Detection and characterization of genetically diverse paramyxoviruses from African bats

by

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Co-supervisors: Prof. L.H. Nel and Dr. J. Weyer

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I, Marinda Mortlock declare that the thesis/dissertation, which I hereby submit for the degree Magister Scientiae Microbiology (M.Sc.) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: _____

Date: _____

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SUMMARY

Detection and characterization of genetically diverse paramyxoviruses from African bats

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In past years, the potential of bats as reservoir for paramyxoviruses was clearly underestimated. Research of the 21st century now provides evidence that bats play an important role as reservoir and host to these viruses. The aim of this study was to detect the presence of any novel paramyxoviruses that may be circulating in bats across Africa. The specific objectives included the screening of specimen panels of insectivorous as well as frugivorous bat species collected from a number of African countries. Two broadly-reactive universal primer sets targeting the *Paramyxovirinae* subfamily and the *Respiro*-, *Morbilli*- and *Henipavirus* genera were used in two semi-nested PCR reactions. Bat kidney was selected as target organ and bats were sampled from several countries across Africa (Cameroon, Democratic Republic of the Congo, Kenya, Nigeria, South Africa and Swaziland). Based on amino acid analysis it was determined that approximately 31 putative viral species were detected. Viruses detected, clustered phylogenetically with known genera namely *Henipavirus*, *Morbillivirus* and the newly proposed *Jeilongvirus*. Several viral sequences clustered outside the known genera and might belong to yet unclassified genera in the *Paramyxovirinae* subfamily. Viral exchange between different bat species was also observed in several occasions where sampling from geographically distant locations was done. The ability of some bat species, *e.g. Eidolon helvum,* to migrate over large distances, likely contributes to the spread of specific virus lineages over significant geographical space. The propensity for many bat species to roost communally is another likely contributor to enhanced virus transmission events. Due to the vast genetic variability among paramyxoviruses in nature, insight into these viruses will be vital in understanding their pathogenic nature and the

possible threat they may pose to public and veterinary health sectors. Propagation and isolation in cell-cultures as well as full-genome sequence analysis will be a foremost requirement in future research of these viruses. Clearly, there are geographical limitations in this study which emphasizes the need for a One Health approach from all African countries that will greatly contribute to future research on paramyxoviruses.

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LIST OF ABBREVIATIONS

3’	:	three prime
5’	:	five prime
AFLP	:	amplified fragment Length polymorphism
AMV	:	avian myeloblastosis virus
AVU	:	*Avulavirus*
BHK	:	baby hamster kidney
bp	:	basepairs
bPMV	:	bovine metapneumovirus
CDC	:	Centers for Disease Control and Prevention
cDNA	:	complementary deoxynucleic acid
CEZD	:	Centre for Emerging Zoonotic Diseases
DNA	:	deoxyribonucleic acid
Dr.	:	Doctor
DRC	:	Democratic Republic of the Congo
DTT	:	dithiotreithol
EDTA	:	ethylenediaminetetraacetic acid
e.g.	:	example
EM	:	electron microscopy
EtOH	:	ethanol
F	:	fusion protein
F1	:	forward primer 1
F2	:	forward primer 2
Ft^2	:	square feet
G	:	gamma sites
g	:	gram
G gene	:	glycoprotein
GA	:	Georgia
GTR	:	generalized time reversible

HCl	:	hydrochloric acid
HEN	:	*Henipavirus*
HG gene	:	hemagglutinin-neuraminidase
HeV	:	Hendra virus
hMPV	:	human metapneumovirus
I	:	invariant sites
ICTV	:	International Committee on Taxonomy of Viruses
i.e.	:	that is
IgM	:	immunoglobulin M
Inc.	:	Incorporated
IUCN	:	International Union for Conservation of Nature
JE	:	Japanese encephalitis
kb	:	kilobases
KCl	:	potassium chloride
KFD	:	Kyasanur forest disease
km	:	kilometre
LB	:	Luria-Beltane
L gene	:	RNA-dependent RNA polymerase
M	:	molar
m	:	meter
M gene	:	matrix protein
M_2 gene	:	trans-membrane protein
$MgCl_2$	:	magnesium chloride
µg/ml	:	micrograms per microliter
µl	:	microliters
ml	:	millilitres
mm	:	millimetre
mM	:	millimolar
MOKV	:	Mokola virus
MOR	:	*Morbillivirus*
N gene	:	nucleoprotein

NaOAc	:	sodium acetate
NCBI	:	National Center for Biotechnology Information
ng/µl	:	nanograms per microliters
NHLS	:	National Health Laboratory Services
NIAID	:	National Institute of Allergy and Infectious Diseases
NiV	:	Nipah virus
NS1/2 gene	:	non-structural proteins
nt	:	nucleotide
P gene	:	phosphoprotein
PAR	:	*Paramyxovirinae*
PCR	:	polymerase chain reaction
pmol	:	picomoles
PMV	:	*Paramyxovirinae*
PNE	:	*Pneumovirinae*
Prof.	:	Professor
R	:	reverse primer
RCA	:	Central African Republic
RES	:	*Respirovirus*
RMH	:	*Respiro*-, *Morbilli*- and *Henipavirus*
RNA	:	ribonucleic acid
rpm	:	revolutions per minute
RSV	:	respiratory syncytial virus
RT	:	reverse transcription
RUB	:	*Rubulavirus*
s	:	seconds
SA	:	South Africa
SARS	:	Severe acute respiratory syndrome
SH gene	:	small hydrophobic protein
SISPA	:	sequence independent single primer amplification
sp.	:	species (singular)
spp.	:	species (plural)
SW	:	Swaziland

SYM	:	symmetrical model
TAE	:	Tris-acetate-EDTA
TIM	:	transmission model
U	:	units
U/µl	:	units per microliter
USA	:	United States of America
UV	:	ultraviolet
v	:	version
V	:	Volts
VIDISCA	:	Virus discovery based on cDNA-AFLP

CHAPTER 1

Literature Review

Introduction

Viral pathogens are among the most important causes of human and animal diseases. Several known epidemic diseases for example rabies, measles, polio and smallpox are all caused by viral agents. Several viruses have emerged or are newly described including Nipah virus in 1997/8, SARS-coronavirus in 2002, Lujo virus in 2008 and a number of bat associated lyssaviruses in the past decade (Chua *et al.,* 2000; Selmi e*t al.,* 2002; Briese *et al.,* 2009; Kuzmin *et al.,* 2010). Viral agents often have a wide host range from avian and terrestrial animals to aquatic ones, depending on the specific virus.

Both known and novel emerging viral diseases are of considerable concern to public and veterinary health sectors (Brown, 2006). The majority of newly defined viral pathogens are zoonotic, implying epidemiological cycles in both human and animal populations (Pekosz & Glass, 2008). Uncertainty of when and where a spill-over event can occur and whether or not it will be catastrophic is of great concern and it is unclear how to stop natural progression of viral transmission, especially in the case of unknown pathogens. There are several steps required for a virus or any other pathogen to establish in a new host (Brown, 2006). These steps are as follows: Exposure, infection, local spread, successful replication, evasion of host defences, manifestation of disease and transmission. Although a spill-over event cannot always be prevented, it can be controlled by intervening at certain steps in the sequence of events for example limiting interaction with reservoirs and or hosts of potential pathogens (*e.g.* limiting bat bushmeat practices) or the use of post-exposure treatment. Although spill-over events might only result in mild disease or no disease presentation at all, unknown viruses should still be considered potential pathogens until proven otherwise.

One prominent example of zoonotic viruses that crossed the species barrier is that of paramyxoviruses and bats. Hendra- and Nipah virus are both known to have bats as natural reservoir from where they spilled over into domestic animals (horses and pigs respectively) and subsequently into humans (Williamson *et al.,* 1998; Chua *et al.,* 2000). Since the emergence of Nipah virus, disease transmission has also adapted to take place directly between bats and humans as well as human-to-human (Gurley *et al.* 2007). Bats are regarded as an important reservoir in the maintenance of viral pathogens when

considering the vast viral diverse that they harbour (Calisher *et al.,* 2006). Several recent studies have described genetically diverse paramyxoviruses from a variety of bat species around the globe (Drexler *et al.,* 2009; Baker *et al.,* 2012; Drexler *et al.,* 2012). Many questions remain regarding the association of paramyxoviruses with bats. The true geographical expanse, the pathogenicity and host-pathogen interactions, the potential for spill-over to other mammals including humans and the resultant public health threats are still unclear. To gain insight into the complex relationship between bats and paramyxoviruses, each should first be discussed separately.

1.1 ORDER CHIROPTERA

Although bats belong to the class Mammalia, they are unique when compared to other mammalian species. Other orders in the class Mammalia did not evolve the ability of true powered flight as with the order Chiroptera. In some instances, for example flying squirrels, they do not fly but merely glide from high to low elevations. The order Chiroptera comprise of nearly 1200 of the approximately 5 000 recognized mammalian species (Teeling *et al.,* 2005; Donaldson *et al.,* 2010) with only the Rodentia order exceeding in species number. The vast number of species and the ability to fly has made bats the most widely distributed mammals found worldwide, excluding the Arctic (Teeling *et al.,* 2005). Tropical and some subtropical regions seem to be most suitable for bats as these regions are species rich in comparison to more temperate regions.

1.1.1 Phylogeny of bats

During the last decade the taxonomic classification of bats has been under debate where several different proposals have been made. Based on morphological data, the order Chiroptera has two extant sub-orders traditionally known as Megachiroptera and Microchiroptera (Teeling *et al.,* 2005) and at least four known extinct clades most closely related to Microchiroptera. Megachiropteran bats are also referred to as megabats or old world fruit bats and occur only in the tropical and sub-tropical regions. This sub-order consisted of a single family named Pteropodidae. Microchiroptera being the larger sub-order contained all of the other bat families.

Based on a more recent phylogenetic analysis, Chiroptera was divided into two sub-orders Yinpterochiroptera containing the old Megachiroptera together with the super-family Rhinolophoidea and Yangochiroptera that includes the rest of the Microchiropteran families

(**Figure 1.1**). The latter sub-order was further divided into three super-familial groups namely Emballonuroidea, Noctilionoidea and Vespertilionoidea (Teeling *et al.,* 2005). Also represented by **Figure 1.1** are the now 19 different families of bats. Each family contains a large number of different species.

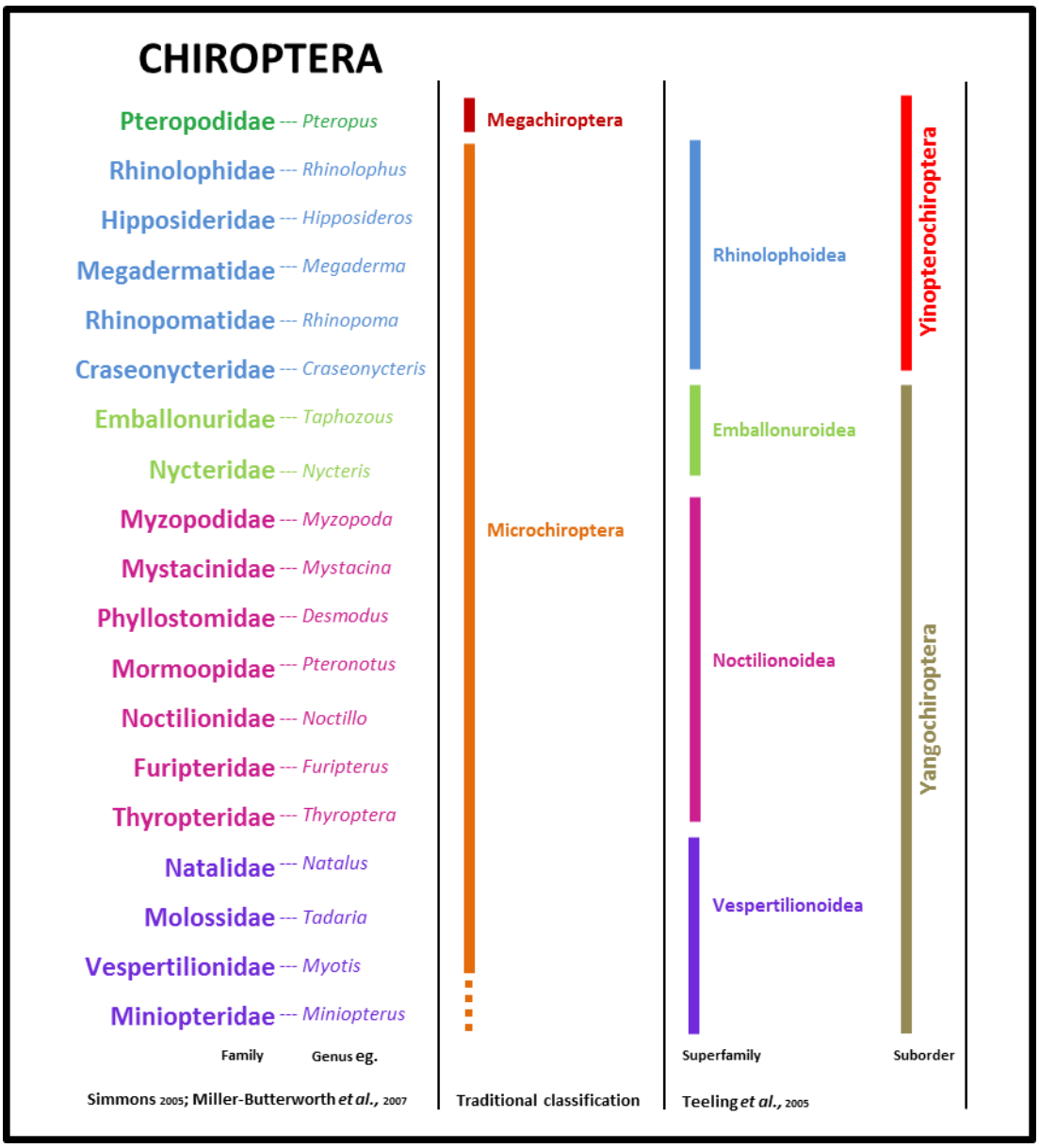

Figure 1.1: Classification of the Chiropteran order. Traditional classification grouped families into two suborders namely Megachiroptera and Microchiroptera which is no longer in use. The Teeling *et al.* (2005) classification groups the families slightly differently but has not yet been accepted as the rule. Species classification mainly still follows Simmons (2005). The dotted line at the traditional classification represents Miniopteridae which was previously not classified as a separate family but formed part of the Vespertilionidae family of bats.

Bats are considered as either frugivorous (Pteropodidae), insectivorous (Rhinolophoidea, Emballonuroidea, Vespertilionoidea) or have a range of food preferences including insects, fruits, blood and even other small animals including rodents or bats (Noctilionoidea) (Monadjem *et al.,* 2010). For an order with such a vast number of mammals, phylogeny and classification is important. Recently, yet another approach for classification was proposed based on echolocation call frequency. This is based on results that the divergence of echolocation call frequency in different bats naturally took place alongside the processes shaping genetic subdivision (Chen *et al.,* 2009).

According to Simmons (2005), the lack of molecular data allows no complete classification of Chiropteran families. Complete classifications similar to those mentioned above were mainly based on morphological data making them out of date. With the current means of obtaining molecular data, a comprehensive analysis of both molecular and morphological data is needed before a complete higher-classification can be made. Simmons (2005) gives a thorough collection of information on members of the Chiropteran order but only from family level down. Up to then, 18 Chiropteran families were known. Recently, the subfamily Miniopterinae from the Vespertilionidae family was reclassified as a family of its own namely Miniopteridae (Miller-Butterworth *et al.,* 2007). The initial classification was solely based on morphological data. With the new taxonomic data presented by Miller-Butterworth *et al.* (2007) Miniopteridae now forms the 19th family in the Chiropteran order. A phylogenetic representation of relatedness between the principle clades of bats are shown in **Figure 1.2**.

1.1.2 Bats and emerging viral diseases

The NIAIDs' (National Institute of Allergy and Infectious Diseases) list of emerging and re-emerging pathogens mentions a number of emerging infectious diseases caused by viruses derived from several bat species (Last updated April 2012 on http://www.niaid.nih.gov/topics/emerging/pages/list.aspx). The majority of these viruses fall into group III for agents with bioterrorism potential (Donaldson *et al.,* 2010).

Up to and including 2011, an increasingly large number of viruses have been isolated from various bat species (Calisher *et al.,* 2008) of which several are zoonotic (Barclay & Paton, 2000; Ksiazek *et al.,* 2003), including, SARS-related coronavirus, Hendra- (HeV) and Marburg virus (MARV) (**Table 1.1**).

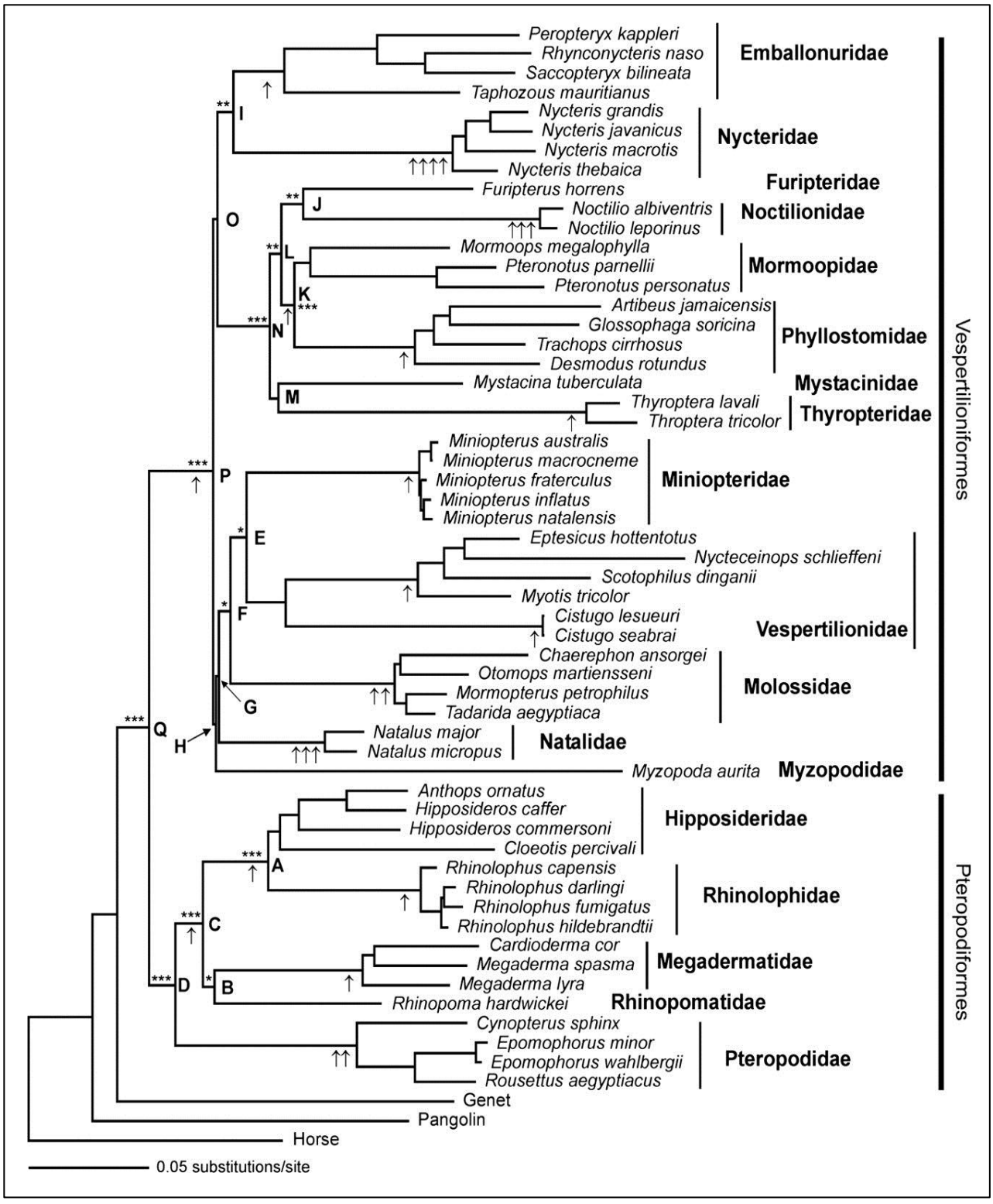

Figure 1.2: **The evolutionary relationship between the principle clades of living bats** (Eick *et al.,* 2005). Four different genetic markers were used for this phylogenetic representation. The number of asterisk is representative of the level of statistical confidence at each particular node. (Licence number: 3026330037630)

Table 1.1: Bat associated viruses, their Chiropteran host species and relative information up to the end of 2011.

Family	Genus/ subfamily (sf)	Virus	Bat species (Virus isolation, RNA/DNA detection, serology)	First isolation/ detection in bats		Target samples	Reference
				Year	Location		
Rhabdoviridae	*Lyssavirus*	Aravan virus	*Myotis blythii*	1991	Krygyzstan	Brain	Kuzmin *et al.,* 1992
		Australian bat virus	*Saccolaimus flaviventris, Pteropus alecto, Pteropus poliocephalus, Pteropus scapulatus, Pteropus conspicullatus*	1996	Australia	Brain	Fraser *et al.,* 1996
		Duvenhage virus	*Miniopterus schreibersii., Nyctalus noctula, Vespertilio murinus, Nycteris thebaica*	1970	Pretoria, South Africa	Brain	Merideth *et al.,* 1971
		European bat lyssavirus 1	*Eptesicus serotinus, Rousettus aegyptiacus, Myotis myotis, Pipistrellus pipistrellus, Miniopterus schreibersii, Myotis nattereri, Rhinolophus ferrumequinum, Vespertilio murinus, Nyctalus noctula*	1984	Poland	Brain	#
		European bat lyssavirus 2	*Myotis myotis, Myotis dasycneme, Myotis nattereri, Miniopterus schreibersii, Rhinolophus ferrumequinum, Myotis daubentonii, Myotis capaccinii*	1985	Finland	Brain	#
		Irkut virus	*Murina leucogaster*	2002	Eastern Siberia, Russia	Brain	Botvinkin *et al.,* 2003
		Khujand virus	*Myotis mystacinus*	2001	Tajikistan, Russia	Brain	Kuzmin *et al.,* 2003
		Lagos bat virus	*Eidolon helvum, Micropteropus pusillu, Epomops dobsonii, Nycteris gambiensis, Epomophorus wahlbergi, Rousettus aegyptiacus, Nycteris gambiensis*	1956	Lagos Island, Nigeria	Brain	Boulger and Porterfield, 1958
		Rabies virus	*Lasionycteris noctivagans, Pipistrellus hesperus, Pipistrellus subflavus, Myotis lucifugus, Tadaris brasiliensis, Dasmodus rotondus, Eptesicus fuscus, Lasiurus borealis, Lasiurus cinereus [Numerous other bat species]*	1954	#	Brain, salivary glands	#
		Shimoni bat virus	*Hipposideros commersoni*	2009	Kenya	Brain	Kuzmin *et al.,*2010
		West Caucasian bat virus	*Miniopterus schreibersi*	2002	Russia	Brain	Botvinkin *et al.,* 2003
	Vesiculovirus	Mount Elgon bat virus	*Rhinolophus hiderbrandtii eloquens*	1964	Mount Elgon, Kenya	Salivary glands	Murphy *et al.,* 1970
	Unassigned	Gossas virus	*Tadarida* sp.	1964	Senegal	#	Calisher *et al.,* 2006
		Kern Canyon virus	*Myotis yumanensis*	1956	California, USA	#	Calisher *et al.,* 2006
		Oita 296	*Rhinolophus cornutus*	1972	Japan	Blood	Iwasaki *et al.,* 2004
Orthomyxoviridae	*Influenzavirus A*	Influenza A virus	*Nyctalus noctula*	#	Kazakhstan	Lung	Lvov *et al.,* 1979
Paramyxoviridae	*Henipavirus*	Hendra virus	*Pteropus alecto, Pteropus poliocephalus, Pteropus scapulatus, Pteropus conspicillatus*	1994	Australia	Nasal swabs, urine, lung, brains	Young *et al.,* 1996
		Nipah virus	*Pteropus hypomelanus, Pteropus vampyrus, Pteropus lylei, Pteropus giganteus, Hipposideros armiger, Eonycteris spelaea, Cynopterus brachyotis, Hipposideros larvatus, Scotophilus kuhlii*	1999	Malaysia	Urine, respiratory secretions	Chua *et al.,* 1999
		BatPV/Eid_hel/GH10/2008	*Eidolon helvum*	2008	Ghana, Africa	Fecal	Drexler *et al.,* 2009
		BatPV/Eid_hel/GH45/2008	*Eidolon helvum*	2008	Ghana, Africa	Fecal	Drexler *et al.,* 2009
		BatPV/Eid_hel/GH48/2008	*Eidolon helvum*	2008	Ghana, Africa	Fecal	Drexler *et al.,* 2009
	Rubulavirus	Bat parainfluenzavirus	*Rousettus leschenaultia*	1966	India	Pooled spleen, brain and salivary glands	Hollinger &Pavri, 1971
		Mapuera virus	*Sturnira lilium*	1979	Brazil	Salivary glands	Karabatsos, 1985
		Menangle virus	*Pteropus poliocephalus, Pteropus alecto, Pteropus conspicillatus, Pteropus scapulatus*	1997	Australia	(Serology, EM of fecal samples)	Chant *et al.,* 1998

Table adapted and improved from information in Calisher *et al.* (2008). References given are first publication on particular virus (as far as possible), more information available in other publications. #: Insufficient data or data not readily available/accessible; ***:** Only available as citations

Table 1.1 [Continued]**:** Bat associated viruses, their Chiropteran host species and relative information up to the end of 2011.

Family	Genus/ subfamily (sf)	Virus	Bat species (Virus isolation, RNA/DNA detection, serology)	First isolation/ detection in bats		Target samples	Reference
				Year	Location		
Paramyxoviridae	*Rubulavirus*	Tioman virus	*Pteropus hypomelanus, Pteropus rufus, Eidolon dupreanum, Rousettus madagascariensis*	2000	Malaysia	Urine	Chua *et al.*, 2001
		Tuhoko virus 1	*Rousettus leschenaultii*	2006	China	Respiratory and alimentary	Lau *et al.*, 2010
		Tuhoko virus 2	*Rousettus leschenaultii*	2006	China	Respiratory and alimentary	Lau *et al.*, 2010
		Tuhoko virus 3	*Rousettus leschenaultii*	2006	China	Respiratory and alimentary	Lau *et al.*, 2010
Coronaviridae	α-*Coronavirus*	*Rh*-BatCoV HKU2	*Rhinolophus sinicus*	2006	China	Anal Swab	Woo *et al.*, 2006
		BtCoV HKU6	*Myotis ricketti*	2006	China	Anal Swab	Woo *et al.*, 2006
		BtCoV HKU7	*Miniopterus magnater*	2006	China	Anal Swab	Woo *et al.*, 2006
		BtCoV/M.sch/BR98-30/08 (BtCoV HKU7-related)	*Miniopterus schreibersi*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/M.sch/BR98-31/08 (BtCoV HKU7-related)	*Miniopterus schreibersi*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		Mi-BatCoV HKU8	*Miniopterus pusillus, Miniopterus magnater, Miniopterus schreibersii*	2006	China	Anal Swab	Woo *et al.*, 2006
		BtCoV/M.sch/BR96-37/08 (*Mi*-BatCoV HKU8-related)	*Miniopterus schreibersi*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/M.sch/BR96-40/08 (*Mi*-BatCoV HKU8-related)	*Miniopterus schreibersi*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/701/05	*Myotis ricketti*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/821/05	*Myotis ricketti*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/970/06	*Rhinolophus ferrumequinum, Rhinolophus pearsoni*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/A773/05	*Miniopterus schreibersi*	2006	China	Anal Swab	Tang *et al.*, 2006
		Mi-BatCoV1	*Miniopterus magnater, Miniopterus pusillus, Miniopterus schreibersii*	2004	Hong Kong	Fecal, respiratory	Poon *et al.*, 2005
		BtCoV/M.sch/BR98-55/08 (*Mi*-BatCoV 1-related)	*Miniopterus schreibersi*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/A911/05	*Miniopterus schreibersi*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV HKU10	*Rousettus lechenaulti*	2007	China	Anal Swab	Woo *et al.*, 2007
		BtCoV/515/05	*Scotophilus kuhlii*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/527/05	*Scotophilus kuhlii*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/1552G1/08	*Scotophilus kuhlii*	2008	Philippines	Large intestine	Watanabe *et al.*, 2010a
		RM-Bat-CoV 65	*Eptesicus fucus*	2006	Rocky Mountains, U. S.	Fecal	Dominguez *et al.*, 2007
		RM-Bat-CoV 3	*Myotis occultus*	2006	Rocky Mountains, U. S.	Fecal	Dominguez *et al.*, 2007
		RM-Bat-CoV 6	*Myotis occultus*	2006	Rocky Mountains, U. S.	Fecal	Dominguez *et al.*, 2007
		RM-Bat-CoV 11	*Myotis occultus*	2006	Rocky Mountains, U. S.	Fecal	Dominguez *et al.*, 2007
		RM-Bat-CoV 27	*Myotis occultus*	2006	Rocky Mountains, U. S.	Fecal	Dominguez *et al.*, 2007
		RM-Bat-CoV 48	*Myotis occultus*	2006	Rocky Mountains, U. S.	Fecal	Dominguez *et al.*, 2007

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				Year	Location		
Coronaviridae	α-*Coronavirus*	BtCoV/M.das/D3.28/07	*Myotis dasycneme*	2008	Bad Segeberg, Germany	Fecal	Gloza-Rausch *et al.*, 2008
		BtCoV/M.das/D3.33/07	*Myotis dasycneme*	2008	Bad Segeberg, Germany	Fecal	Gloza-Rausch *et al.*, 2008
		BtCoV/M.bec/D6.6/07	*Myotis bechsteinii*	2008	Bad Segeberg, Germany	Fecal	Gloza-Rausch *et al.*, 2008
		BtCoV/M.das/NL-VM3/10	*Myotis dasycneme*	2008	Netherlands	Fecal	Reusken *et al.*, 2010
		BtCoV/M.das/NL-VM7/10	*Myotis dasycneme*	2008	Netherlands	Fecal	Reusken *et al.*, 2010
		BtCoV/Hip.sp/GhanaKwam/19/2008	*Hipposideros cf. ruber*	2008	Ghana, Africa	Fecal	Pfefferle *et al.*, 2009
		BtCoV/HIp.sp/GhanaBuo/344/2008	*Hipposideros cf. ruber*	2008	Ghana, Africa	Fecal	Pfefferle *et al.*, 2009
		BtCoV/Hip.sp/GhanaKwam/8/2008	*Hipposideros cf. ruber*	2008	Ghana, Africa	Fecal	Pfefferle *et al.*, 2009
		BtCoV/M.dau/D7.3/07	*Myotis daubetonii*	2008	Bad Segeberg, Germany	Fecal	Gloza-Rausch *et al.*, 2008
		BtCoV/M.dau/NL-VM222/10	*Myotis daubetonii*	2008	Netherlands	Fecal	Reusken *et al.*, 2010
		BtCoV/N.lei/BNM98-30/08	*Nyctalus leisleri*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/N.noc/NL-VM176/10	*Nyctalus noctula*	2008	Netherlands	Fecal	Reusken *et al.*, 2010
		BtCoV/P.nat/D5.16/07	*Pipistrellus nathusii*	2008	Bad Segeberg, Germany	Fecal	Gloza-Rausch *et al.*, 2008
		BtCoV/P.pip/NL-VM312/10	*Pipistrellus pipistrellus*	2008	Netherlands	Fecal	Reusken *et al.*, 2010
		BtCoV/P.pyg/D5.70/07	*Pipistrellus pygmaeus*	2008	Bad Segeberg, Germany	Fecal	Gloza-Rausch *et al.*, 2008
		BtCoV/Rhi.bla/BB98-41/08 (*Rh*-BatCoV HKU2-related)	*Rhinolophus blasii*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Rhi.bla/BB98-15/08	*Rhinolophus blasii*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Rhi.bla/BM48-39/08	*Rhinolophus blasii*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Rhi.eur/BR98-19/08	*Rhinolophus euryale*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Rhi.fer/BM48-28/08 (BtCoV/Rhi.bla/BM98-15/08-related)	*Rhinolophus ferrumequinum*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		Bt-CoV/Trinidad/1CO7B	*Glossaphaga soricina*	2006-2007	Trinidad	Anal Swab	Carrington *et al.*, 2008
		Bt-CoV/Trinidad/1FY2B	*Carollia perspicillata*	2006-2007	Trinidad	Oral and Anal Swabs	Carrington *et al.*, 2008
	β - *Coronavirus*	BtCoV/279/04	*Rhinolophus macrotis*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/273/04	*Rhinolophus ferrumequinum*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/A1018/06	*Rhinolophus sinicus*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/1525G2/08	*Cynopterus brachyotis, Eonycteris spelaea, Ptenochirus jagori, Rousettus amplexicaudatus*	2008	Philippines	Large intestine	Watanabe *et al.*, 2010a
		BtCoV/355/05	*Pipistrellus abramus, Rhinolophus ferrumequinum*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/434/05	*Pipistrellus pipistrellus*	2006	China	Anal Swab	Tang *et al.*, 2006
		BtCoV/133/05	*Tylonycteris pachypus*	2006	China	Anal Swab	Tang *et al.*, 2006
		Bat-SARS-CoV Rf1	*Rhinolophus ferrumequinum*	2004	China	Fecal	Li *et al.*, 2005
		Pi-BatCoV HKU5	*Pipistrellus abramus*	2006	China	Anal Swab	Woo *et al.*, 2006
		Ro-Bat-CoV HKU9	*Rousettus lechenaulti*	2007	China	Anal Swab	Woo *et al.*, 2007
		Ty-BatCoV HKU4	*Tylonycteris pachypus*	2006	China	Anal Swab	Woo *et al.*, *2006*

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Table 1.1 [Continued]**:** Bat associated viruses, their Chiropteran host species and relative information up to the end of 2011.

Family	Genus/ subfamily (sf)	Virus	Bat species (Virus isolation, RNA/DNA detection, serology)	First isolation/ detection in bats		Target samples	Reference
				Year	Location		
Coronaviridae	β - *Coronavirus*	*Sc*-BatCoV 512	*Scotophilus kuhlii*	2006	China	Anal Swab	Tang *et al.*, 2006
		BatCoVDR/2007	*Desmodus rotundus*	2005	São Paulo, Brazil	Fecal	Brandao *et al.*, 2008
		Zaria bat coronavirus	*Hipposideros commersoni*	2008	Zaria, Nigeria	Gastrointestinal tissue	Quan *et al.*, 2010
		BtCoV/P.pip/NL-VM314/10	*Pipistrellus pipistrellus*	2008	Netherlands	Fecal	Reusken *et al.*, 2010
		BtCoV/rhi.bla/BB98-16/08 (SARS*r-Rh*-BtCoV-related)	*Rhinolophus blasii*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Rhi.eur/BB98-43/08 (SARSr-Rh-BtCoV-related)	*Rhinolophus euryale*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Rhi.fer/BM48-34/08	*Rhinolophus ferrumequinum*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		SL01A0066	*Rhinolophus hipposideros*	2008	Slovenia	Fecal	Rihtaric *et al.*, 2010
		SL010050	*Rhinolophus hipposideros*	2008	Slovenia	Fecal	Rihtaric *et al.*, 2010
		SL01A0082	*Rhinolophus hipposideros*	2008	Slovenia	Fecal	Rihtaric *et al.*, 2010
		BtCoV/Rhi.meh/BM48-32/08	*Rhinolophus mehelyi*	2008	Bulgaria	Fecal	Drexler *et al.*, 2010
		BtCoV/Hip.sp/GhanaKwam/20/2008	*Hipposideros cf. ruber*	2008	Ghana, Africa	Fecal	Pfefferle *et al.*, 2009
		BtCoV/Hip.sp/GhanaBuo/348/2008	*Hipposideros cf. ruber*	2008	Ghana, Africa	Fecal	Pfefferle *et al.*, 2009
		SARSr-*Rh*-BatCoV HKU3	*Rhinolophus sinicus*	2004	China	Nasal and anal	Lau *et al.*, 2005
		SARSr-*Rh*-BatCoV RP3	*Rhinolophus sinicus*	2006	China	Fecal	Yuan *et al.*, 2010
Togaviridae	*Alphavirus*	Chikungunya virus	*Rousettus aegyptiacus, Hipposideros caffer , Chaerephon pumilus, Rousettus leschenaultii, Scotophilus nigrita, Scotophilus temminckii*	#	Senegal	Salivary glands	Zhang *et al.*, 1989*****
		Sindbis virus	*Rhinolophidae* sp., *Hipposideridae* sp.	#	#	Organs	Blackburn *et al.*, 1982*****
		Venezuelan equine encephalitis virus	*Desmodus rotundus, Uroderma bilobatum, Artibeus phaeotis, Carollia perspicillata, Carollia subrufa, Artibeus jamaicensis*	1970	Oaxaca, Mexico	Visceral organs	Wong-Chia & Scherer, 1971*****
Flaviviridae	*Flavivirus*	Bukalasa bat virus	*Chaerephon pumilus, Tadarida condylura*	#	Uganda	Salivary glands	Williams *et al.*, 1964
		Carey Island virus	*Cynopterus brachiotis, Macroglossus minimus*	#	Malaysia	#	Calisher *et al.*, 2006
		Central European encephalitis virus	Unidentified bat	#	#	#	Calisher *et al.*, 2006
		Dakar bat virus	*Chaerephon pumilu, Taphozous perforatu, Scotophilus nigrita, Mops condylurus*	#	Senegal	Salivary glands	Williams *et al.*, 1964
		Entebbe bat virus	*Chaerephon pumilu, Mops condylurus*	#	Uganda	Salivary glands	Williams *et al.*, 1964
		Japanese encephalitis virus	*Hipposideros armiger, Miniopterus schreibersii, Rhinolophus cornutus, Hipposideros pomona, Hipposideros speoris, Hipposideros bicolor, Hipposideros cineraceus, Rhinolophus rouxi, Rhinolophus ferrumequinum, Vespertilio superans, Myotis macrodactylus, Pteropus vampyrus*	1970	Japan	Brain, blood, kidney spleen	Sulkin *et al.*, 1970
		Jugra virus	*Cynopterus brachiotis*	#	Malaysia	Blood	Grard *et al.*, 2010
		Kyasanur Forest disease virus	*Rhinolophus rouxi, Cynopterus sphinx , Rousettus leschenaulti*	#	India	#	Pavri & Singh 1968*****
		Montana Myotis leucoencephalitis	*Myotis lucifugus*	1958	Montana	Brain, salivary glands	Bell & Thomas, 1964
		Phnom-Phen bat virus	*Eonycteris spelaea, Cynopterus brachyotis*	1969	Cambodia	Salivary glands, brown fat	Salaün *et al.*, 1974*
		Rio Bravo virus	*Tadarida brasiliensis, Eptesicus fuscus*	1965	California, USA	Salivary glands	Burns & Farinacci, 1956
		St. Louis encephalitis virus	*Tadarida brasiliensis mexicana , Eptesicus fuscus, Myotis lucifugus*	1964	Texas	Blood	Sulkin *et al.*, 1966
		Saboya virus	*Nycteris gambiensis*	#	#	#	Calisher *et al.*, 2006
		Sokuluk virus	*Pipistrellus pipistrellus*	1970	Krygyzstan	Pooled organs	Lvov *et al.*, 1973a

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Table 1.1 [Continued]**:** Bat associated viruses, their Chiropteran host species and relative information up to the end of 2011.

Family	Genus/ subfamily (sf)	Virus	Bat species (Virus isolation, RNA/DNA detection, serology)	First isolation/ detection in bats		Target samples	Reference
				Year	Location		
Flaviviridae	*Flavivirus*	Tamana bat virus	*Pteronotus parnellii*	1974	Trinidad	Salivary glands, spleen	Price, 1978
		Uganda S virus	*Rousettus* sp., *Tadarida* sp.	#	Uganda	#	Calisher *et al.*, 2006
		West Nile virus	*Rousettus aegyptiacus , Eptesicus fuscus, Myotis lucifugus, Myotis septentrionalis*	#	Albany	Blood	Calisher *et al.*, 2006
		Yokose virus	*Miniopterus fuliginosus*	1971	Japan	Blood	Tajima *et al.*, 2005
Bunyaviridae	*Hantavirus*	Hantaan virus	*Eptesicus serotinus, Rhinolophus ferrumequinum*	1992	Korea	Lung	Kim *et al.*, 1994
	Orthobunyavirus	Catu virus	*Molossus obsurus*	#	Amapa, Brazil	#	Calisher *et al.*, 2006
		Guama virus	*Unidentified bat*	#	Brazil	#	Calisher *et al.*, 2006
		Kaeng Khoi virus	*Chaerephon plicata , Taphozous theobaldi*	1969	Thailand	#	Osbourne *et al.*, 2003
		Mojui dos Campos virus	Unidentified bat	1975	Mojui dos Campos, Brazil	Blood	Wanzeller *et al.*, 2002
		Nepuyo virus	*Artibeus jamaicensis, Artibeus lituratus*	#	#	#	Calisher *et al.*, 2006
	Phlebovirus	Rift Valley fever virus	*Micropteropus pusillus , Hipposideros abae, Miniopterus schreibersii, Hipposideros caffer, Epomops franqueti, Glauconycteris argentata*	#	Guinea	#	Boiro *et al.*, 1987
		Toscana virus	*Pipistrellus kuhlii*	1984	Siena	Brain	Vverani *et al.*, 1988
	Nairovirus	Bandia virus	*Scotophilus nigrita*	#	Senegal	#	Calisher *et al.*, 2006
	unassigned	Bangui virus	*Scotophilus* sp., *Pipistrellus* sp., *Tadarida* sp.	#	Central African Republic	#	Calisher *et al.*, 2006
		Bhanja virus	Unidentified bat	#	Krygyzstan	#	Calisher *et al.*, 2006
		Issyk-kul virus	*Nyctalus noctula, Eptesicus serotinus, Pipistrellus pipistrellus, Myotis blythii, Rhinolophus ferrumequinum, Scotophilus kuhlii, Cynopterus brachyotis, Eonycteris spelaea, Chaerephon plicatus, Hipposideros diadema, Taphozous melanopogon, Rhinolophus lepidus, Rhinolophus horsfeldi, Vespertilio pipistrellus*	1970	Issy-Kul, Kyrgyzstan	Visceral organs	Lvov *et al.*, 1973b
		Kasokero virus	*Rousettus aegyptiacus , Scotophilus temminckii*	1985	Uganda	Blood	Kalunda *et al.*, 1986
		Keterah virus	*Scotophilus kuhlii, Myotis blythi, Nyctalus noctula, Eptesicus serotinus, Pipistrellus pipistrellus, Scotopholus temmencki*	#	Malaysia	Blood, pooled brain, liver, spleen, kidney	Calisher *et al.*, 2006
		Yogue virus	*Rousettus aegyptiacus*	#	Senegal	#	Calisher *et al.*, 2006
Reoviridae	*Orbivirus*	Fomede virus	*Nycteris nana, Nycteris gambiensis*	1978	Republic of Guinea, West Africa	#	Calisher *et al.*, 2006
		Ife virus	*Eidolon helvum*	1971	Ife, Africa	Salivary glands, blood, brain	Kemp *et al.*, 1988
		Japanaut virus	*Syconycteris australis*	#	New Guinea	#	Calisher *et al.*, 2006
	Orthoreovirus	Broome virus	*Pteropus alecto, Pteropus scapulatus*	2002	Broome, Western Australia	Pooled lung, liver, kidney and spleen	Thalmann *et al.*, 2010
		Nelson Bay virus	*Pteropus poliocephalus*	1968	Australia	Blood	Gard & Compans, 1970
		Pulau virus	*Pteropus hypomelanus*	2005	Tioman island, Malaysia	Urine	Pritchard *et al.*, 2006
	Rotavirus	Bat/KE4852/07	*Eidolon helvum*	2007	Kenya	Fecal	Esona *et al.*, 2010
Arenaviridae	*Arenavirus*	Tacaribe virus	*Artibeus lituratus, Artibeus jamaicensis*	1961	Trinidad, West Indies	Brain, salivary glands, spleen, liver	Downs *et al.*, 1963

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Table 1.1 [Continued]**:** Bat associated viruses, their Chiropteran host species and relative information up to the end of 2011.

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				Year	Location		
Herpesviridae	*Alphaherpesvirinae (sf)*	CSG248	*Pteropus lylei*	2004	Cambodia	Throat swab	Razafindratsimandresy *et al.,* 2009
		CS732G	*Eidolon dupreanum*	2006	Madagascar	Throat swab	Razafindratsimandresy *et al.,* 2009
		Dak An B N27	Unidentified bat	1965	Central African Republic	Salivary glands	Razafindratsimandresy *et al.,* 2009
		Dak An Y7	*Eidolon helvum*	1971	Cameroon	Organs	Razafindratsimandresy *et al.,* 2009
		Parixa virus	*Lonchophylla thomasi*	1984	Brazil	Blood	Razafindratsimandresy *et al.,* 2009
	Betaherpesvirinae (sf)	Bat betaherpesvirus 1	*Myotis nattereri, Pipistrellus pipistrellus*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat betaherpesvirus 2	*Miniopterus fuliginosus*	2008	Japan	Spleen	Watanabe *et al.,* 2010b
	Gammaherpesvirinae (sf)	Bat gammaherpesvirus 1	*Eptesicus serotinus, Myotis nattereri, Pipistrellus nathusii, Pipistrellus pipistrellus*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat gammaherpesvirus 2	*Myotis myotis, myotis nattereri*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat gammaherpesvirus 3	*Nyctalus noctula, Myotis nattereri, Myotis myotis*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat gammaherpesvirus 4	*Nyctalus noctula*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat gammaherpesvirus 5	*Pipistrellus nathusii*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat gammaherpesvirus 6	*Pipistrellus pipistrellus*	2006	Europe	Lung	Wibbelt *et al.,* 2007
		Bat gammaherpesvirus 7	*Plecotus auritus*	2006	Europe	Lung	Wibbelt *et al.,* 2007
	Unassigned	A cytomegalovirus	*Myotis lucifugus*	1996	Illinois, USA	Salivary glands	Tandler, 1996
		Agua Preta virus	*Carollia subrufa*	#	Brazil	Blood	Karabatsos, 1985
Picornaviridae	Undetermined	Juruaca virus	Unidentified bat	#	#	#	Calisher *et al.,* 2006
Papillomaviridae	New, unnamed	*R. aegyptiacus* papillomavirus type 1	*Rousettus aegyptiacus*	2003	Michigan, USA	Basosquamous carcinoma	Rector *et al.,* 2006
Adenoviridae	*Mastadenovirus*	Ryukyu virus	*Pteropus dasymallus yayeymae*	2007	Japan	Spleen	Maeda *et al.,* 2008
		Bat adenovirus-2 strain *P. pipistrellus* virus 1	*Pipistrellus pipisetellus*	2009	Germany	Intestine	Sonntag *et al.,* 2009
Filoviridae	*Marburgvirus*	Lake Victoria marburgvirus	*Rousettus aegyptiacus, Hipposideros spp. Rhinolophus eloquens*	2007	Gabon	Liver, spleen	Swanepoel *et al.,* 2007
	Ebolavirus	Zaire ebolavirus	*Hypsignathus monstrosus, Epomops franqueti, Myonycteris torquata, Rousettus aegyptiacus*	2005	Gabon	Liver, spleen	Leroy *et al.,* 2005
Astroviridae	*Mammastrovirus*	AFCD337	*Miniopterus magnater, Miniopterus pusillus, Miniopterus schreibersii, Myotis chinensis, Myotis ricketti, Pipistrellus abramus, Rhinolophus rouxi*	2004-2006	Hong Kong	Rectum, throat, fecal	Chu *et al.,* 2008
		Bat astrovirus Ha/GX/L13/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/LG02/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/LH10/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/LS10/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/LS11/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/LY07/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/PD03/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/TD03/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Ha/GX/WW06/07	*Hipposideros armiger*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Hl/GX/LD117/07	*Hipposideros larvaticus*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009
		Bat astrovirus Hl/GX/PX05/07	*Hipposideros larvaticus*	2007	Guangxi, China	Anal Swab	Zhu *et al.,* 2009

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Table 1.1 [Continued]**:** Bat associated viruses, their Chiropteran host species and relative information up to the end of 2011.

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				Year	Location		
Astroviridae	*Mammastrovirus*	Bat astrovirus Hl/GX/WT03/07	*Hipposideros larvaticus*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Hp/GX/BZ9/07	*Hipposideros pomona*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Hp/GX/LC03/07	*Hipposideros pomona*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Hp/GX/LC08/07	*Hipposideros pomona*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Hp/GX/WW08/07	*Hipposideros pomona*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ii/GX/JY10/07	*Ia io*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ii/GX/JY11/07	*Ia io*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ii/GX/JY14/07	*Ia io*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ms/AH/A894/05	*Miniopterus schreibersii*	2005	Anhui, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ms/AH/A909/05	*Miniopterus schreibersii*	2005	Anhui, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ms/FJ/A1196/06	*Miniopterus schreibersii*	2006	Fujian, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ms/GX/A629/05	*Miniopterus schreibersii*	2005	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Ms/HK61/CHN/2007	*Miniopterus schreibersii*	2007	#	Rectal	#
		Bat astrovirus Ms/sy10/CHN/2008	*Miniopterus schreibersii*	2008	#	Rectal	#
		Bat astrovirus Ms/sy37/CHN/2008	*Miniopterus schreibersii*	2008	#	Rectal	#
		Bat astrovirus Ms/sy3/CHN/2008	*Miniopterus schreibersii*	2008	#	Rectal	#
		Bat astrovirus Ms/sy6/CHN/2008	*Miniopterus schreibersii*	2008	#	Rectal	#
		Bat astrovirus Ms/sy8/CHN/2008	*Miniopterus schreibersii*	2008	#	Rectal	#
		Bat astrovirus Ms/YY41/CHN/2008	*Miniopterus schreibersii*	2008	#	Rectal	#
		Bat astrovirus My/GX/JY01/07	*Myotis spp.*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus My/GX/JY06/07	*Myotis spp.*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus My/GX/JY07/07	*Myotis spp.*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Myr/AH/A900/05	*Myotis ricketti*	2005	Anhui, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Myr/JX/A819/05	*Myotis ricketti*	2005	Jiangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Pa/SC/A367/05	*Pipistrellus abramus*	2005	China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Rf/HB/273/04	*Rhinolophus ferrumequinum*	2004	Hubei, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Rf/SD/A977/06	*Rhinolophus ferrumequinum*	2006	Shandong, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Rp/JX/A860/07	*Rhinolophus pearsonii*	2007	Jiangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Tm/GX/LD04/07	*Taphozous melanopogon*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Tm/GX/LD06/07	*Taphozous melanopogon*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Tm/GX/LD113/07	*Taphozous melanopogon*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Tm/GX/LD116/07	*Taphozous melanopogon*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Tm/GX/LD145/07	*Taphozous melanopogon*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Tm/GX/LD98/07	*Taphozous melanopogon*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Bat astrovirus Hp/GX/LC01/07	*Hipposideros pomona*	2007	Guangxi, China	Anal Swab	Zhu *et al.*, 2009
		Numerous other bat astroviruses					
Unclassified	Unknown	Ank 6909 virus	*Nycteris gambiensis*	#	Guinea	#	Konstantinov *et al.*, 2006
		Kolente virus	*Hipposideros* sp.	#	Guinea	#	Konstantinov *et al.*, 2006

Table adapted and improved from information in Calisher *et al.* (2008). References given are first publication on particular virus (as far as possible), more information available in other publications. #: Insufficient data or data not readily available/accessible; ***:** Only available as citations

The number of coronaviruses and astroviruses that have been isolated from bats during 2008-2010 is too great for all to be listed. This just gives an indication of the viral richness of these mammals and their potential to act as successful host and in many cases reservoir.

During the beginning of 2012, Tong *et al.* (2012) reported the discovery of an additional bat associated virus. This new influenza A virus, isolated from *Sturnira lilium* (little yellow-shouldered bat), is considered to be distinct from all other influenza viruses characterized in the past. Several genes have shown to be divergent from other influenza viruses through sequencing and phylogenetic analysis. It has been classified as influenza A sub-type H17. This raises concerns for the emergence of yet another global pandemic in humans that originated from bats (Tong *et al.,* 2012).

