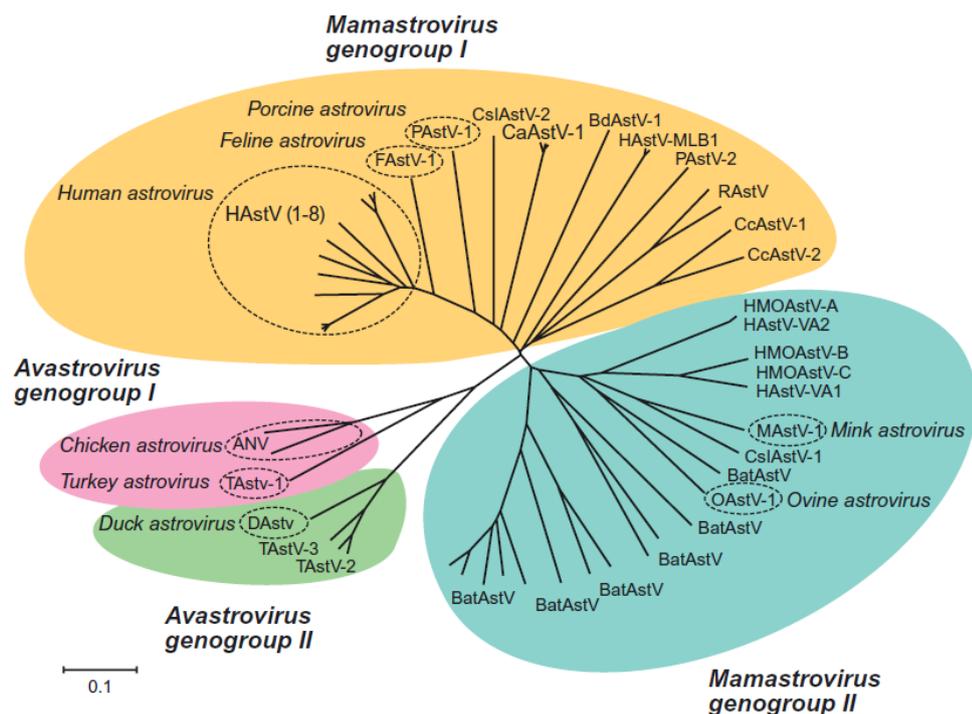
Figure 1.12 Phylogenetic relationship between members of the family *Astroviridae*. Amino acid sequence analysis of capsid polyprotein was performed in MEGA4 using the neighbour-joining method (King *et al.* 2012*) (Image used with permission from Elsevier B. V).*

Mamastroviruses are divided into two genogroups (Figure 1.12): genogroup I consists of human astroviruses (HAstV 1-8 and HAstV-MLB1-2), feline astrovirus (FAstV), Porcine astroviruses (PoAstV 1-2), rat astroviruses (RtAstV) and California sea lion astrovirus 2

(CslAstV 2), dolphin astrovirus (BdAstV), roe deer (CcAstV 1-2) and bovine astrovirus (BoAstV). In genogroup II, are the recently identified human astroviruses HMO (A-C) and VA (1-4) (Finkbeiner *et al.* 2009) cluster with mink (MiAstV) and ovine (OAstV) and CslAstV 1. The genogroup also contains bat astroviruses (BtAstV) identified in various insectivorous bat species (King *et al.* 2012; Tse *et al.* 2011; Xiao *et al.* 2011).

Members of the Avastrovirus genus are divided into two genogroups as well: Chicken astroviruses (ANV) and turkey astroviruses (TAstV 1) cluster together in genogroup I and the other turkey viruses TAstV 2 and 3 cluster with duck astrovirus DAstV (Fu *et al.* 2009; King *et al.* 2012).

1.10.3 Human astroviruses

There are three different clades of astroviruses circulating in human beings; classical HAstVs (1-8), MLB-like and VA1-like viruses. HAstV 1 is the most commonly circulating strain and HAstV 6, 7 and 8 are the least frequently detected (Moser and Schultz-Cherry 2008). Studies conducted in South Africa have detected using EIA and RT-PCR HAstV-1, 3, 5, 6 and 8 in paediatric samples from Pretoria. All 8 classical serotypes were also detected in sewage samples during the same study (Nadan *et al.* 2003). In 2008, novel HAstV, MLB1 was identified in faecal samples from a boy in Australia; subsequently the virus has also been detected in stool specimens from children in the USA (Finkbeiner *et al.* 2008). A second strain was also identified and a study by Chu *et al* (2010) revealed a closely related rat AstV. The third lineage of HAstVs consists of VA-1 and HMO-AstVs; HMOAstVs (A-C) are closely related to ovine and mink astroviruses. HMO-AstVs were identified in a study by Kapoor *et al* (2009) in which paediatric and adult stool samples from Nigeria, Pakistan and Nepal were screened for AstV by RT-PCR. VA-1 identified during an outbreak of diarrhoea in Virginia (USA) is closely related to HMO-AstV C (Finkbeiner *et al.* 2009; Kapoor *et al.* 2009).

Astrovirus disease is associated with the elderly, immunocompromised individuals, but mostly occurs in young children under the age of two (Moser and Schultz-Cherry 2005). Infections occur throughout the year but the highest incidence is observed in autumn and winter. Outbreaks of disease have been correlated with high-density environments such as child care centres, schools and homes for the elderly (Moser and Schultz-Cherry 2008). Co-infections with other enteric pathogens have also been demonstrated (Guix *et al.* 2002; Colomba *et al.* 2006). Astroviruses are transmitted through the faecal-oral route and

epidemiological studies suggest that prevalence of infection is higher in the winter in temperate regions and during the rainy season in tropical regions (Pativada *et al.* 2012).

The onset of symptoms occurs 2-3 days after infection; patients present with diarrhoea, vomiting, abdominal pain, headache and mild dehydration that last for about 3 to 4 days (Moser and Schultz-Cherry 2005). Disease is self-limiting and treatment for diarrhoea includes oral rehydration and intravenous fluids (Glass and Bresee 2011).

1.10.4 Animal astroviruses

1.10.4.1 *Mamastroviruses*

Routine screening for astroviruses is not commonplace and our understanding of infection in animals has been obtained from surveillance studies especially in farm animals such as sheep pigs and cows (Moser and Schultz-Cherry 2008). Recent studies have revealed astrovirus infection in cheetah, deer, dogs, mink, sea lion, bottlenose dolphins as well as bats (Tse *et al.* 2011). Disease has been observed in cats (in Australia, England, Germany, New Zealand and the USA). Disease in kittens presents results in mild diarrhoea and pyrexia. Cheetah AstV was identified in captive animals showing signs of clinical disease; the cheetahs presented with anorexia, lethargy, diarrhoea and regurgitation (Atkins *et al.* 2009). Infection in pigs usually occurs in the young; symptoms are similar to those observed for human infection (Reuter *et al.* 2011).Porcine AstVs have been identified in South Africa, Czech Republic, Colombia, Hungary and Canada. Infection has been detected in apparently healthy animals. Disease has been described in piglets infected with corona-, rota- and calicivirus co-infection (de Benedictis *et al.* 2011).

Bovine and ovine astroviruses are not associated with disease although they have been isolated from faecal material (Tse *et al.* 2011; Reuter *et al.* 2011). A roe deer AstV, CcAstV was identified in a deer suffering from gastroenteritis in Denmark. CcAstV is closely related to BoAstV suggesting that BoAstV and related viruses maybe associated with disease (Smits *et al.* 2010). Zhu *et al* (2011) found an association between astrovirus infection and diarrhoea in dogs; 12% AstV infection was observed in dogs with diarrhoea and no infection in asymptomatic dogs.

Since 2008, astroviruses have been identified in various insectivorous bats in China (Chu *et al.* 2008; Xiao *et al.* 2011) and Germany (Drexler *et al.* 2011). No disease was observed in the positive animals.

1.10.4.2 Avastroviruses

Avian astroviruses have been identified in chickens, turkey and ducks, and disease in poultry is more severe. TAstV1 was identified in Turkey poults suffering from reduced weight, diarrhoea in the early 1980s in the United Kingdom (UK). TAstV1 was identified in the United States along with TAstV2 in the 1980s. A genetically distinct TAstV was isolated and characterised in the late 1990s; it is associated with poult enteritis mortality syndrome (PEMS) (Koci and Schultz-Cherry 2002). Birds develop symptoms within 2 days post infection and viral shedding continues for several weeks (*Fenner's Veterinary Virology* 2011). Avian nephritis virus (ANV) was isolated from asymptomatic broiler chicks and at least two serotypes have been reported so far. Infection causes subclinical disease in most cases although growth depression and mortality has been reported (de Benedictis *et al.* 2011). Disease affects young chicks, and chicks older than 1 month develop resistance to disease (Koci and Schultz-Cherry 2002). Duck AstV have been associated with fatal hepatitis and was known as duck hepatitis virus type 2 and was originally classified as a picornavirus in the 1960s in the UK. DAstV caused a severe outbreak of duck viral hepatitis among 1-2 week old ducklings and had a mortality of 50% in China (Fu *et al.* 2009).

1.10.5 Identification and diagnosis of infection

Astroviruses were first identified by electron microscopy but currently serological assays such as ELISA and immunofluorescence test are commercially available (McIver *et al* 2000). RT-PCR assays based on pan-family primers targeting a conserved region in the *RdRp* gene have been established (Chu *et al.* 2008; Rivera *et al.* 2010) designed to identify known and novel members of the family. Assays with more specific primers have been setup for the determination of causative genotype and strains (Guix *et al*. 2005). Nadan *et al* (2003) used primers specific to classical HAstV strains. Pager and Steele (2002) screened for AstV from adult and paediatric sampmles collected in Johannesburg using commercial EIA kits to detect AstV antibodies. The more recently identified HAstVs, VA1 was identified by pyrosequencing (Finkbeiner *et al.* 2009). Though virus isolation is not used for diagnosis of infection, human astroviruses have successfully been isolated from human kidney epithelial (HEK) cells and later on human carcinoma cell line (Caco2) (de Benedictis *et al.* 2011).

1.10.6 Recombination and zoonotic potential

RNA viruses have high genetic variability as a result of mutations and genetic recombination; this is due to the error-prone RdRp and its tendency to switch from copying one RNA

molecule to the next without releasing the template molecule during the generation of sgRNA molecules (Walter *et al.* 2001). Experimental evidence gathered so far suggests that host selection pressures and evolutionary constrains affect different regions of the virus genome; for instance the capsid protein sites such as sites that interact with the host cell exposed to immune pressure and environmental changes result in positive selection and codon changes (van Hemert *et al.* 2007).

Evidence of recombination events in astroviruses have been described in the literature. Sequence analysis of viral sequence identified in stool samples from children suffering from gastroenteritis, revealed recombinant virus; analysis revealed that ORF1b closely clustered with HAstV3 and ORF2 was related to HAstV5. The authors postulate that the recombination site occurred at the ORF1b/ORF2 junction (Walter *et al.* 2001). More recently Wolfaardt *et al* (2011) described virus identified in Kenya, evolved from multiple recombination events: ORF1a clustered with HAstV 6/7, ORF1b clustered with HAstV3 and ORF2 with HAstV2 suggesting a recombination site in the 3' end of ORF1a and a second in at the ORF1b/ORF2 junction (Wolfaardt *et al.* 2011). Analysis of BAstV and CcAstV provided evidence for the occurrence of at least one recombination event during the evolution of the viruses (Tse *et al.* 2011). Rivera *et al* (2010) reported on a possible recombination event between an AstV identified in the California sea lion and HAstV4, and between PoAstV and HAstV3 suggesting that the viruses evolved from a common ancestor or the occurrence of a cross-species transmission event (Ulloa and Gutiérrez 2010). No zoonotic transmission events have been reported to date, reports in the literature suggest that cross-species transmission of astroviruses is possible. More study is required to determine the zoonotic potential of astroviruses (Chu *et al.* 2009).

1.11 Aims of the study

The aim of this study was to conduct surveillance of local South African small mammals for the presence of viral sequences belonging to the *Astroviridae* and *Arenaviridae* families, the *Coronavirinae* subfamily and the *Hantavirus* genus. Three of these viruses were chosen as the focus of this study because of their implication in recent viral zoonotic events, where cross-species transmission occurred resulting in an outbreak of disease in human beings. *Astroviridae* though not associated with zoonotic events have recently been identified in bats in China and Germany, and the high diversity of bat astroviruses and their inability to cause disease in bat hosts make them a point of interest.

Our objectives were:

> To detect astroviruses, coronaviruses and hantaviruses in South African bat species and to characterise novel viral sequences.

> To detect and characterise arenaviruses, coronaviruses and hantaviruses in rodent and shrew species that occur in South Africa.

The second of aim was to determine the presence and prevalence of hantavirus infection in the human population. The identification of the first hantavirus in Guinea and the subsequent identification of hantaviruses in other West African countries and Ethiopia has led to the search for hantavirus disease in humans as hantavirus disease is well documented in Europe, Asia and the Americas but no cases have been reported in Africa. The current hypothesis is that hantavirus disease most likely occurs in the population but may be misdiagnosed as another disease with a similar clinical presentation especially in areas where other diseases such as Malaria, other viral haemorrhagic fevers and leptospirosis occur.

The specific objectives:

> To determine a clinical case definition for hantavirus disease by recruiting patients severe acute illnesses presenting with febrile illness, respiratory failure, renal and liver failure at Tygerberg and Groote Schuur hospitals

> To determine the prevalence of hantavirus antibodies in the human population using serological assays.

Chapter 2

2 Materials and Methods

2.1 Rodent and shrew samples

Rodent and shrew trapping was conducted by Dr Sonja Matthee's research group at the Department of Conservation Ecology and Entomology at Stellenbosch University, whose research focuses on ectoparasites of small mammals in South Africa.

Rodent and shrew trapping was approved by the Animal Use Committee at Stellenbosch University (Ethics number: 2006B01007). Approval was also obtained from conservation boards in the provinces where trapping was conducted (**Error! Not a valid bookmark self-reference.**). Animals were trapped using Sherman-like live traps baited with peanut butter and oats and euthanised using sodium pentobarbitone (200mg/kg). The aim was to trap 30 to 40 individuals per locality. Each animal was given a reference number and measurements were recorded (Van der Mescht 2011). All personnel handling animals in the field used personal protective equipment which includes a lab coat, disposable latex gloves, surgical mask and goggles (SOP: Handling of tissue samples from small mammals v.02 by Peter Witkowski).

Table 2.1 Permit numbers for trapping for small mammal trapping

Province	Permit Number
Eastern Cape	CRO 117/10CR, CRO 118/10CR
Free-State	01/8091
Gauteng	CPF6 - 0153
Kwa-Zulu Natal	OP 4990/2010
Northern Cape	FAUNA 789/2010, FAUNA788/2010
Western Cape	AAA004-00291-0035
Limpopo (K. Medger)	CPM—333-00002, CPM-002-00002

Additional samples were obtained from groups conducting unrelated research on rodents and shrews at the University of Pretoria: organ samples from 113 *Acomys spinosissimus* and 113 *Elephantulus myurus* trapped in Goro game reserve in the Limpopo province were obtained from Dr Katarina Medger (Department of Zoology and Entomology). Two hundred and fifty-five RNA aliquots from animals trapped in Kruger National Park were provided by Prof

Armanda Bastos (Department of Zoology and Entomology). The breakdown of all samples included in the study is shown in

Table **2.2** (detailed tables showing sample collection in each province is in Appendix B). Trapping by Dr Medger in Limpopo was conducted using Sherman-live traps with the same type of bait but with fish was added. Animals were euthanized with an overdose of halothane (Medger *et al.* 2012).

Table 2.2 Total number of animals specimens collected for the study.

Species	No.
Rhabdomys pumilio	585
Mastomys spp	303
Micaelamys namaquensis	141
Acomys spinosissimus	113
Myotomys spp	91
Mus spp	45
Saccostomys campestris	50
Gerbilliscus spp	47
Steatomys spp	10
Gerbillurus paeba	1
Parotomys	1
Desmodillus spp	5
Grammomus spp	1
Macroscelididae	123
Soricidae	66
Rattus spp	9
Aethomys spp	10
Lemniscomys rosalia	26

2.2   Bat sample

Sampling was conducted by our collaborators Dr Veronika Cottontail (University of Ulm), Ms Leigh Richards (Durban Natural Sciences Museum), Dr Corrie Schoeman (University of KwaZulu-Natal) and Dr Samantha Stoffberg (Evolutionary Genomics Group, Department of Botany and Zoology, Stellenbosch University) (Hantaviruses are unique in the Bunyaviridae family in that, unlike other members of the family, they are not transmitted by arthropods but instead are hosted by a variety of small mammals. Initially found hantaviruses were primarily associated with members of rodent families *Muridae* and *Cricetidae* with a solitary insectivore-borne virus, TPMV (Table 1.1). It was assumed that each hantavirus had co-evolved with a primary reservoir host (Klein and Calisher 2007). In recent years hantaviruses have been identified in a variety of insectivore and bat species (**Error! Not a valid bookmark self-reference.**, **Table 1.2** and **Table 1.3**); at least 24 novel hantavirus species

have been identified in insectivores belonging to the order *Soricomorpha* and at least four bat hantaviruses (**Table 1.4**) have been identified in Brazil (de Araujo *et al.* 2012), China (Guo *et al.* 2013) and West Africa (Sumibcay *et al.* 2012; Weiss *et al.* 2012).

Table 1.1).

Trapping in the Western Cape Province took place at caves in Table Mountain National Park and Garden Route National Park (Permit number: 11LB_SE101) between December 2010 and February 2012. Mist nets were set up at cave entrances before sunset. Bats caught in the nets were transferred to cotton bags before being processed. Bat species were determined using an algorithm based on morphological features; the protocol used was modified from identification keys prepared by Christian Dietz and von Helversen, Tuebingen & Erlangen, Germany (http://iocenosi.dipbsf.uninsubria.it/didattica/bat_key1.pdf and http://mammalwatching.com/Palearctic/Otherreports/Bat%20Key%20for%20Egypt.pdf). Identification of species was performed by Dr Cottontail, who is currently conducting research on bats in Panama.

Mass, sex, age and reproductive status were recorded and faecal pellets, if produced, and from fruit bats also blood were collected and stored in RNAlater® solution (Sigma-Aldrich, Germany). Blood collect from fruit bats was drawn from the cephalic vein by puncturing with a needle and drawing blood with a capillary tube. The blood was then stored in a 1ml EDTA blood tube (Greiner Bio-One, Germany). Samples were transported back to the Division of Medical Virology where they were stored at -80°C until RNA isolation could be performed. Bats were released unharmed.

Trapping at Greyton was conducted by Dr Stoffberg in January 2012 (Cape Nature Permit number: AAA007-00373-0035). Bats were trapped using a harp trap as they emerged from a roost in a house roof. The bats were placed in individual cotton bags prior to recording of measurements, age and sex for each bat. Faecal pellets were collected from each bat and stored in 1ml of RNAlater® (Ambion, France) in cryovials (Greiner Bio one, Germany). Faecal samples were collected from the cotton bags in which the bats were placed or directly from the bat. Once processing and collection of faecal pellets from each bat was complete, the bats were released unharmed.

Bats trapped in KwaZulu Natal were trapped by Ms Richards at Ingwavuma, Phinda private game reserve, Pietermaritzburg, Sappi Mooiplaas and Vryheid (Permit number: OP2021/2011). The bats were euthanized with halothane and were catalogued for inclusion in

the mammal collection at the Durban Natural Science Museum. Faecal pellets were collected and stored in 1ml of RNAlater® solution.

All the animals were handled in guidelines for use of wild animals in research as stated in Sikes *et al* (2011).

Table 2.3 Total number bats collected for this study. Details on trapping locations are in Appendix B.

Family	Species	Number collected
Hipposideridae	*Hipposideros caffer*	6
Miniopteridae	*Miniopterus fraterculus*	6
	Miniopterus natalensis	13
Molossidae	*Chaerephon pumilus*	6
	Mops condylurus	1
	Tadarida aegyptiaca	3
Nycteridae	*Nycteris thebaica*	3
Rhinolophidae	*Rhinolophus clivosus*	8
	Rhinolophus darlingi	2
	Rhinolophus swinnyi	3
Vespertilionidae	*Neoromicia capensis*	10
	Neoromicia nana	6
	Neoromicia zuluensis	1
	Scotophilus viridis	3
Pteropodidae	*Rosettus aegyptiacus*	12

Figure 2.1 *Rhinolophus clivosus* **(A),** *Neoromicia capensis* **(B), collection of faecal pellets (C) and** measurements are taken for a *R. clivosus* bat **(D)** *Rhinolophus clivosus,* trapped at localities in the Western Cape Province. *Photographs by V Cottontail.*

2.3   Human samples

2.3.1   Ethical considerations

Ethical approval the study was obtained from the Human Research Ethics Committee (HREC) at the Faculty of Medicine and Health Sciences (Stellenbosch University): **N09/02/058** (Approval letters from HREC can be found in Appendix A).

2.3.2   Residual sera collection

Residual sera left over after routine testing for various parameters were collected from NHLS laboratories in the Western Cape: Tygerberg, Paarl, Vredendal and Oudtshoorn. The purpose of this screening was to determine whether antibodies are present in the population, therefore samples were anonymised upon receipt and no information on medical conditions and demographics was collected. Minimal patient data as available from the hospital stickers on

the blood tubes including date of birth, gender and source hospital/clinic were recorded. At least 300µl of serum was transferred into 2ml cryovials and stored at -20°C to await screening. A total of 1442 samples were collected between July 2010 and April 2012 (**Error! Not a valid bookmark self-reference.**). The collection strategy was modelled on that of the Health Protection Agency (HPA) seroepidemiology unit (www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1226652136464) in the United Kingdom.

Table 2.4 Residual serum samples collected from NHLS Laboratories for serosurvey.

NHLS laboratory	Number of samples obtained
Oudtshoorn	47
Paarl	192
Tygerberg	1031
Vredendal	172

2.3.3   Collection of samples from acutely-ill patients

Blood samples were collected from acutely ill patients who presented with an otherwise (at least initially) unexplained clinical illness conforming to a case definition for possible hantaviral disease, including fever, acute renal failure or acute hepatitis and / or a rash at Groote Schuur and Tygerberg Hospitals in the Western Cape. Consent forms and pamphlets are included in Appendix F. Case definition was defined from literature published on hantavirus disease (hantavirus disease is described in section 1.7.4, Jonsson *et al.* 2010 and Fulhorst *et al.* 2011).

2.4   Nucleic acid extraction

2.4.1   Isolation of RNA from bat faecal pellets

Bat faecal pellets stored in RNAlater[®] solution were homogenised by vortexing and stirring. One-hundred and forty microliters were used for extractions of RNA using the QIAamp[®] Viral RNA Mini kit (Qiagen, Germany). 560µl of AVL buffer (containing 5.6µl carrier RNA) was added to each sample, and the sample was vortexed for 15 seconds and incubated for 10 minutes at room temperature. Sample was centrifuged briefly and 560µl of 100% molecular biology grade ethanol (Sigma-Aldrich, Germany) was added to the reaction, briefly vortexed and centrifuged. The reaction mix was then transferred to the QIAamp[®] Mini spin column and centrifuged for 1 minute at $6000 \times g$, the supernatant was discarded and the columns

washed by adding 500µl AW1 buffer and centrifuged at 6000×*g*. The supernatant was discarded and 500µl AW2 buffer and centrifuged at 20 000×*g* for 3 minutes. To eliminate the chance of AW2 carryover, the column was centrifuged at 20 000×*g* for 1 minute. The columns were then transferred to clean 1.5ml collection tubes and 50µl of AVE buffer added to the columns, and then incubated at room temperature for 1 minute and then centrifuged at 6000×g for 1 minute to elute the RNA from the column. RNA was stored at -85°C until reverse transcription could be performed.

2.4.2 Isolation of RNA from rodent and shrew tissue

RNA was isolated from lung tissue using the RNeasy® Mini kit (Qiagen, Germany) and the automated spin protocol on the QIAcube® extraction platform. RNA was primarily isolated from lung; when that was not available liver or kidney was used instead. <30mg of tissue was cut from the organ, place in a 2ml microcentrifuge tube and chopped into small pieces using a no.11 scalpel blade. Thereafter, 350µl of RLT buffer (containing 3.5µl β-mercaptoethanol) was added to the sample. The sample was then homogenised by drawing the sample in and out of a 2ml syringe using a 22G needle (avacare®, UK) and transferred to a QIAshredder® Mini column and centrifuged at 20 000×*g* for 5 minutes.

The column was discarded and sample supernatants transferred to a clean 2ml microcentrifuge tubes compatible with the QIAcube® and placed in the sample shaker in the QIAcube®. RNeasy® Columns and collection tubes were loaded into the rotor adaptors and placed in the QIAcube® centrifuge. Buffers RW and RPE, and 70% ethanol required for isolation where placed in the buffer rack in the QIAcube. 1000µl QIAcube filter tips were added to the tip rack and the standard protocol for isolation from animal tissue was selected with the run completed in 30 minutes. RNA was eluted into RNase-free water and stored at -85°C until reverse transcription could be performed.

2.5 Reverse transcription

The cDNA was synthesised using the M-MLV Reverse Transcriptase kit (Invitrogen, USA) and Random Hexamer primer (Bioline, UK). 8µl of RNA was added to a 12µl pre-mix containing 100ng random hexamer and nuclease-free water (Qiagen, Germany) and incubated at 65°C for 5 minutes on the PE 9700 GeneAmp thermocycler (Applied Biosystems, USA). The samples were the quick-chilled on ice before the master mix containing 4µl 5× first strand buffer, Ribolock® RNase inhibitor (Thermoscientific, USA), 0.1µl 0.1M DTT and 1µl 200U/µl M-MLV reverse transcriptase was added to the reaction mix. The reaction was then

incubated under the following conditions: 25°C for 10 minutes, 42°C for 30 minutes, 95°C for 6 minutes.

2.6 Screening PCR assays

Table 2.5 Viruses targeted by the PCR assays used in this study and the animal samples tested.

	Animal specimens		
PCR assay	Bat	Rodent	Insectivore
Arenavirus	—	Lung	Lung
Astrovirus	Faecal	—	—
Coronavirus	Faecal	Lung	Lung
Hantavirus	Faecal	Lung	Lung

2.6.1 Primers

All primers used in this study were synthesised by Integrated DNA Technologies (USA). Primers were analysed through the Basic Local Alignment Sequence Tool (BLAST) on the NCBI website to confirm that they align to the target region used in the PCR assays. Additionally in silico testing of primers was performed against nucleotide alignments of the respective viruses of interest in Geneious R6 (Biomatters Ltd, New Zealand).

2.6.2 PCR positive controls

The positive control for the hantavirus PCR assays was obtained from hantavirus national reference laboratory at Charité Universitätsmedizin Berlin. It was cell-culture derived Dobrava-Slovakia cDNA. *In vitro* transcribed RNA controls for arenavirus, astrovirus and coronavirus PCR assays were provided by the Institute of Virology at the Universitätsklinikum Bonn.

2.6.3 Pan-Old World arenavirus screening assay

Five microliter cDNA of each sample was tested for arenavirus DNA in a PCR assay targeting the L gene (partial *RdRp* fragment) (Vieth *et al.* 2007). The assay was performed using the GoTaq® Hot-start DNA polymerase kit (Promega, USA). The final PCR reaction (total volume 50µl) contained 5µl of 5×GoTaq® Flexi buffer, 3µl of 1.5mM $MgCl_2$, 1µl of 200µM dNTP mix (Bioline, UK), 1µl of MasterAmp® PCR enhancer (Epicentre Biotechnologies, UK), 0.3µM forward primers (LVL3359A, LVL3359D and LVL3359G)

and 0.6μM reverse primers (LVL3754A and LVL3754D), 1.25U of 5U/μl GoTaq® DNA polymerase and 18.8μl nuclease-free water.

Table 2.6 Sequences of primers used in the Pan-Old World arenavirus PCR, targeting a conserved region in the RdRp gene on the L segment.

Oligo Name	5'→3' Oligo Sequences
LVL3359_A	AGA ATT AGT GAA AGG GAG AGC AAT TC
LVL3359_D	AGA ATC AGT GAA AGG GAA AGC AAY TC
LVL3359_G	AGA ATT AGT GAA AGG GAG AGT AAY TC
LVL3754_A	CAC ATC ATT GGT CCC CAT TTA CTA TGR TC
LVL3754_D	CAC ATC ATT GGT CCC CAT TTA CTG TGR TC

The PCR reactions were run on the Veriti® thermocycler (Applied Biosystems, USA) with the temperature profile: 95°C for 2 minutes; 45 cycles at 95°C for 20 seconds, 55°C for 45 seconds and 72°C for 50 seconds; and final extension at 72°C for 5 minutes.

2.6.4   Pan-astrovirus screening assay

Maxima® Hot-Start Master mix (Thermoscientific, USA) was used for pre-nested and hemi-nested amplification. The assay was run using primers from Chu *et al.* 2008, with Astro-F1 and Astro-F2 for the pre-nested, and Astro-HNF1 and Astro-HNF2 in the hemi-nested run, Astro R, was the reverse primer in both reactions.

Table 2.7 Sequences of primers used in the pan-astrovirus PCR assay for the detection of all members of the *Astroviridae* family.

Oligo Name	5'→3' Oligo Sequences
Astro-F1	GAR TTY GAT TGG RCK CGK TAY GA
Astro-F2	GAR TTY GAT TGG RCK AGG TAY GA
Astro-HNF1	CGK TAY GAT GGK ACK ATH CC
Astro-HNF2	AGG TAY GAT GGK ACK ATH CC
Astro-R	GGY TTK ACC CAC ATN CCR AA

2.6.4.1   Pre-nested amplification

The pre-nested reaction was set up as follows: 12.5µl of 1×Maxima® Hot-start Master Mix, 2.5µl of 1µM of each primer (Astro-F1, Astro-F2 and Astro-R), 3µl of nuclease-free water and 2µl of cDNA sample with a total reaction volume of 25µl.

The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, USA) using the following temperature profile: 95°C for 2 minutes, 40 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds and final extension at 72°C for 5 minutes.

2.6.4.2   Hemi-nested amplification

One microliter of the pre-nested PCR product was added to the 49µl of the PCR reaction mix containing 25µl of 1× Maxima Master Mix, 5µl of 1µM of each primer (Astro-HNF1, Astro-HNF2 and Astro-R) and 9µl of nuclease-free water.

The reaction was then run as follows: 95°C for 2 minutes, 30 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 5 minutes.

2.6.5   Pan-coronavirus screening assay

Pre-nested and nested amplification were performed using the GoTaq® Hot-start DNA polymerase kit (Promega, USA) and primers from de Souza Luna *et al.* (2007); Pre-nested primers: PC2S2 (**PCS1** and **PCS2**), PC2As1 (**PCAs1**, **PCAs2** and **PCAs3**) and nested primers: PCS (**PCS3** and **PCS4**) and PCNAs (**PCAs4**). Primer mixes containing equimolar amounts of each component primer were then used for amplification, which was performed on the PE GeneAmp 9700 thermocycler (Applied Biosystems, USA).

Table 2.8 Sequences of primers used in the pan-coronavirus PCR assay for the detection of all members of the *Coronaviridae* family.

Oligo Name		5'→3' Oligo Sequences
PC2S2	**PCS1**	TTA TGG GTT GGG ATT ATC
	PCS2	TGA TGG GAT GGG ACT ATC
PC2As1	**PCAs1**	TCA TCA CTC AGA ATC ATC A
	PCAs2	TCA TCA GAA AGA ATC ATC A
	PCAs3	TCG TCG GAC AAG ATC ATC A
PCS	**PCS3**	CTT ATG GGT TGG GAT TAT CCT AAG TGT AG
	PCS4	CTT ATG GGT TGG GAT TAT CCC AAA TGT GA
PCNAs	**PCAs4**	CAC ACA ACA CCT TCA TCA GAT AGA ATC ATC A

2.6.5.1 *Pre-Nested Amplification*

Five microliter cDNA of each sample was added to 20µl master mix which was prepared as follows: nuclease-free water (Qiagen, Germany) 5µl of 5×GoTaq® colourless buffer, 250µM dNTP mix (Bioline, UK), 2.5mM $MgCl_2$, 1µl 1mg/ml BSA , 0.625U GoTaq® Hot-start DNA polymerase, and 0.2µM of each primer PC2S2(1:2) and PC2As1(1:2:3). Pre-nested amplification was then run as follows: initial denaturation/polymerase activation at 95°C for 2 minutes and touch down amplification for 10 cycles: 95°C for 20 seconds, 60°C for 30 seconds (1°C decrease per cycle), 72°C for 40 seconds. Amplification was then continued for 4o cycles: 95°C for 20 seconds, 54°C for 30 seconds, 72°C for 40 seconds, and final extension at 72°C for 5 minutes.

2.6.5.2 *Nested Amplification*

For nested amplification; 1µl of pre-nested amplification product was added to 49µl master mix which contained nuclease-free water, 10µl 5×GoTaq® Green buffer, 2.5mM $MgCl_2$, 250µM dNTP mix, 1.25U GoTaq® DNA polymerase, and 0.3µM of each primer PCS(3:4) and PCNAs. The product was amplified under the following conditions: 95°C for 2 minutes, 45 cycles: 95°C for 20 seconds, 50°C for 30 seconds, 72°C for 40 seconds, and final extension at 72°C for 5 minutes. Nested amplification products were then visualized by 2% agarose gel electrophoresis with an expected product size of 450bp.

2.6.6   Pan-hantavirus screening assay

Hantavirus pre-nested and nested PCR was conducted with primers from Klempa *et al.* (2006) and the Maxima Hot-start Master Mix (Thermoscientific, USA). The primers target a conserved region in the RdRp gene on the L segment; Han-L-F1 and Han-L-R1 were used in pre-nested amplification, and nested amplification was performed with Han-L-F2 and Han-L-R2.

Table 2.9 Primer sequences for pre-nested and nested amplification; primers target a conserved region in the RdRp gene on the hantavirus L segment.

Oligo Name	5' → 3' Oligo Sequences
Han-L-F1	ATG TAY GTB AGT GCW GAT
Han-L-R1	AAC CAD TCW GTY CCR TCA TC
Han-L-F2	TGC WGA TGC HAC IAA RTG GTC C
Han-L-R2	GCR TCR TCW GAR TGR TGD GCA A

2.6.6.1   Pre-nested amplification

Five microliters of sample cDNA was added to a reaction mix containing 12.5µl of 2× Maxima® Hot-start Mix, 2.5µl of 1µM Han-L-F1 and Han-L-R1, 1µl of MasterAmp® PCR enhancer with betaine (Epicenter Biotechnologies, USA) and 1.5µl of nuclease-free water. The reaction was run under the following cycling conditions on the PE GeneAmp® 9700 thermocycler: 95°C for 2 minutes, 40 cycles at 95°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds, and final extension at 72°C for 6 minutes.

2.6.6.2   Nested amplification

One microliter of pre-nested PCR product was added to a reaction mix containing 25µl of Maxima Hot-start Mix, 5µl of 1µM primers Han-L-F2 and Han-L-R2, 1µl of MasterAmp PCR enhancer with betaine and 13µl of nuclease-free water. Amplification was performed using the following cycling profile: 95°C for 2 minutes, 25 cycles at 95°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds, final extension at 72°C for 6 minutes.

2.7   Amplification of the arenavirus partial *GPC* gene fragment

Samples positive for arenavirus RNA in the screening assay were amplified using S'UTR1 (5'-CGC ACC GGG GAT CCT AGG CA-3') and ARS_3C (5'- TCA AAK AGC CKC AGC

RTG TC-3') primers from Lozano *et al* (1997) in an attempt to obtain a partial GPC gene fragment for analysis. Sample cDNA was added to a reaction mix containing 10μl of 5× GoTaq$^®$ Green Flexi buffer, 1.5mM MgCl$_2$, 10mM (ea.) dNTP mix (Bioline, UK), 500nM primers, 1.25U GoTaq$^®$ Hot-Start DNA polymerase and nuclease-free water. Amplification was performed on the Veriti® 96-well thermal cycler using the following cycling profile: 95°C for 2 minutes, 45×cycles at 95°C for 30 second, 52°C for 50 seconds and 72°C for 1.5 minutes, sand final extension at 72°C for 5 minutes.

2.8   Extension of coronavirus *RdRp* gene fragment

Samples that was positive in the coronavirus screening PCR were amplified to obtain a longer *RdRp* gene fragment (Drexler *et al.* 2010). Five microliters of cDNA was added to a 45μl reaction mix that was composed as follows: 10μl of 5× GoTaq$^®$ Green Flexi buffer, 1.5mM MgCl$_2$, 10mM (ea.) dNTP mix (Bioline, UK), 500nM primers (ext*RdRp*_1 [5'-TTC TTT GCA CAG AAG GGT GAT GC-3'], ext*RdRp*_2 [5'CTT TGC ACA AAA AGG TGA TGC WGC-3'] and PCNAs), 1.25U GoTaq$^®$ Hot-Start DNA polymerase and nuclease-free water. Amplification was performed on the Veriti® 96-well thermal cycler using the following cycling profile: 95°C for 2 minutes, 10 cycles of touchdown at 95°C for 20 seconds, 60°C for 30 seconds (with a 1°C decrease in annealing temperature every cycle) and 72° for 90 seconds, and 45 cycles at 95°C for 20 seconds, 54°C for 30 seconds and 72°C for 90 seconds, and final extension at 72°C for 5 minutes.

2.9   Host species identification by *cytochrome b* PCR

Cytochrome b PCR was performed on the samples that were positive for virus sequences in order to confirm the rodent host species; a modified protocol from Bastos *et al* (2011) was used. Five microliters of cDNA was added to a 20µl reaction mix containing 4µl GoTaq® flexi buffer, 0.2mM of each dNTP, 400nM L14724 (5'-TGA YAT GAA AAA YCA TCG TTG-3') and H15915R (5'-CAT TTC AGG TTT ACA AGA C-3') and 1.25U of GoTaq® DNA polymerase. Amplification was carried out on the PE9700 GeneAmp thermocycler (Applied Biosystems, USA) under the following cycling conditions: 95°C for 2 minutes, 2 cycles: 95°C for 12 seconds, 52°C for 30 seconds and 70°C for 60 seconds, followed by 3 cycles with a lower annealing temperature at 50°C and 45 cycles with another 2°C decrease in the annealing temperature and final extension at 72°C for 5 minutes.

2.10   Visualisation of PCR products

All PCR products were visualised on 2%(m/v) for fragments up to 1kb and 1%(m/v) larger fragments agarose gel. The gel was prepared by mixing 2g of Lonza® LE agarose (BioWhitaker, USA) with milli-Q water to make 100ml mixture. The mixture was heated in the microwave for 3 minutes, the mixture was allowed to cool while stirring and 5µl of Nancy-520 (Sigma-Aldrich, Germany) was added to the mixture. The mixture was poured into an electrophoresis tray and a 1mm gel comb was added, and the gel allowed to set for 30 minutes.

Five microliters of products from PCR reactions run with GoTaq® Flexi green buffer were loaded into the wells directly, and products from the reactions run with the Maxima® Hot-start Master Mix were mixed with 1µl of 6× orange loading dye (Thermoscientific, USA). Once the samples were loaded into the wells, a Generuler® 100bp ladder (Thermoscientific, USA) was loaded to the gel and the electrophoresis tray was connected to a power pack and run at 70V for 50 minutes.

The PCR product was visualised on the UVItec gel documentation system (chemiluminescence and fluorescence system) (UVItec Alliance, UK), the gels were viewed under transilluminator at 254nm and the images were captured using UVIchem software. The images were then enhanced and edited using UVIband software saved and printed.

2.11 PCR product purification

The PCR products were purified before sequencing using the Wizard® SV gel and PCR Clean-up System (Promega, USA) with the centrifugation protocol. Forty-five microliter of membrane binding solution was mixed with the PCR product by pipetting the mixture up and down several times, and the mixture was then transferred to clean-up columns and centrifuged at $16000 \times g$ for 1 minute. The flow-through was discarded and 700µl of membrane wash solution was added to each column, and the columns centrifuged at $16000 \times g$ for 1 minute. The flow-through was discarded and 500µl of membrane wash solution was added to the columns and centrifuged at $16000 \times g$ for 5 minutes. The columns were centrifuged for 1 minute to allow the ethanol in the buffer to evaporate and to prevent it from being carried over into the eluate. The columns were transferred to 1.5ml collection tube and 50µl of nuclease-free water was added to the columns, and the columns incubated at room temperature for 1 minute and then centrifuged at $16000 \times g$ for 1 minute to elute the DNA. The DNA was stored at -20°C until it could be sequenced.

2.12 Sequencing PCR and clean-up

Sequencing reactions were performed using the Big-dye terminator cycle sequencing kit v3.1 (Applied Biosystems, USA). Two microliters of purified with PCR products (15-25ng) was added to a reaction mix containing 5µM of each primer, 1µl Ready-Reaction mix, 2µl sequencing buffer, and water to make up a 10µl final reaction volume. The reaction was set up as follows on the PE GeneAmp® 9700 thermal cycler or the Veriti® (Applied Biosystems, USA) using standard profile as supplied by the manufacturer: 95°C for 1 minute, 30 cycles at 95°C for 10 seconds, 55°C for 5 seconds and 60°C for 4 minutes.

