CIRCULATION, REASSORTMENT AND TRANSMISSION OF NGARI AND BUNYAMWERA VIRUSES IN NORTHERN KENYA.

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

In the Faculty of Health Sciences
Department of Medical Virology

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Ethics

THIS STUDY HAS BEEN APPROVED BY UNIVERSITY OF PRETORIA RESEARCH ETHICS COMMITTEE, PROTOCOL NUMBER **299/2013.**

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 Abbreviations

AA: Amino acid

BATV: Batai virus

BI: Bayesian inference

BUNV: Bunyamwera virus

CCHF: Crimean Congo hemorrhagic fever

cDNA: Complementary DNA

CPE: Cytopathic effects

CVV: Cache Valley virus

DUGV: Dugbe virus

FBS: Fetal bovine serum

HTNV: Hantavirus

ILEV: Ilesha virus

JEEV: Japanese equine encephalitis virus

LACV: Lacrosse virus

LP: Large plaque

MEGA: Molecular Evolutionary Genetics Analysis

NRIV: Ngari virus

PCR: Polymerase chain reaction

PFU: Plaque forming units

PRNT: Plaque reduction neutralization test

RVF: Rift Valley fever

SP: Small plaque

VEEV: Venezuelan equine encephalitis virus

WHO: World Health Organization

WNV: West Nile virus

WT: Wild type

YF: Yellow fever

Abbrevations of amino acids

A: Alanine

C: Cysteine

D: Aspartic acid

E: Glutamic acid

F: Phenylalanine

G: Glycine

H: Histidine

I: Isoleucine

K: Lysine

L: Leucine

M: Methionine

N: Asparagine

P: Proline

Q: Glutamine

R: Arginine

S: Serine

T: Threonine

V: Valine

W: Tryptophan

Y: Tyrosine

Presentations and Publications Related to this Work

Presentations

Collins Odhiambo and Rosemary Sang. Phenotypic and Genotypic Diversity of Bunyamwera Virus. Second Medical Veterinary and Virus Symposium, 8-9 September 2012, Silver Springs Hotel, Nairobi, Kenya.

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Collins Odhiambo, Marietjie Venter, Robert Swanepoel, Rosemary Sang. Circulation, evolution and transmission of Ngari and Bunyamwera orthobunyaviruses in Northern Kenya. The 16th International Congress on Infectious Diseases, 2-5 April 2014, Cape Town, South Africa.

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Collins Odhiambo, Marietjie Venter, Limbaso Konongoi, Robert Swanepoel, Rosemary Sang. Genome sequence analysis of *in vitro* and *in vivo* phenotypes of Bunyamwera and Ngari virus isolates from northern Kenya (2014). PLoS ONE 9(8): e105446.

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Summary

Kenya has experienced severe arboviral outbreaks of public health concern in the recent past, including yellow fever (YF), Crimean Congo hemorrhagic fever (CCHF), chikungunya, and Rift Valley fever (RVF) among others. Most of these infections are under diagnosed and hence neglected due to non-specific nature of their symptoms. Often they are mistaken for endemic tropical diseases such as malaria and typhoid infections and are only recognized during major outbreaks which result in adverse public health and economic consequences to the affected communities. Ongoing inter-epidemic surveillance in RVF virus hotspots in Kenya has indicated continued intense transmission of Bunyamwera virus (BUNV) in the absence or under low level activity of RVF virus. BUNV belongs to the genus *Orthobunyavirus* of the family *Bunyaviridae*. These are segmented RNA viruses whose members have the potential for genetic reassotment and/or drift. Recently, Ngari virus (NRIV), a natural reassortant virus associated with hemorrhagic fever was documented to have emerged from BUNV, which previously was not associated with such symptoms. However, the vectors that are involved in the maintenance and transmission of BUNV and NRIV are diverse and their role in virus maintenance/dynamics is poorly known. It is thus important to investigate the dynamics of BUNV and NRIV in selected transmission foci in an

effort to understand their circulation better in order to be able to control and predict outbreaks.

In this study, we determined the evolutionary and phenotypic diversity of BUNV and NRIV isolates previously obtained from vectors in parts of Kenya. We have provided genetic sequences of two BUNV and three NRIV isolates which contribute to addressing paucity of genetic sequences associated with this group of viruses. Phylogenetic analysis of these sequences in addition to other sequences in GenBank revealed evidence of geographic/temporal clustering that requires further investigation. Next, we demonstrated that plaque purified phenotypes of selected BUNV and NRIV isolates differ in *in vivo* growth kinetics and pathogenicity in mice, possibly related to specific mutations within the genome. The phenotypic changes and identification of mutations possibly associated with these changes support further investigation of specific mutations using site directed mutagenesis. In addition, we determined the competence of some of the mosquito species implicated in their transmission, *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefaciatus* and evaluated the dynamics of their transmission in these vectors. We conclude that *Anopheles gambiae* is likely a more competent vector for NRIV than *Aedes aegypti* and is a moderately competent vector for BUNV, which has implications for animal movement in malaria endemic areas where the vector is present. We also report evidence of BUNV transovarial transmission in both *Aedes aegypti* and *Anopheles gambiae* with the prevalence of transmission related to the ovarian cycle. Finally, we determined the level of human exposure to these viruses in the transmission foci. Orthobunyavirus-specific antibodies were detected by plaque reduction neutralization test in 89 (25.8%) of 345 persons tested. Multivariable analysis revealed age and residence in North Eastern Kenya as risk factors. In conclusion, we propose that acute febrile disease surveillance needs to be implemented in North Eastern Kenya. This study helps identify the virus strains/populations and the vector species that play a critical role in sustenance and transmission of BUNV and NRIV in different ecosystems in the country. All these are important in understanding virus circulation, potential for emergence and risk to populations in areas of circulation, and will help in making decisions for intervention and management. Generated sequence data in this study contributes to global phylogenetic

characterization of Orthobunyaviruses worldwide and their molecular epidemiology. The study also shed light/improve our knowledge on the genetic stability or diversity and evolutionary trends of *Orthobunyavirus* strains in Kenya.

1. Chapter 1

LITERATURE OVERVIEW

1.1 INTRODUCTION

Arthropod-borne viruses (arboviruses), which are RNA viruses except African swine fever virus which is a DNA virus, constitute animal viruses transmitted to vertebrate hosts through blood-sucking arthropod vectors. According to the World Health Organization (WHO), an arbovirus is characterised by, replication in both arthropod and animal host, viral transmission to the vertebrate host by an arthropod vector and the vertebrate host demonstrating viremia and in addition, possible direct and/or transovarial transmission (Kuno and Chang 2005). Arboviruses remain a major public health and veterinary problem in Kenya and worldwide.

While most arboviruses primarily exploit birds and animals, with human infection as a result of a spill-over from zoonotic replication cycles, some like dengue virus, have evolved to exploit humans as primary reservoirs (Forshey et al. 2010). Humans are usually dead-end hosts because they are incapable of transmitting the virus and cannot function as reservoirs for mosquito re-infection (Weaver and Barrett 2004). Human infections may be asymptomatic or result in clinical disease which can range from mild to moderate febrile illness to severe hemorrhagic manifestations and neurological disease. Human exposure worldwide is likely to expand given the increase in activities that encroach on wild arboviral habitat (Forshey et al. 2010). The tropics provide an environment that can support arboviral emergence as significant human pathogens given that arthropods prefer hot and humid conditions and that virus transmission is also enhanced as a result of shortened incubation periods in vectors. Global warming has influenced the emergence and re-emergence of arboviral diseases by providing an enabling environment to mosquito species that were previously restricted to the tropics. Arboviruses exhibit considerable evolution potential with distinct geographical genotypes and enhanced transmissibility, and disease severity may be associated with a particular genotype. A single mutation on the chikungunya virus envelope protein gene resulted in efficient transmission by a less competent vector (*Aedes albopictus*), believed to have resulted

insevere chikungunya fever disease in Reunion island (Tsetsarkin et al. 2007).

1.1 Arboviral transmission cycle

Arboviruses survive in nature through transmission from infected arthropods to susceptible vertebrate host (Weaver and Barrett 2004). Arboviruses require a host in which they replicate, and a vector, such as a mosquito, for transmission to other organisms. Viruses replicate in the invertebrate host, accumulating in the salivary glands and are transmitted to the vertebrate host during blood feeding and are able to transmit the virus to another animal through saliva during a second round of blood feeding (Gray and Banerjee 1999). Three components are necessary for maintaining this transmission cycle: the virus, vertebrate host and invertebrate host (Pfeffer and Dobler 2010). All these three components must be available at the same time and place. Factors influencing transmission include competence of the vector, vertebrate host susceptibility and level of viremia in the infecting host. Changes in viral genetics, host or vector composition and/or dynamics can result in amplification of arboviral population to epidemic levels (Weaver and Reisen 2010). Arboviral outbreaks can be associated with relatively small viral genetic changes or introduction of new strains with increased virulence or amplification potential. These changes may also enhance vector competence and hence increase transmission potential. Environmental change may also affect vector abundance, survival and pathogen replication hence increasing vector contact rate and subsequently enhancing transmission.

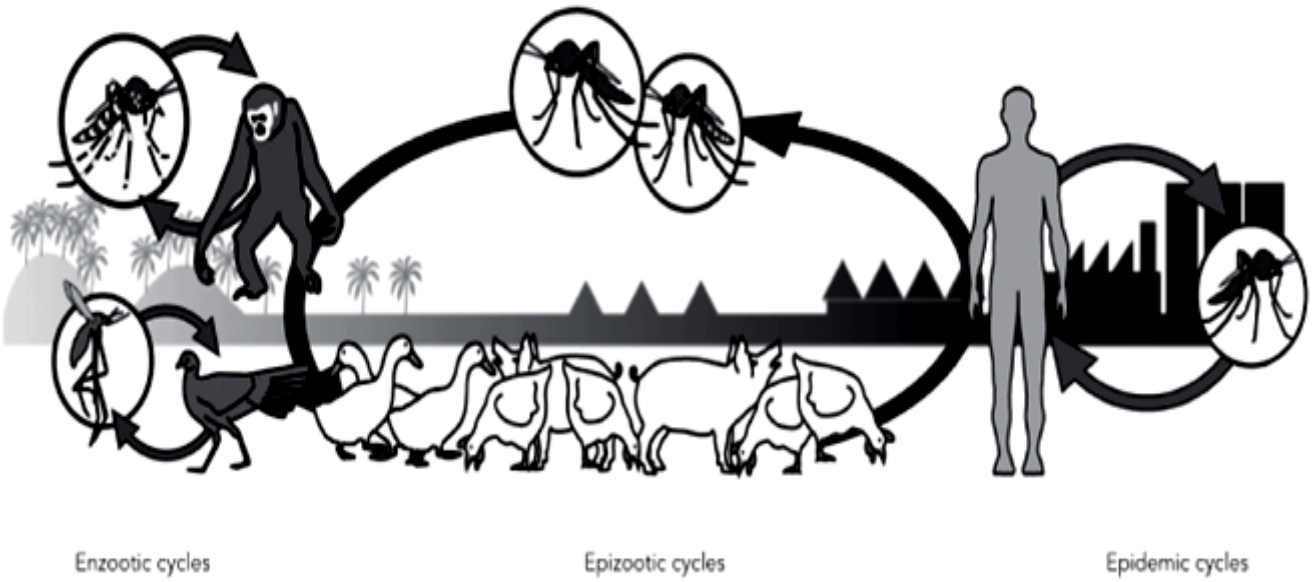


Figure 1: Arboviral transmission cycle (Ellis and Wilcox 2009)

Birds followed by other wild and domestic vertebrates are the most common arbovirus hosts, while humans are usually dead-end hosts, incapable of transmitting the infection and cannot function as reservoirs for re-infection of the vector. However, infection can result in clinical disease. A majority of arboviruses are maintained in enzootic transmission cycles involving birds, rodents or non-human vertebrates as reservoir hosts (Soldan and Gonzalez-Scarano 2005). The enzootic transmission cycles can occur in slyvatic habitats, oscillating between wild animals and the vector e.g. Venezuelan equine encephalitis virus (VEEV) and yellow fever (YF) virus, while others like dengue virus, chikungunya virus and YF virus can cycle in urban or domestic habitats involving domestic animals and the vectors (Figure 1). Human infection and epidemics can result from direct spillover of these enzootic transmission cycles when amplification levels result in tangential transmission or due to human activity in sylvatic enzootic habitats (Gubler 1996, Weaver and Barrett 2004). This spillover transmission may involve the participation of enzootic and bridge vectors.

Mosquitoes are the main transmission vector although other biting arthropods, sand flies, midges

and ticks can also transmit viruses. The arthropod vectors develop a life-long infection and in some cases, infected females can transovarially transmit the virus to their progeny. Alternatively, transmission may result by contamination of the ovum though the micropyle apparatus. Even though transovarial transmission is inefficient, it provides a maintenance survival mechanism for the virus during unfavourable conditions for its vertebrate and vector host (Sang and Dunster 2001). However, transovarial transmission has been documented to be highly efficient for the California serogroup of viruses (Kuno and Chang 2005).

1.2 Arbovirus Classification

Arboviruses including those of medical relevance mainly belong to six families; Togoviridae, Flaviviridae Rhabdoviridae, Orthomyxoviridae, Reoviridae and Bunyaviridae (van Regenmortel et al. 2000, Weaver and Reisen 2010) and are transmitted by arthropods, mainly mosquitoes and ticks. The most prominent examples of arboviruses that have emerged in recent times include dengue virus, WNV, Japanese equine encephalitis virus (JEEV), CCHF virus and chikungunya virus. Arboviruses are classified according to antigenic relationships, morphology, and replicative mechanisms (Alatoom and Payne 2009). The ability of arboviruses to cause epidemics and disease depends on a wide range of factors including viral genetics, vector competence and epidemiology (Weaver and Reisen 2010).

1.3 Arbovirus Disease Burden

Majority of arboviral infections remain undiagnosed due to symptom similarity with other tropical diseases, including malaria, typhoid and other bacterial infections like brucellosis and leptospirosis, the public health threat to the population is greatly underestimated (Sang and Dunster 2001). Currently, there are suggestions to include arboviral infections under the WHO neglected tropical diseases (Table 1). These are diseases that do not receive considerable attention in international public health programs despite their global burden being similar to HIV, malaria and tuberculosis infections (LaBeaud 2008). In 2010, the WHO included dengue fever under this category (WHO,

2010).

Table 1: World Health Organization (WHO) list of Neglected Tropical Diseases (WHO, 2010)

CAUSATIVE PATHOGENS	**DISEASE**
Virus	Dengue/Severe dengue
	Rabies
Protozoa	Chagas disease
	Human African trypanosomiasis (sleeping sickness)
	Leishmaniases
Helminth	Cysticercosis/Taeniasis
	Dracunculiasis (guinea-worm disease)
	Echinococcosis
	Foodborne trematodiases
	Lymphatic filariasis
	Onchocerciasis (river blindness)
	Schistosomiasis
	Soil-transmitted helminthiases
Bacteria	Buruli ulcer
	Leprosy (Hansen disease)
	Trachoma
	Yaws

There has been resurgence in arboviral infections worldwide over the past two decades (Jones et al. 2008). The tropics have been affected most with multiple arboviruses causing epidemics frequently. This resurgence has been driven by a number of factors, including an explosion in population growth leading to unplanned urbanization which has resulted in poor conditions of living that

promote the expansion of mosquito densities. Compounding this problem is the lack of an effective mosquito control program as well as human agricultural practices that have provided suitable larval habitats. International travel and trade has also impacted on the spread of arboviruses and vectors to new habitats (Pfeffer and Dobler 2010). Despite these reports, the actual cumulative impact of arboviral infections on the global diseases burden has not been fully estimated (LaBeaud et al. 2011). Arboviral diseases are characterized by sequelae ranging from asymptomatic infections and undifferentiated fever to encephalitis and hemorrhagic manifestations that can result in significant morbidity and mortality. The East African region is a hotspot for arboviral diseases with epidemics occurring frequently and expected to increase with time. Kenya has had multiple arboviral disease outbreaks within the past two decades resulting in economic losses due to animal trade restrictions and public health distress. These include YF outbreak in 1992 and 1995 (Okello et al. 1993), chikungunya fever outbreak in 2004 (Sergon et al. 2008), and RVF outbreak in 1997 and 2006 (Woods et al. 2002, CDC 2007). The abundance of mosquitoes infected with arboviruses in Kenya has been reported with evidence of simultaneous arboviral circulations (Sang and Dunster 2001, Crabtree et al. 2009, LaBeaud et al. 2011). It is thus probable that cases of arboviral diseases could remain undetected given that during outbreaks suspected cases are attributed to the epidemic arbovirus. Entomological arbovirus surveillance during RVF outbreaks has demonstrated co-circulation of arboviruses including BUNV (Traore-lamizana et al. 2001, Crabtree et al. 2009). It is thus of paramount importance to understand the transmission dynamics of such arboviruses in order to estimate the true disease burden during such outbreaks, improve existing prevention mechanisms, management plans and prevent introduction to new habitats.

1.4 The family *Bunyaviridae*

The Bunyaviridae is the largest of the arbovirus families. It is a diverse group of RNA viruses comprising over 300 viral species including 97 species, 81 possible species, and a large number of isolates, encompassing 19 viruses in seven groups of related viruses and dozens of ungrouped viruses (Briese et al. 2013). The family is divided into five genera; *Orthobunyavirus*, *Nairovirus*,

Tospovirus, *Phlebovirus* and *Hantavirus* (Calisher 1996). The family was characterized in 1975 to classify viruses with diverse lifecycles yet similar morphology and structure (Schmaljohn 1996). Distinction between genera is based on antigenic, serological, molecular and structural differences between the viruses (Calisher 1996). Members of the *Bunyaviridae* family are found all over the world and some are significant animal pathogens while some like the genus *Tospovirus* infect plants (Elliott 2001). A majority are spread through sylvatic transmission cycles between susceptible vertebrate hosts and hematophagus arthropods such as mosquitoes, phlebotomine flies and ticks. The genus *Hantavirus* comprises viruses that do not infect insect vectors and are maintained(Soldan and Gonzalez-Scarano 2005) in nature through persistent, benign infection of their rodent hosts (Tsai, 1987). Others like RVF and CCHF can also be transmitted through a non-vectorial route such as through contact with infected animal tissue or body fluids (Al-Hazmi et al. 2003). The *Bunyaviridae* family is associated with significant human infections globally with diverse clinical manifestations such as severe pediatric encephalitis, associated with La Crosse virus (*Orthobunyavirus*), hemorrhagic, retinal, encephalitic and hepatic disease, associated with RVF virus (*Phlebovirus*) and hemorrhagic fever associated with CCHF virus (*Nairovirus*) (Lambert and Lanciotti 2009).

1.4.1 Virion structure

BUNV was the first virus in this family to be sequenced completely and has a total size of 12294 nucleotides, of which 95.3% encodes amino acids (Elliott 1989). The BUNV genome consists of three negative-sense RNA segments that involve a variety of coding strategies that lead to generation of a limited set of structural and non-structural proteins (Elliot et al. 2000, Schmaljohn and Hooper 2001) (Figure 2-5). The large (L) segment encodes a large protein that consists of the RNA-dependent RNA polymerase activity for replication and transcription of genomic RNA segments. The medium (M) segment encodes a precursor polypeptide which yields the viral surface glycoproteins Gn and Gc and a nonstructural protein NSm, and the S (small) segment encodes the nucleocapsid (NC) and a nonstructural protein NSs in overlapping reading frames (Nunes et al.

2005). The lengths of the S, M and L segments vary among genera, ranging from 0.8-2.9 kb for the S segment, 3.5-5 kb for the M segment and 6.3-13 kb for the L segment (Figure 3-5). The size and coding strategy of proteins encoded by viruses within a genus is similar while the terminal nucleotide sequences at the 3' and 5' ends of the three RNA segments are conserved within a genus but differ from those of other genera demonstrating a molecular basis for the extraordinary diversity of species within the family (Table 2).

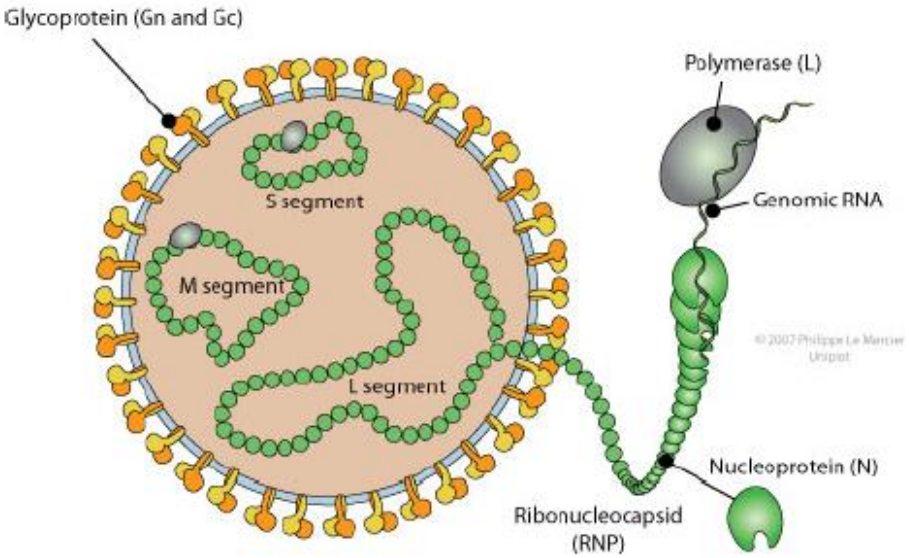


Figure 2: Structure of Bunyavirus virion. The three RNA segments are individually encapsidated by the N protein and the RNA-dependent RNA polymerase associates with the RNA-N complex to form ribonucleoprotein. ViralZone, SIB Swiss Institute of Bioinformatics. http://viralzone.expasy.org/viralzone/all_by_species/250.html

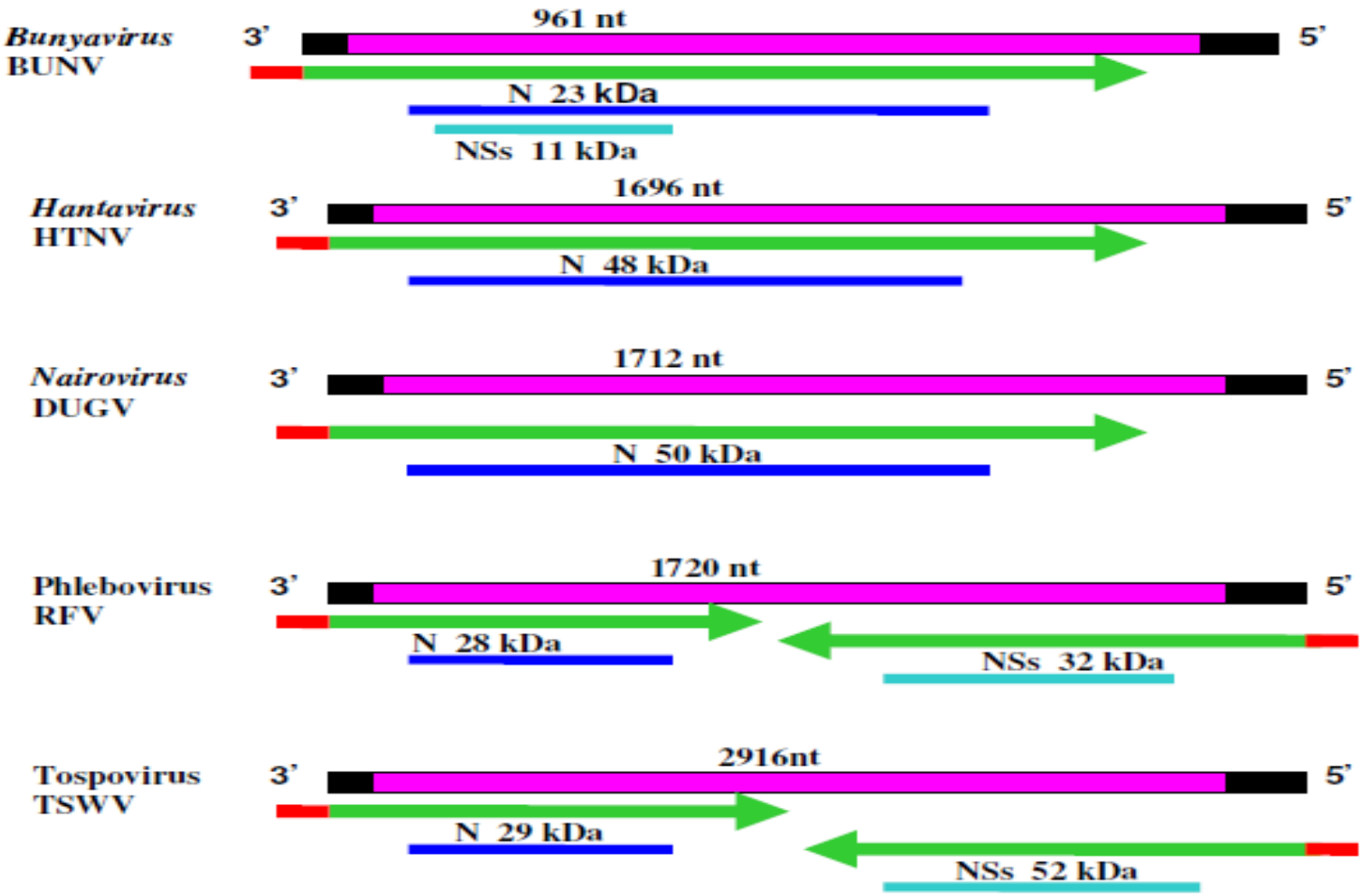


Figure 3: Coding strategies of *Bunyaviridae* S genome segment. Genomic RNAs are represented by purple boxes, black boxes indicate 3'/5' NCR, mRNAs are shown as green boxes, red boxes indicate host-derived primer sequence at 5' end, and arrowheads indicate truncated 3' end, and nt indicates nucleotides. Gene products, with their size in kilodaltons (kDa) are represented by light and dark blue boxes. (Elliot et al. 2000)

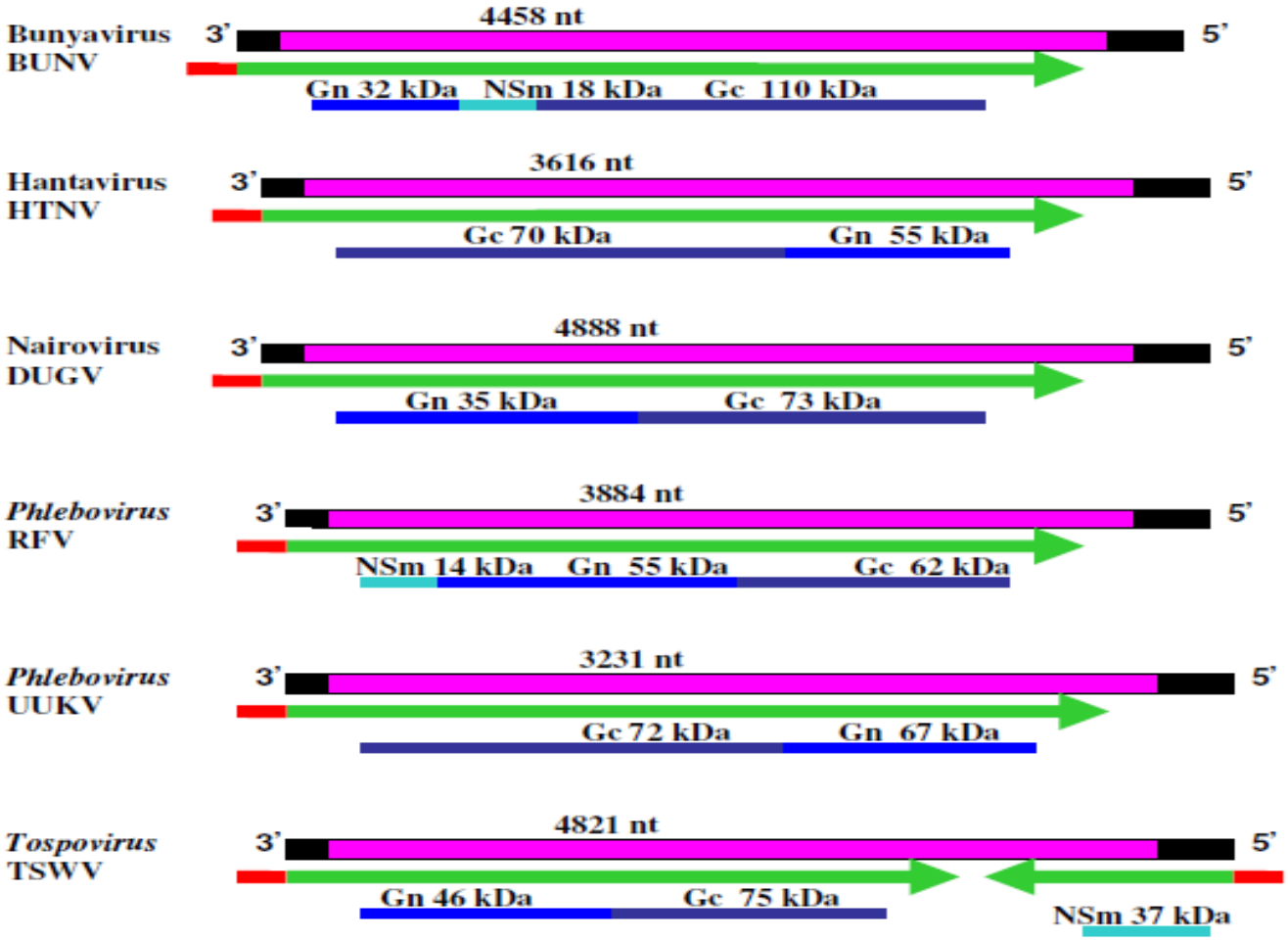


Figure 4: Coding strategies of *Bunyaviridae* M genome segment. Genomic RNAs are represented by purple boxes, black boxes indicate 3'/5' NCR, mRNAs are shown as green boxes, red boxes indicate host-derived primer sequence at 5' end, and arrowheads indicate truncated 3' end, and nt indicates nucleotides. Gene products, with their size in kilodaltons (kDa) are represented by light and dark blue boxes. (Elliot et al. 2000)

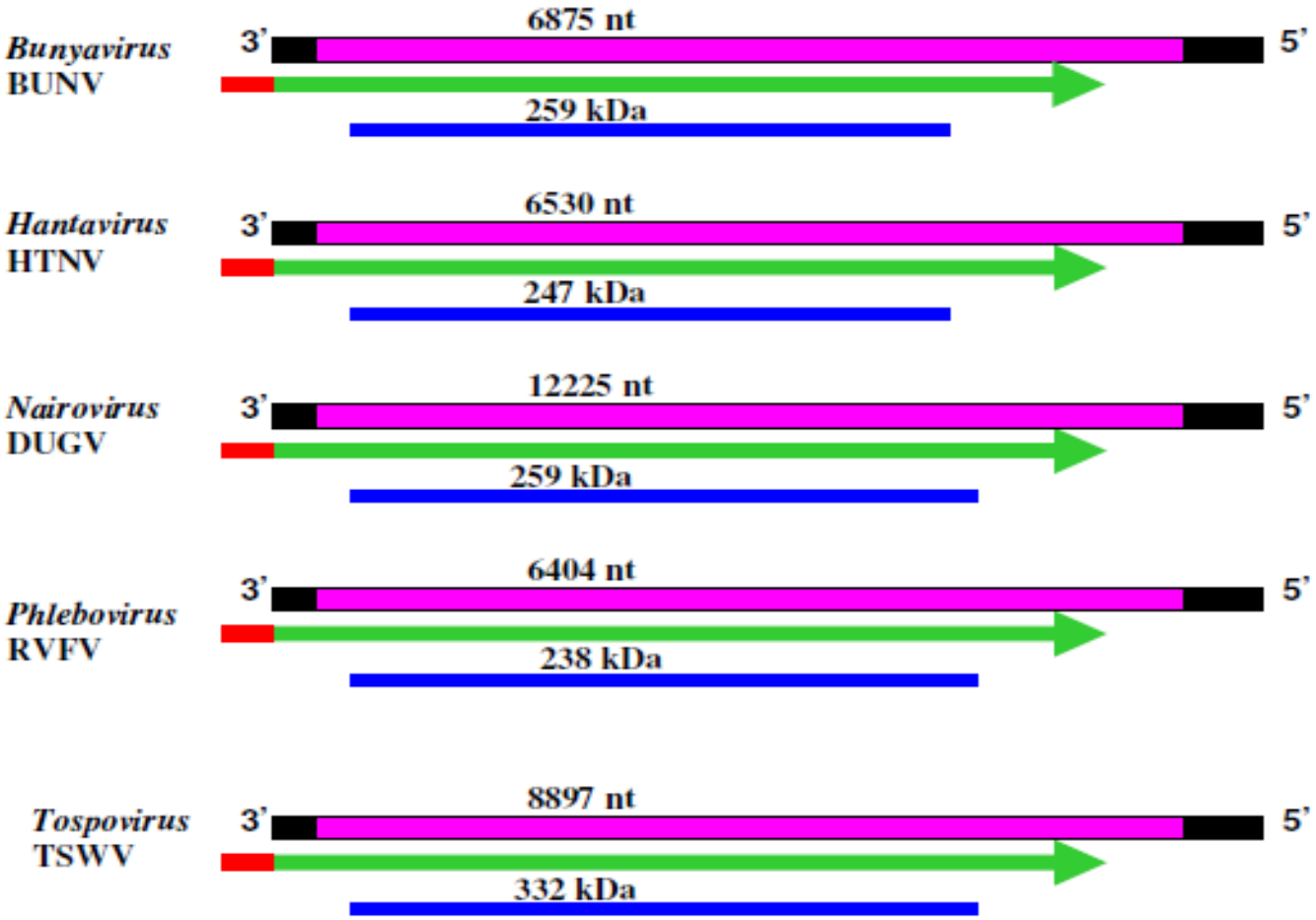


Figure 5: Coding strategies of *Bunyaviridae* L genome segment. Genomic RNAs are represented by purple boxes, black boxes indicate 3'/5' NCR, mRNAs are shown as green boxes, red boxes indicate host-derived primer sequence at 5' end, and arrowheads indicate truncated 3' end, and nt indicates nucleotides. Gene products, with their size in kilodaltons (kDa) are represented by light and dark blue boxes. (Elliot et al. 2000)

The size and coding strategy of proteins encoded by viruses within a genus is similar while the terminal nucleotide sequences at the 3' and 5' ends of the three RNA segments are conserved within a genus but differ from those of other genera demonstrating a molecular basis for the extraordinary diversity of species within the family (Table 2).

