Diversity and Ecology of Astroviruses in South African Bats

by

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Declaration

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Abstract

Emerging infectious diseases are mostly zoonotic in origin and defined as “infections that have newly emerged in a population or have existed but are rapidly increasing in incidence or geographic range". Zoonotic viruses are directly (e.g. bite from a rabid bat) or indirectly (via an intermediate host or vector) transmitted from animals to humans. Bats have received increasing attention as potential hosts for zoonotic diseases. Bats belong to the order Chiroptera, which consists of two suborders: Yinpterochiroptera and Yangochiroptera. More than 1 300 species have been described globally, occurring on almost all continents excluding Antarctica. Specific physiological and ecological characteristics make bats extraordinary evolutionary vessels to carry numerous infectious agents including pathogens.

Astroviruses (AstVs) are amongst the vast array of viruses that have been detected in bats. AstVs are single stranded, positive sense, RNA viruses that are transmitted via the faecal-oral route. Infection with AstVs causes acute diarrhoea, however, more serious clinical presentations such as neurological deficits, stunted growth and encephalitis have also been documented. Bats on the other hand, seem to be asymptomatically infected with AstVs. Little attention has been given to the evolution, phylogenetic relationship, ecology and diversity of AstVs in South African bats. In 2013 the first study in South Africa screening for a variety of viruses in small mammals, including SAn bats, found that bats were frequently co-infected with AstVs and coronaviruses.

The overall aims of the current study were to describe the prevalence, diversity and ecology of AstVs in South African bats, to determine the potential threat to environmental and animal health at wastewater treatment works (WWTW) through testing water and bat samples for the presence of AstVs, to monitor AstV and CoV co-infection in a *Neoromicia capensis* colony over time and to isolate and propagate a bat AstV *in vitro*. The results will be used to determine the potential One Health implications of AstVs in a South African setting.

Sample collection was done via non-invasive capture and release methods by collaborating zoologists. Morphological and ecological data of each bat were recorded. Bat faecal samples (n=500) were screened for AstVs using the hemi-nested screening assay that targets the RNA-dependent RNA polymerase (*RdRP*) gene of the virus. Plasmid positive controls were generated to ensure an optimal AstV screening PCR assay.

The One Health concept emphasizes the interlinkage between human, animal and environmental health. To determine the impact that potential exposure to human AstVs at WWTW might have on animal and environmental health, water samples upstream and downstream of two WWTW were also collected and screened for AstVs.

The overall detection rate of AstVs across bat species was 13%, but it differed significantly between species (*Miniopterus natalensis*, 55%; *Rhinolophus capensis*, 39%; and *R. clivosus*, 17%). Positive samples were further analysed to try and amplify the capsid protein gene (ORF2), which is highly variable and only one ORF2 gene fragment was obtained.

Twenty-five novel AstV *RdRp* sequences and one ORF2 sequence were identified, bringing the total *RdRp* sequences available for South African bat AstVs to forty-four. Maximum likelihood analyses of the *RdRp* gene fragments suggest that South African bat AstVs are not restricted by host species identity or geographical location. Interestingly, the maximum likelihood analyses of the ORF2 sequence suggest that the South African bat AstVs might be more similar to human AstVs from Japan compared to any bat AstVs.

The water samples collected from the WWTW tested negative for the presence of AstVs and only one bat sample collected at the WWTW tested positive for AstV.

Two real-time PCR assays were designed to monitor AstVs and coronaviruses in a *N. capensis* colony over time, as these two viruses regularly co-infect bats. The results indicated that both these viruses had a single amplification peak that was associated with colony formation after migration. Interestingly the peak in viral loads did not correlate with the pupping season of the bats, as was found by another study conducted on these two viruses in Germany.

Statistical analyses of ecological and individual bat factors suggest that being a sexually active adult male bat, species identity and occurrence in the Succulent Karoo biome could contribute to AstV positivity.

The current study was the first ever to successfully isolate and propagate a Miniopterus bat derived AstV *in vitro*. During the isolation attempts three different cell lines were used, human adenocarcinoma, *Neoromicia capensis* kidney and baby hamster kidney cells. Isolation and propagation was only successful in the baby hamster kidney cells. The refined protocol for isolation and propagation of bat AstVs in cell culture will enable future studies to successfully isolate bat AstVs as well as enable genomic and functional studies. The results also gave insight into the potential zoonotic risk of bat AstVs.

The findings of the current study indicated that bat AstVs are diverse and relatively prevalent in South African bats. Phylogenetic analyses of the 24 novel *RdRp* and one ORF2 genes from this study indicated that the virus was not limited by species identity or host geographical range. Furthermore, the phylogenetic analyses of the bat AstV ORF2 gene would suggest that the bat AstV is more similar to human AstVs, which could imply that South African bat AstVs have zoonotic potential. The results of current study gave some potential insights into the One Health implications of AstVs in the SA setting.

Opsomming

Opkomende aansteeklike siektes word gedefinieer as “infeksies wat onlangs verskyn het in ’n populasie of wat al lank reeds teenwoordig was in ’n populasie maar vinnig toeneem in insidensie of geografiese verspreiding”. Die meerderheid van opkomende aansteeklike siektes is zoonoties in oorsprong, wat beteken dat hul oorspronklik in diere voorgekom het, maar nou na mense oorgedra kan word. Zoonotiese virusse kan direk oorgedra word na die mens bv. deur gebyt te raak deur ’n hond met hondsdolheid of indirek deur ’n tussenganger gasheer of vektor. Met die soeke na wilde diere wat dien as gashere van moontlike zoonotiese siektes was vlermuise geïdentifiseer as belangrike gashere vir verskeie potensiële zoonotiese siektes. Vlermuise behoort aan die orde Chiroptera wat verder in twee subordes verdeel word, naamlik Yinpterochiroptera en Yangochiroptera. Die orde Chiroptera is baie divers met meer as 1300 spesies wat globaal beskryf is en voorkom op alle vastelande, uitsluitend Antarktika. Daar is verskeie eienskappe (fisiologies en ekologies) wat vlermuise besonderse evolusionêre vaartuie maak om as draers te dien vir verskeie patogene.

Astrovirusse (AstVs) is een groep van verskeie virusse wat in vlermuise voorkom. AstVs is enkelstring, positiewe sense, RNA virusse wat via die fekale-orale roete versprei word. Infeksie met AstVs veroorsaak gewoonlik akute diarree, maar meer ernstige simptome soos neurologiese afwykings, vertraagde groei asook enkefalitis is al waargeneem. Dit wil voorkom of vlermuise asimptomaties geïnfekteer word deur die virus. Baie min aandag is geskenk aan die evolusie, filogenetiese verwantskappe, ekologie en diversiteit van AstVs wat in Suid Afrikaanse (SA) vlermuise voorkom. Die enigste bestaande studie wat AstVs bestudeer het in SA vlermuise was uitgevoer deur Dr Ithete tydens haar doktorale studie. Die studie deur Ithete (2013) was hoofsaaklik ’n verkenning studie om te bepaal watter virusse in klein soogdiere voorkom in SA, daar was egter nie ’n in-diepte ondersoek na die diversiteit en ekologie van AstVs nie. Tydens die huidige studie was die diversiteit en ekologie van AstVs in SA vlermuise bestudeer deur gebruik te maak van molekulêre-, filogenetiese- en statistiese metodes.

Vlermuis monsters wat gebruik is tydens die huidige studie, was verskaf deur samewerkende dierkundiges met etiese toestemming. Die meerderheid van die monsters was versamel deur nie-indringende metodes, wat vang en vrylating van die vlermuise vereis het. Tydens die versameling van vlermuise morfologiese data was gedokumenteer asook ekologiese data.

’n Totaal van vyfhonderd mis monsters was getoets vir die teenwoordigheid van AstVs deur gebruik te maak van ’n polimerase ketting reaksie (PKR). Die PCR toets teiken die RNA-afhanklike RNA polimerase (*RdRp*) geen van die virus. Om te verseker dat die PKR toets wat reg toegepas word, was ’n plasmied positiewe kontrole geproduseer. Die plasmied positiewe kontrole was *in vitro* getranskribeer na RNA om te verseker dat al die stappe, vanaf omgekeerde transkripsie van onttrekte RNA, gevolg deur die AstV PKR toets, reg verloop. Die virus was opgespoor in 13% van al die monsters wat getoets was vir AstVs. Die opsporing het wel verskil tussen vlermuis spesies, met die hoogste voorkoms van die virus in *Miniopterus natalensis* (55%) gevolg deur *Rhinolophus capensis* (39%) en *Rhinolophus clivosus* (17%). Monsters wat positief getoets het vir AstV is

verder geanaliseer om ’n groter fragment van die virus te probeer bekom, naamlik die kapsied proteïen voorloper geen (ORF2). ’n PKR toets wat ontwikkel is deur Atkins et al., (2009) was gebruik. Aangesien die kapsied proteïen geen baie divers is, het meeste pogings misluk en slegs een ORF2 geen fragment is bekom.

’n Totaal van 25 nuwe AstV *RdRp* gene asook een ORF2 geen is geïdentifiseer. Dit bring die totaal van AstVs *RdRp* geen volgordes wat beskikbaar is vir AstVs van SA vlermuise na 44. Die filogenetiese analise van die *RdRp* gene het gedui dat daar geen definitiewe patroon is in terme van groeperings nie, dit wil voorkom of SA vlermuis AstV nie beperk word deur gasheer spesie of geografiese verspreiding nie. Interessant genoeg het die maksimum waarskynlikheidsanalise van die ORF2 geen aangedui dat die SA vlermuis AstVs meer soortgelyk is aan mens AstVs as aan vlermuis AstVs.

Tydens die studie was daar gebruik gemaak van twee real-time qPCR (werklike tyd kwantitatiewe polimerase ketting reaksie) toetse om AstVs asook caronavirusse te monitor in ’n *Neoromicia capensis* vlermuis kolonie. Die resultate het getoon dat albei virusse een amplifikasie hoogtepunt bereik het na kolonie formasie na migrasie. Die piek in virus lading was nie geassosieer met die geboorte van nuwelinge nie, soos wat voorheen deur ander studies bevind was nie.

Statistiese analises van ekologiese- en individuele vlermuis metings het getoon dat die volgende faktore moontlik kan bydra tot AstV positiwiteit in vlermuise: geslag (seksueel aktiewe mannetjies), spesies identiteit asook bioom (Sukkulente Karoo).

Die huidige studie was die eerste ter wêreld om ’n vlermuis AstV suksesvol te isoleer en propageer *in vitro*. Tydens die isolasie pogings was drie verskillende sellyne gebruik: menslike adenokarsinoom (Caco-2), *Neoromicia capensis* nier selle (NCK) asook hamster nier selle (BHK G43). Die isolasie was slegs suksesvol in die hamster nier selle. Daar is verskeie faktore wat ’n bydra kon lewer tot die sukses van die isolasie poging naamlik die monster tipe wat gebruik was as inokulum, die media komposisie, sellyn tipe asook die inkubasie tydperk na die sellyn geïnokuleer is. Die suksesvolle isolasie van ’n vlermuis AstV *in vitro* sal opkomende studies in staat stel om ook suksesvolle isolasies te doen asook verdere genetiese toetse bv. volledige genoom toetse.

Die bevindinge van die huidige studie toon dat SA vlermuis AstVs relatief volop is en dat hul ook divers is. Vyf-en-twinting *RdRp* geen volgordes en een ORF2 geen volgorde is bekom in die studie. Filogenetiese analises van die *RdRp* geen volgordes het getoon dat die virus nie deur gasheer spesie of geografiese verspreiding beperk word nie. Interessant genoeg het die filogenetiese analise van die ORF2 geen volgorde getoon dat die SA vlermuis AstV nader verwant is aan menslike AstVs as aan vlermuis AstVs, wat moontlike zoonotiese potensiaal impliseer. Die suksesvolle isolasie van ’n vlermuis AstV *in vitro* is ’n groot bydra tot toekomstige studies wat die genoom asook funksionele biologie van die virus verder wil bestudeer.

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Abbreviations

AstV / AstVs	astrovirus / astroviruses
BHK	baby hamster kidney
BSL	biosafety level
Caco-2	human adenocarcinoma
cDNA	complementary DNA
CoV	coronavirus
CPE	cytopathic effect
DEPC	diethylpyrocarbonate
EID	emerging infectious diseases
EM	electron microscopy
FBS	foetal bovine serum
FMI	forearm mass index
GBEB	glycine-beef-extract buffer
GEE	generalized estimating equation
GIT	gastro intestinal tract
GTR	general time reversible model
GTR+G+I	general time reversible model with gamma distribution and invariant sites
HAstV	human astrovirus
ICTV	International Committee on Taxonomy of Viruses
IPTG	isopropyl β-D-1-thiogalactopyranoside
IUCN	International Union for Conservation of Nature
kb	kilobase
KZN	Kwazulu Natal
LB	Luria broth
MEGA	Molecular Evolutionary Genetics Analysis
MERS	Middle East Respiratory Syndrome
ML	maximum likelihood method
NCBI	National Centre for Biotechnology Information
NCK	*Neoromicia capensis* kidney
NGS	next generation sequencing
NHP	non-human primate
NICD	National Institute for Communicable Diseases
NJ	neighbour joining
ORF	open reading frame
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PEG	polyethylene glycol
PPE	personal protective equipment
qRTPCR	Quantitative real time PCR
RdRp	RNA dependent RNA polymerase
RFS	ribosomal frameshift
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
SA / SAn	South Africa / South African
SB	sodium borate
SF	serum free
TGEV	transmissible gastroenteritis virus
USA	United States of America
UTR	untranslated region
UV	ultraviolet
VSV	vesicular stomatitis virus
VTM	viral transport medium
WHO	World Health Organization
WWTW	Wastewater treatment works

Chapter 1 Introduction & Literature Review

1.1 Emerging Infectious Diseases

Emerging infectious diseases (EIDs) are defined as "infections that have newly emerged in a population or have existed but are rapidly increasing in incidence or geographic range" (Morse, 1995). The majority of EIDs are zoonotic in origin. Zoonotic viruses are transmitted from animals to humans, either directly (e.g. bite from a rabid bat) or indirectly via an intermediate host or vector (Ludwig et al., 2003, Halpin et al., 2007; Mendenhall et al., 2015). Emergence of a disease is influenced by various factors which include but are not limited to: acquired resistance of pathogens to antibiotics and other antimicrobial medicine, an increase in the mobility of humans which enhances the spread of disease causing agents, host range, taxonomy of the pathogen, and molecular characteristics of the pathogen (Taylor et al., 2000, Cleveland et al., 2001, Jones et al., 2008, Mendenhall et al., 2015).

1.2 Bats as important hosts of various EIDs

In the search for wildlife that serve as hosts to potentially zoonotic diseases, bats have received increasing attention (Newman et al., 2011, Baker et al., 2013; Mendenhall et al., 2015). Bats belong to the order Chiroptera which consists of two suborders Yinpterochiroptera and Yangochiroptera (Teeling, 2009; Monadjem et al., 2010). Chiropterans are highly speciose, with more than 1300 species described globally, occurring on almost all continents excluding Antarctica (Teeling et al., 2005; Teeling, 2009). This fascinating group of flying mammals have divergent morphologies, ecologies and behaviours (Monadjem et al., 2010). There are ample characteristics (physiological and ecological) that make bats extraordinary evolutionary vessels to carry numerous pathogens, of which the most important will be discussed.

Bats and their associated pathogens have co-evolved for 50 to 100 million years. In rare cases some pathogens carried by bats spilled over to humans, e.g. Hendra virus in Australia (Teeling, 2009; Playford et al., 2010; Wibbelt et al., 2010). This is largely due to habitat destruction and human encroachment of natural habitats, resulting in increased contact between bats and humans, as well as between bats and domesticated animals (Baker et al., 2013).

Some infectious agents carried by bats may be transmitted through direct contact with the infected bat (e.g. a bite from a rabid bat) or indirectly through an intermediate or amplifying host. Findings by Corman et al. (2014) suggest that the Middle East respiratory syndrome coronavirus (MERS-CoV), isolated from camels and humans, potentially originated from bats. This study furthermore indicated

that the virus has undergone host switching events and that the origin of the virus may be in Africa, from where camels are imported into the Arabian Peninsula (Corman et al., 2014). It should however be mentioned that not all pathogens carried by bats will become emerging infectious diseases that will spill over to humans.

Chiropterans are the only flying mammals on earth and this specific characteristic facilitates short- and long-distance dispersal of diseases, e.g. big brown bats (*Eptesicus fuscus*) can travel an average distance of 35 km per night. Migratory bat species are known to travel vast distances and even cross from one continent to another e.g. large flying foxes (*Pteropus vampyrus*) have been recorded to travel distances of up to 2 500 km during migrations, making them extraordinarily good long-distance dispersers of diseases (Halpin et al., 2007; Epstein et al., 2009; Monadjem et al., 2010; Wibbelt et al., 2010; Mendenhall et al., 2015).

Another trait that has also been identified as important to their ability to carry disease is their sociality. Bats display a range of social behaviour such as allogrooming and food sharing. Vampire bats (*Desmodus rotundus*) are known to share blood meals with roost members resulting in the spread of pathogens from one individual to another (Wilkinson, 1984).

Roosting behaviour also differs between bat species, with some bats roost in isolation whilst others form large roosts. The largest known roost is located at Bracken Cave in the United States of America (USA) where millions of Mexican free-tailed bats (*Tadarida brasiliensis*) inhabit the cave. Maternity roosts are a particularly interesting roost type, as the roost consists solely of female bats and their pups. Not only does roosting increase contact rates between individual bats of the same species, but it also allows for interactions with other bat species, which could facilitate pathogen sharing (Calisher et al., 2006; Wibbelt et al., 2010).

While it is evident that bats are important reservoirs for various pathogens, little is known about the underlying mechanisms that drive pathogen maintenance, spread, diversity and epidemiology within individual bats, within colonies and possibly between colonies and between species (Calisher et al., 2006; De Benedictis et al., 2011; Drexler et al., 2011). Even though it is assumed that bats are asymptomatic whilst testing positive for various pathogens, e.g. detection of rabies in apparent healthy bats, a study by Mühldorfer et al. (2011) found that 12 % of bat fatalities investigated during their study could be attributed to infectious agents and parasites. The exact functioning of the bat's immune system is not yet fully understood. Fortunately, research in this particular field is increasing (Kunz & Fenton, 2003; Calisher et al., 2006; Wibbelt et al., 2010; Moratelli & Calisher, 2015). Studies conducted on bat immunology have identified orthologous bat immune genes, which include major histocompatibility class II genes and numerous alleles in *Noctilio albiventris* and *E. fuscus*.

Some of the alleles identified have been linked to parasite burden. Antiviral, innate and interferon genes have also been identified in *Pteropus vampyrus*, *P. lecto* and *Myotis lucifugus*. Microbial pattern recognition receptors have also been detected in *Pteropus* genome scans (Wibbelt et al., 2010). These studies have highlighted the importance of continuing research into bat immunology (Moratelli & Calisher, 2015).

1.3 Introduction to astroviruses

Members of the family *Astroviridae* are non-enveloped, positive-sense, single-stranded RNA (ribonucleic acid) viruses with a characteristic star-like surface structure when observed under an electron microscope (Gray & Desselberger, 2009; De Benedictis et al., 2011; Osborne et al., 2015). The virus was first discovered in 1975 in stool samples from infants suffering from diarrhoea (Appleton & Higgins, 1975).

1.4 Taxonomy

The family *Astroviridae* has been divided into two genera based on the class of host the viruses infect, *avastroviruses* (infect birds) and *mamastroviruses* (infect mammals) (Mendez & Arias, 2007). The *mamastrovirus* genus contains 19 species whilst the *avastrovirus* genus contains three species. The current species classification of astroviruses (AstVs), from both mamastro- and avastrovirus groups is based on the host species from which the virus has been isolated, as such the species do not correspond to genetic phylogenies (Bosch et al., 2014). The classification of these viruses will be redefined based on the complete capsid region at the amino acid level (Boujon et al., 2017). According to the new method AstVs will then be divided into two genogroups: genogroup I and genogroup II (Boujon et al., 2017). The genogroups consist of various genotypes that infect different host species. The mean amino acid genetic distance (p-dist) between two genogroups is 0.704 ± 0.013. The amino acid genetic distance between genotypes within a genogroup ranges between 0.576 and 0.741. Mamastroviruses can also be divided into two genogroups based on the capsid protein amino acids, Genogroup I and Genogroup II (Figure 1.1.). The mean amino acid genetic distance (p-dist) between genogroups is 0.671 ± 0.016 and the genetic distance between genotypes ranges between 0.338 and 0.783 (Bosch et al., 2012). Serotypes within genotypes are defined based on two-way cross-neutralization titres (of 20-fold and greater) and are then given consecutive numbers (Bosch et al., 2014).

However, some evidence suggests that cross-genus and cross-species infection has taken place in the past e.g. an AstV isolated from a stranded California sea lion pup (*Zalophus californianus*) is thought to have originated through recombination of a human AstV isolate (belonging to genotype human

AstV-3 (HAstV-3)) with a marine mammal AstV, resulting in California sea lion AstV-3 (CslAstV-3) (Rivera et al., 2010). Furthermore, inter-genotype recombination has been recorded for HAstV genotypes (Pativada et al., 2011), this has not yet been investigated for bats and other non-human hosts harbouring AstVs. There is also a large gap in the literature with regards to co-infections and the chance of recombination of AstVs strains in non-human hosts (Xiao et al., 2013; Mendenhall et al., 2015).

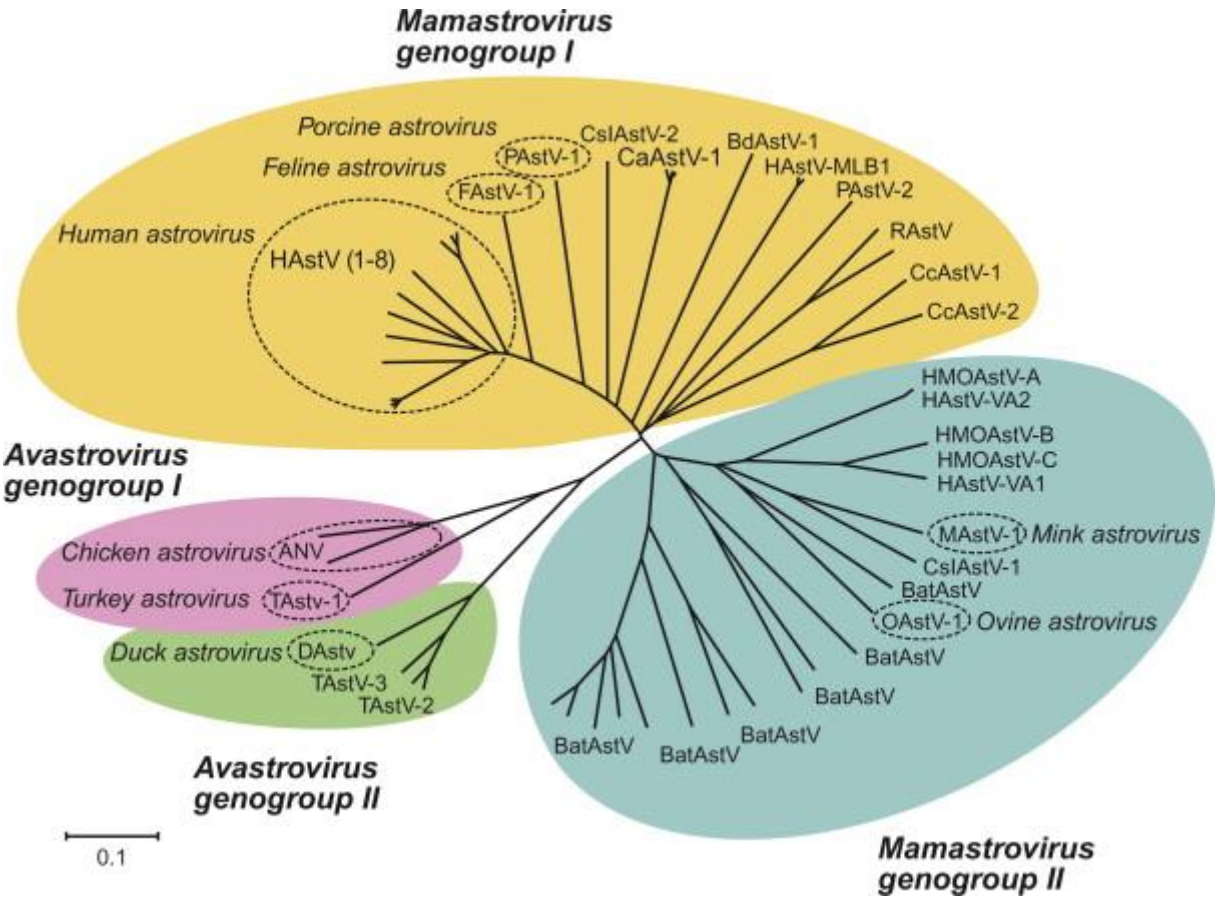


Figure 1.1. Phylogenetic relationship between members of the family Astroviridae based on the analyses of the capsid polyprotein gene (King et al., 2012) (Image used with permission from Elsevier)

1.5 Replication and pathogenesis

Very little is known about AstV attachment and cell entry. Based on what is known about other RNA viruses, it is suggested that multiple receptors are utilized for virus attachment and entry, but no studies have identified the specific receptors. Donelli et al. (1992) suggest that AstVs might enter cells through receptor-mediated endocytosis. Once the virus enters a cell, the virus promotes intracellular membrane rearrangement to produce vesicle-like structures (singe- and double-membrane vesicles), that are associated with protein and viral RNA synthesis as well as virus particle

assembly (Mendez & Arias, 2007). The release mechanism of virus particles from infected cells is also undetermined (Gray & Desselberger, 2009; De Benedictis et al., 2011).

1.6 Genome structure

The length of the AstV genomes varies from 6.1 to 7.3 kilobases (kb) (Mendez & Arias, 2007; Gray & Desselberger, 2009). The genome contains 5' and 3' untranslated regions (UTRs) and three open reading frames (ORFs); ORF1a, ORF1b and ORF2 (Figure 1.2.) (Mendez & Arias, 2007; Strain et al., 2009; Gray & Desselberger, 2009). ORF1a is 2.8 kb in length and encodes an 110kDa polypeptide, which contains an array of conserved motifs (e.g. immunoreactive epitope, putative nuclear localization signal, serine protease) (Mendez & Arias, 2007). The polypeptide is cleaved into five peptides by cellular proteases and viral proteases (Mendez & Arias, 2007). The three ORFs code for different proteins: ORF1a for non-structural proteins, ORF1b codes for RNA-dependent RNA polymerase (*RdRp*) and is separated from ORF1a by a frameshift. Between ORF1a and ORF1b there is an overlap of 70 bases containing sequences that direct ribosomal frameshifting (to a-1 frame) which enables reading of ORF1b (Gray & Desselberger, 2009). ORF1b is the most conserved region between the three ORFs and is usually used in genetic analyses to determine genetic relatedness of viruses (Strain et al., 2009). ORF2 codes for a structural viral polyprotein which is utilized in the production of virions and thus experiences more selective pressures and is therefore more divergent than the other ORFs that code for non-structural proteins (Gray & Desselberger, 2009). Approximately 56 complete AstV genome sequences are available on Genbank (http://www.ncbi.nlm.nih.gov/genome/?term=astrovirus on 1st of January-2019). However, there is a large amount of partial *RdRp* gene sequences available; the *RdRp* gene is the target of the detection assays used in this study which was designed by Chu et al. (2009).

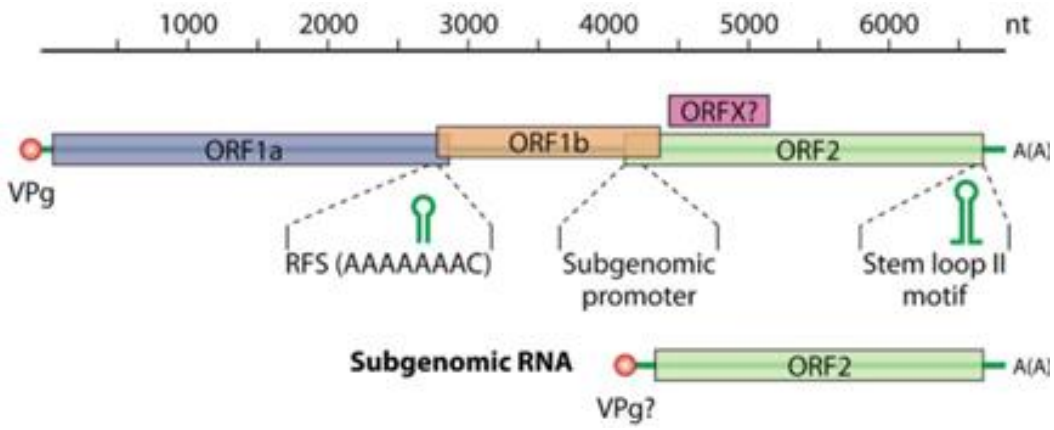


Figure 1.2. Genome organization of human astrovirus (Bosch, Pintó, Guix, 2014). "Genomic and subgenomic RNA organization, with ORF1a, ORF1b, ORF2, and putative ORFX represented as boxes. Nucleotide sequences represent highly conserved sequences located in the ribosomal

frameshift (RFS) signal and upstream of the initiation site of subgenomic RNA transcription. Putative RNA secondary structures conserved in the RFS and in the 3' end of the genome are depicted." With permission from the journal Clinical Microbiology Reviews.

1.7 Astrovirus infection in humans

AstVs are transmitted through the faecal-oral route, either through contact with contaminated surfaces or ingestion of food or water contaminated with infected faecal material (Moser & Schultz-Cherry, 2005; Gray & Desselberger, 2009; De Benedictis et al., 2011). As is the case for many enteric viruses, AstVs are exceptionally durable in the environment, which increases the risk of exposure and infection of other individuals (De Benedictis et al., 2011; Krishnan, 2014; Mendenhall et al., 2015). The incubation period of the infection is two to three days whereafter symptoms appear, such as gastroenteritis and vomiting. More severe symptoms, such as encephalitis and hepatitis, have been documented in immunocompromised patients (Moser & Schultz-Cherry, 2005; Gray & Desselberger, 2009; De Benedictis et al., 2011).

Demographically children, the elderly and immunocompromised individuals are most susceptible to AstV infection (De Benedictis et al., 2011; Bosch et al., 2014; Jeong et al., 2012). These infections occur globally and the reported prevalence amongst children with gastroenteritis ranges from 2 to 9% annually. However, a pilot study conducted in South Africa (SA) screened faecal samples (n = 191 adults and n = 105 children under the age of 5) of individuals with diarrhoea for the presence of AstVs and found a similar incidence in adults (3.1%) and children (4.8%). This study did not disclose whether the samples collected from adults were from individuals that could potentially be immunocompromised (HIV status) (Page, 2002).

A higher incidence of infection is positively correlated with high population density areas, including hostels, old age homes and hospitals (Abad et al., 2001; Moser & Schultz-Cherry, 2008; Bosch et al., 2014). AstV infections occur year-round, however seasonal variation has been documented in humans. In tropical areas high incidence is correlated with the rainy season and in temperate regions infections peak during the winter months (Pativada et al., 2012).

Treatment is supportive as the infection is self-limiting and as such only the accompanying symptoms such as dehydration and vomiting are treated (Glass & Bresee, 2011; Bosch et al., 2014).

1.8 Astrovirus infection in animals

AstV screening in animals is not common practice and most reports of animal AstV infections have resulted from tests conducted on symptomatic infections in livestock and poultry, as well as

opportunistic screening of wildlife (Moser & Schultz-Cherry, 2008). Different AstVs presents with different symptoms in different hosts, e.g. in kittens and puppies, infection may cause mild diarrhoea and pyrexia, whereas in minks the virus causes debilitating shaking mink syndrome. Infected adult pigs and cattle seem to be asymptomatic, whereas infection in juveniles often lead to diarrhoea (Gray & Desselberger, 2009; De Benedictis et al., 2011; Krishnan, 2014). In captive cheetahs infection is associated with anorexia, lethargy, regurgitation and diarrhoea (Atkins et al., 2009).

In avian hosts AstV infection can present as gastroenteritis, but stunted growth and fatal hepatitis have been observed in ducks, as well as interstitial nephritis in chickens (King et al., 2012).

Numerous AstV infections have been described in wildlife species, which include but are not limited to cheetahs, roe deer, crab-eating foxes, sea lions, rodents and bats (Atkins et al., 2009; Chu et al., 2011; Alves et al., 2018). AstV infection in bats seems to be asymptomatic, similar to numerous other viruses that infect this mammal group (Calisher et al., 2006; De Benedictis et al., 2011; Drexler et al., 2011; Mühldorfer et al., 2011).

1.9 Identification and diagnosis of infection

Since the first observation of AstVs in 1975 using electron microscopy (EM), numerous other methods have been developed to identify and diagnose AstV infection in both humans and animals. With the virus being an enteric virus, it is common practice to collect faecal material from the suspected infected individual, however, some studies have also utilized other specimen types, such as the gastro intestinal tract (GIT), brain, sputum and urine samples (Boujon et al., 2017; Fischer et al., 2017; Alves et al., 2018). Although EM was one of the first methods used for diagnosing AstV infection, there are numerous shortcomings, e.g. high virus concentrations (10^7 particles per 10 grams of faecal material) are needed for successful identification, and misidentification is common as only 10% of particles exhibit a star-like surface structure under EM (Busch et al., 2014). Misdiagnosis due to misidentification has therefore led to this method being phased out as a diagnostic tool (Pérot et al., 2017). Other AstV identification methods commonly used include virus isolation, immunodetection and antigenic typing, multiplex reverse transcription polymerase chain reaction (RT-PCR) panels, nanofluidic PCR, microarrays and next-generation sequencing (NGS).

1.9.1. Molecular diagnostics: RT-PCR, Real-time PCR, NGS

Since the development of molecular approaches that amplify the viral genome or transcripts the use of EM, virus isolation and immunoassays was phased out as diagnostic tools for AstV infections (Pérot et al., 2017). Molecular methods are widely used in clinical diagnostic and veterinary laboratories. RT-PCR allows detection and typing of AstVs. There is currently no universal PCR

setup for the detection of all known AstVs. Consensus primers such as those designed by Chu et al. (2011) detect a vast array of AstVs, but not all. The primers are routinely used in bat screening, but human, domesticated animal and wildlife samples usually have their own accompanying set of primers specific for the target of interest. The reason that a universal primer set has not been developed is probably due to high levels of genetic diversity between different AstV strains (Pérot et al., 2017). Real-time PCR methods can diagnose AstVs in a shorter time frame, it reduces the number of false positives and also enable viral load quantification.

Next generation sequencing (NGS) is not routinely used as a diagnostic tool for AstV infection due to the high costs compared to conventional screening RT-PCR or real-time PCR assays. NGS is more commonly used as an exploratory investigative method in research settings and this technology has enabled researchers to obtain partial or complete AstV genomes, which in turn provide invaluable insight into pathogenesis, viral diversity and emergence of novel AstV strains (Pérot et al., 2017). It has successfully been used for both clinical specimens and specimens from wildlife and domesticated animals.

Even though NGS is not usually used as diagnostic tool, it has aided in the diagnosis of neurotropic AstV infections (in humans, minks and cattle), that could not be detected through conventional screening PCR assays (Blomström et al., 2010; Ng et al., 2014; Nagai et al., 2015; Pérot et al., 2017).

1.9.2. Virus isolation

Isolation in cell culture is not routinely used as a diagnostic tool as it is labour intensive, time-consuming and challenging (Pérot et al., 2017). Virus isolation is further complicated by the fact that most wild type AstVs do not grow in standard cell cultures that are routinely used in diagnostic laboratories. However, virus isolation is still a valuable tool to study AstVs, as various AstV strains have been successfully isolated *in vitro* (Table 1.1.), although no *in vitro* attempts to isolate and propagate bat AstVs had been successful to date.

1.10 Current knowledge on bat astroviruses

1.10.1 South African bat astroviruses

From literature it is evident that AstV infectious dynamics has been biased towards human hosts and little attention has been given to the ecology and infection dynamics of AstVs in wild animals, this is particularly true for bat AstVs (Mendenhall et al., 2015). Little attention has been given to the evolution, phylogenetic relationship, ecology and diversity of AstVs in South African (SAn) bats

(Ithete, 2013). The only known study thus far to investigate SA bat AstVs was conducted by Ithete (2013).