Several bat species including for example *Rousettus aegyptiacus*, have been implicated as host for multiple viruses from different families. Pourrut *et al.* (2009) reported the possible co-circulation of Marburg- and Ebola virus from the *Filoviridae* family in this species of bats based on a large serological assay providing evidence of a high seroprevalence to both viruses. *Rousettus aegyptiacus* has also been implicated as host for viruses from several other virus families including *Togaviridae, Flaviviridae, Bunyaviridae* and *Paramyxoviridae* (**Table 1.1**). Towner *et al.* (2009) was able to implicate this bat species as a major natural reservoir of Marburg virus through virus isolation and evidence of long-term virus circulation in bats sampled from Uganda. This is just one of the many examples available which gives an indication of the profound capability of a single bat species to host several genetically diverse viruses. A vast number of viruses that have been detected in bat species have proven to be zoonotic including Hendra- (*Pteropus conspicillatus*), Nipah- (*Pteropus vampyrus)*, Duvenhage- (*Miniopterus schreibersi*) and Rift Valley fever virus (*Micropteropus pusillus)* (**Table 1.1**). The great diversity of human-pathogenic viruses we see in bats should make them a key focus when predicting and preventing possible future emergence of zoonotic diseases.

The order Rodentia should not be underestimated, as they have also proven to harbour several viral pathogens including a high diversity of paramyxoviruses as reported by Drexler *et al.* (2012). Bats and rodents may have been hosts to this diversity of viruses for many centuries, but only described recently as research focusing on these mammals increased. On the other hand one should also keep in mind that these two groups have

been studied more intensively when compared to others. Will we see the same virus diversity in humans or other animals if we study their viromes more intensively?

1.1.3 Characteristics

Bats have certain characteristics that are believed to contribute to their propensity to act as reservoirs of viruses. This includes their ability to fly, hibernate or enter torpor, their relatively long life span, population structure, their roosting behaviours and unique immunological characteristics (Calisher *et al.,* 2006; Kuzmin *et al.,* 2011). These characteristics do however vary depending on the specific bat species for example the fact that some bat populations take part in long distance migration (*Eidolon helvum*) while others do not migrate at all.

They can be either insectivorous, frugivorous or haematophagous as briefly mentioned above (Calisher *et al.,* 2006; Van der Poel *et al.,* 2006). Research regarding these animals has mainly been focused on their habitats, behaviour and morphology (Calisher *et al.,* 2008) but their role in transportation and transmission of pathogens has primarily been researched in the past few years. One such study is that of Streicker *et al.* (2010) on the establishment of rabies virus in bats. Up until recently, no significant research had been done regarding the immunology of bats which allows them to act as reservoirs for various viral pathogens. More recent studies now provide new information regarding this topic (discussed in section ***1.1.3.5***).

1.1.3.1 Self-powered flight

Bats are the only mammals to have evolved the capability of sustained self-powered flight. This unique characteristic is used on a daily basis in search of food (Caviedes-Vidal *et al.,* 2008). Many bat species also take part in seasonal migration over long or short distances. *Eidolon helvum*, the Straw-coloured fruit bat, is known to migrate far-reaching distances over the African continent (Ossa *et al.,* 2012) while *Myotis* sp. only travel between 320-640km from their hibernation sites (Calisher *et al.,* 2006). This allows them to travel to a more suitable climate region during both summer and winter months. Flight clearly present these mammals with several advantages among which their ability to evade predator detection also proves useful.

When considering natural processes in nature, bats also contribute to pollination of several hundred species of plants which wouldn't have been as successful without their

capability of flight (Fleming *et al.,* 1996). From a public health perspective however, flight allows for the possibility of long distance dispersal of viral and other pathogens (Caviedes-Vidal *et al.,* 2008). It can also be hypothesised that the effects of climate change due to global warming will influence the biogeography of bats possibly forcing them to migrate away from the equator more to the poles (Sherwin *et al.,* 2012) which can result in the spread of tropical diseases to countries where it was previously not seen. From a disease emergence perspective, Daszak *et al.* (2013) was able to show, with the use of ecological niche modelling, that the distribution of *Henipavirus* reservoirs and as an effect henipaviruses, are likely to change under climate change scenarios. This is just one of the examples of how climate change can possibly effect the natural distribution of bats and the viruses they harbour.

1.1.3.2 Torpor and hibernation

Torpor is the state where normal metabolic activities are reduced which results in a considerable drop in heart rate and body temperature (Lawrence, 2005). Torpor is divided into two categories that are considered as distinct forms of heterothermia (Geiser & Ruf, 1995). It can occur on a daily basis and is referred to as daily torpor. Short term torpor is mainly related to food availability and ambient temperatures. When occurring on a longer term *e.g.* for several days or weeks it is referred to as hibernation. This is said to be related to day length and hormone changes. Hibernation is thus a prolonged extension of torpor and can be either obligate or facultative. Some bats are considered as facultative hibernators as they can be aroused from hibernation through external stimuli whereas obligate hibernators including certain ground squirrels and other rodents are not.

Although bats are warm blooded animals, some have a remarkable thermolability (Monadjem *et al.,* 2010). To escape cold temperatures during seasonal changes, bats have two options. They either migrate to a more favourable climate as discussed above or they can enter into hibernation. Entering into hibernation allow bats to survive on their stored body fat for several months while food is unavailable. Hibernation and daily torpor are traits of bats belonging to the Vespertilionidae, Rhinolophidae, Hipposideridae families and a few species in the Molossidae family. (Calisher *et al.,* 2006; Monadjem *et al.,* 2010).

The effect of torpor and hibernation on viral maintenance in the reservoir has not been extensively studied. There are however a few hypotheses on the influence of these states on the maintenance as well as pathogenesis of the virus infections in bats (Calisher

et al., 2006). Most bat associated viral infections are asymptomatic in their reservoirs. This persistent infection is believed to be facilitated by hibernation among other things, as recent science reports in LiveScience® 2012 mention the maintenance of rabies virus in hibernating bats (available online at http://www.livescience.com/20442-rabies-snoozes-bats-hibernate.html). Recent research done by George *et al.* (2011) focused on host and viral ecology as determinants of rabies maintenance in bats. They hypothesize that the resultant reduction in metabolic activity causes a reduction in viral replication, which help maintain the bat population and their viral pathogens until the next birthing season where new susceptible juveniles are introduced into the colony. This allows the virus to spread successfully in the colony which in turn would prevent an epizootic fade-out of the virus. Hibernation might only be one of the contributing factors to viral persistence as many non-hibernating bat species are also considered to be capable of viral persistence. Alternatively, these viruses may be benign due to long-term co-evolution where the bat immune system adapted to infection.

1.1.3.3 Longevity

Longevity of bats, provide viruses with a suitable environment for continuous and extensive virus transmission. Their long life span and the fact that they maintain persistent infections allows the viruses to be shed for an extended amount of time compared to other vertebrates (Calisher *et al.,* 2006). Vertical transmission from adult to pup through several generations is also possible. Previous data analysis has shown a correlation between increased life span and factors like hibernation, body mass and cave use as discussed above (Herreid, 1964).

Larger mammals have profoundly longer life spans than smaller mammals *e.g.* mice. For example human have an average lifespan of 77 to 79 years, horses 62, cats 21 and the common house mouse a mere 3 to 4 years. Surprisingly, bats have shown to live between 25 to 35 years (Herreid, 1964; Calisher *et al.,* 2006). The average life span of certain bat species have been shown to be 3.5 times that of terrestrial placental mammals of a similar size (Wilkinson & South, 2002). Wilkinson and South (2002) were able to record individuals from 5 different bat species surviving in the wild with a maximum lifespan of more than 30 years. This places bats on the outside of the traditional range of mammals of the same size (Austad, 2005). Viral pathogens in bats thus do not require a rapid replication strategy to maintain effective transmission. The long lifespan of bats provide a

stable enduring reservoir which can maintain a persistent virus infection for a long time allowing continuous transmission opportunities to other vertebrates.

1.1.3.4 Population size and roosting behaviour

Next to humans and rodents, bats are of the most widely distributed mammals and also occur in large population densities (Calisher *et al.,* 2006). Bats show various roosting habits ranging from trees to houses (hollows and crevices) and more specialized roosts like caves. Examples include *Neoromicia nana, Scotophilus dinganii* and *Rousettus aegyptiacus* respectively (Monadjem *et al.,* 2010). Population size of bats can vary from a few hundred to thousands. It has been showed that certain bats roost in densities of approximately 300 bats per ft^2 in caves in the United States (Constantine, 1967). Population size of cave dwelling bats can reach up to thousands. De Hoop Guano cave in the Western Province is known to harbour the largest bat colony in South Africa, reaching numbers as high as 300 000 individuals (McDonald *et al.,* 1990). Another African example of high population density can be that of *Eidolon helvum,* for which a massive non-breeding colony of approximately 1.5 million individuals have been reported at the Kasanka National Park in Zambia (Sorensen & Halberg, 2001). Colony size has also been shown to vary in specific areas depending on season. Consider *Rousettus aegyptiacus* in the Mission Rocks cave in St. Lucia (South Africa) where colony size drops from over 5000 individuals in colder seasons to a mere 300 in summer (Monadjem *et al.,* 2010). Virus transmission is inevitable under conditions where bats roost in such close proximity. Transmission through aerosols, respiratory droplets and other body fluids cannot be avoided in such an environment.

Different bat colonies have been known to roost together in the same caves. Several examples of this have been reported. In the De Hoop Guano Cave in the western region of South Africa, a vast number of bat species have been recorded to roost together (McDonald *et al.,* 1990). Species include *Myotis tricolor, Miniopterus natalensis, Nycteris thebaica* and *Rhinolophus clivosus*. Roost selection of these bats differs in terms of the humidity, temperature, light intensity and even the height above the cave floor. Interspecies transmission of viruses is thus also possible in these situations. Other roosting habits can also influence transmission of viruses and even spill-over events into human and animal hosts. Bats that roost in the attic of some houses (Drexler *et al.,* 2011), or in trees (Chong *et al.,* 2008) come into close contact with humans and other animals. When bats colonize

houses, humans are more likely to come into contact with their faeces and urine and the chances of being scratched or bitten are also increased. This contributes greatly to the zoonotic potential of bat-associated viruses as many zoonotic pathogens have been detected in the faeces and urine of bats (**Table 1.1**). Bats roosting in trees pose equal opportunity for disease spill-over as they contaminate fruit with urine and faeces. It was hypothesised that contaminated fruit dropping to the ground could have been consumed by scavenging animals, resembling the situation where Nipah virus spilled-over into the pig population. Nipah virus was successfully isolated from swabs taken from partially eaten fruit which supports the hypothesis (Chong *et al.,* 2003).

1.1.3.5 Bat immune system

Allen *et al.* (2009) were able to show that the roosting ecology of *Tadaria brasiliensis* has a profound impact on their immune function. This might well be the case in other bat species. It is hypothesized that bats have a specialized immune system to allow viral-persistence. Although bat-associated viral persistence has not been widely studied, some viral pathogens, for example rabies virus, have been studied with regards to this (Kuzmin *et al.,* 2011). Increased seroprevalence to rabies virus has been observed in female adults and juvenile bats following parturition (Steece & Altenbach, 1989). Steece and Altenbach (1989) performed a study on adult female and juvenile Mexican free-tailed bats. During the study they examined the prevalence of active rabies infection and the immune status of the bats afterwards. With these results it was hypothesised that observed seasonal shift after birthing season may be due to the increased number of susceptible juveniles introduced into the colony as well as regular close contact during feeding. This ensures maintenance of the virus within the colony.

Bats have shown to be able to maintain the majority of viruses asymptomatically, which could indicate the more complex nature of their immune system. Even though there is limited information available regarding the bat immune system, it is clear that they possess certain immunological characteristics allowing them to maintain a virus without being susceptible to infection. Based on work done regarding the maintenance of Hendra virus in its *Pteropus scapulatus* reservoir host, the possibility that the reservoirs immunity wanes over short time periods have been suggested (Plowright *et al.,* 2008). This then possibly supports the persistence of the virus within the population. Another factor that could affect the immunity of an individual is nutritional stress (Plowright *et al.,* 2008). This

has been associated with a risk of increased transmission which could be as a result of a compromised immune system during stress.

A recent study by Zhang *et al.* (2013) compared the genomes of two different bat species, *Pteropus alecto* and *Myotis davidii*, representing the two Chiropteran sub-orders Yinpterochiroptera and Yangochiroptera to gain insight into flight and immunity. They hypothesized that long-term co-evolution took place between bats and viruses. After examining genes involved in the innate immune system of bats, they found that mechanisms of DNA damage response changed during positive selection for flight and could have played a role in the bat immune system adapting to maintain viral pathogens. This corresponds to previous findings that the DNA damage response mechanisms are crucial in the host defences (Turnell & Grand, 2012). Components of this mechanism falls target to virus interaction where they can be selectively activated or repressed. Research regarding bat immunology and virus interaction is now being intensified.

1.1.4 Drivers of bat-associated viral zoonoses

One of the reasons behind emerging infectious disease spill-overs from bats is changes in the ecology of the host (Kuzmin *et al.,* 2011). Kuzmin *et al.* (2011) summarized the drivers involved in this emergence. These drivers were classified as either primary or secondary. Primary drivers include environmental degradation, overpopulation and socioeconomic forces. Drivers considered as secondary, thus as a result of primary drivers, include factory farming, habitat intrusions (encroachment), consumption of bushmeat and the rapid transport of people and animal products.

It is no surprise that increasingly more diseases are spilling over from bats into other hosts due to human activities and encroachment driven by human overpopulation in several countries across the globe. The natural habitat of these animals are being invaded and destroyed due to anthropogenic deforestation for agricultural intensification (Pulliam *et al.,* 2012). An example of this is the study of Chua *et al.* (2002) that suggested the reduction in fruiting forest trees, led to the unexpected invasion of frugivorous bats into established fruit orchards. In the case of Nipah virus this was considered part of the initial transmission chain were urine-contaminated fruit was eaten by pigs scavenging for food on a piggery situated within fruit orchards. This is just one of the many examples of how agricultural and hunting activities of humans led to an increased exposure to this virus-rich order of mammals and to the emergence of deadly viruses.

Bat bushmeat forms an important source of protein in the diets of many under developed countries including Ghana (West Africa), Madagascar and other Old world countries (Jenkins & Racey, 2008; Mickleburgh *et al.,* 2009; Kamins *et al.,* 2011). In Ghana, *Eidolon helvum* is very important in the commodity chain of bushmeat and is sold over a wide geographical range and by multiple vendors (Kamins *et al.,* 2011). In Madagascar, the main bats of choice are *Eidolon dupreanum, Rousettus madagascariensis* and *Hipposideros commersoni* (Jankins & Racey, 2008). Not only is the trade of bat bushmeat a threat to the biodiversity of bats, but also a huge opportunity for possible disease emergence (Kamins *et al.,* 2011).

The majority of bat species being sold as a food commodity have been identified as hosts for several viral pathogens not to mention the yet undiscovered viruses (**Table 1.1**). Contact with raw bat bushmeat and the consumption of undercooked bat meat are ideal opportunities for the spill-over of these viruses into the human or animal population, especially in underdeveloped countries where bat meat is the main source of protein (Weiss *et al.,* 2012). In both underdeveloped and developed countries, the emergence of a novel infectious disease can be devastating and lead to an epidemic of a huge magnitude as the control of novel pathogens is difficult since no treatment or cures will be available and knowledge regarding these pathogens will be lacking. The human population thus clearly contributes towards the emergence and spread of infectious diseases through their hunting and agricultural practices.

1.1.5 Bats on the African continent

It is known that bats have a worldwide distribution and that they have been linked to over a hundred different viruses. In southern Africa alone, more than a hundred species of bats can be found (Monadjem *et al.,* 2010). Several of these bats have been linked to viral diseases over the past few decades. One such virus is Lagos bat virus isolated from bats in South Africa (Markotter *et al.,* 2006). Lagos bat virus is a *Lyssavirus* that belongs to the family *Rhabdoviridae* order *Mononegavirales*, the same order to which the *Paramyxoviridae* family belongs.

It is said that more tropical regions have richer bat species diversity when compared to temperate and arid regions. Climate on the African continent is representative of all three climate types as illustrated in **Figure 1.3** (Peel *et al.,* 2007). Despite the different climate regions on the African continent, it still has high species diversity with bats occurring

widespread. The Northern parts of Africa are considered arid and are home to *Rhinopoma cystops* occurring only in this region and some parts of central Africa (**Table 1.2**).

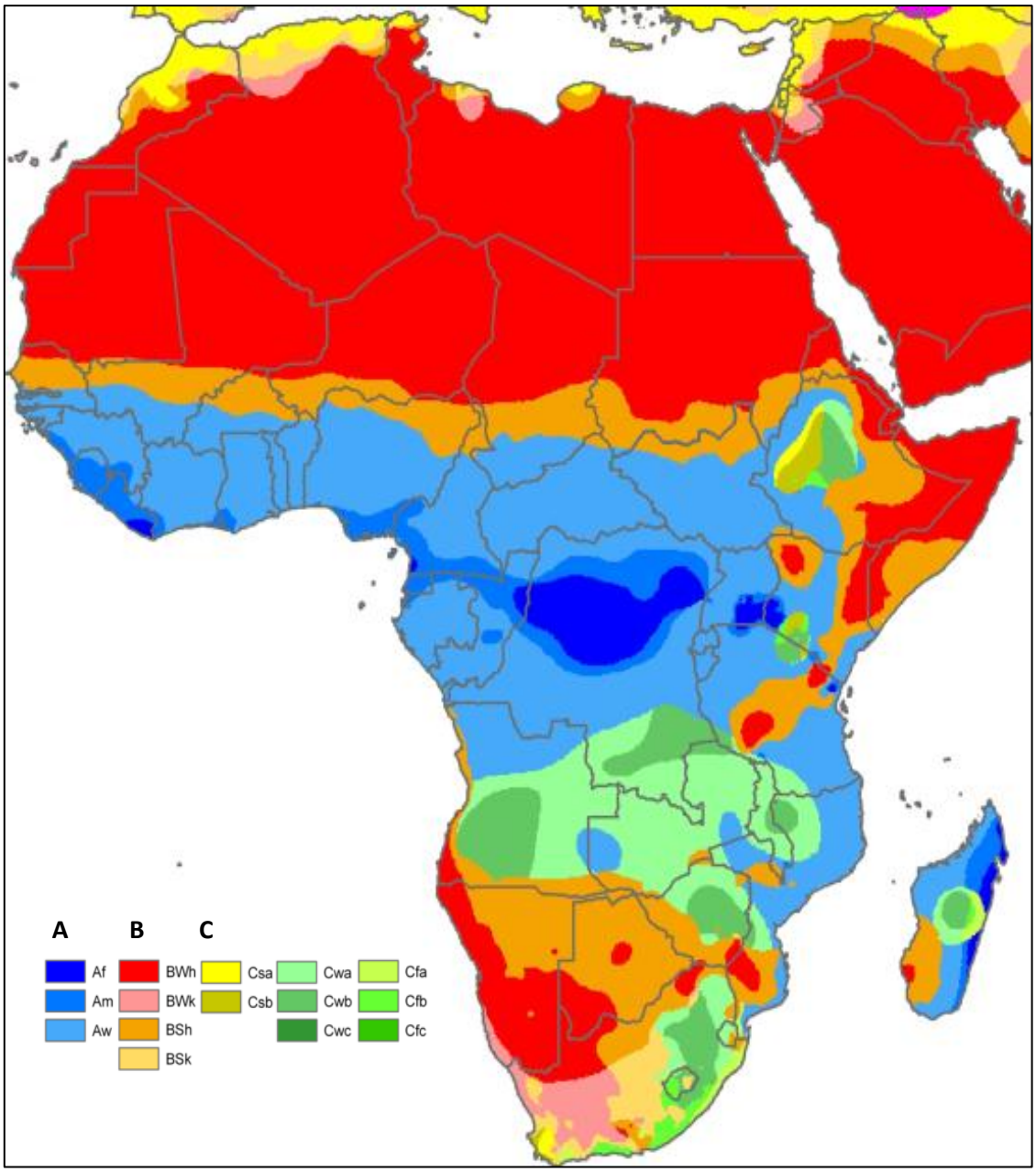


Figure 1.3: Geographical representation of climate sectors on the African continent (Peel *et al.,* 2007). Grouping A: Tropical (blue) with f: rainforest, m: monsoon and w: savannah. Grouping B: Arid (red/orange) with W: desert, S: steppe, h: hot and k: cold. Grouping C: Temperate (green/yellow) with s: dry summer, w: dry winter, f: without dry season, a: hot summer, b: warm summer and c: cold summer.

Table 1.2: Chiropteran families occurring on the African continent with a description of an example species.

Family	Species example		Distribution in African	Roosting	Colony size
	Scientific	Common name			
Emballonuridae	*Coleura afra*	African sheath-tailed bat	Eastern and Western African regions	Caves	Hundreds to thousands
Hipposideridae	*Hipposideros caffer*	Sundevall's leaf-nosed bat	Sub-Saharan Africa but absent from the most of South Africa	Caves, sinkholes	Tens to hundreds
Megadermatidae	*Lavia frons*	Yellow-winged bat	Tropical savannahs across equatorial Africa	Shrubs and trees	Singly
Miniopteridae	*Miniopterus natalensis*	Natal long-fingered bat	Sub-Saharan Africa	Cave	Thousands
Molossidae	*Mops condylurus*	Angolan free-tailed bat	Sub-Saharan Africa	Caves, hollows in trees	Tens to thousands
Myzopodidae	*Myzopoda aurita*	Old World Sucker-footed Bat	Madagascar	Trees	Tens to fifties
Nycteridae	*Nycteris thebaica*	Egyptian slit-faced bat	Throughout the African continent except the Sahara desert	Caves	Several hundreds
Pteropodidae	*Rousettus aegyptiacus*	Egyptian rousette	Sub-Saharan Africa(mostly towards the outer boundaries of the continent)	Caves	Hundreds to thousands
Rhinolophidae	*Rhinolophus darlingi*	Darling's horseshoe bat	Southern Africa, Nigeria and Tanzania	Caves, mine adits	Singly and small groups up to 160
Rhinopomatidae	*Rhinopoma cystops*	Lesser mouse-tailed bat	Northern and Central Africa	Caves, tunnels	Ones to several hundreds
Vespertilionidae	*Scotophilus dinganii*	Yellow house bat	Sub-Saharan Africa	Trees, roofs of buildings	Up to twelve individuals

* Simmons 2005;
* The IUCN Red List Of Threatened Species™ (http://www.iucnredlist.org);
* African Chiroptera report 2011. African Chiroptera Project, Pretoria: i-xvii; 1-4474 (http://www.Africanbats.org).

Central- and partly Western Africa as well as Madagascar have a more tropical climate with *Eidolon helvum, Lavia frons* and *Tadaria condylura* among others species occurring there. Southern Africa consists mainly of arid and temperate regions. Bat species occurring in southern Africa, for example *Epomophorus wahlbergi, Rousettus aegyptiacus, Scotophilus nigrita* and *Hipposideros caffer* have been reviewed by Monadjem *et al.* (2010) in a publication titled 'Bats of Southern and Central Africa'. They report that southern Africa has a rich diversity of bats. In total, a 116 species belonging to nine bat families are known to occur in this part of the African continent, a vast amount of which have shown to be possible reservoir hosts for a number of viruses (**Table 1.1)**.

Species belonging to 11 of the 19 bat families occur on the African continent (African Chiroptera Report 2011). **Table 1.2** summarises a representative for each family showing the diversity in habitat and ecology between different species. As mentioned in section ***1.1.3.4***, population size and roosting behaviour varies significantly between bat species. Some bat species for example *Lavia frons* roost mainly as individuals while *Rousettus aegyptiacus* for example reaches number in the thousands such as in Mission Rock Caves, St. Lucia and caves in Tzaneen in South Africa. *Rhinolophus landeri* can be considered as an intermediate example roosting in caves, and hollow trees with colonies ranging from very few to a mere few dozen.

Jones *et al.* (2008) hypothesized that zoonoses from wildlife animals are correlated to their biodiversity. Their analysis suggested that by reducing anthropogenic activity in areas rich in wildlife biodiversity, the emergence of future zoonotic diseases can also be significantly reduced. As mentioned previously, tropical areas are considerably richer in bat biodiversity than more temperate and arid regions. According to the analysis conducted by Jones *et al.* (2008), tropical Africa is a potential hotspot for disease emergence from bats and other wildlife animals.

1.2 PARAMYXOVIRUSES

The paramyxoviruses are a large group of diverse viruses including several important human and animal pathogens. These include measles-, mumps- and canine distemper viruses. These viruses belong to the family *Paramyxoviridae* which is phylogenetically divided into two sub-families named *Paramyxovirinae* and *Pneumovirinae* (Barclay & Paton, 2000). Viruses belonging to this family are known to infect a wide host range including mammalian (humans, bats, and cattle), avian (chickens, wild birds) and

reptilian (lizard, snake tortoise) hosts (Tong *et al.,* 2008; Virtue *et al.,* 2009) in both terrestrial and aquatic environments.

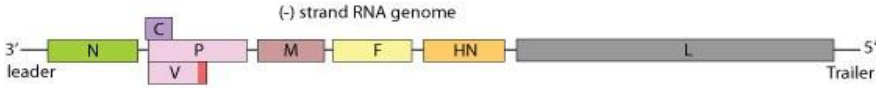
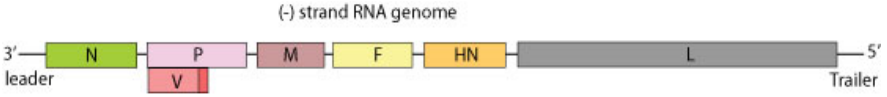
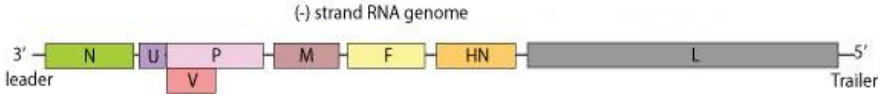
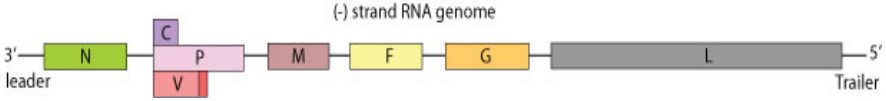
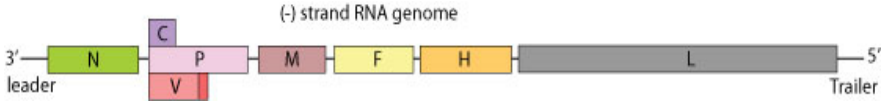
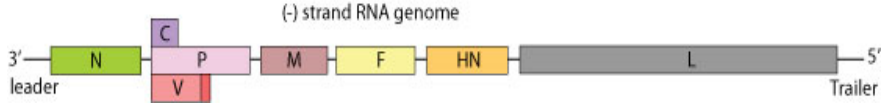
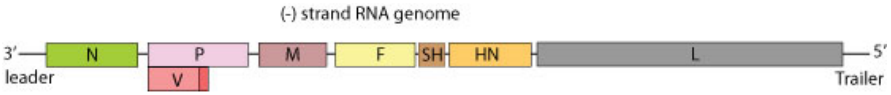
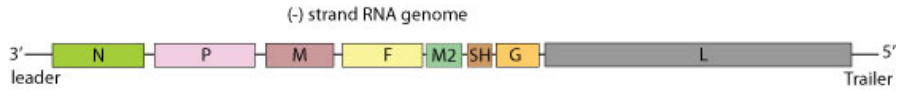
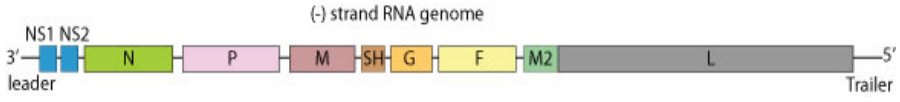
1.2.1 Structure, genome organization and taxonomy

The basic virion structure of paramyxoviruses is irregular and can vary between spherical, filamentous and pleomorphic in shape (Finch & Gibbs, 1970; Barclay & Paton, 2000). Spike-like protrusions visible on the surface of the enveloped virion are formed by the fusion and attachment proteins. Within the capsids, these viruses contain a negative sense single stranded non-segmented RNA genome of approximately 15 to 19kb in size (Morrison, 2003; Bowden & Boyle, 2005). This linear genome contains five to ten genes transcribed by its viral RNA polymerase (Cordey & Roux, 2007). These genes encode several proteins with the basic gene sequence being conserved among members of this family due to transcriptional polarity. The sequence of these genes is the nucleocapsid, phosphoprotein, matrix, fusion, attachment and the large polymerase gene at the 5' end of the genome (**Table 1.3**).

During earlier classification, all paramyxoviruses were grouped together under the *Paramyxoviridae* family. Further research showed significant differences in genetic organization, certain biological properties as well as evolving morphology (Virtue *et al.,* 2009). As previously mentioned, this family is presently subdivided into two sub-families namely *Pneumovirinae* and *Paramyxovirinae.* The former consists of only two genera *Pneumovirus* and *Metapneumovirus* while the latter has seven: *Avula-, Rubula-, Respiro-, Morbilli-, Henipa-, Aquaparamyxo-* and *Ferlavirus* (**Table 1.3**). Another genus has been proposed for this sub-family namely *Jeilongvirus* containing the currently unclassified rodent paramyxoviruses J- and Beilong virus (Li *et al.,* 2006). This genus has however not yet been officially recognised in paramyxovirus classification.

Novel virus classification is initially based on morphology, as discussed above, after which the virus is placed into the *Paramyxoviridae* family. Further classification is based on genetic data. Sequencing of a partial gene and comparison of this fragment with previously identified paramyxoviruses can help determine to which genus the virus might belong but full genome sequencing and sequence comparison and analysis of genes will be required before it is officially placed within a genus or a new species can be named.

Table 1.3: Genome composition and organization of all genera in the *Paramyxoviridae* family.

Sub family	Genus	Genome organization and composition *	Host range	Reference strain	
				Species	Genome size
Paramyxovirinae	***Aquaparamyxovirus***		Fish	Aquatic salmon paramyxovirus	16968 bp
	Avulavirus		Wild birds, chickens, turkey	Newcastle disease virus	15 186 bp
	Ferlavirus		Reptiles	Fer-de-lance virus	15378 bp
	Henipavirus		**Bats**, **humans**, horses, pigs	Hendra virus	18 234 bp
	Morbillivirus		**Humans**, non-human primates, dolphins, dogs, cattle, sheep	Measles virus	15 894 bp
	Respirovirus		Cattle, **humans**, rodents	Sendai virus	15 384 bp
	Rubulavirus		Simians, **humans**, **bats**, pigs, dogs	Mumps virus	15 384 bp
Pneumovirinae	***Metapneumovirus***		**Humans**, birds	Avian metapneumovirus	14 071 bp
	Pneumovirus		**Humans**, cattle, rodents	Human respiratory syncytial virus	15 225 bp

* Illustrations were obtained from ViralZone:www.expasy.org/viralzone, Swiss Institute of Bioinformatics. (Hulo *et al.*, 2010) *[No licencing required]*
Gene proteins encoded: N- nucleoprotein, P- phosphoprotein, M- matrix protein, F- fusion protein, HN- hemagglutinin-neuraminidase, L- RNA polymerase, G- glycoprotein, SH- small hydrophobic protein, M_2- trans-membrane protein, NS1/2 - non-structural proteins.

1.2.2 *Pneumovirinae*

Pneumoviruses (genus *Pneumovirus*) are known to mainly infect humans, cattle and rodents. These viruses have a worldwide distribution with transmission taking place through the respiratory route. Examples of viruses belonging to this genus are human respiratory syncytial virus (RSV) also the type virus in this genus, human metapneumovirus (hMPV) and bovine metapneumovirus (bMPV) (Van den Hoogen *et* al., 2001; International Committee on Taxonomy of Viruses (ICTV) Master species list 2011 available online from http://talk.ictvonline.org/files/ictv_documents/default.aspx). Metapneumoviruses (genus *Metapneumovirus*) also have a worldwide distribution with their transmission taking place through direct contact with airborne droplets from the nose or throat. Avian- and human metapneumoviruses are well known examples of this genus. The sub-family *Pneumovirinae* was created to contain these two genera due to the lack of hemagglutinin and neuraminidase activity of these viruses (Pitt *et al.,* 2000). One morphological difference that also separates the two sub-families is the difference in the structural characteristics of the attachment protein. This sub-family causes respiratory tract diseases, and therefore can be isolated from the respiratory tract.

1.2.3 *Paramyxovirinae*

Avulavirus of the sub-family *Paramyxovirinae* is represented by New-castle disease virus. The natural host for avulaviruses are birds. Morbilliviruses on the other hand are mainly found in human, canid and bovine hosts with the most instantly recognizable member being measles virus (Rima & Duprex, 2006). Sendai virus is the type virus for the *Respirovirus* genus. Its natural host is rodents but humans can also be infected. The fourth genus, *Rubulavirus*, is known to have a wide host range including humans, apes, dogs and pigs. Well known mumps virus belongs to this genus. This virus does not have an animal reservoir indicating that it has entirely adapted to its human hosts (Virtue *et al.,* 2009). The most recent addition to this sub-family of viruses is the *Henipavirus* genus. Currently only two viruses officially belong to this genus namely Hendra- and Nipah virus while a new member, Cedar virus, was recently proposed (Marsh *et al.,* 2012). Hendra and Nipah viruses have caused death in animal as well as high mortality rates in human. Most of the previously identified viruses within the *Paramyxovirinae* sub-family are transmitted through the respiratory route allowing easy spread of the disease through animal and human populations (Kirkland *et al.,* 2001). Evidence of the more recently detected

paramyxoviruses has been found in faecal and urine of bats which allow for other routes of infection.

1.2.4 Paramyxoviruses of bats

Of the long list of viruses in the *Paramyxoviridae* family, only a few have been discovered from chiropteran reservoirs up until a few years ago. Some of these viruses are known zoonotic pathogens and are seen as major public health threats. The most recent members to this group are the henipaviruses that have caused deaths in animals and humans. Although the group of previously identified bat associated viruses is small, they have drawn much attention over the last few years making them one of the focus areas in research.

1.2.4.1 Bat parainfluenza virus (1966 – Rubulavirus)

A 1964 ecological investigation regarding different arboviruses on a stud farm near Poona, India (Pavri *et al.,* 1971) and a preceding rabies outbreak on the same farm in 1962 (Pavri *et al.,* 1964) led to the inclusion of bats in their surveillance due to their known association with these viruses. No isolation of either group of virus was made from the collected bat samples. However, a frugivorous bat *Rousettus leschenaulti* harboured antibodies to Kyasanur Forest disease (KFD) virus, a then novel flavivirus (Pavri *et al.,* 1971). Subsequently the sampling of this particular bat species was increased.

This chain of events that started in 1962, eventually led to the first recorded isolation of a paramyxovirus from a chiropteran host in 1966 (Hollinger & Pavri, 1971). The virus was isolated from a suspension of pooled spleen, brain and salivary glands obtained from *R. leschenaulti.* Several methods including hemagglutination inhibition and neutralization tests were employed for characterization. These tests then concluded that this virus was indeed a new parainfluenza virus strain.

In a small survey of 200 human sera samples, 10% showed antibodies against bat parainfluenza virus (Pavri *et al.,* 1971; Breed, 2008). The answer as to whether the virus was transmitted to humans remains unclear, as the observed antibodies in the human sera might have been the result of serological cross-reaction. There has been no report of human disease as a result of this virus. No further research regarding bat-parainfluenza virus have been published to date.

1.2.4.2 Mapuera virus (1979 - Rubulavirus)

In 1979 a second novel paramyxovirus was discovered in a chiropteran host (Henderson *et al.,* 1995). The virus was isolated from the salivary glands of *Sturnira lilium*, a fruit bat caught in a Brazilian rainforest. No sign of disease was observed in the bat and it was presumed to be otherwise healthy. Initially it was hypothesized that the virus might be an arbovirus but subsequent electron microscopic research conducted by Zeller *et al.* (1989) placed this virus in the *Paramyxoviridae* family of viruses. The virus was eventually termed Mapuera virus in correlation to the area where it was first isolated.

Further classification of this virus was done through the analysis of protein expression patterns and the nucleoprotein gene sequence (Zeller *et al.,* 1989). Results concluded that this virus belonged to the *Rubulavirus* genus. Other known viruses in this genus include mumps virus and human parainfluenza virus 2 and 4. The disease causing potential of Mapuera virus in humans remains unknown (Hagmaier *et al.,* 2007) although intracranial infections of mice performed by Zeller *et al.* (1989), proved to be fatal. The host range and disease causing potential of this virus still remains unknown after all these years as this virus is not widely studied like fellow members in this family (Wang *et al.,* 2007).

Another virus termed porcine rubulavirus was identified as the causal agent of a disease outbreak in pigs during the early 1980's in Mexico (Wang *et al*., 2007). Upon genome comparison of this virus to other paramyxoviruses, it showed a closer relatedness to Mapuera virus than to any other member of the *Rubulavirus* genus. The reservoir host for this virus remains elusive. It is hypothesized that porcine rubulavirus might also originate from a chiropteran host just as its closest relative. The evidence to support this hypothesis is still lacking.

1.2.4.3 Hendra virus (1994 – Henipavirus)

Hendra virus, a novel paramyxovirus belonging to the *Henipavirus* genus, was first documented in September of 1994 following an outbreak of a brief but severe and fatal respiratory disease (Wang *et al.,* 1998). This outbreak took place in Hendra, a suburb of Brisbane, Australia and claimed the lives of thirteen horses and one human (Williamson *et al.,* 1998). For the time being, the virus was named equine morbillivirus but subsequent studies determined that this virus could not easily be classified into the existing genera of the *Paramyxovirinae* subfamily and as a result was renamed to Hendra virus. This name correlates to the suburb in Brisbane in which the initial outbreak occurred.

Findings of subsequent serological studies revealed that Australian fruit bats of the *Pteropus* genus, also known as flying foxes, had high antibody titres to Hendra virus. Antibodies to this virus were not found in any other animal species whilst virus isolation from bat urine consequently implicated them as the natural wildlife reservoir for the virus (Halpin *et al.,* 2000). Up until a few years ago, Hendra virus was found to only infect bats, horses and humans. Early 2011, during a seasonal Hendra virus outbreak in Australia, domestic animals in contact with the infected horses were also tested. A dog living on the same farm as the infected horses tested positive in a serological assay. The dog was subsequently euthanized although no disease symptoms were present (Australian Veterinary Association available online at http://www.ava.com.au/hendra-virus). This was the first report of evidence of natural Hendra virus infection in domestic animals. The epidemiology and pathogenesis of Hendra virus will be discussed in section ***1.2.6.1*** as an example of bat-associated paramyxoviruses.

1.2.4.4 Menangle virus (1997 – Rubulavirus)

In 1997 a piggery in New South Wales (Australia) suffered from to a disease outbreak resulting in stillborn and deformed piglets (Philbey *et al.,* 1998). A novel paramyxovirus, now termed Menangle virus, was soon after isolated from the stillborn piglets. The offspring presented with abnormalities of the brain, spinal cord and skeleton. In some cases the central nervous system was completely undeveloped. No disease was observed in postnatal pigs of any age (Halpin *et al.,* 1999). Philbey *et al.* (1998) were able to amplify the virus from several piglet organ tissues in BHK21 (baby hamster kidney) cells. Based on morphological analysis of viral particles with the use of electron microscopy, the virus was classified as a member of the *Paramyxoviridae* virus family.

During the initial disease outbreak, researchers sampled several animals that could possibly be the reservoir for the virus. Among these were rodents, cattle, sheep, cats and a dog (Philbey *et al.,* 1998). Bats were also considered as reservoir based on the previous evidence that they serve as reservoir for Hendra virus (Halpin *et al.,* 2000). Of these animals, Pteropid bats were the only animals that were seropositive during serological testing. Bat species from which positive results were obtained included *Pteropus poliocephalus, P. alecto* and *P. conspicillatus*. Based on this, and the fact that there was a Pteropid colony established approximately 200m from the affected piggery, it was hypothesized that bats are indeed the reservoir (Philbey *et al.,* 1998). With only serological

evidence implicating Pteropid bats as reservoir, Philbey *et al.* (2008) further examined flying foxes hoping to find evidence of infection. Paramyxovirus-like particles were observed in faecal samples after electron microscopic examination. Reactivity of pooled faecal and urine samples to sera derived from sows exposed to Menangle virus was also observed. Attempts to isolate the virus were however unsuccessful. Recently, Barr *et al.* (2012) was able to provide evidence of a bat origin for Menangle virus after this virus was successfully isolated from the urine of *Pteropus alecto* (black flying fox).

Following the emergence of Menangle virus in pigs, an investigation was launched into the public health risk posed by this virus (Chant *et al.,* 1998). A serological assay was developed to enable testing of animal and human sera for neutralizing antibodies. Approximately 250 human serum samples were analysed of which only two were seropositive with titres of 128 and 512 (Chant *et al.,* 1998; Philbey *et al.,* 1998). Both individuals reported a disease onset within days after coming into contact with the infected animals. The illness was characterized by fever, rash, malaise, sweats, back pain and severe headache (Chant *et al.,* 1998). Weight loss was also recorded in both cases with the illness lasting for about a two week period.

Further classification of Menangle virus was done in 2000 with the use of molecular methods. Bowden *et al.* (2001) obtained nucleotide sequence of Menangle virus and compared this to other members of the *Paramyxovirinae* family. Results indicated that it grouped with viruses in the *Rubulavirus* genus, the same genus to which two other bat associated paramyxovirus belong. It was only in 2005 that the full-genome sequence of this virus was completed and released (Bowden & Boyle, 2005). With this, they were able to confirm the taxonomic classification of this virus into the *Rubulavirus* genus.

1.2.4.5 Nipah virus (1997/8 – Henipavirus)

Nipah virus, yet another novel paramyxovirus, initially emerged in pigs during an outbreak in 1997/8 after which it spilled over into the human population in 1999 in Sungei Nipah village, Malaysia (Chua, 2002). The virus was isolated from cerebrospinal fluid of a fatal human case of encephalitis. It was subsequently linked to the 1998 encephalitis outbreak that started in Malaysia (Chua *et al.,* 2000). During this outbreak, 268 human cases were reported with 105 fatalities and the swine industry suffered a devastating blow. Pigs in these areas developed a respiratory illness and it was attributed to the mosquito-borne viral disease, Japanese encephalitis (JE). JE was also enzootic in the region and

pigs were among the amplified vertebrate hosts. Vaccination against JE and mosquito-control did not appear to affect the outbreak.

Surveillance of wildlife in the area was done in an attempt to discover the natural reservoir. Neutralizing antibodies to Nipah virus were found in several bat species (Lo & Rota, 2008). Searches were expanded to Tioman Island just off the coast of Malaysia to target an island Pteropid bat roosting site. A rate of positivity of 31% in *Pteropus hypomelanus* and 17% for *P. vampyrus* was detected (Johara *et al.* 2001). Multiple isolates of at least two different paramyxoviruses were obtained. One virus was found to be identical to Nipah virus, and the other antigenically related to Menangle virus. The latter virus was subsequently characterized and termed Tioman virus (Chua *et al.,* 2002).

Chong *et al.* (2003) reported that the risk of humans contracting an infection directly from bats is low. This conclusion was made after a survey was done on 8% of the adult population of Tioman Island. This however is not true as evidence of transmission directly from bats to humans was reported in Bangladesh (Luby *et al.,* 2006). During the fruiting season in Bangladesh, young boys would pick fruit from the trees and the fruit was sold in the village. Outbreaks of Nipah virus followed the trail of these salesmen. The way in which pigs initially contracted the disease is believed to be similar through the ingestion of fruit contaminated with viral particles.

Since the emergence of Nipah virus in Malaysia, it has been a recurring threat to human health in Southeast Asia (Lo & Rota, 2008). Hendra- and Nipah virus will be used as examples for the discussion on epidemiology and pathogenesis of bat-associated paramyxoviruses in section ***1.2.6.1***.

1.2.4.6 Tioman virus (2000 – Rubulavirus)

As mentioned above, Tioman virus was only discovered by accident in search for the reservoir host of Nipah virus (Chua *et al.,* 2002). Tioman virus was first isolated from flying foxes on Tioman Island in 2001 (Yaiw *et al.,* 2007). Whole genome characterization placed this virus in the *Rubulavirus* genus of the *Paramyxoviridae* family. Based on molecular and antigenic studies, Tioman virus showed the closest relationship to Menangle virus with an overall 62% similarity at nucleotide level.

As previously mentioned, Menangle virus has been associated with disease outbreaks in pigs and humans, but in the case of Tioman virus it has not been found (Yaiw *et al.,* 2007). Seropositivity found in three inhabitants of Tioman Island, suggest that this

virus or a similar one had infected these humans. Based on these results, Tioman virus is considered to be potentially infective and may cause disease in humans. Surveillance and more in depth research into the pathogenicity of this virus will thus be needed.

1.2.5 Bat paramyxoviruses on the African continent

Pathogenic bat-associated paramyxoviruses (Hendra-, Menangle- and Nipah virus) were not believed to be circulating on the African continent as their distribution was assumed to be limited to that of their Pteropid bat reservoirs. The first report of henipaviruses in Africa was based on serological evidence (Hayman *et al.,* 2008). Hayman *et al.* (2008) tested *Eidolon helvum,* a straw coloured fruit bat found throughout central and southern parts of Africa, as well as a few other fruit-eating bat species including *Epomophorus gambianus, Hypsignathus monstrosus* and *Epomops franqueti*. They reported serological evidence to henipaviruses in these fruit-bats in Ghana, West Africa. *E. helvum* was highly positive for both Hendra- and Nipah virus antibodies (39% for henipaviruses, 39% for Nipah virus and 22% for Hendra virus). *E. gambianus* and *H. monstrosus* however, showed low seroprevalence of 1% and 6% respectively when compared to *E. helvum*. The closest pteropid bat population to the sampling colony in Ghana is approximately 4500km away (Mafia Island, *Pteropus comorensis*). Several research groups took interest in this new development in paramyxovirus ecology. Soon after Drexler *et al.* (2009) also targeted *E. helvum* bats in Ghana with the hope of detecting viral RNA. In the end they amplified RNA of three putative henipaviruses from faecal material of these bats. This was the first viral genomic evidence of these viruses on the African continent.

Based on their previous findings and that of Drexler *et al.* (2009), Hayman *et al.* (2011) hypothesized that domestic animals species in Africa may also have been exposed to *Henipavirus* infection. To test this hypothesis they serologically screened a selection of domestic animals residing in the vicinity of a large *E. helvum* colony in Accra, Ghana. Animals sampled were cats, dogs, sheep, goats and pigs. The largest sample size was derived from pigs making up approximately 77% of serum samples. Their results suggested that the pig population was previously exposed to *Henipavirus*-like viruses as one pig serum sample tested positive for the N protein of Nipah virus in a western blot analysis.

E. helvum is a migratory bat that travels great distances during seasonal changes and thus comes into contact with numerous other bat populations of other genera (Ossa *et al.,* 2012). The transmission of paramyxoviruses to these other bat populations can thus not be excluded. The most southern population of *E. helvum* is found in Maputo, Mozambique. Sporadic appearances of this bat species in the lower southern Africa is believed to be as a result of changes in food supply (Richter & Cunning, 2008) and possibly climate change due to global warming. Further surveillance of *E. helvum* and other bats species of southern Africa should become priority. This will allow the establishment of a wider picture as to the distribution of these viruses and their natural reservoirs on the African continent.

Several bat species in South Africa might prove to be reservoirs for paramyxoviruses due to their relatedness to other bat species that have been associated with these viruses. Some of these bats include *Hipposideros commersoni, H. caffer, Scotophilus nigrita, S. dinganii, S. viridis,* and *Rousettus aegyptiacus.* One can also target closely related genera in the search for reservoir species for example *Epomophorus* which belongs to the same family as the Pteropid reservoir bats (refer to **Figure 1.2**).

The recent detection of henipavirus-related viruses on the African continent, led to an increased interest in these viruses whether it's regarding their geographical distribution, genetic diversity, biodiversity or tissue tropism. *Eidolon helvum* formed the target species of a study conducted by Baker *et al.* (2012) in Accra, Ghana. Urine samples were collected and pooled samples were screened for the presence novel paramyxoviruses. In total, 72 samples were screened of which 31 tested positive (43% positivity). Nucleotide identity of these novel sequences ranged from 57-89% when compared to previously identified paramyxovirus sequences. Notably, some of the novel sequences clustered with mumps virus, a known human pathogen. Several other sequences seemed to be related to known genera, but still formed a separate grouping. These results contributed substantially to the paramyxovirus phylogeny, as the vast number of sequences added indicated the genetic diversity of these viruses in a single species of bats. Previously detected bat paramyxoviruses only belonged to the *Henipa*- and *Rubulavirus* genera. It is also now shown that viruses were grouping with another "unclassified" genus close to the proposed *Jeilongvirus* genus.

The diversity shown by Baker *et al.* (2012) can now be considered as only the tip of the proverbial iceberg. The most recent publication on paramyxovirus diversity in African

bat species by Drexler *et al.* (2012) expands the number of novel putative paramyxovirus species drastically. Here, they report the detection of approximately 66 novel paramyxoviruses based on amino acid similarity between previously and more recently identified sequences. A comparative standard was set by determining the amino acid difference between Hendra and Nipah virus and using this as a guideline for putative species determination. Although this study also targeted *E. helvum*, they took it a few steps further. Unlike the previously mentioned study, they expanded their geographical range of sampling as well as target species. A total of 86 bat species (4954 individuals) were sampled from several African countries as well as some localities in Central America and Europe. African countries sampled included Ghana, Gabon, Congo, Democratic Republic of the Congo (DRC) and Central African Republic (RCA).

Several major discoveries were made during this study i.e. evidence of a possible origin of henipaviruses on the African continent, detection of a virus conspecific to mumps virus and relatives to canine distemper- and respiratory syncytial virus. Full genome sequences from two representative viruses were determined. The first, BatPV/Eid_hel/GH-M74a/GHA/2009 isolated from the spleen of an *Eidolon helvum* bat (GenBank accession number HQ660129) was sequenced as it was closely related to the henipaviruses, the second virus closely related to human mumps virus was isolated from the spleen of an *Epomophorus* sp. (BatPV/Epo_spe/218-ARI/DRC/2009; GenBank accession number HQ660095). These two sequences are the first full genomes available of African bat associated paramyxoviruses. Representatives of paramyxoviruses detected in bats from 2010 to 2012 are summarized in **Table 1.4**.

The majority of henipavirus-related viruses were isolated from bat species belonging to the *Pteropodidae* family (**Figure 1.1 & 1.2**), the same sub-order to which the Pteropid bats, known reservoirs for the henipaviruses, belong. Among these are *Eidolon, Rousettus,* and *Epomophorus.* This shows the diverse nature of paramyxoviruses to be capable of maintenance in not only different bat species but also between genera.

Table 1.4: Representative viruses selected from novel paramyxoviruses detected in bats between 2010 and 2012.