Table 2: Pattern of Bunyaviridae protein sizes (kDa) (Elliott 2001)

RNA	protein	Genus				
		Orthobunyavirus	Hantavirus	Nairovirus	Phlebovirus	Tospovirus
L segment	L	259-263	246-247	459	238-241	330-332
M segment	Gn	29-41	68-76	30-45	50-70	52-58
	Gc	108-120	52-58	72-84	55-75	72-78
	NSm	15-18	none	78-85, 92-115	None or 78	34
S segment	N	19-25	50-54	48-54	24-30	52
	Ns	10-13	none	none	29-31	29

In most of the orthobunyaviruses, the terminal 11 bases of the S, M and L segment are complementary except a mismatch at positions 9 and -9 which facilitate circularization within the ribonucleocapsid (Figure 6) (Elliott 1990).

Orthobunyavirus	3' UCAUCACAU--- 5' AGUAGUGUG---
Hantavirus	3' AUCAUCAUCUG--- 5' UAGUAGUAUGC---
Nairovirus	3' AGAGUUUCU--- 5' UCUCAAAGA---
Phlebovirus	3' UGUGUUUC--- 5' ACACAAAG---
Tospovirus	3' UCUCGUUA--- 5' AGAGCAAU---

Figure 6: Terminal consensus sequences of the S, M and L genome segments of each genus of the family *Bunyaviridae* (Elliott and Wilkie 1986).

1.4.2 Functionality of encoded proteins

1.4.2.1 S segment proteins

The N protein is the most abundant and the first to be expressed in virus-infected cells. The N protein has conserved regions that may be associated with complement fixation antibodies which may cross-react among viruses in the same genus (Calisher 1996). The N protein is used to encapsidate the genomic and antigenomic RNA to form ribonucleoproteins (Jin and Elliott 1991). On the other hand, the NSs protein may contribute to viral pathogenesis by acting as an interferon antagonist that blocks the transcriptional activation of interferons (Weber et al. 2002). The protein also inhibits host cell protein synthesis (Bridgen et al. 2001) and may delay early stage cell death by inhibiting IFN regulatory factor 3 mediated apoptosis (Kohl et al. 2003). Moreover, the NSs protein has been demonstrated to counteract RNA silencing directed against cellular and viral RNA (Soldan et al. 2005).

1.4.2.2 M segment proteins

The M polyprotein has not been detected in infected cells, suggesting that it is co-translationally cleaved to give the mature Gc, Gn and NSm proteins (Lappin et al. 1994). The gene order of the M segment is 5'-Gn-NSm-Gc-3'in the genome-complementary sense (Figure 4) (Fuller and Bishop 1982, Fazakerley et al. 1988, Nakitare and Elliott 1993). There are 2-3 potential glycosylation sites relatively rich in Cysteine and conserved N and C terminal hydrophobic domains that may act as neutralising and protective epitopes (Wang et al. 1993). Conserved regions in the Gn glycoprotein contain type-specific antigenic determinants for hemagglutinating and neutralizing antibodies and are used to classify the viruses into serogroups (Cheng et al. 2000). The Gn glycoprotein contains the Golgi targeting and/or retention signals which may be needed for the Gc-Gn interaction to localize to the Golgi compartment (Lappin et al. 1994, Bupp et al. 1996, Shi et al. 2004, Pollitt et al. 2006). It has also been demonstrated that the N terminal domain of NSm plays a role in virus growth (Pollitt et al. 2006). Additionally, the NSm contains some hydrophobic and non-hydrophobic domains that may be necessary for virus assembly (Shi et al. 2006). On the other hand, the Gc glycoprotein plays a role in fusion activity and may be a major determinant for viral attachment to mammalian cells (Pobjecky et al. 1986, Pekosz et al. 1995). The Gc glycoprotein has also been determined to play a significant role in virulence (Gonzalez-Scarano et al. 1985, Elliott 1990).

1.4.2.3 L segment protein

The L segment encodes the L protein (RNA-dependent RNA polymerase) in a negative-sense coding strategy (Figure 5). Only a small amount of the L protein is detectable in virus-infected cells. The mRNA synthesized from the L segment contains a 5' cap and host derived-primer sequence necessary for transcription, indicating that the L protein has endonuclease activity to mediate the 'cap-snatching' process (Jin and Elliott 1993). The L protein may also have a role in neurovirulence and neurovasiveness as demonstrated in mice infected with California serogroup viruses, but the mechanism has not been elucidated (Endres et al. 1991).

1.4.3 Virus evolution

Co-circulation of these viruses and segmented nature of their genomes provides opportunities for genetic reassortment and recombinations. While reassortment of orthobunyaviruses has been experimentally confirmed to occur between genetically related viruses, natural reassortments have been infrequently described. Segment reassortment of BUNV has recently been associated with human disease outbreaks especially within the East African region (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). NRIV, a natural reassortant virus, was associated with haemorrhagic fever outbreak in Kenya and Somalia. Through sequence analysis, it was determined that the S and L segments of this virus were closely related to BUNV, while the M segment was similar to that of the Batai virus (BATV), an *Orthobunyavirus* first isolated in Malasyia and previously not isolated in humans (Bowen et al. 2001, Briese et al. 2006). This suggests that BATV or a closely related virus is the donor of the NRIV M segment sequence and may have been co-circulating with BUNV.

1.4.4 *Orthobunyavirus*

The *Orthobunyavirus* is the largest genus of the *Bunyaviridae* family with over 172 individual viruses grouped into 18 serogroups (Calisher 1996, Gonz´alez-Scarano et al. 1996). These include Anopheles A, Anopheles B, Bakau, Bunyamwera, Bwamba, Capim, California, Gamboa, Guama, Group C, Koongol, Minatitlan, Nyando, Olifanstlei, Patois, Simbu, Tete and Turlock (Calisher 1996). A majority of the viruses in this genus are transmitted by mosquitoes (Table 3). The majority of human pathogens within this genus are distributed among three serogroups; California serogroup, predominantly in North America and Europe, New World Group C viruses and the Bunyamwera serogroup, predominantly in Africa, Central and South America (Calisher and Karabatsos 1988, Elliot et al. 2000). BUNV is the type member of the *Orthobunyavirus* genus (Yanase et al. 2006). It was first isolated from *Aedes* mosquitoes in Uganda in 1943 (Smithburn et al. 1946) and subsequently from viremic humans in Africa (Karabatsos 1985).

1.4.5 Epidemiology of selected *Orthobunyavirus* serogroups

1.4.5.1 Bunyamwera serogroup

Aside fron Lokern and Main Drain viruses that have been isolated from Culicoides, majority of viruses within this serogroup are transmitted by mosquitoes (Karabatsos 1985). Some viruses including BUNV, NRIV, Ilesha virus, Tensaw virus, Germiston virus, Guaroa virus, BATV, Shokwe virus, Wyeomyia virus and Xingu virus have been isolated from human and are associated with disease (Karabatsos 1985). Main Drain virus has been associated with encephalitis in horses. BUNV was the first isolate discovered in 1943 from *Aedes* mosquitoes in Uganda and has also been isolated from viremic humans in Africa (Smithburn et al. 1946, Karabatsos 1985). Antibodies to BUNV have been detected in humans, livestock, primates, birds and rodents (Gonzalez and Georges 1988).

1.4.5.2 Carlifornia serogroup

California encephalitis virus is the prototype virus of this serogroup, and was first isolated in North America from mosquitoes in 1943 (Hammon et al. 1952). Members of the serogroup are widely distributed globally including Africa, Asia, North and South America and Europe (Calisher 1983). Members of the serogroup have also been isolated from rodents and other animals. Members include California encephalitis, James Canyon, La Crosse, Inkoo, Snowshoe, Tahyna and Trivittatus viruses and may be associated with encephalitis in humans.

1.4.5.3 Group C serogroup

Group C viruses have mainly been isolated from mosquitoes, rodents and marsupials in South and North America (Pinheiro 1981, Karabatsos 1985). Some of the viruses like Carapayu, Oriboca, Itaqui, Nepuyo, Apeu, Marituba, Ossa and Madrid virus are associated with human disease with symptoms including self-limited and dengue-like illness with fever, headache, myalgia, nausea, vomiting and weakness (Nunes et al. 2005).

1.4.5.4 Bwamba serogroup

Most members of this serogroup have been isolated from humans with febrile illness and are

geographically restricted to Africa (Karabatsos 1985). The prototype of this group, Bwamba virus was first isolated in 1937 from an infected human in Uganda. Antibodies to these viruses have been detected in humans, livestock animals, and avian sera in Africa (Bishop and Shope 1979). Bwamba serogroup viruses are serologically related to the California serogroup (Casals 1963).

1.4.5.5 Simbu serogroup

Members of this serogroup, vectored by culicoids and mosquitoes, are widely distributed globally with presence in Asia, Australia, Africa, North and South America(Karabatsos 1985). Isolates have been recovered from birds, cattle and pigs among other vertebrates. Some are human pathogens such as Oropouche and Shuni viruses while others like Akabane and Aino viruses are significant veterinary pathogens (Karabatsos 1985, Bishop 1996). Serological surveys have also revealed that these viruses may also infect monkeys, birds and rodents (Bishop 1996).

1.4.6 Clinical manifestations of Orthobunyaviruses

Clinical manifestations associated with *Orthobunyavirus* genus infections include a nondescript febrile illness with myalgia and arthralgia, and encephalitis. BUNV is associated with febrile illness with headache, arthralgia, rash and infrequent central nervous system involvement (Gonzalez and Georges 1988, Nichol et al. 2005). Oropouche virus (Bowen et al. 2001) causes Oropouche fever, an acute febrile dengue-like illness mainly in South America (Pinheiro et al. 1981). La Crosse virus has been implicated in pediatric encephalitis in the United States (Kappus et al. 1983, Alatoom and Payne 2009) while Cache Valley Virus (CVV) is responsible for a majority of meningitis cases in humans within the same region (Sexton et al. 1997, Campbell et al. 2006).

Table 3: Selected arboviruses belonging to the genus *Orthobunyavirus* of the family *Bunyaviridae*. host, vectors and distribution.

VIRUS SPECIES	DISEASE	PRINCIPAL VECTOR	GEOGRAPHIC DISTRIBUTION
Akabane	Cattle	Mosquito, midge	Africa, Asia, Australia
Bakau	Cattle	Mosquito	Asia
Batai	Human	Mosquito	Africa
Birao	Human	Mosquito	Africa
Bozo	Human	Mosquito	Africa
Bunyamwera	Human	Mosquito	Africa
Bwamba	Human	Mosquito	Africa
Cache Valley	Sheep, cattle	Mosquito	North America
Fort Sherman	Human	Mosquito	South America
Germiston	Human	Mosquito	Africa
Ilesha	Human	Mosquito	Africa
Iquitos	Human	Unknown	South America
La Crosse	Human	Mosquito	North America
Maguari	Human	Mosquito	South America
Mboke	Human	Mosquito	Africa
Ngari	Human	Mosquito	Africa
Nyando	Human	Mosquito	Africa
Oropouche	Human	Midge	South America
Pongola	Human	Mosquito	Africa
Schmallenberg	Cattle, Sheep, Goat	Mosquito, Midge, Tick	Europe
Shamonda	Human	Midge	Africa
Shokwe	Human	Mosquito	Africa
Simbu	Human	Mosquito, Midge	Africa
Tahyna	Human	Mosquito	Europe

Additionally, these viruses also affect livestock for example, Akabane virus and Aino virus are associated with abortions, congenital defects and stillbirths in cattle, sheep and goats, resulting in a devastating economic impact due to their wide distribution (Inaba et al. 1975). CVV is also associated with similar pathogenesis in sheep in the United States, Canada and Mexico (Edwards et

al. 1989). While viruses of the *Orthobunyavirus* genus are known to cause human disease, they were previously not associated with haemorrhagic fevers. However, BUNV reassortant has been implicated in recent outbreaks of haemorrhagic fevers in Kenya and Somalia (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). This is thought to have arisen through genetic reassortment between two segmented viruses co-circulating in the same environment demonstrating that genetic reassortment can profoundly increase viral pathogenicity (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006).

1.5 Evolutionary diversity

Arboviruses are principally transmitted horizontally between arthropod vectors and vertebrate reservoir/susceptible hosts. The majority of arboviruses are RNA viruses that lack polymerases with proofreading activity and thus exhibit error frequencies of 1 mutation in 10^3–10^5 nucleotides per round of replication (Drake and Holland 1999). Their high mutation frequencies, rapid replication, intense transmission and large population sizes allow these viruses to produce variants that can rapidly adapt to fluctuating environments (Ciota and Kramer 2010). Despite this potential for sequence change in RNA viruses, consensus sequences of most arboviruses have remained conserved. This has been attributed to the different selective pressures encountered in the different vertebrate and invertebrate hosts (Woolhouse et al. 2001). Thus, only those mutations that are beneficial to the virus or neutral in either host are maintained, consequently, arboviruses sacrifice the ability to be host specific (Kassen 2002). However, modest variation in terms of number of mutations is not always synonymous with phenotypic outcome of the change. A single mutation can have a major effect in viral replicative ability or infectivity in particular hosts (Ciota and Kramer 2010). The genotype associated with VEEV outbreaks has a single mutation in the E2 gene that increases vector competence or VEEV virulence (Anishchenko et al. 2006). Similarly, the recent chikungunya virus outbreak in the Indian Ocean islands were associated with emergence of a viral strain that shared a single common substitution in the E1 gene and a variable second mutation that resulted in increased competence of *Aedes albopictus* vector (Tsetsarkin et al. 2007, Tsetsarkin et

al. 2009). These examples indicate that even with slower than expected evolutionary rates, arboviruses still retain the capacity to produce variants that can be exploited in new settings.

Given that many of the viruses of the family *Bunyaviridae* use the same arthropod and vertebrate hosts and have segmented genomes, there is a greater possibility for the emergence of both reassortant and recombinant viruses that could result in altered pathogenesis or host range. A large hemorrhagic fever outbreak occurred in Kenya, Tanzania, and Somalia between 1997 and 1998 and was thought to be associated with RVF virus infections (Woods et al. 2002). However, a previously unidentified virus was subsequently isolated from two hemorrhagic fever cases. Moreover, acute infection with this unidentified virus was detected in a quarter of the hemorrhagic fever cases tested. Genetic analysis of the S, M and L segments of the genome revealed that the S and L segments were similar to those of BUNV, while the M segment sequence differed from that of other genetically characterized members of the *Orthobunyavirus* genus, suggesting that the virus was a case of genetic reassortment (Bowen et al. 2001). Known diseases caused by viruses of the *Orthobunyavirus* genus range from uncomplicated febrile illness to fatal encephalitis (Kokernot et al. 1957, Nichol 2001) hence the suggestion that the novel pathogenesis properties of the NRIV is due to the unique nature of the M genomic segment (Zeller et al. 1996).

Borucki et al. demonstrated experimentally that segment reassortment can occur in transovarially infected mosquitoes using LaCrosse virus and snowshoe hare virus both belonging to the Carlifornia serogroup bunyaviruses (Borucki et al. 1999).

1.6 Phenotypic diversity

Plaque phenotypes of RNA viruses have been implicated in virulence (Davis et al. 2004). Small plaque variants of WNV have been characterized to exhibit attenuated properties both *in vivo* and *in vitro*. Small plaque variants of WNV were shown to exhibit low replication efficiency compared to the wild type (Jia et al. 2007). Small plaque variants of other arboviruses such as dengue are known to be temperature sensitive and attenuated in suckling mice (Eckels et al. 1976). Contrary, a small plaque variant of JEEV showed neurovirulence and neuroinvassiveness similar to the parent strain

(Wu et al. 1997). Small plaque variants of YF virus have been shown to be defective in cell penetration, cell to cell spread and reduced growth characteristics (Vlaycheva and Chambers 2002, Vlaycheva et al. 2004, 2005). This was attributed to an amino acid substitution at two positions in the envelope protein.

1.7 Vector competence

Vector competence is the ability or capacity of a vector to acquire, maintain, and transmit a virus to a vertebrate host after an appropriate extrinsic incubation period. Vector competency is determined by isolation of an arbovirus from a naturally infected vector, laboratory demonstration of vector infection following feeding on viremic blood, laboratory evidence of viral transmission during blood feeding and evidence of contact between vector and vertebrate host in nature. Vector competence refers to intrinsic factors such as internal physiological factors that control vector infection and ability to transmit virus as well as behavioral traits including host preference(DeFoliart et al. 1987). Vector competency is further affected by extrinsic factors like rainfall, vertebrate host population, humidity and temperature. Temperature and humidity affect vector development and longetivity. In addition, these two factors influence the rate of virus multiplication in the vector and the time necessary for completion of viral incubation that ultimately results in the ability of the vector to transmit by bite. High virus doses have also been determined to increase vector competence by overcoming barriers to infection for different vector species and viruses (Chamberlain et al. 1959, Jupp et al. 1981, Kramer et al. 1981, Sardelis et al. 2001, Mahmood et al. 2006). Other factors include vector age and strain for example, young *Aedes aegypti* were found to have a higher survival probability compared to older mosquitoes hence more likely to complete the extrinsic incubation period necessary for virus transmission. Duration of the extrinsic incubation period is important in the epidemiology of arboviruses since it determines the length of time a vector must survive after infection for it to efficiently transmit the virus. Thus higher vector population densities or prolonged survival times may be required to maintain transmission cycles of vectors with long extrinsic incubation periods and vice versa (Hardy et al.

1983).

Species of mosquitoes from different geographic regions differ in biological trait and morphologic characteristics (Beams 1985, DeFoliart et al. 1987). *Culex pipiens* has been implicated in RVF outbreak in Egypt (Hoogstraal et al. 1979), *Aedes vexan* in west Africa (Zeller et al. 1997, Traore-lamizana et al. 2001) and Saudi Arabia (Jupp et al. 2002), *Aedes caspius* in Egypt as an enzootic vector (Gad et al. 1999). A competent arboviral vector must be susceptible to infection, abundant, long lived and able to blood feed on and become infected by both amplification and dead-end host. Short-lived mosquitoes rarely serve as competent vectors because they do not have sufficient time to complete the extrinsic incubation period. Laboratory assessment of vector competence can provide an insight on the potentiality of field collected mosquitoes as disease vectors. Such competency studies have been performed for RVF virus (Turell et al. 2007, Moutailler et al. 2008, Turell et al. 2008), WNV (Turell et al. 2001, Balenghien et al. 2008, Reisen et al. 2008, Doyle et al. 2011) , Carlifornia encephalitis virus (Kramer et al. 1992) and VEEV (Turell et al. 2006) among others. Laboratory experiments have shown that several mosquito species can be orally infected with viruses and can subsequently transmit the infection through blood feeding (Turell et al. 1996). These studies, some which use mosquito species not implicated in transmission cycles, have shown the potential for local transmission in the event that the virus is introduced into the environment (Turell and Kay 1998). Given that mosquito control methods differ for different species, it is of public health importance to identify which species of mosquitoes are competent vectors that may be involved in the natural transmission cycle so that appropriate control measures can be applied.

1.7.1 Transmission barriers

Previous studies have documented the existence of a midgut (mesenteron) infection barrier associated with refractoriness when some arboviruses are ingested by certain mosquito species (Chamberlain and Sudia 1961, Murphy 1975). More recent studies have shown the existence of other barriers to infection apart from the midgut barrier. Hardy et al proposed that a series of barriers prevent or reduce further dissemination of virus at various times between its appearance in

the lumen of the midgut and its eventual shedding into the salivary glands (Hardy et al. 1983) (Figure 7). The mosquito lumen is separated from the hemocoel by a single epithelial layer of cells surrounded by a porous multilayered membrane, the basal lamina. The first virus proliferation occurs in the epithelial cells if a sufficient viral dose is ingested. However, infection fails to establish or may be established only at very high viral dose if a mosquito is refractory to a given arbovirus. The second barrier is posed by the basal lamina and is termed the "mesenteronal escape" barrier. A third barrier is the salivary gland infection barrier. Both were experimentally demonstrated with Western equine encephalitis virus in *Culex tarsalis* (Kramer et al. 1981). In 20--30% of infected *C. tarsalis,* the virus did not multiply to normal levels in the mesenteron and did not disseminate into the hemolymph regardless of the length of time. In another 20-45% of infected mosquitoes, the virus multiplied normally and disseminated into the hemolymph, but failed to infect the salivary glands. However, the salivary gland infection barrier in some of these mosquitoes was overcome after an extended incubation period showing that this barrier is both time and dose dependent. Individuals of some species are unable to transmit the virus during blood feeding even at optimal conditions for highly competent vector species (Chamberlain and Sudia 1961, Jupp et al. 1981). This might be related in part to the infecting dose of virus. Studies have shown that a reduction in the infective dose of virus reduces arboviral transmission (Jupp 1974, Watt et al. 1976).

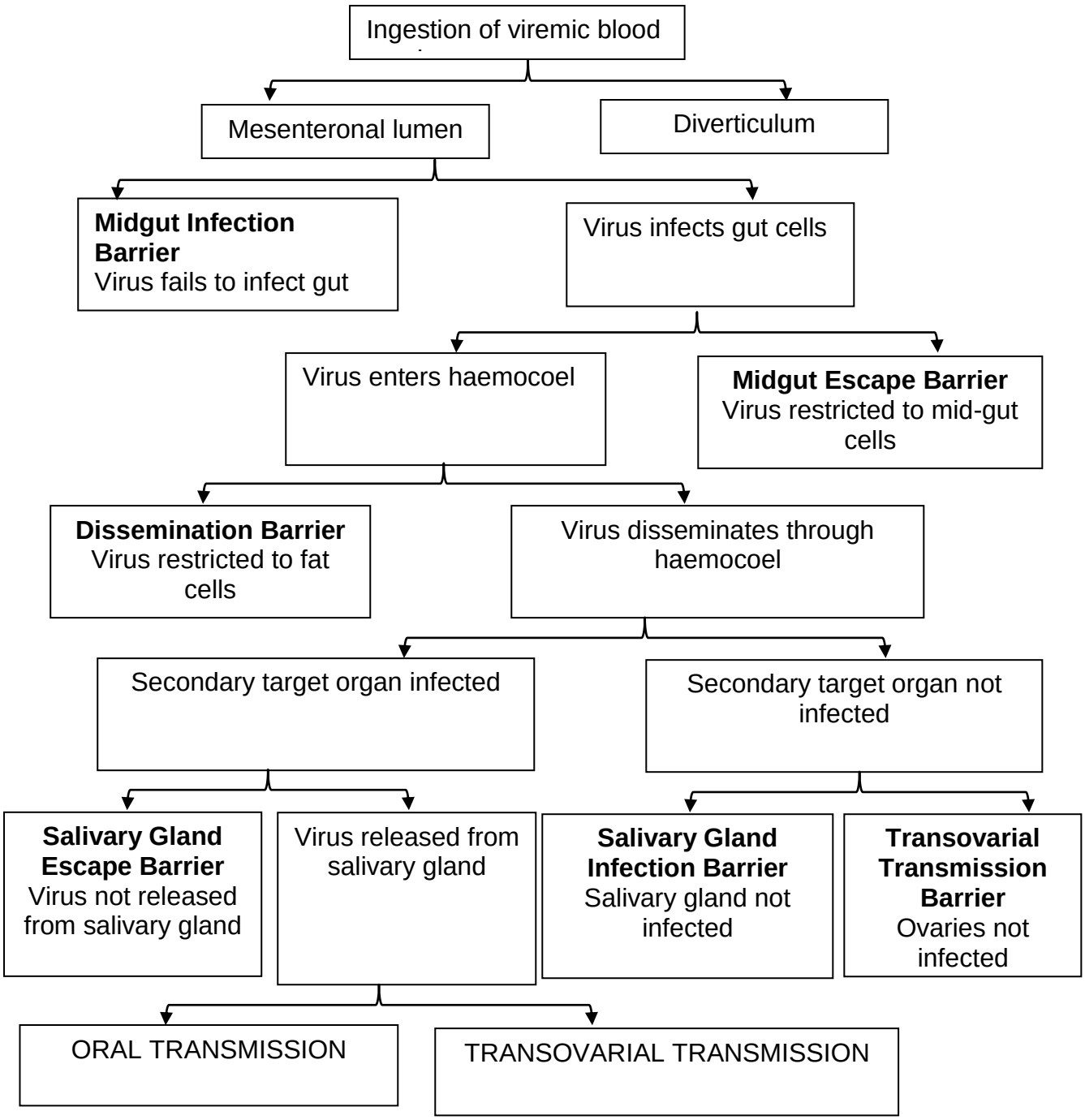


Figure 7: Hypothesizes transmission barriers (bold) to arbovirus infection (Adapted from Hardy et al. 1983).

1.8 Vectors of Orthobunyavirus

BUNV was first isolated from *Aedes* mosquitoes in the Semliki forest in Uganda (Smithburn et al. 1946). However, it was not possible to identify the particular *Aedes* species from which the isolation was made. A second strain of the virus was isolated in 1955 from *Aedes circumluteolus* in

Tongaland, South Africa (Kokernot et al. 1957). Arthropod vectors of medical importance flourish in many parts of Kenya (Linthicum et al. 1985, Sang and Dunster 2001) hence the need for continuous surveillance. Entomological surveys during RVF outbreaks in the recent past have demonstrated co-circulation of arboviruses including BUNV (Traore-lamizana et al. 2001, Crabtree et al. 2009). Ongoing inter-epidemic surveillance has indicated continued intense transmission of BUNV in the absence of or under low level activity of RVF virus. Knowledge on the vectors involved in their transmission is scanty or absent. The surveillance activities incriminated a range of mosquito species associated with BUNV through isolation from field sampled mosquitoes including *Aedes argenteocephalus*, *Aedes dalzieli*, *Aedes vittatus*, *Aedes furcifer-taylori* and *Mansonia africana* and *Mansonia uniformis* as new vectors for this virus in Senegal (Traore-lamizana et al. 2001). In Kenya, BUNV has been isolated from *Aedes ochraceus, Aedes mcintoshi* and *Anopheles funestus* mosquitoes while a related virus, Pongola virus, was isolated from *Aedes mcintoshi and Aedes circumluteolus* (Traore-lamizana et al. 2001, Crabtree et al. 2009, Ochieng et al. 2013). The virus has also been isolated from *Aedes quasiunivittatus* (Logan et al. 1991). Pongola virus was first isolated in South Africa in 1957 from *Aedes circumluteolus* (Kokernot et al. 1957). NRIV has been detected previously in West Africa from a wide diversity of mosquito species including *Anopheles gambiae*, *Anopheles pharoensis*, *Culex antennatus*, *Culex poicillipes* and *Culex tritaeniorhynchus*(Gordon et al. 1992, Zeller et al. 1996). Some of these mosquito species are predominant in different regions of Kenya including *Anopheles gambiae, Anopheles pharoensis and Culex antennatus* (Lutomiah et al. 2013). However, the actual role of the associated mosquito species in the maintenance and transmission of the virus in the environment remains unclear. Surveillance for the vector-borne pathogen and identification of the arthropod vectors that transmit these agents are critical components in estimating the risk of human exposure and understanding the transmission and maintenance mechanism.

1.9 Transovarial transmission

The frequency of transovarial transmission is generally low for most arboviruses (<1%), while

unusually high for some bunyaviruses belonging to the California serogroup (Kuno and Chang 2005). Watts et al. demonstrated transovarial transmission of Lacrosse virus (LACV) experimentally using *Aedes triseriatus* and concluded that this was a mechanism of overwintering (Watts et al. 1973, Watts et al. 1974). This finding in addition to demonstrations in other members of the California serogroup suggests an important mechanism in the maintenance of these arboviruses. Transovarial transmission has also been demonstrated in several tropical bunyaviruses of other serogroups and genera. RVF virus has been isolated from field collected larvae of *Aedes lineatopennis* in Kenya (Linthicum et al. 1985). This mechanism is credited for the long-term survival of RVF virus in the absence of suitable vertebrate hosts, high densities of competent mosquito species and suitable environmental conditions (Moutailler et al. 2008).

1.10 Seroprevalence of Orthobunyaviruses

Diagnostic laboratories rarely test for infections caused by Orthobunyaviruses hence the true prevalence of these viruses remain undetermined (Blitvich et al. 2012). Currently, there is no data on the seroprevalence of Orthobunyaviruses in Kenya. Bunyamwera serogroup viruses associated with human disease have been isolated in Africa and include BUNV, NRIV, Shokwe virus, Ilesha virus and Germiston virus and have overlapping geographic distribution across Africa as shown in Figure 8. Virus transmission usually peak during the rainy season when mosquitoes are abundant and persons of all age groups can be affected. BUNV has been observed to be active in the riverine forests of Nigeria and the Central African Republic (Gonzalez and Georges 1988). For example, all adults living in tropical rain forests of the Democratic Republic of Congo are seropositive for BUNV. Ilesha virus on the other hand, has a higher seropositivity rate in persons living in the Savvanah compared to persons living in the rain forests (Gonzalez and Georges 1988). Germiston virus is endemic in Southern African countries and to date has not been associated with any major outbreak of human disease and is thus considered of minor public health importance (Gonzalez and Georges 1988). All these viruses have also been isolated in Kenya (Johnson et al. 1977, Lwande et al. 2013, Ochieng et al. 2013). Since these viruses comprise a neglected but potentially deadly

group of viruses as evidenced by the implication of NRIV in hemorrhagic fever outbreaks within northern Kenya (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006), there is need to investigate whether these viruses commonly infect humans within this region. BUNV serosurveys suggest wide distribution within sub-Saharan Africa (Gerrard et al. 2004). A previous study on hemorrhagic fever patients during the 1997/1998 RVF outbreak demonstrated NRIV acute infection in 27% of cases (Bowen et al. 2001).

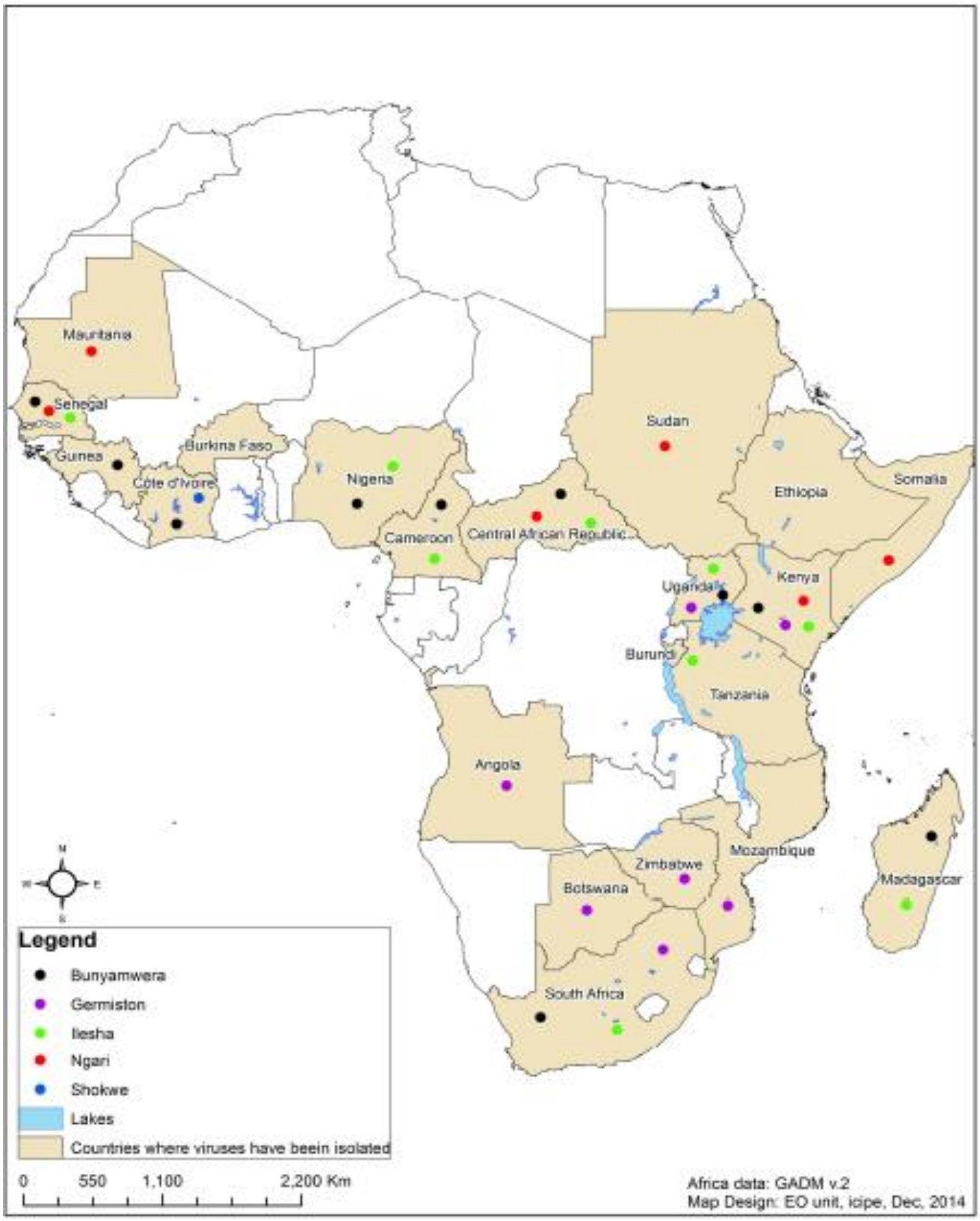


Figure 8: Geographic distribution of Orthobunyaviruses in Africa (Wertheim et al. 2012). Dots represent countries where viruses have been isolated.

1.11 Conclusion

The evolutionary success of some viruses, especially in the *Bunyaviridae* family, is thought to be facilitated by their ability to adapt to varying conditions via genetic drift involving intramolecular genetic changes, and shifts which involve genomic segment reassortment. Genetic drift occurs due to the poor fidelity of the RNA polymerase and lack of proofreading capability resulting in multiple genetic variants or quasispecies. NRIV is a newly emergent reassortant virus thought to be associated with severe disease epidemics within Africa (Gerrard et al. 2004, Briese et al. 2006). Genetic analysis reveals that its S and L RNA segments are derived from BUNV while the M segment is derived from BATV, an *Orthobunyavirus* first isolated in Malaysia. Although genetic drift and reassortment seem to occur in the *Bunyaviridae* family, the epidemiological consequences of these evolutionary events are poorly understood. Because no specific medical treatment exists for arboviral diseases, it is important to continually carry out surveillance in order to understand their transmission trends and dynamics in real time. Genetic reassortments/recombinations are bound to compromise surveillance when new variants and/or genotypes emerge, evading existing diagnostic/detection and management tools. Additionally, entomological arbovirus surveillance during RVF outbreaks has demonstrated co-circulation of RVF virus and other arboviruses including BUNV. In addition, ongoing inter-epidemic surveillance has indicated continued intense transmission of BUNV in the absence of or under low level activity of RVF virus. However, knowledge on the vector competence of mosquito species involved in virus transmission is scanty or absent. The surveillance activities incriminated a range of mosquito species that are associated with BUNV and NRIV through isolation from field sampled mosquitoes but the actual role of the associated mosquito species in the maintenance and transmission of the virus in the environment remains unclear. Efforts to bring these infections under control would not be successful unless we have a full understanding of how these viruses are being maintained and moved in the environment. Moreover, it is necessary to determine whether these viruses represent an unrecognized cause of disease in humans.