Table 1.1. Astroviruses and the cell lines that were successfully used to isolate and culture the virus

AstV	**Cell line for culture**
Human AstV serotypes 1-7	Caco-2 Human colon adenocarcinoma
	T84 Human colon carcinoma
	HT-29 Human colon adenocarcinoma
	SK-CO-1 Human colon adenocarcinoma
	SK-CO-1 Human colon adenocarcinoma
	MA-104c African green monkey foetal kidney
	PLC/PRF/5 cell line
	PLC/PRF/5 hepatoma
HAstV serotype 1 only	HCT-15 Human colon adenocarcinoma
Chicken astrovirus (CAstV) isolates	LMH cell line
Porcine AstV	ESK Porcine embryonic kidney cell
Feline AstV	Feline embryonic kidney cell
Bovine AstV	Bovine embryonic kidney cell

Sources: Shimizu et al., 1990; Taylor et al., 1997; Brinker et al., 2000; Moser & Schultz-Cherry, 2005; Crameri et al., 2009; De Benedictis et al., 2011; Xiao et al., 2011; Xiao et al., 2013

AstVs were identified in seven bat species from the *Miniopteridae*, *Molossidae*, *Rhinolophidae* and *Vespertilionidae* families. The sequences belonged to genus *mamastrovirus* in genogroup II, clustering with bat AstVs found in China (Chu et al., 2009). Genogroup II viruses can further be divided into four clades; clade 1 comprises of Chinese and SAn bat AstVs, clade 2 includes German and Chinese bat AstVs; clade 3 contains bat, mink, human and ovine AstVs and clade 4 consists of bat, rabbit and porcine AstVs. Bat AstVs occur in all four genogroup II clades. Another interesting finding by Ithete (2013) was that AstV strains isolated from a single bat species did not always cluster together, similar to the findings of Drexler et al. (2011). The viral sequences did not cluster according to geographical location and it was found that SAn strains clustered with AstV from China. Viral sequences isolated from *Tadarida aegyptiaca* and *Miniopterus natalensis* bats trapped at various locations in the Western Cape exhibited the highest sequence similarity (97%). The results suggested that BtAstV/MSTM12 and BtAstV/TAr1 could potentially have evolved from a common prototype strain or could have emerged as the result of recombination events (Ithete, 2013).

1.10.2 Bat astrovirus studies in other parts of the world

The first ever study to discover AstVs in insectivorous bats was conducted by Chu et al. (2008) in China. This study built the framework for future bat AstV research studies, by developing a screening PCR that targets a conserved region of the genome, the *RdRp* gene. Since this study, various other research groups have investigated AstVs in bats. The literature shows that bat AstV studies are concentrated in certain areas (Figure 1.3.) and the majority are from China, Europe and West Africa. After the publication by Fischer et al. (2017), AstVs were investigated in three more locations which include Madagascar, Singapore and Mozambique (Lebarbenchon et al., 2017; Mendenhall et al., 2017; Hoarau et al., 2018).

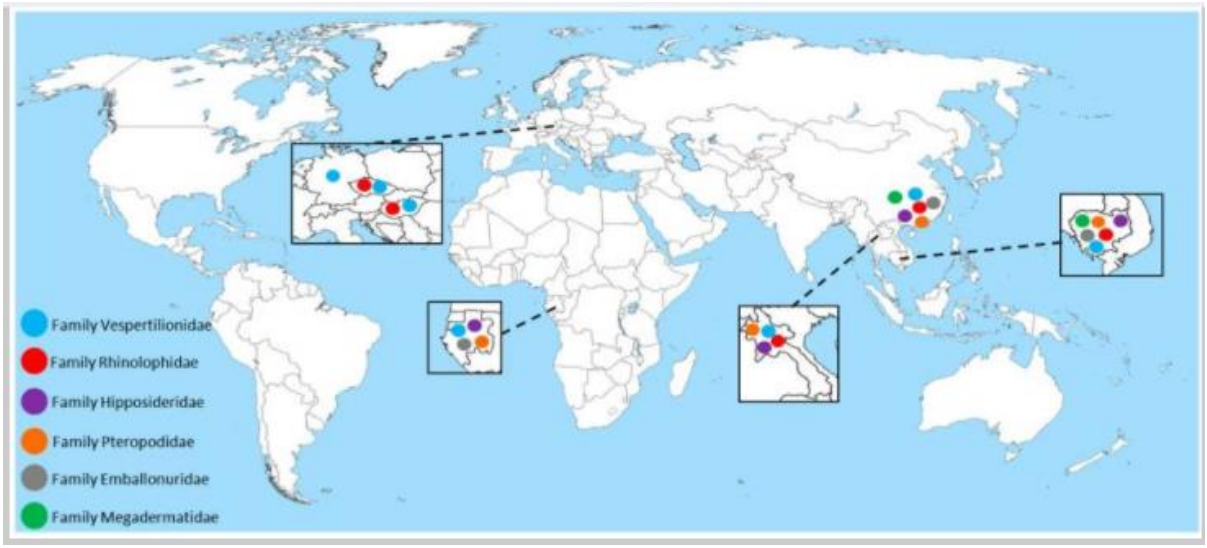


Figure 1.3. Geographic distribution of study sites for the detection of astrovirus RNA in bats (Fischer et al., 2017). Image was used with permission of Elsevier.

AstVs have been identified in one-third of known bat families (Table 1.2.) indicating the diversity of hosts within the order Chiroptera (Fischer et al., 2017). Noted from the literature is that the sequences were highly divergent and that the prevalence differed significantly between species, but also over time (Drexler et al., 2011; Fischer et al., 2017; Lebarbenchon et al., 2017; Mendenhall et al., 2017). It is thought that multiple factors, including ecological as well as host-factors, influence virus prevalence (Mendenhall et al., 2017).

Table 1.2. Species and number of bats that were investigated for the presence of astrovirus RNA/genome by semi-nested RT-PCR. (Adapted from Lebarbenchon et al., 2017; Mendenhall et al., 2017; Fischer et al., 2017; Hoarau et al., 2018)

Family	Species	Region	Number of animals	Positive Results (%)
Vespertilionidae	*Barbastella barbastellus*	Hungary	13	0 (0%)
	Eptesicus nilssonii	Czech Republic	1	0 (0%)
	Eptesicus serotinus	Hungary	3	0 (0%)
		Hungary	7	0 (0%)
		Czech Republic	1	1 (100%)
	Hesperoptenus spp.	Cambodia	1	0 (0%)
	Hypsugo savii	Czech Republic	4	1 (25%)
	Ia io	China	11	4 (36.4%)
		Lao PDR	32	1 (3.1%)
	Miniopterus gleni	Madagascar	2	1 (50%)
	Miniopterus griveaudi	Madagascar	26	15 (57.7%)
	Miniopterus inflatus	Gabon	155	16 (10.3%)
	Miniopterus magnate	China (Hong Kong)	122	67 (54.9%)
	Miniopterus pusillus	China (Hong Kong)	73	31 (42.5%)
	Miniopterus schreibersii	China (Hong Kong)	3	3 (100%)
		China	19	12 (63.2%)
		China	187	22 (11.8%)
		Hungary	15	12 (80%)
	Myotis alcathoe	Hungary	16	0 (0%)
	Myotis bechsteinii	Hungary	22	1 (4.5%)
		Hungary	125	5 (4%)
		Czech Republic	1	0 (0%)
		Germany	321	35 (10.9%)
	Myotis brandtii	Hungary	3	0 (0%)
	Myotis blythii	Hungary	2	0 (0%)
		Hungary	10	0 (0%)
	Myotis chinensis	China (Hong Kong)	9	3 (33.3%)
	Myotis dasycneme	Hungary	11	0 (0%)
	Myotis daubentoniid	Hungary	7	3 (42.9%)
		Hungary	81	6 (7.4%)
		Czech Republic	3	0 (0%)
		Germany	47	30 (63.8%)
	Myotis dasycheme	Hungary	4	0 (0%)
	Myotis emarginatus	Hungary	5	1 (20%)
		Czech Republic	1	1 (100%)
	Myotis horsfieldii	Cambodia	47	20 (42.6%)
	Myotis goudoti	Madagascar	11	1 (9%)
	Myotis	Hungary	6	0 (0%)
		Hungary	29	0 (0%)
	Myotis mystacinus	Hungary	1	0 (0%)
		Czech Republic	1	1 (100%)
	Myotis nattereri	Hungary	4	0 (0%)
		Hungary	37	1 (2.7%)
		Germany	248	99 (39.9%)
	Myotis pilosus	China (Hong Kong)	12	10 (83.3%)
		China	16	2 (12.5%)
		China	1	0 (0%)
	Myotis spp.	China	5	3 (60%)
		Singapore	1	0 (0%)
	Nyctalus leisleri	Hungary	6	0 (0%)

Family	Species	Region	Number of animals	Positive Results (%)
	Nyctalus noctula	Hungary	14	4 (28.6%).
		Czech Republic	7	1 (14.3%)
	Nyctalus plancyi velutinus	China	1	0 (0%)
	Pipistrellus abramus	China (Hong Kong)	2	1 (50%)
		China	20	1 (5%)
	Pipistrellus hesperidus	Madagascar	5	0 (0%)
	Pipistrellus nathusii	Hungary	3	0 (0%)
		Czech Republic	1	0 (0%)
		Germany	22	6 (27.3%)
	Pipistrellus	Hungary	1	0 (0%)
		Hungary	12	0 (0%)
		Czech Republic	12	1 (8.3%)
		Germany	7	0 (0%)
	Pipistrellus pygmaeus	Hungary	6	1 (16.7%)
		Czech Republic	1	1 (100%)
		Germany	12	6 (50%)
	Pipistrellus stenopterus	Singapore	1	0 (0%)
	Pipistrellus spp.	China	5	0 (0%)
		Cambodia	29	0 (0%)
	Plecotus auritus	Hungary	11	1 (9.1%)
		Hungary	29	1 (3.4%)
		Czech Republic	2	0 (0%)
		Germany	118	24 (20.3%)
	Plecotus austriacus	Hungary	3	0 (0%)
		Czech Republic	2	0 (0%)
	Scotophilus kuhlii	China	38	6 (15.8%)
		China	2	0 (0%)
	Scotophilus spp.	Cambodia	524	39 (7.4%)
	Tylonycteris pachypus	China	2	0 (0%)
	Tylonycteris spp.	Cambodia	1	0 (0%)
	Vespertilio murinus	Hungary	3	0 (0%)
		Czech Republic	5	1 (20%)
		TOTAL	**2514**	**486 (19%)**
Rhinolophidae	*Rhinolophus affinis*	China	2	0 (0%)
	Rhinolophus Euryale	Hungary	3	0 (0%)
	Rhinolophus ferrumequinum	China	7	0 (0%)
		China	4	2 (50%)
		Hungary	12	0 (0%)
	Rhinolophus hipposideros	Hungary	3	0 (0%)
		Czech Republic	2	1 (50%)
	Rhinolophus Lepidus	China	11	0 (0%)
		Singapore	1	0 (0%)
	Rhinolophus macrotis	China	2	0 (0%)
		China	1	0 (0%)
	Rhinolophus pearsonii	China	1	1 (100%)
	Rhinolophus rouxii	China (Hong Kong)	8	1 (12.5%)
	Rhinolophus sinicus	China	1	0 (0%)
	Rhinolophus spp.	Cambodia	53	1 (1.9%)
		Lao PDR	102	4 (3.9%)
		TOTAL	**213**	**10 (4.7%)**
Hipposideridae	*Aselliscus stoliczkanus*	China	1	0 (0%)
	Aselliscus spp.	Lao PDR	7	0 (0%)
	Hipposideros armiger	China (Hong Kong)	10	0 (0%)
		China	109	21 (19.3%)
	Hipposideros gigas	Gabon	226	7 (3.1%)

Family	Species	Region	Number of animals	Positive Results (%)
	Hipposideros larvatus	China	29	4 (13.8%)
		China	1	0 (0%)
	Hipposideros Pomona	China	95	13 (13.7%)
		China	15	0 (0%)
	Hipposideros ruber	Gabon	394	17 (4.3%)
	Hipposideros spp.	Cambodia	4	1 (25%)
		Lao PDR	26	1 (3.8%)
	Paratriaenops furculus	*Madagascar*	31	11 (35%)
	Triaenops menamena	*Madagascar*	*40*	0 (0%)
		TOTAL	**988**	**75 (7.5%)**
Pteropodidae	*Cynopterus bra chyotis*	Singapore	144	0 (0%)
	Cynopterus sphinx	China (Hong Kong)	11	0 (0%)
	Cynopterus spp.	Cambodia	321	0 (0%)
		Lao PDR	19	0 (0%)
	Eonycteris spelaea	Singapore	169	30 (17.75%)
	Eonycteris spp.	Cambodia	28	0 (0%)
		Lao PDR	51	3 (5.9%)
	Macroglossus minimus	Singapore	1	0 (0%)
	Macroglossus spp.	Cambodia	21	0 (0%)
		Lao PDR	1	0 (0%)
	Megaerops spp.	Cambodia	29	0 (0%)
		Lao PDR	69	0 (0%)
	Penthetor lucasi	Singapore	79	0 (0%)
	Pteropus spp.	Cambodia	10	0 (0%)
	Rousettus aegyptiacus	Gabon	162	2 (1.2%)
	Rousettus leschenaultia	China	59	1 (1.7%)
	Rousettus madagascariensis	Madagascar	41	2 (4.8%)
	Rousettus spp.	Cambodia	11	1 (9.1%)
		Lao PDR	322	23 (7.1%)
		TOTAL	**1235**	**62 (5%)**
Emballonuridae	*Coleura afra*	Gabon	25	2 (8%)
	Taphozous melanopogon	China	172	160 (93%)
	Taphozous spp.	Cambodia	147	4 (2.7%)
		TOTAL	**344**	**166 (48.3%)**
Megadermatidae	*Megaderma lyra*	China	1	1 (100%)
		Cambodia	21	2 (9.5%)
		TOTAL	**22**	**3 (13.6%)**
Molossidae	*Mormopterus jugularis*	Madagascar	**40**	**0 (0%)**
	Otomops madagascariensis	Madagascar	**6**	**0 (0%)**
		TOTAL	**46**	**0 (0%)**

1.10.3 Cross species transmission and zoonotic potential

Until relatively recently AstV infections were thought to be species-specific. More and more findings are suggesting that the host tropism of AstVs might be wider than previously thought. Currently there exists no evidence of cross-species transmission of a bat AstV to a species of another mammalian order (Fischer et al., 2017). As there is no strict host restriction of bat AstVs within the Chiroptera group it could be that there have been cross-species transmission that are yet undiscovered. There is however evidence of cross-species transmission in other mammalian groups. Livestock farming readily puts different domesticated animals in close proximity to one another, increasing the

probability of spill-over from one species to another. Interestingly, a recent study reported a single AstV species that infected different host organisms in different geographical areas. The neurotropic AstV identified in a sheep in Sweden with encephalitis shared 95% amino acid similarity in the capsid protein gene region, with a bovine AstV identified in Germany (Boujon et al., 2017). As the similarity was so high between the capsid protein gene regions these AstV strains could be seen as the same species according to the International Committee on Taxonomy of Viruses (ICTV) guidelines (Boujon et al., 2017).

Similar to most viruses that originated from animals, the same is true for newly discovered human AstV serotypes, HAstV-MLB1-3, HMO AstVs A, B, and C, and HAstV-VA1-4. These serotypes are phylogenetically more similar to AstVs isolated from animals than the "classical" human AstV serotypes (HAstV1-8) (Bosch et al., 2012).

Another interesting study reported that non-human primates (NHP) harboured diverse AstV strains that were related to human and avian AstVs (Karlsson et al., 2015). This study revealed that members of the avastrovirus group could in fact spill over to mammals, which was thought to be restricted to birds (Karlsson et al., 2015). The study also identified an AstV strain in NHP, which was a recombination between a NHP AstV and a HAstV strain. These NHP were in close contact with human settlements and it is highly plausible that a spill-over event occurred. Also, of interest are the accounts of possible reverse zoonoses that were documented in captive cheetahs that were infected with an AstVs strain that was similar to a human AstV strain (Atkins et al., 2009).

Considering the high diversity of AstVs and their ability to adapt to different environments and different hosts species, it would not be impossible for a bat AstVs to spill over to humans or other animals. This highlights the importance of disease surveillance efforts in both humans and bats.

1.11 One Health Approach

One Health is a concept that recognizes the interconnectedness of human, environmental and animal health (United Nations 2008; Narrod et al., 2012). This concept is not new to the 21st century (Evans & Leighton, 2014) and the first documented reference thereto can already be found in the writings of Hippocrates (460 BCE - 367 BCE) in 'On Airs, Waters and Places', where he discusses the interrelationship of human health and a clean environment (Evans & Leighton, 2014). Even though the fundamental concept of One Health has been acknowledged for hundreds of years, human and animal health remained separate fields of study until the 20th century. In recent years there has been a surge in emerging EID and zoonotic disease studies with a One Health focus (Narrod et al., 2012).

Many of these animal studies have investigated diseases carried by bats, as bats are important hosts for numerous EIDs. Several disease ecology studies have found that anthropogenic disturbances of natural habitats have caused bats to inhabit peri-urban areas, increasing the contact rates between bats and humans, as well as bats and domestic animals (Daszak et al., 2007; Narrod et al., 2012). This in turn leads to an increased likelihood of disease spill-over events, not only from wildlife to humans, but also from humans to wildlife (Daszak et al., 2007).

The objectives of the current study fit well with the One Health concept for various reasons. Firstly, AstVs have been identified to be of medical and veterinary importance; secondly, the virus is regularly used as indicator of environmental contamination with human and animal waste, and lastly the virus has proven zoonotic potential (Meleg et al., 2006).

A subsection of the current study investigated bats trapped at wastewater treatment works (WWTW) in Kwazulu Natal (KZN), to ascertain whether these bats were more likely to be infected with HAstVs, due to exposure to heavy metals and other pollutants. Naidoo (2012) reported that bats foraging at these specific WWTWs accumulated heavy metals in various soft tissue, which in turn reduced their immune system functioning.

1.12 Rationale to proposed research project

During the current study we aimed to investigate the diversity, ecology and zoonotic potential of AstV strains that occur in SAn bats. Information collected during the current study will also help frame the One Health implications of AstVs in SA. The zoonotic potential of bat AstVs is still undetermined, however literature indicates that cross-species transmission is possible (Atkins et al., 2009; Mendenhall et al., 2015; Boujon, 2017; Waruhiu et al., 2017). A study by Xiao et al. (2011) revealed that bat AstVs are phylogenetically closely related to some other mamastroviruses (including HAstVs VA-1, -2 and -3), suggesting possible transmission to humans and other animals. Studying the occurrence, diversity and biology of bat AstVs could provide insight into the origin of viruses that are regarded as “human” viruses and could potentially lead to better preventative and / or management strategies in future.

One might argue that more emphasis should be placed on viruses known to pose an immediate threat to humans, e.g. rabies and Ebola. However, these are the subject of extensive scientific studies already. Therefore, AstVs were chosen for this study for the following reasons: (1) they seem to occur commonly in various bat species (Chu et al., 2008; Drexler et al., 2011; Ithete, 2013; Dufkova et al., 2015; Fischer et al., 2017; Lebarbenchon et al., 2017;); (2) they are RNA viruses like most emerging

viruses; (3) very little is known about AstVs in wild animal hosts; and (4) AstVs have been understudied in SAn bat species.

The main focus of the current study was to investigate the diversity and ecology of AstVs in SA bats. However, there were subsections to the current project which enabled a more holistic approach to AstVs in SA bats, as well as the possible One Health implications of AstVs.

The subsections of the current study were as follows:

1) General surveillance of bats for the presence of AstVs. This was achieved through opportunistic sampling of bats throughout SA. Phylogenetic data (*RdRp* gene sequences and ORF2 sequence data) were used in phylogenetic reconstructions. Ecological and biological information of individual bats recorded during sampling, were statistically analysed to determine factors that could influence AstV positivity.

2) Longitudinal surveillance of a *N. capensis* bat colony for AstVs and CoVs, via monthly faecal collections. This data was used to determine if the colony was co-infected with the virus, as well as how the viral loads fluctuated over time.

3) Surveillance of bats at WWTW for the presence of human AstVs. Samples were collected from bats at the WWTW and from pristine sites that were far enough removed from the WWTW to allow for comparison. Furthermore, environmental samples, in the form of water samples were collected upstream and downstream from the WWTW and were also analysed for the presence of human AstVs.

4) *In vitro* isolation of bat AstV in different cell lines that will enable indirect determination of zoonotic potential.

The results of the four subsections would then be used to discuss the possible One Health implications of AstVs through framing it in the three subsections of the One Health principles; (1) Animal Health, general screening of bats for AstVs across SA; (2) Human health, determining zoonotic risk through *in vitro* isolation and via phylogenetic analyses of bat AstV sequences; and (3) Environmental Health, through collection and screening of WWTW samples for the presence of AstVs in conjunction with screening bats collected at these sites for AstVs to determine the impact of possible exposure to human AstVs.

1.13 Research Question, Hypothesis, Aims & Objectives

1.13.1 Research question

Which environmental and / or host factors drive the diversity and ecology of bat AstVs in selected SAn bat species?

1.13.2 Hypothesis

Host and environmental factors, such as species identity, age, reproductive state, rainfall and biome influence AstV diversity and ecology.

1.13.3 Aims

1. Describe the genetic diversity and ecology of AstVs occurring in various bat species across Southern Africa.
2. Determine ecological and demographical factors (e.g. seasonality, age, reproductive state) that could influence AstV diversity and ecology in SAn bats.
3. Determine the phylogenetic relatedness of SAn bat AstVs in relation to other mammalian AstVs.
4. Describe the results of this study against the background of / in relation to possible One Health implications of AstVs.

1.13.4 Objectives

1. To use sequencing and phylogenetic techniques to characterize novel bat AstV *RdRp* strains detected during current study.
2. To use quantitative real-time PCR to detect and monitor AstVs and CoVs in a *Neoromicia capensis* colony in Velddrif, Western Cape, over time.
3. To analyse biological and ecological data collected on sampled bats to increase understanding of which ecological and demographic factors may drive AstV diversity in SAn bats.
4. To attempt isolation and propagation of one or more bat AstV strains *in vitro* to determine the zoonotic potential of the virus.
5. To determine whether bat populations near WWTW can become infected with human AstV strains by comparing the AstV strains from bats in this study to human AstV strains, as well as virus isolation from water samples collected at the WWTW.

Chapter 2 Materials & Methods

2.1 Biosafety considerations

This study used samples that were collected and stored in RNAlater® (Sigma-Aldrich, St. Louis, USA). These samples were handled under biosafety level (BSL) 2 conditions when standard molecular biology methods were used. Virus isolation attempts and cell culture techniques were performed in a BSL-3 laboratory in the Division of Medical Virology in accordance with various safety standard operating procedures as prescribed by the World Health Organization (World Health Organization 2004).

2.2 Ethical considerations

Samples were collected in collaboration with expert zoologists, Dr. Corrie Schoeman (University of KZN), as well as with the help of public collaborators (Quartus Laubscher and Tanja Jane Kerr). All fieldwork procedures adhered to established best practices under the guidance of trained zoologists with permits obtained from the relevant conservation authorities (**Appendix A**). The majority of samples were collected through non-invasive techniques to minimize disturbance to colonies and harm to animals. Tissue specimens were obtained from bats that were euthanized to obtain voucher specimens by Dr Richards at the Museum of Natural History. Ethics approval for the usage of the samples collected by collaborators was granted by the HREC of Stellenbosch University (SU-ACUD16-00008).

2.3 Sample processing

The samples that were selected for processing (n = 500 faecal samples) during the current study were all processed by members of the emerging viruses research group and me. Where possible, our research group participated with sample collection with collaborating zoologists (especially in the Western Cape and Northern Cape regions). Virus recovery from water samples were also planned and executed by me with assistance from Mr Vurayai Ruhanya. *In vitro* virus isolation and cell culture experiments were all conducted by me.

2.4 Sample availability and collection

2.4.1 Previously collected samples / Archived samples

Aliquots from previously collected samples (2011-2014) were stored in -80°C freezers and were available for use in this study. The available sample repository consisted of an estimated 590 bat

samples and included faecal material (n = 400), oral- (n = 60) and urine- (n = 30) swabs, as well as tissue samples (n = 100).

2.4.2 Sample collection

Samples were collected by collaborating zoologist, Dr Schoeman, from the University of KZN and his research group. Sampling sites were selected based on previous trapping experience of collaborating zoologists. Sample collection took place on two levels; colony surveillance and general surveillance across SA.

General surveillance entailed the collection of samples from various bat species through opportunistic sampling across Southern Africa, aiding in determining the overall diversity of AstVs in various bat species across space and time. Where possible, samples were collected across different geographic locations and biomes in an attempt to obtain a diverse sample set.

Colony surveillance involved a continuous surveillance of known bat colonies. Faecal material was collected monthly from a *N. capensis* colony in the Western Cape Province (Velddrif) with the help of public collaborator, Mr Laubscher. Standardized sampling protocols and personal protective equipment (PPE), such as gloves and masks, were supplied with clear instructions to the collaborating sample collectors. Colony samples were collected for the duration of one calendar year (2015-2016).

2.4.3 Wastewater treatment works samples

In an attempt to determine if bats feeding and drinking at WWTW were infected with human AstVs, bats were sampled from the selected WWTW; i.e. Verulam Wastewater Works (S29º38.38; E31º03.49) which is located on the Mdloti River, and the Umbilo Wastewater Works (S29º50.44; E30º53.31) on the Umbilo River (Naidoo et al., 2015). These two WWTWs both receive industrial and domestic wastewater.

Bat trapping at WWTW

N. nana bats were trapped at WWTW, as well as in pristine downstream localities (3 km away from the WWTW). A total of 50 *N. nana* bats were screened from WWTW and 47 *N. nana* from pristine areas.

Collection of water samples from WWTW

Ten litres of water was collected upstream and downstream of each WWTW. More details are given in Section 2.15.

2.4.4. Bat trapping, data recorded & sample collection

During active sampling, bats were caught with mist nets and / or harp traps (Francis, 1989; Monadjem et al., 2010). Upon capture, bats were retained in a cloth bag until processing by the research team commenced. The following data was recorded: (1) morphological data (measurements of forearm, tail, tragus, wingspan and mass); (2) population demographic data (sex, age, reproductive state); and (3) locality data (GPS coordinates and biome type). Non-invasive sampling techniques were used, such as anal and oral swabs and collection of faecal pellets. Where possible, for further confirmation of species identity, an echolocation recording was taken (EM3 Wildlife Acoustics, Massachusetts, USA). After data collection, bats were released.

2.4.5 Sample handling and storage

Faecal samples of individual bats were placed in separate 2 ml cryogenic vials (Corning, New York, USA) containing either 1 ml RNAlater® (Sigma-Aldrich, USA) solution or 1 ml viral transport medium (VTM), consisting of DMEM (Lonza, Switzerland), Penicillin-Streptomycin (Lonza, Switzerland) and 10% foetal bovine serum (FBS) (Biowest, France). Anal and oral swabs were stored in 5 ml of VTM consisting of DMEM (Lonza, Switzerland), Penicillin-Streptomycin (Lonza, Switzerland) and 10% FBS (Biowest, France).

2.5 Nucleic acid extraction from faecal material

Following faecal disruption and homogenization, RNA was manually extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel, Germany). The extraction protocol for samples collected as part of the colony surveillance required five faecal pellets (Drexler et al., 2011) to be homogenized in 1 000 µl of phosphate-buffered saline (PBS) (Gibco®, California, USA) with five metal beads. For samples acquired from individual bats, one faecal pellet was homogenized in 600 µl of PBS (Gibco®, USA) with five metal beads (Drexler et al., 2011). Faecal pellets were disrupted with the Qiagen TissueLyser LT (QIAGEN, Hilden, Germany), followed by centrifugation for 2 minutes at 11000 x g (5424 Microcentrifuge, Eppendorf, Hamburg, Germany). The supernatant of approximately 150 µl was transferred to a 2 ml safe-lock tube (Eppendorf, Hamburg, Germany) and 600 µl of the provided lysis buffer, RAV1, (RAV1 contains reconstituted carrier RNA) was added and vortexed (S0200, Labnet, New Jersey, USA) for 5 -10 seconds. Samples were incubated at 70°C for 5 minutes. After the incubation step, 600 µl molecular biology grade ethanol (Sigma-Aldrich, USA) was added to the sample and briefly vortexed (S0200, Labnet, USA). The sample was added stepwise to the column and centrifuged for 1 minute at 11 000 x g. The flow-through was discarded and the column placed back in the collection tube. The column was then washed with 500 µl of the

provided buffer, RAW, and centrifuged at 8 000 x g for 1 minute. The flow-through was discarded again and the column was placed in a new collection tube. A second wash step was performed by adding 600 µl RAV3 to the column and centrifuging it for 1 minute at 8000 x g. The flow-through was discarded once again and the column placed in a new collection tube. To ensure that all the residual ethanol was removed, the column was centrifuged again for 5 minutes at 11 000 x g. The column was then placed in a new 2 ml collection tube. Fifty microliters of nuclease-free water was added to the column and incubated at room temperature for 2 minutes and centrifuged for 1 minute at 8 000 x g. The eluted RNA was stored at -80°C until reverse transcription could be performed.

2.6 Reverse transcription

Extracted RNA was reverse transcribed to complementary DNA (cDNA) using RevertAid Reverse Transcriptase (ThermoScientific, USA). The reverse transcription took place in two reactions; the components are listed in Tables 2.1. and 2.2., respectively. Once the reagents listed in Table 2.1. were pipetted (Eppendorf, Germany) into PCR strip-tubes (Nest Scientific Inc., Wuxi, China), the mixture was briefly vortexed and centrifuged. The reaction mixture was incubated (9700 GeneAmp® thermocycler, Applied Biosystems, California, USA) at 65°C for 5 minutes and then chilled on ice for 1 minute before adding the second reaction's master mix that consisted mostly of the enzyme components of the reaction (Table 2.2.). The combined reaction mixture was then incubated at 25°C for 10 minutes and 42°C for 60 minutes. The resulting cDNA was stored at -20°C until PCR screening commenced.

Table 2.1. Reagents for the first reaction of reverse transcription

Reagents	**Stock Concentration**	**Final**	**Volume per run (µl)**
Random hexamer primer (ThermoScientific, USA)	200 ng/µl	400 ng	2
Diethylpyrocarbonate water (DEPC) (ThermoScientific, USA)			13
RNA Template	-	-	10

Table 2.2. Reagents used for the second reaction of reverse transcription

Reagents	**Stock Concentration**	**Final Concentration**	**Volume per run (µl)**
5x RT Buffer	5X	1X	4
dNTP Mix (40mM)	10 mM		2
RNase inhibitor	2 500 µl	20 U	0.5
RevertAid Reverse Transcriptase enzyme	200 µl	200 U	1

2.7 PCR positive control

To ensure optimal running of the AstV screening PCR designed by Chu et al. (2008), plasmid positive DNA controls were generated to be used as DNA positive controls or *in vitro* transcribed to RNA. The process for creating the AstV positive control is described in short below.

A 420bp *RdRp* gene fragment from a *Miniopterus natalensis* derived sequence was used that was obtained from a faecal sample by Ithete (2013). Shortly, the selected fragment was amplified using the PCR assay and primers designed by Chu et al. (2008) and purified with the Rapid PCR Enzyme Cleanup Set (New England Biolabs, USA) described in Section 2.10. The resulting positive control underwent *in vitro* transcription (Section 2.7.7.). The sensitivity of the PCR assay was then established through creating a serial dilution of the quantified positive control RNA (Section 2.7.9.) that underwent reverse transcription and amplification with the hemi-nested AstV *RdRp* PCR (Section 2.8.1. & 2.8.2.).

2.7.1 Ligation reaction

A recombinant plasmid was constructed by covalently connecting the sugar backbone of the vector and the *RdRp* gene by using T4 DNA ligase enzyme (ThermoScientific, USA). The enzyme catalyses the formation of covalent phosphodiester linkages which permanently joins the nucleotides together. The InsTAclone kit (ThermoScientific, USA) was chosen for a few reasons; 1. It allows for relatively easy cloning of Taq-amplified PCR products, 2. It allows cloning of PCR products with TA overhangs and 3. Enables blue / white colony screening.

The reaction was set up as indicated in Table 2.3. The amount of PCR product needed for the ligation reaction was calculated according to the manufacturer's instructions (ThermoScientific, USA) as approximately 86 ng.

Table 2.3. Reagents of the ligation reaction

Reagents	**Volume per reaction**
Vector pTZ57R/T (0.17 pol ends)	3 µl
5X ligation buffer	6 µl
PCR product	made up to 4 µl (~86 ng)
T4 DNA ligase	1 µl
Nuclease-free water	made up to 29 µl
Total volume	**30 µl**

The ligation reaction was run on the 9700 GeneAmp® Thermocycler (Applied Biosystems, USA) using the following temperature profile and cycling parameters as described in the InsTAclone kit user manual: 25°C for 120 minutes, 4 cycles: 4°C for 120 minutes, 1 cycle: 75°C 5 minutes, 4°C hold.

2.7.2 Transformation

After the ligation, the plasmid with the insert DNA is introduced through transformation into competent bacteria, *Escherichia coli* strain JM109. This *E. coli* strain contains a lacZΔM15 deletion mutation, while the plasmid vectors contain a short segment of the lacZ gene that codes for β-galactosidase, an enzyme that metabolizes lactose. The plasmid vectors are manipulated in such a way that this α-complementation process serves as an indicator of successful recombination. Plasmid vector contains a multiple cloning site within the *lacZ* sequence, this sequence is altered by restriction enzymes to insert the desired gene. The transformation reaction was performed as described below.

For transformation, agar plates were prepared by dissolving 10 g LB broth (Sigma-Aldrich, USA) and 7.5 g agar (Sigma-Aldrich, USA) in 500 ml distilled water. LB agar was autoclaved at 121°C for 30 minutes at 100 kPA pressure (STA-400/STA-410, St. Francis, Taipei, Taiwan). After the mixture had cooled sufficiently, to about 60°C, 250 µl ampicillin, to a final concentration of 50-100 µg/ml (Melford Biolaboratories Ltd., Ipswich, UK) was added. Approximately 25 ml of agar was poured into sterile plastic Petri dishes (Gibco®, USA) and allowed to set. To allow for blue / white colony selection, each agar plate was treated with 16 µl X-gal ready to use solution, to a final concentration of 80 µg/ml and 40 µl IPTG, to a final concentration of 20 mM (ThermoScientific, USA) and incubated at 37°C for 30 minutes. Single-use tubes of *Mix & Go* cells (strain: JM109) (Zymo Research Corp, California, USA) were thawed on ice and 1-5 µl of ligation reaction was added and mixed gently for a few seconds. The cells were then incubated on ice for 2-5 minutes. To reduce overgrowth on plates, 25 µl of the transformation reaction was spread per plate. The plates were incubated overnight at 37°C.

2.7.3 Colony picking

The successful transformation of the vector, containing the foreign DNA, into competent cells will lead to the production of dysfunctional β-galactosidase resulting in white colonies. These colonies were picked and cultured in LB broth, to allow for production of more of the bacteria with the gene of interest. The colony picking process is described in detail in the following section.

Luria broth (LB) media was prepared by adding 10 g LB broth (Sigma-Aldrich, USA) to 500 ml distilled water and mixing with magnetic stirrers, until all the particles were dissolved. To sterilize the LB broth, it was autoclaved at 121°C for 30 minutes at 100 kPA pressure (STA-400/STA-410,

St. Francis, Taipei, Taiwan). After the broth had cooled sufficiently to about 60°C, 250 µl of ampicillin (Melford Biolaboratories Ltd., Ipswich, UK) was added to a final concentration of 50-100 µg/ml. Added ampicillin served as an inhibitor for other bacteria that might have been present, as the bacterial strain used during cloning is ampicillin resistant and its growth should not be affected. Five millilitres of LB broth was added to each 14 ml round bottom polypropylene tubes (Falcon™ Corning, New York, USA) and a single white colony was picked using a sterile pipette tip and placed in the Falcon tube (Corning, USA) containing the broth. The tubes with the picked colonies were incubated overnight at 37°C on a shaker at 200-250 rpm.

