Assessment of Bunyavirales activity and circulation in humans, livestock, and peri-domestic rodents in diverse ecologies in Kenya, 2020-2022

By

Dorcus Caroline Achieng Omoga

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Department of Medical Virology,
University of Pretoria

Supervisors:

Prof. Marietjie Venter
Department of Medical Virology
University of Pretoria

Prof. Rosemary Sang
International Centre for Insect Physiology and Ecology (*icipe*)

Dr David P. Tchouassi
International Centre for Insect Physiology and Ecology *(icipe*)

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ETHICS STATEMENT

The author, whose name appears on the title page of this thesis, has obtained, for the research described in this work, the applicable research ethics approval from the Animal Ethics Committee protocol number **568/2020**. Subsequent Ethical approval was granted by the Kenya Medical Research Institute's Scientific and Ethics Review Unit (**SERU No.3312**) after gaining approval from the animal care and use committee.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

Signature:
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DECLARATION

I declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy (Medical Virology) at the University of Pretoria, is my own original work and has not been previously submitted by me for a degree at this or any other tertiary institution.

SIGNED:

DATE:

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LIST OF SYMBOLS AND ABBREVIATIONS

AHSV: Africa Horse sickness virus

AKAV: Akabane virus

BATV: Batai Virus

BLAST: Basic Local Alignment Search Tool

BUNV: Bunyamwera virus

BTV: Bluetongue virus

CPE: Cytopathic effects

CCHF: Crimean-Congo haemorrhagic fever

CCHFV: Crimean-Congo haemorrhagic fever virus

CHIK: Chikungunya

CHIKV: Chikungunya virus

CT: Comparative Threshold cycle

DEN: Dengue

DENV: Dengue Virus

DNA: Deoxyribonucleic acid

DFG: German Research Foundation

EDTA: Ethylenediaminetetraacetic acid

EID: Emerging infectious diseases

ELISA: Enzyme-Linked Immunosorbent Assay

GERV: Germiston virus

GFV: Gabek Forest virus

GTR: General Time Reversible

icipe: International Centre of Insect Physiology and Ecology

IFA: Immunofluorescence Assay

KARV: Karimabad virus

KAPTV: Kaptombes virus

KEMRI: Kenya Medical Research Institute

LASV: Lassa virus

LCMV: Lymphocytic choriomeningitis virus

LD50: The median lethal dose

MAFFT: Multiple Alignment using Fast Fourier Transform

NRIV: Ngari virus

NMK: National Museums of Kenya

NTPV: Ntepes virus

NSDV: Nairobi sheep disease virus

NTDs: Neglected tropical diseases (NTDs)

OIE: World Organization for Animal Health

OROV: Oropouche virus

PCR: Polymerase chain reaction

PFU: Plaque-forming units

PRNT: Plaque Reduction Neutralization Assay

RNA: Ribonucleic Acid

PRNT: Plaque Reduction Neutralization Test

RQ: Relative Quantitation

RVF: Rift Valley fever

RVFV: Rift Valley Fever Virus

SATV:Sathuvachari virus

SBV: Schmallenberg virus

SFV: Semliki Forest virus

SFNV: Sandfly fever Naples virus

SFSV: Sandfly fever Silician virus

SHUV: Shuni virus

TBEV: Tick-borne encephalitis virus

VHF: Viral Haemorrhagic Fever

WGS: Whole genome sequencing

YF: Yellow fever

YFV: Yellow fever virus

ZKV: Zika virus

RESEARCH OUTPUTS

Journal articles

Dorcus C.A. Omoga, David P. Tchouassi, Marietjie Venter, Edwin .O. Ogola, Georg Joachim Eibner, Anne Kopp, Inga Slothouwer, Baldwyn Torto, Sandra Junglen, & Rosemary Sang. (2022). Circulation of Ngari virus in Livestock, Kenya.
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Dorcus C.A. Omoga, David P. Tchouassi, Marietjie Venter, Edwin.O. Ogola, Josephine Osala, Anne Kopp, Inga Slothouwer, Baldwyn Torto, Sandra Junglen, & Rosemary Sang. Transmission dynamics of Crimean Congo Haemorrhagic Fever Virus (CCHFV); Evidence of circulation in Humans, Livestock and Rodents in diverse ecologies in Kenya. *Under review in the journal Transboundary and Emerging Diseases.*

Dorcus C.A. Omoga, David P. Tchouassi, Marietjie Venter, Edwin Ogola, Gibert Rotich, Joseph Njuguna, Dickens Ondifu, Baldwyn Torto, Sandra Junglen, & Rosemary Sang Divergent hantavirus in Somali shrew (*Crocidura somalica*) in semi-arid North Rift, Kenya. *Pathogens 2023, 12(5), 685; https://doi.org/10.3390/pathogens12050685* .

Conference Presentations

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SUMMARY

Viruses in the order *Bunyavirales* are diverse and include zoonotic arthropod- and rodent-borne viruses that cause diseases ranging from mild febrile illnesses to haemorrhagic and/or encephalitis fevers and even death in animals and humans. These viruses contribute significantly to emerging and re-emerging infection threats worldwide as well as neglected tropical diseases as they mainly affect the impoverished and have a long-lasting effect. In tropical and subtropical countries, mainly in Africa, more than 90% of human cases are either undiagnosed or misdiagnosed and treated as other common endemic diseases like malaria. Furthermore, many studies have focused on common arboviruses transmitted by mosquitoes such as the flaviviruses (e.g., yellow fever, Dengue, Chikungunya, Zika viruses), among others, as well as well-known members of the *Bunyavirales* associated with human outbreaks (Rift Valley Fever virus (RVFV) and Crimean Congo Hemorrhagic Fever virus (CCHFV)). Less known yet potentially harmful arboviruses in the order Bunyavirales are mostly ignored despite being zoonotic or having zoonotic potential with the risk of spreading globally. Despite the associated public and veterinary health, social, and economic importance, the impact of these diseases is largely undetermined due to paucity of active surveillance, poor disease reporting systems, and lack of appropriate diagnosis in affected regions. The prevalence, burden of disease and distribution of most members of the *Bunyavirales* remains unknown in African countries where they are endemic. Surveillance is thus essential to determine their importance, provide an early warning of their presence, and guide intervention measures to prevent outbreaks. This thesis instituted “One Health” surveillance in selected pastoralist communities of Kenya (Kajiado and Baringo counties) to assess the circulation and viral activity of *Bunyavirales* among peridomestic rodents, livestock, and humans presenting with febrile illness. Serum samples were analyzed serologically to determine the presence of *Bunyavirales* neutralizing antibodies as well as culture and molecular methods (RT-PCR and whole genome sequencing and phylogenetic analysis) to isolate and characterise known and novel viruses, respectively using methodologies described in chapter 2. In Chapter 3, we report the findings of the screening experiments including the seroprevalence of RVFV, CCHFV, Ngari virus (NRIV), Ntepes virus (NTPV) and Bunyamwera virus (BUNV) as well as the molecular detection of CCHFV, NTPV; NRIV, BUNV, hantavirus, Shamonda virus (SHAV) and some uncharacterised phleboviruses in livestock and peridomestic rodents. Further characterisation of the most important viruses detected is reported in subsequent chapters (4-7). One of the major findings reported herein includes the isolation and circulation of Ngari virus (NRIV),

known to cause outbreaks of haemorrhagic fevers in humans and small ruminants, in apparently healthy cattle, sheep and goats (Chapter 4). Seroprevalence of neutralizing antibodies to the virus ranged 19-52% with growth kinetics on different cells showing efficient replication in cells from sheep and humans and *Aedes albopictus* but weakly on goat cell lines. Phylogenetic analyses of complete-coding sequences of L, M and S segments of four NRIV isolates showed that the Kenyan viruses clustered with a monophyletic clade that is most closely related to a NRIV sequence from a small ruminant from Mauritania. This is the first detection of NRIV in livestock in Kenya. In Chapter 5, active detection of a Nairovirus, Crimean-Congo haemorrhagic fever (CCHF) virus RNA is demonstrated in sheep and rodent sera as virus exposure is serologically confirmed in these hosts and, cattle, goats, and humans. This virus is the causative agent of CCHF, a fatal viral haemorrhagic fever disease in humans. Among livestock, seroprevalence was lowest in goat (8.1%) and highest in cattle (14%). Phylogenetic analyses of partial sequences of the S segment generated from rodent and sheep revealed a high level of nucleotide sequence identity (96-98%) and a close relationship to other pathogenic strains in the CCHFV Africa 3 lineage. We also report for the first time in Kenya the detection of shrew-borne hantaviruses in Somali shrews (*Crocidura somalica)* from Baringo county (Chapter 6). The detected hantaviruses closest relative is an African shrew- borne hantavirus, Tanganya viruses (TGNV) with nucleotide identity of 72-80%.

Finally, through virus isolation and Next Generation Sequencing (NGS) of samples collected in this study, a novel orbivirus was isolated from cattle which is described in Chapter 7 as an additional finding of this thesis. This followed initial isolation in cell culture from the serum of a clinically sick cow aged 2-3 years, presenting signs of emaciation and lethargy. High throughput sequencing revealed the typical orbivirus genome architecture with ten double-stranded RNA segments and a total size of 18,731 bp. A further detection of Kaptombes virus (KPTV) by RT-PCR in three cattle samples and 5% seroprevalence of neutralizing antibodies to the virus, demonstrate its active circulation. This shows the strength of sequence-independent virus discovery methods to discover new pathogens in surveillance study.

Taken together, the findings have provided data on pathogenic bunyavirus prevalence in the two semi-arid regions useful for understanding the virus transmission networks as well as the detection and characterisation of a novel orbivirus. The data herein can be applied in animal health surveillance systems locally to ensure timely and appropriate detection and control as a means of active and continuous animal surveillance and as an early warning sign for zoonotic diseases emergence.

Keywords: Bunyavirales, peri-domestic rodents, livestock, arboviruses, rodent-borne viruses, seroprevalence, surveillance, isolation, zoonotic diseases, Kenya.

Chapter 1

LITERATURE REVIEW

1.1 Introduction

Members of the virus order *Bunyavirales* have linear, segmented, single-stranded, negative-sense or ambisense RNA genome viruses categorized into fourteen families (1,2). Bunyaviruses constitute the largest segmented negative-sense single-stranded RNA (-ssRNA) viruses. This large and diverse group of RNA viruses infects vertebrates, arthropods and plants and consists of important human and animal disease-causing viruses. The majority of pathogenic bunyaviruses are vector-borne (arboviruses), transmitted by arthropods except for arenaviruses (family *Arenaviridae*) and hantaviruses (family *Hantaviridae*) that are rodent-borne (2,3). These viruses are of major economic and public health importance (4–6).

Arboviruses (arthropod-borne viruses) are important pathogens of humans and animals transmitted by hematophagous vectors commonly mosquitoes, ticks, culicoides and sandflies (7,8). Mosquito-borne arboviruses are the most common and extensively studied and characterised of all arboviruses (9). Examples include zika virus (ZKV), Rift Valley fever virus (RVFV), Ngari virus (NRIV), Bunyamwera virus (BUNV), Chikungunya virus (CHIKV), Dengue virus (DENV), West Nile virus (WNV) and Yellow fever virus (YFV) (8,10). Tick-borne arboviruses include Crimean Congo Haemorrhagic fever virus (CCHFV), Jingmen Tick virus (JMTV) and Kemorovo virus (KEMV) among others (11–16). Culicoides borne arboviruses mainly affect livestock, for instance, the locally endemic African horse sickness virus (AHSV), Epizootic haemorrhagic disease virus (EHDV), Bluetongue virus (BTV), Schmallenberg virus (SBV), Akabane virus (AKAV), and Oropouche virus (OROV) amongst others (17–19). Medically important sandfly borne viruses mainly comprise phleboviruses including Toscana virus (TOSV), Sandfly fever Sicilian virus (SFSV) and sandfly fever Naples virus (SFNV) (20–22). Like other arthropod-borne viruses, a growing list of novel viruses are being identified and described, for instance, the recently isolated novel viruses in Kenya like Ntepes virus (NTPV), Embossos virus (EMRV), Bogoria virus (BOGV), Kiborgoch virus (KBGV), and Perkerra virus (PERV) whose public health impact is yet to be determined (20,21) .

Humans get infected with rodent-borne viruses mainly through inhalation of dust that is contaminated with rodent urine or droppings, direct human contact and less commonly through

bite wounds. Pathogenic rodent-borne viruses of public health importance include Lymphocytic Chorio-meningitis (LCMV) whose natural reservoir is the common house mouse (*Mus musculus*), Lassa virus (LSV); reservoir is multi-mammate rat (*Mastomys natalensis* species complex) and orthohantavirus among others (23–26).

Pathogenic bunyaviruses cause serious public health/economic problems and form an important proportion of viruses entering new geographical zones resulting in emerging and re-emerging diseases some of which are zoonotic like CCHFV and RVFV. These viruses significantly affect humans/animal health as well as livestock-dependent food security in developing and developed countries, hence proper knowledge and discovery of these viruses is necessary.

Due to poor surveillance, lack of diagnosis, unreliable reporting systems and improper clinical screening methods, the real health and economic impact of these viruses to humans and livestock remains largely unknown or is grossly underestimated. This is generally the case in much of tropical Africa despite this continent being the origin of several viruses associated with major epidemics globally like RVFV. In Kenya as in most African countries, both epidemiological data to determine the importance of pathogenic bunyaviruses to humans and animals and studies to investigate and describe circulating novel viruses, are limited. These knowledge gaps present a high risk of arboviral disease outbreaks and epidemics. Therefore, implementing a one health surveillance system provides important data on presence of pathogenic bunyaviruses, their prevalence and risk of disease; information that is essential in implementing appropriate disease prevention, control, and managing potential outbreaks.

1.2 Genome Organization of Bunyaviruses

Bunyaviruses are enveloped segmented, linear, single-stranded, negative-sense or ambisense RNA viruses (2). They are characterised by a tripartite (tri-segmented) negative-stranded RNA genome made up of L (Large) segment (encoding the RNA-dependent RNA polymerase), M (Medium) segment (encoding the two glycoproteins, Gn and Gc and a non-structural protein NSm) and S (Small) segment (encoding the nucleocapsid protein, N, and a smaller non-structural protein, NS) (27,28). They are prone to genetic reassortment among related viruses during mixed infection and co-circulation resulting in major phenotypic and genotypic changes, with potential for novel viruses (4). This is because reassortment can occur when two closely related bunyaviruses concurrently infect the same susceptible cell, therefore, their genome segments may be variously incorporated into the progeny viruses (29). Such is the case

of the NRIV (L_{BUNV} M_{BATV} S_{BUNV}), believed to be as a result of reassortment between BUNV and BATV (30–34). Reassortment has been reported in closely and distantly related orthobunyaviruses and also between viruses of same and different serogroups, and has been noted as the underpinning factor in the evolution of bunyaviruses (1,4,30,35–37). The reassortment and recombination mechanism of these viruses in nature has made antigenic classification challenging, confounding their characterisation and limiting detailed description (4,38). Reassortment and generation of new viruses can lead to enhance virulence as is the case of Schmallenberg virus (SBV) and NRIV which are believed to be more virulent than their parental strains; reassortment also widens the host range and geographic spread (29,38).

1.3 Classification of Bunyavirales

Bunyavirales are classified into 14 families but the most important for human and animal health belong to five families namely *Peribunyaviridae, Phenuiviridae, Nairoviridae, Arenaviridae and Hantaviridae* (2). These families are either arthropods or rodent-borne viruses.

1.3.1 Vector-borne Bunyaviruses

Arboviral bunyaviruses of veterinary and human health importance are found in the three families: *Peribunyaviridae*, *Phenuiviridae* and *Nairoviridae*. Those of greatest health importance are found in the genus *Orthobunyavirus* (family *Peribunyaviridae)*, genus *Phlebovirus* (family *Phenuiviridae)* and genus *Orthonairovirus* (family *Nairoviridae)*.

According to the International Committee on Taxonomy of Viruses (ICTV) (2), the genus *Orthobunyavirus* is the largest group in the family *Peribunyaviridae* and consists of 103 viral species including *Bunyamwera orthobunyavirus* and *Shuni orthobunyavirus* species among others. The *Bunyamwera orthobunyavirus* species is the largest in the genus *Orthobunyavirus* and consists of 10 individual viruses including Bunyamwera virus (BUNV), Ngari virus (NRIV) and Germiston virus (GERV) among others (1,38). These viruses are known to cause human and animal diseases; however, with the exception of BUNV which is considered the prototype member of both the genus *Orthobunyavirus* and the order *Bunyavirales* (27,30,39,40). BUNV was first isolated during a yellow fever surveillance programme in the Semliki Forest, Uganda from *Aedes* mosquitoes in 1943 (41). BUNV causes mild symptoms, such as fever, joint pain, and rash, whereas BATV causes flu-like symptoms in humans and genetic defects and abortions in livestock (30,42).

NRIV is a natural reassortant of BATV of *Batai orthobunyavirus* species and BUNV (*Bunyamwera orthobunyavirus* species) (2,32,34). The L and S segments of NRIV originate from BUNV and the M segment from BATV (32–34). NRIV was first isolated in South Eastern Senegal in 1979 from *Aedes simpsoni* mosquitoes (3,36,43) and later recovered from *Aedes* spp., *Anopheles* spp., and *Culex* spp. in many West African countries (44). In humans, the virus was first isolated from two patients in Dakar in 1973 (3,43), and later associated with haemorrhagic fever in two patients in North Eastern Kenya and Southern Somalia during the 1997-1998 RVF outbreaks (30,32–34,45). NRIV is more virulent than the 'parent' viruses and has been confirmed as a probable cause of fatal haemorrhagic fever (3,30,45–47).

BATV belongs to the *Batai orthobunyavirus* species. It was originally isolated from *Culex gelidus* mosquitoes collected in Kuala Lumpur, Malaysia in l955 by the U.S. Army Medical Research Unit. Later, in 1988, it was isolated from patients with malaria like symptoms in Sudan (48,49). BATV is transmitted by several mosquito species, however transmission by ticks and culicoides has also been reported (32). BATV is the most geographically widespread orthobunyavirus occuring in Asia, Europe and Africa and affects both livestock and human (48,50). Other important orthobunyaviruses belong to the *Schmallenberg orthobunyavirus* species. These include Sathuperi virus (SATV), Shamonda virus (SHAV), Douglas virus (DOUV), and Schmallenberg virus (SBV) (1). SBV was first reported from the border of German and Netherlands during an outbreak characterised by abortion and congenital deformities in cattle offspring in 2011 (51,52). Initial studies reported SBV as a reassortant orthobunyavirus between SATV and SHAV but later studies have proved otherwise, although the SBV is known to have a high genetic relationship with the two viruses (53). Shuni virus (SHUV) of the *Shuni orthobunyavirus* species is another important orthobunyavirus. SHUV is a zoonotic orthobunyavirus first isolated in Sokoto, Nigeria from a cow in 1966 as well as in a in a febrile child (54,55). Later, the virus was recovered twice in South Africa (SA), from mosquitoes (*Culex theileri*) and healthy cattle in 1972 and from horses suffering meningoencephalitis. Recently, SHUV has been reported in humans with neurological signs in South Africa as well as in malformed ruminants and neurologically infected cattle during an outbreak in Israel (56–58). SHUV is, therefore, an important zoonotic orthobunyavirus with potential to spread worldwide.

The family *Phenuiviridae* consists of 10 genera of which those of veterinary and human public health belong to the genus *Phlebovirus,* made up of 66 viral species such as *Rift valley fever phlebovirus* of Rift valley fever virus (RVFV), *Sicilian phlebovirus* species of sandfly fever Sicilian virus (SFSV) and *Toscana phlebovirus* species for Toscana virus (TOSV) among

others (1). RVFV is a zoonotic phlebovirus and infects both domesticated livestock and humans, and endemic in some parts of Kenya (59,60). RVFV was first reported in 1931 following a disease outbreak among sheep on a farm in the Kenyan Rift Valley. It causes abortion in pregnant animals and high mortality rates in infant ruminants, especially sheep and goats (61,62). Other than various African countries where outbreaks have been experienced from time to time, RVF was first reported outside Africa in Yemen and Saudi Arabia in the year 2000 (61). The most recent RVF outbreak in Kenya was reported in Murang'a, Garissa, Mandera and Isiolo, counties in 2021 (WHO Report, 2021). During this outbreak, both humans and animals were infected with a reported human case fatality rate (CFR) of 34% (CFR 34 %). Other important phleboviruses include sandfly borne phleboviruses such as TOSV, SFSV and SFNV that have been reported in various parts of the world (63,64) including Kenya (20) and have the potential to infect both humans and animals. TOSV, SFSV and SFNV are endemic in the Mediterranean area and are commonly known to cause febrile illness (65,66). TOSV is a neurotropic arbovirus first isolated in 1971 from the sandfly *Phlebotomus perniciosus* in central Italy and a cause of viral meningitis (66–68). This sandfly borne phleboviruses are among the less studied arboviruses and their human and animal health impact and disease burdens are not well understood (66,69,70), especially in Africa where fewer studies have been done. Recent studies in Baringo county, Kenya revealed the circulation of novel sandfly borne phleboviruses, NTPV with potential to infect humans and livestock and others like PERV, EMRV, BOGV, and KBGV (20,21).

The family *Nairoviridae* is made up of seven genera of which those of importance to human and livestock health belong to the genus *Orthonairovirus* (1, 74). Orthonairoviruses are a significant group of mainly tick-borne viruses that includes pathogens of humans (CCHFV) and livestock (Dugbe virus (DUGV), NSDV) (73). NSDV causes acute haemorrhagic gastroenteritis in sheep and goats, with very high morbidity and mortality rates of up to 90% in susceptible animals (74). CCHFV causes severe disease in human beings, with a reported mortality rate of up to 40% according to the World Health Organization (WHO) (75). In animals, there are no reported cases of illness or death although CCHFV has been isolated from domesticated and wild animals and therefore considered as amplification hosts (76). The symptoms of CCHFV infection in humans and NSDV in sheep are very similar and are characterised by haemorrhage, myalgia, and fever (73).

1.3.2 Rodent-borne Bunyavirales

The important public health zoonotic rodent-borne bunyaviruses are arenaviruses and hantaviruses. Arenaviruses belong to the family *Arenaviridae*, which is made up of four genera (1). The genus *Mammarenavirus* consists of 42 species which include *Lassa mammarenavirus* species, Lassa virus (LASV), and *Lymphocytic choriomeningitis mammarenavirus* species, lymphocytic choriomeningitis virus (LCMV) among others (1,77,78). LASV causes Lassa fever, an acute viral haemorrhagic illness endemic in west Africa (79–81). The natural host of LASV is *Mastomys natalensis* although the virus has also been detected in other *Mastomys* spp. as well as other rodents, like in *Rattus* spp. and *Mus* spp. (82).

Hantaviruses belong to the family *Hantaviridae* which is divided into 4 subfamilies. The sub-family *Mammantavirinae* consists of four genera. Of human importance is the genus *Orthohantavirus,* made up of 38 species including *Hantaan orthohantavirus* species and *Puumala orthohantavirus* species among others (1). Hantaviruses are known to cause Hemorrhagic Fever with Renal Syndrome (HFRS) and Hantavirus Cardiopulmonary Syndrome (HCPS) with up to CFR of 40% (83,84).

Hantavirus has only been reported once in Kenya in an African wood mouse (*Hylomyscus endorobae),* collected from Mount Kenya in 2010 (5), and more recently, a report on seroevidence in patients visiting hospitals in Nairobi (85).

1.4 Bunyavirales Hosts

1.4.1 Invertebrate Hosts

The transmission cycle of arboviral bunyaviruses require an arthropod vector for replication before transmission to other vertebrate hosts (86). This form of transmission is biological, but arthropods are also capable of transmitting viruses mechanically (through virus contaminated mouth parts) where the virus does not undergo any replication in the vector, but it is simply transferred by a vector from an infected to a susceptible host. The direct transfer of the virus during co-feeding from an infected to an uninfected vector on a naïve host has also been reported as in case of CCHFV (87).

Important hematophagous arthropods that transmit viruses to animals are mosquitoes, ticks, sandflies, cimicid bugs and biting midges (88,89). Mosquitoes are the most common vectors responsible for very serious arboviral disease outbreaks like RVF, therefore, they have always been prioritised in various vector monitoring programs followed by ticks, neglecting other blood-feeding vectors such as sandflies (90). Other than RVFV, mosquito-borne bunyaviruses

include BUNV, NRIV, SHUV, BATV (30,91).

Ticks are vectors of some important bunyaviruses like CCHFV that pose a significant threat to human health and others like NSDV that are important to livestock (10,92). The biting midges (*Culicoides*) transmit many arboviruses to humans, livestock, and wildlife some of which are notifiable according to the World Organization for Animal Health (WOAH) (17,93). *Culicoides*-borne bunyaviruses include SBV, Oropouche virus (OROV) and Akabane virus (AKAV). SBV is an orthobunyavirus of veterinary importance first detected in Germany-Netherlands border in 2011 and was initially believed to be as a result of virus reassortment between two other orthobunyaviruses SATV and SHAV (94,95). OROV was first discovered by isolation in Trinidad in 1955. It is the only bunyavirus implicated as primarily transmitted by *culicoides* to and among humans and is responsible for febrile illness major epidemics in the Caribbean and South and Central America (96). Akabane virus is an orthobunyavirus of veterinary importance. It causes late-term abortions, stillbirths and congenital disease in ruminants, mainly goats, sheep, and cattle. It is mainly distributed in parts of Asia, Africa, the Middle East and Australia (97–99).

Sandflies feed on different animals such as cold-blooded vertebrates, birds, and mammals but their trophic preferences vary depending on the sandfly species. They can transmit several phleboviruses like TOSV, SFNV, and SFSV as well as some novel viruses like the recently discovered phleboviruses from Baringo county in Kenya, yet in contrast with a high interest for parasitic diseases such as leishmaniasis, they remain neglected vectors of viral diseases (69). Out of the 66 viral species within the genus *Phlebovirus* currently recognized by the International Committee for the Taxonomy of Viruses (ICTV), the majority are vectored by sand- flies. Other novel unclassified sandfly borne phleboviruses underline the importance of these neglected vectors (100). Sandfly is, therefore, an important vector of human /veterinary diseases and public health importance.

Generally, other than primary vectors known to be responsible for natural transmission of arboviruses, the viruses have been reported in other vectors through detection, isolation as well as laboratory-based studies investigating vector competence. NRIV is primarily known to be transmitted by mosquitoes (*Aedes* spp.), but it has as well been reported in ticks (101). The principal vector for RVFV is floodwater (*Aedes* spp.) though the virus has as well been reported in other mosquito species as well as sandflies (102,103).

For some bunyaviruses, vectors can as well serve as reservoir hosts, in that, they not only transmit the virus, but they can maintain virus for life and transmit it to their offspring via eggs. The eggs later hatch and the progeny vector can transmit the virus. This is the case with tick-

borne CCHFV (*Hyalomm*a ticks) and RVFV flood water transmitting mosquitoes (*Aedes* spp.) (104–107).

1.4.2 Bunyavirales vertebrate hosts

Mammals and birds are the major amplifier hosts for bunyaviruses. The type of vertebrate hosts can determine the potential of virus dispersal, for instance, migratory birds can facilitate virus movement over large distances. Consequently, the wide distribution of BATV has been associated with birds in a bird-mosquito enzootic cycle (48). Whereas, most land-based animal hosts create a restricted virus activity (87), movement of infected volant viraemic host can transfer the virus to a disease-free destination (108).

Generally, wildlife and livestock hosts contribute significantly to arbovirus transmission and maintenance (10). According to Kuno et al., (2017), the viruses circulate in wild animals but only occasionally or incidentally infect humans and /or domestic animals which are in most cases dead-end hosts. This is because the hosts may not be able to maintain virus amplification and spread, however, for some viruses like RVFV, humans and domestic animals may serve as the basis for amplification (8). In RVF cases, mammalian hosts are amplifiers, particularly cattle and sheep (109). Secondary amplification results in high spill over to humans (60,110). Certain adaptations by arboviruses, like alteration of host ranges to include certain domestic animals, enhance virus amplification in proximity to humans (111). Massive arboviral disease outbreaks occur when the reservoir hosts also serve as an amplification host along with the vector in the enzootic cycle (8).

Animal movement either through migration in search of water and pasture, trade or relocation by people play a big role in the introduction and spread of arthropod vectors to new environments (86), therefore, establishing a novel transmission cycle or allowing continuous transmission of a novel arbovirus (61).

Peri-domestic animals like rodents which are known reservoirs of pathogens, including many viral families, some of which live in close proximity to humans and in close contact, thereby presenting serious health implications (112). Rodents are suspected reservoir hosts of certain arboviruses such as TBEV (61) and may play an important role in amplification and transmission of these viruses (113).

Reservoir hosts are not only important for continued virus existence but for virus transmission as well. They can act as either maintenance or amplifier hosts. High titer viraemia produced by virus infected reservoir hosts enables virus transmission to occur. However, reservoir hosts are generally not susceptible to disease (114), for instance; livestock are known to be reservoir

hosts for CCHFV, yet clinical symptoms are not reported in animals infected with the virus. More than one host species may be involved in bunyavirus transmission cycles (87).

Disseminating hosts are responsible for virus movement, for instance, from an area where the virus is actively circulating to a naïve location (115,116). Viraemic birds have been confirmed to play a role in the spread of some bunyaviruses, including TOSV (87,117,118). Animals and human movement can aide the introduction of bunyaviruses into new areas. For livestock, this can occur through trade and for humans, air travel and long distance travel during the viraemic phase is of particular importance (119).

In incidental and dead-end hosts like humans and other vertebrates, the viraemia level is too low to establish infection in a vector and therefore, cannot allow transmission of bunyaviruses to other hosts. Though they get infected, the transmission does not occur with enough regularity for stable maintenance (61). Incidental hosts may be symptomatic or asymptomatic but due to low vireamia virus transmission is less likely/not possible (87).

1.5 Mechanisms of bunyavirus transmission

The transmission cycle of arboviral bunyaviruses can be horizontal, vertical, or venereal. Horizontal transmission is the primary cycle typically occurring in the sylvatic setting in which an infected vector transmits the virus to a vertebrate host notably rodents, birds or non-human primates and naïve vectors acquire infection following a viraemic blood meal. The virus transverses the midgut following ingestion of an infected bloodmeal, and then spreads through the alimentary tract and replicates in the salivary glands before infecting a new vertebrate host during feeding (111). Many bunyaviruses are naturally maintained in a secondary cycle that involves vertical transmission from the female vector to their offspring. Venereal transmission allows for virus transmission from infected males to females during copulation (120).

Bunyaviruses circulate among wild animals whereas humans and/or domestic animals are incidental or dead-end hosts, but in some cases, they may be amplifiers (8). This way the viruses must attain sufficient concentration or viraemia in the amplifying vertebrate host for a permissive arthropod to become infected during a blood meal. In enzootic cycles, dead-end hosts like human and domestic animals might develop only low levels of viraemia with a low chance of passing enough virus to establish an infection in the arthropod vector (121). However, the converse does not always apply; despite cases of high viraemia, for instance, in CCHFV and RVFV infection, there was no evidence of vector transmission to naïve vectors. The reasons for this are not known but may be either to limited exposure to arthropod feeding

or other host/vector factors (122).

Arboviral bunyaviruses may infect humans via a range of mechanisms (111). For instance, replication of RVFV in livestock increases human exposure to the virus. When a reservoir host becomes an amplification host along with the vector in an enzootic cycle, then substantial arboviral disease outbreaks can occur (8).

Arenaviruses and hantaviruses are transmitted through inhalation of aerosolised virus, consumption of contaminated foods and through contact with the virus when shed in saliva, urine, and faeces, but less frequently by a bite from an infected host (123,124) (**Figure 1.1**). Human-to-human transmission may occur through vomitus, blood, infected urine, faeces, saliva, and during organ transfer and blood transfusion (125). However, an exception of hantavirus human-to-human transmissionthrough body fluids and respiratory aerosols during the close contact has been reported for Andes virus (ANDV) in Chile and Argentina (125–127), although a recent review by Toledo et al. 2021, showed some limitation to these reports (128).

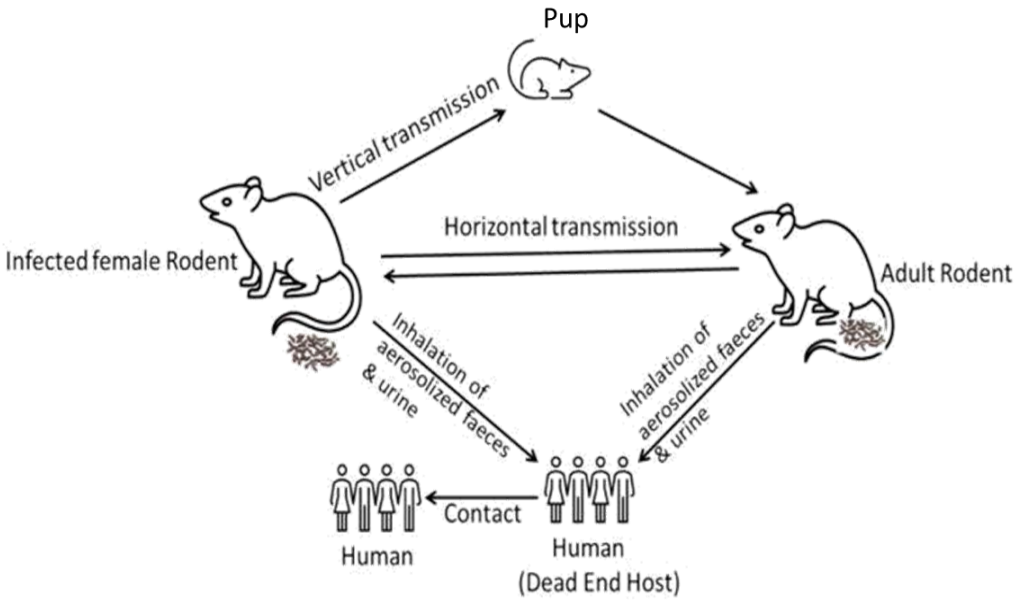


Figure 1.1: Transmission cycle of rodent-borne viruses

1.6 Factors that affect vectorial capacity

Important factors that affect arbovirus transmission include arthropod susceptibility to infection and vector competence, breeding and biting habits, hosts availability, abundance of arthropods, and ecological systems interactions like climate change (87).

1.7 Clinical features of Bunyavirales infection

The major clinical syndromes of bunyaviral infections include systemic febrile disease, haemorrhagic fever, neurological diseases (meningitis, encephalitis, encephalomyelitis), and polyarthralgic illness, and can result in death. However, most infections are asymptomatic or present as nonspecific mild febrile illnesses and only a few cases result into the severe disease. Bunyaviruses like RVFV in severe cases can infect the central nervous system, causing encephalitis (129). Haemorrhage commonly caused by viruses like CCHFV and LASV are among the most serious manifestations of bunyavirus infections and believed to be as a result of interference with blood coagulation factors (130). Other syndromes include abortion in livestock (sheep, goats, and cattle) as observed with RVFV, SBV and BATV infection (10,108).

Generally, systemic febrile illnesses caused by bunyavirus infections are often confused with other common endemic diseases like malaria and typhoid by clinicians in the tropics. They are therefore treated as such or as fever-of-unknown origin (FUO) and are normally misdiagnosed. This has contributed to the underestimation of disease burden attributed to bunyavirus infections (131). Polyarthralgia during these infections is due to inflammation caused by immunological response mediated by the virus (87). Sandfly borne phleboviruses generally cause sandfly fever, a febrile illness but a more severe neuroinvasive disease may occur (66).

Co-infection with bunyaviruses is a common occurrence in different parts of the world, especially those viruses sharing the same hosts and vectors like RVFV, NRIV, BATV, BUNV (33,46). The resultant effect of co-infection with these viruses is still not well understood but different outcomes, like enhanced expressed illness due to synergy, identical clinical presentation due to competition between viruses, or lack of infection due to inhibition of both viruses are expected (132). Human infection with *Bunyamwera orthobunyavirus* and *Cachey Valley orthobunyavirus* can as also result in haemorrhagic and severe acute neurological disease (30,133).

CCHFV causes haemorrhagic fever in humans with a 10-40% CFR according to the World Health Organisation (WHO) report of 2019 (134). No clinical disease has been reported in

wild and domestic animals and are therefore believed to be amplification hosts that play an important role in virus maintenance.

LASV causes Lassa fever, also known as Lassa hemorrhagic fever (LHF). Other than fever, other symptoms include weakness, malaise, and gastrointestinal symptoms. Progression to severe illness includes shock and haemorrhage, and is common in expectant women and may result in abortion (80,81,135). LCMV causes Lymphocytic choriomeningitis (LCM) characterised by flu-like illness or aseptic meningitis, sometimes with rash, arthritis, orchitis, parotitis, or encephalitis (136–138).

Orthohantaviruses typically cause chronic asymptomatic infection in rodents but severe disease such as haemorrhagic cardiopulmonary syndrome (HCPS) and haemorrhagic fever with renal syndrome (HFRS) with up to 40% CFR in humans (25,83,139,140). HFRS is common in Europe and Asia with HPS in the Americas (141).

1.8 Public health and economic importance of Bunyavirales

Bunyaviruses cause human and animal diseases and even death globally, which results in health, social and economic challenges (120). There is a high economic impact from these diseases due to the burden of treatment and hospitalization, and loss of productive hours (142). Diseases like RVF, are Transboundary Animal Diseases (TAD) as they are highly contagious and can spread rapidly across national borders, having a great impact on both local and international animal trade and other agro-industries (143). They cause significant economic losses in domestic animals and are notifiable to the World Organization for Animal Health (WOAH, 2012). These diseases negatively impact the pastoralist communities, farmers, and the nation at large. For instance, in the case of RVF, estimated losses estimated to range from $5 to $470 million USD were reported (143). For instance, in Kenya, the 2006-2007 RVF epidemic resulted in an estimated economic loss of more than 2.1 billion Kenyan Shillings (USD32 million) (144).

The rodent-borne bunyaviruses have the potential to cause epidemics in human populations. Lassa fever is a disease of public health importance in several African countries, especially West Africa. As with other febrile illnesses, Lassa fever initially has nonspecific clinical symptoms and is very contagious and can progress to haemorrhagic disease with a high mortality rate. It causes premature births and spontaneous abortions in pregnant women (79,81).

LCM commonly causes mild febrile illness and is usually not fatal but can progress to

neurological illness and mortality is less than 1% of patients. The infection can result in neurological damage, deafness, and arthritis. LCMV infected pregnant women can pass the infection on to the foetus resulting in foetal death, abortion, birth defects like vision problems and mental retardation (137,138). Hantavirus HFRS and HCPS infections can result in a mortality rate of up to 40% (25,83,139,140).

1.9 Drivers of bunyavirus infection emergence/spread.

The overall representation of arboviral diseases including those caused by bunyaviruses is nearly 30% of all emerging and re-emerging infections worldwide (145), and several factors like socio-economic, competent vectors, vertebrate hosts and environmental factors contribute greatly to their emergence and outbreaks (8,121). Bunyaviral diseases are among neglected tropical diseases (NTDs) which significantly impact humans/animal (146). A high percentage of novel humans emerging infectious diseases (EIDs) are zoonoses caused by viruses that originate from animals, and as humans expand their surroundings by encroaching into wildlife habitat like forests, they come closer into contact and interact more with wildlife reservoirs, and this increases the risk of the emergence of novel viral diseases in domestic animals and humans. Hence, improved discovery and understanding of novel viruses in humans, wildlife and domestic animals through surveillance will act as an early warning sign, assist in developing diagnostic methods and play a major role in effective disease prevention and control (147).