Genus	Virus	Associated bat species	First detection		Reference	Accession number
			Year	Location		
Henipavirus-related	Cedar virus	*Pteropus* sp.	2009	Queensland, Australia	Marsh *et al.*, 2012	JQ001776.1
	BatPV/Eid_hel/GH-M74a/GHA/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	HQ660129.1
	BatPV/Eid_hel/GH-M28/GHA/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	HQ660147.1
	BatPV/Rou_aeg/GB2009/GAB/2005	*Rousettus aegyptiacus*	2005	Gabon, Africa	Drexler *et al.*, 2012	HQ660145.1
	BatPV/Eid_hel/GB1535/GAB/2005	*Eidolon helvum*	2005	Gabon, Africa	Drexler *et al.*, 2012	HQ660141.1
	BatPV/Eid_hel/GH-M67a/GHA/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	HQ660131.1
	BatPV/Myo_tor/CD356/DRC/2009	*Myonycteris torquata*	2009	Democratic Republic of the Congo	Drexler *et al.*, 2012	HQ660126.1
	BatPV/Epo_gam/CD273/DRC/2009	*Epomophorus gambianus*	2009	Democratic Republic of the Congo	Drexler *et al.*, 2012	HQ660122.1
	BatPV/Myo_tor/CO2225/CON/2005	*Myonycteris torquata*	2005	Republic of the Congo	Drexler *et al.*, 2012	HQ660118.1
	Bat Paramyxovirus Pte_par/KCR245H/CR/2010	*Pteronotus parnellii*	2010	Costa Rica	Drexler *et al.*, 2012	JF828297.1
	BatPV/Eid.hel/GH21a/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	FJ971939.1
	Eidolon helvum paramyxovirus clone U42A	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648056.1
	Eidolon helvum paramyxovirus clone U64A	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648077.1
	Eidolon helvum paramyxovirus clone U6B	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648086.1
	Eidolon helvum paramyxovirus clone U67J	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648079.1
	Eidolon helvum paramyxovirus clone U47C	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648063.1
	EPMV_RC09_236s1	*Eidolon helvum*	2009	Congo-Brazzaville	Weiss *et al.*, 2012	HE801055
	EPMV_RC09_214s	*Eidolon helvum*	2009	Congo-Brazzaville	Weiss *et al.*, 2012	HE647822
	EPMV_RC09_239k	*Eidolon helvum*	2009	Congo-Brazzaville	Weiss *et al.*, 2012	HE647829
	EPMV_RC09_215s	*Eidolon helvum*	2009	Congo-Brazzaville	Weiss *et al.*, 2012	HE647823
	EPMV_RC09_236s2	*Eidolon helvum*	2009	Congo-Brazzaville	Weiss *et al.*, 2012	HE801056
	IFBPV46/2011	*Pteropus* sp.	2011	Indonesia	Sasaki *et al.*, 2012	AB691546
Jeilongvirus-related	Eidolon helvum paramyxovirus clone U6A	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648085.1
	IFBPV32/2011	*Pteropus* sp.	2011	Indonesia	Sasaki *et al.*, 2012	AB691544
	IFBPV/01/2010	*Pteropus vampyrus*	2010	Indonesia	Sasaki *et al.*, 2012	AB691542
Morbillivirus-related	BatPV/Des_rot/BR222/BRA/2009	*Desmodus rotundus*	2009	Brazil	Drexler *et al.*, 2012	HQ660189.1
	BatPV/Des_rot/BR21/BRA/2008	*Desmodus rotundus*	2008	Brazil	Drexler *et al.*, 2012	HQ660187.1
	Miniopterus griveaudi paramyxovirus SMG16753	*Miniopterus griveaudi*	2011	Union of the Comoros	Wilkinson *et al.*, 2012	JQ886102
	Miniopterus gleni paramyxovirus SMG16468	*Miniopterus gleni*	2009	Madagascar	Wilkinson *et al.*, 2012	JQ886097
	BatPV/Myo_dau/NMS09-48/GER/2009	*Myotis daubentoni*	2009	Germany, Europe	Drexler *et al.*, 2012	HQ660165.1
	Bat Paramyxovirus Myo_alc/3-320/BGR/2009	*Myotis alcathoe*	2009	Bulgaria	Drexler *et al.*, 2012	HQ660163.1
	BatPV/Hip_caf/GB59-30/GHA/2009	*Hipposideros caffer*	2009	Ghana, Africa	Drexler *et al.*, 2012	HQ660161.1
	Miniopterus griveaudi paramyxovirus SMG16756	*Miniopterus griveaudi*	2011	Union of the Comoros	Wilkinson *et al.*, 2012	JQ886103
	Miniopterus sororculus paramyxovirus SMG16797	*Miniopterus sororculus*	2011	Madagascar	Wilkinson *et al.*, 2012	JQ886104
	Mormopterus acetabulosus paramyxovirus SMG17000	*Mormopterus acetabulosus*	2010	Mauritius	Wilkinson *et al.*, 2012	JQ886105
	Triaenops menamena paramyxovirus SMG16505	*Triaenops menamena*	2009	Madagascar	Wilkinson *et al.*, 2012	JQ886098
	BatPV/Col_afr/GB09478/GAB/2009	*Coleura afra*	2009	Gabon, Africa	Drexler *et al.*, 2012	HQ660155.1
	BatPV/Car_per/BR310/BRA/2009	*Carollia perspicillata*	2009	Brazil	Drexler *et al.*, 2012	HQ660194.1
	BatPV/Des_rot/BR22/BRA/2008	*Desmodus rotundus*	2008	Brazil	Drexler *et al.*, 2012	HQ660188.1

Table 1.4 *(Continued)*: Representative viruses selected from novel paramyxoviruses detected in bats between 2010 and 2012.

Genus	Virus	Associated bat species	First detection		Reference	Accession number
			Year	Location		
Morbillivirus-related	Miniopterus griveaudi paramyxovirus SMG16753	*Miniopterus griveaudi*	2011	Union of the Comoros	Wilkinson *et al.*, 2012	JQ886101
	BatPV/Myo_bec/NM98-46/GER/2008	*Myotis bechsteinii*	2008	Germany, Europe	Drexler *et al.*, 2012	HQ660170.1
	BatPV/Myo_mys/NM98-140/GER/2008	*Myotis mystacinus*	2008	Germany, Europe	Drexler *et al.*, 2012	HQ660168.1
Rubulavirus-related	BatPV Rou_aeg/GB1704/GAB/2005	*Rousettus aegyptiacus*	2005	Gabon, Africa	Drexler *et al.*, 2012	HQ660098.2
	BatPV/Eid.hel/GH31/PNE/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	FJ971960.2
	BatPV/Eid_hel/GB1661-AR/GAB/2005	*Eidolon helvum*	2005	Gabon, Africa	Drexler *et al.*, 2012	HQ660109.2
	BatPV/Eid_hel/GB3378/GAB/2006	*Eidolon helvum*	2006	Gabon, Africa	Drexler *et al.*, 2012	HQ660092.2
	BatPV/Eid.hel/GH1a/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	FJ971943.2
	BatPV/Eid_hel/RCA-P4/RCA/2008	*Eidolon helvum*	2008	Central African Republic	Drexler *et al.*, 2012	HQ660114.1
	BatPV/Hyp_mon/RCA-P17/RCA/2008	*Hypsignathus monstrosus*	2008	Central African Republic	Drexler *et al.*, 2012	HQ660112.1
	BatPV/Rou_aeg/GB1404/GAB/2005	*Rousettus aegyptiacus*	2005	Gabon, Africa	Drexler *et al.*, 2012	HQ660107.1
	BatPV/Hip_caf/GB091001/GAB/2009	*Hipposideros caffer*	2009	Gabon, Africa	Drexler *et al.*, 2012	HQ660099.1
	BatPV/Rou_aeg/GB1415/GAB/2005	*Rousettus aegyptiacus*	2005	Gabon, Africa	Drexler *et al.*, 2012	HQ660090.1
	BatPV/Rou_aeg/CO2784/CON/2006	*Rousettus aegyptiacus*	2006	Republic of the Congo	Drexler *et al.*, 2012	HQ660088.1
	BatPV/Rou_aeg/Bel125/GAB/2009	*Rousettus aegyptiacus*	2009	Gabon, Africa	Drexler *et al.*, 2012	HQ660086.1
	BatPV/Eid.hel/GH13a/2009	*Eidolon helvum*	2009	Ghana, Africa	Drexler *et al.*, 2012	FJ971946.1
	Eidolon helvum paramyxovirus clone U53A	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648070.1
	Eidolon helvum paramyxovirus clone U69D	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648084.1
	Eidolon helvum paramyxovirus clone U9B	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648088.1
	Eidolon helvum paramyxovirus clone U32C	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648055.1
	Eidolon helvum paramyxovirus clone U55C	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648072.1
	Eidolon helvum paramyxovirus clone U67N	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648080.1
	Eidolon helvum paramyxovirus clone U44A	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648059.1
	Achimota virus 1	*Eidolon helvum*	2011	Ghana, Africa	Baker *et al.*, 2013	JX051319
	Achimota virus 2	*Eidolon helvum*	2011	Ghana, Africa	Baker *et al.*, 2013	JX051320
	IFBPV/32/2012	*Acerodon celebensis*	2012	Indonesia	Sasaki *et al.*, 2012	AB710472
	IFBPV/25/2011	*Pteropus* sp.	2011	Indonesia	Sasaki *et al.*, 2012	AB691543
Unknown	Eidolon helvum paramyxovirus clone U50A	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648068.1
	Eidolon helvum paramyxovirus clone U9D	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648089.1
	Eidolon helvum paramyxovirus clone U71C	*Eidolon helvum*	2010	Ghana, Africa	Baker *et al.*, 2012	JN648087.1

Weiss *et al.* (2012) recently investigated bat bushmeat in the Republic of Congo for the presence of henipavirus-related viruses. Similar to previous studies they targeted *E. helvum* as they are the main protein source for the sub-Saharan human population in Africa. Using the *Respiro*-, *Morbilli*- and *Henipavirus* universal primers designed by Tong *et al.* (2008), they screened several organs derived from bats collected by hunters for sale at the market. Fifteen tissue samples derived from eleven individuals, tested positive for paramyxovirus RNA. The *Henipavirus*-like sequences were obtained from spleen, urine, kidney and liver where in one case two genetically different viral sequences were detected in a single individual. This is indicative of the wide tissue tropism of these viruses and points out that sampling of only selected organs may lead to an under estimation of the paramyxovirus diversity in bats.

The fact that hundreds of paramyxovirus-positive bats are being hunted and consumed in Africa is an indication that the human population in Africa is in close contact with these viruses on a daily basis (Weiss *et al.,* 2012). Although human disease and/or deaths as a result of bat-associated paramyxovirus infection have not been documented on the African continent, it cannot be excluded as a possibility. A spill-over event can take place at any moment giving the current situation. It is also possible that this has already taken place but has remained undetected up to now as studies regarding the seroprevalence of these viruses in humans is still lacking. Whether or not these viruses cause benign diseases or are even pathogenic remains to be elucidated.

With this implication of several African bat species (Drexler *et al.,* 2012) as carriers of henipavirus-related viruses and evidence of an origin to Hendra- and Nipah virus on the African continent, public health concerns could become evident in the near future especially in underdeveloped African countries where disease incidence is not reported or misdiagnosed as diseases with similar clinical presentation.

1.2.6 Detection and diagnosis of paramyxoviruses

When focusing on the diagnosis of paramyxoviruses, it is important to take three key points into account. This is the tissue tropism of the viruses, their pathogenicity in the various host species and the method of detection. The success of detection and analysis of these viruses are dependent on these factors.

1.2.6.1 Epidemiology and pathogenesis

The pathogenicity of paramyxoviruses is related to their ability to evade host interferon responses (Williamson & Torres-Velez, 2010). When considering Hendra virus, transmission from bats to humans is believed to be through an intermediate amplification host, as several horses died during the initial outbreak of the virus. Humans contracted the virus after coming into close contact with infected animals. Since then, several sporadic outbreaks have been occurring annually causing disease in horses.

Up to and including 2009, seven human infections have been reported of which four were fatal. This virus thus has a high mortality rate of approximately 57% in humans. The incubation period of this virus in humans ranges from eight days to three weeks before onset of disease. In fatal cases, humans died from either fulminant interstitial pneumonia or non-suppurative encephalitis. After the onset of symptoms, death occurred in most cases within one to three days. Horses developed diseases symptoms within four to 16 days (Murray *et al.,* 1995).

Nipah- and Hendra virus vary with regard to the species they infect and the mode and ease through which transmission takes place (Nichol *et al.,* 2000). Hooper *et al.* (2001) gives a good comparison of disease pathology between Hendra- and Nipah virus. Nipah virus transmission from Pteropid bats to humans initially required an intermediate animal host which in this case is pigs (Field *et al.,* 2001). In terms of human health, Nipah virus is considered to be a larger threat as direct transmission from bats have been reported as well as human-to-human transmission (Gurley *et al.,* 2007). According to statistics provided by the World Health Organization, 477 human infections with Nipah virus have been recorded up to 2008. Of these, 252 were fatal resulting in a mortality rate of 52.8%. Since the initial emergence, annual reports of Nipah virus outbreaks have been reported (Luby *et al.,* 2009). In some outbreaks, the case fatality reached extremes; for example the 100% case fatality reported for India in 2007 (5 cases) and a 92% fatality for Bangladesh in 2005 (12 cases, 11 deaths) (Wahed *et al.,* 2011).

Recently, corpse-to-human transmission was also reported in a case in Bangladesh where two people who came into contact with the corpse of a person who died of Nipah virus infection, contracted the viral disease (Sazzad *et al.,* 2013). The first individual merely hugged the corpse while the second was a caretaker who prepared the corpse for burial and came into contact with the oral secretions and ano-genital excreta of the deceased. It

is clear that this virus is highly infectious even after patients are deceased and transmission is still possible.

Nipah virus infection results in symptoms similar to influenza i.e. fever and muscle pain making it easy to be mistaken for flu. The incubation period for the virus is approximated to range between four and 18 days, which in acute cases resulted in encephalitis. Several cases of relapse have also been reported after recovery from the infection (Wahed *et al.,* 2011).

Both these viruses cause severe human disease, have a relatively high risk of spreading when considering evidence of human-to-human transmission and there is currently no prophylaxis or treatment available (Lam, 2003). Upon definition, this classifies these viruses as class 4 biological agents as recognized by several authorities. The Centre for Disease Control also groups these viruses with Category C critical bioterrorism agents. This emphasizes the severe nature of these viruses and their impact on human health. Research regarding treatment for Hendra- and Nipah virus is on-going.

Menangle virus is known to cause a disease in pigs characterized by brain and spinal cord deterioration as well as skeletal deformation (Yaiw *et al.,* 2008). In humans seroconversion was observed and it was accompanied by a non-specific febrile illness. Humans on Tioman Island presented with antibodies to Tioman virus indicating exposure to or even previous infection with the virus. As mentioned above, experimental Tioman virus infection in both mouse and pig brains resulted in successful replication in these hosts (Yaiw *et al.,* 2007). The possibility that these viruses might be zoonotic cannot be excluded.

1.2.6.2 Host and tissue tropism

When comparing different paramyxoviruses, it is clear that there is a wide host range that can be infected. In a paper by Hooper *et al.* (2001), they report infection of various animals with Hendra- and Nipah virus including horses, cats, guinea pigs, dogs and pigs. Although many of these infections were only laboratory based, it still shows the versatility of these viruses to adapt and infect new hosts. These viruses have been cultured *in vitro* in a wide range of cells including mammalian, avian, reptile, and amphibian as well as fish cells. The difference in cell tropism among viruses in the *Paramyxoviridae* family is as a result of different protein receptors on the capsids surface used for attachment and fusion purposes (Dutch, 2010).

As with the host range, paramyxoviruses also have a wide tissue tropism. When specifically looking at the henipaviruses, each is characterized with a wide tissue tropism (Williamson & Torres-Velez, 2010). **Table 1.5** lists the various species from which the previously described bat-associated paramyxoviruses (section ***1.2.4.1*** - ***1.2.4.6***) have been isolated from or detected in. The wide tissue tropism of these viruses is also summarized in this table. Sample type ranges from respiratory and alimentary (Lau *et al.,* 2010) to salivary and urine (Chua *et al.,* 2001). Although not naturally associated with disease, Tioman virus showed neurotropism during experimental infection of mouse brain (Yaiw *et al.*, 2007). This is just another example of the wide range tissue tropism found in diverse family of viruses.

Table 1.5: Paramyxoviruses previously detected from bats and information regarding detection and/or isolation from different samples of various bat species.

Family	Genus	Virus	Associated bat species	First detection in bats		Target samples
				Year	Location	
Paramyxoviridae	***Henipavirus***	Hendra virus	*Pteropus alecto, Pteropus poliocephalus, Pteropus scapulatus, Pteropus conspicillatus*	1994	Australia	Nasal swabs, urine, lung, brains
		Nipah virus	*Pteropus hypomelanus, Pteropus vampyrus, Pteropus lylei, Pteropus giganteus, Hipposideros armiger, Eonycteris spelaea, Cynopterus brachyotis, Hipposideros larvatus, Scotophilus kuhlii*	1999	Malaysia	Urine, respiratory secretions, kidney
		BatPV/Eid.hel/GH10/2008	*Eidolon helvum*	2008	Africa	Faecal
		BatPV/Eid.hel/GH45/2008	*Eidolon helvum*			
		BatPV/Eid.hel/GH48/2008	*Eidolon helvum*			
	Rubulavirus	Bat parainfluenzavirus	*Rousettus leschenaultia*	1966	India	Pooled spleen, brain and salivary glands
		Mapuera virus	*Sturnira lilium*	1979	Brazil	Salivary glands
		Menangle virus	*Pteropus poliocephalus, Pteropus alecto, Pteropus conspicillatus, Pteropus scapulatus*	1997	Australia	Serology
		Tioman virus	*Pteropus hypomelanus, Pteropus rufus, Eidolon dupreanum, Rousettus madagascariensis*	2000	Malaysia	Urine
		Tuhoko virus 1, 2, 3	*Rousettus leschenaultii*	2006	China	Respiratory and alimentary

The recent study of Drexler *et al.* (2012) also included a few smaller projects based on the primary results of paramyxovirus detection. Among these studies was the evaluation of several different organs of *E. helvum* for the presence of paramyxovirus RNA and the concentrations thereof. Twenty-two different *E. helvum* samples that tested positive for paramyxovirus RNA was selected for further analysis. Both serum and solid organs which included spleen, liver, brain, kidney, lung and intestine were used for analysis. From these results it was clearly observed that the spleen samples contained the highest concentration of viral RNA when compared to other organs. The only exception was sampling number GH90/GHA/2009 from which only the kidney had a detectable virus concentration. Sporadic detection of paramyxovirus RNA was observed in several other organs across the panel of samples, with only one sample testing positive in five of seven tissues tested (F-7/GHA/2009). It was clear that these African strains of paramyxoviruses do not favour neurological tissue or only replicate at concentrations too low for molecular detection, as none of the brain samples tested positive. All bats presented healthy and asymptomatic for paramyxovirus infection. Virus replication showed to be possible in several organs of the same host, while some organs seem to be favoured, *e.g.* the spleen in the case of *Eidolon helvum*.

1.2.6.3 Detection

Detection of viruses with physical evidence can be done through molecular approaches (*e.g.* PCR and sequencing) and other techniques including electron microscopy and virus isolation while serology (e.g. Luminex microsphere assays and enzyme linked immunosorbent assays) provides indirect evidence of virus infection or exposure. The method of choice will largely depend on the aims of the study being conducted. Detection of paramyxoviruses has mainly been focused on the specific virus that researchers targeted. Using the first bat-associated paramyxovirus of major importance, Hendra virus, as an example Murray *et al.* (1995) described the steps followed for the identification of the newly emerged virus. The first approach used was cell culture inoculation followed by electron microscopic (EM) examination. Virus neutralization was used to confirm that the virus responsible for the horse deaths is similar to the virus that caused the death of a human. This was followed by immunofluorescence and a protein immunoblot analysis. Lastly PCR amplification was attempted. Primers specific for morbilliviruses (5'-ATGTTTATGATCACAGCGGT-3' and 5'-ATTGGGTTGCACCACTTGTC-

3'), paramyxoviruses (5'-ACATACAGTGGGATAAGAACC-3' and 5'-CAACCATGAAGCCT CATCAGG-3') and pneumoviruses (5'-AATGGAAAAGAAATGAAATTTG-3' and 5'-CAATC ACTTCATAGAAGCT-3') were used. None of these primer pairs yielded positive results. Based on the results of the serologically, the virus had a weak cross-reaction with rinderpest virus which was used for subsequent primer design to amplify the matrix, fusion and polymerase genes. Only the matrix protein primers (5'-TTCTTAATGGTATAATAGA AG-3' and 5'-TGAAATTGCCGATATGTACCAT-3") produced an amplicon that was subsequently sequenced to yield the first genomic sequence fragment of Hendra virus.

With the outbreak of Nipah virus, the same approach was used. Firstly, cerebrospinal fluid from animals with fatal cases of encephalitis was used to inoculate Vero cells (Chua *et al.,* 2000). After syncytia were observed, cells were subjected to EM and the virus was identified as a member of the *Paramyxovirinae* family of viruses. The same order of events, as with the Hendra virus outbreak, was used for virus identification. An immunofluorescent antibody assay revealed strong cross-reaction to Hendra virus antiserum as opposed to no cross-reaction with other paramyxovirus antisera tested. A paramyxovirus specific primer set (5'-CATTAAAAAGGGCACAGACGC-3' and 5'-TGGACATTCTCCGCAGT-3') targeting the P gene was used for initial amplification of Nipah virus.

In an article by Daniels *et al.* (2001) on laboratory diagnosis of Nipah and Hendra virus infections, several possible approaches are discussed in a bit more detail. The approaches routinely used (as discussed above) include virus isolation, immunohistochemistry (Hooper *et al.,* 1999), electron microscopy, serum neutralization and genome-based assays (PCR amplification and sequencing). Serological and molecular detection of paramyxoviruses will be discussed separately.

1.2.6.3.1 Serological detection

Serological approaches are aimed at detecting antibodies against a pathogen as opposed to nucleotide detection in molecular methods. Velathanthiri *et al.* (2006) for example did a study to compare the efficacy of serology, virus isolation and RT-PCR in the detection of Dengue viral infections. They concluded that of the three methods, serology was the most useful as this method was able to detect Dengue virus infection as early as day 1 of fever onset while only on day 3 for the other two methods. The percentage positivity of serology in this study was 51% compared to the mere 11.5% and 11.9% for

virus isolation and RT-PCR respectively. This is just one of many examples of how serology can be applied in the rapid detection of known viruses. When doing surveillance for the purpose of detecting novel viruses this approach will be lacking due to the fact that this approach cannot distinguish between different viral species. When conducting a broader study to for example determine which virus families are circulating in a given environment, serology can be used as a tool to at least determine the presence of a specific family and/or genus present. For initial surveillance, serology is thus an excellent tool but for further characterization and identification, molecular methods will be required. An important example of this can be the first detection of henipavirus-related viruses on the African continent as detected by Hayman *et al.* (2008) with the use of serological approaches.

Serology has a few associated problems including cross-reactivity and the lack of reactivity (Chua *et al.,* 2001; Miller *et al.* 2010). When in search of previously detected viruses, cross-reactivity can lead to newly yet undiscovered viruses being mistaken for these known viruses. A lack of reactivity as shown by Chua *et al.* (2001) in the case of Tioman virus is yet another drawback. Although Tioman virus is part of the *Paramyxovirus* family, no cross-reactivity was seen between this newly detected virus and several members of this family. It did however cross-react with Menangle virus that was only detected a few years before. Novel viruses might thus not have similar serological properties as known members of a virus family. During surveillance for novel viruses, a lack of reactivity can also result in novel viruses going undetected. Due to cross-reactivity among closely related viruses, molecular approaches are also required to get confirmation of the virus detected. Such an approach would thus not be suitable for the surveillance of novel viruses.

Homaira *et al.* (2010) used a serological approach to investigate Nipah encephalitis in Bangladesh. In their approach they collected blood samples from case-patients suspected of Nipah virus infection. They used an immunoglobulin M (IgM) capture enzyme immunoassay. Serum taken from individuals ten days after the onset of disease presented with detectable Nipah virus antibodies and they were confirmed as infected. Serology can also be used as a useful tool when conducting a longitudinal study to determine distribution and risk factors associated for seropositivity as demonstrated by Rahman *et al.* (2013) in the case of Nipah virus among Pteropid bats in peninsular Malaysia. Serology has also been used in detecting the presence of Hendra virus from bats

(Halpin *et al.,* 2000). Several other such serological assays are available for numerous members of the *Paramyxovirinae* sub-family. Serology is thus a widely use tool in the detection of previously identified paramyxoviruses and the design of a serological assay capable of detecting a wide range of paramyxoviruses can prove helpful in the initial screening for these viruses in bat populations.

1.2.6.3.2 Molecular detection

Currently, molecular methods like PCR and sequencing are the initial detection methods involved in bat-associated paramyxovirus detection as opposed to the above used approach. The more popular PCR based application has become a key step in paramyxovirus detection. Several such assays (RT-PCR and real-time RT-PCR) have been designed for the specific detection of a single paramyxovirus (Smith *et al.,* 2001; Wacharapluesadee & Hemachudha, 2007). Guillaume *et al.* (2004) designed a real-time RT-PCR assay for the detection of Nipah virus to allow rapid and specific identification of the virus and to allow quantification.

Following the accidental discovery of Tioman virus in search of the then unknown Nipah virus, no other bat-associated paramyxovirus was reported up to 2009 when Lau *et al.* (2010) reported the discovery of three novel bat-associated rubulaviruses (Tuhoko viruses 1, 2 and 3). Based on the previous detection of the four rubulaviruses (bat parainfluenza-, Mapuera-, Menangle- and Tioman virus) they designed genus specific primers (5'-GCCAATCATGCWGGNAARTT-3' and 5'-GTTGAATGGATCACCNACATA-3') for this group and screened bats from China. The three novel viral sequences were detected from 15 positive *Rousettus leschenaultia* samples which included alimentary and respiratory tissues. Based on these sequences they went on to design a quantitative RT-PCR specific for these viruses.

Due to the specific nature of these traditional methods and primers, novel viruses are unlikely to be detected. When screening samples for the presence of any paramyxovirus, several reactions and protocols will be needed. For increased ability of detecting novel paramyxoviruses, an approach using universal primers will be the most effective. Such primers are designed to be complementary to a conserved region within the genome of all paramyxoviruses. Several different genome regions have been used as target for molecular detection most of which are sufficient for genus specific primers. The most conserved gene in the *Paramyxoviridae* family has been shown to the L (polymerase)

gene (Tong *et al.,* 2008). Universal primers thus usually target this region of the genome. By using such a universal primer approach, not only can all previously identified paramyxoviruses be detected through only one reaction but the probability of detecting novel paramyxoviruses also increases. In recent years several papers reported the design of such primers.

In 2008, Tong *et al.* published their work done on paramyxovirus detection where they designed a panel of four primer sets as shown in **Table 1.6** (sets include first round and nested primers). All primers were designed to span the L gene sequence of the paramyxovirus genome. A wide panel of paramyxoviruses from all genera including unclassified paramyxoviruses were used in the design of these primers. Bat-associated paramyxoviruses Hendra-, Nipah- and Menangle virus were also included.

These primer sets have been applied in several studies and have proved effective in the amplification of novel paramyxoviruses. Schatzberg *et al.* (2009) applied these primers towards the detection of canine distemper virus from dogs presenting with meningoencephalitis. Two other examples of where these primers have been successfully applied is the studies of Baker *et al.* (2012) and Drexler *et al.* (2012) in the detection of several novel bat-associated paramyxoviruses on the African continent as discussed before. Kurth *et al.* (2012) applied these primers in Europe and Chintapitasakul *et al.* (2012) in Thailand, both detecting novel paramyxoviruses from bats.

Table 1.6: Consensus degenerate primer panel designed by Tong *et al.* (2008) for the detection of paramyxoviruses.

Primer name	Primer sequence (5'-3')	Target
PAR-F1	GAA GGI TAT TGT CAI AAR NTN TGG AC	*Paramyxovirinae*
PAR-F2	GTT GCT TCA ATG GTT CAR GGN GAY AA	
PAR-R	GCT GAA GTT ACI GGI TCI CCD ATR TTN C	
RES-MOR-HEN-F1	TCI TTC TTT AGA ACI TTY GGN CAY CC	*Respirovirus, Morbillivirus, Henipavirus*
RES-MOR-HEN-F2	GCC ATA TTT TGT GGA ATA ATH ATH AAY GG	
RES-MOR-HEN-R	CTC ATT TTG TAI GTC ATY TTN GCR AA	
AVU-RUB-F1	GGT TAT CCT CAT TTI TTY GAR TGG ATH CA	*Avulavirus, Rubulavirus*
AVU-RUB-F1	ACA CTC TAT GTI GGI GAI CCN TTY AAY CC	
AVU-RUB-F1	GCA ATT GCT TGA TTI TCI CCY TGN AC	
PNE-F1	GTG TAG GTA GIA TGT TYG CNA TGC ARC C	*Pneumovirinae*
PNE-F2	ACT GAT CTI AGY AAR TTY AAY CAR GC	
PNE-R	GTC CCA CAA ITT TTG RCA CCA NCC YTC	

Without the use of a universal primer set, detection of these novel paramyxoviruses might not have been possible due to the genetically diverse nature of these novel viruses. In the Baker *et al.* (2012) publication, two of the Tong *et al.* (2008) designed primer sets were selected and used. The one primer set that targeted the *Paramyxovirinae* sub-family (PMV) and the set that targeted the *Respiro-, Morbilli-*, and *Henipavirus* genera (RMH) was used. All samples in this study were screened with both hemi-nested RT-PCR primer sets. Ideally, the sub-family wide primer set should detect all viruses in this sub-family but this was not the case. Some samples only tested positive with the PMV-primers while others were only detected by the RMH-primers. Although this was only observed in a few cases, it shows that even though these primers are successful in paramyxovirus detection, some viruses will not be detected as a result of the genetic diversity observed in this virus family. However, the availability of these primer sets, has allowed the detection of a vast number of novel paramyxovirus sequences.

More recently, Van Boheemen *et al.* (2012) designed a similar pan-paramyxovirus primer set for the detection of all paramyxoviruses in the *Paramyxoviridae.* This assay differs from Tong *et al.* (2008) in the fact that they have one primer set for both sub-families while the other study has separate primers per sub-family. This more recent assay did not include the amplification of the viruses in the *Henipavirus* genus while they do state that theoretically the primers should also bind to viruses in this genus. Whether or not these primers will be as effective as the ones designed by Tong *et al.* (2008), is not known. Given the genetic diversity of bat-associated paramyxoviruses, it can be assumed that the Boheemen *et al.* (2012) primers will amplify some of the novel or yet undiscovered paramyxoviruses. A large number of viruses might still be circulating and due to this genetic diversity, they might not even be detected by any of these universal primer set and will require another approach.

Other possibilities for the detection of novel paramyxoviruses are the use of sequence independent methods. There are several such techniques available. Among these are VIDISCA (Virus discovery based on cDNA-AFLP) (Pyrc, 2007), DNase-SISPA (Sequence independent single primer amplification) (Allander *et al.,* 2001), proteomics (Ye *et al.,* 2010), mass sequencing (Allander *et al.,* 2005), single virus genomics (Allen *et al.,* 2011) and next-generation sequencing that has become a powerful tool for virus discovery (Chiu, 2013). VIDISCA and DNase-SISPA are PCR based methods incorporating restriction enzyme digestion of unknown DNA and the ligation of primer specific adaptors.

Both these methods have been successfully applied in the discovery of viruses including for example coronaviruses (Van der Hoek *et al.,* 2004). These methods are thus also suitable alternatives for the discovery of novel paramyxoviruses not detected by conventional molecular methods. Next-generation sequencing is an agreeable approach for the discovery of viruses although bioinformatics of these results still remain a challenge (Chiu, 2013).

Molecular detection is not considered to be superior to serological methods as the choice of method is based on the particular research question. Currently molecular detection is however the method of choice for use in the surveillance of novel paramyxoviruses circulating as it will be able to discriminate between previously identified and novel viruses based on sequence results.

AIM AND OBJECTIVES

The aim of the study was to investigate the presence of paramyxoviruses in different populations of bats from specimen panels collected from six African countries.

Specific objectives

- Design, test and evaluate existing and new PCR primers sets for the detection of bat associated paramyxoviruses
- Molecular screening of specimen panels from different bat species collected in six African countries
- Describe the genetic diversity and phylogeny of newly and previously described bat associated paramyxoviruses

CHAPTER 2

Paramyxoviruses in African bats

Introduction

For many years, only a limited number of paramyxoviruses have been associated with bats. Three of them Hendra-, Menangle- and Nipah virus have been linked to disease in both human and animal. It was only with the emergence of Hendra virus that attention was given to this family of viruses and their relationship with bats as reservoirs. In all three cases, *Pteropus* spp. was implicated as reservoir for these viruses. Subsequent surveillance mainly took place in the areas where disease outbreaks were reported and the surrounding areas - with the main focus placed on Pteropid bats.

Only recently, the first report was made of henipavirus-related viruses in a host other than *Pteropus* as well as a new geographical location far from the previously reported outbreaks. Hayman *et al.* (2008) provided the first serological evidence of *Henipavirus* infection in West African fruit bats. The following year, Drexler *et al.* (2009) provided sequence evidence to support the findings of Hayman *et al.* (2008) with the amplification of paramyxoviral-RNA from *Eidolon helvum.* Three novel henipavirus-related viral sequences were reported.

Following this evidence, interest into African bats and paramyxoviruses rapidly increased. Baker *et al.* (2012) reported the detection of several putative paramyxovirus species from *Eidolon helvum* sampled in Ghana, West Africa. Drexler *et al.* (2012) expanded their surveillance to other bat species and geographical locations. Not only did they detect putative paramyxovirus species on various continents (including several African countries), but a large number of non-fruit eating bats were also implicated as hosts for paramyxoviruses. Both research groups applied paramyxovirus-specific primers designed by Tong *et al.* (2008) which in both instances, proved to be successful in detecting the genetically diverse population of paramyxoviruses circulating in bats.

In a more recent study conducted by Baker *et al.* (2013), they further investigated two novel rubulaviruses detected from *Eidolon helvum* in their previous study (Baker *et al.,* 2012) named Achimota virus 1 and Achimota virus 2 (GenBank accession numbers JX051319 and JX051320 respectively). They were able to perform virus isolation in cell culture and through the design and application of a serological assay, obtained possible evidence of human infection from Tanzania and Ghana. In this study they concluded that a

zoonotic spill-over could have taken place but went unseen due to poor health surveillance and diagnosis. The zoonotic potential of other novel bat-associated paramyxoviruses should thus also be considered.

Drexler *et al.* (2012) sampled in five African countries namely Gabon, Congo, Democratic Republic of the Congo (DRC), Republic of Central Africa (RCA) and Ghana, all within the tropical region of Africa. In total, 21 species were sampled in the first four countries and 22 species from Ghana. Several frugivorous and insectivorous bats tested positive for paramyxoviral RNA, some with high and others with a low proportion of positives. Although Drexler *et al.* (2012) expanded their search to include African countries other than Ghana and several other bat species, the true distribution of these viruses across the more sub-tropical regions of the African continent is still unknown. Clearly, data regarding the distribution of paramyxoviruses on the African continent is lacking and broader surveillance is required. This study addresses this issue by expanding the molecular screening for these viruses to other African countries and several bat species never screened before. This study will provide additional information on the current distribution of paramyxoviruses on the continent but is still geographically limited.

2.1 MATERIALS AND METHODS

Molecular screening for bat-associated paramyxoviruses on the African continent was done in two segments with regards to where the research was conducted and samples that were made available. Firstly, as part of a guest research visit to the Centers for Disease Control and Prevention (CDC) in Atlanta Georgia, USA, samples from several African countries were screened for the presence of paramyxoviruses under the supervision of Dr. Charles E. Rupprecht and Dr. Ivan V. Kuzmin. The second part of the study was done at the University of Pretoria, where the main focus of molecular screening was South African samples. Methodology differed in some instances between the two parts due to resource availability and will be discussed in two sections.

2.1.1 Section A – Methodology: CDC, Atlanta GA, USA

2.1.1.1 Sample collection

The target organ selected in this study was bat kidney due to the fact that henipaviruses have previously been detected from bat urine. Based on these results, kidneys are thus suitable targets for detection as they form part of the renal system.

Samples from central African countries were made available by the Pox- and Rabies virus Branch, Division of High-Consequence Pathogens and Pathology Rabies at the Centers for Disease Control and Prevention (CDC) in Atlanta GA, USA. These samples were collected as part of previous lyssavirus surveillance and pathogen discovery studies in bats on the African continent which was approved by the Institutional Animal Care and Use Committee (IACUC) of the CDC (reference number 2096) in collaboration with museums and wildlife services of the countries involved. Collection and dissection of samples were performed by the specified institution. Mist nets and/or harp traps were used in collection of the bats. Bats were anesthetized by an intramuscular injection of ketamine hydrochloride (volume of 0.05 to 0.1 mg/g body mass) and subsequently euthanized under sedation according to the field protocol (approved by the IACUC of the CDC).

In-field species identification was done based on morphology. Bat kidney samples from Cameroon, Democratic Republic of the Congo (DRC), Kenya and Nigeria were made available. Sample collection from 2010 to 2011 (depending on the country involved) was screened. Several different locations and bat species were sampled from each country. **Table 2.1** summarizes the information for each country and indicates the specific species sampled and species count for a given year. The majority of samples were available from Kenya as sample collections from two years were made available. Only a limited amount of samples were available for Nigeria. Parallel to the information in **Table 2.1** regarding the locations sampled, **Figure 2.1** provides a map of areas sampled in the countries involved in this study where each location is specifically numbered.

2.1.1.2 Positive and negative controls

The Division of Viral Diseases, National Center for Immunization and Respiratory Diseases at the CDC provided five *Otomops martiensseni* kidney samples from Kenya, known to be positive for novel African paramyxoviruses (Tong *et al.,* 2010; unpublished) for use as positive control during primer design and evaluation. These samples were processed in the same manner discussed below (section ***2.1.1.4 – 2.1.1.8***) and RNA was subsequently used as positive control throughout the screening of samples. Nuclease-free water (Promega, USA) was used as negative control. Sequences obtained from four of the positive samples were aligned with representatives of previously identified paramyxoviruses using the BioEdit Sequence alignment editor v7.0.5.3 (Copyright© 1997-2005 Tom Hall).

Table 2.1: Summary of the samples collected and made available by the CDC for paramyxovirus screening.

Country	Year	Locations sampled #	Bat species	Count
Kenya	2011	Jimba cave[4], Three caves[11] Gilgil mine[3], Suswa cave[13] Kericho cave[5], Kisii[6] Pare cave[11], Shimoni cave[12]	*Coleura afra, Eidolon helvum, Hipposideros commersoni, Miniopterus minor, Miniopterus* sp., *Otomops martiensseni, Rhinolophus landeri, Rhinolophus* sp., *Rousettus aegyptiacus, Taphozous* sp., *Triaenops persicus*	410
Kenya	2010	Asembo -Church[1], Asembo - School[1] Chyulu Hills[2], Vihiga[16], Watamu[17] Gilgil mine[3], Suswa cave[13] Jimba cave[4], Three caves[11] Malindi[7], Meseno KEFRI[8] Mount Elgon: Kitum cave[9] Mount Elgon: Makingeni cave[10] Tsavo East, Ndololo camp[14] Tsavo West, Chyulu camp[15]	*Coleura afra, Eidolon helvum, Epomophorus labiatus, Epomophorus wahlbergii, Hipposideros* sp., *Miniopterus natalensis, Neoromicia* sp., *O. martiensseni, Rhinolophus landeri, Rhinolophus* sp., *Rousettus aegyptiacus, Scotoecus* sp., *Scotophilus dingani, Triaenops persicus*	160
Cameroon	2010	Lanavet[20], Ngong[20] Maya oulu[19], Zoological garden[22] Sodeconton Pitoa[21], Caves[18]	*Chaerephon* sp., *E. helvum, Epomophorus* sp., *Hipposideros* sp., *Rhinolophus* sp., *Scotophilus dingani, Taphozous* sp.	109
Nigeria	2010	College of Agriculture[23] Idanre cave[24]	*Eidolon helvum, Rousettus aegyptiacus, Hipposideros commersoni, Hipposideros* sp., *Lissanycteris angolensis*	60
DRC	2011	Kisantu -Church[29], Kinshasa -School 2[29] Kinshasa -School 1[31] Kisangani, Cimestan[26] Kisangani, Mayele Island[26] Rain forest next to Layoko[27] Rain forest next to Masako[28] UNIKIN Campus Club/house[31] Mbanza Ngungu cave[30] Kisangani -Zoo[26], Kinshasa, N'Dili[25] Kisangani -Abandoned factory[26]	*Chaerephon pumilus, Chaerephon* sp., *E. helvum, Glauconycteris argentata, Hipposideros fuliginosus, Hipposideros gigas, Hypsignatus monstrosus, Megaglossus woermanni, Micropterus pussilus, Mimetillus moloneyi, Miniopterus* sp., *Mops condylurus, Myonycteris torquata, Myotis* sp., *Neoromicia* sp., *Pipistrellus* sp., *Rhinolophus* sp., *Scotophilus dingani*	236

Superscript numbers correspond to numbers indicating specific locations in Figure 2.1

Refer to **Table A1** for a detailed list of species and sample numbers per individual location.

Figure 2.1: **Map of the central part of Africa from where samples were collected.** Sampling was done in Cameroon, DRC, Kenya and Nigeria. Red markers and numbers represent the specific locations listed in Table 2.1. Selected sampling sites were located close to each other and appear as a single red marker and correspond to a single number. For a detailed list of species and sample numbers per location, refer to **Table A1**.

A Neighbour-joining phylogenetic tree was generated from the alignment with the use of the Mega v5.05 software (Copyright© 1993-2011 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S.) to determine the phylogenetic position of these viruses in the *Paramyxovirinae* sub-family. The Jukes-Cantor substitution model was used with a bootstrap of 1000.

2.1.1.3 Primer design and selection

Molecular screening for novel paramyxovirus detection required broadly-reactive primers. Primer design was done by aligning polymerase gene (L gene) sequences of previously identified and where available also novel paramyxovirus sequences detected using the Clustal W multiple alignment function from BioEdit Sequence alignment editor v7.0.5.3 (Copyright© 1997-2005 Tom Hall). This gene was selected as target as it is the most conserved among members of this virus family (Tong *et al.,* 2008). Conserved regions were selected as primers for a hemi-nested PCR. All primers designed in this study were synthesized by the Biotechnology Core Facility Branch, at the Centers for Disease Control and Prevention. Initially, primer design was focused on the *Henipavirus* genus as this genus is believed to contain the zoonotic viruses of most concern to human and animal health. Primers designed and selected for evaluation are listed and summarized in **Table 2.2**.

Primer design of the first two sets of primers (1 and 2) in **Table 2.2** was based on sequences from Hendra- and Nipah virus (GenBank accession numbers NC_001906.2 and NC_002728.1 respectively), as well as the three novel putative henipaviruses detected in Ghana by Drexler *et al.* (2009) in 2008 named BatPV/Eid_hel/GH10; BatPV/Eid_hel/GH45 and BatPV/Eid_hel/GH48 (GenBank accession numbers FJ609191, GQ168929 and FJ609194 respectively) available at that time. Primers were evaluated with Hendra virus RNA received from the Special Viral Pathogens Reference Laboratory in The Centre for Emerging Zoonotic Diseases (CEZD) Unit of the National Health Laboratory Services (NHLS) South Africa. These sets of primers were then subsequently evaluated against the *Otomops* positive samples received.

In a third approach, several semi-conserved regions in the aligned sequences of the three novel Ghana viruses, Hendra- and Nipah virus (primer set 3 **Table 2.2**) were selected by Dr. Ivan Kuzmin (formerly CDC) as possible primer binding sites. Primers designed by Dr. Kuzmin were also evaluated against these positive samples and were used in

conjunction with each other as well as with the selected primers from the above mentioned two sets where feasible, to obtain a possible primer pair capable of amplifying the *Otomops* control samples.

Table 2.2: Designed and evaluated primers for the detection of novel paramyxoviruses.

Set	Name	Sequence (5'-3')	Sequences used for primer design
1	P1F1	GTR TCT ATG ATA GAG CCT TTA G	HeV, NiV *(P1F1,P1F2)*
	P1F2	GKG CAT TTC TRC ATC AYT GC	
	P1R	GCT TTA TCY TTC ATR TAC ATR CTC	HeV, NiV, BatPV/Eid.hel/GH10/2008, BatPV/Eid.hel/GH45/2008, BatPV/Eid.hel/GH48/2008 *(P1R)*
2	P2F1	GAT ARA CAT GGR GGD GYT TGG CC	HeV, NiV, BatPV/Eid.hel/GH10/2008, BatPV/Eid.hel/GH45/2008, BatPV/Eid.hel/GH48/2008
	P2F2	GAG YAT GTA YAT GAA RGA TAA AGC	
	P2R	CTT TYT CYT TKA GAC TRT ANG A	
3	1149F	AGA YAA GGT YCT WGA ATA	HeV, NiV, BatPV/Eid.hel/GH10/2008, BatPV/Eid.hel/GH45/2008, BatPV/Eid.hel/GH48/2008
	1228F	AGR CAT GGR GGA GCY TGG CC	
	4942F	GTC ACA YCC TAG AGT GTT CMR RAG ATT	
	5705R	CAT GTC WCC WGA HCC YTC ACC	
	5195R	CCA AGG WGG YTT ATG GTG ATT TGC	
	5219R	TTC AAT AGA KGT CAK ACC WAG AAT	
4	PAR-F1	GAT ARA CAT GGW GGK GYT KGG C	HeV, NiV, BatPV/Eid.hel/GH10/2008, BatPV/Eid.hel/GH45/2008, BatPV/Eid.hel/GH48/2008, Paramyxovirus bat/E95/2009, Paramyxovirus bat/E20/2009
	PAR-F2	DGA TGA DGM TCT TAS AAY GTR YAG GAA	
	PAR-R	TCT TTY TMT TWT AGA CTV TWN GA	
5	PMV-F1	GAA GGI TAT TGT CAI AAR NTN TGG AC	Most viruses belonging to the *Paramyxovirinae* were used in primer design. Tong *et al.* 2008
	PMV-F2	GTT GCT TCA ATG GTT CAR GGN GAY AA	
	PMV-R	GCT GAA GTT ACI GGI TCI CCD ATR TTN C	
6	RMH-F1	TCI TTC TTT AGA ACI TTY GGN CAY CC	Most viruses belonging to the *Respiro-, Morbilli-* and *Henipavirus* genera were used in primer design. Tong *et al.* 2008
	RMH-F2	GCC ATA TTT TGT GGA ATA ATH ATH AAY GG	
	RMH-R	CTC ATT TTG TAI GTC ATY TTN GCR AA	

Note: 'F'= forward primer, 'R' = reverse primer, 1/2 = primary/nested primer

During primer evaluation of the above primer sets, novel bat-derived paramyxovirus sequences from Europe were released on the GenBank sequence database of The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Two of these sequences (E95 and E20 with GenBank accession numbers JN086954 and JN086953 respectively) were incorporated into the sequence alignment to improve primer

design. From the latter alignment, a new primer set (set 4 **Table 2.2**) was designed and evaluated against the five *Otomops* paramyxovirus positive controls.

The last approach involved the evaluation of two universal primer sets designed by Tong *et al.* (2008) against the positive samples received. The first primer set (set 5 **Table 2.2**) targets the entire *Paramyxovirinae* sub-family (PAR) of viruses while the second (set 6 **Table 2.2**) targets the *Respiro-, Morbilli- and Henipavirus* genera (RMH). As there was already a primer set using the PAR abbreviation in this study (set 4), the PAR abbreviation used in by Tong *et al.* (2008) was renamed to PMV. These two sets were selected as both cover the *Henipavirus* genus containing the bat-associated paramyxoviruses of most public and veterinary health concern. Using the Hendra virus genome (GenBank accession numbers NC_001906.2) as a reference sequence, primer binding regions for these primers were determined. PMV primary primers bind at position 13794 (PMV-F1) and 14428 (PMV-R) while the RMH primers bind upstream at position 12471 (RMH-F1) and 13056 (RMH-R) thus amplifying different regions of the polymerase gene.

2.1.1.4 Sample processing and RNA extraction

Each kidney sample was split into two to allow the retention of original material for future use i.e. proliferation and virus isolation in cell culture in case of virus detection. For this, one section of the kidney tissue was stored away at -80°C until future use. The other section of the kidney was subjected to RNA extraction. Each sample was homogenized using the back end of two Falcon™ swabs (Becton, Dickinson and Company, USA) to allow for better extraction. TRizol® reagent (Invitrogen, USA) was added and extraction proceeded according to the manufacturers' specifications.

2.1.1.5 Reverse transcription

As the target viruses are RNA viruses, an additional step was required to convert viral RNA into cDNA. For this, 1µl of 10pmol of primer RMH-F1 was added to 3µl of extracted RNA, the reaction heated to 94°C for 1 minute and directly placed on ice afterwards. A reverse transcription mix (RTmix1) was prepared by mixing 200µl of 10mM PCR nucleotide mix (Promega, USA), 770µl nuclease-free water (Promega, USA) and 440µl 5x avian myeloblastosis virus (AMV) reverse transcriptase buffer (250mM Tris-HCl, 40mM $MgCl_2$, 150mM KCl and 5mM dithiotreithol (DTT); Roche Diagnostics, Germany). This mix was subsequently used to prepare a second mixture (RTmix2) containing RTmix1,

AMV reverse transcriptase (20U/µl; Roche Diagnostics, Germany) and Protector RNase inhibitor (40U/µl; Roche Diagnostics, Germany) to the ratio of 70µl:1µl:1µl respectively. To the sample RNA and primer reaction prepared above, 7µl of RTmix2 was added. The reaction mixture was incubated at 42°C for 90 minutes and stored at -20°C until use.

2.1.1.6 PCR amplification

For the primary round of PCR amplification, a master mix of 37.45µl nuclease-free water (Promega, USA), 4.45µl 1M Tris (pH8.01; Promega, USA), 1.25U DreamTaq™ DNA polymerase (5U/µl; Fermentas, Inqaba Biotechnologies), 0.54µl RMH-F1 primer (10pmol) and 0.68µl RMH-R primer (10pmol) was prepared as master mix per sample. To the prepared cDNA product, 40µl of the master mix was added. This reaction was subsequently subjected to incubation at 94°C for 2 minutes, 40 cycles of 94°C for 15s, 48°C for 30s and 72°C for 30s followed by a final extension at 72°C for 7 minutes. PCR amplification was performed using a Mastercycler gradient thermocycler (Eppendorf, Germany). After cycle amplification, samples were stored at 4°C until use in a hemi-nested PCR reaction. The master mix for the hemi-nested round of amplification was prepared by mixing 37.30µl nuclease-free water (Promega, USA), 4.45µl 1M Tris (pH8.01; Promega, USA), 1.25U DreamTaq™ DNA polymerase (5U/µl; Fermentas, Inqaba Biotechnologies), 0.68µl each of RMH-F2 primer (10pmol) and RMH-R primer (10pmol) and 7µl of the above prepared RTmix1. Of the prepared reaction, 47µl was added to 3µl of the primary PCR product. This was subsequently subjected to the same cycling conditions as described for the primary PCR reaction.

2.1.1.7 Agarose gel analysis and gel extraction

Due to time constraints and the lack of amplification in the initial PCR of several positive samples, only PCR products of the hemi-nested round of amplification were analyzed on a 2% agarose gel containing 0.5µg/ml ethidium bromide (Merck chemicals) in 1x Tris-acetate-EDTA (Ethylenediaminetetraacetic acid) (TAE) buffer. A 100-1500bp DNA molecular weight marker XIV (Roche Diagnostics, Germany) was run in the first lane to resolve amplicon size upon analysis. Prior to running of the samples, 3µl loading dye (40% sucrose, 0.25% bromophenol blue) was added to 25µl of the hemi-nested PCR product. Subsequently, samples were loaded and the gels were run at 150V using the Power Station 300 Plus (Labnet International Inc.) in a horizontal gel tank in a HE 33 mini

horizontal submarine electrophoresis unit (Hoefer Inc., USA). A UV transilluminator was used to visualize bands after electrophoresis was completed. Amplicon length was determined and products were excised from the gel. With the use of the Wizard® SV gel and PCR clean-up system (Promega, USA) DNA was extracted from the gel according to the specifications of the manufacturer. Extracted DNA was stored at -20°C until use.