2.7.4 Plasmid DNA purification

Following overnight culture of successfully transformed bacterial cells, the plasmid DNA of interest was purified using the GeneJet Plasmid MiniPrep system (ThermoScientific, USA). Falcon® tubes (Corning, USA) containing bacterial growth were centrifuged (Eppendorf, Germany) for 2 minutes at 12 000 rpm. The supernatant was discarded, and the pellet was resuspended in 250 µl of resuspension solution and transferred into a 2 ml microcentrifuge tube. The solution was vortexed (S0200, Labnet, USA) until it was homogenous, where after 250 µl of lysis solution was added and mixed by inverting the tube 4-6 times, until the solution became viscous and slightly clear. The lysis solution was neutralized by adding 350 µl of neutralization solution. The mixture was then centrifuged for 5 minutes at 1 200 x g. The supernatant was transferred to the GeneJet spin column, and centrifuged (Eppendorf, Germany) for 1 minute at 12000 x g. The flow-through was discarded and the column placed back in the collection tube. The column was washed by adding 500 µl of wash solution to the column followed by centrifugation (Eppendorf, Germany) for 1 minute at 12 000 x g. The flow-through was discarded and the column placed back in the collection tube and the wash step was repeated. The column was placed back into the collection tube and centrifuged (Eppendorf, Germany) for an additional 1 minute to remove residual ethanol from the column. To elute the plasmid DNA, the column was placed in a new 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added to the centre of the column and incubated for 2 minutes at room temperature. The column was then centrifuged (Eppendorf, Germany) for 2 minutes at 12 000 x g and the resulting purified plasmid DNA was stored at -20°C.

2.7.5 Linearization and in vitro transcription

Before the plasmid DNA can be transcribed into RNA the plasmid needs to be linearized as the T7 RNA Polymerase enzyme used during transcription will not dissociate from the plasmid and will continue to transcribe around the circular template multiple times. This produces a transcript that is much longer that and that contains mostly vector-derived sequences. Linearization downstream from

the cloned target insert will produce an RNA transcript, of which the length is defined by the 3' end of the template and the transcription product will only contain a small amount of vector sequence. During transcription RNA molecules are synthesized from the DNA sequence by utilizing the T7 promotor sequence in the bacteriophage. Linearization and *in vitro* transcription was conducted as described in Sections 2.7.6. and Section 2.7.7.

2.7.6 Linearization

Linearization was conducted with a restriction enzyme, EcoRI (New England Biolabs, USA), according to the manufacturer's instructions. The reagents for the reaction are listed in the table below (Table 2.4.). Once the reaction was set up it was incubated at 37°C for 1 hour followed by heat inactivation at 65°C for 20 minutes (9700 GeneAmp® thermocycler, Applied Biosystems, USA).

Table 2.4. Reagents used during the linearization of the plasmid

Reagents	**Volume per reaction**
Restriction Enzyme (EcoRI)	1 µl
DNA	1 µl
10X NEBuffer	5 µl
DEPC Water (ThermoScientific, USA)	made up to 18 µl
Total reaction volume	**20 µl**

Purification of linearized product was done to remove excess enzymes as well as to desalt the reaction and concentrate DNA. The purification was done with MinElute Reaction Cleanup Kit (QIAGEN, Germany) as mentioned in Section 2.11.1. with an amendment, instead of adding 5 volumes of the binding buffer to the sample, 300 µl of ERC buffer was added.

2.7.7 *In vitro* Transcription

The purified linearized product was then *in vitro* transcribed by adding the components in Table 2.5 together and centrifuged (Eppendorf, Germany) before incubation at 37°C for 2 hours.

Following *in vitro* transcription residual DNA template was removed through DNAse treatment. For each 1 µg of *in vitro* transcribed DNA 2 U of DNAse I (Thermo Fisher Scientific) was added and the reaction was incubated at 37°C for 15 minutes. The reaction was terminated by RNA purification.

Table 2.5. The components of the in vitro transcription reaction

Reagents	Volume per reaction
5X TranscriptAid reaction buffer	4 µl
ATP/CTP/GTP/UTP mix	8 µl
Template DNA	1 µ1
TranscriptAid enzyme mix	2 µl
DEPC Water (ThermoScientific, USA)	made up to 20 µl
Total reaction volume	**20 µl**

2.7.8 RNA purification

Inactivation of the DNAse I reaction was achieved through RNA purification with the Purelink® RNA Mini Kit (Thermo Fisher Scientific, USA). One volume of lysis buffer was added to one volume of sample and mixed by vortexing. The mixture of sample and lysis buffer was added to the spin column and centrifuged for 15 seconds at 12 000 x g. The flow-through was discarded and two wash steps were performed with 500 µl wash buffer followed by centrifugation at 12 000 x g for 1 minute. The column was then transferred to an elution tube and 35 µl of RNAse free water was added to the centre of the column. The column containing RNAse free water was incubated at room temperature for 2 minutes, whereafter it was centrifuged for 2 minutes at 12 000 x g to elute the RNA from the column.

2.7.9 Quantification of RNA copies

To determine the limit of detection of the screening PCR it was necessary to determine the copy number of the positive control. RNA concentration was determined with the Qubit® 3.0 fluorometer (ThermoScientific, USA). The protocol followed is described below. For each standard and sample, 250 µl of Qubit® working solution was prepared by diluting the Qubit® RNA Reagent (1:200) in Qubit® RNA Buffer. Samples and controls for quantification were prepared according to Table 2.6. The components were added to 0.5 ml Qubit® assay tubes (ThermoScientific, USA), vortexed (S0200, Labnet, USA) for 2-3 seconds and incubated at room temperature for 2 minutes. The Qubit® was calibrated with the standards, followed by readings of the samples by following the prompts on the Qubit® 3.0 fluorometer (ThermoScientific, USA). The readings (ng/µl) were converted to copy number by using online tools (http://cels.uri.edu/gsc/cndna.html). To determine the limit of detection of the screening PCR, serial dilutions were made from the starting concentration of 10^{12} to 10 per µl. However, single use aliquots of concentration of 10^4 of the positive control were frozen and used as is to compensate for degradation of the RNA during freeze thawing.

Table 2.6. Reagents used for RNA quantification in plasmid positive control

Reagent	Volumes for Standards	Volumes for Samples
Working Solution	190 µl	180-199 µl
Standard	10 µl	-
Sample	-	1-20 µl
Total volume	**200 µl**	**200 µl**

2.8 Astrovirus PCR assays

2.8.1 General screening PCR for the detection of astroviruses

The hemi-nested AstV screening PCR developed by Chu et al. (2008) amplifies the *RdRp* gene (422 bp) of bat AstVs but also a host of other mammalian AstVs (Fischer et al., 2017). The hemi-nested PCR was successfully used by Ithete (2013) to detect AstV in SAn bats and as such this protocol was also used during the current study. The screening PCR consists out of a pre-nested amplification and nested amplification, as detailed below.

2.8.2 PCR assay for amplification of the capsid protein gene (ORF2)

AstV species classification and identification is based on the capsid protein gene (ORF2), which is important for virus phylogenetics and classification. The ORF2 region is notoriously difficult to amplify for bat AstVs, as such only a small number of these sequences are available on Genbank® (https://www.ncbi.nlm.nih.gov/nuccore/?term=bat+astrovirus+capsid+protein+gene, visited 10 September 2018) (Fischer et al., 2017). The amplification of this region is either a hit or miss (personal communication Dr Freiden, St Jude Children's Hospital, Memphis USA). Based on some partial ORF2 bat AstV sequences on Genbank®, it was suggested to use the primer set developed by Atkins et al., (2009). AstV samples that were identified as positive by hemi-nested AstV PCR (Chu et al., 2008) and sequencing, were subjected to a second PCR reaction with primers targeting the capsid protein of the virus (Atkins et al., 2009) (Table 2.7.). The PCR assay also entails a pre-nested and nested amplification round as described below. The nested PCR product size is about 800 - 1000 bp.

Table 2.7. Summary table of the primer sets used with different PCR assays during in the amplification of two regions of the AstV genome.

Screening assay & region of amplification	Oligo Name	5'→3' Oligo Sequences
Pre-nested amplification of *RdRp* gene	Pan-Astro_F1	GAR TTY GAT TGG RCK CGK TAY GA
(Chu et al., 2008)	Pan -Astro_F2	GAR TTY GAT TGG RCK AGG TAY GA
	Pan-Astro_R	GGY TTK ACC CAC ATN CCR AA
Nested amplification of the *RdRp* gene	Pan-Astro_HNF1	CGK TAY GAT GGK ACK ATH CC
(Chu et al., 2008)	Pan-Astro_HNF2	AGG TAY GAT GGK ACK ATH CC
	Pan-Astro_R	GGY TTK ACC CAC ATN CCR AA
Pre-nested amplification of ORF2 gene	Astr_4811F	TTTGGNATGTGGGTNAARCC
(Atkins et al., 2009)	Astr_5819R	TCATTNGTGTYNGTNANCCACCA
Nested amplification of ORF2 gene	Astr_5159F	GGAGGGGMGGACCAAAG
(Atkins et al., 2009)	Astr_5819R	TCATTNGTGTYNGTNANCCACCA

2.8.3 Pre-nested amplification of the *RdRp* gene

The pre-nested reaction was set up following the protocol by Chu et al. (2008), with TrueStart Hot Start Taq DNA polymerase (ThermoScientific, USA). The reagents and volumes used are listed in Table 2.8. After the reagents were added to a 2 ml microcentrifuge tube (Nest Scientific Inc., China), the mastermix was vortexed (S0200, Labnet, USA) and centrifuged briefly. A volume of 23 µl of mastermix was pipetted (Eppendorf, Germany) into PCR strip-tubes (Nest Scientific Inc., China) followed by 2 µL of cDNA template. The strip-tubes containing the reaction mixture and cDNA template was briefly vortexed (S0200, Labnet, USA) and centrifuged (Eppendorf, Germany). The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, USA) using the following temperature profile: 95°C for 2 minutes, 40 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds and a final extension at 72°C for 5 minutes.

Table 2.8. Reagents used for the pre-nested amplification reaction of the RdRp gene

Reagents	Stock Concentration	Final Concentration	Volume per run (µl)
Nuclease-free water			10.87
TrueStart Buffer	10X	1X	2.5
$MgCl_2$	25 mM	1.5 mM	1.5
dNTP Mix	10 mM	200 µM	0.5
Pan-Astro_F1	10 µM	1 µM	2.5
Pan-Astro_F2	10 µM	1 µM	2.5
Pan-Astro_R	10 µM	1 µM	2.5
TrueStart *Taq*	5 U/µl	0.625 U	0.125
cDNA template	-	-	2
Total reaction volume			**25µl**

2.8.4 Hemi-nested amplification of the *RdRp* gene

The nested reaction was set up on ice following the protocol of Chu et al. (2008) with TrueStart Hot Start Taq DNA polymerase (ThermoScientific, USA). The reagents and volumes used are listed in Table 2.9. After the reagents were added to a 2 ml microcentrifuge tube (Nest Scientific Inc., China) the mastermix was vortexed (S0200, Labnet, USA) and centrifuged (Eppendorf, Germany) briefly. Each reaction consisted out of 48 µl of mastermix and 2 µL of pre-nested PCR product. The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, USA) using the following temperature profile: 95°C for 2 minutes, 30 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 5 minutes.

Table 2.9. Reagents used for the nested amplification reaction of the RdRp gene

Reagents	**Stock Concentration**	**Final Concentration**	**Volume per run (µl)**
TrueStart Buffer	10X	1X	5
$MgCl_2$	25 mM	1.5 mM	3
dNTP Mix	10 mM	200 µM	1
Primer (Pan-Astro_HNF1)	10 µM	1 µM	5
Primer (Pan-Astro_HNF2)	10 µM	1 µM	5
Primer (Pan-Astro_R)	10 µM	1 µM	5
TrueStart *Taq*	5 U/µl	1.25 U	0.25
Nuclease-free water	-	-	23.75
Pre-nested product	-	-	2
Total reaction volume			**50µl**

2.9 Amplification of AstV capsid protein gene (ORF2) fragment

2.9.1 Pre-nested amplification of the ORF2 gene fragment

The pre-nested reaction was set up on ice following the protocol of Atkins et al. (2009), using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (ThermoScientific, USA). The reagents and volumes used are listed in Table 2.10. After the reagents were added to a 2 ml microcentrifuge tube (Nest Scientific Inc., China), the mastermix was vortexed (S0200, Labnet, USA) and centrifuged briefly. A volume of 23 µl of mastermix was pipetted (Eppendorf, Germany) into PCR strip tubes (Nest Scientific Inc., China) and 2 µl of cDNA template was added. The strip-tube containing the reaction mixture and cDNA template was vortexed (S0200, Labnet, USA) and centrifuged (Eppendorf, Germany) briefly. The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, USA) using the following temperature profile and cycling parameters: 55°C for 30 minutes, 94°C for 2 minutes, 40 cycles: 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 2minutes and a final extension at 68°C for 5 minutes.

Table 2.10. List of reagents used for the pre-nested amplification of the ORF2 gene

Reagents	Stock Concentration	Final Concentration	Volume per run (µl)
2x Reaction Mix	-	-	12.5
Primer (Astr_5159F)	10 µM	1 µM	0.5
Primer (Astr_5819R)	10 µM	1 µM	0.5
SuperScript III RT/Platinum taq mix	-	-	1
Nuclease-free water	-	-	5.5
RNA template	-	-	5
Total reaction volume			**25µl**

2.9.2 Nested amplification of the ORF2 gene fragment

The nested reaction was set following the protocol by Atkins et al. (2009), using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (ThermoScientific, USA). The reagents and volumes used are listed in Table 2.11. After the reagents were added to a 2 ml microcentrifuge tube (Nest Scientific Inc., China), the mastermix was vortexed (S0200, Labnet, USA) and centrifuged briefly. A volume of 20 µl of mastermix was pipetted (Eppendorf, Germany) into PCR strip tubes (Nest Scientific Inc., China) and 5 µl of pre-nested product was added. The strip-tube containing the reaction mixture and pre-nested product was vortexed (S0200, Labnet, USA) and centrifuged (Eppendorf, Germany) briefly. The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, USA) using the following temperature profile and cycling parameters: 55°C for 30 minutes, 94°C for 2 minutes, 40 cycles: 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 2 minutes and final extension at 68°C for 5 minutes.

Table 2.11. List of reagents used for the nested amplification of the ORF2 gene.

Reagents	Stock Concentration	Final Concentration	Volume per run (µl)
2x Reaction Mix	-	-	12.5
Primer (Astr_4811F)	10 µM	1 µM	0.5
Primer (Astr_5819R)	10 µM	1 µM	0.5
SuperScript III RT/Platinum taq mix	-	-	1
Nuclease-free water	-	-	5.5
Pre-nested product	-	-	5
Total reaction volume			**25µl**

2.10 Host species identification by *cytochrome b* gene amplification

Bat species identification based on morphological features and echolocation can be complicated and unreliable, due to the existence of subspecies or cryptic species (Bradley and Baker, 2001; Bastos et al., 2011), thus species identity was confirmed using primers that target the *cytochrome b* gene of the host organism (Table 2.12.). The protocol by Bastos et al. (2011) was used with modifications to the cycling parameters.

Table 2.12. Primer sets used in the amplification of the cytochrome b gene to determine host species identity (Bastos et al., 2011)

Oligo Name	5'→3' Oligo Sequences
L14724	TGA YAT GAA AAA YCA TCG TTG
H15915R	CAT TTC AGG TTT ACA AGA C

The reaction consisted out of the reagents listed in Table 2.13. A volume of 25 µl of mastermix was added to PCR strip tubes (Nest Scientific Inc., China), followed by 5 µl of DNA template. The mixture was vortexed (Labnet, USA) briefly and centrifuged (Eppendorf, Germany).

The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, USA) using the following cycling parameters: 95°C for 2 minutes, 2 cycles: 95°C for 12 seconds, 52°C for 30 seconds and 70°C for 60 seconds, followed by 3 cycles with a lower annealing temperature at 50°C and 45 cycles with the annealing step at 48°C and final extension at 72°C for 5 minutes.

Table 2.13. Reagents used for the amplification of the cytochrome b gene

Reagents	Stock Concentration	Final Concentration	Volume per run (µl)
10x PCR buffer	10X	1X	3.0
Primer (L14724)	10 µM	0.4 µM	1.2
Primer (H15915R)	10 µM	0.4 µM	1.2
$MgCl_2$	50 mM	1.5 mM	0.9
dNTP Mix	10 mM	0.2 mM	0.6
Platinum Taq	-	-	1.2
Nuclease-free water	-	-	17.9
cDNA template	-	-	5
Total reaction volume			**30µl**

2.11 Visualization of PCR products

PCR products were visualized using gel electrophoresis. The DNA electrophoresis workflow that was followed is described below (Brody & Kern, 2004).

Sodium borate (SB) buffer was used during agarose gel electrophoresis, as it has a lower conductivity, runs at higher speeds and produces brighter bands compared to TBE and TAE buffers (Brody & Kern, 2004). A stock solution of 20X SB buffer was prepared by adding 45 g Boric acid and 8 g NaOH to 1 litre of distilled MilliQ water and mixing with magnetic stirrers. The stock solution was then diluted with distilled MilliQ water to produce 1X SB buffer solution that was used during gel electrophoresis.

PCR products were visualised on a 2% agarose gel for fragments up to 1kb, and 1% agarose gel for larger fragments. The gel was prepared by mixing 2 g of Lonza® LE agarose (Lonza BioWhittaker, Verviers, Belgium) with 1X SB buffer to make a 100 ml mixture. The mixture was heated in a microwave for 3 minutes or until no undissolved particles were visible. The mixture was allowed to cool while stirring. Once cooled sufficiently 5 µl of Pronasafe gel dye (Laboratorios CONDA, Madrid, Spain) was added to the gel mixture. The gel mixture was poured into an electrophoresis tray and a gel comb, of approximately 1mm was placed in the tray. The gel was left to set for 30 minutes at room temperature. The comb was removed, and the gel was covered with 1X SB buffer.

Five microliters of PCR product was mixed with 1 µl of 6X orange loading dye (ThermoScientific, USA) and loaded into the wells. Once the samples were loaded into the wells, a Generuler® 100 bp or 1 kb ladder (ThermoScientific, USA) was loaded to the gel as a reference sizing marker. The electrophoresis tray was connected to a power pack and run at 90 volts for 40 minutes.

Following DNA electrophoresis, the PCR products were visualised using the UVItec gel documentation system, chemiluminescence and fluorescence system (UVItec Alliance, Cambridge, UK), using the transilluminator at 254 nm. Images were captured and enhanced using UVIband (UVItec Alliance, Cambridge, UK) software. The expected fragment size for the *RdRp* gene was 422 bp, and the capsid protein gene fragment was 800 bp. Images were saved electronically and printed.

2.12 PCR product purification

Two PCR purification kits were used during the study. The Rapid PCR Enzyme Cleanup Set (New England Biolabs, USA) was used to purify small volumes of PCR product, and the MinElute® PCR purification kit (QIAGEN, Germany) was used when larger volumes of PCR product were purified.

2.12.1 Rapid PCR Cleanup Enzyme Set

Positive PCR products were purified using the Rapid PCR Cleanup Enzyme Set (New England Biolabs, USA). To each 5 µl of PCR product, 1 µl of each enzyme, Exonuclease I and rSAP were added and briefly mixed by pipetting up and down. The reaction was run on the 9700 GeneAmp® thermocycler (Applied Biosystems, California, USA) using the following temperature profile and cycling parameters (per manufacturer's instructions): incubation at 37°C for 5 minutes and heat inactivation at 80°C for 10 minutes. The purified PCR product was stored at -20°C until sequencing was performed.

2.12.3 MinElute® PCR purification kit

PB buffer (100 µl) was added to 20 µl of PCR product and vortexed (S0200, Labnet, USA) briefly. The MinElute® column was placed in a 2 ml collection tube and the sample was added to the column and centrifuged (Eppendorf, Germany) for 1 minute at 17 900 x g. The flow-through was discarded and the column placed back into the collection tube. The column was washed by adding 750 µl of the provided PE buffer, containing ethanol, to the MinElute® column and then centrifuged (Eppendorf, Germany) for 1 minute at 17 900 x g. The flow-through was discarded and the column placed back into the collection tube. The column was centrifuged (Eppendorf, Germany) for an additional 1 minute at maximum speed to remove residual ethanol. The column was placed into a new 1.5 ml collection tube and 10 µl EB buffer was added to the centre of the membrane. The column was incubated for 1 minute at room temperature and centrifuged (Eppendorf, Germany) for 1 minute at 17 900 x g. The purified DNA was stored at -20°C until sequencing commenced.

2.13 Sequencing PCR

Sanger sequencing was performed using the Big-dye terminator cycle sequencing kit v3.1 (Applied Biosystems, California, USA). A mastermix was prepared for each primer used for the specific region of interest being sequenced. According to the manufacturer's instructions 2 µl of purified PCR product (15-25 ng) was added to a reaction mix containing the reagents mentioned in Table 2.14. The reaction was set up as follows on the PE GeneAmp® 9700 thermal cycler or the Veriti® (Applied Biosystems, USA) using the manufacturer's protocol: 95°C for 1 minute, 30 cycles at 95°C for 10 seconds, 55°C for 5 seconds and 60°C for 4 minutes.

Table 2.14. Reagents used for the sequencing PCR setup

Reagents	Stock Concentration	Final Concentration	Volume per run (µl)
BigDye™ Terminator 3.1 Ready Reaction Mix	-	-	4
Primer	2.5 µM	2.5 pmol	1
Nuclease-free water	-	-	up to 10
PCR product	-	-	2
Total reaction volume			**10µl**

2.13.1 Sequencing clean-up

Following the sequencing PCR, the PCR product was purified using the Big-dye X-terminator kit (Applied Biosystems, California, USA). To each sequencing reaction a mixture of 10 µl Big-dye X-terminator and 45 µl SAM solution was added. The reactions were then placed on a shaker at 1800 rpm for 30 minutes. After incubation, the reactions were centrifuged (Rotanta 460R Hettich centrifuge, Massachusetts, USA) at 2 000 × g for 5 minutes and the sequences were read on the 3130*xl* genetic analyser (Applied Biosystems, USA) using the standard protocol. The ORF2 fragments were sent to the Stellenbosch University's Central Analytical Facility for sequencing.

2.14 Sequence and phylogenetic analyses

2.14.1 *RdRp* phylogenetic analyses

The fragment used in the analyses is relatively small (420 bp), however due to the diversity of AstVs amongst bats, this conserved region of the AstV genome is readily used in the literature when performing phylogenetic analyses for bat AstVs (Fischer et al., 2016; Fischer et al., 2017; Lebarbenchon et al., 2017). As the sequences obtained during Ithete (2013) study were only used in a neighbour-joining phylogenetic reconstruction, they will also form part of the subsequent phylogenetic analyses and inferences. Sequences obtained by Ithete (2013) are denoted with NI at the end of the sequence label.

2.14.2 ORF2 phylogenetic analyses

The capsid protein gene is a highly diverse gene in the AstV genome, and notoriously difficult to obtain. During the study numerous attempts were made to amplify the capsid protein gene (ORF2). Twenty-five samples of which the *RdRp* gene of the virus was successfully sequenced, were used to try and amplify the ORF2 gene, however it was only successful for one sample. The ORF2 region highly diverse and difficult to amplify, as such not many bat ORF2 sequences are available on

GenBank (Fischer et al., 2017). One capsid protein gene (ORF2) sequence obtained from a *M. natalensis* bat was used for the phylogenetic analysis. The size of the fragment obtained by PCR was 800 bp long. The ORF2 gene fragment obtained was compared to all the available ORF2 sequences on GenBank. Sequences with a similarity score greater than 50%, query coverage of 50% and above and an e-score value close to 0 were selected for the analyses.

2.14.3 Phylogenetic reconstruction process that was followed

Sequence data files were uploaded to Geneious R10 (Biomatters Inc., New Zealand) and analysed. Primers were trimmed and De Novo assembly function was used to create contigs for each sample, where possible. To determine whether the obtained sequences were bat AstVs, the National Centre for Biotechnology Information's (NCBI) online Basic Local Alignment Sequence Tool (BLAST) was used. The related sequences were downloaded from GenBank®, which is the National Institute of Health's genetic sequence database of all publicly available DNA sequences. It forms part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan, the European Nucleotide Archive and GenBank®. A list of the sequences downloaded from GenBank® is included in Appendix B.

Sequences were imported and edited in Geneious R10 (Biomatters Inc., New Zealand) and aligned using MAFFT online alignment tool. In MEGA7 (Pennsylvania State University, USA) neighbour joining (NJ) trees were created using the percentage distance model. Using the percentage distance model enables inferences about the phylogenetic relationship of the sequences to be made. To validate the accuracy of the consensus tree that was produced, 1 000 bootstrap replicates were performed.

To determine the best model to use for phylogenetic inferences based on maximum likelihood, sequence alignments were run through JModelTest v2.1.4 (Darriba et al., 2012). Based on the results obtained by the program, the best model was selected, and sequence data was used in maximum-likelihood analyses in PhyML v3.1 (Guindon et al., 2010). Tree editing was done in FigTree v1.4.3 (Rambaut, University of Edinburgh, UK).

To determine the nucleotide similarities in the sequences pairwise distance matrixes were generated in MEGA7 (Pennsylvania State University, USA) using p-distance model with 1 000 iterations.

2.15 Quantitative Real-time PCR for monitoring of AstVs and CoVs in a *N. capensis* colony

A bat colony in Velddrif in the Western Cape was monitored over time for the presence of AstVs and CoVs using two qPCR assays. These two viruses have been regularly found to co-infect bat species (Drexler et al., 2011; Kemenesi et al., 2014). Real-time assays were utilized to determine whether the

bat colony was co-infected with these two viruses, as well as how the relative viral loads fluctuated over time. qPCR assays are highly sensitive and more specific than conventional screening PCRs, especially when assays are probe-based. Conventional PCR does not allow for relative viral load quantification, which is needed to determine if there were fluctuations of the viruses over time, hence the use of qPCR assays (Arya et al., 2005). Realtime assays do however have some drawbacks, the region that they amplify is too small to utilize for phylogenetics.

The AstV real-time assay designed was also assessed to determine how it compared in terms of limit of detection and percentage positive sample detection, in comparison with the conventional AstV screening PCR designed by Chu et al. (2008).

2.15.1 Design of AstV qPCR probe and primers

To develop primers and probes for the real-time PCR assay, *RdRp* sequences obtained during the study were aligned in Geneious R10 (Biomatters Inc., New Zealand) and primers were designed to target a fragment approximately 180 bp (Table 2.15.). For real-time PCR, it is important that the amplicon be as small as possible, as smaller amplicons amplify more efficiently and are more resistant to reaction conditions. For this real-time PCR assay, a FAM (6-carboxyfluorescein) reporter dye was attached at the 5' end and a black hole quencher dye at the 3' end of the probe. The following was also considered during primer design; the Tm of the primers should not differ more than 2°C, the GC content of the primers should range between 20-80% and the optimal length for single stranded primers should be between 15 and 20 bp (Arya et al., 2005).

Table 2.15. Astrovirus real-time PCR primers and probe set designed to target a 180 bp of the RdRp gene

Oligo Name	**5'→3' Oligo Sequences**
331_R (Primer)	AGG YCA TGA TYA CAC TCT GT
131_F (Primer)	ACA GGA GAG GTT ACC GTA CA
272 (Probe)	FAM -CGT GAW TGG ZGG AAG TGT GA/3IABkFQ

2.15.2 Betacoronavirus primers and probe

Primers and probe for the real-time PCR assay for beta-CoVs were designed by Dr Ndapewa Ithete and Dr Nadine Cronje of our research group (Table 2.16.). The primer and probe set targets a 200 bp fragment of the *RdRp* gene.

Table 2.16. Real-time PCR primer and probe set designed to target a 200 bp fragment of the RdRp gene of βCoV

Oligo Name	5'→3' Oligo Sequences
2c_RdRp_qPCR_F (Primer)	GTG YGC TCA AGT GYT WAG TGA RTA TGT
2c_RdRp_qPCR_R (Primer)	CCA TTA GCR CYC ATA AGT GCA CTA ACA
2c_RdRp_qPCR_P (Probe)	FAM-GCW TAY GCC/ZEN/ AAT AGT GTY TTT AAC AT/3IABkFQ

2.15.3 Astrovirus and Coronavirus RNA quantification standards

The AstV standard used was created by myself and the CoV standard was created and supplied by Dr Nadine Cronje of our research group. In short, the CoV standard was created from a 900 bp fragment of the *RdRp* gene of a MERS-related beta-CoV detected in a *N. capensis* bat (Cronje, 2017). The AstV standard was generated by cloning the amplified region (3' end of the genome) and *in vitro* transcribing the plasmid (as described in Section 2.7.6.). Quantification of RNA was performed as mentioned in Section 2.7.6. The viral copies per µl was 3.4 x 10^9 for AstV and 1.9 x 10^9 for CoV. Single-use aliquots were stored at -80°C and diluted before use. Ten-fold serial dilutions (10^4, 10^3, 10^2 and 10^1) were made and used as standards for each real-time reaction.

2.15.4 Sample preparation for qPCR

RNA was re-extracted from faecal material as previously described in Section 2.4.1. Single-use aliquots were prepared of each sample and stored at -80°C. Each sample was analysed in duplicate to eliminate technical variability.

2.15.5 Quality of mRNA

To determine the mRNA quality, spectrophotometer measurements are not sufficient for the following various reasons: (1) the measurements give no indication of degradation, it only supplies information about RNA; and (2) most samples contain rRNA and not only mRNA, which is targeted by the qPCR reaction. The spectrophotometer gives a reading of all the RNA (rRNA and mRNA), without being able to distinguish between the two (Eissa et al., 2016). To determine the mRNA integrity of the samples, the Tata Box binding protein housekeeping gene assay was used (Biesold et al., 2011). The reaction was set up with the primer and probe set mentioned in Table 2.17 and reagents mentioned in Table 2.18. Since the RNA was extracted from faecal material no normalization using Ct values of the housekeeping gene could be done as the number of cells shed by bats in their faecal material cannot be standardised. The number of faecal pellets used in the extraction was five pellets.

Table 2.17. Primers and probe of the Tata Box binding gene qPCR assay (Biesold et al., 2011)

Oligo Name	5'→3' Oligo Sequences
bTBP_Fwd (Primer)	TTGCTGCTGTGATCATGAGAATT
bTBP_Rev (Primer)	6-FAM-CCCGGACCACGGCCCTGA-TAMRA
bTBP_Pr (Probe)	ACACCATCTTCCCAGAACTGAAG

2.15.6 Real-time RT-PCR reaction

The 20 µl SensiFAST™ Probe No-ROX One-Step kit (Bioline, London, United Kingdom) reaction was set up (on ice) as indicated in Table 2.18. The master mix and sample RNA was added to a Bio-Rad Hard-Shell® 96 well plate (Bio-Rad, California, USA). The plate was sealed with optically clear heat seal (Bio-Rad, USA) using the PX1 PCR plate sealer (Bio-Rad, USA) and covered with tinfoil, due to the light sensitivity of the probe. The plate was centrifuged (MPS 1000 mini plate spinner, Labnet, USA) for 2 minutes at 1 200 rpm to ensure mixing of RNA and master mix and to eliminate air bubbles. Following the Bio-Rad CFX program manager prompts, the plate setup was entered, and the cycling parameters were selected. The different cycling parameters of each qPCR assay is given below in Sections 2.14.8-2.14.10.

Table 2.18. Reagents used for the qPCR reactions

Reagents	Stock Concentration	Final Concentration	Volume per run (µl)
2x SensiFAST™ No-ROX One-Step Mix	-	1X	10
Forward primer	10 µM	400 nM	0.8
Reverse primer	10 µM	400 nM	0.8
Reverse transcriptase	-	-	0.2
RiboSafe RNAse Inhibitor	-	-	0.4
EEPC-treated water (TermoScientific, USA)	-	-	3.6
RNA template	-	-	4
Total reaction volume			**20µl**

2.15.7 Cycling parameters of the AstV qPCR

The cycling parameters were as follows; 45°C for 20 minutes, 95°C 2 minutes, 45 cycles: 95°C for 5 seconds, 51.9°C for 20 seconds. Due to a probe being used, no melting curve analyses was conducted.

2.15.8 Cycling parameters of the CoV qPCR assay

The cycling parameters were as follows; 45°C for 20 minutes, 95°C 2 minutes, 40 cycles: 95°C for 5 seconds, 60°C for 20 seconds.

2.15.9 Cycling parameters of the housekeeping gene assay

The cycling parameters were as follows; 95°C for 30 seconds, 45 cycles: 95°C for 5 seconds, 58°C for 30 seconds.

2.15.10 Comparing qPCR and conventional AstV screening PCR detection results

To compare the sensitivity and detection for each of the screening methods used during this study, 150 samples were randomly selected to be screened using the AstV qPCR screening assay. The percentage difference in detection was calculated between the assays.

2.16 WWTW water sample collection and analyses

To determine how potential exposure to HAstVs through ingestion of water from wastewater works affects bat AstV diversity, water samples were collected from these WWTW and analysed for the presence of AstVs. As viruses are highly diluted in water bodies, the viruses needed to be concentrated by using glass wool and polyethylene glycol (PEG).

2.16.1 Water sample collection and preparation

Water samples were collected upstream and downstream from each WWTW (Umbilo (S29º50.44; E30º53.31) and Verulem (S30º04.29; E30º51.26)). Ten litres of water was collected upstream and downstream from each site. Samples were kept at 4°C and shipped overnight from the University of KZN to the Division of Medical Virology, Faculty of Medicine and Health Sciences, Stellenbosch University.

2.16.2 Viral adsorption-elution (VIRADEL) using glass wool

To concentrate viruses from large volumes of water samples, a widely used method of glass wool filtration was employed. Custom filter casings needed to be built to allow for work with the glass wool. The filter casings were constructed out of irrigation piping with a length of 20 cm and internal diameter of 30 mm. Each column was packed with 15 g of Sodocalcic glass wool (Merck, Darmstadt, Germany). To positively charge glass wool to allow for adsorption of virus particles, the glass wool was chemically pre-treated by soaking it in sterile distilled water, and then consecutively treating it with 40 ml 1M HCl (Merck, Germany), 100 ml sterile distilled water, and 40 ml 1M NaOH (Merck,

Germany). The pH of the charged glass wool was adjusted to 7 using distilled water (Venter, 2004; Vivier et al., 2004).

2.16.3 Seeding experiment to validate filtration method

To validate the glass wool filtration method, 1 litre of sterile reverse osmosis water was spiked with 0.5 ml of Coxsackie virus B6 (CV-B6 TCID50 1.5 x 10^7 /ml), provided by National Health Laboratory Service (NHLS) Tygerberg Medical Virology laboratory. A qPCR was performed on the samples by the NHLS and the sample tested positive for the virus (estimated copy number 1.48 x 10^2).

2.16.4 Filtration of water samples

No pre-treatment of water samples was necessary due to the use of positively charged glass wool filters. Water samples were filtered through the glass wool columns with the use of a vacuum pump (2522 WOB-L Welch®, Welch-Ilmvac, Illinois, USA) and vacuum trap, constructed out of a five litre Erlenmeyer flasks and rubber stoppers with serological pipettes.

2.16.5 Elution

Sterile glycine-beef-extract buffer pH 9.5 (GBEB: 3.754 g/l glycine (Merck, Germany); 5 g/l beef extract powder (Merck, Germany)) was used to elute viruses that bound to the glass wool column. The elution buffer (GBEB) was left in contact with the glass wool for 15 minutes before being passed through the filter under vacuum. The pH of the eluate was adjusted to 7.0 with 1 M HCl (Merck, Germany). The flow-through was collected and used for secondary precipitation using PEG (Abcam, Cambridge, USA) (Vivier et al., 2004, Venter, 2004).

2.16.6 Secondary virus precipitation

A secondary virus precipitation method is routinely employed in conjunction with glass wool filtration. Precipitating the virus after filtration contributes to the successful detection of the virus in downstream analyses. Various viruses have been successfully precipitated using PEG 8000 as precipitation agent (Lewis and Metcalf, 1998; Vilaginès et al., 1997; Mattison and Bidawid, 2009). For each 10 ml of water sample, 2.5 ml of PEG (Abcam, USA) solution was added and vigorously vortexed and incubated at 4°C overnight on a shaker. Samples were centrifuged (Eppendorf, Germany) at 3 200 x g for 30 minutes. The supernatant was removed, and the white pellet was resuspended with 100 µl of virus resuspension solution (Abcam, USA). Samples were stored at -80°C until RNA extraction could be performed.

2.16.7 RNA extraction, cDNA generation and Screening by PCR

Following the precipitation of virus using PEG, viral RNA was extracted using the same protocol as mentioned in Section 2.4.1. using the NucleoSpin® RNA virus kit (Macherey-Nagel, Germany). cDNA was made using the protocol described in Section 2.5 using RevertAid reverse transcriptase (ThermoScientific, USA). The AstV screening PCR described in Section 2.6. was used to screen for all known mammalian AstVs in the water samples. Extracted RNA was screened for the presence of AstVs using the hemi-nested screening PCR of Chu et al. (2008) and the AstV qPCR assay designed during the current study.