The present study was undertaken to extend the currently available full sequencing data on the S, M and L RNA segments of BUNV and NRIV. Generating sequencing data on the three segments of these two viruses from disparate historic, host and geographic origins will further contribute to phylogenetic characterization and molecular epidemiology of these viruses, including tracking their movement, identification of the sources of outbreaks, and aid investigations of reassortment events. We also characterize and monitor emergent arboviral genotypes of BUNV and NRIV from previous surveillance exercises in Kenya, determine their seroprevalence among patients presenting with febrile illness within selected regions of Kenya and determine the vector competence of selected mosquito species that that may mediate transmission in order to design effective detection and control measures.

1.12 Hypothesis

1. Bunyamwera and Ngari viruses are genetically stable in spite of isolation from different mosquito host, time or space, implicated mosquito species are not efficient vectors of these viruses and human exposure to the two viruses is minimal among febrile patients in the northern Kenya region.

1.13 Objective

To understand the dynamics of Ngari and Bunyamwera virus circulation and transmission in northern Kenya.

1.13.1. Specific objectives

1. To investigate the genotypic diversity and reassortments of Bunyamwera and Ngari virus isolates in the northern Kenya ecozone with respect to vector, time and space.
2. To evaluate the growth characteristics and virulence of Bunyamwera and Ngari virus isolates obtained from diverse geographic region, time and vector species in Kenya.

3. To determine the infection, dissemination and transmission rates of *Aedes*, *Culex* and *Anopheline* mosquito species exposed to Bunyamwera and Ngari virus infected-blood meal and identify transmission barriers.

4. To determine transovarial transmission of Bunyamwera and Ngari viruses in selected mosquito vectors as a mechanism of virus maintenance in nature

5. To determine the seroprevalence of Bunyamwera and Ngari viruses in febrile patients in northern Kenya from 2009 to 2012.

Chapter 2

PHYLOGENETIC ANALYSIS OF BUNYAMWERA AND NGARI VIRUSES (FAMILY *BUNYAVIRIDAE*; GENUS *ORTHOBUNYAVIRUS*) ISOLATED IN KENYA

2.1 INTRODUCTION

The Bunyaviridae family is divided into five genera; *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus* and *Tospovirus* (Calisher 1996) . The genus *Orthobunyavirus* is composed of over 172 viruses that infect humans and are transmitted by mosquitoes, midges and ticks from reservoir animals like rodents and livestock. Members of the *Orthobunyavirus* genus are tri-segmented, negative-sense, single-stranded RNA viruses responsible for mild to severe human ans animal diseases. The L segment encodes a large protein that consists of the RNA-dependent RNA polymerase activity for replication and transcription of genomic RNA segments. The M segment encodes a precursor polypeptide which yields the virion surface glycoproteins Gn and Gc and a nonstructural protein NSm, and the S segment encodes the nucleocapsid (NC) and a nonstructural protein (NSs) in overlapping reading frames (Soldan and Gonzalez-Scarano 2005). BUNV is the prototype virus of the *Orthobunyavirus* genus as well as the *Bunyaviridae* family of arboviruses. BUNV is associated with febrile illness with headache, arthralgia, rash and infrequent central nervous system involvement (Gonzalez and Georges 1988). Viruses of the *Orthobunyavirus* genus were previously not associated with haemorrhagic symptoms until NRIV was implicated in haemorrhagic fever outbreaks in Kenya and Somalia, and retrospectively in Sudan (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). NRIV was determined to be a reassortment between two segmented viruses (BUNV and BATV) co-circulating within the same environment. Subsequent analysis of isolate sequences from outbreak samples showed that the L and S segment sequences closely matched those of BUNV while the M segment was identical to that of BATV (Briese et al. 2006).

Surveillance activities during RVF outbreaks have demonstrated co-circulation of arboviruses including BUNV (Crabtree et al. 2009). Additionally, ongoing inter-epidemic surveillance activities

have indicated continued intense transmission of BUNV (Lutomiah et al. 2014). During a RVF outbreak in Kenya and Somalia in 1997/1998, NRIV was isolated from the blood of two patients and antibody detected in several others with hemorrhagic fever (Briese et al. 2006). Retrospectively, two isolations were made from the blood of two patients during an earlier outbreak in the Sudan with IgM and IgG detected in 7% and 61% of patients (Nashed et al. 1993).

Co-circulation of viruses within the same serogroup is likely to provide opportunities for genetic reassortments. Efforts to bring these infections under control and predict their emergence would not be successful unless we have a full understanding of how these viruses are being maintained and moved in the environment. However, characterization of these emergent arboviral species has been hampered by paucity of full genome sequences in the Genbank that in turn make it impossible to accurately estimate their evolutionary trend and public health burden. Moreover, an in depth study of the transmission and evolutionary history of BUNV and NRIV in Kenya has not been reported. We have recently isolated BUNV and NRIV from surveillance exercises in Kenya based on short diagnostic sequences (Ochieng et al. 2013, Lutomiah et al. 2014). Our main objective was to provide complete coding sequence of some of these isolates as well as, in addition to sequences already deposited in the GenBank, investigate their genetic diversity with respect to time, geographic location and vector species of isolation.

2.2 METHODS

2.2.1 Cells and viruses

Vero cells (African green monkey kidney) (ATCC® CCL-81™) were used for growing virus stocks for quantification by plaque assay. The cells were maintained in Eagles Minimum Essential Media (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 2 mM L-glutamine (Invitrogen). BUNV and NRIV isolates were obtained from previous surveillance exercises in Kenya. The virus isolates used in this study are listed in Table 4 along with their geographical origin (Figure 9 and 10), host source and year of isolation.

Table 4: Bunyamwera and Ngari virus isolates included in phylogenetic analysis of the full coding sequences of selected orthobunyaviruses.

Virus	Specimen code	Site of isolation	Collection date	Isolation source
Bunyamwera	**BUNV_GSA/S4/11232**	Kenya (Garissa)	2009	*Aedes Mcintoshi*
	BUNV_MGD/S1/12060	Kenya (Magadi)	2010	*Anopheles Funestus*
	BUNV prototype	Uganda	1943	*Aedes*
	BUNV_ArB29051	Central African Republic	1994	*Mansoni uniformis*
	BUNV_(AF325122)	Australia	2000	
	BUNV_ArB28215	Central African Republic	1992	*Anopheles sp*
	BUNV_(JF961341)	Kenya (Garissa)	2011	*Aedes Mcintoshi*
	BUNV_PE-7.0014	Peru	1997	*Mosquito*
Ngari	**NRIV_TND/S1/19801**	Kenya (Tana-delta)	2011	*Anopheles Funestus*
	NRIV_GSA/TS7/5170	Kenya (Garissa)	2009	*Amblyomma gemma*
	NRIV_ISL/TS2/5242	Kenya (Isiolo)	2009	*Rhipicephalus pulchellus*
	NRIV_DakArD28542	Senegal (Dakar)	1979	*Aedes simsoni*
	NRIV_9800535	Kenya	1998	*Homo sapiens*
	NRIV_9800521	Somalia	1998	*Homo sapiens*
	NRIV_SUD-HKV141	Sudan	1988	*Homo sapiens*
	NRIV_SUD-HKV66	Sudan	1988	*Homo sapiens*
	NRIV_Adrar	Mauritania	2010	*Small ruminant*

* Isolates sequenced in this study are bolded

2.2.2 Propagation of virus

Freshly confluent Vero cells (ATCC® CCL-81™) grown in 75 cm^2 flasks were infected with 1 ml of each isolate to make virus stock for the study. The cultures were observed daily for cytopathic effects (CPE) and harvested when more than 75% cells showed CPE. The virus was harvested by freezing and thawing of the cultures followed by centrifugation at 880 *g* for 10 min at 4^oC to remove the cell debris/lysate. The supernatant was aliquoted into 500µl each into cryotubes and stored at -70^oC**,** until use. Final virus isolates sequenced were low passage (Vero 3).

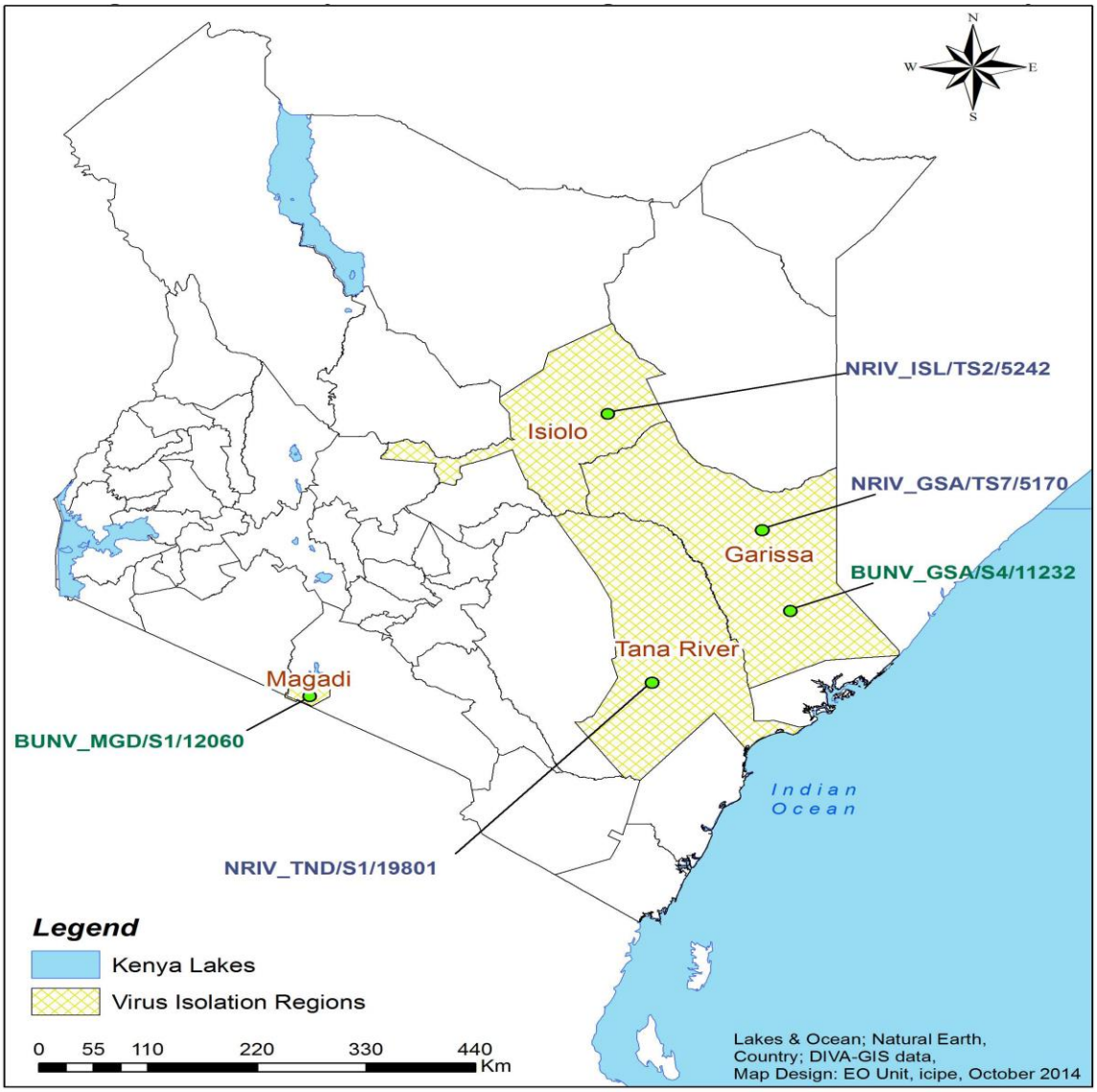


Figure 9: Geographic regions of Bunyamwera and Ngari virus isolations in Kenya

2.2.3 RNA isolation and cDNA synthesis

For RNA extraction, we used the MagNA Pure LC RNA Isolation Kit I (Roche Applied Science, Indianapolis, IN). Complementary DNA (cDNA) was synthesised using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) with 2 µl Random hexamers followed by PCR using Phusion High-Fidelity PCR Kit (Finnzyme OY, Espoo, Finland) and appropriate primers. Primers (Appendix 2, Table 19) for each segment were either designed based on sequences of BUNV, BATV and NRIV available in GenBank or obtained from previous publications (Yanase et al. 2006, Jost et al. 2011).

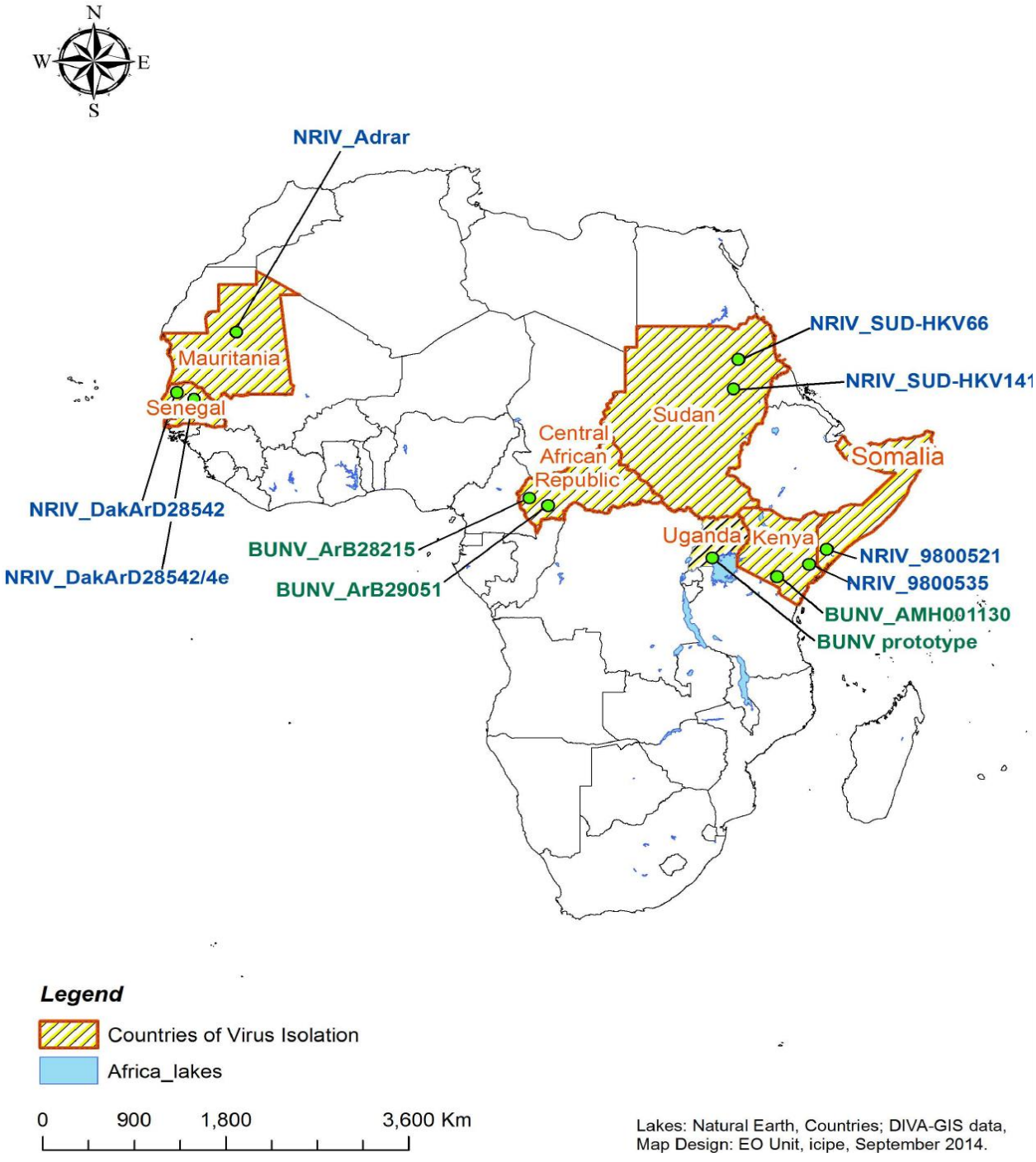


Figure 10: Geographic regions of Bunyamwera and Ngari virus isolations in Africa

Reactions were performed in thin-walled 0.5 ml tubes (Thermo Scientific™) using a Bio-Rad thermal cycler with heated lid. The total volume per reaction mixture was 20µl.

Phusion PCR reaction:

5× PCR reaction buffer……..4µl

dNTPs (10 mM each)…….0.4µl

Template DNA ….....2µl

Primer F (10µM).....1µl

Primer R (10µM).....1µl

DMSO (Fisher Scientific).....0.6µl

Polymerase (1U/ µl)…….....0.2µl

dH_2O……. 10.8µl

PCR programme:

Initial denaturing……...98°C……30s

Strand separation……..98°C…….10 s

Annealing…….57-65°C…..15s

Elongation……. 72°C…….45s

40-45 cycles

Final extension……..68°C…….7 mins

PCR products were mixed with 6X loading buffer (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0) (Promega)) and loaded in the wells of the agarose gel. An electric current was applied to the gel (70-130 volts) for approximately 45 minutes in order to separate different fragments of nucleic acids. As a size marker, two different DNA ladders were used; 1 Kb and 100 bp ladders (Promega). Amplified DNA fragments were visualized by electrophoresis on a 1.5% agarose gel (Figure 9). The Amplified DNA was purified and prepared for sequencing using ExoSAP-IT PCR clean-up kit (USB Corp, Cleveland, OH) according to the manufacturer's instructions and stored at -20^{o}C.

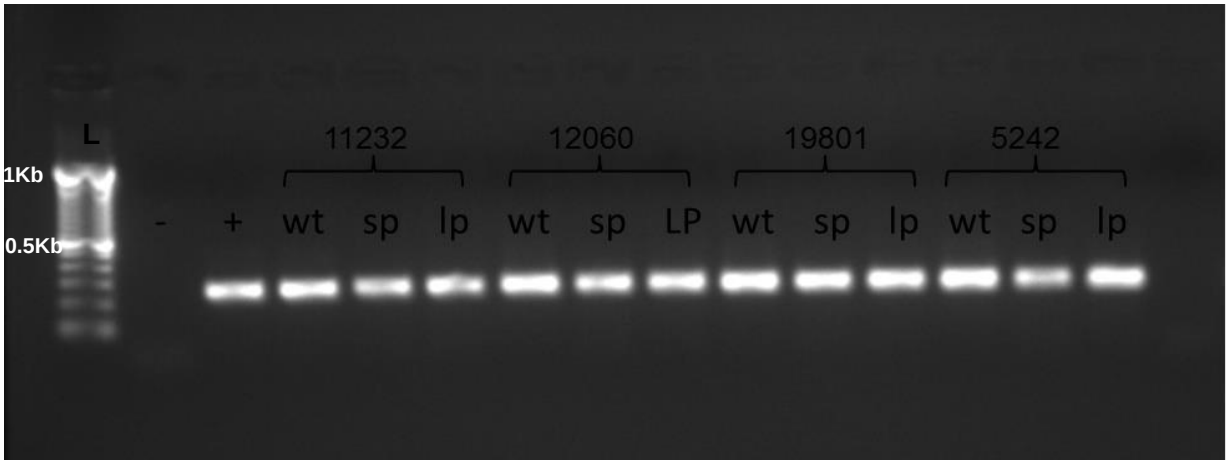


Figure 11: RT-PCR gel showing amplified S genome fragments of Bunymawera and Ngari virus isolates in the current study. wt; wild type, sp: small plaque, lp: large plaque, +: Bunyamwera virus control, -: no template added.

2.2.4 Sequencing and phylogenetic analysis

Sequencing was performed using different sets of primers for S, M and L segments as defined above using Big Dye V3.1 kit (Applied biosystems) according to manufacturer's instructions and run on the 3500XL genetic analyser (Applied Biosystems, Foster city, California, USA). Purification of sequencing products before injection was done with the Zymo research sequencing clean-up kit. The sequences obtained were cleaned and edited using Bioedit software, USA for both the reads from the forward and reverse primers. Sequences obtained were subjected to BLAST searches in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast) to identify similar sequences. The clean sequences of each segment were aligned against a selection of corresponding segment sequences of Bunyamwera serogroup viruse including BUNV, BATV and NRIV using MAFFT sequence alignment program (Standley 2013). MrModeltest version 2.3 (Nylander 2004) in cooperation with PAUP*4b10 (Swofford 2002) using the Akaike information criterion (AIC) (Akaike 1973) was used to predict the best parameters in reconstructing Bayesian trees. The MrModeltest predicted that the general time reversible (GTR) evolutionary model would be the best for the set of sequences for the three segments (L, M, S) in the Bayesian phylogenies' analyzing the relationships among all BUNV and NRIV isolates whose sequence was determined. However, only data for S segment were subject to a gamma distribution with a proportion of invariable sites. The

MrBayes software package 3.1.2 (Ronquist and Huelsenbeck 2003) was then used to run MCMC Bayesian inference (BI). The program was set to run for 10,000,000 generations with sampling every 1,000 generations. A 50% majority rule consensus tree was created from the trees remaining after a 10% burn-in removal. The included Bayesian sets of trees were sampled after likelihood scores reached convergence and the mean split difference values were almost 0.01. Trees were visualized using Figtree v 1.4.0 (Rambaut 2006-2012) with nodal support evaluated by posterior probabilities (PP) for the BI. La Crosse virus of the California serogroup was used as outgroup for the phylogenetic analysis of all the three segments of BUNV and NRIV. Nucleotide and amino acid similarity and genetic distance between the Kenyan isolates and selected isolates from diverse regions (Table 5) were computed in MEGA v5.20 using the p-distance method (Tamura et al. 2011).

2.2.4 Nucleotide sequence accession numbers

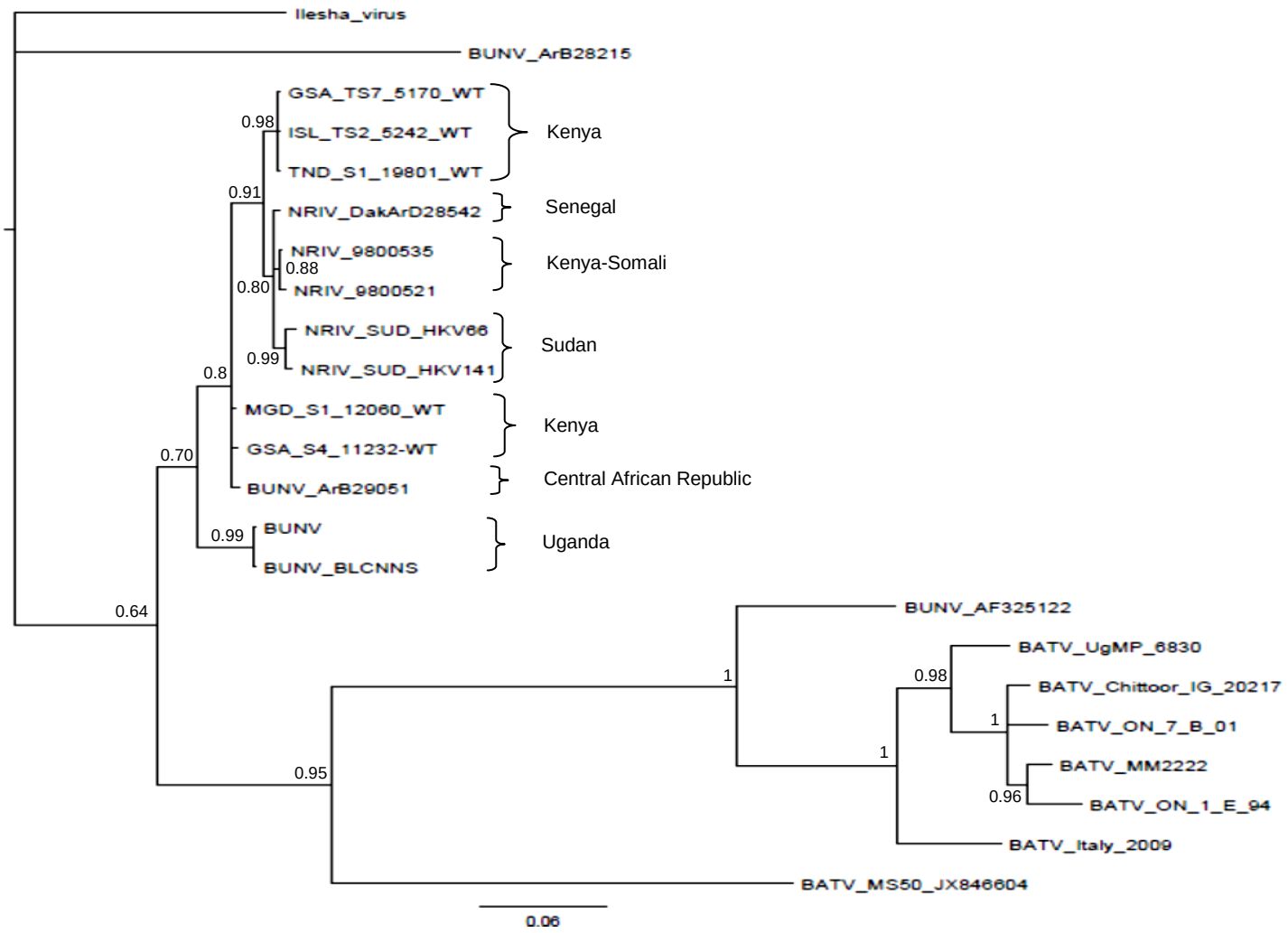
The genome sequences determined in this study were deposited in GenBank under the following accession numbers (S segment, M segment, and L segment): KM507344, KM507340 and KM507338 (BUNV_MGD_S1_12060_WT); KM507345, KM507339 and KM507337 (BUNV_GSA_S4_11232_WT); KM507343, KM514679 and KM507335 (NRIV_TND_S1_19801_WT); KM507341, KM514677 and KM507336 (NRIV_GSA_TS7_5170_WT); KM507342, KM514678 and KM507334 (NRIV_ISL_TS2_5242_WT.

2.3 RESULTS

To support the molecular characterization efforts (Chapters 2 and 3), 15 full length S, M and L coding nucleotide and amino acid sequences of the 5 viruses sequenced in this study and their alignment with other viruses from GenBank are presented (Appendix 1) As previously reported by Groseth et al, the S segment of the NRIV and BUNV isolates formed entirely separate phylogenetic

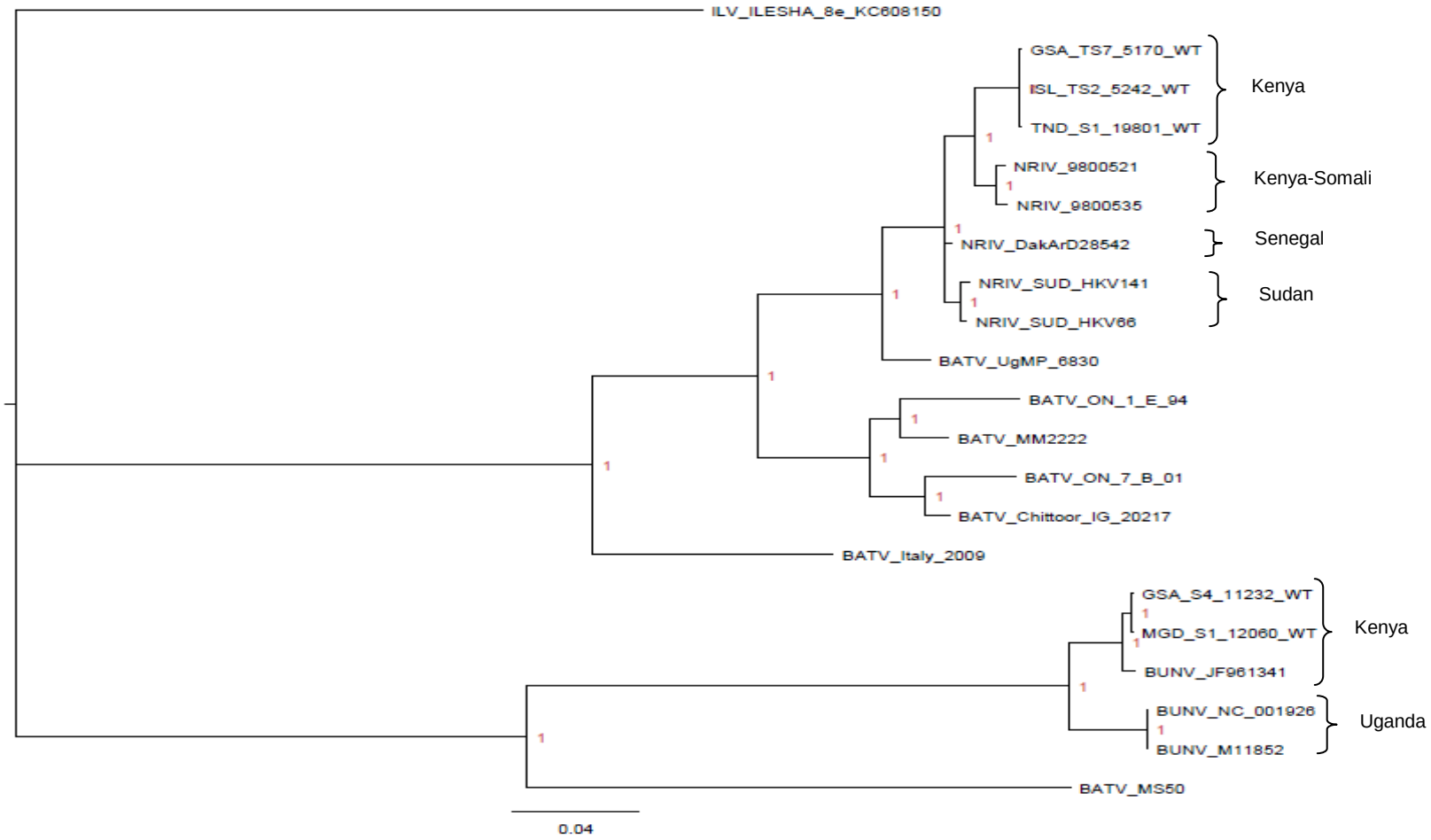
branches (Groseth et al. 2012). The Kenyan NRIV isolates sequenced in the current study, regardless of the vector species of origin, geographic region and year of isolation, formed a monophyletic group with strong bootstrap support (98%), distinct from the other NRIV isolates from Sudan and Senegal and were more closely related to the NRIV isolates associated with the 1997/1998 hemorrhagic fever outbreak in East Africa, NRIV_9800535 and NRIV_9800521 (Figure 12). The Kenyan BUNV isolates sequenced in the current study, (MGD_S1_12060WT and GSA_S4_11232WT) clustered together with the Central African Republic isolate, BUNV_ArB29051 (AM709778) and with the Kenyan BUNV_AMH001130 (JF961342) isolated in 2011 but all these isolates were divergent from the prototype virus isolated from Uganda, BUNV (NC_001925) which is ancestral to all the NRIV isolates. The Australian BUNV isolate, BUNV_M11852 (AF325122) clustered more closely with BATV and Calovo virus isolates distinct from the African BUNV isolates. Likewise, BUNV isolate BUNV_ArB28215 (AM711130) was divergent from the African isolates despite isolation from Central African Republic and was closest to Nyando and Bozo virus isolates (Figure 12).

Phylogenetic analysis of the M and L segments followed a similar pattern as the S segment with the Kenya NRIV isolates sequenced in the current study forming a monophyletic group with 100% bootstrap support (Figure 13 and 14). The Kenyan isolates clustered closest to the two isolates associated with the 1997/1998 hemorrhagic fever outbreak in the Kenya Somali border, NRIV_9800521 and NRIV_9800535 as well as NRIV_Adrar, recently isolated from a small ruminant in Mauritania. Phylogenetic analysis of the L segment indicated that the Kenyan BUNV isolates were divergent from the prototype virus from Uganda (NC.001925). The two BUNV isolates in the current study clustered closest with the 2011 Kenyan isolate BUNV_AMH001130 (JF961341) but distinct from the prototype virus from Uganda (Figure 14).