Following the PCR unincorporated ddNTPs and other components of the sequencing PCR were removed using the Big-dye X-terminator kit (Applied Biosystems). Ten microliters of Big-dye X-terminator particles mixed with 45µl of SAM solution was added to each sequencing reaction, and the reactions were then incubated on a shaker at 1800 rpm for 30 minutes. After incubation, the reactions were centrifuged at $2000 \times g$ for 5 minutes and the sequences were read on the 3130*xl* genetic analyser (Applied Biosystems, USA) using the standard protocol.

2.13 Sequence and phylogenetic analysis

Sequence chromatogram files were uploaded into Geneious R6 (Biomatters Inc. New Zealand); the sequences were trimmed and contigs were assembled for each sample. The contigs were run through the Basic Local Alignment Sequence Tool (BLAST) on the NCBI website to determine the whether the sequences obtained were indeed the targeted viruses in the PCR assays. .

Viral sequences available on Genbank were downloaded and imported into MEGA v5.1 software (http://www.megasoftware.net) with our sequences, complete gap deletion was performed and Clustal algorithm was used to align the sequences. A neighbour-joining tree was performed using the percentage distance model to infer phylogenetic relationship of the sequences and 1000 bootstrap replicates were included in order to validate the accuracy of the consensus tree produced.

The sequence alignments were then run through JModelTest 2.1 (Posada 2008) to determine the most appropriate model for phylogenetic inference. The programme makes use Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) to compare different models simultaneously and chooses the most appropriate model for analysing the data set (Lemey *et al.* 2009). Maximum-likelihood and Bayesian analysis was performed in MEGA5, Geneious R6 and MrBayes v3.1 according to the model selected by the JModelTest 2.1.

2.14 Serology

2.14.1 Antigens and positive control material

Recombinant nucleocapsid protein antigen for Dobrava, Puumala and Sangassou viruses were prepared at the Institute of Virology, Charité - Universitätsmedizin Berlin, Germany as described in Razanskiene *et al* (2004). These antigens were used in the in-house ELISA and Western blot assays. Positive control sera were provided by the hantavirus study group at the Institute of Virology as well. Positive controls are composed of patient sera positive for Dobrava and Puumala virus.

2.14.2 Preparation of buffers for ELISA

2.14.2.1 Coating buffer

Coating buffer was prepared by adding 1.53g sodium carbonate (Sigma-Aldrich, Germany) to milli-Q water to make up 500ml solution with 0.05M and pH9.6. The buffer was stored at 4°C and was stable for one week.

2.14.2.2 Dilution buffer and blocking buffer

One gram of Fraction V bovine serum albumin (BSA) (Sigma-Aldrich, Germany) was mixed with 1× Lonza® Dulbecco's phosphate buffered saline (DPBS) (BioWhitaker, USA) (without Mg^{2+} and Ca^{2+}) to make a 1%(m/v) 100ml dilution buffer.

Three grams of Fraction V BSA was mixed with 1×DPBS to make a 3%(m/V) 100ml blocking buffer solution. Buffers were stored at 4°C.

2.14.2.3 Wash buffer

One molar Tris-Cl solution was prepared by adding to 33.33g Ultrapure TRIS and 21ml of concentrated hydrochloric acid (Sigma-Aldrich, Germany) was added to mill-Q water to make 300ml solution, and the solution was autoclaved at 121°C for 30 minutes.

For the preparation of 10×wash buffer: 100ml of 1.0 M Tris-Cl, 175.6g of NaCl (Sigma-Aldrich, Germany) and 10ml Tween 20® (Sigma-Aldrich, Germany) was added to mill-Q water to make 1L 10× stock solution.

Working solution of wash buffer was prepared by diluting 10× wash buffer stock solution in milli-Q water by adding 100ml of stock solution to 900ml of water.

2.14.2.4 Stop solution

One molar sulphuric acid was prepared by diluting concentrated (18N) sulphuric acid (Sigma-Aldrich, Germany) 18× in milli-Q water: 1ml of acid was added to 17ml of water.

2.14.3 Direct coating IgG ELISA

Samples were screened for hantavirus antibodies using recombinant nucleocapsid protein derived Dobrava, Puumala and Sangassou viruses (Meisel *et al.* 2006). The plates were coated with 100µl of antigens diluted in coating buffer Na_2CO_3, pH 9.6 to a concentration of 0.3µg/ml, and control well for each sample was filled with dilution buffer (as illustrated in the template below). The plates were then incubated for 1 hour at 37°C, thereafter the

contents of wells were discarded and 150μl of blocking buffer was added to each well, and the plate incubated at room temperature for 1 hour. Samples and controls were diluted in dilution buffer 1:200, for samples and negative control and 1:1000 for the positive controls.

The plates were then washed four times with 1×wash buffer on the MultiWash II plate washer from Tricontinent (USA) and then 100μl of samples and controls were added to their respective wells as illustrated in figure and the plate incubated for 1 hour at 37°C. After incubation the plates were washed four times and then 100μl of goat antihuman-IgG HRP conjugated antibody (Promega, USA) diluted to a concentration of 0.5μg/ml in dilution buffer. The plates were then incubated at 37°C for 1 hour, after which they were washed once again, and then 100μl of TMB 1-component microwell substrate solution (KPL, USA). The plates were then incubated in the dark for 10 minutes and 100μl of Stop solution was added. The plates were then read on the Microplate reader (Worldwide Diagnostics, USA) at 450nm.

The net absorbance values for samples was calculated by subtracting the absorbance value for the control well from the absorbance values obtained in the wells coated with each antigen. Test OD450 − Control OD450 = antibody activity (net OD450), samples were classified as positive if they had an OD450 greater than or equal to 0.150 as determined in Meisel *et al* (2006) and Klempa *et al (*2010).

2.14.4 Preparation of buffers for Western blot

2.14.4.1 *Separating gel buffer*

Separating gel buffer was prepared by adding deionised water to 45.42g of Tris (Roth, Germany) to make 200ml solution. The pH was adjusted to 8.8 using 1M hydrochloric acid, then 9.6ml of 10(m/v)% SDS was added to the solution. Deionised water was added to make a solution with a final volume of 250ml.

2.14.4.2 *Stacking buffer*

To make stacking gel buffer, 15.14g of TRIS was mixed with water to make a 200ml solution. The pH of the solution was adjusted to 6.8 using 1M hydrochloric acid and then 10ml of 10 %(m/v) SDS and water was added to obtain a 250ml solution.

2.14.4.3 Running buffer

10 × SDS-PAGE stock buffer was prepared by mixing 10g TRIS with 144g Glycine and 10g SDS, and then deionised water was added to make a 1L solution. Running buffer was prepared by adding 900ml of water to 100ml 10× SDS-PAGE stock.

2.14.4.4 Loading buffer

Stock solution was prepared by mixing 1M TRIS (pH 6.8), 1.5g SDS, 15ml glycerine and 1ml bromophenol blue, water was then added to make up 50ml. Ready-to-use 2× buffer was prepared by mixing 1ml of the stock solution with 250µl DTT. This solution is only stable for 1 week.

2.14.4.5 Blotting buffer

One litre of blotting buffer was made up of 5.8g TRIS, 2.9g Glycine, 0.4g SDS and 200ml methanol was mixed with deionised water.

2.14.4.6 TBST buffer

Twenty times stock solution of Tris-buffered saline (TBS) was prepared by adding water to 350.8g sodium chloride and 121.2g TRIS to make a 2L solution. Fifty millilitres of 20× solution was mixed with 1ml Tween®20, to which water was added to make a 1L solution, the pH was adjusted to 7.3 using 1M hydrochloric acid.

2.14.5 In-house IgG Sangassou recombinant immunoblot assay

15% separating gel was prepared by adding 3.25ml separating gel buffer with 6.25ml of 30% ProtoGel® acrylamide (National Diagnostics, USA) and 2.85ml of deionised water. One millilitre of the separating gel was then mixed with 5µl TEMED (Bio-Rad) and 25µl ammonium persulphate (APS) (Carl Roth, Germany). This mixture was then used to seal the bottom of the plates. The rest of the separating gel was mixed with 250µl APS and 25µl TEMED, poured into the plates; it was then overlaid with ¬500µl 2-propanol and water, and then allowed to set for 30 minutes.

Stacking gel was prepared by mixing 2.5ml stacking gel buffer with 1.3ml 30% ProtoGel® acrylamide, 6.1ml water, 50µl 10% APS and 10µl TEMED. The water and 2-propanol was discarded and the stacking gel was poured into the plates on top of the set separating gel, and was allowed to set for 30 minutes.

Recombinant antigens (proteins) were prepared for loading: each antigen was mixed 1:1 ratio with the ready-to-use Lammeli loading buffer. 1μg per sample well was prepared. The Sangassou antigen stock had a starting concentration of 268μg/ml which was diluted to 20μg/ml in water by mixing 7.5μl antigen stock with 92.5μl of water. The diluted antigen was then mixed with 100μl loading buffer and incubated at 100°C for 5 minutes to denature the protein, after which it was place on a cooling block.

The gel was then transferred to a vertical electrophoresis tank, and the tank filled with running buffer. The antigen/loading buffer mixture was then loaded onto the gel. The tank was connected to a power supply set to 75V and run at this voltage until the antigen passed through the stacking gel into the separating gel, at which point the voltage was increased to 100V and run until the loading buffer ran off the gel completely.

Blotting paper and Protran® nitrocellulose membrane (Whatman, USA) were cut to the correct dimensions (12.5cm length × 5.5cm height) and equilibrated in blotting buffer for at least 2 minutes before preparing the blot. The separating gel was cut to the same dimensions as the paper and membrane and equilibrated in blotting buffer as well. The paper, gel and membrane were stacked in the following order on the transfer unit: 3 pieces of blotting paper, the separating gel, the nitrocellulose membrane and 3 pieces of blotting paper. Bubbles were removed from the stack by rolling over the stack with a glass rod. The transfer unit was then connected to a power supply with current set to 68mA (this value is obtained by multiplying the height and length of the stack) and run for 1:15 hours.

Once the transfer was complete, the membrane is incubated in blocking buffer (5%(m/v) milk powder in TBST buffer) at 4°C overnight with agitation. Following overnight incubation, the membrane was then washed with TBST buffer 4× for 10 minutes each. After washing the membrane was cut into strips and placed in blot wells containing 2ml of dilution buffer (2% milk powder in TBST buffer). Twenty microlitres of sample and 2μl of positive control (rabbit anti-Sangassou polyclonal antibodies) were added to each well, and the plates were incubated for 2 hours at room temperature with agitation. The washing step was repeated as described above, after which sheep anti-human IgG POD-conjugated antibody and anti-rabbit POD-conjugated antibody was added to the respective wells. The plates were incubated at room temperature for 1 hour with agitation, thereafter the strips were washed.

After washing the plates were arranged on a transparency, and the substrate solution was prepared; Luminol/enhancer solution and stable peroxide solution (Lumi-Light^PLUS Western

blotting substrate) from Roche (USA) were mixed with HPLC water 1:1:1 to a final volume of 600μl. The blot was then read under chemiluminescent light at different exposure times (ranging from 1 second to 1 minute) and photographs were taken of the blot.

2.14.6 *recom*Line Bunyavirus IgG

All samples that were negative on the in-house Sangassou assay were screened using the *recom*Line Bunyavirus IgG blotting kit from Mikrogen (Germany). Ready-to-use wash buffer was prepared by mixing 100ml of 10× wash buffer A, 5g skim milk powder and 900ml deionised water. 2ml of ready-to-use buffer A was aliquoted into wells in an incubation tray. Test strips were then added to each well and 20μl patient serum was added to each well, the trays were then incubated at room temperature on a shaker for 1 hour. After incubation, the strips were washed 3× with buffer A on a shaker for 5 minutes each time. IgG conjugate solution was prepared by diluting it 1:10 in ready-to-use buffer A, and 2ml of conjugate solution was added to each well and the strips were incubated as described above for 45 minutes. The strips were then washed as previously described and then 1.5 ml substrate solution was added to each well, and the strips incubated for 5-10 minutes at room temperature. Thereafter, the strips were washed at least 3× with deionised water and allowed to dry for 2 hours between two layers of absorbent paper, before results could be read by comparing the protein bands to the cut-off band (according to Table 2.10 below).

Table 2.10 Key for the assessment of band intensity (in relation to the cut-off band) for the determination of results on the *recom*Line Bunyavirus IgG assay. Samples were considered positive of a low intensity band was obtained for one of the hantaviruses on the strip.

Stain intensity of the bands Assessment	
No reaction	−
Very low intensity (lower than the cut-off band)	±
Low intensity (equivalent to the cut-off band)	+
High intensity (higher than the cut-off band)	++
Very strong intensity	+++

2.14.7 Indirect immunofluorescence test

Immunofluorescence test was performed using slides prepared in-house at the Institute of Virology, Charité - Universitätsmedizin Berlin. Slides prepared had twelve wells coated with infected and non-infected Vero-E6 cells, and prepared for the following viruses: Dobrava, Hantaan, Puumala and Sangassou. Patient sera and negative, and positive controls were

91

diluted in 1×PBS (Roth AG, Germany), 1:20 and 1:100 respectively. Twenty microliters of diluted sample and controls was added to each well, and the slides were incubated in a moisture chamber, at room temperature for 30 minutes. Following incubation, slides were rinsed and washed 2× in a bijou with 1×PBS on a shaker for 5 minutes, and air dried at room temperature. Polyclonal rabbit anti-human FITC (Dako, Denmark) was diluted 1:40 in PBS and 20µl added to each well. The slides were then incubated in a moisture chamber, at room temperature for 30 minutes. After incubation, the slides were washed as previously described and dried in the dark. A small drop of mounting medium (Argene, France) was added to each well and the slide was covered with a coverslip before viewing under a fluorescence microscope at 20× and 40× magnification.

Chapter 3

3   Results

3.1   Identification and characterisation of arenaviruses in South African rodents

3.1.1   Detection of arenaviruses in South African rodents

Arenavirus sequences were detected in eight out of 1651 rodent and insectivore lung samples (Table 3.1 and Figure 3.1 and Appendix B). *Cytochrome b* analysis confirmed the host species of all the positive samples; *Myotomys irroratus*, *Micaelamys namaquensis* and *Rhabdomys pumilio*.

3/4 *M. irroratus* trapped in East London, 2/15 *M. namaquensis* trapped in Goegap, 1/25 *R. pumilio* trapped at Agama tented camp, 1/8 *M. irroratus* trapped at Buffels Jags rivier, 1/4 *M. namaquensis* trapped in Van Rhynsdorp were positive for arenavirus RNA in the Pan-OW arenavirus PCR; details about the trapping location including the GPS coordinates are listed below (

Table **3.1** and Figure 3.1).

Table 3.1 Samples positive in the Pan-Old World arenavirus PCR assay. Samples are listed with species name and trapping location. Host species was confirmed by *cytochrome b* PCR.

Samples	Species	Trapping Location	GPS coordinates	Location on Map
ATCRp18	*Rhabdomys pumilio*	Agama tented camp, Garies	30° 25' 23" S, 17° 53' 01" E	2
BJROr1	*Myotomys irroratus*	Buffel Jags Rivier, Swellendam	34° 3' 9.56" S, 20° 32' 57.54" E	4
ELOr2	*Myotomys irroratus*	East London		5
ELOr3	*Myotomys irroratus*	East London	32° 58' 11" S,	
ELOr4	*Myotomys irroratus*	East London	27° 52' 12" E	
GpMn1	*Mus minutoides*	Goegap, Springbok	29° 39' 59" S,	1
GpMn5	*Micaelamys namaquensis*	Goegap, Springbok	17° 53' 1" E	
VRWMn1	*Micaelamys namaquensis*	Vanrhynsdorp	31° 36' 14.7168" S, 18° 44' 24.4932" E	3

93
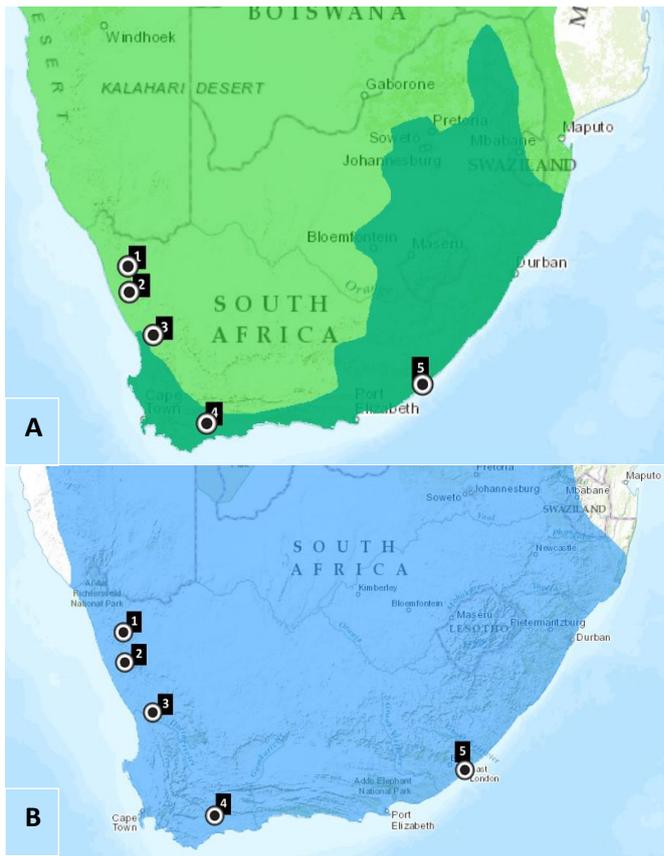
Figure 3.1 Locations at which the rodents positive for Arenavirus were trapped. (A)The light green area represents the geographical range of *Micaelamys namaquensis* and the dark green is the range for *Myotomys irroratus*. (B) Geographical range of *Rhabdomys pumilio* in South Africa (see Appendix C for the full range). Map was drawn using ArcGIS Explorer and data from the IUCN http://www.iucnredlist.org (Coetzee, Griffin, and Taylor 2008a; Coetzee and van der Straeten 2008b; Taylor, Maree, and Monadjem 2008)

3.1.2 Sequence and phylogenetic analysis of novel arenavirus sequences
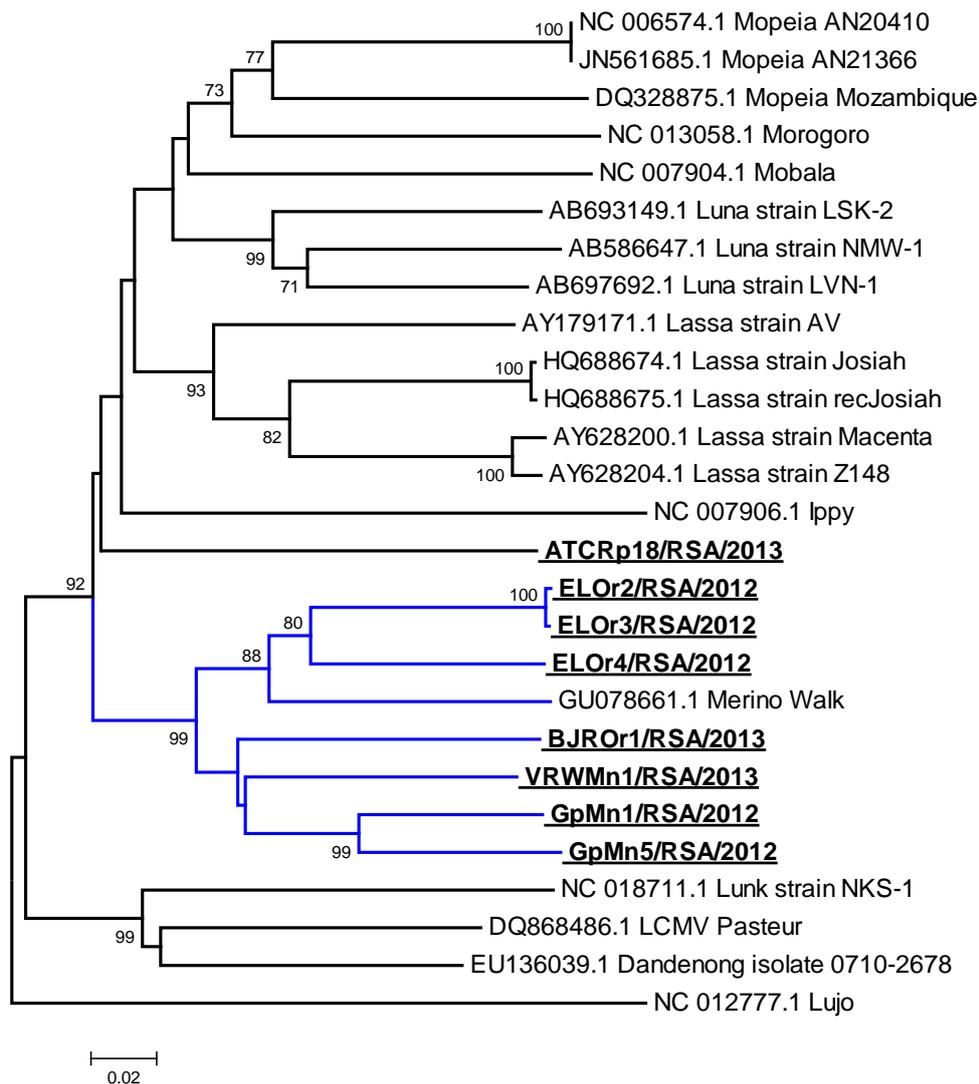
Figure 3.2 Phylogenetic analysis of Old World arenaviruses including novel viral sequences identified in our study. A 395bp partial *RdRp* gene fragment nucleotide sequence was used for analysis performed using the Neighbour-joining method and the p-distance model in MEGA5. Evolutionary relationship between taxa was confirmed with 1000 bootstrap replicates. Distance values at the branch nodes lower than 70% were excluded from the tree. Novel South African viral sequences from this study are highlighted in bold and underlined.

Neighbour-joining analysis showed that ELOr2, 3, 4, VRWMn1 and BJROr1, GpMn5 and GpMn1 cluster together with Merino Walk virus (MWV) identified in a *Myotomys unisulcatus* in the Eastern Cape (Palacios *et al.* 2010). Pairwise sequence difference among the MWV-related sequences ranges from 0.3% between ELOr2 and 3, to 24% between GpMn5 and ELOr3. The closest relative to MWV is ELOr2 with a difference of 17% between the two sequences. GpMn5 is furthest from MWV with a difference of 23%. Viral

sequences clustered with Merino Walk virus form two geographical clusters, suggesting that Merino Walk and the related viruses diverge geographically rather than based on host species. The viral sequence identified in *Rhabdomys pumilio* ATCRp18 is a unique lineage and does not cluster with any known viruses in the Lassa-LCMV sero-complex (Figure 3.2).

An attempt was made to confirm phylogeny observed in the partial *RdRp* gene fragment by analysing a partial *GPC* fragment. Amplicons and subsequent sequences could only be obtained for two out of 8 samples: ELOr3/RSA/2012 and ELOr4/RSA/2012. A ~920bp GPC fragment was obtained for both samples. Clustal alignment with S segment of Old World arenaviruses using the Blosum model. Phylogeny was then inferred with the Neighbour-joining method using the Poisson model in MEGA5. Analysis confirmed the relationship of our sequences with Merino Walk virus (**Figure** 3.3).
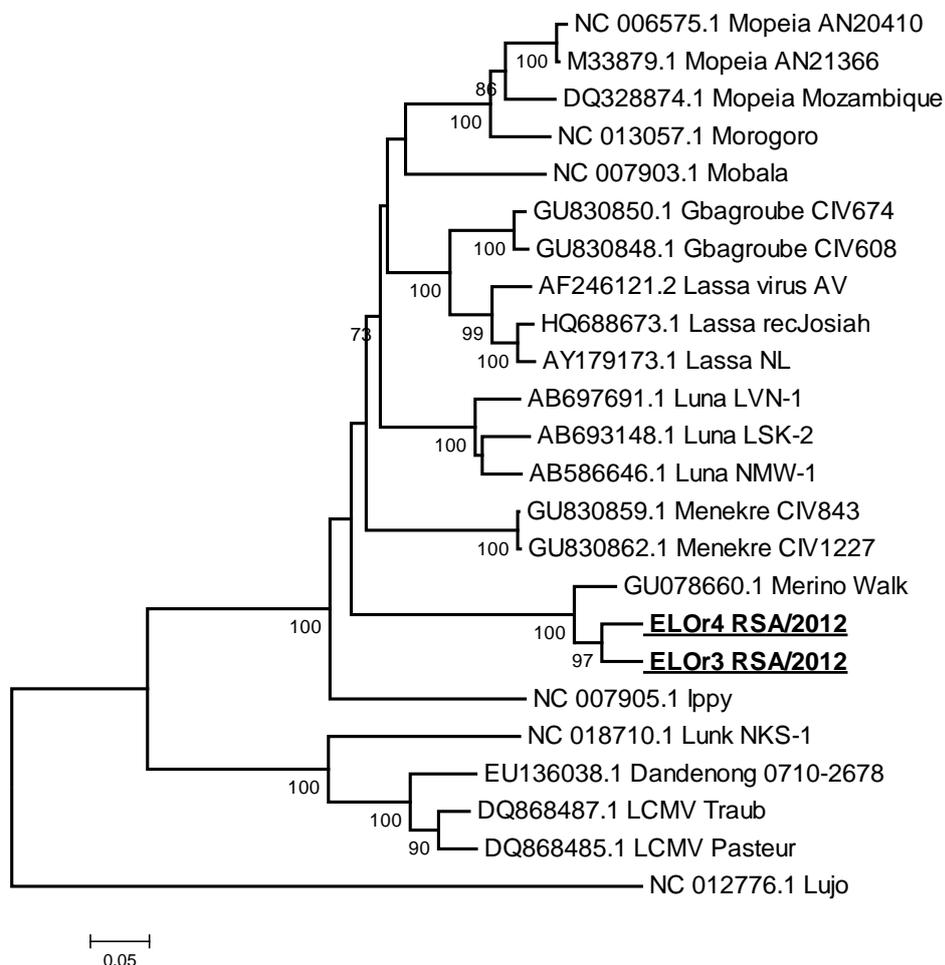
Figure 3.3 Partial *GPC* gene phylogeny including the novel Merino-Walk related viral sequences ELOr3/RSA/2012 and ELOr4 /RSA/2012 from *Myotomys irroratus*. Neighbour-joining method using the Poisson model with 1000 bootstrap replicates was used to infer phylogenetic relationship in MEGA5 using 340aa sequence. Branches corresponding to partitions reproduced in less than 50%

bootstrap replicates are collapsed. Numbers at the branch nodes represent amino acid substitutions per site and all values lower than 70 were excluded from the tree. The novel viral sequences are shown in bold and underlined.

3.2   Identification and characterisation of astroviruses

3.2.1   Detection of astroviruses in bats

Astrovirus RNA was detected in 35 faecal samples from various insectivorous bat species (Table 3.2), in insectivorous bats from the families *Hipposideridae, Miniopteridae, Molossidae*, *Rhinolophidae* and *Vespertilionidae*.

Table 3.2 Species in which astrovirus viral RNA was detected by PCR and confirmed by sequencing.

Family	Species	No. tested	PCR positives
Hipposideridae	*Hipposideros caffer*	6	**2**
Miniopteridae	*Miniopterus natalensis*	13	**12**
	Miniopterus fraterculus	6	**6**
Molossidae	*Chaerephon pumilus*	6	0
	Tadarida aegyptiaca	3	**2**
	Mops condylurus	1	0
Nycteridae	*Nycteris thebaica*	3	0
Rhinolophidae	*Rhinolophus clivosus*	8	**1**
	Rhinolophus darlingi	2	0
	Rhinolophus swinnyi	3	**1**
Vespertilionidae	*Neoromicia capensis*	10	**8**
	Neoromicia nana	6	**1**
	Neoromicia zuluensis	1	0
	Scotophilus viridis	3	0
Pteropodidae	*Rosettus aegyptiacus*	12	0

3.2.2   Sequence and phylogenetic analysis of astroviruses

Phylogenetic inference for astrovirus sequences was obtained by the Neighbour-joining method using the p-distance model. Evolutionary relationship between viral taxa was confirmed by 1000 bootstrap replicates (**Error! Reference source not found.**).

All bat astrovirus sequences identified during this study belong to the mamastrovirus genogroup II along with bat astroviruses identified in China and Germany. It can be seen from the tree that viruses identified in the same host species do not necessarily cluster together. Astrovirus sequences identified in South African bats are dispersed among Chinese

astrovirus sequences. No clusters based on host species or geographic location could be observed.

Pairwise distance analysis of sequences identified in bats of the same host species and from the same colony showed high diversity. *Miniopterus natalensis* bats (MSTM) trapped in a cave on Table Mountain showed a percentage nucleotide difference between 21.8% (between MSTM9 and MSTM 14) and 30.9% (MSTM 8 and MSTM 12) in the analysed sequence. Viral sequences from *M. fraterculus* (Mf) trapped in Pietermaritzburg had % difference range of 14.8% (Mf 5 and Mf6) to 27.6% (Mf3 and Mf4). The % difference among viral sequences identified in *Neoromicia capensis* (trapped in Greyton) varied between 1.7% (between GreytonNC1 and NC7) and 33% (between GrNC3 and GrNC4). When all the South African sequences are taken into account, the lowest % difference was observed between MSTM1 and GreytonTAr1(3%) and the highest (34.8%) between GreytonNC3 and MSTM12. The Pairwise distance matrix can be found in Appendix D.
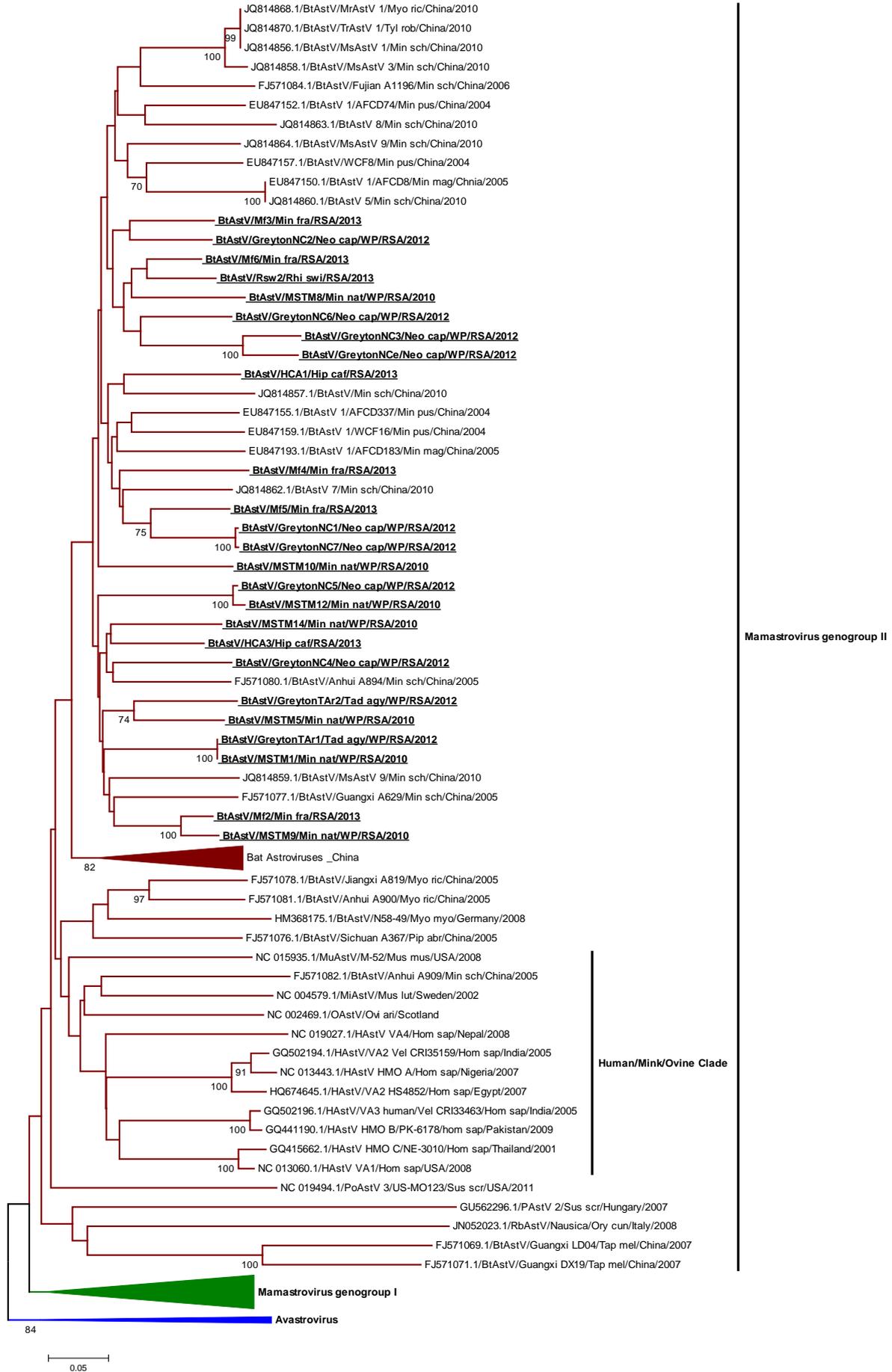
Figure 3.4 Phylogenetic relationship of novel astroviruses with in the *Astroviridae* family. A partial *RdRp* fragment (corresponding to positions 3659-4041bp in HAstV NC_001943.1) was used to construct a neighbour-joining tree using the percentage distance model on MEGA5. Mamastrovirus Genogroup I and Avastrovirus were collapsed to provide a better view of the relationship of taxa within genogroup II to which our novel bat astroviruses belong. Full tree including all taxa in the collapsed clades is in Appendix E.

3.3   Identification and characterisation of coronaviruses

3.3.1   Detection of coronaviruses

Eighty-three faecal samples representing 15 different bat species were screened for coronavirus RNA. Thirteen out of 83 were positive; sequences analysis and preliminary phylogenetic analysis showed that the 11 of the viral sequences belong to the *Alphacoronavirus* genus and 1 to the *Betacoronavirus* genus (**Error! Not a valid bookmark self-reference.**).

Alphacoronaviruses identified in *M. natalensis* were identified in MSTM2, an adult female, and MSTM6, a sub-adult male. Viral sequences identified in *N.capensis*, GrNC1, GrNC3 and GrNC6 were identified in post-lactating females and GrNC2, GrNC4, GrNC5 and GrNC7 were identified in adult males. GrNC8 escaped before measurements and data could be recorded. NCL_MCO1 *M. condylurus* and VHNC2 *N. nana* was identified in adult male bats trapped at Ndumo and Vryheid in KwaZulu Natal. The Betacoronavirus sequence was identified in a pregnant female from Phinda in KwaZulu Natal. Maps showing the distribution of each species in Africa can be found in Appendix C.

Table 3.3 Bats screened for coronaviruses by PCR. The first two columns indicate bat family and species tested in this study. The third column shows the number of individuals of each species tested, followed by the number of PCR positive samples and the genus to which the identified virus belongs.

Family	Species	No. tested	PCR positives	Genus
Hipposideridae	*Hipposideros caffer*	6		
Miniopteridae	*Miniopterus natalensis*	13	2	α
	Miniopterus fraterculus	6		
Molossidae	*Chaerephon pumilus*	6		
	Tadarida aegyptiaca	3		
	Mops condylurus	1	1	α
Nycteridae	*Nycteris thebaica*	3		
Rhinolophidae	*Rhinolophus clivosus*	8		
	Rhinolophus darlingi	2		
	Rhinolophus swinnyi	3		
Vespertilionidae	*Neoromicia capensis*	10	8	α
	Neoromicia nana	6	1	α
	Neoromicia zuluensis	1	1	β
	Scotophilus viridis	3		
Pteropodidae	*Rosettus aegyptiacus*	12		

3.3.2   Sequence and phylogenetic analysis of novel coronavirus sequences

The generated sequences were aligned with coronavirus sequences available in the NCBI Genbank database using the ClustalW algorithm in MEGA v.5.1. Neighbour-joining method was used to infer a preliminary phylogenetic relationship between the viral sequences (**Error! Reference source not found.**).

Positive samples were re-amplified in an assay obtain a longer *RdRp* gene fragment for confirmation of results; this had been found to give better resolution for grouping coronaviruses confidently (Drexler *et al.* 2010).

101

3.3.2.1 *Alphacoronaviruses*
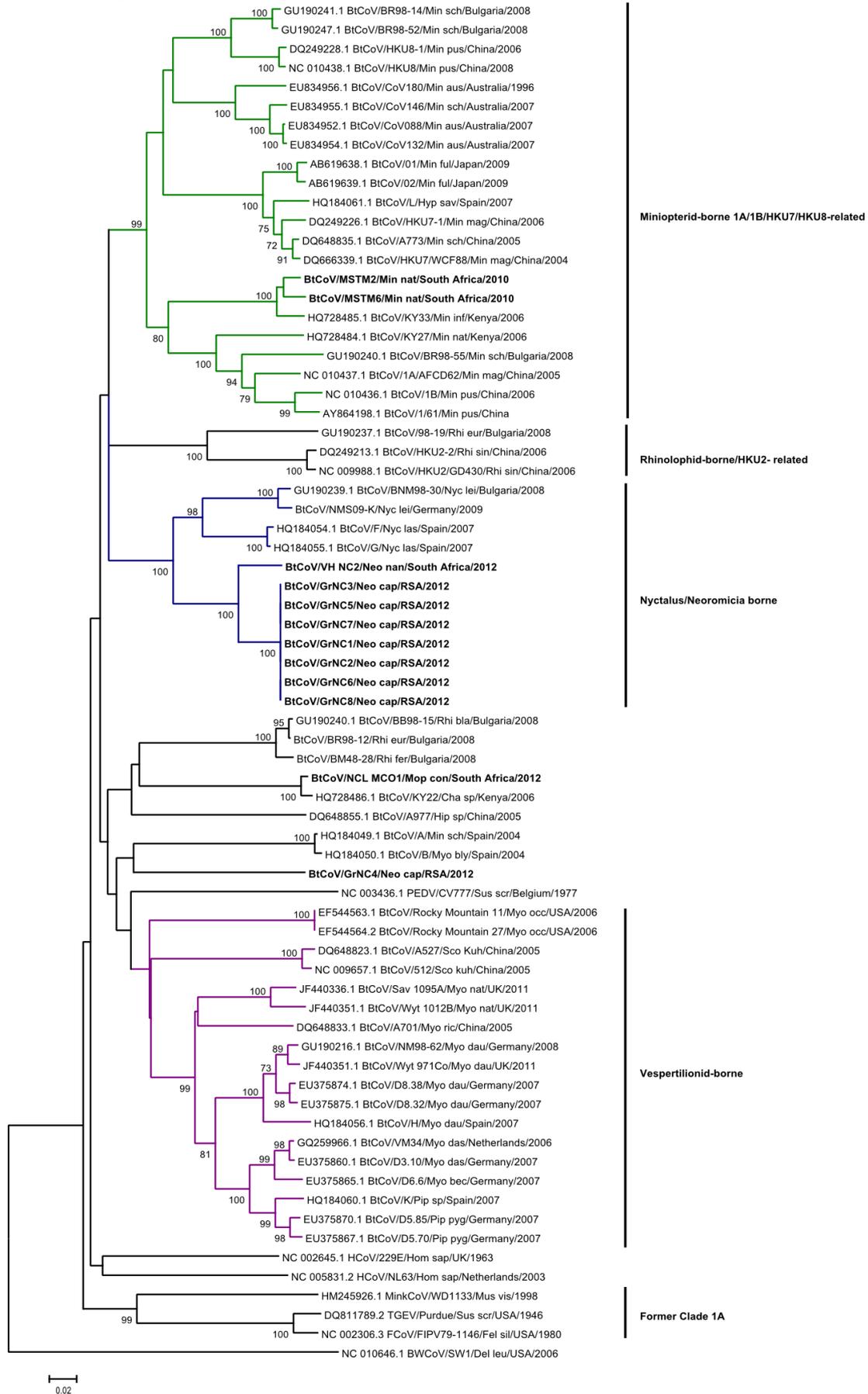
Figure 3.5 Partial *RNA-dependent RNA polymerase* gene phylogeny including the novel alphacoronaviruses from South African bat species. Phylogenetic inference analysis using a 395bp partial *RdRp* gene sequence (which corresponds to positions 14092-14487bp in MinkCoV HM245926.1 sequence) was performed in MEGA v5.1 with the Neighbour-joining method. The percentage distance model was used to determine evolutionary distance between taxa and consensus tree was inferred from 1000 bootstrap replicates. The Beluga whale coronavirus (BWCoV/SW1/Del leu/USA/2006_NC_010646.1) was used as an outgroup. Taxa are indicated at the end of the branches providing information on the host and country in which the sequence was identified. Genbank accession numbers are indicated and host bat family/genus is indicated to the right of the taxa. Novel sequences identified in this study are bold typed.