2.17 Virus isolation *in vitro*

AstVs are classified as a BSL II virus, however isolation and propagation were performed in a BSL 3 laboratory (Marvin, Meliopoulos and Schults-Cherry. 2014). Experiments were conducted in duplicate and negative controls were also utilized to ensure no contamination has taken place. More details about the experimental design is given in Appendix I.

2.17.1 Isolation and propagation of bat astroviruses in human cell line

To determine whether bat AstVs are capable of infecting human cells, and as such pose a possible zoonotic threat, human intestinal adenocarcinoma cells (Caco-2) were used during attempted isolation and propagation. The Caco-2 cells used were supplied by the University of the Western Cape, Faculty of Biomedical Sciences (ATCC, catalogue number: HTB-37).

Caco-2 cells are the most preferred cell lines for studying AstVs in cell culture, due to their ability to form differentiated intestinal epithelium that mimics that of the human intestine (Brinker, Blacklow, and Herrmann, 2000).

2.17.2 Propagation and isolation attempt of bat astroviruses in bat cells and BHK G43 cells

Bat AstVs have not yet been successfully isolated and propagated in cell culture. A bat derived cell line (*N. capensis* kidney cells (NCK)) established by Dr Tasnim Suliman in collaboration with the Institute of Virology, University of Bonn Medical Centre, Germany, was used to attempt isolation of bat AstVs *in vitro*.

Baby hamster kidney cells (BHK), specifically the strain G43, was used in bat AstV isolation attempts.

2.17.3 Cell line resuscitation and maintenance

Cryovials containing aliquots of cell lines of interest, previously frozen in DMSO, were removed from liquid nitrogen storage and thawed in a dry bath (AccuBlock digital dry bath, Labnet, USA) set to 37°C. Cells were gently swirled in the dry bath while defrosting. The vial containing the cells was transferred to a laminar flow hood (NU-425-400 Series-24, NuAire, Minnesota, USA) where it was sterilized with 70% ethanol. Complete media, pre-warmed to 37°C, was added into a 15 ml Falcon® tube (Corning, USA), cells were transferred to the tube dropwise. The suspension was centrifuged (Rotanta 460R Hettich centrifuge, Beverley, Massachusetts, USA) at 200 x g for 5 minutes. The supernatant was removed via aspiration (Gilson Safe Aspiration Station F110741, Gilson, Wisconsin, USA) without disturbing the cell pellet. The cell pellet was gently resuspended in complete media and transferred into a cell culture flask (Corning, USA). Cells were placed in an incubator (Air-Jacketed DHD Autoflow Automatic CO_2 incubator, NuAire, Minnesota, USA) at 37°C with 5% CO_2. Cells were inspected daily under a microscope (Nikon, Eclipse TS 100, Minato, Tokyo, Japan) to establish the state of their growth and confluence. Media was changed when necessary. Media was aspirated (Gilson, Wisconsin, USA), and cells were washed with 1x PBS (Gibco®, USA). Fresh complete media was then added to the flask (Table 2.19.).

Table 2.19. Recipes for the different media used during cell culture maintenance and inoculation of cell cultures with virus

Media type	Reagents
Complete Media used for cell line growth and maintenance	DMEM (Lonza BioWhittaker®, Verviers, Belgium) solution with: – 1% penicillin/streptomycin mixture (Lonza BioWhittaker®, Belgium) – 1% non-essential amino-acids (Lonza BioWhittaker®, Belgium) – 1% sodium pyruvate (Lonza BioWhittaker®, Belgium) – 1% L-glutamine (Lonza BioWhittaker®, Belgium) – 10% FBS (foetal bovine serum) (Gibco®, USA)
Serum Free Media (SF) used for inoculation of cell cultures with virus	DMEM (Lonza BioWhittaker®, Belgium) solution with: – 1% penicillin/streptomycin mixture (Lonza BioWhittaker®, Belgium) – 1% non-essential amino-acids (Lonza BioWhittaker®, Belgium) – 1% sodium pyruvate (Lonza BioWhittaker®, Belgium) – 1% L-glutamine (Lonza BioWhittaker®, Belgium)

2.17.4 Passaging of cells

When cells reached 80-90% confluency they were passaged. The supernatant was aspirated (Gilson, Wisconsin, USA), and the cells washed with 1X PBS (Gibco®, USA). Cells were then detached using

Accutase® (Biowest, France) or 1X trypsin EDTA (Gibco®, USA) and incubated at 37°C for 2-5 minutes, or until all cell detached from the surface of the cell culture flask. The cell suspension was then transferred to a Falcon tube (Corning, USA) and centrifuged (Rotanta 460R Hettich centrifuge, Massachusetts, USA) at 1 000 x g for 5 minutes. The supernatant was aspirated (Gilson, Wisconsin, USA), and the cell pellet resuspended in DMEM (see Table 2.20. for volumes). For the specific protocol used for the propagation of AstVs in cell culture (Marvin, Meliopoulos, and Schults-Cherry, 2014), a specific number of cells needed to be seeded into new flasks. Cell counting, and seeding was performed as described in Section 2.15.5.

2.17.5 Cell counting and seeding

Cells were counted manually using a haemocytometer and microscope (Nikon, Tokyo, Japan). The haemocytometer and coverslip were cleaned using 90% ethanol (Sigma-Aldrich, USA). The coverslip was moistened and affixed to the haemocytometer. Newton's refraction rings were used to determine if adhesion was successful before continuing. The protocol used for counting of cells is given in Appendix C.

Table 2.20. Volumes of reagents per volume flask for the different treatment steps during passaging of cells

Treatment	Reagent	Volume of reagent per volume of flask
Wash	PBS (1x PBS) if Accutase® is used for detachment)	T25 ~ 1 ml
	Repeat wash X 3 (1x PBS) if trypsin EDTA is used for detachment	T75 ~ 7 ml
		T175 ~ 25 ml
Detach cells	Trypsin EDTA or Accutase®	T25 ~ 1 ml
		T75 ~ 3 ml
		T175 ~ 7 ml
Resuspension	DMEM Supplemented	T25 ~ 4 ml
		T75 ~ 7 ml
		T175 ~ 21 ml
Seeding	DMEM Supplemented (+ cell suspension)	T25 ~ 5 ml total
		T75 ~ 18 ml total
		T175 ~ 25 ml total

2.18 Bat astrovirus isolation and propagation attempt

2.18.1 Identification and selection of astrovirus positive bat samples

Samples stored in VTM that were confirmed to be AstV positive were identified and used during the isolation and propagation attempts. Saliva and urine swabs were used from two bat species (*Rhinolophus clivosus* and *M. natalensis*) from the same locality, Steenkamps Kraal in the Northern Cape. For each attempt approximately 500 µl sample was filter sterilized with a 0.4 µm syringe filter, before infecting cell lines to reduce chances of contamination with bacteria and other pathogens that could be present in the sample. The filtered sample was then supplemented with 2.5 ml SF media containing 5 µg/ml porcine trypsin (Sigma-Aldrich, USA).

2.18.2 Human astrovirus positive control

PCR-confirmed AstV-positive human stool samples were supplied as positive controls for the experiment. The stool samples were courier overnight from the National Institute of Communicable Diseases (NICD) in Johannesburg to the Division of Medical Virology, Faculty of Medicine and Health Sciences, Stellenbosch University. Samples were filter sterilized before infecting cell lines.

2.18.3 Protocol for the propagation of astroviruses in cell culture

T75 flasks (Corning, USA) were seeded with 2.5-5 x 10^6 various cells lines in complete media (Table 2.19.). Two flasks were prepared for each cell line, one for the virus infection experiment and one to serve as negative control. For the human positive control one flask of Caco-2 cells were prepared.

Cells were grown at 37°C with 5% CO_2 for 3-4 days until cells reached 100% confluency. Media was aspirated (Gilson, Wisconsin, USA), and cells were washed with 1x PBS (Gibco®, USA).

AstV positive samples that were prepared as mentioned in Section 2.15.6.1. were added to the cells and incubated for 90 minutes at 37°C (Air-Jacketed DHD Autoflow Automatic CO_2 incubator, NuAire, Minnesota, USA).

The infective media was aspirated and 7 ml SF media with 10 µg/ml porcine trypsin (Sigma-Aldrich, USA) was added.

Cells were then incubated for 3-4 days at 37°C with 5% CO_2 (Air-Jacketed DHD Autoflow Automatic CO_2 incubator, NuAire, Minnesota, USA).

Supernatant and cells were collected 3-4* days post inoculation and stored at -80°C. For cells infected with bat AstV positive samples supernatant was collected 5 days post-inoculation.

2.18.4 Blind passaging of bat astrovirus infected cells and supernatant

AstV isolation in cell culture is further complicated by the fact that cytopathic effects are rarely observed, thus the presence of the virus could only be confirmed molecularly. Inoculated cells and supernatant were passaged blindly to increase the chances of successful virus isolation. Cells were prepared as mentioned earlier (Section 2.15.2-2.15.5.4). Once the cells reached 90-100% confluency, 1 ml AstV infected cells and supernatant were used to infect the new cultures. The protocol mentioned in Section 2.15.6.3. was followed and supernatant and cells were removed 5 days post infection and stored at -80°C.

2.19 Screening of cells and supernatant for AstV

Viral RNA was extracted as mentioned in Section 2.4. and screened for the presence of AstVs using the qPCR assay (Section 2.14.).

2.20 Data analyses

Statistical analyses were conducted in R v3.4.3 (Foundation for Statistical Computing, Vienna, Austria) and Statistica R13.3 (Tibco, California, USA).

The morphological and ecological data recorded for individual bat samples (n = 495) were used for the analyses, the database used for the analyses is included in Appendix D. The data was divided into bat biological data (bat individual measurements; sex, age, forearm mass index (FMI), species identity) and ecological data (biome, altitude and rainfall) and analysed separately to determine if either host or environment have a significant effect on AstV positivity.

2.20.1 Bat morphological and biological data recorded

Measurements taken on the individual bat level included sex, age and forearm mass index (FMI). Sex is a relatively straight forward category to comprehend, bats were classified as either male or female. The reproductive statuses for males were: scrotal, non-scrotal. For females the reproductive statuses were: pregnant, not-pregnant, lactating, post-lactation.

Bat age is determined by epiphyseal-diaphyseal fusion. By examining the closure or fusion of the cartilaginous epiphyseal growth plates of the fourth metacarpal-phalangeal joint, through illumination with a flashlight the age of the bat is determined. If the joint was fused, the bat was classified as adult and if the joint was not fully fused, the bat was classified as sub-adult or juvenile (Brunet-Rossinni and Wilkinson, 2009). As only a small number of juvenile bats (n = 10) were sampled, age was not used in any analyses.

FMI is a relatively new method of determining the overall body condition of the bat (Meng et al., 2016). It is similar to the body condition index that is used as a proxy for human health. FMI calculation was made by using the following formula:

$$\text{FMI} = \frac{weight\ of\ the\ bat\ (kg)}{forearm\ length\ (m^2)}$$

2.20.2 Statistical analyses of bat morphological and biological data

Most of the variables recorded on the individual bat level were classified as categorical variables (sex, reproductive status, species identity), except FMI which was classified as a continuous variable. Categorical variables take on a value or within a specified set of categories (Joshi, 1990). As opposed to continuous variables that can take on an infinite number of variables (Joshi, 1990).

The database of bat morphological and biological variables was imported from Excel into statistical software, R v3.4.3 (Foundation for Statistical Computing, Vienna, Austria).

Individual Chi-squared analyses were conducted on the variable sets where the dependent variable (AstV status) and independent variable (sex, reproductive status, species identity) were classified as categorical.

- **Sex, reproductive status and AstV positivity**

Sex and reproductive status were used in individual Chi-squared analyses. To account for dependencies of observations within trapping site (locality), the Roa-Scott adjustment was used.

- **Species identity and AstV positivity**

To account for differences in sample size between bat species, the screening results (AstV status per species) were converted to percentages and then used in a Chi-squared analyses. Due to small counts in the cells for the crosstab table, the generalized Fisher exact test was also performed. Both the Fisher exact test and the Chi-squared analysis indicated that species identity was a significant ($p = 0.01$) factor for AstV positivity. To account for repeated measures within locality (trapping site), Roa-Scott adjustment was incorporated.

- **FMI and AstV positivity**

FMI was the only continuous variable in the dataset. The FMI measurements were used in a mixed model one-way ANOVA with Restricted Maximum Likelihood (RMl). To account for repeated measures in the dataset, locality (trapping site) was selected as a random effect in the analyses.

2.20.3 Ecological variables

The biome variable was classified as one of seven different vegetation types: Succulent Karoo, Nama Karoo, Savanna, Grasslands, Forests or Albany thicket. Altitude was a measure of the trapping location's position in meters above sea level. This was determined by taking the GPS coordinates of the trapping location and importing it into GPS visualizer software to determine the altitude. The database of ecological variables was imported from Excel into statistical software, R v3.4.3 (Foundation for Statistical Computing, Vienna, Austria).

2.20.4 Statistical analyses of ecological variables

The Generalized Estimating Equations (GEE) approach was used to determine which ecological factors (biome, altitude and rainfall) might have contributed significantly to AstV positivity. GEE was selected to take into account repeated measures within the locality (trapping site) units. AstV status was the dependent variable and also binary in nature and therefore the Binomial distribution was selected as underlying distribution. Dependent variables included in the analyses were biome, altitude and rainfall. GEE results indicated that biome significantly ($p = 0.01$) influenced AstV positivity. Fisher LSD post-hoc test was used to determine the significance between the different biomes.

Chapter 3 Results

Chapter Outline

This chapter is structured in such a way as to reflect the outline of Chapter 2 as far as possible. This chapter starts with the AstV PCR screening results and sample localities where bats that tested positive for AstV RNA were trapped. Thereafter the phylogenetic relationships of the obtained *RdRp* sequences and ORF2 sequence follows. Based on the sequences obtained, real-time PCR assays were designed to monitor the amplification of AstVs and CoVs in a colony over time. To determine how AstV diversity in bats might be impacted by exposure to human AstVs at WWTW, bat faecal material and water samples were screened for the presence of AstVs. *In vitro* isolation of bat AstVs were attempted and the findings presented. The last section of the results focuses on the statistical analyses of the screening results and the comparison between the screening results of the qPCR and conventional PCR assay.

3.1 Identification and characterization of astroviruses in South African bats

3.1.1 Screening results of individual bat samples

During the current study 500 individual bat faecal samples were screened for the presence of AstV RNA. The overall prevalence of the virus across the nine species was 13%. Prevalence on species level was highly variable ranging from the highest of 55% for *M. natalensis* to the lowest of 4% in *N. nana* (Table 3.1.). The only other available study that investigated bat AstVs in SA is that of Dr Ithete (2013). The screening results of Ithete (2013) and that of the current study were combined in a summary table to depict all SAn bat species that have tested positive for AstV RNA (Table 3.2.). The spatial distribution of sample localities across SA is given in Figure 3.1.a), and the localities where bats tested positive for AstV RNA is given in Figure 3.1.b). A detailed list of sample localities and GPS coordinates is included in Appendix E**.**

Table 3.1. Prevalence of AstV RNA detected in different SA bat species using primers designed by Chu et al. (2008) that targets the RdRp gene of the virus

Family	Species	Total Screened	AstV Negative	AstV Positive	Prevalence (%)
Miniopteridae	*M. natalensis*	22	10	**12**	55
Molossidae	*Chaerephon pumilus*	13	13	0	0
	Mops midas	6	6	0	0
	Myotis bocagii	5	5	0	0
	Tadarida aegyptiaca	15	13	**2**	14
Rhinolophidae	*R. capensis*	23	14	**9**	39
	R. clivosus	121	101	**20**	17
	R. darling	10	10	0	0
	R. denti	10	10	0	0
	R. simulator	14	14	0	0
	R. smithersi	1	1	0	0
	R. swinnyi	10	10	0	0
Vespertilionidae	*Myotis tricolor*	16	15	**1**	7
	N. capensis	120	107	**13**	11
	N. nana	47	45	**2**	4
	Pipistrellus hesperidus	56	50	**6**	11
	Kerivoula lanosa	1	1	0	0
	Scotophilus dinganii	10	10	0	0

Table 3.2. Summary of all South African bat species that have tested positive for AstV RNA during the current study and during the study by Ithete (2013)

Family	Species	Total Screened	PCR Positive	Prevalence (%)
Hipposideridae	*Hipposideros caffer*	6	**2**	33
Miniopteridae	*M. natalensis (Ithete, 2013)*	13	**12**	92
	M. natalensis	22	**12**	55
	M. fraterculus (Ithete, 2013)	6	**6**	100
Molossidae	*Tadarida aegyptiaca (Ithete, 2013)*	3	**2**	67
	Tadarida aegyptiaca	15	**2**	13
Rhinolophidae	*R. clivosus (Ithete, 2013)*	8	**1**	13
	R. clivosus	121	**20**	17
	R. swinnyi (Ithete, 2013)	3	**1**	33
	R. capensis	23	**9**	39
Vespertilionidae	*N. nana (Ithete, 2013)*	6	**1**	7
	N. nana	47	**2**	4
	N. capensis (Ithete, 2013)	10	**8**	80
	N. capensis	120	**13**	11
	Myotis tricolor	16	**1**	6
	Pipistrellus hesperidus	56	**6**	11

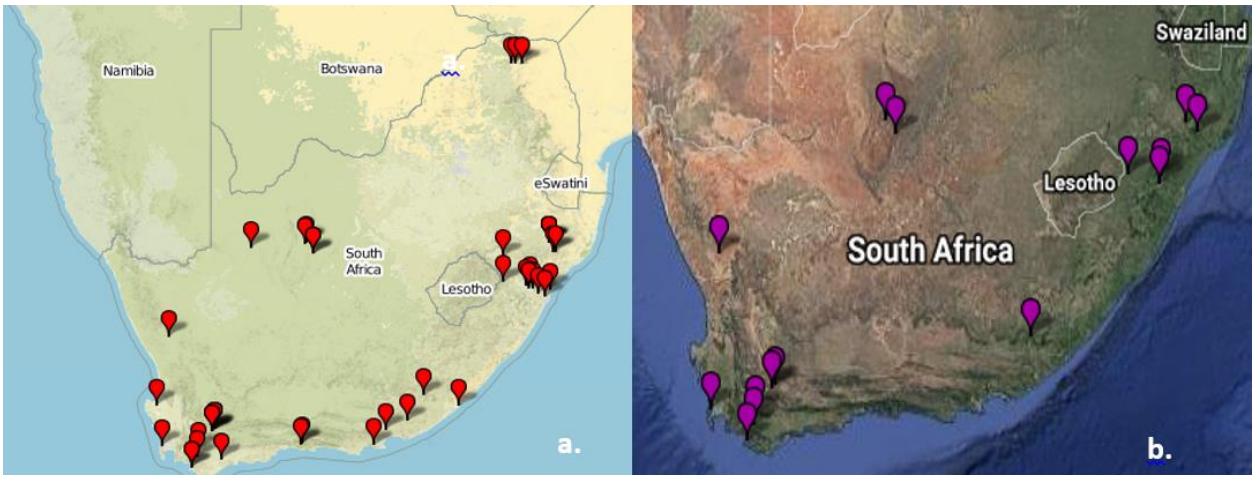


Figure 3.1. a) Localities sampled during the course of this study;
b) Spatial distribution of all PCR positive sample localities in SA including those detected by Ithete (2013). Maps were created using GPS visualizer online software

3.2 Phylogenetic analyses

3.2.1 Phylogenetic analyses based on the RNA dependant RNA polymerase (RdRp) gene

Using the conventional hemi-nested AstV screening PCR (Chu et al., 2008) which targets a 420 bp fragment of the AstV genome, a total of 25 novel *RdRp* sequences were obtained during the current study. Bringing the total number of SA bat AstV *RdRp* sequences to 44 (Ithete, 2013). The phylogenetic relationship of all available SA bat AstVs and their related sequences is depicted in Figure 3.2., and magnifications are presented in Figures 3.3-3.5.

The novel *RdRp* sequences were also used to determine their relationship within the larger scope of AstV sequences available on GenBank, a ML tree was constructed that included members of *Mamastrovirus* genogroup I and genogroup II (Appendix F).

The evolutionary divergence of the *RdRp* gene sequences as determined by pairwise distance matrix analysis is given in Appendix G.

3.2.2 Phylogenetic inferences based on the *RdRp* gene fragment

Based on the phylogenetic reconstruction depicted in Figure 3.2., the following observations can be made; the sequences are highly diverse with numerous clusters having poor bootstrap support, there is not one clear trend with regards to the clustering of sequences with some sequences seen to cluster according to bat genera whilst others cluster according to locality. Figure 3.2. was split into three sections for better illustration of the clustering of sequences and each section will be discussed in the following subsections. Only clusters with bootstrap support values above 70 were discussed.

- **Section A**

Section A (Figure 3.3.) is a magnification of Figure 3.2. and the grouping of sequences will be discussed systematically from the bottom (avastrovirus outgroup) to the top, numbered from 1-5. Sequences at position 1 indicate two sequences isolated from the same bat species, *N. capensis*, from the same colony trapped in Greyton in the Western Cape (BatAstV/Greyton/NC7 & BatAstV/Greyton/NC1). From Figure 3.3, the cluster of sequences from SA at position 1 appears to share a common ancestor with several sequences obtained from *Miniopterus* bats from China (Chu et al., 2008; Wu et al., 2012) (Genbank IDs: EU847157.1, EU847150.1 & JQ814860.1).

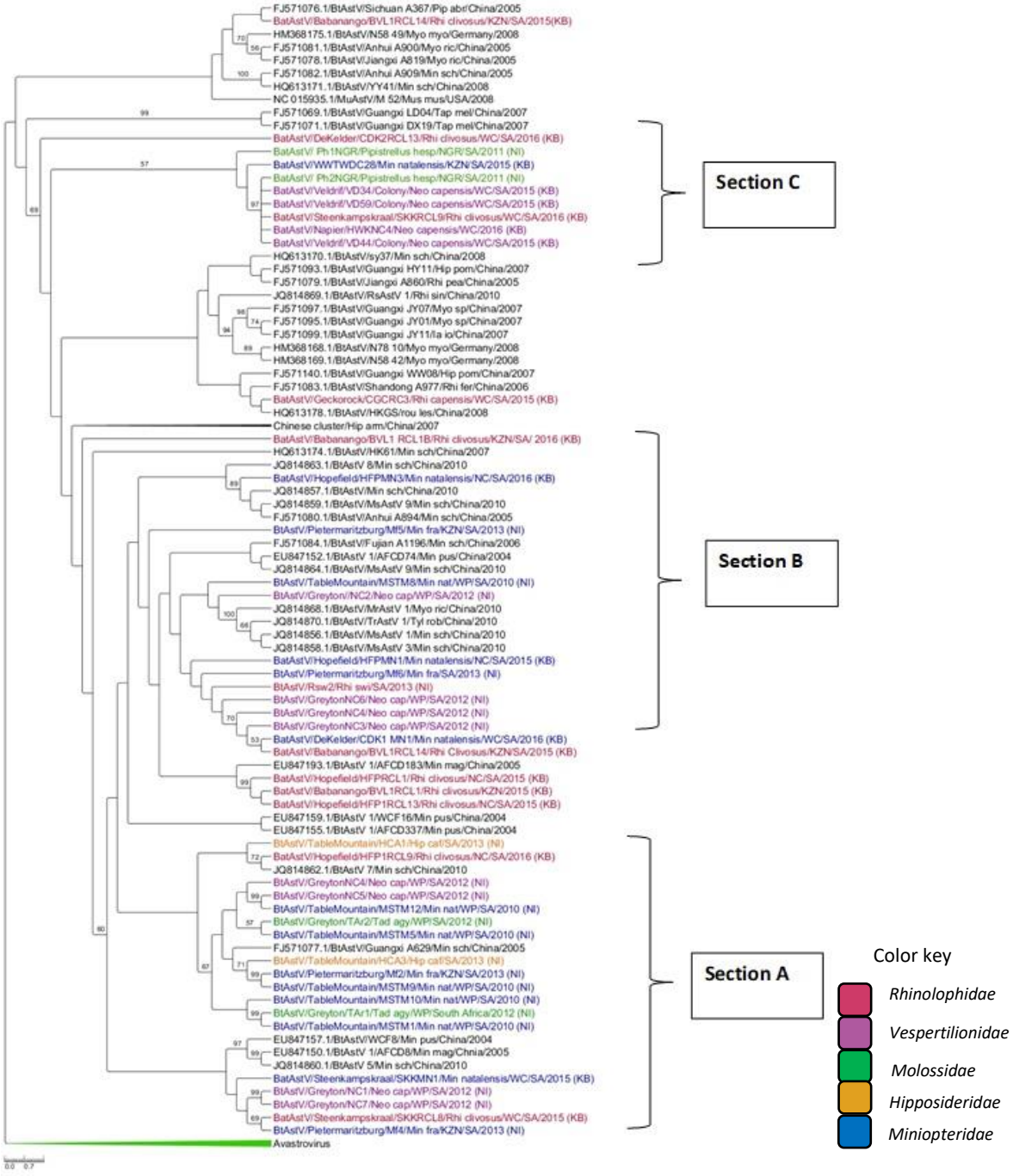


Figure 3.2. Phylogenetic relationship of SAn bat AstVs with related bat AstV sequences. The RdRp fragment (420 bp) (corresponding to positions 3659-4041 bp in HAstV NC_001943.1) was used to construct a maximum likelihood tree on the nucleotide level in PhyML. The evolutionary history was inferred using the General Time Reversible model with Gamma distribution and invariant sites (GTR+G+I) (Nei & Kumar 2000) with 1000 Bootstrap replicates. Bootstrap support values above 50% are indicated at supported nodes. The tree is outgroup rooted with avastrovirus sequences. Each sequence is designated by a unique sequence name comprising of GenBank accession number, virus abbreviation (Appendix H), bat host species abbreviation, individual sample ID, country of origin and year when the sample was collected. The SAn bat sequences are colour coordinated according to bat family.

The sequence clustering indicated by number 2 is two SAn bat AstV sequences (BatAstV/TableMountain/MSTM1 & BatAstV/Greyton/Tar1) from different bat species, *M. natalensis* and *T. aegyptiaca*, sampled at different locations, Greyton and Table Mountain. The next two sequences at position 3 were obtained from the same bat species, *M. natalensis*, from two different provinces in SA, Pietermaritzburg in KZN and Table Mountain in the Western Cape. In Figure 3.3, above the two *Miniopterus* sp.-derived sequences at position 3, is a sequence (BatAstV/TableMountain/HC3) from a different bat species, *H. caffer*, also from Table Mountain. The two *Miniopterus* derived sequences possibly share a common ancestor with the *Hipposoderus*-derived sequence. At position 4 two sequences (BatAstV/TableMountainMSTM12 & BatAstV/Greyton/NC5) obtained from two bat species from different locations, *M. natalensis* and *N. capensis*, cluster together. The *Miniopterus*-derived sequence was from a bat trapped at Table Mountain and *Neoromicia*-derived sequence from a bat trapped at Greyton. At position 5, a SAn bat AstV sequence (BatAstV/Hopefield/HFP1RCL9) from a *R. clivosus* bat appears to cluster with a bat AstV sequence from a *M. schreibersci* bat from China (GenBank ID: JQ814862.1) (Wu et al., 2012).

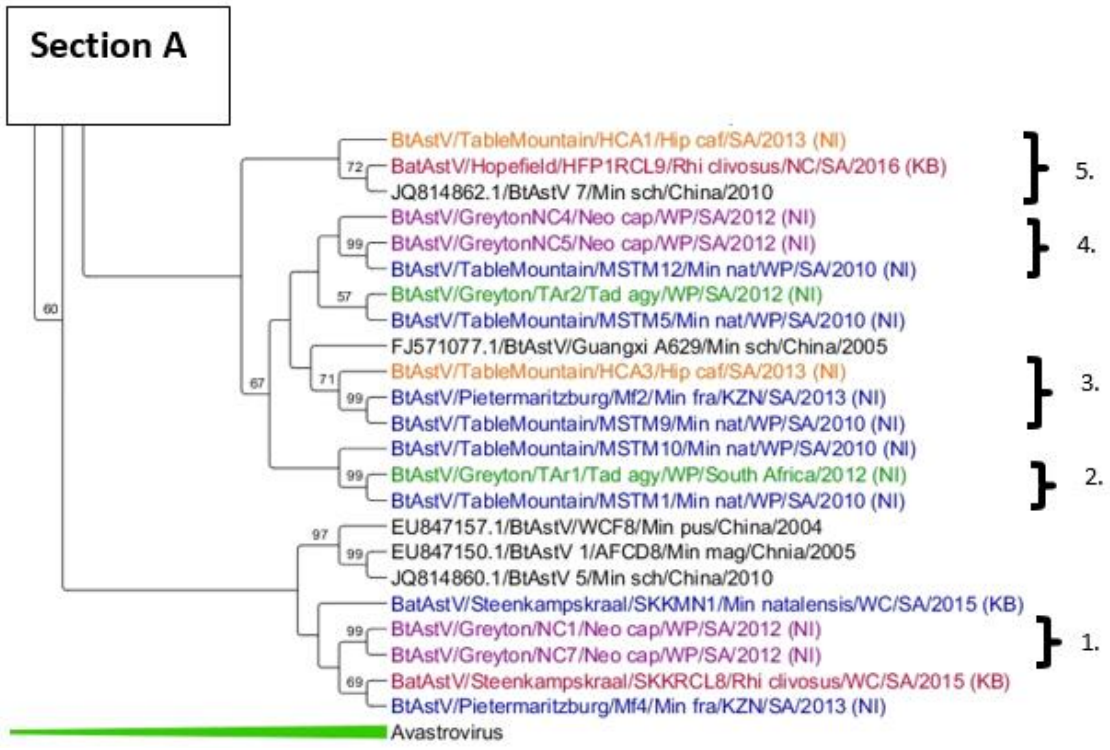


Figure 3.3. Magnification of section A of Figure 3.2. Sequence groupings with significant bootstrap support (>70%) were numbered 1-5.

- **Section B**

The magnified section B (Figure 3.4.) of Figure 3.2. shows various sequences obtained from bats in SA clustering with Chinese bat AstV sequences (Chu et al., 2008; Zhu et al., 2009; Wu et al., 2012). The cluster of sequences at position 1 in Figure 3.4. shows SA bat AstV sequences (BatAstV/Hopefield/HFPRCL1, BatAstV/Hopefield/HFPRCL13 & BatAstV/Babanango/BVL1RCL1) appearing to share a common ancestor with a Chinese bat AstV sequences (Genbank ID: EU847193.1). The SAn bat sequences at position 1 were all obtained from bats within the same genus, *Rhinolophus.* Interestingly, the Chinese sequence (GenBank ID: EU847193.1) with which these sequences appear to cluster was obtained from a bat from a different family, *Miniopteridae*. Although the SAn sequences at position 1 were all obtained from *R. clivosus* bats, sampling took place at different localities, namely Hopefield in the Northern Cape, and Babanango in KZN. The second cluster of interest in Section B of Figure 3.2. at position 2 depicts a SAn bat AstV sequence (BatAstV/Hopefield/HFPMN3) obtained from *M. natalensis*, being ancestral to Chinese sequences (Genbank IDs: JQ814868.1, JQ814870.1, JQ814856.1 & JQ814858.1) that were also obtained from species within the *Miniopteridae* family.

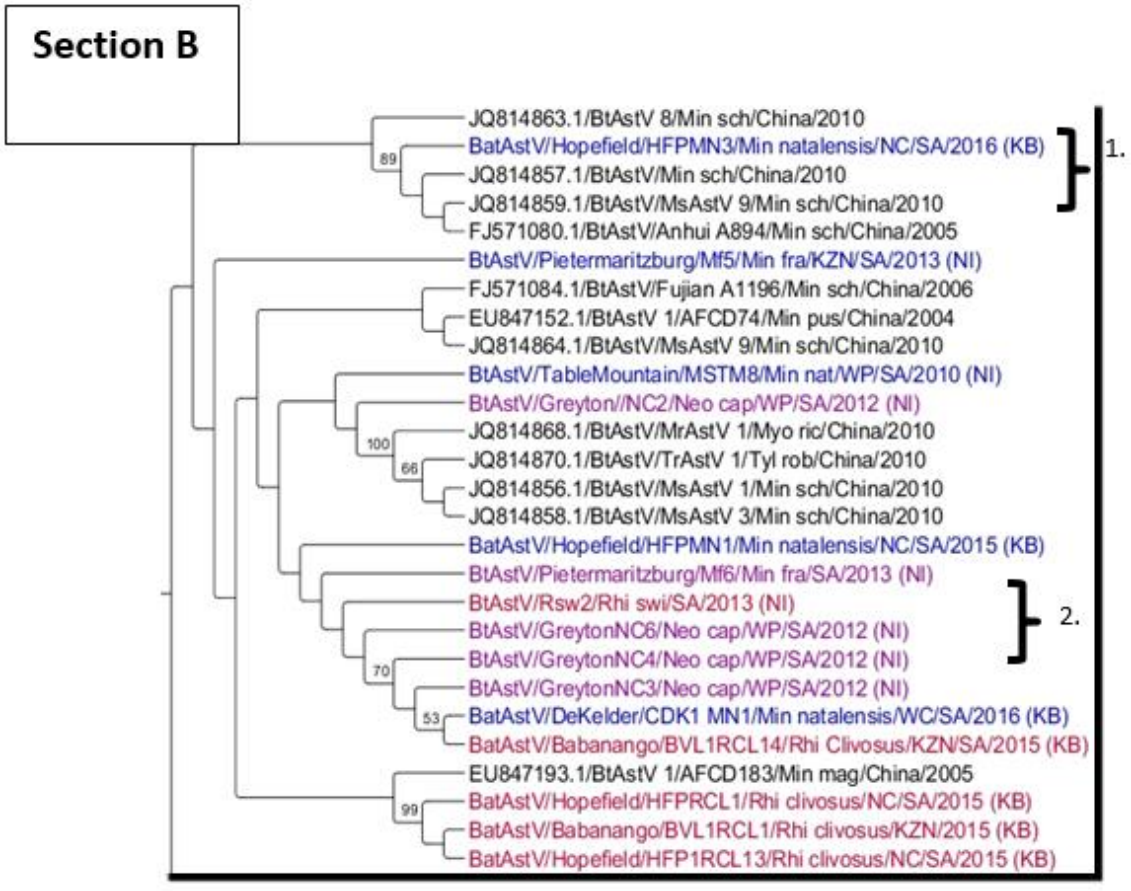


Figure 3.4. Magnification of section B of Figure 3.2. Sequence groupings with significant bootstrap support (>70%) were numbered 1-2.

- **Section C**

At position 1 in Section C of Figure 3.5., is a single cluster of SA AstV sequences of particular interest. The cluster consisted of six sequences, four sequences obtained from *N. capensis* bats, one from a *R. clivosus* and one from *Pipestrellus hesperidus*. Three of the four *N. capensis* sequences in the cluster were obtained from a single colony in Velddrif (BatAstV/Velddrif/VD34, BatAstV/Velddrif/VD44 and BatAstV/Velddrif/VD59) in the Western Cape of SA. The other *N. capensis* sequence (BatAstV/Napier/HWKNC4) in the cluster was obtained from the same bat species but from a different locality, Napier in the Western Cape. The *Rhinolophus* derived sequences (BatAstV/Steenkampskraal/SKKRCL9) was obtained from a *R. clivosus* bat collected from a decommissioned radioactive mine in the Northern Cape. There was also an AstV sequence obtained from a *P. hesperidus* bat (BatAstV/Ph2NGR/Pip hesperidus) that was collected in Greyton.

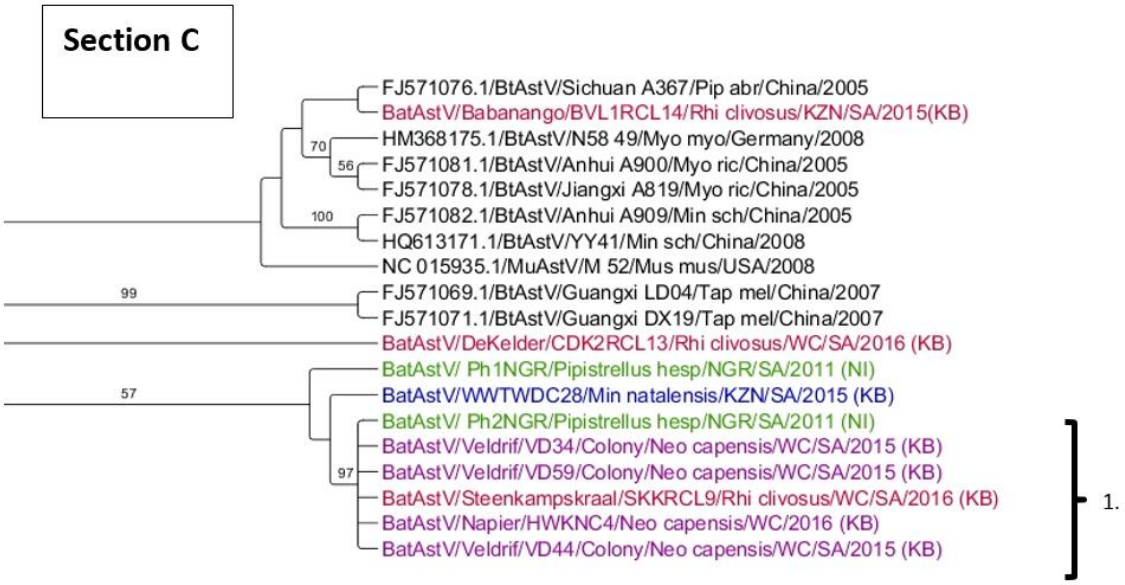


Figure 3.5. Magnification of section C of Figure 3.2. The sequence grouping with significant bootstrap support (>70%) was numbered.