1.10 Laboratory methods for Bunyavirales diagnosis

Pathogenic bunyavirales infections initially present as generalized febrile illnesses and have been in many cases confused with other common and endemic infections in the tropics like malaria and typhoid and treated as such, resulting in misdiagnosis. This has contributed to the underestimation of disease burden on the population. Another challenge to detection/diagnosis of these diseases is the lack of diagnostic capability (expertise, availability of right equipment and proper guidelines on case definition) in routine health care and cross-reactivity of available and affordable serological techniques (ELISA, IFA) due to close antigenic relatedness/similarity among related viruses resulting to nonspecific detection/identification (131). However, different methods are applicable for diagnosis:

1.10.1 Virus culture, isolation, and characterisations

Cell culture is routinely and widely used for the isolation of viruses. Detection and identification of viruses after cell culture can be done in different ways including observation of morphological changes like cytopathic effects (CPE), haemadsorption, heterologous interference, and transformation due to oncogenic viruses in the cultured cells in which the virus replicates in (148). Virus isolation has been used to detect various novel viruses including bunyaviruses before subsequent characterisation. It is therefore the gold standard method for virus diagnosis and detection. Different methods of virus isolation include tissue culture, use of embryonated eggs and laboratory animals.

After isolation, the virus can be characterised further through next generation sequencing allowing for the development of before specific primers to screen samples and determine the prevalence of that novel virus. Like in metagenomics, screening of samples through virus culture is not specific to any virus and in the process different unknown viruses can be isolated. For instance, during this study, a novel orbivirus was isolated through virus isolation in cell culture and subsequently sequenced and characterised. Specific primers were then designed, and samples tested retrospectively to determine the prevalence of the virus. This wasn't targeted as part of the study objective; however, the findings are important as it provides a basis for establishing the molecular epidemiology of the novel virus as well as pathogenicity studies**.**

Isolation of viruses and pathogenicity studies by animal inoculation involves the use of animals like mouse, rabbits, hamsters, newborn or suckling rodents and inoculation is done through intracerebral, subcutaneous, intraperitoneal, or intranasal routes depending on the type of the virus, after which the animals are observed for signs of disease or death (149). This method is important in establishing virus pathogenicity and host range. Mice are commonly used in many studies, for instance, in establishing the pathogenicity of the novel orbivirus detected in this study.

When arboviruses are isolated in cell cultures, they can easily be identified by monoclonal antibody binding in immunofluorescence antibody (IFA), enzyme immunoassay (EIA) or neutralisation assays.

The gold-standard method in conducting pathogenicity studies of viruses detected by other methods like metagenomics is virus isolation either through cell culture using a variety of cell lines or suckling mice inoculations (112).

1.10.2 Serological methods

Serological diagnosis remains the main routine diagnostic method for most virus infections, and it involves the detection of antibodies against viruses. This includes enzyme immunoassays, immunofluorescence, haemagglutination inhibition, neutralisation and complement assays (150). These techniques can detect different antibodies, including neutralizing antibodies. Recent/Active infections can be confirmed by either increase in antibody levels or screening for specific antibodies known to be produced during an active infection like Immunoglobulin M (IgM) for RVFV infections (87). Cross-reactivity of closely related viruses is a problem with serological assays and may lead to a misdiagnosis. The two serological methods employed in this study were Enzyme Linked Immunosorbent Assay (ELISA) and Plaque Reduction Neutralization Test (PRNT). ELISA was specifically for CCHFV seroprevalence studies. PRNT is the gold standard serological method for viruses and based on the neutralization of live viruses by antibodies present in serum samples. It is mainly applicable for viruses that do not require the highest level of containment.

1.10.3 Molecular Based methods

Molecular methods used in detection and discovery of viruses including arboviruses are hybridization-based methods like microarray techniques, PCR-based methods, and metagenomics (151). These methods can be used to detect viruses that cannot be cultured and to confirm the identity of cultured viruses.

Polymerase chain reaction (PCR)-based methods

PCR, one of the main methods employed in this study is used for virus antigen detection/discovery through identification of viral nucleic acid. It allows the detection of virus antigen as well as non-viable viruses through the use of virus-specific primers designed to amplify a specific region of the virus genome and therefore requires prior knowledge of targeted or closely related viral genomes (148). The result of most of these PCR methods is amplified DNA that may require definitive identification by sequencing; using the Sanger method and bioinformatics analysis, such as phylogenetics, to provide detailed genetic information (112,129).

Even though bunyaviruses exhibit a very short viraemia, the virus can be detected in blood, but it can be technically challenging. However, various sample enrichment methods like centrifugation, nuclease treatment, host DNA depletion (HDD) and filtration can be used to increase the availability and yield of virus nucleic acid from the blood and other biological

samples. Other samples that can be used for virus detection include Cerebrospinal Fluid (CSF) and tissues samples including postmortem tissues (87).

1.10.4 Virus discovery and Metagenomics

Metagenomics is an important method in detection of novel viral species including arboviruses that can be of potential threat to human and animal health (112). This method is important in virus discovery specifically for viruses that cannot grow, or are difficult to grow in cell culture, or which do not induce a cytopathic effect in cells. On the other hand, when PCR can be used in detection, developing primers becomes a challenge due to lack of common viral genes that can be targeted and high genetic diversity between viruses; in this case metagenomics is applied (147).

Although high throughput sequencing is expensive and the resultant bioinformatics data analysis demanding, this technique does not require prior knowledge on virus genome as there are no specific antibodies or primers required and therefore allows for discovery and characterisation of novel viruses (152,153).

1.11 Geographical distribution of Bunyavirales and associated epidemiological factors.

Bunyaviruses are distributed worldwide with a high proportion of those associated with human and animal diseases circulating in tropical and subtropical regions, where vectors are abundant. The virus circulates almost throughout the year and in broad seasonal peaks (121,129). In temperate climates, the disease is common in warmer months and absent in colder months while in cooler climates; the virus persists by overwintering in eggs (130). However, there are several reports of these viruses in new areas where they have not been recorded before.

Several factors that contribute to the emergence and broad distribution of these infections include socio-economic, environmental, ecological and biological factors like vector and virus mutations (121). Global warming and climate change are contributing to the emergence, reemergence and spread of infectious diseases including bunyaviral infections (86,154). For instance, climate change may lead to increased rainfall and flooding presenting perfect breeding grounds for flood water mosquitoes known to transmit bunyaviruses like RVF and NRIV and can result in disease outbreaks. Increased rainfall promotes farming and food production, the farms present a conducive habitat for rodents and increased risks of rodent-borne diseases. Animal and human movement has as well contributed to the spread of various bunyaviruses like CCHFV as ticks engorged on livestock can be transported over a wide area

resulting in introduction of disease in new environments as well as naïve populations. Livestock trade largely contributes to the spread of arbovirus vectors. Therefore, the introduction of animals may play a role in the introduction of arthropod vectors, and associated pathogens, to new environments (86).

Some arboviral bunyaviruses originated and are actively circulating in Africa. These viruses include RVFV, BUNV, GERV, ILEV, NRIV, SHUV and CCHFV, amongst others (8,61,155,156). Sandfly borne phleboviruses have been primarily reported in the Middle East and Central Asia, Africa, the Mediterranean region, and the Indian subcontinent (66). TOSV has been isolated in northern African countries like Morocco, Tunisia, Algeria, and Sandfly Fever Silician Virus (SFSV) has been reported in Ethiopia (157).

Kenya has in the past, experienced several outbreaks of endemic arboviruses as well as sporadic cases or evidence of exposure to less known arboviruses like BUNV, NRIV, CCHFV, GERV and others have been reported (20,91,129,131,158–161). Data from serological and virus isolation from vector studies have also shown a wide distribution of bunyaviruses throughout the country, indicating the potential local public health risk posed by to these viruses (20,91,158,159,162,163). There has been an increase in undiagnosed febrile fever cases in hospitals and unexplained livestock disease outbreaks in different parts of the country that would necessitate active investigation. The recent RVF outbreak in Kenya in June 2018 according to the WHO report of 2018, resulted in humans CFR of 23% in Wajir with many cases confirmed in designated RVF high-risk counties like Marsabit and Wajir, but also affecting low-risk areas like Siaya (164). In high-risk areas, increased deaths were reported in camels and goats. I n recent year, RVF cases have increased in endemic areas like Murang'a and confirmed cases in low-risk areas like Nyandarua have been confirmed, suggesting the possibility of increased virus and vector activity, an indication that the virus could be becoming more virulent and the vectors more competent, therefore, expanding their biological distribution to new ecological regions (59,60,164). This could be generally due to socio-economic, environmental, and genetic factors. As RVFV cases continue to be realised in new environments, there is a possibility that other closely related phleboviruses and other bunyavirales could also be too in circulation as was the case during the 1997-1998 and 2006-2007 outbreaks where BUNV and NRIV were also isolated, and co-circulation of these viruses was confirmed (91,161,165). Therefore, there is a need to determine the circulation of these neglected, less known yet potentially harmful arboviruses. Other animal arboviruses that affect livestock like NSDV may be in circulation yet their true prevalence is not known and some

could be the cause of undiagnosed livestock death that is very common in different parts of the country especially among the pastoralist communities (131).

Toscana virus, sandfly fever Naples viruses (SFNV) and sandfly fever Silician virus (SFSV) are found in northern Africa and the Mediterranean region (65,166,167). However, serological evidence of humans infections with these viruses and others like Karimabad virus (KARV) and the Gabek Forest virus (GFV) have been reported in Sub-Saharan Africa, including Uganda, Ethiopia, Somalia and Djibouti (168,169). The emergence and re-emergence of various bunyaviruses in different hosts and parts of the world, provide a clear indication of the continuous threat of the emergence of arboviruses.

Urbanization, deforestation, and global warming have created a conducive environment for vectors and led to the spread and emergence of vector-borne diseases. An increase in human population has led to increased international travel, tourism, shipping, and industrialization which has in turn widens the distribution of the diseases.

Due to the widespread distribution of rodents, rodent-borne viruses are widely distributed and have the potential to emerge and re-emerge in different parts of the world (26). Based on geographical location and antigenic properties, mammarenaviruses have been divided into Old World (OW)/Lassa–lymphocytic choriomeningitis serocomplex) that include viruses indigenous to Africa, Asia and the worldwide ubiquitous lymphocytic choriomeningitis virus (LCMV), and New World (NW)/Tacaribe serocomplex which include viruses from the Americas (77). In Africa, hantaviruses have been reported more widely compared to arenaviruses which are mainly reported in West Africa (5,23,170,171). Lassa virus is endemic in some countries in West Africa (79). In Kenya, there are few studies that have reported the presence of hantaviruses and arenaviruses (5,85,172).

1.12 Bunyavirales prevention, management, and control

The two main methods of controlling arboviral bunyavirus infections are vaccination and vector control, as there are no specific therapeutic antiviral agents available other than supportive and symptomatic treatment offered to relieve signs and symptoms (87).

Several vaccines have been developed for well-studied and characterised arboviruses like YFV and others, like DENV, are at different stages of development including transmission-blocking vaccines (TBV) (173), yet there is little on neglected arboviruses like bunyaviruses, the only exception being for RVFV (87). The RVFV inactivated vaccine is available for livestock use,

however, for humans, it is limited to individuals at higher risk of infection such as laboratory workers and veterinarians, not available for general use.

Vector control is methods are the most widely used approach for the prevention and control of arboviruses. The methods include elimination of vector breeding sites, use of indoor and outdoor insecticides to kill vectors and reduce human vector contact, and biocontrol (use of predators, parasitoids or infectious microbial agents to eliminate vectors) (129). However, vector control programs are not easy to sustain due to the costs involved and the negative effect on the environment, and in spite of all the measures that have been put in place, arboviruses continue to emerge in new geographical regions partly due to factors like resistance to insecticides and global warming. Therefore, new approaches like the use of Genetically Modified Vectors (GMVs) that are being exploited should be incorporated to prevent arbovirus emergence and spread (174–177).

Transmission of hantaviruses and arenaviruses from rodents to humans can be prevented by avoiding contact with rodent hosts, safely storing food in rodent-proof containers, and avoiding the use of rodents as food. Cleaning living areas including homes and the surroundings can discourage rodents from living and breeding therefore reducing their populations. Human to human transmission can be prevented by controlling contact with those infected through patient isolation, use of personal protective equipment (PPE) such as masks and gloves and sterilisation contaminated PPEs and equipment. Currently, vaccines are not available for hantaviruses or arenaviruses but several studies are presently underway to develop some (178–180).

Public education and awareness to enlighten people on these important bunyaviruses including control and prevention measures as well as developing robust diagnostic tests, drugs and vaccines can greatly limit spillover, infection and spread of diseases.

1.13 Conclusion

Bunyaviruses are co-circulating in the selected pastoralist dominated areas in Kenya in a transmission network involving livestock with potential health impact on human populations. However, these viruses evade detection due to prevailing poor surveillance and diagnosis, lack of reliable reporting systems and improper clinical screening methods. These challenges have ensured that most of these infections circulate undetected until an outbreak occurs. Therefore, there is a need for routine one health surveillance incorporating molecular and serological techniques of diagnosis as well as advanced methods of metagenomics and isolation attempts. Further research to understand the impact of these viruses on animal health and potential human exposure including during animal handling as is the case for RVFV, should be conducted.

1.14 Research Problem

Bunyaviruses cause serious public health/economic problems, form part of emerging and re-emerging diseases and contribute significantly to worldwide economic and disease burden. Irrespective of the negative impact of these viruses, only limited knowledge on the circulation and transmission networks among multiple host systems is available.

As a result of poor surveillance and laboratory diagnosis methods, unreliable reporting systems and improper clinical screening methods, the real public health and economic impact of these viruses to humans and livestock is most likely not known or is underestimated, generally in parts of Africa where major epidemics have originated and are endemic. In these African countries, Kenya included, there is limited epidemiological data available to determine the importance of these viruses to humans and animals and potential for emergence of novel viruses remains underestimated. This presents a high risk of large-scale arboviral disease outbreaks and epidemics. Focused surveillance system to provide information on arbovirus presence, prevalence, transmission networks and risk to public and animal health is essential in implementing appropriate disease control measures and managing future outbreaks.

1.15 Aim and Objectives

1.15.1 Aim

The aim of this study was to assess Bunyavirales activity and circulation in humans, livestock, and peri-domestic rodents in diverse ecologies in Kenya.

1.15.2 Objectives

1) To determine the seroprevalence of phleboviruses, orthobunyaviruses, and nairoviruses in domesticated livestock and peri-domestic rodents in selected ecological sites
2) To determine the seroprevalence of phleboviruses, orthobunyaviruses, and nairoviruses in febrile patients visiting health facilities in the same ecological sites
3) To identify viable circulating phleboviruses, orthobunyaviruses, and nairoviruses in humans, livestock and peridomestic rodents through cell culture
4) To detect and establish the molecular epidemiology and genetic diversity of circulating viruses in the order Bunyavirales found in humans, domesticated livestock and peridomestic rodents

Chapter 2

METHODOLOGY

Methodology for investigating humans, livestock, and rodents for the presence of Bunyavirales and novel viruses.

2.1 Introduction

The methods described in this chapter were used to address the aim of this study which is to screen rodents, livestock, and humans for viruses in the order Bunyavirales. The subsequent chapters will refer to these methods.

2.2 Study site, design, and sample size

Baringo (0.4695° N, 35.9833° E) and Kajiado (1.7617° S, 36.0255° E) counties are located in the Kenyan Rift Valley and have semi-arid ecologies with diverse populations and a history of arbovirus circulation (20,21,181–184) **(Figure 2.1**). Both ecologies are inhabited mostly by nomadic pastoralist communities.

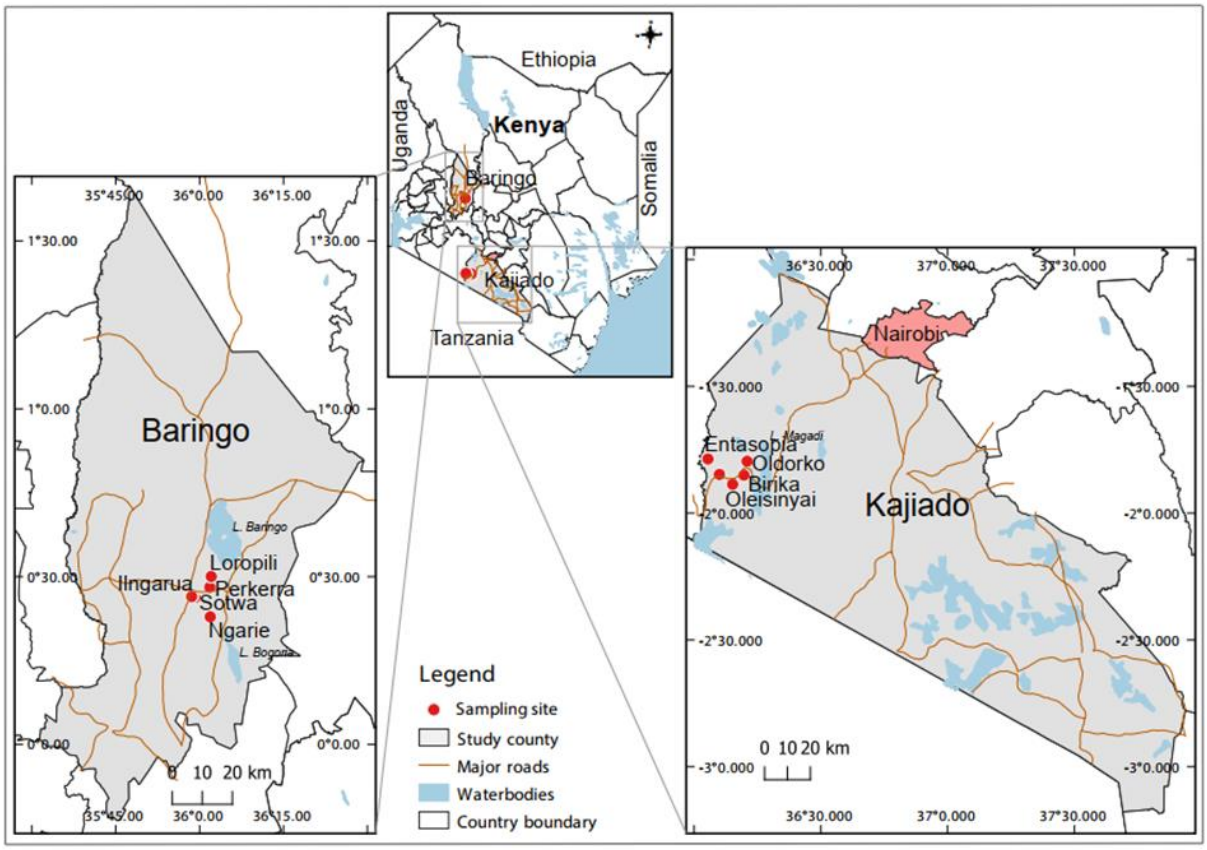


Figure 2.1: A map of Kenya showing the study sites in Baringo and Kajiado counties respectively shaded in grey. The red spots represent the selected locations within the counties where sampling was conducted. The maps were created in the open source GIS software, QGIS 3.22 using GPS co-ordinates and shape files derived from Natural Earth (http://www.naturalearthdata.com/, a free GIS data source) and Africa Open data (https://africaopendata.org/dataset/kenya-counties-shapefile, license Creative Commons) (185).

The study was a cross-sectional laboratory based, descriptive survey. The study was hospital-based, cross-sectional, descriptive survey of febrile malaria-like cases who report to the selected health facilities located in the selected study sites.

Sample size (*n*) was determined based on the livestock population in each area using the following formula:

$n = (z^{2)}\, p\, (1-p)/d^2$

where *n* is the sample size,

z is the z value for the confidence level (1.96 for 95% confidence level)

p is the expected prevalence/proportion of the virus in the target population (Estimated to be 50% as there is no data on the actual disease prevalence, $p=0.5$)

d the required level of precision/ confidence interval/margin of error, taken to be 5% (expressed as $d= 0.05$ means (±5))

Correction for a finite population used in calculating the sample size:

$n_2 = n/ (1+n-1/pop.)$

pop. is the livestock population.

Baringo South subcounty (Marigat) livestock population was goats (n=100,000), cattle (n=65,000) and sheep (n=60,000); Kajiado West subcounty (Nguruman) population was goats (n=70,000), cattle (n=80,000) and sheep (n=40,000). Based on this population, 95% confidence level, 5% confidence interval, and 50% estimated prevalence, the sample size was: goats-383, cattle -382, and sheep -382.

The sample size for rodents was dependent on the frequency of capture.

Livestock and rodent sampling were done concurrently twice a year for two weeks after the rainy seasons. Human sampling was done for one year during the period.

2.3 Sample collection

Humans, livestock, and rodent sampling was conducted in different locations within Baringo and Kajiado counties.

2.3.1 Livestock Sampling

A total of 2039 apparently healthy/asymptomatic livestock were assessed in this study, of which 1,239 heads were from Baringo county and 800 from Kajiado county. This total was made up of cattle (n=715), goats (n=680) and sheep (n=644), aged 1-3 years sampled by a team that included a registered veterinarian and/ or animal health technician. Sampling was done twice a year after the rainy seasons when the vector and arboviral activity were presumed to be high. Approximately 5mL whole blood was collected aseptically from the jugular vein of each animal into 10mL BD Vacutainer® blood collection with EDTA. Blood for serum was collected into 10mL BD Vacutainer® blood collection plain vacutainers precoated with serum activator. The samples were processed, aliquoted into cryovials and appropriately labelled. All samples were transported on dry ice from the field to the Martin Lüscher Emerging Infectious Diseases (ML-EID) Laboratory at ICIPE for immediate testing and/or storage at -80˚C until further screening.

2.3.2 Rodent Sampling

Rodents were trapped using the LFAHD Folding Live Capture Rodent/Rat/Mouse Traps (3 x 3.5 x 9” (7.62 x 8.89 x2 2.86 CM)) and SFA Small Folding Live Capture Rodent/Vole/Shrew/Mouse Traps (2 x 2.5 x 6.5” (5.08 x 6.35 x 16.51 CM)) (https://www.shermantraps.com/animal-traps/) set inside homes and their surroundings according to the guidelines set by the National Museum of Kenya (NMK). The traps were baited with a mixture of locally available peanut butter and white oats, opened at dusk, checked every morning, and left empty, and closed during the day. Two to four traps were set per room/surrounding environment for the whole night depending on observed rodent activity and inspections were carried out each morning. Each trapped specimen was placed in a handling bag, weighed, identified to the genus or species level based on morphological and geographical criteria according to Kingdom guide to African mammals and East African mammals as reference guides and further by molecular analyses (186,187).

Parameters like species, sex, age, weight were recorded from each trapped specimen before being euthanatized by cervical dislocation. Thereafter, the tissues (kidney, spleen, lungs, heart,

and liver samples) and blood if adequate were collected in 1.8 mL cryovials and 5 mL BD Vacutainer® tubes, respectively. The blood samples were thereafter processed, through centrifugation at 3000 rpm for 5 min and the serum aliquoted in cryovials. All samples were stored in liquid nitrogen before transportation to Emerging Infectious Disease (EID) Laboratory at *icipe*, Nairobi, where they were stored at -80˚C until screening.

A total of 489 peridomestic small mammals (rodents and shrews) were captured of which, 63.8%, (312/489) were captured in Nguruman and 36.2%, (177/489) in Marigat. Eleven of all the small mammals captured were shrews (2.5%) and the remaining 478 (97.5%) were rodents.

2.3.3 Human sampling

Human sampling was conducted in Marigat Subcounty Hospital (Baringo County) and Entasopia Health Centre (Kajiado County). Febrile patients (male and female) ≥5 years presenting with a clinical case definition of acute febrile illness characterised by fever (body temperature ≥ 38˚C) and with at least one of the following clinical manifestations: cough, joint pains, headache, chills, general body malaise, and any signs of bleeding and neurological abnormalities, who visited one of the two health care facilities, were formally recruited into the study after obtaining their written consent or assent for children from guardians.

A total of 5 mL of blood was collected from each participant into BD Vacutainer® serum tubes with a clot activator by a trained and licenced phlebotomist. Serum was then processed from the blood by centrifugation in Eppendorf™ 5702 Series Centrifuge at 1500 rpm for 10 min and aliquoted into 1 mL volumes. Samples were stored in liquid nitrogen at the health facilities until collection and transportation on dry ice to the EID Laboratory at *icipe*, Nairobi, where they were stored at -80˚C until screening.

In total, 493 human samples were collected, of which 323 (65.5%) were from Marigat and 170 (34.5%) from Nguruman, with a higher proportion of females (n=295, 59.8%) than males (n=198, 40.2%) being represented.

2.4 Screening methods

2.4.1 Serological methods for antibody detection

Serological methods were carried out to determine the prevalence of different bunyavirales in humans, livestock, and rodents.

Plaque Reduction Neutralisation Test (PRNT)

This method was used for detection of RVFV, BUNV and NTPV antibodies in human, livestock, and rodents as well as to confirm all the NRIV seropositive serum samples after IFA

to rule out any false-positive results that could be due to cross-reactivity of viruses in the same family.

Serum samples, suspected virus ($TCID_{50}$), Gibco DMEM media enriched with (10% Gibco FBS, 2% Gibco™ Antibiotic-Antimycotic (100X), Gibco™ MEM Non-Essential Amino Acids Solution (100X), Gibco™ L-Glutamine (200 mM) were used in the study. Vero cell line (ATCC® CCL-81) grown in 12 well tissue culture plates, 2.5% H7509 Sigma-Aldrich methyl cellulose (viscosity 2,600-5,600 cP), 3.7% (v/v) F8775 Sigma-Aldrich formaldehyde solution, 0.5% (w/v) C0775 Sigma-Aldrich Crystal Violet and Gibco Dulbecco's PBS pH 7 were used for the assay. Equipment used included a New Brunswick Galaxy 170S incubator, Leica DMi1 LED Cell Culture Microscope, 1300 Series A2 Class II bio safety cabinets from Thermo Fisher and Airstream® Class II Type B2 Biological Safety Cabinet.

Vero cells at a concentration of $1x10^6$ cells per mL were seeded in 12 well tissue culture plates at a volume of 2 mL per well and incubated overnight at 37˚C in a 5% CO_2 New Brunswick Galaxy 170S incubator. The cells were observed to ensure 70-90% confluency and even cell distribution before inoculation. The sera were aliquoted and heat-activated at 56˚C for 30 minutes in a water bath before serial dilution in a minimum essential medium (MEM). Two-fold serial dilutions (1:2) of sera samples were prepared in a microtiter plate, then 50 µL of the serially diluted serum was mixed with 50 µls $TCID_{50}$ of the suspected virus (constant amount), titer determined by plaque assay and the mixtures incubated at 37˚C in the presence of 5% CO_2 for 1 hour.

A co-incubated mixture of antibody and virus was used to infect seeded 12 wells culture plates containing confluent monolayers of Vero cells after pouring off the growth media and incubated at 37°C for 60-90 minutes in a 5% CO_2 incubator. After incubation, 2mL of 2.5% methylcellulose was added to each well and incubated at 37˚C in a 5% CO_2 incubator for 4-14 days depending on the virus. The plate was fixed with 3.7% (v/v) formalin by pipetting 2mL per well for a minimum of 2 hours and then stained with 0.5% (w/v) crystal violet solution at 2mL per well overnight, washed, dried and the plaques counted. The number of plaques in an individual plate was divided by the original number of virions to calculate the percentage neutralization. The neutralizing titer (the reciprocal of the highest dilution of serum or antibody at which the virus is neutralized in 50% of the wells) was determined. PRNT90 positive samples were determined as the reciprocal of the serum dilution, giving ≥90% reduction in plaque counts (188).

Indirect Immunofluorescent Assay (IFA)

This assay was carried out to detect the presence of NRIV antibodies in humans and livestock serum samples. Custom made NRIV-antigen coated IFA slides were used to test for the antibodies in serum. One hundred and forty-four randomly selected serum samples from cattle, sheep and goats were screened for anti-NRIV IgG antibodies using a modified NRIV IIFA slide test kit (Euroimmun; Lübeck, Germany). The slides contained a mixture of KE_O93 NRIV isolate and non-infected Vero E6 cells in a 1:1 ratio fixed on each well except the negative control well contained non-infected Vero E6 cells. The serum samples were diluted at a ratio of 1:10 with sampling buffer, and 25 µL of the diluted samples applied to the biochip and incubated for 30 min at room temperature. After incubation, the slides were washed twice, 5 min each in wash buffer containing phosphate-buffered saline (PBS), pH 7.2 and 0.2% Tween 20. Twenty-five microlitres of Alexa 488 labelled donkey anti-sheep IgG antibodies, donkey anti-goat IgG antibodies, and goat anti-bovine IgG antibodies (Dianova, Hamburg, Germany) for sheep, goat, and cattle respectively, diluted 1:200 in PBS were applied to each well according to the sample type. The slides were incubated in the dark at room temperature for 30 min, washed twice with wash buffer for 5 min each and then rinsed in distilled water for 2 min. A drop of Prolong Gold Antifade Reagent with DAPI was added, covered with a coverslip, and allowed to dry. Finally, the slides were examined on a fluorescence microscope (Zeiss Flourescence Microscope). Confirmation of all reactive sera was done using PRNT.

Plaque Assay

Plaque assay was used to determine the virus titer before use in PRNT.

Vero cells grown in Growth media (Gibco DMEM media enriched with (10% Gibco FBS, 2% Gibco™ Antibiotic-Antimycotic (100X), Gibco™ MEM Non-Essential Amino Acids Solution (100X), Gibco™ L-Glutamine (200 mM)) at a concentration of 2 x10^6 cells per mL was seeded in each well of the 12 well plates at a volume of 2 mL per well and incubated overnight at 37˚C in a 5% CO_2 New Brunswick Galaxy 170S incubator. The cells were observed to ensure 70-90% confluency and even cell distribution before inoculation.

A serial dilution of the virus in maintenance media (Gibco DMEM media enriched with 2% Gibco FBS, 2% Gibco™ Antibiotic-Antimycotic (100X), Gibco™ MEM Non-Essential Amino Acids Solution (100X), Gibco™ L-Glutamine (200 mM)) was diluted up to 10^{-6}. Then 100 µL of each dilution was inoculated in each well after pouring out the media, incubated at 37°C for one hour and rocking the plate after every 15 minutes. Approximately 2 mL of methyl cellulose overlay was added to the wells and incubated at 37°C for a period of 4-14 days depending on the virus. When the plaques were well visible, the overlay was removed, cells

were fixed with 2 mL per well of a 3.7% (v/v) formalin solution overnight and then stained with 2 mL 0.5% (w/v) crystal violet solution per well overnight and then washed off.

The virus titer: plaque forming unit (pfu) was determined by the formula:

Pfu/mL = No of Plaques/D x V

Where D is the dilution factor and V is the volume of the diluted virus added to the well.

2.4.2 Molecular methods for Bunyavirus detection

RNA extraction, RT-PCR assays, and Sanger sequencing

This approach was undertaken to detect the presence of various viruses' nucleic acid in humans, livestock, and rodent samples.

The following equipment and reagents were used: Applied Biosystems 7900HT real-time PCR system, Applied Biosystem 7500H Thermocycler, pipettes and pipettes tip, virus-specific Primers, Thermofischer PCR plates and tubes, QIAGEN Viral RNA Kit, Invitrogen™ SuperScript™ III Reverse Transcriptase and E7023 Sigma-Aldrich absolute ethanol.

Human and rodent samples were screened as individually, whereas livestock samples were screened in pools of five to seven individual serum samples pooled on the basis of species and site, (100 µL per sample) for RNA extraction. Viral RNA was extracted from 140 µL of either individual or pooled sample using the QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany) according to the manufacturer's protocol. A volume of 50 µL of RNA was obtained and used as a template for cDNA synthesis by Invitrogen SuperScript™ III Reverse Transcriptase (1 x 10,000 units (200 U/µL)). A 20 µL cDNA reaction was prepared by adding 10 µL extracted RNA to 1µL Random Hexamers (50 mM), 1.5 µL of Invitrogen™ RT-PCR Grade water and 0.5 µL Thermo Scientific™ dNTP Mix (25 mM) to make a total volume of 13 µL reaction (Mixture 1) and incubated at 65°C for 5 min, then placed on ice for 1 min. Mixture 1 was then added to 7 µL of mixture 2, containing 1 µL Invitrogen™ SuperScript™ III Reverse Transcriptase (1 x 10,000 units (200 U/µL)), 1µL Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor (5,000 U (40 U/µL)), 1µL Thermo Scientific™ USB Dithiothreitol (DTT) and 4 µL 5X RT First Strand buffer, and incubated at 15°C for 20 min, then 50°C for 60 min, and finally at 85°C for 5 min. The cDNA was stored at -80°C until further use.

Samples were screened by PCR assays using various bunyavirales pan-genus as well as specific primers targeting either the L, M or S segments (**Table 2.1**). The reaction volume (25 µL) comprised 15.65 µL water, 2.50 µL 10x-Buffer, 1.25 µL Mg (50 mM), 0.50 µL dNTPs

(10mM), 1.5 µL of 10 µM forward and reverse primers, 0.10 µL Platinum-Taq polymerase and 2.0 µL template (cDNA). For nested PCRs, subsequent nested reactions were performed using the PCR product of the first round PCR as a template. The PCR conditions varied with the virus being investigated. The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide and positive samples were purified using ExoSAP-IT™ PCR product Clean-up Reagent (Applied Biosystems) according to the manufacturer's instructions, then sequenced in both directions.

The sequencing services were outsourced from Macrogen, Europe B.V. Further, RNA was extracted from positive individual serum samples and screened as described.

Table 2.1: Primers used in the study.

Primer name	Primer Type	Primer sequences	Target Genus/virus/gene	Source/ Reference
Pan-Ortho_F4	Forward	CAAARAACAGCAAAAGAYAGRGARA	*Orthobunyavirus*	(189)
Pan-Ortho_F5	Forward	ATGATTAGYAGRCCDGGHGA	*Orthobunyavirus*	(189)
Pan-Ortho_R5	Reverse	TTCAAATTCCCYTGIARCCARTT	*Orthobunyavirus*	(189)
Pan-Ortho_R6	Reverse	CTTGACATRTCWGCATTDATYTC	*Orthobunyavirus*	(189)
Pan-Phlebo_F1	Forward	TCAARAAGAMICAACATGGTGG	*Phlebovirus*	(64)
Pan-Phlebo_R1	Reverse	TATGCCYTGCATCATYCCWG	*Phlebovirus*	(64)
Pan-Phlebo_F2	Forward	GGACTTAGAGAGATYTAYGTITTGG	*Phlebovirus*	(64)
Pan-Phlebo_R2	Reverse	ACATGRTGACCYTGRTTCCA	*Phlebovirus*	(64)
CCHFV_F2	Forward	TGGACACCTTCACAAACTC	CCHFV	(190)
CCHFV_R3	Reverse	GACAAATTCCCTGCACCA	CCHFV	(190)
HAN-L_F1	Forward	ATGTAYGTBAGTGCWGATGC	*Hantavirus*	(191)
HAN-L-R1	Reverse	AACCADTCWGTYCCRTCATC	*Hantavirus*	(191)
HAN-L_F2	Forward	TGCWGATGCHACIAARTGGTC	*Hantavirus*	(191)
HAN-L_R2	Reverse	GCRTCRTCWGARTGRTGDGCAA	*Hantavirus*	(191)
LVL3359A_F1	Forward	AYNGGNACNCCRTTNGC	*Lassavirus*	(192)
LVL3754A_R1	Reverse	TCHTAYAARGARCARGTDGGDGG	*Lassavirus*	(192)
LVL3359D_F2	Forward	GGNACYTCHTCHCCCCANAC	*Lassavirus*	(192)
LVL3754D_R2	Reverse	AGYAARTGGGGNCCNAYKATG	*Lassavirus*	(192)
KPTV_F	Forward	AGCGAGGTGGATAGTGAAGA	Kaptombes virus	This study
KPTV_R	Reverse	CTCCGCCCTAACATCCAATAAA	Kaptombes virus	This study
sorcytb365F	Forward	CAGTAATAGCCACTGCCTTTATAGG	CytB	(193)
sorcytb969R	Reverse	CATTGGCTGAATGGGCGGAATATTAT	CytB	(193)
Bat L5310	Forward	CCTACTCRGCCATTTTACCTATG	CO1	(194)
R6036R	Reverse	ACTTCTGGGTGTCCAAAGAATCA	CO1	(194)

2.4.3 Cell culture methods for virus detection

This assay was undertaken to detect viable virus in humans, livestock and rodents from sera and tissues for further characterisation. Serum samples were first heat inactivated at 56°C in a water bath before inoculation. Homogenized rodent tissues treated with Gibco™ Antibiotic-

Antimycotic (100X) to prevent bacterial and fungal growth respectively were processed by filtering using 0.2 µm pore size VWRTM Avantor syringe filters before inoculation.

Vero CCL-81 (ATCC® CCL-81™) cells were seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark) to 80% confluency in Gibco Dulbecco's modified Eagle's medium (DMEM) containing 10% Gibco™ Fetal Bovine Serum (FBS), 2% Gibco™ L-Glutamine (200 mM), 2% Gibco™ Antibiotic-Antimycotic (100X). The cells were rinsed with Gibco™ PBS, pH 7.4, and 50 µL serum was added followed by incubation at 37°C, 5% CO_2 (New Brunswick™ Galaxy® 170 R CO_2 Incubator Series, Eppendorf, USA) for one hour, rocking after every 15 mins to allow virus adsorption. After incubation, Gibco Dulbecco's modified Eagle's medium (DMEM) maintenance medium (MM) with 5% Gibco™ Fetal Bovine Serum (FBS), 2% Gibco™ L-Glutamine (200 mM), and 2% Gibco™ Antibiotic-Antimycotic (100X) was added. Cells were incubated at 37°C, 5% CO_2 (New Brunswick™ Galaxy® 170 R CO_2 Incubator Series, Eppendorf, USA) and observed daily for signs of cytopathic effects (CPE). The CPE positive sample was passaged in 25-cm^2 cell culture flasks (Nunc, Roskilde, Denmark) and frozen at -80°C before harvesting by thawing and centrifuging at 3000 rpm for 10 mins. The infectious supernatant was stored at -80°C until further use.

CPE positive samples were confirmed by either PCR or NGS.

2.4.4 Next Generation Sequencing (NGS)

Clarified infectious cell culture supernatant, twenty selected serum samples, and hantavirus PCR positive rodent tissue homogenates were filtered using 0.22-µm filters (Merck Millipore Co., MA, USA) to remove possible cellular residues and contaminants. RNA was extracted from filtered samples as well as CCHFV positive human and livestock serum samples using QIAamp viral RNA minikit (QIAGEN, Hilden Germany) following the manufacturer's recommended protocol. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit RNA 2.0 fluorometer using the Qubit RNA HS assay kit (Invitrogen, USA). The RNA was then prepared for Illumina library preparation.

Libraries for sequencing were prepared using TruSeq stranded mRNA kit (Illumina, USA), following the manufacturer's recommended protocol with the modification to exclude the poly(A)-containing mRNA purification steps. Reverse transcription was done using Superscript III reverse transcriptase (Invitrogen, USA) and random hexanucleotide primers (Invitrogen, USA). This was followed by second-strand synthesis using DNA polymerase I and RNase H, provided with the library preparation kit. Purification was performed using AMPure XP beads (Beckman Coulter, USA) after which the purified double-stranded cDNA fragments

were end repaired by adding a single A nucleotide to the 3′ end of the blunt fragments to prevent the formation of chimeras and improve adapter ligation efficiency. Ligation of the adapters was performed, and the products purified and enriched by PCR to create the final library. Libraries were normalized, pooled, and sequenced using the Illumina platform.

2.5 Data Management and Analysis

2.5.1 Seroprevalence data

The seroprevalence studies data were entered into Microsoft Excel v. 2016, cleaned then imported to R version 4.2.0 for analysis. Comparison of KPTV seroprevalence between the different livestock species was done using Fisher exact test. The 95% confidence intervals (CIs) were estimated using the Agresti-Coull method. All tests were performed at a 5% significance level.