Phylogenetic analysis was based on the 395bp PCR fragment because sequences available for analysis on Genbank were mostly for this conserved region of the *RdRp* gene (**Error! Reference source not found.**). The difference between BtCoV/MSTM2/Min nat/South Africa/2010 and BtCoV/MSTM6/Min nat/South Africa 2010 is 2.5%. The closest relative of these *Miniopterus* viral sequences were identified in Kenyan *Miniopterus* bats (*M. inflatus* and *M. natalensis*) with BtCoV/KY33/Min_inf/Kenya/2006_HQ728485.1 and BtCoV/KY27/Min_nat/Kenya/2006_ _HQ728484.1; 3.8% and 20.3% respectively. MSTM2 and MSTM6 cluster with bat coronavirus 1A (NC_010437.1) and 1B (NC_010436.1) (and related viruses) which were both identified in Chinese Miniopterus bats *M. magnater* and *M. pusillus*. MSTM2 and 6 are part of a monophyletic clade which consists of viral sequences identified in bats from Europe, Africa, Asia and Oceania which belong to the same family *Miniopteridae*.

Other sequences identified in this study BtCoV/GrNC1/Neo cap/RSA/2012 to BtCoV/GrNC8/Neo cap/RSA/2012 and BtCoV/VHNC2/Neo nan/RSA/2012 were identified in serotine bats *N. capensis* and *N. nana*; these bats belong to the *Vespertilionidae* family. Their sequences cluster with sequences identified in *Nyctalus* bats from Europe. All coronaviral sequences identified *in N. capensis* are identical with BtCoV/GrNC4/Neo cap/RSA/2012 which does not cluster with the other serotine viruses. It represents a unique alphacoronavirus lineage and is most closely related to viral sequences identified in Spanish bats *M. schreibersii* and *Myotis blythii* with a pairwise difference of 24.6% and 25.1 % respectively.

103

A viral sequence was also identified in *Mops condylurus*, the sequence is most closely related to BtCoV/KY22/Cha_sp/Kenya/2006_HQ728486.1 identified in *Chaerephon pumilus* in Kenya with a sequence difference of only 4% in the analysed fragment. Both bats belong to the same family *Molossidae.*

3.3.2.2 *Betacoronaviruses*
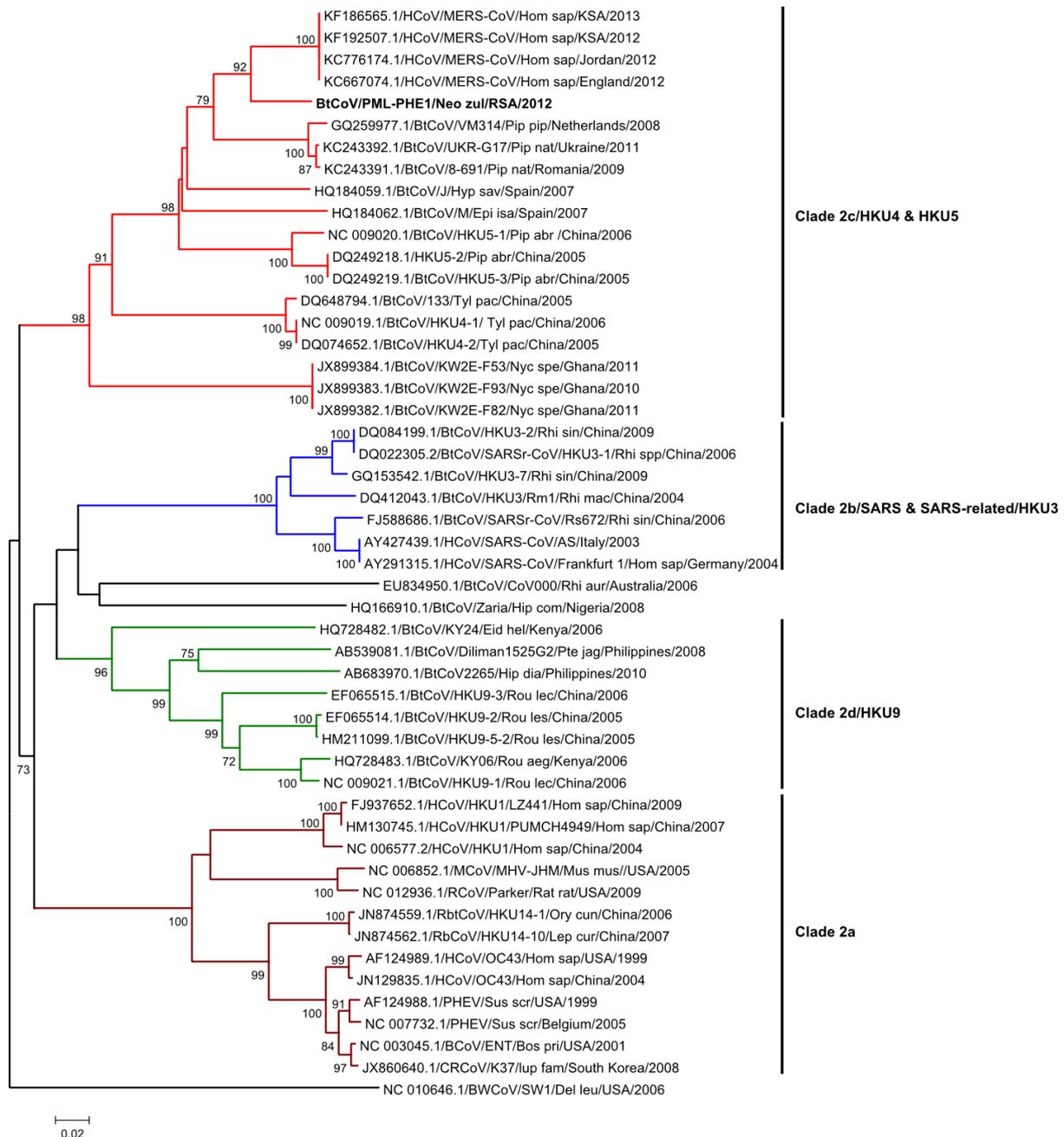
Figure 3.6 Evolutionary relationship of coronaviruses including the novel betacoronvirus identified in *N. zuluensis* (BtCoV/PML/2011/Neo zul/RSA/2012). Neighbour-joining method using the percentage distance model was used to infer the phylogenetic relationship between taxa;

relationship between taxa was confirmed by 1000 bootstrap replicates. A 395bp partial RdRp fragment was used in analysis and the Beluga Whale coronavirus (NC_010646.1/BWCoV/SW1/Del leu/USA/2006) was used as the outgroup in the analysis.

One betacoronavirus was identified in this study; PML-PHE1 was identified in a pregnant female bat trapped at Phinda in KwaZulu Natal. Phylogenetic analysis showed that the virus clusters with clade 2c betacoronaviruses; HKU4 and 5 bat viruses and the recently identified Middle East Respiratory Syndrome coronavirus (MERS-CoV). PML-PHE1 most closely related to MERS-CoV based on 395bp partial *RdRp* gene fragment neighbour-joining (Figure 3.6). A longer *RdRp* fragment (GenBank accession no. KC869678) was obtained for analysis (Figure 3.7). Phylogenetic inference was obtained by Maximum-likelihood analysis in MEGA5 and Bayesian analysis in MrBayes v3.1 (Bayesian inference is in Appendix E). Results observed in *RdRp* analysis were confirmed by analysis of a partial *spike* gene fragment (Figure 3.8) (Ithete *et al.* 2013).
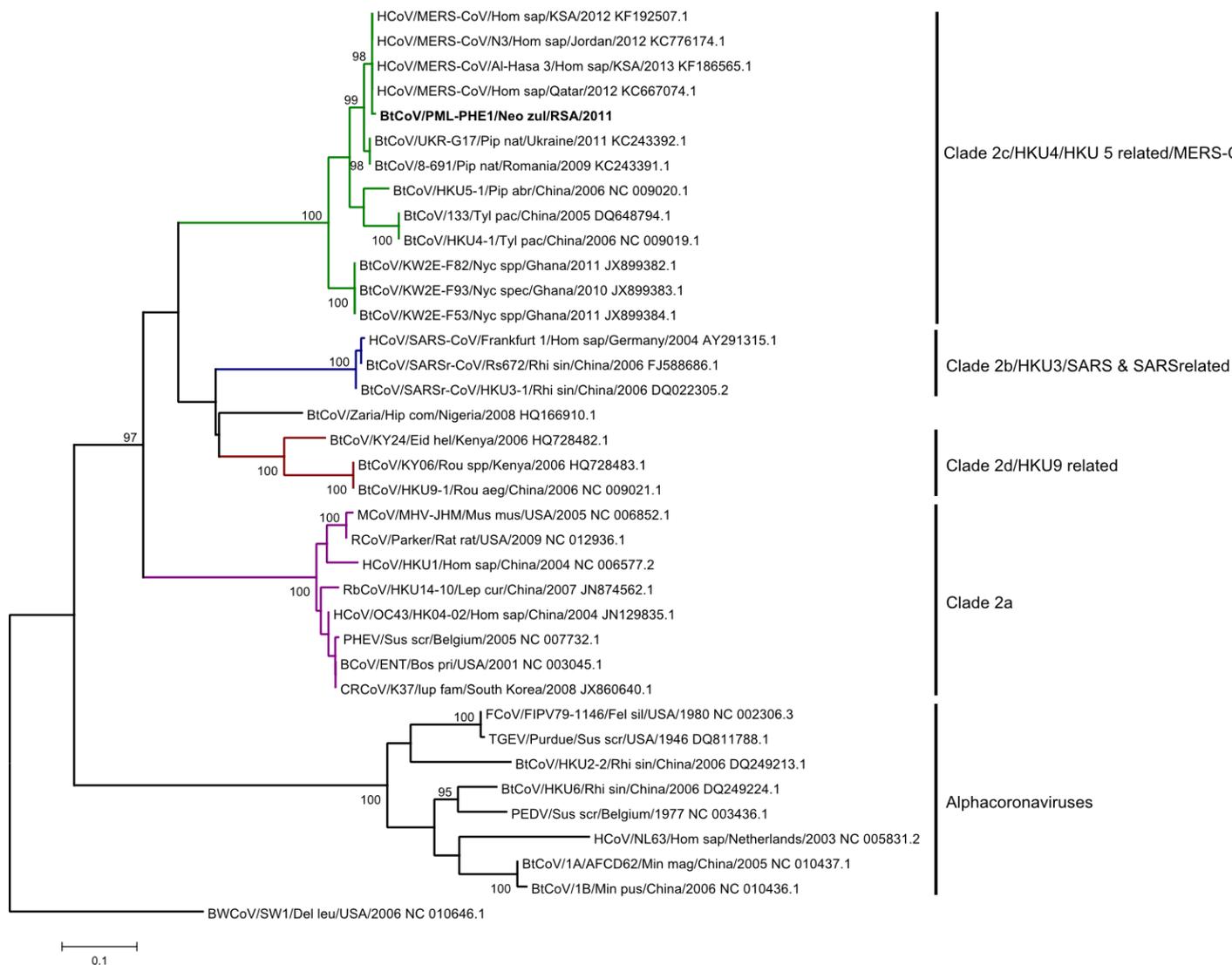
Figure 3.7 Partial *RdRp* gene phylogeny of coronaviruses including the novel PML-PHE1 betacoronavirus identified in *Neoromica zuluensis*. Maximum likelihood was performed using translated partial *RdRp* fragment using the Whelan and Goldman substitution model and the consensus tree was inferred from 1000 bootstrap replicates. The percentage of replicates confirming relationship between taxa are indicated at the branch nodes and values lower than 90% were excluded from the tree; Blue Whale coronavirus (NC_010646.1) was used as an outgroup and the novel viral sequence is underlined and bold typed. Taxa are listed with Genbank accession numbers and the coronavirus clades are depicted to the right of the taxa.
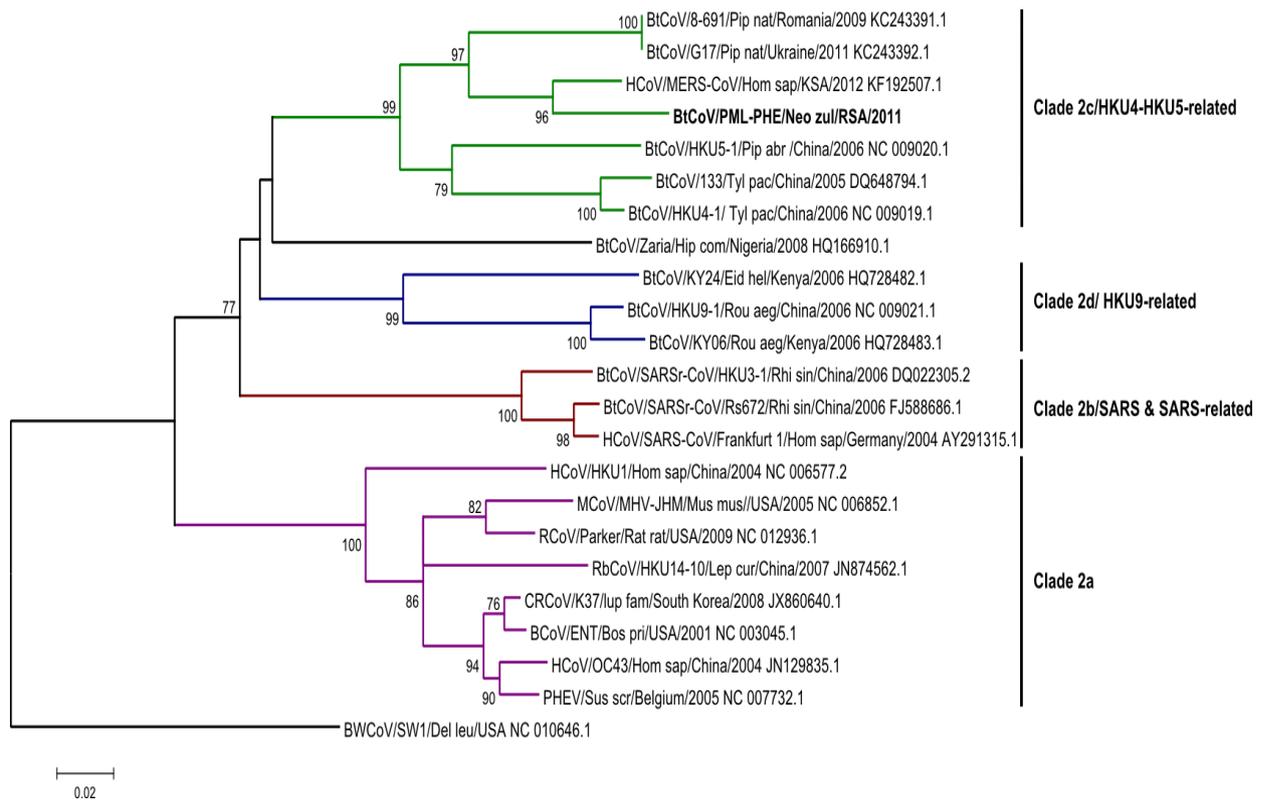
Figure 3.8 Phylogenetic relationship between members of the *Betacoronavirus* genus using a partial spike fragment. The Neighbour-joining method using the percentage distance model with complete gap deletion and the consensus tree was inferred from 1000 bootstrap replicates. The Beluga whale coronavirus (NC_010646.1/BWCoV/SW1/Del leu/USA/2006) was used as the outgroup and a 231nt fragment was used for analysis. The coronavirus identified in *Neoromicia zuluensis* (BtCoV/PML-PHE1/Neo zul/RSA/2012) is highlighted in bold and underlined. The values at the branch nodes represent the bootstrap replicates supporting the relationship between the taxa analysed.

Pairwise comparison of the 395bp gene fragment nucleotide sequence showed that PML-PHE1 differs from MERS-CoV by 8.4%, by comparison the difference between PML-PHE1 and prototype clade 2c bat viruses HKU 4 (*Tylonycteris pachypus*) and HKU5 (*Pipistrellus abramus*) was 22.5% and 16.9% respectively. The closest bat relative to PML-PHE1 are

106

VM314 which was identified in *P. pipistrellus* from the Netherlands, UKR-G17 and 8-691 identified in *P. nathusii* from Ukraine (12.8%) and Romania (13.0%) respectively. The closest African relatives were identified in *Nycteris* bats from Ghana; which differ from PML-PHE1 by 26.7%.

Pairwise analysis of the amino acid alignment of the 816bp RdRp fragment showed that PMl-PHE1 and MERS-CoV share a 99.6% sequence similarity, with one amino acid difference (Asparagine in PML-PHE1 and Serine in MERS-CoV at position 266 [see Appendix D] in the alignment.

3.4   Hantaviruses in South Africa

3.4.1   Detection of hantaviruses in small mammals

All 1651 lung specimens from rodents and insectivores from South Africa, and 83 bat faecal specimens tested for hantavirus RNA using a Pan-PCR assay targeting a conserved region in the *RdRp* gene on the L segment were negative.

3.4.2   Detection of hantavirus infection in South Africa

3.4.2.1   *Patient screening by direct-coating IgG ELISA*

In this study 1442 residual sera were collected from National Health Laboratory Service laboratories at Tygerberg, Paarl, Oudtshoorn and Vredendal between August 2010 and April 2012. Sera used were sent in for routine diagnostic testing. Of the 1442 samples tested, 210 (14.6%) were reactive against recombinant antigens DOB-rN and PUU-rN (see Appendix G for table); 152 were reactive against DOB-rN, 111 against PUUrN and 53 against both antigens.

Samples from acutely ill patients were collected from Groote Schuur Hospital and Tygerberg Hospital between November 2011 and February 2013. The samples were tested for IgG antibodies against DOB-rN and PUU-rN, and 7 out of 10 were reactive in the assay (Appendix G).

3.4.2.2   *Western Blot*

All samples that were reactive in the IgG ELISA were tested further for confirmation. Samples were first screened with the *recom*Line IgG Bunyavirus immunoblot kit (Mikrogen Diagnostik, Germany); samples that were negative on the commercial kit were tested against a fifth hantavirus antigen using the in-house immunoblot blot assay in which sera were tested against Sangassou virus recombinant nucleocapsid protein (SANG-rN). Thirty-seven of the

ELISA reactive serosurvey samples tested positive in the Western blot assays (Table 3.4). All seven ELISA reactive samples obtained from acutely-ill patients were non-reactive in both immunoblot assays and against all antigens.

Table 3.4 Samples that tested positive on the *recom*Line Bunyavirus IgG Immunoassay and the in-house Sangassou Western blot assay. Samples were tested against recombinant N proteins from hantaviruses DOBV, HTNV, PUUV, SANGV and SEOV.

Samples	*recom*Line Bunyvirus IgG					In-house WB
	PUUV+HTNV	**PUUV**	**HTNV**	**DOBV**	**SEOV**	**SANGV**
Han34	+	-	+	++	-	
Han72	-	-	-	-	-	+
Han155	±	-	±	+	-	
Han157	-	-	-	-	-	+
Han187	-	-	-	-	-	+
Han198	-	-	-	+	-	
Han213	-	-	-	+	-	
Han217	±	±	±	±	-	+
Han242	-	-	-	++	-	
Han255	-	-	-	-	-	+
Han298	-	-	-	++	-	
Han311	-	-	+	+	-	
Han338	-	-	-	-	-	+
Han362	-	-	-	+	-	
Han479	-	-	-	++	-	
Han553	-	-	-	-	-	+
Han555	-	±	-	-	-	+
Han696	-	-	-	-	-	+
Han713	+	+	-	-	-	
Han733	-	-	-	++	-	
Han745	+	-	+	+	-	
Han751	-	-	-	+	-	
Han761	-	-	-	++	-	
Han845	-	-	-	++	-	
Han859	-	-	-	-	-	+
Han921	-	-	-	-	-	+
Han933	-	-	-	-	-	+
Han944	-	-	-	-	-	+
Han982	-	-	-	++	-	
Han991	-	-	-	++	-	
Han1033	-	-	-	+	-	
Han1097	-	-	-	-	-	+
Han1153	-	-	-	-	-	+

Han1230	-	+	-	-	-		
Han1289	-	-	-	-	-	+	
Han1316	-	-	-	++	-		
Han1323	-	-	-	++	-	1	

3.4.2.3 *Indirect immunofluorescence test*

Western blot positive sera were tested by IIFT for four hantaviruses; results are shown below (**Error! Not a valid bookmark self-reference.**). Immunofluorescence images for some of the samples are shown in Figure 3.9 to Figure 3.11. Samples were considered to be positive if a grainy fluorescence was observed in the cytoplasm of the infected cells. Of the 37 samples tested, 14 were reactive against hantaviruses.

Samples which showed strong signal in the initial IIFT tests were titrated by serially diluting the sera (1:20 to 1:160). Samples 217, 1033 and 1439 showed antibody reactivity at 1:160 dilution: Han217 and 1033 against HTNV, 1033 and 1439 against DOBV and SANGV (

Table 3.6).

Table 3.5 Summary of results obtained in IIFT assay. Patient sera were tested against for hantaviruses: DOBV, HTNV, PUUV and SANGV. Sera that tested positive on IIFT are highlighted in bold

Sample	DOBV	HTNV	PUUV	SANGV
Han31	-	-	-	-
Han34	**-**	**-**	**-**	**+**
Han53	-	-	-	-
Han63	-	-	-	-
Han66	-	-	-	-
Han72	**-**	**-**	**+**	**-**
Han86	-	-	-	-
Han121	-	-	-	-
Han155	-	-	-	-
Han187	**-**	**+**	**-**	**+**
Han198	-	-	-	-
Han217	**+**	**+**	**-**	**++**
Hsn242	-	-	-	-
Han311	-	-	-	-
Han399	-	-	-	-
Han479	**-**	**-**	**+**	**+**
Han499	-	-	-	-
Han540	-	-	-	-
Han544	-	-	-	-
Han555	-	-	-	-
Han556	-	-	-	-
Han591	-	-	-	-
Han595	-	-	-	-
Han713	**-**	**-**	**-**	**+**
Han733	-	-	-	-
Han745	**-**	**-**	**-**	**+**
Han754	-	-	-	-
Han761	-	-	-	-
Han784	-	-	-	-
Han845	-	-	-	-
Han933	**+**	**+**	**-**	**-**
Han944	**-**	**+**	**-**	**-**
Han982	-	-	-	-
Han991	-	-	-	-
Han1033	**+**	**+**	**-**	**++**
Han1097	-	-	-	-
Han1153	-	-	-	-
Han1289	**-**	**+**	**-**	**+**
Han1316	**-**	**-**	**-**	**+**

Han 1323	+	-	-	-
Han1439	+	+	-	++
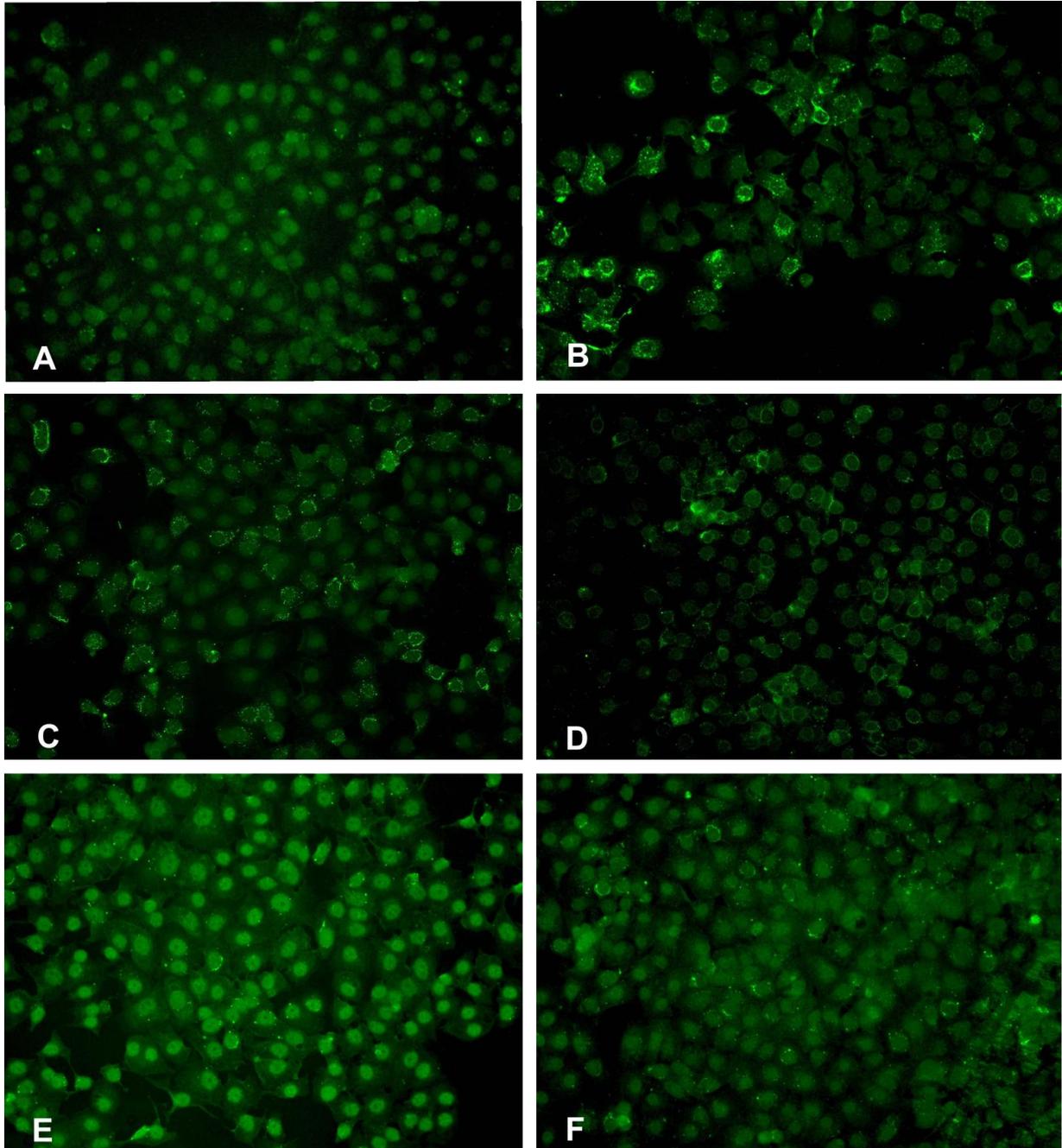
Figure 3.9 Immunofluorescence slides for Sangassou virus. Positive samples are shown along with positive and negative controls: A Negative control, B Positive control, C Han 217, E Han 242 and F Han 713.
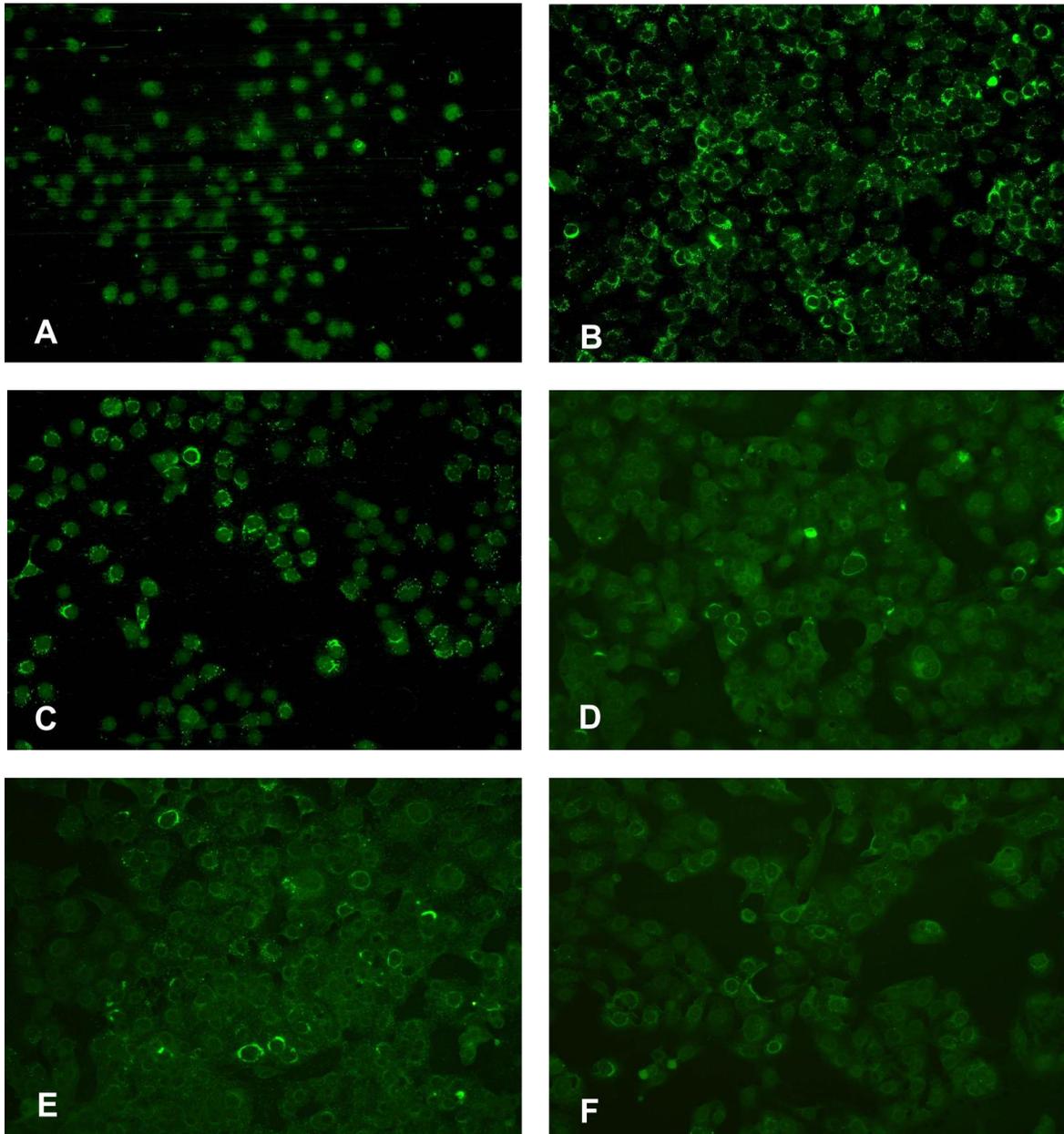
Figure 3.10 IIFT slides for Hantaan virus; the images above were obtained for samples A Negative control, B Positive control, C Han 217, D Han 944, E Han 1033, and D Han 1439.
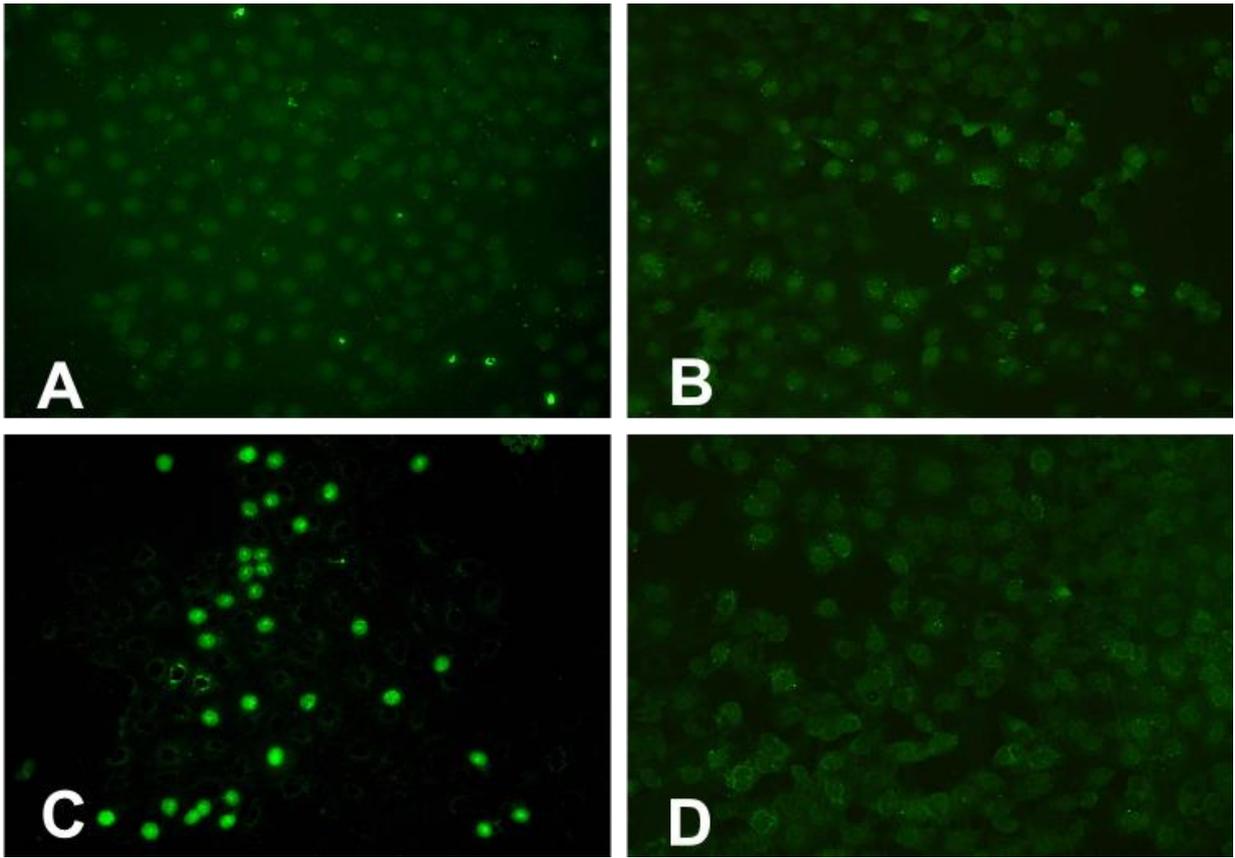
Figure 3.11 IIFT slides for Dobrava (strain Slovakia); above are the images obtained for samples A Negative control, B Positive control, C Han 34 and D Han 1439.

Table 3.6 IIFT results for titrated samples. Patient sera were diluted (1:20 to 1:160) to determine the highest dilution at which antibody reaction is still detectable. The gender and age of the patients from which samples were obtained as well as the source laboratory are included.

Samples	Gender	Age	Source	Virus titres			
				DOBV	HTNV	PUUV	SANGV
Han 34	M	51	TYG	–	–	–	1:40
Han 72	F	27	TYG	–	–	1:80	–
Han 187	F	56	TYG	–	1:20	–	1:20
Han 217	F	68	TYG	1:20	1:160	–	1:80
Han 479	F	18	TYG	–	–	1:20	1:20
Han 713	M	57	TYG	–	–	–	1:20
Han 745	F	66	TYG	–	–	–	1:20
Han 933	F	41	TYG	–	1:80	–	–
Han 944	F	34	TYG	–	1:20	–	–
Han 1033	F	27	PAA	1:160	1:160	–	1:160
Han 1289	F	58	OUD	–	–	–	1:40
Han 1316	M	29	PAA	–	–	–	1:40
Han 1323	M	41	PAA	1:40	–	–	–
Han 1439	M	–	VRE	1:160	1:80	–	1:160
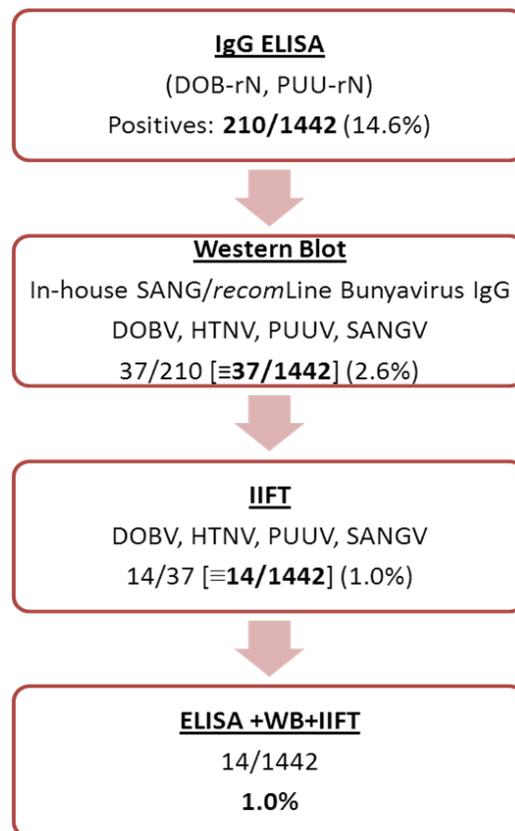
Figure 3.12 Screening algorithm and summary of serosurvey results. A total of 1442 samples were screened by IgG ELISA; 210 samples were reactive against recombinant antigens DOB-rN and PUU-rN and were confirmed by Western blot assays indicated. Samples positive by Western blot were then tested by IIFT. The final seroprevalence value (1.0%) was obtained by combining the results of all the assays used.

The results of the screening and IgG ELISA and the subsequent confirmatory assays were combined using a screening algorithm used in (Klempa *et al.* 2010; 2012b) for seroprevalence studies conducted in Guinea; a seroprevalence of 1.0% was observed in the study population (**Figure 3.12**).

Chapter 4

4   Discussion and Conclusions

4.1   Identification of bat-borne coronaviruses

Following the SARS outbreak a decade ago and the subsequent identification of bats as reservoir hosts of SARS/SARS-related cluster of coronaviruses (Li *et al.* 2005), numerous bat coronaviruses have been identified wherever bats occur; the ICTV currently recognises 5 bat alphacoronavirus and 9 bat betacoronavirus species (King *et al.* 2012). Coronaviruses are also known from various other mammal and bird species. Phylogenetic analysis of known coronaviruses suggests that bats are the source of mammalian viruses in the *Alphacoronavirus* and *Betacoronavirus* genera, and that birds are the source of coronaviruses belonging to the *Gammacoronavirus* and the tentative *Deltacoronavirus* genus (Woo *et al.* 2012a). Recent studies provide evidence for the bat origin of the human coronaviruses HCoV-229E (approximately 200 years ago) and NL63 in the more distant past (approximately 500-800 years ago) (Pfefferle *et al.* 2009; Huynh *et al.* 2012). The recently emerged dangerous human-pathogenic MERS-CoV clusters closely with bat viruses HKU4 and HKU5 (van Boheeman *et al.* 2012) suggesting that like SARS-CoV it may have emerged from a bat source, presumably via an intermediate host.

In this study, 13 coronavirus sequences were identified in various South African bat species; all rodent and shrew samples screened were negative for coronavirus RNA. To date, no rodent coronaviruses of zoonotic potential have been identified, but the close relationship between HCoV-HKU1 and murine hepatitis virus, suggest that they share a common ancestor (Woo *et al.* 2012a).

 Alphacoronaviruses were identified in three different bat species*: Miniopterus natalensis, Mops condylurus* and *Neoromicia capensis* trapped at various localities in the Western Cape and KwaZulu-Natal provinces. A betacoronavirus was identified in *Neoromicia zuluensis* trapped in KwaZulu-Natal.

4.1.1   Alphacoronaviruses

Phylogenetic analysis shows that the alphacoronaviruses identified in this study belong to three difference lineages within the genus: (BtCoV/KY22/Cha pum/Kenya/2006) identified in *Chaerephon pumilus*, a Molossid bat from Kenya (Tong *et al.* 2009). Eight alphacoronavirus sequences BtCoV/VH_NC2/Neo nan/South Africa/2012 and BtCoV/GrNC1 to NC8 (94.9% sequence similarity) identified in *N. capensis* clustered together with sequences identified in *Nyctalus* genus bats from Europe (Drexler *et al.* 2010; Falcón *et al.* 2011). BtCoV/GrNC4/Neo cap/RSA/2012 does not cluster with viral sequences from the same colony and is most closely related to viral sequences BtCoV/A/Min sch/Spain/2004_HQ184049.1 and BtCoV/B/Myo bly/Spain/2004_HQ184050.1 from *Miniopterus schreibersii* and *Myotis blythii*.