3.3 Phylogenetic analyses based on the Capsid Protein Gene (ORF2)

3.3.1 Phylogenetic inferences based on ORF2 fragment

The SA bat ORF2 sequence obtained from a *Miniopterus natalensis* bat appears to share a common ancestor with human AstV isolates from Japan, albeit with very weak Bootstrap support (Figure 3.6).

Figure 3.6. Phylogenetic relationship of AstVs based on a partial capsid protein precursor sequence using the ML method on the nucleotide level. A ML phylogenetic tree was constructed using AstV capsid protein precursor sequences (800 bp in size) (corresponding to positions 4583-5383 bp in HAstV NC_001943.1) including the sequence obtained during this study. The evolutionary history was inferred by using the General Time Reversible model with Gamma distribution and invariant sites (GTR+G+I) (Nei & Kumar 2000) with 1000 Bootstrap replicates. Bootstrap support values above 40% are indicated at supported nodes. The tree is outgroup rooted (AstV). Each sequence is designated by a unique sequence name containing the isolate or species name, virus abbreviation, host species abbreviation, country of origin and year when the sample was collected. The SAn bat ORF2 sequence is highlighted in green.

3.3.2 Pairwise distance matrix

To further investigate the evolutionary distance between the SAn bat AstV ORF2 sequences and related ORF2 sequences, a pairwise distance matrix was constructed. Pairwise distance matrixes measure the number of nucleotide substitutions occurring between the sequences in question. This matrix analysis of the ORF2 sequences indicated that the SA bat ORF2 sequence was in fact more similar to bat AstV sequences than to the human AstV sequences (Table 3.3.) The bat AstV ORF2 sequences with the highest similarity to the SAn bat ORF2 sequence were FJ571069 (22%) and EU847155 Mamastrovirus isolate number 18 (22%). The sequences that differed the most from the SAn bat AstV ORF2 sequence was the Turkey AstV sequences (47%), followed by the human AstV isolates (32%) (Table 3.3.).

Table 3.3. Estimates of Evolutionary Divergence between ORF2 Sequences. The number of base differences per site from between sequences are shown. The analysis involved 15 nucleotide sequences using the p-distance model with 1000 iterations. Evolutionary analysis was conducted in MEGA7 (Kumar et al., 2016).

1	**Turkey_AstV**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
2	AB025808_Human AstV4/isolate: _O-24/93-439	48%													
3	AB025812_HumanAstV4/isolate: _O-33/87-25	48%	0%												
4	AB025811_HumanAstV4/isolate: _O-29/90-276	48%	1%	1%											
5	AB025809_HumanAstV4/isolate: _O-25/93-197	48%	0%	0%	0%										
6	FJ571074_BatAstV/Guangxi/LC03/2007	51%	36%	36%	37%	37%									
7	FJ571068_BatAstV_Ha/Guangxi/LS11/2007	50%	36%	36%	36%	36%	28%								
8	EU847155_Mamastro18/isolate_AFCD337	50%	39%	39%	39%	39%	27%	27%							
9	Bat AstV/HFPMN1/South Africa	47%	32%	32%	32%	32%	32%	27%	22%						
10	FJ571069_BatAstV/Guangxi/LD04/2007	46%	38%	38%	38%	38%	27%	25%	21%	22%					
11	FJ571072_BatAstV/Guangxi/LD45/2007	48%	39%	39%	39%	39%	27%	20%	22%	25%	15%				
12	FJ571073_BatAstV/Guangxi/LD54/2007	48%	37%	37%	38%	38%	28%	30%	23%	26%	15%	17%			
13	FJ571065_BatAstV/Guangxi/LD38/2007	47%	36%	36%	36%	36%	28%	26%	20%	25%	17%	17%	15%		
14	FJ571070_BatAstV/Guangxi/LD27/2007	48%	38%	38%	38%	38%	28%	25%	23%	26%	16%	18%	16%	11%	
15	FJ571071_BatAStV/Guangxi/DX19/2007	48%	38%	38%	38%	38%	29%	25%	23%	26%	16%	17%	16%	11%	0%

3.3 Real-time PCR results

3.3.1 Amplification of AstVs and CoVs in a bat colony

The amplification of AstV and CoV was monitored in a *N. capensis* bat colony in the Western Cape of SA, using real-time PCR assays. During the month of January AstV RNA was detected in the colony, but CoV RNA was absent (Figure 3.7.). From February until April, no AstV RNA was detected. During April CoV RNA was present in the colony. The relative viral loads (AstV 240 per 5 faecal pellet and CoV 155 per 5 faecal pellets) indicated that the amplification of both viruses increased significantly in September and steadily declined towards the end of November (Figure 3.7.). The absence of samples from May until August is due to the bats migrating from the roost. During September, the bats recolonized the roost again.

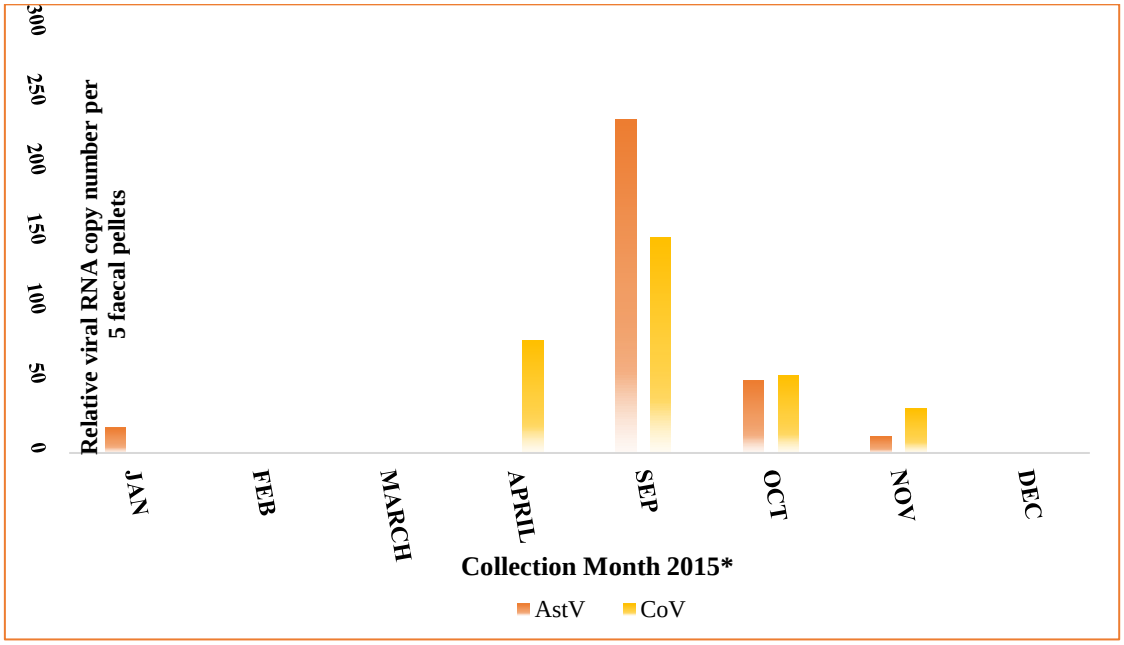


Figure 3.7. AstrV and CoV amplification in a bat colony over the span of a calendar year (2015). AstV is indicated in orange and CoV in yellow. The gap from May until August corresponds with the migration of the bats from the roost and as such no samples were collected during this time. The y-axis represents the relative viral copy number per 5 faecal pellets, the amount of faecal material was used as a normalizer.

3.3.2 Real-time PCR screening assay versus conventional PCR screening assay

To determine whether the AstV real-time PCR assay designed during the current study could serve as an alternative improved screening and detection tool, 150 selected samples were re-screened using both the conventional hemi-nested AstV PCR assay (Chu et al., 2008) and the AstV qPCR assay designed during the current study.

The results indicated that the real-time assay was more sensitive, as it was able to detect as low as 10 viral RNA copies per reaction, compared to the conventional PCR only being able to detect 10^2 viral RNA copies per reaction. The results of both assays are given in Table 3.4. The samples that were found to be positive by conventional AstV screening PCR were also positive using the qPCR screening and the qPCR detected more positives than the conventional AstV screening assay.

Table 3.4. Comparison of the screening results of the conventional AstV screening assay with the Real-time PCR assay

Calculated	Conventional PCR	Real-time PCR
Positives	17	47
Negatives	133	103
% positives	11.4	31.5
% negatives	88.6	68.5
% difference in positives		20

3.4 Wastewater treatment works samples

3.4.1 Water samples

Water samples that were collected upstream and downstream from two WWTWs in KZA; Verulam Wastewater Works (S29º38.38; E31º03.49) and Umbilo Wastewater Works (S29º50.44; E30º53.31) were analysed for the presence of AstVs as described in Section 2.14. The water collected from the two facilities tested negative for the presence of AstVs.

3.4.2 Bat samples

The *N. nana* samples collected from WWTW only delivered one positive sample out of 50 (field code DC 28 for reference in phylogenetics). No *N. nana* bats tested positive in the pristine localities.

3.5 *In vitro* virus isolation

Three different cell lines were used during the attempts: Caco-2, BHK-G43 and NCK cells. A human AstV positive stool sample was used as a cell culture positive control to infect Caco-2 cells. Images were taken of the cell cultures before and after infection (Zeiss Axiocam ERc 5 S, Zeiss, Oberkochen, Germany), however AstV infected cells do not exhibit cytopathic effects (CPE) (Brinker et al., 2000). It should be noted that in the post-infection images (Figures 3.8.-3.10.), cells are clumping and detached, this is due to the minute amounts of porcine trypsin which was added to the SF media. The porcine trypsin activates the proteins in the capsid of the virus, which should enable infection.

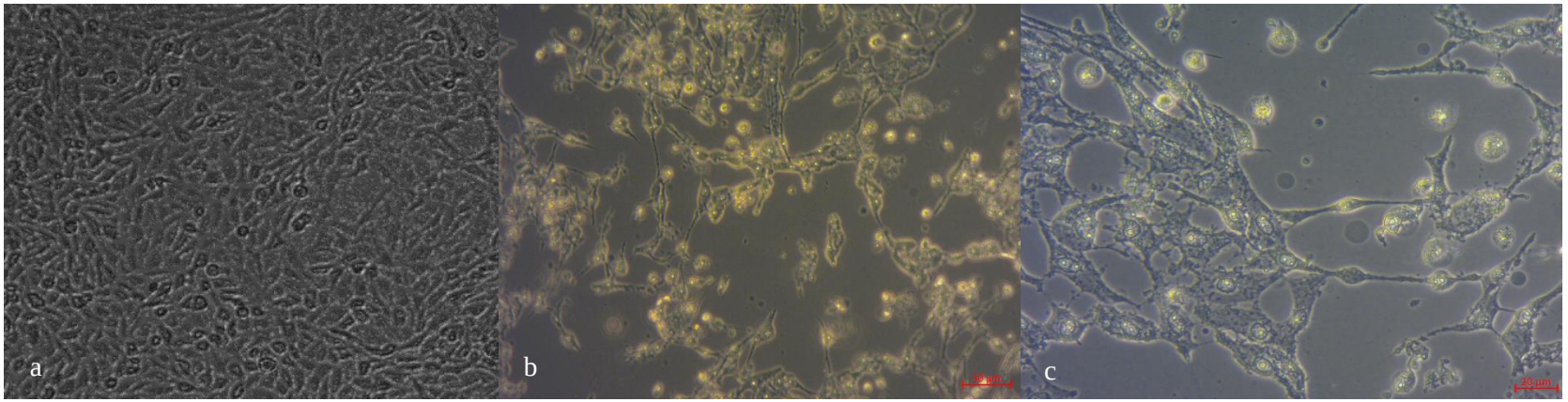


Figure 3.8. *a) Human colon adenocarcinoma cells (Caco-2) at 90-100% confluency, pre-infection.*
b) Caco-2 cells three days post infection with a human AstV positive faecal sample.
c) Caco-2 cells four days post infection with human AstV positive faecal sample. Images were taken with Zeiss Axiocam ERc 5 S (Zeiss, Oberkochen, Germany).

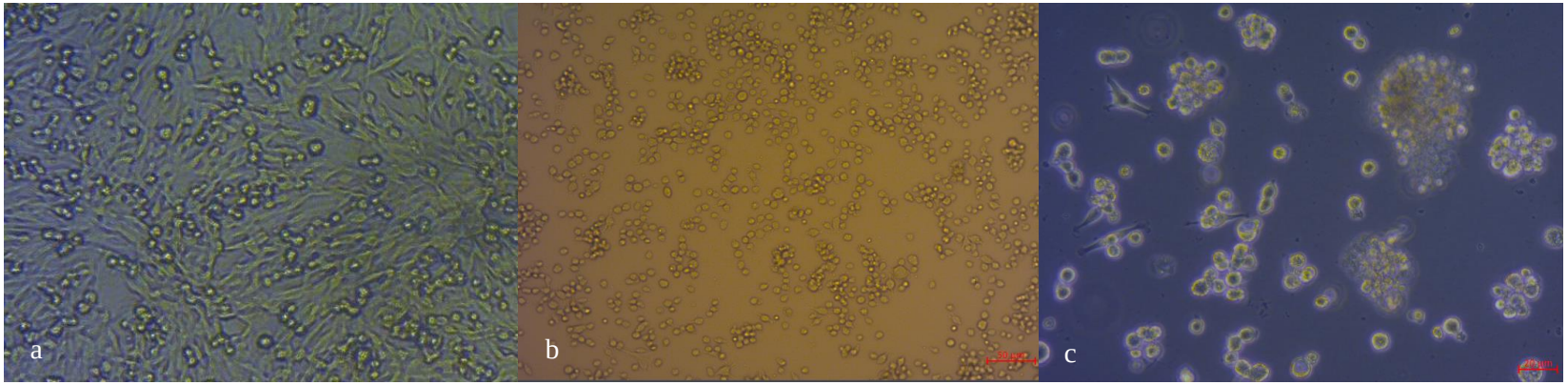


Figure 3.9. *a) Baby hamster kidney cells (BHK-G43) at 90-100% confluency pre-infection.*
b) BHK-G43 cells day three post infection with an AstV positive bat sample.
c) BHK-G43 cells day five post infection with AstV positive bat sample. Images were taken with Zeiss Axiocam ERc 5 S (Zeiss, Oberkochen, Germany).

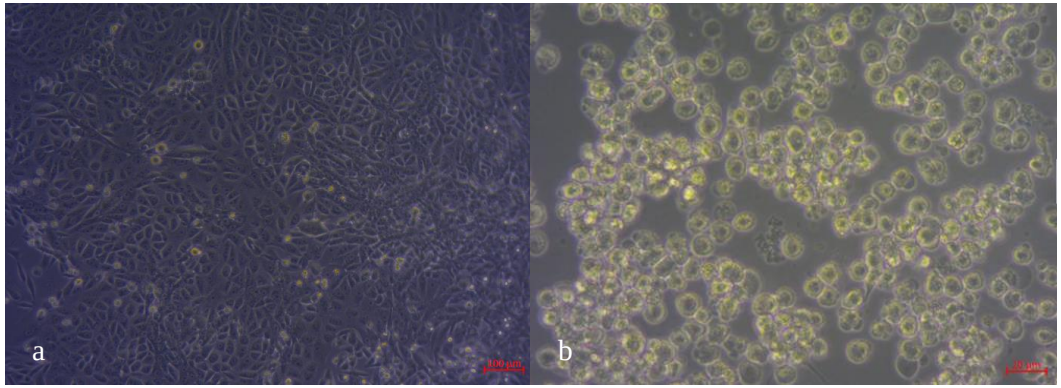


Figure 3.10. a) Neoromicia capensis kidney cells (NCK) at 90-100% confluency pre-infection.

b) NCK cells five days post infection with AstV positive bat sample. Images were taken with Zeiss Axiocam ERc 5 S (Zeiss, Oberkochen, Germany).

To determine whether the cells were infected with AstV, the supernatant and cells were harvested on day five post-infection. Extracted RNA was screened using both conventional hemi-nested AstV screening PCR assay (Chu et al., 2008) and the AstV qPCR assay developed during the current study (Section 2.13.). The qPCR results indicated that a bat AstV from a *M. natalensis* bat (field code SKKMN1) was successfully isolated and propagated in BHK-G43 cells after the first passage (Figures 3.11. & 3.12.). These results were also confirmed with Sanger sequencing. The cell culture positive control was also confirmed with the hemi-nested AstV screening PCR and Sanger sequencing.

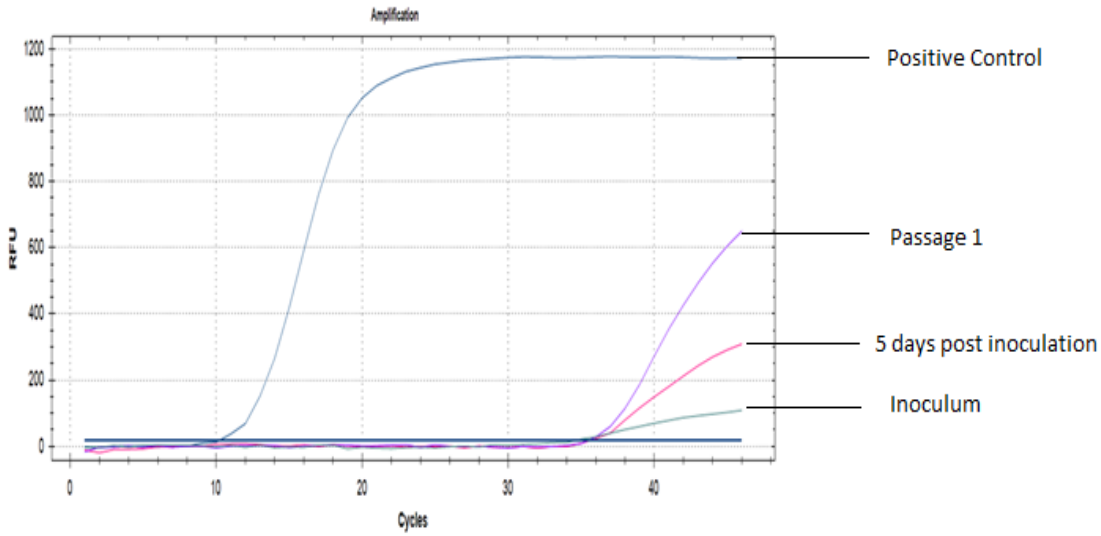


Figure 3.11. Amplification of bat AstV (SKKMN1) in BHK-G43 cells five days post-inoculation (bright pink curve) and five days post inoculation after the first blind passage (purple curve). The lowest amplification was that of the supernatant of the infectious material that was removed after the 90-minute incubation on the cells during infection.

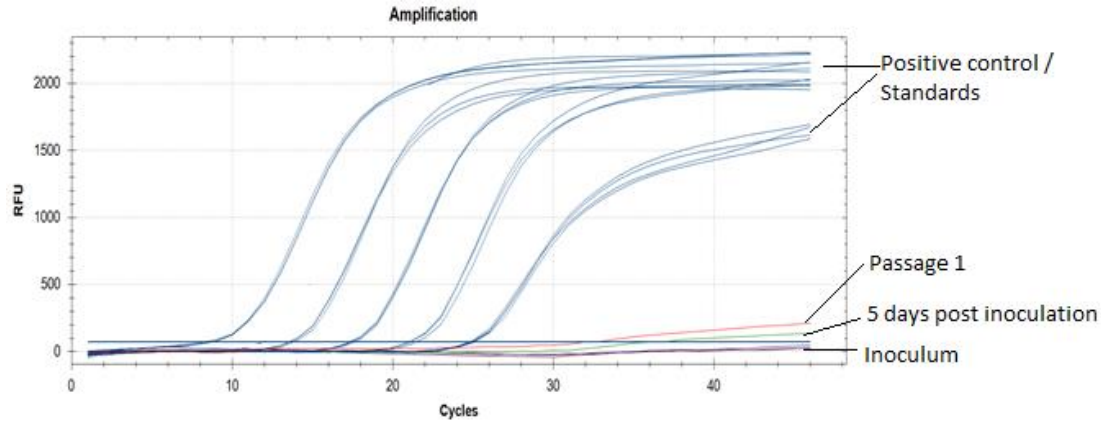


Figure 3.12. Amplification of bat Astrovirus SKKMN1 in BHK G43 cells and supernatant. Passage one of SKKMN1, day five post inoculation (red curve), day five post inoculation (green curve), inoculum (purple curves).

3.6 Statistical analyses of factors associated with astrovirus positivity

For the statistical analyses, only individual bat samples with host biological data documented, were included (n = 497) in the database. The database consisted of ecological data collected about the trapping location (altitude, rainfall, biome) and biological and morphological data of the individual bats sampled (sex, age, reproductive status, FMI, weight).

3.6.1 Morphological and biological variables of individuals bats

Statistical analyses were conducted on the data collected from individual bats. Most of the factors were categorical variables, excluding FMI, and were used in individual Chi-squared analyses.

3.6.1.1 Sex, reproductive status and astrovirus positivity

Chi-squared analysis of the individual bat data indicated that scrotal (sexually reproductive male) bats, were significantly ($p < 0.05$) more likely to be positive for AstVs than females of all reproductive stages. A visual representation of the results is given in Figure 3.13.

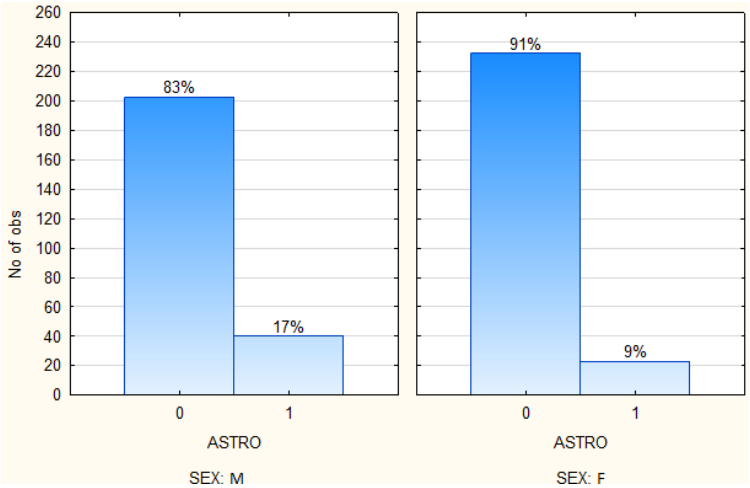


Figure 3.13. Visual representation of differences in AstV positivity between male and female bats. On the x-axes AstV positive status is indicated by 1 and a negative status by 0.

3.6.1.2 Species identity and astrovirus positivity

Chi-squared analyses and Fisher exact tests of the screening results between bat species indicated that species identity was a significant ($p = 0.01$) factor for AstV positivity. When adjusting for repeated measures within locality (trapping site) with Roa-Scott adjustment, the species identity was not significant ($p > 0.05$). A visual representation of screening results per species is given in Figure 3.14.

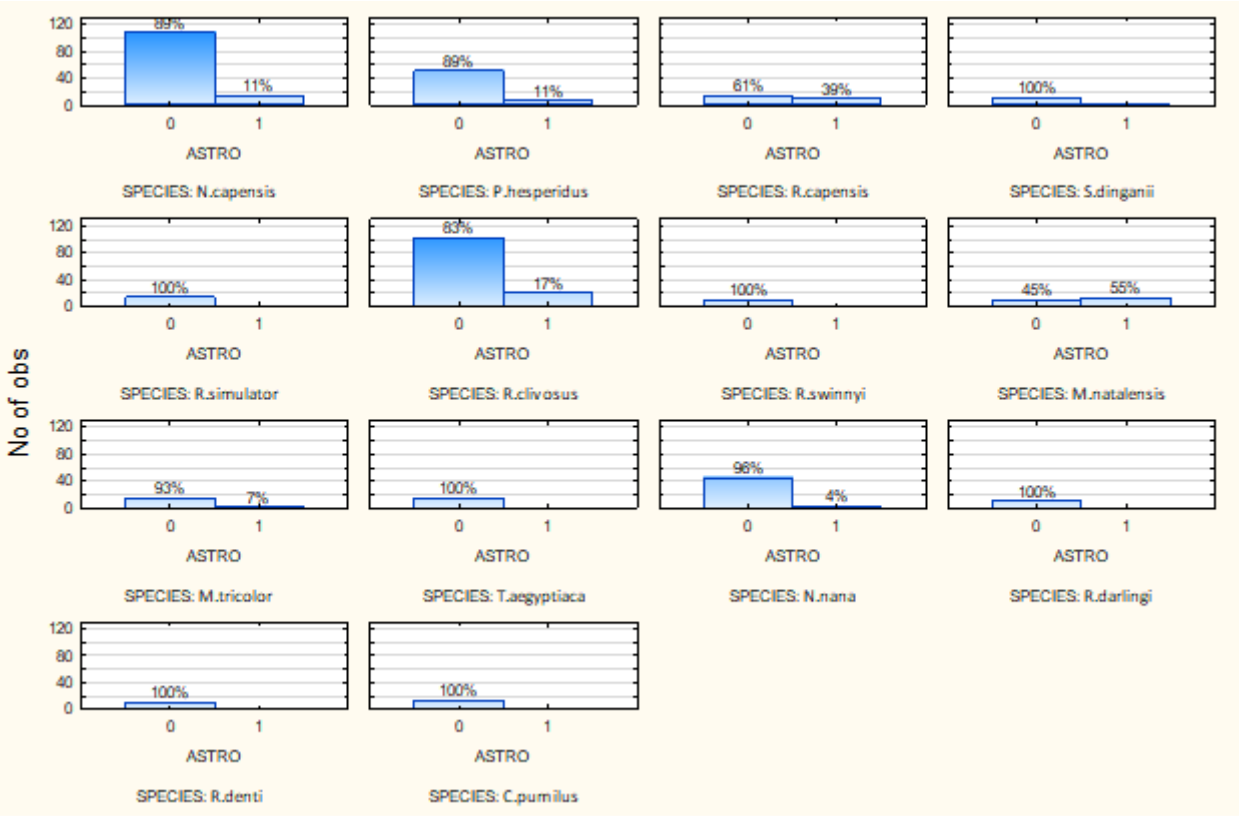


Figure 3.14. AstV screening results per bat species. On the y-axes of the graphs are the number of bats sampled (number of observations) and on the x-axes is the AstV status, positivity is demarcated with 1 and negativity with 0.

3.6.1.3 FMI

The mixed model one-way ANOVA with AstV positivity as the fixed effect and location (trapping site) as random effect, indicated a weak trend ($p = 0.07$) between lower FMI measurements and AstV positivity.

3.6.2 Environmental factors

GEE results indicated that biome significantly ($p = 0.01$) influenced AstV positivity (Table 3.5.). The best model that predicts AstV positivity is biome (Model: Astro~Biome) (Table 3.5.). The LSD post-hoc analyses indicated that bats trapped in the Succulent Karoo (SK) were more likely ($p < 0.05$) to be positive than bats trapped in other biomes (Figure 3.15.).

Table 3.5. GEE model output for environmental factors

Distribution: binomial Model: ASTRO ~ ALT+RAIN+Biome			
	Df	**Wald**	**p value**
ALT	1	2.15	0.14
RAIN	1	0.23	0.63
BIOME	6	5 573.3	0.01*

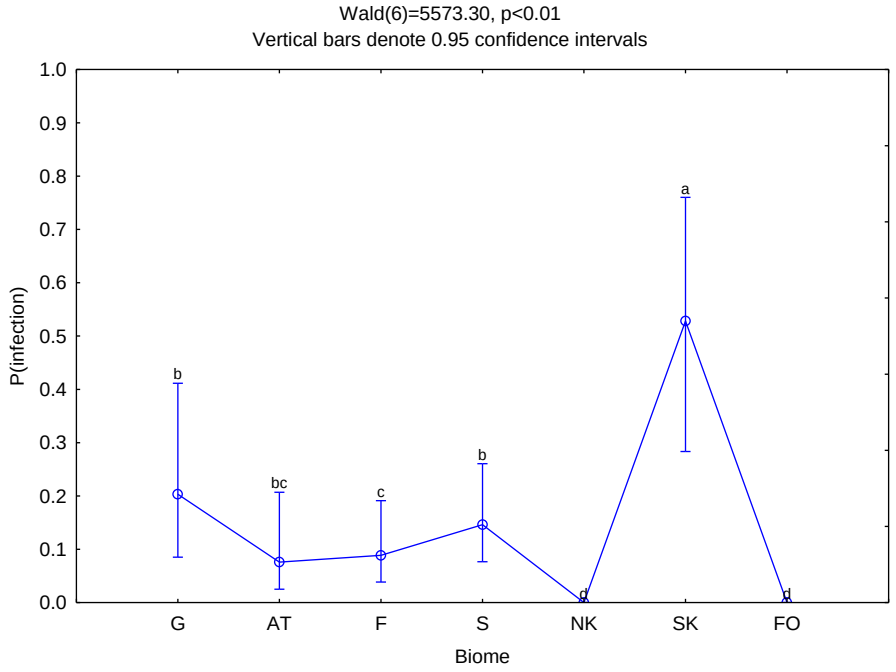


Figure 3.15. LSD post-hoc analyses results of GEE on AstV positivity in different biomes. Abbreviations of biome names: G: Grasslands, AT: Albany Thicket, F: Fynbos, S: Savannah, NK: Nama Karoo, SK: Succulent Karoo and FO: Forest.

Chapter 4 Discussion & Concluding Remarks

Chapter Outline

The overall objective of this study was to investigate the ecology and diversity of AstVs in SAn bats using molecular, phylogenetic and statistical tools. This study was only the second to investigate AstVs in SAn bats and builds on the initial study by Dr Ndapewa Ithete in 2013 (Ithete, 2013). The structure of this chapter will mirror that of the methods and results chapters as far as possible.

4.1 Prevalence of AstV RNA detected in South African bats compared to other studies

During this study, AstVs were detected in eleven different bat species belonging to four families*;* *Miniopteridae*, *Molossidae, Rhinolophidae* and *Vespertilionidae* (Table 3.1.). The overall prevalence of AstV RNA across all individual bat samples screened was 13% (65/500). A summary of the prevalence rates recorded by various studies is given in Table 4.1. There are substantial differences in the prevalence rates of the virus reported by different published studies (Xiao et al., 2011; Hu et al., 2014; Kemenesi et al., 2014; Rougeron et al., 2016; Lacroix et al., 2017). The cause for this variability is unclear, but it seems that study design, host species sampled, sample type, geographical location, and time of year when sampling occurred could be the most important contributing factors (Fischer et al., 2017).

The highest AstV prevalence was reported by Chu et al. (2008) with 46% (121 positives out of 264 samples) across the nine different bat species sampled. Similar results were obtained by Zhu et al. (2009) who detected AstV RNA in 44.8% of 500 individual bat samples. Other studies that recorded higher prevalences compared to the results of the current study, include Fisher et al. (2011) with an overall prevalence of 25.8% in 653 bats sampled in Germany, with the highest prevalence noted in one colony at 65%. A recent study conducted in Madagascar reported an overall prevalence of 22% (Lebarbenchon et al., 2017). It is difficult to interpret these differences as these studies were conducted in different parts of the world, with their own unique species and habitat types, utilizing different sampling and testing protocols, all of which are factors that could influence the prevalence of virus detection. A summary of all presently published bat AstV studies listing the study location, number of samples, sample type, prevalence recorded, and sample handling is presented in Table 4.1.

A recent meta-analysis of bat virus discovery studies found that the following factors significantly influenced the probability of a single sample testing positive for a virus: (1) specimen type; (2) detection methods; (3) viral family tested; and (4) number of specimens tested (Young & Olival, 2016). AstV studies were included in the meta-analysis and it clearly showed that the optimal

specimen type for AstV detection was faecal material. Even though AstVs were also detected in urine, this sample type had a much lower median viral prevalence (Young & Olival, 2016). All the studies mentioned in Table 4.1. used the primer sets developed by Chu et al. (2008). The study by Young and Olival (2016) did however not elaborate whether the use of different DNA polymerases may affect the probability of detection of a virus. Some of the studies used different DNA polymerases, in conjunction with the PCR assay developed by Chu et al. (2008), which could also significantly affect virus detection, as experienced in our own research group. For example, Dr Cronje found that using Maxima Hot Start Taq DNA Polymerase (ThermoScientific, New York, USA) worked better for the detection of CoVs compared to Go Taq (Promega, Wisconsin, USA) (Cronje, 2017). During the current study TrueStart Hot Start Taq DNA polymerase (ThermoScientific, USA) worked best for the detection of the *RdRp* gene fragment of AstVs, compared to Go Taq (Promega, Wisconsin, USA). For the amplification of the larger ORF2 gene fragment SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (ThermoScientific, USA).

4.2 Variation in the detection rate of astroviruses between bat species

The results obtained by this study showed a significant difference in detection rates of AstV RNA between bat species. The highest detection was found in *M. natalensis* with 55% (12/22), followed by *R. capensis* with 39% (9/23) and *R. clivosus* with 17% (20/121). Interestingly only two Rhinolophid individuals (2/7) tested positive for AstV RNA during Ithete's study (2013), while during the current study it was found abundantly in both Rhinolophid species screened (*R. capensis* in 39% and *R. clivosus* in 17%). Ithete (2013) detected AstV RNA abundantly in members of the *Miniopterus* genus, with a 100% (6/6) detection rate in *M. fraterculus* followed by a 92% (12/13) detection rate in *M. natalensis*. Overall a higher prevalence rates were noted across the species screened by Ithete (2013), even though the sample size (n = 82) was much smaller than that of the current study (n = 500). This could in part be due to the fact that most individuals from one species were trapped at a single location belonging to a single roost. Interestingly during the current study and Ithete's (2013) study, the highest detection rates for AstV RNA were documented in species belonging to the *Miniopterus* genus. Various other studies have also found that species of *Miniopterus* genus have the highest detection rates of the virus (Zhu et al., 2009; Xiao et al., 2011; Hu et al., 2014; Rougeron et al., 2016). The study by Rougeron et al. (2016) conducted in Gabon found that the detection rate was significantly higher in *M. inflatus* (10.9%) compared to *Coleura afra* (8%), *R. aegyptiacus* (1.23%), *Hipposideros cf. ruber* (4.31%) and for *H. gigas* (3.10%).