2.1.1.8 Sequence amplification and sequencing

Preparation for sequencing amplification of amplicons was done by mixing 1µl of either the RMH-R primer (10pmol) or RMH-F2 primer (10pmol), 3µl of BigDye®Terminator v3.1 kit (Applied Biosystems®, Life Technologies) with 7µl of purified amplicon DNA. Reactions were cycled in a Mastercycler gradient thermocycler (Eppendorf, Germany). Cycling conditions involved 1 cycle at 94°C for 1 minute, 25 cycles of 94°C for 10s, 50°C for 5s and 60°C for 4 minutes. Following sequencing amplification, products were purified using Centri-sep columns (Princeton Separations Inc.) according to manufacturers' instructions. To the purified product, 50µl 100% formamide (Promega, USA) was added and the sample was incubated at 96°C for 1 minute. The samples were subsequently loaded and analysed on an ABI 3100 automated capillary sequence analyser. Both the forward and reverse sequences were run for each sample.

2.1.1.9 Additional Eidolon helvum analysis

Based on results implicating *Eidolon helvum* as host for henipavirus-related as well as other paramyxoviruses in numerous occasions (Drexler *et al.,* 2009; Baker *et al.,* 2012; Drexler *et al.,* 2012), the spleen and faecal of 20 *E. helvum* samples were selected for additional screening (five per location sampled). These organs were selected based on results by Drexler *et al.* (2012) indicating a higher virus concentration in the spleen compared to other organs. Processing of these samples took place in the same manner as described above for the kidney samples.

2.1.2 Section B – Methodology: University of Pretoria, South Africa

2.1.2.1 Sample collection

As part of previous research on lyssaviruses in bats conducted by the University of Pretoria, bats were caught from all over South Africa either with mist nets or harp traps. The study was funded by the National Research Foundation and done in collaboration with the Gauteng and Northern Region Bat Interest Group, KwaZulu-Natal Bat Interest Group,

North West Nature Conservation and Wilderness Safaris. Kidney samples were made available for this paramyxovirus study. For tissue collection, captured bats were anaesthetized using a mixture of ketamine and xylazine after which they were bled out by exsanguination from the heart. Finally, bats were euthanized by cervical dislocation. **Table 2.3** lists the different countries in South Africa as well as Swaziland where sampling took place - all species sampled and sample numbers were included. The several different locations sampled are represented by **Figure 2.2** and corresponds to the numbering in **Table 2.3**. Species identification of the bats was done through the analysis of morphological characteristics based on the specifications provided by Monadjem *et al.* (2010) by Dr. Teresa Kearny from Ditsong National Museum of Natural History. Specimens were also deposited as museum vouchers. Sampling permit number 000039 NW-07 was obtained for sampling at Taung (North West), permit number RB/2010/04 for Pafuri (Kruger National Park, Limpopo) and OP 500/2010 for Rocktail Bay (St. Lucia, KwaZulu-Natal).

2.1.2.2 Primer selection

No surveillance or molecular screening of paramyxoviruses in bats has been done in the southern part of Africa. It would thus be more efficient to target a wider range of paramyxoviruses in this area as paramyxovirus presence and diversity is still unknown. The *Paramyxovirinae (*PMV) primer set designed by Tong *et al.* (2008) would thus be best suited. However, work done by Baker *et al.* (2012) showed that novel viruses detected with the RMH set of primers was not always detected by the PMV primer set. Thus only using either the *Paramyxovirinae* sub-family wide primer set or the RMH primer set designed by Tong *et al.* (2008) might not detect all the diversity. Based on the lack of coverage in southern Africa and this primer difference, it was decided to use both primer sets from Tong *et al.* (2008) for maximum coverage in southern Africa. Primer sequences for PMV and RMH primers are listed in **Table 2.2** (sets 5 and 6).

2.1.2.3 Positive and negative controls

DNA from the previously discussed *Otomops positive* samples was used as positive control for the RMH PCR reactions. Measles virus RNA was supplied by the Special Viral Pathogens Reference Laboratory in The Centre for Emerging Zoonotic Diseases (CEZD) Unit of the National Health Laboratory Services for use as positive control for the PMV PCR reactions. RNA was received in nuclease-free water.

Table 2.3: The location and bat species sampled in the southern African region.

Country	Province	Location sampled #	Species sampled	Count
South Africa	Gauteng	Irene cave [6] Pretoria zoo [14] Midrand [10]	*Miniopterus* sp., *Neoromicia capensis*	39
	KwaZulu-Natal	Rocktail bay: St Lucia [15] 28 Edinburgh Street, Umkomaas [2] 27 Edinburgh Street, Umkomaas [2] 26 Edinburgh Street, Umkomaas [2] Ethekwini unicity [5] 23 Star Street, Chatsworth [1] 24 Star Street, Chatsworth [1] 81 Winfield drive [3]	*Epomophorus wahlbergi, Chaerephon pumilus, Nycteris thebaica, Scotophilus dinganii, Chaerephon ansorgei, Pipistrellus hesperidus, Kerivoula argentata, Glauconycteris variegata, Pipistrellus hesperidus*	40
	Limpopo	Pafuri, Kruger National Park [13] Buzzard mountain [4] Orrie/Matlapitsi cave [12]	*Epomophorus walbergi, Rousettus aegyptiacus, Taphozous mauritianus, Glauconycteris variegata, Nycticeinops schlieffeni, Scotophilus dinganii, Neoromicia nana, Rhinolophus fumigatus, Mops condylurus, Scotophilus leucogaster, Scotophilus nigrita, Pipistrellus rusticus, Chaerephon pumilus, Neoromicia helios, Scotophilus viridis, Hipposideros caffer, Rhinolophus landeri, Neoromicia zuluensis, Neoromicia rueppellii, Chaerephon ansorgei, Epomophorus gambianus, Miniopterus* sp., *Rhinolophus* sp., *Rousettus* sp.	109
	North West	Taung [16] Mooiplaats 94 KP, Madikwe Game reserve [11] Kgaswane [8] Kalkfontein 111 KP, Madikwe Game Reserve [7] Leeuwenhoek 112 KP, Madikwe Game Reserve [9]	*Tadaria aegyptiaca, Rhinolophus denti, Neoromicia capensis, Eptesicus hottentotus, Rhinolophus darlingi, Rhinolophus simulator, miniopterus natalensis, Nycteris thebaica, Sauromys petrophilus, Scotophilus* sp.	50
Swaziland	n/a	Mlawula Nature Reserve[17]	*Nycteris thebaica*	4

Superscripts correlate to numbering on Figure 2.2 giving the relative position of each sampling site

Refer to **Table A1** for a detailed list of species and sample numbers per individual location.

Figure 2.2: Map of southern Africa indicating sampling sites. Sampling took place in four South African provinces as well as in Swaziland (SW). Red markers and numbers represent the regions sampled in each province/country and correspond to numbering in Table 2.3. For a detailed list of species and sample numbers per location, refer to **Table A1**.

Because of the high concentration of RNA, a 1:5 dilution was prepared with a final concentration of 4.4ng/µl. RNA of the L gene was transcribed and amplified with PMV primers. After sequence confirmation, the amplified DNA was cloned into the p-GEM®-T Easy Vector system (Promega, USA) according to the manufacturers' specifications. Ten white colonies were selected and transferred into 25ml of Luria-Bertani (LB) broth (2g NaCl (Sigma-Aldrich, USA), 2g tryptone (Merck chemicals) and 1g yeast extract (BioLab Diagnostics)) supplemented with ampicillin (0.1µg/µl) (Roche Diagnostics, Germany). The broth was incubated overnight at 37°C at 200 rpm in an orbital shaker incubator (Yihder, LM-530R. Taiwan).

Plasmid extraction was performed using the GeneJet™ Plasmid miniprep kit (Thermo Scientific, Fermentas, USA) according to the manufacturers' specifications. This was performed in duplicate after which one tube containing the extracted plasmid was stored at -20°C and the other at -70°C. The plasmids (4.2ng/µl) were subsequently used as positive control for the PMV PCR reactions. As negative control for both PCR reactions, nuclease-free water (Promega, USA) was used.

2.1.2.4 Sample processing and RNA extraction

Samples were processed similarly to as described in section ***2.1.1.4***. For RNA extraction, the tissue was added to 750µl TRizol® reagent (Invitrogen, USA). Two sterile stainless steel beads (5mm, Qiagen®, USA) was added to each tube and the tissue was lysed using the TissueLyser II system (Qiagen®, USA). Beads were subsequently removed with a magnet and samples were further processed in the TRizol® reagent (Invitrogen, USA) as per manufacturers' instructions.

2.1.2.5 Reverse transcription to gel extraction

The process involved from reverse transcription up to the analysis and extraction of positive samples from the gel was the same as described in section ***2.1.1.5*** - ***2.1.1.7***. Two separate PCR reactions were however done for each extracted sample, one RMH reaction and one PMV reaction.

2.1.2.6 Sequence amplification and sequencing

Sequencing amplification was done in the same manner as described in section ***2.1.1.8***. Sequencing purification was performed according to the standard sequencing protocol of the Virology Research Group, Department of Microbiology and Plant Pathology,

University of Pretoria incorporating the EDTA/NaOAc/EtOH method (BigDye® Terminator v3.1 kit cycle sequencing protocol, Applied Biosystems, 2002). Briefly, 1µl of 125µM EDTA (Ethylenediaminetetraacetic acid) was added to each sample. This was followed by 1µl of 3M sodium acetate (NaOAc) and 25µl of 100% non-denatured ethanol (EtOH, Merck chemicals). Samples were vortexed and incubated at room temperature for 15 minutes. The samples were subsequently centrifuged at 10 000 g in a minispin centrifuge (Eppendorf, Germany) for 25 minutes. The supernatant was removed and 100µl of 70% EtOH (Merck chemicals) was added. Tubes were again subjected to centrifugation at the same speed for 15 minutes. Supernatant was then removed. For a more purified product the latter three steps were repeated. Pellets were dried by incubating samples for 1 minute at 94°C using an AccuBlock™ Digital Dry Bath (Labnet International, Inc) and stored at -20°C until sequencing. Samples were subsequently submitted to the sequencing facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria and analysed on an ABI 3100 automated capillary sequencer analyser.

2.1.3 Data analysis of detected paramyxovirus sequences

2.1.3.1 Sequence editing

Raw sequence data generated was analysed with BioEdit Sequence alignment editor v7.0.5.3 (Copyright© 1997-2005 Tom Hall). Manual base-calling was done where needed. Visual analysis of sequences was done to trim off low quality sections and primer sequences on both the 5' and 3' ends. All trimmed reverse sequences were reverse-complemented to obtain the complementary sequence in the right orientation. For each sample, the forward and reverse trimmed sequences were pairwise aligned using the Clustal W multiple alignment function in BioEdit Sequence alignment editor to obtain the consensus sequence. The consensus sequence of each positive sample was subsequently used for phylogenetic analysis. For the PMV-primer derived sequences, the average length after trimming was 510bp while the average length for the RMH-derived primers was 460bp.

2.1.3.2 Phylogenetic analysis

For phylogenetic analysis, several representative viruses per paramyxovirus genus were selected for comparison (**Table 2.4**). Due to the vast amount of bat-associated paramyxoviruses detected in past two or so years, only a few representatives were

selected for analysis along with the more established paramyxoviruses (**Table 2.5**). Where only a few sequences were available per research output, all were used for phylogenetic analysis while a representative from each clearly visible cluster was selected from Baker *et al.* (2012) and Drexler *et al.* (2012) research (**Table 2.5**).

Table 2.4: Previously identified paramyxoviruses used for phylogenetic analysis.

Genus	Virus	Host range	GenBank accession #
Avulavirus	Avian paramyxovirus 6	Birds	NC_003043
	Goose paramyxovirus SF02	Geese	NC_005036
	Newcastle disease virus	Domestic/wild birds	NC_002617
Henipavirus	Hendra virus	Bats, horses, humans	NC_001906
	Nipah virus	Bats, pigs, humans	NC_002728
	Cedar virus	Bats	JQ001776
Morbillivirus	Canine distemper virus	Dogs	NC_001921
	Feline morbillivirus	Cats	JQ411014
	Measles virus	Humans	NC_001498
	Peste-des-petits-ruminants virus	Sheep, goats	NC_006383
	Rinderpest virus	Cattle	NC_006296
Respirovirus	Bovine parainfluenza virus 3	Cattle	NC_002161
	Human parainfluenza virus 1	Humans	NC_003461
	Sendai virus	Mice, hamsters, rats	NC_001552
	Swine parainfluenza virus 3	Pigs	EU439429
Rubulavirus	Mapuera virus	Bats	NC_009489
	Menangle virus	Bats, pigs	NC_007620
	Mumps virus	Humans	NC_002200
	Porcine rubulavirus	Pigs	NC_009640
	Simian virus 41	Monkey, apes	NC_006428
	Tioman virus	Bats	NC_004074
	Tuhoko virus 1	Bats	GU128080
	Tuhoko virus 2	Bats	GU128081
	Tuhoko virus 3	Bats	GU128082
Ferlavirus	Fer-de-Lance virus	Reptiles	NC_005084
*Jeilongvirus**	Beilong virus	Rats	NC_007803
	J-virus	Rats	NC_007454
Unclassified	Mossman virus	Rats	NC_005339
	Nariva virus	Rats	FJ362497
	Tupaia paramyxovirus	Shrew	NC_002199

* Proposed genus (Li *et al.* 2006)

Table 2.5: Selected newly detected paramyxoviruses used in phylogenetic analysis.

Selected newly detected paramyxoviruses used for PMV-sequence analysis		
Reference	**Virus**	**Accession #**
Kurth *et al.,* 2012	Paramyxovirus bat/E95/2009	JN086954
	Paramyxovirus bat/E20/2009	JN086953
	Paramyxovirus bat/E155/2009	JN086952
Baker *et al.,* 2012	Eidolon helvum PMV U9D	JN648089
	Eidolon helvum PMV U50A	JN648068
	Eidolon helvum PMV U42B	JN648057
	Eidolon helvum PMV U6A	JN648085
	Eidolon helvum PMV U69D	JN648084
	Eidolon helvum PMV U53A	JN648070
	Eidolon helvum PMV U52C	JN648069
Weiss *et al.,* 2012	EPMV_RC09_236u1	HE647835
	EPMV_RC09_239s	HE647831
Sasaki *et al.,* 2012	Paramyxovirus IFBPV01/2010	AB691542
	Paramyxovirus IFBPV39/2011	AB691545
	Paramyxovirus IFBPV32/2012	AB710472
	Paramyxovirus IFBPV25/2011	AB691543
Selected newly detected paramyxoviruses used for RMH-sequence analysis		
Reference	**Virus**	**Accession #**
Weiss *et al.,* 2012	EPMV_RC09_222k	HE647825
	EPMV_RC09_237s	HE647828
	EPMV_RC09_236u	HE647827
Sasaki *et al.,* 2012	Paramyxovirus IFBPV46/2011	AB748560
	Paramyxovirus IFBPV01/2010	AB748561
	Paramyxovirus IFBPV32/2011	AB748559
Baker *et al.,* 2012	Eidolon helvum PMV U61A	JN862578
	Eidolon helvum PMV U58B	JN862583
Wilkinson *et al.,* 2012	Min_gleni SMG16468	JQ886097
	Tri_menamena SMG16462	JQ886096
	Mor_acetabulosus SMG17000	JQ886105
	Min_griveaudi SMG16753	JQ886102
Drexler *et al.,* 2012	Paramyxovirus bat/GH36/2008	FJ609192
	Paramyxovirus bat/GH27a/2009	FJ971940
	Bat PMV Col_afr/GB09478/GAB/2009	HQ660155
	Bat PMV Hip_caf/GB59-30/GHA/2009	HQ660161

The polymerase gene for each virus was obtained from GenBank (NCBI). These sequences were aligned with the primers used for amplification to obtain the same gene region amplified in the newly detected sequences. Alignments were done using the MAFFT v6 online alignment tool (Copyright © 2011 Kazutaka Katoh available online at

mafft.cbrc.jp/alignment/software/; Katoh *et al.,* 2002; Katoh & Toh, 2008). This alignment tool provides a superior quality alignment when using large data sets. Two alignments sets were obtained from the two different primer sets used. Primer sequences were trimmed on both the 5' and 3' end where needed. This resulted in a nucleotide region of approximately 444nt for the RMH alignment and 556nt for the PMV alignment of the genome regions each primer set corresponds to (section ***2.1.1.3***). In the case of the selected set of novel representative viruses, only a short fragment of the polymerase gene sequence was available on GenBank. These fragments however span the same gene regions as the ones targeted in this study and could thus be used in phylogenetic analysis, as most researchers used the same primer sets.

Phylogenetic tree construction of the nucleotide sequences was done using a Bayesian approach. BEAST v1.7.4 and associated programs included in this package (available online from http://beast.bio.ed.ac.uk) was used for tree construction. Briefly, all trimmed nucleotide sequences of previously identified and novel paramyxoviruses were aligned with the positives samples detected in this study using the MAFFT online alignment tool (Copyright© 2011 Kazutaka Katoh, Katoh *et al.,* 2002; Katoh & Toh, 2008). Two final alignments for phylogenetic analysis were obtained. Each alignment was analysed with the jModelTest software v.0.1.1. (Posada, 2008) to determine the best fit model for each alignment set. The best fit model for the RMH data subset was determined to be GTR+G while for the PMV subset the GTR+G+I model was determined as most suitable. The BEAUTi tool included in the BEAST phylogenetic package was subsequently used to convert the alignment files to a usable format required for the BEAST analysis. A BEAST phylogenetic analysis was run for each subset with the corresponding model.

2.1.3.3 Comparison with parallel research

For a more in depth comparison of the results obtained in this study and the parallel study that was done by Drexler *et al.* (2012), sequences believed to group within the *Henipavirus*, *Morbillivirus* and *Jeilongvirus* genera was compared to corresponding sequences reported by Drexler *et al.* (2012). Sequences believed to belong to the same genus were aligned using the MAFFT online alignment tool (Copyright© 2011 Kazutaka Katoh, Katoh *et al.,* 2002; Katoh & Toh, 2008) and subsequently subjected to the jModelTest software v.0.1.1 (Posada, 2008) to determine the best fit model. This was done for all alignments. According to this test the best fit models for the henipavirus-related data

set was the TIM2+I (transition model) and SYM+I+G (symmetrical model) for the morbilli-/jeilongvirus-related data set. Phylogenetic analysis was subsequently conducted in the same manner as described in section ***2.1.3.2***.

2.1.3.4 Amino acid analysis

An amino acid analysis was also performed on recently detected RMH-derived paramyxovirus sequences from this study. Briefly, the nucleotide sequences were converted to the amino acid sequences in the correct reading frame using the BioEdit Sequence alignment editor v7.0.5.3 (Copyright© 1997-2005 Tom Hall) translate function. Amino acid sequences were subsequently aligned together with Hendra- and Nipah virus amino acid sequence from this region as reference, using the MAFFT online alignment tool (Copyright© 2011 Kazutaka Katoh, Katoh *et al.,* 2002; Katoh & Toh, 2008). Sequences were subsequently trimmed to obtain the same length (115 amino acids). A sequence identity matrix of this data was produced in BioEdit Sequence alignment editor v7.0.5.3 (Copyright© 1997-2005 Tom Hall).

Based on work done by Drexler *et al.* (2012) the amino acid similarity between Hendra and Nipah virus for this region was determined to range between 93 and 92.5% (7.0-7.5% dissimilarity). This approach was used as guideline for the maximum amino acid similarity between two sequences classified as different species. Due to shorter nucleotide sequence as a result of trimming bad quality ends, the amino acid region used for the comparison in this study was shorter than the 186 amino acid length of Drexler *et al.* (2012). The amino acid similarity between Hendra and Nipah virus for this specific trimmed region was subsequently determined to also be 93% and sequences sharing a similarity of any value above this were considered to be the same species.

To give relative information regarding similarity between different genera, an amino acid similarity analysis was also conducted between representative members of each established genus as well as the unclassified viruses. The exact procedure and genome region used for the previously mentioned amino acid analysis was used for genus comparison. Amino acid sequences from this study considered to be putative viral species were also analysed to determine the similarity between them as well as a representative virus from the *Henipavirus* (Hendra virus NC_001906), *Morbillivirus* (measles virus NC_001498) and *Jeilongvirus* (Beilong virus NC_007803) genera.

As a last approach, the amino acid sequences of the presumed putative sequences detected in this study were analysed with that of the sequences detected by Drexler *et al.* (2012). All sequences considered to the same species were removed from the data set of this analysis and residual sequences were compared to Baker *et al.* (2012), Weiss *et al.* (2012), Wilkinson *et al.* (2012) and Sasaki *et al.* (2012). This was done to determine whether these sequences are unique or considered as the same viral species as that of previously described bat-associated paramyxoviruses. All amino acid analysis was done in the same manner as discussed above using the exact genome region as before.

2.1.4 Phylogeography of paramyxoviruses across Africa

For more information regarding paramyxoviruses on the African continent, all RMH-derived paramyxovirus sequences from African bats that were available on the GenBank sequence database of The National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) (Baker *et al.,* 2012; Drexler *et al.,* 2012; Weiss *et al.,* 2012; Wilkinson *et al.,* 2012; **Table A4**) as well as those detected in this study were used for analysis (**Table A2**). RMH-derived sequences were selected over PMV-derived sequences as the former group had more available sequences. All sequences were aligned using the MAFFT v6 online alignment tool (Copyright © 2011 Kazutaka Katoh, Katoh *et al.,* 2002; Katoh & Toh, 2008) available online at mafft.cbrc.jp/alignment/software/) and subsequently edited in BioEdit Sequence alignment editor v7.0.5.3 (Copyright© 1997-2005 Tom Hall). During editing of sequences, they were trimmed to obtain sequences of a similar length. The alignment was subsequently used for further analysis

The jModelTest software v.0.1.1 (Posada, 2008) was used to determine the best fitting DNA substitution model for the data set. The GTR + G model was selected and used for phylogenetic analysis. BEAST v1.7.4 (available online from http://beast.bio.ed.ac.uk) was used to infer the phylogeographic patterns evident from the phylogenetic relationships between viruses detected in different countries. This was achieved by importing the above alignment along with the country of detection as trait (location) into the BEAUTi software (included in the BEAST phylogenetic package). A partition was created from the trait and the expansion growth curve was selected as demographic model. Locations for the internal nodes were inferred using an asymmetric continuous-time Markov model. All parameters were estimated in a Monte Carlo Markov Chain of 50 million steps, saving 10 000 samples. The same model and alignment were used to repeat the analysis with another chain of 50

million steps (Lemey *et al.,* 2009). Both chains were visually inspected using the Tracer software (available online from http://tree.bio.ed.ac.uk/software/tracer/) to check for convergence and signs of auto-correlation. These chains were subsequently combined using LogCombiner v1.7.4 (part of the BEAST phylogenetic package) with a burn-in of 10%. The combined chain was summarized as a maximum clade credibility tree using TreeAnnotator v1.7.4 (BEAST package) and visualized using FigTree v1.4.0 (2006-2012 Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh).

2.2 RESULTS

2.2.1 Primer selection

Due to the availability of only a short sequence from the L gene of these recently detected novel viruses, conserved regions for primer design was limited. The first set of primers (P1F1, P1F2 and P2R) was designed in such a way that both forward primers would bind outside the available sequence of the newly detected viruses while the reverse primer was specific for all 5 sequences used (**Table 2.2**) as seen in the primer alignment in **Figure 2.3A**. Due to the primer bias of the two forward primers towards the *Henipavirus* genus, these primers were unsuccessful in the amplification of the *Otomops* positive samples. Following this, the second set of degenerate primers (P2F1, P2F2 and P2R) was designed to all bind to available sequences of these three novel African paramyxoviruses as shown in **Figure 2.3B**. Although the bias towards the henipaviruses was removed with these primers, they also failed to amplify the positive controls.

With only short sequences of three novel bat-associated paramyxovirus L genes from the African continent available, finding conserved regions for primer design proved to be challenging. The several semi-conserved regions selected by Dr. Kuzmin as primer binding sites did not provide a usable set of primers in any of the combinations used as none of the positive controls were amplified in either the first or the second round of amplification. During evaluation of the above primer sets, novel bat-associated paramyxoviruses from Europe were published on the GenBank sequence database of The National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The sequences provided were of the same L gene region as the African bat paramyxoviruses and could subsequently be used in the same alignment to develop more specific primer sequences (**Figure 2.4**). These primers, termed PAR-F1, PAR-F2 and PAR-R (**Table 2.2**), were only capable of amplifying two of the five positive *Otomops* positive samples.

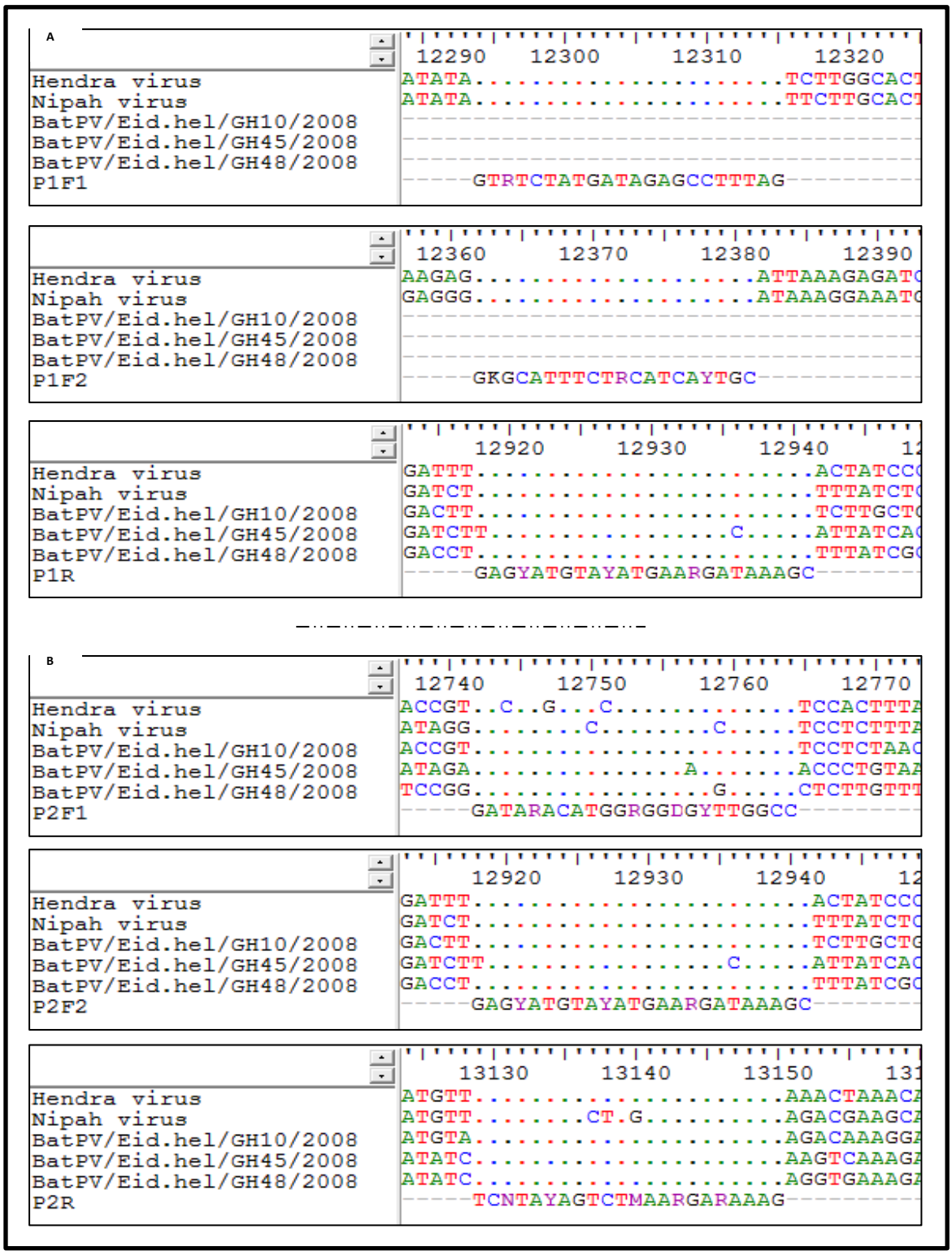


Figure 2.3: **Primer alignments with selected paramyxovirus sequences** of the polymerase gene region. **A** – Primer set 1 (P1F1, P1F2 and P1R); **B** – Primer alignment of primer set 2 (P2F1, P2F2 and P2R). (GenBank accession numbers available in section ***2.1.1.3***)

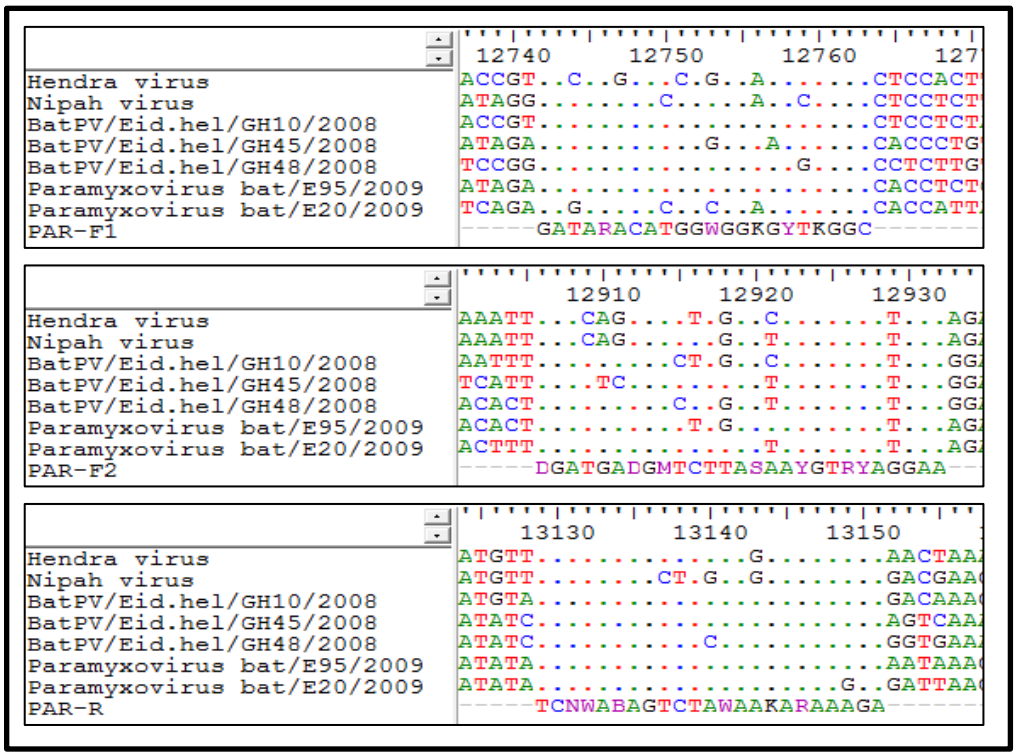


Figure 2.4: Primer alignment of primer set 4 (PAR-F1, PAR-F2 and PAR-R). (GenBank accession numbers to all sequences used are available under section ***2.1.1.3***).

Due to the lack of or limited amplification of the positive controls with the above primer sets, two of the universal primer sets (set 5 and 6 **Table 2.2**) designed by Tong *et al.* (2008) were selected and compared to each other. The *Paramyxovirinae* specific primers (PMV) were also only capable of amplifying two of the positive control samples while the RMH primers successfully amplified four out of five samples. Differences in the ability to amplify virus from the *Otomops* positives samples led to the selection of the RMH- and not PMV-primer set for paramyxovirus screening of the African bat kidney samples.

2.2.2 Analysis of positive controls

Sequences derived from the *Otomops martiensseni* positive samples, grouped outside of proposed *Jeilongvirus* genus **Figure 2.5**. Results showed that the sequences were closely related to each other. The fifth known positive received that did not produce an amplicon upon PCR amplification also groups outside of this genus but separately from the other four known positives (Tong *et al.,* unpublished).

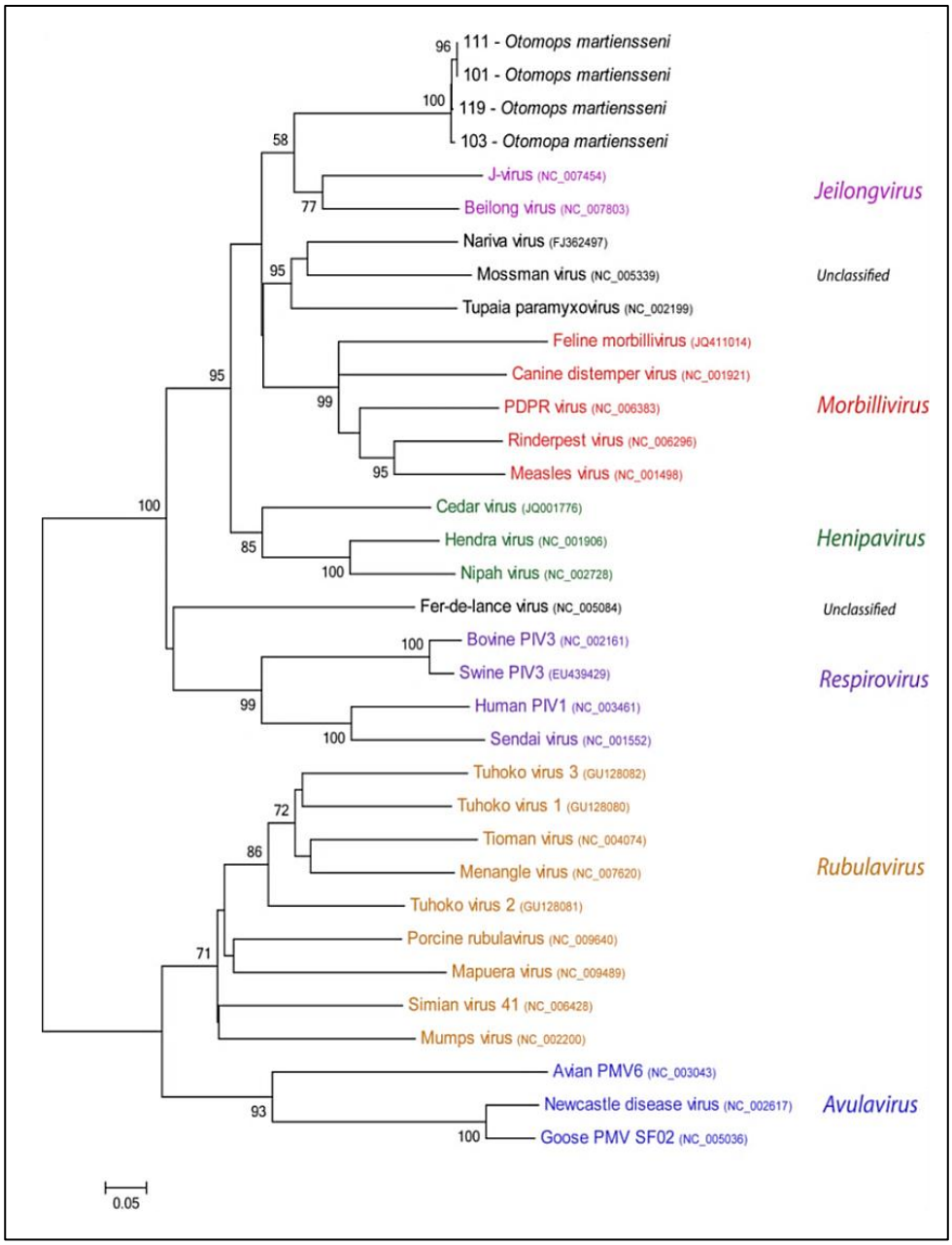


Figure 2.5: Neighbour-joining analysis of paramyxoviral positive control sequences. Sequences were derived from four *Otomops martiensseni* positive samples. Analysis was done with a bootstrap value of 1000 (Mega v5.05 software).

2.2.3 Sample screening

A total of 1220 bat kidney samples were screened for the presence of paramyxoviruses. All samples were screened with the RMH-primer set, while only the 243 samples derived from South Africa (SA) and Swaziland were additionally screened with the PMV-primer set. A summary per region is given in **Table 2.6**. Percentage positivity per

country increased with the number of samples screened ranging between 0% to a high 10.7%. Swaziland, where only four samples were available, no positives were detected while Kenya with the highest number of samples screened produced the highest percentage positivity.

Table 2.7 gives a more detailed list of the various species sampled, the number of samples screened per species and the number of positive samples detected (GenBank accession numbers to all sequences detected are listed in **Table A2**). At least 48 different bat species were represented in this study. This number excludes bats that were only classified down to genus level.

Table 2.8 gives a more detailed description of each individual positive sample. Positive samples obtained, originated from thirteen Chiropteran genera belonging to multiple families. Of the thirteen genera, at least 14 different bat species tested positive for paramyxoviral RNA. This number might be higher as several samples were only classified down to genus level. From the South African samples, both PMV and RMH derived sequences were obtained. Five samples only tested positive with PMV primers, six with both primer sets and five with only the RMH primers. The voucher numbers of samples kept at the Ditsong National Museum of Natural Science is listed in **Table A3**.

Table 2.6: Molecular screening summary per country.

Country	Cameroon	DRC	Kenya	Nigeria	SA	Swaziland
Samples screened	109	238	570	60	239	4
Positives detected	5	25	63	1	16	0
Positivity	4.6%	10.5%	11%	1.7%	6.6%	0%
Species sampled	7	18	19	5	37	1

Table 2.7: African bat species represented in this study and results.

AFRICAN BAT SPECIES SAMPLED
(number sampled/number positive)

South Africa

Chaerephon ansorgei (2/0)
Chaerephon pumilus (8/0)
Epomophorus gambianus (2/0)
Epomophorus walbergi (15/0)
***Eptesicus hottentotus* (2/1)**
Glauconycteris variegata (5/0)
Hipposideros caffer (4/0)
***Kerivoula argentata* (1/1)**
Miniopterus natalensis (5/0)
Miniopterus sp. (37/0)
Mops condylurus (7/0)
Neoromicia capensis (16/0)
Neoromicia helios (6/0)
***Neoromicia nana* (7/2)**
Neoromicia rueppellii (1/0)
Neoromicia zuluensis (1/0)
***Nycteris thebaica* (12/2)**
Nycticeinops schlieffeni (9/0)
Pipistrellus hesperidus (5/0)
Pipistrellus rusticus (5/0)
Pipistrellus sp. (5/0)
Rhinolophus darlingi (5/0)
***Rhinolophus denti* (5/3)**
Rhinolophus fumigatus (2/0)
***Rhinolophus landeri* (1/1)**
Rhinolophus simulator (2/0)
***Rhinolophus* sp. (6/5)**
Rousettus aegyptiacus (18/0)
Scotophilus sp. (12/0)
Sauromys petrophilus (1/0)
Scotophilus dinganii (25/0)
Scotophilus leucogaster (2/0)
Scotophilus nigrita (1/0)
Scotophilus dinganii (1/0)
Scotophilus viridis (3/0)
Tadaria aegyptiaca (5/0)
***Taphozous mauritianus* (3/1)**

Kenya

***Coleura afra* (27/10)**
Eidolon helvum (15/0)
Epomophorus labiatus (6/0)
Epomophorus wahlbergi (2/0)
Hipposideros commersoni (71/0)
***Hipposideros* sp. (8/1)**
***Miniopterus minor* (151/14)**
Miniopterus natalensis (15/0)
***Miniopterus* sp. (77/13)**
Neoromicia sp. (25/0)
***Nycteris* sp. (2/1)**
***Otomops martiensseni* (40/9)**
Rhinolophus landeri (12/0)
Rhinolophus sp. (14/0)
***Rousettus aegyptiacus* (84/2)**
Scotoecus sp. (2/0)
Scotophilus dinganii (2/0)
Taphozous sp. (1/0)
***Triaenops persicus* (16/12)**

Nigeria

Eidolon helvum (20/0)
Hipposideros commersoni (8/0)
***Hipposideros* sp. (3/1)**
Lissanycteris angolensis (8/0)
Rousettus aegyptiacus (21/0)

DRC

Chaerephon pumilus (25/0)
Chaerephon sp. (22/0)
Eidolon helvum (22/0)
Glauconycteris argentata (1/0)
***Hipposideros fuliginosus* (21/3)**
Hipposideros gigas (2/0)
Hypsignathus monstrosus (2/0)
Megaglossus woermanni (10/0)
Micropteropus pussilus (1/0)
Mimetillus moloneyi (1/0)
***Miniopterus* sp. (41/2)**
Mops condylurus (33/0)
Myonycteris torquata (8/0)
Myotis sp. (3/0)
Neoromicia sp. (1/0)
***Pipistrellus* sp. (40/20)**
Rhinolophus sp. (1/0)
Scotophilus dinganii (2/0)

Cameroon

Chaerephon sp. (32/0)
Eidolon helvum (15/0)
Epomophorus sp. (1/0)
***Hipposideros* sp. (39/1)**
***Rhinolophus* sp. (9/1)**
Scotophilus dinganii (1/0)
***Taphozous* sp. (12/3)**

Swaziland

Nycteris thebaica (4/0)

Table 2.8: Summary of paramyxovirus positive samples.

Sample number	Bat species	Country	Location	Year sampled
Cam-45	*Rhinolophus* sp.	Cameroon	Caves[18]	2010
Cam-49	*Taphozous* sp.	Cameroon	Caves[18]	2010
Cam-84	*Taphozous* sp.	Cameroon	Caves[18]	2010
Cam-88	*Hipposideros* sp.	Cameroon	Caves[18]	2010
Cam-99	*Taphozous* sp.	Cameroon	Caves[18]	2010
DRC-04	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-08	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-09	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-10	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-11	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-51	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-54	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-72	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-75	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-77	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-79	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-82	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-83	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-85	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-86	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-90	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-92	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-94	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-112	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-113	*Pipistrellus* sp.	DRC	Kinshasa, school 2[29]	2011
DRC-216	*Miniopterus* sp.	DRC	Mbanza Ngungu Cave[30]	2011
DRC-231	*Miniopterus* sp.	DRC	Mbanza Ngungu Cave[30]	2011
DRC-328	*Hipposideros fuliginosis*	DRC	Kisangani rain forest, Layoko[27]	2011
DRC-388	*Hipposideros fuliginosis*	DRC	Kisangani rain forest, Layoko[27]	2011
DRC-399	*Hipposideros fuliginosis*	DRC	Kisangani rain forest, Layoko[27]	2011
Ken-170	*Triaenops persicus*	Kenya	Three caves[11]	2010
Ken-181	*Triaenops persicus*	Kenya	Three caves[11]	2010
Ken-217	*Hipposideros* sp.	Kenya	Jimba cave[14]	2010
Ken-219	*Coleura afra*	Kenya	Jimba cave[14]	2010
Ken-221	*Coleura afra*	Kenya	Jimba cave[14]	2010
Ken-241	*Triaenops persicus*	Kenya	Jimba cave[14]	2010
Ken-243	*Triaenops persicus*	Kenya	Jimba cave[14]	2010
Ken-279	*Coleura afra*	Kenya	Jimba cave[14]	2010
Ken-292	*Triaenops persicus*	Kenya	Jimba cave[14]	2010
Ken-298	*Coleura afra*	Kenya	Jimba cave[14]	2010
Ken-300	*Triaenops persicus*	Kenya	Jimba cave[14]	2010
Ken-345	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-355	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-402	*Coleura afra*	Kenya	Three caves[11]	2011

Table 2.8 [Continued]: Summary of paramyxovirus positive samples.

Sample number	Bat species	Country	Location	Year sampled
Ken-412	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-414	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-415	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-434	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-435	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-438	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-439	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-462	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-474	*Triaenops persicus*	Kenya	Three caves[11]	2011
Ken-484	*Coleura afra*	Kenya	Three caves[11]	2011
Ken-490	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-491	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-492	*Otomops martiensseni*	Kenya	Suswa cave[13]	2011
Ken-514	*Otomops martiensseni*	Kenya	Suswa cave[13]	2010
Ken-534	*Otomops martiensseni*	Kenya	Suswa cave[13]	2010
Ken-678	*Miniopterus minor*	Kenya	Three caves[11]	2011
Ken-681	*Triaenops persicus*	Kenya	Three caves[11]	2011
Ken-708	*Miniopterus minor*	Kenya	Three caves[11]	2011
Ken-709	*Triaenops persicus*	Kenya	Three caves[11]	2011
Ken-712	*Nycteris* sp.	Kenya	Three caves[11]	2011
Ken-718	*Triaenops persicus*	Kenya	Three caves[11]	2011
Ken-721	*Coleura afra*	Kenya	Three caves[11]	2011
Ken-740	*Miniopterus* sp.	Kenya	Kericho cave[5]	2011
Ken-747	*Miniopterus* sp.	Kenya	Kericho cave[5]	2011
Ken-756	*Miniopterus* sp.	Kenya	Kericho cave[5]	2011
Ken-757	*Miniopterus* sp.	Kenya	Kericho cave[5]	2011
Ken-765	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-766	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-769	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-776	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-787	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-789	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-793	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-794	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-795	*Miniopterus* sp.	Kenya	Gilgil mine[3]	2011
Ken-803	*Miniopterus minor*	Kenya	Jimba cave[14]	2011
Ken-804	*Miniopterus minor*	Kenya	Jimba cave[14]	2011
Ken-808	*Triaenops persicus*	Kenya	Jimba cave[14]	2011
Ken-809	*Triaenops persicus*	Kenya	Jimba cave[14]	2011
Ken-814	*Coleura afra*	Kenya	Jimba cave[14]	2011
Ken-815	*Coleura afra*	Kenya	Jimba cave[14]	2011
Ken-839	*Rousettus aegyptiacus*	Kenya	Three caves[11]	2011
Ken-841	*Rousettus aegyptiacus*	Kenya	Three caves[11]	2011
Ken-856	*Coleura afra*	Kenya	Three caves[11]	2011

Table 2.8 [Continued]: Summary of paramyxovirus positive samples.

Sample number	Bat species	Country	Location	Year sampled
Ken-857	*Miniopterus minor*	Kenya	Jimba cave[14]	2011
Ken-877	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-887	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Ken-898	*Miniopterus minor*	Kenya	Pare cave[11]	2011
Nig-955	*Hipposideros* sp.	Nigeria	College of Agriculture[23]	2010
SA-160	*Rhinolophus denti*	South Africa	Taung [16]	2007
SA-163*	*Rhinolophus denti*	South Africa	Taung [16]	2007
SA-170*	*Eptseicus hottentotus*	South Africa	Taung [16]	2007
SA-172	*Rhinolophus denti*	South Africa	Taung [16]	2007
SA-190	*Nycteris thebaica*	South Africa	Madikwe Game Reserve [11]	2007
SA-712*	*Taphozous mauritianus*	South Africa	Pafuri, Kruger National Park [13]	2010
SA-724	*Neoromicia nana*	South Africa	Pafuri, Kruger National Park [13]	2010
SA-844*	*Kerivoula argentata*	South Africa	Rocktail bay, St Lucia [15]	2010
SA-855	*Nycteris thebaica*	South Africa	Rocktail bay, St Lucia [15]	2010
SA-922	*Neoromicia nana*	South Africa	Pafuri, Kruger National Park [13]	2010
SA-947	*Rhinolophus landeri*	South Africa	Pafuri, Kruger National Park [13]	2010
SA-1485*	*Rhinolophus* sp.	South Africa	Orrie/Matlapitsi cave [12]	2012
SA-1486*	*Rhinolophus* sp.	South Africa	Orrie/Matlapitsi cave [12]	2012
SA-1493	*Rhinolophus* sp.	South Africa	Orrie/Matlapitsi cave [12]	2012
SA-1494	*Rhinolophus* sp.	South Africa	Orrie/Matlapitsi cave [12]	2012
SA-1495	*Rhinolophus* sp.	South Africa	Orrie/Matlapitsi cave [12]	2012

***** *Tested positive with both primer sets (a=PMV and b=RMH)*
Location numbers as indicated in the table can be viewed in ***Figure 2.1*** *and* ***Figure 2.2***.

2.2.4 Phylogenetic analysis

2.2.4.1 PMV-primer derived sequence analysis

PMV derived sequences from the South African (SA) positive samples, were analysed separately along with previously identified and the more recent bat associated paramyxovirus sequences of the same L gene region (**Table 2.5**). SA positive samples all cluster together. The European bat paramyxoviruses detected by Kurth *et al.* (2012), E95 and E20 were most closely related. These newly detected viral sequences group outside of any of the established paramyxovirus genera, but formed a cluster with rat derived J- and Beilong viruses from the newly proposed *Jeilongvirus* genus (**Figure 2.6**). A noticeable grouping of *Rhinolophus* derived sequences was observed with the exception of SA-1485-*Rhinolophus* sp. grouping with the SA-712-*Taphozous mauritianus* positive sample.

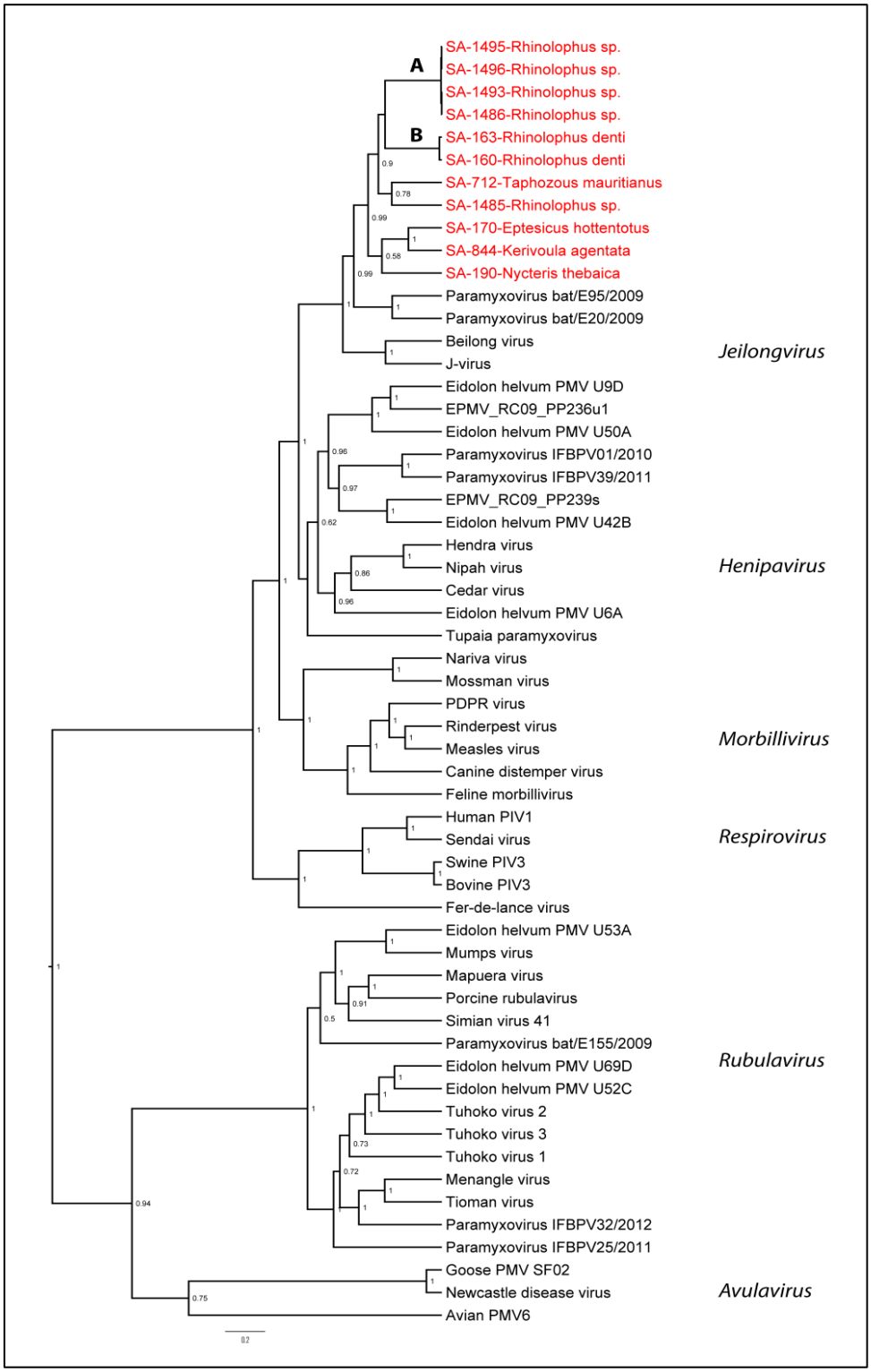


Figure 2.6: Phylogenetic analysis of novel viral sequences (510bp) detected in South Africa using the *Paramyxovirinae* specific primer set (PMV) targeting the L gene region. South African positives are indicated in red. GenBank accession numbers of established paramyxovirus sequences are listed in Table 2.5 and that of newly detected sequences are listed in Table A2 (Appendix). A and B designates the two geographical *Rhinolophus* clusters. (A= Orrie/Matlapitsi cave[12]; B= Taung[16])

The latter *Rhinolophus* sample (SA-1485) was sampled at the same time and location as the other *Rhinolophus* sp. samples (Orrie/Matlapitsi cave[12] in 2012) represented by **A** in **Figure 2.6** (**Table 2.8**). A geographical grouping was also observed for both *Rhinolophus* sp. and *Rhinolophus denti* (**Figure 2.6 A** and **B** respectively). The former group was detected in Limpopo province (Orrie/Matlapitsi cave[12]) while the latter was detected from North West Province (Taung[16]).