The sequences identified in *Miniopterus natalensis* BtCoV/MSTM2/Min nat/South Africa/2010 and BtCoV/MSTM6/Min nat/South Africa/2010 (sequence similarity of 97.5%) clustered with sequences from bats of the genus *Miniopterus*, most closely with BtCoV/KY33/Min inf/Kenya/2006 (with a pairwise sequence similarity of 96.2%). The virus sequences cluster together to form a monophyletic clade, suggesting that all alphacoronaviruses from *Miniopterus* bats originated from one common ancestor. *Miniopterus* bats belong to the sole genus *Miniopterus* within the family *Miniopteridae*, which was previously a subfamily within the *Vespertilionidae* family (Eick *et al.* 2005). The miniopterid bats form two separate monophyletic clusters; African and European taxa cluster together, while Asian and Australasian taxa form a separate cluster (Miller-Butterworth *et al.* 2005). Although there is currently no evidence implicating alphacoronaviruses hosted by *Miniopterus natalensis* in zoonotic transmission, this illustrates the evolution of closely related viruses in geographically separated host populations, which may have implications for potential zoonotic viruses hosted by *Miniopterus* bats. For example, Swanepoel *et al* detected Marburg viruses in *Miniopterus inflatus* bats in the Democratic Republic of the Congo (Swanepoel *et al.* 2007). Negredo *et al* (2011) described a new filovirus Lloviu (LLOV) in dead *M. schreibersii* following an unexplained mass die-off in Spain. Though a causal relationship between could not be established between infection and bat death, the identification of a novel filovirus distinct from Ebola and Marburg has other implications. *M. schreibersii* has a wide geographic distribution spreading from west Europe to China. *Miniopterus* bats occur in Europe, Africa and as far as south East Asia with the exception of a few islands in Oceania. The monophyly observed for Miniopterid alphacoronaviruses may be

117

observed for other viruses they host. More unidentified filoviruses may therefore be present in other geographically separated *Miniopterus* species thus presenting a potential threat. Further investigations are required on filovirus diversity in bats is to draw any definite conclusions.

Two sequences from Falcón *et al* (2011) do not fit this picture: BtCoV/A/Min sch/Spain/2004_HQ184049.1 clusters with coronaviruses from *Myotis* and *Pipistrellus* bats and not with those from *Miniopterus* bats, whereas BtCoV/L/Hyp sav/Spain/2007_HQ184061.1 clusters with the *Miniopterus* virus sequences. Pairwise analysis of BtCoV/A/Min sch/Spain/2004 with BtCoV/B/Myo bly/Spain/2004, its closest relative, shows a sequence similarity of 99.5%. This suggests that an inter-species spillover may have occurred; an alternative explanation would be a mix-up of samples, species allocations or test results. The bats from which the sequences were isolated were trapped in the same location (Falcón *et al*. 2011). Spillover between different bat species has been reported; Lau *et al* (2012) reported the transmission of HKU10 from the fruit bat *Rousettus leschenaulti* bat to the insectivorous bat *Hipposideros pomona*.

A recently published study by Geldenhuys *et al* (2013) identified three novel alphacoronaviruses in South African bat species. Phylogenetic inferences including the 277bp sequences available was performed in MEGA5 (Appendix E). BtCoV/Irene/Min spp/RSA/2007_JQ519817.1 from a bat trapped in Irene (Gauteng province) did not cluster with BtCoV/MSTM2/Min nat/South Africa/2010 and BtCoV/MSTM6/Min nat/South Africa/2010 but it falls within the Miniopterid clade; suggesting that the bat may be from another *Miniopteru*s species that occurs in South Africa. BtCoV/1364/Mop spp/RSA/2011_ JQ519819.1 identified in a Mops bat from Limpopo province clusters with BtCoV/NCL_MCO/Mop con/RSA/2012 identified in *Mops condylurus* and BtCoV/KY22/Cha spp/Kenya/2006_HQ728486.1, as with the *Miniopterus* viruses, our virus is more closely related to the Kenyan virus. Geldenhuys *et al* also identified a virus in a *Neoromicia* bat, BtCoV/167/Neo spp/RSA/2007_JQ519818.1 which clusters with BtCoV/VH_NC2/Neo nan/RSA/2012 in the *Nyctalus/Neoromicia* clade.

Two different lineages of alphacoronaviruses were identified in the *N. capensis* trapped at Greyton. Seven of the viral sequences are identical (this was confirmed by two PCR assays) and the eighth (GrNC4) represents a previously undescribed lineage most closely related to viral sequences identified in *M. blythii* and *M. schreibersii* identified in Spain (Falcón *et al*. 2011).

118

The divergence among South African viruses especially those hosted by closely related bat species suggests that diversity of coronaviruses hosted by South African bats species is high. Since only a short sequence was used for this analysis, more studies are required to obtain additional sequence information on these viruses to get a clearer picture on bat-borne coronaviruses in South Africa.

The occurrence of two or more different lineages in bats from a single colony confirms findings by other groups of the co-circulation of different coronavirus lineages (Chu *et al* 2008). Similarly, studies conducted in *Eidolon helvum* colonies in Ghana also showed that diverse paramyxoviruses can occur in a single colony. Recombination between coronaviruses in bat colonies is theoretically possible, but it has not been reported. Type II feline coronavirus strains emerged as a result of recombination between Canine coronavirus and type I feline coronavirus (Herrewegh *et al.* 1998).

4.1.2   Betacoronaviruses

Only one of the novel bat coronaviruses (BtCoV/PML-PHE1/Neo zul/South Africa/2012) identified in our study is a betacoronavirus. Neighbour-joining analysis of the 395bp PCR fragment showed that the viral sequence clustered with clade 2c (HKU4/HKU5) viruses identified in *Tylonyceteris* and *Pipistrellus* bats in China, Ghana, Netherlands and Ukraine (Annan *et al.* 2013). Of all known clade 2c viruses it was the one clustering most closely with the recently emerged MERS-CoV. To confirm this, the PCR fragment was extended at the 5' end in order to obtain an 816bp fragment, and a partial *Spike* fragment was also amplified, sequenced and analysed to confirm the close relatedness using a structural gene. The sequence similarity between MERS-CoV and the South African bat PML-PHE1 is comparable to that between SARS-CoV and bat SARSr-CoV-Rs672 which also has a similarity of 99.6 % with a single amino acid difference at position 266 (asparagine in BtCoV/PML-PHE1/Neo zul/South Africa/2012 versus serine in MERS-CoV) and 173 (asparagine in SARS-CoV versus serine in bat SARSr-CoV) respectively [Appendix D]. The high sequence similarity between MERS-CoV and bat-derived clade 2c coronaviruses supports the hypothesis that like SARS-CoV, MERS-CoV emerged from an ancestor virus hosted by *Vespertilionidae* bats (both *Pipistrellus* and *Neoromicia* bats are vespertilions). Furthermore, the very close relatedness between MERS-CoV and PML-PHE1, compared to clade 2c viruses from European and Asian bats, might suggest that MERS-CoV may have originated in Africa. However this would have to be confirmed by more extensive sampling

119

and extensive sequencing of the viral genome of vesper bat species. Past exportation of infectious disease from Africa to the Arabian Peninsula, such as Rift Valley fever (Bird *et al.* 2007), proves that such a scenario is plausible. The most likely scenario however is that the MERS-CoV progenitor is hosted by a closely related bat species occurring in the Arabian Peninsula. Memish *et al.* (2013) reported on a MERS-related viral sequence in bat from Saudi Arabia. Phylogenetic analysis using a partial *RdRp* alignment 190 nucleotides long shows that the sequence identified in *Taphozous peforatus* (Egyptian tomb bat) *Emballonuridae* family, is the closest related animal-derived sequence to MERS-CoV. Two independent studies (Reusken *et al.* 2013 and Perera *et al.* 2013) conducted on dromedary camels from the Middle East and North Africa, detected neutralising antibodies against MERS-CoV. The detection of neutralising antibodies does not prove that the virus circulating in camels is MERS-CoV, but it might be a closely related virus. One might hypothesise that bats are the natural host of the ancestor virus of MERS-CoV, which was then transmitted to camels, who in turn act as intermediate hosts transmitting the virus to humans. However, more surveillance of domestic animals and bats in the region needs to be conducted in an attempt to isolate the virus from an animal source. In depth case reports for all MERS patients are required, in order to determine the mode of transmission and source of human infections.

4.2   Astroviruses in South African bats

This study is the first to identify astroviruses in South African bats. All other known bat astroviruses were found in different bat species in China and Germany; the first study to report a bat astrovirus was Chu *et al* (2008), followed by two more studies in China (Zhu *et al.* 2009; Chu *et al.* 2010), and Drexler *et al.* (2011) reported on bat astroviruses in Germany. In our study 33 out of a total 83 bats tested were positive for astrovirus RNA; however not all PCR positive samples could be sequenced and analysed despite multiple attempts made.

We identified astroviruses in seven different bat species belonging to the families *Miniopteridae, Molossidae, Rhinolophidae* and *Vespertilionidae*. All viruses identified in our study belong to genogroup II in the *Mamastrovirus* genus and cluster with other bat astroviruses. Although statistical support for the base nodes is weak, genogroup II can be divided into four clades. The first clade consists of Chinese and South African bat astrovirus sequences, the second contains Chinese and German bat astroviruses, the third consists of bat, mink, ovine and human (VA, HMO) astroviruses and the fourth contains bat, rabbit and

porcine astroviruses (Figure 3.4). Bat astroviruses occur in all four clades; the first two clades consist of bat viruses only.

Viral sequences from the same bat species do not necessarily cluster together in the same clade. The same phenomenon was observed for bat astroviruses from China and Germany. Additionally, the viral sequences do not cluster according to geographical location. South African viral sequences are dispersed with viruses from China. The high genetic diversity observed among viral sequences derived from bats of the same species and roosting site was observed by other groups (Chu *et al.* 2008; Drexler *et al.* 2011). The highest sequence similarity observed in our study was between viral sequences from *Tadarida aegyptiaca* and *Miniopterus natalensis* (97%) both trapped in the Western Cape but at different locations; a similar observation was made among Chinese sequences where sequences from *Tylonycteris robustula, Myotis ricketii* and *Miniopterus schreibersii* had similarities higher than 95%. As in South Africa, all three bats in which AstVs were identified were trapped in different locations (Hainan, Jiangxi and Hong Kong) (Wu *et al.* 2011). The BtAstV/MSTM12 and BtAstV/TAr1 may have evolved from a common prototype strain. Alternatively recombination events may have occurred in the past, although this could not be proved in our study.

We attempted to obtain an ORF1b-ORF2 fragment for recombination analysis by rapid genome sequencing (Mizutani *et al.* 2007) and PCR (Farkas *et al.* 2012), however this attempt was unsuccessful, possibly due to insufficient viral loads in the samples. We could not attempt virus isolation as our samples were collected and stored in RNAlater® solution making it impossible to retrieve viable virus from the samples. Recombination, although not proven by this study, has occurred over time in the evolution of astroviruses and has made determination of relationships between members of the family difficult. Phylogenetic criteria for this are yet to be established.

Amplification of an ORF1b-ORF2 fragment would have allowed us to perform analysis to determine if recombination points were present in our sequences. Recombination has been reported in classic HAstVs (1-8) (Wolfaardt *et al* 2011); analysis of a HAstV strain clustered with HAstV 6/7 when a partial ORF1a fragment was used for phylogenetic inference, it clustered with HAstV 3 when ORF1b fragment was used for analysis and clustered with HAstV 2 when phylogeny was inferred using ORF2. Chu *et al*. (2011) performed recombination analysis on bat astrovirus sequences in their study but did not find evidence of recombination events.

The high positivity rate in *Miniopterus natalensis* from Table Mountain, *M. fraterculus* from Pietermaritzburg and *Neoromicia capensis* from Greyton suggest that the bats are persistently infected, but more studies including longitudinal surveillance of natural bat colonies over time are required to confirm this observation. Chu *et al.* (2011) suggest that the clustering of viral sequences from different host species may indicate that some bat astroviruses are able to cross the species barrier quite easily. One might therefore speculate that some bat astroviruses have zoonotic potential.

4.3   Co-infection of bats by astroviruses and coronaviruses

Neoromicia bats trapped at Greyton and *Miniopterus* bats trapped on Table Mountain were positive for both coronaviruses and astroviruses. Drexler *et al* (2011) reported on the occurrence of astroviruses, adenoviruses and coronaviruses in a *Myotis* colony in Germany. MSTM2 was co-infected with astrovirus and coronavirus as were all the *Neoromicia* bats from Greyton. The simultaneous occurrence of multiple viruses in healthy bats and detection of viruses over three years of study (Drexler *et al.* 2011) suggests that infection in bats is persistent and maybe lifelong.

4.4   Identification of arenaviruses in rodents

Arenaviruses are hosted by rodents in the *Murinae, Neotominae* and *Sigmodontinae* subfamilies; the Tacaribe virus is the only known bat arenavirus. Ten members of the genus are associated with serious viral haemorrhagic fever disease in Africa and the Americas while LCMV infection in humans may range from asyptomatic to severe disease with meningitis (Gonzalez *et al.* 2007).

The most recently identified human-pathogenic arenavirus is the Lujo virus which originated in Zambia and caused a nosocomial outbreak in South Africa (Paweska *et al.* 2009). There have been no outbreaks of disease in humans since nor has a reservoir host been identified. Surveillance studies (Figure 1.6) have been conducted in southern Africa in the wake of the Lujo outbreak to determine the diversity of arenaviruses in Southern African rodent species; in the process, two novel viruses, Luna and Lunk, were identified in *Mastomys natalensis* and *Mus minutoides* respectively (Ishii *et al.* 2011). The Luna virus is closely related to Mopeia virus which is non-pathogenic (Table 4.1) and Lunk virus represents a unique lineage in the Lassa-LCMV serocomplex.

The first South African arenavirus was identified by Palacios *et al.* (2010) from samples collected in the 1980s: Merino Walk virus in *Myotomys unisulcatus* from Merino Walk in the

Eastern Cape Province. The virus was only identified in one animal and is not known to be associated with human disease.

Table 4.1 Novel arenaviruses identified in Africa in the last decade.

Virus	Host	Country	Reference
Gbagroube	*Mus (Nannomys) setulosus*	Cote d'Ivoire	Coulibaly N'Golo *et al.* 2011
Lujo	*unknown*	Zambia, South Africa	Paweska *et al.* 2008
Luna	*Mastomys natalensis*	Zambia	Ishii *et al.* 2011
Lunk	*Mus minutoides*	Zambia	Ishii *et al.* 2012
Kodoko	*Mus Nannomys minutoides*	Guinea	Lecompte *et al.* 2007
Menekre	*Hylomyscus spp*	Cote d'Ivoire	Coulibaly N'Golo *et al.* 2011
Merino Walk	*Myotomys unisulcatus*	South Africa	Palacios *et al.* 2010
Morogoro	*Mastomys natalensis*	Tanzania	Günther *et al.* 2009

Our study identified eight arenavirus sequences in 1651 South African rodent and shrew lung samples screened in the rodent species *Myotomys irroratus, Micaelamys namaquensis* and *Rhabdomys pumilio*.

Phylogenetic relationship based on partial L *RdRp* gene fragment (Figure 3.2) between Merino Walk-related viruses suggests that there may be some geographical clustering: ELOr 2, 3, 4 identified in *M. irroratus* trapped in East London are most closely related to Merino Walk virus which was identified in *M. unisulcatus* trapped in Merino Walk; the trapping locations are 156km apart both in the Eastern Cape. Viral sequences from animals trapped in the Northern Cape cluster together even though they were identified in two different rodent species. Clustering appears to be geographical rather than based on host phylogeny. Bowen *et al* (2000) and Weaver *et al* (2000) demonstrated geographic clustering for Lassa virus in West Africa and Guanarito virus in Venezuela, however more viral sequences are required to make any conclusions about the distribution of Merino Walk and related viruses.

Phylogenetic analysis based on a partial *GPC* gene confirmed the relationship between the sequence identified in East London and Merino Walk. Despite exhaustive modifications to the protocol, including switching enzymes used for reverse transcription as well as PCR; amplicons could not be obtained for the other 6 samples. For future studies virus isolation will have to be attempted in order to obtain high viral titres for RNA isolation and subsequent sequence analysis.

Figure 4.1 is a map showing the geographical range of the rodents species in which arenavirus sequences were identified; *M. namaquensis* occurs over most of southern Africa, the range of *M. unisulcatus* extends from southern Namibia to parts of the Eastern Cape, and

M.irroratus occurs over most of the Western Cape, along the southern Cape and east coast of the country as far as Limpopo in the northeast of South Africa.
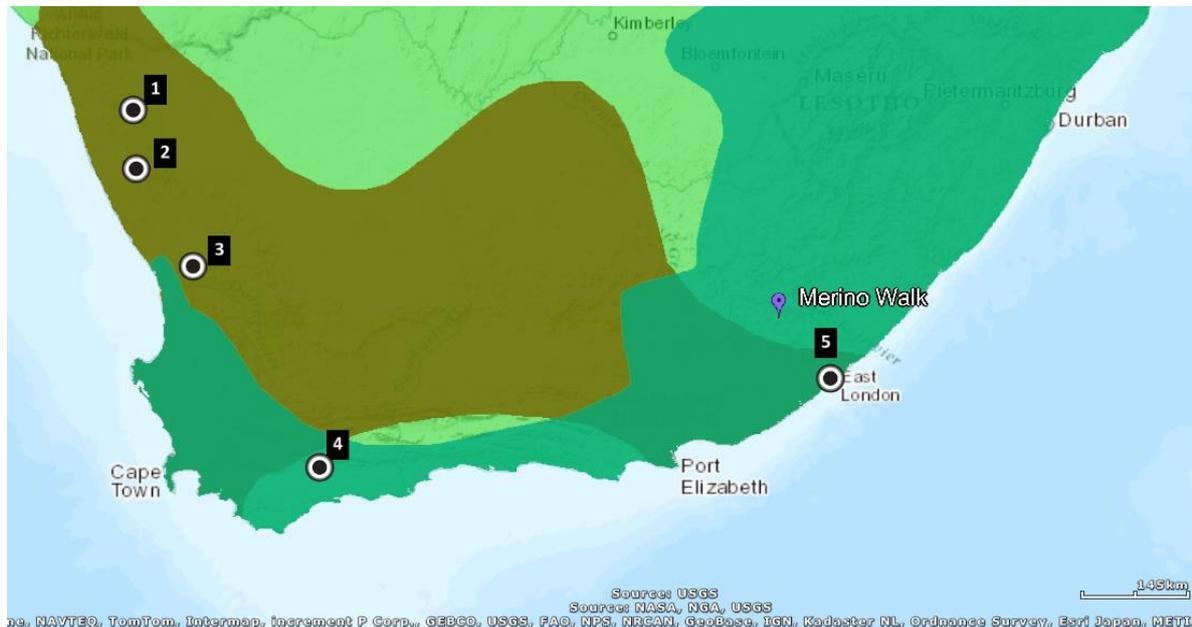
Figure 4.1 Map showing the geographical range of rodent species in which Merino Walk-related viral sequences were identified. The range for *Micaelamys namaquensis* is shown in green, *Myotomys irroratus* in blue and *Myotomys unisulcatus* is shown in orange. Map was drawn with ArcGIS Explorer using data obtained from the IUCN http://www.iucnredlist.org (Coetzee and van der Straeten 2008b; Taylor, Maree, and Monadjem 2008a; Taylor and Monadjem 2008b). Numbers 1, 3-5 are the trapping locations at which animals positive for Merino Walk- related viruses, and number 2 is the location where *R. pumilio* was trapped (distribution is highlighted in orange).

In West Africa, a similar phenomenon has been observed for Lassa virus which is hosted by two species from the *Mastomys* genus, *M. natalensis* and *M. huberti* whose geographical range overlaps (Gonzalez *et al.* 2007). Junin virus which causes VHF in Argentina has two primary hosts, *Calomys musculinus* and *C. laucha*, and *Akodon azarae* as a secondary host.

ATCRp18, identified in *Rhabdomys pumilio*, represents a novel and unique lineage in the Old World arenavirus clade. Phylogenetic inference shows that the viral sequence roots the main Lassa/Mopeia/Mobala clade which now includes Luna and Morogoro, recently identified in *Mastomys natalensis* in Zambia and Tanzania respectively (Ishii *et al.* 2011; Günther *et al.* 2009). Out of the 30 samples sampled at this location, ATC Rp18 was the only sample positive for arenavirus. More sampling and testing is required to determine whether *R. pumilio* is indeed the host of this novel virus.

This study found four viral sequences related to Merino Walk virus from *M. irroratus*, the same genus as the rodent in which Merino Walk virus was found. Additionally two viral sequences were identified in *M. namaquensis* not previously described as an arenavirus host. The seventh sequence identified is a unique lineage in the *Arenaviridae* family and also associated with a new arenavirus rodent host species *R. pumili*o. All the rodents from which arenaviral sequences were obtained are endemic to southern Africa.

4.5   Detection of hantavirus in South Africa

Up until 2006, hantaviruses were exclusively known from rodents belonging to the *Murinae, Arvicolinae, Neotominae* and *Sigmondontina*e subfamilies in Asia, Europe and the Americas. The only virus in the genus not hosted by a rodent was Thottapalayam virus (TPMV) hosted by the Asian house shrew (*Suncus murinus*) (Henttonen *et al.* 2008). Hantaviruses were phylogenetically divided into New World and Old World viruses with TPMV forming a distinct lineage. Since 2006, 22 new hantaviruses hosted by insectivores from the *Soricidae* and *Talpidae* families have been found, and more recently hantaviruses were also found in *Nycteridae*, *Rhinolophidae* and *Vespertilionidae* bats in Asia and Africa (Weiss *et al.* 2012; Guo *et al.* 2013). The identification of such a diversity of hantaviruses in such different mammal species suggests that there are more as yet undiscovered hantaviruses. The table below lists hantaviruses recently described from various mammals in Africa (Table 4.2).

Table 4.2 Hantaviruses identified in Africa since 2006; Magboi and Mouyassué were identified in bats, Azagny and Tanganya in shrews, and Sangassou and Tigray were identified in rodents belonging to the *Murinae* subfamily.

Virus	Host	Country	Reference
Azagny	*Crocidura obscurior*	Cote d'Ivoire	Kang *et al.* 2011
Magboi	*Nycteris hispida*	Sierra Leone	Weiss *et al.* 2012
Mouyassué	*Neoromicia nana*	Cote d'Ivoire	Sumibcay *et al.* 2012
Sangassou	*Hylomyscus simus*	Guinea	Klempa *et al.* 2006
Tanganya	*Crocidura theresae*	Guinea	Klempa *et al.* 2007
Tigray	*Stenocephalemus abipes*	Ethiopia	Meheretu *et al.* 2012

4.5.1   Hantaviruses in small-mammals

In this study 1651 samples from various bat, rodent and shrew species from South Africa were screened for hantavirus by genus-specific PCR. All samples tested negative, positive control was included in all PCR runs and this assay has been used to identify a diverse range of novel hantaviruses in bat (Weiss *et al.* 2012), rodent (Cao *et al.* 2010; Meheretu *et al.*

2012) and shrew (Radosa *et al.* 2013) viruses from different continents. The majority of samples were from *Rhabdomys pumilio* (35.4%), followed by *Mastomys spp (coucha* and *natalensis)* (20.3%). A total of 66 shrews (*Soricidae*) and 123 Elephant shrews (*Macroscelididae*) were also tested as were 83 bats.

Detection of hantavirus RNA in small animal is usually conducted using tissue or blood, and ideally from lung or liver tissue. Most of the bat samples tested in this study were faecal samples obtained during catch and release studies. Previous studies during which bat hantaviruses were identified tested liver samples (Sumibcay *et al.* 2011, Weiss *et al.* 2012 and Guo *et al.* 2013).

Our findings do not necessarily indicate the absence of hantaviruses in small mammals in southern Africa. Most studies during which general screening was conducted screened large numbers of specimens before a positive result was obtained (Klempa *et al.* 2006; 2007; 2012). In the Guinea study 1649 samples representing 19 different rodent and shrew species were screened for hantavirus; 2 out 6 *Hylomyscus spp* were positive for Sangassou virus (Klempa *et al.* 2012a). The variety of species screened here is minute when considering the number of rodent, shrew and bat species that occur in South Africa. There are more than 60 rodent species, more than 17 shrews and more than 50 bat species that occurs in South Africa (http://www.biodiversityexplorer.org/mammals/placentalia.htm).

4.5.2   Seroprevalence of hantavirus in South Africa

Detection of hantavirus infection in humans is mostly by serological tests as viraemia is short-lived during the acute phase of disease, making RT-PCR unsuitable for patient diagnosis. Most serological assays are based on the nucleocapsid (N) protein which is the major antigenic target of the humoral response (Sjölander and Lundkvist 1999). Serological methods employed are immunofluorescence assay (IFA), ELISA and Western blot: viral antigens for antibody assays can either be obtained from cell culture or use full-length or truncated N protein expressed in *E.coli, S. cerevisiae* and insect cells (Krüger *et al.* 2001). Studies have shown that N-specific antibodies occur early in infection and persistence is probably life-long. The N protein is highly conserved and therefore cross-reactive between different hantaviruses which allows for the detection of the majority of hantavirus infections including those caused by yet unknown viruses (Meisel *et al.* 2006).

In our study, IgG ELISA using recombinant N proteins from Dobrava and Puumala viruses (DOB-rN and PUU-rN) found 210 (14.6%) of 1442 residual sera collected from various

laboratories in the Western Cape Province reactive. Direct-coating IgG ELISA has high sensitivity but low specificity (Krüger *et al.* 2001) which may result in false positive results. ELISA reactive sera were therefore subjected to more specific confirmatory assays, namely Western blot and IIFT. First, ELISA reactive sera were tested using a commercial assay to detect antibodies against DOBV, HTNV, PUUV and SEOV and in-house assay to detect antibodies against SANGV. Thirty-seven out of 210 were reactive and subsequently tested in IIFT using slides prepared with cells infected with DOBV, HTNV, PUUV and SANGV. Fourteen of the samples were positive on IIFT and subsequent titration experiments were performed using sera diluted from 1:20 to 1:160. To determine the true seroprevalence in our study population a screening algorithm (Figure 3.12) used by our collaborators in their studies in Guinea was employed; ELISA reactive samples had to be reactive in Western blot and IIFT assays to be considered positive (Klempa *et al.* 2010; 2012b). Using this stringent approach, a seroprevalence of 1% was determined in our study.

Similar studies have been conducted in Brazil (de Souza *et al.* 2011), Spain (Lledo *et al.* 2003; Sanfeliu *et al.* 2011), and Germany (Schilling *et al.* 2007). The study conducted by Sanfeliu *et al* (2011) screened sera from patients admitted to hospital for minor surgery and non-infectious diseases. All sera were tested by IFA only and yielded a hantavirus seroprevalence of 1.8%. The authors indicated that the results they obtained were comparable to those observed in other surveys conducted in Spain and other European countries; Belgium (1.4%), Holland (0.9%) and Germany (1.7%). A higher seroprevalence was reported from a study conducted in the border region between Argentina and Brazil. The investigators surveyed a population in an agricultural area where forest has been destroyed to make land available for farming. Using a similar approach to ours, sera were initially tested using IgG ELISA based on recombinant antigen from Araraquara hantavirus and reactive sera were then tested by IFA. They reported a seroprevalence rate of 3.5%. They also reported a higher proportion of seroreactive individuals among farmworkers suggesting an occupational risk of infection (de Souza *et al* 2011). Higher seroprevalence rates have been reported in endemic areas in Scandinavia and the Balkans; seroprevalence rates as high as 20% are observed in Finland, 8% in northern Sweden and 9% in Estonia (Vapalahti *et al.* 2003).

Ten acutely ill patients were included in this study; eight out of the ten samples were reactive in the IgG ELISA. However none of these could be confirmed by subsequent Western blot testing. This is not unexpected because the ELISAs have high sensitivity and low specificity,

which is why all samples reactive in the ELISA assays are re-tested with more specific assays such as Western Blot and IIFT (Klempa *et al.* 2010).

That no acute cases of clinically symptomatic hantavirus infection were detected could be due to the low number of patients recruited. Alternative explanations could be that no hantaviruses occur in southern Africa; that hantaviruses do occur but do not infect human beings; that they infect humans but do not cause disease; or that the disease manifestation was not captured by our case definition. We believe that hantaviruses occur globally and will be identified with sufficient efforts; we have found evidence for human hantavirus infection during our serosurveillance study so we have evidence for human hantavirus infections occurring.

Future studies will have to actively recruit patients with febrile illness of unknown aetiology. A study in Indonesia recruited more than 400 such patients at two hospitals; one patient with high antibody titres was identified and in a follow-up study rodent trapping near the patient's home and workplace identified rats infected with Seoul virus (Alisjahbana *et al.* 2012).

4.6   General conclusions

4.6.1   Pathogen surveillance in bats and rodents

RNA viruses account for the majority of zoonoses that have emerged in recent years. In the last 20 years numerous viral diseases that have their origin in rodents and bats have emerged in human beings different parts of the world. Hantavirus cardiopulmonary syndrome (HCPS) was recognised in the United States in the early 1990s and since then several hantaviruses causing HCPS have been found in the Americas (Nichol *et al.* 1993; Hjelle *et al.* 1994). Hendra and Nipah emerged in the second half of the 1990s in Australia and south-east Asia from *Pteropus* fruit bats with transmission to humans involving an intermediate host, horses and pigs, respectively (Wild 2009). In the 2000s SARS-CoV emerged from an ancestor SARS-related virus hosted by Chinese horseshoe bats, again through an intermediate host, triggering enormous interest in bats as a source of emerging viral zoonoses (Li *et al.* 2005). In our part of the world an outbreak of haemorrhagic fever occurred in Zambia and resulting in several cases of life-threatening human disease in South Africa (Paweska *et al.* 2009). The causative agent Lujo is an arenavirus but the – presumably rodent – reservoir host remains unknown while the search for it continues.

These examples show that disease emergence is ongoing and that its likelihood has if anything increased through various factors, especially human activity such as deforestation,

encroachment and agricultural intensification leading to increased contact between human beings and wildlife (Brown 2004; Greger 2007). In all above mentioned examples of disease emergence the infectious agent and its host were identified only in the aftermath of the outbreak, during scientific searches for the source. In the case of SARS animals were implicated as a source of infection after the realisation that most of the infected individuals were restaurant workers or worked at live animal ("wet") markets (Breiman *et al.* 2003; Guan *et al.* 2003). It was another two years of study before the identification of bats as the natural reservoir hosts of SARS-CoV (Li *et al.* 2005). That MERS-CoV might be the result of zoonotic events was determined within months of the outbreak because of the availability of genetic information on related viruses (van Boheeman *et al.* 2012; Woo *et al.* 2012). This information was available as a result of bat surveillance studies (Woo *et al.* 2006; Woo *et al.* 2007) conducted in the years since SARS emerged.

Screening of animals for potential zoonotic viruses in this study was performed using sequence-dependent method. Pan-PCR primers used in this study to detect novel viral sequences were designed from sequence alignments of characterised members that belong to the virus genus/subfamily/family of interest. Sequence-independent methods do not require information on known viruses and are therefore useful in detecting novel viruses for which no genetic information is available. Traditional sequence-independent methods such as suppression subtractive hybridisation, representational difference analysis and Sequence-independent single primer amplification are time-consuming and thus not suitable for high-throughput screening (Mokili *et al.* 2012). Viral metagenomics presents a high throughput approach that may be used for the detection of unknown-disease associated viruses and for the discovery of novel viruses. The high cost of platforms, reagents and maintenance, long sample preparation time and intensive data analysis are the main disadvantages attributed to NGS (Barzon *et al.* 2011).

Surveillance not only provides viral genetic information that may be useful in identifying a novel virus in the event of an outbreak but also allows us to study virus dynamics in terms of virus-host interactions in individual animals and at population levels and hopefully helps to elucidate the events that lead to the emergence of a new infectious disease.

In this study, opportunistic random sampling was employed, whereby I screened readily available samples, which only allows for the detection of viruses that are present in the samples at the time of collection. To determine if an animal is truly the host of the species and is not infected as a result of spill-over transmission from the true host that occurs in the

same habitat. It is therefore necessary to conduct longitudinal surveillance which involves monitoring a study population over time and sampling at regular intervals. This way we can monitor the virus-host relationship, and note changes in diversity and viral load in the population in relation to changes in the host population, food availability and environmental changes. However surveillance is difficult in naturally infected populations for a number of reasons; including the need to achieve a balance between sufficient sampling and minimising disturbance or damage to the study animals as well as their habitat (FAO 2011). Rodent- and insectivore-borne viruses

In this study, we identified eight novel arenavirus sequences in three different South African rodent species *Myotomys irroratus, Micaelamys namaquensis* and *Rhabdomys pumilio* that belong to the *Murinae* subfamily. Seven of the sequences are related to the Merino Walk virus previously identified in *Myotomys unisulcatus.* This is the first description of Merino Walk virus related sequences since the identification of MWV (Palacios *et al.* 2010) and our findings suggest that MWV may be hosted by more than one rodent species. All three rodent species have overlapping geographical ranges (Figure 4.1). So far, MWV has not been associated with human infection or disease but only seroprevalence studies using assays based on recombinant N proteins can determine if transmission to human beings occurs. Additional studies in the animal hosts are required to determine if infection is persistent and also to determine which of the three rodent hosts acts as the primary host.

We identified an arenavirus sequence representing a novel lineage in the Lassa-LCMV serocomplex in *R. pumilio*. Further studies are needed including surveys of rodents in the location at which the animal was trapped to determine if *R. pumilio* is the host of this novel virus. Virus isolation on cell culture should also be attempted to obtain sufficient viral genetic material for full genome sequencing.

No South African hantaviruses could be identified in this study; samples were collected in eight provinces but the majority of samples were from Western Cape and the least from the Free State province. *R. pumilio* accounted for the majority of samples collected, followed by *Mastomys spp.*; for species such as *Parotomys, Desmodillus* and *Steatomys spp.* less than forty samples were included. *Soricomorpha/Eulipotyphla* were underrepresented with only 66 samples included; possibly due to the type of bait (peanut butter and oats mixture) used which is optimised for rodent trapping. To optimise shrew trapping traps should be baited with fish bait such as worms since shrews feed mainly on insects and worms (van der Merwe *et al.* 2011; US Fish and Wildlife Services).

The opportunistic serosurvey for hantavirus antibodies yielded a 1% seroprevalence rate in the 1442 human samples screened. Although at the low end of the scale, this rate is comparable to results observed in similar studies conducted in Guinea, Europe and South America, i.e. in areas where hantaviruses are known to occur. Higher seroprevalence rates have been reported in areas where hantavirus disease is endemic (Argentine pampas, the Balkans and certain parts of Scandinavia). However the lack of an indigenous virus hampers efforts to obtain a true picture of human hantavirus infection in South Africa, as by necessity all seroprevalence studies so far detect antibody cross-reactivity only.

Future studies should target risk populations such as farmers and farm workers, rangers, field biologists and mammalogist who have increased contact with rodents. Vapalahti *et al* (1999) showed that farmers in Finland had increased risk of contracting Puumala virus infection. A survey in Brazil found the highest prevalence among rural farm workers (de Souza *et al.* 2011).

4.6.2   Bat-borne viruses

Novel astroviruses were identified in 35 out of 83 bat samples collected for this study from species belonging to five different families of insectivorous bats. All astrovirus sequences identified belong to mamastrovirus genogroup I and cluster in a clade with Chinese bat AstVs. Viral sequences identified in the same species from the same colony do not necessarily cluster together and in some cases viral sequences from different species share sequence similarities higher than 90%. The same phenomenon was observed in China (Wu *et al.* 2011) but more studies are clearly needed to provide a clearer picture of astrovirus evolution, recombination, inter-colony transmission among bats and their potential as zoonotic viruses.

Coronaviruses were identified in four different species from the *Miniopteridae* and *Vespertilionidae* bat families. Ten viral sequences belong to the *Alphacoronavirus* genus and one to the *Betacoronavirus* genus. Viral sequences identified in *Miniopterus natalensis* clustered with other Miniopterid bat viruses in a monophyletic clade which includes the prototype coronaviruses 1A and 1B identified in Chinese *Miniopterus* bats. Viral sequences identified in *Neoromicia capensis* cluster with *Nyctalus spp.* sequences from Europe, with the exception of one sequence that represents a unique lineage. The closest relative so far known of the recently emerged MERS-CoV was found in this study in a *Neoromicia* cf. *zuluensis* bat from KwaZulu-Natal (Ithete *et al.* 2013). This finding strongly suggests that the ancestor of

MERS-CoV occurs in *Vespertilionidae* bats in Africa or adjacent areas. Since the viral sequence was identified in one bat, more bats from the same area need to be tested, as well as closely related bats from elsewhere including the Arabian Peninsula. More studies should be conducted to obtain more genome sequences for further analysis and to investigate the natural ecology of these viruses in relation to their hosts.

4.6.3   Future perspectives

As human activities such as encroachment into forests, hunting for bushmeat, and agricultural intensification continue, contact with wildlife sources of potentially zoonotic potential will lead to the emergence of new infectious diseases in human beings. Surveillance of wildlife species such as bats and rodents is important for monitoring the diversity of viruses circulating, understanding the nature of infection and the evolution of reservoir hosts and potential pathogens.

The occurrence of disease outbreaks for which the source of infection is initially unknown highlights the importance of surveillance to identify viruses and their reservoir hosts. For instance, it is most likely that the hosts for Lujo virus and Sabia virus are rodent species that occur locally in the outbreak area but have not been identified yet.

Longitudinal studies of naturally infected reservoirs are important. For instance, why is the geographical range of some reservoir hosts larger than the disease range: Lassa is endemic to West Africa yet *M. natalensis* occurs over most of sub-Saharan Africa (see Appendix C)? *M. natalensis* in other parts of Africa host other arenaviruses Mopeia (Mozambique), Morogoro (Tanzania) and Luna (Zambia) but none of these viruses is associated with human infection. The same is true for Puumala hantaviruses: Its host occurs over wide parts of Europe whereas only some populations seem to be infected.

The high species diversity within the orders investigated in this study points to an equally high diversity of hosted microbes. The difficulty is determining how and which of these viruses identified during surveillance are potential zoonoses and therefore relevant to public health and agriculture. Any surveillance studies conducted must therefore aim to determine virus-host interaction, infection dynamics within a naturally infected population, and the effects of anthropogenic factors such as habitat fragmentation and destruction on the reservoir host (Morse *et al.* 2012).

The data presented in this study contributes new information on the diversity of viruses hosted by South African small mammals and to the global surveillance of potential zoonotic

emerging viruses in wildlife. Future investigation on the novel viruses identified should be multidisciplinary studies in order to better understand how the interactions between viral and host genetics, virus-host relationship within a host and at a population level and ecological factors enable cross-species transmission and disease emergence in humans. It is therefore necessary for the collaborative efforts to include ecologists, zoologists and virologists to understand the dynamics of viral emergence.