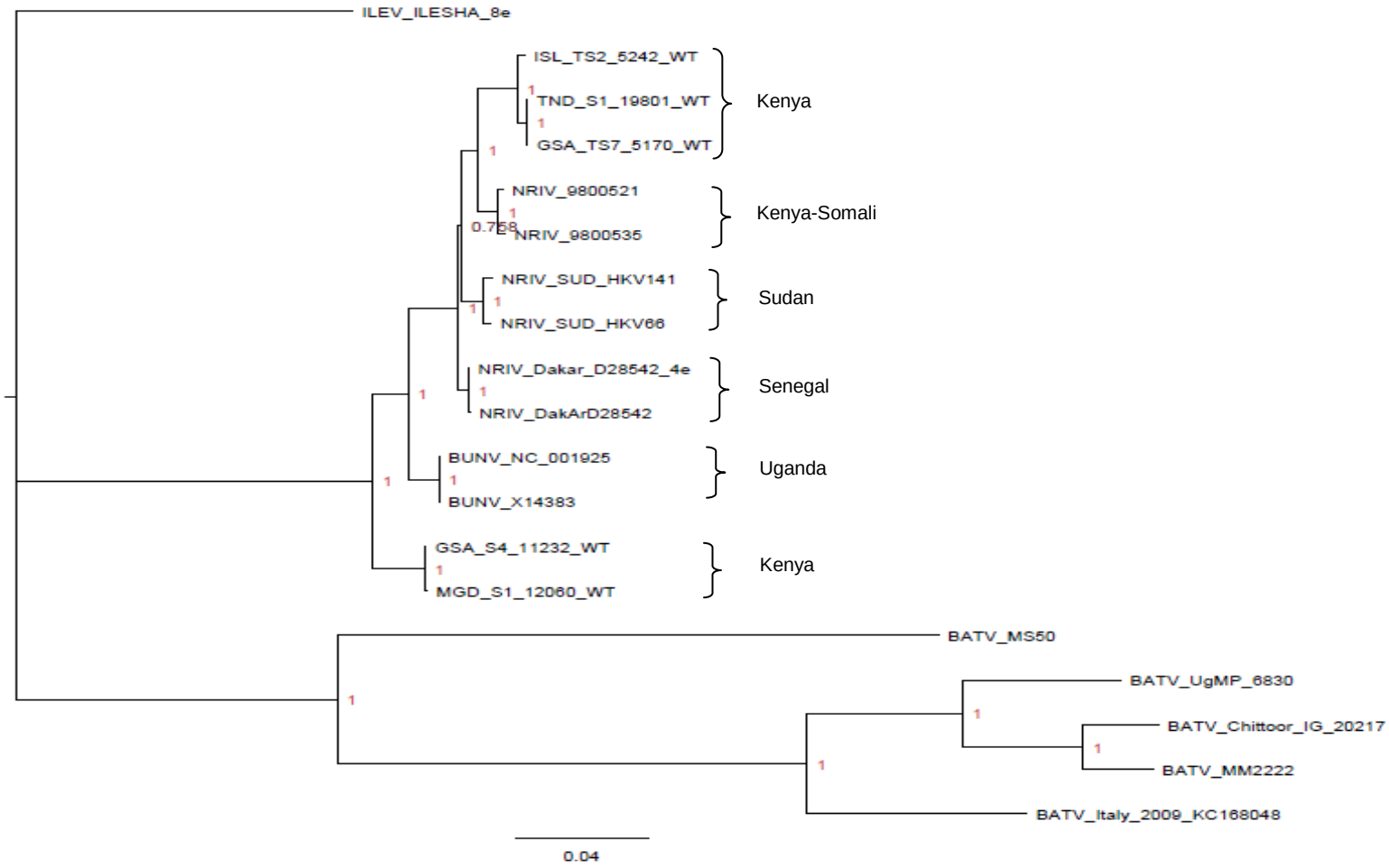
Bayesian analysis of the coding region of the S segment of Bunyamwera and Ngari virus isolates from diverse regions. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree.All Orthobunyavirus abbreviations are used according to (ICTV, 2005). BUNV: Bunyamwera virus, NRIV: Ngari virus, BATV: Batai virus

Figure 12: Bayesian analysis of the coding region of the S segment of Bunyamwera and Ngari virus isolates from diverse regions. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree. Kenyan isolates sequenced in this study are indicated by solid dots. All Orthobunyavirus abbreviations are used according to (ICTV, 2005). Batai virus, BIRV: Birao virus, BOZOV: Bozo virus, BUNV: Bunyamwera virus, CVV: Cache Valley virus, CVOV: Calovo virus, FSV: Fort Shernan virus, GERV: Germiston virus, ILEV: Ilesha virus, KIRV: Kairi virus, LACV: La Crosse virus, NDOV: Nyando virus, NOLAV: Nola virus, NRIV: Ngari virus, PGAV: Pongola virus, POTV: Potosi virus, SHOV: Shokwe virus, TSV: Tensaw virus, WYOV: Wyomyia virus, XINV: Xingu virus. S segment accession numbers; Abbey_lake_orthobunyavirus_Cu20_XJ (KJ710424), BATV_Chittoor_IG_20217 (JX846598), BATV_MM2222 (JX846595), BATV_MS50 (JX846604), BATV_NM_12 (KJ187040), BATV_UgMP_6830 (JX846601), BATV_XQ_B (KJ398936), BIRV_ArB2198 (AM711131), BOZOV_ArB13529 (AM711132), BUNV_BLCNNS (D00353), BUNV_Pe7_0014 (AJ697960), BUNV_ArB29051(AM709778), BUNV_NC (NC_001927), BUNV_ArB28215(AM711130), BUNV_M11852 (AF325122), BUNV_AMH1130 (JF961342), CVOV_134 (KJ542624), CVOV_138_pool_468 (KC608157), CVOV_8020 (KJ542630), CVOV_8040 (KJ542633), CVOV_JAn_MS3 (KJ542627), CVV_MNZ_92011 (KC436108), FSV_86MSP18 (EU564829), GERV_BLCSA (M19420), ILEV_ArB16282 (AM709780), ILEV_HB80P125 (AM709779), ILEV_ILESHA_8e (KC608151), ILEV_R5964 (KF234073), KRIV_Mex07 (EU879063), LACV_Dallas_TX_2009 (GU591164), NDOV_ArB16055 (AM709781),NOLAV_ArB2882 (AM711134), NRIV_9800521_Somalia_1998 (JX857325), NRIV_9800535 (JX857328), NRIV_Adrar (KJ716848), NRIV_D28542/4e (KC608154), NRIV_DakArD28542 (JX857316), NRIV_HKV141(JX857322), NRIV_HKV66 (JX857319), PGAV_SAAr1 (EU564828), POTV_AY729652_1 (AY729652), SHOV_SAAr_4042 (EU564831), TSV_TSV_FL06 (FJ943507), WYOV_JN572082 (JN572082), XINV_BeH388464_e (EU564830)



Bayesian analysis of the M polyprotein of Bunyamwera and Ngari virus isolates from diverse regions. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree.All Orthobunyavirus abbreviations are used according to (ICTV, 2005). BUNV: Bunyamwera virus, NRIV: Ngari virus, BATV: Batai virus

Figure 13: Bayesian analysis of the M polyprotein of Bunyamwera and Ngari virus isolates from diverse regions. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree. Kenyan isolates sequenced in this study are indicated by solid dots. All Orthobunyavirus abbreviations are used according to (ICTV, 2005). Batai virus, BIRV: Birao virus, BOZOV: Bozo virus, BUNV: Bunyamwera virus, CVV: Cache Valley virus, CVOV: Calovo virus, FSV: Fort Shernan virus, GERV: Germiston virus, ILEV: Ilesha virus, KIRV: Kairi virus, LACV: La Crosse virus, NDOV: Nyando virus, NOLAV: Nola virus, NRIV: Ngari virus, PGAV: Pongola virus, POTV: Potosi virus, SHOV: Shokwe virus, TSV: Tensaw virus, WYOV: Wyomyia virus, XINV: Xingu virus. M segment accession numbers: BATV_ON_7_B_01 (AB257765), BATV_ON_1_E_94 (AB257764), BATV_NM_12 (KJ187039), TSV_FE3_66FB (FJ943508), CVV_AF082576 (AF082576), TSV_FL06 (FJ943506), GERV_BLCMPOLY (M21951), CVV_MI80_1_450 (AF186241), ILEV_KO_2 (AY859372), CVV_807270 (AF186243), CVV_CK102 (AF186242), BUNV_BLCMA (M11852), MAGV_AY286443 (AY286443), MAGV_ts8 (AY286444), CVV_082576 (AF082576), ILEV_R5964 (KF234074), POTV_89_3380 (EU004189), NRIV_Dakar_D28542/4e (KC608153), ILEV_ILESHA_8e (KC608150), NRIV_Adrar (KJ716849), BATV_XQ+B (KJ398937), Abbey_lake_orthobunyavirus_Cu20_XJ (KJ710423), BATV_MS50 (JX846605), BATV_UgMP_6830 (JX846602), BATV_Chittoor_IG_20217 (JX846599), BATV_MM2222 (JX846596), NRIV_9800521 (JX857326), NRIV_9800535 (AY593725), NRIV_DakArD28542 (JX857317), NRIV_HKV141 (JX857323), NRIV_HKV66 (JX857320), CHLV_CHLV_Mex07 (JN808310), CVOV_138_pool_468 (KC608156) GROV_BeH22063 (AY380581), CVV_MNZ_92011 (KC436107), BATV_Italy_2009 (KC168047), IACOV_BeAn314206 (JN572066), LACV_Human_78 (AF528166), BUNV_NC (NC_001926), BUNV_AMH001130 (JF961341), CVOV_JAnMS3 (KJ542628),CVOV_134 (KJ542625), CVOV_184 (DQ334335)



Bayesian analysis of the L protein of Bunyamwera and Ngari virus isolates from diverse regions. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree.All Orthobunyavirus abbreviations are used according to (ICTV, 2005). BUNV: Bunyamwera virus, NRIV: Ngari virus, BATV: Batai virus

Figure 14: Bayesian analysis of the L protein of Bunyamwera and Ngari virus isolates from diverse regions. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree. Kenyan isolates sequenced in this study are indicated by solid dots. All Orthobunyavirus abbreviations are used according to (ICTV, 2005). Batai virus, BIRV: Birao virus, BOZOV: Bozo virus, BUNV: Bunyamwera virus, CVV: Cache Valley virus, CVOV: Calovo virus, FSV: Fort Shernan virus, GERV: Germiston virus, ILEV: Ilesha virus, KIRV: Kairi virus, LACV: La Crosse virus, NDOV: Nyando virus, NOLAV: Nola virus, NRIV: Ngari virus, PGAV: Pongola virus, POTV: Potosi virus, SHOV: Shokwe virus, TSV: Tensaw virus, WYOV: Wyomyia virus, XINV: Xingu virus. L segment accession numbers: Abbey_lake_orthobunyavirus_Cu20_XJ (KJ710425), NRIV_Dakar_D28542/4e (KC608152), ILEV_ILESHA_8e (KC608149), BUNV_AMH001130 (F961340), NRIV_9800521 (JX857327), NRIV_9800535 (JX857330), NRIV_DakArD28542 (JX857318), NRIV_HKV141 (JX857324), NRIV_HKV66 (JX857321), ILEV_R5964 (KF234075), LACV_Human_78 (AF528165), BATV_NM_12 (KJ187038), BATV_MS50 (JX846606), BATV_UgMP_6830 (JX846603), CVOV_138-pool_468 (JX846600), BATV_MM2222 (JX846597), BATV_Italy_2009 (KC168048), CVOV_8040 (KJ542635), CVOV_8020 (KJ542632), CVOV_JAn_MS3 (KJ542629), CVOV_134 (KJ542626), TSV_TSV_FE3_66FB (FJ943510), TSV_TSV_FL06 (FJ943509), CVV_MNZ_92011 (KC436106), BUNV_14383 (X14383), NRIV_Adrar (KJ716850), BUNV_NC_Uganda (NC001925)

The Kenyan NRIV isolates sequenced in the current study were closely related with nucleotide sequence identity of over 98% for all segments and nucleotide diversity no more than 2.7% (Tables 5-8). When comparing with other NRIV isolates from the Kenya-Somali border, Sudan and Senegal nucleotide sequence identities ranged from 98.5-99.3, 98.5-99.3 and 97.3-98.0% for the S, M and L segments respectively. Similarly, the Kenyan BUNV isolates in the current study were closely related with nucleotide sequence identity of over 99.6% for all segments. However, Comparing the Kenyan BUNV isolates to other Bunyamwera viruses, nucleotide sequence identity ranged from 82.2-96.4 , 96.0-99.4 and 96.6% for the S, M and L segments respectively. Similarly, nucleotide diversity was no more than 3.8%, 6.3% and 3.4% for the S, L and M segments except isolates BUNV_ArB29051 from Central African Republic and BUNV_M11852 from Australia which were more than 17% divergent in the S segment. This was confirmed by p-distance analysis of nucleotide and amino acid sequences which reveal that these two viruses are closer to Nyando and Colovo viruses respectively (Table 9)

The encoded proteins were highly conserved among the Kenyan NRIV isolates with similarity not less than 99.5% for the N protein, Ns protein, M polyprotein, and L protein (Tables 5-8). The NSs protein was the most conserved when compared among NRIV isolates from the different geographic regions (Table 6). For BUNV, amino acid similarity among the Kenyan isolates were no less than 99.0% for the N protein, NS protein, M polyprotein and L protein (Tables 5-8). The least amino acid similarity was observed with the two BUNV isolates, the Central African Republic BUNV_ArB28215 and the Australian BUNV_M11852 which were less than 93% identical in the N and Ns proteins (Tables 5 and 6).

Table 5: Table Ss protein nucleotide/amino acid sequence identities among selected Bunyamwera serogroup viruses. Nucleotide and amino acid sequence identities are shown below and above the diagonal, respectively. Comparison of nucleotide sequences is based on the complete S segment (Kenyan isolates in this study are bolded).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	**GSA/TS7/5170_WT**		100	100	100	100	100	100	100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
2	**ISL/TS2/5242/WT**	100		100	100	100	100	100	100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
3	**TND/S1/19801/WT**	100	100		100	100	100	100	100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
4	NRIV_DakArD28542	99.6	99.6	99.6		100	100	100	100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
5	NRIV_SUD-HKV141	99.6	99.6	99.6	99.3		100	100	100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
6	NRIV_SUD-HKV66	99.6	99.6	99.6	99.3	100.0		100	100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
7	NRIV_9800521	100	100	100	99.6	99.6	99.6		100	100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
8	NRIV_9800535	100	100	100	99.6	99.6	99.6	100		100	99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
9	**MGD/S1/12060/WT**	100	100	100	99.6	99.6	99.6	100	100		99.0	100	100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
10	**GSA/S4/11232/WT**	99.6	99.6	99.6	99.3	99.3	99.3	99.6	99.6	99.6		99.0	99.0	99.0	85.1	92.0	94.0	88.1	88.1	87.1	87.1	87.1	87.1	93.0
11	BUNV_(NC_001927)	100	100	100	99.6	99.6	99.6	100	100	100	99.6		100	100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
12	BUNV_BLCNNS_	100	100	100	99.6	99.6	99.6	100	100	100	99.6	100		100	86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
13	BUNV_ArB29051	100	100	100	99.6	99.6	99.6	100	100	100	99.6	100	100		86.1	93.0	95.0	89.1	89.1	88.1	88.1	88.1	88.1	94.0
14	BUNV_(AF325122)	92.7	92.7	92.7	93.0	93.0	93.0	92.7	92.7	92.7	92.4	92.7	92.7	92.7		83.1	84.1	95.0	93.0	94.0	94.0	94.0	94.0	84.1
15	BUNV_ArB28215_	94.3	94.3	94.3	94.0	94.0	94.0	94.3	94.3	94.3	94.0	94.3	94.3	94.3	88.7		91.0	85.1	84.1	84.1	84.1	84.1	84.1	89.1
16	BATV_MS50_	95.7	95.7	95.7	95.3	95.3	95.3	95.7	95.7	95.7	95.3	95.7	95.7	95.7	89.4	92.7		85.1	87.0	86.0	86.0	86.0	86.0	93.0
17	BATV_UgMP-6830	93.0	93.0	93.0	93.3	93.3	93.3	93.0	93.0	93.0	92.7	93.0	93.0	93.0	97.6	89.1	89.4		98.0	99.0	97.0	99.0	99.0	85.1
18	BATV_Chittoor/IG-20217	92.0	92.0	92.0	92.4	92.4	92.4	92.0	92.0	92.0	91.7	92.0	92.0	92.0	96.0	88.4	90.3	98.3		99.0	97.0	99.0	99.0	87.0
19	BATV_MM2222	91.4	91.4	91.4	91.7	91.7	91.7	91.4	91.4	91.4	91.0	91.4	91.4	91.4	96.0	88.7	90.3	98.3	99.3		98.0	100.0	100	86.0
20	BATV_Italy-2009	91.0	91.0	91.0	91.4	91.4	91.4	91.0	91.0	91.0	90.7	91.0	91.0	91.0	95.0	89.1	89.6	96.6	97.6	97.6		98.0	98.0	86.0
21	BATV_ON-7/B/01	91.7	91.7	91.7	92.0	92.0	92.0	91.7	91.7	91.7	91.4	91.7	91.7	91.7	96.3	88.4	90.0	98.6	99.6	99.6	98.0		100	86.0
22	BATV_ON-1/E/94	92.4	92.4	92.4	92.7	92.7	92.7	92.4	92.4	92.4	92.0	92.4	92.4	92.4	96.6	88.4	90.6	98.6	99.0	99.0	97.3	99.3		86.0
23	ILEV_ILESHA/8e	95.7	95.7	95.7	95.3	95.3	95.3	95.7	95.7	95.7	95.3	95.7	95.7	95.7	90.0	91.4	94.6	90.0	91.0	90.3	90.0	90.6	90.6	

Table 6: N protein nucleotide/amino acid sequence identities among selected Bunyamwera serogroup viruses. Nucleotide and amino acid sequence identities are shown below and above the diagonal, respectively. Comparison of nucleotide sequences is based on the complete S segment (Kenyan isolates in this study are bolded).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	**GSA/TS7/5170_WT**		100	100	99.5	100	100	99.5	99.5	100	100	100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
2	**ISL/TS2/5242/WT**	100		100	99.5	100	100	99.5	99.5	100	100	100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
3	**TND/S1/19801/WT**	100	100		99.5	100	100	99.5	99.5	100	100	100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
4	NRIV_DakArD28542	99.1	99.1	99		99.5	99.5	99.1	99.1	99.5	99.5	99.5	99.5	99.1	93.1	91.4	90.1	93.1	92.7	92.2	93.1	92.7	93.1	93.9
5	NRIV_9800535	99.1	99.1	99	99.7		100	99.5	99.5	100	100	100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
6	NRIV_9800521	98.9	98.9	99	99.5	99.8		99.5	99.5	100	100	100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
7	NRIV_SUD-HKV141	98.9	98.9	99	99.2	99.2	99.1		100	99.5	99.5	99.5	99.5	99.1	93.1	91.4	90.1	93.1	92.7	92.2	93.1	92.7	93.1	93.9
8	NRIV_SUD-HKV66	98.5	98.5	99	99.1	99.1	98.9	99.5		99.5	99.5	99.5	99.5	99.1	93.1	91.4	90.1	93.1	92.7	92.2	93.1	92.7	93.1	93.9
9	**MGD/S1/12060/WT**	98.2	98.2	98	98.2	98.2	98.1	97.8	97.7		100	100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
10	**GSA/S4/11232/WT**	97.9	97.9	98	97.9	97.9	97.8	97.5	97.4	99.7		100	100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
11	BUNV_(NC_001927)	95.8	95.8	96	95.5	95.5	95.7	95.7	95.5	96.4	96.4		100	99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
12	BUNV_BLCNNS	95.8	95.8	96	95.5	95.5	95.7	95.7	95.5	96.4	96.4	100		99.5	92.7	91.8	90.5	92.7	92.2	91.8	92.7	92.2	92.7	94.4
13	BUNV_ArB29051_	98.1	98.1	98	98.1	98.1	97.9	97.7	97.5	99.5	99.5	96.5	96.5		92.2	91.8	90.1	92.2	91.8	91.4	92.2	91.8	92.2	93.9
14	BUNV_(AF325122)	86.4	86.4	86	85.9	85.6	85.5	86.1	85.6	85.9	85.9	85.9	85.9	86.1		87.9	89.2	98.2	97.8	97.4	97.4	97.8	97.8	90.9
15	BUNV_ArB28215_	86.9	86.9	87	86.8	87.1	87.2	86.5	86.8	87.5	87.5	87.8	87.8	87.5	83.5		86.6	87.9	87.5	87.9	88.8	87.5	87.9	88.8
16	BATV_MS50_	85.6	85.6	86	85.1	85.1	84.9	85.4	85.4	85.2	84.9	85.8	85.8	85.1	90.5	82.6		88.8	88.4	88.8	87.9	88.4	88.8	88.4
17	BATV_UgMP-6830	86.6	86.6	87	86.5	86.8	86.9	86.4	86.4	86.9	86.9	86.8	86.8	86.8	83.9	84.6	84.1		99.5	99.1	98.2	99.5	99.1	90.5
18	BATV_Chittoor/IG-20217	85.5	85.5	86	85.5	85.5	85.4	85.8	85.8	85.1	84.8	84.8	84.8	84.9	89.9	82.4	95.5	83.9		99.5	97.8	100	99.5	90.1
19	BATV_MM2222	85.2	85.2	85	84.9	84.9	84.8	85.2	85.2	84.5	84.2	83.8	83.8	84.1	89.5	82.4	94.7	83.4	97.5		97.4	99.5	99.1	89.6
20	BATV_Italy-2009	84.6	84.6	85	84.4	84.4	84.5	84.4	84.4	84.5	84.2	84.8	84.8	84.1	89.4	83.8	93.2	84.4	92.9	92.9		97.8	97.4	90.5
21	BATV_ON-7/B/01	85.1	85.1	85	85.4	85.4	85.2	85.4	85.1	84.6	84.4	84.2	84.2	84.2	90.1	82.4	94.7	83.5	97.7	97.2	92.8		99.5	90.1
22	BATV_ON-1/E/94	85.9	85.9	86	85.9	85.9	85.8	86.2	86.2	85.8	85.5	84.9	84.9	85.4	89.6	82.5	94.4	84.4	96.5	97.2	92.7	95.8		89.6
23	ILEV_ILESHA/8e	89.8	89.8	90	89.8	90.1	90.2	89.9	90.1	89.2	89.2	88.9	88.9	89.4	83.4	86.5	84.2	83.9	83.1	82.8	82.6	82.2	82.6	

Table 7: M polyprotein nucleotide/amino acid sequence identities among selected Bunyamwera serogroup viruses. Nucleotide and amino acid sequence identities are shown below and above the diagonal, respectively. Comparison of nucleotide sequences is based on the complete M segment (Kenyan isolates in this study are bolded).

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1	**GSA/TS7/5170_WT**		100.0	99.9	98.8	98.7	98.6	98.7	98.6	63.8	63.8	63.5	63.5	61.2	95.2	97.8	95.0	94.9	63.6	95.3	93.1	68.6
2	**ISL/TS2/5242_WT**	99.9		99.9	98.8	98.7	98.6	98.7	98.6	63.8	63.8	63.5	63.5	61.2	95.2	97.8	95.0	94.9	63.6	95.3	93.1	68.6
3	**TND/S1/19801_WT**	99.9	99.9		98.7	98.6	98.5	98.6	98.5	63.7	63.7	63.4	63.4	61.1	95.1	97.7	94.9	94.9	63.6	95.2	93.0	68.6
4	NRIV_DakArD28542	97.4	97.4	97.4		99.7	99.6	98.7	98.6	63.7	63.7	63.5	63.5	61.1	95.4	98.6	95.5	95.3	63.8	95.7	93.4	68.9
5	NRIV_SUD-HKV141	96.9	96.9	96.9	99.0		99.7	98.6	98.5	63.8	63.8	63.5	63.5	61.2	95.5	98.7	95.6	95.4	63.9	95.8	93.5	69.0
6	NRIV_SUD-HKV66	96.9	97.0	96.9	99.1	99.5		98.5	98.3	63.7	63.7	63.4	63.4	61.0	95.2	98.4	95.3	95.1	63.8	95.5	93.2	68.8
7	NRIV_9800521	97.7	97.7	97.7	97.9	97.4	97.4		99.5	63.8	63.8	63.4	63.4	61.2	94.9	97.7	95.1	94.8	63.6	95.3	93.1	68.6
8	NRIV_9800535	97.6	97.6	97.6	97.8	97.3	97.4	99.3		63.9	63.9	63.5	63.5	61.2	94.8	97.6	94.9	94.8	63.6	95.1	93.2	68.6
9	**MGD/S1/12060_WT**	64.0	64.0	63.9	64.0	63.9	63.9	63.9	63.8		99.9	98.1	98.1	95.4	64.6	63.9	64.4	64.6	78.6	64.3	64.4	63.3
10	**GSA/S4/11232_WT**	64.0	64.0	64.0	64.0	63.9	63.9	64.0	63.9	99.9		98.2	98.2	95.5	64.6	63.9	64.4	64.6	78.6	64.3	64.4	63.2
11	BUNV_(NC_001926)	63.9	63.9	63.9	63.9	63.7	63.7	63.7	63.7	95.7	95.7		100.0	94.4	64.2	63.7	64.0	64.2	78.5	63.9	64.1	62.8
12	BUNV_(M11852)	63.9	63.9	63.9	63.9	63.7	63.7	63.7	63.7	95.7	95.7	100.0		94.4	64.2	63.7	64.0	64.2	78.5	63.9	64.1	62.8
13	BUNV_(JF961341)	61.7	61.7	61.7	61.6	61.5	61.5	61.6	61.5	95.3	95.3	91.7	91.7		61.9	61.3	61.7	61.9	76.1	61.7	61.9	61.0
14	BATV_MM2222	88.9	88.9	88.8	89.5	89.2	89.3	88.7	88.6	64.6	64.6	64.1	64.1	62.0		96.0	98.1	97.1	64.3	98.1	93.3	69.2
15	BATV_UgMP-6830	94.7	94.7	94.7	96.4	96.0	96.1	95.0	94.9	64.0	64.1	63.8	63.8	61.5	90.3		95.8	95.8	64.1	96.2	93.8	68.9
16	BATV_ON-1/E/94	88.1	88.1	88.0	88.8	88.5	88.4	88.0	87.9	64.2	64.3	63.9	63.9	61.8	94.9	89.1		96.4	64.5	97.3	93.3	69.2
17	BATV_ON-7/B/01	88.0	88.0	87.9	88.6	88.5	88.6	87.9	88.0	64.2	64.3	64.0	64.0	61.7	93.5	89.3	91.9		64.0	98.1	93.1	68.8
18	BATV_MS50	63.8	63.8	63.8	63.8	63.8	63.8	63.8	63.7	72.8	72.8	72.7	72.7	70.0	64.4	63.9	64.2	64.4		64.0	64.5	64.3
19	BATV_Chittoor/IG-20217	88.7	88.7	88.7	89.5	89.2	89.3	88.7	88.7	64.4	64.4	64.1	64.1	61.9	95.4	89.9	93.4	96.4	64.5		93.3	68.8
20	BATV_Italy-2009	84.1	84.1	84.1	84.7	84.5	84.4	84.2	84.3	64.5	64.5	64.1	64.1	62.0	84.7	84.8	84.6	84.0	64.7	84.2		68.9
21	ILEV_ILESHA/8e	68.7	68.7	68.7	68.6	68.7	68.7	68.6	68.6	65.0	65.0	64.9	64.9	62.9	68.2	68.2	67.9	68.2	65.3	68.4	68.1	

Table 8: L protein nucleotide/amino acid sequence identities among selected Bunyamwera serogroup viruses. Nucleotide and amino acid sequence identities are shown below and above the diagonal, respectively. Comparison of nucleotide sequences is based on the complete L segment (Kenyan isolates in this study are bolded).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	**GSA/TS7/5170_WT**		99.8	100	99.4	99.1	99.3	99.4	99.3	99.1	99.2	99	99	81.8	81.5	82.7	81.8	81.5	91.8
2	**ISL/TS2/5242_WT**	99.5		99.8	99.3	99.1	99.2	99.3	99.2	98.9	99.1	98.8	98.8	81.9	81.6	82.7	81.9	81.5	91.7
3	**TND/S1/19801_WT**	100	99.5		99.4	99.1	99.3	99.4	99.3	99.1	99.2	99	99	81.8	81.5	82.7	81.8	81.5	91.8
4	NRIV_DakArD28542	97.7	97.7	97.7		99.4	99.5	99.6	99.5	99.4	99.5	99.3	99.3	81.6	81.4	82.6	81.6	81.3	91.6
5	NRIV_SUD-HKV141	97.3	97.3	97.3	98.6		99.6	99.2	99.1	99	99.1	99	99	81.6	81.4	82.7	81.6	81.3	91.8
6	NRIV_SUD-HKV66	97.3	97.4	97.3	98.7	99.4		99.3	99.2	99.1	99.2	99.1	99.1	81.8	81.5	82.7	81.8	81.5	91.7
7	NRIV_9800521	97.8	98	97.8	98.5	97.9	97.9		99.8	99.1	99.2	99	99	81.7	81.4	82.7	81.7	81.4	91.7
8	NRIV_9800535	97.7	97.9	97.7	98.4	97.8	97.8	99.6		99	99.1	98.9	98.9	81.6	81.3	82.6	81.6	81.3	91.7
9	**MGD/S1/12060_WT**	94.9	94.4	94.9	95.7	95.2	95.2	94.8	94.8		99.8	99.5	99.5	81.7	81.5	82.7	81.7	81.5	91.7
10	**GSA/S4/11232_WT**	95	94.5	95	95.7	95.3	95.3	94.9	94.9	99.9		99.6	99.6	81.7	81.4	82.7	81.7	81.5	91.8
11	BUNV_(NC_001925)	96	95.9	96	97.3	96.8	96.9	96.4	96.3	96.5	96.5		100	81.5	81.4	82.7	81.5	81.4	91.5
12	BUNV_(X14383)	96	95.9	96	97.3	96.8	96.9	96.4	96.3	96.5	96.5	100		81.5	81.4	82.7	81.5	81.4	91.5
13	BATV_MM2222	73.5	73.6	73.5	73.5	73.6	73.7	73.4	73.3	73.6	73.6	73.6	73.6		98.1	81.3	99.1	96.6	81.4
14	BATV_UgMP-6830	73.5	73.7	73.5	73.5	73.6	73.7	73.6	73.5	73.5	73.5	73.3	73.3	90.5		81.2	98.2	96.1	80.9
15	BATV_MS50	73.8	73.9	73.8	74.1	74.1	74.1	74	74	74	74	74.1	74.1	72.7	72.7		81.2	81	81.8
16	BATV_Chittoor/IG-20217	73.4	73.4	73.4	73.5	73.5	73.7	73.3	73.3	73.5	73.4	73.5	73.5	95.7	90.4	72.7		96.6	81.1
17	BATV_Italy-2009	73.6	73.7	73.6	73.6	73.6	73.7	73.5	73.5	73.8	73.8	73.7	73.7	85.9	86.1	72.8	85.8		80.9
18	ILEV_ILESHA/8e	81.4	81.3	81.4	81.6	81.5	81.4	81.3	81.3	81.3	81.3	81.6	81.6	73.7	73.9	74.5	73.5	74.3	

Table 9: Between group mean distance comparisons for the S segment of selected virus isolates included in this study.

	Virus Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	**Batai**		0.352	0.341	0.400	0.684	0.383	0.231	0.160	0.336	0.308	0.377	0.308	0.350	0.693
2	**Batai_MS50**	0.163		0.269	0.327	0.684	0.316	0.327	0.324	0.265	0.281	0.311	0.283	0.301	0.694
3	**Bunyamwera**	0.152	0.130		0.217	0.654	0.274	0.311	0.338	**0.056**	0.082	0.238	0.085	0.237	0.670
4	**Bozo**	0.196	0.177	0.109		0.653	0.255	0.398	0.401	0.240	0.265	0.278	0.263	0.240	0.663
5	**Bunyamwera_PE-7.0014**	0.351	0.371	0.353	0.364		0.679	0.684	0.715	0.671	0.684	0.675	0.676	0.679	**0.189**
6	**Bunyamwera_ARB28215**	0.175	0.153	0.123	0.123	0.354		0.347	0.353	0.270	0.281	0.256	0.291	**0.199**	0.663
7	**BUNV_M11852**	**0.098**	0.159	0.139	0.196	0.363	0.163		**0.243**	0.311	0.301	0.346	0.310	0.337	0.689
8	**Calovo**	0.072	0.156	0.151	0.193	0.361	0.162	**0.106**		0.340	0.335	0.385	0.338	0.328	0.709
9	**Bunyamwera_(this_study)**	0.152	0.129	**0.021**	0.117	0.356	0.123	0.139	0.151		0.051	0.242	0.058	0.250	0.686
10	**Ngari_(this_study)**	0.145	0.131	0.031	0.126	0.363	0.129	0.134	0.150	**0.019**		0.237	**0.019**	0.255	0.689
11	**Ilesha**	0.175	0.168	0.113	0.153	0.375	0.139	0.170	0.178	0.113	0.110		0.234	0.233	0.665
12	**Ngari**	0.147	0.133	0.034	0.126	0.361	0.130	0.141	0.151	0.022	0.012	0.108		0.253	0.691
13	**Nyando**	0.160	0.156	0.101	0.127	0.361	**0.099**	0.153	0.147	0.104	0.106	0.113	0.109		0.658
14	**Wyeomyia**	0.363	0.384	0.366	0.370	**0.069**	0.353	0.371	0.365	0.371	0.371	0.375	0.373	0.364	

NB: Mean distances in bold represent lowest mean distance between virus groups

2.4 DISCUSSION

In this chapter, we present phylogenetic analysis of full coding sequences of three NRIV and two BUNV isolates originating from diverse regions of Kenya and from different vector and potential host species (Ochieng et al. 2013, Lutomiah et al. 2014). We also investigate whether genetic diversity is correlated with year, geographic location and vector species of isolation.

Our data seems to support the hypothesis of a geographic/temporal association among NRIV isolates evidenced by phylogenetic analyses of the three genomic segments generally grouping isolates from the same region and collection year together, although there were some exceptions to this grouping. We observed a temporal/geographic variation in the rate of evolution of the L and M segment between the NRIV isolates. The 1979 NRIV_DakArD28542 prototype virus from Senegal was closest to the last common ancestor followed by the 1988 Sudan isolates (NRIV_SUD_HKV141 and NRIV_SUD_HKV66), the 1998 Kenya-Somali isolates (NRIV_9800521 and NRIV_9800535) which also mimics the geographical distance between these regions (Figure 10). The temporal/geographic clustering rather than by source of isolation is further supported by the observation of the 1988 NRIV isolates from Sudan (SUD-HKV66 and SUD-HKV141) and the 1998 Kenya-Somali border isolates (NRIV_9800535 and NRIV_9800521) clustering separately for all segments despite isolation from human hosts. However, we do not have information on how these humans, who are likely dead end hosts, acquired these infections. Temporal clustering is further supported by the 2011 Mauritania isolate (NRIV_Adrar) clustering closely with the NRIV isolates in the current study despite being isolated from diverse regions. However, it is possible this isolate may have been introduced into Mauritania from the East African region through infected livestock. Modelling of Rift Valley fever infections suggest movement of Rift Valley fever virus (RVF) virus from the East African region to Senegal and Mauritania possibly due to animal trade (Soumare et al. 2012).

The S segment did not display a similar pattern as observed for the L and M segments and this may be related to difference in mutation rates between the various segments. The slow evolution of the S

segment is corroborated by the highly conserved amino acid sequences observed for the N and Ns protein in the current study. NRIV isolates from different regions; Kenya, Kenya-Somali border, Sudan and Senegal clustered separately, distinct for each region of isolation. The Kenyan NRIV isolates in the current study clustered closest to the 1998 NRIV isolates (NRIV_9800521 and NRIV_9800535) from the Kenya-Somali border indicating that the current Kenyan NRIV isolates may have been introduced into the various regions in Kenya from the Kenya-Somali border. The amino acid positions with substitutions unique to the Kenyan isolates in both the M polyprotein and L protein may represent geographical/temporal motifs. This is given further credence by unique substitutions identified for the Kenyan NRIV isolates in the current study and the two 1998 NRIV isolates mentioned above and confirmed by phylogenetic analysis.