2.5.2 Sanger sequence phylogenetic analysis

The Sanger sequences of the PCR positive samples were imported into Geneious Prime software (https://www.geneious.com). The sequences were cleaned using Geneious Prime software inbuilt parameters and queried against the GenBank-NCBI database (195,196) using the Basic Local Alignment Search Tool (BLAST) (197). Related sequences were downloaded from the GenBank-NCBI database and multiple sequence alignment were performed by MUSCLE (198). Phylogenetic analysis was performed in Geneious Prime (http://www.geneious.com) with PhyML (199), General-time-reversible (GTR) substitution models substitution model applying 1000 bootstrap replicates. The inferred phylogenies were visualized in Figtree v1.4.4.

2.5.3 NGS data analysis

Raw sequence reads were initially subjected to cleaning using Trim Galore v0.6.5 to remove adapters and Prinseq Lite v0.20.4 to remove low-quality reads using the following parameters: minimum length, 50 bp; maximum length, 301 bp; and minimum mean Q score, 30. Further, filtering of the reads was performed by using ribo Picker v0.4.3, to remove rRNA sequences by comparing them to the SILVA rRNA database, release 138.1. Paired-end reads were merged using PEAR 0.9.8, and preliminary analysis was performed using the MG-RAST server to identify reads taxonomically. *De-novo* assembly of cleaned reads was done using a trinity program with default parameters. The cleaned reads were mapped back to the assembled

contigs and filtered to retain only contigs in which at least 90% of nucleotides had a 5 times coverage. Contigs that met this criterion were first compared to the NCBI viral database using the BLASTx program. Potential viral contigs were further compared to the entire NCBI nr database using the BLASTx program to filter out all nonviral sequences. Sequences that were confirmed to be of viral origin were translated, and ORF predictions were performed using the Expasy server. Phylogenetic reconstruction was performed based on 1,000 bootstrap estimates and 1,000 approximate-likelihood-ratio tests. The inferred phylogenies were visualized in Figtree v1.4.4

Chapter 3

RESULTS

Findings of molecular and serological screening for the presence of bunyavirales in livestock, humans, and rodents.

3.1 Introduction

This chapter presents the results obtained through targeted molecular and serological screening of bunyavirales in the three hosts in the study sites in Kenya. Molecular detection of viruses was achieved through targeted genus-specific polymerase chain reactions, and a combined virus isolation-NGS sequencing approach. This allowed for the detection and characterisation of certain phleboviruses, nairoviruses, orthobunyaviruses and hantaviruses and for genomic characterization of NRIV in cattle and sheep and Kaptombes virus (KPTV) in cattle. In addition, a metagenomics approach in which selected samples suspected of being positive for any virus were subjected to deep sequencing by Illumina and this allowed for the detection of a pegivirus and a hepacivirus in human and cattle, respectively. Serological screening of specific viruses representing various genera was carried out to determine the prevalence of RVFV, BUNV, CCHFV, and NRIV in humans and animals in the study areas.

3.2 Materials and Methods

Methods as described in chapter 2 were used for testing samples.

3.3 Results

2.3.1 Summary of the samples collected.

A total of 2039 livestock, 493 febrile patients and 489 rodents were sampled during the study. The demographics of each species are summarised in **Table 3.1-3.3**.

Table 3.1: Demographics of febrile patients sampled in the two hospitals.

Parameter	Level	N (%)
Location	Marigat	323 (65.5)
	Nguruman	170 (34.5)
Gender	Female	295 (59.8)
	Male	198 (40.2)
Age	5-10	49 (9.9)
	10≤18	114 (23.1)
	≥18	330 (66.9)
Occupation	Artisan-Mason	1 (0.2)
	casual work	2 (0.2)
	Farmer	104 (21.1)
	Non-Student	17 (3.4)
	Nurse	2 (0.4)
	Pastoralist	2 (0.4)
	Peasant	71 (14.4)
	Businesspersons	27 (5.5)
	Security	2 (0.4)
	Student	169 (34.3)
	Unemployed	97 (19.7)

Table 3.2: Demographics of livestock sampled.

Livestock Species	N	Location		Sex	
		Marigat n (%)	Nguruman n (%)	Females n (%)	Males n (%)
Cattle	715	415 (58)	300 (42)	465 (65)	250 (35)
Goats	660	420 (63.6)	240 (36.4)	462 (70)	198 (30)
Sheep	664	404 (60.8)	260 (39.2)	458 (69)	206 (31)
Total	**2039**	**1239 (60.8)**	**800 (39.2)**	**1385 (67.9)**	**276 (32.1)**

Table 3.3: Demographics of peri-domestic small mammals (rodents and shrews) sampled.

Parameter	Description	Site		Total
		Marigat	Nguruman	N (%)
Sex	Female	102 (42.1)	140 (57.9)	242 (49.5)
	Male	75 (30.4)	172 (69.6)	247 (50.5)
Age	Sub-Adult	36 (23.4)	118 (76.6)	154 (31.5)
	Adult	141 (42.1)	194 (57.9)	335 (68.5)
Species	*Acomys* spp	1	48	49 (10)
	Aethomys spp	1	6	7 (1.4)
	Arvicanthis spp	0	15	15 (3.1)
	Oenomys spp	1	0	1 (0.2)
	Lemniscomys spp	0	2	2 (0.4)
	Gerbilliscus spp	3	2	5 (1)
	Grammomys spp	2	0	2 (0.4)
	Graphiurus spp	0	1	1 (0.2)
	Mastomys spp	47	217	264 (54)
	Mus spp	73	0	73 (15)
	Paraxerus spp	0	1	1 (0.2)
	Gerbillus spp	0	1	1 (0.2)
	Crocidura spp	11	0	11 (2.2)
	Rattus spp	38	19	57 (11.7)

3.3.2 Seroprevalence of Bunyavirales in febrile patients, livestock, and peridomestic rodents

Bunyavirales seroprevalence was determined by PRNT for all the viruses except CCHFV, where ELISA was used. We found divergent seroprevalence for different members of the Bunyavirales (**Table 3.4**)

Table 3.4: Seroprevalence of screened viruses in the order Bunyavirales

Virus	Screening method	Seroprevalence (%)				
		Cattle	Goats	Sheep	Rodents	Human
CCHFV	ELISA	14 (44/310)	8 (24/295)	9.8 (29/295)	6.5 (6/93)	5.9 (29/493)
RVFV	PRNT	18 (129/715)	39.7 (262/660)	40 (267/664)	11.3 (14/124)	7.3 (36/492)
BUNV	PRNT	1.3 (9/715)	4.1 (27/660)	2 (13/664)	4 (5/124)	1.2 (6/492)
NRIV	PRNT	41.6 (25/60)	52.4 (22/42)	19 (8/42)	-	23.3 (14/60)
NTPV	PRNT	1 (7/715)	<1 (5/660)	<1 (6/664)	-	10 (49/492)

3.3.3 Molecular detection of Bunyavirales by PCR and phylogenetic analysis

Screening by molecular methods including PCR, sequencing and confirmed through sequencing and phylogenetic analysis aimed at detecting and establishing the molecular epidemiology and genetic diversity of circulating viruses in the order *Bunyavirales* in human, domesticated livestock and peridomestic rodents, we detected different bunyaviruses. (**Table**

3.5). Through genus specific PCRs for orthobunyaviruses we identified Bunyamwera viruses in goats in Marigat as well as Ngari virus in goats, sheep, and cattle as well as rodents in both Marigat and Nguruman while SHAV was identified in cattle in Marigat. CCHFV, which represented the nairoviruses was detected in sheep from Marigat and Nguruman and rodents from Nguruman. Screening for phleboviruses using genus-specific primers resulted in the detection of sandfly borne phlebovirus in cattle from Nguruman and uncharacterised phleboviruses in cattle and sheep from Nguruman and Marigat. More in-depth analysis is included in chapters 3-5.

Table 3.5: Bunyaviruses detected in the study by PCR.

Virus detected		**Animal Species**	**Origin**	**No of samples positive**
Genus	**Species**			
Orthobunyavirus	BUNV	Goats	Marigat	2/415
	NRIV	Cattle	Marigat	3/415
		Goats	Nguruman	2/240
			Marigat	2/420
		Sheep	Marigat	2/404
		Rodents	Marigat	1/177
	SHAV	Cattle	Marigat	1/415
Nairovirus	CCHFV	Sheep	Marigat	3/404
			Nguruman	1/260
		Rodents	Marigat	4/177
Phlebovirus	Sandfly borne phleboviruses	Cattle	Nguruman	2/300
	Uncharacterised phlebovirus	Cattle	Marigat	3/415
		Sheep	Marigat	1/404
Orthohantavirus	Hantavirus	Shrews	Marigat	4/11

3.4 Genetic characterization and phylogenetic analysis of detected Bunyavirales

PCR products were further investigated through Sanger sequencing and phylogenetic analysis of partial sequences or by full genome analysis for sequences obtained through NGS using PhyML algorithm based on the maximum likelihood (ML) sequence analysis and 1000 bootstrap replicates.

Phylogenetic analysis of the sand-fly borne phleboviruses detected in cattle from Kajiado county (Nguruman) clustered with other identified phleboviruses with 78 and 99% nucleotide identity across the partial L segment that was characterised (**Figure 3.1**).

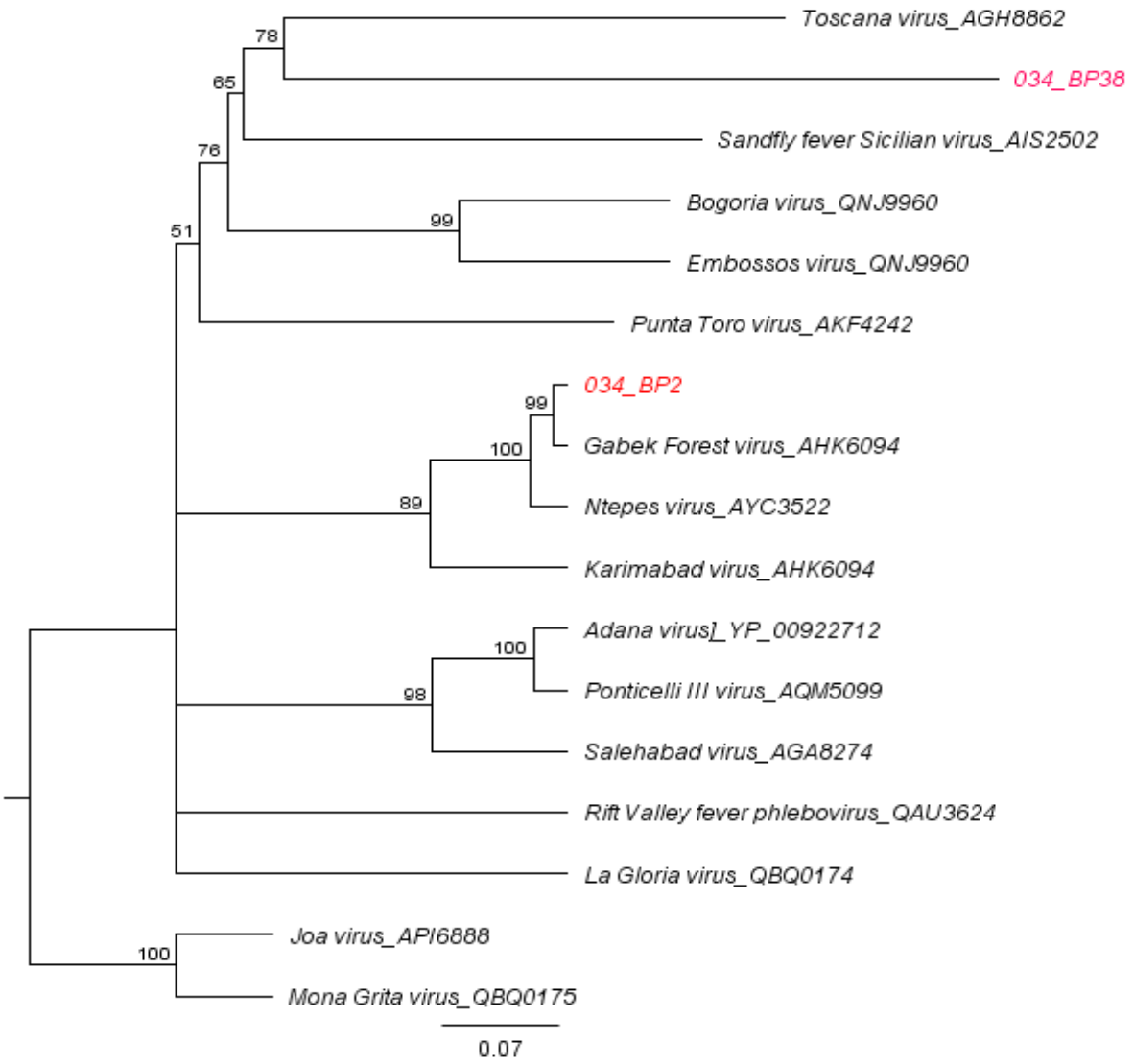


Figure 3.1: Maximum Likelihood tree based on the 252 nt partial sequence of the phlebovirus L segment showing the relationships between detected sandfly borne phleboviruses and other related phleboviruses sequences obtained from GenBank. Phylogenetic tree was inferred from MAFFT alignment using PhyML v. 2.2.4 with General-time-reversible (GTR) substitution models employing 1000 bootstrap replicates. The detected sandfly borne phlebovirus sequences identified in this study are highlighted in red. All bootstrap support values are shown.

Phylogenetic analysis of the uncharacterised phleboviruses detected in cattle and sheep from Baringo county (Marigat) within a well-supported monophyletic lineage (90% bootstrap support), within a broader clade containing Gouleako virus and Cumuto virus (82% support). The viruses characterised in this study had up to 72% nucleotide sequence identity to Gouleako virus across the analysed partial L segment region (**Figure 3.2**).

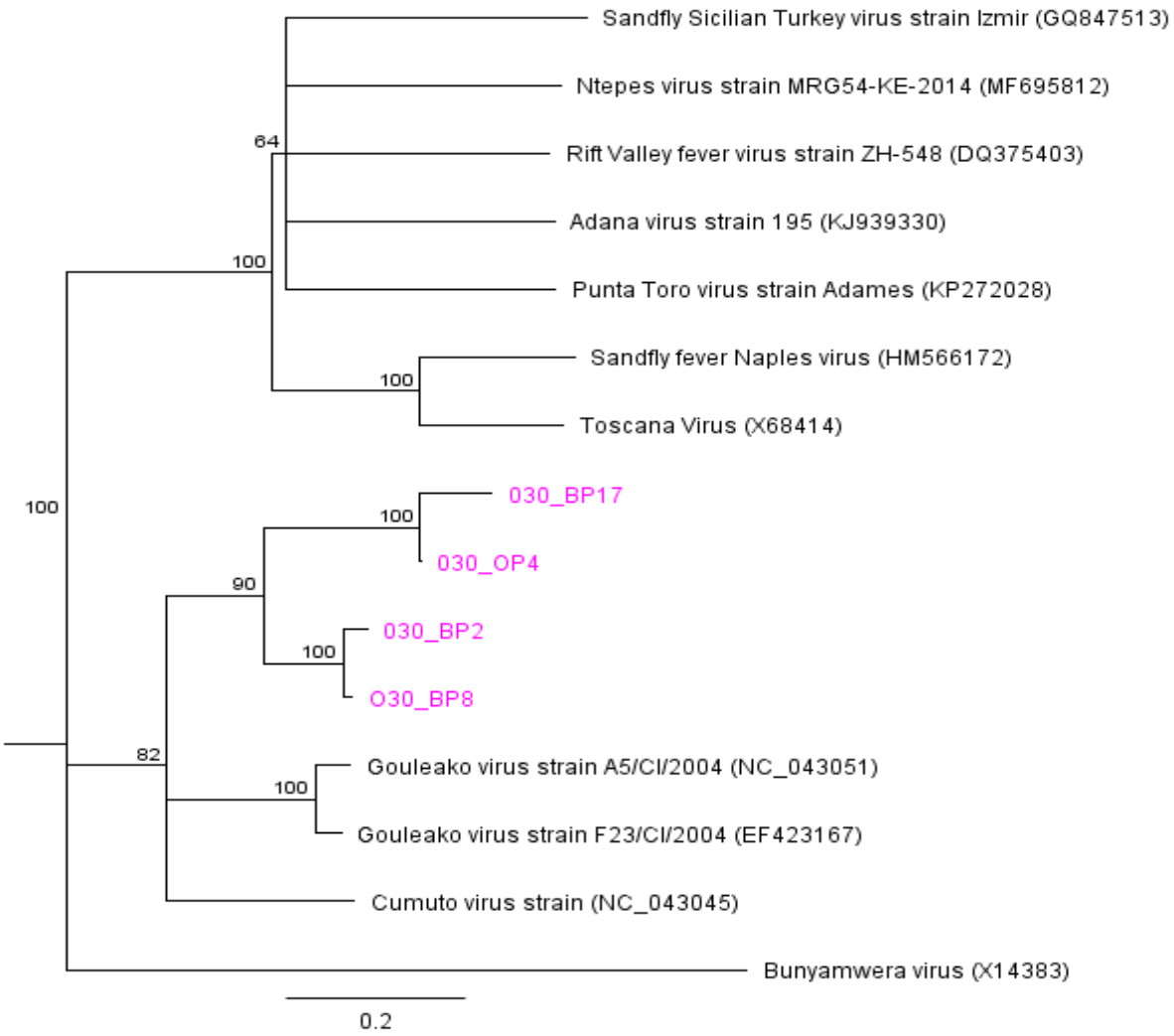


Figure 3.2: Maximum Likelihood tree based on the 252 nt partial sequence of the phlebovirus L segment showing the relationships between detected uncharacterised phleboviruses and other related phlebovirus sequences obtained from GenBank. The phylogenetic tree was inferred from MAFFT alignment using PhyML v. 2.2.4 with General-time-reversible (GTR) substitution models employing 1000 bootstrap replicates. The detected phlebovirus sequences identified in this study are highlighted in pink. All bootstrap support values are shown.

Phylogenetic Analysis of CCHFV detected in sheep and rodents.

CCHFV was detected in sheep and two rodent genera (*Mus* and *Rattus*). The detected viruses belonged to the Africa 3 lineage, clustering together with other CCHFV from Africa. They exhibited 98-100% identity to CCHFV isolated from humans in South Africa and Sudan, across the S segment that codes the nucleoprotein (np) (**Figure 3.3**).

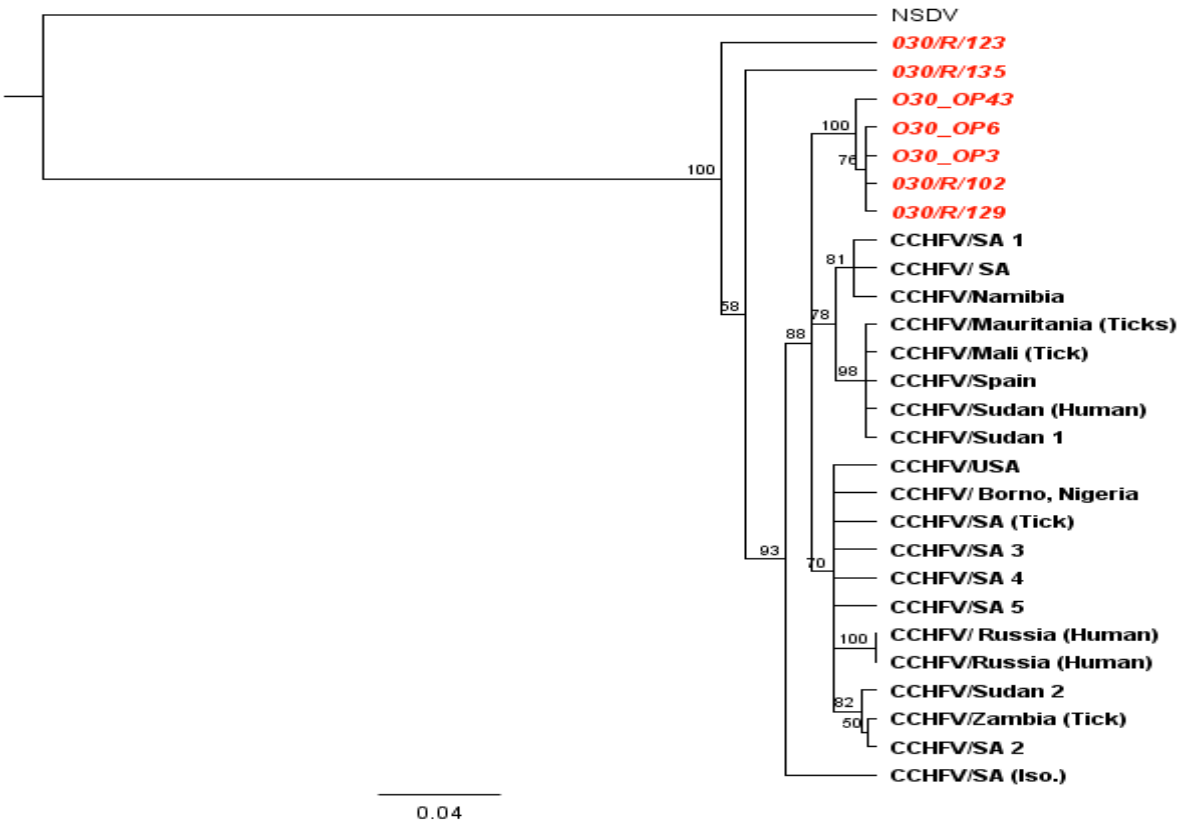


Figure 3.3: Maximum Likelihood tree based on the 536 nt partial sequence of the CCHFV S segment showing the relationships between detected CCHFV and other related sequences obtained from GenBank. The tree is rooted to NSDV. Phylogenetic tree was inferred from the MAFFT alignment using PhyML v. 2.2.4 with General-time-reversible (GTR) substitution models and employing 1000 bootstrap replicates to assess nodal support. The detected CCHFV sequences identified in this study are highlighted in red. All bootstrap support values are shown.

Phylogenetic Analysis of NRIV detected in cattle, goats, and sheep.

NRIV was detected in several livestock samples before isolation attempts. The detected NRIV confirms a close relationship with the virus detected in livestock in Mauritania in 2010 and 2015 based on available L-segment sequence data (**Figure 3.4**).

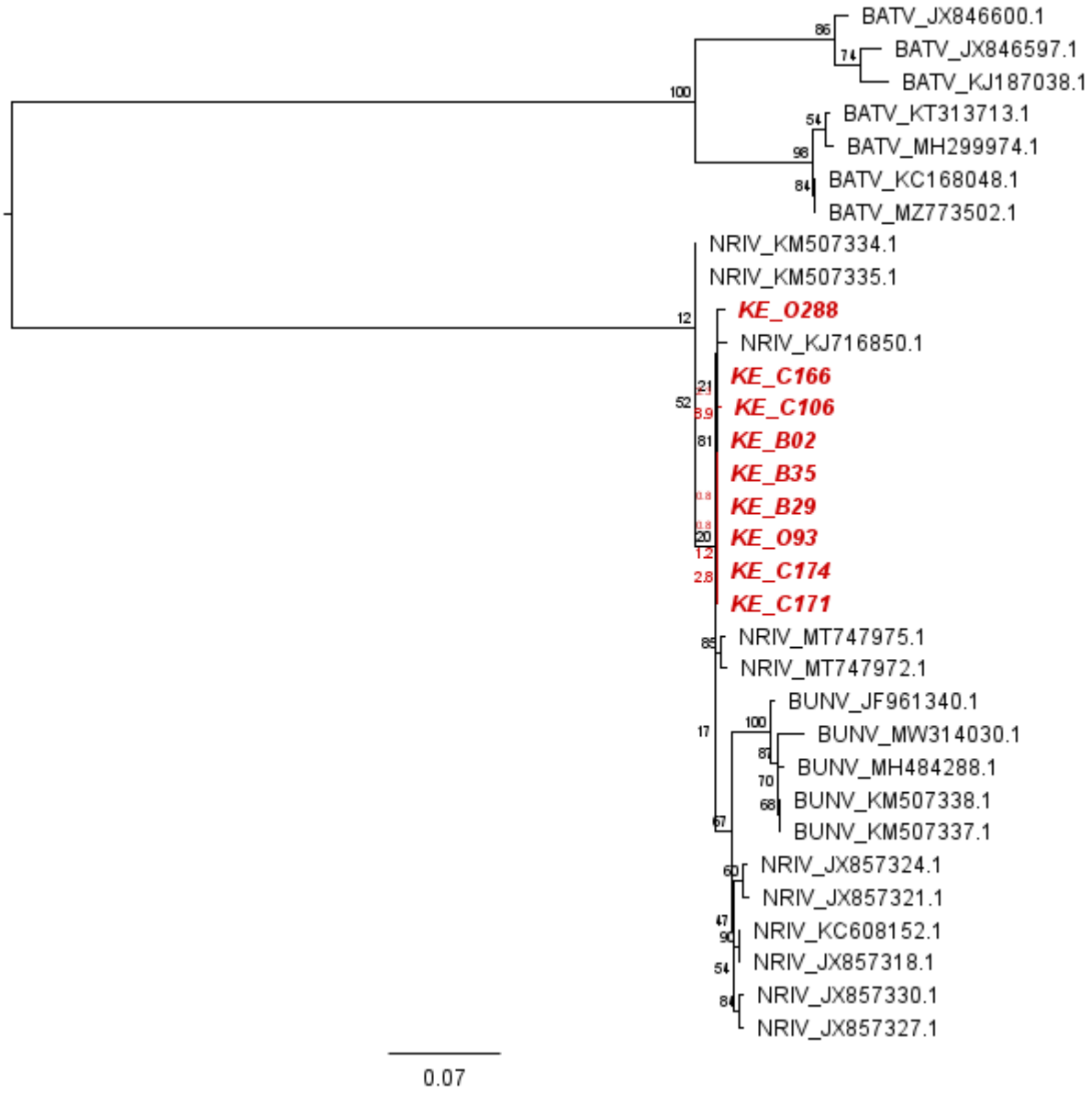


Figure 3.4: Maximum likelihood tree based on the 513 nt partial sequence of the orthobunyavirus L segment showing the relationships between detected NRIV and other related orthobunyaviruses obtained from GenBank. The phylogenetic tree was inferred from MAFFT alignment using PhyML v. 2.2.4 with General-time-reversible (GTR) substitution models employing 1000 bootstrap replicates. The detected NRIV sequences identified in this study are highlighted in red. All bootstrap support values are shown.

3.5 Virus isolation and NGS

We aimed to isolate circulating phleboviruses, orthobunyaviruses, and nairoviruses in human, livestock and peridomestic rodents through cell culture. The NGS was important to obtain whole genome sequences for further characterisation and determination of molecular epidemiology and genetic diversity of the isolates. Despite several attempts, only one bunyavirus, NRIV was successfully isolated from cattle, goats, and sheep. A novel orbivirus was also isolated from cattle and sequenced (**Table 3.7**). The two virus isolates are further characterised in chapters 4 and 7, respectively.

Table 3.6: Cell culture isolated viruses

Virus isolated		**Animal Species**	**No of isolates**
Genus	**Species**		
Orthobunyavirus	NRIV	Cattle	3/715
		Goats	2/660
		Sheep	2/604
Orbivirus	Kaptombes virus (KPTV)	Cattle	1/1500

Phylogenetic analysis of the NRIV isolates.

To further characterise and determine the genetic diversity of the isolates, phylogenetic analysis of the four NRIV sequenced isolates based on the full sequence of the L segment was performed (**Figure 3.5**). In-depth characterisation based on the 3 segments is described in chapter 4.

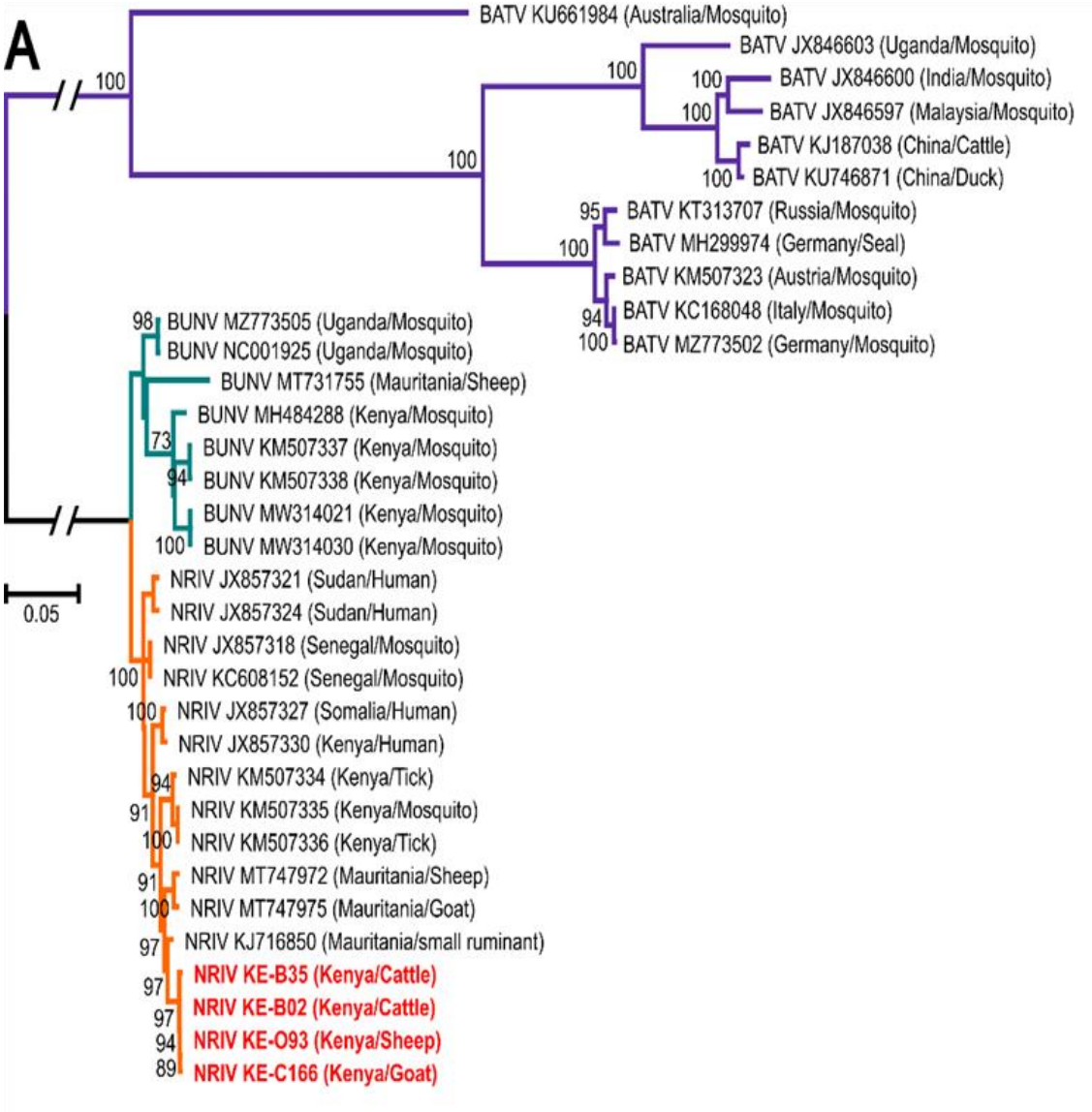


Figure 3.5: Phylogenetic analysis of the isolated NRIV based on the L segment (6717nt) compared to other orthobunyaviruses. The sequences in this study are highlighted in red. The sequences were aligned using MAFFT with E-insi and 100PAM. The maximum likelihood (ML) tree was inferred using IQtree via CIPRES (Geneious plug-in at www.phylo.org) and non-parametric bootstrapping was used. All the bootstrap values are displayed. The viruses isolated and characterised in this study are indicated in red.

As an additional finding to this thesis, a novel 10 segmented orbivirus named Kaptombes virus (KPTV) from cattle, isolated in Vero cells and sequenced through NGS was characterised as well. The most closely related virus to KPTV is Sathuvachari virus (SVIV), previously reported in India and Japan in a bird (Brahminy myna) and cattle, respectively. **Figure 3.6** shows the relationship of the novel virus to SVIV (100% bootstrap support) and with other orbiviruses. The KPTV-SVIV clade is sister to the tick-borne orbivirus clade.

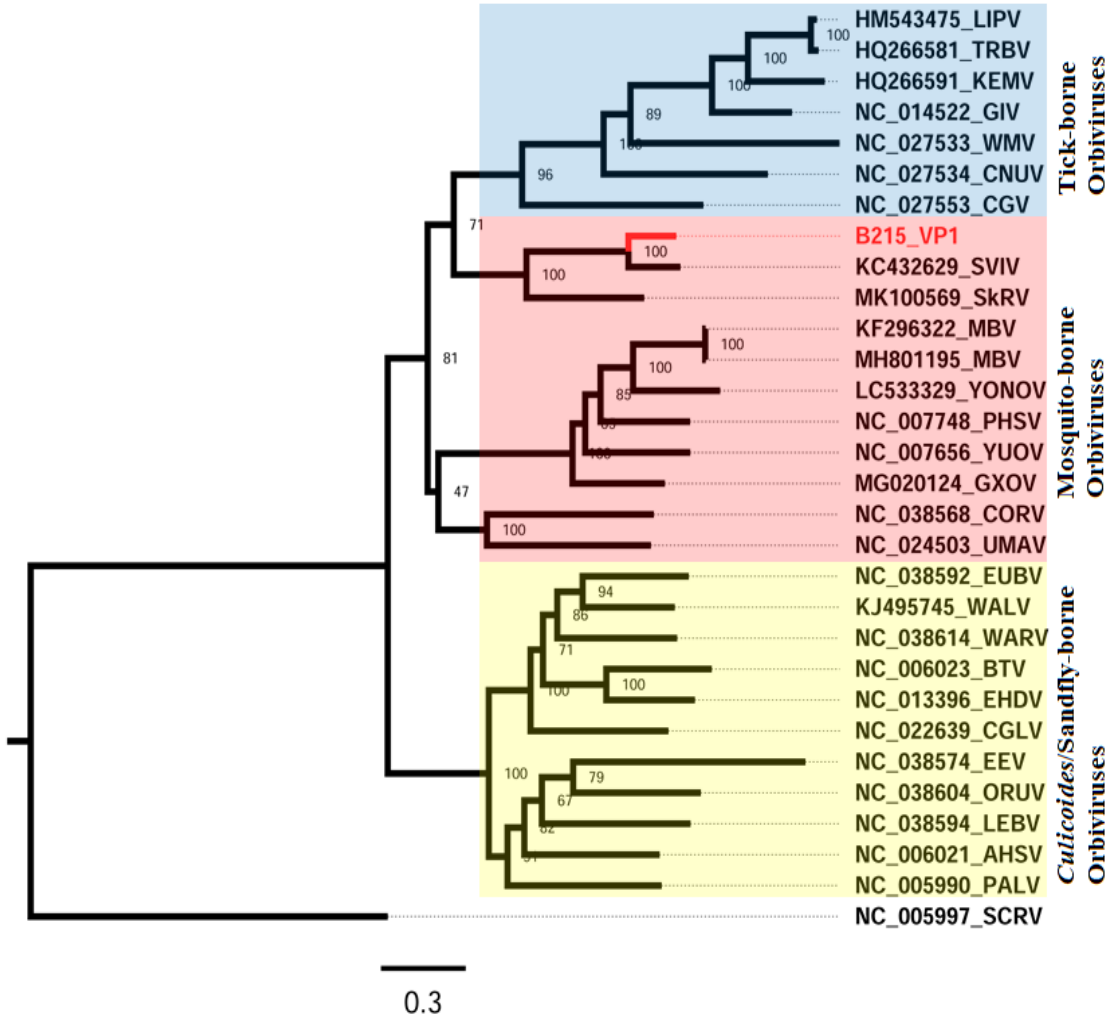


Figure 3.6: Phylogenetic analysis of isolated novel Kaptombes virus based on the conserved segment 3 which encodes the major sub core structural protein (VP3), compared to other orbiviruses. The phylogenetic tree was inferred from MAFFT alignment using PhyML v. 2.2.4 with General-time-reversible (GTR) substitution models employing 1000 bootstrap replicates. The sequence characterised in this study is highlighted in red. All the bootstrap values are displayed. More in-depth analysis is described in Chapter 7.

Metagenomic analysis through direct sequencing by NGS of clinical samples resulted in the detection of certain viruses not in the order *Bunyavirales* (**Table 3.7**).

Table 3.7: Viruses detected through NGS.

Virus detected		Animal Species	No of detection
Genus	**Species**		
Pegivirus	Pegivirus	Human	1
Hepacivirus	Hepacivirus	Cattle	1

3.6 Discussion

Viruses in the order *Bunyavirales* are diverse and contribute significantly to animal and human health. These viruses are widely spread and are among emerging and re-emerging infectious disease agents. In this study, we report the active circulation of known Bunyavirales in already known and new hosts as well as their prevalence. We also made attempts to isolate viable viruses from the hosts, perform whole genome sequencing and characterisation.

Surveillance of hosts for infection with zoonotic Bunyavirales viruses is important for early detection and intervention measures to prevent outbreaks. As many viruses, including members of the order Bunyavirales have short viraemia in their hosts, virus detection is challenging, and seroprevalence surveillance is important in determining exposure of a population to the virus and the degree of virus circulation.

Seroprevalence rates were determined for CCHFV, BUNV, NRIV, RVFV, and NTPV while the primers used for molecular detection were able to detect all viruses within the genera Orthobunyavirus and Phlebovirus. For the genus Nairovirus, only CCHFV was screened for as attempts to ensure broader representation of the entire genus through optimisation of the pan-Nairovirus primers were not successful.

Phleboviruses are widely spread and cause disease in animals and humans. In livestock, the highest seroprevalence realized was for RVFV; 18% in cattle, 39.7% in goats and 40% in sheep, across all sites. The high seroprevalence can be attributed not only to the active circulation of the virus but also frequent vaccination of the animals as RVFV is endemic in different parts of Kenya. The overall RVFV seroprevalence in febrile patients was 7.3% (36/492), and there was no significant difference in human RVFV seroprevalence in the two sites; Marigat 8% (25/311) and Nguruman 6.1% (11/181), p=0.48. The contribution of RVFV to febrile illness and active circulation of the virus in the human population cannot be underestimated as the PRNT results used to determine seroprevalence were determined at a high serum dilution of 1:320. Considering the absence of RVFV vaccination for humans, these results confirm high levels of exposure of the febrile patients to the virus. However, RVFV RNA was not detected in all the hosts screened.

The seroprevalence of the recently discovered sandfly borne phlebovirus, NTPV was low (1% in cattle and <1% in goats and sheep). In humans, the seroprevalence was higher at 10% (49/492). There was no significant difference between the two sites for levels of human exposure: Marigat 9%, 28/311 and Nguruman 11.6% 21/181 p=0.35. As cross reactivity with other phleboviruses like RVFV is less suspected, pathogenicity studies in livestock are

recommended to further understand the NTPV circulation. NTPV RNA was detected in cattle from Nguruman, and another sand-fly borne phlebovirus with 78% nucleotide identity to Toscana virus was detected in cattle from the same area. This confirms the active circulation of the sand fly borne viruses in the area. Considering the initial isolation of NTPV in Marigat and the present detection of the virus in cattle from Nguruman, the results confirm that cattle are susceptible to NTPV, and they could act either as reservoirs or amplification hosts. Other than RVFV and the sand-fly-borne phleboviruses, an uncharacterised phlebovirus was detected in cattle and sheep from Marigat. The virus has 82% nucleotide identity to Gouleako virus (GOUV) previously isolated from west African mosquitoes. Attempts to isolate the virus and obtain WGS for characterisation were unsuccessful but other methods of sample enrichment before sequencing are being employed.