References

Alisjahbana, B., Antonjaya, U., Blair, P. J., Hoo, Y., Ibrahim, I. N. and Kosasih, H., *et al.,* 2011, 'Evidence of human hantavirus infection and zoonotic investigation hantavirus prevalence on rodents in Western Java, Indonesia', *Vector-Borne and Zoonotic Diseases*, **11**(6), pp. 709-713

Anderson, M. G., Frenkel, L D., Homann, S. and Guffey, J., 2003, 'A case of severe monkeypox virus disease in an American child: emerging infections and changing professional values', *Pedaitric Infectious Diseases Journal*, **22**, pp. 1093-1096

Annan, A., Baldwin, H. J., Corman, V. M., Klose, S. M., Nkrumah, E. E., Badu, E. K., *et al.,* 2013, 'Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe', *Emerging Infectious Diseases*, **19**(3), 456-459

Arai, S., Bennett, S. N., Sumibcay, L., Cook, J. A., Song, J-W., Hope, A., *et al.,* 2008, 'Short Report: Phylogenetically distinct hantaviruses in the masked shrew (*Sorex cinereus*) and dusky shrew (*Sorex monticolus*) in the United States', *American Journal of Tropical Medicine and Hygiene*, **78**(2), pp. 348-351

Arai, S., Nguyen, S. T., Boldgiv, B., Fukui, D., Araki, K., Dang, C. N., *et al.,* 2013, 'Novel Bat-borne hantavirus, Vietnam', *Emerging Infectious Diseases*, **19**(7), pp 1159-1161

Atkins, A., Wellehan Jr., J. F. X., Chidress, A. L., Archer, L. L., Fraser, W. A. and Citino, S. B., 2008, 'Characterization of an outbreak of astroviral diarrhea in a group of cheetahs (*Acinonyx jubatus*)', *Veterinary Microbiology*, **136**, pp. 160-165

Baker, S. C., 2008, 'Coronaviruses: Molecular Biology', In B.W.J. Mahy and M.H.V. van Regenmortel (eds), *Encyclopedia of Virology* (3rd Edition), Academic Press, Oxford, pp. 554-562

Ball et al 2005, 'The Positive Sense Single Stranded RNA Viruses', *Virus Taxonomy*, Academic Press, San Diego

Barzon, L., Lavezzo, E., Millitello, V., Toppo, S. and Palú, G., 2011, 'Applications of Next-Generation Sequencing Technologies to Diagnostic Virology', *International Journal of Molecular Sciences*, **12**, pp. 7861-7884

Barzon, L., Lavezzo, E., Constanzi, G., Franchin, E., Toppo, S. and Palú, G., 2013, 'Next-generation sequencing technologies in diagnostic virology', *Journal of Clinical Virology*, http://dx.doi.org/10.1016/j.jcv.2013.03.003

Bastos, A. D., Nair, D., Taylor, P. J., Brettschneider, H., Kirsten, F., Mostert, E., *et al.,* **2011**, 'Genetic monitoring detects an overlooked cryptic species and reveals the diversity and distribution of three invasive Rattus congeners in South Africa', *BMC Genetics*, **12**: 26, doi:10.1186/1471-2156-12-26

Beck, E. T. and Henrickson, K. J., 2010, 'Molecular diagnosis of respiratory viruses', *Future Microbiology*, **5**(6), pp. 901-916

Belouzard, S., Millet, J. K., Licitra, B. N., and Whitaker, G. R., 2012, ' Mechanisms of Coronavirus Cell Entry Mediated by the Viral Spike Protein', *Viruses*, **4**, pp. 1011-1033

Bender, S. J., and Weiss, S. R., 2010, 'Pathogenesis of Murine Coronavirus in the Central Nervous System', *Journal of Neuroimmune Pharmacology*, **5**(3), pp. 336-354

Bengis, R. G., Leighton, F. A., Fischer, J. R., Artois, M., Mörner, T. and Tate, C. M., 2004, 'The role of wildlife in emerging and re-emerging zoonoses', *Revue scientifique et technique,* **23**(2), pp. 497-511

Berger, A., Drosten, C., Doerr, H. W., Stürmer, M. and Preiser, W., 2004, 'Severe acute respiratory syndrome (SARS)-paradigm of an emerging viral infection', *Journal of Clinical Virology*, **29**, pp. 13-22

Bermingham, A., Chand, M. A., Brown, C. S., Aarons, E., Tong, C., Langrish, C., *et al.,* 2012, 'Severe respiratory illness caused by a novel coronavirus in a patient transferred to the United Kingdom from the Middle East, September 2012', *Euro Surveillance*, 17(40), pii=20290, http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20290

Bexfield, N. and Kellam, P., 2011, 'Metagenomics and the molecular identification of novel viruses', *The Veterinary Journal*, **190**, pp. 191-198

Bi, Z., Formenty, P. B. H. and Roth C. E., *et al.,* 2008, 'Hantavirus infection: a review and global update', *Journal of Infection in Developing Countries*, **2**(1), pp. 3-23

Bird, B. H., Khristova, M. L., Rollin, P. E., Ksiazek, T. G. and Nichol, S. T., 2007, 'Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever

virus strains reveals widespread virus movement and low genetic diversity due ot recent common ancestry', *Journal of Virology*, **81**(6), pp. 2805-2816

Bolles, M., Donaldson, E., and Baric, R., 2011, 'SARS-CoV and emergent coronaviruses: viral determinants of interspecies transmission', *Current Opinion in Virology*, **1**, pp. 624-634

Bosch, A., Giux, S., Krishna, N. K., Méndez, E., Monroe, S.S., Patin-Jackwood, M. and Schultz-Cherry, S., 2010, Nineteen new species in the genus Mamastrovirus in the *Astroviridae* family, ICTV 2010.0.17abV

Boudreau, E. F., Josleyn, M., Ullman, D., Fisher, D., Dalrymple, L., Sellers-Myers, K., *et al.,* 2012, 'A Phase 1 clinical trial of hantaan virus and puumala virus M-segment DNA vaccines for hemrrhagic fever with renal syndrome', *Vaccine*, **30**, 1951-1958

Bowen, M. D., Rollin, P. E., Ksiazek, T. G., Hustad, H. L., Bausch, D. G., Demby, A. H., *et al.,* 2000, 'Genetic diversity among Lassa virus strains', *Journal of Virology*, **74**(15), pp. 6992-7004

Branco, L.M., Matschiner, A., Fair, J. N., Goba, A., Sampey, D. B., Ferro, P. J., *et al.,* 2008, 'Bacterial-based systems for expression and purification of recombinant Lassa virus proteins of immunological relevance', *Virology Journal*, **5**(74), doi:10.1186/1743-422X-5-74

Bredenbeek, P. J., Molenkamp, R., Spaan, W. J. M., Deubel, V., Marianneau, P., Salvato, M. S., *et al.,* 2006,'a recombinant Yellow Fever 17D vaccine expressing Lassa virus glycoproteins', *Virology*, **345**(2), pp. 299-304

Breiman, R. F., Evans, M. R., Preiser, W., Maguire, J., Schnur, A., Li, A., *et al.,* 2003, 'Role of China in the quest to define and control severe acute respiratory syndrome', *Emerging Infectious Diseases*, **9**(9), pp. 1037-1041

Briese, T., Paweska, J. T., McMullan, L. K., Hutchison, S. K., Street, C., Palacios, G., *et al.,* 2009, 'Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from Southern Africa', *PLos Pathogens*, **5**(5), e1000455, doi:10.1371/journal.ppat.1000455

Brinker, J. P., Blacklow, N. R. and Herrmann, J. E., 2000, 'Human astrovirus isolation and propagation in multiple cell lines', *Archives of Virology*, **145**, pp. 1847-1856

Brown, C., 2004, 'Emerging zoonoses and pathogens of public health significance – an overview', *Revue scientifique et technique,* **23**(2), pp. 435-442

Buchmeier, M.J., 2002, 'Arenaviruses: Protein Structure and Function', in M.B.A Oldstone (ed.), *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 159-173

Buchmeier, M.J., de la Torre, J.C. and Peters, C.J., 2006, 'Arenaviridae: The Viruses and Their Replication' in D.M. Knipe and P.M. Howley (eds.), *Field's Virology (fifth edition)*, pp. 1791-1827

Cajimat, M. N. B., Milazzo, M. L., Hess, B. D., Rood, M. P., and Fulhorst, C. F., 2007, 'Principal host relationships and evolutionary history of the North American arenaviruses', *Virology*, **367**(2), pp. 235-243

Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V., and Schountz, T., 2006, 'Bats: Important reservoir hosts of emerging viruses', *Clinical Microbiology Reviews*, **19**(3), pp. 531-545

Cao, Z-W., Zuo, S-Q., Gong, Z-D., Zhan, L., Bian, C-L., Zhang, P-H., *et al.,* 2010, 'Genetic analysis of a hantavirus strain carried by Niviventer confucianus in Yunnan province, China', *Virus Research*, **153**, pp. 157-160

Carrion Jr., R., Bredenbeek, P., Jiang, X., Tretyakova, I., Puahko, P., and Lukashevich, I. S., 2012, 'Vaccine Platforms to Control Arenaviral Hemorrhagic Fevers', *Journal of Vaccines and Vaccinations*, **3**(7), http://dx.doi.org/10.4172/2157-7560.1000160

Cavanagh, D. and Britton, P., 2008, 'Coronaviruses: General Features', In B.W.J. Mahy and M.H.V. van Regenmortel (eds), *Encyclopedia of Virology (3rd Edition)*, Academic Press, Oxford, pp.549-554

Cavanagh, D., 2004. Coronaviruses and Toroviruses, In A. J. Zuckerman, J. E. Banatvala, J. R. Pattison, P. D. Griffiths and B. D. Schoub (eds), *Principles and Practice of Clinical Virology (5th Edition)*, John Wiley & Sons Ltd, pp. 379-397

Cavanagh, D., 2007, 'Coronavirus avian infectious bronchitis virus', *Veterinary Research*, **38**, pp. 281-297

CDC, 2000, 'Fatal Illnesses Associated With a New World Arenavirus – California, 1999-2000', *Morbidity and Mortality Weekly Report*, August, **49**(31), 709

Chan, J. F. W., Li, K. S. M., To, K.K. W., Cheng, V. C. C., Chen, H. and Yuen, K-Y., 2012, 'Is the discovery of the novel human betacoronavirus 2c EMC/2012 (HCoV-EMC) the beginning of another SARS-like epidemic?', *Journal of Infection*, **65**, pp. 477-489.

Chan, P. K. S., Ng, K-C., Chan, R. C. W., Lam, R. K. Y., Chow, V. C. Y., Hui, M., *et al.,* 2004, 'Immunofluorescence assay for serologic diagnosis of SARS', *Emerging Infectious Diseases*, **10**(3), 530-532

Chapter 24: *Coronaviridae***,** 2011, In N. J.Maclachlan and E. J. Dubovi (eds), *Fenner's Veterinary Virology (4th Edition)*, Academic Press, San Diego, pp. 393-413

Chapter 28: *Astroviridae*, 2011, In N. J. Maclachlan and E. J. Dubovi (eds), *Fenner's Veterinary Virology (4th Edition)*, Academic Press, San Diego, pp. 451-454

Charrel, R. N. and de Lambarellie, X., 2010, 'Zoonotic aspects of arenavirus infections', *Veterinary Microbiology*, **140**, pp. 213-220

Charrel, R. N., de Lamballerie, X. and Emonet, S., 2008, 'Phylogeny of the genus Arenavirus', *Current Opinion in Microbiology*, **11**, pp. 362-368

Charrel, R. N., de Lamballerie, X. and Fulhorst, C. F., 2001, 'The Whitewater Arroyo virus: Natural evidence for genetic recombination among Tacaribe serocomplex viruses (family *Arenaviridae*)', *Virology*, **283**, pp. 161-166

Charrel, R.N. and de Lamballerie, X., 2003, 'Arenaviruses other than Lassa virus', *Antiviral Research*, **57**, 89-100

Charrel, R. N., Feldman, H., Fluhorst, C. F., Khelifa, R., de Chesse, R. and de Lamballerie, X., 2002, 'Phylogeny of New World arenaviruses based on the complete coding lineage produced by intrasegmental recombination', *Biochemical and Biophysical Research Communications*, **296**, 1118-1124

Childs, J. E., Richt, J. A. and Mackenzie, J. S., 2007, 'Introduction: Conceptualizing and partitioning the emergence process of zoonotic viruses from wildlife to humans', in J.E. Childs, J. S. Mackenzie and J. A. Richts (eds), *Wildlife and emerging zoonotic diseases: The biology, circumstances and consequences of cross-species transmission CTMI 315 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 1-31

Chomel, B. B., Belotto, A. and Meslin, F-X., 2007, 'Wildlife, exotic pets, and emerging zoonoses', *Emerging Infectious Diseases*, **13**(1), pp. 6-11

Chu, D. K. W., Chin, A. W. H., Smith, G. J., Chan, K-H., Guan, Y., Peiris, J. S. M. and Poon, L. L. M., 2010, 'Detection of novel astroviruses in urban brown rats and previously unknown astroviruses in humans', *Journal of General Virology*, **91**, pp. 2457-2462

Chu, D. K. W., Peiris, J. S. M. and Poon, L. L. M., 2009, 'Novel Coronavirus and Astroviruses in Bats', *Virologica Sinica*, **24**(2), pp. 100-104

Chu, D. K. W., Poon, L. L. M., Guan, Y. and Peiris, J. S. M., 2008, 'Novel Astroviruses in Insectivorous Bats', *Journal of Virology*, **82**(18), pp. 9107-9114

Chu, D. K. W., Poon, L. L.M., Chan, K. H., Chen, H., Guan, Y., Yuen, K. Y. and Peiris, J. S. M., *et al.,* 2011, 'Coronaviruses in bent-winged bats (*Miniopterus spp.*)', *Journal of General Virology*, **87**, 2461-2466

Clegg, J.C.S., 2002, 'Molecular Phylogeny of the Arenaviruses', in M.B.A Oldstone (ed.), *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 1-24

Cleveland, S., Haydon, D. T. and Taylor, L., 2007, 'Overviews of pathogen emergence: Which pathogens emerge, when and why?' in J.E. Childs, J. S. Mackenzie and J. A. Richts (eds), *Wildlife and emerging zoonotic diseases: The biology, circumstances and consequences of cross-species transmission CTMI 315 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 85-111

Coetzee, N., Griffin, M. and Taylor, P.J., 2008a, *Aethomys namaquensis*, In *IUCN Red List of Threatened Species,* Version 2013.1. <www.iucnredlist.org>. Downloaded on 16 July 2013.

Coetzee, N. and van der Straeten, E., 2008b, *Rhabdomys pumilio*, In *IUCN Red List of Threatened Species*, Version 2013.1. <www.iucnredlist.org>. Downloaded on 16 July 2013.

Colomba, C., de Grazia, S., Giammanco, G. M., Saporito, L., Scarlata, F., Titone, L. and Arista, S., 2006, 'Viral gastroenteritis in children hospitalised in Sicily, Italy' *European Journal of Clinical Microbiology and Infectious Diseases*, **25**, pp. 570-575

Corman, V. M., Eckerle, I., Bleicker, T., Zaki, A., Landt, O., Eschbach-Bludau, M., *et al.,* 2012, 'Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction', *Eurosurveillance*, **17**(39), pii=20285, http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20285

Coulaud, X., Chouaib, E., Georges, A.J., Rollin, P. and Gonzalez, J. P., 1987, 'First human case of haemorrhagic fever with renal syndrome in the Central African Republic', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **81**, pp. 686

Coulibaly-N' Golo, D., Allali, B., Kouassi, S. K., Fichet-Calvet, E., Becker-Ziaja, B., Rieger, T., 2011, 'Novel Arenavirus Sequences in *Hylomyscus sp.* and *Mus (Nannomys) setulosus* from Côte d'Ivoire: Implications for Evolution of Arenaviruses in Africa', *PLoS ONE*, **6**(6), e20893,doi:10.1371/journal.pone.0020893

Cowley, T. J., and Weiss, S. R., 2010, 'Murine coronavirus neuropathogenesis: determinants of virulence', *Journal of Neurovirology*, **16**(6), pp. 427-434

Davis, S., Calvet, E. and Leirs, H. *et al.,* 2005, 'Fluctuating rodent populations and risk to humans from rodent-borne zoonoses', *Vector-borne and Zoonotic Diseases*, **5**(4), 305-314

Dawood, S., Jain, S., Finelli, L., Shaw, M. W., Lindstrom, S., Garten, L. V., *et al.,* 2009, 'Emergence of a novel swine-origin influenza A(H1N1) virus in humans', *New England Journal of Medicine*, **360**, pp. 2605-2615

De Araujo, J., Thomazelli, L M., Henriques, D. A., Lautenschalager, D., Ometto, T., Dutra, L. M. *et al.,* 2012, 'Detection of hantavirus in bats from remaining rain forest in São Paolo, Brazil', *BMC Research Notes*, **5**: 690, http://www.biomedcentral.com/1756-0500/5/690

De Benedictis, P., Schultz-Cherry, S., Burnham, A. and Catolli, G., 2011, 'Astrovirus infections in humans and animals – Molecular biology, genetic diversity, and interspecies transmissions' , *Infections, Genetics, and Evolution*, **11**, pp. 1529-1544

De Figueiredo, G. G., Figueiredo, L. T. M., Machado, A. M., and dos Santos, G. S., *et al.,* 2009, 'Laboratory diagnosis of human hantavirus infection: novle insights and future potential', *Future Virology*, **4.4**, pp. 383, http://dx.doi.org.ez.sun.ac.za/10.2217/fvl.09.15

De Souza Luna, L. K., Heiser, V., Regamey, N., Panning, M., Drexler, J. F., Mulangu, S., *et al.,* 2007, 'Generic detection of coronaviruses and differentiation at het prototype strain level by reverse transcription PCR and nonfluorescent low-density microarray', *Journal of Clinical Microbiology***, 45**(3), pp. 1049-1052

De Souza, W. M., Machado, A. M., Figueredo, L. T. M. and Boff, E. *et al.,* 2011, 'Serosurvey of hantavirus infection in humans in the border region between Brazil and Argentina', *Revista da Sociedade Brasileira de Medicina Tropical*, **44**(2), pp. 131-135

Dearing, M. D. and Dizney, L., 2010, 'Ecology of hantavirus in a changing world', *Annals of the New York Academy of Sciences*, **1195**, pp. 99-112

Decaro, N., Desario, C., Elia, G., Mari, V., Lucenta, M. S., Cordioli, P., et al., 2007, 'Serological and molecular evidence that canine respiratory coronavirus is circulating in Italy', *Veterinary Microbiology*, **121:** 225-230

Decaro, N., Campolo, M., Desario, C., Cirone, F., D'abramo, M., Lorusso, E., *et al,.* 2008, 'Respiratory disease associated with bovine coronavirus infection in cattle herds in Southern Italy', *Journal of Veterinary Diagnostic Investigation*, **20**, pp. 28-32

Delwart, E., 2012, 'Animal virus discovery: improving animal health, understanding zoonoses, and opportunities for vaccine development', *Current Opinion in Virology*, **2**, pp. 1-9

Derlet, R.W. and Richards, J.R., 2000, 'Haemorrhagic Fever in California', *The California Journal of Emergency Medicine*, **1**(2), 9

Djikeng, A., Halpin, R., Kuzmickas, R., DePasse, J., Feldblyum, J., Sengamalay, N., *et al.,* 2008, 'Viral genome sequencing by random priming methods', *BMC Genomics*, **9**:5, doi:10.1186/1471-2164-9-5

Donelli, G., Superti, F., Tinari, A. and Marziano, M. L., 1992, 'Mechanism of astrovirus entry into Graham 293 cells', *Journal of Medical Virology*, **38**(4), pp. 271-277

Douady, C. J., Chatelier, P. I., Madsen, O., de Jong, W. W., Catzeflis, F., Springer, M. S. and Stanhope, M. J., *et al.,* 2002, 'Molecular phylogenetic evidence confirming the Eulipotyphla concept and in support of hedgehogs as the sister group to shrews', *Molecular Phylogenetics and Evolution*, **25**, pp. 200-209

Downs, W. G., Anderson, C. R., Spence, L., Aitken, T. H. G. and Greenhall, A. H., 1963, 'Tacaribe virus, a new agent isolated from Artibeus bats and mosquitoes in

Trinidad, West Indies', *American Journal of Tropical Medicine and Hygiene*, **12**, pp. 640-646

Drexler, J. F., Corman, V. M., Gloza-Rausch, F., Seebens, A., Annan, A., Ipsen, A., *et al.,* 2009, 'Henipavirus RNA in African bats', *PLoS ONE*, **4**(7), e6367, doi:10.1371/journal.pone.0006367

Drexler, J. F., Corman, V. M., Wegner, T., Tateno, A. F., Zerbinati, R. M., Gloza-Rausch, F., et *al.,* 2011, 'Amplification of Emerging Viruses in a Bat Colony', *Emerging Infectious Diseases*, **17**(1), pp. 449-456

Drexler, J. F., Gloza-Rausch, F., Glende, J., Corman, V. M., Muth, D., Goettsche, M., *et al.,* 2010, 'Genomic characterisation of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene seqeunces', *Journal of Virology*, **84**(21), pp. 11336-11349

Drosten, C., Günther, S., Preiser, W., van der Werf, S., Bordt, H-R., Becker, S., et al., 2003, 'Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome', *New England Journal of Medicine*, **348**, pp. 1967-76

Eick, G. N., Jacobs, D. S. and Matthee, C. A., *et al.,* 2005, 'A nuclear DNA phylogenetic perspective on the evolution of echolocation and historical biogeography of extant bats (Chiroptera), *Molecular Biology and Evolution*, **22**(9), pp. 1869-1886

Emonet, S., Lemasson, J-J., Gonzalez, J-P., de Lamballerie, X., and Charrel, R. N., 2006, 'Phylogeny and evolution of old world arenaviruses', *Virology*, **350**, pp. 251-257

Emonet, S.F., de la Torre, J.C., Domingo, E. and Sevilla, N., 2009, 'Arenavirus genetic diversity and its biological implications', *Infections, Genetics and Evolution*, **9**, 417-429

Enria, D. A., Briggiler, A. M. and Feuilade, M. R., 1998, 'An overview of the epidemiological, ecological and preventive hallmarks of Argentine haemorrhagic fever (Junin virus)', *Bulletin de l'Institut Pasteur*, **96**, pp. 103-114

Enria, D. A., Mills, J. N., Bausch, D., Shieh, W-J., and Peters, C. J., 2011, 'Chapter 68 – Arenavirus Infections, *Tropical Infectious Diseases: Principles, Pathogens, and Practice (3rd Edition)*, W. B. Saunders, Edinburgh, pp. 449-461.

Escutenaire, S., Chalon, P., de Jaegere, F., Karelle-Bui, L., Mees, G., Brochier, B., *et al.,* 2002, 'Behavioural, physiologic, and habitat influences on the dynamics of Puumala virus infection in bank voles (*Clethrionomys glareolus*)', *Emerging Infectious Diseases*, **8**(9), pp. 930-936

Esper, F., Weibel, C., Ferguson, D., Landry, M. L., and Kahn, J. S., 2006, 'Coronavirus HKU1 Infection in the United States', *Emerging Infectious Diseases*, **12**(5), pp.775-779

Falcon, A., Vázquez-Morón, S., Casas, I., Aznar, C., Ruiz, G., Pozo, F., *et al.,* 2011, 'Detection of alpha and betacoronaviruses in multiple Iberian bat species', *Archives of Virology*, **156**, pp. 1883-1890

Farkas, T., Fey, B., Keller, G., Martella, V. and Egyed, L., *et al.,* 2012, 'Molecular detection of novel astroviruses in wild and laboratory mice', *Virus Genes*, **45**, pp. 518-525

Field, H. E., 2009, 'Bats and emerging zoonoses: Henipaviruses and SARS', *Zoonoses and Public Health*, **59**, pp. 278-284

Field, H. E., Mackenzie, J. S. and Daszak, P., 2007, 'Henipaviruses: Emerging paramyxoviruses associated with fruit bats', in J.E. Childs, J. S. Mackenzie and J. A. Richts (eds), *Wildlife and emerging zoonotic diseases: The biology, circumstances and consequences of cross-species transmission CTMI 315 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 133-159

Fielding, B. C., 2011, 'Human coronavirus NL63: a clinically important virus?', *Future Microbiology*, **6**(2), pp. 153-159

Finkbeiner, S. R., Kirkwood, C. D. and Wang, D., 2008, 'Complete genome sequence of a highly divergent astrovirus isolated from a child with diarrhea', *Virology Journal*, **5**, pp. 117

Finkbeiner, S. R., Li, Y., Ruone, S., Conrardy, C., Gregoricus, N., *et al.,* 2009, 'Identification of a novel astrovirus (astrovirus VA1) associated with an outbreak of acute gastroenteritis', *Journal of Virology*, **20**, pp. 10836-10839

Food and Agricultural Organisation of the United Nations, 2011, 'Investigating the role of bats in emerging zoonoses: Balancing ecology, conservation and public health

interests', S. H. Newman, H. E. Field, C. E. de Jong and J. H. Epstein (eds), *FAO Animal Production and Health Manual*, No. 12, Rome

Fouchier, R. A. M., Hartwig, N. G., Besteboer, T. M., Niemeyer, B., de Jong, J. C., Simon, J. H., *et al.,* 2004, 'A previously undescribed coronavirus associated with respiratory disease in humans', *Proceedings of the National Academy of Science*, **101**(16), pp. 6212-6216

Frame, J. D., Baldwin Jr, J. M., Gocke, D. J. and Troup, J. M., 1970, 'Lassa fever, a new virus disease of man from West Africa', *American Journal of Tropical Medicine and Hygiene*, **19**(4), 670-675

Fu, Y., Pan, M., Wang, X., Xu, Y., Xie, X., Knowles, N. J., *et al.,* 2009, 'Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings', *Journal of General Virology,* **90**, pp. 1104-1108

Fukushi, S., Tani, H., Yoshikawa, T., Saijo, M., and Morikawa, S., *et al.,* 2012, 'Serological assays based on recombinant viral proteins for the diagnosis of arenavirus hemorrhagic fevers', *Viruses*, **4**, pp. 2097-2114

Fulhorst, C. F., Bowen, M. D., Salas, R. A., Duno, G., Utrera, A., Ksiazek, T. G., *et al.,* 1999, 'Natural rodent host associations of Guanarito and Pirital viruses (family *Arenaviridae*) in central Venezuela', *American Journal of Tropical Medicine and Hygiene*, **61**(2), pp. 325-330

Fulhorst, C. F., Koster, F. T., Enria, D. A. and Peters, C. J., 2011, 'Chapter 71-hantavirus infections' in *Tropical Infectious Diseases: Principles, Pathogens, and Practice (3rd Edition)*, W. B. Saunders, Edinburgh, pp. 470-480.

Fulton, R. W., Ridpath, J. F. and Burge, L. J., *et al.,* 2012, 'Bovine coronaviruses from the respiratory tract: Antigenic and genetic diversity', *Vaccine*, **31**, pp 886-892

Garcin, D. and Kolakofsky, D., 1992, 'Tacaribe Arenavirus RNA Synthesis In Vitro Is Primer Dependent and Suggests an Unusual Model for the Initiation of Genome Replication', *Journal of Virology*, May, 1370-1376.

Garin , D., Peyrefitte, C., Crance, J-M., Le Faou, A., Jouan, A. and Bouloy, M., *et al.,* 2001, 'Highly sensitive Taqman® PCR detection of Puumala hantavirus', *Microbes and Infection*, **3**(9), pp.739-745

Gavrilovskaya, I. N., Persleni, T., Geimonen, E. and Mackow, E. R., 2002, 'Pathogenic hantaviruses selectively inhibit β3 integrin directed endothelial cell migration', *Archives of Virology*, **147**, pp. 1913-1931

Geldenhuys, M., Weyer, J., Nel, L. H. and Markotter, W., *et al.,* 2013, 'Coronaviruses in South African bats', *Vector-Borne and Zoonotic Diseases*, **13**(7), 516-519

Glass, R. I. and Bresee, J. S., 2011, 'Chapter 63 - Astroviruses, Enteric Adenoviruses, and Other Gastroenteritis Viral Infections', in *Tropical Infectious Diseases: Principles, Pathogens and Practice (3rd Edition*), W.B. Saunders, Edinburgh, pp. 416-419

Gonzalez, J. P., Emonet, S., de Lamballerie, X., and Charrel, R., 2007, 'Arenaviruses', in J. E. Childs, J. S. Mackenzie and J. A Richt (eds), *Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission CTMI 315 Current Topics in Microbiology and Immunology*, 2007, Springer-Verlag Berlin, Heidelberg, pp. 253-288

Gonzalez, J. P., Josse, R., Johnson, E. D., Merlin, M., Georges, A. J., Abandja, J., *et al.,* 1989, 'Antibody prevalence against haemorrhagic fever viruses in randomized representative central African populations', *Research Virology*, **140**, pp. 319-331

Gonzalez, J. P., McCormick, J. B., Baudon, D., Gautun, J. P., Meunier, D. Y., Dournon, E. and Georges, A. J., 1984, 'Serological evidence for hantaan-related virus in Africa', *The Lancet*, 1036-1037

Grajkowska, L. T., Pedras-Vasconcelos, J. A., Sauder, C., Verthelyi, D. and Puig, M., 2009, 'High-throughput real-time PCR for early detection and quantitation of arenavirus Tacaribe', *Journal of Virological Methods*, **159**, pp. 239-243

Greger, M., 2007, 'The human/animal interface: Emergence and resurgence of zoonotic infectious diseases', *Critical Reviews in Microbiology*, **33**, pp. 243-299

Guan, Y., Zheng, B. J., He, Y. Q., liu, X. L., Zhuang, Z. X., Cheung, C. L., *et al.,* 2003, 'Isolation and characterisation of viruses related to the SARS coronavirus from animals in Southern China', *Science*, **302**, pp. 276-278

Guarner, J., Johnson, B. J., Paddock, C. D., Shieh, W-J., Goldsmith, C. S., Reynolds, M. G., *et al.,* 2004, 'Monkeypox transmission and pathogenesis in prairie dogs', *Emerging Infectious Diseases*, **10**(3), pp. 426-431

Guix, S., Bosch, A. and Pintó, R. M., 2005, 'Human astrovirus diagnosis and typing: current and future prospects', *Letters in Applied Microbiology*, **41**, pp. 103-105

Guix, S., Caballero, S., Villena, C., Bartolomé, R., Latorre, C., Rabella, N., *et al.,* 2002, 'Molecular epidemiology of astrovirus infection in Barcelona, Spain', *Journal of Clinical Microbiology*, **40**(1), pp. 133-139

Günther, S., Hoofd, G., Charrel, R., Röser, C., Becker-Ziaja, B., Lloyd, G., *et al.,* 2009, 'Mopeia virus-related arenavirus in multimammate mice, Morogoro, Tanzania', *Emerging Infectious Diseases*, **15**(2), 2008-2012

Guo, W-P., Lin, X-D., Wang, W., Tian, J-H., Cong, M-L., Zhang, H-L., *et al.***,** 2013, 'Phylogeny and Origins of Hantaviruses Harboured by Bats, Insectivores, and Rodents', *PLoS Pathogens*, **9**(2): e1003159. doi:10.1371/journal.ppat.1003159

Guy, J. S., Breslin, J. J., Breuhaus, B., Vivrette, S. and Smith, L. G., 2000, 'Characterization of a coronavirus isolated from a diarrheic foal', *Journal of Clinical Microbiology*, **38**(12), pp. 4523-4526

Hammerbeck, C. D., Wahl-Jensen, V. and Hooper, J. W., 2009, 'Chapter 23 – Hantavirus', in A. D. T. Barrett and L. R., Stanberry (eds), *Vaccines for Biodefense and Emerging and Neglected Diseases*, Academic Press, London, pp. 379-411

Hamre, D. and Procknow, J. J., 1966, 'A new virus isolated from the human respiratory tract', *Experimental Biology and Medicine*, **121**(1), 190-193

Hansen, J., Sato, M., Ruedy, R., Lo, K., Lea, D. W. and Medina-Elizade, M., 2006, 'Global temperature change', *Proceedings for the National Academy of Science*, **103**(39), pp. 14288-14293

Henttonen, H., Buchy, P., Suputtamongkel, Y., Jittapalapong, S., Herbreteau, V., Laakokonen, J., *et al.,* 2008, 'Recent discoveries of new hantaviruses wident their range and question their origins', *Animal Biodiversity and Emerging Diseases***, 1149**, 84-89

Hepojoki, J., Strandin, T., Lankin, H., and Vaheri, A., 2012, 'Hantavirus structure – molecular interactions behind the scene', *Journal of General Virology*, **93**, pp. 1631-1644

Herrewegh, A. A. P. M., Smeenk, I., Horzinek, M. C., Rottier, P. J. M., and de Groot, R. J., 1998, 'Feline Coronavirus Type II Strains 79-1683 and 79-1146 Originate from a Double Recombination between Feline Coronavirus Type I and Canine Coronavirus', *Journal of Virology*, **72**(5), pp. 4508-4514

Heyman, P., Plyusnina, A., Berny, P., Cochez, C., Artois, M., Zizi, M., *et al.,* 2004, 'Seoul hantavirus in Europe: first demonstration of the virus genome in wild Rattus norvegicus captured in France', *European Journal of Clinical Microbiology and Infectious Diseases*, **23**, pp. 711-717

Hilbe, M., Herrsch, R., Kolodziejek, J., Nowotny, N., Zlinsky, K. and Ehrensperger, F., *et al.,* 2006, 'Shrews as reservoir hosts of borna disease virus', *Emerging Infectious Diseases*, **12**(4), 675-677

Hinson, E. R., Shone, S. M., Zink, C., Glass, G. E. and Klein, S. L., 2004, 'Wounding: The primary mode of Seoul virus transmission among male Norway rats', *The American Journal of Tropical Medicine and Hygiene*, **70**(3), pp. 310-317

Hjelle, B., Jenison, Torrez-Martin, N., Yamada, T., Nolte, K., Zumwalt, R., *et al.,* 1994, 'A novel hantavirus associated with an outbreak of fatal respiratory disease in the southwestern United States: evolutionary relationships to known hantaviruses', *Journal of Virology*, **68**(2), pp. 592-596

Ho, M-S., 2011, 'Chapter 59 - Severe Acute Respiratory Syndrome (SARS)', *Tropical Infectious Diseases: Principles, Pathogens and Practice (3*rd* Edition)*, W.B. Saunders, Edinburgh, pp. 392-397

Holmes, E. C. and Drummond, A. J., 2007, 'The evolutionary genetics of viral emergence', ' in J.E. Childs, J. S. Mackenzie and J. A. Richts (eds), *Wildlife and emerging zoonotic diseases: The biology, circumstances and consequences of cross-species transmission CTMI 315 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 51-66

Holmes, E. C., 2009, 'The origin of RNA viruses' in *The evolution and emergence of RNA viruses*, Oxford Series in Ecology and Evolution, Oxford University Press, UK, pp. 15-36

Hon, C-C., Lam, T-Y., Shi, Z-L., Drummond, A. J., Yip, C-W., Zheng, F., 2008, 'Evidence of the recombinant origin of a bat severe acute respiratory syndrome (SARS)-like coronavirus and its implications on the direct ancestor of SARS coronavirus', *Journal of Virology*, **82**(4), pp. 1819-1826

Hooper, J. and Schmaljohn, C., 2001, 'Hantavirus', in C. A. Tidona and G. Darai (eds), *The Springer index of viruses*, Berlin, Heidelberg, New York, pp. 150-156

Howard, C. R., 2008, 'Arenaviruses', in In A. J. Zuckerman, J. E. Banatvala, J. R. Pattison, P. D. Griffiths and B. D. Schoub (eds), *Principles and Practice of Clinical Virology (5th Edition)*, John Wiley & Sons Ltd, pp. 733-754

Hugot, J. P., Gonzalez, J. P. and Denys, C., 2001, 'Evolution of the Old World arenaviridae and their rodent hosts: generalized host-transfer or association by descent', *Infection, Genetics and Evolution*, **1**, pp. 13-20

Hui, E. **K-W.,** 2006, 'Reasons for the increase in emerging and re-emerging viral infectious diseases' *Microbes and Infection*, **8**, pp. 905-916

Hussein, I. T. M., Haseeb, A., Haque, A., and Mir, M. A., 2011, 'Recent Advances in Hantavirus Molecular Biology and Diseases', *Advances in Applied Microbiology*, **74**, pp. 35-75

Huynh, J., Li, S., Yount, B., Smith, A., Sturges, L., Olsen, J. C., *et al.,* 2012, *'Evidence supporting a Zoonotic Origin of Human Coronavirus Strain NL63', Journal of Virology,* **86**(23): 12816-12825

Irwin, N. R., Bayerlova, M., Missa, O. and Martinkova, N., 2012, 'Complex patterns of host switching in New World arenaviruses', *Molecular Ecology*, **21**, pp. 4137-4150

Ishii, A., Thomas, Y., Moonga, L., Nakamura, I., Ohnuma, A., Hang'ombe, B., Takada, A., Mweene, A., and Sawa, H., 2011, 'Novel Arenavirus, Zambia', *Emerging Infectious Diseases*, **17**(10), pp. 1921-1924

Ithete, N. L., Stoffberg, S., Corman, V. M., Cottontail, V. M., Richards, L. R., Schoeman, M. C., *et al.,* 2013, 'Close relative of human Middle East Respiratory Syndrome coronavirus in bat, South Africa', *Emerging Infectious Diseases*, **19**(10), http://dx.doi.org/10.3201/eid1910.130946

Jin, M., Park, J., Lee, S., Park, B., Shin, J., Song, K-J., *et al.,* 2002, 'Hantaan Virus Enters by Clathrin-Dependent Receptor-Mediated Endocytosis', *Virology*, **294**, pp. 60-69.

Jonsson, C. B., Figueiredo, L. T.M., and Vapalahti, O., 2010, 'A Global Perpective on Hantavirus Ecology, Epidemiology, and Disease', *Clinical Microbiology Reviews*, **23**(2), pp. 412-441

Kallio, E. R., Begon, M., Henttonen, H., Koskela, E., Mappes, T., Vaheri, A. and Vapalahti, O., 2010, 'Hantavirus Infections in fluctuating populations: the role of maternal antibodies', *Proceedings of the Royal Society B*, **277**, pp. 3783-3791

Kang, H. J., Bennett, S.N., Sumibcay, L., Arai, S., Hope, A. G., Mocz, G., *et al.,* 2009, 'Evolutionary insights from a genetically divergent hantavirus harboured by the European common mole (*Talpa europea*)', *PLoS ONE*, **4**(7), e6149. doi:10.1371/journal.pone.0006149

Kang, H. J., Bennett, S. N., Hope, A. G., Cook, J. A. and Yanagihara, R., 2011a, 'Shared ancestry between a newfound mole-bonre hantavirus and hantaviruses harboured by Cricetid rodents', *Journal of Virology*, **85**(15), pp. 7496-7503

Kang, H. J., Kadjo, B., Dubey, S., Jacquet, F. and Yanagihara, R., *et al.,* 2011b, 'Molecular evolution of Azagny virus, a newfound hantavirus harboured by the West African pygmy shrew (*Crocidura obscurior*) in Côte d'Ivoire', *Virology Journal*, **8**:373, http://www.virologyj.com/content/8/1/373

Kapoor, A., Li, L., Victoria, J., Oderinde, B., Mason, C., Pandey, P., *et al.,* 2009, 'Multiple novel astrovirus species in human stool', *Journal of General Virology*, **90**, pp. 2965-2972

Kellam, P., 1999, 'Chapter 24 - Emerging viruses: their diseases and identification', in, A. Dalgleish, R. Weiss, R. A. Weiss and R. A. Weiss (eds), *HIV and the New Viruses (2nd edition)*, Academic Press, San Diego, pp. 521-537

King A. M. Q., Adams M. J., Carstens E. B., Lefkowitz E. J. (eds), 2012, *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. San Diego: Elsevier Academic Press.

Kinunen, P. M., Palva, A., Vaheri, A. and Vapalahti, O., et al., 2013, 'Epidemiology and host spectrum of Borna disease virus infections', *Journal of General Virology*, **94**, pp. 247-262

Klein, S. L. and Calisher, C. H., 2007, 'Emergence and Persistence of Hantaviruses', in J. E. Childs, J. S. Mackenzie, J. A. Richt (eds), *CTMI 315 Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission*, Springer-Verlag, Heidelberg, pp.217-252

Klempa B., Witkowski, P. T., Popugaeva, E., Auste, B., Koivogui, L., Fichet-Calvet, E., *et al.,* 2012a, 'Sangassou virus, the first hantavirus isolate from Africa, displays genetic and functional properties distinct from those of other Murinae-associated hantaviruses', *Journal of Virology*, **86**(7), pp. 3819-3827

Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., et al., 2006, 'Hantavirus in African wood mouse, Guinea', *Emerging Infectious Diseases*, **12**(5), pp. 838-840

Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., *et al.,* 2007, 'Novel hantavirus sequence in shrew, Guinea', *Emerging Infectious Diseases*, **13**(3), pp.520-522

Klempa, B., Koivogui, L., Sylla, O., Koulemou, K., Auste, B., Krüger, D. H. and ter Meulen, J., 2010, 'Serological evidence of human hantavirus infections in Guinea, West Africa', *Journal of Infectious Diseases*, **201**, pp. 1031-1034

Klempa, B., Koulemou, K., Auste, B., Emmerich, P., Thomé-Bolduan, C., Günther, S., *et al.,* 2012b, 'Seroepidemiological study reveals regional co-occurrence of Lassa- and hantavirus antibodies in upper Guinea, West Africa', *Tropical Medicine amd International Health*, **18**(3), pp. 366-371

Klempa, B., Tkachenko, E. A., Dzagurova, T. K., Yunicheva, Y. V., Morozov, V. G., Okulova, N. M., et al., 2008, 'Hemorrhagic fever with renal syndrome casued by 2 lineages of Dobrava hantavirus, Russia', *Emerging Infectious Diseases*, **14**(4), pp. 617-625

Koci, M. D. and Schultz-Cherry, S., 2002, 'Avian astroviruses', *Avian Pathology*, **31**, pp. 213-227a

Kramski, M., Meisel, H., Klempa, B., Krüger, D. H., Pauli, G. and Nitsche, *et al.,* 2007, 'Detection and typing of human pathogenic hantaviruses by real-time reverse transcription and pyroseqeuncing', *Clinical Chemistry*, **53**(11), pp. 1899-1905

Krautkrämer, E. and Zeier, M., 2008, 'Hantavirus causing hemorrhagic fever with renal syndrome enters from the apical surface and requires decay-accelerating factor (DAF/CD55)', *Journal of Virology*, **82**(90, pp. 4257-4264

Krüger, D. H., Ulrich, R. and Lundkvist, Å., *et al.,* **2001,** 'Hantavirus infections and their prevention', *Microcbes and Infection*, **3**, pp. 1129-1144

Ksiazek, T. G., Erdman, D., Goldsmith, C., Zaki, S. R., Peret, T., Emery, S., *et al.,* 2003, 'A novel coronavirus associated with severe acute respiratory syndrome', *New England Journal of Medicine*, **348**, pp.1953-1966

Kuenzi, A. J., Douglass, R. J., White Jr, D., Bond, C. W. and Mills, J. N., 2001, 'Antibody to Sin Nombre virus in Rodents associated with peridomestic habitats in west central Montana', *American Journal of Tropical Medicine and Hygiene*, **64**(3, 4), pp. 137-146

Kunz, S., Borrow, P. and Oldstone, M.B.A., 2002, 'Receptor Structure, Binding, and Cell Entry of Arenaviruses', in M.B.A Oldstone (ed.), *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, 2002, Springer-Verlag, Berlin, pp. 111-137

Lau, S. K. P., Li, K. S. M., Tsang, A. K. L., Shek, C-T., Wang, M., Choi, G. K. Y., *et al.,* 2012, 'Recent transmission of a novel alphacoronavirus, bat coronavirus HKU10, from Leschnault's Rousettes to Pomona leaf-nosed bats: first evidence of interspecies transmission of coronavirus between bats of different suborders', *Journal of Virology*, **86**(21), pp. 11906-11918

Lee, H. W., French, G. R., Lee, P. W., Baek, L. J., Tsuchiya, K. and Foulke, R. S., *et al.,* 1981, 'Observations on natural and laboratory infection of rodents with the etiologic agent of Korean hemorrhagic fever', *American Journal of Tropical Medicine and Hygiene*, **30**, pp. 477-482

Lee, H. W., Lee, P. W. and Johnson, K. M., 1978, 'Isolation of the Etiologic Agent of Korean Hemorrhagic Fever', *Journal of Infectious Diseases*, **137**(3), pp. 298-308

Lee, P-W., Amyx, H. L., Yanagihara, R., Gajdusek, D. C., Goldgaber, D. and Gibbs Jr, C. J., *et al.,* 1985, 'Partial characterization of Prospect Hill virus isolated from meadow voles in the United States', *Journal of Infectious Diseases*, **152**(4), pp. 826-829

Lee, P.W., Park, M. S., Keen, G. A., Noveljic, Z., Tucker, T. J., Ryst, E. V., Viljoen, J. I., Pretorius, A. M. and Oelofsen, M., 1999, 'Seroepidemiologic evidence for the presence of hantavirus in South Africa', *Journal of Korean Society of Virology*, **29**,pp. 11-22 (Abstract only).