2.2.4.2 RMH-primer derived sequence analysis

The majority of paramyxovirus sequences in this study correspond to the genome region amplified by the RMH-primer set. The majority of newly detected bat-associated paramyxoviruses over the past few years were also amplified using the same primers and thus allowed the inclusion of several representatives of these sequences in the phylogenetic analysis (**Table 2.5**). For an improved analysis of the large data set, the phylogenetic tree was circularized (**Figure 2.7**).

Similar to what was observed in the PMV-derived sequence analysis, a vast number of sequences group outside of the known genera of this family. Several different clusters were observed within the phylogenetic tree. Aside from the *Avula*-, *Rubula*- and *Respirovirus* genera at the base of the tree, the other known genera and new clusters were labelled. At least six major clusters were present. The clusters were labelled with A-F and colour coded (**Figure 2.7**) to indicate each cluster.

Cluster A was referred to as the *Henipavirus* and -related viruses (Drexler *et al.,* 2012). Two of the Kenya sequences Ken-839 and Ken-841 derived from *Rousettus aegyptiacus,* phylogenetically grouped in this cluster. Two smaller clusters are observed in this larger cluster, one to which the known henipaviruses belong and the other into which the two Kenya samples cluster. All viral sequences in cluster A were derived from fruit bats. Cluster B was formed by the *Morbillivirus* genus as well as a cluster of unclassified viruses (Mossman, Nariva and Tupaia paramyxovirus). None of the newly detected sequences in this study grouped within this cluster.

Phylogenetic analysis showed the formation of a cluster (**Figure 2.7** C) between the *Morbillivirus* and proposed *Jeilongvirus* genus and could be considered as separate from these two genera. This cluster is enlarged in **Figure 2.8** where phylogenetic position was more evident. The majority of sequences in this cluster were derived from the *Miniopterus* genus.

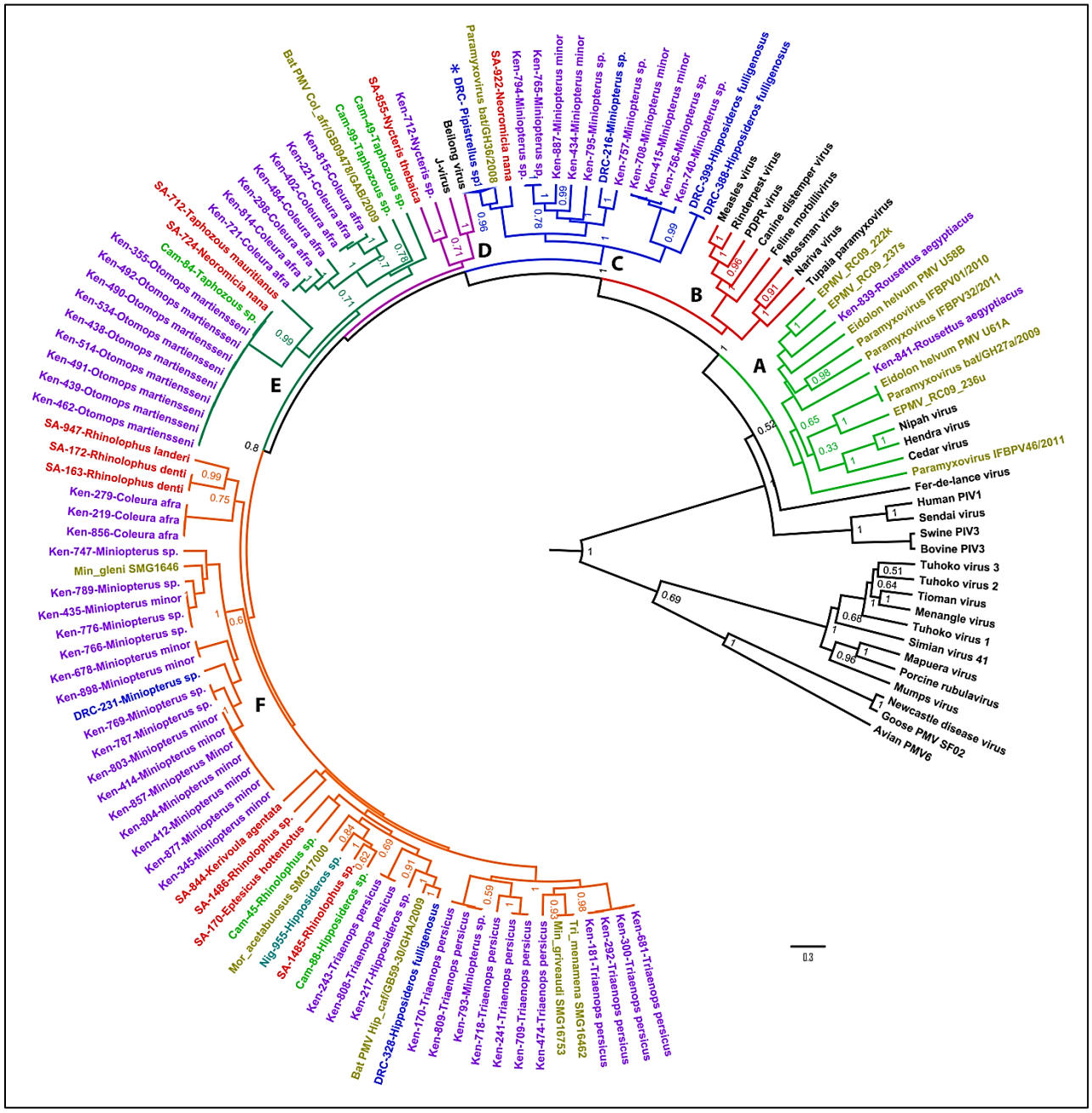


Figure 2.7: Bayesian phylogenetic analysis based on the partial polymerase (L) gene (430bp) of novel viral sequences detected in several countries across Africa using the *Respiro*-, *Morbilli*- and *Henipavirus* specific primer set (RMH). Letters A to F represents established and possibly novel paramyxovirus genera (each cluster region indicated by colour). Accession numbers to all newly detected paramyxovirus sequences are listed in Table A2. *Colour of labels– Purple: Kenya; Blue: DRC; Green: Cameroon; Red: South Africa; Turquoise: Nigeria; Gold: representative viruses recently detected; Black: Formerly identified paramyxoviruses.*

Many of these samples were taken in locations geographically close to each other (*e.g.* Three caves[11] and Pare cave[11] in Kenya). All DRC-*Pipistrellus* derived sequences grouped together as identical sequences and were most closely related to the Paramyxovirus

bat/GH36/2008 sequence from Drexler *et al.* (2012) classified as a morbillivirus-related virus. One South African sequence from *Neoromicia nana* (SA-922) grouped together in the smaller cluster with the DRC-*Pipistrellus* sequences and the GH36 sequence from Drexler *et al.* (2012).

In cluster D (**Figure 2.8**), representing the proposed *Jeilongvirus* genus (Li *et al.,* 2006), two newly detected sequences (SA-855-*Nycteris thebaica* and Ken-712-*Nycteris* sp.) group with J- and Beilong virus. These two new sequences were both derived from the *Nycteris* genus. Based on phylogenetic clustering these two novel viruses were considered as jeilongvirus-related viruses.

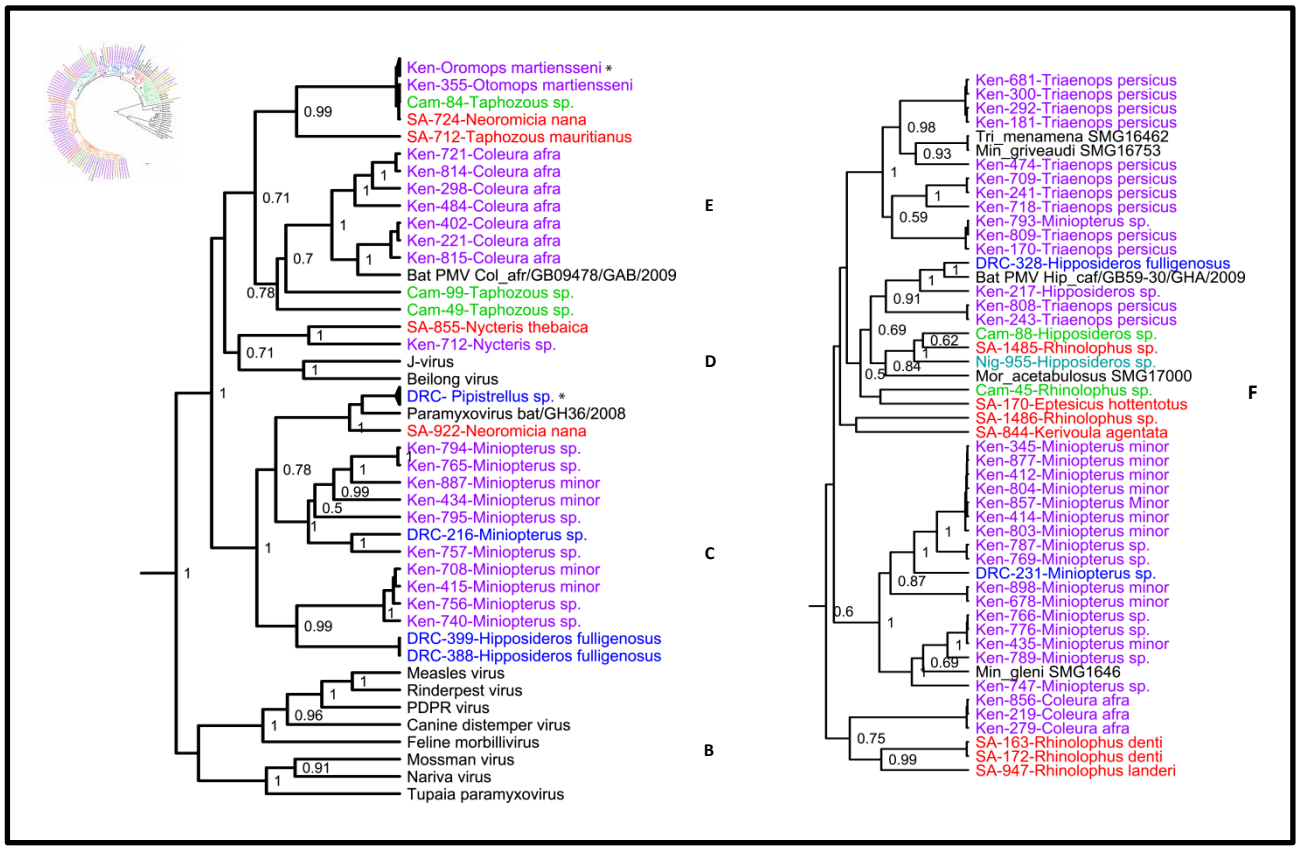


Figure 2.8: Enlarged view of clusters B to F observed in the RMH-sequence analysis. Stars represent clusters of identical viruses that were collapsed. Refer to Figure 2.7 for complete phylogenetic analysis.

The fifth cluster of interest (cluster E) also grouped separate from the established paramyxovirus genera. In this cluster three *Taphozous* sp. samples from the same location in Cameroon (Caves[18]) produced genetically diverse sequences. Similar to what was observed in the DRC-*Pipistrellus* colony in cluster C, several genetically identical viral

sequences were isolated from *Otomops martiensseni* individuals sampled from Suswa cave[13] in Kenya (**Figure 2.7**). These sequences were obtained from samples collected during two consecutive years (2010-2011). A large number of *Coleura afra* derived sequences also grouped in this cluster. These sequences were derived from two locations (Three caves[11] and Jimba cave[14] in Kenya). An identical virus was also isolated in two consecutive years from this species (Ken-221 in 2010 and Ken-402 in 2011). These sequences also group closely to the *Coleura afra* sequence (GB09478) detected by Drexler *et al.* (2012) from Gabon which was classified as a morbillivirus-related sequence. It was observed that an almost identical sequence was derived from Ken-355, Cam-84 and SA-724 representative of three different locations and three different species.

The last and biggest cluster, F (**Figure 2.8**), did not seem to be related to any of the established paramyxovirus genera and contained multiple smaller clusters. Similar to what was observed in **Figure 2.6**, geographical grouping was again observed in the *Rhinolophus* derived sequences (SA-172 and 163 from Taung[16]; SA-947 from Pafuri[13] in South Africa). A small *Coleura afra* cluster that formed, showed genetically identical viruses isolated from two consecutive years as well as from two different locations (Three caves[11] and Jimba cave[14] in Kenya). A large cluster consisting only of *Miniopterus*-derived sequences from Kenya was also noticed. This cluster contained a DRC positive and grouped with a *Miniopterus gleni*-derived sequence detected by Wilkinson *et al.* (2012) from Madagascar. The *Miniopterus* cluster contained samples derived from different locations. Two identical sequences (Ken-898 and Ken-678) were obtained from two different caves that are geographically close to each other. With the availability of more RMH-derived paramyxovirus sequences than that available for the PMV analysis, SA-844-*Kerivoula argentata* and SA-170-*Eptesicus hottentotus* grouped differently than observed in **Figure 2.6**. SA-170 now grouped closer to Cam-45 derived from *Rhinolophus.* A *Hipposideros fuliginosus* positive from DRC (DRC-328) and one from Kenya (Ken-217) grouped with a *Hipposideros caffer* positive (GB59-30) from Ghana detected by Drexler *et al.* (2012). This sequence was also considered as morbillivirus-related (Drexler *et al.,* 2012). Cluster F contained all the positive sequences derived from *Triaenops persicus* and showed the formation of several smaller clusters. Detection of the same sequence in two consecutive years was again observed (Ken-681 and Ken-300). Ken-474 grouped with two sequences detected by Wilkinson *et al.* (2012) from *Miniopterus griveaudi* and *Triaenops menamena*.

When considering the phylogenetic tree as a whole (**Figure 2.7**) it was also observed that a vast genetic divergence was present in the same location/colony sampled in numerous occasions. For example, this was observed in two different *Coleura afra* colonies in Kenya (Ken-814 and Ken-815 from Jimba cave[14] and Ken-484, Ken-721 and Ken-402 from Three caves[11]). Another example was that of *Miniopterus* for samples Ken-747, Ken-789 and Ken-766 sampled from Gilgil mine[3].

2.2.5 Comparison with parallel research

Comparison of sequences derived in this study with that of Drexler *et al.* (2012) was done in two parts. The first was a comparison of all the henipavirus-related viruses reported by Drexler *et al.* (2012) with the related sequences detected in this study. The phylogenetic relationship between these sequences is represented in **Figure 2.9**.

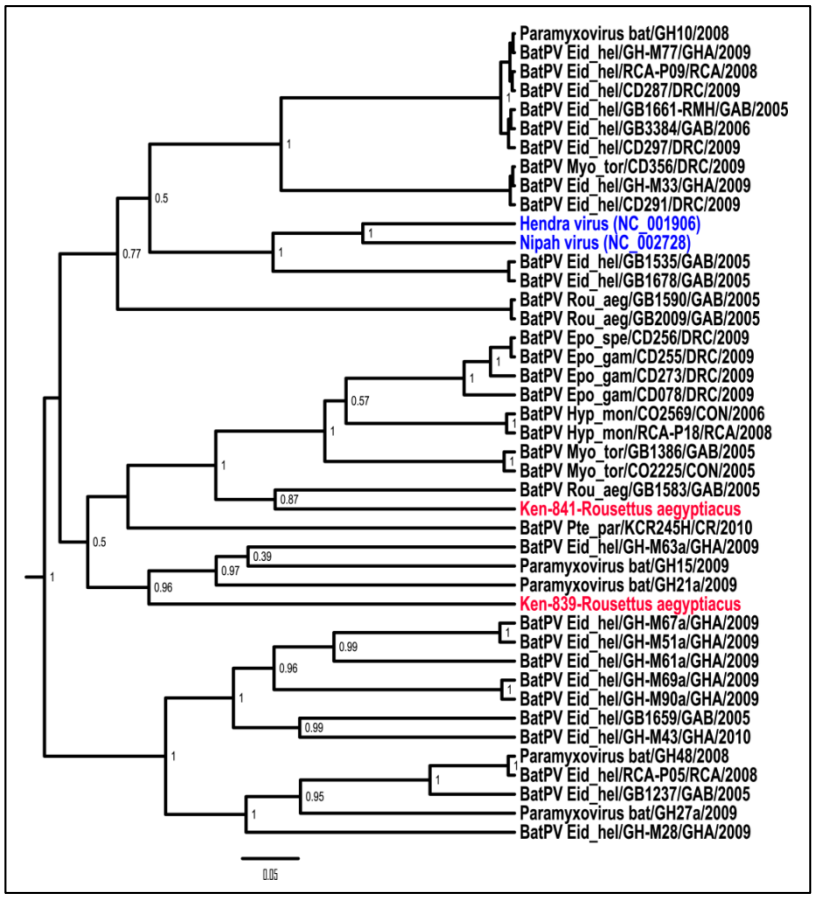


Figure 2.9: Phylogenetic comparison of henipavirus-related viruses (467bp). Bayesian phylogenetics were used to compare the partial L-gene sequences (amplified by the RMH-primers) detected by Drexler *et al.* (2012) with that detected in this study. Sequences in red were identified in this study, black detected by Drexler *et al.* (2012) and blue sequences represents Hendra and Nipah virus.

The first henipavirus-related isolate detected in this study, Ken-841-*Rousettus aegyptiacus* was most related to another sequence derived from the same species (GB1583). The genetic distance between these two sequences is higher than observed between Hendra and Nipah virus for the gene region analysed. The second sequence detected, Ken-831, also showed a vast genetic distance from any of the previously detected sequences, but was most closely related to three sequences derived from *Eidolon helvum* that was sampled in Ghana. Ken-831 specifically grouped with GH21a for which Drexler *et al.* (2012) determined that it had the order-wide GDNQ motif in the polymerase gene and not the GDNE motif seen in the recently established *Henipavirus* genus. Both sequences grouped in a sister-cluster compared to other known henipaviruses.

The second comparison was made between the proposed morbillivirus-related viruses detected by Drexler *et al.* (2012) and viruses detected in this study that belong to clusters B to E (**Figure 2.7**). The phylogenetic tree is represented by **Figure 2.10**. All sequences detected in this study grouped away from both the *Morbillivirus* and the proposed *Jeilongvirus* genus. None of the sequences detected in this showed a close genetic relationship with the sequences detected by Drexler *et al.* (2012). It was clearly noticed in the tree that there was species- and genus-specific clustering taking place in numerous occasions. A few major cluster examples were that of *Miniopterus* from Kenya, *Myotis* from Germany, and *Hipposideros* from West Africa. Two smaller *Coleura afra* clusters can be considered as one larger cluster if it wasn't for the *Hipposideros abae* sequence grouping between them. It was also observed that BatPV Hip_caf/GB59-30/GHA/2009 grouped away from the *Morbillivirus* genus (**Figure 2.10**) with the sequences from cluster F (**Figure 2.8**). The close genetic distance observed between the proposed *Jeilongvirus* genus and the two *Nycteris* sequences (SA-855 and Ken-712) in **Figure 2.8**, was increased with the inclusion of the vast number of sequences from Drexler *et al.* (2012).

2.2.6 Amino acid analysis

Table A5 provides the nucleotide similarity values (a score of 1 being identical) of all sequences detected in this study. Similarity as high as 100% was observed in several samples as indicated with red text in **Table A5**. The two *Rousettus* derived sequences (Ken-839 and Ken-841) were the only two sequences with a similarity of 50-60% compared to the other viruses detected in the study.

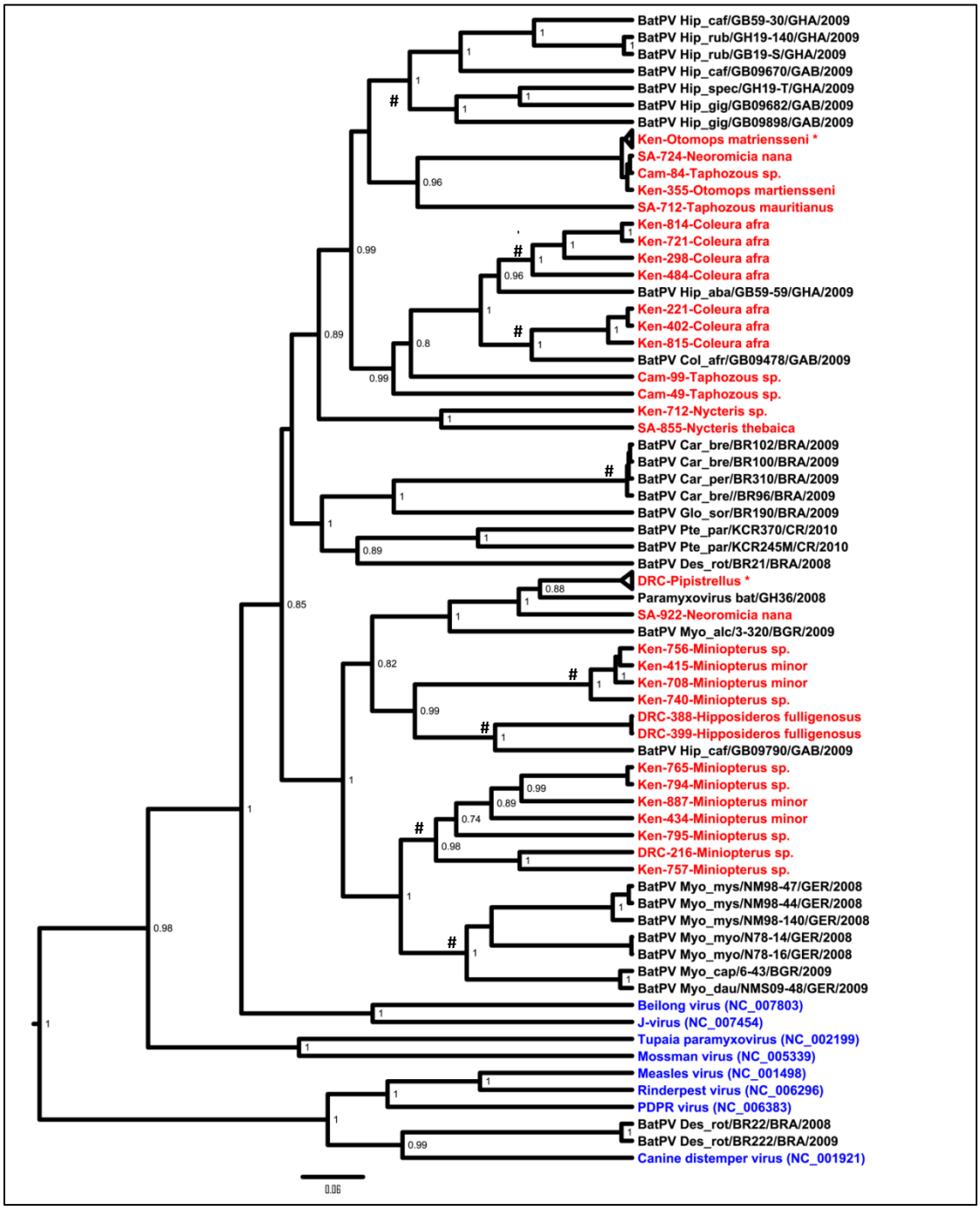


Figure 2.10: Phylogenetic comparison of unclassified sequences. Comparison was between partial L gene sequences detected by Drexler *et al.* (2012) and that detected in this study. Blue represents known paramyxoviruses, red the sequences detected in this study and black that of Drexler *et al.* (2012). Asterisks represent collapsed clusters of identical sequences. The # indicates species specific clusters.

All other sequences in this study shared a similarity of above 60%.The lowest similarity between all sequences analysed was 51%. This was seen between the positive Ken-839 from *Rousettus aegyptiacus* (grouping with henipavirus-related viruses) and Ken-298 from

Coleura afra (grouping outside of the proposed *Jeilongvirus* genus). Amino acid similarities between individuals from the same colony also varied in several cases for example the *Miniopterus* sp. colony in Gilgil mine[3]. Some sequences presented with a 100% similarity (Ken-766 and Ken-776) while a low similarity of 68% was observed between Ken-765 and Ken-769.

The amino acid similarity between Hendra and Nipah virus for the specific region used for analysis was determined to be approximately 93% (0.930). The maximum identity between two sequences while still considered as separate species was thus 93% with all values higher treated as the same species. Upon analysing the sequence identity matrix in **Table A5**, it was determined that the viruses detected in this study represent at least 36 putative bat-associated paramyxovirus sequences.

An amino acid similarity matrix of several representative paramyxoviruses is represented in **Table 2.9**. The same genomic region as in the previous discussed matrix was used. The highest similarity observed between two viruses (measles and Nipah virus) classified into two different genera (*Morbilli-* and *Henipavirus* respectively) was 62%. A higher similarity of 63% was also observed between canine distemper virus (*Morbillivirus*) and Tupaia paramyxovirus that remains unclassified to genus level. When using these values as a guideline for between genus similarity only the *Rousettus aegyptiacus* samples (Ken-839 and Ken-841) were considered to belong to a separate genus from the other sequences detected in this study, while based on the genus cut-off value determined, all other sequences supposedly group in a single genus. A summary of all sequences considered to be representative of a putative virus species is listed in **Table 2.10**. On amino acid level each putative sequence was compared to a representative of the *Henipavirus, Morbillivirus* and *Jeilongvirus* genus (**Table 2.11**).

When using the proposed genus cut-off value determined (**Table 2.9**), the majority of sequences from the putative species group with the *Jeilongvirus* genus. In two instances (Ken-757 and Ken-841) the same similarity was observed with Hendra- and Beilong virus. The only sequence with a value below the proposed cut-off was Ken-839.

Table 2.9: Amino acid similarity matrix of representative viruses in the *Paramyxovirinae* sub-family (L gene fragment).

Genus	Sequence	APMV 6	GPMV	NDV	Menangle	PRV	Mumps	SPV5	SV40	Tioman	Beilong	J-virus	BPIV 3	CDV	FDLV	HPIV1	HPIV3	Measles	Mossman	RPV	Sendai	Tupaia	Nipah	Hendra
Avula	NC_003043.1 **Avian paramyxovirus 6**	*	0.43	0.43	0.43	0.41	0.42	0.43	0.47	0.47	0.24	0.25	0.31	0.28	0.25	0.27	0.31	0.26	0.23	0.27	0.26	0.29	0.26	0.26
Avula	NC_005036.1 **Goose paramyxovirus SF02**		*	0.94	0.35	0.40	0.36	0.36	0.41	0.38	0.26	0.24	0.22	0.26	0.21	0.20	0.21	0.25	0.26	0.24	0.18	0.25	0.25	0.24
Avula	NC_002617.1 **Newcastle disease virus B1**			*	0.35	0.39	0.36	0.36	0.41	0.36	0.24	0.24	0.23	0.25	0.22	0.21	0.22	0.25	0.24	0.24	0.19	0.25	0.27	0.25
Rubula	NC_007620.1 **Menangle virus**				*	0.54	0.52	0.60	0.62	0.74	0.31	0.29	0.32	0.32	0.29	0.28	0.31	0.31	0.29	0.34	0.29	0.31	0.35	0.35
Rubula	NC_009640.1 **Porcine rubulavirus**					*	0.62	0.65	0.63	0.60	0.32	0.29	0.32	0.30	0.31	0.30	0.32	0.27	0.30	0.28	0.29	0.29	0.33	0.34
Rubula	NC_002200.1 **Mumps virus**						*	0.66	0.69	0.64	0.31	0.33	0.36	0.31	0.33	0.31	0.35	0.31	0.31	0.33	0.30	0.33	0.34	0.33
Rubula	AF_052755.1 **Simian parainfluenza virus 5**							*	0.78	0.61	0.31	0.32	0.36	0.31	0.34	0.33	0.35	0.29	0.31	0.28	0.32	0.31	0.35	0.34
Rubula	NC_006428.1 **Simian virus 41**								*	0.66	0.30	0.31	0.38	0.34	0.33	0.34	0.37	0.30	0.32	0.31	0.32	0.29	0.34	0.35
Rubula	NC_004074.1 **Tioman virus**									*	0.30	0.30	0.35	0.31	0.32	0.32	0.34	0.31	0.30	0.32	0.31	0.31	0.32	0.31
Jeilong	NC_007803.1 **Beilong virus**										*	0.80	0.45	0.61	0.51	0.50	0.45	0.60	0.64	0.62	0.48	0.61	0.58	0.56
Jeilong	NC_007454.1 **J-virus**											*	0.47	0.60	0.51	0.49	0.47	0.63	0.63	0.62	0.52	0.64	0.57	0.56
Respiro	NC_002161.1 **Bovine parainfluenza virus 3**												*	0.49	0.52	0.64	0.93	0.48	0.48	0.50	0.64	0.48	0.48	0.47
Morbilli	NC_001921.1 **Canine distemper virus**													*	0.49	0.44	0.48	0.77	0.60	0.81	0.46	**0.63**	0.61	0.60
Unknown	NC_005084.2 **Fer-de-lance virus**														*	0.55	0.53	0.47	0.50	0.47	0.55	0.45	0.48	0.48
Respiro	NC_003461.1 **Human parainfluenza virus 1**															*	0.66	0.46	0.48	0.47	0.89	0.44	0.46	0.45
Respiro	NC_001796.2 **Human parainfluenza virus 3**																*	0.47	0.48	0.49	0.68	0.47	0.48	0.48
Morbilli	NC_001498.1 **Measles virus**																	*	0.65	0.86	0.47	0.64	0.62	0.59
Unknown	NC_005339.1 **Mossman virus**																		*	0.64	0.49	0.69	0.57	0.56
Morbilli	NC_006296.2 **Rinderpest virus**																			*	0.48	0.65	**0.62**	0.61
Respiro	NC_001552.1 **Sendai virus**																				*	0.42	0.47	0.48
Unknown	NC_002199.1 **Tupaia paramyxovirus**																					*	0.58	0.58
Henipa	NC_002728.1 **Nipah virus**																						*	0.93
Henipa	NC_001906.2 **Hendra virus**																							*

Table 2.10: Summary putative viral species detected in this study.

Putative species representative	Sequences considered as same species (partial L-gene sequences)			Similarity to members of established genera (partial L-gene sequences)		
	Sequence numbers	Highest similarity	Lowest similarity	Hendra virus	Measles virus	Beilong virus
Cam-45	*None*	-	-	0.565	0.600	0.669
Cam-49	*None*	-	-	0.556	0.608	0.678
Cam-88	*None*	-	-	0.565	0.608	0.704
Cam-99	*None*	-	-	0.565	0.591	0.721
DRC-231	*None*	-	-	0.539	0.591	0.669
DRC-328	Ken-217	0.947	0.947	0.573	0.565	0.713
DRC-399	DRC-388	1.000	1.000	0.608	0.626	0.686
Ken-402	Ken-221, 815	1.000	1.000	0.556	0.582	0.678
Ken-474	*None*	-	-	0.547	0.626	0.669
Ken-681	Ken-181, 292, 300	1.000	0.991	0.556	0.617	0.686
Ken-712	*None*	-	-	0.539	0.591	0.695
Ken-718	Ken-241, 300	0.973	0.973	0.547	0.608	0.713
Ken-756	Ken-415, 708, 740	0.965	0.956	0.573	0.643	0.678
Ken-757	DRC-216	0.934	0.934	0.547	0.626	0.626
Ken-789	Ken-435, 747, 766, 776	1.000	0.965	0.565	0.617	0.695
Ken-795	*None*	-	-	0.591	0.626	0.678
Ken-808	Ken-243	1.000	1.000	0.582	0.608	0.660
Ken-809	Ken-170, 973	1.000	0.991	0.573	0.617	0.686
Ken-814	Ken-298, 484, 721	0.991	0.956	0.565	0.591	0.669
Ken-839	*None*	-	-	0.573	0.556	0.582
Ken-841	*None*	-	-	0.626	0.565	0.626
Ken-856	Ken-219, 279	1.000	1.000	0.573	0.626	0.686
Ken-877	Ken-345, 412, 414, 769, 787, 803, 804, 857	1.000	0.965	0.556	0.600	0.695
Ken-887	Ken-434, 765, 794	0.947	0.939	0.556	0.634	0.669
Ken-898	Ken-678	1.000	1.000	0.547	0.626	0.686
Nig-955	*None*	-	-	0.565	0.608	0.704
SA-170	*None*	-	-	0.556	0.565	0.669
SA-172	Ken-163	1.000	1.000	0.530	0.582	0.695
SA-712	*None*	-	-	0.582	0.573	0.695
SA-724	Cam-84, Ken-514, 534, 355, 438, 439, 462, 490, 491, 492	1.000	0.982	0.556	0.591	0.730
SA-844	*None*	-	-	0.504	0.608	0.695
SA-855	*None*	-	-	0.539	0.547	0.695
SA-922	DRC-*Pipistrellus* (all)	0.965	0.965	0.582	0.634	0.669
SA-947	*None*	-	-	0.530	0.565	0.695
SA-1485	*None*	-	-	0.565	0.626	0.704
SA-1486	*None*	-	-	0.556	0.600	0.713

** 'None' indicates no other sequence considered to be the same viral specie.*

** Red indicates the highest similarity of each particular sequence to the established paramyxoviruses.*

Table 2.11: Amino acid similarity matrix of putative viral species detected in this study (L gene fragment).

Sequence	C45	C49	C88	C99	D231	D328	D399	K402	K474	K681	K712	K718	K756	K757	K789	k795	K808	K809	K814	K839	K841	K856	K877	K887	K898	N955
C45	*	0.739	0.826	0.713	0.730	0.791	0.695	0.669	0.756	0.773	0.704	0.765	0.695	0.643	0.747	0.686	0.739	0.747	0.678	0.539	0.591	0.721	0.739	0.704	0.739	0.791
C49		*	0.782	0.756	0.713	0.756	0.686	0.756	0.756	0.739	0.756	0.808	0.678	0.695	0.791	0.713	0.765	0.765	0.756	0.565	0.626	0.747	0.704	0.730	0.756	0.773
C88			*	0.730	0.739	0.826	0.704	0.713	0.739	0.739	0.704	0.808	0.704	0.695	0.765	0.721	0.808	0.791	0.721	0.565	0.591	0.721	0.756	0.747	0.756	0.904
C99				*	0.739	0.730	0.678	0.765	0.713	0.721	0.721	0.747	0.643	0.669	0.765	0.695	0.695	0.747	0.773	0.591	0.591	0.713	0.721	0.678	0.730	0.739
D231					*	0.765	0.669	0.686	0.747	0.721	0.686	0.730	0.678	0.660	0.817	0.678	0.739	0.773	0.660	0.547	0.565	0.678	**0.913**	0.695	0.808	0.730
D328						*	0.739	0.704	0.730	0.765	0.721	0.782	0.686	0.678	0.765	0.713	0.834	0.756	0.713	0.565	0.582	0.704	0.791	0.721	0.756	0.817
D399							*	0.660	0.643	0.652	0.704	0.660	0.782	0.704	0.695	0.765	0.678	0.686	0.669	0.556	0.634	0.730	0.678	0.773	0.721	0.713
K402								*	0.695	0.695	0.713	0.695	0.643	0.652	0.739	0.678	0.713	0.765	0.904	**0.530**	0.582	0.695	0.713	0.678	0.747	0.756
K474									*	0.904	0.686	0.817	0.660	0.660	0.782	0.660	0.756	0.860	0.678	0.547	0.573	0.713	0.765	0.686	0.782	0.747
K681										*	0.713	0.860	0.660	0.652	0.747	0.652	0.765	0.834	0.695	0.556	0.582	0.695	0.739	0.678	0.730	0.765
K712											*	0.765	0.704	0.652	0.713	0.713	0.704	0.704	0.704	0.565	0.643	0.669	0.660	0.695	0.704	0.713
K718												*	0.643	0.678	0.756	0.669	0.765	0.843	0.713	0.600	0.617	0.704	0.739	0.695	0.756	0.782
K756													*	0.695	0.686	0.756	0.678	0.652	0.643	0.556	0.634	0.660	0.695	0.747	0.695	0.695
K757														*	0.695	0.834	0.678	0.695	0.669	**0.530**	0.573	0.678	0.686	0.860	0.678	0.686
K789															*	0.695	0.756	0.817	0.721	0.565	0.600	0.704	0.843	0.713	0.886	0.756
K795																*	0.721	0.678	0.669	0.608	0.600	0.721	0.686	0.878	0.686	0.730
K808																	*	0.782	0.695	0.539	0.573	0.695	0.739	0.713	0.756	0.782
K809																		*	0.739	0.556	0.582	0.704	0.800	0.713	0.817	0.773
K814																			*	**0.530**	0.600	0.704	0.669	0.669	0.704	0.747
K839																				*	0.678	0.573	**0.530**	0.591	0.539	0.582
K841																					*	0.634	0.547	0.608	0.565	0.608
K856																						*	0.669	0.704	0.721	0.713
K877																							*	0.686	0.852	0.730
K887																								*	0.704	0.756
K898																									*	0.739
N955																										*

Table 2.11 [Continue]: Amino acid similarity matrix of putative viral species detected in this study (L gene fragment).

Sequence	SA-170	SA-172	SA-712	SA-724	SA-844	SA-855	SA-922	SA-947	SA-1485	SA-1486	Beilong	Measles	Hendra
C45	0.747	0.739	0.704	0.686	0.756	0.660	0.660	0.721	0.826	0.773	0.669	0.600	0.565
C49	0.747	0.747	0.765	0.704	0.782	0.713	0.669	0.747	0.791	0.791	0.678	0.608	0.556
C88	0.782	0.739	0.730	0.765	0.773	0.686	0.704	0.704	0.904	0.852	0.704	0.608	0.565
C99	0.695	0.713	0.739	0.739	0.739	0.704	0.678	0.730	0.756	0.730	0.721	0.591	0.565
D231	0.721	0.739	0.678	0.721	0.756	0.695	0.669	0.730	0.730	0.765	0.669	0.591	0.539
D328	0.773	0.747	0.713	0.756	0.765	0.686	0.686	0.730	0.826	0.791	0.713	0.565	0.573
D399	0.686	0.686	0.660	0.704	0.686	0.634	0.756	0.678	0.704	0.695	0.686	0.626	0.608
K402	0.686	0.713	0.747	0.747	0.704	0.695	0.686	0.713	0.747	0.704	0.678	0.582	0.556
K474	0.747	0.739	0.713	0.686	0.791	0.660	0.643	0.730	0.782	0.765	0.669	0.626	0.547
K681	0.756	0.747	0.713	0.704	0.791	0.669	0.643	0.756	0.791	0.739	0.686	0.617	0.556
K712	0.713	0.713	0.678	0.695	0.713	0.826	0.678	0.730	0.739	0.704	0.695	0.591	0.539
K718	0.739	0.773	0.721	0.730	0.773	0.704	0.669	0.773	0.808	0.817	0.713	0.608	0.547
K756	0.695	0.660	0.652	0.704	0.713	0.669	0.730	0.660	0.686	0.713	0.678	0.643	0.573
K757	0.660	0.669	0.626	0.643	0.678	0.669	0.765	0.669	0.678	0.721	0.626	0.626	0.547
K789	0.756	0.765	0.756	0.773	0.765	0.704	0.686	0.730	0.765	0.791	0.695	0.617	0.565
k795	0.686	0.695	0.669	0.730	0.704	0.704	0.782	0.704	0.721	0.721	0.678	0.626	0.591
K808	0.765	0.739	0.713	0.756	0.773	0.678	0.660	0.721	0.782	0.800	0.660	0.608	0.582
K809	0.765	0.747	0.756	0.739	0.765	0.695	0.695	0.730	0.782	0.791	0.686	0.617	0.573
K814	0.669	0.660	0.730	0.713	0.695	0.686	0.686	0.695	0.756	0.704	0.669	0.591	0.565
K839	0.530	0.565	0.626	0.591	0.547	0.556	0.556	0.582	0.600	0.565	0.582	0.556	0.573
K841	0.547	0.617	0.617	0.582	0.556	0.591	0.643	0.600	0.591	0.600	**0.626**	**0.565**	**0.626**
K856	0.721	0.721	0.713	0.695	0.721	0.660	0.686	0.730	0.739	0.747	0.686	0.626	0.573
K877	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.695	0.600	0.556
K887	0.713	0.704	0.669	0.704	0.730	0.678	0.773	0.730	0.739	0.747	0.669	0.634	0.556

Table 2.11 [Continue]: Amino acid similarity matrix of putative viral species detected in this study (L gene fragment).

Sequence	SA-170	SA-172	SA-712	SA-724	SA-844	SA-855	SA-922	SA-947	SA-1485	SA-1486	Beilong	Measles	Hendra
K898	0.765	0.773	0.739	0.782	0.747	0.713	0.695	0.747	0.765	0.765	0.686	0.626	0.547
N955	0.739	0.721	0.721	0.756	0.773	0.660	0.704	0.704	0.904	0.782	0.704	0.608	0.565
SA-170	*	0.739	0.730	0.730	0.800	0.747	0.643	0.730	0.765	0.808	0.669	0.565	0.556
SA-172		*	0.747	0.730	0.747	0.704	0.634	0.826	0.730	0.765	0.695	0.582	0.530
SA-712			*	0.747	0.704	0.721	0.617	0.713	0.747	0.704	0.695	0.573	0.582
SA-724				*	0.704	0.704	0.678	0.730	0.730	0.756	0.730	0.591	0.556
SA-844					*	0.695	0.660	0.773	0.791	0.817	0.695	0.608	0.504
SA-855						*	0.634	0.747	0.686	0.695	0.695	0.547	0.539
SA-922							*	0.626	0.686	0.695	0.669	0.634	0.582
SA-947								*	0.713	0.756	0.695	0.565	0.530
SA-1485									*	0.791	0.704	0.626	0.565
SA-1486										*	0.713	0.600	0.556
Beilong											*	0.600	0.556
Measles												*	0.591
Hendra													*

The highest similarity observed between a putative viral species sequence and one of the established viruses was 73% between SA-724 and Beilong virus. Data regarding the highest and lowest similarity observed between sequences considered as the same viral species, showed varying results. In some instances the two sequences fall just within the range of same species *e.g.* Ken-757 and DRC-216, sharing a similarity of 93.4% for the particular gene region. Opposite to that, some sequences only differed by 0.09% (Ken-809 and Ken-170). The complete amino acid similarity matrix of all putative viral species can be found in **Table 2.11**. The highest similarity observed between the putative species was 91% between Ken-877 and DRC-231 (**Table 2.11** in red). The lowest similarity observed between these sequences was 53% (**Table 2.11** in blue).

Amino acid similarity analysis of the 36 putative sequences detected in this study compared to the sequences of Drexler *et al.* (2012), revealed 4 sequences from this study to have a similarity of more than 93% to sequences detected by Drexler *et al.* (2012) (**Table 2.12** indicated by red). These sequences were represented by Cam-88, DRC-328, SA-922 and Ken-402 and therefore considered as the same species. The data set used for comparison in the second round consisted of 32 putative viral species. **Table 2.13** gives the amino acid similarity for this analysis. Only one sequences (Ken-789) had a similarity of 94% and 96% with two bat paramyxovirus sequences detected by Wilkinson *et al.* (2012). Based on these results, at least 31 putative paramyxovirus species were detected in this study. Most sequences that showed similarity to that detected in other studies was similar to sequences derived from the same bat genus.

2.2.7 *Eidolon helvum* analysis

Screening of *Eidolon helvum* kidney samples resulted in no virus detection (**Table 2.7**). The same results were seen for both the spleen and faecal samples screened. A total of 72 kidney, 20 spleen and 20 faecal samples tested negative for paramyxovirus RNA with the use of the RMH primer set. Samples were not available for re-screening with the PMV-primer set.

Table 2.12: Amino acid similarity of putative viral species from this study and that of Drexler *et al*. (2012) (L gene fragment).

Sequence	Eid_hel/GH-M77/GHA/2009	Eid_hel/RCA-P09/RCA/2008	Eid_hel/CD287/DRC/2009	GH10/2008	Eid_hel/CD297/DRC/2009	Eid_hel/GB1661-RMH/GAB/2005	Eid_hel/GB3384/GAB/2006	Eid_hel/CD291/DRC/2009	Myo_tor/CD356/DRC/2009	Eid_hel/GH-M33/GHA/2009	Eid_hel/GB1678/GAB/2005	Eid_hel/GB1535/GAB/2005	Epo_gam/CD078/DRC/2009	Epo_gam/CD255/DRC/2009	Epo_spe/CD256/DRC/2009	Epo_gam/CD273/DRC/2009	Myo_tor/CO2225/CON/2005	Myo_tor/GB1386/GAB/2005	Hyp_mon/CO2569/CON/2006	Hyp_mon/RCA-P18/RCA/2008	Rou_aeg/GB1583/GAB/2005	GH15/2009
Cam-45-*Rhinolophus* sp.	0.56	0.56	0.55	0.56	0.56	0.56	0.56	0.59	0.59	0.59	0.54	0.54	0.64	0.63	0.63	0.63	0.60	0.60	0.63	0.63	0.59	0.57
Cam-49-*Taphozous* sp.	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.61	0.62	0.62	0.56	0.56	0.65	0.66	0.66	0.66	0.64	0.64	0.66	0.66	0.61	0.61
Cam-88-*Hipposideros* sp.	0.58	0.58	0.59	0.58	0.58	0.58	0.58	0.63	0.63	0.63	0.55	0.55	0.65	0.64	0.64	0.64	0.61	0.62	0.64	0.64	0.59	0.57
Cam-99-*Taphozous* sp.	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.60	0.60	0.60	0.57	0.57	0.63	0.64	0.64	0.64	0.61	0.62	0.63	0.63	0.61	0.61
DRC-231-*Miniopterus* sp.	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.63	0.63	0.63	0.53	0.53	0.61	0.61	0.61	0.62	0.60	0.59	0.60	0.60	0.57	0.54
Ken-474-*Triaenops persicus*	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.59	0.59	0.59	0.51	0.51	0.57	0.57	0.57	0.57	0.60	0.59	0.58	0.58	0.57	0.57
Ken-681-*Triaenops persicus*	0.58	0.58	0.57	0.58	0.58	0.58	0.58	0.59	0.59	0.59	0.54	0.54	0.56	0.57	0.57	0.57	0.59	0.59	0.57	0.57	0.58	0.57
Ken-712-*Nycteris* sp.	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.59	0.59	0.59	0.53	0.53	0.66	0.68	0.69	0.66	0.67	0.67	0.66	0.66	0.64	0.59
Ken-718-*Triaenops persicus*	0.57	0.57	0.58	0.57	0.57	0.57	0.57	0.60	0.61	0.61	0.56	0.56	0.62	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.62	0.57
Ken-756-*Miniopterus* sp.	0.58	0.58	0.57	0.58	0.58	0.58	0.58	0.55	0.55	0.55	0.54	0.54	0.63	0.62	0.62	0.61	0.62	0.62	0.63	0.63	0.62	0.59
Ken-757-*Miniopterus* sp.	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.51	0.51	0.55	0.56	0.55	0.56	0.53	0.53	0.56	0.56	0.55	0.57
Ken-789-*Miniopterus* sp.	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.64	0.64	0.64	0.55	0.55	0.63	0.64	0.64	0.64	0.63	0.63	0.63	0.63	0.58	0.59
Ken-795-*Miniopterus* sp.	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.60	0.60	0.60	0.56	0.56	0.64	0.66	0.65	0.65	0.60	0.60	0.63	0.63	0.62	0.61
Ken-808-*Triaenops persicus*	0.59	0.59	0.60	0.59	0.59	0.59	0.59	0.60	0.60	0.60	0.53	0.53	0.62	0.63	0.63	0.63	0.61	0.61	0.63	0.63	0.58	0.53
Ken-809-*Triaenops persicus*	0.56	0.56	0.57	0.56	0.56	0.56	0.56	0.59	0.59	0.59	0.53	0.53	0.59	0.61	0.61	0.61	0.61	0.60	0.61	0.61	0.58	0.58
Ken-814-*Coleura afra*	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.59	0.60	0.60	0.55	0.55	0.60	0.61	0.61	0.61	0.57	0.57	0.59	0.59	0.58	0.58
Ken-839-*Rousettus aegyptiacus*	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.55	0.55	0.55	0.66	0.66	0.65	0.66	0.65	0.65	0.63	0.63	0.63	0.63	0.65	0.65
Ken-841-*Rousettus aegyptiacus*	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.62	0.62	0.70	0.71	0.71	0.70	0.71	0.71	0.70	0.70	0.75	0.64
Ken-856-*Coleura afra*	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.57	0.57	0.57	0.57	0.57	0.63	0.63	0.63	0.63	0.61	0.61	0.63	0.63	0.61	0.61
Ken-877-*Miniopterus minor*	0.57	0.57	0.58	0.57	0.57	0.57	0.57	0.63	0.63	0.63	0.55	0.55	0.59	0.59	0.59	0.60	0.58	0.57	0.58	0.58	0.56	0.54
Ken-887-*Miniopterus minor*	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.58	0.58	0.58	0.54	0.54	0.58	0.59	0.59	0.59	0.58	0.58	0.61	0.61	0.60	0.61
Ken-898-*Miniopterus minor*	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.61	0.61	0.61	0.54	0.54	0.62	0.63	0.63	0.63	0.61	0.60	0.62	0.62	0.56	0.56
DRC-328-*Hipposideros fuliginosis*	0.55	0.55	0.56	0.55	0.55	0.55	0.55	0.62	0.63	0.63	0.56	0.56	0.61	0.62	0.62	0.61	0.59	0.59	0.62	0.62	0.60	0.56
DRC-399-*Hipposideros fuliginosis*	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.57	0.56	0.56	0.57	0.57	0.63	0.64	0.64	0.63	0.63	0.63	0.67	0.67	0.62	0.58
Nig-955-*Hipposideros* sp.	0.60	0.60	0.59	0.60	0.60	0.60	0.60	0.63	0.63	0.63	0.57	0.57	0.63	0.63	0.63	0.63	0.60	0.61	0.63	0.63	0.61	0.59
SA-170-*Eptesicus hottentotus*	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.57	0.57	0.57	0.53	0.53	0.59	0.58	0.58	0.58	0.57	0.57	0.59	0.59	0.56	0.57
SA-172-*Rhinolophus denti*	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.63	0.63	0.63	0.54	0.54	0.63	0.64	0.64	0.64	0.63	0.63	0.63	0.63	0.57	0.55
SA-712-*Taphozous mauritianus*	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.61	0.61	0.61	0.57	0.57	0.66	0.67	0.67	0.67	0.65	0.65	0.67	0.67	0.63	0.59
SA-724-*Neoromicia nana*	0.58	0.58	0.59	0.58	0.58	0.58	0.58	0.61	0.61	0.61	0.59	0.59	0.63	0.63	0.63	0.63	0.60	0.61	0.62	0.62	0.59	0.57
SA-844-*Kerivouls argentata*	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.60	0.59	0.59	0.50	0.50	0.59	0.58	0.58	0.58	0.59	0.58	0.60	0.60	0.57	0.56
SA-855-*Nycteris thebaica*	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.57	0.57	0.57	0.53	0.53	0.63	0.63	0.63	0.61	0.62	0.61	0.62	0.62	0.61	0.56
SA-922-*Neoromicia nana*	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.55	0.55	0.55	0.55	0.55	0.58	0.60	0.60	0.58	0.57	0.58	0.60	0.60	0.60	0.58
SA-947-*Rhinolophus landeri*	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.53	0.53	0.61	0.62	0.62	0.62	0.60	0.60	0.60	0.60	0.58	0.57
SA-1485-*Rhinolophus* sp.	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.63	0.63	0.63	0.55	0.55	0.64	0.65	0.65	0.65	0.62	0.63	0.65	0.65	0.61	0.57
SA-1486-*Rhinolophus* sp.	0.57	0.57	0.58	0.57	0.57	0.57	0.57	0.59	0.59	0.59	0.53	0.53	0.63	0.63	0.63	0.63	0.60	0.61	0.63	0.63	0.58	0.57
Ken-402-*Coleura afra*	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.61	0.61	0.61	0.54	0.54	0.60	0.62	0.62	0.62	0.58	0.58	0.60	0.60	0.57	0.59

Table 2.12 [Continue]: Amino acid similarity of putative viral species from this study and that of Drexler *et al.* (2012) (L gene fragment).