Phylogenetic analyses of BUNV isolates were hampered by the lack of complete sequences for all segments. However, there was evidence of geographic clustering for example the Kenyan BUNV isolates in the current study shared unique amino acid substitutions with the previously isolated 2011 Kenyan BUNV isolate (BUNV_AMH001130 (JF961341) from the same region when compared with the BUNV prototype from Uganda. The current Kenyan BUNV isolates in the current study clustered closest to the 1994 Central African Republic BUNV_ArB29051 but divergent from the Australian isolate BUNV_M11852. Similar observations have been made for BATV in which correlation between geographic and genetic diversity has been suggested (Jost et al. 2011, Huhtamo et al. 2013). BATV isolates from Europe, Asia and Africa generally cluster independently within strongly supported groups with country specific viruses clustering closely (Huhtamo et al. 2013). This was more or less similar for the BUNV isolates where the S segment of the Australian isolate BUNV_M11852, was divergent from the Kenyan, Central African Republic and Ugandan BUNV isolates and clustered more closely with BATV and Calovo virus isolates. An exception was the 1992 Central African Republic isolate BUNV_ARB28215 which was divergent from the other African isolates and clustered closest to Nyando and Bozo virus isolates. Similar observation for this isolate has been reported in a previous study (Yandoko et al. 2007) suggesting

that it may have been erroneously named BUNV since, unlike the other African isolates, BUNV_ARB28215 has a shorter non-coding region and may probably be a different virus. Similarly, for the M segment, the Kenyan BUNV isolates sequenced in the current study formed a monophyletic clade with the 2011 Kenyan isolate, BUNV_AMH001130, divergent from the prototype virus from Uganda. The 1979 NRIV prototype virus NRIV_DakArD28542 from Senegal is at the base of all other NRIV isolates in all three genomic segments. BUNV_PE-7.0014 isolate from Peru clusters closest to Wyeomyia virus of the California serogroup as previously reported by Mores et al (Mores et al. 2009), distinct from the African BUNV isolates analyzed in the current study. This is further supported by the short distance observed between the two viruses in the p-distance analysis of both nucleotide and amino acid sequences.

Our data did not support the existence of distinct virus isolates circulating in specific host species, for example, we did not find any major differences in the NRIV isolates from mosquito and tick vectors indicating that the same virus strain may be circulating within northern Kenya in both ticks and mosquitoes and diverse vertebrate host species. Similarly, we did not identify significant differences in the BUNV isolates from different mosquito genus and species. While RNA viruses have the potential to undergo sequence changes due to their plasticity and high error rate of their RNA polymerases (Woolhouse et al. 2001, Kassen 2002), our isolates may represent the consensus genotype of the virus. Such consensus genotypes arise from the different selective pressures encountered within the different host systems. Alternatively, ticks, (from which NRIV was isolated) may have fed on viremic host and may not be competent vectors for NRIV. This calls for tick vector competence studies for NRIV to be undertaken.

In summary, our analysis suggests a rough geographic/temporal association of NRIV isolates and does not support any evolutionary clustering of viral isolates based upon the host source of isolation of the virus; viral isolates from mosquitoes, ticks or humans sources did not group together. Data from this study therefore do not support the existence of distinct virus isolates circulating in specific host species. There is a need to sequence all three segments of available isolates to correctly

categorize them. It is likely that BATV_MS50 (JX846604) is misclassified as a BATV as observed by recent studies (Dilcher et al. 2013, Huhtamo et al. 2013). It would be of interest to also re-look at the BUNV isolate from Central African Republic, BUNV ArB28215 (AM711130) which is distinct from the prototype virus in our study as well as Yandoko et al, (Yandoko et al. 2007), where it clustered more closely with Bozo virus. Likewise, the Australian BUNV isolate, BUNV_M11852 and Peru isolate BUNV_PE-7.0014 need to be re-considered given their association with BATV and Wyeomyia virus, respectively.

Despite the relative slow rate of evolution of these viruses, the possibility of genetic shift and drift remains real with continued isolation of orthobunyaviruses from previously unknown vectors and hosts such as ticks (Lwande et al. 2013) and birds (Tauro et al. 2009). While there was evidence of a a geographic/temporal correlation with genetic diversity, a more in depth analysis including identification of signature motifs that might be representative of a given geographic clade is restricted by limited full length sequences of these viruses. Furthermore there is need for more studies on pathogenesis and wider distribution of these viruses. While public health efforts have focused on well characterized viruses such as RVF, WNV, chikungunya and dengue viruses, the emergence of Orthobunyaviruses such as NRIV and Schmallenbergvirus as human and veterinary pathogens emphasizes there is need for in-depth characterization of these viruses and determination of their true public health impact.

Chapter 3

GENOME SEQUENCE ANALYSIS OF *IN VITRO* AND *IN VIVO* PHENOTYPES OF BUNYAMWERA AND NGARI VIRUS ISOLATES FROM NORTHERN KENYA.

3.1 INTRODUCTION

BUNV is the prototype virus of the *Orthobunyavirus* genus of the *Bunyaviridae* family of arboviruses. The virus is associated with febrile illness with headache, arthralgia, rash and infrequent central nervous system involvement (Gonzalez and Georges 1988). While viruses of the *Orthobunyavirus* genus are known to cause human disease, they were previously not associated with hemorrhagic manifestations. However, NRIV has been implicated in recent outbreaks of hemorrhagic fevers in Kenya and Somalia (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). NRIV is thought to have arisen through genetic reassortment between bunyaviruses co-circulating in the same environment (Gerrard et al. 2004).

Like other viruses within the *Bunyaviridae* family, the BUNV genome consists of three negative-sense RNA segments that employ a variety of coding strategies leading to generation of a limited set of structural and non-structural proteins (Elliot et al. 2000, Schmaljohn and Hooper 2001). The L (large) segment encodes a large protein that comprises the RNA-dependent RNA polymerase for replication and transcription of genomic RNA segments. The M (medium) segment encodes a precursor polypeptide which yields the viral surface glycoproteins Gn and Gc and a nonstructural protein NSm, and the S (small) segment encodes the nucleocapsid (NC) and a nonstructural protein (NSs) in overlapping reading frames (Schmaljohn and Hooper 2001). The prevalence of members of the *Bunyaviridae* family are likely underestimated because of lack of detection tools arising partly from their high level of diversity, limited phenotypic and genetic characterization and segmented nature of their genome.

Orthobunyaviruses are mostly isolated and amplified in interferon defective African green monkey kidney epithelial Vero cell line that may result in mutations yielding substrains that are

phenotypically different from the parental wild type virus (Sundstrom et al. 2011). Such observations have been reported among other viruses of the family *Bunyaviridae*, including Puumala virus where the large plaque (LP) grows to higher titers than the small plaque (SP) and the parental wild type (WT) virus in interferon defective cells but no difference in growth when interferon competent cells were used (Sundstrom et al. 2011). Genome sequencing analysis revealed differences at two positions in the NC protein and two positions in the L protein (Sundstrom et al. 2011). Attenuation both *in vivo* and *in vitro* has also been observed for the SP phenotype of West Nile virus (WNV) (Davis et al. 2004). Attenuated pathogenesis has also been reported for dengue and Japanese encephalitis virus in mice experiments (Eastman and Blair 1985, Blaney Jr et al. 2001, Blaney Jr et al. 2002, Blaney Jr et al. 2003). Thus, understanding the mutation distribution in a heterogeneous arbovirus population is important, given that any variant can be favored by selection which ultimately affects fitness. We hypothesize that natural mutations may accumulate during passage of BUNV and NRIV isolates, obtained from entomological surveillance exercises in Kenya (Ochieng et al. 2013). Such mutations may yield substrains with genotypic and phenotypic differences between each other and with the parental WT strains (likely composed of several different substrains). In analyzing the viral phenotypes, we studied the kinetics of replication following infection of Vero cells. Additionally, we studied pathogenesis of viral strains after peritoneal inoculation of mice. We also sequenced the coding regions of the three RNA segments to determine whether difference in phenotype is associated with particular mutations in the genome. We report that BUNV and NRIV substrains compared to each other and to the parental wild type display contrasting phenotypes.

3.2 METHODS

3.2.1 Ethics statement

The study protocol (number SSC 2677) was approved by the Animal Care and Use Committee of the Kenya Medical Research Institute and by the Animal Ethics Committee of the University of Pretoria (Protocol number H012-13). All animal experiments were carried out in accordance with

the regulations and guidelines of the Kenya Medical Research Institute and University of Pretoria Animal Ethics Committees.

3.2.2 Virus stock preparation

The site in Kenya and vector species from which the 5 virus isolates used in the study were obtained is summarized in Table 10.

Table 10: Virus isolates obtained from diverse geographical regions and species in Kenya.

Specimen identity	**Specimen code**	**Isolation site**	**Isolation date**	**Mosquito/tick species**	**Passage history**
Bunyamwera viruses	GSA/S4/11232	Garissa	2009	*Aedes mcintoshi*	Vero 3
	MGD/S1/12060	Magadi	2010	*Anopheles funestus*	Vero 3
Ngari viruses	TND/S1/19801	Tana-delta	2011	*Anopheles funestus*	Vero 3
	GSA/TS7/5170	Garissa	2009	*Amblyomma gemma*	Vero 3
	ISL/TS2/5242	Isiolo	2009	*Rhipicephalus pulchellus*	Vero 3

Vero cells (ATCC® CCL-81™) were grown in T-75 culture flasks containing Eagle's minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (Gibco-BRL), 2% L-glutamate (Sigma) and 2% penicillin/streptomycin (Gibco-BRL). Confluent cells were rinsed with sterile phosphate buffered saline, and 0.1 mL clarified homogenate of field collected mosquitoes were added followed by incubation at 37^{o}C for one hour to allow virus adsorption with constant rocking. After incubation, maintenance medium (MEM with Earle's salts, 2% FBS, 2% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B) was added and cells

incubated at 37^{o}C and observed daily for CPE. Each isolate was grown individually to avoid cross-contamination and supernatants were harvested when approximately 75% of the cells exhibited CPE. The culture supernatants were aliquoted and stored at -80^{o}C until used. The stock concentrations were determined by plaque assay titration as described below.

3.2.3 Plaque assay and purification

Vero cells were seeded on 6 well plates and incubated in a humidified CO_2 incubator at 37^{0}C overnight before use. The cells were used when they attained 75–90% confluence. Ten-fold dilutions of the virus isolates were made in maintenance media. Media was carefully aspirated from the cells on the 6-well plates using sterile transfer pipettes and 100 µl of the appropriate viral dilution added to each of duplicate wells of 6-well plates with gentle rocking to evenly distribute the virus. Plates were incubated at 37^{o}C for 1 hour after which media was carefully aspirated and 3 ml of 1.25% methylcellulose solution gently added to each plate. Plates were placed in a CO_2 humidified incubator and incubated for 5 days. Development of plaques was monitored by visualization under an inverted microscope (Leica DM IL LED). To facilitate visualization of plaques, methylcellulose solution (Sigma Aldrich) was carefully aspirated using transfer pipette followed by fixation in 10% formaldehyde (Sigma Aldrich) after which plates were stained with crystal violet solution (Sigma Aldrich).

Plaque purification

The LP and SP phenotypes of isolates GSA/S2/11232WT and TND/S4/19801WT (previously passaged 3 times on Vero cells) with a titer of 1x10^{9} PFU/ml were diluted in maintenance media to approximately 10 PFU/ml. Then, confluent Vero cells in 24-well plates were infected with 100 µl of diluted virus per well. After adsorption for 1 h at 37°C, the cells were overlaid with 1 ml of 1.25% methylcellulose. Five days later, the methylcellulose medium was aspirated and pasture pipettes used to pick plaques from wells with single plaques and placed in 500ul of maintenance media, then propagated on Vero cells and the procedure was repeated twice more, without intermediate

amplification, for each of the plaque isolates. The purified isolates were then amplified by propagation on confluent Vero cells and then frozen at -80^{o}C until use.

3.2.4 *Invitro* growth kinetics

Our viral isolates including the parental WT, i.e. mixture of SP and LP, were used to infect 90% confluent monolayers of Vero cells at a multiplicity of infection of 0.01 and incubated for one hour to allow virus adsorption. Infected monolayers were washed twice with sterile PBS and overlaid with maintenance medium and incubated at 37^{0}C. An aliquot of tissue culture fluid (0.5 ml) was collected every 12 hours for the first 2 days and once on day 3 of infection, mixed 1:10 with maintenance media and frozen at -80^{0}C until use. Daily samples were titrated by plaque assay as described above (Section 3.2.3). The statistical package R (R Development Core Team 2008) was used for fitting exponential growth data using the Kruskal–Wallis test(Kruskal and Wallis 1952). The detection of correlated error structure in the growth curve data was carried out as follows; the log-transformed data was fit to linear mixed effects models using R, and an AR1 model was found to fit the data better than a repeated measures model.

3.2.5 Molecular characterization of plaque purified phenotypes

RNA isolation and cDNA synthesis

For RNA extraction, we used the MagNA Pure LC RNA Isolation Kit I (Roche Diagnostics, Indianapolis, IN). Complementary DNA (cDNA) was synthesised using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) with Random hexamers followed by PCR using Phusion High-Fidelity PCR Kit (Finnzyme OY, Espoo, Finland) and appropriate primers as described in section 2.2.3. Primers for each segment were either designed based on sequences of BUNV, BATV and NRIV available in GenBank or obtained from publications (Yanase et al. 2006, Jost et al. 2011) (Appendix 2). Amplified DNA fragments were visualized by electrophoresis on a 1.5% agarose gel. The Amplified DNA was purified and prepared for

sequencing using ExoSAP-IT PCR clean-up kit (USB Corp, Cleveland, OH) according to the manufacturer's instructions and stored at -20^{o}C.

Sequence analysis of viral genomes

Sequencing was performed using different sets of primers for the S, M and L segments as defined in section 2.2.3 above using Big Dye V3.1 kit (Applied biosystems) and injection on a 3500XL genetic analyser (Foster city, California, USA). The sequences obtained were cleaned and edited using Bioedit software (www.mbio.ncsu.edu/BioEdit/BioEdit.html, USA) for both the reads from the forward and reverse primers. Sequences obtained were compared to those in the gene bank using the Basic Local Alignment Search tool (BLAST) (Altschul et al. 1990) in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast) to identify similar sequences. The clean sequences of each segment of each phenotype were aligned against the corresponding segment sequences of the wild type virus isolate using Bioedit.

3.2.6 Clinical disease in mice

Pathogenicity of the plaque phenotypes was evaluated in Swiss Albino suckling (1-4 days old) and 6 week old mice obtained from the KEMRI animal house. To obtain the required sample size for the mice experiments, we assumed that equal sample sizes are required for BUNV and NRIV, respectively. Then to calculate the minimum number of mice required for each viral arm, the base sample size was estimated using the formula (Kirkwood and Sterne 2003):

$$n=\frac{\left(Z_{1-\alpha/2}\sqrt{2\bar{p}(1-\bar{p})}+Z_{1-\beta}\sqrt{p_n(1-p_n)+p_b(1-p_b)}\right)^2}{(p_n-p_b)^2}$$

Such that:

$$\bar{p}=(p_n-p_b)/2$$

n = minimum mice required per group

p_n & p_b= expected proportion of mice dying due to NRIVand BUNV infection, respectively.

$Z_{1-\alpha/2}$ & $Z_{1-\beta}$ = Z statistic for a level of significance and power, respectively.

Preliminary work as recorded in the international catalogue of arboviruses (http://wwwn.cdc.gov/arbocat/catalog-listing.asp?VirusID=79&SI=1) has shown that NRIV and BUNV can lead to 100% and 50% mortality of infected mice, respectively by day two of infection. Considering a critical value of 1.96 and a power of 84% the base sample size becomes 10.5 ≈ 11 mice per group or viral treatment.

Mice were inoculated intraperitoneally with 100µl of 10^9 PFU/ml of wild type or amplified plaque purified virus isolates (LP and SP) in maintenance media (Figure 15). As control, mice were injected with an equal volume of maintenance media. All mice were carefully observed twice daily up to 14 days for clinical disease which included characteristic tremors and hind-limb paralysis. Survival functions were graphed for the two sets of viruses. Pairwise comparisons of survival curves were made using the Wilcoxon-Breslow test to test for equality of survivor functions

Figure 15: Picture showing intraperitoneal inoculation of 1-4 day old suckling mice with experimental virus diluted in maintenance media.

3.3 RESULTS

3.3.1 Isolation and Purification of Plaque phenotypes

Plaque titration of NRIV and BUNV yielded plaques of two significantly distinct phenotypes, LP (Range: 0.88-1.21 mm) and SP (Range: 0.47-0.66 mm) (Figure 16). Each plaque phenotype was sub-cloned twice and purified each time by inoculation onto new Vero cells. The plaque phenotypes retained their plaque size after amplification by single passage in cell culture to generate viral stocks with high titers for onward experimentation. The cloned LP substrain produced larger plaques than the SP suggesting that the former was more efficiently replicated in Vero cells than the latter.

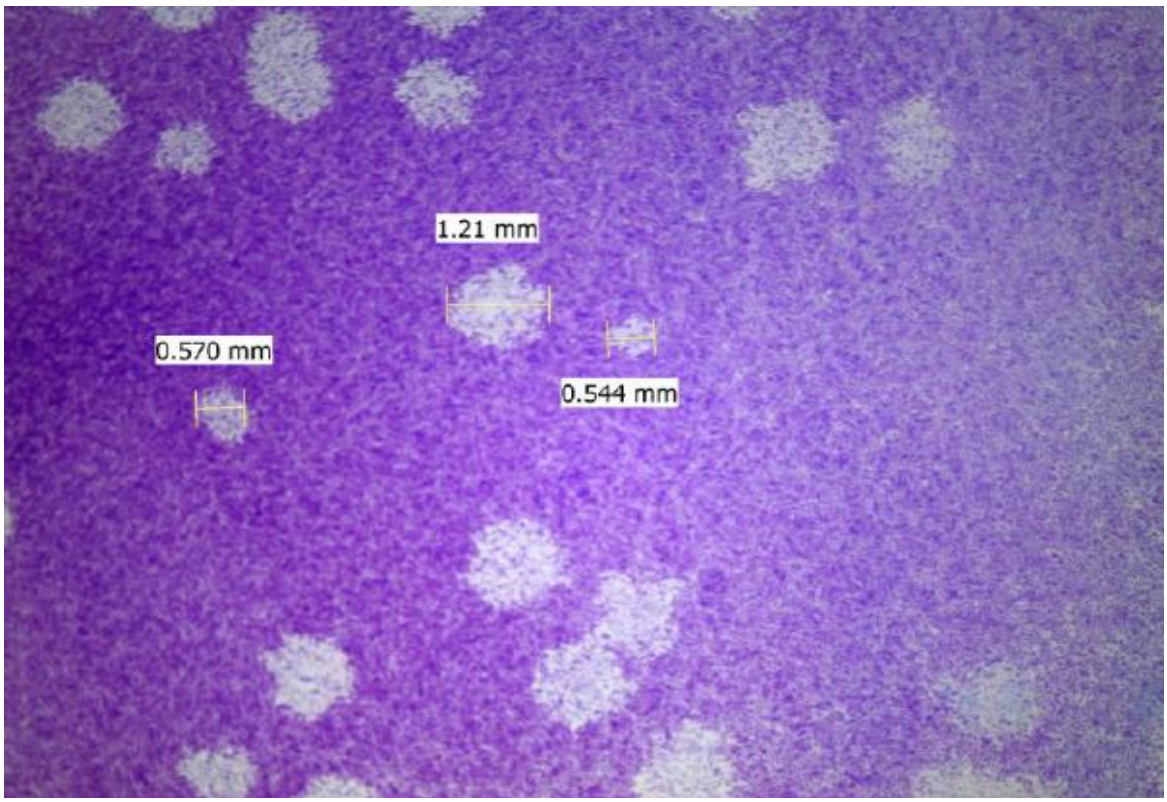


Figure 16: Photograph of crystal violet-stained Bunyamwera virus infected Vero cell monolayer showing plaque phenotypes.

3.3.2 *In vitro* growth curves

In general, the LP phenotype of BUNV isolates grew at a faster rate and to a significantly higher titre ($p=0.009$) than the SP phenotype by day 3 of infection (Figures 17 and 18). The BUNV WT phenotype reached approximately 5-logs higher than the virus titre of the SP and LP phenotypes by day 3 post-infection. However, the difference in growth of the SP and LP phenotypes was insignificant. BUNV WT isolates generally grew to a higher titre than NRIV WT isolates.

For the NRIV isolates (Figure 19-21), the difference in titre between the WT and plaque phenotypes at 3 days post-infection was not more than 1 log except for isolate GSA/TS7/5170 (Figure 20). However, the difference in titer between the WT and plaque variants was not significant.

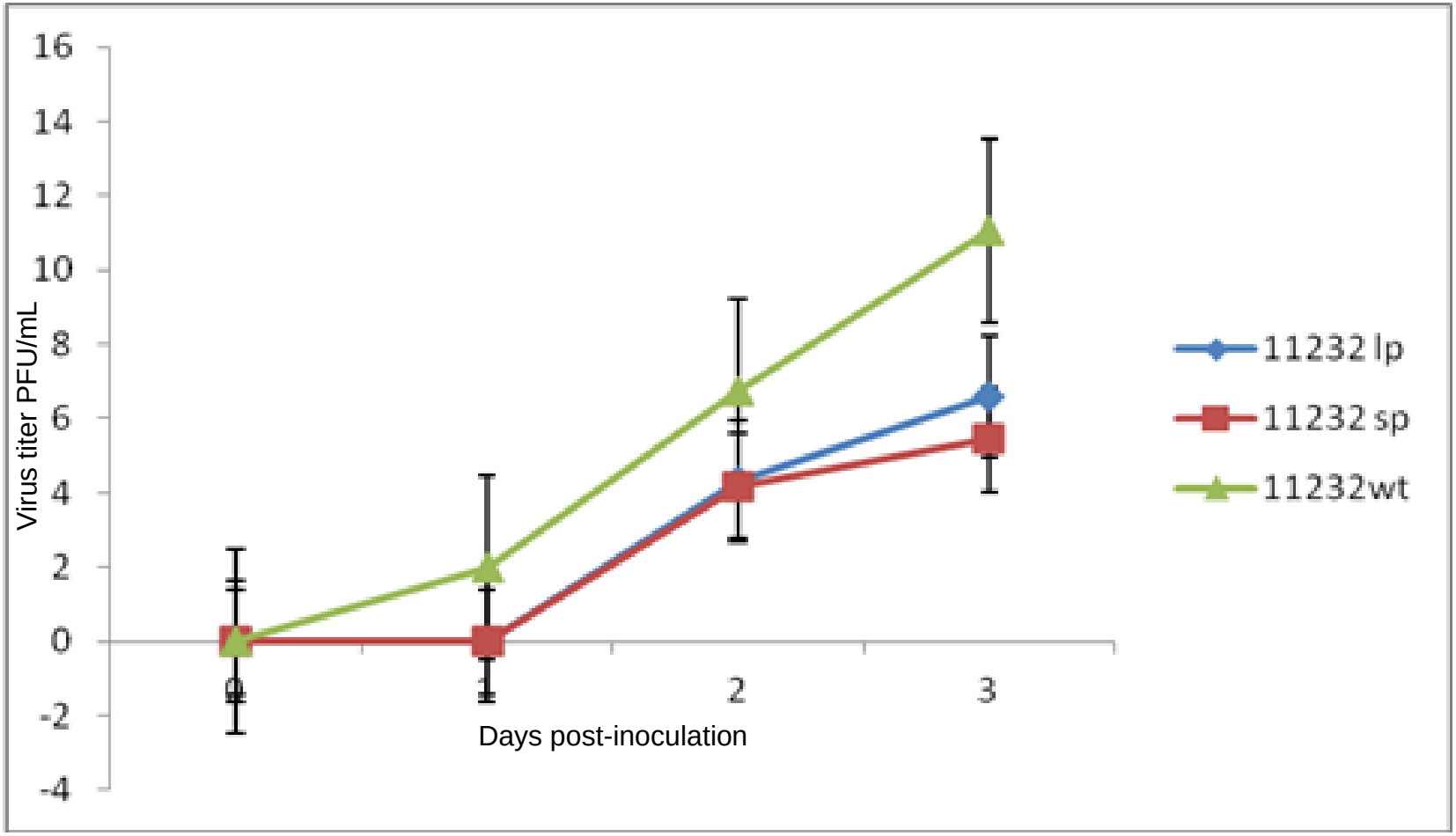


Figure 17: Growth kinetics of wild type parental and plaque purified phenotypes of Bunyamwera virus isolate GSA/S4/11232. There was a significant difference between the WT and the plaque phenotypes by day 3 post-inoculation.

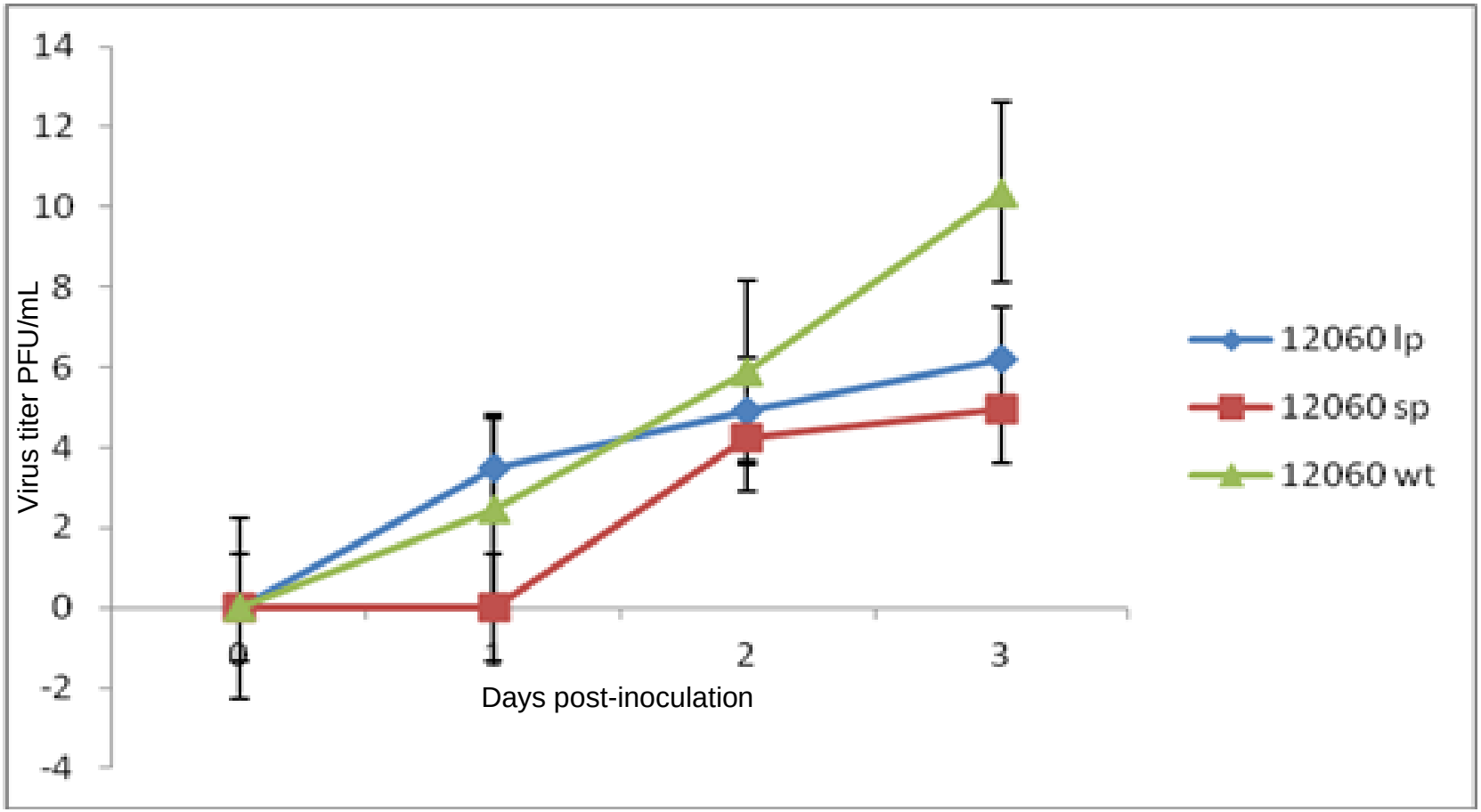


Figure 18: Growth kinetics of wild type parental and plaque purified phenotypes of Bunyamwera virus isolate MGD/S1/12060. There was a significant difference between the WT and the plaque phenotypes by day 3 post-inoculation.

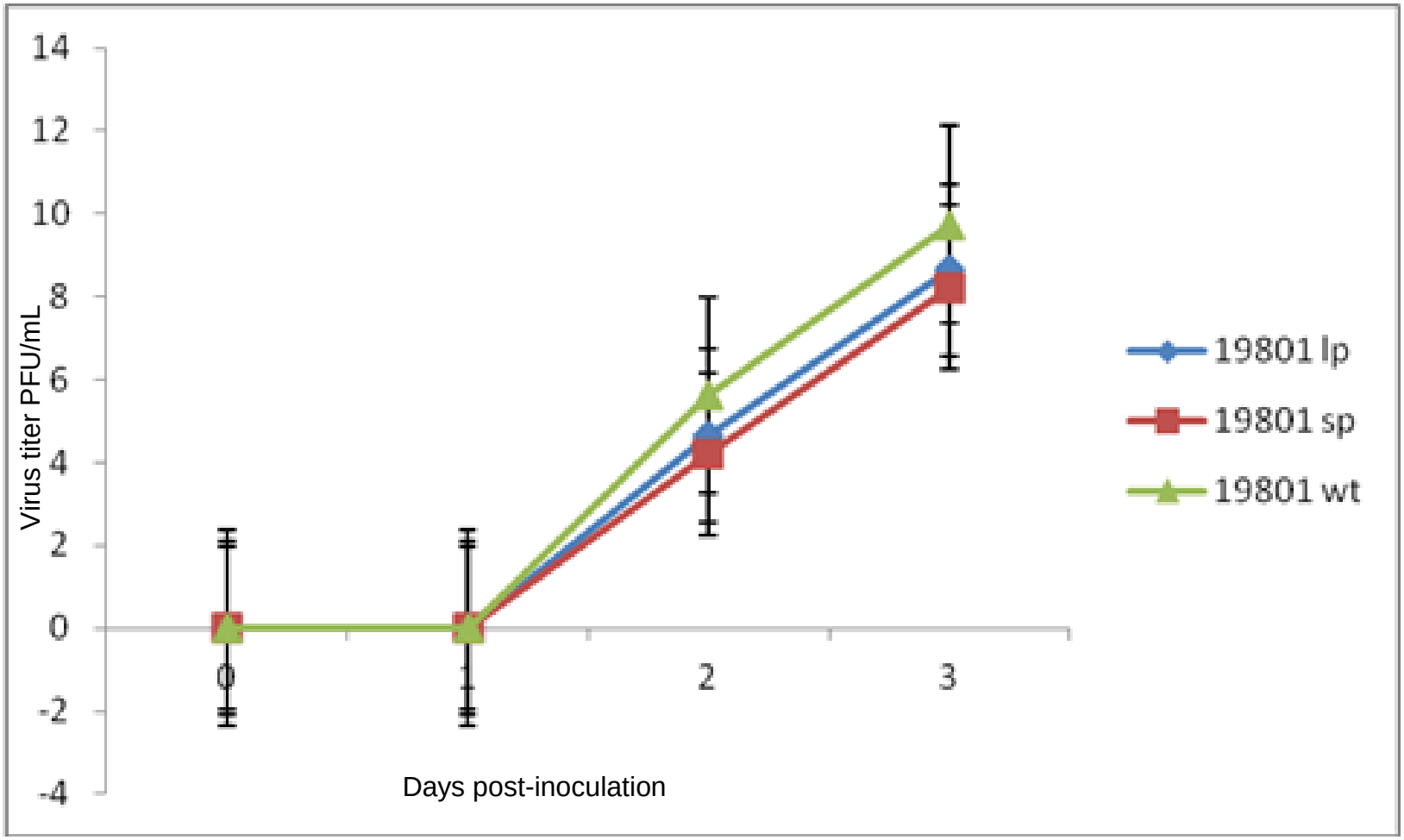


Figure 19: Growth kinetics of wild type parental and plaque purified phenotypes of Ngari virus isolate TND/S1/19801. There was no significant difference between the WT and plaque phenotypes at any time point.

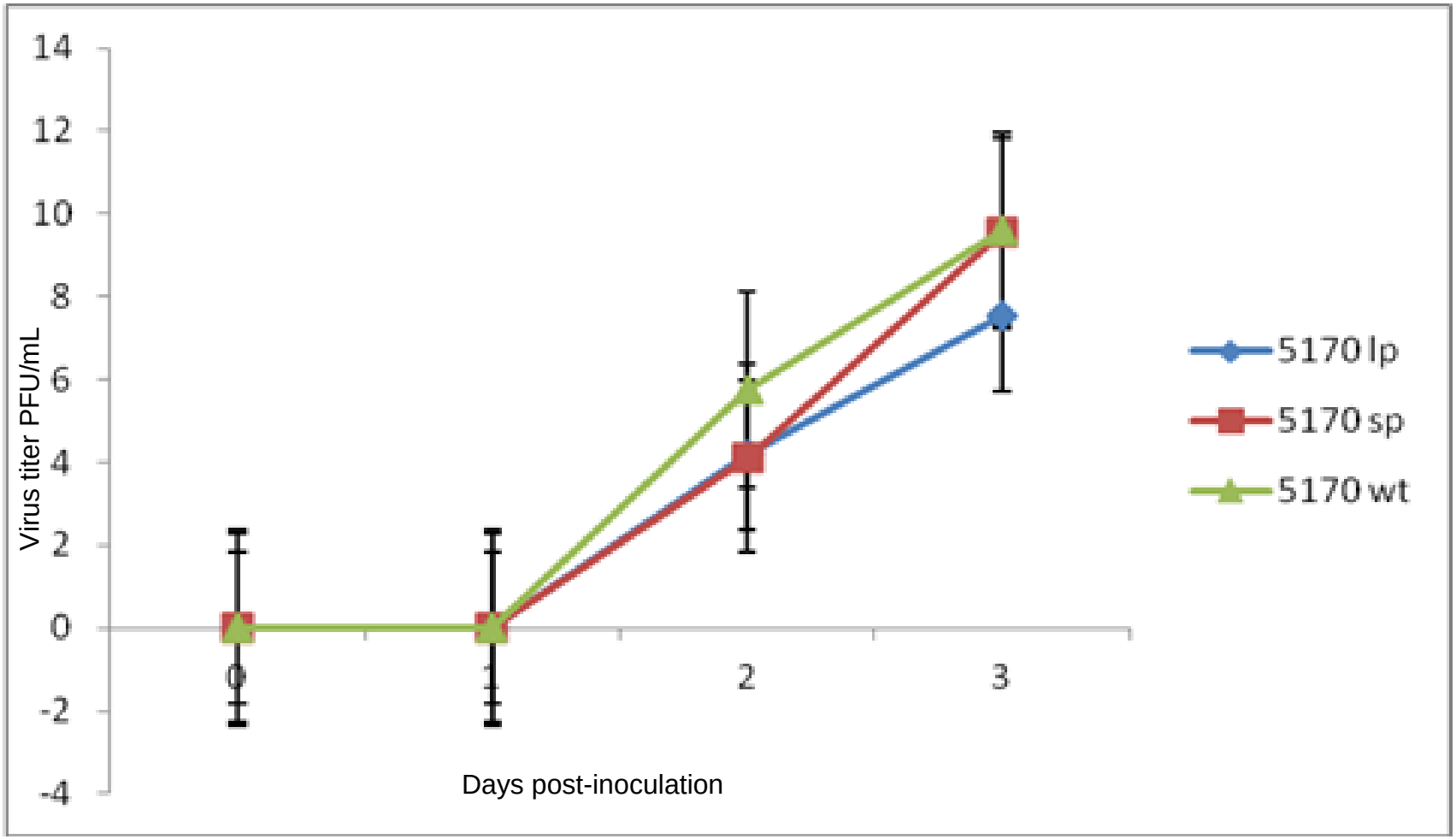


Figure 20: Growth kinetics of wild type parental and plaque purified phenotypes of Ngari virus isolate GSA/TS7/5170. There was no significant difference between the WT and plaque phenotypes at any time point.