Lemey, P., Rambaut, A., Drummond, A. J. and Suchard, M. A., *et al.,* 2009, 'Bayesian phylogeography finds its roots', *PLoS Computational Biology*, **5**(9), e1000520. doi:10.1371/journal.pcbi.1000520

Leroy, E. M., Epelboin, A., Mondonge, V., Pourrut, X., Gonzalez, J-P., Muyembe-Tamfum, J-J. and Formenty, P., 2009, 'Human ebola outbreak resulting from direct

exposure to fruit bats in Luebo, Democratic Republic of Congo, 2007', *Vector-Borne and Zoonotic Diseases*, **9**(6), pp. 723-728

Leslie, M. J. and McQuiston, J. H., 2007, 'Chapter 8 - Surveillance for zoonotic diseases', in N. M. M'ikanatha, R. Lynfield, C. A. van Beneden and H. de Valk (eds), *Infectious Disease Surveillance*, Blackwell Publishing Inc., USA, pp. 93-106

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., *et al.,* 2005, 'Bats are the natural reservoirs of SARS-like coronaviruses', *Science*, **310**, pp. 676-679

Lipkin, W. I., and Firth, C., 2013, 'Viral surveillance and discovery', *Current Opinion in Virology*, **3**, pp. 199-204

Lledo, L., Klingström, J., Gegúndez, M. I, Plyusnina, A., Vapalahti, O., Saz, J. V., et al., 2003, 'Hantavirus infections in Spain: analysis of sera from the general population and from patients with pneumonia, renal disease and hepatitis', *Journal of Clinical Virology*, **27**, pp. 296-307

Lopez, N. and Franze-Fernandez, M.T., 2007, 'A single stem-loop structure in Tacaribe arenavirus intergenic region is essential for transcription termination but not required for a correct initiation of transcription and replication', *Virus Research*, **124**, pp. 237-244

Lozano, M. E., Posik, D. M., Albariño, C. G., Schujman, G., Ghiringheli, P. D., Calderon, G., Sabatitini, M., and Romanowski, V., 1997, 'Characterization of arenaviruses using a family-specific primer set for RT-PCR amplification and RFLP analysis Its potential use for detection of uncharacterized arenaviruses', *Virus Research*, **49**, pp. 79-89

Luis, A. D., Hayman, D. T. S., O'Shea, T. J., Cryan, P. M., Glibert, A. T., Pulliam, J. R. C., 2013, 'A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special?', *Proceedings of the Royal Society B Biological Sciences*, **280,** 20122753

Lukashov, V. V. and Goudsmit, J., 2002, 'Evolutionary relationships among Astroviridae', *Journal of General Virology*, **83**, pp. 1397-1405

Machado, A. M., Figueirredo, G. G., Campos, G. M., Lozano, M. E., Machado, A. R. S. R., and Figueiredo, L. T. M., 2010, 'Standardization of an ELISA test using a recombinant nucleoprotein from the Junin virus as the antigen and serological screening for arenavirus among the population of Nove Xavantina, State of Mato Grosso', *Revista da Sociedade Brasileira de Medicina Tropical* **43**(3), pp.229-233

Madeley, C. R. and Cosgrove, B. P., 1975, '28nm particles in faeces in infantile gastroenteritis' *Lancet II*, pp. 451-452

Maes, P., Klempa, B., Clement, J., Mattijnssens, J., Gajdusek, D. C., Krüger, D. H. and Van Ranst, M., 2009, 'A proposal for new criteria for the classification of hantaviruses based on S and M segment protein seqeunces', *Infection, Genetics and Evolution*, **9**, pp. 813-820

Maiztegui, J. I., 1975, 'Clinical and epidemiological patterns of Argentine haemorrhagic fever', *Bulletin of the World Health Organisation*, **52**, 567-575

Martinez, V. P., Bellomo, C., San Juan, J., Pinna, D., Forlenza, R., Elder, M. and Padula, P. J., 2005, 'Person-to-person transmission of Andes virus', *Emerging Infectious Diseases*, **11**(12), pp. 1848-1853

Mather, S., Scott, S., Temperton, N., Wirght, E., King, B. and Daly, J., 2013, 'Current progress with serological assays for exotic emerging/re-emerging viruses', *Future Virology*, **8**, pp. 745-755

McCormick, J. B. and Fischer-Hoch, S. P., 2002, 'Lassa Fever', in M.B.A Oldstone (ed.), *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, 2002, Springer-Verlag, Berlin, pp. 75-109

McCormick, J. B., Sasso, D. R., Palmer, E. L. and Kiley, M. P., et *al***.,** 1982, 'Morphological identification of the agent of Korean haemorrhagic fever (Hantaan virus) as a member of the *Bunyaviridae* family', *Lancet*, **3**, 765-768

McIntosh, K., Dees, J. H., Becker, W. B., Kapikian, A. Z. and Chanock, R. M., 1967, 'Recovery in tracheal organ culture of novel viruses from patients with respiratory disease', *Proceedings of the National Academy of Sciences*, **57**(4), 933-940

McIver, C. J., Palombo, E. A., Doultree, J. C., Mustafa, H., Marshall, J. A. and Rawlinson, W. D., 2000, 'Detection of astrovirus gastroenteritis in children', *Journal of Virological Methods*, **84**, pp. 99-105

McMichael, A. J., 2004, 'Environmental and social influences on emerging infectious diseases: past, present and future', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **359**, pp. 1049-1058

Medger, K., Chimimba, C. T. and Bennett, N. C., 2012, 'Seasonal reproduction in the eastern rock elephant shrew: influenced by rainfall and ambient temeperature?' *Journal of Zoology*, **288**(4), pp. 283-294

Meerburg, B. G., Singleton, G. R., and Kijlstra, A., *et al.,* 2009, 'Rodent-borne diseases and their risks for public health', *Critical Reviews in Microbiology*, **35**(3), pp. 221-270

Meheretu, Y., Čížková, D., Těšíková, J., Welegerima, K., Tomas, Z., Kidane, Z., *et al.,* 2012, 'High diversity of RNA viruses in rodents, Ethiopia'*, Emerging Infectious Diseases*, **18**(12), pp. 2047-2050

Meisel, H., Wolbert, A., Razanskiene, A., Marg, A., Kazaks, A., Sasnauskas, K., *et al.,* 2006, 'Development of novel Immunoglobulin G (IgG), IgA, and IgM enzyme immunoassays based on recombinant Puumala and Dobrava hantavirus nucleocapsid proteins', *Clinical and Vaccine Immunology*, **13**(12), pp. 1349-1357

Memish, Z.A., Mishra, N., Olival, K. J., Fagbo, S. F., Kapoor, V., Epstein, J. H., *et al.,* 2013, 'Middle East Respiratory Syndrome Coronavirus in Bats, Saudi Arabia', *Emerging Infectious Diseases*, **19**(11), http://dx.doi.org/10.3201/eid1911.131172 (In Press)

Méndez, E., Murillo, A., Velázquez, R., Burnham, A. and Arias, C. F., 2013, 'Chapter 2 − Replication cycle of astroviruses', in S. Schultz-Cherry (ed), *Astrovirus Research: Essential Ideas, Every Impacts, Future Directions*, Springer, New York, pp. 19-45

Mercado, R. R., 1975, 'Rodent control programmes in areas affected by Bolivian haemorrhagic fever', *Bulletin of the World Health Organisation*, **52**, pp. 691- 696

Meyer, B. J. and Schmaljohn, C. S. *et al.,* 2000, 'Persistent hantavirus infections: characteristics and mechanisms', *Trends in Microbiology*, **8**(2), pp 61-67

Meyer, B.J., de la Torre, J.C. and Southern, P.J., 2002, 'Arenaviruses: Genomic RNAs, Transcription, and Replication', in M.B.A Oldstone (ed.)**,** *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 139-157

Mickleburgh, S., Waylen, K. and Racey, P., 2009, 'Bats as bushmeat: a global review', *Fauna and Flora International, Oryx*, **43**(2), pp. 217-234

Miller-Butterworth, C. M., Eick, G., Jacobs, D. S., Schoeman, M. C. and Harley, E. H., *et al.,* 2005, 'Genetic and phenotypic differences between South African long-fingered

bats, with a global Miniopterins phylogeny', *Journal of Mammalogy*, **86**(6), pp. 1121-1135

Miller-Butterworth, C. M., Murphy, W. J., O'Brien, S. J., Jacobs, D. S., Springer, M. S. and Teeling, E. C., 2007, 'A Family Matter: Conclusive Resolution of the Taxonomic Position of the Long-Fingered Bats, Miniopterus', *Molecular Biology and Evolution*, **24**(7), pp. 1553-1561

Mills, J. N. and Childs, J. E., 1998, 'Ecologic studies of rodent reservoirs: their relevance for human health', *Emerging Infectious Diseases*, **4**(4), pp. 529-537

Mizutani, T., Endoh, D., Okamoto, M., Shirato, K., Shimizu, H., Arita, M., *et al.,* 2007, 'Rapid genome sequencing of RNA viruses', *Emerging Infectious Diseases*, **13**(2), pp. 322-324

Mokili, J. L., Rohwer, F. and Dutilh, B. E., 2012, 'Metagenomics and future perspectives in virus discovery', *Current Opinion in Virology*, **2**, pp. 63-77

Monadjem, A., Taylor, P. J., Cotterill, F. P. D. and Schoeman, M. C., 2010, *Bats of Southern and Central Africa: A biogeographic and taxonomic synthesis,* Wits University Press, Johannesburg

Moraz, M-L. and Kunz, S., 2011, 'Pathogenesis of arenavirus hemorrhagic fevers', *Expert Review of Anti-infective Therapy*, **9**(1), pp. 49-59

Morse, S. S., 1995, 'Factors in the emergence of infectious diseases', *Emerging Infectious Diseases*, **1**(1), pp. 7-15

Morse, S. S., Mazet, J. A. K., Woolhouse, M., Parrish, C. R., Carroll, D., Karesh, W. B., *et al.,* 2012, 'Prediction and prevention of the next pandemic zoonsis', *Lancet*, 380, pp. 1956-1965

Moser, L. A. and Schultz-Cherry, S., 2005, 'Pathogenesis of Astrovirus Infection', *Viral Immunology*, **18**(1), pp. 4-10

Moser, L. and Schultz-Cherry, S., 2008, 'Astroviruses', Encyclopaedia of Virology, pp. 204-210

Moya, A., Holmes, E. C. and González-Candelas, 2004, 'The population genetics and evolutionary epidemiology of RNA viruses', *Nature Reviews Microbiology*, **2,** pp. 279-288

Müller, M. A., Paweska, J. T., Leman, P. A., Drosten, C., Grywna, K., Kemp, A., *et al,* 2007, 'Coronavirus antibodies in African bat species', *Emergnig Infectious Diseases*, **13**(9), 1367-1370

Muranyi, W., Bahr, U., Zeier, M. and van der Woude, F. J., 2005, 'Hantavirus infection', *Journal of the American Society of Nephrology*, **16**, pp. 3669-3679

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A., Gleeson, L., *et al.,* 1995, 'A Morbillivirus that caused fatal disease in horses and humans', *Science,* **268**, pp. 94-97

Nadan, S., Walter, J. E., Grabow, W. O. K., Mitchell, D. K. and Taylor, M. B., 2003, 'Molecular Characterization of Astroviruses by Reverse Transcriptase PCR and Sequence Analysis: Comparison of Clinical and Environmental Isolates from South Africa', *Applied and Environmental Microbiology*, **69**(2), pp. 747-753

Negredo, A., Palacios, G., Vázquez-Morón, S., González, F., Dopazo, H., Molero, F., *et al.,* 2011, 'Discovery of an Ebola-like filovirus in Europe', *PLos Pathogens*, 7(10): e1002304. doi:10.1371/journal.ppat.1002304

Nichol, S. T., Spiropoulou, C. F., Morzunov, S., Rolin, P. E., Ksiazek, T. G., Feldmann, H., *et al.,* 1993, 'Genetic Identification of a Hantavirus Associated with an Outbreak of Acute Respiratory Illness', *Science*, **262**, pp. 914-917

Ogbu, O., Ajuluchukwu, E. and Uneke, C. J., *et al.,* 2007, 'Lassa fever in West African sub-region: an overview', *Journal of Vector-borne Diseases*, **44**, pp. 1-11

Okumura, M., Yoshimatsu, K., Kumperasart, S., Nakamura, I., Ogino, M., Taruishi, M., *et al.,* 2007, 'Development of serological assays for Thottapalayam virus, an insectivore-borne hantavirus', *Clinical and Vaccine Immunology*, **14**(2), pp. 173-181

Olsson, G. E., Leirs, H. and Henttonen, H., et al., 2010, 'Hantaviruses and their hosts in Europe: Reservoirs here and there, but not everywhere?', *Vector-borne and Zoonotic Diseases*, **10**(6), pp. 594-561

Padula, P.J., Edelstein, A., Miguel, S. D.L., López, N. M., Rossi, C. M. and Rabinovich, R. D., *et al.,* 1998, 'Hantavirus pulmonary syndrome outbreak in Argentina: Molecualr evidence for person-to-person transmission of Andes virus', *Virology,* **241**, pp. 323-330

Pager, C. T., and Steele, A. D., 2002, 'Astrovirus-associated diarrhea in South African adults', *Clinical Infectious Diseases*, **35**, pp. 1452-1453

Palacios, G., Savji, N., Hui, J., da Rosa, A., Popov, V., Briese, T., Tesh, R. and Lipkin, W. I., 2010, 'Genomic and phylogenetic characterization of Merino Walk virus, a novel arenavirus isolated in South Africa', *Journal of General Virology*, **91**, pp. 1315-1324

Pattamadilok, S., Lee, B-H., Kumperasart, S., Yoshimatsu, K., Okumura, M., Nakamura, I., *et al.,* 2006, 'Geographical distribution of hantaviruses in Thailand and potential human health significane of Thailand virus', *American Journal of Tropical Medicine and Hygiene*, **75**(5), pp. 994-1002

Pativada, M., Nataraju, S. M., Ganesh, B., Rajendran, K., Ramamurthy, T., Ganguly, S., *et al.,* 2012, 'Emerging trends in the epidemiology of human astrovirus infection among infants, children and adults hospitalized with acute watery diarrhea in Kolkata, India', *Infection, Genetics and Evolution*, **12**, pp. 1685-1693

Patz, J. A., Olson, S. H., Uejio, C. K. and Gibbs, H. K., 2008, 'Disease emergence from global climate and land use change', *Medical Clinics of North America*, **92**, pp. 1473-1491

Paweska, J.T., Sewlall, N.H., Ksiazek, T.G., Blumberg, L.H., Hale, M.J., Lipkin, W.I., et al., 2009, 'Nosocomial Outbreak of Novel Arenavirus Infection in Southern Africa', *Emerging Infectious Diseases*, 15(10), 1598-1602

Peeters, M., Courgnaud, V., Abela, B., Auzel, P., Pourrut, X., Bibollet-Ruche, F., *et al.,* 2002, 'Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat', *Emerging Infectious Diseases*, **8**(5), pp. 451-457

Peiris, J. S. M. and Poon, L. L. M., 2009, 'Coronaviruses and Toroviruses', in A. J. Zuckerman, J. E. Banatvala, J. R. Pattison, P. D. Griffiths and B. D. Schoub (eds), *Principles and Practice of Clinical Virology (5*th *Edition)*, John Wiley & Sons Ltd, pp. 511-531

Perera, R. A., Wang, P., Gomaa, El-Shesheny, R., Kandell, A., Bagato, O., *et al.,* 2013, 'Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013', *Eurosurveillance*, **18**(36): pii=20574, http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20574

Perlman, S. and Dandekar, A. A., 2005, 'Immunopathogenesis of coronavirus infections: implications for SARS', *Nature Reviews Microbiology*, **5**, 917-927

Peters, C. J., 2002, 'Human infection with arenaviruses in the Americas', in M.B.A Oldstone (ed.), *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 65-74

Pfefferle, S., Oppong, S., Drexler, J. F., Gloza-Rausch, F., Ipsen, A., Seebens, A., *et al.,* 2009, 'Distant relatives of severe acute respiratory syndrome coronavirus and close relatives og human coronaviruses 229E in bats, Ghana', *Emerging Infectious Diseases*, **15**(9), 1377-1384

Plyusnin, A. and Morzunov, S., 2001, 'Virus evolution and genetic diversity of hantaviruses and their rodent hosts, in C. Schmaljohn and S. N. Nichol (eds), *Current Topics in Microbiology and Immunology 256 Hantaviruses*, Springer-Verlag, Berlin, Heidelberg, pp. 47-75

Plyusnin, A., 2002, 'Genetics of hantaviruses: Implications to taxonomy', *Archives of Virology*, **147**, pp. 665-682

Poon, L. L. M., Wong, O. K., Luk, W., Yuen, K. Y., Peiris, J. S. M. and Guan, Y., *et al.,* 2003, 'Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS)', *Clinical Chemistry*, **49**(6), pp. 953-955

Posada, D., 2008, 'jModelTest: phylogenetic model averaging', *Molecular Biology and Evolution*, **25**(7), 1253-1256

Quan, P-L., Firth, C., and Street, C., *et al.,* 2010, 'Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf-nosed bat in Nigeria', *mBio* **1**(4): e00208-10. doi:10.1128/mBio.00208-10.

Quiroga, M. A., Cappuccio, J., Piñeyro, P., Basso, W., Moré, G., Kienast, M., et al., 2008, 'Hemagluttinating Encephalomyelitis Coronavirus Infection in Pigs, Argentina', *Emerging Infectious Diseases*, **14**(3), pp. 484-486

Rabozzi, G., Boizzi, L., Crespi, E., Somaruga, C., Sokooti, M., Tabibi, R. *et al.* 2012, 'Emerging Zoonoses: the "One Health Approach"', *Safety and Health at Work*, **3**, pp. 77-83.

Radosa, L., Schlegel, M., Gebauer, P., Ansorge, H., Heroldova, M., Jánova, E., *et al.,* 2013, 'Detection of shrew-borne hantavirus in Eurasian pygmy shrew (*Sorex Minutus*) in Central Europe', *Infection, Genetics and Evolution*, http://dx.doi.org/10.1016/j.meegid.2013.04.008

Ramanathan, H. N. and Jonsson, C. B., 2008, 'New and old world hantaviruses differentially utilize host cytoskeletal components during their life cycles', *Virology*, **374**, pp.138-150

Razanskiene, A., Schmidt, J., Geldmacher, A., Ritzi, A., Niedrig, M., Lundkvist, Å., *et al.,* 2004, 'High yields of stable and highly pure nucleocapdis proteins of different hantaviruses can be generated in the yeast *Saccharomyces cerevisiae'*, *Journal of Biotechnology*, **111**, pp. 319-333

Reuter, G., Pankovics, P. and Boros, A., 2011, 'Identification of a novel astrovirus in a domestic pig in Hungary', *Archives of Virology*, **156**, pp. 125-128

Riber, A. B., 2006, 'Habitat use and behaviour of European hedgehog Erinaceus europaeus in a Danish rural area', *Acta Theriologica*, **51**(4), pp. 363-371

Richman, D. D., 1993, 'Virus detection systems', in S. S. Morse (ed), *Emerging Viruses*, Oxford University Press, New York, pp. 91-99

Rivera, R., Nollens, H. N., Venn-Watson, S., Gulland, F. M. D. and Wellehan Jr, J. F. X., 2010, 'Characterization of phylogenetically diverse astroviruses of marine mammals', *Journal of General Virology*, **91**, pp. 166-173

Rodas, J. D. and Salvato, M. S., 2006, 'Tales of mice and men: Natural history of arenaviruses', *Revista Columnbiana de Ciencias Pecuarias*, **19**(4), pp. 382-400

Rodier, G., Soliman, A. K., Bouloumié, J. and Kremer, D., 1993, 'Presence of antibodies to Hantavirus in rat and human populations of Djibouti', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, pp. 160-161

Roingeard, P., 2008, 'Viral detection by electron microscopy: past, present and future', *Biology of the Cell*, **100**(8), pp. 491-501

Salah, S., Fox, E., Abbatte, E. A., Constantine, N. T., Asselin, P. and Soliman, A. K., 1988, 'A negative human serosurvey of haemorrhagic fever viruses in Djibouti', *Annales de l'Institut Pasteur. Virology*, **139**, pp. 439-442

Salazar-Bravo, J., Ruedas, L.A. and Yates, T.L., 2002, 'Mammalian Reservoirs of Arenaviruses', in M.B.A Oldstone (ed.)**,** *Arenaviruses I CTMI 262 Currrent Topics in Microbiology and Immunology*, 2002, Springer-Verlag, Berlin, pp. 25-63

Sanfeliu, I., Nogueras, M. M., Gegúndez, M. I., Segura, F., Lledo, L., Font, B., and J. V., *et al.,* 2011, 'Seroepidemiologicalsurvey of hantavirus infection in health people in Vallés Occidental, Barcelona', *Vector-borne and Zoonotic Diseases*, **11**(6), 697-700

Schilling, S., Emmerich, P., Klempa, B., Auste, B., Schnaith, E., Schmitz, H., *et al.,* 2007, 'Hantavirus disease outbreak in Germany: Limitations of routine serological diagnostics and clustering of virus sequences of human and rodent origin', *Journal of Clinical Microbiology*, **45**(9), pp. 3008-3014

Schmaljohn, C. S., Hasty, S. E., Dalrymple, J. M., LeDue, J. W., Lee, H. W., Von Bonsdorff, C-H., *et al.,* 1985, 'Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome', *Science*, **227**(4690), pp. 1041-1044

Schmaljohn, C., 2009, 'Vaccines for hantaviruses', *Vaccine*, **27**, pp. D61-D64

Schwarz, A. C., Ranft, U., Piechotowski, I., Childs, J. E. and Brockmann, S. O., *et al.,* 2010, 'Risk factors for human infection with Puumala virus, Southwestern Germany', *Emerging Infectious Diseases*, **15**(7), pp. 1032-1039

Settergren, B., 2000, 'Clinical aspects of nephropathia epidemica (Puumala virus infection) in Europe: a review', *Scandinavian Journal of Infectious Diseases*, **32**, pp. 125-132

Sharp, P. M., Bailes, E., Chaudhuri, R. R., Rodenburg, C. M. Santiago, M. O. and Hahn, B. H., 2001, 'The origins of acquired immune deficiency syndrome viruses: Where and when?', *Philosophical Transcations: Biological Sciences*, **356**(1410), pp. 867-876

Sikes, R. S., Gannon, W. L., and the Animal care and use committee of the American society of mammalogists, 2011, 'Guidelines of the American society of mammalogists for the use of wild mammals in reaserch', *Journal of Mammalogy*, **92**(1), pp. 235-253

Simpson, S. Q., Spikes, L., Patel, S. and Faruqi, I., *et al.* 2010, 'Hantavirus pulmonary syndrome', *Infectious Diseases Clinics of North America*, **24**, pp. 159-173

Sjölander, K. B. and Lundkvist, Å., 1999, 'Dobrava virus infection: serological diagnosis and cross-reactions to other hantaviruses', *Journal of Virological Methods*, **80**, 137-143

Sloots, T. P., McErlean, P., Speicher, D. J., Arden, K. E., Nisssen, M. D., and MacKay, I. M., 2006, 'Evidencec of human coronavirus HKU1 and human bocavirus in Australian children', *Journal of Clinical Virology*, **35**, pp. 99-102

Smits, S. L., van Leeuwen, M., Kuiken, T., Hammer, A. S., Simon, J. H. and Osterhaus, A. D. M. E., 2010, 'Identification and characterization of deer astroviruses', *Journal of General Virology*, **91**, pp. 2719-2722

Smuts, H. and Hardie, D., 2006, 'Human bocavirus in hospitalised children, South Africa', *Emerging Infectious Diseases*, **12**(9), 1457-1458

Song, J-W., Baek, L. J., Schmaljohn, C. S., and Yanagihara, R., 2007, 'Thottapalayam Virus, a Prototype Shrewborne Hantavirus', *Emerging Infectious Diseases,* **13**(7), pp. 980-985

Song, D. and Park, B., 2012, 'Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines', *Virus Genes*, **44**, pp. 167-175

Strain, E., Kelley, L. A., Schultz-Cherry, S., Muse, S. V. and Koci, M. D., 2008, 'Genomic analysis of closely related astroviruses', *Journal of Virology*, 82(10), pp. 5099-5103

Sumibcay, L., Kadjo, B., Gu, S. H., Kang, H. J., Lim, B. K., Cook, J. A., *et al.*, 2012, 'Divergent lineage of a novel hantavirus in the banana pipistrelle (Neoromicia nana) in Cote d'Ivoire', *Virology Journal*, **9**(34), doi:10.1186/1743-422X-9-34

Suzán, G., Marcé, E., Giermakowski, J. T., Armién, B., Pascale, J., Mills, J., *et al.*, 2008, 'The effect of habitat fragmentations and species diversity loss on hantavirus prevalence in Panama', *Animal Biodiversity and Emerging Diseases*, **1149**, pp. 80-83

Swanepoel, R., Smit, S. B., Rollin, P. E., Formenty, P., Leman, P. A., Kemp, A., *et al.,* 2007, 'Studies of reservoir hosts for Marburg virus'*, Emerging Infectious Diseases*, **13**(2), 1847-1851

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., 2011, 'MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods', *Molecular Biology and Evolution* **28**, pp.2731-2739

Taylor, L. H., Latham, S. M. and Woolhouse, M. E. J., 2001, 'Risk factors for human disease emergence', *Philosophical transactions of the Royal Society of London Series B, Biological sciences*, **356**, pp. 983-989

Taylor, P.J. and Monadjem, A., 2008b, *Otomys unisulcatus*, In *IUCN Red List of Threatened Species*, Version 2013.1. <www.iucnredlist.org>. Downloaded on 16 July 2013.

Taylor, P.J., Maree, S. and Monadjem, A., 2008a, *Otomys irroratus*. In *IUCN Red List of Threatened Species*, Version 2013.1. <www.iucnredlist.org>. Downloaded on **16 July 2013**

Ter Meulen, J., Lukashevich, I., Sidibe, K., Inapogui, A., Marx, M., Dorlemann, A., et al., 1996, 'Hunting of peridomestic rodents and consumption of their meat as possible risk factors for rodent-to-human transmission of Lassa virus in the Republic of Guinea', *The American Journal of Tropical Medicine and Hygiene*, **55**(6), 661-666

Tersago, K., Verhagen, R., Servais, A., Heyman, P., Ducoffre, G., and Leirs, H., 2009, 'Hantavirus disease (nephropathia epidemica) in Belgium: effects of tree seed production and climate', *Epidemiology and Infection*, **137**, pp. 250-256

Toffan, A., Jonassen, C. M., De Battisti, C., Schiavon, E., Kofstad, T., Capua, I. and Cattoli, G., 2009, 'Genetic characterization of a new astrovirus detected in dogs suffering from diarrhoea', *Veterinary Microbiology*, **139**, pp. 147-152

Tomori, O., Morikawa, S., Matsuura, Y. and Kitamura, T., 1986, 'Antibody to Japanese strain of haemorrhagic fever with renal syndrome (HFRS) virus in Nigerian sera', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, pp. 1008

Tong, S., Conrardy, C., Ruone, S., Kuzmin, I. V., Guo, X., Tao, T., et al., 2009, 'Detection of novel SARS-like and other coronaviruses in bats from Kenya', *Emerging Infectious Diseases*, **15**(3), pp. 482-485

Tse, H., Chan, W-M., Tsoi, H-W., Fan, R. Y. Y., Lau, C. C. Y., Lau, S. K. P., et al., 2011, 'Rediscovery and genomic characterization of bovine astroviruses', *Journal of General Virology*, **92**, pp. 1888-1898

Turmell, A. S. and Olival, K. J., 2009, 'Correlates of viral richness in bats (order Chiroptera)', *EcoHealth*, **6**, 522-539

Tyrell, D. A. and Bynoe, M. L., 1965, 'Cultivation of a novel type of common-cold virus in organ cultures', *British Medical Journal*, **5448**, 1467-1470

Ulloa, J. C. and Gutiérrez, M. F., 2010, 'Genomic analysis of two ORF2 segments of new porcine astrovirus isolates and their close relationship with human astroviruses', *Canadian Journal of Microbiology*, **56**, pp. 569-577

US Fish and Wildlife Services, Survey Protocol for Determining Presence of the Buena Vista Lake Ornate Shrew, http://www.fws.gov/sacramento/ES/Survey-Protocols-Guidelines/es_survey.htm

Vabret, A., Dina, J., Gouarin, S., Petitjean, J., Corbet, S., and Freymuth, F., 2006, 'Detection of the New Human Coronavirus HKU1: A Report of 6 Cases', *Clinical Infectious Diseases*, **42**, pp. 634-639

Van Boheemen, S., de Graaf, M., Lauber, C., Besteboer, T. M., Raj, S., Zaki, A. M., et al, 2012, 'Genomic Characterization of a Newly Discovered Coronavirus Associated with Acute Respiratory Distress Syndrome in Humans', mBio 3(6): http://dx.org/doi:10.1128/mBio.00473-12

Van der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-Oost, W., Berkhout, R. J. M., Wolthers, K. C., *et al.* 2004, 'Identification of a new human coronavirus', *Nature Medicine*, **10**(4), pp. 368-373

Van der Merwe, I., Oosthuizen, M. K., Chimimba, C. T. and Bennett, N. C., *et al.,* 2011, 'Circadian rhythms of locomoter activity in the reddish-grey musk shrew (Eulipotyphla: Soricidae) from South Africa', *Journal of Zoology*, **284**(2), pp. 124-132

Van der Mescht, L., 2011, 'Ectoparasite assemblage of the four-striped mouse, Rhabdomys pumilio: the effect of anthropogenic habitat transformation and temporal variation', Thesis – Stellenbosch University, http:// ir1.sun.ac.za/handle/10019.1/6850

Van Hemert, F. J., Berkhout, B. and Lukashov, V. V., 2007, 'Host-related nucleotide composition and codon usage as driving forces in the recent evolution of the Astroviridae', *Virology*, **361**, pp. 447-454

Vandegrift, K. J., Wale, N. and Epstein, J. H., 2011, 'An Ecological and conservation perspective on advances in the applied virology of zoonoses', *Viruses*, **3**, pp. 379-397

Vapalahti, O., Lundkvist, Å., Fedorov, V., Conroy, C. J., Hirvonen, S., Plyusnina, A., *et al.,* 1999, 'Isolation and characterisation of a hantavirus from Lemmus sibiricus: evidence for host switching during hantavirus evolution', *Journal of Virology*, **73**(7), 5586-5592

Vapalahti, O., Mustonen, J., Lundkvist, Å., Henttonen, H., Plyusnin, A. and Vaheri, A., *et al.,* 2003, 'Hantavirus infections in Europe', *Lancet Infectious Diseases*, **3**, pp. 653-661

Vieth, S., Drosten, C., Lenz, O., Vincent, M., Omilabu, S., Hass, M., Becker-Zaija, B., ter Meulen, J., Nichol, S. T., Schmitz, H., and Günther, S., 2007, 'RT-PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **101**, pp. 1253-1264

Vijgen, L., Keyaerts, E., Moës, E., Thoelen, I., Wollants, E., Lemey, P., *et al.,* 2005, 'Complete genomic sequence of human coronavirus OC43: Molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event', *Journal of Virology*, **79**(3), pp. 1595-1604

Walter, J. E., Briggs, J., Guerrero, M. L., Matson, D. O., Pickering, L. K., Ruiz-Palacios, *et al.,* 2001, 'Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children', *Archives of Virology*, **146**, pp. 2357-2367

Wang, L-F. and Daniels, P., 2012, 'Diagnosis of Henipavirus infection: Current capabilities and future directions', in B. Hur and P. A. Rota (eds), *Henipavirus CTMI 359 Current Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, pp. 179-196

Weaver, S. C., Salas, R. A., de Manzione, N., Fluhorst, C. F., Duno, G., Utrera, A., *et al.,* 2000, 'Guanarito virus (*Arenaviridae*) isolates from endemic and outlying localities in Venezueala: sequence comparison among and within strains isolated from Venezuelan haemorrhagic fever patients and rodents', **Virology**, **266**, pp. 189-195

Weiss, S., Witkowski, P.T., Auste, B., Nowak, K., Weber, N., Fahr, J., *et al.,* 2012, 'Hantavirus in Bat, Sierra Leone', *Emerging Infectious Diseases*, **18**(1), pp. 159-161

Wevers, B. A. and van der Hoek, L., 2009, 'Recently Discovered Human Coronaviruses', *Clinical Laboratory Medicine*, **29**, pp. 715-724.

Wild, T. F., 2009, 'Henipaviruses: A new family of emerging Paramyxoviruses', *Virus émergents*, **57**(2), pp. 188-196

Wolfaardt, M., Kiulia, N. M., Mwenda, J. M. and Taylor, M. B., 2011, 'Evidence of a Recombinant Wild-Type Human Astrovirus Strain from a Kenyan Child with Gastroenteritis', *Journal of Clinical Microbiology*, **49**(2), pp. 728-731

Wong, S., Lau, S., Woo, P. and Yuen, K-Y., 2007, 'Bats as a continuing source of emerging infections in humans', *Reviews in Medical Virology*, **17**, pp. 67-91

Woo, P. C. Y., Lau, S. K. P. and Yuen, K., 2006, 'Infectious diseases emerging from Chinese wet-markets: zoonotic origins of severe respiratory viral infections', *Current Opinion in Infectious Diseases*, **19**, pp. 401-407

Woo, P. C. Y., Lau, S. K. P., Chu, C-M., Chan, K-H., Tsoi, H-W., Huang, Y., et al., **2005,** 'Characterization and Complete Genome Sequence of a Novel Coronavirus HKU1, from Patients with Pneumonia', *Journal of Virology*, **79**(2), pp.884-895.

Woo, P. C. Y., Lau, S. K. P., Huang, Y., and Yuen, K-Y., 2009, 'Coronavirus Diversity, Phylogeny and Interspecies Jumping', *Experimental Biology and Medicine*, **234**, pp. 1117-1127

Woo, P. C. Y., Lau, S. K. P., Li, K. S. M., Tsang, A. K. L. and Yuen, K-Y., *et al,.* 2012b, 'Genetic relatedness of the novel human group C betacoronavirus to Tylonycteris bat coronavirus HKU4 and Pipistrellus bat coronavirus HKU5', *Emerging Microbes and Infection*, **1**, e35; doi:10.1038/emi.2012.45

Woo, P. C. Y., Lau, S. K. P., Wong, B. H. L., Chan, K-H., Hui, W-T., Kwan, G. S. W., *et al.,* 2004, 'False-positive results in a recombinant severe acute respiratory syndrome-associated coronaviruses (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide', *Journal of Clinical Microbiology*, **42**(12), pp. 5885-5888

Woo, P. C. Y., Wang, M., Lau, S. K. P., Xu, H., Poon, R. W. S., Guo, R., *et al.,* 2007, 'Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features', *Journal of Virology*, **81**(4), pp. 1574-1585

Woo, P.C., Lau, S. K., Lam, C. S. F., Lau, C. C. Y., Tsang, A. K. L., Lau, J. H. N., et al., 2012a, 'Discovery of Seven Novel Mammalian and Avian Coronaviruses in the Genus Deltacoronavirus Supports Bat Coronavirus as the Gene Source of Alphacronavirus and Betacoronavirus and Avian Coronaviruses as the Source of Gammacoronavirus and Deltacoronavirus', *Journal of Virology*, **86**(7), pp. 3995-4008

Woolhouse, M. E. J. and Gaunt, E., 2007, 'Ecological origins of novel human pathogens', *Critical Reviews in Microbiology*, **33**, pp. 231-242

Woolhouse, M. E. J. and Gowtage-Sequeria, S., 2005, 'Host Range and Emerging and Reemerging pathogens', *Emerging Infectious Diseases*, **11**(12), pp. 1842-1847

Wu, Z., Ren, X., Yang, L., Hu, Y., Yang, J., He, G., *et al.,* 2011, 'Virome analysis for identification of novel mammalian viruses in bat species from Chinese provinces', *Journal of Virology*, **86**(20), pp. 10999-11012

Xiao, J., Li, J., Hu, G., Chen, Z., Wu, Y., Chen, Y., et al., 2011, 'Isolation and phylogenetic characterization of bat astroviruses in southern China', *Archives of Virology*, **156**, pp. 1415-1423

Yamada, T., Hjelle, B., Lanzi, R., Morris, C., Anderson, B. and Jenison, S., *et al.,* 1995, 'Antibody responses to Four Corners hantavirus infections in the deer mouse (Peromyscus maniculatus): identification of an immunodominant region of the viral nucleocapsid protein', *Journal of Virology*, **69**(3), pp. 1939-1943

Yates, T. L., Mills, J. N., Parmenter, C. A., Ksiazek, T. G., Parmenter, R. R., Vande Castle, J. R., *et al.,* 2002, 'The ecology and evolutionary history of an emergent disease: hantavirus pulmonary syndrome', *BioScience*, **52**(11), pp. 989-998

Zaki, A. L., van Boheeman, S., Besteboer, T. M., Osterhaus, A. D. M. E. and Fouchier, R. A. M., *et al.,* 2012, 'Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia', *New England Journal of Medicine*, **367**, pp. 1814-1820

Zapata, J.C. and Salvato, M.S., 2013, 'Arenavirus Variations Due to Host-Specific Adaptation', *Viruses*, **5**, 541-278

Zhang, J., Guy, J. S., Snijder, E. J., Denniston, D. A., Timoney, P. J., and Balasuriya, U. B. R., 2007, 'Genomic characterisation of equine coronavirus', *Virology,* **369**, pp. 92-104

Zhang, Y-Z., Zou, Y., Fu, Z. F. and Plyusnin, A., *et al.,* 2010, 'Hantavirus infections in humans and animals, China', *Emerging Infectious Diseases*, **16**(8), pp. 1195-1203

Zhu, A. L., Zhao, W., Yin, H., Shan, T. L., Zhu, C. X., Hua, X. G., *et al.,* 2011, 'Isolation and characterization of canine astrovirus in China', *Archives of Virology*, **156**, pp. 1671-1675

Zhu, H. C., Chu, D. K. W., Liu, W., Dong, B. Q., Zhang, S. Y., Zhang, J. X., *et al.,* 2009, 'Detection of diverse astroviruses from bats in China', *Journal of General Virology*, **90**, pp. 883-887

Appendices

Appendix A Ethical Approval

Hantavirus seroprevalence study

27 February 2009   **MAILED**

Miss N Ithete
Department of Medical Virology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Miss Ithete

"Molecular Identification and Characterisation of Rodent-and Shrew-borne Hantaviruses."

<u>ETHICS REFERENCE NO: N09/02/058</u>

<u>RE : PROVISIONAL APPROVAL</u>

It is my pleasure to inform you that the abovementioned project has been provisionally approved on
26 February 2009 for a period of one year from this date. You may start with the project, but this approval will however be
submitted at the next meeting of the Committee for Human Research for ratification, after which we will contact you again.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of
more information that they might deem necessary to make their final decision.

Please quote the abovementioned project number in all future correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before
the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary).
Annually a number of projects may be selected randomly and subjected to an external audit.