Sequence	Eid_hel/GH-M63a/GHA/2009	GH21a/2009	Pte_par/KCR245H/CR/2010	Eid_hel/GB1659/GAB/2005	Eid_hel/GH-M43/GHA/2010	Eid_hel/GH-M51a/GHA/2009	Eid_hel/GH-M67a/GHA/2009	Eid_hel/GH-M61a/GHA/2009	Eid_hel/GH-M69a/GHA/2009	Eid_hel/GH-M90a/GHA/2009	GH48/2008	Eid_hel/RCA-P05/RCA/2008	Eid_hel/GB1237/GAB/2005	GH27a/2009	Eid_hel/GH-M28/GHA/2009	Rou_aeg/GB2009/GAB/2005	Rou_aeg/GB1590/GAB/2005	Myo_mys/NM98-140/GER/2008	Myo_mys/NM98-44/GER/2008	Myo_mys/NM98-47/GER/2008	Myo_cap/6-43/BGR/2009	Myo_dau/NMS09-48/GER/2009
Cam-45-*Rhinolophus* sp.	0.55	0.51	0.60	0.59	0.61	0.55	0.54	0.57	0.60	0.60	0.55	0.55	0.56	0.57	0.53	0.56	0.56	0.67	0.66	0.66	0.67	0.65
Cam-49-*Taphozous* sp.	0.57	0.55	0.61	0.58	0.59	0.56	0.57	0.55	0.60	0.60	0.62	0.62	0.63	0.57	0.57	0.59	0.59	0.68	0.67	0.67	0.71	0.70
Cam-88-*Hipposideros* sp.	0.57	0.53	0.61	0.58	0.60	0.55	0.56	0.56	0.59	0.59	0.60	0.60	0.61	0.57	0.57	0.58	0.58	0.70	0.69	0.69	0.70	0.69
Cam-99-*Taphozous* sp.	0.58	0.57	0.62	0.59	0.60	0.57	0.57	0.57	0.57	0.57	0.61	0.61	0.63	0.60	0.59	0.57	0.57	0.66	0.65	0.65	0.67	0.66
DRC-231-*Miniopterus* sp.	0.54	0.53	0.62	0.57	0.59	0.54	0.53	0.57	0.54	0.54	0.56	0.56	0.56	0.56	0.56	0.58	0.58	0.67	0.66	0.66	0.67	0.66
Ken-474-*Triaenops persicus*	0.52	0.50	0.58	0.57	0.58	0.58	0.57	0.57	0.56	0.56	0.57	0.57	0.57	0.56	0.52	0.58	0.58	0.69	0.70	0.70	0.69	0.69
Ken-681-*Triaenops persicus*	0.51	0.50	0.58	0.57	0.57	0.57	0.56	0.55	0.54	0.54	0.54	0.54	0.55	0.53	0.50	0.59	0.59	0.64	0.65	0.65	0.66	0.65
Ken-712-*Nycteris* sp.	0.58	0.53	0.58	0.62	0.61	0.56	0.56	0.56	0.57	0.57	0.58	0.58	0.59	0.57	0.57	0.57	0.57	0.67	0.66	0.66	0.69	0.68
Ken-718-*Triaenops persicus*	0.55	0.52	0.60	0.59	0.59	0.58	0.59	0.57	0.57	0.57	0.61	0.61	0.62	0.55	0.55	0.61	0.61	0.63	0.63	0.63	0.66	0.65
Ken-756-*Miniopterus* sp.	0.56	0.52	0.58	0.65	0.62	0.55	0.54	0.55	0.53	0.53	0.57	0.57	0.57	0.57	0.57	0.56	0.56	0.74	0.73	0.73	0.76	0.74
Ken-757-*Miniopterus* sp.	0.51	0.50	0.54	0.57	0.57	0.57	0.57	0.56	0.56	0.56	0.58	0.58	0.59	0.56	0.54	0.56	0.56	0.83	0.83	0.83	0.85	0.83
Ken-789-*Miniopterus* sp.	0.60	0.58	0.59	0.62	0.62	0.57	0.57	0.57	0.60	0.60	0.62	0.62	0.62	0.58	0.59	0.59	0.59	0.70	0.71	0.71	0.73	0.72
Ken-795-*Miniopterus* sp.	0.60	0.56	0.58	0.61	0.60	0.56	0.55	0.58	0.55	0.55	0.61	0.61	0.62	0.63	0.61	0.59	0.59	0.87	0.85	0.86	0.87	0.85
Ken-808-*Triaenops persicus*	0.57	0.50	0.58	0.55	0.54	0.53	0.54	0.53	0.56	0.56	0.57	0.57	0.57	0.57	0.53	0.54	0.54	0.68	0.67	0.67	0.68	0.67
Ken-809-*Triaenops persicus*	0.55	0.52	0.58	0.60	0.61	0.59	0.58	0.58	0.57	0.57	0.58	0.58	0.59	0.56	0.54	0.60	0.60	0.69	0.70	0.70	0.71	0.70
Ken-814-*Coleura afra*	0.56	0.57	0.57	0.58	0.58	0.59	0.58	0.55	0.59	0.59	0.60	0.60	0.61	0.56	0.57	0.57	0.57	0.66	0.67	0.67	0.69	0.68
Ken-839-*Rousettus aegyptiacus*	0.70	0.69	0.61	0.63	0.63	0.60	0.59	0.63	0.57	0.57	0.59	0.59	0.60	0.62	0.58	0.58	0.58	0.55	0.55	0.54	0.56	0.55
Ken-841-*Rousettus aegyptiacus*	0.69	0.60	0.61	0.66	0.66	0.63	0.63	0.61	0.65	0.65	0.63	0.63	0.64	0.59	0.61	0.58	0.58	0.60	0.59	0.59	0.60	0.59
Ken-856-*Coleura afra*	0.57	0.53	0.63	0.59	0.63	0.57	0.57	0.60	0.60	0.60	0.60	0.60	0.61	0.61	0.61	0.59	0.59	0.69	0.68	0.68	0.70	0.69
Ken-877-*Miniopterus minor*	0.53	0.54	0.62	0.57	0.58	0.55	0.54	0.57	0.56	0.56	0.57	0.57	0.57	0.58	0.55	0.58	0.58	0.70	0.70	0.70	0.72	0.70
Ken-887-*Miniopterus minor*	0.58	0.54	0.57	0.62	0.60	0.57	0.56	0.60	0.56	0.56	0.58	0.58	0.59	0.61	0.59	0.59	0.59	0.88	0.87	0.87	0.90	0.88
Ken-898-*Miniopterus minor*	0.57	0.54	0.60	0.61	0.58	0.56	0.55	0.57	0.58	0.58	0.58	0.58	0.60	0.59	0.57	0.57	0.57	0.69	0.70	0.70	0.74	0.73
DRC-328-*Hipposideros fuliginosis*	0.56	0.50	0.58	0.57	0.56	0.53	0.52	0.56	0.55	0.55	0.58	0.58	0.58	0.60	0.56	0.57	0.57	0.68	0.67	0.67	0.69	0.68
DRC-399-*Hipposideros fuliginosis*	0.60	0.50	0.56	0.64	0.63	0.57	0.56	0.61	0.57	0.57	0.59	0.59	0.60	0.62	0.61	0.57	0.57	0.77	0.75	0.76	0.77	0.76
Nig-955-*Hipposideros* sp.	0.57	0.56	0.58	0.58	0.60	0.57	0.56	0.57	0.59	0.59	0.62	0.62	0.63	0.58	0.59	0.58	0.58	0.71	0.70	0.70	0.72	0.70
SA-170-*Eptesicus hottentotus*	0.54	0.50	0.58	0.57	0.58	0.55	0.54	0.53	0.55	0.55	0.56	0.56	0.57	0.57	0.55	0.56	0.56	0.66	0.67	0.67	0.69	0.69
SA-172-*Rhinolophus denti*	0.57	0.52	0.61	0.60	0.59	0.56	0.57	0.56	0.60	0.60	0.58	0.58	0.59	0.57	0.56	0.53	0.53	0.68	0.69	0.69	0.69	0.69
SA-712-*Taphozous mauritianus*	0.59	0.57	0.59	0.56	0.59	0.53	0.52	0.55	0.60	0.60	0.59	0.59	0.60	0.58	0.57	0.53	0.53	0.63	0.63	0.64	0.69	0.68
SA-724-*Neoromicia nana*	0.57	0.57	0.58	0.57	0.57	0.53	0.52	0.54	0.56	0.56	0.58	0.58	0.58	0.63	0.58	0.56	0.56	0.67	0.68	0.68	0.70	0.69
SA-844-*Kerivouls argentata*	0.55	0.53	0.62	0.59	0.57	0.56	0.55	0.55	0.56	0.56	0.57	0.57	0.57	0.57	0.57	0.58	0.58	0.69	0.68	0.68	0.70	0.70
SA-855-*Nycteris thebaica*	0.57	0.53	0.59	0.57	0.59	0.53	0.52	0.53	0.54	0.54	0.54	0.54	0.55	0.57	0.54	0.54	0.54	0.65	0.66	0.66	0.70	0.70
SA-922-*Neoromicia nana*	0.55	0.50	0.57	0.61	0.58	0.57	0.56	0.57	0.57	0.57	0.62	0.62	0.63	0.56	0.57	0.59	0.59	0.80	0.79	0.79	0.79	0.77
SA-947-*Rhinolophus landeri*	0.56	0.56	0.61	0.59	0.60	0.57	0.56	0.56	0.57	0.57	0.57	0.57	0.57	0.59	0.58	0.58	0.58	0.67	0.66	0.66	0.70	0.70
SA-1485-*Rhinolophus* sp.	0.60	0.54	0.60	0.58	0.61	0.56	0.55	0.57	0.59	0.59	0.59	0.59	0.60	0.59	0.58	0.60	0.60	0.70	0.69	0.69	0.70	0.69
SA-1486-*Rhinolophus* sp.	0.57	0.50	0.63	0.57	0.61	0.57	0.58	0.57	0.57	0.57	0.60	0.60	0.61	0.57	0.57	0.57	0.57	0.70	0.69	0.69	0.71	0.70
Ken-402-*Coleura afra*	0.57	0.56	0.57	0.60	0.58	0.57	0.56	0.55	0.58	0.58	0.58	0.58	0.59	0.57	0.56	0.55	0.55	0.67	0.68	0.68	0.70	0.70

Table 2.12 [Continue]: Amino acid similarity of putative viral species from this study and that of Drexler *et al.* (2012).

Sequence	Myo_myo/N78-14/GER/2008	Myo_myo/N78-16/GER/2008	Pip_nan/GH36/2008	Myo_alc/3-320/BGR/2009	Hip_caf/GB09790/GAB/2009	Col_afr/GB09478/GAB/2009	Hip_aba/GB59-59/GHA/2009	Hip_caf/GB09670/GAB/2009	Hip_caf/GB59-30/GHA/2009	Hip_rub/GB19-S/GHA/2009	Hip_rub/GH19-140/GHA/2009	Hip_gig/GB09682/GAB/2009	Hip_spec/GH19-T/GHA/2009	Hip_gig/GB09898/GAB/2009	Des_rot/BR21/BRA/2008	Pte_par/KCR245M/CR/2010	Pte_par/KCR370/CR/2010	Car_bre//BR96/BRA/2009	Car_per/BR310/BRA/2009	Car_bre/BR100/BRA/2009	Car_bre/BR102/BRA/2009	Glo_sor/BR190/BRA/2009
Cam-45-*Rhinolophus* sp.	0.65	0.65	0.66	0.65	0.67	0.67	0.66	0.83	0.79	0.80	0.80	0.74	0.75	0.74	0.69	0.63	0.65	0.58	0.58	0.58	0.58	0.63
Cam-49-*Taphozous* sp.	0.70	0.70	0.66	0.69	0.69	0.77	0.73	0.78	0.75	0.77	0.77	0.78	0.77	0.74	0.69	0.66	0.68	0.62	0.62	0.62	0.62	0.65
Cam-88-*Hipposideros* sp.	**0.69**	**0.69**	**0.70**	**0.70**	**0.70**	**0.71**	**0.70**	**0.95**	**0.81**	**0.83**	**0.83**	**0.78**	**0.80**	**0.75**	**0.70**	**0.69**	**0.70**	**0.58**	**0.58**	**0.58**	**0.58**	**0.63**
Cam-99-*Taphozous* sp.	0.66	0.66	0.68	0.69	0.66	0.78	0.76	0.75	0.72	0.74	0.74	0.71	0.71	0.70	0.67	0.70	0.71	0.58	0.58	0.58	0.58	0.62
DRC-231-*Miniopterus* sp.	0.66	0.66	0.67	0.67	0.65	0.71	0.70	0.73	0.76	0.77	0.77	0.77	0.76	0.80	0.70	0.66	0.67	0.57	0.57	0.57	0.57	0.62
Ken-474-*Triaenops persicus*	0.68	0.68	0.66	0.66	0.64	0.70	0.69	0.76	0.75	0.74	0.74	0.80	0.77	0.76	0.67	0.63	0.67	0.60	0.60	0.60	0.60	0.68
Ken-681-*Triaenops persicus*	0.69	0.69	0.66	0.63	0.63	0.70	0.70	0.77	0.78	0.77	0.77	0.77	0.77	0.73	0.67	0.65	0.67	0.60	0.60	0.60	0.60	0.66
Ken-712-*Nycteris* sp.	0.70	0.70	0.67	0.70	0.69	0.73	0.70	0.71	0.71	0.71	0.71	0.69	0.69	0.70	0.70	0.71	0.71	0.62	0.62	0.62	0.62	0.67
Ken-718-*Triaenops persicus*	0.66	0.66	0.67	0.68	0.66	0.71	0.70	0.81	0.77	0.78	0.78	0.78	0.78	0.77	0.69	0.69	0.70	0.59	0.59	0.59	0.59	0.67
Ken-756-*Miniopterus* sp.	0.76	0.76	0.72	0.70	0.78	0.66	0.65	0.70	0.69	0.69	0.69	0.70	0.70	0.70	0.70	0.63	0.65	0.56	0.56	0.56	0.56	0.63
Ken-757-*Miniopterus* sp.	0.83	0.83	0.78	0.75	0.70	0.66	0.64	0.70	0.68	0.70	0.70	0.72	0.74	0.70	0.65	0.68	0.64	0.55	0.55	0.55	0.55	0.60
Ken-789-*Miniopterus* sp.	0.71	0.71	0.70	0.68	0.70	0.74	0.74	0.78	0.77	0.77	0.77	0.81	0.80	0.82	0.71	0.66	0.68	0.61	0.61	0.61	0.61	0.62
Ken-795-*Miniopterus* sp.	0.83	0.83	0.79	0.79	0.77	0.70	0.68	0.73	0.70	0.72	0.72	0.73	0.75	0.73	0.72	0.70	0.70	0.59	0.59	0.59	0.59	0.63
Ken-808-*Triaenops persicus*	0.70	0.70	0.66	0.66	0.66	0.73	0.72	0.78	0.82	0.82	0.82	0.81	0.78	0.74	0.71	0.64	0.67	0.60	0.60	0.60	0.60	0.62
Ken-809-*Triaenops persicus*	0.69	0.69	0.71	0.67	0.67	0.77	0.76	0.80	0.76	0.76	0.76	0.80	0.80	0.79	0.70	0.68	0.68	0.59	0.59	0.59	0.59	0.64
Ken-814-*Coleura afra*	0.69	0.69	0.69	0.66	0.67	0.90	0.92	0.75	0.71	0.72	0.72	0.70	0.70	0.69	0.65	0.70	0.68	0.60	0.60	0.60	0.60	0.58
Ken-839-*Rousettus aegyptiacus*	0.56	0.56	0.56	0.58	0.56	0.55	0.54	0.58	0.56	0.57	0.57	0.56	0.57	0.58	0.57	0.61	0.63	0.57	0.57	0.57	0.57	0.57
Ken-841-*Rousettus aegyptiacus*	0.59	0.59	0.63	0.61	0.64	0.60	0.58	0.62	0.57	0.57	0.57	0.57	0.58	0.59	0.62	0.63	0.62	0.56	0.56	0.56	0.56	0.58
Ken-856-*Coleura afra*	0.70	0.70	0.68	0.69	0.70	0.71	0.68	0.74	0.70	0.71	0.71	0.72	0.72	0.74	0.74	0.72	0.75	0.60	0.60	0.60	0.60	0.63
Ken-877-*Miniopterus minor*	0.70	0.70	0.72	0.69	0.66	0.71	0.71	0.74	0.79	0.79	0.79	0.80	0.79	0.79	0.70	0.64	0.66	0.58	0.58	0.58	0.58	0.62
Ken-887-*Miniopterus minor*	0.84	0.84	0.77	0.75	0.75	0.70	0.67	0.76	0.71	0.73	0.73	0.76	0.78	0.73	0.69	0.69	0.68	0.58	0.58	0.58	0.58	0.63
Ken-898-*Miniopterus minor*	0.72	0.72	0.71	0.67	0.70	0.73	0.72	0.77	0.77	0.77	0.77	0.82	0.80	0.83	0.72	0.68	0.70	0.60	0.60	0.60	0.60	0.63
DRC-328-*Hipposideros fuliginosis*	0.67	0.67	0.69	0.68	0.72	0.73	0.71	0.83	**0.98**	**0.98**	**0.98**	0.78	0.79	0.71	0.73	0.67	0.70	0.59	0.59	0.59	0.59	0.61
DRC-399-*Hipposideros fuliginosis*	0.73	0.73	0.74	0.72	0.92	0.69	0.68	0.71	0.73	0.74	0.74	0.70	0.70	0.71	0.71	0.69	0.72	0.57	0.57	0.57	0.57	0.65
Nig-955-*Hipposideros* sp.	0.71	0.71	0.70	0.70	0.71	0.76	0.75	0.90	0.82	0.83	0.83	0.77	0.79	0.74	0.70	0.71	0.71	0.62	0.62	0.62	0.62	0.63
SA-170-*Eptesicus hottentotus*	0.68	0.68	0.66	0.61	0.66	0.69	0.67	0.79	0.78	0.78	0.78	0.80	0.80	0.75	0.71	0.67	0.71	0.60	0.60	0.60	0.60	0.60
SA-172-*Rhinolophus denti*	0.70	0.70	0.64	0.66	0.68	0.70	0.69	0.73	0.76	0.76	0.76	0.78	0.77	0.74	0.69	0.66	0.69	0.66	0.66	0.66	0.66	0.60
SA-712-*Taphozous mauritianus*	0.68	0.68	0.63	0.63	0.63	0.74	0.73	0.75	0.72	0.72	0.72	0.72	0.73	0.70	0.69	0.65	0.68	0.61	0.61	0.61	0.61	0.59
SA-724-*Neoromicia nana*	0.70	0.70	0.70	0.67	0.70	0.75	0.73	0.77	0.76	0.75	0.75	0.77	0.79	0.77	0.70	0.68	0.70	0.63	0.63	0.63	0.63	0.63
SA-844-*Kerivouls argentata*	0.71	0.71	0.65	0.63	0.67	0.72	0.70	0.77	0.77	0.77	0.77	0.83	0.80	0.77	0.68	0.69	0.70	0.61	0.61	0.61	0.61	0.64
SA-855-*Nycteris thebaica*	0.70	0.70	0.64	0.65	0.63	0.69	0.66	0.70	0.70	0.69	0.69	0.71	0.71	0.70	0.70	0.68	0.69	0.62	0.62	0.62	0.62	0.63
SA-922-*Neoromicia nana*	0.77	0.77	**0.96**	0.84	0.76	0.70	0.68	0.72	0.68	0.69	0.69	0.69	0.70	0.70	0.69	0.68	0.68	0.54	0.54	0.54	0.54	0.59
SA-947-*Rhinolophus landeri*	0.70	0.70	0.62	0.63	0.64	0.71	0.68	0.72	0.72	0.74	0.74	0.80	0.78	0.77	0.72	0.68	0.68	0.64	0.64	0.64	0.64	0.63
SA-1485-*Rhinolophus* sp.	0.70	0.70	0.70	0.69	0.70	0.75	0.74	0.93	0.82	0.83	0.83	0.77	0.77	0.74	0.71	0.70	0.73	0.60	0.60	0.60	0.60	0.63
SA-1486-*Rhinolophus* sp.	0.70	0.70	0.70	0.70	0.70	0.72	0.70	0.82	0.77	0.79	0.79	0.90	0.90	0.82	0.71	0.69	0.70	0.61	0.61	0.61	0.61	0.65
Ken-402-*Coleura afra*	0.70	0.70	0.70	0.65	0.66	**0.96**	0.90	0.72	0.71	0.71	0.71	0.74	0.75	0.70	0.66	0.67	0.68	0.63	0.63	0.63	0.63	0.59

Table 2.13: Amino acid similarity of putative species with other recently detected bat paramyxoviruses (L gene fragment).

Sequence	PMV SMG16462	PMV SMG16505	PMV SMG16753	PMV SMG16723	PMV SMG16756	PMV SMG16753	PMV SMG16723	PMV SMG17000	PMV SMG16797	PMV SMG16468	EPMV_RC09_210s	EPMV_RC09_215s	EPMV_RC09_216s	EPMV_RC09_222k	EPMV_RC09_226s	EPMV_RC09_236s1	EPMV_RC09_236s2	EPMV_RC09_236u	EPMV_RC09_237s	EPMV_RC09_239k	EPMV_RC09_239l	EPMV_RC09_239s	EPMV_RC09_240s	EPMV_RC09_241s
Cam-45	0.74	0.74	0.76	0.76	0.64	0.75	0.64	0.77	0.74	0.73	0.59	0.50	0.56	0.57	0.53	0.57	0.55	0.59	0.52	0.57	0.59	0.58	0.74	0.74
Cam-49	0.74	0.74	0.75	0.75	0.70	0.75	0.70	0.74	0.77	0.77	0.58	0.55	0.60	0.56	0.57	0.56	0.62	0.58	0.54	0.57	0.57	0.57	0.74	0.74
Cam-99	0.72	0.70	0.74	0.74	0.66	0.73	0.66	0.71	0.75	0.77	0.59	0.51	0.57	0.62	0.54	0.57	0.61	0.58	0.56	0.58	0.59	0.57	0.72	0.70
DRC-231	0.74	0.75	0.83	0.83	0.67	0.75	0.67	0.74	0.80	0.83	0.57	0.52	0.57	0.56	0.55	0.56	0.56	0.57	0.53	0.56	0.57	0.57	0.74	0.75
DRC-399	0.66	0.65	0.70	0.70	0.71	0.67	0.71	0.71	0.69	0.69	0.64	0.51	0.56	0.60	0.53	0.62	0.59	0.64	0.51	0.63	0.64	0.63	0.66	0.65
Nig-955	0.75	0.75	0.76	0.76	0.70	0.76	0.70	0.77	0.74	0.74	0.58	0.55	0.60	0.55	0.57	0.56	0.62	0.58	0.56	0.57	0.58	0.57	0.75	0.75
Ken-474	0.90	0.91	0.78	0.78	0.68	0.91	0.68	0.77	0.76	0.78	0.57	0.52	0.57	0.53	0.55	0.56	0.57	0.57	0.51	0.57	0.57	0.56	0.90	0.91
Ken-681	0.90	0.92	0.77	0.77	0.65	0.91	0.65	0.73	0.73	0.74	0.57	0.53	0.58	0.52	0.56	0.55	0.54	0.57	0.50	0.56	0.57	0.55	0.90	0.92
Ken-712	0.69	0.71	0.70	0.70	0.65	0.70	0.65	0.69	0.70	0.70	0.62	0.53	0.58	0.58	0.56	0.59	0.58	0.61	0.54	0.61	0.61	0.60	0.69	0.71
Ken-718	0.84	0.84	0.76	0.76	0.67	0.85	0.67	0.75	0.74	0.74	0.59	0.52	0.57	0.57	0.55	0.57	0.61	0.59	0.51	0.58	0.58	0.57	0.84	0.84
Ken-756	0.64	0.64	0.70	0.70	0.70	0.65	0.70	0.72	0.69	0.69	0.65	0.53	0.58	0.58	0.56	0.63	0.57	0.65	0.53	0.63	0.65	0.63	0.64	0.64
Ken-757	0.66	0.64	0.68	0.68	0.93	0.67	0.93	0.65	0.70	0.68	0.57	0.51	0.56	0.52	0.53	0.56	0.58	0.57	0.50	0.57	0.57	0.56	0.66	0.64
Ken-789	0.75	0.76	0.90	0.90	0.70	0.76	0.70	0.77	**0.96**	**0.94**	0.62	0.52	0.57	0.61	0.55	0.59	0.62	0.62	0.57	0.61	0.61	0.60	0.75	0.76
Ken-795	0.67	0.64	0.68	0.68	0.83	0.68	0.83	0.69	0.70	0.69	0.61	0.52	0.57	0.57	0.54	0.58	0.61	0.61	0.56	0.59	0.61	0.59	0.67	0.64
Ken-808	0.78	0.75	0.77	0.77	0.70	0.79	0.70	0.74	0.77	0.77	0.55	0.54	0.59	0.53	0.57	0.52	0.57	0.55	0.50	0.54	0.55	0.54	0.78	0.75
Ken-809	0.87	0.87	0.79	0.79	0.70	0.88	0.70	0.79	0.80	0.82	0.60	0.50	0.56	0.56	0.53	0.58	0.58	0.60	0.51	0.60	0.60	0.58	0.87	0.87
Ken-814	0.69	0.69	0.71	0.71	0.68	0.70	0.68	0.69	0.71	0.70	0.58	0.48	0.53	0.57	0.50	0.56	0.60	0.57	0.56	0.58	0.58	0.57	0.69	0.69
Ken-839	0.55	0.55	0.54	0.54	0.53	0.55	0.53	0.57	0.53	0.53	0.63	0.49	0.52	0.67	0.50	0.63	0.59	0.63	0.68	0.63	0.63	0.62	0.55	0.55
Ken-841	0.57	0.59	0.57	0.57	0.57	0.58	0.57	0.57	0.57	0.57	0.66	0.55	0.59	0.71	0.57	0.65	0.63	0.65	0.60	0.64	0.66	0.64	0.57	0.59
Ken-856	0.70	0.70	0.70	0.70	0.69	0.71	0.69	0.69	0.68	0.68	0.59	0.50	0.56	0.58	0.53	0.57	0.60	0.58	0.52	0.58	0.59	0.57	0.70	0.70
Ken-877	0.75	0.74	0.86	0.86	0.69	0.76	0.69	0.77	0.84	0.87	0.57	0.52	0.57	0.56	0.55	0.55	0.57	0.57	0.54	0.56	0.57	0.56	0.75	0.74
Ken-887	0.69	0.68	0.70	0.70	0.86	0.70	0.86	0.72	0.70	0.69	0.62	0.52	0.57	0.56	0.54	0.59	0.58	0.62	0.53	0.60	0.62	0.60	0.69	0.68
Ken-898	0.75	0.76	0.90	0.90	0.71	0.76	0.71	0.79	0.90	0.91	0.61	0.51	0.57	0.57	0.54	0.58	0.58	0.61	0.54	0.61	0.60	0.60	0.75	0.76
SA-170	0.76	0.77	0.75	0.75	0.68	0.77	0.68	0.77	0.76	0.74	0.57	0.50	0.55	0.52	0.52	0.54	0.56	0.57	0.49	0.56	0.57	0.56	0.76	0.77
SA-172	0.75	0.77	0.80	0.80	0.66	0.76	0.66	0.72	0.76	0.78	0.60	0.56	0.61	0.58	0.58	0.57	0.58	0.60	0.52	0.58	0.59	0.59	0.75	0.77
SA-712	0.70	0.71	0.73	0.73	0.63	0.71	0.63	0.73	0.75	0.74	0.56	0.51	0.57	0.61	0.54	0.53	0.59	0.55	0.57	0.56	0.56	0.55	0.70	0.71
SA-724	0.70	0.69	0.79	0.79	0.64	0.70	0.64	0.76	0.77	0.77	0.57	0.53	0.58	0.60	0.57	0.55	0.58	0.57	0.56	0.56	0.57	0.57	0.70	0.69
SA-844	0.78	0.78	0.79	0.79	0.70	0.79	0.70	0.75	0.75	0.77	0.59	0.52	0.57	0.53	0.55	0.57	0.57	0.59	0.53	0.57	0.59	0.57	0.78	0.78
SA-855	0.66	0.68	0.69	0.69	0.67	0.67	0.67	0.70	0.71	0.71	0.57	0.50	0.55	0.54	0.52	0.54	0.54	0.56	0.52	0.56	0.57	0.56	0.66	0.68
SA-947	0.72	0.75	0.76	0.76	0.69	0.73	0.69	0.69	0.75	0.74	0.59	0.55	0.60	0.57	0.57	0.57	0.57	0.58	0.54	0.57	0.58	0.58	0.72	0.75
SA-1486	0.77	0.77	0.77	0.77	0.70	0.77	0.70	0.77	0.78	0.77	0.57	0.52	0.57	0.57	0.55	0.56	0.60	0.57	0.50	0.56	0.57	0.56	0.77	0.77

Table 2.13 [Continue]: Amino acid similarity of putative species with other recently detected bat paramyxoviruses (L gene fragment).

Sequence	EPMV_RC09_240s	EPMV_RC09_241s	EPMV_RC09_247k	PMV U5A	PMV U6A	PMV U42A	PMV U45A	PMV U49B	PMV U50B	PMV U51A	PMV U53A	PMV U54A	PMV U58B	PMV U59A	PMV U61A	PMV U62A	PMV U63A	PMV U64A	PMV U66A	PMV U68A	PMV U71A	PMV U72A	PMV IFBPV46/2011	PMV IFBPV01/2010
Cam-45	0.59	0.50	0.56	0.54	0.59	0.54	0.55	0.58	0.58	0.58	0.54	0.57	0.57	0.55	0.57	0.55	0.58	0.57	0.56	0.54	0.54	0.55	0.59	0.48
Cam-49	0.58	0.52	0.62	0.57	0.62	0.57	0.60	0.63	0.64	0.64	0.57	0.56	0.61	0.56	0.57	0.58	0.64	0.63	0.60	0.55	0.55	0.56	0.62	0.50
Cam-99	0.59	0.54	0.60	0.57	0.60	0.57	0.56	0.62	0.63	0.63	0.60	0.62	0.63	0.60	0.60	0.61	0.63	0.61	0.57	0.57	0.57	0.57	0.62	0.47
DRC-231	0.57	0.51	0.55	0.53	0.63	0.53	0.57	0.59	0.59	0.59	0.55	0.56	0.59	0.54	0.56	0.56	0.59	0.57	0.57	0.53	0.53	0.54	0.56	0.48
DRC-399	0.64	0.49	0.58	0.59	0.56	0.59	0.56	0.61	0.62	0.62	0.58	0.60	0.57	0.57	0.62	0.59	0.62	0.60	0.56	0.56	0.56	0.57	0.64	0.43
Nig-955	0.58	0.54	0.62	0.57	0.63	0.57	0.59	0.63	0.63	0.63	0.62	0.55	0.60	0.60	0.58	0.63	0.63	0.62	0.60	0.56	0.56	0.57	0.63	0.47
Ken-474	0.57	0.49	0.57	0.51	0.59	0.51	0.57	0.61	0.61	0.61	0.51	0.53	0.58	0.56	0.56	0.52	0.61	0.59	0.57	0.57	0.57	0.58	0.57	0.45
Ken-681	0.57	0.48	0.54	0.50	0.59	0.50	0.58	0.60	0.60	0.60	0.53	0.52	0.61	0.56	0.53	0.54	0.60	0.58	0.58	0.56	0.56	0.57	0.58	0.48
Ken-712	0.62	0.51	0.57	0.57	0.59	0.57	0.58	0.60	0.61	0.61	0.57	0.58	0.60	0.56	0.57	0.57	0.61	0.59	0.58	0.55	0.55	0.56	0.66	0.44
Ken-718	0.59	0.50	0.61	0.54	0.61	0.54	0.57	0.60	0.60	0.60	0.57	0.57	0.62	0.57	0.55	0.57	0.60	0.58	0.57	0.57	0.57	0.58	0.62	0.49
Ken-756	0.65	0.50	0.56	0.55	0.55	0.55	0.57	0.62	0.63	0.63	0.57	0.58	0.55	0.57	0.57	0.57	0.63	0.61	0.58	0.54	0.54	0.55	0.63	0.44
Ken-757	0.57	0.49	0.57	0.50	0.56	0.50	0.55	0.58	0.58	0.58	0.53	0.52	0.54	0.56	0.56	0.54	0.58	0.57	0.56	0.56	0.56	0.57	0.62	0.44
Ken-789	0.62	0.56	0.61	0.59	0.64	0.59	0.57	0.65	0.65	0.65	0.57	0.61	0.63	0.58	0.58	0.57	0.65	0.63	0.57	0.57	0.57	0.57	0.60	0.50
Ken-795	0.61	0.54	0.60	0.59	0.60	0.59	0.56	0.63	0.64	0.64	0.58	0.57	0.58	0.61	0.63	0.59	0.64	0.63	0.57	0.55	0.55	0.56	0.65	0.45
Ken-808	0.55	0.49	0.57	0.56	0.60	0.56	0.59	0.59	0.59	0.59	0.56	0.53	0.57	0.56	0.57	0.57	0.59	0.57	0.59	0.52	0.52	0.53	0.59	0.47
Ken-809	0.60	0.50	0.58	0.54	0.59	0.54	0.56	0.61	0.61	0.61	0.56	0.56	0.60	0.56	0.56	0.57	0.61	0.59	0.56	0.58	0.58	0.59	0.60	0.47
Ken-814	0.58	0.54	0.59	0.55	0.60	0.55	0.53	0.61	0.62	0.62	0.61	0.57	0.60	0.56	0.56	0.62	0.62	0.60	0.53	0.58	0.58	0.59	0.58	0.43
Ken-839	0.63	0.66	0.59	0.70	0.55	0.70	0.52	0.66	0.68	0.68	0.58	0.67	0.68	0.63	0.62	0.59	0.68	0.66	0.52	0.59	0.59	0.60	0.65	0.57
Ken-841	0.65	0.57	0.63	0.68	0.59	0.68	0.58	0.64	0.65	0.65	0.62	0.71	0.68	0.61	0.59	0.63	0.65	0.63	0.59	0.63	0.63	0.63	0.68	0.57
Ken-856	0.59	0.50	0.60	0.56	0.57	0.56	0.56	0.65	0.65	0.65	0.58	0.58	0.63	0.59	0.61	0.59	0.65	0.63	0.56	0.57	0.57	0.57	0.62	0.47
Ken-877	0.57	0.52	0.56	0.52	0.63	0.52	0.57	0.62	0.62	0.62	0.54	0.56	0.60	0.53	0.58	0.55	0.62	0.60	0.57	0.54	0.54	0.55	0.56	0.48
Ken-887	0.62	0.51	0.58	0.57	0.58	0.57	0.56	0.63	0.63	0.63	0.57	0.56	0.57	0.61	0.61	0.58	0.63	0.62	0.57	0.56	0.56	0.57	0.65	0.46
Ken-898	0.61	0.52	0.57	0.56	0.61	0.56	0.57	0.63	0.63	0.63	0.55	0.57	0.61	0.55	0.59	0.56	0.63	0.62	0.57	0.55	0.55	0.56	0.57	0.48
SA-170	0.57	0.47	0.56	0.53	0.57	0.53	0.55	0.62	0.62	0.62	0.56	0.52	0.57	0.54	0.57	0.57	0.62	0.61	0.55	0.54	0.54	0.55	0.59	0.46
SA-172	0.60	0.50	0.58	0.56	0.63	0.56	0.60	0.62	0.62	0.62	0.54	0.58	0.61	0.56	0.57	0.55	0.62	0.60	0.61	0.55	0.55	0.56	0.62	0.47
SA-712	0.56	0.55	0.59	0.58	0.61	0.58	0.57	0.65	0.66	0.66	0.57	0.61	0.63	0.55	0.58	0.58	0.66	0.64	0.57	0.52	0.52	0.53	0.63	0.47
SA-724	0.57	0.55	0.57	0.57	0.61	0.57	0.57	0.62	0.63	0.63	0.56	0.60	0.61	0.57	0.63	0.57	0.63	0.63	0.58	0.52	0.52	0.53	0.62	0.50
SA-844	0.59	0.51	0.57	0.54	0.59	0.54	0.57	0.61	0.61	0.61	0.56	0.53	0.56	0.57	0.57	0.57	0.61	0.60	0.57	0.55	0.55	0.56	0.59	0.45
SA-855	0.57	0.50	0.53	0.57	0.57	0.57	0.55	0.60	0.61	0.61	0.53	0.54	0.61	0.54	0.57	0.54	0.61	0.59	0.55	0.52	0.52	0.53	0.60	0.46
SA-947	0.59	0.52	0.57	0.55	0.60	0.55	0.59	0.63	0.63	0.63	0.54	0.57	0.60	0.57	0.59	0.55	0.63	0.61	0.60	0.56	0.56	0.57	0.60	0.45
SA-1486	0.57	0.49	0.61	0.56	0.59	0.56	0.57	0.62	0.62	0.62	0.55	0.57	0.58	0.57	0.57	0.56	0.62	0.60	0.57	0.57	0.57	0.57	0.61	0.47

2.2.8 Phylogeography of paramyxoviruses across Africa

Phylogeographic analysis of these African bat-associated paramyxoviruses allowed the study of the geographic spread of the viruses involved. **Figure 2.11** gives the phylogenetic relationship between all available RMH-derived paramyxovirus sequences detected from bats on the African continent. The genetic diversity among these viruses was clearly observed with a distinct split into two clusters. The bottom cluster (**Figure 2.11** no. 1) was formed by the rubulavirus-related viruses while the other cluster (no. 2-5) sub-divided into smaller clusters belonging to the henipavirus-, morbillivirus- and jeilongvirus-related groups as well as possible new genera. The majority of these viruses grouped outside of the proposed *Jeilongvirus* genus (situated at the base of cluster 4). Coloured branches represented the country from where each sequence was detected. In some instances small clusters originating from the same country formed. The DRC and Kenya were two such examples. Opposite to this, in several occasions genetically related viruses were isolated from several different countries. The top end of the fifth cluster was a clear illustration of this.

For ease of analysis, the phylogeographic tree was converted to a cladogram (to only display topology) in which terminal clusters of two or more sequences from the same location were collapsed and countries of origin were added as labels (**Figure 2.12**). Based on this geographical analysis, the first evidence of paramyxoviruses on the African continent was in Gabon from where it eventually was introduced into Ghana (**Figure 2.12** split near the root of the tree). The most ancestral cluster (no. 1) of the tree belongs to the rubulavirus-related viruses all of which originated from Central Africa (Gabon) and then introduced into DRC and Ghana. The split near the root of the tree into Ghana (no. 2) was eventually introduced into Kenya (**Figure 2.12** no. 3-5) resulting in the formation of a third major cluster. These three major clusters were referred to as the Gabon-, Ghana- and Kenya clusters. In the Ghana cluster, introductions seemed to be mainly limited to West and Central African countries with only two introductions into East Africa (Kenya). From the branching point resulting in the Kenya cluster, several introductions into a number of countries were observed (**Figure 2.12** no. 3-5). **Figure 2.13** presents a geographical map with the links between countries based on data derived from the phylogeographic analysis. The three main clusters (Kenya, Gabon and Ghana) are clearly marked.

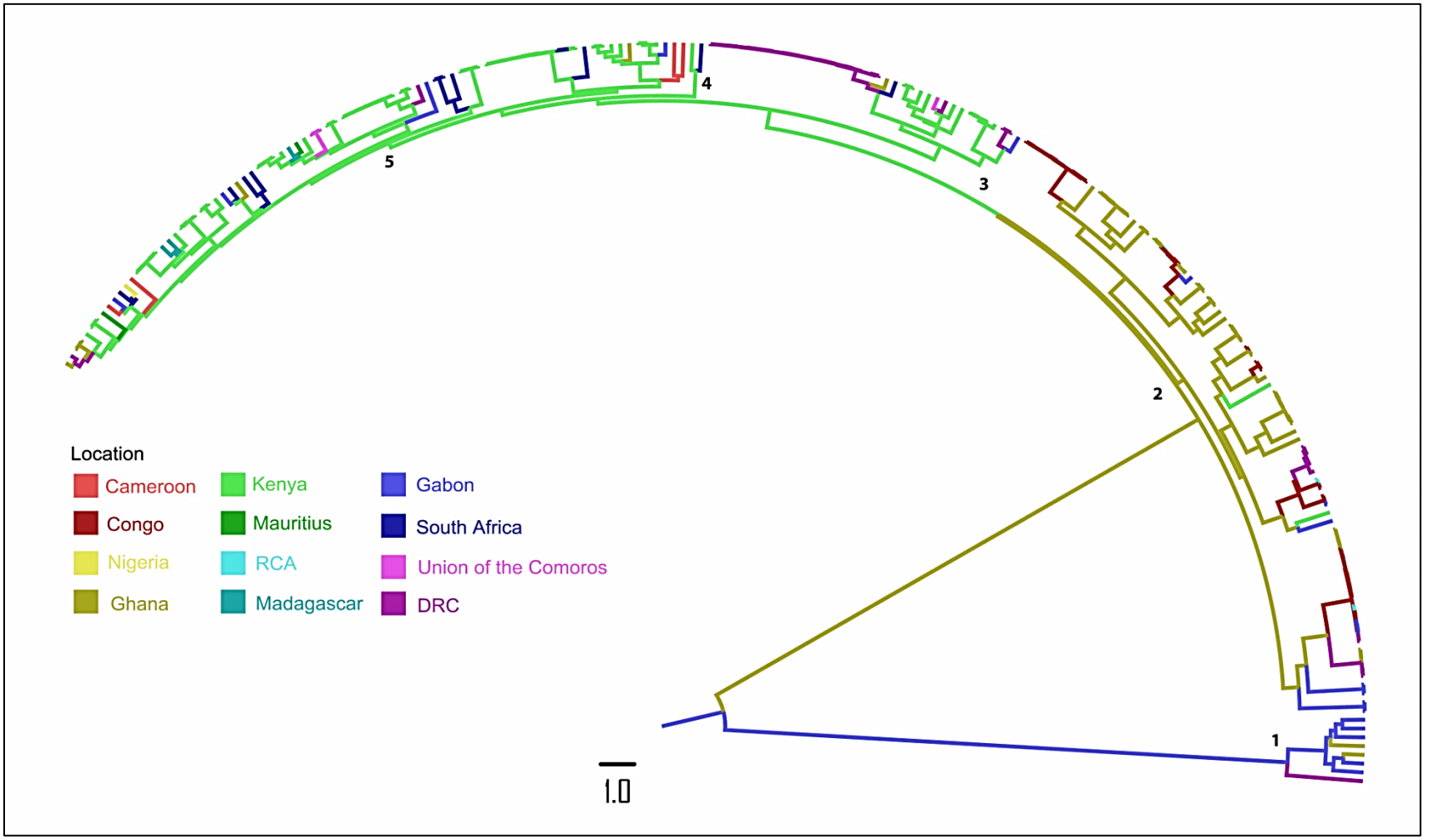


Figure 2.11: Phylogeography of bat paramyxoviruses detected on the African continent (partial L-gene sequences). The phylogenetic relationship between African bat-associated paramyxoviruses is depicted. All sequences were derived from the RMH universal primer set designed by Tong *et al.* (2008). Bayesian phylogenetics was used to incorporate the country where each sequence was detected. (1: Rubulavirus-related; 2: Henipavirus-related; 3: Morbillivirus-related (Based on classification of Drexler *et al.,* 2012); 4: Jeilongvirus-related; 5: Possible novel genera). Refer to **Figure 2.12** for a cladogram of this tree for an increased resolution of branches and **Figure 2.14** for specific sequence labels).

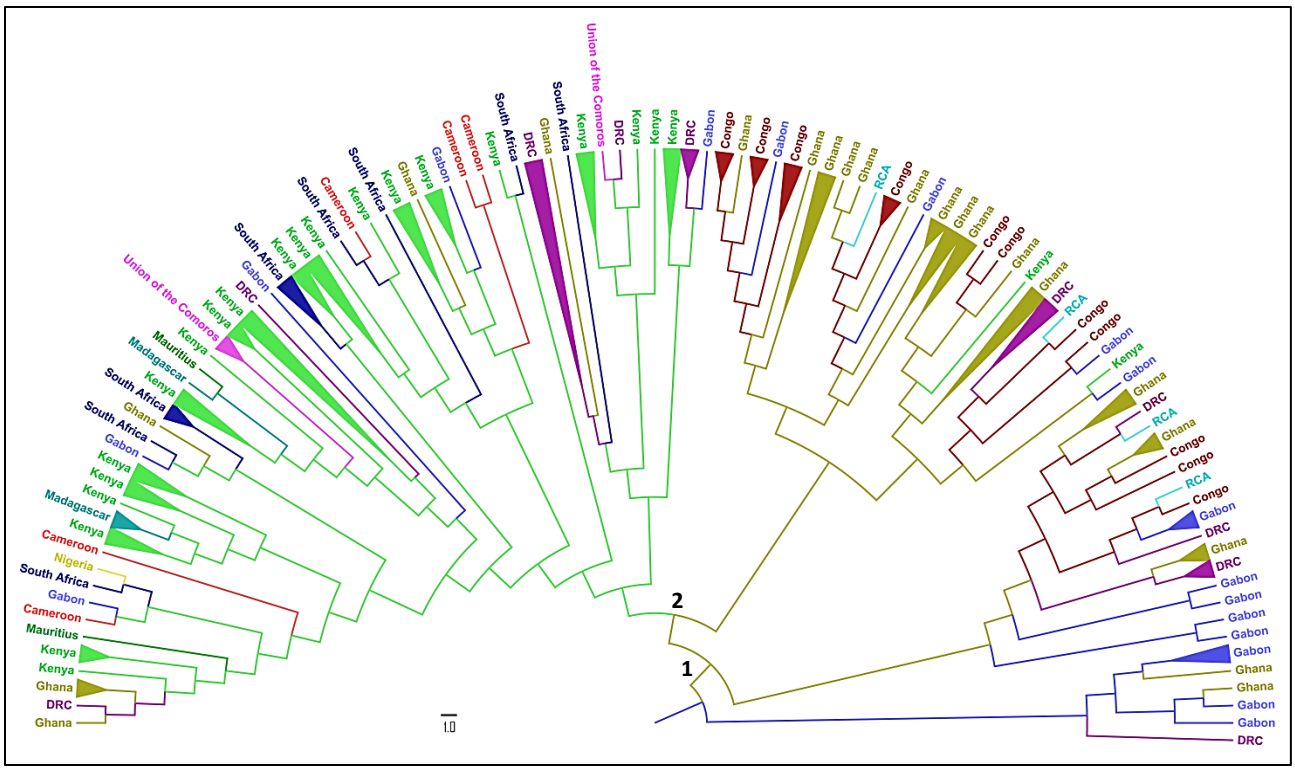


Figure 2.12: Cladogram based on the phylogeography of paramyxoviruses on the African continent (partial L-gene sequences) (also refer to **Figure 2.14**). Based on this, the possible directions of spill-over events between countries can be observed. (Numbers 1 and 2 indicate major branching points resulting in three main clusters referred to as the *Gabon*, *Ghana* and *Kenya* clusters).

The number of sequences that were available for analysis per region is represented by the size of the circles at each country. The majority of available sequences were derived from Kenya and Ghana (Baker *et al.,* 2012; Drexler *et al.,* 2012). Preliminary results showed that introduction into West African countries took place at a slow rate. All South African as well as off-coast island introductions originates from Kenya. Introductions into other countries mainly originate directly from Kenya.

The cladogram depicted by **Figure 2.14** also represents the specific viral sequence detected from where bat species can derived. All sequences belonging to the Gabon and Ghana cluster (rubula- and henipavirus-related), originated from fruit bats (*Pteropodidae*) although several non-fruit bat species were also sampled in countries that grouped within these clusters (Drexler *et al.,* 2012). The first evidence of paramyxoviruses on the African continent up to this point was from fruit bats. Opposite to this, the Kenya cluster was representative of viral sequences isolated mainly from other bat families and a few fruit bats. The isolates from the Kenya cluster all group within the *Morbillivirus*- and

jeilongvirus-related genera as well as within possible new genera. Results were also indicative of several other clusters forming (**Figure 2.14** no.5) that could be potential novel genera in the *Paramyxovirinae* sub-family.

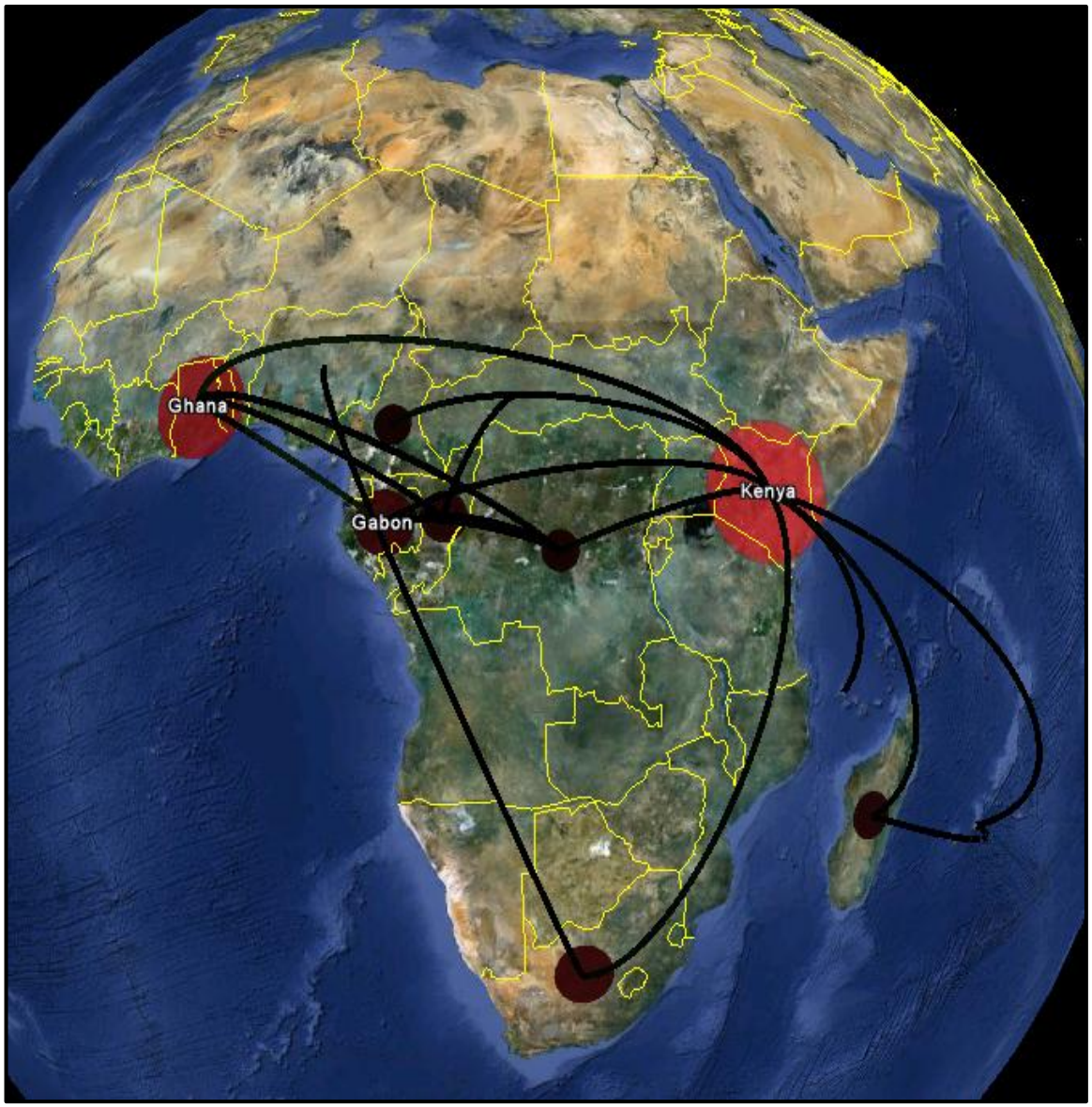


Figure 2.13: **African Paramyxovirus transmission between the different countries sampled.** The three major clusters (Gabon, Ghana and Kenya) from where most introductions originate from are clearly depicted in the figure. Lines connecting countries is indicative of virus spread between the two involved countries. Based on current findings the directionality of spread is from West Africa to East Africa and to other countries from hereon (Image generated with Google Earth®).