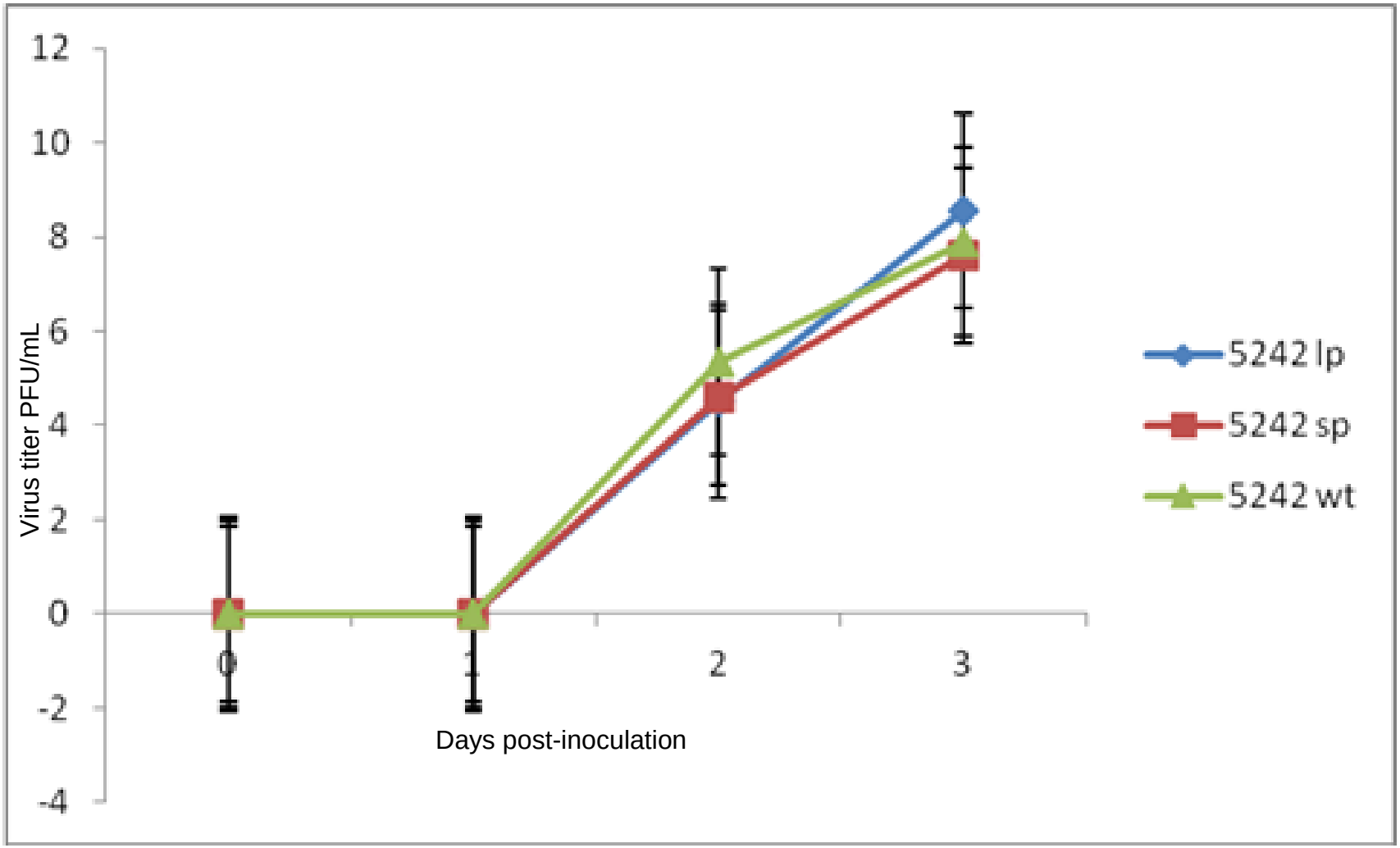


Figure 21: Growth kinetics of wild type parental and plaque purified phenotypes of Ngari virus isolate ISL/TS2/5242. There was no significant difference between the WT and plaque phenotypes at any time point.

3.3.3 Genetic characterization of plaque phenotypes

Comparison of the full genome nucleotide and amino acid sequences of low passage NRIV isolates revealed little or no divergence within the S segments (Table 11).

Table 11: Nucleotide differences between wild type parental and Plaque purified phenotypes of Bunyamwera and Ngari virus isolates.

Specimen identity	**Virus Phenotype**	**Passage history**	**Nucleotide (amino acid) substitution in indicated Bunyamwera/Ngari virus segment**		
			S	**M**	**L**
Bunyamwera viruses	**GSA/S4/11232SP**	Vero 7	G320C (G79R)	A1503G (K485E) C2009T	No changes
	GSA/S4/11232LP	Vero 7	T244C C559T	G3593A	G129A (A27T) T1523C C2623T (T858I)
	MGD/S1/12060SP	Vero 7	A803T C915T	C82T (A11V) G1503A (E485K) T2601C (S853P) G3423A (E1127K) T4229C	G278T A6099C (Q217K)
	MGD/S1/12060LP	Vero 7	A803T C915T	C1319T G1503A (E485K) T4229C	A6099C (Q217K)
Ngari viruses	**TND/S1/19801SP**	Vero 7	No changes	No changes	No changes
	TND/S1/19801LP	Vero 7	No changes	No changes	G300A (D84N)
	GSA/TS7/5170SP	Vero 7	No changes	T3272C	No changes
	GSA/TS7/5170LP	Vero 7	No changes	No changes	No changes
	ISL/TS2/5242SP	Vero 7	No changes	No changes	G869A G2741A G2993A
	ISL/TS2/5242LP	Vero 7	No changes	G3839A	No changes

* LP = large plaque; SP =small plaque

The N and NSs proteins of NRIV isolates were 100% conserved between the phenotypes. However, all BUNV isolate phenotypes exhibited nucleotide substitutions in all segments compared

to the WT except the L segment of isolate GSA/S4/11232SP. There was a single nucleotide change in the M segment of isolate GSA/TS7/5170SP and ISL/TS2/5242LP and three changes on the L segment of isolate ISL/TS2/5242SP. All these single nucleotide changes were synonymous. There were no changes in the nucleotide sequences of L segments of other NRIV isolates except isolate TND/S1/19801LP which had one nucleotide substitution resulting in a non-synonymous change in the amino acid sequence (D84N).

For the BUNV isolates, there were more transversions than transitions resulting in several non-synonymous codons. The 5' and 3' genome ends of the BUNV and NRIV phenotypes were highly conserved for all the three segments (Figures 22-24). All NRIV phenotypes were identical for the 5' non-coding region of the S and M segments while for the L segment, isolate ISL/TS2/5242 phenotypes differed from the other two NRIV isolates at 4 positions (Figure 22). Isolate TND/S1/19801 WT and TND/S1/19801SP phenotype differed from the two NRIV isolates at a single position. Similarly, there were only two nucleotide differences in the 5' non-coding region of the S segment between isolate MGD/S1/12060WT and all the other phenotypes of all BUNV isolates and none for the M and L segments (Figure 23). However, there was a 5' nucleotide deletion at positions 6815-6817 in the non-coding region of the L segment of isolate GSA/S4/11232LP (Figure 23).

The 3' non-coding region for all the BUNV and NRIV isolates were identical for all three segments except 1 nucleotide difference for the former in the L segment (Figure 24).

5’ NCR S segment Ngari

```
ISL/TS2/5242/WT             GCAGGGAAGCATTTTAAATCGGGCTAAAATCATCAGTTTTAATTTGGCTAAAAGGGTTGTTTCAACCCACAAAATAACAGCTGTTTGGGTGGGTGGTTGGGGACAGAAAG 110
ISL/TS2/5242/SP             ..........................................................................................................  110
ISL/TS2/5242/LP             ..........................................................................................................  110
GSA/TS7/5170_WT             ..........................................................................................................  110
GSA/TS7/5170_SP             ..........................................................................................................  110
GSA/TS7/5170_LP             ..........................................................................................................  110
TND/S1/19801/WT             ..........................................................................................................  110
TND/S1/19801/SP             ..........................................................................................................  110
TND/SA/19801/LP             ..........................................................................................................  110

ISL/TS2/5242/WT             ACAGCGGACTAAATTAACATTACATTATTAATGGTATTTTAAGTTTTAGGTGGAGCACACTACT 174
ISL/TS2/5242/SP             .................................................................. 174
ISL/TS2/5242/LP             .................................................................. 174
GSA/TS7/5170_WT             .................................................................. 174
GSA/TS7/5170_SP             .................................................................. 174
GSA/TS7/5170_LP             .................................................................. 174
TND/S1/19801/WT             .................................................................. 174
TND/S1/19801/SP             .................................................................. 174
TND/SA/19801/LP             .................................................................. 174
```

5’ NCR M segment Ngari

```
ISL/TS2/5242_WT    TTTTTTAAGAAATCATCATTTTAGTTTTAAAAATTTTATATGTTAGCCTTAGGGCAAATTAGCTGTTATTATATCGGTAGCACACTACT 89
ISL/TS2/5242_SP    ........................................................................................... 89
ISL/TS2/5242_LP    ........................................................................................... 89
GSA/TS7/5170_WT    ........................................................................................... 89
GSA/TS7/5170_SP    ........................................................................................... 89
GSA/TS7/5170_LP    ........................................................................................... 89
TND/S1/19801_WT    ........................................................................................... 89
TND/S1/19801_SP    ........................................................................................... 89
TND/S1/19801_LP    ........................................................................................... 89
```

5’ NCR L segment Ngari

```
ISL/TS2/5242_WT    AGCATATCTTCATTGGTTTATTTAATTGGACATTCCAAAAGCCACTATGTGGCAAAAATGATGACAGCATCAAAAAAAGTACAATTTTCTTATGTAGGAGCACACTACT 109
ISL/TS2/5242_SP    ........................................................................................................... 109
ISL/TS2/5242_LP    ........................................................................................................... 109
GSA/TS7/5170_WT    .A.....................................................A.......TC..................................... 109
GSA/TS7/5170_SP    .A.....................................................A.......TC..................................... 109
GSA/TS7/5170_LP    .A.....................................................A.......TC..................................... 109
TND/S1/19801_WT    .A.......................A.....................................A.......TC..................................... 109
TND/S1/19801_SP    .A.......................A.....................................A.......TC..................................... 109
TND/S1/19801_LP    .A.....................................................A.......TC..................................... 109
```

Figure 22: Alignment of 5' non-coding region of S, M and L genome segments of Kenyan Ngari virus isolates.

5’ NCR S segment Bunyamwera

```
MGD/S1/12060/WT          GCAGGGAAGCATTTTAAATCGGGCTAAAATCATCAGTTTTAATTTGGCTAAAAGGGTTGTTTCAACCCACAAAATAACAGCTGTTTGGGTGGGTGGTTGG 100
MGD/S1/12060/SP          ..............T.................................................................................... 100
MGD/S1/12060/LP          ..............T.................................................................................... 100
GSA/S4/11232/WT          ..............T.................................................................................... 100
GSA/S4/11232/SP          ..............T.................................................................................... 100
GSA/S4/11232/LP          ..............T.................................................................................... 100

MGD/S1/12060/WT          GGACAGAAAGACAGCGGACTAAATTAACATTACATTATTAATGGTATTTTAAGTTTTAGGTGGAGCACACTACT 174
MGD/S1/12060/SP          ........................T....................................................... 174
MGD/S1/12060/LP          ........................T....................................................... 174
GSA/S4/11232/WT          ........................T....................................................... 174
GSA/S4/11232/SP          ........................T....................................................... 174
GSA/S4/11232/LP          ........................T....................................................... 174
```

5’ NCR M segment Bunyamwera

```
MGD/S1/12060_WT   AAATTAGACGGTTATTAATTCATTATTATATACATTCAAAATTCATATTGACACATTGTGTCAAAAAACAAGGCTGTTTTGTTATCGGTAGCACACTACT 100
MGD/S1/12060_SP   .................................................................................................... 100
MGD/S1/12060_LP   .................................................................................................... 100
GSA/S4/11232_WT  .................................................................................................... 100
GSA/S4/11232_SP  .................................................................................................... 100
GSA/S4/11232_LP  .................................................................................................... 100
```

5’ NCR L segment Bunyamwera

```
MGD/S1/12060_WT   AACATATCTTCATTGGTTTATTTAATTGGACATTCCAAAAGCACTATGTGGCAAAAATGATGACAGCATCAAAAAAAGTACAATTTTCTTATGTAGGAGCACACTACT 108
MGD/S1/12060_SP   .................................................................................................... 108
MGD/S1/12060_LP   .................................................................................................... 108
GSA/S4/11232_WT  .................................................................................................... 108
GSA/S4/11232_SP  .................................................................................................... 108
GSA/S4/11232_LP  ..........................................--.......................................................... 108
```

Figure 23: Alignment of 5' non-coding region of S, M, and L genome segments of Kenyan Bunyamwera virus isolates.

3’ NCR Bunyamwera S segment

```
GSA/S4/11232/WT          AGTAGTGTACTCCACACTACAAACTTGCTATTGTTGAAAATCGCTGTGCTATCAAATCTAACAGAAAGTCATTAAAGGCTCTTTA 85
GSA/S4/11232/SP          ..................................................................................... 85
GSA/S4/11232/LP          ..................................................................................... 85
MGD/S1/12060/WT          ..................................................................................... 85
MGD/S1/12060/SP          ..................................................................................... 85
MGD/S1/12060/LP          ..................................................................................... 85
```

3’ NCR Bunyamwera M segment

```
MGD/S1/12060_WT   AGTAGTGTACTACCGATACATCACAAACCTTTCAGAGACACATCTTTATTTTCAAG 56
MGD/S1/12060_SP   ........................................................... 56
MGD/S1/12060_LP   ........................................................... 56
GSA/S4/11232_WT   ........................................................... 56
GSA/S4/11232_SP   ........................................................... 56
GSA/S4/11232_LP   ........................................................... 56
```

3’ NCR Bunyamwera L segment

```
MGD/S1/12060_WT   AGTAGTGTACTCCTACATATAGAAAATTTAAAAACATAACCAGTAGGAGT 50
MGD/S1/12060_SP   .................................................. 50
MGD/S1/12060_LP   .................................................. 50
GSA/S4/11232_WT   ..........................C....................... 50
GSA/S4/11232_SP   .................................................. 50
GSA/S4/11232_LP   .................................................. 50
```

Figure 24: Alignment of 3' non-coding region of S, M, and L genome segments of Kenyan Bunyamwera virus isolates.

3.3.4 Mice pathogenesis

We selected the wild type and plaque phenotypes of isolates GSA/S2/11232 and TND/S7/19801 for the mice pathogenesis experiments. Six-week old mice were not susceptible to infection by either virus isolate. However, newborn mice were susceptible to infection with both BUNV and NRIV isolates and displayed clinical symptoms such as hind limb paralysis, tremors, disorientation and mortality beginning 2-3 days post inoculation. Figure 25 shows a picture of virus inoculated newborn mouse displaying partial paralysis of the hind limb compared to control mouse.

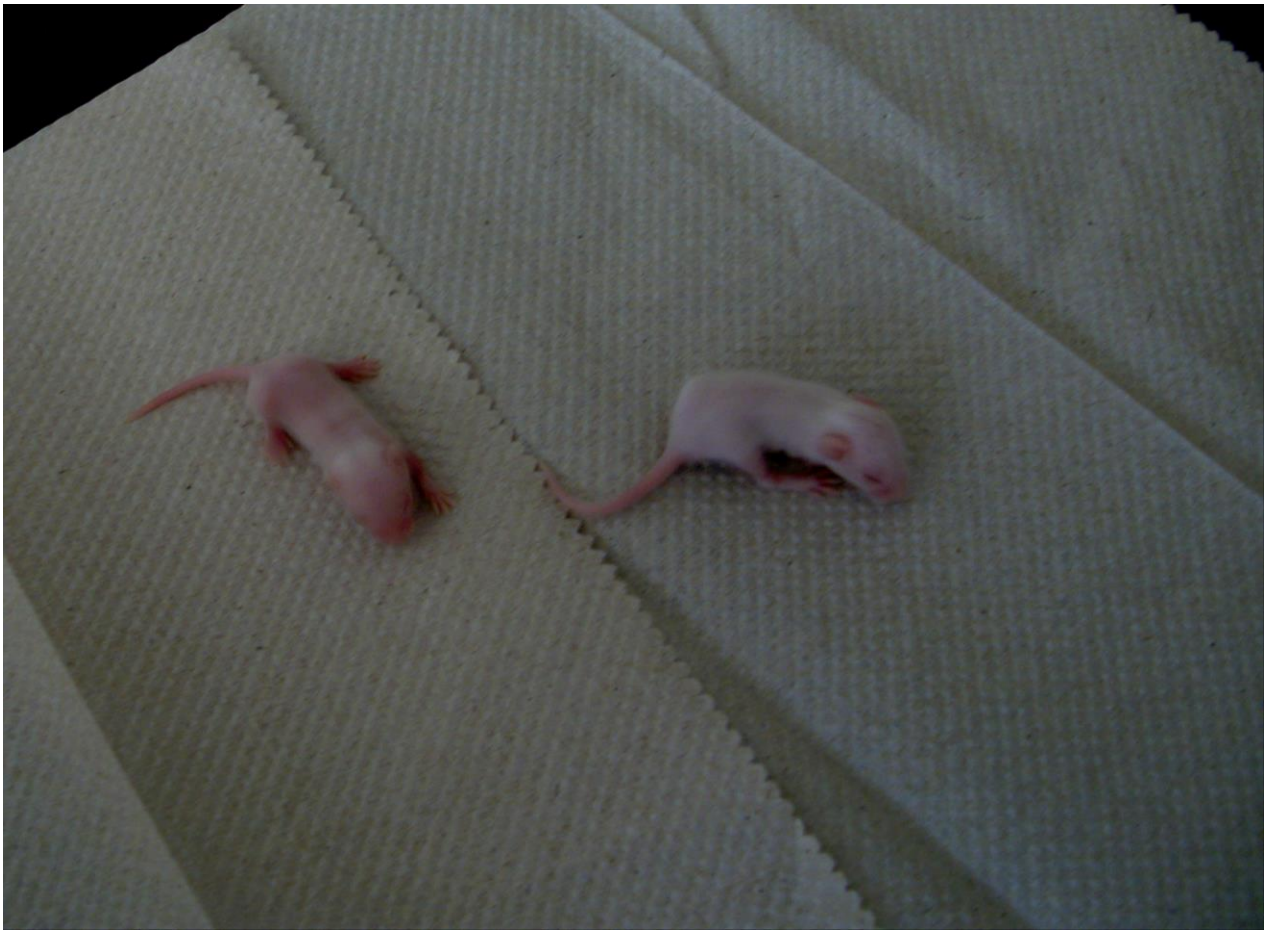


Figure 25: Picture showing comparison between control mice (left) and virus inoculated mice (right). The virus infected mice is showing right hind limb paralysis.

Mice inoculated with tisolate GSA/S4/11232SP all died 3 days post inoculation compared to only 50% of mice inoculated with the LP phenotype (Figure 26). However, the rest of the mice died by day 4 post inoculation. The difference in mortality rate between the SP phenotype and the WT and LP was significant (p = 0.0110). The converse was true for mice inoculated with NRIV isolate TND/S1/19801 where the LP phenotype was more lethal than the SP phenotype (Figure 27). Mice inoculated with the SP phenotype were alive 4 days post inoculation whereas all mice inoculated with the LP were dead. The difference in mortality was not significant (p= 0.3579).

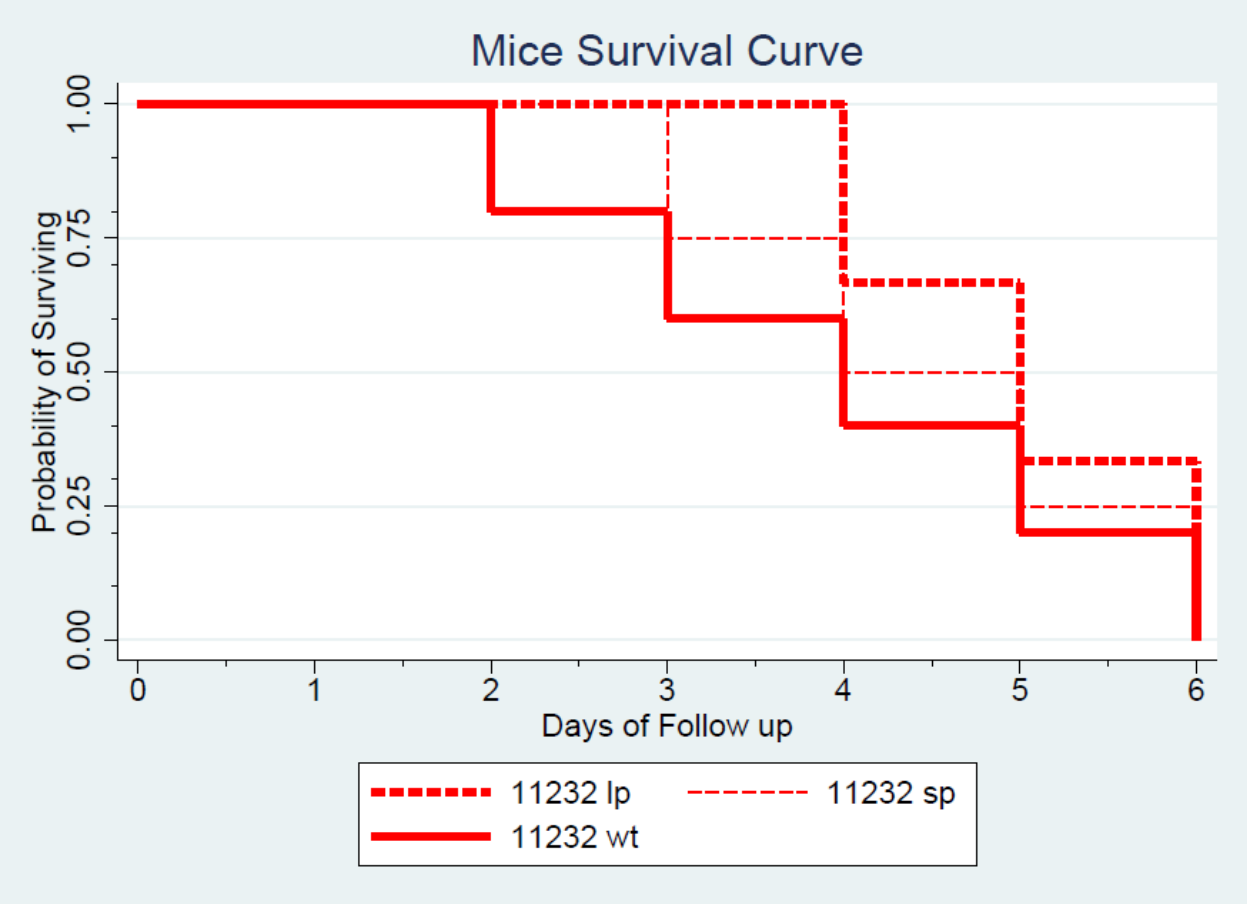


Figure 26: Survival curve comparison of wild type parental and Plaque purified phenotypes of Bunyamwera and Ngari virus isolate infection in mice.

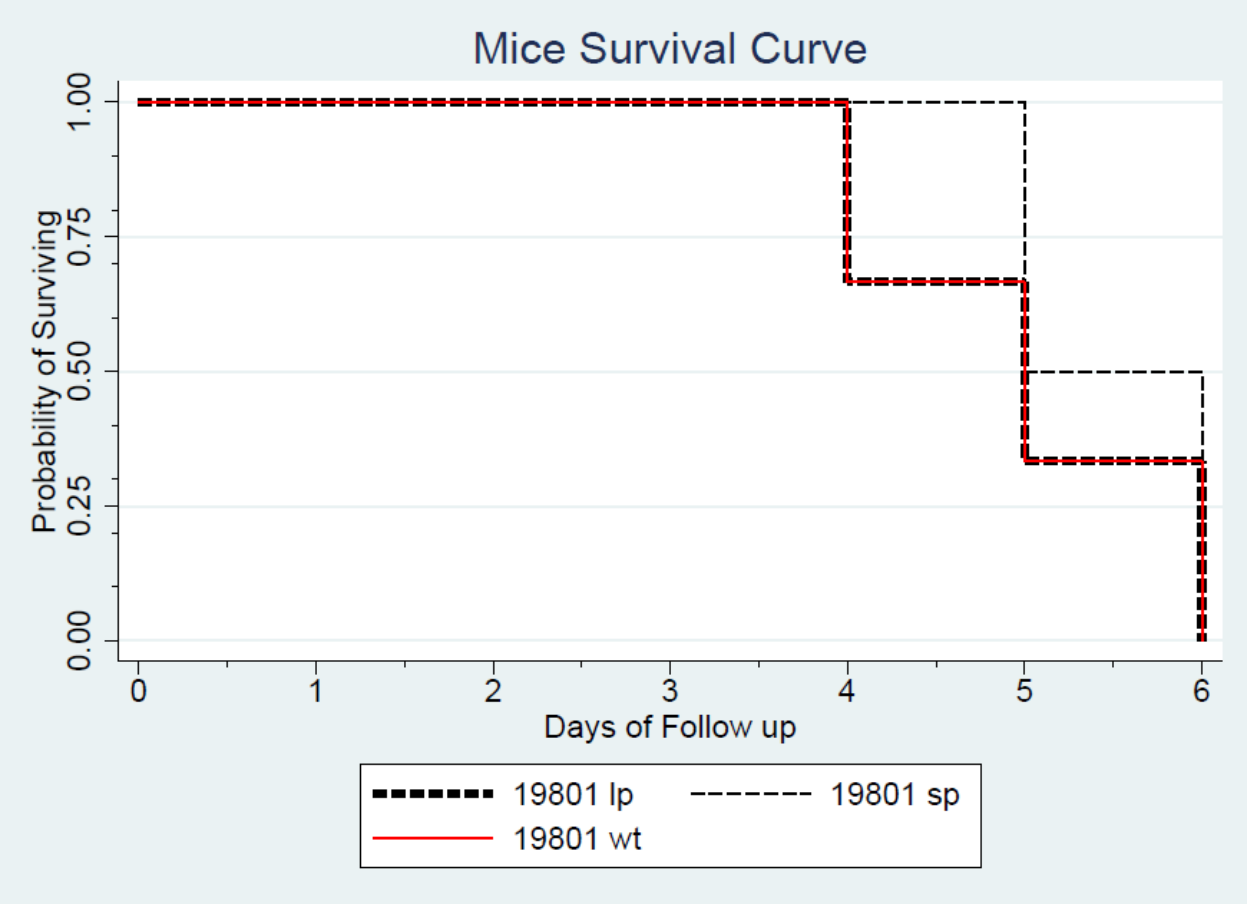


Figure 27: Survival curve comparison of wild type parental and Plaque purified phenotypes of Ngari virus isolate TND/S1/19801 infection in mice.

3.4 Discussion

In the current chapter we evaluate the genetic diversity of plaque purified phenotypes of BUNV and NRIV isolates by full genome sequencing. We also determine the rate of *in vivo* growth in Vero cells and evaluate pathogenesis of the viral phenotypes in Swiss Albino mice. Difference in growth was more pronounced in BUNV than NRIV isolates. This may be explained by the more mutations observed for the former possibly due to the extra passages which the SP and LP phenotypes underwent compared to the WT. In contrast, NRIV phenotypes had fewer mutations despite undergoing extra passages than the WT during the purification and amplification

processes. As expected, the LP phenotypes grew to a higher titer than the SP phenotypes for both BUNV and NRIV isolates. Previous studies of other viruses have correlated plaque size to replication rate with the LP phenotypes displaying faster replication rates than SP phenotypes (Ramsingh et al. 1995, Zhang et al. 1995, Kanno et al. 1998). The LP phenotypes were generally more virulent than SP phenotypes and this would be expected to be the same both *in vitro* and *in vivo* on the assumption that LP phenotypes would produce larger foci of cell destruction (Kanno et al. 1999). However, inoculation of mice with selected BUNV and NRIV isolate phenotypes resulted in discordant observations in the present study. While mice inoculated with the SP phenotype of NRIV isolate, TND/S1/19801, survived longer than the LP phenotype, the reverse was true for BUNV isolate GSA/S4/11232 in which mice inoculated with the SP phenotype died 3 days post-inoculation compared to 4 days post-inoculation for the LP phenotype and this difference in mortality rate was significant. This difference in neurovirulence for phenotypes of BUNV isolate GSA/S4/11232 in mice cannot fully be accounted for by the rate of replication as shown in the one-step growth curves. Previous neurovirulence studies of viruses within the *Orthobunyavirus* genus have mapped such differences to the L segment (Endres et al. 1991). The study by Endres et al., was designed to identify molecular determinants responsible for attenuation of a variant California serogroup virus. Another study investigating the biological function of BUNV L protein demonstrated that mutations in the polymerase genome affect the ability of BUNV to replicate in different cells (Shi and Elliott 2009). Thus, the discordant observed in the current study may have resulted from the unusual deletion observed in the 5' non-coding region of the L segment of the LP phenotype. The non-coding regions are known to play a role in viral RNA and protein synthesis, nucleocapsid formation and virion assembly (Mazel-Sanchez and Elliott 2012). However, reverse genetic studies have shown that non-

conserved sequences of the non-coding region result in attenuation only when large sections are excised (Lowen and Elliott 2005, Mazel-Sanchez and Elliott 2012). This is corroborated by the observed higher growth rate of the GSA/S4/11232 virus isolate LP phenotype compared to the SP phenotype. Moreover, the deletion did not occur between the terminal nucleotides, 20 and 33 where the important packaging elements have been mapped (Kohl et al. 2006). Hence, it is more likely that pathogenesis may have been dependent on the single nucleotide substitutions that were present in the different segments of the BUNV isolate.

It is interesting that all the nucleotide substitutions on the M segment while resulting in non-synonymous amino acid changes, involved substitution of amino acids with the same properties thus a significant difference in protein function would be unexpected. The M segment substitutions resulted in exchange of positively charged amino acids, glutamic acid for lysine. However, two mutations in the L segment of the LP phenotype involved substitution of amino acids with different properties which are likely to alter the functionality of the L protein. Thus for isolate GSA/S4/11232, the LP attenuated pathogenesis may be mapped to any of the 2 non-synonymous mutations on the L segment. The T858I mutation resulting in amino acid substitution of a polar for a non-polar amino acid, occurring within the predicted catalytic site of the L protein (AA 597-1330) seems the most plausible cause of the observed attenuation in pathogenesis. Mutation within the catalytic site of the L protein has been demonstrated to abolish polymerase activity in a previous study (Shi and Elliott 2009).

With regard to isolate TND/S1/19801SP phenotype which was attenuated in mice but genetically similar to the WT virus, it is likely that the SP phenotype was present in a higher quantity in the WT virus, which is a mixture of both LP and SP phenotypes, and could have preferentially been sequenced. However, in the mice pathogenesis experiment, it is likely that the LP phenotype in

the WT grew at a faster rate as expected and resulted in earlier death of mice compared to the SP phenotype. However, we did not isolate the infecting virus from mice to confirm this observation. Another limitation was the use of interferon defective Vero cells for the one step growth curve analysis which may have limited our comparison with mice pathogenesis as the GSA/S4/11232 SP phenotype may have been better at counteracting the interferon response.

In summary, we have identified a mutation in the L segment of BUNV isolate GSA/S4/11232 LP phenotype which may be associated with decreased pathogenesis in suckling mice and virus replication in Vero cells. In addition, we have identified other natural mutations whose role in viral growth and pathogenesis should be determined. Site directed mutagenesis studies may clarify the exact mutation involved in the observed phenotypic changes.

Chapter 4

VECTOR COMPETENCE OF SELECTED MOSQUITO SPECIES IN KENYA FOR NGARI AND BUNYAMWERA VIRUSES.

4.1 INTRODUCTION

BUNV is the type species on the *Orthobunyavirus* genus, the largest of the 7 genera within the *Bunyaviridae* family with 18 different serogroups(Calisher and Karabatsos 1988, Gonzalez-Scarano and Nathanson 1996, Yanase et al. 2006). However, the majority of human pathogens within the genus are distributed among three serogroups; Carlifornia serogroup, predominantly in North America and Europe, New World Group C viruses and the Bunyamwera serogroup, predominantly in Africa, Central and South America (Calisher and Karabatsos 1988, Elliot et al. 2000). The majority of viruses within the Bunyamwera serogroup are transmitted by mosquitoes.

The clinical manifestations associated with BUNV infection include febrile illness with headache, athralgia, rash and infrequent central nervous system involvement (Gonzalez and Georges 1988). While viruses of the *Orthobunyavirus* genus are known to cause human disease, they were previously not associated with hemorrhagic manifestation. However, during recent outbreaks of hemorrhagic fevers in Kenya and Somalia, NRIV, a reassortant virus was suspected to have contributed to part of the human hemorrhagic cases (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). Genetic characterization of NRIV revealed that the medium (M) segment was similar to that of BATV, an *Orthobunyavirus* first isolated in Malaysia and not associated with human infection, while the S and L segment were related to BUNV (Traore-

lamizana et al. 2001). This was thought to have occurred due to segment reassortment as a result of co-circulation of BATV and BUNV.