Please note that in line with the recent changes to research ethics guidelines, including the Declaration of Helsinki, the CHR
requires that all researchers specifically request and motivate for a "waiver of informed consent" for retrospective clinical
audits.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Committee for Human Research complies with the SA National Health Act No.61 2003 as it pertains to health research
and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and
principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as
well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

27 February 2009 11:14   Page 1 of 2

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352

Yours faithfully

PROF PIERRE DE VILLIERS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: +27(0)21 9389449 / E-mail: pjtdv@sun.ac.za
Fax: +27(0)21 9389153

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352

02 April 2009

MAILED

Miss N Ithete
Department of Medical Virology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Miss Ithete

"Molecular Identification and Characterisation of Rodent-and Shrew-borne Hantaviruses."

ETHICS REFERENCE NO: N09/02/058

RE : RATIFICATION

At a meeting that was held on 01 April 2009, the Committee for Human Research ratified the approval of the above project by the Chairperson.

Yours faithfully

MRS ELVIRA ROHLAND
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9677 / E-mail: elr@sun.ac.za
Fax: 021 931 3352

02 April 2009 11:52

Page 1 of 1

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

Ethics Letter

02-Apr-2013

Ethics Reference #: N09/02/058
Title: Molecular Identification and Characterisation of Rodent-and Shrew-borne Hantaviruses.

Dear Miss Ndapewa Ithete,

At a review panel meeting of the Health Research Ethics Committee that was held on 4 March 2013, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 4 March 2013 Expiry Date: 4 March 2014

If you have any queries or need further help, please contact the REC Office 0219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

Ethical approval for bat, rodent and shrew studies

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

25 March 2009

Miss NL Ithete
Division of Medical Virology

Dear Miss Ithete

RESEARCH PROJECT : "Molecular identification and characterisation of rodent and shrew borne   hantaviruses."

PROJECT NUMBER : P09/01/002

At a meeting of the Committee for Experimental Animal Research that was held on 25 February 2009 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 25 March 2009 **for a period of one year from this date.** This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary

Yours faithfully

FRANKLIN WEBER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9657 / E-mail: fweb@sun.ac.za

R:\KPN\PROJEKTE\P0901002_ITHETE_CONDITIONAL.DOC

Fakulteit Gesondheidswetenskappe • Faculty of Health Sciences

Verbind tot Optimale Gesondheid • Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun • Research Development and Support Division
Posbus/PO Box 19063 • Tygerberg 7505 • Suid-Afrika/South Africa
Tel: +27 21 938 9677 • Faks/Fax: +27 21 931 3352
E-pos/E-mail: rdsdinfo@sun.ac.za

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

8 November 2011

Prof T Seifert
Forest and Wood Science
Faculty of Agrisciences
Stellenbosch University

E-MAILED and **MAILED**

Dear Prof Seifert

Application for an Amendment to study:
Catching and blood taking of bats.
Ref: 11LB_SEI01

Your application for ethics clearance has been approved by the Research Ethics Committee. Please note that this clearance is only valid for a period of twelve months. Ethical clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document will be available on the Division for Research Development's website www.sun.ac.za/research **, shortly.**

Please feel free to contact me if any additional information is needed.

Kind regards

WA Beukes (Secretariat: REC: ACU)

APPROVED
0 8 NOV 2011

DIE UNIVERSITEIT VAN STELLENBOSCH
0 8 NOV 2011
THE UNIVERSITY OF STELLENBOSCH

Mr · Mnr WA Beukes
Afdeling: Navorsingsontwikkeling • Division: Research Development
Privaat Sak/Private Bag·Matieland 7602·Suid-Afrika/ South Africa
Tel +27 21 808 9003 • E-mail wabeukes@sun.ac.za • Faks/Fax: +27 021 808 4537

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

2 March 2012

Prof W Preiser
Department of Pathology
Faculty of Health Science
Tygerberg Campus
Stellenbosch University

E-MAILED and **MAILED**

Dear Prof Preiser

Application for Ethical Clearance:
Catching and non-invasive sampling of bats
Ref: SU-ACUM12-00001

Your application for ethics clearance has been approved by the Research Ethics Committee: Animal Care and Use (REC: ACU). Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Development's website www.sun.ac.za/research .

Please feel free to contact me if any additional information is needed.

Kind regards
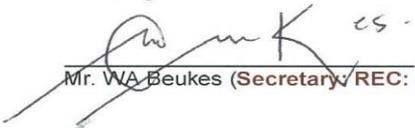
Mr. WA Beukes (Secretary/REC: ACU)

APPROVED
0 2 MAR 2012

DIE UNIVERSITEIT VAN STELLENBOSCH
0 2 MAR 2012
THE UNIVERSITY OF STELLENBOSCH

Mr · Mnr WA Beukes
Afdeling: Navorsingsontwikkeling • Division: Research Development
Privaat Sak/Private Bag·Matieland 7602·Suid-Afrika/ South Africa
Tel +27 21 808 9003 • E-mail wabeukes@sun.ac.za • Faks/Fax: +27 021 808 4537

175

Appendix B Animal samples collected during this study

Rodents and insectivores screened in the study

Eastern Cape	Species	AL	EL	ELS	FB	HB	TCS	TS
	Shrew spp.	–	–	3	5	1	3	2
	Mastomys natalensis	15	–	–	16	–	–	–
	Micealamys namaquensis	–	15	–	–	–	–	–
	Mus domesticus	–	–	–	–	–	–	–
	Mus munitoides	–	–	–	2	–	–	–
	Mus musculus	–	5	–	–	4	–	1
	Myotomys irroratus	8	4	8	6	2	15	
	Parotomys sp	–	–	–	–	–	–	1
	Rattus norvegicus	–	–	–	–	–	–	2
	Rattus rattus	2	–	–	–	2	–	–
	Rhabdomys pumilio	6	20	30	18	10	25	1
	Sacostomys campestris	–	–	–	1	–	–	–

Free State	Species	Be	DS
	Micealamys namaquensis	19	4
	Elephant shrew	2	–
	Rhabdomys pumilio	3	3
	Sacostomys campestris	2	2
	Desmodillus auricularis	–	1
	Gerbillurus paeba	–	1
	Mus domesticus	–	1
	Myotomys unisulcatus	–	2

Gauteng	Species	RV	KP
	Crocidura sp	9	9
	Mastomys sp	18	39
	Myotomys sp	2	1
	Rhabdomys pumilio	24	66

KwaZulu-Natal	Species	AF	CH	IN	OG	VC	VH
	Crocidura spp.	1	8	2	6	7	2
	Grammomus dolichurus	–	–	–	–	–	1
	Lemniscomys rosalia	–	–	–	–	–	1
	Mastomys natalensis	18	4	9	–	–	5
	Micealamys namaquensis	–	–	5	4	3	3
	Mus munitoides	–	–	–	–	–	1
	Myosorex spp.	–	–	2	–	–	–
	Myotomys spp.	2	3	3	–	–	–
	Rattus rattus	–	–	3	–	–	–
	Rhabdomys pumilio	–	23	9	2	16	4

Northern Cape	Species	ATC	JK	Gp	GH
	Desmodylus	–	–	4	–
	Elephant shrew	1	3	3	–
	Micaelamys namaquensis	14	17	15	–
	Mus minutoides	–	2	1	–
	Myotomys irroratus	–	–	–	–
	Myotomys unisulcatus	3	5	2	3
	Rhabdomys pumilio	25	31	23	32

North-West	Species	Mn	Ws	Ze
	Aethomys chrysophilus	8	–	2
	Lemniscomus rosalia	13	–	2
	Mastomys sp	21	–	2
	Mus indutus	–	4	–
	Mus minutoides	7	–	–
	Crocidura sp	3	–	–
	Steatomys pratensis	10	–	–
	Tatera brantsii	–	20	–

Western Cape	Species	AB	BJR	Df	FH	GB	HT	JM	K	Lk	Rp	SS	Sw	VRW
	Crocidura sp.	1	1	–	–	–	–	–	–	–	–	–	1	–
	Elaphantulus edwardii	1	–	–	–	–	–	–	–	–	–	–	–	–
	Gerbilliscus leucogaster	–	–	1	–	–	–	–	–	–	–	–	–	–
	Mastomys coucha	–	–	2	–	–	–	–	–	–	–	–	–	–
	Micealamys namaquensis	6	–	–	–	–	20	–	–	–	12	–	–	4
	Mus minutoides	–	–	–	–	–	1	–	–	–	–	–	–	2
	Mus musculus	–	–	1	–	–	–	–	–	–	13	–	–	–
	Myotomys irroratus	2	8	–	–	–	1	–	–	–	–	–	–	–
	Myotomys unisulcatus	11	–	–	–	–	–	–	–	–	–	–	–	–
	Rhabdomys pumilio	33	27	7	8	26	9	30	18	7	12	19	–	18
	Sacostomys campestris	–	–	–	–	–	–	–	–	–	4	–	–	–

Abbreviation	Location
Eastern Cape	
AL	Alice
EL/ELS	East London
FB	Fort Beaufort
HB	Hogsback
TCS	The Croft
TS	Tsitsikamma
Free State	
Be	Betulie
DS	Drie susters
Gauteng	
KP	Kaalplaas
RV	Rietvlei
KwaZulu Natal	
AF	Albert falls
CH	Chlemsford
IN	Inkunzi
OG	Oribi gorge
VC	Vernon Crookes
VH	Vryheid
Northern Cape	
ATC	Agama tented camp
JK	Jakobskraal
Gp	Goegap
GH	Groblershoop
North West	
Mn	Mooinooi
Ws	Woodside
Ze	Zeerust
Western Cape	
AB	Anysberg
BJR	Buffel jags rivier
Df	Dronsford
FH	Franschoek
GB	Gordons baai
HT	Hottentots-Holland
JM	Jan Marais
K	Khayelitsha
Lk	
RP	Rocherpan
SS	Somerset Wes
Sw	Swellendam
VRW	VanRhynsdorp

Additional Samples obtained from Prof A Bastos and Dr K Medger at the University of Pretoria.

	Trapping Locations			
Prof A Bastos	**Kruger National Park**	**Tsh**	**LS 2**	**LS 3**
	Mastomys natalensis/coucha	106	23	25
	Saccostomys campestris	35	5	1
	Lemniscomys rosalia	6	3	1
	Tatera leucogaster	21	5	—
Dr K Medger	**Goro Game Reserve**			
	Acomys spinosissimus	113		
	Elephantulus edwardii	113		

Abbreviation	Location
Tsh	Tshokwane
LS2	Lower Sabie 2
LS3	Lower Sabie 3

Bat samples collected and sampled in this study

KwaZulu Natal	Locations				
Species	Phinda	Vryheid	Pieter-maritzburg	Sappi Mooiplaas	Ingwavuma
Chaerephon pumilus	5	–	–	–	1
Hipposideros caffer	4	–	–	2	–
Miniopterus fraterculus		–	6	–	–
Mops condylurus	1	–	–	–	–
Neoromicia capensis	2	–	–	–	–
Neoromicia nana		4	–	–	–
Neoromicia zuluensis	1	–	–	–	–
Nycteris thebaica	1	–	–	2	–
Rhinolophus clivosus		–	6	–	–
Rhinolophus darlingi	2	–	–	–	–
Rhinolopphus swinnyi		–	3	–	–
Scotophilus viridis	3	–	–	–	–
Tadarida aegyptiaca	1	–	–	–	–

Western Cape	Locations		
Species	Table Mountain	Greyton	Rheenendal
Miniopterus natalensis	13	–	–
Neoromicia capensis	–	8	2
Rhinolophus clivosus	–	–	2
Rossettus aegyptiacus	12	–	–
Tadarida aegyptiaca	–	2	–

Appendix C Geographic distribution of bats and rodents
Bats
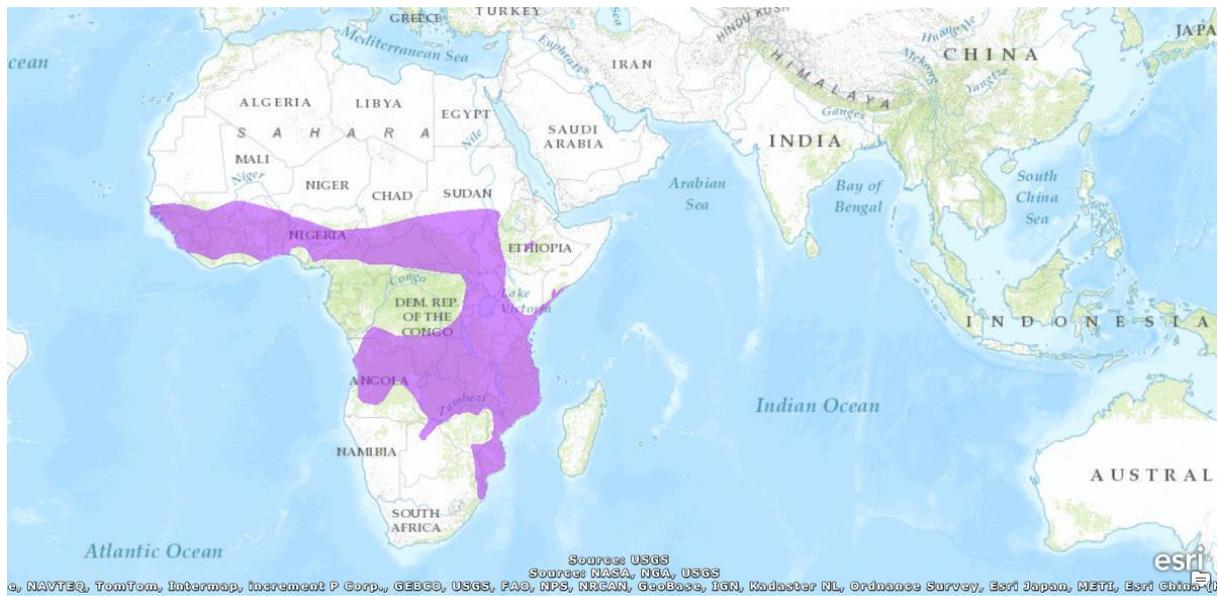
Mops condylurus
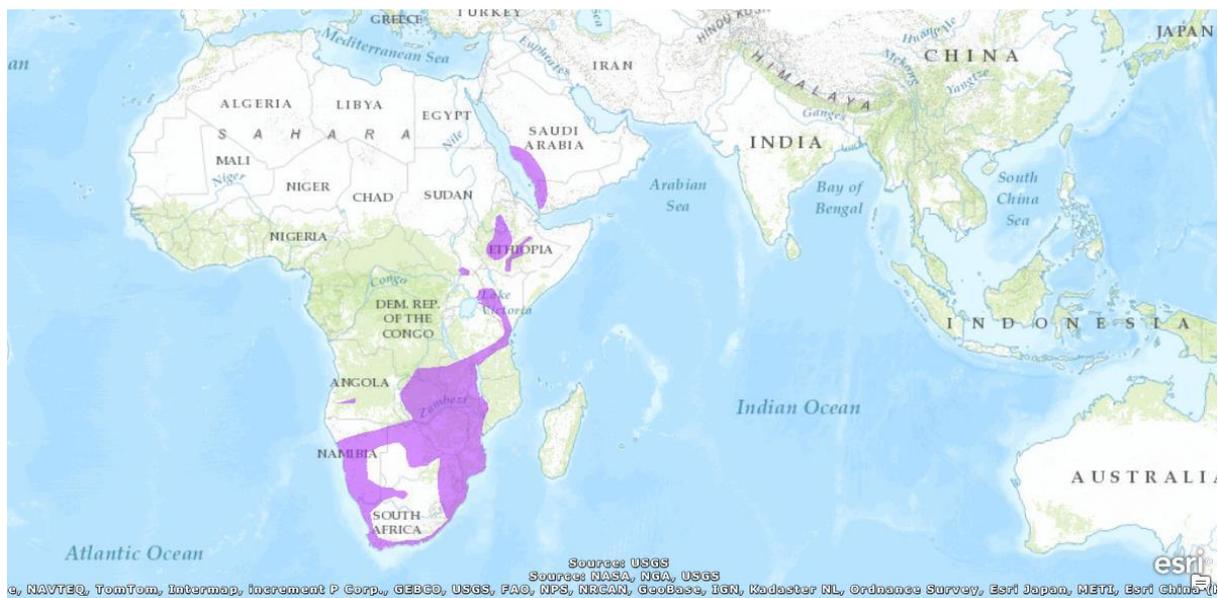
Miniopterus natalensis
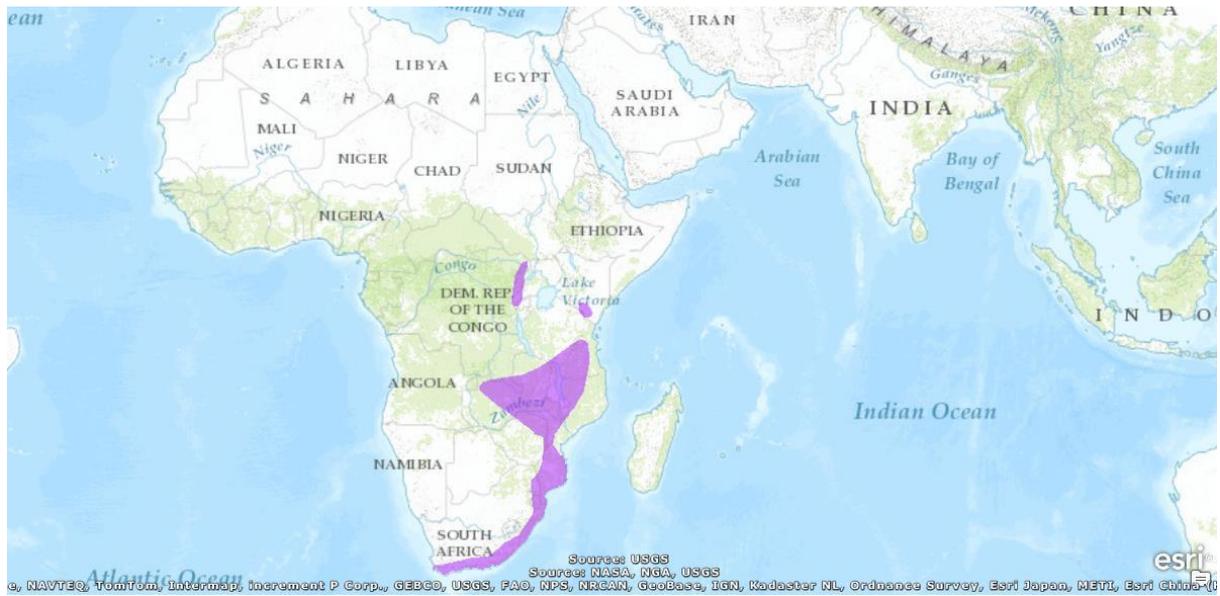
Miniopterus fraterculus
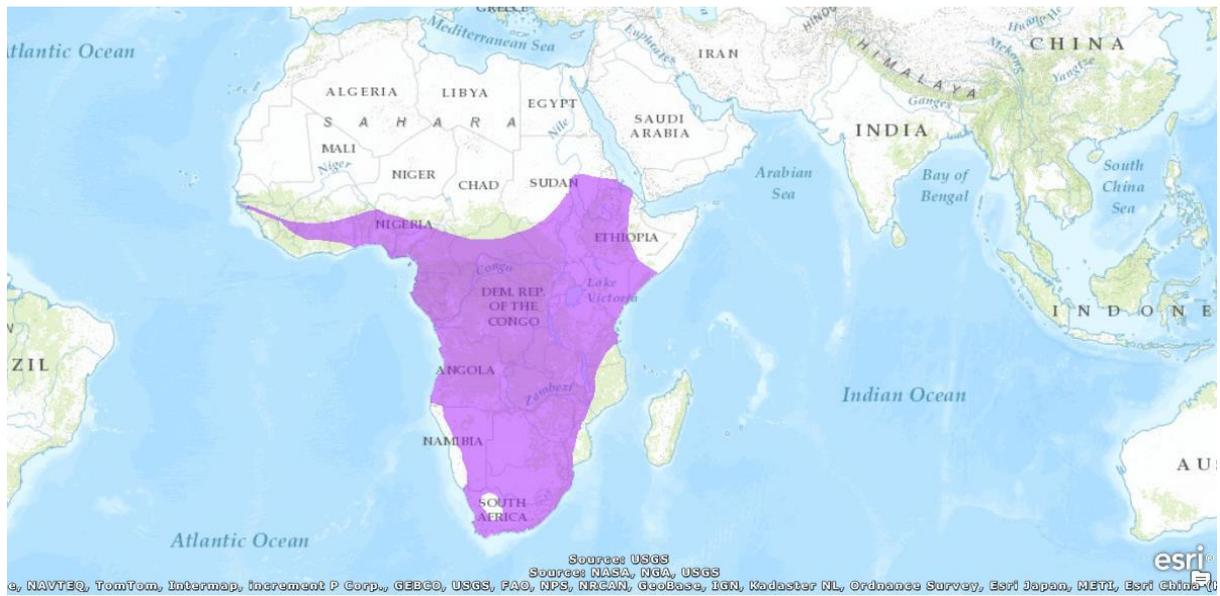
Neoromicia capensis
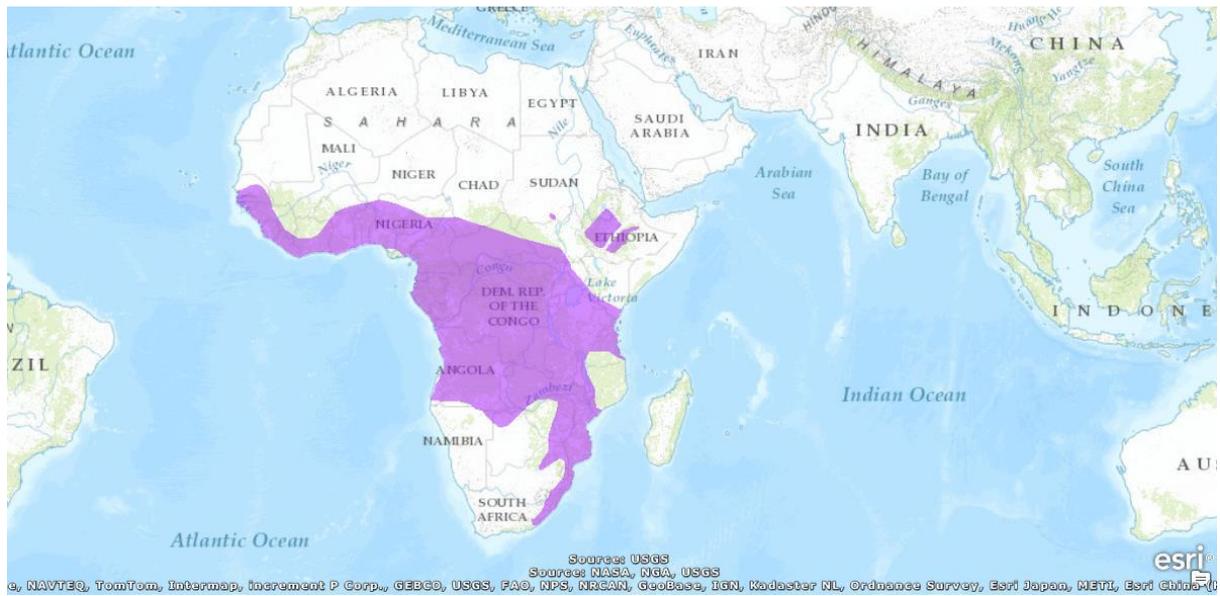
Neoromicia nana
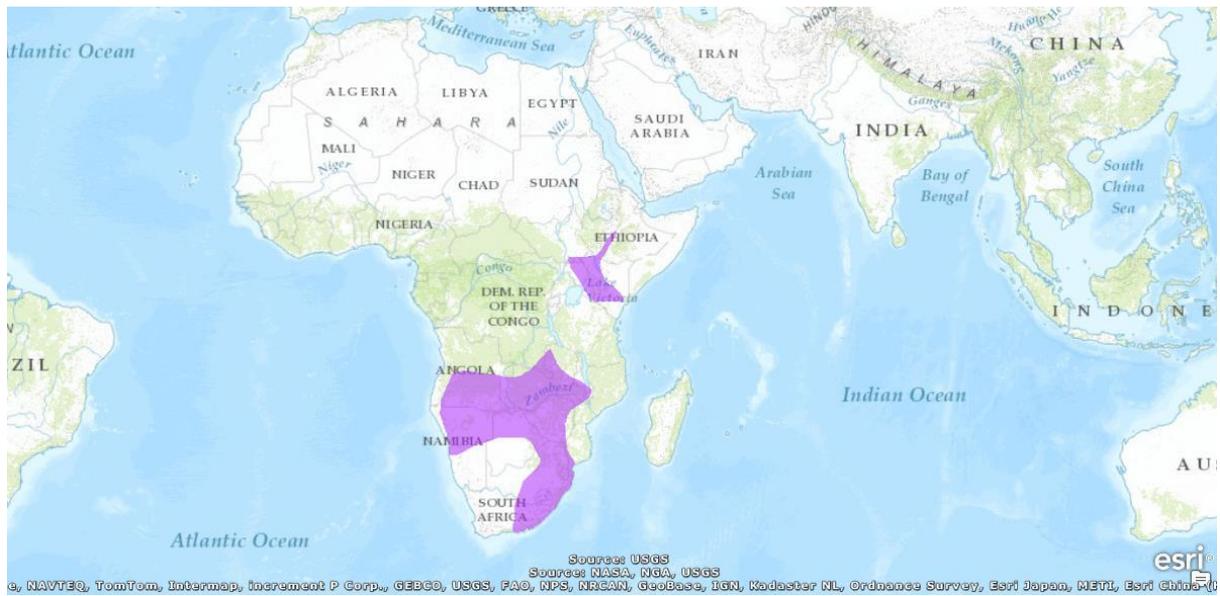
Neoromicia zuluensis

Rodents
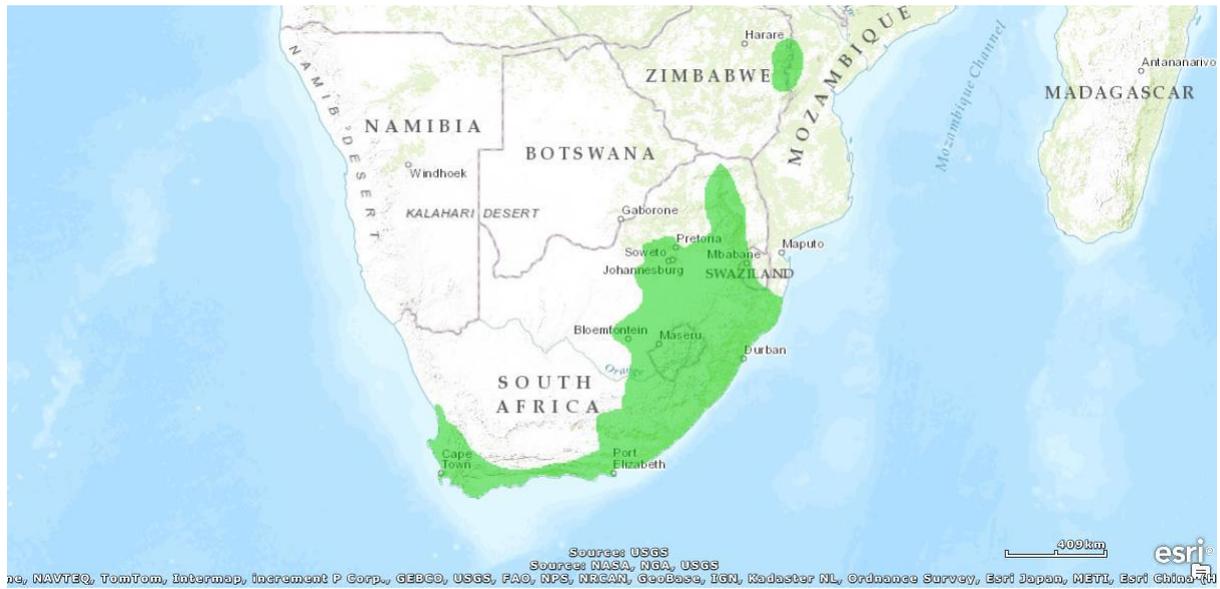
Myotomys irroratus
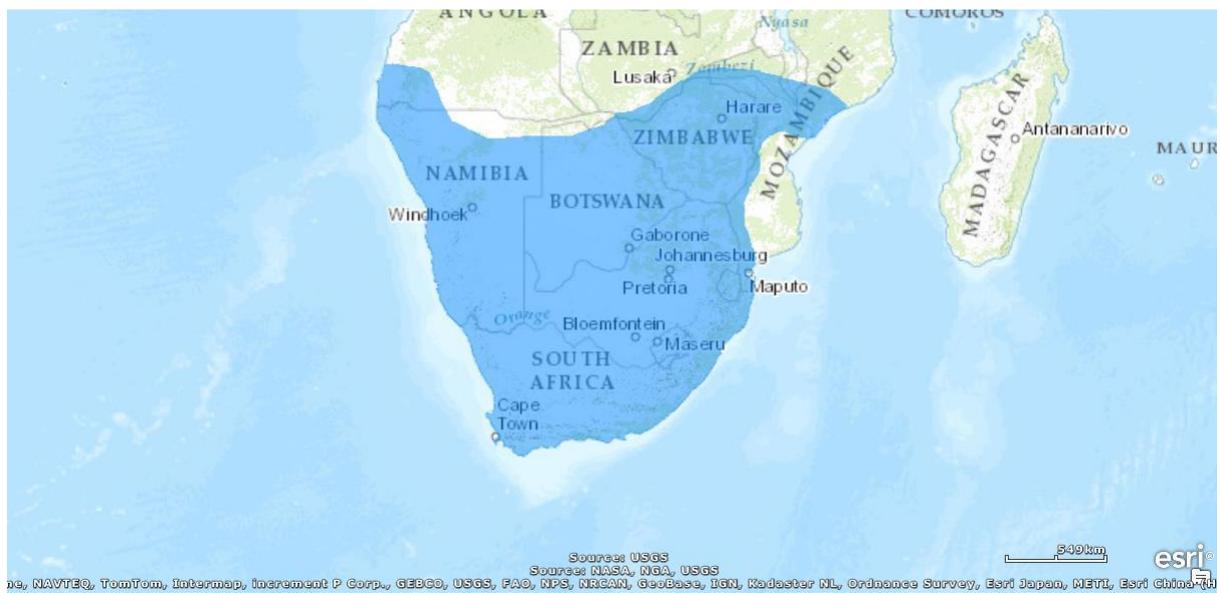
Micaelamys namaquensis
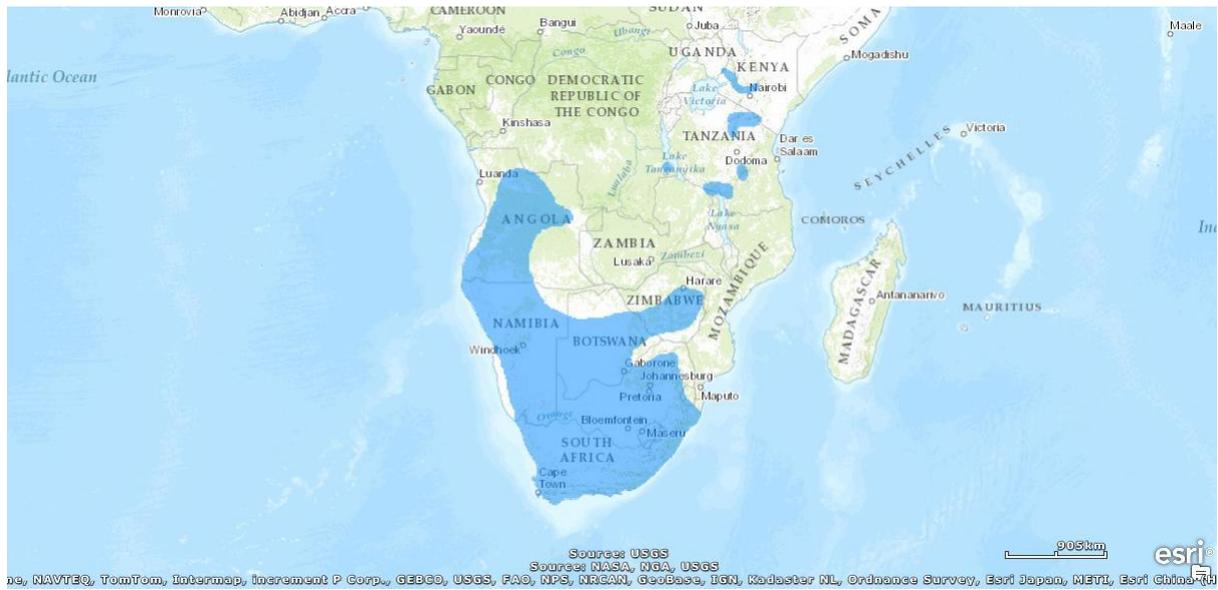
Rhabdomys pumilio
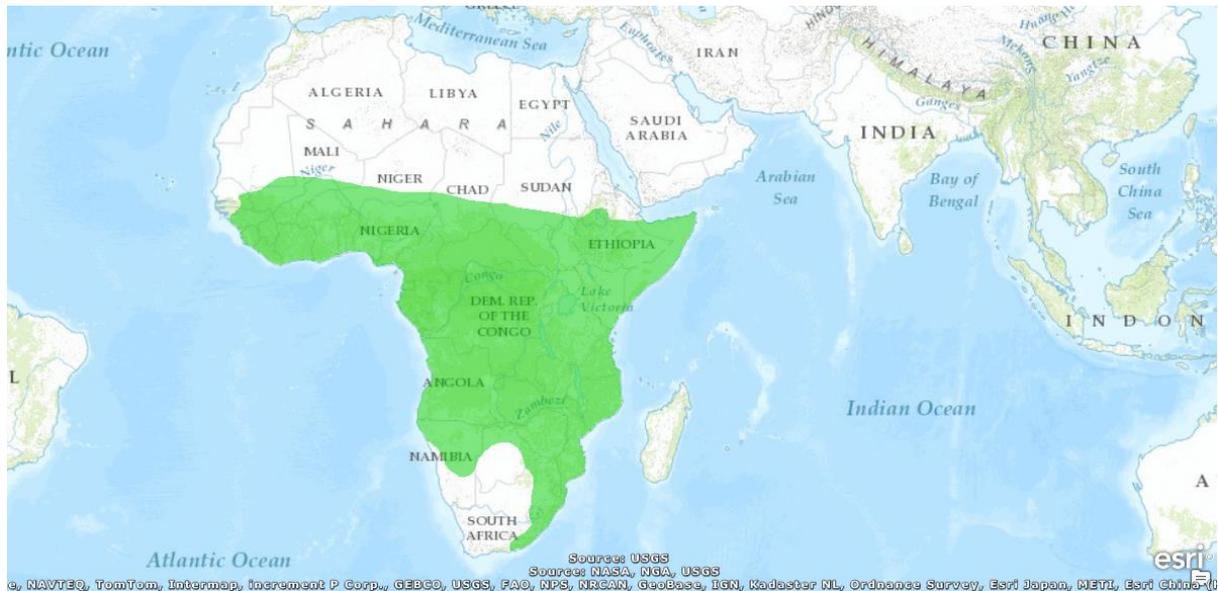
Mastomys natalensis

All maps were drawn in ArcGIS Explorer. Species distribution data was obtained from the IUCN red list website, IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. <www.iucnredlist.org>. Downloaded on **15 July 2013**

Appendix D Pairwise distance Matrices

Estimates of evolutionary divergence between sequences for astroviruses based on Clustal alignment of partial *RdRp* fragment (395bp).