Table 4.1. Summary table of all known bat astrovirus research studies conducted globally adapted from Fischer et al. (2017)

Study location	Sample size	Overall Prevalence	Type of specimens screened	Sample storage condition	Kits
Mozambique (Hoarau et al., 2018)	Total n=259 Breakdown per site: **Mayotte =79** **Mozambique= 180**	20.1%	**Mayotte:** Rectal swabs (21) & faecal samples (58) **Mozambique:** Rectal swabs (180) & oral swabs (180)	VTM and flash frozen in liquid Nitrogen	Extraction: QIAamp Viral RNA Mini Kit (Qiagen, California, USA) cDNA&screening: ProtoScript II Reverse Transcriptase (New England BioLabs, USA) **Chu et al., 2008 hemi-nested screening PCR**
Cambodia & Lao PDR (Lacroix et al., 2017)	Total n=1876 Breakdown per site: **Cambodia=1247** **Lao PDR=629**	5.5%	Faecal pellets (187), oral swabs (1211), rectal swabs (1684) & 328 organs (328)	Phase 1 collection: VTM and flash frozen in liquid Nitrogen Phase 2: collection from guano farms – RNA*later*. Dead bats RNA*later* / VTM	RNA Extraction: QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and RNeasy Mini Kit (Qiagen, Hilden, Germany) cDNA & screening: SuperScript III kit (Invitrogen, USA) **Chu et al., 2008 hemi-nested screening PCR**
Madagascar (Lebarbenchon et al., 2017)	178	22%	Rectal swabs (178)	Swabs were placed in brain heart infusion medium (Conda, Spain) supplemented with penicillin G (1000 units/mL), streptomycin (1 mg/mL), kanamycin (0.5 mg/mL), gentamicin (0.25 mg/mL) and amphotericin B (0.025 mg/mL). Frozen in liquid nitrogen. Stored in − 80°C freezer @ research facility.	RNA Extraction: QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription: ProtoScript II Reverse Transcriptase (New England BioLabs, USA) Screening PCR: GoTaq G2 Hot Start Green Master Mix (Promega, Wisconsin, USA) **Chu et al., 2008 hemi-nested screening PCR**

Study location	Sample size	Overall Prevalence	Type of specimens screened	Sample storage condition	Kits
Singapore (Mendenhall et al., 2017)	431	44.9% (faecal pellets), 9.9% (oral & rectal swabs)	Faecal pellets, oral swabs & rectal swabs	Not mentioned.	Extraction: QIAamp virus RNA mini kit (Qiagen, Germany) Super Script III One Step RT-PCR Kit (Invitrogen, USA) Hemi-nested: Accuprime Taq DNA polymerase (Invitrogen, USA) **Chu et al., 2008 hemi-nested screening PCR assay**
Kenya (Waruhiu et al., 2017)	1029	12.8%	Faecal pellets Collected at roost site, did not sample individual bats (1029)	Collection with polythene sheets underneath bat roosts (left at roost about 11 hours). Individual faecal pellets per tube with RNA*later* (Qiagen, Germany). Samples were transported from sample site in cooled iceboxes. Stored at -80℃ at lab. Identification of bat species at roost site by chiroptologist & *cytochrome b*.	RNA Extraction: High pure Viral RNA kit (Roche, Mannheim, Germany) RT-PCR & Screening: Invitrogen OneStep-RT PCR kit using gene specific primers (Chu et al., 2008) **Chu et al., 2008 hemi-nested screening PCR**

Study location	Sample size	Overall Prevalence	Type of specimens screened	Sample storage condition	Kits
Germany (Fischer et al., 2016)	775 samples (653 individual bats)	23.5%	Oral swabs (47), urine swabs (430) & faecal pellets (480)	Cell culture media (Minimal Essential Medium; Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institute, Germany) RNA*later*	Extraction: Viral RNAMini Kit (Qiagen, Germany) cDNA & Pre-nested: Super Script III One Step RT-PCR Kit (Invitrogen, USA) Hemi-nested PCR: PWO DNA Polymerase Kit (Roche, Germany) **Chu et al., 2008 hemi-nested screening PCR**
Gabon, Central Africa (Rougeron et al., 2016)	962	4.57%	Organs – intestine samples (962)	Trapping locations were in caves. Organs were frozen in field and transported to lab and frozen @ -80℃	Extractions: EZ1 RNA Tissue Mini Kit (Qiagen, Germany) cDNA & Screening: Superscript III One-step RT-PCR kit (Invitrogen, USA) **Chu et al., 2008 hemi-nested screening PCR assay**
Czech Republic (Dufkava et al., 2015)	43	20.9%	Intestine samples of deceased bats (40) & 3 pooled faecal samples	40 deceased animals 3 Mist netted individuals No information with regards to storage.	Extraction: QIAamp Viral RNA Mini Kit (Qiagen, Germany) cDNA: Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) **Chu et al., 2008 hemi-nested screening PCR**

Study location	Sample size	Overall Prevalence	Type of specimens screened	Sample storage condition	Kits
China (Hu et al., 2014)	620	7.6%	Rectal swabs (620)	Rectal swabs placed in RNA*later* and stored at -80℃	Extraction: QIAamp virus RNA mini kit (Qiagen, Germany) Super Script III One Step RT-PCR Kit (Invitrogen, USA) Hemi-nested: Accuprime Taq DNA polymerase (Invitrogen, USA) **Chu et al., 2008 hemi-nested screening PCR**
Hungary (Kemenesi et al., 2014)	60	8.3% (5/60)	Faecal pellets (60)	Faecal pellets collected from individual caught bats, placed in bat bags for approx. 30 min. medium stored in is unclear.	Extraction: DiaExtract Viral RNA Isolation Kit (DIAGON Ltd., Hungary) cDNA & Screening: OneStep RT-PCR Kit and Dia Taq Kit (DIAGON Ltd., Hungary) **Chu et al., 2008 hemi-nested screening PCR**
Hungary (Kemenesi et al., 2014)	447	6.93%	Faecal pellets (447)	Faecal pellets collected from individual caught bats, placed in bat bags for approx. 30 min. Pellets were stored in PBS and kept on dry ice. Stored at -80℃ @ research facility.	Extraction: DiaExtract Viral RNA Isolation Kit (DIAGON Ltd., Hungary) cDNA & Screening: OneStep RT-PCR Kit and Dia Taq Kit (DIAGON Ltd., Hungary) **Chu et al., 2008 hemi-nested screening PCR**

Study location	Sample size	Overall Prevalence	Type of specimens screened	Sample storage condition	Kits
Southern China (Xiao et al., 2011)	321	9% (29/321)	Rectal swabs (321)	Rectal swabs placed in RNA*later* and stored at -80℃	Extraction: Roche High Pure Viral RNA Kit (Roche, Germany) cDNA: AMV Reverse Transcriptase (Promega, USA) Pre-nested and Hemi-nested PCR: Ex Taq Hot Start Version Kit (TaKaRa) **Chu et al., 2008 hemi-nested screening PCR**
China (Zhu et al., 2009)	500	44.8% (224/500)	Rectal swabs (500)	Rectal swabs placed in VTM	QIAamp virus RNA mini kit (Qiagen, Germany) Super Script III One Step RT-PCR Kit (Invitrogen, USA) Hemi-nested: Accuprime Taq DNA polymerase (Invitrogen, USA) **Chu et al., 2008 hemi-nested screening PCR**
China (Chu et al., 2008)	Total=262 bats were sampled	46% rectal swabs (116/250) 8% oral swabs (19/246)	Rectal swabs (250) & oral swabs (246)	Swabs placed in VTM	QIAamp virus RNA mini kit (Qiagen, Germany) Super Script III One Step RT-PCR Kit (Invitrogen, USA) Hemi-nested: Accuprime Taq DNA polymerase (Invitrogen, USA) **Chu et al., 2008 hemi-nested screening PCR**

4.3 South African bat Astrovirus diversity based on phylogenetic analyses

During this study, 25 AstV *RdRp* sequences and one capsid protein (ORF2) sequence were obtained, bringing the available bat AstV sequences from SA to 44 *RdRp* sequences and one ORF2 sequence (not yet available on GenBank) (Ithete, 2013). Multiple attempts were made to obtain more *RdRp* gene sequences and ORF2 sequences, but some were of very poor quality even after attempting to clone them. According to the ICTV AstVs are classified as species based on the capsid protein gene (ORF2) (Fauquet et al., 2005). The ORF2 region of the AstV genome is more variable compared to the conserved *RdRp* gene (Fischer et al., 2017; Rougeron et al., 2016; Chu et al., 2008). This region has successfully been obtained for many other AstVs infecting mammals and birds, but amplification of the ORF2 gene of bat AstVs has been relatively challenging as this region is highly diverse (Shimizu et al., 1990; Koci & Schultz-Cherry, 2002; Chu, 2011; Karlsson et al., 2015; Fischer et al., Eloit, 2017; Alves et al., 2018).

Only a few studies have managed to obtain the ORF2 sequence of bat AstVs as reflected by the limited number of bat AstV ORF2 sequences available on GenBank (approximately 14 ORF2 sequences are available on GenBank, (https://www.ncbi.nlm.nih.gov/nuccore/?term=bat+astrovirus+capsid+protein/ visited 23 January 2019)) compared to the number of *RdRp* sequences (approximately 600) (Chu et al., 2008; Zhu et al., 2009; Rougeron et al., 2016; Fischer et al., 2017). It is therefore common practice to use the smaller, more conserved *RdRp* region of the genome for diversity and phylogenetic analyses (Chu et al., 2008; Chu, 2011; Rougeron et al., 2016; Lebarbenchon et al., 2017; Mendenhall et al., 2017; Waruhiu et al., 2017; Hoarau et al., 2018).

4.3.1 Phylogenetic inferences based on the *RdRp* phylogeny

ML analyses of the *RdRp* gene sequences of SAn bat AstVs (Ithete, 2013) place them in genogroup II in the *Mamastrovirus* genus. The SA bat *RdRp* sequences were separate from all other mammalian AstV sequences. The sequences obtained were not closely related to any other mammalian AstV sequence. Many studies on bat AstV sequences have similar results (Chu et al., 2008; Chu, 2011; Rougeron et al., 2016; Lebarbenchon et al., 2017; Waruhiu et al., 2017; Hoarau et al., 2018), however there are studies that have found that their bat AstV sequences were closely related to sequences originating from avian and other mammalian hosts. The bat AstV sequences found by Fischer et al. (2016) were closely related to AstV sequences originating from humans, birds, foxes and rodent hosts.

The results of the current study support the suggestions made by Xiao et al. (2011) that bat AstVs do not exhibit strict host tropism and could potentially infect other host species. Our results further

suggest that clustering does not follow geographical location, as sequences from different localities were observed clustering. Similar findings were made by Rougeron et al. (2016) in Gabon where sequences obtained from different bat species inhabiting three different roosts clustered together. Furthermore, the results of the current study also indicated that sequences did not cluster according to host species. Hoarau et al. (2018) also found that AstVs sequences from different bats species from different geographic locations clustered together, suggesting that host species restriction is limited. The SAn bat AstVs were found to be highly diverse with no apparent trend in terms of restriction of sequence clustering. Poor bootstrap support was also noted for most sequence clusters, which is a common phenomenon noted in AstV phylogenetic analyses (Rougeron et al., 2016; Waruhiu et al., 2017; Hoarau et al., 2018). The poor resolution of the phylogenetic trees can be attributed to limited sequences available for SA bat AstVs, the size of the *RdRp* gene used for the phylogenetic analyses and the high saturation of their genome (Mendenhall et al., 2015; Hoarau et al., 2018). Groupings that had significant bootstrap support of more than 70% will be discussed (Efron et al., 1996).

Three types of sequence clusters were noted: (1) clusters of sequences from the same species from the same location; (2) clusters of sequences from species within the same bat family from different locations; and (3) clusters of sequences from different species from different locations. The phylogenetic tree was split into three sections (A, B, & C) for ease of reference. Groupings with significant bootstrap support will be discussed.

4.3.1.1 Clustering of sequences from the same species from the same locality

The first grouping of interest in Section A (Figure 3.3.) is that of two *RdRp* sequences derived from *N. capensis* bats (BatAstV/Greyton/NC7 and BatAstV/Greyton/NC1) from the same locality, Greyton in the Western Cape. The pairwise distance matrix indicated that these two sequences are highly similar, with a 1% difference at the nucleotide level (Appendix G). This grouping could potentially suggest that these two bats from the same roost had two very similar, if not the same, AstV strain. It was not uncommon for bats from the same roost to be infected with the same AstV strain, e.g. the *Myotis myotis* colony studied by Drexler et al. (2011) was infected with the same AstV strain. AstV strains discovered in Kenyan bats were highly diverse, but there were sequences from the same bat species, *Cardioderma cor* from the same location that clustered together (Waruhiu et al., 2017).

4.3.1.2 Clustering of AstVs *RdRp* sequences derived from bat species belonging to the same genus from different localities across South Africa

There was also a grouping in Section B (Figure 3.4.) of sequences from members of the same bat genus from different geographic localities; i.e. *M. fraterculus* from Pietermaritzburg

(BtAstV/Pietermaritzburg/Mf2/Min fra/KZN) and *M. natalensis* from Table Mountain (BtAstV/TableMountain/MSTM9/Min nat/WP). According to the pairwise distance matrix these two sequences had a nucleotide similarity of 94% (Appendix G). These sampling locations are very far apart (approximately 1 500 km) and it would be unlikely for individuals to migrate between these areas (Monadjem et al., 2010). Rougeron et al. (2016) reported that bats that inhabited different cave systems had similar AstV strains circulating in them, and that individuals from the same cave roost had more divergent strains. The most plausible explanation is that these sequences evolved through convergent evolution.

4.3.1.3 Clustering of sequences from different species sampled from different geographical locations

In Section A (Figure 3.3.), there are two sequence clusters that include sequences found in bats from Greyton and bats occurring on Table Mountain. The different clusters were between *T. aegyptiaca* (BatAstV/GreytonTAr1) and *M. natalensis* (BatAstV/TablemountainMSTM1), and *N. capensis* (BatAstV/Greyton/NC5) and *M. natalensis* (BatAstV/TableMountainMSTM12) sequences. Based on the pairwise distance matrix these sequence clusters were highly similar with a 0% difference on the nucleotide level. The two sampling locations are approximately 160 km apart. It is however documented in the literature that *M. natalensis* can migrate distances of up to 250 km between roosts, this could suggest that *M. natalensis* co-roosted with *T. aegytiaca* in Greyton and migrated back to the roost on Table Mountain. The study by Voigt et al., (2014) found that *M. natalensis* exhibited seasonal elevational movements in search of cold hibernacula at higher elevations at Mount Kilimanjaro. This could also be the case for *M. natalensis* in the Western Cape, where they might utilize caves on Table Mountain as winter hibernacula (Voigt et al., 2014), making it more plausible that *M. natalensis* transmitted the virus between multiple bat colonies. As with many bat species little information is available on *T. aegyptiaca's* home range size or migratory ecology (Monadjem et al., 2010). As such it is speculated that *M. natalensis* might be the carrier of the virus between the different roosts.

The third cluster of interest in Section C (Figure 3.5.) consisted of six sequences obtained from three different bat species, belonging to different bat genera, sampled at four different locations. Based on the pairwise distance matrix these sequences were 100% similar on nucleotide level (Appendix G). Four of the six sequences were obtained from *N. capensis*, one sequence from a *R. clivosus* and one sequence from a *P. hesperidus*. Three of the four *N. capensis* sequences in the cluster were obtained from a single colony in Velddrif located in the Western Cape of SA (BatAstV/Velddrif/VD34, BatAstV/Velddrif/VD44 and BatAstV/Velddrif/VD59). The other *N. capensis* sequence

(BatAstV/Napier/HWKNC4) in the cluster was obtained from a different sampling locality, Haarwegskloof Nature Reserve in the Overberg in the Western Cape. The *Rhinolophus*-derived sequence (BatAstV/Steenkampskraal/SKKRCL9) was obtained from a *R. clivosus* bat collected from a decommissioned radioactive mine in the Northern Cape. There was also an AstV sequence obtained from a *P. hesperidus* bat (BatAstV/Ph2NGR/Pip hesperidus) that was collected in Greyton. These four localities are far apart from each other, about 280-350 km. It would be most unlikely for the bats to fly these distances between the roosts. A more likely scenario is that these bat colonies have co-evolved with a common AstV strain.

These findings are in contrast with those by Dufkova et al. (2015). AstV sequence similarity was greater between different bat species at the same location compared to sequences derived from the same species, but from different geographical locations (Dufkova et al., 2015). The study by Zhu et al. (2009) also suggested that bat AstVs group according to host bat species, family or genera; this was not the case during the current study. Our findings were more similar to those of Rougeron et al. (2016), who found that their bat AstVs were not species-specific or limited by geography of the bat species. This could be suggestive of a long evolutionary history between bats and AstVs. As the *RdRp* gene region is highly conserved, it is possible that the different bat species co-evolved with a common prototype AstV strain. To ascertain whether these sequences are truly as similar as suggested by the phylogenetic reconstruction of the *RdRp* region, it will be invaluable to obtain the capsid protein gene (ORF2) sequences as this region is more variable.

4.3.1.4 Clustering of South African bat astrovirus sequences with sequences from China

In Section A (Figure 3.3.) a SAn *R. clivosus*-derived sequence (BatAstV/Hopefield/HFP1RCL9*)* appears to share a common ancestor with a sequence derived from a Chinese *M. schreibersci* bat. In Section B (Figure 3.4.) a cluster of SAn *Rhinolophus*-derived sequences (BatAstV/Hopefield/HFPRCL1, BatAstV/Hopefield/HFPRCL13 & BatAstV/Babanango/BVL1RCL1) share a common ancestor with sequence (GenBank ID: EU847193.1) derived from a bat within the *Miniopteridae* family from China. In section B (Figure 3.4.) a second cluster of SAn and Chinese sequences was noted. The SAn sequence (BatAstV/Hopefield/HFPMN3) appears to be ancestral to the Chinese bat AstV sequences (GenBank ID's: JQ814868.1, JQ814870.1, JQ814856.1 & JQ814858.1) that were obtained from *M. pteridae*.

The phylogenetic analyses of the current study found that there was no strict level of host restriction or geographical distance. The findings further mirror the suggestions and findings of other studies in that bat AstVs are highly diverse, adaptable to new environments and new host species, and that they have a long evolutionary history with bats (Mendenhall et al., 2015; Mendenhall et al., 2017). To be

able to make more meaningful deductions about the co-evolution between bats and AstVs, more studies are needed on the basic ecology of SAn bats, as there is a large gap in available knowledge (Monadjem et al., 2010; MacEwan et al., 2016; African Bats NPC, 2018). To better understand the true diversity of AstVs in SAn bats, or bats in general, it is of utmost importance to obtain more sequences of the ORF2 gene, which is a better reflection of true diversity, as it is under constant evolutionary pressure (Bosch et al., 2014).

4.3.2 Phylogenetic analyses of ORF2

The SAn ORF2 bat AstV sequence was found to be more similar to HAstV sequences than bat AstV sequences. This is not an uncommon finding, as Fischer et al. (2016) discovered two sequences from *M. nattereri* bats clustering with human AstVs. The cluster of the SA bat AstV ORF2 sequence and the HAstV strains had weak bootstrap support, possibly due to the fact that there are not many ORF2 bat sequences available for analysis and that the SAn bat-derived sequence is more similar to fragments in the human ORF2 sequences compared to other known bat ORF2 sequences. Another factor that could have influenced the results could be the different lengths of the sequences used during the analysis. The ORF2 fragment obtained during the current study was shorter (1 kb) than the other sequences used (2 kb – 2,5 kb) (Xia, 2016). Furthermore, the alignment of multiple highly divergent sequences could result in poor sequence alignment that in turn affected the subsequent phylogenetic analyses (Xia, 2016).

Interestingly the pairwise distance matrix analysis of the ORF2 genes (on the nucleotide level) indicated contrasting results, suggesting that the bat ORF2 sequences were more similar (similarity of 73-78%) compared to the human ORF2 sequences (similarity of 68%). One plausible explanation for the difference between the phylogenetic output is the fact that the phylogenetic models used for the ML analysis takes different aspects into account and is more in-depth than the pairwise distance matrix analysis (Tamura et al., 2010; Kumar et al., 2016; Xia, 2016). The ORF2 ML analysis further suggests that SAn bat AstVs could be more diverse than depicted by the shorter *RdRp* gene analyses, highlighting the importance to attempt to obtain more ORF2 sequences in future studies, perhaps through NGS methods.

4.4 Astrovirus and Coronavirus amplification within a *Neoromicia capensis* colony

A colony of *N. capensis* bats residing in bat boxes on a farm in Velddrif, on the West Coast of SA, were monitored over a period of twelve months during 2015. The bat boxes were affixed to the outside of a farm storage building. The colony consisted of both sexes and the colony size was estimated at 50 individuals. Faecal droppings were collected monthly by a lay collaborator. It was noted that the

colony migrated during the winter months (May-July) and only recolonized the roost in late August. The colony was monitored for two different viruses, AstVs and MERS-related beta-CoVs, using real-time PCR assays (Section 2.13.1, Methods Chapter), as bats are regularly co-infected with these two viruses (Chu, 2008; Drexler et al., 2011; Seltmann et al., 2017). A single amplification peak was noted for both viruses, with the relative viral loads reaching a peak during September, correlating with the recolonization of the roost (Figure 3.7.). The increase in viral loads of both species did not seem to correspond to changes in seasonality, however, seasonal variation cannot be excluded as there was a gap in sampling during the winter months. During the current study there was no correlation between the increase in viral loads and the pupping season of *N. capensis*, which usually takes place during November (Taylor, 2000; Monadjem et al., 2010). This could in part be due to the drought that was experienced in the area. A study by Adams (2010) documented that a reduction in precipitation and water available as drinking sources for bats can lead to a 50% decline in bat reproduction. Furthermore, the survival of pups during drought periods are very low, hence it is plausible that pups that might have been born during November might have succumbed and not contributed immunologically naïve individuals to the colony that could have led to an increase in viral loads (Voigt & Kingston, 2016).

Another study that also monitored the amplification of AstVs and CoVs in a bat colony was conducted by Drexler et al. (2011). The study conducted by Drexler et al. (2011) span over three years and the amplification of AstVs and CoVs were monitored in a *Myotis myotis* maternal colony. The results of their study indicated that the AstVs also had a single amplification peak which is similar to the results of the current study, except for the third year of the study where a second amplification peak was noted and correlated to the introduction of a novel AstV strain. The peak in amplification of the AstV was associated with colony formation and parturition. There were differences between the current study and the one conducted in Germany. The *Myotis myotis* colony monitored by Drexler et al. (2011) was much larger in size (maximum population size 200 individuals compared to 50) and was a maternity roost, only consisting out of female bats and their pups (Drexler et al., 2011). Another aspect that differed between the two roosts was that the roost monitored by Drexler et al. (2011) was enclosed within a roof of a building, with samples collected from plastic sheets placed on the roof floor, i.e. inside, whereas the *N. capensis* roost was in a bat box affixed to a barn and pellets were collected from below the bat box, subjecting the samples to possible UV radiation, heat etc. The results also indicated that the viral loads were much lower (highest viral load 240 for AstVs and 150 for CoVs) than that found by Drexler et al. (2011), ranging from 10^3 to 10^8 for AstVs and 10^3 to 10^{10} for CoVs. This could be attributed to colony size, differences in sample collection and sample exposure to elements at the Velddrif colony. During the winter months the SAn colony migrated, and

recolonization only started during the end of August, thus no virus data was recorded to rule out that the viral loads were not subject to seasonal changes. A study by Seltmann et al. (2017) noted that AstV shedding fluctuated seasonally and was positively correlated with the rainy season. For the current study it seems that both viruses reached their peak amplification during the end of September, this is most likely when the roost was recolonized, and the population reached the critical population size for the virus to replicate and spread (Chu, 2011, Drexler et al., 2011). However, 2015 was also the first year of a four-year draught cycle that was experienced in the Western Cape of SA. It was noted that the bats left the roosts completely during 2016, which could be attributed to the drought. This halted the long-term surveillance of AstVs and CoVs in the colony.

4.5 Virus isolation *in vitro*

Various mammalian and avian AstVs have been successfully isolated and propagated in cell culture (Lee & Kurtz, 1981; Shimizu et al., 1990; Brinker et al., 2000; Fischer et al., 2017). The only available account of a failed bat AstV isolation attempt *in vitro* is that of Chu (2011). The present study describes the first isolation and propagation of what is likely to be a bat AstV in BHK-G43 cells. The sample material used as inoculum during the isolation attempts were saliva and urine samples collected from *R. clivosus* and *M. natalensis* bats, the samples were pre-screened for AstVs using hemi-nested screening PCR (Chu et al., 2008) and confirmed as a bat AstV through Sanger sequencing. The inoculum that led to the successful isolation was the saliva sample collected from a *M. natalensis* bat (field code SKKMN1). Three days post-inoculation changes were observed in the cell cultures, cells started to detach and clump, it was difficult to ascertain whether the changes observed were due to cytopathic effects of the virus or due to the porcine trypsin (Figures 3.8-3.10.). The isolation results were confirmed by Sanger sequencing and qPCR. Sanger sequencing confirmed that it was in fact a bat AstV that was isolated and not contamination. The qPCR results confirmed that the viral loads increased following the first passage and that it was not merely residual virus particles left behind after inoculation. Factors that could have played a role in the successful isolation include sample type used as inoculum, cell line, media constitution and post infection incubation period.

4.5.1 Samples used as inoculum

Most AstV isolation studies conducted on mammalian AstVs have used faecal material as inoculum for cell cultures (Shimizu et al., 1990; Brinker et al., 2000; Moser & Schultz-Cherry, 2005; Crameri et al., 2009; De Benedictis et al., 2011; Xiao et al., 2011; Xiao et al., 2013). During the current study's attempts, saliva and urine swabs stored in VTM at -80°C were used to infect cell cultures. A HAstV

positive stool sample was used as a positive isolation control. The human stool sample was used to inoculate Caco-2 cells (Marvin, Meliopoulos, and Schults-Cherry, 2014).

4.5.2 Cell lines used for virus isolation attempts

From the literature successful AstV isolation attempts utilized either kidney or colon derived cell lines (Shimizu et al., 1990; Brinker et al., 2000; Moser & Schultz-Cherry, 2005; Crameri et al., 2009; De Benedictis et al., 2011; Xiao et al., 2011; Xiao et al., 2013). As mentioned in the literature review, the cell receptors used by AstVs to gain entry into the cell are still undetermined, contributing to the complexity of *in vitro* isolation of this virus (Brinker et al., 2000). During the current study three cell lines were experimented with during the isolation attempts namely, Caco-2, BHK-G43 and NCK cells. Isolation and propagation were only successful in BHK-G43 cells, while isolation in NCK and Caco-2 cell lines could not be achieved. It is unclear why the NCK cells were not permissive to infection, it could be that the cells were not adapted to grow in SF media. Another possibility could be that bat cell lines are more resistant to infection compared to the baby hamster kidney cell line used. A study by Hoffman et al. (2013) found that lung and kidney bat cell lines from both *Yinpterochiroptera* and *Yangochiroptera* were not susceptible to infection by transmissible gastroenteritis virus (TGEV), a porcine CoV or SARS-CoV. They propose that bat cell lines might be more resistant to infection with the CoVs through receptor-dependent restriction. This might also be the case for the *Neoromicia* cell line used in the current study. BHK-G43 cells are transgenic BHK-21 cell clones that can be manipulated to express vesicular stomatitis virus (VSV) G protein with mifepristone (Moreira et al., 2016). During the current isolation attempts the BHK-G43 cells were not manipulated to express VSV G proteins, as such they can be regarded as BHK-21 cells (Kalhoro et al., 2009). *in Vitro* bat AstV isolation attempts by Chu (2011) made use of primary cell lines (lung and kidney) derived from *M. magnater*, as well as Caco-2 cells. All attempts of AstV isolation and propagation by Chu (2011) were unsuccessful. Table 4.2. compares the methods used during the current study with those used by Chu (2011).

Table 4.2. Comparison between bat astrovirus isolation and propagation attempts

Cell culture factors	**Current study**	**Chu (2011)**
Cell lines	Caco-2, BHK-G43 & NCK	*M. magnater* primary cell lines (lung & kidney) & Caco-2
Media type	DMEM	MEM
Sample type (as inoculum)	Urine & saliva	Not specified
Porcine trypsin added	5 µg / ml during inoculation step 10 µg / ml during post-inoculation incubation	10 µg / ml
Antibiotics	Yes	Yes

Incubation post-inoculation	5 days	14 days
Passaging	Yes, once five days post inoculation. Both cells and supernatant were passaged.	Yes, once 14 days post-inoculation. Not mentioned

4.5.3 Media

Serum free media has been used for the isolation and / or propagation of numerous viruses. Scientists were able to propagate a chimeric parainfluenza virus type 3 respiratory syncytial virus to 100-fold higher titres in Vero cells with the use of SF media compared to serum-rich media (Yuk et al., 2006). Numerous AstVs have been successfully isolated in cell culture by using SF media with the addition of porcine trypsin (Lee & Kurtz, 1981; Shimizu et al., 1990; Brinker et al., 2000; Marvin et al., 2014). It is postulated that porcine trypsin activates the proteins in the capsid of the virus which might play a role in the attachment of the virus to the cell and cell entry (Lee & Kurtz, 1981). Experiments have shown that CPE-causing AstVs ceased to cause CPE in the absence of porcine trypsin (Lee & Kurtz, 1981; Brinker et al., 2000). It was also found that more virus particles were released from cells when porcine trypsin was added to the media, resulting in higher virus titres (Lee & Kurtz, 1981). Many publications were not forthcoming with the exact concentration of porcine trypsin that should be added to the SF media, which could have hampered previous bat AstV isolation attempts. The virus isolation protocol used during this study is routelinely used for the isolation of HAstVs *in vitro* (Marvin et al., 2014). The exact composition of the SF media with the addition of porcine trypsin plays an important role in isolation attempts (Lee & Kurtz, 1981). For isolation of laboratory strains of viruses, no antibiotic and antimycotics are added, however during the current isolation attempts antibiotics and antimycotics were added, as the sample material was collected from animals and other microorganisms could be present in the sample. AstVs have been successfully isolated from faecal samples of pigs with diarrhoea with the use of SF media with antibiotics and antimycotics added (Shimizu et al., 1990).

4.5.4 Incubation period post inoculation

Another factor that might have played a role is the incubation period post-inoculation of the cell cultures. The protocol that was used during this study was based on HAstVs strains with high viral titres infecting Caco-2 cells, according to the protocol cells should be incubated three to four days post-inoculation. However, it was suggested by collaborating AstV isolation expert Dr. Meliopoulos (St. Jude's Children's Research Hospital, Memphis, USA) that the bat AstV infected cells be incubated for at least five days, as the titre of the virus in the sample was unknown. Furthermore, there are no known protocols for the successful isolation and propagation of bat AstVs. The cells were therefore incubated for five days post-inoculation. Cells and supernatant were used to inoculate

a second batch of BHK-G43 cells, resulting in the first blind passage. These cells were also incubated for five days following inoculation. The blind passage might play an important role in increasing the viral load, as the qPCR results indicate that the amplification was higher in the second passage compared to the initial inoculation.

Some viruses might take months to be isolated successfully in cell culture, and as there is no protocol for isolation of bat AstVs *in vitro* previous attempts could have failed due to the incubation period post-inoculation being too short or too long. The bat AstV isolation attempt by Chu (2011) reported a post-inoculation incubation period of 14 days. This period might have been too long. The optimal post-inoculation period should be investigated in future studies.

4.6 Statistical analyses

Statistical analyses correlating viral presence with host biological measurements or environmental measurements are useful tools to gain insight into the ecology of viruses in their hosts. To make more meaningful assumptions, long-term longitudinal studies need to be conducted. The statistical analyses indicated that bat species identity, sex and biome where the bats occurred in were significant contributors to AstV positivity. Statistical analyses on the ecological and biological data recorded during the current study provided a snapshot in time of factors that could possibly have played a role in virus prevalence, as the ecological fallacy points out some factors might be overshadowed due to the scale of the investigation. “The ecological fallacy consists in thinking that relationships observed for groups necessarily hold for individuals” (Freedman et al., 1998).

4.6.1 Individual bat factors that possibly play a role in astrovirus positivity

Species identity was indicated as a possible significant contributing factor in terms of AstV positivity in bats sampled in SA. A closer look at the ecology of the three bat species with the highest AstV prevalence might provide additional information on the ecology of the virus itself.

4.6.1.1 *Miniopterus natalensis*

The highest detection rate of AstV RNA was recorded in *M. natalensis* (55%). This species is widely distributed throughout Africa and the Arabian Peninsula. The extent of occurrence of *M. natalensis* is estimated to be 1 387 139 km^2 (Figure 4.1.), due to its large extent of occurrence and adaptability this species is classified as least concern by the IUCN (Monadjem et al., 2010; MacEwan et al., 2016). This species is predominantly cave roosting, but they have also been documented roosting in crevice type roosts. Interestingly, they make use of two different cave systems, cooler cave systems are used as winter hibernacula and warmer caves are utilized during the summer as maternity roosts (MacEwan

et al., 2016). The females migrate between the cave systems and the distances between caves can range up to 260 km. The size of the colony in a specific roost can vary from a few individuals to more than 2 500 animals; at De Hoop Guano Cave on the Western Cape's south coast the colony size is estimated to be 200 000 during certain seasons (Monadjem et al., 2010). The migratory nature of this species coupled with its ability to form large roosts could be important factors in the maintenance and spread of AstVs. Another interesting aspect of their ecology that could play a role in virus dissemination is the use of different cave systems during the year.

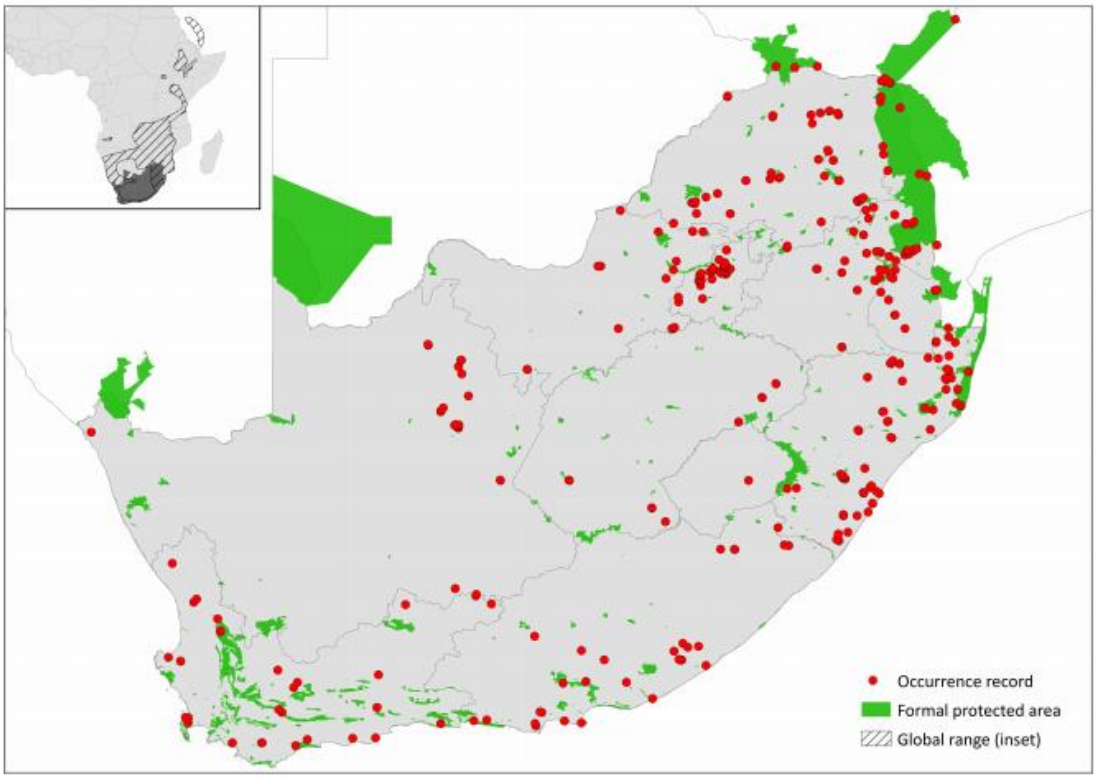


Figure 4.1. Distribution of Miniopterus natalensis in South Africa (MacEwan et al., 2016)

4.6.1.2 *Rhinolophus capensis*

R. capensis was the species with the second highest detection rate of AstV RNA in the current study (39%). This species is endemic to Southern Africa and is restricted in distribution to the coastal belt of the Southern Cape (Figure 4.2.) and is readily found in the Succulent Karoo and Fynbos biomes (Monadjem et al., 2010). According to the IUCN red list *R. capensis* is of least concern, due to the documentation of large colonies and their occurrence within protected areas (MacEwan et al., 2016). The estimated extent of occurrence of this species is 639 540 km^2. This species prefers roosting in coastal sea caves but is also readily found in abandoned mines and dark lofts. They readily co-roost with *R. clivosus* and *M. natalensis* (Monadjem et al., 2010). This species is not known for long-distance migrations and will only travel short distances of about 10 km. As this species occurs in

coastal areas the temperatures allow for abundant prey during the winter months, hence the rarity of hibernation in this species (Monadjem et al., 2010). Ecological factors that could possibly be of importance for the spread and maintenance of AstVs is their co-roosting behaviour, colony size and potentially roost sites (Nunn et al., 2015). Co-roosting behaviour could enhance interspecies transfer of the virus resulting in recombinant strains. As mentioned earlier, *M. natalensis* is a migratory species, and as these two species readily occur together *M. natalensis* could serve as a source of novel viruses when returning to communal roosting sites after migration.