Arthropod borne viruses flourish in many parts of Kenya (Linthicum et al. 1985, Sang and Dunster 2001) and entomological surveys during hemorrhagic fever outbreaks have demonstrated co-circulation of arboviruses, including BUNV (Traore-lamizana et al. 2001, Crabtree et al. 2009). BUNV has been isolated from a range of mosquito species in surveys including *Aedes mcintoshi, Aedes ochraceus* and *Aedes quasiunivittatus* (Logan et al. 1991, Crabtree et al. 2009, Ochieng et al. 2013). However, the actual role of these mosquito species in the maintenance and transmission of the virus remains unclear. The identification of mosquito species that are efficient in transmitting these viruses is critical components in estimating the risk of human exposure and understanding the transmission and maintenance mechanism. Additionally, while there is evidence that genetic reassortment can profoundly increase viral pathogenicity (Bowen et al. 2001, Briese et al. 2006); there is lack of data on how this may affect mosquito vector competence. Even a single nucleotide mutation can have a major effect on viral infectivity or replication in particular hosts (Ciota and Kramer 2010). For example, a single mutation in the E2 gene of VEEV can increase vector competence or virulence to cause outbreaks (Anishchenko et al. 2006). Similarly, the recent chikungunya virus outbreak in the Indian Ocean islands was associated with emergence of a virus strain that shared a single common substitution in the E1 gene and a variable second mutation that resulted in increased competence of *Aedes albopictus* as a vector (Tsetsarkin et al. 2007, Tsetsarkin et al. 2009). Likewise, the RVF vaccine, MP-12 virus, is an attenuated virus resulting from accumulation of mutations in its L and M segments (Caplen et al. 1985).

Laboratory assessment of vector competence can provide an insight into the potential of field collected mosquitoes as disease vectors. These studies are intended to assess variations in vector ability to transmit viruses (Hardy et al. 1983). Given that mosquito control methods differ depending on the species type, it is of public health importance to identify which species of mosquitoes are competent vectors that may be involved in the natural transmission cycle so that appropriate measures can be applied. Thus, we set out to determine the vector competence of, *Aedes aegypti*, *Culex quinquefaciatus* and *Anopheles gambiae* for transmission of recent Kenyan isolates of BUNV and NRIV because they are the most abundant mosquito species in many ecological zones in Kenya. Secondarily, we determined the possible effect of genetic reassortment on vector competence in the selected mosquito species.

4.2 MATERIALS AND METHODS

4.2.1 Mosquitoes

Mosquitoes tested included established laboratory colonies of *Aedes aegypti* (F>35), *Anopheles gambiae* (F>36) and *Culex quinquefaciatus* (F>40) selected because of their abundance in Kenya, ease of laboratory rearing as well as being sources of previous isolations of BUNV and NRIV (Karabatsos 1985). Additionally, we used field collected *Aedes aegypti* from Rabai, in the Kenyan coastal region to establish a low generation colony (F4-8). Mosquitoes were maintained in 4-liter plastic cages in the biological safety level-2 insectary at 28^{o}C, relative humidity 75-80% and a 12:12 (light: dark) hour photoperiod. They were provided with 10% sucrose solution on cotton pads as a carbohydrate source until used in the study. The selected mosquito species were evaluated for their susceptibility to, and competence in transmitting the two viruses to newborn mice.

4.2.2 Viruses

The viruses used in the study were isolated during previous surveillance exercises in the northeastern Kenya ecozone (Ochieng et al. 2013). Viral stocks were prepared by inoculating the viruses on confluent monolayers of Vero cells and harvested when showing over 75% cytopathic effects. The culture fluids were clarified by centrifugation at 5000g and the supernatants stored frozen at -70^{o}C, after determination of the PFU/mL titers by plaque assay titration on Vero cells, until used (Gargan 2nd et al. 1983).

4.2.3 Vector competence

The selected viral isolates were diluted in defibrinated rabbit blood to a final concentration of approximately 10^{10} PFU/mL. Female mosquitoes, starved for 24 hours, were allowed to feed on infected blood through a Hematok membrane feeder (Discovery Workshops, Accrington, the United Kingdom) (Figure 28). After 1 hour, engorged mosquitoes were separated from unfed mosquitoes and placed into new cages while non-engorged mosquitoes were destroyed. The engorged mosquitoes were maintained for up to 14 days at 28^{o}C, 12:12 (Light: Day) hour and provided 10% sucrose as a carbohydrate source until assayed for infection and dissemination, and transmission potential.

Figure 28: Mosquito feeding experimentation using Hematok membrane feeder.

At days 7 and 14, a subset of mosquitoes was sampled and the extent of virus infection determined by plaque assay of triturated leg and abdomen on Vero cells as previously described (Turell et al. 1984). Thus, detection of virus in the mosquito abdomen only was considered as a non-disseminated infection limited to its midgut while detection in both abdomen and leg was considered a disseminated infection (Turell et al. 1984).

Moist paper in ovicups was placed in the different cages to stimulate oviposition (Figure 29).

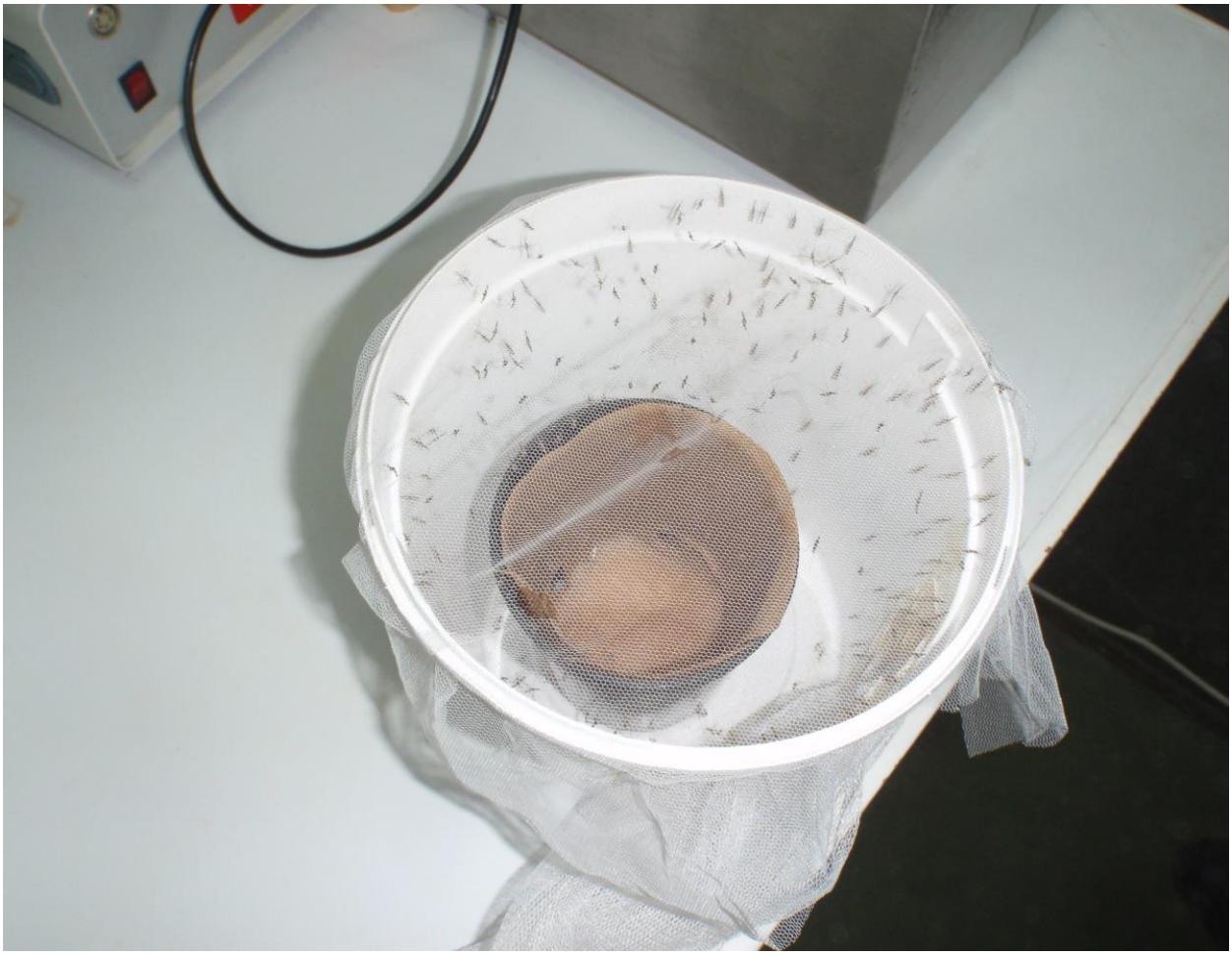


Figure 29: Picture showing ovicup with moist filter paper for mosquito oviposition.

4.2.4 Oral transmission

For the transmission experiments, fully engorged day 14 infected mosquitoes (infection dose, approx. 10^{10} PFU/mL) were selected from the pool of fed mosquitoes and placed in cages either singly (Figure 30) or in groups of 2-5 as described by Turrell et al (Turell et al. 2007) and allowed to feed on Swiss Albino suckling mice (1-4 days old) . The study protocol (number SSC 2677) was approved by the Animal Care and Use Committee of the Kenya Medical Research

Institute.

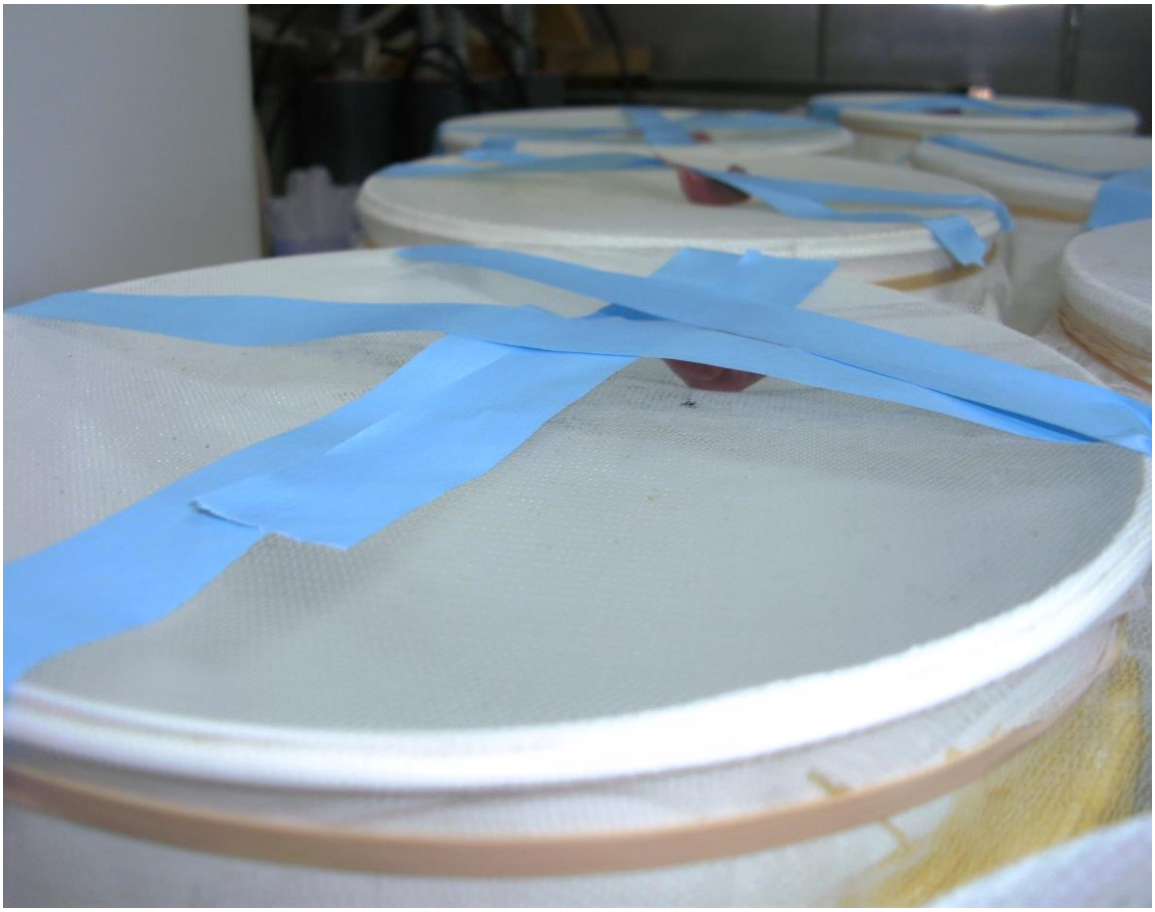


Figure 30: Mosquito virus transmission experiment showing mosquito feeding on immobilized mice.

Immediately after the transmission assay, mosquitoes were sampled and their feeding status determined. The legs and abdomen of engorged mosquitoes were separated and assayed by plaque assay as described in section 3.2.3 above. The development of clinical symptoms or death in exposed mice was an indication of successful transmission.

4.2.5 Statistical analysis

Sample size for the vector competence studies was calculated as follows;

A priori power analyses based on chi-square analyses was conducted to determine the appropriate sample sizes required to detect significant differences in mosquito infection and dissemination rates among virus treatment groups using G power software (GPower, http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/) (Faul et al. 2007). Cohen's effect size *w*, a measure of the size of the difference between the null and alternative hypotheses (Cohen 1992), was applied. Based on this, we calculated the sample sizes necessary to detect a small to medium difference ($w = 0.2$) of effect size with 99% power (Richards et al. 2007). Thus, for the effect of geographical region, dose, phenotype and species ($P = 0.05$, degrees of freedom [df] = 3), a total sample size of about 589 mosquitoes was required.

The infection rate was determined for each mosquito species as the percentage of mosquitoes with virus in the abdomen, dissemination rate as the percentage of infected mosquitoes with a disseminated infection (virus in the legs), and the transmission rate as the percentage of infected mosquitoes that refed on newborn mice and transmitted virus by bite. Statistical analyses were performed using the R statistical software (R Development Core Team 2008). The Fisher exact test was used to compare virus infection and dissemination rates among mosquito species. Significance was tested at a level of alpha ≤ 0.05.

4.3 RESULTS

All the mosquito species tested ingested virus-infected blood. However, different barriers were present in different species (Table 12). This was dependent on the virus species and the infection and dissemination rates were dependent on intrinsic incubation period. We did not observe any difference in infection rates between established laboratory colony of *Aedes aegypti* and the newly established colony hence these data were combined. *Aedes aegypti* was moderately susceptible to BUNV with infection rates of 30.1% and 44.1% at day 7 and day 14 respectively,

developing a midgut infection. The infection rate was similar regardless of the incubation period. Over 60% of *Aedes aegypti* with a midgut infection developed disseminated infection at both time points. Approximately, 17% more mosquitoes developed a disseminated infection at day 14 compared to day 7 and this was statistically significant (p=0.004). However, the picture was completely different when *Aedes aegypti* was fed on blood infected with NRIV, with under 4.2% of exposed mosquitoes developing a midgut infection regardless of the intrinsic incubation period (Table 12). None of the few mosquitoes with a midgut infection developed disseminated infection.

Table 12: Infection and dissemination rates for mosquitoes orally exposed to Bunyamwera and Ngari viruses (infectious dose=10^{10} PFU/ml).

Virus	Mosquito species	Incubation period (days post-infection)	Number tested	Midgut infection N (%)	p-value	Disseminated infection N (%)	P value
Bunyamwera GSA/S1/11232	***Aedes aegypti***	**7**	103	31 (30.1)	0.33	19 (18.4)	0.004
		14	143	63 (44.1)		51 (35.7)	
	Anopheles gambiae	**7**	96	24 (25.0)	0.76	15 (15.6)	0.137
		14	84	32 (38.1)		21 (25.0)	
	Culex quinquefaciatus	**7**	72	1 (1.4)	ND	ND	ND
		14	80	0		ND	
Ngari TND/S4/19801	***Aedes aegypti***	**7**	58	2 (3.4)	1.0	0	ND
		14	96	4 (4.2)		0	
	Anopheles gambiae	**7**	96	32 (33.3)	0.611	8 (8.3)	0.032
		14	63	24 (38.1)		13 (20.6)	
	Culex quinquefaciatus	**7**	102	0	ND	ND	ND
		14	87	0			

ND: Not done

In contrast, *Anopheles gambiae* was moderately susceptible to both viruses with 25.0% and 38.1% developing a midgut infection at day 7 and day 14 with BUNV. A similar rate was observed for NRIV with 33.3% and 38.1% of blood fed mosquitoes developing a midgut

infection at day 7 and day 14 respectively. The dissemination rate was 15.6% and 25.0% for BUNV at both day 7 and day 14 time points and was not significantly influenced by the incubation period. However, for NRIV, dissemination rate was 8.3% at day 7 and more than doubled to 20.6% by day 14 (Table 12). This difference in dissemination rate was statistically significant (p=0.032). *Culex quinquefaciatus* was refractory to both BUNV and NRIV with only one (1.4%) mosquito developing a midgut infection with BUNV at day 7 which was not disseminated (Table 12). Since, infection rate for NRIV was zero, we did not perform any dissemination experiments for *Culex quinquefaciatus* at days 7 and 14.

4.3.1 Oral Transmission

Twelve fully engorged 'infected' mosquitoes at day 14 intrinsic incubation, previously starved for 24 hours, were allowed to feed on 12 singly restrained suckling mice separately for one hour. Eight mice (80%) among those fed on by 10 *Aedes aegypti* mosquitoes (83.3%) with a disseminated infection of BUNV isolate, GSA/S4/11232, developed clinical symptoms characterized by tremors and partial paralysis by day 2 post-transmission (Table 13).

Likewise, 5 (41.7%) mice exposed to BUNV isolate and 5 (100%) exposed to NRIV isolate, TND/S1/19801 infection through bite by *Anopheles gambiae* with disseminated infection developed clinical symptoms by day 2 post-infection. There was no significan difference in dissemination or transmission rates between the mosquito and viral species ($p>0.05$). The mice recovered fully by day 5 post-infection and remained healthy throughout the 14 days of observation. However, for mice exposed to groups of mosquitoes, mortality was observed by day 4 post-infection whenever two or more mosquitoes with a disseminated infection fed.

Table 13: Transmission rates for mosquitoes with disseminated infection after oral exposure to Bunyamwera and Ngari virus isolates

Virus isolate	Mosquito species	No of mosquito tested	No with disseminated infection	Percent Transmission
GSA/S1/11232	*Aedes aegypti*	12	10 (83.3)	8 (80.0)
GSA/S1/11232	*Anopheles gambiae*	12	7 (58.3)	5 (71.4)
TND/S4/19801	*Anopheles gambiae*	12	5 (41.7)	5 (100%)

4.4 DISCUSSION

Implication of a particular mosquito species as a vector of a particular virus requires demonstration of mosquito vector competence and transmission in addition to detection of virus in field collected mosquitoes (Kramer et al. 1992). This is the first report on the ability of mosquitoes in Kenya to transmit NRIV and BUNV. In this study we examined the vector competence of three common mosquito species in Kenya for field isolated BUNV and NRIV. All mosquito species tested ingested blood but different barriers were observed per species with midgut barrier associated with low infection rates and midgut escape barrier associated with a small percentage of infected mosquitoes developing a disseminated infection as described by Turrel et al. (Turell et al. 2008). The results indicate that *Anopheles gambiae* is a potentially efficient vector of NRIV. Most isolations of NRIV have been obtained from *Anopheles gambiae* as a single species (Karabatsos 1985), although one of the study isolates (GSA/S4/11232) was obtained from *Anopheles funestus* (Ochieng et al. 2013). *Anopheles gambiae* was moderately susceptible for both viruses with *Aedes aegypti* moderately competent for BUNV. Although

Anopheles gambiae may be a potential vector of NRIV and BUNV, other factors including feeding preference and abundance, need to be considered in implicating the species rather than merely transmission by bite (Turell et al. 2001). Additionally, other mosquito species need to be tested for their competence for NRIV which has been detected previously in West Africa from a wide diversity of mosquito species (Gordon et al. 1992, Zeller et al. 1996). NRIV was the most common isolate from various mosquito species that normally feed on both humans and domestic ungulates including *Anopheles gambiae*, *Anopheles pharoensis*, *Culex antennatus*, *Culex poicillipes* and *Culex tritaeniorhynchus* which suggests that humans and domestic animals may be involved in the ecology of this virus (Gordon et al. 1992). Moreover, NRIV has also been isolated from sick sheep in southern Mauritania and in a survey conducted to monitor RVF virus following the outbreak of 1987 in West Africa. Some of these mosquito species are predominant in different regions in Kenya including *Anopheles gambiae*, *Anopheles pharoensis and Culex antennatus* (Lutomiah et al. 2013) hence a risk in case NRIV is introduced in those regions through movement of infected persons, vectors or animals. For instance, RVF outbreak in the Kenyan coast region was attributed to movement of infected animals from the Northern part of Kenya (Nguku et al. 2010). As previously determined, *Aedes aegypti* was a competent vector for BUNV and most isolations have been made from *Aedes* species (Karabatsos 1985). *Culex quinquefaciatus* was incompetent for either virus. This species has been reported to be poorly susceptible to RVF virus, a member of the family *Bunyaviridae* (McIntosh et al. 1980, Turell and Kay 1998). This was attributed to the existence of a major midgut infection barrier (Turell et al. 2008) and which we confirmed as there was almost non-existent mosquito midgut infection for either virus. Moreover, *Culex quinquefaciatus* has a preference for avian species (Elizondo-Quiroga et al. 2006, Garcia-Rejon et al. 2010) from which no NRIV has been isolated although a

recent study has documented the presence of BUNV antibodies in birds in Argentina (Tauro et al. 2009). For a mosquito to be an efficient vector, it must primarily feed on the susceptible host or be a general feeder to act as a bridging vector. However, it is possible that with continuous exposure of these viruses to avian immune system may in future result in mutations that alter vector competence of the vector species for these viruses.

Although *Aedes aegypti* and *Anopheles gambiae* varied in their susceptibility to NRIV and BUNV, over 70% of mosquitoes that developed a disseminated infection transmitted virus by bite. This suggests that midgut infection and escape barriers may be the principal factors controlling vector competence with these viruses (Kramer et al. 1981). One limitation of our study is that we did not use low or moderate viremia that may represent natural viremia in animals. Additionally, we did not test field collected mosquitoes, however, we did not observe a significant difference in infection, dissemination and transmission rates between low and high generation *Aedes aegypti*.

In conclusion, the study suggests that *Anopheles gambiae* is a more competent vector for NRIV than *Aedes aegypti* possibly due to the indirect contribution of genetic reassortment. In addition, *Anopheles gambiae* is also moderately competent for BUNV. This has major implication in the light of continued animal trade and travel especially into malaria endemic regions where *Anopheles gambiae* is more prevalent. In the likely event of introduction of the viruses in such regions, it would pose a challenge to public health authorities due to symptom similarities with other tropical illnesses including malaria. Public health authorities should continually monitor emergent arboviral genotypes circulating within particular regions as well as identify vectors mediating these transmissions in order to preempt and prevent their adverse effects.

Chapter 5

TRANSOVARIAL TRANSMISSION OF NGARI AND BUNYAMWERA VIRUS ISOLATES IN NORTHERN KENYA

5.1 INTRODUCTION

NRIV (family *Bunyaviridae,* genus *Orthobunyavirus*) emerged as a significant human pathogen during previous RVF outbreaks in Africa (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). NRIV was isolated from two hemorrhagic patients and antibodies detected in several others with hemorrhagic fever during the RVF outbreak of 1997/1998 in the Kenya-Somali border region (Gerrard et al. 2004, Briese et al. 2006). Similar observations were made when NRIV was retrospectively isolated from samples of hemorrhagic patients from the 1987/1988 RVF outbreak in the Sudan (Nashed et al. 1993). NRIV is a reassortant composed of the S and L segments of BUNV and the M segment of BATV (Bowen et al. 2001), the later viruses not associated with hemorrhagic symptoms.

Arthropod-borne viruses flourish in many parts of Kenya (Linthicum et al. 1985, Sang and Dunster 2001) and entomological surveys during hemorrhagic fever outbreaks have demonstrated co-circulation of arboviruses, including BUNV (Traore-lamizana et al. 2001, Crabtree et al. 2009). BUNV has been isolated from a range of mosquito species in surveys including *Aedes mcintoshi, Aedes ochraceus* and *Aedes quasiunivittatus* (Logan et al. 1991, Traore-lamizana et al. 2001, Crabtree et al. 2009, Ochieng et al. 2013). Likewise, NRIV has been detected previously in West Africa from a wide diversity of mosquito species including *Anopheles gambiae, Anopheles pharoensis, Culex antennatus, Culex poicillipes* and *Culex tritaeniorhynchus* (Gordon et al. 1992, Zeller et al. 1996). Some of these mosquito species are predominant in different regions of Kenya including *Anopheles gambiae, Anopheles pharoensis and Culex antennatus* (Lutomiah et al. 2013). However, the actual role of these mosquito species in the maintenance and transmission of the virus in the environment remains unclear. Surveillance for the vector-borne pathogen and identification

of the arthropod vectors that transmit these agents are critical components in estimating the risk of human exposure and understanding the transmission and maintenance mechanism.

Vertical transmission, a means of viral persistence in nature, is important not only for viral survival in nature but also for their role in the establishment of biological transmission. Some Orthobunyaviruses within the Carlifornia serogroup are known to be mainatained in nature through vertical transmission and is unusually high ranging from 20% to over 90% (Turell and Kay 1998, Kuno and Chang 2005). However, there is paucity of data regarding vertical transmission in Bunyamwera serogroup viruses. We have recently determined the vector competence of selected mosquito species (*Aedes aegypti*, *Culex quinquefaciatus* and *Anopheles gambiae*) for BUNV and NRIV isolates in Kenya (Chapter 4). *Aedes aegypti* and *Anopheles gambiae* were determined to be competent vectors for BUNV while the later was competent for NRIV. In this study we assess the ability of competent mosquito species, as determined in the preceeding chapter (Chapter 4), to transmit BUNV and NRIV transovarially to their progeny.

5.2 MATERIALS AND METHODS

5.2.1 Mosquitoes

Mosquitoes tested included established laboratory colonies of *Aedes aegypti* (F>4-8), and *Anopheles gambiae* (F>36). Mosquitoes were maintained in 4-liter plastic cages in the biological safety level-2 insectary at 28^{o}C, relative humidity 75-80% and a 12:12 (light: dark) hour photoperiod. They were provided with 10% sucrose solution on cotton pads as a carbohydrate source until used in the study.

5.2.2 Viruses

The viruses used in the study were isolated during previous surveillance exercises in northeastern Kenya (Ochieng et al. 2013). Two Kenyan isolates were employed; these were the BUNV isolate GSA/S1/11232, from a pool of *Aedes mcintoshi* and NRIV isolate TND/S1/19801 obtained from a pool of *Anopheles funestus*. Viral stocks were prepared by inoculating the viruses on confluent

monolayers of Vero cells and harvested when showing over 75% cytopathic effects. The culture fluids were clarified by centrifugation at 5000g and the supernatants stored frozen at -70^{o}C, after determination of the PFU/mL titers by plaque assay (Section 3.2.3) titration on Vero cells, until used (Gargan 2nd et al. 1983).

5.2.3 Transovarial transmission

The selected viral isolates were diluted in defibrinated rabbit blood to a final concentration of approximately 10^{10} PFU/mL. Female mosquitoes, starved for 24 hours, were allowed to feed on infected blood through a Hematok membrane feeder (Discovery Workshops, Accrington, the United Kingdom) (Figure 28). After 1 hour, engorged mosquitoes were separated from unfed mosquitoes and placed into new cages while non-engorged mosquitoes were destroyed. The engorged mosquitoes were maintained at 28^{o}C, 12:12 (L: D) hour for 6-7 days and provided 10% sucrose as a carbohydrate source. Moist paper in ovicups was placed in the different cages to stimulate oviposition (Figure 29). After oviposition, mosquitoes were fed on uninfected mice to stimulate egg production and fresh ovicups placed into the cages (Figure 31). This was repeated again to achieve the third ovarian cycle. All egg rafts on filter paper were collected and allowed to hatch in distilled water (Figure 32). Pupae were removed, washed in water, and placed for emergence in 1-liter cardboard containers with netting secured over the open end. Emerged adult mosquitoes were contained in a mosquito rearing container (Figure 33) and maintained for 4-8 days at 26^{0}C and then triturated in pools of 25 mosquitoes each in 1 ml of diluent. These were frozen at -70^{0}C until assayed by plaque assay on Vero cell monolayers.

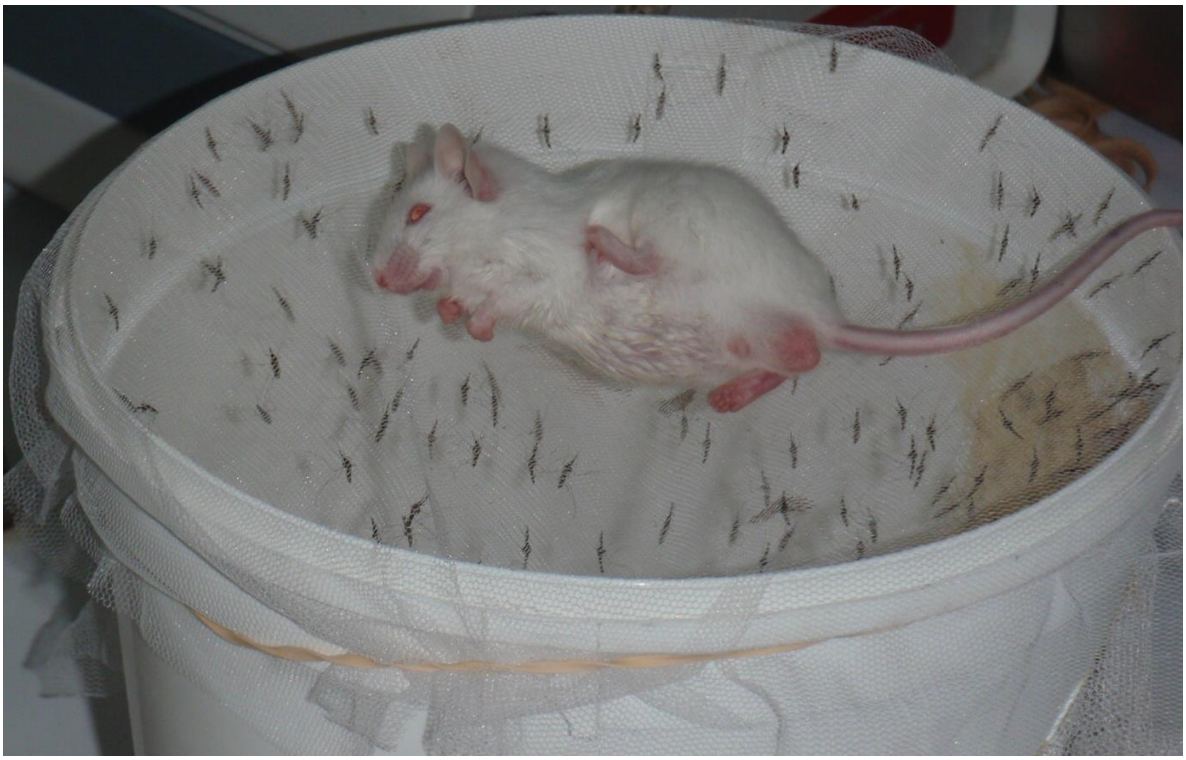


Figure 31: Photograph showing mosquito feeding on anesthesized mice to stimulate oviposition.

Figure 32: Photograph showing filter papers with mosquito eggs placed in distilled water to allow for hatching.

Figure 33: Photograph showing mosquito rearing container and shelves for adult mosquito containment

5.3 RESULTS

Using plaque assay to identify infected mosquito pools, 12 pools comprising 291 orally infected *Aedes aegypti* mosquitoes, 9 pools (75.0%) were positive for BUNV during the first ovarian cycle (Table 14)

.Table 14: Comparison between ovarian cycle and vertical transmission rates of Bunyamwera virus isolate GSA/S4/11232 among *Aedes aegypti* and *Anopheles gambiae* colonies.

	Number of infected pools/total pools (%)	
Ovarian cycle	***Aedes aegypti***	***Anopheles gambiae***
1	9/12 (75.0%)	3/9 (33.3%)
2	3/12 (25.0%)	3/6 (50.0%)

There was a drastic reduction during the second ovarian cycle with only 3 pools (25.0%) of adult mosquitoes being positive. For *Anopheles gambiae* mosquitoes however, 3 pools (33.3%) out of 9 pools in the first ovarian cycle were positive for BUNV while 3 pools (50%) of adult mosquitoes were positive in the second ovarian cycle.

5.4 DISCUSSION

Our study confirms that *Aedes aegypti* and *Anopheles gambiae* mosquitoes are able to vertically transmit BUNV in contrasting fashion. The efficiency of Bunyavirus transovarial transmission is thought to result from direct infection of ovarian tissues hence ova are already infected before oviposition as opposed to virus infecting eggs during oviposition in other viruses (Beaty and Thompson 1978, Rosen 1988, Rosen et al. 1989, Dohm et al. 2002). The higher transovarial transmission rates in our study are thus not unexpected as determined previously (Turell et al. 1982a). Transovarial transmission of BUNV by *Anopheles gambiae* was consistent with previous studies that indicate vertical transmission inefficiency during the first ovarian cycle after oral exposure (Miller et al. 1979). However, *Aedes aegypti* displayed a different picture with transovarial transmission more efficient during the first ovarian cycle and subsequently reduced in the second and third ovarian cycles. This is similar to a previous study of vertical transmission of California encephalitis virus by two *Aedes* mosquito species where filial infection rate was highest in the first ovarian cycle progeny and declined with increasing ovarian cycles (Turell et al. 1982a). Both mosquito species displayed transovarial transmission in the first ovarian cycle and the rates were reflective of the infection and dissemination rates determined in the preceeding chapter (Chapter 4) where *Aedes aegypti* had a more permissive midgut infection and escape barrier for BUNV compared to *Anopheles gambiae*. The same transmission barriers have been implicated in the vector competence of *Aedes aegypti* and *Aedes albopictus* species for La Crosse virus (Hughes et al. 2006). Transovarial transmission to the first ovarian cycle progeny may be attributed to the development of a stabilized infection in these mosquito species, where nearly all cells are infected including the germinal tissue as determined for some Carlifornia serogroup viruses (Tesh and

Shroyer 1980, Turell et al. 1982b). The difference in vertical transmission rate in the different mosquito species needs to be investigated to determine the exact mechanism.