Nairoviruses are important to both human and animal health. In this study, we screened for CCHFV, one of the most important nairoviruses. We report seroprevalence of 5.9% (29/490), 11.9% (113/951), and 6.5% (6/93) among humans, livestock, and peri-domestic rodents respectively from the two semi-arid ecologies within the Kenyan Rift Valley. The observations are consistent with previous detection of antibodies against CCHFV in febrile patients (162,181,182) and cattle (200,201) as well as wildlife (200). The detection of CCHFV RNA in sheep is an indication of active circulation at the sampling sites in the two counties and in peri-domestic rodents, a novel finding potentially implicating rodents in CCHFV epidemiology in the country. These findings together with the widespread abundance of tick vector species (159,202) suggest that the virus might be endemic in diverse parts of the country.

The genus *Orthobunyavirus* is the largest group in the order *Bunyavirales.* Orthobunyaviruses are distributed worldwide and known to infect both humans and animals. We determined the seroprevalence of two orthobunyaviruses; NRIV and BUNV in livestock and febrile patients and screened for orthobunyaviruses using the genus-based pan-orthobunyavirus primers.

We report the previously undetected circulation of NRIV in apparently healthy cattle, sheep, and goats in Kenya. NRIV is associated with outbreaks of hemorrhagic fever in humans and small ruminants. We demonstrate the isolation of infectious viruses from several animals as well as presence of neutralizing antibodies in 38% of the tested animals. Our data indicate active virus circulation and endemicity likely having important implications for human and animal health. NRIV has been detected in mosquitoes and ticks from different parts of the country and several vectors were shown to be able to transmit the virus in laboratory experiments (44,101,158,161,203–205). Thus, depending on the distribution and feeding habits of these vectors, NRIV could be more abundant than available data would suggest. Herein, we

found high seroprevalence rates in the three most common livestock species and detected several viraemic animals which did not show any obvious signs of disease, a clear indication that the virus is endemic in the country and actively circulating. Importantly, our data indicates the unreported circulation of a NRIV in the three most common livestock species (cattle, goats, and sheep). We did not detect NRIV RNA in febrile patients.

NRIV seroprevalence rates in cattle, goats, and sheep were: 41.6% (95% CI 30-54.3), 52.4% (95% CI 37.7–66.6), and 19% (95% CI 9.7-33.6), respectively. The proportion was higher in goats and lowest in sheep in both sites. NRIV antibody prevalence in livestock did not differ significantly between Baringo (33.3%, 24/72) and Kajiado counties (43.1%, 31/72; Fisher exact test odds ratio [OR] 1.4, 95% CI 0.83–2.66; p=0.18). In febrile patients, a seroprevalence rate of 23.3%, 14/60 was realized. The proportion was higher in males than in females but there was no significance difference in seroprevalence between the two genders: female 15%, 6/40 (95% CI 7-29), and males 40%, 8/20 (95% CI 21.9-61.3), p=0.05. The seroprevalence in febrile patients in Marigat was 20%, 6/30 (95% CI 9.5-37.3) and in Nguruman it was 26.7% 8/30 (95% CI 14.2-44.5), p=0.76.

Another orthobunyavirus that was detected in livestock was BUNV. Two goat samples from Marigat were positive. Although BUNV has not been reported previously in livestock in Kenya, it has been reported in Rwanda. Attempts to isolate the virus were unsuccessful but phylogenetic analysis revealed a 95% nucleotide identity to BUNV isolated from mosquitoes from Uganda. Seroprevalence among livestock was highest in goats at 4%, sheep at 2% and cattle at 1%. The higher seroprevalence could explain the detection of BUNV RNA only in goats confirming higher levels of circulation in this species. BUNV seroprevalence in rodents was 4%. However, the possibility of cross reactivity with other orthobunyaviruses like NRIV which was detected in rodents but not screened for antibodies due to depletion of samples, can not be ruled out. BUNV seroprevalence in febrile patients was 1.2% (6/492), and prevalence did not differ significantly (p=0.67) between Marigat (1%, 3/311) and Nguruman (1.7%, 3/181), sampling sites. Although the virus was not detected in febrile patients' serum, the seroprevalence of 1.2% (95% CI 0.5-2.6) was confirmed at a very high dilution of 1: 320 and having ruled out the chances of cross reactivity, this suggests a very high possibility of a contribution of this virus to febrile illness.

Shamonda virus (SHAV), an African simbu group virus, was detected in cattle from Marigat. SHAV was first detected in cattle and Culicoides in Nigeria in 1960 and is known to cause congenital deformities and abortions in animals. The detection of the virus confirms a wider circulation of a virus that has not been previously reported in Kenya. This therefore would

necessitate the need to undertake targeted surveillance specifically during unresolved outbreaks as the virus could be a contributing factor. Further studies to determine the seroprevalence of the virus is recommended.

Additional findings

During virus isolation attempts, a novel Orbivirus was isolated from cattle and identified through whole genome sequencing using a next generation sequencing approach. This data has been added to this thesis as an additional finding. Metagenomics through NGS also resulted in the detection and generation of whole genome sequences for two viruses in the Flaviviridae family, viz. a Pegivirus in a human sample and a Hepacivirus in a cattle sample.

Chapter 4

CIRCULATION OF NGARI VIRUS IN LIVESTOCK, KENYA

This chapter presents the results obtained through molecular and serological screening of orthobunyaviruses in livestock in the two study sites. Molecular detection through genus specific pan-orthobunyavirus PCR primers, virus isolation and NGS sequencing allowed the detection and characterisation of the Ngari virus in livestock. Serological screening through IFA and PRNT was carried out to determine the prevalence in livestock.

4.1 Introduction

Ngari virus (NRIV) belongs to the *Bunyamwera orthobunyavirus* species (genus *Orthobunyavirus*, family *Peribunyaviridae*, order Bunyavirales) and is the only known naturally occurring reassortant in the Bunyamwera serogroup (2). The *Bunyamwera orthobunyavirus* species comprises 10 viruses, including Bunyamwera virus (BUNV), Germiston virus (GERV) and Shokwe virus (SHOV) (1,2,30,206). The tripartite genome of orthobunyaviruses is composed of linear, segmented single-stranded, negative-sense RNA. The three segments are named small (S), medium (M), and large (L) according to their length. The S segment encodes the nucleocapsid (N) protein and the NSs non-structural protein, the M segment encodes the two glycoproteins Gn and Gc and a non-structural protein NSm, and the L segment, the RNA-dependent RNA-polymerase (3,32,34,161).

NRIV is a reassortant of BUNV (*Bunyamwera orthobunyavirus* species) and Batai virus (BATV, *Batai orthobunyavirus* species) (2,32,34) containing the L and S segments from BUNV and the M segment from BATV (32–34). Genetic reassortment among orthobunyaviruses has occasionally been reported and is considered the main evolutionary force leading to the emergence of new strains and species (1,4,30,35–37). Whereas BATV and BUNV cause mild symptoms, such as fever, joint pain, and rash in humans, NRIV is more virulent and can induce severe and fatal hemorrhagic fever (3,30,45–47). However, BATV can cause a more severe form of disease in livestock, resulting in genetic defects and abortions (30,42).

NRIV was first isolated from *Aedes simpsoni* mosquitoes in 1979 in South Eastern Senegal (3,36,43). It was later recovered from *Aedes* spp., *Culex* spp., and *Anopheles* spp. in Senegal, Burkina Faso, Central African Republic and Madagascar (44). NRIV has also been detected in engorged ixodid ticks (*Amblyomma variegatum*, *Rhipicephalus geigyi* and *Rh. (Boophilus)*

spp.) collected from cattle in Guinea but there is no evidence that ticks can transmit the virus (207). The virus was first associated with human disease following isolation from two patients in Senegal in 1973 (3,43), and subsequently implicated as a cause of hemorrhagic fever during a large hemorrhagic fever outbreak in Northeastern Kenya and southern Somalia in 1997-1998 (32–34). In a later study conducted between 2009 and 2012 in Northeastern Kenya, the presence of NRIV neutralizing antibodies was reported among febrile patients (91), indicating circulation of an understudied virus. In regard to animal infections, NRIV has only been isolated from small ruminants in Mauritania during the 2010 and 2015 RVF outbreaks (45,46,63), confirming co-circulation with RVF and other orthobunyaviruses, BATV and BUNV on two separate occasions.

BUNV and NRIV have mainly been identified in Africa. BUNV is considered endemic in certain African countries including Rwanda, Kenya, Nigeria, Senegal, Uganda, Tanzania, Mozambique, Guinea, South Africa, DRC and Madagascar (30,45–47). BUNV has also been reported in Mexico and Argentina in birds and mosquitoes and more recently as a cause of neurological disease and abortion in horses (39,207,208). BATV is distributed worldwide, though commonly found in Asia and Europe (3,39,207,208). Although the geographic range of these viruses suggests a restricted distribution, there is potential for spread due to globalization, human and animal movement and environmental changes due to global warming. NRIV has been found to circulate concurrently with RVFV during outbreaks and has been clinically misdiagnosed as RVF as the case during the 1997/98 RVF outbreak in Kenya and Somalia and as malaria in Sudan during the two-year same period (32,43). The misdiagnosis was partly due to similarities in symptom presentation. Thus, its distribution and associated health impact could be grossly underestimated due to the paucity of active surveillance, poor disease reporting systems, and lack of appropriate diagnosis.

In Kenya, since its initial isolation in humans, several studies have isolated or detected NRIV in different mosquito species, including *Anopheles funestus* (Tana Delta), *Aedes mcintoshi* (Garissa), and different tick species, including *Amblyomma gemma* (Garissa) and *Rhipicephalus pulchellus* (Isiolo) (44,101,158,161,203). Diverse tick and mosquito species have been found to be competent vectors for the virus in laboratory experiments (204,205). However, there has not been any report of NRIV circulation in any livestock species outside of Mauritania.

Against the backdrop of poor surveillance, this study was initiated to improve our understanding of the circulation of NRIV in selected predominantly pastoral ecosystems in Kenya. We aimed to detect and characterise NRIV in serum samples collected from a network

of livestock hosts, such as goat, sheep, and cattle in dryland ecosystems in Kenya. Findings of the study have implications for the sources of NRIV outbreaks in humans and for surveillance and control of NRIV.

4.2 Materials and Methods for detection, isolation and seroprevalence of NRIV.

4.2.1 Study design and sites.

The study, as part of a bigger project meant to improve understanding of arbovirus transmission networks in Kenya, was conducted as a cross-sectional field and laboratory-based survey. Sampling was performed twice a year in May/June and September/October between 2020 and 2021 immediately at the end of the rainy seasons (March to May, and August to September) in selected sites of Baringo and Kajiado counties in Kenya's Rift Valley. The two ecologies are semi-arid and inhabited by pastoral communities providing high interactions of humans, livestock, and wildlife. Several arboviruses are endemic to Baringo county, like yellow fever virus (183), RVFV and the recently discovered phleboviruses Ntepes virus, Perkerra virus, Embossos virus, Bogoria virus and Kiborgoch virus (20,21).

4.2.2 Livestock Sampling.

A total of 2039 apparently healthy/asymptomatic cattle (n=715), goats (n=680) and sheep (n=644) aged 1-3 years were sampled from the two study sites by a team including a registered veterinarian and/ or animal health technician. Sampling was done twice a year after the rainy seasons when the vector and arboviral activity were presumed to be high. Approximately 5mL whole blood was collected aseptically from the jugular vein of each animal into 10mL BD Vacutainer® blood collection with EDTA. Blood for serum was collected into 10mL BD Vacutainer® blood collection plain vacutainers precoated with serum activator. The samples were processed, aliquoted into cryovials and appropriately labelled. All samples were transported on dry ice from the field to the Martin Lüscher Emerging Infectious Diseases (ML-EID) Laboratory at *icipe* for immediate testing and/or storage at -80˚C until further screening.

4.2.3 PCR screening.

Five to seven individual livestock serum samples were pooled depending on the species and site (100 µL per sample) for RNA extraction. Viral RNA was extracted from 140 µL of pooled serum using the QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany) according to the manufacturer's protocol. A volume of 50 µL of RNA was obtained and used as a template for

cDNA synthesis by Invitrogen SuperScript™ III Reverse Transcriptase. Each 20 µL cDNA reaction was prepared by adding 10 µL of extracted RNA to 1µL random hexamers (50 mM), 1.5 µL of Invitrogen™ RT-PCR Grade water and 0.5 µL Thermo Scientific™ dNTP Mix (25 mM) to make a 13 µL reaction (Mixture 1) and incubated at 65°C for 5 min, then placed on ice for 1 min. Mixture 1 was then added to 7 µL of mixture 2, containing 1µL Invitrogen™ SuperScript™ III Reverse Transcriptase, 1µL Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor, 1 µL Thermo Scientific™ USB Dithiothreitol (DTT) and 4 µL 5X RT First Strand buffer, incubated at 15°C for 20 min, 50°C for 60 min, then 85°C for 5 min. The cDNA was stored at -80°C until further use.

Samples were screened by a generic PCR assay using established pan-orthobunyavirus primers targeting the L segment (189), as well as other arboviruses. The reaction volume (25 µL) comprised 15.65 µL PCR water, 2.50 µL 10x-Buffer, 1.25 µL Mg (50 mM), 0.50 µL dNTPs (10mM), 1.5 µL of 10 µM forward and reverse primers, 0.10 µL Platinum-Taq polymerase (2 units/µl) and 2.0 µL template (cDNA). Subsequent nested PCR was performed using the PCR product of the first round PCR as a template. The PCR conditions were 95°C for 3 min, touch down of 0.5°C per cycle for 10 cycles starting with 95°C for 15 s, 55°C for 20 s and 72°C for 40 s, followed by 35 cycles of 95°C for 15 s, 50°C for 20 s, 72°C for 40 s and a further extension of 72°C for 10 min. The PCR products were electrophoresed in a 2% agarose gel stained with ethidium bromide and positive samples were purified using ExoSAP-IT™ PCR product Clean-up Reagent (Applied Biosystems) according to the manufacturer's instructions and submitted for bidirectional sequencing.

The sequencing services were outsourced from Macrogen, Europe B.V. RNA was subsequently extracted from individual serum samples that made up the initial positive pool and rescreened as previously described in order to identify the individual positive serum sample.

4.2.4 Viral isolation.

A confluent monolayer of Vero cells (ATCC® CCL-81) grown in 24-well tissue culture plates (Nunc, Roskilde, Denmark) in Growth Media (GM) containing Gibco Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% Gibco™ Foetal Bovine Sera (FBS), 2% Gibco™ Antibiotic-Antimycotic (100X), and 2% Gibco™ L-Glutamine (200 mM) was used for initial inoculations. Fifty microlitres of individual serum samples from NRIV PCR positive pools were inoculated into each well of the confluent monolayer and incubated in a 5% CO_2 incubator (New Brunswick™ Galaxy® 170 R CO_2 Incubator Series, Eppendorf, USA) at 37˚C for 1 h, rocking after every 15 min. After adsorption, maintenance media (MM) containing 100 µL

DMEM supplemented with 5% Gibco™ FBS, 2% Gibco™ Antibiotic-Antimycotic (100X), and 2% Gibco™ L-Glutamine (200 mM) was added to each well and incubated at 37°C in a 5% CO_2 incubator for up to 14 days, observing the cells daily for cytopathic effects (CPE) using a Leica DMi1 LED Cell inverted microscope. The positive samples were passaged in a T-25 flask containing confluent Vero cells up to the second passage. The virus was harvested by freeze thawing the infected cells then centrifuging at 3000 rpm for 10 min. The supernatant was used for RNA extraction and downstream RT-PCR reaction, next generation sequencing (NGS), and stored at -80°C until further use.

4.2.5 *In vitro* viral growth kinetics of Ngari virus.

To understand the growth characteristics of the NRIV isolates, *in vitro* growth kinetics was performed in vertebrate cell lines: human, HEK293-T (human embryonic kidney cells); sheep, Llu-L; goat, ZnR (zinc sensing receptor cells); primate, VeroE6 (African green monkey, kidney cells); as well as insect cell lines: U4.4 (*Aedes albopictus*), CxT (*Culex tarsalis*), Aag2 (*Aedes aegypti*), AS (*Anopheles stephensi*), and C6/36 (*Aedes albopictus* larvae); sand fly, PP-9 (*Phlebotomus papatasi*). Insect and vertebrate cells were infected with NRIV in duplicates at a multiplicity of infection (MOI) of 0.1 and 0.01, respectively. Aliquots of infectious cell culture supernatants of vertebrate and insect cells were harvested every 24 hours for a period of six and three days, respectively. Viral genome copy numbers were quantified by real-time RT-PCR using plasmid-based quantification standards.

4.2.6 Genome sequencing and analysis.

The clarified supernatant of isolates from sheep, KE_O93, goat KE_C166 and cattle KE_B02 and KE_B35 all from Baringo county were filtered using 0.22-µm filters to concentrate the viral particles and remove any host “contaminants” and bacteria. RNA was extracted from the isolates and libraries prepared using the KAPA HyperPlus kit (Roche, Penzberg, Germany), sequenced using the Illumina MiSeq HTS platform with a designated yield of ~25 million paired-end reads (21,64,209).

Raw NGS reads were trimmed, assembled, and analysed in Geneious Prime (http://www.geneious.com), Biomatters. The reference-assisted assembly was done using default parameters to obtain a full-length genome sequence of the isolates. The obtained contigs were reconfirmed using BLASTn in the NCBI database (209,210). Confirmed sequences were translated, and CDS predictions were performed in Geneious Prime. Phylogenetic analysis was performed based on the nucleotide sequences of the L, M, and S segments. Related sequences

were downloaded from the GenBank-NCBI database and multiple sequence alignment performed by MAFFT (198). Phylogenetic analysis was performed in Geneious Prime (http://www.geneious.com) with PhyML (199), and the GTR model of sequence evolution. Nodal support was assessed by applying 1000 bootstrap replicates. To understand the virus evolutionary relationships, the isolated viruses' closest homologs based on each of the three segments were downloaded from GenBank and used as references in sequence analysis. The inferred phylogenies were visualized in Figtree v1.4.4.

4.2.7 Indirect immunofluorescence assay (IIFA).

One hundred and forty-four randomly selected serum samples from cattle (n=60), sheep (n=42) and goats (n=42) were screened for anti-NRIV IgG antibodies using a modified NRIV IIFA slide test kit. The slides contained a mixture of KE_O93 NRIV isolate and non-infected Vero E6 cells in a 1:1 ratio fixed on each well except the negative control well which contained non-infected Vero E6 cells. The serum samples were diluted at a ratio of 1:10 with sampling buffer, and 25 µL of each diluted sample was applied to the biochip and incubated for 30 min at room temperature. After incubation, the slides were washed twice for 5 min in a wash buffer containing phosphate-buffered saline (PBS), pH 7.2 and 0.2% Tween 20. Twenty-five microlitres of Alexa 488 labelled donkey anti-sheep IgG antibodies, donkey anti-goat IgG antibodies, and goat anti-bovine IgG antibodies (Dianova, Hamburg, Germany) for sheep, goat, and cattle respectively, diluted 1:200 in PBS were applied to each well according to the sample type. The slides were incubated at room temperature in the dark for 30 min, washed twice with wash buffer for 5 min each and then rinsed in distilled water for 2 min. A drop of Prolong Gold Antifade Reagent with DAPI was added, covered with a coverslip, and allowed to dry. Finally, the slides were examined on a fluorescence microscope (Zeiss Flourescence Microscope).

4.2.8 Plaque Reduction Neutralization Test (PRNT).

All NRIV IIFA positive samples were confirmed using PRNT. Vero cells grown in Growth media (GM) were seeded in 24-well plates at a concentration of $1x10^6$ cells per well at a volume of 1 mL per well and incubated overnight at 37˚C in a 5% CO_2. The cells were observed under the microscope after a day to ensure 70-90% confluency and even cell distribution before inoculation. The IIFA positive serum samples were then aliquoted and heat-activated at 56˚C in a water bath before serial dilution in maintenance media (MM). Two-fold serial dilutions 1:10 -1:320 of IFA positive serum samples were prepared in a microtiter plate, then 30 µL of each serially diluted serum sample was mixed with an equal amount of NRIV KE_O93 isolate

diluted to a plaque assayed standard concentration that gave 20–50 plaques. The mixtures were incubated at 37˚C in the presence of 5% CO_2 for 1 h.

A co-incubated mixture of antibody and NRIV virus was used to infect seeded 24-well culture plates containing confluent monolayers of Vero cells after pouring off the growth media and incubated at 37°C for 60-90 minutes in a 5% CO_2 incubator. After incubation, 2 mL of 2.5% H7509 Sigma-Aldrich methylcellulose (viscosity 4000cP) was added to each well and incubated at 37˚C in a 5% CO_2 incubator for 7 days. The plates were then fixed by 3.7% (v/v) F8775 Sigma-Aldrich formaldehyde solution prepared in Gibco Dulbecco's PBS pH 7 by pipetting 2 mL per well for a minimum of 2 h and then stained with 0.5% (w/v) C0775 Sigma-Aldrich crystal violet prepared in absolute ethanol at 2 mL per well overnight, washed, and dried, after which the plaques counted manually. PRNT90 positive samples were determined as the reciprocal of the serum dilution, giving ≥90% reduction in plaque counts (188).

4.2.9 Statistical Analysis.

The seroprevalence data was analyzed using R version 4.2.0. Comparison of NRIV seroprevalence between the two study sites and different species was done using Fisher exact test. The 95% confidence intervals (CIs) were estimated using the Agresti-Coull method. All tests were performed at a 5% significance level.

4.3 Sequence Accession Numbers.

The four isolates (KE_C166, KE_O93, KE_B02, and KE_B35) L, M, and S segments sequences were deposited in GenBank under the accession numbers ON755192–ON755203.

4.4 Results

Ngari virus infects sheep, goat, and cattle in Kenya. A total of 2039 sera from apparently healthy cattle (n= 715), goats (n= 680) and sheep (n= 644) aged 1-3 years were sampled from selected sites inhabited by pastoral communities of Baringo and Kajiado counties in Kenya's Rift Valley (**Figure 2**). Of the total, 1239 samples (60.8%) originated from Baringo county (cattle, n= 415 samples, goats, n= 420 samples, and sheep, n= 404 samples) and 800 samples (39.2%) originated from Kajiado county (cattle, n=300 samples, goats, n=260 samples, and sheep, n= 240 samples). NRIV was detected in eleven individual samples (11/2039, 0.54% (95% CI 0.29-0.98): three from cattle (3/715, 0.42%), three from goats (3/680, 0.44%) and four from sheep (4/644, 0.62/%) by RT-PCR of which two goat sera were from Kajiado county and

the other nine from Baringo county (**Table 4.1**). None of the eleven NRIV positive samples tested positive for any other arbovirus, including RVFV and BUNV.

Table 4.1: Origin of NRIV positive livestock samples

Sample-ID	Livestock species	Age (Yrs)	Sex	Place of origin (County/location)	Dates of sample collection
KE-O52	Sheep	2	Female	Baringo (Sotwa)	22/09/2020
KE-O56	Sheep	2	Female	Baringo (Sotwa)	22/09/2020
KE-O93	Sheep	2	Male	Baringo (Loropilli)	23/09/2020
KE-O288	Sheep	2	Female	Baringo (Elketeiyo Sintaan)	19/05/2021
KE-C166	Goat	2	Male	Baringo (Lokuru)	26/09/2020
KE-C177	Goat	2	Male	Baringo (Lokuru)	27/09/2020
KE-C106	Goat	1	Female	Kajiado (Oldorko)	13/10/2020
KE-C174	Goat	2	Male	Kajiado (Entasopia)	15/10/2020
KE-B02	Cattle	1	Male	Baringo (Ilngarua)	16/09/2020
KE-B29	Cattle	2	Male	Baringo (Perkerra)	17/09/2020
KE-B35	Cattle	2	Male	Baringo (Perkerra)	17/09/2020

NRIV tropism appears restrictive to *Aedes* cells, while vertebrate cells are broadly susceptible. We next attempted to isolate the virus from the eleven PCR-positive serum samples in cell culture. Seven samples from Baringo county consistently displayed similar cytopathic effects (CPE) 2-4 days post-inoculation (dpi). Sequence analysis of extracted RNA from the supernatant of the isolates (goat: KE_166, KE_177; sheep: KE_O93 and KE_O288; cattle: KE_B02, KE_B29, and KE_B35) confirmed the isolation of NRIV in cell culture.

Albeit that, NRIV has been detected in various mosquito species from different genera, *in vitro* growth analyses showed that NRIV replicated only in cell lines derived from *Aedes albopictus* (C6/36 and U4.4) but not in cells derived from *Anopheles* or *Culex* mosquitoes nor *Phlebotomus* sandflies (**Figure 4.1A**) suggesting a vector specificity of NRIV towards *Aedes* mosquitoes. In contrast, vertebrate cells were broadly susceptible for NRIV infection with peak genome copy numbers in cells derived from human, sheep, and non-human primates and 1,000 – 10,000-fold lower replication rates in goat cells (**Figure 4.1B**).

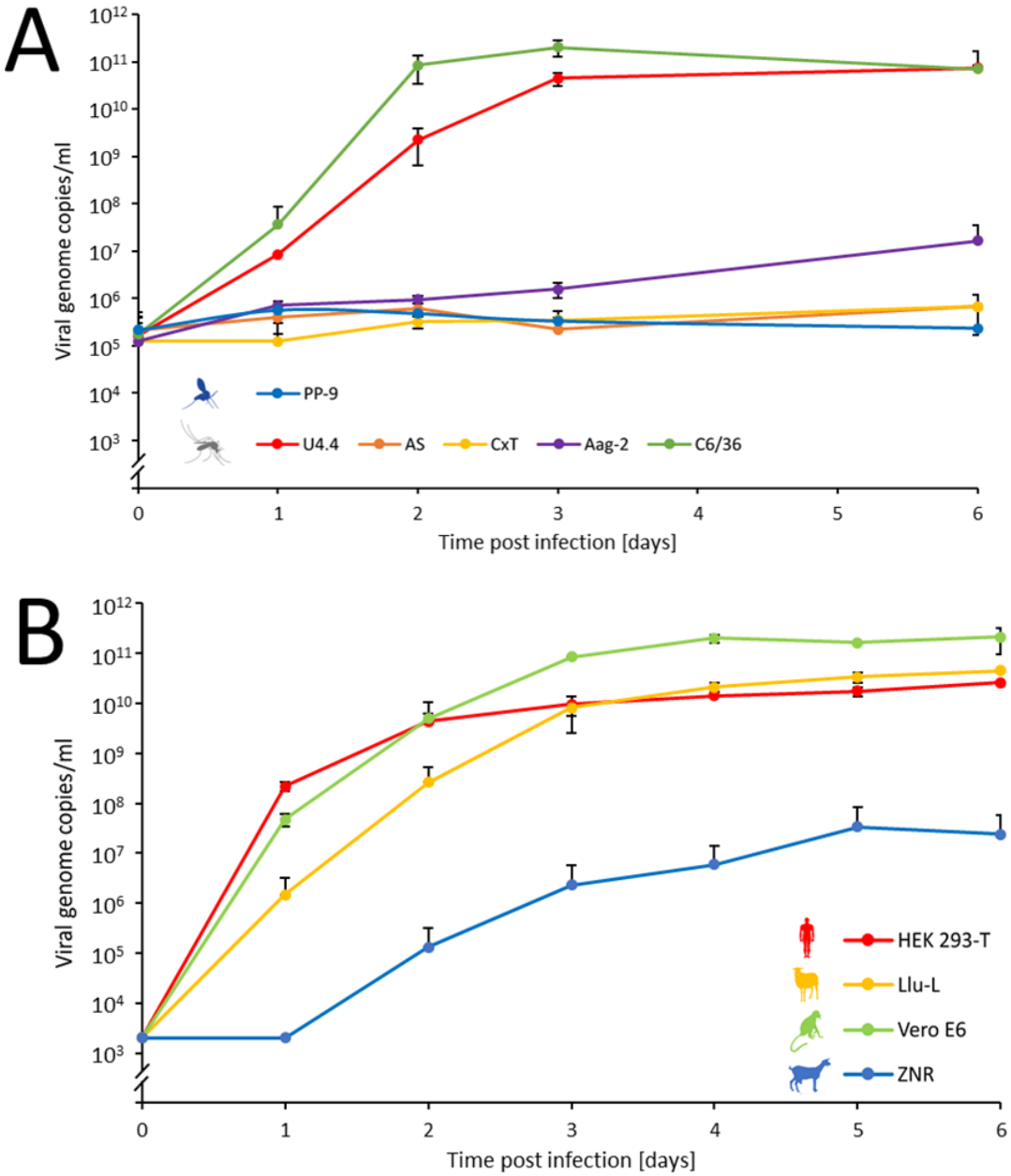


Figure 4.1: In vitro growth kinetics of NRIV in different cell lines. **A**) Insect cell lines (U4.4, AS, CxT, Aag-2, C6/36, mosquito; and PP-9, sand fly) were infected with NRIV in duplicates with a multiplicity of infection (moi) of 0.1. **B**) Vertebrate cell lines (HEK293-T, human; Llu-L, sheep; VeroE6, primate; ZN-R, goat) were infected with NRIV in duplicates with a moi of 0.01. Infectious cell culture supernatants were collected at the indicated time points and viral genome copies were measured by real-time reverse transcription PCR.

The Kenyan NRIV isolates are most closely related to NRIV isolates from Mauritania. Entire NRIV genomes were sequenced from four virus isolates derived from sheep (KE_093), goat (KE_C166), and cattle (KE_B02 and KE_B35). All four genomes showed similar length for the three segments of L=6717 nucleotides (nt), M=4305 nt, and S=702 nt and were closely

related to each other with nucleotide identities of 99.7-100%. Phylogenetic analyses based on nucleotide sequences of entire RdRp, glycoprotein and nucleoprotein ORFs showed that the four Kenyan strains shared a most recent common ancestor (MRCA) with the NRIV Adrar strain isolated from a goat in Mauritania in 2010 (45) (**Figure 4.2**). All NRIV sequences formed a monophyletic clade with a sister relationship to BUNV sequences based on L and S segment derived phylogenies, whereas the NRIV M segment sequences were placed as a sister clade to BATV sequences, as has been observed in previous studies.

Figure 4.2: Phylogenetic analysis of the isolated NRIV in livestock from Baringo County based on the cds nucleotide sequences of the: A) L segment (6717nt), B) M segment (4305 nt); and C) S segment (702 nt) compared to other orthobunyaviruses. The samples in this study are highlighted in red. The sequences were aligned using MAFFT with E-insi and 100PAM. The ML tree was calculated using IQtree via CIPRES (Geneious plug-in at www.phylo.org) and non-parametric 1000 bootstrapping was used.

Undetected circulation of NRIV in livestock in Kenya. To assess the proportion of animals that have been exposed to NRIV, sera from both study sites were tested for presence of antibodies against NRIV. Analyses of 144 sera by indirect immunofluorescence assay (IIFA) identified 60 reactive samples (41.6%; 95% CI 0.34-0.5) of which 55 (38.2%; 95% CI 30.2-46.8) were confirmed by Plaque Reduction Neutralization Test (PRNT) with titers ranging from 1:40 to 1:320 (**Figure 4.3**). NRIV seroprevalence rates in cattle, goats, and sheep were: 41.6% (95% CI 30-54.3), 52.4% (95% CI 37.7–66.6), and 19% (95% CI 9.7-33.6), respectively. The proportion was higher in goats and the lowest in sheep in both sites (**Table 4.2**). NRIV antibody prevalence did not significantly differ between Baringo (33.3%, 24/72) and Kajiado counties (43.1%, 31/72; Fisher exact test odds ratio [OR] 1.4, 95% CI 0.83–2.66; p = 0.18).

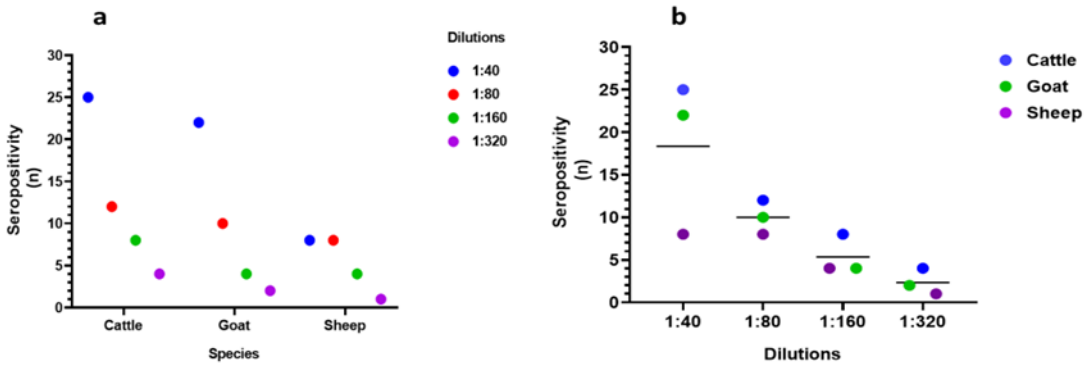


Figure 4.3: Number of livestock samples positive for NRIV neutralizing antibodies per species (a) and at various PRNT dilutions (b). The n on the Y-axis represents the number of individuals per species that tested positive. The horizontal line within the graph represents the mean of individuals that tested positive. The graph was constructed using GraphPad Prism 9.3.1.

Table 4.2: Summary of NRIV-neutralizing serum samples from cattle, sheep, and goats.

Sites				**Species**			**Sex**		**Age (years)**				
Baringo	**Location**	**Total sample**	**Total No. Pos**	**Cattle**	**Goats**	**Sheep**	**Female**	**Male**	**3**	**2**	**1**	**Prevalence per Location (%)**	**95% CI**
	Ilngarua	31	10	5(21)	5(10)	0(0)	7(19)	3(12)	2(2)	6(10)	2(19)	32.3	18.6-49.9
	Perkerra	4	3	3(4)	0(0)	0(0)	3(4)	0(1)	1(1)	2(3)	0(0)	75	30.1-95.4
	Ngarie	6	3	0(0)	3(6)	0(0)	2(4)	1(2)	1(2)	2(3)	0(1)	50	18.8-81.2
	Lokuru	5	2	0(0)	2(5)	0(0)	2(4)	0(1)	0(0)	1(3)	1(2)	40	11.8-76.9
	Sotwa	5	3	3(5)	0(0)	0(0)	2(4)	1(1)	0(0)	2(4)	1(1)	60	23.1-88.2
	Sintaan	10	0	0(10)	0(0)	0(0)	0(9)	0(1)	0(0)	0(10)	0(0)	0	0-27.7
	Loropilli	11	3	0(0)	0(0)	3(11)	2(6)	1(5)	1(2)	2(6)	0(3)	27.3	9.8-56.7
Kajiado	Olesinyai	39	15	14(30)	0(0)	1(9)	14(33)	1(6)	3(8)	9(23)	3(8)	38.5	24.9-54.1
	Oldorko	16	8	0(0)	8(10)	0(6)	7(9)	1(1)	3(6)	5(9)	0(1)	50	28-72
	Entasopia	11	4	0(0)	4(11)	0(0)	2(9)	2(2)	0(0)	4(6)	0(5)	36.7	15.2-64.6
	Birika	6	4	0(0)	0(0)	4(6)	3(4)	1(2)	3(3)	0(1)	1(2)	66.7	30-90.3
Total		**144**	**55**	**25(60)**	**22(42)**	**8(42)**	**43(108)**	**12(36)**	**13(23)**	**30(79)**	**12(42)**	**33.8**	

(): Total in different categories

4.5 Discussion

We report the circulation of NRIV in livestock (cattle, goats, and sheep) from two selected pastoralist ecologies of Kenya. This is the first report of the isolation, detection, and characterisation of the virus in livestock hosts in Kenya. So far, the virus has been detected sporadically during RVF outbreaks associated with hemorrhagic fever and this is the first detection apart from an outbreak situation. The virus was first reported in the country during the RVF outbreak in East Africa in 1997 and1998 in hemorrhagic fever patients from North Eastern Kenya (30,32–34,45). Since then, there has been only one study that reported the presence of NRIV antibodies in febrile patients from different health centers in North Eastern Kenya between 2009-2012 (91), but no further report of NRIV infection in humans or livestock, perhaps due to the lack of specific active screening and surveillance of the virus. However, NRIV has been detected in mosquitoes and ticks from different parts of the country and several vectors were shown to be able to transmit the virus in laboratory experiments (44,101,158,161,203–205). Thus, depending on the distribution and feeding habits of these vectors, NRIV could be more abundant than available data would suggest. Herein, we found

high seroprevalence rates in the three most common livestock species and detected several viraemic animals which did not show any obvious signs of disease, a clear indication that the virus is endemic in the country and actively circulating. Importantly, our data indicate the unreported circulation of a NRIV in the three most common livestock species.

There have been few studies carried out testing livestock for NRIV infections, and consequently the epidemiology of NRIV in Kenya as well as the entire African continent remains unclear. To the best of our knowledge, there are only two reports available where NRIV was detected in small ruminants (goats and sheep) during RVF outbreaks in Mauritania in 2010 and 2015-2016 (45,46). Seroprevalence rates below 9% with antibody titers of up to 1:2560 were detected in small ruminants of varied ages (1-10 years) during the Mauritanian RVF outbreak in 2015-2016 (46). In contrast, we observed an overall NRIV seroprevalence rate of 38.2%, in small and large ruminants (cattle 41.7%, goats 52%, and sheep 19%) at the age of one or two years with lower titres of up to 1:320 confirming high exposure of the three livestock species to the virus. The seroprevalence was high in goats at both sites and less in sheep, but whether this difference is attributed to animal species-specific susceptibility cannot be ascertained due to the small sample size screened and lack of wider geographic scope. There was no significant difference in the seroprevalence rate between the two sites, Baringo county (33.3%) and Kajiado county (43.1%) (Table 2). None of the samples screened were positive for either RVF or BUNV antibodies. However, seroprevalence against BATV was not performed and though the possibilities of virus cross reactivity cannot be ruled out, unlike NRIV and BUNV which have been repeatedly detected in mosquitoes in the country, BATV is yet to be reported. Our inference nonetheless is based on a small number of samples, requiring validation of our results through extended surveys employing larger datasets.

We document active circulation of the virus in apparently healthy members of three livestock species: cattle, goats, and sheep. This pattern is inconsistent with previous detections of NRIV during RVF outbreaks (45,46). Our detection of NRIV in apparently asymptomatic animals in different areas in Kenya suggests that a wider geographic circulation that has so far gone unnoticed, is likely. Whether the virus contributes to the disease burden within the livestock population could not be ascertained. Hence, further studies including experimental challenges may be required to understand NRIV impact on livestock health, if any, as well as to investigate the role of livestock in the epidemiology of NRIV and the possible impact on the associated human population.

Phylogenetic analyses revealed that the NRIV strains detected in this study were genetically most closely related to strains found in small ruminants in Mauritania in 2010 and 2015 (45,46),

an observation that is not surprising considering limited sequence data available for NRIV from livestock. However, the close relationship of the two strains could as well suggest either low genetic diversity of NRIV, which is supported by the low phylogenetic diversification of the entire NRIV clade, and an expansive geographic circulation in Africa or, alternatively possible exchange of infected animals (or vectors) between the two countries. Interestingly, the available NRIV strains did not cluster according to geographic origin or associated host.

Although NRIV has been isolated from various mosquito species of different genera and a large vector range is suggested, our *in vitro* growth kinetics rather suggest a vector specificity to *Aedes* mosquitoes.Several studies detected NRIV in *Aedes* mosquitoes including the first isolation of NRIV from *Aedes simpsoni* mosquitoes in 1979 in South Eastern Senegal (3,36,43). NRIV has been reported to co-circulate with RVFV in Kenya and with RVF, BATV and BUNV in Mauritania (32,34,45,46). Factors contributing to the co-circulation are not well understood. However, these viruses infect similar vectors and hosts facilitating simultaneous transmission of multiple viruses between vectors and hosts. The regular isolation of BUNV and NRIV from pools of flood water *Aedes* mosquitoes in northern Kenya supports the hypothesis of simultaneous transmission of multiple viruses by mosquitoes (203). The implementation of differential diagnosis for NRIV, RVFV, BATV and BUNV would be important whenever symptoms of disease compatible with infections with these viruses occur in endemic areas (32,33,45,211). The most recent RVFV outbreak in Kenya was reported in Mandera, Isiolo, Garissa, and Murang'a counties in 2021, according to the WHO Report, 2021 (212). It affected animals and humans with a reported case fatality rate of 34% and 55% in humans and livestock, respectively. Surprisingly, only a subset, (20/120) of the samples collected from suspected animal cases tested positive for RVFV according to Central Veterinary Laboratories (CVL) Kabete Lab Report (unpublished) and WHO report, 2021 (212) suggesting the potential contribution of pathogens of unknown aetiologies to the outbreak. Moreover, considering that frequent RVFV outbreaks occur in these areas despite RVFV vaccination campaigns, it would be important to test for the potential contribution of other pathogenic arboviruses known to circulate in the region, such as NRIV, BATV and BUNV, through specific laboratory screening.