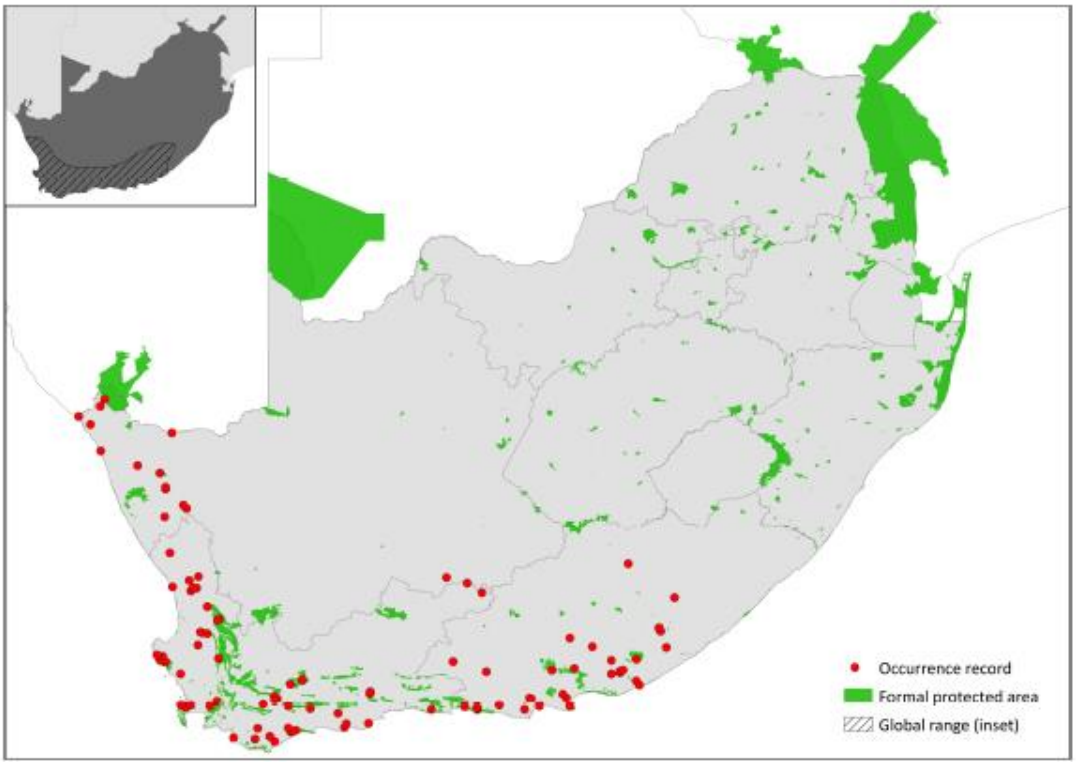


Figure 4.2. Distribution of Rhinolophus capensis in South Africa (MacEwan et al., 2016)

4.6.1.3 *Rhinolophus clivosus*

During the current study AstV RNA detection was recorded at 17% in *R. clivosus* bats. *R. clivosus* share many ecological similarities with *R. capensis*. This species roosts in caves, rock crevices, abandoned mines, rural and urban buildings and hollow baobab trees (*Adansonia* species) (Monadjem et al., 2010). They also make use of feeding roosts during the evening, where they eat their insect prey before returning to their roosts (Monadjem et al., 2010). Unlike *R. capensis* this species' distribution is not limited to coastal areas or even to SA (Stoffberg et al., 2012; Stoffberg, 2013). They occur in a wide variety of biomes throughout SA and greater Africa and is classified as least concern by the IUCN. The estimated extent of occurrence is 1 196 606 km^2 (Figure 4.3.), however due to the existence of cryptic species this range will probably be re-assessed in the future (Stoffberg

et al., 2012; Stoffberg, 2013). Another fascinating difference between *R. clivosus* and *R. capensis* is that *R. clivosus* undergoes prolonged periods hibernation, which is less common in *R. capensis*. Hibernation could also play an important role in virus maintenance and spread. A study by George et al. (2011) on hibernation's effect on rabies in big brown bats (*E. fuscus*), found that the incubation period of the rabies virus and the reduced metabolic effect of colder temperatures during hibernation supress virus activity. This causes a temporal maintenance reservoir of the rabies virus, keeping the virus dormant until the hosts emerge from hibernation and naïve individuals are introduced into the colony via parturition (George et al., 2011).

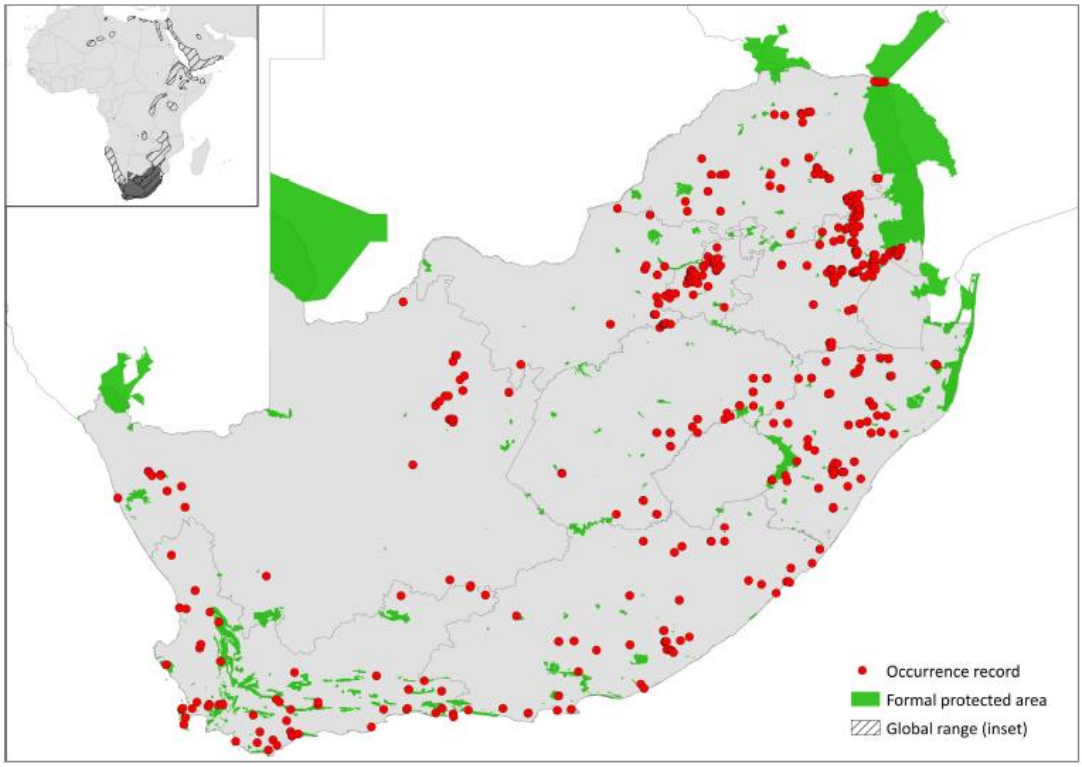


Figure 4.3. Distribution of Rhinolophus clivosus in South Africa (MacEwan et al., 2016)

Observations made by collaborating zoologists who conducted the sampling of bats, could also shed some light as to why *Rhinolophus* bats were found to have high detection rates of AstV RNA. One sampling locality where numerous *R. clivosus* bats tested positive for AstV RNA was located in an abandoned mine in Babanango in KZN. It was noted that most of the cave system where the bats roosted was waterlogged. It was postulated that these colonies of hundreds of individuals, defecate in the stagnant water, which could then serve as a reservoir for virus maintenance within the cave system, as these bats might drink the water.

Another interesting observation made is that of colony size at trapping locations. Sites with larger colonies (100 individuals and more) tended to have higher detection rates of AstV RNA; however,

colony size was not recorded for all sampling localities and could therefore not be used in statistical modelling.

4.6.2 Sex a significant predictor of astrovirus positivity

The findings of the current study suggest that sexually active male bats were more likely to have active AstV infections (40/242, 17%) than females (23/255, 9%). Male-biased infection rates of AstVs in bats were also documented by Mendenhall et al. (2017). However, several other bat AstVs studies did not document a significant difference in infection between male and female bats (Mendenhall et al., 2017; Hoarau et al., 2018).

Various physiological and behavioural differences exist between male and female bats, which could significantly influence their susceptibility to different infections (Christe et al., 2007). Hormones could play an important role; testosterone is a known immunosuppressant and it is widely documented that the production of this hormone increases susceptibility to infections (Klein, 2012).

Monadjem et al. (2010) noted differences in the roosting behaviour between male and female bats of *M. natalensis*. At De Hoop Guano cave, it was found that males and females of the species *M. natalensis* inhabited different sections of the cave. These different sections have different temperatures and most likely different microclimates, which could be important to AstV transmission. Furthermore, roosting behaviour during different times of the year differ significantly between the sexes. As mentioned in the previous section, it is documented that female *M. natalensis* bats migrate from the coastal regions of the Western Cape to more inland locations during the winter, to induce hibernation, whereas the males tend to stay behind at the roost.

Differences in torpor behaviour between male and female bats could also play a role. Torpor is a physiological mechanism bats use to reduce their energy consumption by lowering their metabolism and body temperature. It is suggested that intrahost pathogen replication could be temperature-dependent, and that seasonal torpor could suspend virus replication (Sadler & Enright, 1959; Sulkin et al., 1960; Luis & Hudson, 2006). Thus, species and sex differences in torpor behaviour might affect the co-evolution of pathogen variants and their transmission.

4.6.3 Environmental factors

The results of the generalized estimating equation suggest that biome type, specifically Succulent Karoo, could play an important role in AstV positivity in bats. This could be linked to the species in which the virus was found during this study. AstV RNA was frequently detected in *R. capensis* and *R. clivosus* that were often trapped in localities in this biome. The Succulent Karoo biome only occurs

in SA and no data currently exists that could explain why AstVs might be more prevalent in bats trapped in this biome. Further studies are required to elucidate these findings in the future.

4.7 qPCR vs conventional astrovirus screening PCR

During this study a real-time PCR assay was designed based on the bat AstV *RdRp* sequences obtained. The qPCR assay was a probe-based assay that enhances specificity. The qPCR assay was used to monitor the relative viral load fluctuations within a bat colony over time as well as to assess the virus isolation attempts. The assay was also assessed to determine if it could provide a more sensitive detection tool compared to the conventional hemi-nested screening PCR developed by Chu et al. (2008). For the subset of samples that were rescreened (n = 150), the real-time assay detected 20% more positives than Chu's assay, including all the samples in the subset that had been positive via the screening PCR. The results indicated that the real-time PCR assay developed is highly sensitive and able to detect the presence of AstVs at low copy numbers, suggesting that qPCR assays could be alternative AstV screening tools. Studies that have investigated AstVs in sewage and WWTW routinely use real-time assays, as they are able to detect AstVs at low concentrations (Le Cann et al., 2004). qPCR assays could also be used to detect AstV in samples that might have lower viral loads, which are not detectible with the conventional hemi-nested screening PCR assay. However, it is important to consider that sequence data cannot be generated from qPCR reactions, and that the sequence fragment would be insufficient for phylogenetic analysis due to the small size of the amplicon. If positives are identified they can be re-screened using the hemi-nested PCR assay developed by Chu et al. (2008) to allow for Sanger sequencing.

4.8 Possible One Health implications of AstVs in South Africa

The One Health concept acknowledges that the health of humans, animals and the environment are interlinked and dependant on one another. One of the aims of the study was to frame the results as possible One Health implications.

During the current study the host species that was studied was bats (*Chiroptera*) and the AstVs that they harbour. The zoonotic potential of wildlife AstVs, including those harboured by bats, has not yet been determined, however numerous studies suggest that the zoonotic potential of wildlife AstVs is high due to limited host species restriction, cross species transmissions, as well as the error proneness of the viral genome. However, numerous factors influence the zoonotic potential of a virus, including the number of viruses harboured by the primary host organisms, phylogenetic proximity between the host organism and humans, contact rates between the host and humans, habitat destruction and virus factors (Olival et al., 2017).

The possible zoonotic potential of SAn bat AstVs were deduced from the results obtained from the phylogenetic analyses of the ORF2 gene fragment and the *in vitro* isolation and propagation attempts. The phylogenetic results of the ORF2 gene suggest that bat AstVs are more closely related to HAstVs than to bat AstVs and could potentially pose a threat to human health. As the analyses only included one SAn bat AstV ORF2 gene, it would be advisable to obtain more ORF2 gene fragments to support this theory. Furthermore, as clinical samples of patients with gastroenteritis are not routinely screened for the presence of AstVs there is not a lot of genetic data available on SAn HAstVs (Pager, 2002; Nadan et al., 2003). To better understand the phylogenetic relationship between SAn bat AstVs and SAn HAstVs it will be crucial to obtain more SAn HAstV sequences.

The *in vitro* isolation and propagation attempt of the bat AstV revealed that it was not capable of propagating in Caco-2 cell lines, but the virus was however capable of propagating in BHK-G43 cells. This could suggest that the virus could make use of an intermediate rodent or murine host through which it can then potentially adapt to infect humans. It could also suggest that the Caco-2 cell line does not have the correct receptors to allow bat AstV entry and replication. More in-depth research should be conducted to further elucidate the true zoonotic risk of bat AstVs.

The potential impact of AstVs on environmental and animal health in terms of anthropogenic alteration of environments through the possible contamination of water sources with HAstVs were investigated at WWTW in KZN. Bats were trapped at various WWTW in KZN and tested for the presence of AstV RNA. In conjunction with bat samples, water samples were also collected from the WWTW sites and tested for the presence of AstV RNA. Interestingly only one bat (out of 50) tested positive from these sites. Furthermore, the phylogenetics of the *RdRp* sequence derived from the bat, were also not closely related to HAstVs, suggesting limited reverse zoonotic potential of the virus. The WWTW water samples were analysed and tested negative for the presence of AstV RNA. This could be indicative that the WWTW that were investigated were functioning optimally and posed limited threat in terms of environmental contamination with HAstVs.

4.7 Conclusion

The current study aimed to investigate the diversity and ecology of AstVs in SAn bats using phylogenetic-, molecular-, cell culture- and statistical methods.

The results of the current study demonstrated the diversity of AstVs in SA bats, as predicted in the hypothesis. Twenty-five novel *RdRp* sequences and one ORF2 sequence were obtained during the current study bringing the available bat AstV *RdRp* sequences to forty-four. The phylogenetic analyses highlighted how diverse these sequences are, as clustering was not restricted by species

identity or host geographic distribution. The phylogenetic analysis of the ORF2 gene fragment suggests that SAn bat AstVs might be more similar to human AstVs than bat AstVs.

The monitored *N. capensis* colony was co-infected with AstVs and CoVs, but the amplification of these viruses was not correlated to seasonality. The single peak in virus amplification corresponded with the recolonization of the roost after migration during September, and not with seasonality. Furthermore, the peak in virus amplification was not associated with the pupping season of *N. capensis*, which was the case for a *Myotis myotis* bat colony monitored by Drexler et al. (2011). These results disproved the hypotheses that AstVs and CoV infection in this specific bat colony would be correlated to seasonality. The results did however support the hypothesis that the *N. capensis* colony would be co-infected with AstVs and CoVs.

Host and environmental factors were found to influence AstV detection in bats. The statistical analyses indicated that bat species identity, being a sexually active male bat and occurring in the Succulent Karoo, are important factors in bat AstV positivity. This also supports the hypotheses that both host factors and environmental factors influence AstVs positivity.

The current study was the first to successfully isolate and propagate a bat AstV *in vitro* using a saliva sample collected from a *M. natalensis* bat in BHK-G43 cells. The methods used during the isolation attempts might enable other studies to successfully isolate bat AstVs *in vitro*. The successful isolation of a bat AstV *in vitro* will enable future studies to obtain larger fragments of the genome or full genomes with NGS technology.

The results of the current study provided some insight into the possible One Health implications of bat AstVs in SA. Phylogenetic analysis of the ORF2 gene suggests that bat AstVs are more similar to HAstVs than to bat AstVs, and as such could potentially pose a threat to human health. The *in vitro* isolation and propagation of a bat AstV *in vitro* demonstrated that the virus is capable of infecting cells originating from hamsters, which suggest that the virus can easily cross the species barrier and can be of possible veterinary importance. The analyses of water samples from WWTW for the presence of AstVs were indicative that these treatment works were functioning optimally and pose little threat environmentally.

The current study contributes significantly towards the body of knowledge of AstVs in SAn bats in terms of phylogenetic networks and factors that influence AstVs positivity. Furthermore, an optimized protocol for the successful isolation of bat AstVs *in vitro* was developed, which is invaluable for future functional studies.

4.7.1 Shortcomings of the current study

The current study investigated AstVs in numerous insectivorous bats occurring in SA, however no fruit bat samples were screened, as these bats were not sampled by our collaborators. Future studies should attempt to include as many bat species as possible to enable comparability with other studies. Furthermore, the samples used during the current study was collected opportunistically and could obscure the true prevalence of the virus. Direct sampling of sufficient numbers of each bat species will give a more accurate indication of virus prevalence and diversity.

Only a few juvenile bats were sampled during the current study, as such the influence of age on AstV prevalence in bats could not be determined. Future studies should include more juvenile bats to elucidate the influence of age on AstV prevalence.

Even though the study was the first to obtain a SAn bat AstV ORF2 fragment, only one sequence was obtained and only limited phylogenetic inferences could be made. It would have been advantageous to explore NGS methods to obtain longer ORF2 fragments or complete genome sequences, however due to time and financial constraints these methods could not be employed during the current study.

The longitudinal surveillance study of AstVs and CoVs in a *N. capensis* colony provided some valuable insights into the ecology of both viruses, however it would have been more advantageous if the monitoring could have been for two or three years. With the extreme drought conditions in the area the bats completely vacated the roost, resulting in the shorter timeframe for monitoring.

4.7.2 Future directions

One very important aspect that is lacking in bat virus research in general is the lack of information on the ecology and habits of the bats. To fully understand the intricate role bats play as carriers of various zoonotic diseases, it is of utmost importance to understand their ecology and behaviour. This could be achieved through in-depth longitudinal research that focuses on species specific colonies that documents their ecology and behaviour using radio telemetry or GPS trackers, as well as behavioural observations by researchers.

To obtain a better understanding of potential One Health implications of bat AstVs, the surveillance of this virus in bats should be coupled with screening of clinical as well as environmental samples. It would be advantageous to test bat colonies that live in close proximity to humans, and then also screen the human population and even domesticated animals for the presence of AstVs.

The optimised protocol developed in this study for the isolation and propagation of a bat AstV *in vitro*, can be used in future studies to obtain whole genome sequences of these viruses and virus isolates for phenotypic studies, in which cell tropism and cell receptor usage can be determined.

References

Abad, F.X., Villena, C., Guix, S., Caballero, S., Pinto, R.M., Bosch A. 2001. "Potential role of fomites in the vehicular transmission of human astroviruses.", *Applications of Environmental Microbiology,* 67:3904–3907.

Adams, R.A. 2010. "Bat Reproduction Declines When Conditions Mimic Climate Change Projections for Western North America." *Ecology,* 91 (8): 2437–45.

Alves, C.D.B.T., da Fontoura Budaszewski R., Cibulski, S.P., Weber, M.N., Mayer, F., Bianchi, M.V., Zafalon-Silva, B. *et al*. 2018. "Mamastrovirus 5 Detected in a Crab-Eating Fox (Cerdocyon Thous): Expanding Wildlife Host Range of Astroviruses." *Comparative Immunology, Microbiology and Infectious Diseases,* 58: 36–43.

Appleton, H. & Higgins P.G. 1975. Letter: "Viruses and gastroenteritis in infants.", *Lancet* 7 (1):1297.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., and Patel, H.R.H. 2005. "Basic Principles of Real-Time Quantitative PCR.", *Expert Review of Molecular Diagnostics,* 5 (2): 209–19.

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

Baker, M.L., Schountz, T. and Wang, L.F. 2013. "Antiviral Immune Responses of Bats: A Review.", *Zoonoses and Public Health* 60, 104–116.

Bastos, A.D., Nair, D., Taylor, P.J., Brettschneider, H., Kirsten, F., Mostert, E., von Maltitz, E. *et al*. 2011. "Genetic Monitoring Detects an Overlooked Cryptic Species and Reveals the Diversity and Distribution of Three Invasive Rattus Congeners in South Africa.", *BMC Genetics* 12: 26.

Biesold, S.E., Ritz, D., Gloza-Rausch, F., Wollny, R., Drexler, J.F., Corman, V.M., Kalko, E.K.V., Oppong, S., Drosten, C. and Müller, M.A. 2011. "Type I Interferon Reaction to Viral Infection in Interferon-Competent, Immortalized Cell Lines from the African Fruit Bat *Eidolon Helvum*.", *PLOS one* 6 (11): e28131.

Boujon, C.L., Koch, M.C., Wüthrich, D., Werder, S., Jakupovic, D., Bruggmann, R. and Seuberlich, T. 2017. "Indication of Cross-Species Transmission of Astrovirus Associated with Encephalitis in Sheep and Cattle.", *Emerging Infectious Diseases* 23 (9): 1604–6.

Bosch, A., Pintó, R.M., Guix, S. 2012. "Human astroviruses.", *Clinical Microbiology Reviews* 27 (4): 1048-1074.

Bradley, R.D., and Baker, R.J. 2001. "A Test of the Genetic Species Concept: Cytochrome-b Sequences and Mammals.", *Journal of Mammalogy* 82 (4): 960–73.

Brinker, J.P., Blacklow, N.R. and Herrmann, J.E. 2000. "Human Astrovirus Isolation and Propagation in Multiple Cell Lines.", *Archives of Virology* 145 (9): 1847–56.

Brody, J.R., & Kern, G.S. 2004. "History and Principles of Conductive Media for Standard DNA Electrophoresis.", *Analytical Biochemistry* 333 (1): 1–13.

Brunet-Rossinni, A.K., and Wilkinson, G.S. 2009. “Methods for Age Estimation and the Study of Senescence in Bats.” In Kunz, T. and Parsons, S. (eds) *Ecological and Behavioural Methods for the Study of Bats*, Second Edition, Baltimore: The Johns Hopkins University Press ,315–19.

Calisher, C., Childs, J.R., Holmes, K. and Schountz, T. 2006. "Bats: important reservoir hosts of emerging viruses.", *Clinical Microbiology Review* 19 (3): 531-545.

Chu, D.K.W. 2011. “Diversity and Evolution of Coronaviruses and Astroviruses in Bat, Wild Birds and Rodents.” *PhD Dissertation*, University of Hong Kong, Hong Kong.

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

Chu, D. K. W., Poon, L. L. M., Guan, Y. and Peiris, J. S. M. 2008. “Novel Astroviruses in Insectivorous Bats.”, *Journal of Virology* 82 (18): 9107–14.

Cleveland, S., Haydon, D. T. and Taylor, L. 2007. "Overviews of pathogen emergence: Which pathogens emerge, when and why?" in Childs, J.E., Mackenzie, J. S. and Richts, J. A. (eds), *Wildlife and emerging zoonotic diseases: The biology, circumstances and consequences of cross-species transmission CTMI 315 Current Topics in Microbiology and Immunology*, Springer-Verlag, Berlin, 85-111. http://www.abcam.com/protocols/counting-cells-using-a-haemocytometer.

Corman, V.M., Ithete, N.L., Richards, L., Schoeman, M.C., Preiser, W., Drosten, C. and Drexler, J.F. 2014. "Rooting the phylogenetic tree of MERSCoronavirus by characterization of a conspecific virus from an African Bat.", *Journal of Virology* 1498-1514.

Crameri, G., Todd, S., Grimley, S., McEachern, J.A., Marsh, G.A., Smith, G., Tachedjian, M., De Jong, C., Virtue, E.R., Yu, M., Bulach, D., Liu, J.P., Michalski, W.P., Middleton, D., Field, H.E. and Wang, L.F. 2009. "Establishment, immortalisation and characterisation of Pteropod bat cell lines.", *PLOS one* 4 (12): 1-9.

Cronje, N. 2017. “The diversity of coronaviruses in southern African bat populations.”, *PhD dissertation*, Stellenbosch University, South Africa.

Darriba, D., Guillermo L.T., Ramón D., and David P. 2012. “JModelTest 2: More Models, New Heuristics and Parallel Computing.”, *Nature Methods* 9 (8): 772.

Daszak, P., Epstein, J. H., Kilpatrick, A. M., Aguirre, A. A., Karesh, W. B., Cunningham, A. A. 2007. “*Collaborative Research Approaches to the Role of Wildlife in Zoonotic Disease Emergence*.” Consortium for Conservation Medicine, 460 West 34th Street, 17th Floor, New York, USA.

De Benedictis, P., Schultz-Cherry, S., Burnham, A. and Cattoli, G. 2011. "Astrovirus infection in humans and animals: molecular biology, genetic diversity and interspecies transmissions.", *Infection, Genetics and Evolution* 11 (7): 1529-1544.

Drexler, J.F., Corman, V. M., Wegner, T., Tateno, A.F., Zerbinati, R.M., Gloza-Rausch, F., Seebens, A., Müller, M. A., and Drosten, C. 2011. “Amplification of Emerging Viruses in a Bat Colony.”, *Emerging Infectious Diseases* 17 (3): 449–56.

Epstein, J. H., K.J., Olival, J.R.C., Pulliam, C. Smith, C., Westrum, J., Hughes, T., Dobson, A.P., Zubaid, A., Rahman, S.B., Basir, M.M., Field, H.E. and Daszak, P. 2009. "Pteropus vampyrus, a hunted migratory species with a multinational home-range and a need for regional management.", *Journal of Applied Ecology* 46: 991–1002.

Efron, B., Halloran, E., and Holmes. S. 1996. "Bootstrap Confidence Levels for Phylogenetic Trees.", *Proceedings of the National Academy of Sciences USA* 6: 45-52.

Eissa, N., Hussein, H., Wang, H., Rabbi, M.F., Bernstein, C.N. and Ghia, J. 2016. "Stability of Reference Genes for Messenger RNA Quantification by Real-Time PCR in Mouse Dextran Sodium Sulphate Experimental Colitis.", *PLOS one* 11 (5):87-93.

Evans, B.R. and Leighton, F.A. 2014. "A history of One Health.", Scientific and Technical Review of the Office International des Epizooties 33 (2): 413-420.

FAO, Food and Agriculture Organisation of the United Nations. 2011. "Investigating the role of bats in emerging zoonoses: Balancing ecology, conservation and public health interests", in Newman, S. H. (eds) *FAO Animal Production and Health Manual No.12*. Rome: Food and Agriculture Organisation of the United Nations.

Fischer, K., Pinho dos Reis, V., and Balkema-Buschmann, A. 2017. "Bat Astroviruses: Towards Understanding the Transmission Dynamics of a Neglected Virus Family.", *Viruses* 9 (2): 118-126.

Fischer, K., Zeus, V., Kwasnitschka, L., Kerth, G., Haase, M., Groschup, M.H. and Balkema-Buschmann, A. 2016. "Insectivorous Bats Carry Host Specific Astroviruses and Coronaviruses across Different Regions in Germany.", *Infection, Genetics and Evolution* 37: 108–16.

Freedman, D.A., Klein, S.P. Ostland, M. and Roberts, M.R. 1998. "Review of a Solution to the Ecological Inference Problem.", *Journal of the American Statistical Association* 94: 352–57.

George, D.B., Webb, C.T., Farnsworth, M.L., O'Shea, T.J., Bowen, R.A., Smith, D.L., Stanley, T.R., Ellison, L.E., and Rupprecht, C.E. 2011. "Host and Viral Ecology Determine Bat Rabies Seasonality and Maintenance.", *Proceedings of the National Academy of Sciences of the United States of America* 108 (25): 10208–13.

Glass, R. I. & Bresee, J. S. 2011. "Astroviruses, Enteric Adenoviruses, and Other Gastroenteritis Viral Infections." In *Tropical Infectious Diseases: Principles, Pathogens and Practice, 3rd Edition*, Saunders, Edinburgh, 416-419.

Gray, J. & Desselberger, U. 2009. "Rotaviruses". In A. J. Zuckerman, J. E. Banatvala, B. D. Schoub, P. D. Griffiths and P. Mortimer (eds), *Principles and practice of clinical virology*, Sixth Edition, John Wiley & Sons, Ltd, Chichester, UK, 83-119.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. 2010. "New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0.", *Systematic Biology* 59 (3): 307–21.

Halpin, K., Hyatt, A.D., Plowright, R.K., Epstein, J.H., Daszak, P., Field, H.E., Wang, L. and Daniels, P.W. 2007. "Emerging viruses: coming in on a wrinkled wing and a prayer.", *Clinical Infectious Diseases* 44 (5): 711-718.

Hoarau, F., Le Minter, G., Joffrin, L., Schoeman, C.M., Lagadec, E., Ramasindrazana, B., Santos, A.D. *et al*. 2018. "Bat Astrovirus in Mozambique.", *Virology Journal* 15 (1): 104.

Hu, B., Ge, X., Wang, L.F. and Shi, Z. 2015. "Bat origin of human coronaviruses." *Virolology Journal* 12 (1): 221.

ICTV, International Committee on Taxonomy of Viruses. 2005. *Virus taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*. Edited by C. Fauquet *et a*l. Academic Press.

Ithete, N.L. 2013. *"Investigation of small mammal-borne viruses with zoonotic potential in South Africa." PhD dissertation*, Stellenbosch University, South Africa.

Jeong, H.S., Jeong, A. and Cheon, D.S. 2012. "Epidemiology of astrovirus infection in children.", *Korean Journal of Pediatrics* 55 (3): 77-82.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J. L., *et al.* 2008. "Global trends in emerging infectious diseases.", *Nature* 451 (7181): 990–3.

Joshi, K.D. 1990. *Foundations of Discrete Mathematics*. New Age International Limited.

Kalhoro, N.H., Veits, J., Rautenschlein, S., and Zimmer, G. 2009. "A Recombinant Vesicular Stomatitis Virus Replicon Vaccine Protects Chickens from Highly Pathogenic Avian Influenza Virus (H7N1).", *Vaccine* 27 (8): 1174–83.

Karlsson, E.A., Small, C.T., Freiden, P., Feeroz, M.M., Matsen, F.A., San, S., Hasan, M.K., Wang, D., Jones-Engel, L. and Schultz-Cherry, S. 2015. "Non-Human Primates Harbor Diverse Mammalian and Avian Astroviruses Including Those Associated with Human Infections.", *PLoS Pathogens* 11 (11).

Kemenesi, G., B. Dallos, T. GöRföL, S. Boldogh, P. EstóK, K. Kurucz, M. Oldal, *et al*. 2014. "Novel European Lineages of Bat Astroviruses Identified in Hungary.", *Acta Virologica* 58 (01): 95–98.

Kemenesi, G., Dallos, B., Görföl, T., Boldogh, S., Estók, P., Kurucz, K., Kutas, A. *et al*. 2014. "Molecular Survey of RNA Viruses in Hungarian Bats: Discovering Novel Astroviruses, Coronaviruses, and Caliciviruses.", *Vector-Borne and Zoonotic Diseases* 14 (12): 846–55.

Krishnan, T. 2014. "Novel human astroviruses: challenges for developing countries.", *Virus Disease* 25 (2), 208–214.

Kumar, S., Stecher, G. and Tamura, K. 2016. "MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets." , *Molecular Biology and Evolution* 33(7): 1870–4.

Kunz, T.H. & Fenton, M. B. 2003. *Bat ecology*. The University of Chicago Press, Chicago, USA, 16-49.

Lacroix, A., Duong, V., Hul, V., San, S., Davun, H., Omaliss, K., Chea, S. *et al*. 2017. "Diversity of Bat Astroviruses in Lao PDR and Cambodia.", *Infection, Genetics and Evolution* 47: 41–50.

Lacroix, A., Duong, V., Hul, V., San, S., Davun, H., Omaliss, K., *et al.* 2017. "Genetic diversity of coronaviruses in bats in Lao PDR and Cambodia." *Infection, Genetics and Evolution* 48: 10–18.

Le Cann, P., Ranarijaona, S., Monpoeho, S., Le Guyader, F. and Ferré, V. 2004. "Quantification of Human Astroviruses in Sewage Using Real-Time RT-PCR." *Research in Microbiology* 155 (1): 11–15.

Lebarbenchon, C., Ramasindrazana, B., Joffrin, L., Bos, S., Lagadec, E., Le Minter, G., Gomard, Y. *et al*. 2017. "Astroviruses in Bats, Madagascar.", *Emerging Microbes & Infections* 6 (6): e58.

Lewis, G.D. & Metcalf, T.G., 1998. "Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A and human rotaviruses, from oyster water and sediment samples.", *Applied and Environmental Microbiology* 54: 1983-1988.

Ludwig, B., Kraus, F.B., Allwinn, R., Doerr, H.W. and Preiser, W. 2003. "Viral zoonoses - a threat under control?", *Intervirology*, 46 (2): 71-8.

MacEwan, K., Richards, L.R. Cohen, L., Jacobs, D., Monadjem, A., Schoeman, M.C., Sethusa, T. and Taylor, P.J. 2016. "A Conservation Assessment of *Miniopterus Natalensis*." In *The Red List of Mammals of South Africa, Swaziland and Lesotho.* South African National Biodiversity Institute and Endangered Wildlife Trust, South Africa.

Marvin, S., Meliopoulos, V. and Schults-Cherry, S. 2014. "Human Astrovirus Propagation, Purification and Quantification.", *Bio-Protocol* 4 (6): 1–8.

Mattison, K. & Bidawid, S. 2009. "Analytical methods for food and environmental viruses.", *Food and Environmental Virology* 1:107-122.

Meleg, E., Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szucs, G.2006. "Human astroviruses in raw sewage samples in Hungary.", *Journal of Applied Microbiology* 101 (5): 1123-9.

Mendenhall, I.H., Skiles, M.M., Neves, E.S., Borthwick, S.A., Low, D.H.W., Liang, B., Lee, B.P.Y.H., Su, Y.C.F. and Smith, G.J.D. 2017. "Influence of Age and Body Condition on Astrovirus Infection of Bats in Singapore: An Evolutionary and Epidemiological Analysis.", *One Health* 4: 27–33.

Mendenhall, I.H., Smith, G.J.D. and Vijaykrishna, D. 2015. "Ecological Drivers of Virus Evolution: Astrovirus as a Case Study." Edited by S. Schultz-Cherry. *Journal of Virology* 89 (14): 6978–81.

Mendez, E. & Arias, C. F. 2007. Astroviruses. In Howley, P. M. , Knipe, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B. & Straus, S. E. *Fields Virology*, 5th edition,Lippincott Williams & Wilkins, Philadelphia, USA.

Meng, F., Zhu, L., Huang, W., Irwin, D.M. and Zhang, S. 2016. "Bats: Body Mass Index, Forearm Mass Index, Blood Glucose Levels and *SLC2A2* Genes for Diabetes.", *Scientific Reports* 6: 29960.

Monadjem, A., Taylor, P.J., Cotterill, F.P.D. and Schoeman, M.C. 2010. *Bats of Southern and Central Africa: A Biogeographic and Taxonomic Synthesis.* University of the Witwatersrand Press, Johannesburg, South Africa.

Moratelli, R. & Calisher, C. H. 2015. "Bats and zoonotic viruses: can we confidently link bats with emerging deadly viruses?", *Memórias do Instituto Oswaldo Cruz* 110 (1): 1–22.

Moreira, É.A., Locher, S., Kolesnikova, L., Bolte, H., Aydillo, T., García-Sastre, A., Schwemmle, M., and Zimmer, G. 2016. "Synthetically Derived Bat Influenza A-like Viruses Reveal a Cell Type- but Not Species-Specific Tropism.", *Proceedings of the National Academy of Sciences* 113 (45): 12797–802.

Morse, S.S. 1995. "Factors in the emergence of infectious diseases.", *Emerging Infectious Diseases* 1(1): 7-11.

Moser, L. & Schultz-Cherry, S. 2008. "Astroviruses", *Encyclopaedia of Virology*, 204-210.

Moser, L.A. & Schultz-Cherry, S. 2005. "Pathogenesis of astrovirus infection.", *Viral Immunology* 18 (1): 4-10.

Mühldorfer, K., Speck, S., Kurth, A., Lesnik, R., Freuling, C., Müller, T., Kramer-Schadt, S. and Wibbelt, G. 2011. "Diseases and causes of death in European bats: dynamics in disease susceptibility and infection rates.", *PLOS one* 6 (12): 12-18.

Nadan, S., Walter, J.E., Grabow, W.O.K., Mitchell, D.K., Taylor, M.B. 2003."Molecular Characterization of Astroviruses by Reverse Transcriptase PCR and Sequence Analysis: Comparison of Clinical and Environmental Isolates from South Africa.", *Applied and Environmental Microbiology* 6 (2): 747–753.

Nagai, M., Omatsu, T., Aoki, H., Otomaru, K., Uto, T., Koizumi, M., Minami-Fukuda, F. *et al*. 2015. "Full Genome Analysis of Bovine Astrovirus from Fecal Samples of Cattle in Japan: Identification of Possible Interspecies Transmission of Bovine Astrovirus.", *Archives of Virology* 160 (10): 2491–2501.