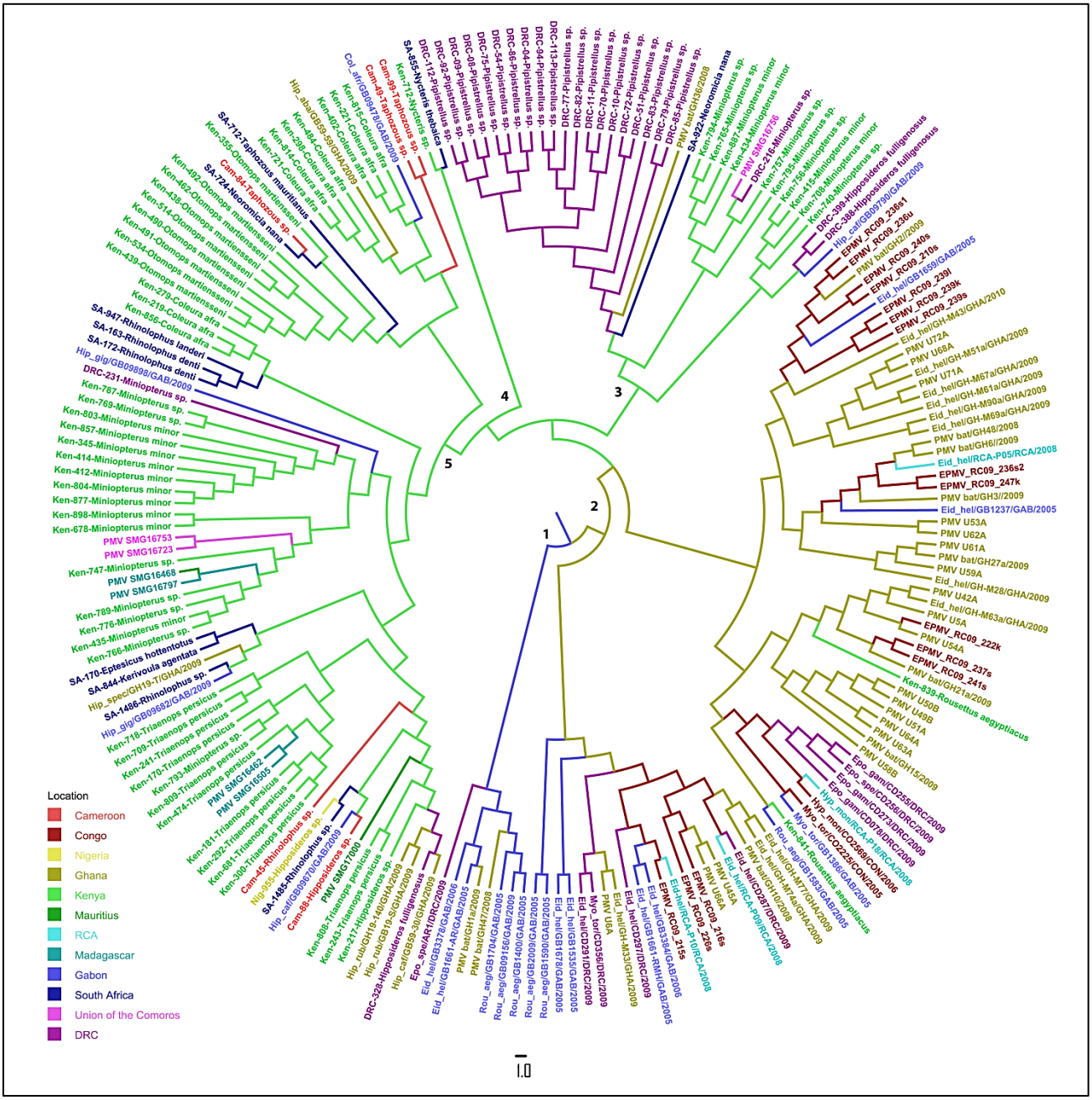


Figure 2.14: Cladogram of the phylogeography and the specific sequence labels of paramyxoviruses detected on the African continent (partial L-gene sequences). This tree allows the deduction of bat species involved at the specific locations and species initially implicated as host on the continent prior to spill-over between bat species. (1:Rubulavirus-related; 2:Henipavirus-related; 3:Morbillivirus-related; 4:Jeilongvirus-related; 5:Possible novel genera)

2.3 DISCUSSION

The only two viruses in the *Paramyxoviridae* family of zoonotic origin, Hendra and Nipah virus, are associated with bats as their reservoir host (Dutch, 2010). Both these viruses are highly pathogenic in humans. As a result of agricultural intensification and hunting practices among other things, humans are increasingly coming into close contact with these animals. The possibility of bats to harbour other potential zoonotic paramyxoviruses cannot be excluded. This emphasizes the importance of detecting the presence and determining the genetic diversity of these viruses in their bat hosts. When this is established, these viruses can be further studied with regards to pathogenicity and zoonotic potential.

In past years, when considering paramyxoviruses and bats the terms fruit bats and henipaviruses were most popular. Supplementary to other publications *e.g.* that of Baker *et al.* (2012) and Drexler *et al.* (2012), the vast genetic variability of paramyxoviruses is also reported in this study. Detection of these genetically diverse viruses would not have been possible without the use of degenerate universal primers. Such primers, designed by Tong *et al.* (2008), proved to be superior to the primers designed in this study and a useful tool in paramyxovirus detection. Initial primer design in this study had limited or no amplification capabilities of the positive control sequences. This can be a result of primers designed to focus more on the *Henipavirus* genus while the positive *Otomops* controls grouped away from this genus. Although these primers did not prove useful in this study, they have the potential to be used in henipavirus-related surveillance studies.

The *Paramyxovirinae*-specific primers (PMV) appear superior to the *Respiro-*, *Morbilli-* and *Henipavirus* (RMH) primers when considering the henipavirus-related viruses detected by Baker *et al.* (2012). The majority of henipavirus-related viruses in that study were amplified with the use of the PMV primers. The RMH primer set proved capable of detecting sequences grouping with and outside of the proposed new *Jeilongvirus* genus. This was also observed during the evaluation of these primer sets (PMV and RMH) using the *Otomops* positive samples as positive control. Here only two positives were amplified with the PMV-primers as opposed to the RMH-primers amplifying four of the five. Clearly the genetic diversity of paramyxoviruses present in bats cannot be detected by a single primer set, and will require multiple approaches incorporating different primers to enable a relatively good coverage of what is circulating in nature. Other sequence independent

methods as previously discussed can also provide coverage for the genetic diversity as shown by Van der Hoek *et al.* (2004) in the case of coronaviruses.

The difference in sequence specificity of the two primer sets may have influenced the results of this study. Through only using the RMH primers for screening of the samples received from the CDC, this study was limited to the extent of what could have been detected. By also screening the samples obtained from the CDC with the PMV primers, a more concrete conclusion would have been possible with regards to the effectiveness of the two primers as well as a better resolution of the phylogenetic relationship between different sequences (where sequences were obtained from both primers).

The full genome of at least four newly detected paramyxoviruses derived from fruit bats has been completed. The first two were completed by Drexler *et al.* (2012), one being conspecific with human mumps virus (BatPV/Epo_spe/218-AR1/DRC/2009) and another grouping closely with the *Henipavirus* genus (BatPV/Eid_hel/GH-M74a/GHA/2009). Baker *et al.* (2013) also successfully sequenced the full-genome of two novel rubulaviruses Achimota paramyxovirus 1 and 2. With the availability of an increased number of full-genome sequences of the recently detected paramyxoviruses, current primers can be greatly improved and other primer binding sites can be identified (for other genes or regions of the genome) when targeting more specific clusters.

2.3.1 *Eidolon helvum* as possible reservoir

Most literature on African bat-paramyxoviruses based their search on the findings of Drexler *et al.* (2009) in which *Eidolon helvum* was implicated as host to the first bat-associated paramyxoviruses in Africa. The possible pathogenicity of these viruses, as they are considered henipavirus-related, played a major role in the exponential search for paramyxoviruses in bats across the globe. The majority of novel paramyxoviral sequences detected since the initial discovery were derived from this particular bat species. With the detection of a vast number of new sequences also considered to be henipavirus-related, the phylogenetic grouping of these initial henipavirus-related sequences has shifted. Sequences previously considered to be closely relate to the henipaviruses, now cluster in sister-groups to this genus.

Despite screening of three different tissue types and 72 different *E. helvum* specimens, no positives were obtained in this study. This could either been due to the use of only the RMH primers during sample screening at the CDC. As previously mentioned,

these primers seem to have limited capability when compared to that of the PMV primers in detecting henipavirus-related viruses that seem to be solely linked to fruit bats. Several non-henipaviruses were also detected by Baker *et al.* (2012) from this bat species. The positivity rate in *E. helvum* detected by Drexler *et al.* (2012) on the African continent was one in every eight samples screened. Based on this, the expected positivity of 11.6% was not seen for this species in this particular study despite sampling from the same region (DRC). Two henipavirus-related viruses were however detected in *Rousettus aegyptiacus* with the use of the RMH-primers (Ken-839 and Ken-841) indicating that this primer set does possess the capability of amplifying selected henipavirus-related viruses. These two sequences did however prove to be unique and not particularly similar to *Eidolon helvum* derived sequences. The likelihood of detecting viral RNA closely related to these sequences was thus possible with the RMH-primers although not observed for *Eidolon helvum* in this study. The possibility that selected individuals from the 72 samples were in fact positive and went undetected due to primer choice cannot be excluded. This assumption can only be supported once these samples are re-tested with the PMV-primers for which they were unfortunately not available.

Sampling by Drexler *et al.* (2012) from other species, implicated several other bat species, frugivorous and insectivorous, as hosts for paramyxoviruses. This showed that although *E. helvum* was the first to be implicated as a reservoir and has high paramyxovirus prevalence, it is not superior to other species. Several insectivorous bat species are now also recognised hosts of paramyxoviruses on the African continent.

2.3.2 Other bat species as possible reservoirs

Paramyxovirus studies on the African continent were mainly focused on the fruit bat *Eidolon helvum* as mentioned previously. One such study conducted by Drexler *et al.* (2012) also explored a bit further into other bat species (including insectivorous bats) on the African continent. In this study, a number of insectivorous species were confirmed as hosts to paramyxoviruses. Chintapitasakul *et al.* (2012) conducted a similar study regarding cave dwelling bats in Thailand and was able to implicate several more bat species. The current study was the first to shift the focus to non-frugivorous bat species as reservoirs for paramyxoviruses and can be considered the most extensive molecular screening study on the African continent in terms of species diversity and regions sampled. The large sample number of 1241 bats is representative of at least 48 different

species. Several species did not test positive for example *Tadaria aegyptiaca* and *Glauconycteris variegata* which could be due to the limited sampling of that particular species as only 5 samples for each species were available for screening. The opposite is also true in the case of *Hipposideros commersoni* for which a large number of 79 kidneys were sampled, all negative for paramyxoviruses. This species can thus be paramyxovirus free or be host to paramyxoviruses whose genetic sequence is too diverse to be amplified by the current primer panel. Another possibility can also be a very low prevalence of these viruses as seen with *Rousettus aegyptiacus* from which only two samples tested positive out of the 123 screened.

Tissue type could also be considered a huge limitation when conducting surveillance studies. Information regarding tissue tropism of these viruses is still lacking. A different tissue tropism might be seen between these viruses and might also depend on the bat species involved. Drexler *et al.* (2012) reported the various concentrations of paramyxoviruses in the different organs of *Eidolon helvum* samples that tested positive. For this particular species, the spleen presented as the best tissue for surveillance as in most instances, the highest paramyxovirus concentration was detected here. In one instance however, a detectable concentration was only found in the kidney of one individual (GH90/GHA/2009). The tissue tropism for species sampled in this study remains unknown and kidney might not have been the main organ in which these viruses proliferate. Screening of multiple organs from individual bats may prove useful in determining if they in fact are positive for paramyxoviruses and not presumed negative as a result of limited tissue sampling. Tissue type should thus be considered as a factor influencing positivity of species.

When considering the sampling of bats, another limiting factor that should be considered is the conservation of bat populations. Many bat species are seen as threatened and specimen collection is not a viable option. For the sampling of paramyxoviruses, non-invasive sampling as described by Chua (2003) is a viable option for these threatened species. The collection of urine and faeces has previously aided in the detection of paramyxoviruses from *Eidolon helvum* (Baker *et al.,* 2012). Non-invasive sampling does however have its own limitations but from a bat conservation perspective, it would be a more suitable approach than capturing and killing bats for organ harvesting.

Molecular screening of this vast number of bat species in Africa allowed the first report of several new genera implicated as host and possible reservoir for paramyxoviruses which include *Eptesicus (*Vespertilionidae*), Nycteris (*Nycteridae*), Rhinolophus (*Rhinolophidae*) and Otomops (*Molossidae*)* among others. Several new species within previously implicated genera also tested positive for paramyxoviruses *e.g. Taphozous mauritianus*, *Hipposideros fuliginosus* and *Miniopterus minor* (Chintapitasakul *et al.,* 2012; Drexler *et al.,* 2012 and Wilkinson *et al.,* 2012).

Some species presented with a much higher prevalence than others. *Pipistrellus* sp. sampled in the DRC had 50% positivity as compared to *Rousettus aegyptiacus* ranging at 1.6%. Opposite to these results Drexler *et al.* (2012) reported a positivity of 8.5% for *R. aegyptiacus* and 5.9% positivity from *Pipistrellus* species sampled. Difference in positivity between these independent studies might be due to the difference in geographic sampling locations or perhaps a difference in species sampled within this genus.

The high positivity in *Pipistrellus* may be a result of the virus being actively transmitted horizontally or vertically between individuals as all *Pipistrellus* samples were derived from a single colony in the DRC. This particular virus appears to have established itself in this specific colony. For a better understanding of the relationship between this particular virus species and the particular bats, it will be crucial to determine the specific bat species involved as these bats were only classified down to genus level. The difference in *R. aegyptiacus* positivity on the other hand appears to be related to region. Samples collected in Kenya and South Africa presented with a low prevalence compared to samples collected in Gabon by Drexler *et al.* (2012) presenting with several positive samples. This bat species occurs in several regions across the continent (The IUCN Red List of Threatened Species v2012.2, http://www.iucnredlist.org) with vast distances between the Gabon, Kenya and South African locations. Virus transmission between individuals of this species in these different countries would not be possible as they do not migrate vast distances as seen for *Eidolon helvum.* Migration of this bat species has however been recorded for distances up to 500km, which presents ample opportunity for virus transmission between individuals within this geographical distance. Individuals in Gabon however might have been exposed to several other bat species occurring in the region from where virus spill-over could have taken place. *Rousettus aegyptiacus* is known to co-roost in caving systems with several other bat species including *Miniopterus spp.* and *Rhinolophus* spp. The possibility of virus transmission between bat species and genera is

thus possible. Based on the phylogenetic results however, none of the *Rousettus aegyptiacus* positive sequences, group closely with these or other insectivorous bat sequences detected.

The above variance in positivity between this study and that of Drexler *et al.* (2012) is also true for several other species screened including *Coleura afra*. This could again be as a result of the geographical distances between colonies from different countries or the establishment of a virus within bats from a particular region. Other possible reasons for different prevalence between bat species and between the separate studies could be the time when sampling took place, virus titres might be linked to birthing season or seasonality, or be attributable to the genetics of the different viruses for example difference in virulence and transmissibility. One could also argue that some species are more likely to be primary hosts (high prevalence) as opposed to incidental hosts (low prevalence due to limited transmissibility). To determine this, a more intensive longitudinal study will be required focussing on specific bat species and the viruses they harbour.

The health status of all bats sampled was noted. No significant conclusions could be drawn. The majority of bats that tested positive were deemed healthy upon capture. Several, but not all, *Coleura afra* individuals that tested positive were however noted to be sick or dead. Whether or not paramyxoviruses are the etiological agents, remain to be determined. It could be debated that the presumed healthy individuals that tested positive might have been in the early stage of disease where symptoms were not yet prominent. On the other hand, these paramyxoviruses might be present but only cause disease when these bats were stressed (immune compromised) by external factors, including environmental conditions, food availability or even during the birthing season. A more intensive molecular screening approach coupled with pathogenicity studies (Koch's postulates) will be required to determine the true impact these viruses have on their Chiropteran hosts and whether they cause disease.

2.3.3 Phylogenetic clustering of paramyxoviruses

For phylogenetic analysis, representative sequences were selected for each genus. The type virus as well as several other members per genus and several unclassified viruses were included. It can be debated whether or not this selection of sequences introduced a bias, but resulting phylogenetic placing, grouped the majority of newly detected sequences outside any of the known genera. Inclusion of viruses yet to be

classified to genus level also reduced the level of bias if one was present. Phylogenetic clustering would thus not have been influenced by including more of the previously described established paramyxovirus sequences.

Phylogenetic analysis revealed an extensive distribution of the newly detected paramyxovirus sequences throughout the *Paramyxovirinae* sub-family. Six clusters of interest were noted. Many studies stress the importance of henipavirus-related viruses and how they can have zoonotic potential. The importance of the grouping of the two *Rousettus*-derived sequences within this cluster is debatable. With the inclusion of all sequences considered to be henipavirus-related, this larger cluster is divided into at least three other smaller clusters. The two *Rousettus* sequences, cluster into a sister-group to the Hendra- and Nipah virus cluster. Whether these sister groups should be considered as separate genera or not, remains to be determined.

Drexler *et al.* (2012) pointed out that the henipaviruses contain an amino acid change in the highly conserved GDNQ motif in the polymerase gene to GDNE. Several of their sequences considered as henipavirus-related still contain the GNDQ motif. The newly described *Henipavirus,* Cedar virus, does not contain this GDNE motif but the ancestral GDNQ. It was observed by Marsh *et al.* (2012) that challenge studies with Cedar virus in ferrets and guinea pigs resulted in no clinical manifestations although virus replication took place. This difference in the GDNQ motif might play a role in the pathogenicity of Hendra- and Nipah virus and the lack thereof in the case of Cedar virus. The two *Rousettus*-derived sequences cluster with sequences from Drexler *et al.* (2012) determined to have the GDNQ motifs. Although the pathogenicity of these newly detected viruses from *Rousettus* remains unknown, it is hypothesized that novel henipavirus-related viruses containing a GDNE motive, for example BatPV Eid_hel/GB1535/GAB/2005 detected by Drexler *et al.* (2012), should be of most concern.

Opposed to what was observed in the analysis of *Morbillivirus* and related viruses by Drexler *et al.* (2012), no direct clustering of isolates from this study took place with this genus. Interestingly, one of the sequences classified as morbillivirus-related by Drexler *et al.* (2012) (Bat PMV Hip_caf/GB59-30/GHA/2009), groups in the cluster furthest from this genus in the RMH-phylogenetic analysis. Inclusion of the proposed *Jeilongvirus* genus in the analysis by Drexler *et al.* (2012) would have drastically changed the grouping of this virus (among others) as they would have been considered as jeilongvirus-related or unrelated to any of the established genera. Inclusion of all sequences detected in this

study to the analysis of the sequences from Drexler *et al.* (2012), a distinct phylogenetic split was observed, implicating that these viruses are neither morbillivirus- nor jeilongvirus-related but rather form part of one or more novel genera. Based on phylogenetic clustering observed from this study and through various comparisons with other studies, it is hypothesized that at least two novel genera can be described for the *Paramyxovirinae* sub-family.

Comparative to the results of Drexler *et al.* (2012), all henipa- and related viruses seem to be linked solely to fruit bats (*Eidolon, Rousettus, Epomophorus etc.*). This might be due to limited screening of insectivorous bats, screening with primers limited in their capability of detecting henipaviruses or it could be indicative of the specificity of these viruses to fruit bats.

2.3.4 Geographical and species/genus clustering

A clear geographical grouping was observed from sequences derived from *Rhinolophus* sampled in different locations across South Africa. This might be as a result of the different viruses establishing in the different location and adapting to that specific species and/or colony. The phylogenetic analysis between sequences from this study and that of Drexler *et al.* (2012) provided evidence of species and genus clustering in numerous occasions. With detection of more paramyxovirus sequences from other bat species it would provide supplementary information to this feature of these viruses. Adaptation of specific viruses to specific bat species and/or genera might also be as a result of differences in immune system or availability/presence of specific receptors in these bat species.

2.3.5 Novel paramyxoviruses in bats

Zhu *et al.* (2009) detected a vast number of genetically diverse astroviruses in bats from China. Paramyxoviruses clearly share this diversity in their bat hosts. This study alone obtained approximately 36 unique paramyxovirus sequences (five of which were similar to that of previous publications) not to mention the 66 detected by Drexler *et al.* (2012), those reported by Baker *et al.* (2012) from Ghana, Sasaki *et al.* (2012) from Indonesia, Weiss *et al.* (2012) from the Congo and Wilkinson *et al.* (2012) from Southwest Indian Ocean Islands. Amino acid comparison of all newly detected bat-associated paramyxoviruses resulted in the conclusion that at least 31 putative viral species were

detected in this study. This number may increase with full-genome sequencing, as several sequences lie just above the cut-off border of 93% amino acid similarity and may prove to be more different in other regions of their genome.

With the increasing number of bat derived paramyxoviruses detected, a drastic change in the phylogenetics of this family of viruses is taking place. Where the *Henipavirus* genus only consisted of two viruses clustering together, we now see several clusters forming around this genus. This is also true for several novel viruses and other genera in this family. The question is whether these viruses truly belong to these genera or whether they in fact form part of novel yet to be classified genera? When considering the amino acid similarity matrix of previously identified paramyxoviruses (**Table 2.9**), viruses from different genera share above 60% similarity (*Morbilli-*, *Henipa-* and *Jeilongvirus*). The majority of newly detected paramyxoviruses share a similarity of above 60%. Given the current analysis based on this genome region, almost all these viruses belong to the same genus and in some instances share the same similarity with two established viruses from different genera. Using such an approach to determine possible genus classification is thus not feasible especially when comparing such a short sequence from the most conserved gene in this family. This can perhaps only give an indication as to the closest related genus to the specific sequence. Determining the genus classification of these putative viral species, can only be done through analysis of longer length, less conserved regions of the genome or ideally full-genome sequence comparison.

Determination of genus classification based on sequence divergence can become problematic, as discussed above, when considering the vast genetic diversity observed in this subfamily. Full-genome sequencing will thus form key to classification of these newly detected viral species into existing genera or possibly into novel genera.

2.3.6 Genetic diversity and virus circulation

Similar to what was observed by Chintapitasakul *et al.* (2012) a wide genetic diversity was observed at similar sites. Why particular virus populations within a single colony or location mutates at an alarming rate, resulting in this genetic diversity, is not yet clear. According to observations made by Chintapitasakul *et al.* (2012), human interference at roosting sites of bats greatly increase the mutation rates of viruses within those particular colonies. This might perhaps be due to increased stress placed on these animals causing a drop in immune protection, subsequently allowing a more active

replication of the virus in the host. This then allows replication errors to be incorporated or it might be new introductions into the colonies as opposed to mutations taking place.

Evidence of virus circulation was obtained from an *Otomops martiensseni* colony in Kenya. A genetically identical virus was detected from the same colony in two consecutive years. *Miniopterus minor* is also a good example of virus movement between individuals roosting in different locations. Individuals from two different caves presenting with a genetically identical virus can indicate the movement of this particular species between different roosting sites. The unrestricted host range of related paramyxoviruses as well as the more restricted paramyxoviruses capable only of infecting specific species or members in a specific genus was clearly demonstrated in this study.

Contamination during the analysis of these samples was excluded as a possibility, as screening was conducted per location before moving to samples from the next location. This is also true for Ken-793-*Miniopterus* sp. harbouring the same paramyxoviral RNA as *Triaenops persicus* samples from Kenya, as these samples were processed on different dates.

2.3.7 Paramyxovirus distribution across Africa

A unique characteristic that contributes to the success of bats as reservoirs is their ability to fly (Calisher *et al.,* 2006). Breed *et al.* (2010) was able to study the long-distance movements of several *Pteropus* species indicating the vast distances travelled by these animals over a certain amount of time. This is also true for the African fruit bat species *Eidolon helvum* who spends the majority of each year partaking in long-distance migration (Ossa *et al.,* 2012). Ossa *et al.* (2012), using a different approach to that described by Breed *et al.* (2012), were able to show that some individuals were able to travel a distance of approximately 2000 km.

Bat movement, migration (as mentioned above) and roost sharing between species play a major role in the distribution of viruses across the continent and between different species through horizontal transmission. The phylogeography of novel paramyxoviruses indicates their widespread distribution across the African continent. The occurrence of similar viruses in geographically distant countries can be ascribed to the long (and short) migration of different bat species allowing contact between species residing in different locations. Based on findings by Ossa *et al.* (2012), the distribution and movements of *Eidolon helvum* could be traced and mapped out (**Figure 2.15**).

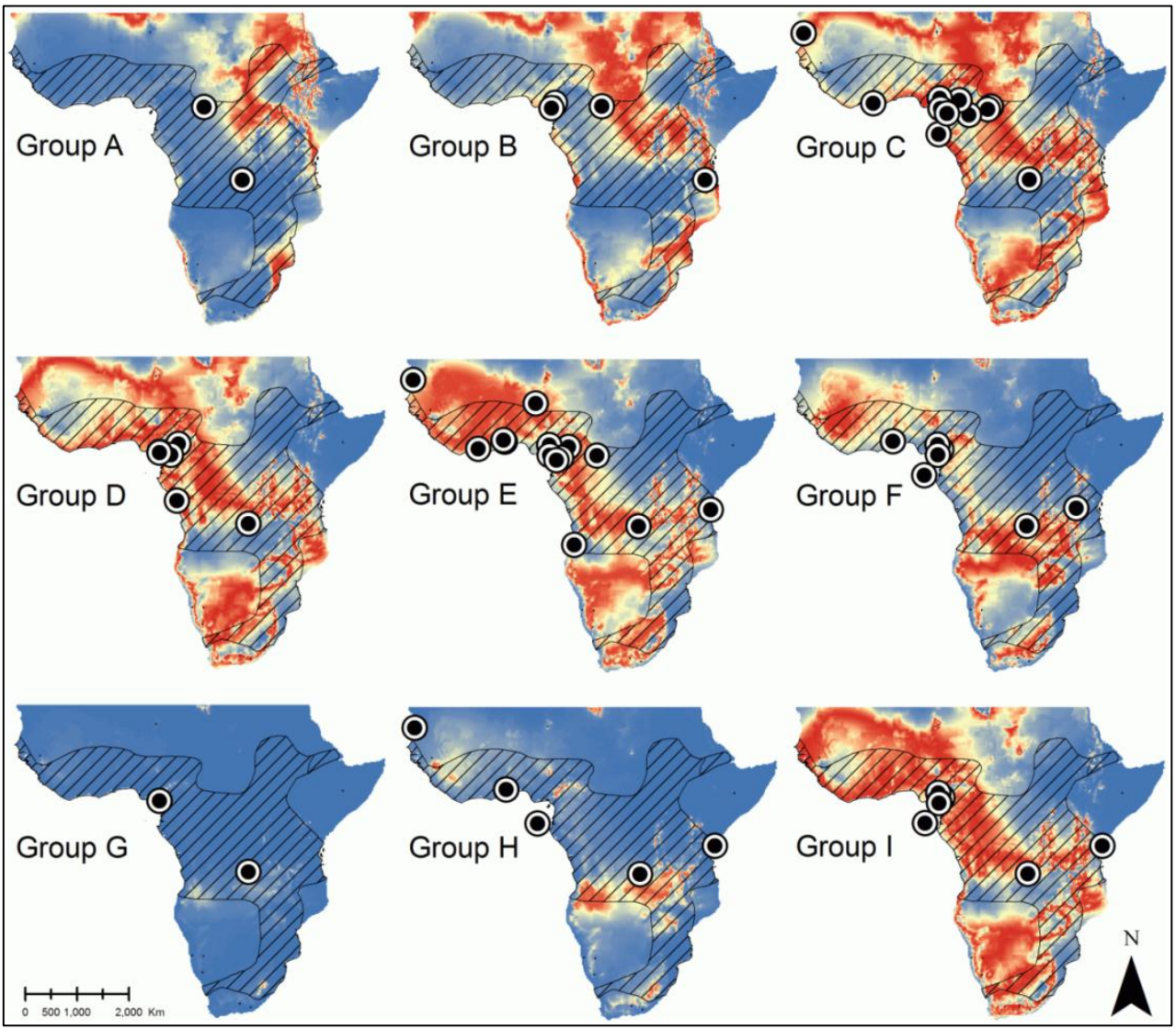


Figure 2.15: Mean probability maps of *Eidolon helvum* migration as presented by Ossa *et al.* (2012). Diagonal lines indicate the IUCN species distribution range. High to low probability of origin based on the isoscape model is indicated from red to blue respectively. Pale yellow shading represents intermediate probabilities. Group A-I represents nine mean probability maps. Black dots represent probable locations where these bats have been based on the isotope content in their fur upon analysis.

The shaded area of **Figure 2.15** represents the geographical distribution of this species as reported by the IUCN Red List of Threatened Species™ (http://www.iucnredlist.org) while black dots indicate specific locations confirmed by Ossa *et al.* (2012). In light of the fact that this species is one of the major migrants on the African continent and considering the movement to specific countries, it can be correlated to the path of transmission of several novel paramyxoviruses as indicated by the phylogeograpic analysis. It can thus be

hypothesised that *Eidolon helvum* is one of the main species involved in virus transmission and spread of novel paramyxoviruses across Africa. The long distance migration of *E. helvum* as well as their link to some of the most ancestral bat-associated paramyxoviruses (Drexler *et al.,* 2012) detected on the African continent to date, strengthens this hypothesis.

In light of this phylogeographical analysis, Gabon in central Africa seems to be the point of origin for paramyxoviruses on the continent. Rubulaviruses (most ancestral cluster) appears also to be restricted to West and Central Africa. This finding could however be skewed due to sampling. Broader surveillance and screening across other countries might strengthen this finding or alternatively prove a wider distribution of bat-associated rubulaviruses than currently observed. Gabon, Ghana and Kenya clearly formed key countries from where paramyxovirus spread took place. Interestingly, these three countries correlate to locations along the migratory movement of *Eidolon helvum* (Ossa *et al.,* 2012). **Figure 2.15E** shows the distribution and specific locations where one group of *Eidolon helvum* was traced and includes the three major countries (Gabon, Ghana and Kenya). The transmission incidence between the countries is shown in **Figure 2.13** and can be compared to the findings of Ossa *et al.* (2012) as shown in **Figure 2.15**.

Phylogeographic analysis presents strengthening proof of the involvement of Africa countries north of the equator (0-20°) as the proverbial ‘hot zone’ for paramyxovirus spread and movement from where introduction into other countries like South Africa take place at a lower rate than in this zone. It is thus hypothesised that the highest paramyxovirus prevalence and most phylogenetically ancestral viruses will be seen in this zone when sampling is done in other countries of this region as opposed to extreme northern and southern countries.

The true phylogeography of paramyxoviruses across the continent will only be achieved once more countries and bat species in Africa have been screened. Surveillance in countries surrounding Ghana, Gabon and Kenya, may provide better resolution in terms of virus transmission and spread across the continent.

2.3.8 South African perspective

This study provided the first evidence of paramyxoviruses this far south on the African continent. Although South Africa is considered a sub-tropical or temperate region (with a large arid area across the Northern Cape Province) (Peel *et al.,* 2007), it has a

surprisingly high diversity of bat species. Sampling in South Africa produced a 24% positivity among species sampled (33 sampled, 8 positive). While only small sample numbers were available in some instances (ranging from 1-12 per species), these species tested positive. This could be an indication of the high prevalence of paramyxoviruses in these bat species from this region.

Sampling in South Africa only took place in the north-eastern part of the country. Mozambique is adjacent to this part of the country and houses the most southern colony of *Eidolon helvum* on the African continent. If the hypothesis that this species forms key to transmission and spread of paramyxoviruses across the continent is true, one would expect a lower prevalence of paramyxoviruses in bats species as you sample more to the south-western part of the country. This would also be true for the more arid countries Namibia and Botswana (southern Africa) and the majority of countries in northern Africa that do not form part of the geographical distribution of *Eidolon helvum* (The IUCN Red List Of Threatened Species™; http://www.iucnredlist.org). Such a study in these countries will provide strengthening evidence to support this hypothesis if a noticeable decrease in prevalence is observed.

Several insectivorous bat species are also known to partake in migration, however not to the extent that we see with *E. helvum. Miniopterus natalensis* for example, have been documented to migrate distances of up to 260km (Mondajem *et al.* 2010). Distribution and spread of paramyxoviruses over shorter distances across South Africa and Africa can thus also be possible through the insectivorous bat populations. When considering the fact that many insectivorous bat species co-roost with other bats including *Rousettus aegyptiacus,* as seen in many cave systems in South Africa (Mondajem *et al.* 2010), the possibility of virus spread between bat families is possible. This however is yet to be proven and will only be possible through more intensive surveillance in these co-roosting bats.

CHAPTER 3

Concluding remarks

The emerging role that insectivorous bats play as host for paramyxoviruses should not be underestimated. This study was able to extend our knowledge regarding these bats and the vast geographical range of bat-associated paramyxoviruses across the African continent. Genetic diversity among these viruses is incredible when considering the vast number of putative species detected in the last few years. Similar studies on other continents may provide comparable results considering the almost worldwide distribution of bats and their clear association with paramyxoviruses. The hypothesis of Chintapitasakul *et al.* (2012) that co-evolution took place between paramyxoviruses and their bat hosts is strengthened by the results of this study. Clearly paramyxoviruses have proven to be adaptable to a wide range of bat species which might be a result of their capability to mutate and to produce an extensive panel of genetically diverse viruses. Africa now also proves to be a rich source of paramyxoviruses in its natural bat populations.

Although only based on hypothesis drawn from data, *Eidolon helvum* may prove to be one of the main species involved in paramyxovirus spread across Africa. On numerous occasions, this species has been implicated as possible reservoir to several viral species. With the availability of more detailed information regarding the migration of these bats and species interaction during these movements, with both migrating and non-migrating bat species, determination of virus spread between countries might become more feasible. Research regarding bat-virus interaction and the bat immune system, might also prove helpful in determining the role of *Eidolon helvum* in transmission of paramyxoviruses to other bat species and spread to other countries. The recent study by Ossa *et al.* (2012) regarding the movement and distribution of *Eidolon helvum* across the continent already provided useful information.

Phylogeographic information places the origin of paramyxoviruses on the African continent in Gabon (West Africa). Countries on either side of the equator have also shown to play an important role in the spread and movement of paramyxoviruses between countries. This can be expected as this tropical region houses a large diversity of bat populations and is known for the movements of migratory bats. More recently evolved viruses are present in countries further from the equator. Spread of these viruses thus clearly originates from the proverbial ‘hot zone’ of the continent. This however still remains

speculative as skewed sampling largely affects the current picture observed in analysis. Data obtained through sampling of other countries and bat species may change the picture we see entirely.

With the now known diversity of paramyxoviruses in nature, what is the health and veterinary health implications that should be considered? Spill-over of the henipaviruses into domestic animal and human populations proved to be catastrophic (Wang *et al.,* 1998; Chua *et al.,* 2000; Luby *et al.* 2006). Several newly detected paramyxoviruses closely related to and grouping with the *Henipavirus* genus and other known pathogenic viruses, raise concern. Not much is known about the host range and disease causing potential of these viruses and the threat they pose to both human and veterinary health.

Contact of humans and domestic animals with bats is also of concern. The bat bushmeat industry in Africa poses the greatest threat and opportunity for spill-over of these viruses into the human population. This interface is considered extremely important as this forms the major source of protein in several developing countries on the African continent. Humans come into contact with the live bats and are at risk of being bitten, scratched and infected through excretions. They get direct exposure to the vast diversity of viruses in bats, providing ample opportunity for a spill-over event to occur. Other contact opportunities are tourism and traditional practices in caves, also providing opportunity for introduction into the human population. The role of human activity (*e.g.* encroachment and bushmeat practices) largely influences the rate at which diseases can emerge. Considering the vast amount of paramyxoviruses as well as other pathogenic viruses linked to bats, the human population should strive to limit their contact and interaction with bats as a step towards disease prevention. Many underdeveloped and developing countries in Africa lack the infrastructure to detect diseases caused by these viruses. A main reason for this is misdiagnoses of the emerging disease as other diseases with similar disease presentation. Constant surveillance of paramyxoviruses will be useful in detecting possible pathogenic species and serve as an early warning system.

Baker *et al.* (2013) obtained results of possible human infection with two of the newly detected and characterised rubulaviruses (Achimota virus 1 and 2) with the use of a serological approach specifically targeting these viruses. This might well be true for several of the other newly described paramyxoviruses which emphasize the importance of future research in the field of bat paramyxoviruses.

Molecular screening for novel paramyxoviruses is only the first step in preventing disease emergence or spread. Considering that the vast genetic diversity of paramyxoviruses was discovered only recently, it is clear that most aspects regarding the pathogenicity and host range of these viruses remain to be elucidated. For a better understanding of the novel paramyxoviruses, an approach similar to that used by Baker *et al.* (2013) will be required. As previously mentioned, through further analysis of the two novel rubulaviruses, they were able to do virus isolation, proliferation and analysis in cell culture as well as full-genome sequence analysis and characterization. Such an approach will provide the means to study these viruses and obtain insight into pathogenicity and perhaps the host range. By developing serological assays for individual viruses, surveillance in human and animal (wild and domestic) may be useful to evaluate any likely transmission and zoonoses events.

Future research on these newly detected paramyxoviruses should follow a similar approach to that of Baker *et al.* (2013). Coupled with this, spill-over experimental studies at cell line level seem equally important to determine the possible risk of human and animal populations to these viruses. Such studies are likely to provide rapid, informative results. A multi-disciplinary approach between virologists, zoologists and the medical sectors, whether human or veterinary, i.e. the One Health approach, will be essential for future progress.

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APPENDIX

Table A1: Detailed record of sampling per individual location.

Country	Location (Figure 2.1)	Bat species	Number/ positive
Kenya	Asembo-Church and School[1]	*Rhinolophus labiatus*	6/0
		Neoromicia sp.	23/0
		Scotoecus sp.	1/0
	Chyulu Hills[2]	*Miniopterus* sp.	1/0
	Gilgil mine[3]	*Miniopterus* sp.	42/9
		Rhinolophus sp.	2/0
	Jimba cave[4]	*Coleura afra*	19/6
		Hipposideros sp.	8/1
		Miniopterus minor	48/3
		Triaenops persicus	9/6
	Kericho cave[5]	*Miniopterus* sp.	15/4
	Kisii[6]	*Eidolon helvum*	8/0
	Malindi[7]	*Eidolon helvum*	5/0
	Meseno KEFRI[8]	*Eidolon helvum*	1/0
	Mount Elgon-Kitum cave[9]	*Miniopterus natalensis*	15/0
		Rhinolophus sp.	6/0
	Mount Elgon-Makingeni cave[10]	*Rousettus aegyptiacus*	24/0
	Pare cave[11]	*Miniopterus minor*	53/9
		Rhinolophus sp.	5/0
	Three caves[11]	*Coleura afra*	8/4
		Nycteris sp.	2/1
		Triaenops persicus	7/6
		Miniopterus minor	50/2
		Hipposideros commersoni	50/0
		Rousettus aegyptiacus	50/2
	Shimoni cave[12]	*Hipposideros commersoni*	21/0
		Taphozous sp.	1/0
	Suswa cave[13]	*Miniopterus* sp.	19/0
		Otomops martiensseni	40/9
		Rhinolophus landeri	9/0
		Rhinolophus sp.	1/0
	Tsavo East[14]	*Epomophorus wahlbergi*	2/0
		Scotoecus sp.	1/0

Table A1 [Continued]: Detailed record of sampling per individual location.

Country	Location (Figure 2.1)	Bat species	Number/ positive
Kenya	Tsavo East[14]	*Scotophilus dinganii*	2/0
	Tsavo West[15]	*Neoromicia* sp.	2/0
		Rhinolophus labnderi	3/0
	Vihiga[16]	*Eidolon helvum*	1/0
	Watamu[17]	*Rousettus aegyptiacus*	10/0
Cameroon	Caves[18]	*Hipposideros* sp.	39/1
		Rhinolophus sp.	9/1
		Taphozous sp.	12/3
	Maya oulu[19]	*Eidolon helvum*	9/0
	Lanavet[20]	*Chaerephon* sp.	20/0
		Scotophilus dinganii	1/0
	Ngong[20]	*Chaerephon* sp.	11/0
	Sodeconton Pitoa[21]	*Eidolon helvum*	3/0
		Epomophorus sp.	1/0
	Zoological garden[22]	*Eidolon helvum*	3/0
Nigeria	College of Agriculture[23]	*Eidolon helvum*	20/0
		Hipposideros sp.	3/1
		Lissanycteris angolensis	8/0
	Idanre cave[24]	*Hipposideros commersoni*	8/0
		Rousettus aegyptiacus	21/0
Democratic Republic of the Congo	Kinshasa N'Dili[25]	*Myonycteris torquata*	1/0
	Kisangani-Chimestan[26]	*Eidolon helvum*	4/0
	Kisangani-Mayele Island[26]	*Eidolon helvum*	18/0
	Kisangani-Zoo[26]	*Myonycteris torquata*	2/0
		Myotis sp.	2/0
	Kisangani-Abandoned factory[26]	*Mimetillus moloneyi*	1/0
	Rain forest next to Layoko[27]	*Glauconycteris argentata*	1/0
		Hipposideros fuliginosus	21/3
		Hipposideros gigas	1/0
		Hypsignathus monstrosus	2/0
		Megaglossus woermanni	4/0
		Myonycteris torquata	5/0
		Myotis sp.	1/0

Table A1 [Continued]: Detailed record of sampling per individual location.

Country	Location (Figure 2.1)	Bat species	Number/ positive
Democratic Republic of the Congo	Rain forest next to Layoko[27]	*Neoromicia* sp.	1/0
	Rain forest next to Masako[28]	*Hipposideros gigas*	1/0
		Megaglossus woermanni	6/0
	Kisantu-Church[29]	*Chaerephon pumilus*	25/0
		Chaerephon sp.	1/0
		Micropteropus pussilus	1/0
		Mops condylurus	4/0
	Kisantu-School 2[29]	*Pipistrellus* sp.	40/20
		Chaerephon sp.	13/0
	Mbanza Ngungu cave[30]	*Miniopterus* sp.	41/2
		Rhinolophus sp.	1/0
	Kinshasa-School 1[31]	*Chaerephon* sp.	8/0
		Mops condylurus	27/0
	UNIKIN Campus Club/house[31]	*Mops condylurus*	2/0
		Scotophilus dinganii	2/0
Country	**Location (Figure 2.2)**	**Bat species**	**Number/ positive**
South Africa	***Gauteng***		
	Irene cave[6]	*Miniopterus* sp.	32/0
	Pretoria zoo[14]	*Neoromicia capensis*	2/0
	Midrand[10]	*Neoromicia capensis*	2/0
	KwaZulu-Natal		
	Rocktail bay: St Lucia[15]	*Chaerephon ansorgei*	1/0
		Chaerephon pumilus	3/0
		Epomophorus walbergi	3/0
		Glauconycteris variegata	2/0
		Kerivoula argentata	1/1
		Nycteris thebaica	6/1
		Pipistrellus hesperidus	5/0
		Scotophilus dinganii	10/0
	28 Edinburgh Street, Umkomaas[2]	*Chaerephon pumilus*	1/0
	27 Edinburgh Street, Umkomaas[2]	*Epomophorus walbergi*	2/0
	26 Edinburgh Street, Umkomaas[2]	*Epomophorus walbergi*	1/0
	Ethekwini unicity[5]	*Epomophorus walbergi*	1/0

Table A1 [Continued]: Detailed record of sampling per individual location.

Country	Location (Figure 2.2)	Bat species	Number/ positive
South Africa	***KwaZulu-Natal***		
	23 Star Street, Chatsworth[1]	*Epomophorus walbergi*	2/0
	24 Star Street, Chatsworth[1]	*Epomophorus walbergi*	1/0
	81 Winfield drive[3]	*Epomophorus walbergi*	1/0
	Limpopo		
	Pafuri, Kruger National Park[13]	*Chaerephon ansorgei*	1/0
		Chaerephon pumilus	4/0
		Epomophorus gambianus	2/0
		Epomophorus walbergi	3/0
		Glauconycteris variegata	3/0
		Hipposideros caffer	4/0
		Mops condylurus	6/0
		Neoromicia capensis	2/0
		Neoromicia helios	6/0
		Neoromicia nana	7/2
		Neoromicia rueppellii	1/0
		Neoromicia zuluensis	1/0
		Nycticeinops schlieffeni	9/0
		Pipistrellus rusticus	5/0
		Rhinolophus fumigatus	2/0
		Rhinolophus landeri	1/1
		Rousettus aegyptiacus	9/0
		Scotophilus dinganii	13/0
		Scotophilus leucogaster	2/0
		Scotophilus nigrita	1/0
		Scotophilus viridis	3/0
		Taphozous mauritianus	3/1
	Buzzard mountain[4]	*Epomophorus walbergi*	1/0
	Matlapitsi cave[12]	*Miniopterus* sp.	5/0
		Rhinolophus sp.	6/5
		Rousettus aegyptiacus	9/0
	North West		
	Taung[16]	*Eptesicus hottentotus*	2/1
		Neoromicia capensis	6/0

Table A1 [Continued]: Detailed record of sampling per individual location.

Country	Location (Figure 2.2)	Bat species	Number/ positive
South Africa	***North West***		
	Taung[16]	*Rhinolophus darlingi*	1/0
		Rhinolophus denti	5/3
		Tadaria aegyptiaca	4/0
	Mooiplaats 94 KP, Madikwe Game reserve[11]	*Miniopterus natalensis*	5/0
		Nycteris thebaica	2/1
		Rhinolophus darlingi	4/0
		Rhinolophus simulator	2/0
	Kgaswane[8]	*Neoromicia capensis*	1/0
		Pipistrellus sp.	2/0
		Scotophilus sp.	12/0
	Kalkfontein 111 KP, Madikwe Game Reserve[7]	*Neoromicia capensis*	2/0
		Sauromys petrophilus	1/0
	Leeuwenhoek 112 KP, Madikwe Game Reserve[9]	*Scotophilus dinganii*	1/0
Swaziland	Mlawula Nature Reserve[17]	*Nycteris thebaica*	4/0

Table A2: Viral sequence names and accession number as on GenBank (partial L-gene).

Sample	Primer set	GenBank sequence name	Accession number
Cam-45	RMH	BatPV/Rhi_sp./Cam-45/2010	KC578678
Cam-49	RMH	BatPV/Tap_sp./Cam-49/2010	KC578679
Cam-84	RMH	BatPV/Tap_sp./Cam-84/2010	KC578680
Cam-88	RMH	BatPV/Hip_sp./Cam-88/2010	KC578681
Cam-99	RMH	BatPV/Tap_sp./Cam-99/2010	KC578682
DRC-04	RMH	BatPV/Pip_sp./DRC-04/2011	KC578653
DRC-08	RMH	BatPV/Pip_sp./DRC-08/2011	KC578654
DRC-09	RMH	BatPV/Pip_sp./DRC-09/2011	KC578655
DRC-10	RMH	BatPV/Pip_sp./DRC-10/2011	KC578656
DRC-11	RMH	BatPV/Pip_sp./DRC-11/2011	KC578657
DRC-51	RMH	BatPV/Pip_sp./DRC-51/2011	KC578658
DRC-54	RMH	BatPV/Pip_sp./DRC-54/2011	KC578659
DRC-72	RMH	BatPV/Pip_sp./DRC-72/2011	KC578660
DRC-75	RMH	BatPV/Pip_sp./DRC75/2011	KC578661
DRC-77	RMH	BatPV/Pip_sp./DRC77/2011	KC578662
DRC-79	RMH	BatPV/Pip_sp./DRC-79/2011	KC578663
DRC-82	RMH	BatPV/Pip_sp./DRC-82/2011	KC578664
DRC-83	RMH	BatPV/Pip_sp./DRC-83/2011	KC578665
DRC-85	RMH	BatPV/Pip_sp./DRC-85/2011	KC578666
DRC-86	RMH	BatPV/Pip_sp./DRC-86/2011	KC578667
DRC-90	RMH	BatPV/Pip_sp./DRC-90/2011	KC578668
DRC-92	RMH	BatPV/Pip_sp./DRC-92/2011	KC578669
DRC-94	RMH	BatPV/Pip_sp./DRC-94/2011	KC578670
DRC-112	RMH	BatPV/Pip_sp./DRC-112/2011	KC578671
DRC-113	RMH	BatPV/Pip_sp./DRC-113/2011	KC578672
DRC-216	RMH	BatPV/Min_sp./DRC-216/2011	KC578673
DRC-231	RMH	BatPV/Min_sp./DRC-231/2011	KC578674
DRC-328	RMH	BatPV/Hip_ful/DRC-328/2011	KC578675
DRC-388	RMH	BatPV/Hip_ful/DRC-388/2011	KC578676
DRC-399	RMH	BatPV/Hip_ful/DRC-399/2011	KC578677
Ken-170	RMH	BatPV/Tri_per/Ken-170/2010	KC578640
Ken-181	RMH	BatPV/Tri_per/Ken-181/2010	KC578641
Ken-217	RMH	BatPV/Hip_sp./Ken-217/2010	KC578642
Ken-219	RMH	BatPV/Col_afr/Ken-219/2010	KC578643
Ken-221	RMH	BatPV/Col_afr/Ken-221/2010	KC578644
Ken-241	RMH	BatPV/Tri_per/Ken-241/2010	KC578645
Ken-243	RMH	BatPV/Tri_per/Ken-243/2010	KC578646
Ken-279	RMH	BatPV/Col_afr/Ken-279/2010	KC578647
Ken-292	RMH	BatPV/Tri_per/Ken-292/2010	KC578648
Ken-298	RMH	BatPV/Col_afr/Ken-298/2010	KC578649
Ken-300	RMH	BatPV/Tri_per/Ken-300/2010	KC578650
Ken-345	RMH	BatPV/Min_min/Ken-345/2011	KC578591
Ken-355	RMH	BatPV/Oto_mar/Ken-355/2011	KC578592

Table A2 [Continued]: Viral sequence names and accession number as on GenBank (partial L-gene).