Chapter 5

TRANSMISSION DYNAMICS OF CRIMEAN CONGO HAEMORRHAGIC FEVER VIRUS (CCHFV); EVIDENCE OF CIRCULATION IN HUMANS, LIVESTOCK AND RODENTS IN DIVERSE ECOLOGIES IN KENYA.

This chapter presents the results obtained through molecular and serological screening for CCHFV in the three discrete host groups: humans, livestock, and rodents. Molecular screening through CCHFV specific primers and sequencing allowed the detection and characterisation of CCHFV in rodents and sheep, and serological screening through ELISA was carried out to determine the prevalence in the three host groups.

5.1 Introduction

Crimean Congo haemorrhagic fever virus (CCHFV) is a tick-borne zoonotic virus in the species Crimean-Congo haemorrhagic fever orthonairovirus, genus Orthonairovirus, family Nairoviridae in the order Bunyavirales (1,2). The virus can induce symptoms that range from mild febrile illness up to severe haemorrhagic fever in humans. It was first described in Crimea in 1944 and given the name Crimean haemorrhagic fever and later, in 1969, recognized as the same pathogen responsible for an illness identified in 1956 in the Congo and was thus designated as Crimean-Congo Haemorrhagic Fever (12,213,214). CCHF has a case fatality rate of 10-40% according to the World Health Organisation (WHO) (134). Of the seven CCHFV genetic lineages (Africa 1, 2 and 3, Asia 1 and 2 and Europe 1 and 2), all are associated with severe disease in humans except the Europe 2 lineage (215).

Several studies have confirmed the presence of antibodies and even detection of infectious virus in domestic animals from different parts of the world (131,134,162,216). Belobo *et al*., 2021(217), reported an overall worldwide CCHFV seroprevalence of 12.0% in animal species and Spengler *et al*., 2016 (104), seroprevalence in domesticated animals of 19.3% in cattle, 21.7% in donkeys, 28.1% in goats and 23.9% in sheep [15][16]. Much higher seroprevalence rates occur in Africa among livestock species in different countries including Uganda (36.5%), Zimbabwe (37%), Mauritania (67%), South Africa (74.2%) and Senegal (32.5%), amongst others countries (218–221). However, no clinical disease has been reported in wild and domestic animals thus far and it is believed that they serve as amplification hosts that play an important role in the virus maintenance cycle.

Rodents, especially mice, can be infected with CCHFV and serve as animal models for infection and pathogenicity studies (216). In nature, they may also contribute to CCHFV transmission and spread as they are highly exposed to tick bites. Rodents are widely distributed in peri-domestic habitats and contact with human populations often occurs with a potential risk of various disease transmission including CCHF (222). The rodent species commonly found in human settlement e.g., *Rattus rattus*, *Mastomys natalensis*, *Crocidura spp*. and house mice (*Mus spp*.) are known to be vectors of medically important hantaviruses and arenaviruses but also arboviruses like Wesselsbron and Usutu viruses (USUV) (222–224). However, the role of rodents in CCHFV transmission is not well understood. Rodents can become infected through parasitized infected immature tick species which in turn could infect/transmit CCHFV to humans that live in close proximity (225). Previous studies have reported presence of CCHFV antibodies in the mouse species *Apodemus sylvaticus, A. agrarius, A. sylvaticus* (Hungary), brown rat *Rattus norvegicus* (Pakistan); Bushveld gerbil (*Gerbilliscus leucogaster*), *Aethomys namaquensis, Rhabdomys pumilio* and *Mastomys spp*. (South Africa/Zimbabwe); *Rattus rattus* (Pakistan); *Arvicanthis niloticus* and *Mastomys erythroleucus* (Mauritania); unidentified rodents in Iraq and Iran, amongst others (216,219,225–228).

How CCHFV is maintained in an ecosystem is a subject of interest. *Hyalomma* ticks of the family *Ixodidae* have been implicated as vectors and reservoirs of the virus (12,214,219). The virus has also been detected in other tick species in the genera *Rhipicephalus, Amblyomma,* and *Dermacantor*, although their role in virus maintenance and transmission has yet to be determined (13,214,229). Ticks are also considered as reservoirs supporting long-term survival of the virus in contrast to short-term viremia observed in vertebrate hosts (104,217,230,231), e.g. non-systemic transmission of the virus in ticks can occur through co-feeding on a non-viraemic host as well as via venereal routes which both ensure prolonged virus survival (116,214,225,232–234).

CCHFV has been detected in ticks sampled from various domesticated livestock and the presence of antibodies against CCHFV in livestock indicates their participation in the amplification cycle, yet few studies have detected CCHFV genome copies in these hosts, most probably due to short viraemia (158,159,200–202). Animals and humans become infected with CCHFV via bites of an infected tick. In addition, human infection can also occur through contact with blood, secretions, organs or other body fluids of infected persons and livestock during slaughter. Consequently, this makes people who are constantly in contact with animals such as pastoralists, veterinarians, and abattoir workers, more prone to CCHFV infections, although the viraemia is short in the majority of hosts, lasting 2-7 days (13,235). The vertebrate

hosts amplifies the virus and supports the virus spread from one tick to another (11,236). CCHFV is widely distributed and endemic in some parts of Europe, the Middle East, Africa, and in Asia. This geographic spread is believed to be influenced by the distribution of the primary *Hyalomma* tick vector, the prolonged maintenance of the virus in ticks through horizontal and vertical transmission, long distance movement of livestock as well as long feeding times associated with ticks (12,155,214).

In Kenya, CCHFV was first detected in *Rh. pulchellus* in 1970 from a dying sheep at Kabete veterinary laboratories (229). The first documented case of acute human infection with CCHFV was in 2000 in a farmer in western Kenya (157,225). Subsequent studies have detected the virus in diverse tick species infesting livestock and humans through serosurveys (159,162,182,202,237). For instance, in Northeastern Kenya, the virus was detected in *Hy. rufipes* and *Hy. truncatum* infesting cattle and camel, and more recently in *Rh. decoloratus* in western Kenya (159,202). A recent study in Baringo and Kajiado counties confirmed livestock infestation with *Rhipicephalus spp.* and *Hyalomma spp.* ticks (184), which represent potential risk factors for CCHFV circulation. Serosurveys have revealed CCHFV prevalence among febrile patients ranging from 14 to 35% in different geographical areas in Kenya (162,181,182). To date, few studies have monitored infection rates of the virus in livestock and wildlife in Kenya (200,201). Fewer still have examined the role of rodents in the epidemiology of the virus.

In this study, a one health surveillance of CCHFV among a network of vertebrate hosts, including humans, was implemented. The study was instituted in two pastoralist dominated areas, Kajiado and Baringo counties, in the Kenyan Rift Valley. Specifically, evidence of infection with the virus and exposure inapparently asymptomatic livestock, including cattle, goat, and sheep, and peridomestic rodents, were assessed. Furthermore, syndromic surveillance of the virus in patients with febrile illness was established in the study areas.

5.2 Materials and Methods

Methods unique to this chapter are described here, the rest are as listed in chapter 2.

5.2.1 Study Site

Humans, livestock, and rodent sampling was conducted in different locations within Baringo (0.4695° N, 35.9833° E) and Kajiado (1.7617° S, 36.0255° E) counties. Both sites are located in the Kenyan Rift Valley and have semi-arid ecologies with diverse populations and a history

of arbovirus circulation (20,21,181–184). Both ecologies are inhabited mostly by nomadic pastoralist communities.

5.2.2 Sample Collection

Human sampling

Human sampling was conducted in Marigat Subcounty Hospital (Baringo County) and Entasopia Health Centre (Kajiado County). Febrile patients (male and female) ≥5 years presenting with a clinical case definition of acute febrile illness characterised by fever (body temperature ≥ 38˚C) and with any of these clinical manifestations: joint pains, headache, chills, general body malaise, cough, and any signs of bleeding and neurological abnormalities, were formally recruited into the study after obtaining their written consent or assent for children from guardians.

A total of 5 mL of blood was collected from each participant into BD Vacutainer® serum tubes with a clot activator by a trained and licenced phlebotomist. Serum was then processed from the blood by centrifuging in an Eppendorf™ 5702 Series Centrifuge at 1500 rpm for 10 min and aliquoted into 1 mL volumes. Processed samples were stored in liquid nitrogen at the health facilities until collection and transportation on dry ice to the Emerging Infectious Disease (EID) Laboratory at *icipe*, Nairobi, to be stored at -80˚C until further analysis.

Livestock sampling

Targeted sampling of domesticated livestock aged 1-3 years was conducted twice a year after the short and long rains when viral exposure in animals through abundant vectors are likely to be high. Sampling at both sites was performed by a registered veterinarian and/or animal health technician after obtaining verbal consent from the farmers. From each animal, two sets of blood samples were collected aseptically from the jugular vein, one into a 10 mL BD Vacutainer® with EDTA for whole blood and another into a 10 mL BD Vacutainer® precoated with serum activator for serum. The two sets of samples were transported on dry ice to the EID Laboratory at *icipe*, Nairobi, and then stored at -80˚C until screening.

Sampling of peri-domestic rodents

Rodents were trapped using the LFAHD Folding Live Capture Rodent/Rat/Mouse Traps (3 x 3.5 x 9” (7.62 x 8.89 x2 2.86 CM)) and SFA Small Folding Live Capture Rodent/Vole/Shrew/Mouse Traps (2 x 2.5 x 6.5” (5.08 x 6.35 x 16.51 CM)) (https://www.shermantraps.com/animal-traps/) set inside homes and their surrounding according to the National Museum of Kenya’s (NMK) set guidelines. The traps were baited with a mixture of locally available peanut butter and white oats, opened at dusk, checked every

morning, and left closed during the day. Two to four traps were set per room/surrounding environment for the whole night depending on observed rodent activity and inspections were carried out each morning. Each trapped specimen was placed in a handling bag, weighed, identified to the genus or species level based on morphological and geographical criteria according to Kingdom guide to African mammals and East African mammals as reference guides and further by molecular analyses (186,187).

Parameters like species, sex, age, weight were recorded from each trapped specimen before being euthanatized by cervical dislocation. Thereafter, the tissues (kidney, spleen, lungs, heart, and liver samples) and blood if adequate were collected in 1.8 mL cryovials and 5 mL BD Vacutainer® tubes, respectively. The blood samples were thereafter processed, through centrifugation at 3000 rpm for 5 min and the serum aliquoted in cryovials. The samples were preserved in liquid nitrogen before transportation to the EID Laboratory at *icipe*, Nairobi, and kept at -80˚C until testing.

5.2.3 Seroprevalence of CCHFV in Humans, Livestock and Rodents

The seroprevalence of CCHFV in humans was performed by ELISA using commercial ELISA kits (VectoCrimean-CHF-IgG/IgM, Vector-Best, Russia), (https://vector-best.ru), based on the recombinant CCHFV antigen and human IgM monoclonal antibodies, respectively, according to the manufacturer's instructions. The reported diagnostic sensitivity and specificity of the IgG kit is 100% (95% CI 96.2–100) and 100% (95% CI 96.0–100) respectively, and IgM kit 100% (95% CI 96.4–100) and 100% (95% CI 96.9–100) respectively.

Livestock and rodent serum samples were screened for CCHFV antibodies by ELISA kit (ID Vet, https://www.id-vet.com) based on the recombinant purified nucleoprotein antigen (NP), according to the manufacturer's instructions.

The tests were performed in 96-well microplates and the Optical density (OD) values were determined using a BioTek ELX800 Microplate reader at 450 nm. Data analysis and interpretation were carried out according to the kits manufacturers' instructions.

5.2.4 RNA Extraction, PCR Screening and Sequencing

Five to seven individual livestock blood samples were pooled (100 µL per sample) per animal type and site for RNA extraction. Viral RNA was extracted from 140 µL of each pooled livestock serum sample, individual human sera and homogenized rodent tissues using the QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany) according to the manufacturer's protocol. A volume of 50 µL of RNA was obtained and used as a template for cDNA synthesis

by Invitrogen SuperScript™ III Reverse Transcriptase according to the manufacturer's instructions (Thermo Fisher Scientific). The cDNA was stored at -80°C until further use.
Samples were screened by RT- PCR using established CCHFV primers targeting the small (S) segment that encodes the nucleocapsid protein: CCHF F2 (5′-TGGACACCTTCACAAACTC-3′) and CCHFV_R3 (5′-GACAAATTCCCTGCACCA-3′), positions 135–153 and 653- 670, respectively, amplicon size of 536 bp (190). The PCR products were electrophoresed in a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich Chemie GmbH) and positive samples were purified using ExoSAP-IT™ PCR Product Clean-up Reagent (Applied Biosystems) according to the manufacturer's instructions, prior to bidirectional sequencing at Macrogen, Europe B.V. NGS libraries were prepared from PCR positive samples and whole genome sequencing (WGS) was attempted using the Illumina MiSeq platform.

5.2.5 Phylogenetic analysis

The sequences were cleaned in Geneious Prime software (https://www.geneious.com) and queried against the GenBank-NCBI database (195,196) using the Basic Local Alignment Search Tool (BLAST) (197). Related sequences were downloaded from the GenBank-NCBI database and multiple sequence alignment was performed in MAFFT (198). A phylogenetic tree was inferred using PhyML (199) under the general time reversible (GTR) model and applying 1000 bootstrap replicates to assess modal support. All analyses were performed in Geneious Prime (http://www.geneious.com) using the default parameters.

5.2.6 Statistical data analysis

The seroprevalence data were analysed using R version 4.2.0. Comparison of CCHFV seroprevalence between the two study sites and different hosts and species was done using a chi-square test. The 95% confidence intervals (CIs) were estimated using the Agresti-Coull method. All tests were performed at a 5% significance level. Multiple logistic regression analysis was performed to understand the predictive factors of CCHFV infection among livestock species, humans, and rodents as well as other species specific parameters investigated such as age and gender.

5.3 Results

CCHFV Virus Detection

A total of 493 human and 480 rodent samples, as well as 2039 livestock samples corresponding to 280 pools, were screened for CCHFV by RT-PCR. Of these, four sheep pools (Marigat=3, Nguruman=1), and four rodent samples (all from Marigat) tested positive for CCHF viral RNA. One of the four positive rodent samples was *Rattus rattus, and* the rest were *Mus musculus* (**Table 5.1**). Attempts to obtain full genomes from the positive samples were not successful.

Table 5.1: Individual CCHFV Livestock positive samples

Sample Identity	Origin	Host species	GPS Coordinates
030_0P3	Marigat	Sheep	N00.440679°E035.97664°
030_0P6	Marigat	Sheep	N00.53997° E036.03925°
030_OP43	Marigat	Sheep	N00.46214° E036.10767°
034_0P28	Nguruman	Sheep	S001.84785°E36.099044°
030_R102	Marigat	Rodent (*Mus musculus)*	N00.46868° E036.03048°
030_R123	Marigat	Rodent (*Rattus rattus)*	N00.46868° E036.03048°
030_R129	Marigat	Rodent (*Mus musculus)*	N00.47570° E036.03346°
030_R135	Marigat	Rodent (*Mus musculus)*	N00.47570° E036.03346°

Phylogenetic Analysis

The partial CCHFV S segment sequences (536 bp) generated both from sheep and rodents revealed high levels of sequence identity (96-98%) to other pathogenic strains in the CCHFV Africa 3 lineage and 80-99% nucleotide sequence identity among the detected viruses. The sequences formed a sub-clade that grouped within the Africa lineage 3 cluster together with other CCHFV strains reported from South Africa, Sudan, Mauritania, and West Africa (**Figure 5.1**).

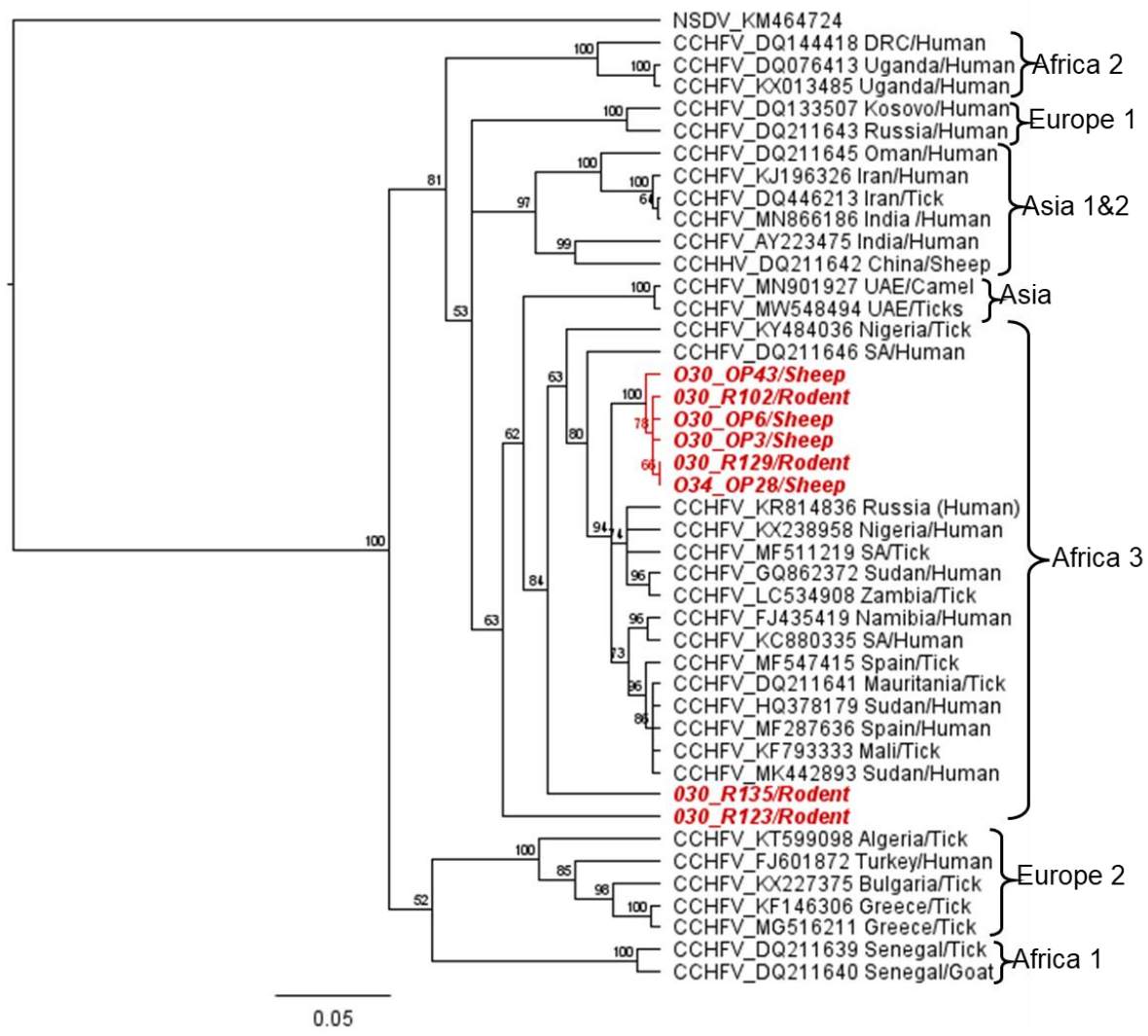


Figure 5.1: The maximum likelihood (ML) phylogenetic analysis based on the 536-nucleotide fragment of the nucleoprotein (S segment). All the sequences generated from sheep and rodents in this study shown in red, and were aligned to other orthonairovirus reference sequences obtained from the GenBank, using MAFFT and the tree inferred. Nodal support values obtained from 1000 boostrap replicatates were obtained from a using PhyML v. 2.2.4 analysis with GTR substitution model.

Seroprevalence of CCHFV in Humans, Livestock and Rodents

A total of 493 human samples were analyzed, of which 323 (65.5%) were from Marigat and 170 (34.5%) were from Nguruman. There was a higher proportion of females (295,59.8%) than males (198, 40.2%) in the sample set (**Table 5.2**).

Of the 493 human samples screened, 29 (5.9%) were positive for CCHFV IgG antibodies with a higher proportion observed in Marigat (22/29, 75.9%) than Nguruman (7/29, 24.1%), however, there was no significant difference ($p=0.23$) between the sampling sites (**Table 5.2**).

Prevalence rates did not differ by sex or age categories (**Table 5.2**). Also, occupation was a poor predictor of CCHFV exposure in humans based on IgG antibodies. Although being in contact with domesticated animals did not pose a risk to CCHF infection (*p=0.62*), those in contact with these animals were 1.4 times more likely to be infected. Location, age group, gender, and occupation, had no statistically significant effect on CCHFV seroprevalence and incidence but increased risk was observed in males and farmers **(Table 5.2 and 5.3**). In terms of symptoms, there was a significant positive correlation between CCHFV seropositivity and retro-orbital pain (*p*=0.042) (**Table 5.4**).

Table 5.2: Influence of demographics on CCHFV seroprevalence among febrile patients from Marigat Sub-County and Entasopia hospitals.

Parameter	Level	N (%)	Contact with animals. n (%)	IgG Positive	χ^2, df	P value
Location	Marigat	323 (65.5)	289 (89.5)	22 (6.8)	1.46, 1	0.23
	Nguruman	170 (34.5)	138 (81.2)	7 (4.1)		
Gender	Female	295 (59.8)	255 (86.4)	13 (4.4)	2.89, 1	0.09
	Male	198 (40.2)	172 (86.9)	16 (8.1)		
Age	5-10	49 (9.9)	41 (83.7)	3 (6.1)	1.53, 2	0.47
	10≤18	114 (23.1)	98 (60.1)	4 (3.5)		
	≥18	330 (66.9)	288 (87.3)	22 (6.7)		
Occupation	Artisan-Mason	1 (0.2)	1 (100)	0	6.82, 10	0.74
	casual work	2 (0.2)	0	0		
	Farmer	104 (21.1)	94 (90.4)	6 (5.8)		
	Non-Student	17 (3.4)	11 (64.7)	0		
	Nurse	2 (0.4)	1 (50)	0		
	Pastoralist	2 (0.4)	2 (100)	0		
	Farmhand	71 (14.4)	64 (90.1)	8 (11.3)		
	Businesspersons	27 (5.5)	22 (81.5)	2 (7.4)		
	Security personnel	2 (0.4)	2 (100)	0		
	Student	169 (34.3)	147 (87)	10 (5.9)		
	Unemployed	97 (19.7)	83 (85.6)	3 (3.1)		

Table 5.3: Variation in CCHF incidence by Gender and Age and Location.

	Level	N	Ig M	χ^2, df	P-value
Gender	Female	13	4 (30.8)	2.890,1	0.27
	Male	16	5 (31.3)		
Age	5-10	3	2 (66.7)	1.993, 2	0.37
	10≤18	4	1 (25)		
	≥18	22	6 (27.3)		
Location	Marigat	22	8 (36.4)	1.209, 1	0.27
	Nguruman	7	1 (14.3)		

Table 5.4: Correlation between CCHFV seroprevalence and incidence versus symptoms of febrile patients.

		Age	Gender	Joint pain	Retro-orbital pain	Headache	Abdominal pain
Results: IgG	Pearson Correlation	0.070	0.077	-0.008	.091	0.030	-0.022
	Sig. (2-tailed)	0.119	0.090	0.867	**0.043***	0.510	0.620
	N	493	493	493	493	493	493
Results: IgM	Pearson Correlation	-0.184	0.005	-0.141	.380	0.183	0.025
	Sig. (2-tailed)	0.339	0.979	0.467	**0.042***	0.343	0.896
	N	29	29	29	29	29	29

*. Correlation is significant at the 0.05 level (2-tailed)

Nine hundred and fifty-one (951) livestock serum samples were screened for antibodies against CCHFV. These samples composed of cattle (n=310), goats (n=295), sheep (n=295) and donkey (n=51) (**Table 5.5**). More of the samples were from Marigat (n=551) than Nguruman (n=400). Analysis showed an overall CCHFV seroprevalence of 11.9% (113/951); 95% CI, 10-14.1 (Table 6). Seroprevalence did not differ between the Marigat and Nguruman sampling sites (12.7% vs 10.8%;95% CI, 1.2 (0.80-1.80), p= 0.36) or between the sex (11.6% in females vs 12.7% in males; 95% CI, 1.1 (0.72-1.70), p= 0.63) **(Table 5.6**). For each animal type, the odds of testing positive for CCHFV antibodies were similar in males and females (OR=1.1, df 1, p=0.24). Although there was no significant difference in CCHFV seroprevalence between the sites *(p=0.36*), livestock in Marigat were 1.2 times more likely to be seropositive for CCHFV as compared to those in Nguruman.

Table 5.5: Demographics of livestock sampled

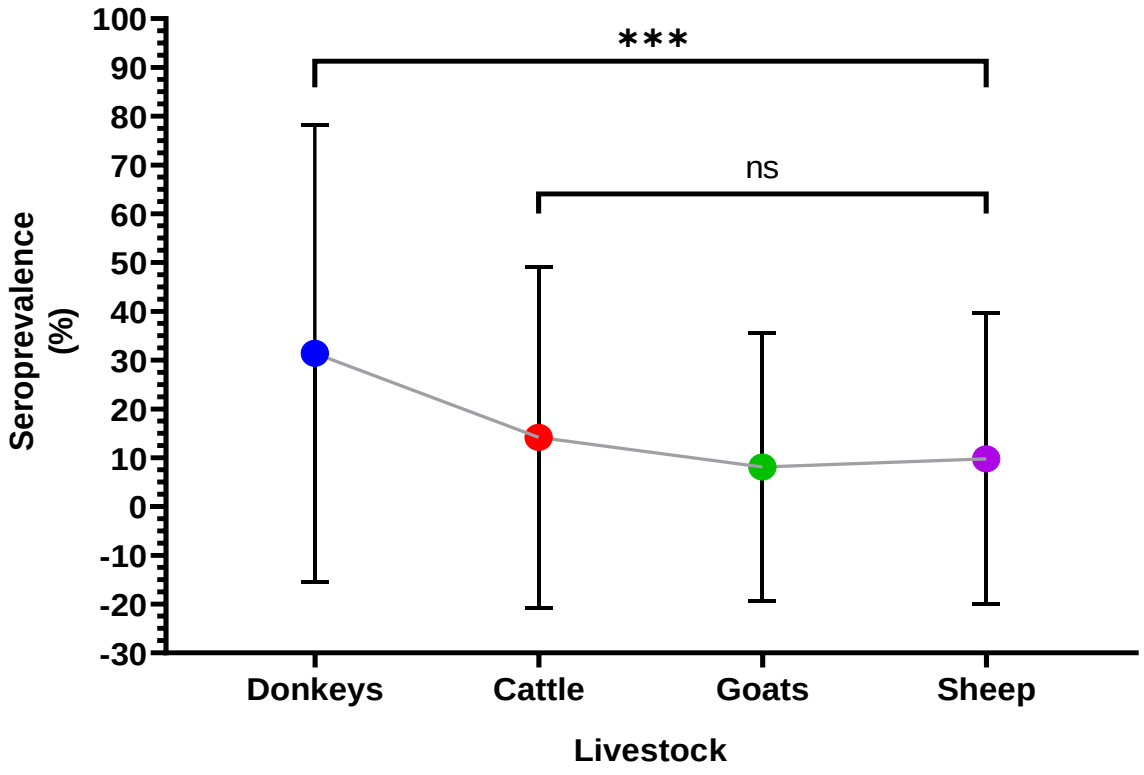
Livestock Species	N	Location		Sex		Age (yrs)		
		Marigat n (%)	Nguruman n (%)	Females n (%)	Males n (%)	1 n (%)	2 n (%)	3 n (%)
Cattle	310	170 (54.8)	140 (45.2)	207 (66.8)	103 (33.2)	70 (22.6)	177 (57.1)	63 (20.3)
Goats	295	165 (55.9)	130 (44.1)	219 (74.2)	76 (25.8)	75 (25.4)	171 (58)	49 (16.6)
Donkeys	51	51 (100)	0	40 (78.4)	11 (21.6)	6 (11.8)	36 (70.6)	9 (17.6)
Sheep	295	165 (55.9)	130 (44.1)	209 (70.8)	86 (29.2)	64 (21.7)	182 (61.7)	49 (16.6)
Total	**951**	**551 (57.9)**	**400 (42.1)**	**675 (71)**	**276 (29)**			

Table 5.6: Seroprevalence of CCHFV in the Livestock population, gender, and species

Species	N	Within population n (%)	Among the total population n (%)	Sex	Seropositive n (%)	χ^2, df	P-Value	OR (95% CI)
Cattle	310	44 (14.19)	44 (4.63)	Female⁑	32 (15.5)	0.82, 1	0.365	1.4 (0.68-2.74)
				Male	12 (11.7)			
Goats	295	24 (8.14)	24 (2.52)	Female	14 (6.4)	3.46, 1	0.063	2.2 (0.94-5.19)
				Male⁑	10 (13.2)			
Donkeys	51	16 (31.37)	16 (1.68)	Female⁑	13 (32.5)	0.11, 1	0.7407	1.3 (0.28-5.04)
				Male	3 (27.3)			
Sheep	295	29 (9.83)	29 (3.05)	Female	19 (9.1)	0.44, 1	0.665	1.3 (0.60-2.93)
				Male⁑	10 (11.6)			

⁑: Reference category for the odds ratio.

There was a significant difference in the proportion of infections per livestock species (χ^2=15.20, df =3, p=0.001). Seroprevalence was highest in donkey (31.4%, 16/51) and least in sheep (8.1%, 24/295) (**Figure 5.2**).



***: Highly significant, ns: not significant at the 0.05 level.

Figure 5.2: Seroprevalence of CCHFV in various livestock species within the livestock population.

Ninety-two rodent sera were screened that included 37 from Marigat and 56 from Nguruman (**Table 5.7**). Analysis revealed an overall seroprevalence of 6.5% (6/93) that was higher in Nguruman (5/56,8.9%) but which did not differ significantly when compared to Marigat (1/37,2.7%) (95% CI, 3.5 (0.4 - 42.6), p= 0.23). Similarly, seroprevalence was higher in females (5/54,9.3%) than in males (1/39,2.5%) (95% CI, 3.9 (0.5-46.7) but the difference was not significant (p= 0.19) (**Table 5.8**). Risk of CCHFV seropositivity did not vary by age group (RR=1.0, df 1, p=0.9705) but there was variation by gender and location, with female rodents being 3.9 times more likely to test positive for CCHFV compared to males, and rodents sampled from Nguruman being 3.4 times more likely to test positive than those from Marigat (**Table 5.8**).

Table 5.7: Information on rodent demographics in relation to CCHFV seroprevalence

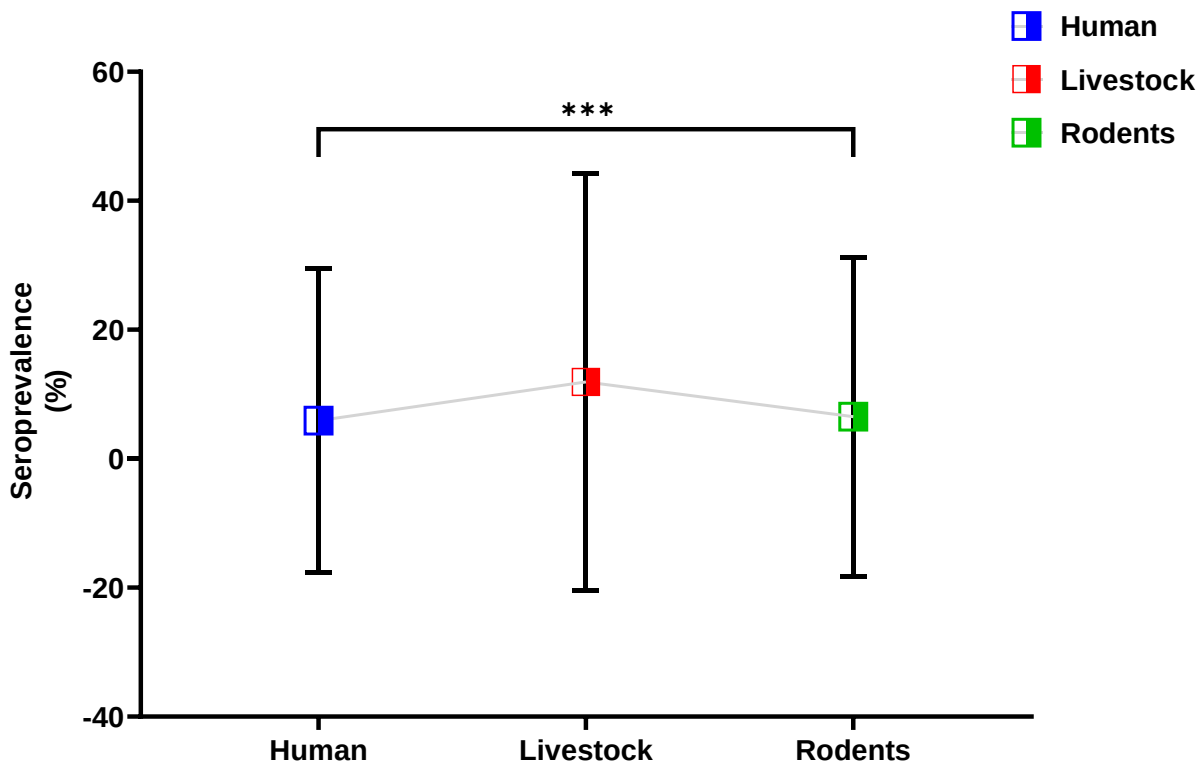
Parameter	**Level**	**N (%)**	**Positives (%)**
Gender	Female	54 (58.1)	5 (9.3)
	Male	39 (41.9)	1 (2.5)
Age	Sub-Adult	78 (83.9)	5 (6.4)
	Adult	15 (16.1)	1 (6.7)
Location	Marigat	37 (39.8)	1 (2.7)
	Nguruman	56 (60.2)	5 (8.9)
Species	*Acomys* sp.	1 (1.1)	0
	Aethomys spp.	4 (4.3)	0
	Arvicanthis spp.	6 (6.5)	0
	Gerbilliscus spp.	3 (3.2)	0
	Grammomys spp	2 (2.2)	0
	Graphiurus sp.	1 (1.1)	0
	Mastomys spp.	48 (51.6)	5 (10.4)
	Mus sp.	1 (1.1)	0
	Paraxerus sp.	1 (1.1)	0
	Rattus spp.	26 (28)	1 (3.8)

Table 5.8: Effect of location, gender, and age on CCHFV seroprevalence in rodents

Parameter	**Variables**	**N (%)**	**Positives**	**χ^2, df**	**P-Value**	**OR 95% CI**	**RR**
Gender	Female-Ref	54 (58.1)	5 (9.3)	1.682, 1	0.1947	3.9 (0.5-46.7)	1.5 (0.8-2.0)
	Male	39 (41.9)	1 (2.5)				
Age	Sub-Adult	78 (83.9)	5 (6.4)	0.001370, 1	0.9705	1.0 (0.1-12.0)	1.0 (0.5-1.2)
	Adult	15 (16.1)	1 (6.7)				
Location	Marigat	37	1 (2.7)	1.431, 1	0.2316	3.5(0.4- 42.6)	2.5 (0.7-13.9)
	Nguruman	56	5 (8.9)				

Comparison of CCHFV seropositivity rate by animal groups

Generally, there was a significant difference in CCHFV seropositivity when disaggregated by animal group *viz*: livestock (11.9%), rodents (6.5%), and human (5.9%) (p=0.001) (**Figure 5.3**). There was a significantly higher probability of CCHFV infection in livestock than humans (OR=95% CI; 2.2 (1.4-3.3), RR=1.7 (1.3-2.5), df=1, p=0.0003). However, rates did not differ significantly between humans and rodents (OR=95% CI; 1.1 (0.5-2.6), RR=1.0 (0.9-1.3), df=1, p=0.83) nor and between livestock and rodents (OR=95% CI; 2.0 (0.9-4.2), RR=1.0 (0.9-1.1), df=1, p=0.12).



***: Highly significant at the 0.05 level.

Figure 5.3: Overall seroprevalence of CCHFV in humans, livestock, and rodents. The seroprevalence was significantly higher in livestock.

5.4 Discussion

We confirm CCHFV circulation among humans, livestock, and peri-domestic rodents from two semi-arid ecologies within the Kenyan Rift Valley. The observations are consistent with previous detection of antibodies against CCHFV in febrile patients (162,181,182) and cattle (200,201) as well as wildlife (200). The detection of CCHFV RNA in sheep is an indication of active circulation at the sampling sites in the two counties. The detection of viral RNA in peri-domestic rodents constitutes a novel finding potentially implicating them in CCHFV epidemiology in the country. These findings together with the widespread abundance of tick vector species (159,202) suggest that the virus might be endemic in diverse parts of the country. CCHFV infection in humans is known to be highly lethal, depending on the infecting strain. Our analysis of samples from humans with fevers found the presence of CCHFV IgM antibodies (1.8% (9/493)), indicating evidence of active infection and likely contribution of the virus as the cause of febrile illness in the study areas. The contribution of other aetiologies cannot be discounted as fever is a common symptom of other local disease conditions often with similarity in clinical presentation to those of arboviral pathogens (3,21,168). CCHFV incidence was not affected by gender but by location, being higher in Marigat than Nguruman, 8/9 88.9% (95% CI 54.3-100) and 1/9, 11.1% (95% CI (1-45.6), respectively, as well as by age, being higher in the age group ≥18 years (6/9, 66.7% (95% CI 35.1-88.3). Geographic difference in risk profile could be attributed to other factors including abundance of specific tick vectors, exposure bites to humans and perhaps the degree of contact with animals. The higher disease incidence and seroprevalence in older patients could be attributed to the type of occupation and long-term involvement in activities that require frequent contact with livestock. When asked about the risk pathways, our data show that humans in contact with animals were more likely to be CCHFV seropositive. Interestingly, there was a strong positive association between CCHFV seropositivity (IgG and IgM) and retro-orbital pain, an indication that this symptom could be an important predictor of CCHFV infection among humans in the study areas. Detailed socio-economic analyses are required to quantify the human virus infection burden.

In general, seroprevalence of 11.9% was realized in livestock herein (95% CI 9.9-14.1) while higher rates of 28% were detected in cattle in Narok and Laikipia counties in a previous study (201). Prevalence was particularly high in donkeys (31%) while the lowest rates were observed among the small ruminants (sheep and goats). These findings highlight potential differential exposure rates of different species (201). We suspect that the difference in seroprevalence rates

between our study and the previous study could be related to the age difference and number of animals sampled, although requiring additional studies for conclusive evidence. Age and infection risk of an animal with CCHFV is positively, correlated, as the likelihood of getting into contact with an infected tick increases over time. This hypothesis is consistent with our findings of increasing CCHFV seropositivity in older animals: 1 year (10.2%), 2 years (11.1%), and 3 years (16.5%). The increase in seropositivity with age is consistent with other studies both in Africa and beyond (134,220,238). Worldwide, an average seroprevalence of up to 80% in livestock has been estimated based on geographic area, type of test used, and livestock species (104). Higher CCHFV seroprevalence rates than observed in this study were reported in different parts of Africa, e.g. in Uganda 75.0% (218), Senegal (57.1%) (220), Mali (13-95%) (239), Sudan (19.1-21%) (240), Mauritania (15-80%) (238,241), South Africa (12.7%) (240,242). However, lower rates were reported in Egypt (3.13%) (243). The differences could be ascribed to other processes including well managed tick control programs both by use of acaricides and dips, which commonly target livestock species, except camels. Alternatively, tick species composition infesting livestock could vary. Ogola et al., 2021 (184), found differential infestation rates of *Hyalomma marginatum*, the principal vector and reservoir of CCHFV, on sheep and no other livestock species from the same areas that was used in this study. Whether this could have contributed to the recovery of CCHF viral RNA only from sheep in our present study merits further elucidation. Phylogenetic analysis showed that the partial CCHFV sequences from this study formed a sub-clade within the Africa lineage 3 clade which is known to cause disease in humans (240,244–247). This highlights a potential risk of CCHFV transmission to humans in the study regions. However, there is a need for generating whole genome sequences to allow for detailed genetic characterisation and fine scale inferences on the genetic diversity of circulating strains in Kenya.