Novel sequences identified in this study are highlighted in green and blue. Numbers highlighted in red represent the highest and lowest nucleotide difference in the among the South African astroviruses

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	BtAstV/MSTM1/Min_nat/WP/RSA/2010																								
2	BtAstV/MSTM5/Min_nat/WP/RSA/2010	0.225																							
3	BtAstV/MSTM8/Min_nat/WP/RSA/2010	0.293	0.295																						
4	BtAstV/MSTM9/Min_nat/WP/RSA/2010	0.223	0.249	0.275																					
5	BtAstV/MSTM10/Min_nat/WP/RSA/2010	0.226	0.251	0.288	0.252																				
6	BtAstV/MSTM14/Min_nat/WP/RSA/2010	0.246	0.245	0.244	0.218	0.251																			
7	BtAstV/MSTM12/Min_nat/WP/RSA/2010	0.278	0.297	0.309	0.277	0.271	0.286																		
8	BtAstV/Mf2/Min_fra/RSA/2013	0.233	0.230	0.236	0.054	0.240	0.191	0.278																	
9	BtAstV/Mf3/Min_fra/RSA/2013	0.241	0.271	0.229	0.236	0.295	0.251	0.284	0.223																
10	BtAstV/Mf4/Min_fra/RSA/2013	0.290	0.317	0.288	0.306	0.285	0.276	0.312	0.276	0.261															
11	BtAstV/Mf5/Min_fra/RSA/2013	0.264	0.274	0.234	0.264	0.253	0.239	0.292	0.234	0.220	0.221														
12	BtAstV/Mf6/Min_fra/RSA/2013	0.253	0.272	0.191	0.243	0.249	0.208	0.294	0.203	0.191	0.229	0.148													
13	BtAstV/HCA1/Hip_caf/RSA/2013	0.260	0.268	0.283	0.273	0.244	0.290	0.287	0.254	0.237	0.246	0.199	0.210												
14	BtAstV/HCA3/Hip_caf/RSA/2013	0.217	0.235	0.249	0.168	0.230	0.183	0.244	0.146	0.233	0.276	0.218	0.216	0.272											
15	BtAstV/Rsw2/Rhi_swi/RSA/2013	0.268	0.264	0.198	0.244	0.274	0.235	0.315	0.219	0.186	0.255	0.190	0.136	0.260	0.222										
16	BtAstV/GreytonNC1/Neo_cap/WP/RSA/2012	0.248	0.247	0.269	0.278	0.260	0.277	0.322	0.246	0.250	0.259	0.188	0.181	0.235	0.250	0.242									
17	BtAstV/GreytonNC2/Neo_cap/WP/RSA/2012	0.288	0.282	0.205	0.282	0.266	0.246	0.306	0.251	0.181	0.250	0.197	0.180	0.242	0.226	0.179	0.235								
18	BtAstV/GreytonNC3/Neo_cap/WP/RSA/2012	0.331	0.329	0.287	0.305	0.344	0.309	0.348	0.291	0.268	0.332	0.259	0.247	0.311	0.290	0.244	0.295	0.291							
19	BtAstV/GreytonNC4/Neo_cap/WP/RSA/2012	0.260	0.272	0.286	0.269	0.259	0.256	0.278	0.253	0.260	0.297	0.241	0.248	0.244	0.211	0.280	0.280	0.279	0.331						
20	BtAstV/GreytonNC5/Neo_cap/WP/RSA/2012	0.278	0.293	0.306	0.267	0.266	0.279	0.018	0.263	0.272	0.294	0.276	0.292	0.282	0.232	0.306	0.317	0.292	0.330	0.259					
21	BtAstV/GreytonNC6/Neo_cap/WP/RSA/2012	0.301	0.316	0.223	0.290	0.298	0.269	0.290	0.255	0.166	0.255	0.212	0.179	0.284	0.251	0.183	0.243	0.194	0.247	0.311	0.276				
22	BtAstV/GreytonNC7/Neo_cap/WP/RSA/2012	0.247	0.245	0.268	0.279	0.261	0.278	0.323	0.247	0.254	0.250	0.177	0.185	0.234	0.251	0.244	0.017	0.220	0.299	0.273	0.310	0.245			
23	BtAstV/GreytonNCe/Neo_cap/WP/RSA/2012	0.315	0.320	0.267	0.317	0.337	0.298	0.327	0.300	0.230	0.303	0.234	0.234	0.294	0.271	0.238	0.286	0.254	0.110	0.309	0.305	0.160	0.273		
24	BtAstV/GreytonTAr1/Tad_agy/WP/RSA/2012	0.003	0.228	0.294	0.226	0.228	0.248	0.283	0.230	0.242	0.285	0.262	0.248	0.257	0.219	0.270	0.249	0.285	0.332	0.249	0.274	0.301	0.236	0.308	
25	BtAstV/GreytonTAr2/Tad_agy/WP/RSA/2012	0.275	0.212	0.309	0.264	0.284	0.294	0.271	0.243	0.280	0.288	0.279	0.253	0.280	0.252	0.272	0.285	0.285	0.315	0.241	0.257	0.301	0.272	0.307	0.264

Estimates of evolutionary divergence between sequences for old world arenaviruses based on Clustal alignment of partial *RdRp* fragment (395bp). Novel South African viruses identified in this study are highlighted in blue.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	ELOr2/RSA/2012																										
2	ELOr3/RSA/2012	0.003																									
3	ELOr4/RSA/2012	0.142	0.143																								
4	BJROr1/RSA/2013	0.220	0.223	0.182																							
5	VRWMn1/RSA/2013	0.189	0.194	0.167	0.178																						
6	GpMn1/RSA/2012	0.208	0.212	0.213	0.181	0.171																					
7	GpMn5/RSA/2012	0.235	0.240	0.203	0.190	0.179	0.115																				
8	ATCRp18/RSA/2013	0.250	0.253	0.278	0.260	0.239	0.281	0.281																			
9	GU078661.1_Merino_Walk	0.170	0.173	0.182	0.230	0.191	0.213	0.232	0.345																		
10	AB586647.1_Luna_NMW-1	0.288	0.291	0.304	0.271	0.274	0.271	0.274	0.325	0.380																	
11	AB693149.1_Luna_LSK-2	0.260	0.258	0.271	0.304	0.276	0.279	0.274	0.333	0.387	0.153																
12	AB697692.1_Luna_LVN-1	0.273	0.273	0.276	0.281	0.261	0.263	0.281	0.333	0.385	0.175	0.198															
13	NC_018711.1_Lunk_NKS-1	0.304	0.309	0.296	0.320	0.317	0.325	0.304	0.377	0.411	0.412	0.397	0.385														
14	NC_007904.1_Mobala	0.278	0.273	0.291	0.263	0.281	0.286	0.304	0.335	0.379	0.328	0.319	0.327	0.400													
15	NC_006574.1_Mopeia_AN20410	0.311	0.309	0.291	0.289	0.276	0.269	0.279	0.358	0.388	0.310	0.306	0.318	0.394	0.294												
16	JN561685.1_Mopeia_AN21366	0.311	0.309	0.291	0.289	0.276	0.269	0.279	0.358	0.389	0.309	0.307	0.319	0.393	0.294	0.003											
17	DQ328875.1_Mopeia_Mozambique	0.281	0.283	0.291	0.286	0.291	0.269	0.281	0.333	0.380	0.315	0.318	0.308	0.415	0.295	0.213	0.212										
18	NC_013058.1_Morogoro	0.296	0.301	0.304	0.286	0.289	0.309	0.324	0.358	0.368	0.313	0.330	0.317	0.414	0.304	0.247	0.247	0.263									
19	HQ688674.1_Lassa_Josiah	0.250	0.255	0.278	0.278	0.264	0.258	0.261	0.283	0.379	0.343	0.335	0.344	0.398	0.349	0.346	0.347	0.335	0.338								
20	HQ688675.1_Lassa_recJosiah	0.253	0.258	0.278	0.281	0.264	0.258	0.261	0.285	0.378	0.343	0.335	0.344	0.400	0.349	0.346	0.347	0.335	0.338	0.001							
21	AY628200.1_Lassa_Macenta	0.263	0.265	0.273	0.265	0.261	0.258	0.279	0.314	0.366	0.338	0.343	0.346	0.400	0.359	0.342	0.343	0.335	0.326	0.150	0.151						
22	AY628204.1_Lassa_Z148	0.253	0.255	0.268	0.260	0.256	0.258	0.279	0.317	0.368	0.338	0.347	0.344	0.397	0.364	0.345	0.346	0.333	0.329	0.149	0.150	0.016					
23	AY179171.1_Lassa_AV	0.247	0.250	0.258	0.276	0.269	0.271	0.276	0.317	0.357	0.322	0.320	0.318	0.397	0.334	0.345	0.346	0.356	0.333	0.219	0.218	0.200	0.204				
24	NC_007906.1_Ippy	0.306	0.319	0.289	0.312	0.294	0.307	0.291	0.365	0.401	0.368	0.349	0.359	0.410	0.352	0.353	0.356	0.362	0.371	0.343	0.343	0.360	0.361	0.354			
25	DQ868486.1_LCMV_Pasteur	0.273	0.278	0.281	0.281	0.284	0.299	0.307	0.371	0.394	0.384	0.376	0.388	0.295	0.396	0.379	0.379	0.407	0.395	0.398	0.397	0.401	0.404	0.385	0.422		
26	EU136039.1_Dandenong_0710-2678	0.273	0.278	0.286	0.307	0.302	0.279	0.294	0.342	0.403	0.389	0.383	0.392	0.303	0.393	0.390	0.389	0.404	0.384	0.373	0.372	0.394	0.396	0.390	0.407	0.212	
27	NC_012777.1_Lujo	0.355	0.360	0.342	0.366	0.372	0.350	0.372	0.390	0.432	0.417	0.426	0.435	0.411	0.409	0.431	0.433	0.419	0.448	0.422	0.422	0.408	0.413	0.400	0.426	0.392	0.403

Estimates of evolutionary divergence between sequences for alphacoronaviruses based on Clustal alignment of partial RdRp fragment (395bp). Novel South African sequences identified in this study are highlighted in green.

|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1 BtCoV/MSTM2/Min_nat/RSA/2010																													
2 BtCoV/MSTM6/Min_nat/RSA/2010	0.025																												
3 BtCoV/NCL_MCO1/Mop_con/RSA/2012	0.278	0.278																											
4 BtCoV/VH_NC2/Neo_nan/RSA/2012	0.248	0.253	0.276																										
5 BtCoV/GrNC1/Neo_cap/RSA/2012	0.251	0.251	0.285	0.054																									
6 BtCoV/GrNC2/Neo_cap/RSA/2012	0.251	0.251	0.285	0.054	0.000																								
7 BtCoV/GrNC3/Neo_cap/RSA/2012	0.251	0.251	0.285	0.054	0.000	0.000																							
8 BtCoV/GrNC4/Neo_cap/RSA/2012	0.294	0.296	0.275	0.265	0.257	0.257	0.257																						
9 BtCoV/GrNC5/Neo_cap/RSA/2012	0.251	0.251	0.283	0.054	0.000	0.000	0.000	0.256																					
10 BtCoV/GrNC6/Neo_cap/RSA/2012	0.251	0.251	0.285	0.054	0.000	0.000	0.000	0.257	0.000																				
11 BtCoV/GrNC7/Neo_cap/RSA/2012	0.251	0.251	0.285	0.054	0.000	0.000	0.000	0.257	0.000	0.000																			
12 BtCoV/GrNC8/Neo_cap/RSA/2012	0.251	0.251	0.285	0.054	0.000	0.000	0.000	0.257	0.000	0.000	0.000																		
13 AB619638.1_BtCoV/01/Min_ful/Japan/2009	0.251	0.251	0.284	0.281	0.291	0.291	0.291	0.281	0.291	0.291	0.291	0.291																	
14 AB619639.1_BtCoV/02/Min_ful/Japan/2009	0.251	0.251	0.272	0.281	0.291	0.291	0.291	0.294	0.291	0.291	0.291	0.291	0.012																
15 GU190240.1_BtCoV/BR98-55/Min_sch/Bulgaria/2008	0.213	0.221	0.310	0.282	0.269	0.269	0.269	0.307	0.269	0.269	0.269	0.269	0.232	0.232															
16 GU190247.1_BtCoV/BR98-52/Min_sch/Bulgaria/2008	0.190	0.192	0.280	0.215	0.213	0.213	0.213	0.260	0.211	0.213	0.213	0.213	0.208	0.208	0.216														
17 GU190241.1_BtCoV/BR98-14/Min_sch/Bulgaria/2008	0.190	0.192	0.280	0.215	0.215	0.215	0.215	0.269	0.215	0.215	0.215	0.215	0.217	0.217	0.221	0.010													
18 DQ666339.1_BtCoV/HKU7/WCF88/Min_mag/China/2004	0.233	0.235	0.264	0.249	0.258	0.258	0.258	0.269	0.256	0.258	0.258	0.258	0.058	0.064	0.221	0.191	0.197												
19 DQ249226.1_BtCoV/HKU7-1/Min_mag/China/2006	0.243	0.241	0.259	0.259	0.268	0.268	0.268	0.284	0.266	0.268	0.268	0.268	0.052	0.052	0.223	0.201	0.207	0.025											
20 NC_010438.1_BtCoV/HKU8/Min_pus/China/2008	0.182	0.184	0.272	0.236	0.239	0.239	0.239	0.277	0.239	0.239	0.239	0.239	0.207	0.207	0.221	0.069	0.069	0.192	0.200										
21 DQ249228.1_BtCoV/HKU8-1/Min_pus/China/2006	0.172	0.180	0.256	0.234	0.243	0.243	0.243	0.276	0.241	0.243	0.243	0.243	0.208	0.208	0.231	0.070	0.071	0.188	0.198	0.008									
22 NC_010437.1_BtCoV/1A/AFCD62/Min_mag/China/2005	0.185	0.192	0.297	0.240	0.230	0.230	0.230	0.314	0.229	0.230	0.230	0.230	0.223	0.223	0.109	0.198	0.197	0.206	0.216	0.211	0.214								
23 AY864198.1_BtCoV/1/61/Min_pus/China	0.197	0.213	0.279	0.244	0.255	0.255	0.255	0.305	0.253	0.255	0.255	0.255	0.220	0.226	0.107	0.213	0.220	0.214	0.219	0.216	0.216	0.067							
24 NC_010436.1_BtCoV/1B/Min_pus/China/2006	0.203	0.213	0.300	0.259	0.267	0.267	0.267	0.302	0.265	0.267	0.267	0.267	0.220	0.226	0.109	0.229	0.231	0.206	0.206	0.231	0.236	0.093	0.053						
25 DQ648835.1_BtCoV/A773/Min_sch/China/2005	0.230	0.233	0.261	0.246	0.261	0.261	0.261	0.279	0.259	0.261	0.261	0.261	0.058	0.064	0.223	0.191	0.197	0.013	0.028	0.190	0.183	0.211	0.219	0.211					
26 EU834956.1_BtCoV/CoV180/Min_aus/Australia/1996	0.201	0.217	0.257	0.225	0.212	0.212	0.212	0.282	0.212	0.212	0.212	0.212	0.199	0.199	0.223	0.153	0.158	0.166	0.169	0.165	0.161	0.223	0.212	0.220	0.166				
27 EU834952.1_BtCoV/CoV088/Min_aus/Australia/2007	0.185	0.192	0.263	0.243	0.233	0.233	0.233	0.284	0.233	0.233	0.233	0.233	0.187	0.187	0.231	0.152	0.154	0.162	0.165	0.151	0.154	0.218	0.225	0.228	0.162	0.070			
28 EU834955.1_BtCoV/CoV146/Min_sch/Australia/2007	0.182	0.190	0.266	0.235	0.230	0.230	0.230	0.289	0.230	0.230	0.230	0.230	0.193	0.193	0.231	0.157	0.159	0.170	0.172	0.151	0.154	0.213	0.223	0.230	0.170	0.075	0.020		
29 EU834954.1_BtCoV/CoV132/Min_aus/Australia/2007	0.187	0.195	0.266	0.246	0.235	0.235	0.235	0.284	0.235	0.235	0.235	0.235	0.187	0.187	0.231	0.154	0.157	0.162	0.165	0.153	0.157	0.218	0.225	0.228	0.162	0.072	0.003	0.023	
30 HQ184061.1_BtCoV/L/Hyp_sav/Spain/2007	0.234	0.231	0.270	0.250	0.264	0.264	0.264	0.278	0.263	0.264	0.264	0.264	0.064	0.070	0.227	0.199	0.205	0.038	0.043	0.201	0.199	0.220	0.220	0.212	0.035	0.180	0.175	0.178	0.175

Estimates of Evolutionary Divergence between in Clade 2b and 2c of *Betacoronavirus* genus, 275 amino acid sequences were compared in MEGA5 using the Poisson correction model. The number of amino acid substitutions per site between sequences are shown. The amino acid difference between MERS-CoV and PML-PHE1 and difference between human SARS-CoV and SARSr-CoV/Rs672 are bold typed in red.

BtCoV/PML-PHE1/Neo_zul/RSA/2011	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
BtCoV/KW2E-F93/Nyc_spec/Ghana/2010_JX899383.1	0.080																		
BtCoV/KW2E-F82/Nyc_spp/Ghana/2011_JX899382.1	0.080	0.000																	
BtCoV/KW2E-F53/Nyc_spp/Ghana/2011_JX899384.1	0.080	0.000	0.000																
HCoV/MERS-CoV/Al-Hasa_3/Hom_sap/KSA/2013_KF186565.1	**0.004**	0.080	0.080	0.080															
HCoV/MERS-CoV/N3/Hom_sap/Jordan/2012_KC776174.1	**0.004**	0.080	0.080	0.080	0.000														
HCoV/MERS-CoV/Hom_sap/Qatar/2012_KC667074.1	**0.004**	0.080	0.080	0.080	0.000	0.000													
HCoV/MERS-CoV/Hom_sap/KSA/2012_KF192507.1	**0.004**	0.080	0.080	0.080	0.000	0.000	0.000												
BtCoV/HKU4-1/Tyl_pac/China/2006_NC_009019.1	0.080	0.100	0.100	0.100	0.080	0.080	0.080	0.080											
BtCoV/HKU5-1/Pip_abr/China/2006_NC_009020.1	0.061	0.096	0.096	0.096	0.061	0.061	0.061	0.061	0.065										
BtCoV/133/Tyl_pac/China/2005_DQ648794.1	0.080	0.100	0.100	0.100	0.080	0.080	0.080	0.080	0.000	0.065									
BtCoV/UKR-G17/Pip_nat/Ukraine/2011_KC243392.1	0.019	0.080	0.080	0.080	0.019	0.019	0.019	0.019	0.084	0.057	0.084								
BtCoV/8-691/Pip_nat/Romania/2009_KC243391.1	0.019	0.080	0.080	0.080	0.019	0.019	0.019	0.019	0.084	0.057	0.084	0.000							
HCoV/SARS-CoV/Frankfurt_1/Hom_sap/Germany/2004_AY291315.1	0.273	0.259	0.259	0.259	0.273	0.273	0.273	0.273	0.259	0.288	0.259	0.268	0.268						
BtCoV/SARSr-CoV/Rs672/Rhi_sin/China/2006_FJ588686.1	0.273	0.254	0.254	0.254	0.273	0.273	0.273	0.273	0.254	0.283	0.254	0.268	0.268	**0.004**					
BtCoV/SARSr-CoV/HKU3-1/Rhi_sin/China/2006_DQ022305.2	0.268	0.249	0.249	0.249	0.268	0.268	0.268	0.268	0.245	0.278	0.245	0.263	0.263	0.011	0.007				
BtCoV/Zaria/Hip_com/Nigeria/2008_HQ166910.1	0.254	0.235	0.235	0.235	0.259	0.259	0.259	0.259	0.259	0.263	0.259	0.249	0.249	0.208	0.203	0.199			
BtCoV/HKU9-1/Rou_aeg/China/2006_NC_009021.1	0.293	0.259	0.259	0.259	0.293	0.293	0.293	0.293	0.288	0.298	0.288	0.283	0.283	0.231	0.231	0.226	0.203		
BtCoV/KY06/Rou_spp/Kenya/2006_HQ728483.1	0.293	0.259	0.259	0.259	0.293	0.293	0.293	0.293	0.288	0.298	0.288	0.283	0.283	0.231	0.231	0.226	0.203	0.000	
BtCoV/KY24/Eid_hel/Kenya/2006_HQ728482.1	0.283	0.254	0.254	0.254	0.283	0.283	0.283	0.283	0.278	0.288	0.278	0.278	0.278	0.240	0.235	0.231	0.203	0.105	0.105

Amino acid differences between human SARS-CoV & Bat SARSr-CoV and MERS-CoV BtCoV/PML-PHE1/Neo_zul/RSA/2011: A single amino acid difference is observed at position 266 (asparagine in BtCoV/PML-PHE1/Neo zul/South Africa/2012 versus serine in MERS-CoV) and 173 (asparagine in SARS-CoV versus serine in bat SARSr-CoV)
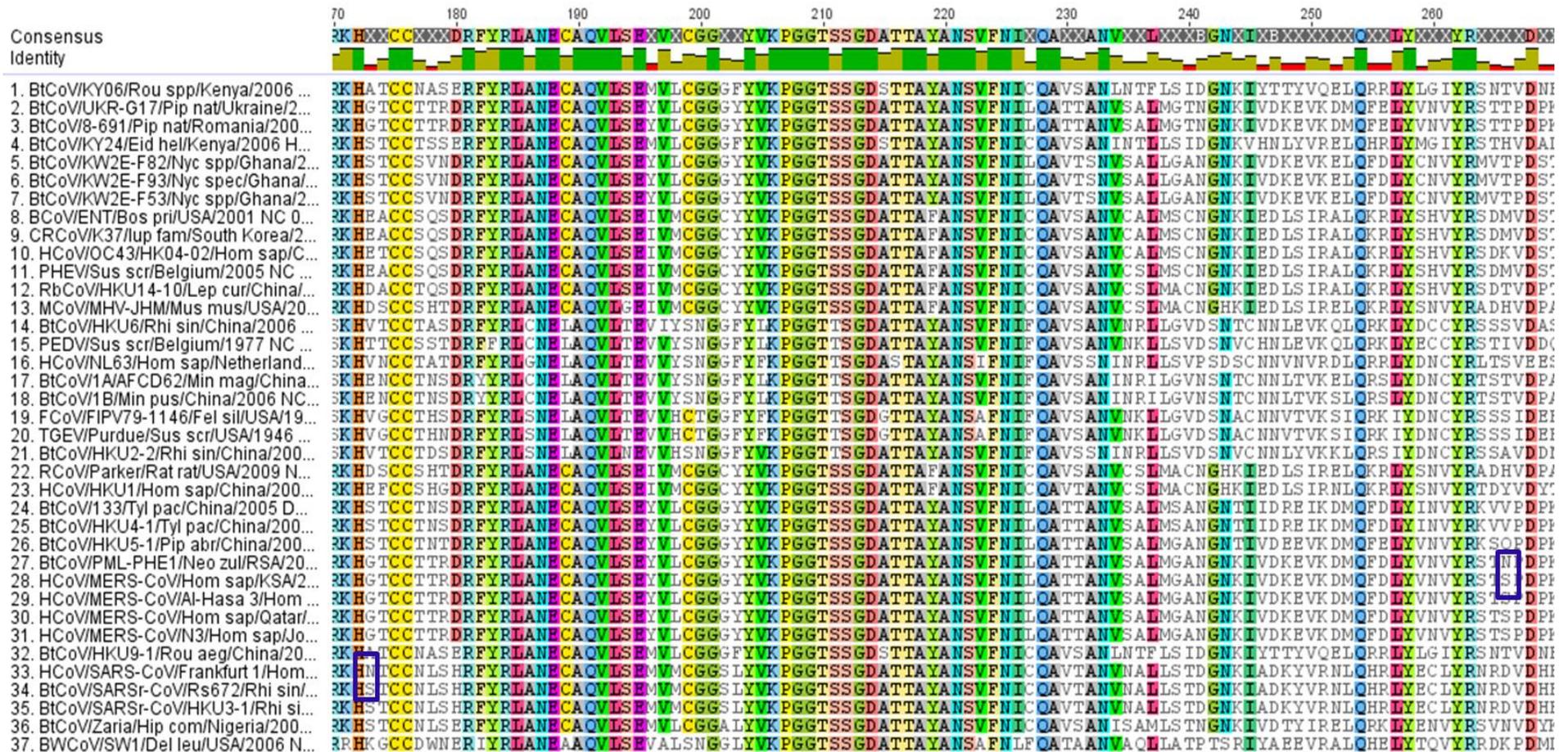
Appendix E Additional phylogenetic trees
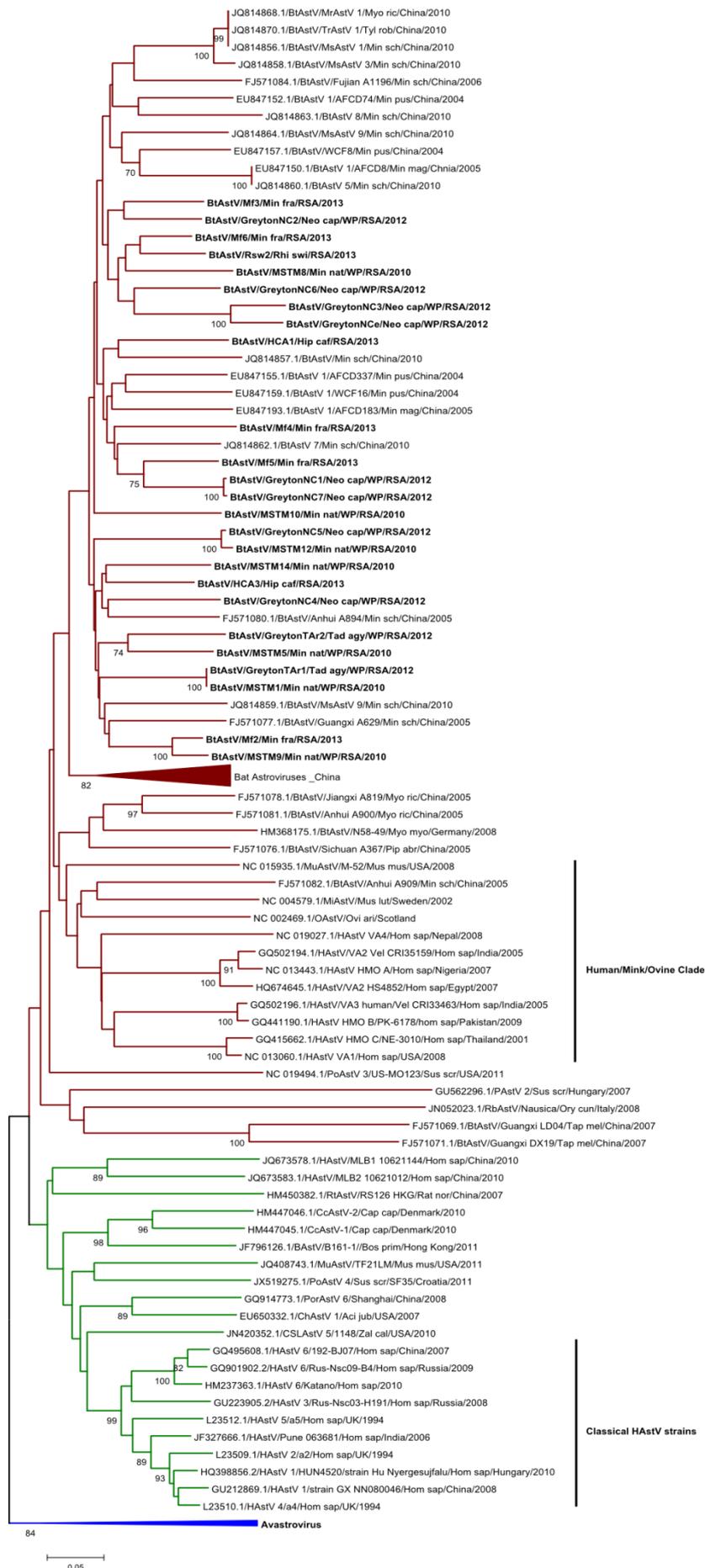
Phylogenetic relationship of novel astroviruses with in the *Astroviridae* family. The neighbour-joining method was employed in MEGA5 using the percentage distance model. Novel astroviruses identified in this study are bold typed.

Neighbour-joining tree for alphacoronaviruses drawn using the p-distance model based on 277bp *RdRp* fragment. South African sequences identified by Geldenhuys *et al* (2013) were included in the analysis

Partial RNA-dependent RNA polymerase (RdRp) gene phylogeny, including the novel betacoronavirus from a *Neoromicia cf. zuluensis* bat in South Africa (GenBank accession no. KC869678). The Bayesian inference was done on a translated 816-nt RdRp gene sequence fragment in MrBayes V3.1 with a WAG substitution model. Assumptions over 2,000,000 generations were sampled every 100 steps, resulting in 20,000 trees, of which 25% were discarded as burn-in. A whale gammacoronavirus (EU111742) was used as an outgroup. The novel *N. zuluensis* bat virus is highlighted in grey. Values at deep nodes represent statistical support from posterior probabilities. Only values >0.9 are shown. Coronavirus clades are depicted to the right of taxa. Scale bar represents genetic distance (Ithete *et al.* 2013).

Appendix F Pamphlet and consent form for hantavirus study

Research project: Do hantaviruses occur in Southern Africa?

Hantaviruses are members of the *Bunyaviridae* family. Their natural hosts are rodents and shrews. So far, more than 30 different hantavirus species have been found infecting different species of rodent or shrew.
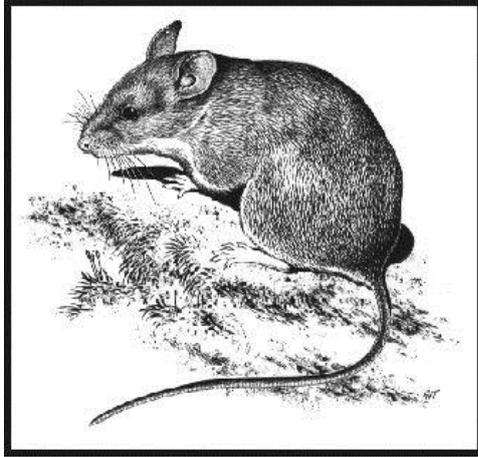
Some types of hantaviruses may cause zoonotic disease in human beings. Humans are typically infected by inhaling aerosolized excreta of infected rodents, e.g. when cleaning rodent-infested buildings. The two recognised hantavirus-associated human disease entities are haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS).
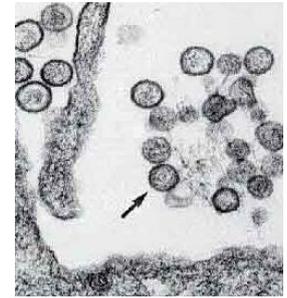
Until recently hantaviruses were known to occur in Asia, Europe and the Americas. However, in 2006 the first African hantavirus was identified in and isolated from the African wood mouse (*Hylomyscus simus*) in Guinea, West Africa. The new virus was named Sangassou, after the area where it was first detected. Soon afterwards genetic evidence for another hantavirus was detected in a Therese's shrew (*Crocidura theresae*), also in West Africa, and named Tanganya virus.

The identification of these two African hantaviruses has highlighted the possibility that there may be yet other hantaviruses in Africa that are yet to be identified. These viruses might infect and cause disease in human beings. It is possible that infections and diseases caused by yet unknown hantaviruses may be confused with other severe diseases such as rickettsiosis, severe pneumonia, leptospirosis, viral haemorrhagic fevers etc. Without specific laboratory testing, a hantavirus aetiology may not be recognised (as was the case with HFRS in Eurasia and HCPS in America before the responsible viruses were discovered).

Our study aims to identify hantaviruses and possible hantavirus diseases in southern Africa. So far, the occurrence of these viruses and any possibly related illnesses is not known from our region. The study has been approved by the ethics committee of the University of Stellenbosch. We would like to test patients with clinical disease that might possibly be due to hantavirus infection, to see whether hantavirus infections do occur at all and if so, whether they may be causing disease. If we find evidence of hantavirus infection in patients a clinical case definition and diagnostic assays can be developed to help diagnose future cases.

We are looking for patients with clinical symptoms that might be indicative of hantavirus disease. These symptoms are:

- **Fever of unknown origin**, not responding to antibiotic treatment,

PLUS

- any of the following, **of unknown aetiology**: **acute renal failure, acute respiratory failure, acute liver failure, pneumonia and/or mucosal bleeding.**

We need EDTA blood samples from patients fulfilling this case definition. Blood samples will be tested for the presence of hantavirus antibodies by an in-house ELISA assay. A patient information sheet and consent form has been developed, as well as a study sample form (asking for a minimum set of clinical information) is available.

We look forward to your participation! Please contact:

Miss Ndapewa Ithete
Division of Medical Virology
021 938 9360
14567008@sun.ac.za

Prof Wolfgang Preiser
Division of Medical Virology
021 938 9353
preiser@sun.ac.za

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: MOLECULAR IDENTIFICATION AND CHARACTERISATION OF RODENT- AND SHREW-BORNE HANTAVURSES

REFERENCE NUMBER: **N02/09/058**

PRINCIPAL INVESTIGATOR: **Ndapewa Ithete**

ADDRESS: **Division of Medical Virology, Department of Pathology, Faculty of Health Sciences, Stellenbosch University.**

CONTACT NUMBER: **021 938 9360**

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

This study aims to identify hantaviruses and hantavirus diseases in southern Africa. So far, the occurrence of these viruses and any possibly related illnesses is not known from our region. The idea is to test patients with clinical disease that might possibly be due to hantavirus infection for the evidence of this, to see whether hantavirus infections do occur at all and if so, whether they may be causing disease.

Patients will be recruited at two hospitals: Tygerberg Hospital and Groote Schuur Hospital in Cape Town.

Blood samples from patients with clinical symptoms that might be indicative of hantavirus disease will be collected. Such symptoms include fever of unknown origin not responding to antibiotic treatment, plus any of the following: acute renal failure, acute respiratory failure, and acute liver failure, mucosal bleeding, all of unknown aetiology. Blood samples will be obtained at different time points after onset of disease, frozen and later tested for hantaviruses. In addition, if and when biopsies (e.g. renal biopsies in cases of acute renal failure) are performed for routine diagnostic purposes, residual sample material will also be obtained for hantavirus testing.

The aim of this project is to identify and characterise novel hantaviruses in patients whose clinical illness matches that seen in patients with proven hantavirus infection in other parts of the world. Patients will be selected based on clinical criteria and patient samples will be tested by molecular and serological methods. If we find evidence of hantavirus infection in such patients this will help us to define a clinical case definition and to develop diagnostic assays that might in future help to diagnose hantavirus-infected patients.

When routine blood samples are taken from the participating patients, another (extra) tube of blood will be collected for the purposes of this study. This specimen will then be tested for the presence of anti-hantavirus antibodies and of hantavirus genome.

Why have you been invited to participate?

You have been chosen to participate because you are currently suffering from a disease which resembles that caused by hantaviruses in other parts of the world.

What will your responsibilities be?

To provide us with an extra blood sample when blood is taken for routine purposes anyway. One tube with 10 ml (2 to 3 teaspoons) of blood will be collected.

Will you benefit from taking part in this research?

You will not benefit from this research directly yourself. Your participation would however be very valuable as it might lead to the identification of new viruses. This contributes to medical knowledge in general and will allow us to develop a clinical case definition of hantavirus disease in South Africa.

Possible identification of hantaviruses will allow the development of diagnostic assays to be used to determine whether future patients have hantavirus infection.

Are there in risks involved in your taking part in this research?

There is minimal risk involved in your participation in this study as the extra blood specimen to be taken will be obtained at the same time as one or more for routine diagnostic testing (no extra needle prick involved).

If you do not agree to take part, what alternatives do you have?

Whether you wish to take part in this study or not will not affect your clinical care in any way.

Who will have access to your medical records?

All personal information collected will be treated as confidential and access to it will be strictly controlled and limited to the investigators. All identifying information will be anonymised at the earliest possible time point. All patient specimens will be assigned numbers for identification purposes when used in a publication or thesis.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

This is not applicable, as the only inconvenience is drawing an extra tube of blood from you at the same time as samples are being obtained for routine purposes anyway.

Will you be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study, and you will not incur any costs either.

Is there any thing else that you should know or do?

➢ You can contact Ms Ndapewa Ithete at telephone 021 938 9360 if you have any further queries or encounter any problems.

➢ You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

➢ You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ………………………………..…………. agree to take part in a research study entitled **Molecular Identification and Characterisation of Rodent- and Shrew-borne Hantaviruses.**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

- I have had a chance to ask questions and all my questions have been adequately answered.

- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ....……………. on (*date*) …………...……….. 2010.

....
Signature of participant

....
Signature of witness

Declaration by investigator

I (*name*) …………………………………………..……… declare that:

- I explained the information in this document to …………………..……………..

- I encouraged him/her to ask questions and took adequate time to answer them.

- I am satisfied that he/she adequately understands all aspects of the research, as discussed above

- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ....……………. on (*date*) …………...……….. 2010.

....
Signature of investigator

....
Signature of witness

Declaration by interpreter (if applicable)

I (*name*) …………………………………………..……… declare that:

- I assisted the investigator (*name*) ……...............……………………………. to explain

  the information in this document to (*name of participant*)

  ....…………..…………………………. using the language medium of

  Afrikaans/Xhosa (delete as applicable).

- We encouraged him/her to ask questions and took adequate time to answer them.

- I conveyed a factually correct version of what was related to me.

- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ....……………. on (*date*) …………...……….. 2010

....
Signature of interpreter

....
Signature of witness

Appendix G Hantavirus IgG ELISA results – Optical Density values

Serosurvey results

Specimen number	DOB-rN	PUU-rN	Specimen number	DOB-rN	PUU-rN
Han 31	0.192	0.014	Han 497	0.131	0.174
Han 34	0.674	0.006	Han 499	0.198	0.025
Han 49	0.279	0.011	Han 510	0.172	0.136
Han 54	0.215	0.014	Han 514	0.273	0.094
Han 55	0.174	0.007	Han 519	0.153	0.091
Han 63	0.320	0.050	Han 528	0.152	0.151
Han 66	0.250	0.044	Han 537	0.107	0.151
Han 72	0.016	0.162	Han 540	0.141	0.235
Han 84	0.078	0.504	Han 544	0.137	0.159
Han 86	0.214	0.092	Han 553	0.244	0.248
Han 88	0.085	0.166	Han 554	0.094	0.164
Han 91	0.168	0.078	Han 555	0.245	0.320
Han 101	0.266	0.099	Han 556	0.165	0.200
Han 107	0.190	0.152	Han 577	0.183	0.168
Han 115	0.067	0.162	Han 591	0.171	0.177
Han 121	0.191	0.132	Han 593	0.171	0.165
Han 125	0.165	0.129	Han 595	0.152	0.169
Han 143	0.231	0.106	Han 602	0.198	0.177
Han 147	0.158	0.154	Han 618	0.184	-0.015
Han 149	0.153	0.073	Han 626	0.016	0.237
Han 151	0.285	0.247	Han 648	0.059	0.290
Han 155	0.544	0.041	Han 649	0.050	0.234
Han 157	0.271	0.203	Han 665	-0.019	0.307
Han 164	0.287	0.087	Han 696	0.166	0.000
Han 170	0.048	0.166	Han 713	0.018	0.151
Han 171	0.199	0.150	Han 733	0.109	0.260
Han 177	0.422	0.072	Han 745	0.228	0.093
Han 179	0.582	0.025	Han 751	0.207	0.067
Han 180	0.141	0.204	Han 754	0.261	0.046
Han 187	0.184	0.163	Han 761	0.237	0.044
Han 198	0.160	0.104	Han 784	0.014	0.204
Han 205	0.286	0.119	Han 819	0.231	0.052
Han 213	0.184	0.011	Han 834	0.072	0.231
Han 217	0.532	0.032	Han 844	0.137	0.150
Han 225	0.337	0.063	Han 845	0.281	0.024
Han 227	0.094	0.156	Han 848	0.173	0.035
Han 242	0.444	0.034	Han 859	0.159	0.054
Han 255	0.375	0.049	Han 877	-0.013	0.249
Han 259	0.069	0.188	Han 896	0.025	0.363
Han 269	0.057	0.184	Han 921	0.175	0.070
Han 275	0.083	0.153	Han 932	0.263	0.238
Han 267	0.159	0.096	Han 933	0.220	0.113
Han 298	0.242	0.074	Han 935	0.159	0.058
Han 311	0.277	0.088	Han 937	0.181	0.196
Han 338	0.138	0.204	Han 939	0.280	0.090
Han 342	0.050	0.171	Han 944	0.282	0.288
Han 362	0.156	0.017	Han 945	0.112	0.241
Han 373	0.556	0.367	Han 946	0.172	0.108
Han 399	0.313	0.186	Han 947	0.266	0.205
Han 444	0.167	0.004	Han 949	0.208	0.127
Han 467	0.044	0.288	Han 954	0.219	0.351
Han 479	0.396	0.041	Han 957	0.220	0.179
Han 453	0.046	0.440	Han 960	0.109	0.156
Han 459	0.202	-0.002	Han 962	0.211	0.146

Specimen number	DOB-rN	PUU-rN	Specimen number	DOB-rN	PUU-rN
Han 963	0.093	0.192	Han 1201	0.006	0.172
Han 967	0.297	0.385	Han 1202	0.009	0.173
Han 973	0.304	0.035	Han 1203	0.134	0.235
Han 979	0.089	0.428	Han 1205	0.310	0.271
Han 982	0.938	0.096	Han 1207	0.155	0.281
Han 983	0.332	-0.062	Han 1209	0.200	0.235
Han 991	0.222	0.043	Han 1211	0.225	0.103
Han 995	0.251	0.115	Han 1213	0.215	0.159
Han 997	0.836	0.035	Han 1218	0.046	0.212
Han 999	0.179	0.202	Han 1225	0.052	0.163
Han 1000	0.304	0.045	Han 1229	0.082	0.249
Han 1001	0.010	0.438	Han 1230	0.104	0.366
Han 1007	0.072	0.483	Han 1231	0.128	0.208
Han 1009	0.174	0.058	Han 1232	0.076	0.253
Han 1011	0.087	0.347	Han 1234	-0.113	0.666
Han 1014	0.171	0.046	Han 1243	0.037	0.273
Han 1021	0.188	0.064	Han 1255	0.035	0.252
Han 1024	0.177	0.060	Han 1256	0.184	0.163
Han 1025	0.170	0.052	Han 1258	0.024	0.161
Han 1027	0.278	0.287	Han 1263	0.161	0.175
Han 1030	0.206	0.135	Han 1271	0.170	0.153
Han 1033	0.208	0.201	Han 1272	0.212	0.246
Han 1041	0.223	0.110	Han 1275	0.156	0.228
Han 1045	0.135	0.151	Han 1277	0.222	0.331
Han 1046	0.499	0.149	Han 1285	-0.023	0.193
Han 1048	0.153	0.317	Han 1288	0.159	0.511
Han 1049	0.192	0.258	Han 1289	0.040	0.168
Han 1051	0.169	0.141	Han 1306	0.168	0.071
Han 1054	0.202	0.068	Han 1308	0.272	0.051
Han 1055	0.213	0.333	Han 1314	0.264	0.379
Han 1058	0.178	0.139	Han 1315	0.154	0.171
Han 1059	0.175	0.116	Han 1316	0.367	0.101
Han 1060	0.181	0.126	Han 1319	0.033	0.294
Han 1088	0.048	0.196	Han 1323	0.309	-0.017
Han 1090	0.250	0.118	Han 1325	0.176	0.566
Han 1095	0.255	-0.062	Han 1337	0.225	0.054
Han 1097	0.213	-0.026	Han 1344	0.179	-0.012
Han 1116	0.135	0.200	Han 1345	0.165	0.106
Han 1120	0.157	0.123	Han 1348	0.171	0.107
Han 1123	0.067	0.462	Han 1352	0.296	0.271
Han 1132	0.170	0.174	Han 1363	0.054	0.167
Han 1136	0.097	0.197	Han 1364	0.195	0.180
Han 1137	0.092	0.242	Han 1404	0.190	0.202
Han 1139	0.040	0.158	Han 1433	0.180	0.205
Han 1141	0.245	0.190	Han 1438	0.160	0.203
Han 1145	0.036	0.258	Han 1439	2.338	0.022
Han 1148	0.289	0.101	Han 1442	0.181	0.023
Han 1153	0.186	-0.034	Han 1457	0.254	0.062
Han 1154	0.387	0.138			
Han 1155	0.271	0.310			
Han 1171	0.651	0.297			
Han 1175	0.034	0.175			
Han 1192	0.393	-0.002			
Han 1197	0.163	0.130			

Acutely ill patients recruited

Samples	Hospital	DOB-rN	PUU-rN
Han Ac1	GSH	0.203	0.135
Han Ac2	GSH	0.095	0.156
Han Ac3	GSH	0.189	0.132
Han Ac4	GSH	0.371	0.142
Han Ac5	TYG	0.240	0.037
Han Ac6	GSH	0.206	0.040
Han Ac7	GSH	0.317	0.144
Han Ac8	GSH	0.033	0.083
Han Ac9	GSH	0.359	0.205

Appendix H Permission numbers for the use of copyrighted images and tables

Figures /Tables	Reference	Permission Number
Figure 1.2	**Hepojoki** *et al.* 2012, *J Gen Virol* **93**: 1631-1644	11104938
Figure 1.4	**Meyer** 2002, *CTMI Arenaviruses I*, 262: 139-157	3240641446214
Figure 1.7	**Perlman and Dandekar** 2005, *Nat Rev Immunol,* **5**: 917-927	3181270459092
Figure 1.8	**Baker** 2008, Encyclopedia of Virology (3rd Ed.)	3240750222074
	King *et al.* 2012, *Virus Taxonomy: 9th Report of the ICTV* , 806-828	3240760600968
Figure 1.10	**Toffan** *et al.* 2009, *Vet Microbiol*, **139**: 147-152	3181360955291
Figure 1.12	**King** *et al.* 2012, *Virus Taxonomy: 9th Report of the ICTV* , 953-959	3181301156316
Table 1.1	**Klein and Calisher** 2007, C*TMI Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission, 315* : 217-252	3240761290121
Table 1.6	**Salazaar-Bravo, Ruedas and Yates** 2002, *CTMI Arenavirus I*, 262: 25-63	3240780342836