Sample	Primer set	GenBank sequence name	Accession number
Ken-402	RMH	BatPV/Col_afr/Ken-402/2011	KC578593
Ken-412	RMH	BatPV/Min_min/Ken-412/2011	KC578594
Ken-414	RMH	BatPV/Min_min/Ken-414/2011	KC578595
Ken-415	RMH	BatPV/Min_min/Ken-415/2011	KC578596
Ken-434	RMH	BatPV/Min_min/Ken-434/2011	KC578597
Ken-435	RMH	BatPV/Min_min/Ken-435/2011	KC578598
Ken-438	RMH	BatPV/Oto_mar/Ken-438/2011	KC578599
Ken-439	RMH	BatPV/Oto_mar/Ken-439/2011	KC578600
Ken-462	RMH	BatPV/Oto_mar/Ken-462/2011	KC578601
Ken-474	RMH	BatPV/Tri_per/Ken-474/2011	KC578602
Ken-484	RMH	BatPV/Col_afr/Ken-484/2011	KC578603
Ken-490	RMH	BatPV/Oto_mar/Ken-490/2011	KC578604
Ken-491	RMH	BatPV/Oto_mar/Ken-491/2011	KC578605
Ken-492	RMH	BatPV/Oto_mar/Ken-492/2011	KC578606
Ken-514	RMH	BatPV/Oto_mar/Ken-514/2010	KC578651
Ken-534	RMH	BatPV/Oto_mar/Ken-534/2010	KC578652
Ken-678	RMH	BatPV/Min_min/Ken-678/2011	KC578607
Ken-681	RMH	BatPV/Tri_per/Ken-681/2011	KC578608
Ken-708	RMH	BatPV/Min_min/Ken-708/2011	KC578609
Ken-709	RMH	BatPV/Tri_per/Ken-709/2011	KC578610
Ken-712	RMH	BatPV/Nyc_sp./Ken-712/2011	KC578611
Ken-718	RMH	BatPV/Tri_per/Ken-718/2011	KC578612
Ken-721	RMH	BatPV/Col_afr/Ken-721/2011	KC578613
Ken-740	RMH	BatPV/Min_sp./Ken-740/2011	KC578614
Ken-747	RMH	BatPV/Min_sp./Ken-747/2011	KC578615
Ken-756	RMH	BatPV/Min_sp./Ken-756/2011	KC578616
Ken-757	RMH	BatPV/Min_sp./Ken-757/2011	KC578617
Ken-765	RMH	BatPV/Min_sp./Ken-765/2011	KC578618
Ken-766	RMH	BatPV/Min_sp./Ken-766/2011	KC578619
Ken-769	RMH	BatPV/Min_sp./Ken-769/2011	KC578620
Ken-776	RMH	BatPV/Min_sp./Ken-776/2011	KC578621
Ken-787	RMH	BatPV/Min_sp./Ken-787/2011	KC578622
Ken-789	RMH	BatPV/Min_sp./Ken-789/2011	KC578623
Ken-793	RMH	BatPV/Min_sp./Ken-793/2011	KC578624
Ken-794	RMH	BatPV/Min_sp./Ken-794/2011	KC578625
Ken-795	RMH	BatPV/Min_sp./Ken-795/2011	KC578626
Ken-803	RMH	BatPV/Min_min/Ken-803/2011	KC578627
Ken-804	RMH	BatPV/Min_min/Ken-804/2011	KC578628
Ken-808	RMH	BatPV/Tri_per/Ken-808/2011	KC578629
Ken-809	RMH	BatPV/Tri_per/Ken-809/2011	KC578630
Ken-814	RMH	BatPV/Col_afr/Ken-814/2011	KC578631
Ken-815	RMH	BatPV/Col_afr/Ken-815/2011	KC578632
Ken-839	RMH	BatPV/Rou_aeg/Ken-893/2011	KC578633

Table A2 [Continued]: Viral sequence names and accession number as on GenBank (partial L-gene).

Sample	Primer set	GenBank sequence name	Accession number
Ken-841	RMH	BatPV/Rou_aeg/Ken-841/2011	KC578634
Ken-856	RMH	BatPV/Col_afr/Ken-856/2011	KC578635
Ken-857	RMH	BatPV/Min_min/Ken-857/2011	KC578636
Ken-877	RMH	BatPV/Min_min/Ken-877/2011	KC578637
Ken-887	RMH	BatPV/Min_min/Ken-887/2011	KC578638
Ken-898	RMH	BatPV/Min_min/Ken-898/2011	KC578639
Nig-955	RMH	BatPV/Hip_sp./Nig-955/2010	KC538903
SA-160	PMV	BatPV/Rhi_den/RSA-160/2007	KC578569
SA-163	PMV	BatPV/Rhi_den/RSA-163a/2007	KC578570
	RMH	BatPV/Rhi_den/RSA-163b/2007	KC578571
SA-170	PMV	BatPV/Ept_hot/RSA-170a/2007	KC578572
	RMH	BatPV/Ept_hot/RSA-170b/2007	KC578573
SA-172	RMH	BatPV/Rhi_den/RSA-172/2007	KC578574
SA-190	PMV	BatPV/Nyc_the/RSA-190/2007	KC578575
SA-712	PMV	BatPV/Tap_mau/RSA-712a/2010	KC578576
	RMH	BatPV/Tap_mau/RSA-712b/2010	KC578577
SA-724	RMH	BatPV/Neo_nan/RSA-724/2010	KC578578
SA-844	PMV	BatPV/Ker_age/RSA-844a/2010	KC578579
	RMH	BatPV/Ker_age/RSA-844b/2010	KC578580
SA-855	RMH	BatPV/Nyc_the/RSA-855/2010	KC578581
SA-922	RMH	BatPV/Neo_nan/RSA-922/2010	KC578582
SA-947	RMH	BatPV/Rhi_lan/RSA-947/2010	KC578583
SA-1485	PMV	BatPV/Rhi_sp./RSA-1485a/2012	KC578584
	RMH	BatPV/Rhi_sp./RSA-1485b/2012	KC578585
SA-1486	PMV	BatPV/Rhi_sp./RSA-1486a/2012	KC578586
	RMH	BatPV/Rhi_sp./RSA-1486b/2012	KC578587
SA-1493	PMV	BatPV/Rhi_sp./RSA-1493/2012	KC578588
SA-1494	PMV	BatPV/Rhi_sp./RSA-1494/2012	KC578589
SA-1495	PMV	BatPV/Rhi_sp./RSA-1495/2012	KC578590

Table A3: Available museum voucher numbers of positive South African samples.

Sample number	Voucher number (Field number)
SA-160-*Rhinolophus denti*	TM48034
SA-163-*Rhinolophus denti*	TM48036
SA-170-*Eptesicus hottentotus*	TM48039
SA-172-*Rhinolophus denti*	TM48041
SA-190-*Nycteris thebaica*	TM48014
SA-712-*Taphozous mauritianus*	(ECJS-15/2010)
SA-724-*Neoromicia nana*	(ECJS-12/2010)

Table A4: Accession numbers to sequences used in phylogeographic analysis (partial L-gene sequences).

Sequence	GenBank Accession
BatPV/Col_afr/GB09478/GAB/2009	HQ660155
BatPV/Eid_hel/CD287/DRC/2009	HQ660123
BatPV/Eid_hel/CD291/DRC/2009	HQ660124
BatPV/Eid_hel/CD297/DRC/2009	HQ660125
BatPV/Eid_hel/GB1237/GAB/2005	HQ660140
BatPV/Eid_hel/GB1535/GAB/2005	HQ660141
BatPV/Eid_hel/GB1659/GAB/2005	HQ660142
BatPV/Eid_hel/GB1661-AR/GAB/2005	HQ660109
BatPV/Eid_hel/GB1661-RMH/GAB/2005	HQ660143
BatPV/Eid_hel/GB1678/GAB/2005	HQ660144
BatPV/Eid_hel/GB3378/GAB/2006	HQ660092
BatPV/Eid_hel/GB3384/GAB/2006	HQ660146
BatPV/Eid_hel/GH10/GHA/2008	FJ609191
BatPV/Eid_hel/GH15/GHA/2009	FJ971935
BatPV/Eid_hel/GH1a/GHA/200	FJ971943
BatPV/Eid_hel/GH2/GHA/2009	FJ971944
BatPV/Eid_hel/GH21a/GHA/2009	FJ971939
BatPV/Eid_hel/GH27a/GHA/2009	FJ971940
BatPV/Eid_hel/GH47/GHA/2008	FJ609195
BatPV/Eid_hel/GH48/GHA/2008	FJ609194
BatPV/Eid_hel/GH6/GHA/2009	FJ971945
BatPV/Eid_hel/GH-M28/GHA/2009	HQ660147
BatPV/Eid_hel/GH-M33/GHA/2009	HQ660148
BatPV/Eid_hel/GH-M43/GHA/2010	HQ660127
BatPV/Eid_hel/GH-M51a/GHA/2009	HQ660132
BatPV/Eid_hel/GH-M61a/GHA/2009	HQ660133
BatPV/Eid_hel/GH-M63a/GHA/2009	HQ660136
BatPV/Eid_hel/GH-M67a/GHA/2009	HQ660131
BatPV/Eid_hel/GH-M69a/GHA/2009	HQ660135
BatPV/Eid_hel/GH-M74a/GHA/2009	HQ660129
BatPV/Eid_hel/GH-M77/GHA/2009	HQ660130
BatPV/Eid_hel/GH-M90a/GHA/2009	HQ660134
BatPV/Eid_hel/RCA-P05/RCA/2008	HQ660150
BatPV/Eid_hel/RCA-P09/RCA/2008	HQ660151
BatPV/Eid-hel/RCA-P10/RCA/2008	HQ660149
BatPV/Epo_gam/CD078/DRC/2009	HQ660128
BatPV/Epo_gam/CD255/DRC/2009	HQ660120
BatPV/Epo_gam/CD273/DRC/2009	HQ660122
BatPV/Epo_spe/AR1/DRC/2009	HQ660095

Table A4 [Continued]: Accession numbers to sequences used in phylogeographic analysis (partial L-gene sequences).

Sequence	GenBank Accession
BatPV/Epo_spe/CD256/DRC/2009	HQ660121
BatPV/Hip_aba/GB59-59/GHA/2009	HQ660162
BatPV/Hip_caf/GB09670/GAB/2009	HQ660156
BatPV/Hip_caf/GB09790/GAB/2009	HQ660158
BatPV/Hip_caf/GB59-30/GHA/2009	HQ660161
BatPV/Hip_gig/GB09682/GAB/2009	HQ660157
BatPV/Hip_gig/GB09898/GAB/2009	HQ660159
BatPV/Hip_rub/GB19-S/GHA/2009	HQ660160
BatPV/Hip_rub/GH19-140/GHA/2009	HQ660153
BatPV/Hip_spec/GH19-T/GHA/2009	HQ660154
BatPV/Hyp_mon/RCA-P18/RCA/2008	HQ660152
BatPV/Myo_tor/CD356/DRC/2009	HQ660126
BatPV/Myo_tor/CO2225/CON/2005	HQ660118
BatPV/Myo_tor/GB1386/GAB/2005	HQ660137
BatPV/Pip_nan/GH36/GHA/2008	FJ609192
BatPV/Rou_aeg/CO2569/CON/2005	HQ660094
BatPV/Rou_aeg/GB09156/GAB/2009	HQ660100
BatPV/Rou_aeg/GB1400/GAB/2005	HQ660106
BatPV/Rou_aeg/GB1583/GAB/2005	HQ660138
BatPV/Rou_aeg/GB1590/GAB/2005	HQ660139
BatPV/Rou_aeg/GB1704/GAB/2005	HQ660098
BatPV/Rou_aeg/GB2009/GAB/2005	HQ660145
Eidolon helvum paramyxovirus clone U45A	JN862582
Eidolon helvum paramyxovirus clone U49B	JN862572
Eidolon helvum paramyxovirus clone U50B	JN862580
Eidolon helvum paramyxovirus clone U51A	JN862564
Eidolon helvum paramyxovirus clone U53A	JN862568
Eidolon helvum paramyxovirus clone U58B	JN862583
Eidolon helvum paramyxovirus clone U59A	JN862570
Eidolon helvum paramyxovirus clone U5A	JN862592
Eidolon helvum paramyxovirus clone U61A	JN862578
Eidolon helvum paramyxovirus clone U62A	JN862567
Eidolon helvum paramyxovirus clone U63A	JN862574
Eidolon helvum paramyxovirus clone U64A	JN862575
Eidolon helvum paramyxovirus clone U66A	JN862581
Eidolon helvum paramyxovirus clone U68A	JN862584
Eidolon helvum paramyxovirus clone U6A	JN862565
Eidolon helvum paramyxovirus clone U71A	JN862585

Table A4 [Continued]: Accession numbers to sequences used in phylogeographic analysis (partial L-gene sequences).

Sequence	GenBank Accession
Eidolon helvum paramyxovirus clone U72A	JN862576
Eidolon paramyxovirus_RC09_210s	HE647821
Eidolon paramyxovirus_RC09_215s	HE647823
Eidolon paramyxovirus_RC09_216s	HE647824
Eidolon paramyxovirus_RC09_222k	HE647825
Eidolon paramyxovirus_RC09_226s	HE647826
Eidolon paramyxovirus_RC09_236s1	HE801055
Eidolon paramyxovirus_RC09_236s2	HE801056
Eidolon paramyxovirus_RC09_236u	HE647827
Eidolon paramyxovirus_RC09_237s	HE647828
Eidolon paramyxovirus_RC09_239k	HE647829
Eidolon paramyxovirus_RC09_239l	HE647830
Eidolon paramyxovirus_RC09_239s	HE647831
Eidolon paramyxovirus_RC09_240s	HE647832
Eidolon paramyxovirus_RC09_241s	HE647833
Eidolon paramyxovirus_RC09_247k	HE647834
Miniopterus gleni paramyxovirus SMG16468	JQ886097
Miniopterus griveaudi paramyxovirus SMG16723	JQ886099
Miniopterus griveaudi paramyxovirus SMG16753	JQ886101
Miniopterus griveaudi paramyxovirus SMG16756	JQ886103
Miniopterus sororculus paramyxovirus SMG16797	JQ886104
Mormopterus acetabulosus paramyxovirus SMG17000	JQ886105
Triaenops menamena paramyxovirus SMG16462	JQ886096
Triaenops menamena paramyxovirus SMG16505	JQ886098

Table A5: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Cam-45	Cam-49	Cam-84	Cam-88	Cam-99	DRC-04	DRC-08	DRC-09	DRC-10	DRC-11	DRC-51	DRC-54	DRC-72	DRC-75	DRC-77	DRC-92	DRC-113	DRC-94	DRC-79	DRC-82	DRC-83	DRC-85	DRC-86	DRC-70	DRC-112	DRC-216
Cam-45	*	0.739	0.686	0.826	0.713	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.678	0.643
Cam-49		*	0.704	0.782	0.756	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.669	0.686
Cam-84			*	0.765	0.739	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.634
Cam-88				*	0.730	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.713	0.686
Cam-99					*	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.686	0.652
DRC-04						*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-08							*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-09								*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-10									*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-11										*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-51											*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-54												*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-72													*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-75														*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-77															*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-92																*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-113																	*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-94																		*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-79																			*	1.000	1.000	1.000	1.000	1.000	1.000	0.756
DRC-82																				*	1.000	1.000	1.000	1.000	1.000	0.756
DRC-83																					*	1.000	1.000	1.000	1.000	0.756
DRC-85																						*	1.000	1.000	1.000	0.756
DRC-86																							*	1.000	1.000	0.756
DRC-70																								*	1.000	0.756
DRC-112																									*	0.756
DRC-216																										*

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	DRC-231	Ken-241	Ken-170	Ken-181	Ken-217	Ken-219	Ken-221	Ken-243	Ken-279	Ken-292	Ken-298	Ken-300	Ken-514	Ken-534	DRC-328	DRC-388	DRC-399	Ken-345	Ken-355	Ken-412	Ken-414	Ken-415	Ken-434	Ken-435	Ken-438	Ken-439
Cam-45	0.730	0.773	0.739	0.765	0.808	0.721	0.669	0.739	0.721	0.765	0.660	0.773	0.686	0.678	0.791	0.695	0.695	0.739	0.686	0.739	0.739	0.686	0.713	0.739	0.686	0.686
Cam-49	0.713	0.817	0.756	0.739	0.765	0.747	0.756	0.765	0.747	0.739	0.721	0.739	0.704	0.704	0.756	0.686	0.686	0.704	0.704	0.704	0.704	0.669	0.739	0.782	0.704	0.704
Cam-84	0.721	0.747	0.747	0.704	0.747	0.695	0.747	0.756	0.695	0.704	0.713	0.704	1.000	0.991	0.756	0.704	0.704	0.756	1.000	0.756	0.756	0.695	0.713	0.765	1.000	1.000
Cam-88	0.739	0.826	0.782	0.739	0.826	0.721	0.713	0.808	0.721	0.739	0.695	0.739	0.765	0.756	0.826	0.704	0.704	0.756	0.765	0.756	0.756	0.695	0.747	0.756	0.765	0.765
Cam-99	0.739	0.747	0.747	0.713	0.747	0.713	0.765	0.695	0.713	0.713	0.747	0.721	0.739	0.739	0.730	0.678	0.678	0.721	0.739	0.721	0.721	0.643	0.713	0.765	0.739	0.739
DRC-04	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-08	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-09	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-10	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-11	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-51	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-54	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-72	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-75	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-77	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-92	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-113	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-94	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-79	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-82	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-83	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-85	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-86	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-70	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-112	0.678	0.669	0.695	0.652	0.695	0.686	0.686	0.669	0.686	0.652	0.669	0.652	0.686	0.695	0.695	0.747	0.747	0.713	0.686	0.713	0.713	0.765	0.808	0.695	0.686	0.686
DRC-216	0.660	0.660	0.678	0.626	0.678	0.686	0.643	0.695	0.686	0.626	0.643	0.626	0.634	0.634	0.660	0.704	0.704	0.678	0.634	0.678	0.678	0.704	0.843	0.686	0.634	0.634

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-462	Ken-474	Ken-484	Ken-490	Ken-491	Ken-492	Ken-678	Ken-681	Ken-708	Ken-709	Ken-712	Ken-718	Ken-721	Ken-740	Ken-747	Ken-756	Ken-757	Ken-765	Ken-766	Ken-769	Ken-776	Ken-789	Ken-793	Ken-794	Ken-795	Ken-803
Cam-45	0.686	0.756	0.669	0.695	0.686	0.686	0.739	0.773	0.686	0.773	0.704	0.765	0.678	0.695	0.739	0.695	0.643	0.704	0.747	0.730	0.747	0.747	0.747	0.704	0.686	0.739
Cam-49	0.704	0.756	0.756	0.695	0.704	0.704	0.756	0.739	0.669	0.817	0.756	0.808	0.747	0.678	0.773	0.678	0.695	0.739	0.791	0.695	0.791	0.791	0.765	0.739	0.713	0.704
Cam-84	1.000	0.686	0.721	0.982	1.000	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Cam-88	0.765	0.739	0.704	0.747	0.765	0.765	0.756	0.739	0.695	0.826	0.704	0.808	0.713	0.704	0.747	0.704	0.695	0.747	0.765	0.730	0.765	0.765	0.791	0.747	0.721	0.756
Cam-99	0.739	0.713	0.782	0.730	0.739	0.739	0.730	0.721	0.643	0.747	0.721	0.747	0.773	0.652	0.739	0.643	0.669	0.678	0.765	0.704	0.765	0.765	0.747	0.678	0.695	0.721
DRC-04	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-08	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-09	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-10	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-11	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-51	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-54	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-72	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-75	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-77	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-92	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-113	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-94	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-79	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-82	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-83	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-85	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-86	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-70	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-112	0.686	0.652	0.695	0.678	0.686	0.686	0.704	0.652	0.765	0.669	0.678	0.678	0.686	0.773	0.678	0.739	0.782	0.773	0.695	0.695	0.695	0.695	0.704	0.773	0.800	0.713
DRC-216	0.634	0.660	0.643	0.626	0.634	0.634	0.704	0.626	0.704	0.660	0.652	0.652	0.652	0.704	0.686	0.695	0.930	0.808	0.695	0.652	0.695	0.695	0.686	0.808	0.817	0.678

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-787	Ken-804	Ken-808	Ken-809	Ken-814	Ken-815	Ken-839	Ken-841	Ken-856	Ken-857	Ken-877	Ken-887	Ken-898	Nig-955	SA-163	SA-170	SA-172	SA-712	SA-724	SA-844	SA-855	SA-922	SA-947	SA-1485	SA-1486	Ken-402
Cam-45	0.730	0.739	0.739	0.747	0.678	0.669	0.539	0.591	0.721	0.730	0.739	0.704	0.739	0.791	0.739	0.747	0.739	0.704	0.686	0.756	0.660	0.660	0.721	0.826	0.773	0.669
Cam-49	0.695	0.704	0.765	0.765	0.756	0.756	0.565	0.626	0.747	0.695	0.704	0.730	0.756	0.773	0.747	0.747	0.747	0.765	0.704	0.782	0.713	0.669	0.747	0.791	0.791	0.756
Cam-84	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Cam-88	0.730	0.756	0.808	0.791	0.721	0.713	0.565	0.591	0.721	0.739	0.756	0.747	0.756	0.904	0.739	0.782	0.739	0.730	0.765	0.773	0.686	0.704	0.704	0.904	0.852	0.713
Cam-99	0.704	0.721	0.695	0.747	0.773	0.765	0.591	0.591	0.713	0.704	0.721	0.678	0.730	0.739	0.713	0.695	0.713	0.739	0.739	0.739	0.704	0.678	0.730	0.756	0.730	0.765
DRC-04	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-08	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-09	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-10	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-11	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-51	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-54	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-72	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-75	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-77	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-92	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-113	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-94	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-79	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-82	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-83	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-85	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-86	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-70	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-112	0.695	0.713	0.669	0.704	0.686	0.686	0.573	0.626	0.686	0.713	0.713	0.773	0.704	0.713	0.634	0.652	0.634	0.626	0.686	0.669	0.634	0.965	0.626	0.704	0.704	0.686
DRC-216	0.660	0.678	0.695	0.686	0.652	0.643	0.521	0.556	0.686	0.669	0.678	0.843	0.704	0.678	0.669	0.669	0.669	0.617	0.634	0.678	0.660	0.739	0.669	0.669	0.721	0.643

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	DRC-231	Ken-241	Ken-170	Ken-181	Ken-217	Ken-219	Ken-221	Ken-243	Ken-279	Ken-292	Ken-298	Ken-300	Ken-514	Ken-534	DRC-328	DRC-388	DRC-399	Ken-345	Ken-355	Ken-412	Ken-414	Ken-415	Ken-434	Ken-435	Ken-438	Ken-439
DRC-231	*	0.747	0.765	0.721	0.765	0.678	0.686	0.739	0.678	0.721	0.669	0.721	0.721	0.713	0.765	0.669	0.669	0.913	0.721	0.913	0.913	0.686	0.730	0.817	0.721	0.721
Ken-241		*	0.860	0.834	0.782	0.704	0.695	0.791	0.704	0.834	0.669	0.843	0.747	0.739	0.782	0.669	0.669	0.747	0.747	0.747	0.747	0.660	0.713	0.782	0.747	0.747
Ken-170			*	0.843	0.739	0.695	0.756	0.773	0.695	0.843	0.721	0.843	0.747	0.756	0.747	0.678	0.678	0.791	0.747	0.791	0.791	0.652	0.713	0.808	0.747	0.747
Ken-181				*	0.765	0.704	0.695	0.765	0.704	1.000	0.704	0.991	0.704	0.713	0.756	0.652	0.652	0.739	0.704	0.739	0.739	0.669	0.686	0.756	0.704	0.704
Ken-217					*	0.721	0.721	0.808	0.721	0.765	0.713	0.773	0.747	0.739	0.947	0.739	0.739	0.782	0.747	0.782	0.782	0.695	0.747	0.756	0.747	0.747
Ken-219						*	0.695	0.695	1.000	0.704	0.686	0.695	0.695	0.695	0.704	0.730	0.730	0.669	0.695	0.669	0.669	0.678	0.730	0.704	0.695	0.695
Ken-221							*	0.713	0.695	0.695	0.869	0.695	0.747	0.739	0.704	0.660	0.660	0.713	0.747	0.713	0.713	0.634	0.686	0.730	0.747	0.747
Ken-243								*	0.695	0.765	0.721	0.765	0.756	0.747	0.834	0.678	0.678	0.739	0.756	0.739	0.739	0.669	0.713	0.747	0.756	0.756
Ken-279									*	0.704	0.686	0.695	0.695	0.695	0.704	0.730	0.730	0.669	0.695	0.669	0.669	0.678	0.730	0.704	0.695	0.695
Ken-292										*	0.704	0.991	0.704	0.713	0.756	0.652	0.652	0.739	0.704	0.739	0.739	0.669	0.686	0.756	0.704	0.704
Ken-298											*	0.704	0.713	0.721	0.713	0.678	0.678	0.678	0.713	0.678	0.678	0.643	0.652	0.704	0.713	0.713
Ken-300												*	0.704	0.713	0.765	0.652	0.652	0.739	0.704	0.739	0.739	0.669	0.686	0.747	0.704	0.704
Ken-514													*	0.991	0.756	0.704	0.704	0.756	1.000	0.756	0.756	0.695	0.713	0.765	1.000	1.000
Ken-534														*	0.747	0.695	0.695	0.747	0.991	0.747	0.747	0.686	0.704	0.756	0.991	0.991
DRC-328															*	0.739	0.739	0.791	0.756	0.791	0.791	0.678	0.730	0.756	0.756	0.756
DRC-388																*	1.000	0.678	0.704	0.678	0.678	0.791	0.791	0.686	0.704	0.704
DRC-399																	*	0.678	0.704	0.678	0.678	0.791	0.791	0.686	0.704	0.704
Ken-345																		*	0.756	1.000	1.000	0.704	0.721	0.843	0.756	0.756
Ken-355																			*	0.756	0.756	0.695	0.713	0.765	1.000	1.000
Ken-412																				*	1.000	0.704	0.721	0.843	0.756	0.756
Ken-414																					*	0.704	0.721	0.843	0.756	0.756
Ken-415																						*	0.756	0.678	0.695	0.695
Ken-434																							*	0.721	0.713	0.713
Ken-435																								*	0.765	0.765
Ken-438																									*	1.000
Ken-439																										*

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-462	Ken-474	Ken-484	Ken-490	Ken-491	Ken-492	Ken-678	Ken-681	Ken-708	Ken-709	Ken-712	Ken-718	Ken-721	Ken-740	Ken-747	Ken-756	Ken-757	Ken-765	Ken-766	Ken-769	Ken-776	Ken-789	Ken-793	Ken-794	Ken-795	Ken-803
DRC-231	0.721	0.747	0.678	0.713	0.721	0.721	0.808	0.721	0.686	0.747	0.686	0.730	0.660	0.686	0.817	0.678	0.660	0.704	0.817	0.913	0.817	0.817	0.773	0.704	0.678	0.913
Ken-241	0.747	0.834	0.686	0.739	0.747	0.747	0.782	0.843	0.660	1.000	0.765	0.973	0.686	0.669	0.765	0.652	0.669	0.704	0.782	0.730	0.782	0.782	0.852	0.704	0.678	0.747
Ken-170	0.747	0.852	0.739	0.730	0.747	0.747	0.808	0.843	0.652	0.860	0.713	0.852	0.730	0.660	0.791	0.643	0.686	0.704	0.808	0.773	0.808	0.808	0.991	0.704	0.669	0.791
Ken-181	0.704	0.913	0.695	0.695	0.704	0.704	0.730	0.991	0.669	0.834	0.713	0.852	0.695	0.660	0.756	0.660	0.652	0.686	0.756	0.739	0.756	0.756	0.834	0.686	0.652	0.739
Ken-217	0.747	0.747	0.695	0.739	0.747	0.747	0.791	0.773	0.695	0.782	0.713	0.782	0.721	0.704	0.765	0.704	0.695	0.739	0.765	0.782	0.765	0.765	0.747	0.739	0.721	0.782
Ken-219	0.695	0.713	0.713	0.686	0.695	0.695	0.721	0.695	0.678	0.704	0.669	0.704	0.704	0.686	0.686	0.660	0.678	0.713	0.704	0.669	0.704	0.704	0.704	0.713	0.721	0.669
Ken-221	0.747	0.695	0.921	0.730	0.747	0.747	0.747	0.695	0.634	0.695	0.713	0.695	0.904	0.643	0.739	0.643	0.652	0.686	0.739	0.704	0.739	0.739	0.765	0.686	0.678	0.713
Ken-243	0.756	0.756	0.704	0.747	0.756	0.756	0.756	0.765	0.669	0.791	0.704	0.765	0.695	0.660	0.773	0.678	0.678	0.704	0.756	0.721	0.756	0.756	0.782	0.704	0.721	0.739
Ken-279	0.695	0.713	0.713	0.686	0.695	0.695	0.721	0.695	0.678	0.704	0.669	0.704	0.704	0.686	0.686	0.660	0.678	0.713	0.704	0.669	0.704	0.704	0.704	0.713	0.721	0.669
Ken-292	0.704	0.913	0.695	0.695	0.704	0.704	0.730	0.991	0.669	0.834	0.713	0.852	0.695	0.660	0.756	0.660	0.652	0.686	0.756	0.739	0.756	0.756	0.834	0.686	0.652	0.739
Ken-298	0.713	0.678	0.939	0.695	0.713	0.713	0.695	0.704	0.643	0.669	0.678	0.686	0.965	0.634	0.713	0.652	0.652	0.660	0.713	0.669	0.713	0.713	0.730	0.660	0.652	0.678
Ken-300	0.704	0.904	0.695	0.695	0.704	0.704	0.730	1.000	0.669	0.843	0.713	0.860	0.695	0.660	0.747	0.660	0.652	0.686	0.747	0.739	0.747	0.747	0.834	0.686	0.652	0.739
Ken-514	1.000	0.686	0.721	0.982	1.000	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Ken-534	0.991	0.678	0.721	0.973	0.991	0.991	0.773	0.713	0.686	0.739	0.686	0.739	0.721	0.695	0.765	0.695	0.652	0.721	0.765	0.721	0.765	0.765	0.747	0.721	0.721	0.747
DRC-328	0.756	0.730	0.695	0.747	0.756	0.756	0.756	0.765	0.678	0.782	0.721	0.782	0.704	0.686	0.773	0.686	0.678	0.721	0.765	0.773	0.765	0.765	0.756	0.721	0.713	0.791
DRC-388	0.704	0.643	0.669	0.695	0.704	0.704	0.721	0.652	0.791	0.669	0.704	0.660	0.678	0.800	0.678	0.782	0.704	0.791	0.695	0.660	0.695	0.695	0.686	0.791	0.765	0.678
DRC-399	0.704	0.643	0.669	0.695	0.704	0.704	0.721	0.652	0.791	0.669	0.704	0.660	0.678	0.800	0.678	0.782	0.704	0.791	0.695	0.660	0.695	0.695	0.686	0.791	0.765	0.678
Ken-345	0.756	0.765	0.686	0.747	0.756	0.756	0.852	0.739	0.704	0.747	0.660	0.739	0.669	0.704	0.852	0.695	0.686	0.695	0.843	0.965	0.843	0.843	0.800	0.695	0.686	1.000
Ken-355	1.000	0.686	0.721	0.982	1.000	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Ken-412	0.756	0.765	0.686	0.747	0.756	0.756	0.852	0.739	0.704	0.747	0.660	0.739	0.669	0.704	0.852	0.695	0.686	0.695	0.843	0.965	0.843	0.843	0.800	0.695	0.686	1.000
Ken-414	0.756	0.765	0.686	0.747	0.756	0.756	0.852	0.739	0.704	0.747	0.660	0.739	0.669	0.704	0.852	0.695	0.686	0.695	0.843	0.965	0.843	0.843	0.800	0.695	0.686	1.000
Ken-415	0.695	0.669	0.626	0.686	0.695	0.695	0.686	0.669	1.000	0.660	0.695	0.652	0.634	0.991	0.678	0.965	0.713	0.756	0.678	0.704	0.678	0.678	0.660	0.756	0.765	0.704
Ken-434	0.713	0.695	0.678	0.704	0.713	0.713	0.730	0.686	0.756	0.713	0.730	0.704	0.669	0.765	0.713	0.747	0.860	0.921	0.730	0.704	0.730	0.730	0.721	0.921	0.913	0.721
Ken-435	0.765	0.782	0.721	0.756	0.765	0.765	0.878	0.747	0.678	0.782	0.704	0.756	0.713	0.686	0.956	0.678	0.704	0.713	0.991	0.826	0.991	0.991	0.817	0.713	0.686	0.843
Ken-438	1.000	0.686	0.721	0.982	1.000	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Ken-439	1.000	0.686	0.721	0.982	1.000	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-787	Ken-804	Ken-808	Ken-809	Ken-814	Ken-815	Ken-839	Ken-841	Ken-856	Ken-857	Ken-877	Ken-887	Ken-898	Nig-955	SA-163	SA-170	SA-172	SA-712	SA-724	SA-844	SA-855	SA-922	SA-947	SA-1485	SA-1486	Ken-402
DRC-231	0.913	0.913	0.739	0.773	0.660	0.686	0.547	0.565	0.678	0.895	0.913	0.695	0.808	0.730	0.739	0.721	0.739	0.678	0.721	0.756	0.695	0.669	0.730	0.730	0.765	0.686
Ken-241	0.730	0.747	0.791	0.852	0.695	0.695	0.608	0.608	0.704	0.730	0.747	0.704	0.782	0.782	0.773	0.747	0.773	0.739	0.747	0.782	0.713	0.660	0.773	0.808	0.843	0.695
Ken-170	0.773	0.791	0.773	0.991	0.730	0.756	0.565	0.591	0.695	0.773	0.791	0.704	0.808	0.765	0.756	0.756	0.756	0.765	0.747	0.765	0.704	0.686	0.739	0.773	0.782	0.756
Ken-181	0.739	0.739	0.765	0.834	0.695	0.695	0.547	0.591	0.704	0.721	0.739	0.678	0.730	0.765	0.756	0.756	0.756	0.713	0.704	0.791	0.669	0.643	0.756	0.791	0.747	0.695
Ken-217	0.782	0.782	0.808	0.747	0.730	0.721	0.565	0.573	0.721	0.765	0.782	0.739	0.791	0.834	0.765	0.773	0.765	0.713	0.747	0.791	0.678	0.686	0.747	0.834	0.800	0.721
Ken-219	0.669	0.669	0.695	0.704	0.704	0.695	0.573	0.634	1.000	0.660	0.669	0.704	0.721	0.713	0.721	0.721	0.721	0.713	0.695	0.721	0.660	0.686	0.730	0.739	0.747	0.695
Ken-221	0.704	0.713	0.713	0.765	0.904	1.000	0.530	0.582	0.695	0.704	0.713	0.678	0.747	0.756	0.713	0.686	0.713	0.747	0.747	0.704	0.695	0.686	0.713	0.747	0.704	1.000
Ken-243	0.721	0.739	1.000	0.782	0.695	0.713	0.539	0.573	0.695	0.721	0.739	0.713	0.756	0.782	0.739	0.765	0.739	0.713	0.756	0.773	0.678	0.660	0.721	0.782	0.800	0.713
Ken-279	0.669	0.669	0.695	0.704	0.704	0.695	0.573	0.634	1.000	0.660	0.669	0.704	0.721	0.713	0.721	0.721	0.721	0.713	0.695	0.721	0.660	0.686	0.730	0.739	0.747	0.695
Ken-292	0.739	0.739	0.765	0.834	0.695	0.695	0.547	0.591	0.704	0.721	0.739	0.678	0.730	0.765	0.756	0.756	0.756	0.713	0.704	0.791	0.669	0.643	0.756	0.791	0.747	0.695
Ken-298	0.669	0.678	0.721	0.730	0.956	0.869	0.513	0.591	0.686	0.669	0.678	0.652	0.695	0.721	0.669	0.660	0.669	0.721	0.713	0.704	0.660	0.669	0.678	0.730	0.686	0.869
Ken-300	0.739	0.739	0.765	0.834	0.695	0.695	0.556	0.582	0.695	0.721	0.739	0.678	0.730	0.765	0.747	0.756	0.747	0.713	0.704	0.791	0.669	0.643	0.756	0.791	0.739	0.695
Ken-514	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Ken-534	0.721	0.747	0.747	0.747	0.721	0.739	0.582	0.591	0.695	0.747	0.747	0.695	0.773	0.747	0.721	0.730	0.721	0.739	0.991	0.695	0.704	0.686	0.730	0.721	0.747	0.739
DRC-328	0.773	0.791	0.834	0.756	0.713	0.704	0.565	0.582	0.704	0.773	0.791	0.721	0.756	0.817	0.747	0.773	0.747	0.713	0.756	0.765	0.686	0.686	0.730	0.826	0.791	0.704
DRC-388	0.669	0.678	0.678	0.686	0.669	0.660	0.556	0.634	0.730	0.678	0.678	0.773	0.721	0.713	0.686	0.686	0.686	0.660	0.704	0.686	0.634	0.756	0.678	0.704	0.695	0.66
DRC-399	0.669	0.678	0.678	0.686	0.669	0.660	0.556	0.634	0.730	0.678	0.678	0.773	0.721	0.713	0.686	0.686	0.686	0.660	0.704	0.686	0.634	0.756	0.678	0.704	0.695	0.66
Ken-345	0.973	1.000	0.739	0.800	0.669	0.713	0.530	0.547	0.669	0.982	1.000	0.686	0.852	0.730	0.756	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.713
Ken-355	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Ken-412	0.973	1.000	0.739	0.800	0.669	0.713	0.530	0.547	0.669	0.982	1.000	0.686	0.852	0.730	0.756	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.713
Ken-414	0.973	1.000	0.739	0.800	0.669	0.713	0.530	0.547	0.669	0.982	1.000	0.686	0.852	0.730	0.756	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.713
Ken-415	0.695	0.704	0.669	0.660	0.634	0.634	0.573	0.643	0.678	0.704	0.704	0.756	0.686	0.686	0.652	0.686	0.652	0.643	0.695	0.704	0.660	0.756	0.652	0.678	0.721	0.634
Ken-434	0.704	0.721	0.713	0.721	0.669	0.686	0.591	0.617	0.730	0.713	0.721	0.939	0.730	0.747	0.721	0.721	0.721	0.669	0.713	0.739	0.686	0.782	0.713	0.756	0.747	0.686
Ken-435	0.826	0.843	0.747	0.817	0.713	0.730	0.565	0.600	0.704	0.826	0.843	0.704	0.878	0.747	0.756	0.747	0.756	0.747	0.765	0.756	0.695	0.686	0.721	0.756	0.791	0.73
Ken-438	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Ken-439	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-462	Ken-474	Ken-484	Ken-490	Ken-491	Ken-492	Ken-678	Ken-681	Ken-708	Ken-709	Ken-712	Ken-718	Ken-721	Ken-740	Ken-747	Ken-756	Ken-757	Ken-765	Ken-766	Ken-769	Ken-776	Ken-789	Ken-793	Ken-794	Ken-795	Ken-803
Ken-462	*	0.686	0.721	0.982	1.000	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Ken-474		*	0.678	0.678	0.686	0.686	0.782	0.904	0.669	0.834	0.686	0.817	0.678	0.660	0.782	0.660	0.660	0.678	0.782	0.773	0.782	0.782	0.860	0.678	0.660	0.765
Ken-484			*	0.704	0.721	0.721	0.721	0.695	0.626	0.686	0.704	0.695	0.956	0.634	0.713	0.634	0.652	0.678	0.730	0.678	0.730	0.730	0.747	0.678	0.678	0.686
Ken-490				*	0.982	0.982	0.773	0.695	0.686	0.739	0.686	0.721	0.695	0.695	0.765	0.695	0.634	0.713	0.765	0.721	0.765	0.765	0.721	0.713	0.721	0.747
Ken-491					*	1.000	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Ken-492						*	0.782	0.704	0.695	0.747	0.695	0.730	0.713	0.704	0.773	0.704	0.643	0.730	0.773	0.730	0.773	0.773	0.739	0.730	0.730	0.756
Ken-678							*	0.730	0.686	0.782	0.704	0.756	0.704	0.695	0.895	0.695	0.678	0.713	0.886	0.843	0.886	0.886	0.817	0.713	0.686	0.852
Ken-681								*	0.669	0.843	0.713	0.860	0.695	0.660	0.747	0.660	0.652	0.686	0.747	0.739	0.747	0.747	0.834	0.686	0.652	0.739
Ken-708									*	0.660	0.695	0.652	0.634	0.991	0.678	0.965	0.713	0.756	0.678	0.704	0.678	0.678	0.660	0.756	0.765	0.704
Ken-709										*	0.765	0.973	0.686	0.669	0.765	0.652	0.669	0.704	0.782	0.730	0.782	0.782	0.852	0.704	0.678	0.747
Ken-712											*	0.765	0.704	0.704	0.695	0.704	0.652	0.704	0.713	0.660	0.713	0.713	0.704	0.704	0.713	0.660
Ken-718												*	0.704	0.660	0.739	0.643	0.678	0.695	0.756	0.721	0.756	0.756	0.843	0.695	0.669	0.739
Ken-721													*	0.643	0.704	0.643	0.669	0.678	0.721	0.660	0.721	0.721	0.739	0.678	0.669	0.669
Ken-740														*	0.669	0.956	0.721	0.765	0.686	0.704	0.686	0.686	0.669	0.765	0.773	0.704
Ken-747-															*	0.686	0.678	0.704	0.965	0.834	0.965	0.965	0.800	0.704	0.678	0.852
Ken-756																*	0.695	0.747	0.686	0.695	0.686	0.686	0.652	0.747	0.756	0.695
Ken-757																	*	0.826	0.695	0.669	0.695	0.695	0.695	0.826	0.834	0.686
Ken-765																		*	0.721	0.678	0.721	0.721	0.713	1.000	0.860	0.695
Ken-766																			*	0.826	1.000	1.000	0.817	0.721	0.695	0.843
Ken-769																				*	0.826	0.826	0.782	0.678	0.678	0.965
Ken-776																					*	1.000	0.817	0.721	0.695	0.843
Ken-789																						*	0.817	0.721	0.695	0.843
Ken-793																							*	0.713	0.678	0.800
Ken-794																								*	0.860	0.695
Ken-795																									*	0.686
Ken-803																										*

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-787	Ken-804	Ken-808	Ken-809	Ken-814	Ken-815	Ken-839	Ken-841	Ken-856	Ken-857	Ken-877	Ken-887	Ken-898	Nig-955	SA-163	SA-170	SA-172	SA-712	SA-724	SA-844	SA-855	SA-922	SA-947	SA-1485	SA-1486	Ken-402
Ken-462	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Ken-474	0.773	0.765	0.756	0.860	0.678	0.695	0.547	0.573	0.713	0.747	0.765	0.686	0.782	0.747	0.739	0.747	0.739	0.713	0.686	0.791	0.660	0.643	0.730	0.782	0.765	0.695
Ken-484	0.678	0.686	0.704	0.747	0.956	0.921	0.530	0.591	0.713	0.678	0.686	0.669	0.721	0.747	0.669	0.678	0.669	0.739	0.721	0.695	0.678	0.695	0.695	0.739	0.695	0.921
Ken-490	0.721	0.747	0.747	0.721	0.695	0.730	0.582	0.573	0.686	0.747	0.747	0.686	0.773	0.739	0.721	0.721	0.721	0.730	0.982	0.695	0.695	0.678	0.721	0.721	0.747	0.73
Ken-491	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Ken-492	0.730	0.756	0.756	0.739	0.713	0.747	0.591	0.582	0.695	0.756	0.756	0.704	0.782	0.756	0.730	0.730	0.730	0.747	1.000	0.704	0.704	0.678	0.730	0.730	0.756	0.747
Ken-678	0.852	0.852	0.756	0.817	0.704	0.747	0.539	0.565	0.721	0.834	0.852	0.704	1.000	0.739	0.773	0.765	0.773	0.739	0.782	0.747	0.713	0.695	0.747	0.765	0.765	0.747
Ken-681	0.739	0.739	0.765	0.834	0.695	0.695	0.556	0.582	0.695	0.721	0.739	0.678	0.730	0.765	0.747	0.756	0.747	0.713	0.704	0.791	0.669	0.643	0.756	0.791	0.739	0.695
Ken-708	0.695	0.704	0.669	0.660	0.634	0.634	0.573	0.643	0.678	0.704	0.704	0.756	0.686	0.686	0.652	0.686	0.652	0.643	0.695	0.704	0.660	0.756	0.652	0.678	0.721	0.634
Ken-709	0.730	0.747	0.791	0.852	0.695	0.695	0.608	0.608	0.704	0.730	0.747	0.704	0.782	0.782	0.773	0.747	0.773	0.739	0.747	0.782	0.713	0.660	0.773	0.808	0.843	0.695
Ken-712	0.652	0.660	0.704	0.704	0.704	0.713	0.565	0.643	0.669	0.652	0.660	0.695	0.704	0.713	0.713	0.713	0.713	0.678	0.695	0.713	0.826	0.678	0.730	0.739	0.704	0.713
Ken-718	0.721	0.739	0.765	0.843	0.713	0.695	0.600	0.617	0.704	0.721	0.739	0.695	0.756	0.782	0.773	0.739	0.773	0.721	0.730	0.773	0.704	0.669	0.773	0.808	0.817	0.695
Ken-721	0.660	0.669	0.695	0.739	0.991	0.904	0.530	0.600	0.704	0.660	0.669	0.669	0.704	0.739	0.660	0.669	0.660	0.730	0.713	0.704	0.686	0.686	0.695	0.747	0.704	0.904
Ken-740	0.695	0.704	0.660	0.669	0.643	0.643	0.582	0.652	0.686	0.704	0.704	0.765	0.695	0.695	0.643	0.695	0.643	0.652	0.704	0.695	0.669	0.765	0.652	0.686	0.730	0.643
Ken-747-	0.834	0.852	0.773	0.800	0.704	0.739	0.539	0.582	0.686	0.834	0.852	0.695	0.895	0.739	0.782	0.747	0.782	0.747	0.773	0.765	0.695	0.669	0.739	0.747	0.773	0.739
Ken-756	0.686	0.695	0.678	0.652	0.643	0.643	0.556	0.634	0.660	0.695	0.695	0.747	0.695	0.695	0.660	0.695	0.660	0.652	0.704	0.713	0.669	0.730	0.660	0.686	0.713	0.643
Ken-757	0.669	0.686	0.678	0.695	0.669	0.652	0.530	0.573	0.678	0.678	0.686	0.860	0.678	0.686	0.669	0.660	0.669	0.626	0.643	0.678	0.669	0.765	0.669	0.678	0.721	0.652
Ken-765	0.678	0.695	0.704	0.713	0.678	0.686	0.608	0.626	0.713	0.704	0.695	0.947	0.713	0.773	0.704	0.713	0.704	0.678	0.730	0.739	0.660	0.773	0.713	0.747	0.739	0.686
Ken-766	0.826	0.843	0.756	0.817	0.721	0.739	0.565	0.600	0.704	0.826	0.843	0.713	0.886	0.756	0.765	0.756	0.765	0.756	0.773	0.765	0.704	0.686	0.730	0.765	0.791	0.739
Ken-769	0.991	0.965	0.721	0.782	0.660	0.704	0.530	0.539	0.669	0.947	0.965	0.669	0.843	0.721	0.739	0.713	0.739	0.686	0.730	0.730	0.678	0.686	0.713	0.730	0.739	0.704
Ken-776	0.826	0.843	0.756	0.817	0.721	0.739	0.565	0.600	0.704	0.826	0.843	0.713	0.886	0.756	0.765	0.756	0.765	0.756	0.773	0.765	0.704	0.686	0.730	0.765	0.791	0.739
Ken-789	0.826	0.843	0.756	0.817	0.721	0.739	0.565	0.600	0.704	0.826	0.843	0.713	0.886	0.756	0.765	0.756	0.765	0.756	0.773	0.765	0.704	0.686	0.730	0.765	0.791	0.739
Ken-793	0.782	0.800	0.782	1.000	0.739	0.765	0.556	0.582	0.704	0.782	0.800	0.713	0.817	0.773	0.747	0.765	0.747	0.756	0.739	0.765	0.695	0.695	0.730	0.782	0.791	0.765
Ken-794	0.678	0.695	0.704	0.713	0.678	0.686	0.608	0.626	0.713	0.704	0.695	0.947	0.713	0.773	0.704	0.713	0.704	0.678	0.730	0.739	0.660	0.773	0.713	0.747	0.739	0.686
Ken-795	0.669	0.686	0.721	0.678	0.669	0.678	0.608	0.600	0.721	0.686	0.686	0.878	0.686	0.730	0.695	0.686	0.695	0.669	0.730	0.704	0.704	0.782	0.704	0.721	0.721	0.678
Ken-803	0.973	1.000	0.739	0.800	0.669	0.713	0.530	0.547	0.669	0.982	1.000	0.686	0.852	0.730	0.756	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.713

Table A5 [Continued]: Amino acid sequence similarity matrix generated from positives samples in this study (partial L-gene).

Sequence	Ken-787	Ken-804	Ken-808	Ken-809	Ken-814	Ken-815	Ken-839	Ken-841	Ken-856	Ken-857	Ken-877	Ken-887	Ken-898	Nig-955	SA-163	SA-170	SA-172	SA-712	SA-724	SA-844	SA-855	SA-922	SA-947	SA-1485	SA-1486	Ken-402
Ken-787	*	0.973	0.721	0.782	0.660	0.704	0.530	0.539	0.669	0.956	0.973	0.669	0.852	0.721	0.739	0.713	0.739	0.686	0.730	0.730	0.678	0.686	0.721	0.730	0.739	0.704
Ken-804		*	0.739	0.800	0.669	0.713	0.530	0.547	0.669	0.982	1.000	0.686	0.852	0.730	0.756	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.713
Ken-808			*	0.782	0.695	0.713	0.539	0.573	0.695	0.721	0.739	0.713	0.756	0.782	0.739	0.765	0.739	0.713	0.756	0.773	0.678	0.660	0.721	0.782	0.800	0.713
Ken-809				*	0.739	0.765	0.556	0.582	0.704	0.782	0.800	0.713	0.817	0.773	0.747	0.765	0.747	0.756	0.739	0.765	0.695	0.695	0.730	0.782	0.791	0.765
Ken-814					*	0.904	0.530	0.600	0.704	0.660	0.669	0.669	0.704	0.747	0.660	0.669	0.660	0.730	0.713	0.695	0.686	0.686	0.695	0.756	0.704	0.904
Ken-815						*	0.530	0.582	0.695	0.704	0.713	0.678	0.747	0.756	0.713	0.686	0.713	0.747	0.747	0.704	0.695	0.686	0.713	0.747	0.704	1.000
Ken-839							*	0.678	0.573	0.530	0.530	0.591	0.539	0.582	0.565	0.530	0.565	0.626	0.591	0.547	0.556	0.556	0.582	0.600	0.565	0.53
Ken-841								*	0.634	0.547	0.547	0.608	0.565	0.608	0.617	0.547	0.617	0.617	0.582	0.556	0.591	0.643	0.600	0.591	0.600	0.582
Ken-856									*	0.660	0.669	0.704	0.721	0.713	0.721	0.721	0.721	0.713	0.695	0.721	0.660	0.686	0.730	0.739	0.747	0.695
Ken-857										*	0.982	0.678	0.834	0.730	0.747	0.704	0.747	0.686	0.756	0.721	0.669	0.704	0.730	0.713	0.747	0.704
Ken-877											*	0.686	0.852	0.730	0.756	0.721	0.756	0.695	0.756	0.739	0.686	0.704	0.739	0.730	0.765	0.713
Ken-887												*	0.704	0.756	0.704	0.713	0.704	0.669	0.704	0.730	0.678	0.773	0.730	0.739	0.747	0.678
Ken-898													*	0.739	0.773	0.765	0.773	0.739	0.782	0.747	0.713	0.695	0.747	0.765	0.765	0.747
Nig-955														*	0.721	0.739	0.721	0.721	0.756	0.773	0.660	0.704	0.704	0.904	0.782	0.756
SA-163															*	0.739	1.000	0.747	0.730	0.747	0.704	0.634	0.826	0.730	0.765	0.713
SA-170																*	0.739	0.730	0.730	0.800	0.747	0.643	0.730	0.765	0.808	0.686
SA-172																	*	0.747	0.730	0.747	0.704	0.634	0.826	0.730	0.765	0.713
SA-712																		*	0.747	0.704	0.721	0.617	0.713	0.747	0.704	0.747
SA-724																			*	0.704	0.704	0.678	0.730	0.730	0.756	0.747
SA-844																				*	0.695	0.660	0.773	0.791	0.817	0.704
SA-855																					*	0.634	0.747	0.686	0.695	0.695
SA-922																						*	0.626	0.686	0.695	0.686
SA-947																							*	0.713	0.756	0.713
SA-1485																								*	0.791	0.747
SA-1486																									*	0.704
Ken-402																										*