This study presents the first known detection of CCHFV among rodents in Kenya in species known to inhabit human dwellings, such as house rats (*Rattus rattus*) and house mice (*Mus musculus*). *Rattus rattus* hosts a wide variety of harmful internal and external parasites, like fleas and tick larvae that live on these rats and harbour disease-causing microorganisms that can infect humans, livestock, and other animals resulting to serious diseases (223,224). *Mus musculus* (house mice) live as a human commensal in close association with humans, in houses and granaries. They also occupy cultivated fields, fencerows, and even wooded areas, but they rarely stray far from buildings and human habitation (113,248,249). The close interaction of these rodents with humans could therefore present a perfect setting for disease transmission either through a vector or directly as in case of rodent-borne viruses like Lassa or Hantaviruses.

Thus, these rodents can introduce CCHFV into human dwellings and livestock pasture, facilitating transmission. Although the role of rodents in CCHFV transmission is not well understood, various studies have shown the presence of antibodies in rodents from different continents: Africa (South Africa/Zimbabwe, Senegal, Mauritania and Central Africa Republic (CAR)), Asia (Pakistan, Iraq and Iran) and Europe (Hungary) (104,216,219,226,227,229). In this study, we report an overall seroprevalence of 6/93, 6.5% (95% CI 2.7-13.6) of which five (83.3%) were *Mastomys natalensis* from Nguruman and only one positive animal (16.7%) was a *Rattus rattus* from Marigat. The presence of antibodies in multimammate mice (*Mastomys* spp.) has also been reported in Mauritania (27%) and South Africa/ Zimbabwe (0.3%) (219,228). Herein we report the detection of CCHFV in *Rattus rattus* and *Mus* spp. supporting the hypothesis of rodents as hosts for CCHFV (250). However, the small sample size of this study prevents conclusive inferences results and further studies with a larger sample size and concomitant collection of ticks in different life stages are needed to get a better understanding of the enzootic CCHFV maintenance cycle.

Chapter 6

DIVERGENT HANTAVIRUS IN SOMALI SHREW (*CROCIDURA SOMALICA*) IN SEMI-ARID NORTH RIFT, KENYA.

This chapter presents the results obtained through molecular screening of hantaviruses in rodents in the two study sites. Molecular screening through genus specific hantavirus primers allowed for the detection and characterisation of hantavirus in shrews from Marigat.

6.1 Introduction

Hantaviruses are single-stranded, enveloped, tripartite, negative-sense RNA viruses in the order Bunyavirales, family *Hantaviridae*, genus *Orthohantavirus* (1). They have been reported in diverse small mammals, including rodents, shrews, moles and bats (191,193,251–255). However, the recently reported hantaviruses have been found mainly in shrews and moles as well as bats in Africa and Asia (139,254,256). Hantaviruses typically cause chronic asymptomatic infection in rodents but severe disease such as haemorrhagic fever with renal syndrome (HFRS) and haemorrhagic cardiopulmonary syndrome (HCPS) with up to 40% case-fatality rate in humans (25,83,139,140). HFRS is common in Europe and Asia, while HCPS occurs in the Americas (141).

Humans get infected with hantaviruses through aerosolized virus inhalation, consuming contaminated foods and contact with the virus that is shed in urine, faeces, and saliva, and less frequently by a bite from an infected rodent host (123,124). Human-to-human transmission may occur via infected urine, faeces, saliva, vomitus, blood and during organ transfer and blood transfusion (125). However, the only exception of hantavirus human-to-human transmission through body fluids and direct contact has been reported in Andes virus (ANDV) in Chile and Argentina (125–128).

Temperate weather conditions are favourable to small mammal populations, while harsh weather conditions such as drought might drive these animals indoors in search of food and thus increase contact with human beings. Availability of foods also increases population size, for instance, rodent population notably during harvesting due to the abundancy of food and increased reproduction. Rodent-borne disease incidence directly correlated to rodent population abundance.

Hantaviruses are broadly distributed worldwide and well characterised in Asia, Europe, and Americas but not in Africa, especially Sub-Saharan Africa. Shrew-borne hantaviruses have

been reported in various shrew species from different parts of the world. This includes Seewis virus (SWSV) from the Eurasian common shrew (*Sorex araneus*) in Switzerland (257), Boginia virus (BOGV) from the Eurasian water shrew (*Neomys fodiens*) in Poland (258) among others. Sangassou virus (SANGV), the first hantavirus to originate from Africa was isolated from the African wood mouse (*Hylomyscus simus*) (84), and later a shrew-borne hantavirus, Tanganya virus (TNGV) was detected in *Crocidura theresae* (259), both in Guinea. Other shrew-borne hantaviruses that have been reported in Africa include Azagny virus (AZGV) from a West African Pigmy shrew (*Crocidura obscurior*) from Cote d'voire (255), Kilimanjaro virus (KMJV) and Uluguru virus (ULUV), detected in the Kilimanjaro mouse shrew *(Myosorex zinki*) and Geata mouse shrew (*Myosorex geata*) respectively in Tanzania among others (260). Seroepidemiological studies have the revealed presence of hantavirus antibodies in humans including febrile patients with fever of unknown origin (FUO), for instance, in Guinea where a 4.4% seroprevalence was realized (261), presenting a possibility that hantaviruses might be contributing to human disease burden.

In Kenya, hantavirus RNA was once detected in African wood mouse (*Hylomyscus endorobae*) (5) and recently, a study conducted among febrile patients in Kibera informal settlement, Nairobi reported a seroprevalence of 8.1%, an indication that the virus is actively circulating among people (85).

Herein we report for the first time the detection of hantavirus in shrews in Kenya.

6.2 Methodology

Methods unique to this chapter are described here, the rest are as listed in chapter 2.

6.2.1 Study sites

Sampling of peridomestic rodents and shrews was performed in diverse ecologies along the Rift Valley in Kenya. Marigat (0.4695° N, 35.9833° E) in Baringo County and Nguruman (1.7617° S, 36.0255° E) in Kajiado County are semi-arid areas with diverse populations and high humans/livestock/wildlife interactions. The two ecologies are both inhabited by the semi-nomadic, agro-pastoralist communities, who keep livestock and grow maize, millet, and fruits in irrigated areas. Availability of these foods support rodent populations, which are high during harvesting periods.

6.2.2 Peridomestic rodent and shrew sampling

Live Folding Sherman box traps were set inside dwelling places and their surroundings following the guidelines set by the National Museum of Kenya (NMK) for trapping and sampling rodent populations in Kenya (186). The peanut butter and white oats baited traps were opened at dusk, checked every morning, and left closed during the day. Varied number of traps were set per room/surrounding environment for the whole night depending on observed rodent activity and inspections carried out each morning. Each trapped specimen was placed in a handling bag, weighed, and initially identified to the genus or species level based on morphological and geographical criteria according to Kingdom guide to African mammals and East African mammals as reference guides. Subsequent molecular analyses were used to definitively assign species status (186,193,262).

Parameters like species, sex, age, length, weight were recorded for each of the trapped individuals after being euthanatized by cervical dislocation. Immediately after ensuring that the mice are completely euthanatized, blood samples were collected from the heart through cardiac puncture and thoracotomy first, then, other tissue samples (kidney, spleen, lungs, heart, and liver samples) collected, appropriately labelled before transportation in liquid nitrogen to EID Laboratory at *icipe*- Nairobi and kept at -80˚C until testing.

6.2.3 RNA extraction, cDNA synthesis and PCR detection of hantaviruses

Tissues (lungs, spleen, kidney, and liver) of individual rodent were pooled and homogenised in PBS. RNA was extracted from 140 µL of homogenized rodent tissues using the QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany) according to the manufacturer's protocol. A volume of 50 µL of RNA was obtained and used as a template for cDNA synthesis by Invitrogen SuperScript™ III Reverse Transcriptase as per the manufacturer and resultant cDNA preserved at -80°C until further use.

The samples were screened for the hantavirus RNA by an optimized assay targeting partial polymerase (L) gene sequences of the hantavirus genome using primers Han-L-F1:5'-ATGTAYGTBAGTGCWGATGC-3'; Han-L-R1:5´-AACCADTCWGTYCCRTCATC-3´; Han-L-F2:5'-TGCWGATGCHACIAARTGGTC-3'; and Han-L-R2:5'-GCRTCRTCWGARTGRTGDGCAA-3' (191). The reaction volume (25 µL) comprised 15.65 µL PCR water, 2.50 µL 10x-Buffer, 1.25 µL Mg (50 mM), 0.50 µL dNTPs (10mM), 1.5 µL of 10 µM forward and reverse primers, 0.10 µL Platinum-Taq polymerase (2 U/µL) and 2.0 µL template (cDNA).

The following conditions were used for the PCR reaction: 98°C for 30 s; 35 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s; and 72°C for 7 min followed by a hold at 4°C.
The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide (Sigma-Aldrich Chemie GmbH) and positive samples were purified using ExoSAP-IT™ PCR Product Clean-up Reagent (Applied Biosystems) according to the manufacturer's instructions, prior to submitting samples for bidirectional sequencing.

6.2.4 Sanger sequencing and phylogenetic analysis

The Sanger sequencing services were outsourced to Macrogen, Europe B.V. for both forward and reverse strands using the Han-L-F2: 5'-TGCWGATGCHACIAARTGGTC-3'; and Han-L-R2: 5'-GCRTCRTCWGARTGRTGDGCAA-3' primers. The sequences were cleaned in Geneious Prime software (https://www.geneious.com) and queried against the GenBank-NCBI database (195,196) using the Basic Local Alignment Search Tool (BLAST) (197). Related sequences were downloaded from the GenBank-NCBI database and multiple sequence alignment performed by MAFFT (198). Phylogenetic analysis with PhyML (199) was performed using the general time reversible (GTR) model applying 1000 bootstrap replicates. All analyses were performed in Geneious Prime (http://www.geneious.com) using default parameters.

6.2.5 Rodents and Shrews Identification

Species identification of both rodents and the shrews was done by extracting DNA from tissue samples using Qiagen DNeasy Blood & Tissue extraction kit. The shrews barcoding was performed using forward primer sorcytb365F (5'-CAGTAATAGCCACTGCCTTTATAGG-3') and reverse primer sorcytb969R (5'-CATTGGCTGAATGGGCGGAATATTAT-3') that target a 500 bp region of the cytochrome *b* (cyt *b* gene (193). For rodent identification, cytochrome c oxidase 1 gene (COI) barcode region, specific primers BatL5310: 5′-CCTACTCRGCCATTTTACCTATG-3′ and R6036R: 5′-ACTTCTGGGTGTCCAAAGAATCA-3′ were used (194)(263)(264). The following conditions were used for the PCR reaction: 95°C for 3 min; 35 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 7 min followed by a hold at then 4°C.
PCR products were separated by 1.5% agarose gel electrophoresis, sized against a molecular weight marker, and visualised by ethidium bromide staining (Sigma-Aldrich Chemie GmbH) under UV light. Products of the correct size were purified using ExoSAP-IT™ PCR Product Clean-up Reagent (Applied Biosystems), and submitted to Macrogen, Europe B.V. for

bidirectional sequencing. Phylogenetic analyses were performed in Geneious Prime (https://www.geneious.com).

6.3 Results

Rodent distribution

A total of 489 peridomestic small mammals (rodents and shrews) were captured; 312 (63.8%) in Nguruman and 177 (36.2%) in Marigat. Eleven of all the small mammals captured were shrews (2.5%) and the remaining 478 (97.5%) were rodents. The captured small mammals belonged to different species (**Table 6.1 and Figure 6.1**). Of the 312 small mammals captured in Nguruman, 48% were captured indoors and 52% outdoors and more males (55%) than females (45%) were captured (**Table 6.1**). In contrast, at Marigat, out of the 177 small mammals captured, 80% were captured indoors and 20% outdoors, and more females (58%) than the males (42%) were captured (**Table 6.1**).

Table 6.1: Distribution of the small mammals from the two study sites

Parameter	**Group**	**Site**		**Totals**
		Marigat	**Nguruman**	**Total**
Sex	Female	102	140	242
	Male	75	172	247
Age	Sub-Adult	36	118	154
	Adult	141	194	335
Place of Capture	Indoor	137	150	287
	Outdoor	40	162	202
Species	*Acomys* spp	1	48	49
	Aethomys spp	1	6	7
	Arvicanthis spp	0	15	15
	Oenomys spp	1	0	1
	Lemniscomys spp	0	2	2
	Gerbilliscus spp	3	2	5
	Grammomys spp	2	0	2
	Graphiurus spp	0	1	1
	Mastomys spp	47	217	264
	Mus spp	73	0	73
	Paraxerus spp	0	1	1
	Gerbillus spp	0	1	1
	Crocidura spp	11	0	11
	Rattus spp	38	19	57

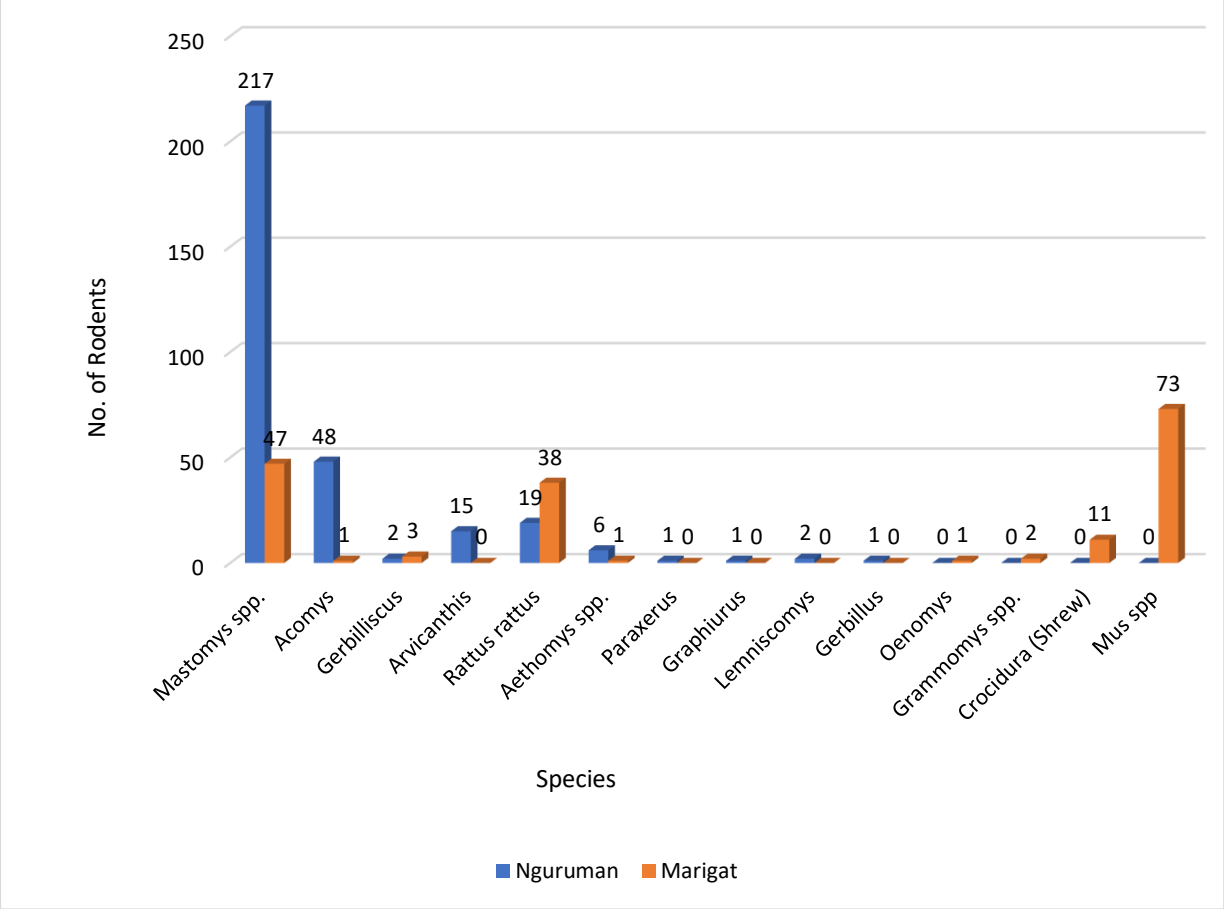


Figure 6.1: Distribution of small mammal species from the two study sites. Mastomys spp. were prevalent in Nguruman and Mus spp. in Marigat.

Hantavirus detection, characterisation, and phylogenetic analysis

We detected hantaviruses in four out of the eleven shrews captured (4/11, 36%). The amplicon sequences revealed notable genetic diversity among the detected virus strains (74.7- 92.8%) and a low level (72-80%) of nucleotide identity to other shrew-borne hantaviruses like TNGV, AZGV and SWSV, across the partial polymerase gene region characterised in this study. No hantavirus RNA was detected in any of the rodent species (**Figure 6.2**).

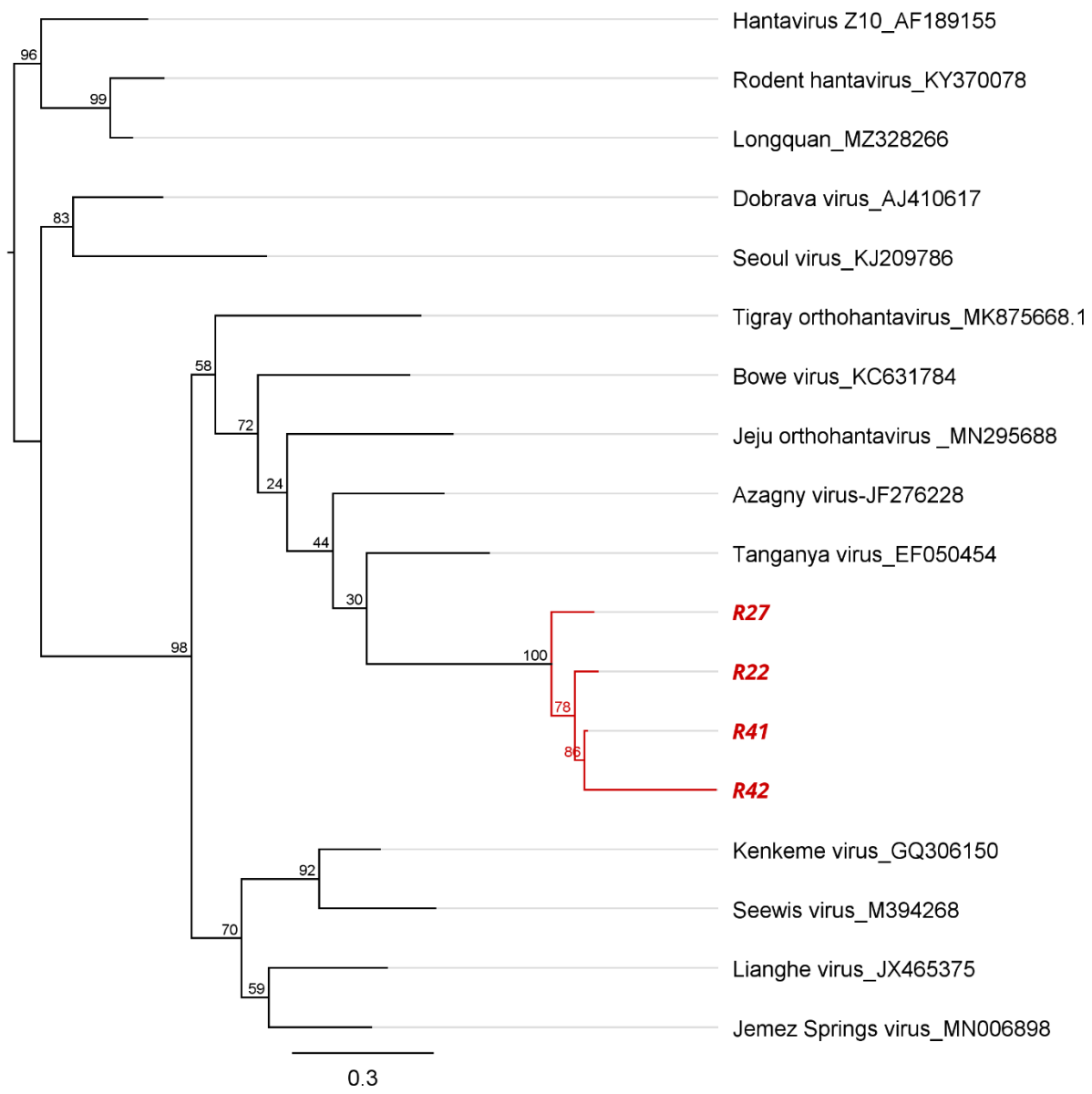


Figure 6.2: The maximum likelihood tree depicting phylogenetic relationships of Hantaviruses detected in this study relative to other members of the family Hantaviridae, genus Orthohantavirus, based on a partial 385 nucleotide region of the RNA dependent RNA polymerase (L segment). All the sequences generated from the shrews in this study are indicated in red. Taxon names include the virus name, accession number, host, and country origin (where available). The tree was inferred using PhyML v. 2.2.4 in Geneious Primer, under the general time reversible (GTR) substitution model employing 1000 non-parametric bootstrap replicates.

Barcoding identification

The sequencing of all the eleven shrew samples revealed the identity as Somali shrew *Crocidura somalica*, with 99-100% identity to shrews previously captured in the same area (265). The obtained sequences had 99-100% nucleotide identity among the sequences. Phylogenetic analysis revealed the formation of a monophylogenetic clustering with shrews obtained from Kenya but with two sister clades (**Figure 6.3**).

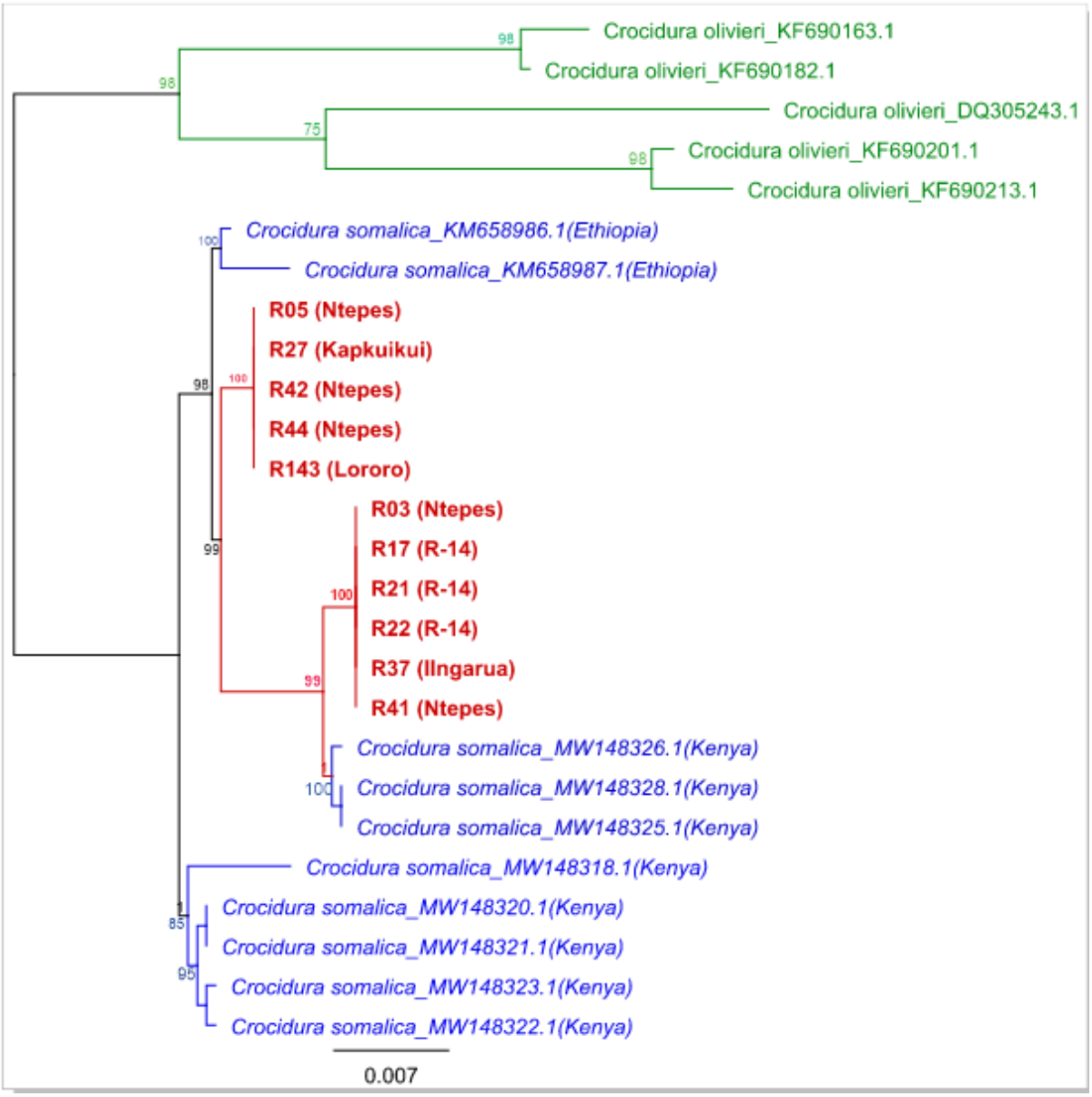


Figure 6.3: Maximum likelihood phylogenetic tree depicting the cytochorme b gene relationships of *Crocidura somalica* based on the 500 nucleotide region characterised in this study. *Crocidura olivieri* is included as an outgroup and samples characterised in this study are indicated in red font. The phylogeny was inferred using PhyML v. 2.2.4 with the GTR substitution model and 1000 bootstrap replications.

6.4 Discussion

Herein we report for the first time the circulation of hantavirus in Somali shrew (*Crocidura somalica*) as well as the pattern of distribution of peri-domestic small mammals in the two ecosystems in the Rift Valley of Kenya. To our knowledge, this is the first report of shrew-borne hantavirus detection in Kenya.

Peridomestic small mammals share habitats with humans and represent a risk for transmitting zoonotic diseases. Human activities like farming and building of structures and houses present opportunities for rodent infestation and for increased contact and disease spillover. For instance, house mice are adapted to inhabit human structures and farming activities not only provide a conducive environment for habitation but food as well, mainly during harvesting periods. Consequently, surveillance to determine the potential to harbour and transmit zoonotic disease- causing pathogens by small synanthropic mammals cannot be underestimated.

Hantaviruses are widely distributed, and the highest infections have been reported in Asia and least in Africa. Whether this is because of enhanced surveillance, improved diagnosis, and reporting systems in Asia, can only be ascertained when similar and comparable investigations in the two areas are implemented. Other than rodent and bat-borne hantaviruses, shrew-borne hantaviruses have been reported in Asia and Europe but occasionally in Africa as well where TGNV was isolated in *Crocidura theresea* in Guinea between 2002-2004 and later AZGV from West Africa pigmy shrews (*Crocidura obscurior*) in Ivory coast, amongst others (255,266).

The detected hantaviruses were shown to have the highest level of sequence identity to TGNV with up to 77% nucleotide identity across the partial gene region characterised in this study. However, the phylogeny did not support a sister relationship to TGNV. Instead, the four viruses detected in *Crocidura somalica* formed a monophyletic lineage (100% bootstrap support) and clustered within a clade containing Bowe, Jegu, Azagny and Tanganya viruses (72% bootstrap support). The genetic distance together with a confirmation of monophyly indicate that the virus strains circulating in Kenya represent a novel species that is distinct for strains reported in other regions of Africa as well as other parts of the world (255,266). However, whilst there is support for the genetic distinctiveness of the Kenyan strains, the relationship of this novel lineage to other viruses recognised variants remains unresolved and indicates that the partial L segment is inadequate for conclusive resolution of relationships. Expanded studies aimed at generating full-length sequences that allow for genome comparison studies are recommended. In this study, attempts to obtain full sequences from the tissue samples were not successful and

virus isolation could not be attempted due to the high containment laboratory requirement for hantavirus isolation.

Overall, there have not been many reported cases of shrew-borne hantavirus infection in humans in Africa but serological evidence of infection in humans from Côte d'Ivoire and Gabon has been reported (267). However, this cannot be concluded as inability of the detected viruses to cause disease in human unless wider studies are carried out to rule it out. Therefore, continuous surveillance and pathogenicity studies would be necessary to have a proper understanding of these viruses.

The geographical distribution of shrews in Kenya is undetermined due to limited studies. One previous study found the same shrew species from the same area (265), an indication that *Crocidura somalica* commonly inhabit the region. Although widely distributed and known to have adapted to a wide variety of environments, its natural habitat is dry savanna (268). In this study, the shrews were only captured in Marigat area (Baringo South sub-county) and not in Nguruman (Kajiado West sub-county). The ecosystem determines the type of small mammal species that are likely to inhabit an area. As *Crocidura somalica* is mainly found in arid habitats, such as dry savanna and semi-desert areas, their presence at the study sites is anticipated (269). Comparing the two study sites that are similarly semi-arid, Marigat has a more settled human population with permanent houses/structures and therefore the rodents/shrews mainly found in this area could have adapted to living in/around houses and closely interacting with human. For instance, the common house mouse (*Mus musculus*) and the *Crocidura somalica* shrew were only captured in Marigat and not in Nguruman. In contrast, in the settlement in Nguruman that consists of temporary shelters inhabited by the semi-nomadic pastoralists the environment might not be conducive for species that have adapted to living in/around houses or more permanent settings as seen in Marigat. For instance, *Mastomys natalensis*, the most common rodent species, was more prominent in Nguruman; this could be attributed to the shrubland ecosystem. Overall, this can explain the preference and adaptation of the shrews to certain environments.

The virus characterisation results reported herein were limited by the inability to obtain the full genome sequence. Whilst this could have been addressed through virus isolation, the requirement for high containment to attempt this was not available. The reported results are nonetheless important for confirming the presence of hantaviruses in these arid regions and for identifying the role of shrews in disease ecology. The findings call for further surveillance to be carried out to improve our understanding of hantavirus ecology and epidemiology in Kenya.

Chapter 7

CHARACTERISATION OF A NOVEL ORBIVIRUS FROM CATTLE REVEALS ACTIVE CIRCULATION OF A PREVIOUSLY UNKNOWN AND PATHOGENIC ORBIVIRUS IN RUMINANTS IN KENYA.

This chapter presents the results obtained through virus isolation and next generation sequencing (NGS) that allowed the isolation and characterisation of a novel orbivirus in cattle. Further molecular screening using virus-specific primers allowed for the detection of the virus in additional cattle from Marigat.

7.1 Introduction

Orbiviruses are arthropod-borne viruses (arboviruses) that are commonly transmitted by mosquitoes, sandflies, biting midges, and ticks (270–273) to a wide range of vertebrates (271,274–280) The genus *Orbivirus* (family *Sedoreoviridae*, order Reovirales) consists of 22 classified virus species (270,271). About 15 further potential orbivirus species await classification by the International Committee on Taxonomy of Viruses (ICTV). The genus contains several viruses that can cause severe disease in wild and domestic animals, such as Bluetongue virus (BTV), African horse sickness virus (AHSV), and Epizootic haemorrhagic disease virus (EHDV). BTV mainly causes a disease with high mortality in sheep but can also infect cattle, goats, buffalo, antelope, deer, elk and camels (10,281,282) AHSV can lead to fatal disease in horses and mules and also infects donkeys, zebras and other equines. EHDV mainly infects wild and domesticated ruminants causing haemorrhagic disease in deer while domestic ruminants are subclinically infected.

The virion of orbiviruses is non-enveloped, icosahedral and has a triple-capsid structure. The genome is composed of 10 linear segments of double-stranded RNA (dsRNA). The segments encode seven structural proteins (virion proteins VP1–VP7) and five non-structural proteins (NS1-NS5) (274,283,284). Encoded proteins of these segments vary between viruses of different species and the pattern of segment-encoded proteins is linked to the arthropod vector (285). The VP2 (OC1) and the VP5 (OC2) proteins form the outer capsid and mediate serological reactivity. The VP3 (T2) protein forms the inner capsid, which controls the size and organization of the capsid structure and interacts with internal proteins (270). VP3 (T2) is either encoded by segment 2 in tick- and mosquito-borne orbiviruses or segment 3 in Culicoides-borne orbiviruses (286–290). In some studies, the genome segment has been used for

classification rather than the encoded protein, resulting in some level of non-uniformity and confusion (283,286). The intermediate capsid protein VP7 (T13) is widely used for virus serotype and species-specific identification (287,291). Orbivirus genome segments have terminal non-coding regions (NCRs) which are partially conserved within species (287,290). Orbiviruses are distributed worldwide and their emergence and distribution depend on the abundance of competent vectors and environmental conditions that favour virus transmission between vectors and hosts (8,86,292). In East Africa and Kenya, the most common orbiviruses are the culicoides-borne BTV that causes Bluetongue disease (BT) and AHSV that causes African horse sickness (AHS), both notifiable diseases according to the World Organization of Animal Health (WOAH). BTV has been reported in different parts of Kenya in various livestock species including sheep, goats, cattle and camels with prevalence rates of up to >80% signifying widespread exposure (293–295). AHS has been found in donkeys and horses (296). In this study, we report the discovery and molecular and phenotypic characterisation of a novel orbivirus designated Kaptombes virus (KPTV) from a symptomatic cow and provide evidence of a wider circulation in cattle in Kenya.

7.2 Materials and Methods

Methods unique to this chapter are described here, the rest are as listed in chapter 2.

7.2.1 Livestock Sample Collection

Sample collection was done as part of a larger project aimed at understanding arbovirus transmission networks in Kenya. Blood samples were collected from randomly selected cattle, sheep, and goats between 2020-2022, twice a year after the short and long rains in the semi-arid areas of Marigat, Baringo County (0.4695° N, 35.9833° E) and Nguruman, Kajiado County (1.7617° S, 36.0255° E) in Kenya. Two sets of blood samples, whole blood and serum were collected from other asymptomatic animals and transported in dry ice to the Martin Lüscher Emerging Infectious Diseases (ML-EID) Laboratory at *icipe*.

7.2.2 Virus Isolation in cell culture

Vero CCL-81 (ATCC® CCL-81™) cells were seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark) to 80% confluency in Gibco Dulbecco's modified Eagle's medium (DMEM) containing 10% Gibco™ Fetal Bovine Serum (FBS), 2% Gibco™ L-Glutamine (200 mM), and 2% Gibco™ Antibiotic-Antimycotic (100X). The cells were rinsed with Gibco™ PBS, pH 7.4, and 50µL serum was added followed by incubation at 37°C, 5% CO_2 (New

Brunswick™ Galaxy® 170 R CO_2 Incubator Series, Eppendorf, USA) for one hour, rocking after every 15 min to allow virus adsorption. After incubation, Gibco Dulbecco's modified Eagle's medium (DMEM) maintenance medium (MM) with 5% Gibco™ Fetal Bovine Serum (FBS), 2% Gibco™ L-Glutamine (200 mM), and 2% Gibco™ Antibiotic-Antimycotic (100X) was added. Cells were incubated at 37°C, 5% CO_2 (New Brunswick™ Galaxy® 170 R CO_2 Incubator Series, Eppendorf, USA) and observed daily for signs of cytopathic effects (CPE). The CPE-positive sample was passaged in 25-cm^2 cell culture flasks (Nunc, Roskilde, Denmark) and frozen at -80°C before harvesting by thawing and centrifuging at 3000 rpm for 10 mins. The infectious supernatant was stored at -80°C until further use.

7.2.3 Next Generation Sequencing

Clarified infectious cell culture supernatant was filtered using 0.22-µm filters (Merck Millipore Co., MA, USA) to remove possible cellular residues and contaminants. RNA was extracted from 140 µL cell supernatant using QIAamp viral RNA minikit (QIAGEN, Hilden Germany) following the manufacturer's recommended protocol. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit RNA 2.0 fluorometer using the Qubit RNA HS assay kit (Invitrogen, USA). Then 1 µg total RNA was prepared for Illumina library preparation using TruSeq stranded mRNA kit (Illumina, USA), following the manufacturer's recommended protocol with the modification to exclude the poly(A)-containing mRNA purification steps. Reverse transcription was done using Superscript III reverse transcriptase (Invitrogen, USA) and random hexanucleotide primers (Invitrogen, USA). This was followed by second-strand synthesis using DNA polymerase I and RNase H, provided with the library preparation kit. Purification was performed using AMPure XP beads (Beckman Coulter, USA) after which the purified double-strand cDNA fragments were end repaired by adding a single A nucleotide to the 3′ end of the blunt fragments to prevent the formation of chimeras and improve adapter ligation efficiency. Ligation of the adapters was performed, and the products were purified and enriched by PCR to create the final library. Libraries were normalized, pooled, and sequenced using the Illumina platform.

7.2.4 Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis (SDS-PAGE)

(i) RNA Extraction

For dsRNA segment analyses, 50 µL of prewarmed 1M sodium acetate (NaAc) containing 1% sodium dodecyl sulphate (SDS), pH 5.0 was added to 450 µL of infectious cell culture supernatant from either KPTV or the BTV BLUVAX vaccine isolate from KEVEVAPI (Kenya

Veterinary Vaccine Production Institute) isolate in an Eppendorf tube, vortexed for 10 seconds and incubated for 15 mins at 37°C. Five hundred microlitres of phenol/chloroform (1:1) was added to the tube, vortexed for 1 min then incubated for 15 mins at 56°C in a water bath. After incubation, the sample was vortexed for 1 min then centrifuged for 5 mins at 12000 rpm. The upper aqueous phase containing the dsRNA was pipetted into a clean Eppendorf tube and 40 µL (~1/10 volume) 3M NaAc was added and the tube was filled with ice-cold (-20°C) absolute ethanol, mixed gently by turning the tube over and over 4-6 times then incubated at -20°C overnight. The tubes were centrifuged at 4°C for 20 mins at 12000 rpm to pellet the dsRNA, the supernatant was poured off immediately and the tubes allowed to air dry upside down on a piece of paper towel. After drying, the pellet was resuspended in 30 µL PAGE sample dye containing 10 mg bromophenol blue, 5 mL of Spacer gel buffer and 1 mL of glycerol before loading on a PAGE gel.

(ii) PAGE Analysis

Ten percent 1.5 mm Resolving Gel was prepared using 15.8 mL distilled water, 10 mL of 30% acrylamide stock, 3.75 mL resolving buffer (pH 8.9) containing 36.3 g Tris Base and 48 mL 1M HCL, 15 µL Tetramethylethylenediamine (TEMED), and 450 µL of 10% Ammonium persulphate. The gel was immediately poured between the thick gel spacers up to the gel mark after cleaning with 96% ethanol then overlayed with 1 mL layer of water before polymerization to ensure the formation of an even interface as well as exclusion of oxygen. The resolving gel was allowed to polymerise for 2 hours, after which the water was poured off and 1.5mm 3% spacer gel prepared by 6.8 mL distilled water, 1.6 mL of 30% acrylamide stock, 1.25 mL spacer buffer (pH 6.7) containing 5.98 g Tris Base and 50 mL distilled water, 5 µL TEMED, and 150 µL of 10% Ammonium persulphate was poured on top after positioning the combs and allowed to polymerize for 45 mins. After polymerization, the combs were removed, and the glass plate assembled onto the electrophoresis apparatus with electrophoresis buffer prepared by 200 mL 5% Tris-glycerine buffer to 800 mL of distilled water. The dsRNA of KPTV and BTV BLUVAX vaccine strain extracted as earlier described and diluted in 30 µL PAGE-dye were loaded onto the wells and run for 18 hours at 100V.