Naidoo, S., Vosloo, D. and Schoeman, M.C. 2015. "Haematological and genotoxic responses in an urban adapter, the banana bat, foraging at wastewater treatment works.", *Ecotoxicology and Environmental Safety* 114: 304–311.

Narrod, C., Zinsstag, J. and Tiongco, M. 2012. "A One Health framework for estimating the economic cost of zoonotic diseases on society.", *EcoHealth* 9 (2): 150-162.

Nei, M. & Kumar, S. 2000. "*Molecular Evolution and Phylogenetics."* Oxford University Press, New York, USA.

Ng, T.F., Rodrigo J., Mesquita, M., Nascimento, S.J., Kondov, N.O., Wong, W., Reuter, G., Knowles, N.J. *et al*. 2014. "Feline Fecal Virome Reveals Novel and Prevalent Enteric Viruses.", *Veterinary Microbiology* 171 (1): 102–11.

Nunn, C.L., Jordán, F., McCabe, C.M., Verdolin, J.L. and Fewell, J.H. 2015. "Infectious Disease and Group Size: More than Just a Numbers Game.", *Philosophical Transactions of the Royal Society B: Biological Sciences* 370 (1669): 82-95.

Osborne, C.M., Montano, A.C., Robinson, C.C., Schultz-Cherry, S. and Dominguez, S.R. 2015. "Viral gastroenteritis in children in Colorado 2006-2009.", *Journal of Medical Virology* 87 (6): 931-9.

Pager, C.T. & Steele, A. D. 2002. "Astrovirus-Associated Diarrhea in South African Adults." *Clinical infectious diseases* 35(11): 44-49.

Pativada, M.S., Chatterjee, D., Mariyappa, N. S., Rajendran, K., Bhattacharya, M.K., Ghosh, M., Kobayashi, N. and Krishnan, T. 2011. "Emergence of unique variants and inter-genotype recombinants of human astroviruses infecting infants, children and adults in Kolkata, India.", *International Journal of Molecular Epidemiology and Genetics* 2 (3): 228–235.

Pativada, M., Nataraju, S. M., Ganesh, B., Rajendran, K., Ramamurthy, T., Ganguly, S., *et al.* 2012. "Emerging trends in the epidemiology of human astrovirus infection among infants, children and adults hospitalized with acute watery diarrhea in Kolkata, India.", *Infection, Genetics and Evolution* 12: 1685-1693.

Playford, E.G., McCall, B., Smith, G., Slinko, V., Allen, G., Smith, I., Moore, F., Taylor, C., Kung, Y. and Field, H. 2010. "Human hendra virus encephalitis associated with equine outbreak, Australia, 2008.", *Emerging Infectious Diseases* 16 (2): 219-223.

Rivera, R., Nollens, H.H., Venn-Watson, S., Gulland, F.M. and Wellehan, J.F. 2010. "Characterization of phylogenetically diverse astroviruses of marine mammals.", *Journal of General Virology*, 91 (Pt 1), 166–173.

Seltmann, A., Corman, V.M., Rasche, A., Drosten, C., Czirják, G.A., Bernard, A., Struebig, M.J. and Voigt, C.C. 2017. "Seasonal Fluctuations of Astrovirus, But Not Coronavirus Shedding in Bats Inhabiting Human-Modified Tropical Forests.", *EcoHealth* 14 (2): 272–84.

Shimizu, M., Shirai, J., Narita, M. and Yamane, T. 1990. "Cytopathic astrovirus isolated from porcine acute gastroenteritis in an established cell line derived from porcine embryonic kidney.", *Journal of Clinical Microbiology* 28 (2): 201-206.

Strain, E., Kelley, L.A., Schultz-Cherry, S., Muse, S.V. and Koci, M.D. 2009. "Genomic analysis of closely related astroviruses.", *Journal of Virology* 82 (10): 5099-103.

Sulkin, S.E., Allen, R. and Sims, R.A. 1960. "Lipotropism in Pathogenesis of Encephalitis Viruses in Insectivorous Bats.", *Virology* 11: 302–3.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2010. "MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods.", *Molecular Biology and Evolution* 28(10): 2731–39.

Teeling, E.C., Springer, M.S., Madsen, O., Bates, P., O'Brien, S.J. and Murphy, W.J. 2005. "A molecular phylogeny for bats illuminate's biogeography and the fossil record.", *Science* 307: 580–584.

Teeling, E.C. 2009. *Bats (Chiroptera). The timetree of life*. Oxford University Press, New York, USA.

United Nations.2008. Contributing to One World, One Health: A strategic framework for reducing risk of infectious diseases at the animal-human-ecosystem interface. FAO/OIE/WHO/UNICEF/UNSIC/World Bank. Available: http://un-influenza.org/files/OWOH_14Oct08.pdf. Accessed 24 April 2009.

Venter, J.M.E. 2004. The incidence of hepatitis A virus in selected water sources and associated risk of infection in South Africa. *MSc dissertation*. University of Pretoria, South Africa.

Vivier, J.C., Ehlers, M.M., Grabow W.O.K. 2004. "Detection of enteroviruses in treated drinking water.", *Water Research* 38:2699-2705.

Vilaginés, P.H., Sarrette, B., Husson, G., Vilaginés, R. 1997. "Glasswool for virus concentration from water at ambient pH levels.", *Water Science and Technology* 35:445-449.

Voigt, C.C., & Kingston, T. 2016. "Bats in the Anthropocene: Conservation of Bats in a Changing World*.",* Springer open access, 36-49.

Voigt, C.C., Helbig-Bonitz, M., Kramer-Schadt, S., Kalko, E.K.V. 2014. "The third dimension of bat migration: evidence for elevational movements of *Miniopterus natalensis* along the slopes of Mount Kilimanjaro.", *Oecologia* 174 (3):751-64.

Waruhiu, C., Ommeh, S., Obanda, V., Agwanda, B., Gakuya, F., Ge, X., Yang, X., et al. 2017. "Molecular Detection of Viruses in Kenyan Bats and Discovery of Novel Astroviruses, Caliciviruses and Rotaviruses.", *Virologica Sinica* 32 (2): 101–14.

Wibbelt, G., Moore, M.S., Schountz, T. and Voigt, C.C. 2010. "Emerging diseases in Chiroptera: why bats? ", *Biological Letters in Pathogen biology* 6 (4):438-440.

Wilkinson, G.S. 1984. "Reciprocal food sharing in the vampire bat.", *Nature* 308: 181-184.

Wittemyer, G., Elsen, P., Bean, W.T., Coleman, A., Burton, O., *et al.* 2008. "Accelerated human population growth at protected areas edges.", *Science* 321: 123–126.

World Health Organization, ed. 2004. *Laboratory Biosafety Manual*. 3rd ed. Geneva: World Health Organization.

Wu, Z., Ren, X., Yang, L., Hu, Y., Yang, J., He, G., Zhang, J. et al. 2012. "Virome Analysis for Identification of Novel Mammalian Viruses in Bat Species from Chinese Provinces.", *Journal of Virology* 86 (20): 10999–12.

Xia, X. 2016. "PhyPA: Phylogenetic Method with Pairwise Sequence Alignment Outperforms Likelihood Methods in Phylogenetics Involving Highly Diverged Sequences.", *Molecular Phylogenetics and Evolution* 102: 331–43.

Xiao C.T., Gimenez-Lirola, L.G., Gerber, P.F., Jiang, Y.H., Halbur, P.G. and Opriessnig, T. 2013. "Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types.", *Journal of General Virology* 94 (3): 570–582.

Xiao, J., Li, J., Hu, G., Chen, Z., Wu, Y., Chen, Y., Chen, Z. et al. 2011. "Isolation and Phylogenetic Characterization of Bat Astroviruses in Southern China.", *Archives of Virology* 156 (8): 1415–23.

Young, C.C.W., and Olival, K.J. 2016. "Optimizing Viral Discovery in Bats.", *PLOS one* 11 (2): 18-26.

Zhu, H. C., Chu, D.K.W., Liu, W., Dong, B.Q., Zhang, S.Y., Zhang, J.X. Li, F.L. et al. 2009. "Detection of Diverse Astroviruses from Bats in China.", *The Journal of General Virology* 90 (Pt 4): 883–87.

Appendix A - Ethics approval of collaborators for trapping of bats

Cape Nature Permit number: 0056-AAA041-00091

Ezemvilo Permit number: 3899/2015

Biodiversity of Northern Cape permit number: FAUNA 1541/2014

Appendix B - Genbank identification number and description of sequences downloaded for RdRp phylogenetic analyses

JQ814870 Tylonycteris robustula astrovirus 1 RNA-dependent RNA polymerase (RdRp) gene, partial cds

JQ814856 Miniopterus schreibersii astrovirus 1 RNA-dependent RNA polymerase (RdRp) gene, partial cds

JQ814868 Myotis ricketti astrovirus 1 RNA-dependent RNA polymerase (RdRp) gene, partial cds

EU847198 Bat astrovirus 1 isolate AFCD271 polyprotein 1AB gene, partial cds

EU847207 Bat astrovirus 1 isolate AFCD94 polyprotein 1AB gene, partial cds

KU510453 Bat astrovirus strain 09GB409Mi RNA-dependent RNA polymerase-like (RdRp) gene, partial sequence

KY575651 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

KY575647 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

KY575652 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

FJ571065 Bat astrovirus Tm/Guangxi/LD38/2007 non-structural polyprotein 1AB (pol) gene, partial cds; and capsid protein precursor (ORF2) gene, complete cds

KU510460 Bat astrovirus strain 09GB552Hg RNA-dependent RNA polymerase-like (RdRp) gene, partial sequence

KY575670 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

EU847211 Bat astrovirus 1 isolate AFCD198 polyprotein 1AB gene, partial cds

EU847213 Bat astrovirus 1 isolate AFCD254 polyprotein 1AB gene, partial cds

EU847203 Bat astrovirus 1 isolate AFCD317 polyprotein 1AB gene, partial cds

EU847154 Bat astrovirus 1 isolate WCF214 polyprotein 1AB gene, partial cds

EU847164 Bat astrovirus 1 isolate WCF96 polyprotein 1AB gene, partial cds

EU847195 Bat astrovirus 1 isolate AFCD208 polyprotein 1AB gene, partial cds

JQ814871 Miniopterus schreibersii astrovirus 13 RNA-dependent RNA polymerase (RdRp) gene, partial cds

KJ571393 Bat astrovirus Mm/NX29/Hainan nonstructural polyprotein 1AB gene, partial cds

EU847219 Bat astrovirus 1 isolate AFCD293 polyprotein 1AB gene, partial cds

KY575661 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

JQ814865 Miniopterus schreibersii astrovirus 10 RNA-dependent RNA polymerase (RdRp) gene, partial cds

JQ814862 Miniopterus schreibersii astrovirus 7 RNA-dependent RNA polymerase (RdRp) gene, partial cds

EU847147 Bat astrovirus 1 isolate WCF98 polyprotein 1AB gene, partial cds

KJ571381 Bat astrovirus Ms/LS007/Hainan nonstructural polyprotein 1AB gene, partial cds

EU847191 Bat astrovirus 1 isolate AFCD166 polyprotein 1AB gene, partial cds

KU510456 Bat astrovirus strain 09GB438Mi RNA-dependent RNA polymerase-like (RdRp) gene, partial sequence

EU847159 Bat astrovirus 1 isolate WCF16 polyprotein 1AB gene, partial cds

KU510474 Bat astrovirus strain 09GB1224Mi RNA-dependent RNA polymerase-like (RdRp) gene, partial sequence

FJ571077 Bat astrovirus Ms/Guangxi/A629/2005 RNA-dependent RNA polymerase (pol) gene, partial cds

KJ571431 Bat astrovirus Myr/QX60-2/Guangxi nonstructural polyprotein 1AB gene, partial cds

BtAstV/CGC/Neo_capensis4/WC/South_Africa

KY575665 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

KY575657 Astrovirus sp. RNA-dependent RNA polymerase gene, partial cds

Avastrovirus1 (isolate_MPJ1601)

Appendix C - Protocol for counting cells with haemocytometer

Preparing the cell suspension for cell counting

Media was aspirated (Gilson, Wisconsin, USA) from the cell culture flask and cells were washed with 1x PBS (Gibco®, USA). Trypsin EDTA was added and the flask was incubated for 2-5 minutes, or until all the cells completely detached from the flask's surface. Media was added to neutralize the trypsin. Cells were then transferred to a 50 ml Falcon tube (Corning, USA). Cells were resuspended by gently pipetting up and down. The cell suspension was centrifuged (Rotanta 460R Hettich centrifuge, Massachusetts, USA) for 5 minutes at 1 000 x g. The supernatant was aspirated (Gilson, Wisconsin, USA), and care was taken not to disrupt the cell pellet. The cell pellet was resuspended with growth media to the original volume used in the starting culture and 0.5 ml of cell suspension was transferred to a sterile 2 ml tube (Eppendorf, Germany). In a new 2 ml tube (Eppendorf, Germany), 100 µl of cell suspension was added to 400 µl (0.4%) Trypan Blue (MBL international, Massachusetts, USA) and mixed gently by inverting the tube ("Counting cells using a haemocytometer | Abcam," n.d.).

Counting of cells

A volume of 100 µl of the Trypan Blue (MBL international, USA) treated cell suspension was added to the chambers underneath the coverslip of the haemocytometer. The haemocytometer was viewed under the 10x objective of the microscope (Nikon, Eclipse TS 100, Minato, Tokyo, Japan). Cells that were unstained, indicative of live cells, were counted in all 4 sets of 16 squares (Figure 1.) ("Counting cells using a haemocytometer | Abcam," n.d.).

Cells were counted in the highlighted squares

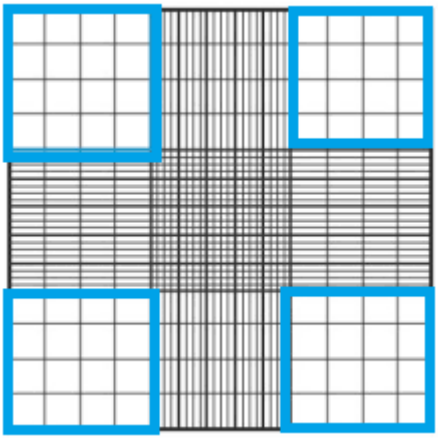


Figure 1. Representation of the 4 grids of the haemocytometer used during manual cell counting

Calculating the number of viable cells per ml

The following equation was used to determine the number of viable cells per ml:

$$\text{Viable cells per ml} = \left(\frac{cell\ count}{4} \times 10^4\right) \times 5\ (to\ correct\ for\ the\ dilution\ with\ Trypan\ Blue)$$

Appendix D - Snapshot of the datasheet used for data analyses

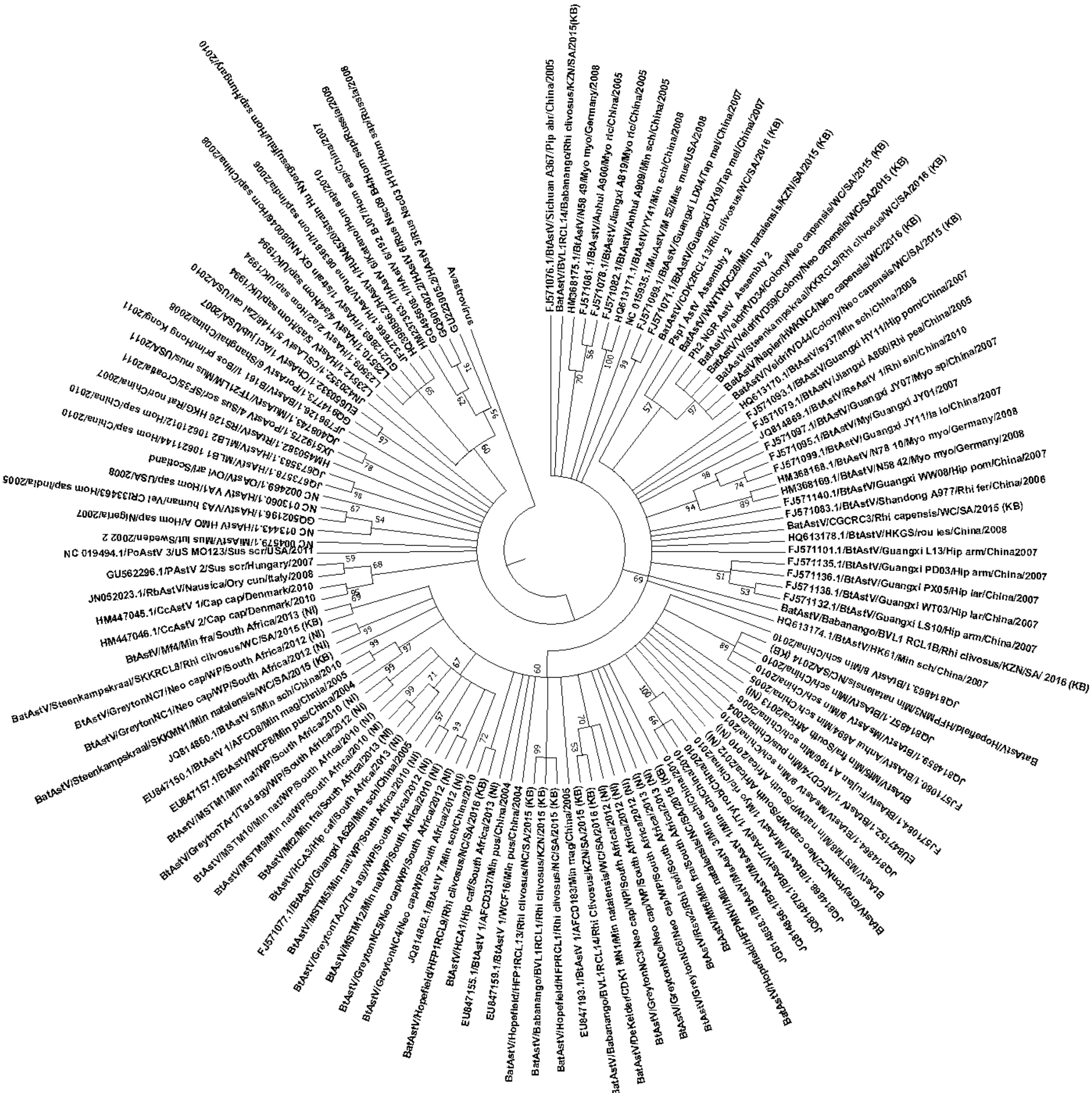
month	Prov	site	sample_ code	alt	bio	sex	age	rep. state	mass	FA	FMI	AstV	Train
January	KZN	LNR	NC11	1568	G	F	SA	NP	6	34	5.190311	0	130.2
October	EC	ABA	PH10	57	AT	M	A	NS	4.5	33	4.132231	0	25.4
October	EC	ABA	PH13	57	AT	F	A	P	8	34	6.920415	0	25.4
October	EC	ABA	PH9	57	AT	F	A	NP	5.5	34.5	4.620878	0	25.4
October	EC	ABA	PH5	57	AT	F	A	P	8.5	34	7.352941	0	25.4
July	EC	ABA	NC1	57	AT	M	A	NS	7.5	34	6.487889	0	108.4
July	EC	ABA	PH1	57	AT	F	A	NP	8.5	36	6.558642	0	108.4
July	EC	ABA	NC2	57	AT	M	A	NS	6.5	34	5.622837	0	108.4
July	EC	ABA	NC3	57	AT	M	A	NS	6.5	35	5.306122	0	108.4
July	EC	ABA	NC4	57	AT	M	A	NS	7.5	34	6.487889	0	108.4
July	EC	ABA	NC5	57	AT	M	A	NS	6	34	5.190311	0	108.4
July	EC	ABA	NC7	57	AT	M	A	NS	6	33.5	5.346402	0	108.4
October	EC	ABA	PH11	57	AT	F	A	P	7.5	34	6.487889	0	25.4
October	EC	ABA	PH2	57	AT	M	A	S	5.5	33	5.050505	0	25.4
October	EC	ABA	PH4	57	AT	F	A	P	9	33.5	8.019603	0	25.4
October	EC	ABA	PH6	57	AT	F	A	P	8.5	35	6.938776	0	25.4
October	EC	ABA	PH7	57	AT	F	A	P	8.5	36.5	6.380184	0	25.4
October	EC	ABA	PH8	57	AT	F	A	P	9.5	35	7.755102	0	25.4
October	EC	AEL	PH4	83	AT	F	A	NP	8.5	34	7.352941	0	94.6
October	EC	AEL	PH3	83	AT	F	A	P	8	34	6.920415	0	94.6
January	EC	AHHB	NC4	1227	G	M	A	NS	7	33	6.427916	0	52.2
January	EC	BCHB	NC1	1229	G	M	A	NS	6	33	5.509642	0	52.2
January	EC	BCHB	NC2	1229	G	F	A	PL	5	34	4.32526	0	52.2
January	WC	CCK	NC1	17	F	F	A	NP	8	37	5.843682	0	9.6
January	WC	CCK	NC7	17	F	F	A	NP	9	38	6.232687	0	9.6
January	WC	CCK	NC3	17	F	M	A	S	8	37	5.843682	0	9.6
January	WC	CCK	NC8	17	F	F	A	NP	9	38	6.232687	0	9.6

Appendix E - Table with Bat trapping sites. All bat trapping sites, with abbreviated site codes, where samples were collected.

Province	**Site and code in brackets**	GPS coordinates
Eastern Cape	Aardvark Backpackers, Addo (ABA)	-33.5350 25.6955
	Arena Resort East London (AEL)	-32.8839 28.0612
	Arminel Hotel Hogsback (AHHB)	-32.5919 26.9332
	Sleepy Hollow Maitland (SHM)	-33.9568 25.3132
	Table Farm Grahamstown (TFG)	-33.2853 26.4276
KwaZulu-Natal	Albert Falls (AF)	-29.4452 30.4301
	Babanango Exploratory Mine 2 (BVLEM2)	-28.2852 31.0137
	Babanango Exploratory Mine 1 (BVLEM1)	-28.2871 31.0129
	Babanango Main Mine (BVLMM)	-28.2867 31.0133
	Babango Valley (BVL)	-28.2867 31.0133
	Buffelsdrift (BDF)	-29.7567 30.6791
	Doornhoek Mine (DHM)	-29.6000 30.5200
	Hilton Train Tunnel (HTT)	-29.5497 30.2958
	Inkunzi Lodge. Babanango (ILB)	-28.5617 31.2404
	Lotheni Nature Reserve (LNR)	-29.4375 29.5150
	Mooiplaas Gold Mine (MPG)	-28.5582 31.1653
	Spionkop Lodge (SKL)	-28.6950 29.5355
	Umbilo WWTW (DC2)	-29.8455 30.8919
	Verulum WWTW (DC1)	-29.6439 31.0636

Limpopo	Royal Macadamia (LRM)	-23.0553 30.1495
	Kim's Farm (LKF)	-23.0222 29.7989
	Peter Taylor's House (LPT)	-23.0326 29.9296
Northern Cape	La Fugue Guesthouse (LFU)	-28.4402 21.2945
	Blinkklip Grotte (BKP)	-28.3001 23.1156
	Hopefield Farm 1 (HFP1)	-28.6188 23.3242
	Hopefield Farm 2 (HFP2)	-28.6305 23.3397
Western Cape	Steenkampskraal Mine (SKK)	-30.9750 18.6343
	Cloeteskraal Farm Velddrif * (CCK)	-32.8732 18.2236
	De Kelders Cave 1 (CDK1)	-34.5556 19.3642
	De Kelders Cave 2 (CDK2)	-34.5500 19.3710
	Drie Kuilen Nature Reserve (CDK)	-33.5815 20.0312
	Forest Edge Knysna (FEK)	-33.9294 22.9386
	Gecko Rock Cottage (CGC)	-33.5184 20.1310
	Gecko Rock Main House (CGR)	-33.5184 20.1188
	Haarwegskloof Nature Reserve (HWK)	-34.3383 20.3261
	Knysna Millwood Mines (KMM)	-33.8900 22.9910
	May's Lane Greyton (GML)	-34.0548 19.6054

Appendix F - Phylogenetic relationship of novel astroviruses with in the Astroviridae family. ML analyses based on the RdRp gene with 1 000 iterations



Appendix G - Estimates of evolutionary divergence between RdRp sequences. The number of base differences per site between sequences are shown. The analysis involved 45 nucleotide sequences using the p-distance model with 1000 iterations. Evolutionary analysis was conducted in MEGA7 (Kumar et al., 2016)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
1	GreytonTAr1Tad_agy_WP_SA2012																																												
2	MSTM1Min_nat_WP_SA2010	0%																																												
3	BtAMSTM10Min_nat_WP_SA2010stV	21%	21%																																										
4	BtAstVMSTM9Min_nat_WP_SA2010	16%	16%	23%																																									
5	Mf2Min_fra_SA2013	16%	16%	21%	6%																																							
6	HCA3_Hip_caf_SA2013	16%	16%	21%	8%	7%																																						
7	GreytonTAr2Tad_agy_WP_SA2012	18%	18%	34%	18%	18%	17%																																					
8	MSTM5Min_nat_WP_SA2010	22%	22%	27%	27%	23%	21%	17%																																				
9	GreytonNC4_WP_SA2012	23%	23%	27%	23%	15%	20%	16%	25%																																			
10	CDK2RCL13	33%	33%	41%	31%	31%	39%	23%	37%	28%																																		
11	M-52Mus_mus_USA2008	29%	29%	44%	25%	29%	31%	28%	49%	34%	27%																																	
12	HWKNC4	27%	27%	29%	32%	24%	22%	34%	38%	30%	29%	37%																																
13	VD_44	27%	27%	29%	32%	24%	22%	34%	38%	30%	29%	37%	0%																															
14	VD_34	27%	27%	29%	32%	24%	22%	34%	38%	30%	29%	37%	0%	0%																														
15	VD59	27%	27%	29%	32%	24%	22%	34%	38%	30%	29%	37%	0%	0%	0%																													
16	SKKRCL9	27%	27%	29%	32%	24%	22%	34%	38%	30%	29%	37%	0%	0%	0%	0%																												
17	DC28	28%	28%	33%	23%	20%	26%	38%	40%	24%	23%	32%	14%	14%	14%	14%	14%																											
18	CGCNC4	26%	26%	25%	25%	20%	23%	29%	36%	27%	23%	41%	21%	21%	21%	21%	21%	19%																										
19	GreytonNC5_WP_SA2012	23%	23%	25%	12%	18%	19%	16%	29%	22%	25%	27%	48%	48%	48%	48%	48%	35%	34%																									
20	MSTM12Min_nat_WP_SA2010	23%	23%	25%	12%	18%	19%	16%	29%	22%	25%	27%	48%	48%	48%	48%	48%	35%	34%	0%																							
21	GreytonNC2_WP_SA2012	23%	23%	23%	25%	23%	22%	27%	28%	25%	30%	42%	27%	27%	27%	27%	27%	25%	20%	25%	25%																							
22	HFPMN1	29%	29%	29%	23%	18%	23%	28%	23%	19%	19%	38%	21%	21%	21%	21%	21%	18%	13%	29%	29%	11%																						
23	Rsw2_Rhi_swi_SA2013	23%	23%	25%	23%	18%	16%	26%	24%	23%	30%	41%	19%	19%	19%	19%	19%	20%	18%	34%	34%	8%	8%																					
24	GreytonNC6_WP_SA2012	32%	32%	26%	26%	21%	25%	35%	35%	29%	26%	36%	20%	20%	20%	20%	20%	25%	22%	29%	29%	15%	11%	11%																				
25	Mf5Min_fra_SA2013	24%	24%	25%	31%	25%	25%	33%	30%	18%	19%	39%	14%	14%	14%	14%	14%	13%	17%	34%	34%	14%	12%	13%	15%																			
26	Mf6Min_fra_WP_SA2013	17%	17%	25%	24%	19%	21%	28%	30%	21%	20%	39%	20%	20%	20%	20%	20%	16%	16%	33%	33%	10%	10%	7%	12%	7%																		
27	BVL1RCL1	32%	32%	33%	27%	29%	27%	31%	37%	29%	29%	39%	29%	29%	29%	29%	29%	34%	22%	29%	29%	20%	13%	19%	17%	25%	19%																	
28	HFPRCL1	32%	32%	33%	27%	29%	27%	31%	37%	29%	29%	39%	29%	29%	29%	29%	29%	34%	22%	29%	29%	20%	13%	19%	17%	25%	19%	0%																
29	HFP1RCL13	32%	32%	33%	27%	29%	27%	31%	37%	29%	29%	39%	29%	29%	29%	29%	29%	34%	22%	29%	29%	20%	13%	19%	17%	25%	19%	0%	0%															
30	MSTM8Min_nat_WP_SA2010	25%	25%	30%	23%	14%	19%	30%	30%	19%	28%	40%	30%	30%	30%	30%	30%	22%	18%	27%	27%	15%	13%	13%	16%	16%	13%	14%	14%	14%														
31	GreytonNC3_WP_SA2012	34%	34%	38%	35%	33%	33%	29%	35%	31%	30%	48%	30%	30%	30%	30%	30%	35%	34%	39%	39%	27%	23%	21%	24%	22%	24%	36%	36%	36%	27%													
32	GreytonNC_WP_SA2012	34%	34%	38%	35%	33%	33%	29%	35%	31%	30%	48%	30%	30%	30%	30%	30%	35%	34%	39%	39%	27%	23%	21%	24%	22%	24%	36%	36%	36%	27%	0%												
33	CDK1_MN1	47%	47%	41%	37%	30%	30%	33%	40%	34%	30%	49%	26%	26%	26%	26%	26%	31%	23%	42%	42%	31%	19%	25%	26%	20%	28%	38%	38%	38%	27%	11%	11%											
34	BVL1RCL14	58%	58%	64%	48%	44%	53%	47%	56%	53%	35%	64%	37%	37%	37%	37%	37%	40%	39%	61%	61%	36%	23%	33%	32%	32%	35%	44%	44%	44%	41%	24%	24%	22%										
35	HCA1_Hip_caf_SA2013	27%	27%	29%	32%	25%	29%	29%	31%	22%	19%	36%	17%	17%	17%	17%	17%	21%	12%	35%	35%	13%	10%	14%	14%	8%	11%	18%	18%	18%	18%	32%	32%	32%	39%									
36	HFP1RCL9	24%	24%	25%	29%	23%	23%	29%	25%	25%	21%	44%	19%	19%	19%	19%	19%	21%	10%	39%	39%	17%	10%	11%	16%	11%	11%	24%	24%	24%	23%	32%	32%	28%	38%	9%								
37	Mf4_Min_fra_SA2013	29%	29%	29%	30%	26%	29%	33%	34%	30%	33%	53%	27%	27%	27%	27%	27%	30%	24%	28%	28%	12%	22%	18%	20%	18%	16%	30%	30%	30%	22%	42%	42%	41%	51%	15%	19%							
38	SKKRCL8	24%	24%	21%	27%	18%	21%	26%	28%	18%	22%	47%	19%	19%	19%	19%	19%	21%	15%	25%	25%	13%	14%	19%	21%	11%	12%	23%	23%	23%	18%	41%	41%	35%	47%	9%	11%	11%						
39	SKKMN1	29%	29%	22%	31%	31%	29%	32%	36%	25%	21%	37%	30%	30%	30%	30%	30%	27%	19%	24%	24%	14%	21%	23%	20%	15%	13%	31%	31%	31%	27%	35%	35%	33%	49%	18%	16%	18%	13%					
40	GreytonNC1_WP_SA2012	21%	21%	18%	32%	23%	28%	32%	28%	23%	28%	47%	21%	21%	21%	21%	21%	23%	18%	31%	31%	10%	17%	16%	15%	8%	10%	29%	29%	29%	15%	29%	29%	28%	47%	13%	15%	13%	10%	14%				
41	GreytonNC7_WP_SA2012	21%	21%	19%	32%	23%	28%	32%	28%	23%	28%	47%	21%	21%	21%	21%	21%	23%	18%	33%	33%	11%	17%	16%	15%	8%	10%	29%	29%	29%	15%	29%	29%	28%	47%	13%	15%	14%	11%	16%	1%			
42	BVL1_RCL1_(B)	34%	34%	28%	41%	34%	39%	40%	41%	26%	29%	47%	16%	16%	16%	16%	16%	23%	25%	47%	47%	29%	27%	25%	22%	14%	21%	32%	32%	32%	30%	37%	37%	41%	57%	19%	23%	27%	23%	23%	18%	18%		
43	BVL1RCL12	31%	31%	20%	25%	18%	27%	34%	27%	21%	23%	35%	18%	18%	18%	18%	18%	16%	20%	37%	37%	23%	14%	18%	18%	13%	16%	27%	27%	27%	19%	32%	32%	26%	37%	17%	17%	26%	19%	21%	16%	16%	19%	
44	HFPMN3	21%	21%	21%	31%	29%	25%	29%	26%	21%	19%	37%	17%	17%	17%	17%	17%	19%	14%	34%	34%	11%	10%	13%	15%	5%	7%	18%	18%	18%	19%	25%	25%	27%	34%	6%	8%	19%	11%	13%	11%	11%	19%	15%
45	CGCRC3	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	92%	100%	100%	94%	107%	95%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	88%	95%	100%	99%	100%	100%

Appendix H - Abbreviations used in sequence names in Figure 3.2.

The names of the sequences were as follows: abbreviation of the type of virus (BtAstV)/locality/Species abbreviation/province/continent/ year (Sequenced by KB or NI)

BtAstV- Bat astrovirus

Species abbreviations used

South African bat species

Hip caf: Hipposideros caffer

Neo cap: Neoromicia capensis

Pip hesperidus: Pipistrellus hesperidus

Rhi capensis: Rhinolophus capensis

Rhi clivosus: Rhinolophus clivosus

Other bat species abbreviations

Min pus: Miniopterus pusillus

Min sch: Miniopterus schreibersii

Him arm: Hipposideros armiger

Hip pom: Hipposideros pomona

Min mag: Miniopterus magnater

Myo Myo: Myotis myotis

Myo ric: Myotis ricketti

Pip abr: Pipistrellus abramus

Rhi fer: Rhinolophus ferrumequinum

Rhi pea: Rhinolophus pearsonii

Rhi sin: Rhinolophus sinicus

Rou les: Rou les

Tap mel: Taphozous melanopogon

Tyl rob: Tylonycteris pachypus

SA province abbreviations

WC: Western Cape

NC: Northern Cape

KZN: KwaZulu-Natal

Appendix I - Outline of experimental designs for different subsections of the project

1. General surveillance of AstVs across SAn bat species

Bats were sampled opportunistically across SA by collaborating Zoologists. A total of 500 individual bat samples were used during the current study. The bat faecal material was screened for the presence of AstVs using the hemi-nested screening PCR assay designed by Chu et al. (2008).

Positive and negative controls were used during each step from extraction of RNA as well as during the screening PCR and sequencing process.

If the PCR positive controls did not come up during gel electrophoresis (indicating possible failure of RNA extraction or cDNA failure), the entire process was repeated for the batch of samples. If negative PCR controls came up as positive during gel electrophoresis (indicating possible contamination), the entire process was repeated for the batch of samples in question.

2. Surveillance of a specific colony of *N. capensis* bats in the Western Cape of SA for the presence of AstVs and CoVs

Faecal material was collected on a monthly basis over the period of one calendar year (January-December 2015). These samples were screened for the presence of AstVs and CoVs by means of two qPCR assays. Standard curve approach was used to determine relative viral loads for each virus. Negative controls were included in each qPCR run to rule out contamination. To further rule out any inconsistencies, samples were analysed in triplicate during each run.

3 Screening of bats (*N. nana*) at WWTW for the presence of AstVs

N. nana bats were sampled at WWTW (n=50) and pristine areas 3km away from WWTW (n=47). These samples were screened for the presence of AstVs using the hemi-nested screening PCR assay designed by Chu et al. (2008), as well as with the AstV qPCR assay designed. Positive and negative controls were utilized during each step of the experiment, from extraction of RNA as well as during the screening PCR and sequencing process.

During qPCR runs, samples were run in triplicate to rule out any pipetting inconsistencies.

4 Bat AstV isolation *in vitro*

During isolation attempts of the bat AstV in different cell lines. Negative controls for each experiment (each cell line) was included. Negative cell culture controls contained no sample material and were utilized to rule

out contamination. A cell culture positive control (HAstV positive faecal sample) was also utilized to establish that the isolation protocol was performed correctly.

To minimize the possibility of contamination between the different cell lines used, cells were not handled at the same time in the laminar flow hood. Cleaning was also performed after working with each cell line.

To further justify positive findings the isolation and propagation attempts were performed in duplicate.