After electrophoresis, the gel was washed, fixed with 200 mL fixing solution 1; prepared with 80 mL ethanol, 110 mL distilled water and 10 mL acetic acid, then incubated for 30 mins on an orbital shaker. The fixing solution 1 was drained off and replaced with 200 mL of fixing solution 2 containing 20 mL ethanol, 180 mL distilled water and 1 mL acetic acid and incubated for 30 mins on the orbital shaker. The fixing solution 2 was drained off, and the gel was stained

for 30 mins using silver nitrate ($AgNO_3$) staining solution then washed twice in distilled water for 2 mins each wash. To remove the black precipitate, approximately 50 mL of developing solution (7.5g NaOH, 250mL dH_2O, 2 mL 36% Formaldehyde) was added, agitated for 30 seconds, poured off then 200 mL added and incubated for 5 mins, and drained off before adding stopping solution (10 mL acetic acid and 200 mL dH_2O) to prevent further colour development for 10 mins after which the gel was rinsed in distilled water for 10 mins. Distilled water was added to the gels and left until ready to dry.

7.2.5 Primer design, PCR screening, and Sanger sequencing

KPTV specific primers (F 5'-AGCGAGGTGGATAGTGAAGA-3' and R 5'-CTCCGCCCTAACATCCAATAAA-3') and real time RT-PCR primers (F 5'-TTGGGACGGAAGCGACTTAG-3', R 5'-ATCTCCTCCTGCATGACACG-3' and probe FAM/AAACTCTAC/ZEN/TCTGAT CGCAAATTCG/3IABkFQ) were designed based on Segment 2 using the IDT PrimerQuest™ Tool.

A total of 248 livestock serum pools made up of 83 (33.5%) cattle, 85 (34.3%) goats and 80/248 (32.2%) sheep from Baringo county were screened for the presence of KPTV RNA. Each pool consisted of five to seven individual samples that were pooled based on species and location (100 µL per sample). Viral RNA was extracted from 140 µL of each pooled serum sample and the CPE positive sample (KPTV) with the QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany). A final volume of 50 µL of RNA was obtained and used as a template for cDNA synthesis. The cDNA was synthesized by SuperScript™ III Reverse Transcriptase and stored at -80°C until further use.

The samples were tested for presence of KPTV genome copies using a 25 µL PCR reaction containing 15.65 µL PCR water, 2.50 µL 10x PCR reaction Buffer, 1.25 µL Mg (50 mM), 0.50 µL 10 mM dNTPs, 1.5 µL of 10 µM forward and reverse primers, 0.10 µL Platinum-Taq polymerase (2 U/µL) and 2.0 µL cDNA template. Cycling conditions were 95°C for 3 mins, followed by 40 cycles of 95°C for 15 seconds, 55°C for 40 seconds, and 72°C for 1 min and a further extension of 72°C for 10 min, then 4°C for infinity. The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide (Sigma-Aldrich Chemie GmbH) and positive samples purified using ExoSAP-IT™ PCR Product Clean-up Reagent (Applied Biosystems) according to the manufacturer's instructions, then sequenced in both directions. The sequencing services were outsourced to Macrogen, Europe B.V.

The qPCR was used for virus quantification: The 25µL qRT-PCR total reaction volume included 16.05 µL of water, 0.75µL forward and reverse primer each, 0.25µL FAM™ target

probe, 2µL Mg (50mM), 2.5µL 10X Buffer, 0.5µL dNTPS (10mM), 0.2µL Invitrogen Platinum^{TM} Taq and 2µL cDNA template. The thermal cycling conditions involved initial denaturation for 2 min at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at 60°C and a hold of 5 s at 40°C.

7.2.6 Screening for KPTV neutralizing antibodies using Plaque Reduction Neutralization Test (PRNT)

A total of 200 livestock samples from Baringo county (cattle n=100, sheep n= 50 and goats n=50) were aliquoted in volumes of 30 µL, heat inactivated at 56°C for 30 min and then tested for neutralizing antibodies to Kaptombes virus in two-fold serial dilutions from 1:20 to 1:320 using $PRNT_{90}$ as previously described (131,188,297).

7.3 Data Management and Analysis

Seroprevalence: in vivo and in vitro data

The seroprevalence, *in vivo* and *in vitro* studies data were entered into Microsoft Excel v. 2016, cleaned then imported to R version 4.2.0 for analysis. Comparison of KPTV seroprevalence between the different livestock species was done using Fisher exact test. The 95% confidence intervals (CIs) were estimated using the Agresti-Coull method. All tests were performed at a 5% significance level.

Sanger sequencing and phylogenetic analysis

The Sanger sequences of the PCR positive samples were imported into Geneious Prime software (https://www.geneious.com). The sequences were cleaned using Geneious Prime software inbuilt parameters and queried against the GenBank-NCBI database (195)(196) using the Basic Local Alignment Search Tool (BLAST) (197). Related sequences were downloaded from the GenBank-NCBI database and multiple sequence alignment performed by MUSCLE (198). Phylogenetic analysis was performed in Geneious Prime (http://www.geneious.com) with PhyML (199), the General-time-reversible (GTR) substitution models substitution model applying 1000 bootstrap replicates. The inferred phylogenies were visualized in Figtree v1.4.4.

NGS data analysis.

Raw sequence reads were initially subjected to cleaning using Trim Galore v0.6.5 to remove adapters and Prinseq Lite v0.20.4 to remove low-quality reads using the following parameters: minimum length, 50 bp; maximum length, 301 nucleotides; and minimum mean Q score, 30. Further, filtering of the reads was performed by using ribo Picker v0.4.3, to remove rRNA

sequences by comparing them to the SILVA rRNA database, release 138.1. Paired-end reads were merged using PEAR 0.9.8, and preliminary analysis was performed using the MG-RAST server to identify reads taxonomically. *De-novo* assembly of cleaned reads was done using the trinity program with default parameters. The cleaned reads were mapped back to the assembled contigs and filtered to retain only contigs in which at least 90% of nucleotides had a 5 times coverage. Contigs that met this criterion were first compared to the NCBI viral database using the BLASTx program. Potential viral contigs were further compared to the entire NCBI nr database using the BLASTx program to filter out all nonviral sequences. Sequences that were confirmed to be of viral origin were translated, and ORF predictions were performed using the Expasy server. Phylogenetic reconstruction was performed based on 1,000 bootstrap estimates and 1,000 approximate-likelihood-ratio tests. The inferred phylogenies were visualized in Figtree v1.4.4.

7.4 Results

Isolation and genome analysis of Kaptombes virus

In total, 1500 serum samples from cattle, goats, and sheep (500 each) were inoculated in Vero CCL-81 cells for blind virus isolation. One serum sample obtained from a cow with symptoms of emaciation and lethargy located in Kaptombes village in Kapkuikui, Baringo County (**Figure 7.1**), induced a cytopathic effect (CPE) between 4-6 days post infection (dpi). The CPE was consistent in further passages. Infectious cell culture supernatant was applied to high throughput sequencing (HTS) revealing the genome of an orbivirus with 10 segments of dsRNA (**Table 7.1**). The total genome consisted of 18,731 bp with segment (S) sizes ranging from 781 bp (S10) to 3987 bp (S1), and each predicted to encode a single protein (**Table 7.1**). Sequence comparison to sequences available in the NCBI database using BLAST revealed that the highest similarity of the detected virus was to Sathuvachari virus (SVIV), a mosquito-borne orbivirus, which was first isolated from a starling bird (*Brahminy myna*) in India in 1963, and later found in healthy cattle from Japan in 2005 (278,285) (**Table 7.1**). S1, predicted to encode the RNA-dependent RNA polymerase (VP1), showed 77.5% nucleotide identity to the VP1 of SVIV. Similar to what is observed for mosquito- and tick-borne orbiviruses, S2 is predicted to encode the inner capsid protein VP3 (T2) and showed 80.7% nucleotide identity to the VP3 of SVIV. Segments S3, S4, S5, S6, S7, S8, S9 and S10 of the isolated virus were predicted to encode VP2, VP4, NS1, VP5, NS2, VP7, VP6 and NS3 proteins, respectively, as is the case for other mosquito-borne orbiviruses. The nucleotide identity of VP7 was the highest (83.64%)

to SVIV VP7forms the outer core surface and is encoded by S8. The outer capsid protein VP2, encoded by S3, had the lowest nucleotide identity (73.3%) to that of SVIV.

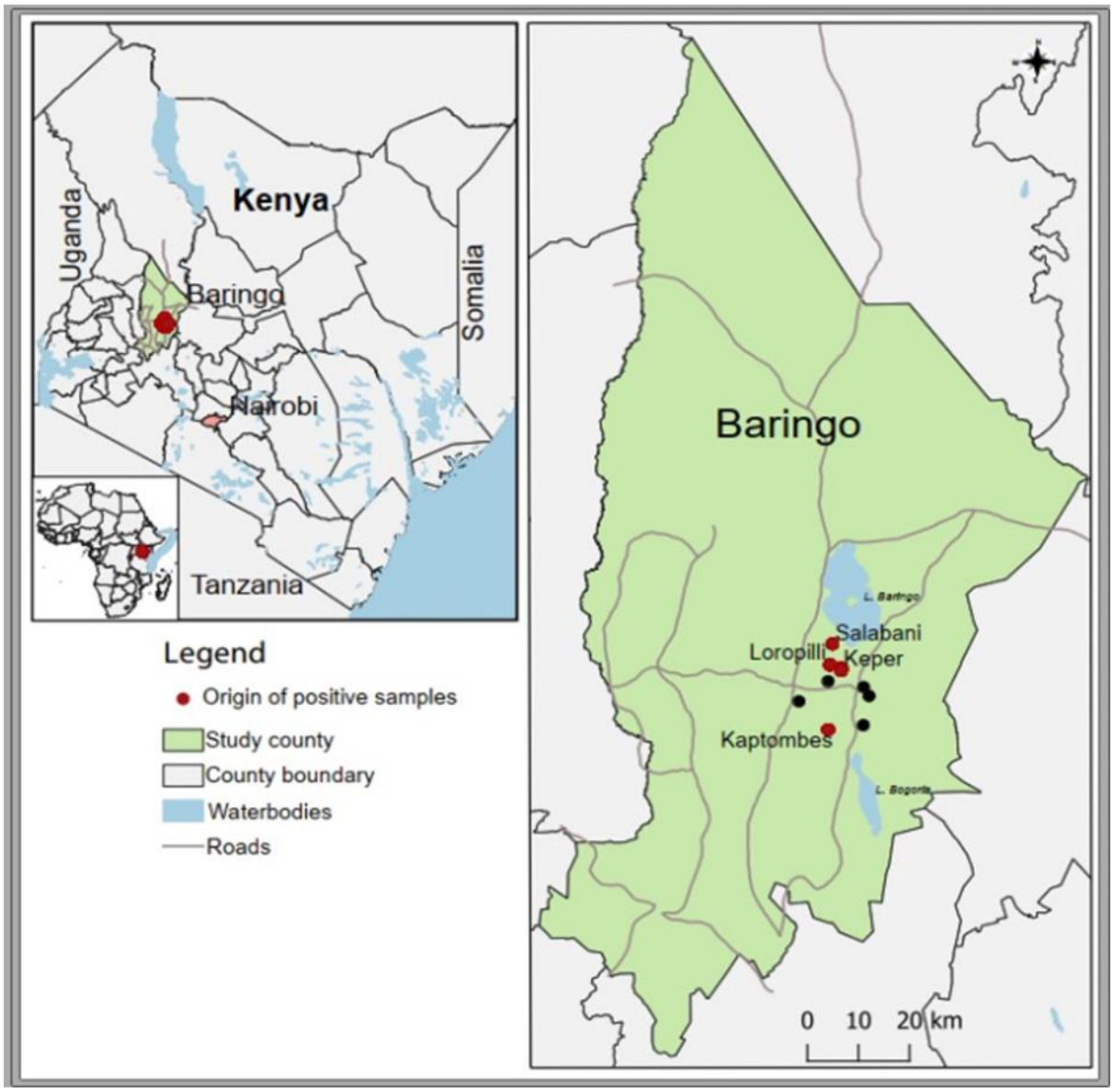


Figure 7.1: A map of Kenya showing the sampling sites within Baringo county. Red spots represent sites where KPTV-positive samples (virus and antibodies) originate from. Black dots represent other sampling sites. The maps were created in the open source GIS software, QGIS 3.22 using GPS co-ordinates and shape files derived from Natural Earth (*http://www.naturalearthdata.com/*, a free GIS data source) and Africa Open data (*https://africaopendata.org/dataset/kenya-counties-shapefile*, license Creative Commons) (185).

Table 7.1: Comparison of KPTV genome segments and proteins with Sathuvachari virus (SVIV) strain Ian-66411 and BTV Serotype 1 (BTV-1)

Genome segment	Encoded Proteins			Segment Size (bp)			Protein size (No. of amino acids)			Identity to Sathuvachari virus (SVIV IAn-66411)	
	KPTV	SVIV IAn-66411	BTV-1	KPTV	SVIV IAn-66411	BTV-1	KPTV	SVIV IAn-66411	BTV-1	nt identity%	aa identity%
Seg.1	VP1 (Pol)	VP1 (Pol)	VP1 (Pol)	3987	4015	3944	1320	1321	1302	77.46	89.5
Seg.2	VP3 (T2)	VP3 (T2)	VP2 (OC1)	2843	2860	2953	924	919	956	80.71	94.44
Seg.3	VP2(OC1)	VP2(OC1)	VP3 (T2)	2367	2393	2772	776	777	901	73.73	80.03
Seg.4	VP4 (Cap)	VP4 (Cap)	VP4 (Cap)	2011	1997	1980	641	644	644	77.36	89.43
Seg.5	NS1 (TuP)	NS1 (TuP)	VP5 (OC2)	1788	1789	1769	559	562	552	78.05	87.63
Seg.6	VP5(OC2)	VP5 (OC2)	VP6 (Hel)	1622	1644	1638	525	522	526	80.89	93.68
Seg.7	NS2 (ViP)	NS2 (ViP)	VP7 (T13)	1167	1205	1156	371	366	349	81.03	92.08
Seg.8	VP7 (T13)	VP7 (T13)	NS1 (TuP)	1155	1188	1125	351	351	354	83.64	98.29
Seg.9	VP6 (Hel)	VP6 (Hel)	NS2 (ViP)	1010	933	1049	281	287	329	75.60	60.14
Seg.10	NS3	NS3	NS3	781	810	822	213	213	229	82.4	91.04
Total				**18,731**	**18,834**	**19,208**	**5961**	**5962**	**6142**		

According to the orbivirus sequence related species demarcation criteria of the ICTV, distinct species share less than 78% amino acid identity in their RdRp proteins and less than 83% amino acid identity (<76% nucleotide identity) in their VP3 protein. The closest genetic relative of KPTV was SVIV with nucleotide (nt) and amino acid (aa) identities respectively being 77.46% and 89.5% for VP1 (RdRp), and 80.71% and 94.44% for VP3 (T2), suggesting that both viruses belong to the same virus species. The orbivirus outer-core VP7 (T13) is more conserved than the outer-capsid proteins, VP2 (OC1) and VP5 (OC2), and is considered virus-species specific and therefore targeted for serological assays (284,287,298). VP7 of KPTV showed nt and aa identities of 83.64 and 98.29% to the VP7 of SVIV (strain IAn-66411). Phylogenetic analyses based on KPTV VP1 (Pol), VP3 (T2), and VP7 (T13) protein sequences showed that KPTV is sister to the monophyletic SVIV sequence clade and that the genetic distance of KPTV to all SVIV sequences was greater than that within the SVIV clade, clearly separating the African KPTV from its Asian relative (**Figure 7.2**).

An SDS-PAGE analysis confirmed the presence of 10 dsRNA segments of different sizes in KPTV and in the BTV BLUVAX vaccine strain, which was used as a control (**Figure 7.3**). Genome segments of KPTV generated a 6-2-2 migration pattern: all genome segments migrated separately, except for S7 and S8, which are almost equal in size and co-migrated in the gel system. In contrast, the BTV BLUVAX vaccine strain showed a 3-3-3-1 migration pattern.

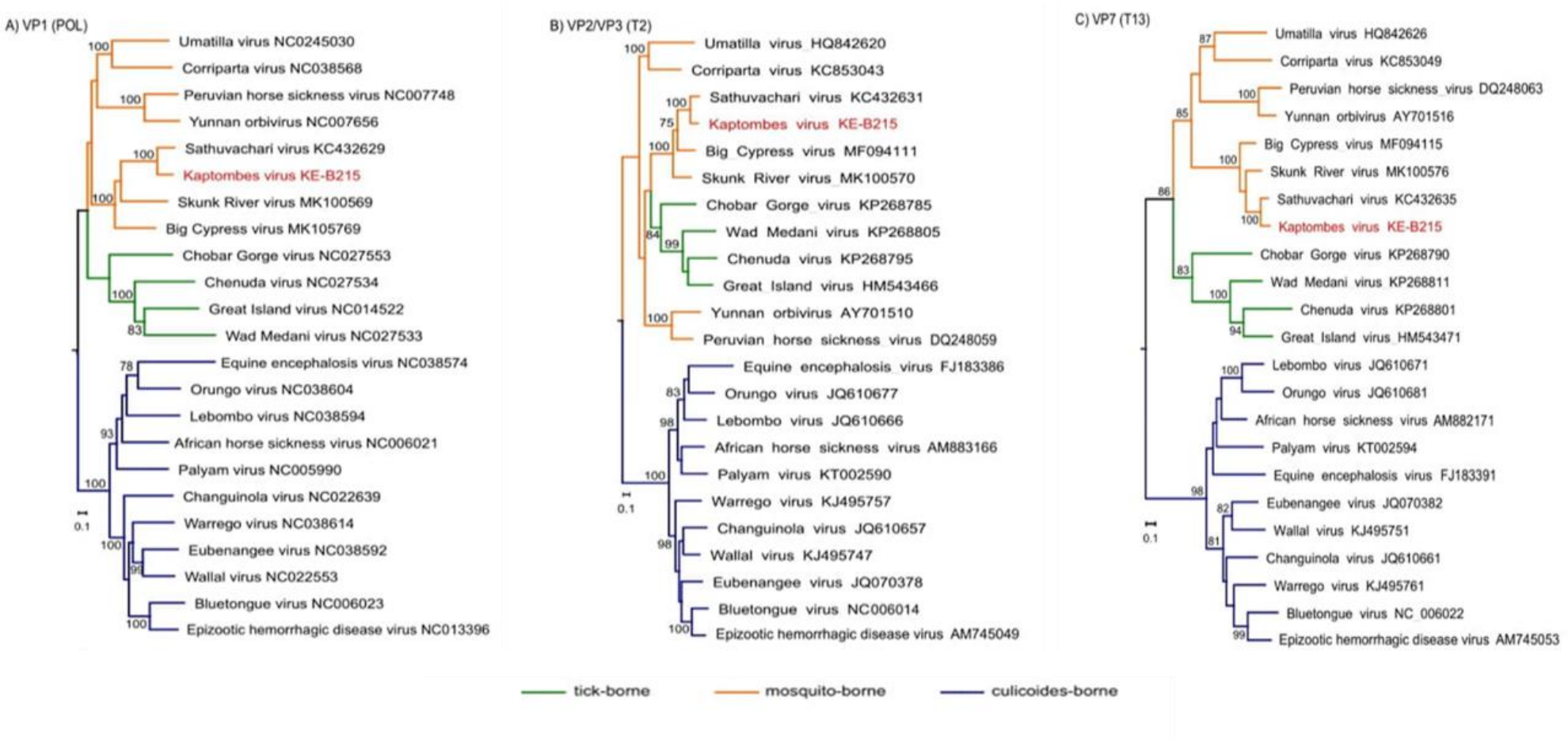


Figure 7.2: Maximum likelihood phylogenetic analysis of KPTV. Trees were based on the RdRp (Pol 1) protein (a), the VP3 (T2) protein (b) and the VP7 (T13) (c) protein. Sequences were aligned using MAFFT and trees inferred using PhyML v. 2.2.4 with the GTR substitution model employing 1000 bootstraps replicates.

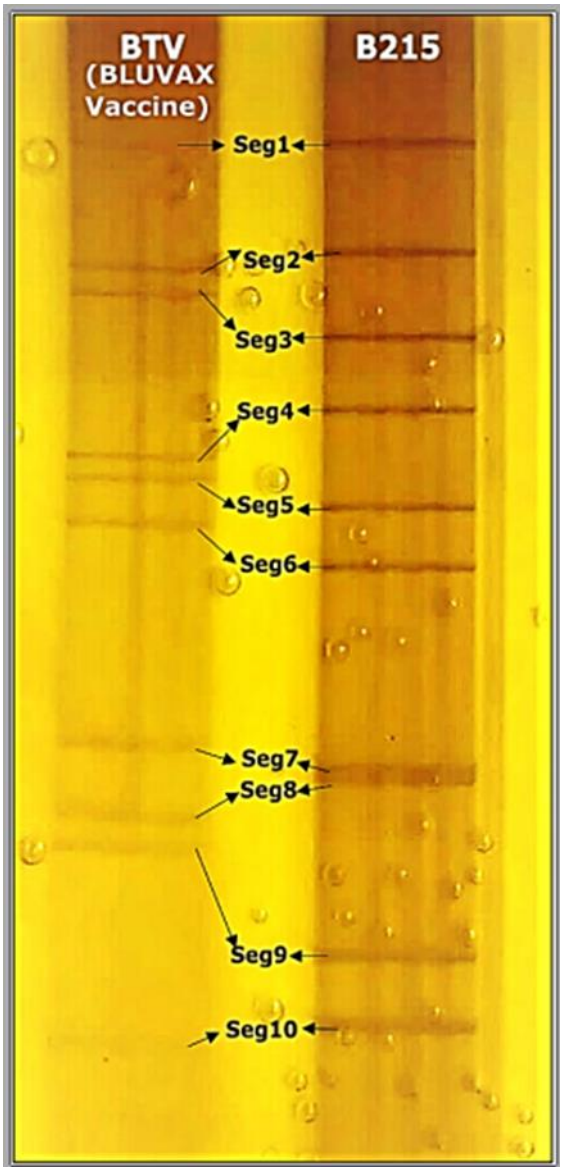


Figure 7.3: Electrophoretic profile of dsRNA of KPTV and BTV. Viral RNA of KPTV and BTV was separated on a 10% SDS-PAGE gel and stained with silver nitrate ($AgNO_3$). KPTV displayed a 6-2-2 migration pattern in contrast to the 3-3-3-1 migration pattern displayed by BTV.

***In vitro* pathogenicity studies of Kaptombes virus**

We next sought to gain insight into the cell tropism of KPTV and its potential pathogenicity. *In vitro* growth analyses were performed in the vertebrate cell lines Vero E6, MDBK and BHK 21 and the mosquito cell lines C636 and Aag2 to determine permissiveness. Insect and vertebrate cells were broadly susceptible for KPTV infection with highest replication in mosquito and hamster cell lines (**Figure 7.4**). These findings suggest a mosquito and vertebrate transmission cycle for KPTV, similar to those of other mosquito-borne orbiviruses like SVIV, Umatilla virus and Skunk River Virus, amongst others (275,285,287,299).

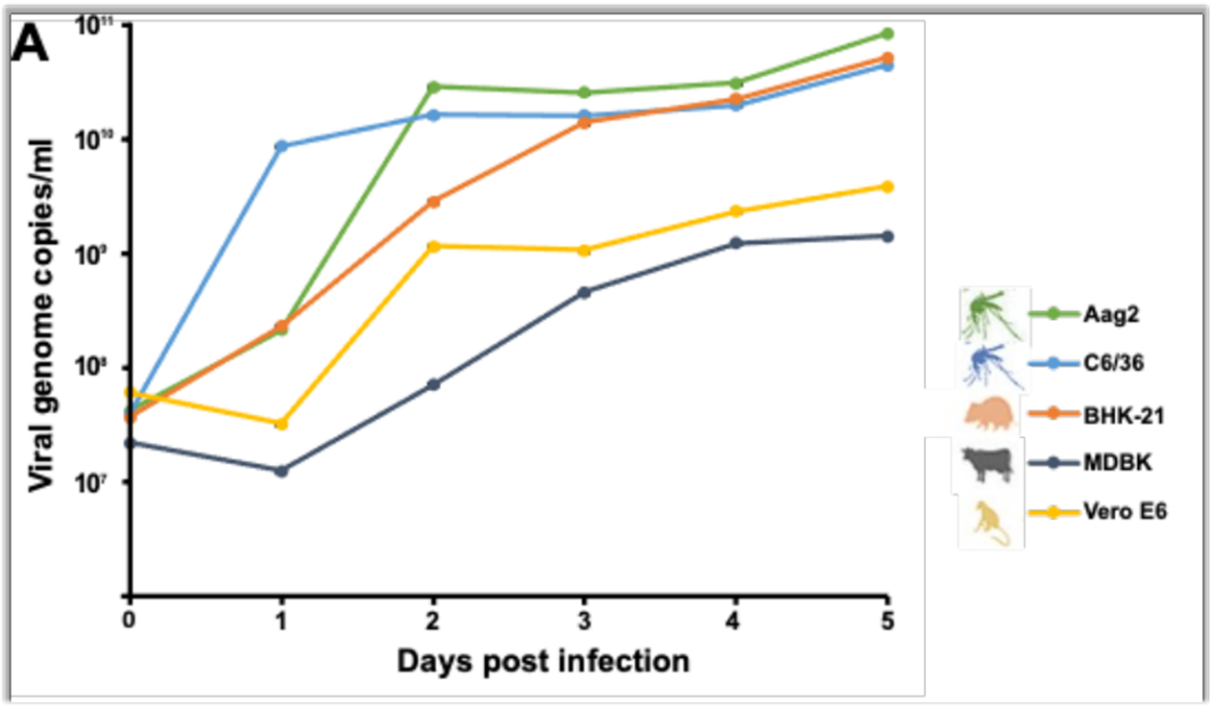


Figure 7.4: *In vitro* growth characteristics of KPTV. A) C6/36, Aag-2, BHK-21, MDBK and VeroE6 cell lines were infected with KPTV in duplicate at a MOI of 0.1 and the supernatant was harvested and quantified by real-time RT-PCR every 24 hours for a period of five days post infection.

Circulation of Kaptombes virus in livestock from Baringo county

To evaluate if KPTV is circulating in livestock in Kenya, we tested 248 serum samples from cattle, sheep, and goats for acute infection by RT-PCR. Surprisingly, KPTV RNA was detected in three additional samples from asymptomatic cattle (1.2%, 3/248) collected at four different locations in 2020 and 2021 (**Table 7.2**). Phylogenetic analyses based on the obtained KPTV sequence fragments, 735 nt in size, revealed that the Kenyan sequences (KE-B215-2020, KE-B97-2020, KE-B364-2021, and KE-B128-2020) formed a well-supported sister clade to all SVIV sequences (**Figure 7.5**).

Table 7.2: KPTV positive livestock samples.

Sample ID	Origin	Species	Age	Sex	Year of collection
KPTV KE-B97	Loropilli	Cattle	1	Female	2020
KPTV KE-B128	Keper	Cattle	2	Male	2020
KPTV KE-B215	Kaptombes	Cattle	2	Female	2020
KPTV KE-B364	Salabani	Cattle	2	Male	2021

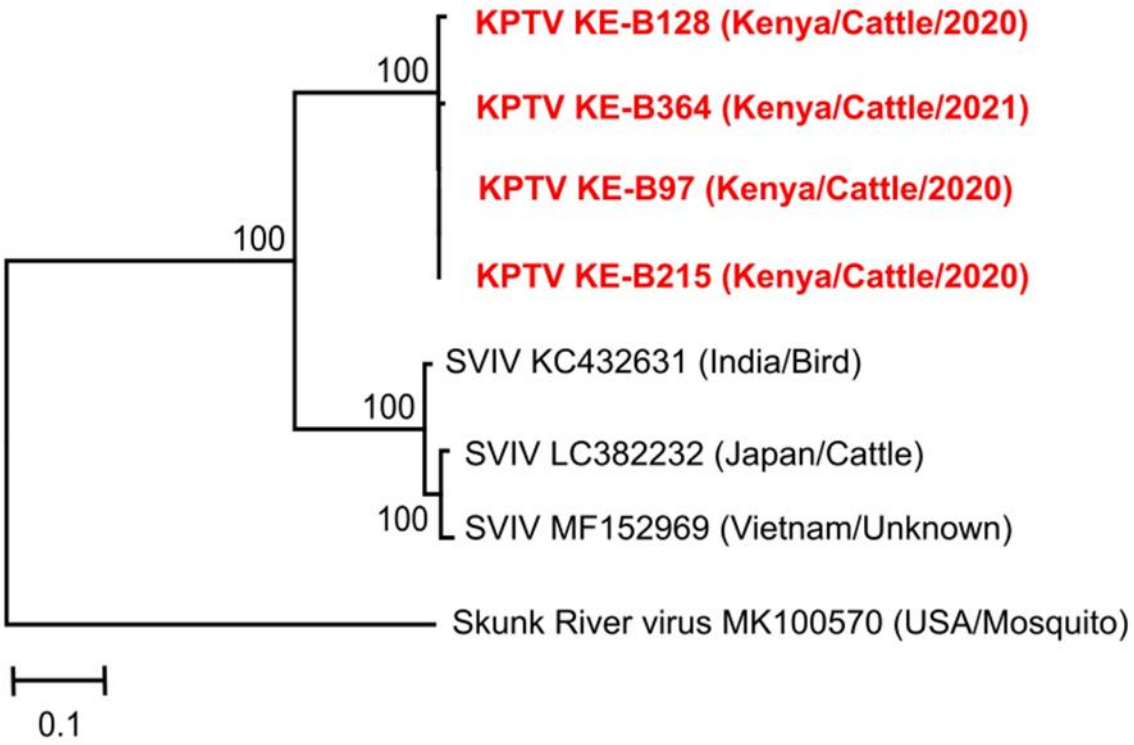


Figure 7.5: The ML tree of the 735 nt partial sequence of the VP3 (T2) sequence showing the relationships between detected KPTV and other closely related orbiviruses. The phylogenetic tree was inferred in PhyML v. 2.2.4 with General-time-reversible (GTR) substitution models employing 1000 bootstrap replicates. KPTV sequences are highlighted in red. All bootstrap support values are shown.

To further assess the extent of KPTV field presence/exposure, we randomly selected 200 livestock serum samples from the same geographical area where KPTV originated and screened them by PRNT for neutralizing reactivity against KPTV. In total, twelve samples (6% 95% CI 3.14-10.25) neutralized the virus with titres ranging from 1:20 to 1:320. The presence of neutralizing antibodies was highest in cattle (5%,10/200) compared to both sheep and goats (n=2, 0.5%,), and representing a significant difference in exposure between cattle and small ruminants (Fisher exact test odds ratio [OR] 5.4, 95% 1.16 to 25.52; p = 0.0316). All antibody positive samples were negative for KPTV RNA by qPCR and none of the PCR-positive samples contained KPTV neutralizing antibodies.

7.5 Discussion

Herein the detection and isolation of a novel putative mosquito-borne orbivirus designated Kaptombes virus from cattle in Kenya is reported. KPTV was initially isolated from a clinically sick cow aged 2-3 years, presenting signs of emaciation and lethargy. The virus was subsequently also detected in two cows sampled in the same year in other animal herds and in a cow from another location sampled one year later. Neutralizing antibodies against KPTV were detected in cattle and, to a small extent, in small ruminants. Together, these findings indicate active circulation of a previously unknown orbivirus in ruminants in Kenya.

At the time of sampling, the cow in which the virus was initially detected had signs of poor health including emaciation, with no response to different antibiotic treatments administered for almost 3 months according to the farmer. An aliquot of the sample was sent to the Central Veterinary Laboratory (CVL) Kabete, Kenya for routine diagnostics where no positive results were obtained for tested diseases including RVF (CVL, Lab report). At this time, we cannot confirm the orbivirus infection to be the cause nor dismiss its contribution to the disease symptoms of the animal. The other animals from which the virus was later detected in serum samples showed no symptoms of disease.

In the present study, KPTV RNA was only detected in cattle and not in other livestock species (sheep and goats) screened. However, neutralising antibodies were also detected in one sheep and one goat, although at a significantly lower prevalence than in cattle. Our data support previous reports of, SVIV (the closest virus relative) in cattle, signifying a probable higher susceptibility of cattle to the virus or a narrow livestock vertebrate host range. An overall seroprevalence of 6% (12/200, 95% CI 3.14-10.25) was found with a significantly higher exposure of cattle to KPTV at 5% compared to the 0.5% exposure confirmed in small ruminants (goats and sheep), (P = 0.0316). Orbivirus specificity to a certain vertebrate host is common and has been observed among some host species, notably, horses which are known to be more susceptible to AHSV and sheep which are more susceptible to BTV. However, AHSV and BTV can also infect many other host species (10,281,282). This could also be the case for KPTV implying that cattle may be more susceptible to the virus than sheep and goats. Nonetheless, we recommend further studies to understand its pathogenicity and susceptibility of various livestock species to KPTV. In addition, a larger sample size from different parts of the country will be required to assess distribution and prevalence rates of KPTV.

KPTV's closest relative (75% nucleotide-identity) is the mosquito-borne SVIV orbivirus, which was first isolated in 1963 from a starlings bird (*Brahminy myna*) in India, and later

isolated from sera of healthy cattle from Japan in 2005 (278,285). SVIV has also been isolated from a mosquito pool collected from Vietnam in 1966 (300). The KPTV 6-2-2 dsRNA segment migration pattern in electrophoretic analysis (**Figure 7.3**), is consistent to that of SVIV as presented by Kato et al., 2018 (278) further confirming the close genetic relationship of the two viruses. According to the 10th report of the ICTV for orbivirus classification, viruses of the same species have more than 76% nt identity and 83% aa identity in their major sub core structural protein VP3 (T2) and more than 78% aa identity in their VP1 (Pol) proteins. Amino acid sequence identities of 94% and 89,5% between KPTV and SVIV in the T2 and polymerase proteins were found, respectively, suggesting that both may belong to the same species (270,299,301). However, the nt and aa identities of the VP2 (OC1) protein to that of SVIV revealed a high levels of genetic variability of nucleotide and amino acid levels; 73.73% and 80.3%, respectively (302). Other species demarcation criteria, such as serological cross reactivity in conserved antigens and cross-hybridization of conserved genome segments, could not be investigated as no SVIV isolate was available. Currently, all SVIV strains are considered belonging to the same serotype due to high sequence identities (97.49%/98.58% nt/aa) in the VP2 (OC1) protein (278). However, the KPTV VP2 shows a much lower sequence identity (73.73%/80.03%, nt/aa, respectively), when compared to SVIV and thus may be serologically distinct. Further, KPTV is well separated from all currently known SVIV sequences in the phylogenetic analyses supporting the existence of two discrete genetic lineages that may indicate the presence of two different species. Whilst the common ancestry of KPTV and SVIV is clear, it is not known whether these viruses share common vectors and host species; this component requires further investigation. Also, the pathogenicity of the two viruses is currently not well understood. SVIV was shown to be pathogenic for new-borne mice (300), though the bovid from which it was isolated in Japan were apparently healthy. The finding that the KPTV was isolated from an emaciated and lethargic animal highlights pathogenetic potential and should be further investigated.

Orbiviruses are known to have non-coding regions (NCRs) at the termini of all segments, the total percentage of which varies depending on the virus. Mosquito-borne orbiviruses have overall NCRs of 5.03-5.695% (285,287). The overall NCRs in the entire KPTV genome was 4.675%, similar to that of SVIV strain IAn-66411 with 4.874%. Although for both, virus transmission by mosquitoes is hypothesized, the range of NCRs is less than the reported range for other mosquito-borne orbiviruses. However, the NCRs percentages are within the range for tick-borne orbiviruses of 4.47-4.9%, and is likely attributable attributed to common ancestry, although other unknown factors cannot be ruled out (275,287,288). Orbiviruses exhibit varied

G+C content based on the transmitting arthropod vector. The documented range for mosquito-borne orbiviruses is between 36.7% for PHSV to 45.55% for YUOV (287). The G+C content of SVIV is 42.3% (285). The tick borne orbiviruses generally exhibit a higher G+C content of over 50% and as much as 58.1% for Great island virus (GIV) (288), while the *Culicoides*/sandfly borne orbiviruses have an intermediate G+C content of 39.89%-45.89% (287,288,303). KPTV has a G+C content of 43.14%, thus falling within the range of mosquito-borne orbiviruses. Phylogenetic analyses based on VP1 (Pol), VP3 (T2), and VP7 (T13) proteins placed KPTV in the same cluster with mosquito-borne orbiviruses. Mosquito cell lines were found to be susceptible to infection with KPTV further supporting the idea of a mosquito transmission cycle.

SVIV has so far only been reported in three Asian countries (India, Japan and Vietnam) (278,285,300). The occurrence of a related virus, KPTV in Kenya raises questions about the evolutionary origin of the two viruses and their spatial and temporal spread, possibly through movement of host species like migratory birds. SVIV was first detected in birds (285), and annual north-south bird migration/movement trends provide an opportunity for a possible spread of the virus to other geographical regions (304–306). KPTV was detected in Baringo county, which is home to two freshwater lakes (Lake Bogoria and Lake Baringo) that host large populations of diverse migratory bird species. Additional genome sequences are needed to understand the spatial and temporal spread of KPTV and its relatives in Africa and Asia and to evaluate the role of birds, if any, in KPTV epidemiology.

Chapter 8

CONCLUDING REMARKS

The order Bunyavirales contains a diverse group of viruses that affect different hosts. Animal and human pathogenic bunyaviruses are either rodent or insect-borne (arboviruses). Pathogenic arboviral bunyaviruses like RVFV, NRIV and BUNV are zoonotic and responsible for disease in both human and livestock. Others like CCHFV and TOSV are among the viruses that severely affects humans and although they have animal reservoir or replication hosts, these non-human hosts do not show clinical signs to our knowledge. Hantaviruses and Lassa viruses are rodent borne bunyaviruses that are pathogenic to humans. Assessment of circulation of these viruses through a one health approach and active surveillance system in thus important to understand virus circulation and transmission dynamics. Knowledge on circulation will provide information important in diagnosis and in developing and implementing effective disease control strategies to prevent future unprecedented outbreaks.

In this study, a cross-sectional laboratory-based survey was performed to assess Bunyavirales activity and circulation in humans, livestock, and peri-domestic rodents in diverse ecologies in Kenya using molecular screening, serology to determine the prevalence in different species and NGS sequencing for whole genome characterisation and virus discovery. In chapter 3, we reported the overall findings of the screening process for all families investigated here in humans, livestock, and rodents.

Overall, the study confirmed the circulation of various bunyaviruses in the three hosts through virus detection, presence of antibodies and virus isolation. These viruses include both a wide range of arboviral bunyaviruses and rodent borne hantaviruses detected in shrews. Confirmation of a wide circulation of the virus in various hosts, was revealed for CCHFV and this therefore supports the importance of implementing one health surveillance.

The detection of uncharacterised phleboviruses in livestock clearly confirms circulation of unknown viruses and necessitates the need for continuous surveillance, characterisation, and pathogenicity studies to ascertain the degree to which these novel viruses contribute to disease burden. In chapters 4-7, the most important findings of the screening process are described.

Chapter 4 reports the first detection of NRIV in livestock in Kenya. Here, we show NRIV to be actively circulating in apparently asymptomatic cattle, sheep, and goats in two pastoralist-dominated areas in Kenya. Furthermore, livestock and humans showed high seroprevalence rates of up to 38.2% and 23.3%, respectively suggesting unnoticed virus circulation. Our results demonstrate active and silent circulation of NRIV in the three most common livestock species

highlighting the need for an active one health surveillance of host networks including humans, livestock, and vectors and the urgent need to establish diagnostic tools to investigate the potential health impact of NRIV on livestock and human populations.

In chapter 5, the detection of CCHFV RNA and antibody in rodents and three main livestock species and humans confirm the active circulation of the virus in Kenya. The report provides the first report of CCHFV in rodents in Kenya. These findings emphasize the value of one health active surveillance in different potential hosts to understand the transmission network of different zoonotic viruses while considering monitoring of other small mammals like rodents and shrews that live near humans and have in most cases been ignored. The data of this study provide an indication of CCHFV-associated disease occurrence in humans and a potential risk for outbreaks.

In Chapter 6, the study confirms the circulation of rodent-borne hantavirus in shrews. Few studies have targeted rodent borne viruses locally and this report provide the baseline information and first evidence of circulation of the virus in shrews. The contribution of the shrew-borne hantavirus to human disease burden in the area and nationwide remains unknown. The information herein can be used for further studies to ascertain the ecology of the virus and its pathogenicity. Evaluation of circulation in humans is thus necessary.

Chapter 7 demonstrates the importance of robust methods employed in the study. Here we revealed the circulation of a novel orbivirus in cattle through virus isolation in cell lines. These findings expand our knowledge of circulating orbiviruses in livestock. Cattle represents the most important/high value livestock species in the farming industry and are often the main source of livelihoods in rural areas of Africa. Thus, infectious diseases of cattle may have major economic consequences and are highly relevant. The data presented here form a baseline for further research including active surveillance of KPTV to understand its geographic distribution, susceptible vertebrate hosts, pathology, mode of transmission, its potential to cause outbreaks, and implications for animal and human health. This is necessary to assess if KPTV needs to be added to the list of notifiable diseases.

The findings of this research confirm the active circulation of pathogenic Bunyavirales with potential to spread and cause epidemics not only in livestock but humans as well. It also emphasizes the importance of active one health surveillance of known zoonotic and novel viruses to provide useful information that can be used to prevent unprecedented disease outbreaks by implementing timely and effective disease control measures.

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Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

12 August 2022

Approval Certificate
Annual Renewal

Dear Ms DCA Omoga,

Ethics Reference No.: 568/2020 – Line 2
Title: Assessment of Bunyavirales activity and circulation in humans, livestock and peri-domestic rodents in diverse ecologies in Kenya, 2020-2022

The Annual Renewal as supported by documents received between 2022-07-26 and 2022-08-10 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-08-10 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-08-12.
- Please remember to use your protocol number (568/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

_____

ıe FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix 2: Isolated Ngari virus L, M, and S segment distance matrix

L segment

Distance matrix of the RdRp protein showing representative viruses and samples sequenced in this study. Light grey to dark shades indicates nucleotide distances.

	NRIV A...	KE-093...	KE-C16...	KE-B02...	KE-B35...	NRIV _...	NRIV_M...	NRIV_K...	NRIV_K...	NRIV_K...	NRIV_JX...	NRIV_JX...	NRIV_JX...	NRIV_JX...	NRIV_JX...	BUNV_...	BUNV_...	BUNV_...	BUNV_...	BUNV_...	BUNV_J...	BUNV_...	Bozo vir...	BTV_M...	BTV_N...	BTV_JX8...	BTV_JX...	BTV_KT...	BTV_KC...
NRIV Adrar_KJ716850.1...		99.0%	98.9%	99.0%	98.9%	98.7%	98.7%	98.3%	98.5%	98.1%	98.1%	97.6%	97.7%	98.1%	98.2%	96.3%	94.7%	95.1%	94.7%	94.8%	94.6%	94.0%	75.2%	74.0%	73.9%	73.7%	73.8%	73.6%	73.9%
KE-093_NRIV_L	99.0%		100.0%	100.0%	99.9%	98.2%	98.3%	97.8%	98.0%	97.7%	97.7%	97.2%	97.3%	97.6%	97.7%	95.9%	94.4%	94.8%	94.5%	94.6%	94.3%	93.7%	75.1%	73.7%	74.0%	73.6%	73.9%	73.7%	74.0%
KE-C166_NRIV_L	98.9%	100.0%		100.0%	99.8%	98.2%	98.3%	97.8%	97.9%	97.7%	97.7%	97.2%	97.3%	97.6%	97.7%	95.9%	94.4%	94.8%	94.5%	94.5%	94.3%	93.7%	75.1%	73.7%	74.0%	73.6%	73.8%	73.7%	74.0%
KE-B02_NRIV_L	99.0%	100.0%	100.0%		99.9%	98.2%	98.3%	97.8%	98.0%	97.7%	97.7%	97.2%	97.3%	97.6%	97.7%	95.9%	94.4%	94.8%	94.5%	94.6%	94.3%	93.7%	75.2%	73.7%	74.0%	73.6%	73.8%	73.6%	74.0%
KE-B35_NRIV _L	98.9%	99.9%	99.8%	99.9%		98.2%	98.2%	97.8%	97.9%	97.7%	97.7%	97.2%	97.3%	97.6%	97.7%	95.9%	94.4%	94.8%	94.5%	94.5%	94.2%	93.7%	75.2%	73.7%	74.0%	73.6%	73.8%	73.6%	74.0%
NRIV_MT747972.1(Ma...	98.7%	98.2%	98.2%	98.2%	98.2%		99.5%	97.9%	98.1%	97.7%	97.6%	97.1%	97.2%	97.7%	97.9%	95.8%	94.4%	94.8%	94.6%	94.6%	94.4%	93.7%	75.2%	74.1%	74.0%	73.7%	74.0%	73.6%	73.9%
NRIV_MT747975.1(Mau...	98.7%	98.3%	98.3%	98.3%	98.2%	99.5%		98.0%	98.2%	97.7%	97.7%	97.2%	97.3%	97.8%	97.9%	95.9%	94.5%	94.9%	94.5%	94.6%	94.4%	93.8%	75.2%	74.0%	73.9%	73.7%	73.9%	73.5%	73.8%
NRIV_KM507335.1(Ken...	98.3%	97.8%	97.8%	97.8%	97.8%	97.9%	98.0%		99.5%	97.8%	97.7%	97.3%	97.4%	97.8%	97.9%	96.1%	94.7%	95.1%	95.0%	95.0%	94.3%	93.9%	75.1%	73.9%	73.8%	73.6%	73.7%	73.6%	73.9%
NRIV_KM507334.1(Ken...	98.5%	98.0%	97.9%	98.0%	97.9%	98.1%	98.2%	99.5%		97.8%	97.8%	97.4%	97.4%	97.9%	98.0%	95.9%	94.5%	94.9%	94.5%	94.5%	94.3%	93.7%	75.1%	74.0%	73.9%	73.6%	73.9%	73.6%	73.9%
NRIV_KC608152.1(Sene...	98.1%	97.7%	97.7%	97.7%	97.7%	97.7%	97.7%	97.8%	97.8%		100.0%	98.7%	98.7%	98.4%	98.5%	97.4%	95.7%	96.0%	95.8%	95.8%	95.5%	95.0%	75.2%	74.2%	74.0%	73.7%	73.8%	73.6%	73.9%
NRIV_JX857318.1(Sene...	98.1%	97.7%	97.7%	97.7%	97.7%	97.6%	97.7%	97.7%	97.8%	100.0%		98.7%	98.7%	98.4%	98.5%	97.3%	95.7%	96.0%	95.7%	95.8%	95.4%	95.0%	75.2%	74.2%	73.9%	73.7%	73.7%	73.5%	73.8%
NRIV_JX857324.1(Suda...	97.6%	97.2%	97.2%	97.2%	97.2%	97.1%	97.2%	97.3%	97.4%	98.7%	98.7%		99.5%	97.9%	97.9%	96.8%	95.3%	95.6%	95.3%	95.3%	95.0%	94.6%	75.3%	74.2%	74.0%	73.7%	73.8%	73.5%	73.8%
NRIV_JX857321.1(Suda...	97.7%	97.3%	97.3%	97.3%	97.3%	97.2%	97.3%	97.4%	97.4%	98.7%	98.7%	99.5%		97.9%	98.0%	96.9%	95.3%	95.7%	95.3%	95.3%	95.0%	94.7%	75.3%	74.2%	74.2%	73.9%	73.9%	73.5%	73.9%
NRIV_JX857330.1(Kenya...	98.1%	97.6%	97.6%	97.6%	97.6%	97.7%	97.8%	97.8%	97.9%	98.4%	98.4%	97.9%	97.9%		99.7%	96.4%	95.0%	95.3%	94.9%	94.9%	94.6%	94.1%	75.0%	74.1%	73.8%	73.5%	73.6%	73.4%	73.7%
NRIV_JX857327.1(Soma...	98.2%	97.7%	97.7%	97.7%	97.7%	97.9%	97.9%	97.9%	98.0%	98.5%	98.5%	97.9%	98.0%	99.7%		96.4%	95.0%	95.3%	94.9%	94.9%	94.6%	94.2%	75.0%	74.1%	73.8%	73.5%	73.7%	73.5%	73.7%
BUNV_X14383.1	96.3%	95.9%	95.9%	95.9%	95.9%	95.8%	95.9%	96.1%	95.9%	97.4%	97.3%	96.8%	96.9%	96.4%	96.4%		96.4%	96.8%	96.5%	96.6%	96.2%	95.7%	75.2%	74.2%	73.7%	73.7%	73.5%	73.6%	74.0%
BUNV_MW314030.1(Ke...	94.7%	94.4%	94.4%	94.4%	94.4%	94.4%	94.5%	94.7%	94.5%	95.7%	95.7%	95.3%	95.3%	95.0%	95.0%	96.4%		98.3%	98.1%	98.1%	97.6%	94.5%	75.2%	74.1%	73.8%	73.6%	73.8%	73.7%	74.0%
BUNV_MH484288.1(Ke...	95.1%	94.8%	94.8%	94.8%	94.8%	94.8%	94.9%	95.1%	94.9%	96.0%	96.0%	95.6%	95.7%	95.3%	95.3%	96.8%	98.3%		98.2%	98.2%	97.9%	94.6%	75.3%	74.0%	74.1%	73.8%	74.1%	73.8%	74.1%
BUNV_KM507338.1(Ke...	94.7%	94.5%	94.5%	94.5%	94.5%	94.6%	94.5%	95.0%	94.5%	95.8%	95.7%	95.3%	95.3%	94.9%	94.9%	96.5%	98.1%	98.2%		99.9%	98.8%	94.4%	75.3%	74.1%	73.9%	73.6%	73.7%	73.9%	74.0%
BUNV_KM507337.1(Ke...	94.8%	94.6%	94.5%	94.6%	94.5%	94.6%	94.6%	95.0%	94.5%	95.8%	95.8%	95.3%	95.3%	94.9%	94.9%	96.6%	98.1%	98.2%	99.9%		98.8%	94.4%	75.3%	74.1%	73.9%	73.6%	73.7%	73.8%	74.0%
BUNV_JF961340.1(Keny...	94.6%	94.3%	94.3%	94.3%	94.2%	94.4%	94.4%	94.3%	94.3%	95.5%	95.4%	95.0%	95.0%	94.6%	94.6%	96.2%	97.6%	97.9%	98.8%	98.8%		94.0%	74.5%	73.2%	73.4%	73.3%	73.7%	73.5%	74.0%
BUNV_MT731755.1(Ma...	94.0%	93.7%	93.7%	93.7%	93.7%	93.7%	93.8%	93.9%	93.7%	95.0%	95.0%	94.6%	94.7%	94.1%	94.2%	95.7%	94.5%	94.6%	94.4%	94.4%	94.0%		75.3%	74.1%	73.5%	73.2%	73.3%	73.5%	73.6%
Bozo virus strain DakAr...	75.2%	75.1%	75.1%	75.2%	75.2%	75.2%	75.2%	75.1%	75.1%	75.2%	75.2%	75.3%	75.3%	75.0%	75.0%	75.2%	75.2%	75.3%	75.3%	75.3%	74.5%	75.3%		73.8%	72.4%	72.8%	72.7%	73.0%	73.0%
BTV_MS50 NC_043580....	74.0%	73.7%	73.7%	73.7%	73.7%	74.1%	74.0%	73.9%	74.0%	74.2%	74.2%	74.2%	74.2%	74.1%	74.1%	74.2%	74.1%	74.0%	74.1%	74.1%	73.2%	74.1%	73.8%		72.7%	72.9%	72.8%	72.6%	73.0%
BTV_NM/12_KJ187038....	73.9%	74.0%	74.0%	74.0%	74.0%	74.0%	73.9%	73.8%	73.9%	74.0%	73.9%	74.0%	74.2%	73.8%	73.8%	73.7%	73.8%	74.1%	73.9%	73.9%	73.4%	73.5%	72.4%	72.7%		95.3%	91.0%	85.6%	85.8%
BTV_JX846600.1(India/...	73.7%	73.6%	73.6%	73.6%	73.6%	73.7%	73.7%	73.6%	73.6%	73.7%	73.7%	73.7%	73.9%	73.5%	73.5%	73.7%	73.6%	73.8%	73.6%	73.6%	73.3%	73.2%	72.8%	72.9%	95.3%		90.5%	85.6%	85.9%
BTV_JX846603.1(Ugan...	73.8%	73.9%	73.8%	73.8%	73.8%	74.0%	73.9%	73.7%	73.9%	73.8%	73.7%	73.8%	73.9%	73.6%	73.7%	73.5%	73.8%	74.1%	73.7%	73.7%	73.7%	73.3%	72.7%	72.8%	91.0%	90.5%		86.2%	86.2%
BTV_KT313704.1(Russi...	73.6%	73.7%	73.7%	73.6%	73.6%	73.6%	73.5%	73.6%	73.6%	73.6%	73.5%	73.5%	73.5%	73.4%	73.5%	73.6%	73.7%	73.8%	73.9%	73.8%	73.5%	73.5%	73.0%	72.6%	85.6%	85.6%	86.2%		97.6%
BTV_KC168048.1(Italy/...	73.9%	74.0%	74.0%	74.0%	74.0%	73.9%	73.8%	73.9%	73.9%	73.9%	73.8%	73.8%	73.9%	73.7%	73.7%	74.0%	74.0%	74.1%	74.0%	74.0%	74.0%	73.6%	73.0%	73.0%	85.8%	85.9%	86.2%	97.6%	

M segment

Distance matrix of the glycoprotein showing representative viruses and samples sequenced in this study. Light grey to dark shades indicates nucleotide distances.

	BUNV_...	BUNV_...	BUNV_...	BUNV_J...	BUNV_...	Bozo vir...	NRIV A...	KE-093...	KE-C16...	KE-B02...	KE-B35...	NRIV_M...	NIRV_M...	NRIV_K...	NRIV_K...	NRIV_K...	NRIV_J...	NRIV_A...	NRIV_J...	NRIV_JX...	NRIV D...	BTV_X8...	BTV_D...	BTV_KU...	BTV_KJ1...	BTV_JX8...	BTV_AY...	BTV_D...	BTV_JX8...	BTV_D...	BTV_FJ4...	BTV_M...	BTV_KT...
BUNV_MH484289.1(Ke...		98.0%	98.0%	97.8%	96.0%	74.5%	64.0%	64.1%	64.1%	64.0%	64.1%	64.0%	64.0%	64.5%	64.5%	64.5%	64.4%	64.3%	64.3%	64.3%	64.5%	64.4%	64.5%	64.2%	64.1%	64.7%	64.7%	64.9%	64.5%	64.8%	64.4%	64.7%	64.6%
BUNV_KM507340.1(Ke...	98.0%		100.0%	99.3%	95.8%	75.2%	64.2%	64.2%	64.2%	64.1%	64.2%	64.1%	64.1%	64.5%	64.5%	64.5%	64.5%	64.4%	64.4%	64.4%	64.6%	64.5%	64.5%	64.4%	64.3%	64.9%	64.9%	65.1%	64.8%	65.1%	64.7%	64.9%	64.8%
BUNV_KM507339.1(Ke...	98.0%	100.0%		99.3%	95.8%	75.2%	64.2%	64.2%	64.2%	64.2%	64.3%	64.2%	64.1%	64.5%	64.6%	64.6%	64.5%	64.5%	64.4%	64.4%	64.6%	64.5%	64.5%	64.4%	64.4%	65.0%	65.0%	65.2%	64.9%	65.2%	64.7%	64.9%	64.8%
BUNV_JF961341.1(Keny...	97.8%	99.3%	99.3%		95.7%	75.2%	64.2%	64.2%	64.2%	64.1%	64.3%	64.2%	64.1%	64.6%	64.6%	64.7%	64.5%	64.5%	64.4%	64.4%	64.5%	64.4%	64.4%	64.3%	64.2%	64.7%	64.7%	64.7%	64.8%	64.8%	64.6%	64.8%	64.7%
BUNV_MZ773506.1 (Ug...	96.0%	95.8%	95.8%	95.7%		74.5%	63.8%	63.9%	63.9%	63.9%	63.9%	63.7%	63.7%	64.3%	64.4%	64.4%	64.2%	64.2%	64.1%	64.4%	64.2%	64.2%	64.2%	63.9%	63.8%	64.4%	64.4%	64.6%	64.6%	64.9%	64.5%	64.8%	64.6%
Bozo virus DakArB _MH...	74.5%	75.2%	75.2%	75.2%	74.5%		77.2%	77.9%	77.9%	77.9%	77.9%	77.2%	76.5%	77.2%	77.2%	77.2%	77.2%	77.2%	76.5%	76.5%	76.5%	77.2%	77.2%	75.8%	76.5%	77.2%	77.2%	77.2%	75.2%	75.2%	75.8%	71.1%	70.5%
NRIV Adrar_KJ716849.1...	64.0%	64.2%	64.2%	64.2%	63.8%	77.2%		98.5%	98.5%	98.5%	98.4%	98.2%	98.0%	97.1%	97.2%	97.1%	97.5%	97.4%	96.8%	96.8%	97.3%	94.5%	94.4%	88.6%	88.3%	88.7%	88.7%	88.7%	88.5%	88.5%	88.1%	84.2%	84.3%
KE-093_NRIV_M	64.1%	64.2%	64.2%	64.2%	63.9%	77.9%	98.5%		100.0%	99.9%	99.8%	97.8%	97.8%	96.8%	96.9%	96.8%	97.2%	97.1%	96.5%	96.4%	96.9%	94.4%	94.4%	88.7%	88.4%	88.7%	88.7%	88.7%	88.5%	88.6%	88.1%	84.4%	84.5%
KE-C166_NRIV_M	64.1%	64.2%	64.2%	64.2%	63.9%	77.9%	98.5%	100.0%		100.0%	99.8%	97.9%	97.8%	96.8%	96.9%	96.9%	97.2%	97.1%	96.5%	96.4%	96.9%	94.4%	94.4%	88.7%	88.4%	88.7%	88.7%	88.7%	88.6%	88.6%	88.1%	84.5%	84.6%
KE-B02_NRIV_M	64.0%	64.1%	64.2%	64.1%	63.9%	77.9%	98.5%	99.9%	100.0%		99.7%	97.9%	97.8%	96.8%	96.8%	96.8%	97.2%	97.0%	96.5%	96.4%	96.9%	94.4%	94.3%	88.7%	88.3%	88.6%	88.6%	88.7%	88.5%	88.6%	88.1%	84.5%	84.6%
KE-B35_NRIV_M	64.1%	64.2%	64.3%	64.3%	63.9%	77.9%	98.4%	99.8%	99.8%	99.7%		97.7%	97.7%	96.8%	96.8%	96.8%	97.1%	97.0%	96.4%	96.3%	96.8%	94.3%	94.3%	88.6%	88.3%	88.7%	88.7%	88.7%	88.5%	88.6%	88.1%	84.4%	84.5%
NRIV_MT747973.1(Mau...	64.0%	64.1%	64.2%	64.2%	63.7%	77.2%	98.2%	97.8%	97.9%	97.9%	97.7%		99.3%	96.8%	96.8%	96.8%	97.4%	97.3%	96.7%	96.6%	97.1%	94.4%	94.4%	88.5%	88.2%	88.6%	88.6%	88.6%	88.4%	88.4%	88.0%	84.3%	84.4%
NIRV_MT747976.1(Mau...	64.0%	64.1%	64.1%	64.1%	63.7%	76.5%	98.0%	97.8%	97.8%	97.8%	97.7%	99.3%		96.8%	96.8%	96.8%	97.4%	97.2%	96.6%	96.5%	97.0%	94.4%	94.4%	88.4%	88.1%	88.5%	88.5%	88.5%	88.2%	88.2%	87.8%	84.2%	84.3%
NRIV_KM514679.1(Ken...	64.5%	64.5%	64.5%	64.6%	64.3%	77.2%	97.1%	96.8%	96.8%	96.8%	96.8%	96.8%	96.8%		100.0%	99.9%	97.7%	97.6%	97.0%	96.9%	97.4%	94.8%	94.8%	88.9%	88.6%	88.9%	88.9%	88.9%	88.7%	88.7%	88.4%	84.3%	84.4%
NRIV_KM514678.1(Ken...	64.5%	64.5%	64.6%	64.6%	64.4%	77.2%	97.2%	96.9%	96.9%	96.8%	96.8%	96.8%	96.8%	100.0%		100.0%	97.8%	97.7%	97.0%	97.0%	97.5%	94.8%	94.8%	89.0%	88.7%	88.9%	88.9%	88.9%	88.8%	88.8%	88.4%	84.4%	84.5%
NRIV_KM514677.1(Ticks)	64.5%	64.5%	64.6%	64.7%	64.4%	77.2%	97.1%	96.8%	96.9%	96.8%	96.8%	96.8%	96.8%	99.9%	100.0%		97.8%	97.7%	97.0%	96.9%	97.4%	94.8%	94.8%	89.0%	88.7%	88.9%	88.9%	89.0%	88.8%	88.8%	88.5%	84.4%	84.5%
NRIV_JX857326.1(Som...	64.4%	64.5%	64.5%	64.5%	64.2%	77.2%	97.5%	97.2%	97.2%	97.2%	97.1%	97.4%	97.4%	97.7%	97.8%	97.8%		99.4%	97.5%	97.4%	97.9%	95.0%	95.0%	88.7%	88.5%	88.7%	88.7%	88.7%	88.8%	88.7%	88.2%	84.5%	84.5%
NRIV_AY593725.1(Keny...	64.3%	64.4%	64.5%	64.5%	64.2%	77.2%	97.4%	97.1%	97.1%	97.0%	97.0%	97.3%	97.2%	97.6%	97.7%	97.7%	99.4%		97.4%	97.4%	97.9%	95.0%	95.0%	88.7%	88.5%	88.7%	88.6%	88.6%	88.8%	88.8%	88.3%	84.5%	84.5%
NRIV_JX857320.1(Suda...	64.3%	64.4%	64.4%	64.4%	64.2%	76.5%	96.8%	96.5%	96.5%	96.5%	96.4%	96.7%	96.6%	97.0%	97.0%	97.0%	97.5%	97.4%		99.5%	99.1%	96.2%	96.2%	89.3%	89.1%	89.3%	89.3%	89.3%	89.4%	89.4%	88.7%	84.5%	84.6%
NRIV_JX857323.1(Suda...	64.3%	64.4%	64.4%	64.4%	64.1%	76.5%	96.8%	96.4%	96.4%	96.4%	96.3%	96.6%	96.5%	96.9%	97.0%	96.9%	97.4%	97.4%	99.5%		99.0%	96.1%	96.1%	89.2%	89.0%	89.2%	89.2%	89.2%	89.2%	89.2%	88.6%	84.5%	84.7%
NRIV DakArD_JX857317...	64.5%	64.6%	64.6%	64.5%	64.4%	76.5%	97.3%	96.9%	96.9%	96.9%	96.8%	97.1%	97.0%	97.4%	97.5%	97.4%	97.9%	97.9%	99.1%	99.0%		96.4%	96.4%	89.6%	89.4%	89.6%	89.6%	89.6%	89.6%	89.5%	88.9%	84.8%	84.9%
BTV_X846602.1(Ugand...	64.4%	64.5%	64.5%	64.4%	64.2%	77.2%	94.5%	94.4%	94.4%	94.4%	94.3%	94.4%	94.4%	94.8%	94.8%	94.8%	95.0%	95.0%	96.2%	96.1%	96.4%		100.0%	90.1%	89.9%	90.3%	90.3%	90.3%	90.0%	89.9%	89.2%	85.0%	85.1%
BTV_DQ4364608.1(Uga...	64.5%	64.5%	64.5%	64.4%	64.2%	77.2%	94.4%	94.4%	94.4%	94.3%	94.3%	94.4%	94.4%	94.8%	94.8%	94.8%	95.0%	95.0%	96.2%	96.1%	96.4%	100.0%		90.0%	89.9%	90.3%	90.3%	90.3%	90.0%	89.9%	89.3%	85.0%	85.1%
BTV_KU746870.1(China...	64.2%	64.4%	64.4%	64.3%	63.9%	75.8%	88.6%	88.7%	88.7%	88.7%	88.6%	88.5%	88.4%	88.9%	89.0%	89.0%	88.7%	88.7%	89.3%	89.2%	89.6%	90.1%	90.0%		98.7%	95.5%	95.5%	95.5%	95.5%	95.5%	94.2%	84.1%	84.3%
BTV_KJ187039.1(China/...	64.1%	64.3%	64.4%	64.2%	63.8%	76.5%	88.3%	88.4%	88.4%	88.4%	88.3%	88.3%	88.2%	88.1%	88.6%	88.7%	88.7%	88.5%	88.5%	89.1%	89.0%	89.4%	89.9%	89.9%	98.7%		95.3%	95.3%	95.3%	95.3%	95.3%	94.1%	84.4%	84.4%
BTV_JX846596.1(Malay...	64.7%	64.9%	65.0%	64.7%	64.4%	77.2%	88.7%	88.7%	88.7%	88.7%	88.6%	88.7%	88.6%	88.5%	88.9%	88.9%	88.9%	88.7%	88.7%	89.3%	89.2%	89.6%	90.3%	90.3%	95.5%	95.3%		100.0%	99.9%	95.5%	95.5%	94.1%	84.6%	84.9%
BTV_AY772534.1(Malay...	64.7%	64.9%	65.0%	64.7%	64.4%	77.2%	88.7%	88.7%	88.7%	88.6%	88.7%	88.6%	88.5%	88.9%	88.9%	88.9%	88.7%	88.6%	89.3%	89.2%	89.6%	90.3%	90.3%	95.5%	95.3%	100.0%		99.9%	95.5%	95.5%	94.1%	84.6%	84.9%
BTV_DQ375394.1(Mala...	64.9%	65.1%	65.2%	64.7%	64.6%	77.2%	88.7%	88.7%	88.7%	88.7%	88.7%	88.6%	88.5%	88.9%	88.9%	89.0%	88.7%	88.6%	89.3%	89.2%	89.6%	90.3%	90.3%	95.5%	95.3%	99.9%	99.9%		95.5%	95.5%	94.1%	84.7%	85.0%
BTV_JX846599.1(India/...	64.5%	64.8%	64.9%	64.8%	64.6%	75.2%	88.5%	88.5%	88.6%	88.5%	88.5%	88.4%	88.2%	88.7%	88.8%	88.8%	88.8%	88.8%	89.4%	89.2%	89.6%	90.0%	90.0%	95.5%	95.3%	95.5%	95.5%	95.5%		99.9%	97.7%	84.2%	84.4%
BTV_DQ341311.1(India...	64.8%	65.1%	65.2%	64.8%	64.9%	75.2%	88.5%	88.6%	88.6%	88.6%	88.6%	88.4%	88.2%	88.7%	88.8%	88.8%	88.7%	88.8%	89.4%	89.2%	89.5%	90.0%	89.9%	95.5%	95.3%	95.5%	95.5%	95.5%	99.9%		97.6%	84.3%	84.5%
BTV_FJ436799.1(India/P...	64.4%	64.7%	64.7%	64.6%	64.5%	75.8%	88.1%	88.1%	88.1%	88.1%	88.1%	88.0%	87.8%	88.4%	88.4%	88.5%	88.2%	88.3%	88.7%	88.6%	88.9%	89.2%	89.3%	94.2%	94.1%	94.1%	94.1%	94.1%	97.7%	97.6%		83.9%	84.2%
BTV_MH299973.1(Ger...	64.7%	64.9%	64.9%	64.8%	64.8%	71.1%	84.2%	84.4%	84.5%	84.5%	84.4%	84.3%	84.2%	84.3%	84.4%	84.4%	84.5%	84.5%	84.5%	84.5%	84.8%	85.0%	85.0%	84.1%	84.1%	84.6%	84.6%	84.7%	84.2%	84.3%	83.9%		98.7%
BTV_KT313694.1(Russi...	64.6%	64.8%	64.8%	64.7%	64.6%	70.5%	84.3%	84.5%	84.6%	84.6%	84.5%	84.4%	84.3%	84.4%	84.5%	84.5%	84.5%	84.5%	84.6%	84.7%	84.9%	85.1%	85.1%	84.3%	84.4%	84.9%	84.9%	85.0%	84.4%	84.5%	84.2%	98.7%	

S segment

Distance matrix of the nucleoprotein showing representative viruses and samples sequenced in this study. Light grey to dark shades indicates nucleotide distances.

	NRIV_JX...	NRIV_A...	NRIV_JX...	NRIV_A...	NRIV_JX...	NRIV_A...	NRIV_JX...	NRIV_JX...	NRIV_K...	BUNV_...	BUNV_...	NRIV_M...	NRIV_M...	KE-O93...	KE-C16...	KE-B35...	KE-B02...	NRIV_KJ...	BUNV_...	BUNV_...	BUNV_...	BUNV_...	BATV_K...	BATV_J...	BATV_F...	BATV_A...	BATV_N...	Bozo vir...
NRIV_JX857325.1(Soma...		100%	99.9%	99.8%	99.6%	99.6%	99.1%	99.0%	99.0%	99.0%	99.0%	98.3%	98.4%	98.1%	98.1%	98.1%	98.1%	97.9%	98.3%	97.9%	86.6%	85.6%	85.6%	84.9%	85.3%	85.3%	87.0%	86.0%
NRIV_AF398346.1(Som...	100%		99.8%	99.8%	99.6%	99.6%	98.9%	98.7%	98.5%	98.5%	98.5%	97.6%	97.8%	97.6%	97.6%	97.6%	97.6%	97.3%	97.3%	96.9%	83.0%	81.6%	81.6%	81.4%	81.6%	81.6%	81.6%	82.3%
NRIV_JX857328.1(Kenya...	99.9%	99.8%		100%	99.7%	99.8%	99.3%	99.1%	99.1%	99.1%	99.1%	98.4%	98.6%	98.3%	98.3%	98.3%	98.3%	98.0%	98.4%	98.0%	86.5%	85.8%	85.8%	85.0%	85.5%	85.5%	86.9%	85.9%
NRIV_AF398345.1(Keny...	99.8%	99.8%	100%		99.8%	99.8%	99.1%	98.9%	98.7%	98.7%	98.7%	97.8%	98.0%	97.8%	97.8%	97.8%	97.8%	97.6%	97.6%	97.1%	82.7%	81.9%	81.9%	81.6%	81.9%	81.9%	81.4%	82.1%
NRIV_JX857316.1(Sene...	99.6%	99.6%	99.7%	99.8%		100%	99.3%	99.1%	99.1%	99.1%	99.1%	98.7%	98.9%	98.6%	98.6%	98.6%	98.6%	98.3%	98.4%	98.0%	86.8%	86.0%	86.0%	85.0%	85.5%	85.5%	86.6%	85.9%
NRIV_AY593729.1(Sene...	99.6%	99.6%	99.8%	99.8%	100%		99.3%	99.1%	99.1%	99.1%	99.1%	98.4%	98.6%	98.2%	98.2%	98.2%	98.2%	98.0%	98.2%	97.7%	84.8%	83.9%	83.9%	83.2%	83.6%	83.6%	84.3%	84.1%
NRIV_JX857322.1(Suda...	99.1%	98.9%	99.3%	99.1%	99.3%	99.3%		99.6%	99.0%	98.7%	98.7%	98.0%	98.1%	97.9%	97.9%	97.9%	97.9%	98.1%	98.0%	97.6%	86.9%	86.2%	86.2%	85.3%	85.8%	85.5%	86.5%	85.3%
NRIV_JX857319.1(Suda...	99.0%	98.7%	99.1%	98.9%	99.1%	99.1%	99.6%		98.6%	98.6%	98.6%	98.1%	98.3%	98.0%	98.0%	98.0%	98.0%	98.3%	97.9%	97.4%	86.9%	85.8%	85.8%	85.3%	85.8%	85.2%	86.5%	85.6%
NRIV_KM507341.1(Ken...	99.0%	98.5%	99.1%	98.7%	99.1%	99.1%	99.0%	98.6%		98.6%	98.6%	98.1%	98.3%	98.0%	98.0%	98.0%	98.0%	97.7%	97.9%	98.0%	87.3%	86.5%	86.5%	85.3%	85.5%	85.2%	86.8%	85.9%
BUNV_MW314032.1(K...	99.0%	98.5%	99.1%	98.7%	99.1%	99.1%	98.7%	98.6%	98.6%		100%	97.9%	98.0%	97.7%	97.7%	97.7%	97.7%	97.4%	98.1%	97.7%	86.5%	85.6%	85.6%	84.3%	84.8%	84.8%	86.3%	85.5%
BUNV_MW314023.1(Ke...	99.0%	98.5%	99.1%	98.7%	99.1%	99.1%	98.7%	98.6%	98.6%	100%		97.9%	98.0%	97.7%	97.7%	97.7%	97.7%	97.4%	98.1%	97.7%	86.5%	85.6%	85.6%	84.3%	84.8%	84.8%	86.3%	85.5%
NRIV_MT747977.1(Mau...	98.3%	97.6%	98.4%	97.8%	98.7%	98.4%	98.0%	98.1%	98.1%	97.9%	97.9%		99.9%	99.6%	99.6%	99.6%	99.6%	99.0%	97.4%	97.3%	86.6%	85.6%	85.6%	84.9%	85.9%	85.3%	86.3%	85.6%
NRIV_MT747974.1(Mau...	98.4%	97.8%	98.6%	98.0%	98.9%	98.6%	98.1%	98.3%	98.3%	98.0%	98.0%	99.9%		99.7%	99.7%	99.7%	99.7%	99.1%	97.6%	97.4%	86.6%	85.8%	85.8%	85.0%	85.8%	85.5%	86.3%	85.8%
KE-O93_NRIV_S	98.1%	97.6%	98.3%	97.8%	98.6%	98.2%	97.9%	98.0%	98.0%	97.7%	97.7%	99.6%	99.7%		100%	100%	100%	99.1%	97.3%	97.2%	86.3%	85.8%	85.8%	85.0%	85.8%	85.5%	86.0%	85.8%
KE-C166_NRIV_S	98.1%	97.6%	98.3%	97.8%	98.6%	98.2%	97.9%	98.0%	98.0%	97.7%	97.7%	99.6%	99.7%	100%		100%	100%	99.1%	97.3%	97.2%	86.3%	85.8%	85.8%	85.0%	85.8%	85.5%	86.0%	85.8%
KE-B35_NRIV_S	98.1%	97.6%	98.3%	97.8%	98.6%	98.2%	97.9%	98.0%	98.0%	97.7%	97.7%	99.6%	99.7%	100%	100%		100%	99.1%	97.3%	97.2%	86.3%	85.8%	85.8%	85.0%	85.8%	85.5%	86.0%	85.8%
KE-B02_NRIV_S	98.1%	97.6%	98.3%	97.8%	98.6%	98.2%	97.9%	98.0%	98.0%	97.7%	97.7%	99.6%	99.7%	100%	100%	100%		99.1%	97.3%	97.2%	86.3%	85.8%	85.8%	85.0%	85.8%	85.5%	86.0%	85.8%
NRIV_KJ716848.1(Mauri...	97.9%	97.3%	98.0%	97.6%	98.3%	98.0%	98.1%	98.3%	97.7%	97.4%	97.4%	99.0%	99.1%	99.1%	99.1%	99.1%		97.0%	96.9%		86.8%	85.5%	85.5%	85.0%	85.8%	85.2%	86.0%	85.3%
BUNV_MH484290.1(Ke...	98.3%	97.3%	98.4%	97.6%	98.4%	98.2%	98.0%	97.9%	97.9%	98.1%	98.1%	97.4%	97.6%	97.3%	97.3%	97.3%	97.3%	97.0%		99.3%	86.5%	86.2%	86.2%	84.0%	85.2%	84.9%	86.9%	85.8%
BUNV_KM507345.1(Ke...	97.9%	96.9%	98.0%	97.1%	98.0%	97.7%	97.6%	97.4%	98.0%	97.7%	97.7%	97.3%	97.4%	97.2%	97.2%	97.2%	97.2%	96.9%	99.3%		86.9%	86.0%	86.0%	84.3%	84.8%	84.5%	87.0%	86.0%
BUNV_KP063900.1(Arg...	86.6%	83.0%	86.5%	82.7%	86.8%	84.8%	86.9%	86.9%	87.3%	86.5%	86.5%	86.6%	86.6%	86.3%	86.3%	86.3%	86.3%	86.8%	86.5%	86.9%		89.0%	89.0%	88.5%	88.2%	87.6%	85.0%	85.5%
BUNV_AF325122.1(Aus...	85.6%	81.6%	85.8%	81.9%	86.0%	83.9%	86.2%	85.8%	86.5%	85.6%	85.6%	85.6%	85.8%	85.8%	85.8%	85.8%	85.8%	85.5%	86.2%	86.0%	89.0%		100%	89.6%	89.9%	90.2%	84.0%	84.9%
BATV_KU661980.1(Aus...	85.6%	81.6%	85.8%	81.9%	86.0%	83.9%	86.2%	85.8%	86.5%	85.6%	85.6%	85.6%	85.8%	85.8%	85.8%	85.8%	85.8%	85.5%	86.2%	86.0%	89.0%	100%		89.6%	89.9%	90.2%	84.0%	84.9%
BATV_JX846595.1(Mala...	84.9%	81.4%	85.0%	81.6%	85.0%	83.2%	85.3%	85.3%	85.3%	84.3%	84.3%	84.9%	85.0%	85.0%	85.0%	85.0%	85.0%	85.0%	84.0%	84.3%	88.5%	89.6%	89.6%		97.4%	97.3%	83.5%	83.3%
BATV_FJ436806.1(India...	85.3%	81.6%	85.5%	81.9%	85.5%	83.6%	85.8%	85.8%	85.5%	84.8%	84.8%	85.9%	85.8%	85.8%	85.8%	85.8%	85.8%	85.8%	85.2%	84.8%	88.2%	89.9%	89.9%	97.4%		97.6%	83.9%	83.3%
BATV_AB257762.1(Japa...	85.3%	81.6%	85.5%	81.9%	85.5%	83.6%	85.5%	85.2%	85.2%	84.8%	84.8%	85.3%	85.5%	85.5%	85.5%	85.5%	85.5%	85.2%	84.9%	84.5%	87.6%	90.2%	90.2%	97.3%	97.6%		83.6%	83.5%
BATV_NC_043581.1(Ma...	87.0%	81.6%	86.9%	81.4%	86.6%	84.3%	86.5%	86.5%	86.8%	86.3%	86.3%	86.3%	86.3%	86.0%	86.0%	86.0%	86.0%	86.0%	86.9%	87.0%	85.0%	84.0%	84.0%	83.5%	83.9%	83.6%		84.2%
Bozo virus DakArB 734...	86.0%	82.3%	85.9%	82.1%	85.9%	84.1%	85.3%	85.6%	85.9%	85.5%	85.5%	85.6%	85.8%	85.8%	85.8%	85.8%	85.8%	85.3%	85.8%	86.0%	85.5%	84.9%	84.9%	83.3%	83.3%	83.5%	84.2%	