Our study was not devoid of limitations, including the use of long colonized laboratory strains of the vectors (*Anopheles gambiae*). Hence, these results need confirmation using newly colonized strains. Our efforts to determine the rate of vertical transmission of NRIV was frustrated by the inability to obtain adult mosquitoes from the larvae which died during experimentation. This may be attributed to retardation of larval development by the infecting virus. This warrants further investigation to identify the exact cause of larval retardation. Additionally, we did not attempt to isolate virus from the larvae as confirmation of successful transovarial transmission.

In conclusion, *Aedes aegypti* and *Anopheles gambiae* are competent vector of BUNV with contrasting vertical transmission rates depending on the ovarian cycle. Additionally, our results suggest that BUNV may persist longer in *Anopheles gambiae* mosquito species, making it a more competent vector in sustaining the virus during dry periods.

Chapter 6

SEROPREVALENCE OF ORTHOBUNYAVIRUSES IN SELECT PARTS OF RIFT VALLEY AND NORTH EASTERN KENYA

6.1 INTRODUCTION

BUNV and NRIV have recently been isolated from mosquitoes and ticks in the north eastern pastoral zones of Kenya (Lwande et al. 2013, Ochieng et al. 2013). Likewise, Ilesha and Germiston viruses have been previously isolated from mosquitoes in western Kenya (Johnson et al. 1977). These viruses belong to the *Orthobunyavirus* genus of the family *Bunyaviridae* and possess a tripartite, single-stranded, negative-sense RNA genome. Some members of the genus including these four viruses are known to cause clinical disease in humans with diverse pathological consequences. BUNV, the prototype member of the family, is associated with febrile illness with headache, arthralgia, rash and infrequent central nervous system involvement (Gonzalez and Georges 1988, Grimstad 1988). Ilesha virus has been associated with febrile illness and erythema, and in some cases, fatal meningoencephalitis and hemorrhagic fever (Morvan et al. 1994). Germiston virus is characterized by mild disease with fever, rash and headache (Karabatsos 1985). Genetic reassortments between members of serogroups within the *Orthobunyavirus* genus are known to occur in nature leading to emergence of new viruses with altered pathogenesis (Bowen et al. 2001, Saeed et al. 2001, Gerrard et al. 2004, Aguilar et al. 2011, Blitvich et al. 2012). NRIV, a reassortant of BUNV and BATV, has been implicated in recent outbreaks of hemorrhagic fevers in Kenya and Somalia (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006). NRIV genome comprises the L and S segment of BUNV and the M segment of BATV, the two viruses not associated with hemorrhagic manifestations (Briese et al. 2006).

While BUNV, NRIV, Ilesha virus and Germiston virus have been isolated from mosquito and/or tick vectors during surveillance exercises in Kenya, no clear evidence exists for incidence of human

infection with these viruses as diagnostic laboratories seldom test for *Orthobunyavirus* infections. The actual disease burden of these viruses in Kenya remains to be determined. Moreover, viruses of the genus comprise a neglected but potentially deadly group of viruses as evidenced by the recent outbreak of Schmallenberg virus in Europe that resulted in malformations in ruminants (Lutomiah et al. 2013) and the implication of NRIV in hemorrhagic fever outbreaks within northern Kenya (Bowen et al. 2001, Gerrard et al. 2004, Briese et al. 2006, Lutomiah et al. 2013).

The International Centre of Insect Physiology and Ecology (ICIPE) is conducting a project entitled; An Integrated Response System for Emerging Infectious Diseases in East Africa, also known as Arbovirus Infection and Diversity (AVID) project. The project brings together a consortium of implementing institutions consisting of health, veterinary, wildlife and vector experts to take an integrated approach to arbovirus surveillance and research. The main aim of the project is to improve the prediction and prevention of RVF and other emerging arboviruses (CCHF, YF, Dengue, Onyong-nyong, WN, CHIK, Semliki Forest viruses etc.) and hoping to develop a model for response that could be expanded to other emerging diseases in the East African region. The project is interested in the discovery of both known and unknown viruses causing Emerging Infectious Diseases. The present analysis that uses samples from the AVID project aims to determine 1) the prevalence of *Orthobunyavirus* antibodies among febrile patients randomly selected from an ongoing febrile surveillance in three different regions of Kenya, as an indication of past clinical or subclinical infections and, 2) the demographic risk factors associated with exposure to these viruses.

6.2 METHODS

6.2.1 Study site

The study was conducted in Ijara District, Garissa County of northern Kenya (Figure 34). The region is an arid to semi-arid region with a majority of inhabitants practicing nomadic pastoralism with cattle, goats, sheep, donkeys and camels. Human whole blood specimens were drawn from febrile patients attending Sangailu Dispensary and Ijara Health Centre, the main health facilities that

serve most of the inhabitants. Additionally, blood specimens were drawn from Naivasha, a town within the Rift Valley Province of Kenya where these viruses have not been previously isolated, but a known RVF endemic region (Lutomiah et al. 2013).

6.2.2 Viruses

The BUNV (GSA/S4/11232) and NRIV (TND/S1/19801) strains used in this study were isolated from *Aedes mcintoshi* and *Anopheles funestus* respectively, during surveillance in the northern Kenya pastoral zone (Ochieng et al. 2013). Ilesha and Germiston viruses were obtained from the Centers of Disease Control and Prevention, Fort Collins, Colorado.

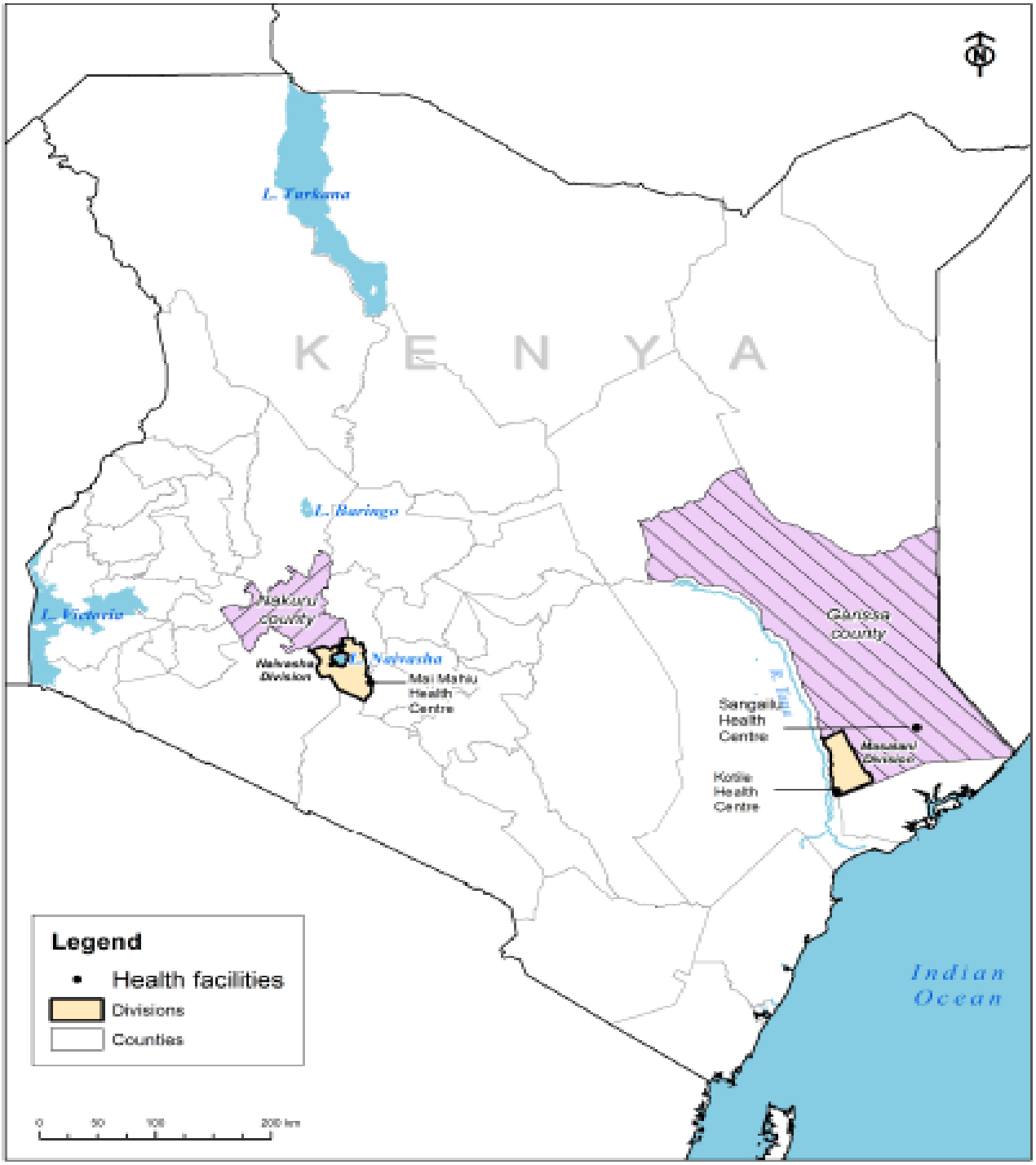


Figure 34: A map showing study sites in Garissa and Nakuru Counties, Kenya.

6.2.3 Study population

This was a retrospective study utilizing randomly selected 345 serum specimens collected from febrile patients in Sangailu Dispensary (n=94), Kotile Health Centre (n=118) and Naivasha health centre (n=133). The surveillance was conducted from January 2009 through April 2012. Blood

blood specimens were obtained from patients presenting with acute febrile illness, including symptoms like chills, cough, headache, joint aches, general body weakness, and any hemorrhagic signs. Inclusion criteria included patients greater than 5 years of age, referred for laboratory blood test by clinicians and consented to participate in the study. Demographic data was obtained from participants during the consenting process using a standard questionnaire (Appendix 4).

6.2.4 Ethical consideration

The samples used in this study were drawn from consenting patients and were de-identified. A signed consent form was obtained from participants 18 years and above while for patients younger than 18, a written assent was obtained from the parent or guardian (Appendix 3A-C). The human use protocol was approved by the Ethical Review Committees of the Kenya Medical Research Institute, Kenya (Protocol 2677) and the University of Pretoria, South Africa (Protocol 299/2013).

6.2.5 Neutralizing antibody prevalence

Each serum specimen was screened for anti-viral antibodies using the plaque reduction neutralization test (PRNT) as previously described (Rodrigues et al. 2011, Blitvich et al. 2012). Briefly, virus isolates were diluted to a standard concentration yielding 20-50 plaques. Sera were heat-inactivated at 56^{o} for 30 minutes and diluted at 1:20 concentration in maintenance media (minimum essential media) (Sigma) with Earle's salts, 2% fetal bovine serum, 2% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B). The serum dilutions were added to microcentrifuge tubes containing the standard concentration of the diluted virus and incubated for 1hour at 37^{0}C. The virus–antibody mixtures were then added to a 24-well plate with confluent Vero cell monolayer and incubated for 1 hour for un-neutralized virus adsorption after which an overlay of 1.25% methylcellulose (Sigma Aldrich) was added and incubated for 5 days. The plates were stained with 0.25% crystal violet (Sigma Aldrich) in absolute ethanol (Sigma Aldrich) and plaques counted. All reactive sera from the initial screening were further serially diluted to determine the endpoint titer, highest dilution that neutralizes 90% or greater of the virus

relative to a serum-free control (Figure 35). For serum specimens that were reactive with more than one virus, a four-fold or higher $PRNT_{90}$ antibody titer difference was required to determine the infecting virus.

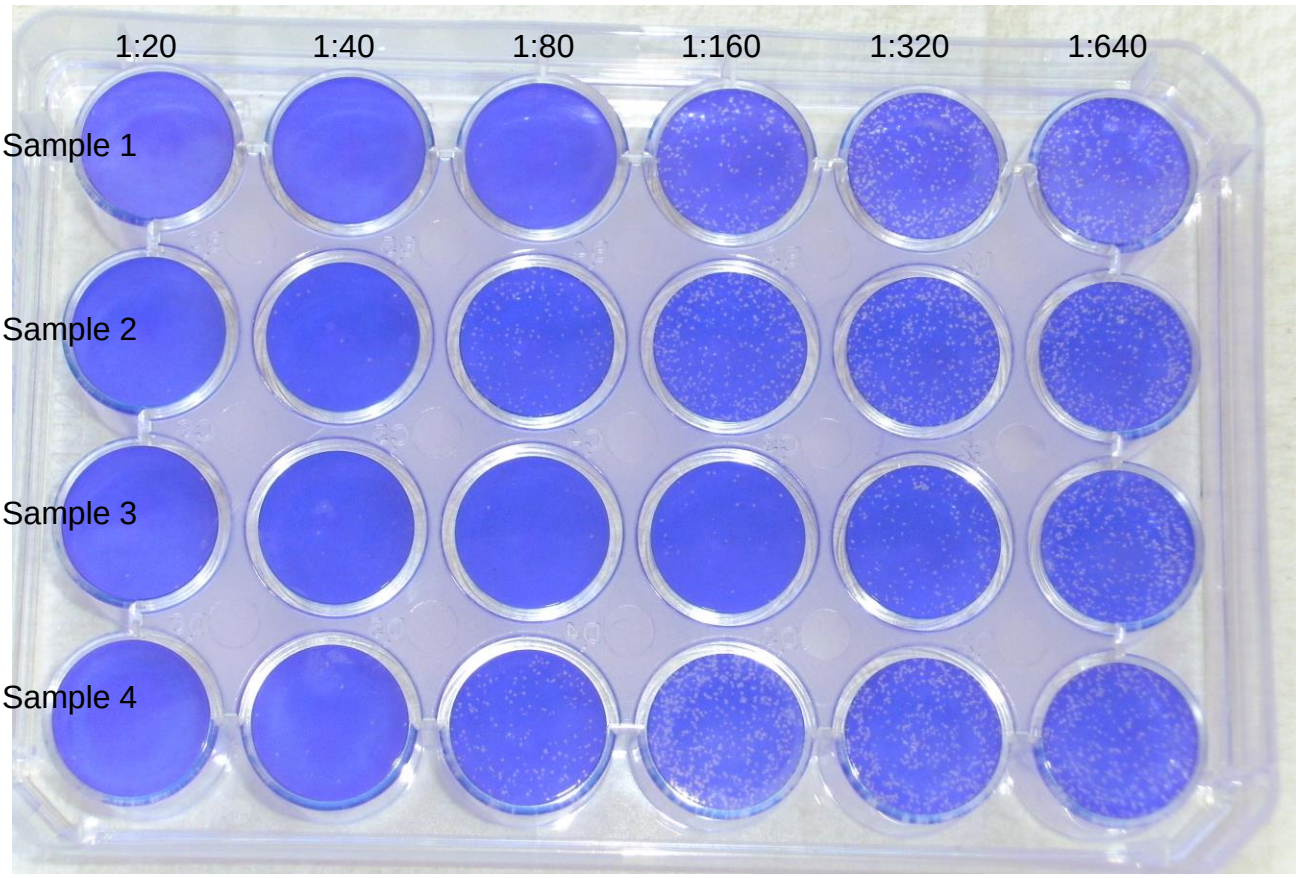


Figure 35: Picture showing comparative Plaque Reduction Neutralizing Test performed on a 24-well plate

6.2.6 Statistical analysis

Statistical analysis was performed using R statistical package (R Development Core Team 2008). The proportions of reactive specimens (PRNT≥20) were calculated with their respective 95% confidence intervals. Proportions were compared using Pearson Chi Square or Fischer exact tests where cell counts were less than 5. Bivariate analysis was done and variables that had a $p \leq 0.25$ were included in the final multivariate model. Variables that fit in the final model were age, occupation and district. Associations were reported using odds ratios. Variables that had a $p \leq 0.05$ in the final model were reported as significant.

6.3 RESULTS

6.3.1 PRNT antibody seroprevalence and risk factors

Overall, 89/345 (25.8%) febrile patients had neutralizing antibody to at least one of the four orthobunyaviruses with prevalences of 11.9%, 15.9%, 8.7% and 3.2% for BUNV, NRIV, Ilesha virus and Germiston virus respectively. Thirty three patients (9.6%) had neutralizing antibodies to more than one virus with majority of cross-neutralization between Ilesha virus and BUNV (Table 15). These 33 specimens were titrated and analyzed by comparative PRNT to identify the *Orthobunyavirus* responsible for the seropositivity. Eight specimens were resolved based on this analysis while 25 specimens remained undetermined (Table 15). Patients with neutralizing antibodies against any *Orthobunyavirus* in this study were significantly older than those without neutralizing antibodies; BUNV (35 versus 25 years; Chi Square test; $P < 0.0001$), NRIV (30 versus 25 years Chi Square test; $P = 0.033$), Ilesha virus (46 versus 28 years; $p=0.000$) and Germiston virus (41 verus 30 years; $p=0.019$).

Seroprevalence were significantly higher in Sangaila district for both BUNV and NRIV followed by Kotile district (25.5% vs 12.7% and 33.0% vs 20.8% respectively) (Table 16) while Naivasha district had the least prevalence with 1.5% for BUNV and no evidence of exposure to NRIV (Figures 36 and 37). However, for Ilesha virus, seroprevalence was relatively similar between Sangailu and Kotiel districts (14.9% versu 13.6%) while higher in the latter for Germiston virus (2.1% versus 7.6%).

Table 15: Endpoint titers of serum samples collected from persons in North Eastern Kenya and analyzed using comparative PRNT

	Demographic characteristics				$PRNT_{90}$ titers				
Patient ID	**Collection year**	**Residence**	**Age (y)**	**Sex**	**BUNV**	**NRIV**	**ILEV**	**GERV**	**Diagnosis**
HSA010062	2009	Sangailu	50	M	160	160	160	80	UND
HSA010005	2009	Sangailu	50	F	160	-	20	-	BUNV
HSA010010	2009	Sangailu	60	M	-	40	40	-	UND
HSA010074	2010	Sangailu	30	M	20	160	-	-	NRIV
HSA010094	2010	Sangailu	20	F	160	80	-	-	UND
HSA010109	2010	Sangailu	60	F	40	20	-	-	UND
HSA010303	2010	Sangailu	60	M	40	-	40	-	UND
HSA010312	2010	Sangailu	20	F	40	80	320	160	UND
HSA010314	2010	Sangailu	20	F	80	-	40	-	UND
HSA010389	2010	Sangailu	47	M	80	40		-	UND
HSA010395	2010	Sangailu	60	M	80	-	20	-	UND
HSA010752	2011	Sangailu	16	M	20	160	-	-	NRIV
HSA010779	2011	Sangailu	30	F	20	40	-	-	UND
HSA010780	2011	Sangailu	50	F	80	40	40	-	UND
HSA010856	2011	Sangailu	80	F	40	-	20	-	UND
HSA010888	2011	Sangailu	29	M	80	-	40	-	UND
HSA010918	2011	Sangailu	30	M	20	160	40	-	NRIV
HSA010947	2011	Sangailu	90	F	20	80	40	-	UND
HSA010988	2012	Sangailu	38	M	-	160	40	-	NRIV
HSA050018	2011	Kotile	17	M	80	-	20	-	BUNV
HSA050075	2011	Kotile	37	M	40	40	-	20	UND
HSA050083	2011	Kotile	35	M	80	-	80	40	UND
HSA050102	2011	Kotile	36	M	80	160	40	80	UND
HSA050124	2011	Kotile	30	M	-	≥640	160	-	NRIV
HSA050137	2011	Kotile	24	F	160	20	160	40	UND
HSA050146	2011	Kotile	19	M	20	80	-	-	NRIV
HSA050220	2011	Kotile	76	M	-	80	80	40	UND
HSA050222	2011	Kotile	73	M	-	80	80	160	UND
HSA050225	2011	Kotile	30	M	80	320	160	160	UND
HSA050286	2012	Kotile	37	F	80	160	160	80	UND
HSA050308	2012	Kotile	8	F	40	20	20	-	UND
HSA050388	2012	Kotile	55	F	20	-	40	-	UND
HSA050399	2012	Kotile	50	F	20	40	-	-	UND

*PRNT, plaque reduction neutralization test; BUNV, Bunyamwera virus; NRIV, Ngari virus; ILEV, Ilesha virus; GERV, Germiston virus; –, titer <20; UND, undetermined orthobunyavirus.

Neutralizing antibody prevalence for both BUNV and NRIV was similar among males and females (12.7% versus 11.4% and 15.7% versus 16.1%, respectively; $p<0.05$) (Table 16). However, seroprevalence was more than twice higher in males for Ilesha and Germiston viruses (12.7% versus 6.2% and 6.0% versus 1.4%, respectively). Seroprevalence was significantly higher in participants above 50 years of age for BUNV and Ilesha viruses while for NRIV and Germiston viruses, seroprevalence peaked in participants of ages between 30 and 49 years. Participants 19 years and below had the lowest seroprevalence of either virus (3.0%).

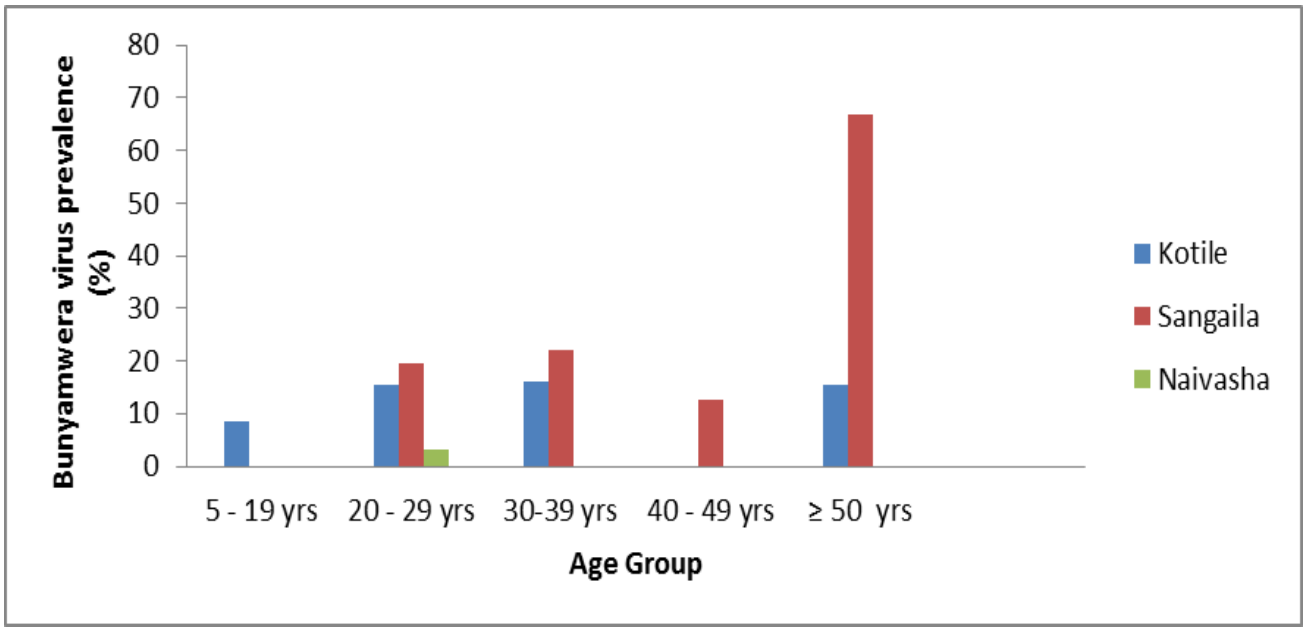


Figure 36: Bunyamwera virus seroprevalence by age groups in health facilities in Kotile, Sangailu and Naivasha health facilities 2009-2012.

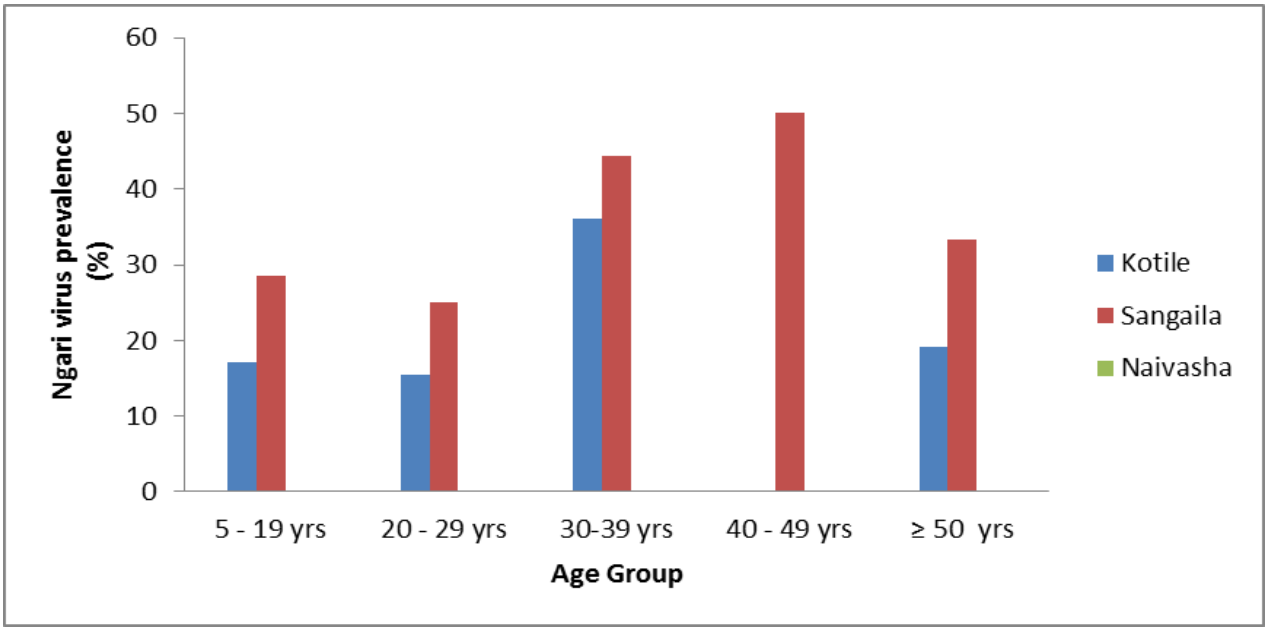


Figure 37: Ngari virus seroprevalence by age groups in health facilities in Kotile, Sangailu and Naivasha health facilities 2009-2012.

Table 16: Demographic characteristics of persons with positive PRNT for Bunyamwera and Ngari viruses in Kenya.

Characteristic	**BUNV $PRNT_{90}$**	**NRIV $PRNT_{90}$**	**ILEV $PRNT_{90}$**	**GERV $PRNT_{90}$**	
District	**N (%)**	**N (%)**			**Total**
Sangailu	24 (25.5)	31 (33.0)	14 (14.9)	2 (2.1)	94
Naivasha	2 (1.5)	0 (0)	0 (0)	0 (0)	133
Kotile	15 (12.7)	24 (20.3)	16 (13.6)	9 (7.6)	118
Gender					
male	17 (12.7)	21 (15.7)	17 (12.7)	8 (6.0)	134
female	24 (11.4)	34 (16.1)	13 (6.2)	3 (1.4)	211
Age					
5-19	3 (4.1)	10 (13.5)	2 (2.7)	0 (0)	74
20-29	13 (10.5)	13 (10.5)	6 (4.8)	2 (1.6)	124
30-39	8 (10.5)	17 (22.4)	7 (9.2)	6 (7.9)	76
40-49	1 (5.6)	4 (22.2)	0 (0)	0 (0)	18
≥50	16 (30.2)	11 (20.8)	15 (28.3)	3 (5.7)	53
Occupation					
Farmer	6 (14.0)	5 (11.6)	7 (16.3)	2 (4.7)	43
Herdsman	7 (21.2)	9 (27.3)	6 (18.2)	1 (3.0)	33
Housewife	17 (13.7)	25 (20.2)	7 (5.6)	2 (1.6)	124
Student	4 (12.1)	5 (15.2)	1 (3.0)	0 (0)	33
Businessman	1 (1.9)	3 (5.7)	3 (5.7)	3 (5.7)	53
Formal employment	2 (10.0)	2 (10)	0 (0)	1 (5.0)	20

In terms of occupation, seroprevalence was highest among herdsmen for BUNV, NRIV and Ilesha viruses (21.2%, 27.3% and 18.2% respectively). Businessmen had the lowest seroprevalence for BUNV and NRIV (1.9% vs 5.7% respectively).

Univariate and Multivariate logistic regression indicated that age and location were significantly associated with *Orthobunyavirus* seroprevalence (Table 17 and 18). The odds of *Orthobunyavirus* infections increased by approximately 1.0 for a 1-year increase in age after controlling for other factors. The odds of BUNV seropositivity were higher in Sangailu than Kotile (OR 3.76, 95% CI 1.45, 9.73). Similarly, the odds for NRIV seropositivity were higher in Sangailu compared to Kotile (OR 2.83, 95% CI 1.27, 6.33). Further analysis revealed that occupation and gender of patients was not significantly associated with BUNV and NRIV seropositivity. However, gender was associated with Ilesha and Germiston virus seropositivity with males more than twice likely to have antibodies against the two viruses.

Table 17: Bivariable analysis of risk factors associated with *Orthobunyavirus* infections in Kenya.

	Bunyamwera virus		**Ngari virus**		**Ilesha virus**		**Germiston virus**	
Variable	**OR** [95% CI]	**p-value**	**OR** [95% CI]	**p-value**	**OR** [95% CI]	**p-value**	**OR** [95% CI]	**p-value**
Gender		0.7145		0.9129				
Females	*ref.*		*ref.*		*ref.*		*ref.*	
Males	1.13 [0.58 – 2.20]	0.714	0.967 [0.535- 1.750]	0.913	2.23(1.04-4.76)	**0.037**	4.42(1.15-17.00)	**0.03**
Age (years)	1.04 [1.02 - 1.06]	< **0.0001**	1.017 [0.9999 - 1.0342]	**0.052**	1.06[1.03 - 1.08]	**0.000**	1.04[1.00 - 1.07]	**0.025**
Age Category (Years)		**0.0006**		0.1427		**0.000**		**0.053**
5 - 19	*ref.*		*ref.*		*ref.*		*ref.*	
20 - 29	2.77 [0.76 - 10.07]	0.121	0.750 [0.311 - 1.807]	0.521	0.58(0.11 - 3.03)	0.523	0.20(0.03-1.26)	0.087
30 - 39	2.78 [0.71 - 10.94]	0.142	1.844 [0.782 - 4.347]	0.162	1.60(0.31 - 8.13)	0.574	1.43(0.34-6.00)	0.63
40 - 49	1.39 [0.14 -14.23	0.78	1.829 [0.500 -6.681]	0.361			1	
≥ 50	10.23 [2.80 -37.39	< **0.0001**	1.676 [0.654 -4.294]	0.282	6.12(1.30 - 28.82)	**0.022**	1	
Location		< **0.0001**		**0.004**		0.782		**0.060**
Sangailu	2.35 [1.15-4.80]	0.019	1.927 [1.036- 3.587]	0.038	1.12[0.51 - 2.42]	0.782	*ref.*	
Naivasha	0.10 [0.02-0.47]	0.003						
Kotile	*ref.*		*ref.*		*ref.*		3.80(0.80-18.02	0.090
Occupation		**0.0794**		0.077		**0.008**		
Businessman	0.17 [0.02- 2.03]	0.162	0.54 [0.083- 3.499]	0.518	64[2.83 - 1446.88]	**0.009**	1.14[0.11-11.65]	0.912
Farmers	1.46 [0.27- 7.96]	0.662	1.184 [0.209- 6.7]	0.848	1.92[0.19 - 19.27]	0.579	0.93[0.08-10.86]	0.952
Herdsman	2.42 [0.45- 13.03]	0.303	3.375 [0.648- 17.566]	0.148	6.22[0.73 - 53.35]	0.095	0.59[0.04-10.05]	0.718
Housewife	1.43 [0.30- 6.72]	0.651	2.273 [0.494- 10.447]	0.291	7.11[0.81 - 62.79]	0.078	0.31[0.03-3.60]	0.350
Student	1.24 [0.21- 7.48]	0.813	1.607 [0.281- 9.188]	0.594	1.91[0.23 - 16.13]	0.550		
Formal employment	*ref.*		*ref.*		*ref.*		*ref.*	

Table 18: Multivariable analysis of risk factors associated with *Orthobunyavirus* infections in Kenya

	Bunyamwera virus		**Ngari virus**		**Ilesha virus**		**Germiston virus**	
Variable	**AOR** [95% CI]	**p-value**	**AOR** [95% CI]	**p-value**	**AOR** [95% CI]	**p-value**	**AOR** [95% CI]	**p-value**
Gender								
Females					*ref*		*ref*	
Males					2.61(0.74 - 9.29)	0.137	8.00(1.89-33.74)	**0.005**
Age (years)								
Age Category (Years)								
5 - 19	*ref.*				*ref*		*ref*	-
20 - 29	2.16 [0.22 - 21.57]	0.513	0.636 [0.183 - 2.21]	0.476	0.15(0.02 - 1.25)	0.080	0.35(0.05-2.30)	0.272
30 - 39	2.21 [0.19 - 26.21]	0.531	1.877 [0.532 - 6.628]	0.328	0.37(0.04 - 3.18)	0.368	2.49(0.53-11.84)	0.250
40 - 49	8.57 [0.71- 103.03]	0.0910	0.981 [0.183- 5.253]	0.9820			1	-
≥ 50	31.56 [2.96- 336.12]	0.0040	0.700 [0.171 - 2.868]	0.6200	0.74(0.09 - 6.15)	0.778	1	-
Location								
Sangailu	3.76 [1.45- 9.73]	0.006	2.829 [1.265- 6.326]	0.011			*ref*	
Naivasha	0.04 [0.01- 0.37]	0.004	--				-	
Kotile	*ref.*						5.77[1.13 - 29.61]	**0.036**
Occupation								
Businessman	0.11 [0.01 - 1.75]	0.118	0.869 [0.080- 9.479]	0.908	2.98[0.20 - 44.89]	0.430		
Farmers	0.24 [0.03 - 2.11]	0.196	0.577 [0.063 - 5.268]	0.626	7.23[0.53 - 98.53]	0.138		
Herdsman	0.18 [0.02 -1.61]	0.126	0.534 [0.063 - 4.539]	0.565	6.53[0.4 - 92.98]	0.166		
Housewife	0.12 [0.02- 0.86]	0.035	0.396 [0.0538 - 2.915]	0.363	4.60[0.31 - 68.28]	0.267		
Student	2.39 [0.15 - 38.46]	0.54	0.722 [0.065 - 8.014]	0.728	*ref.*			
Formal employment	*ref.*		*ref.*		-			

6.3.3 Malaria diagnosis

Of 87 patients with atleast one *Orthobunyavirus* seropositivity, only 43 (49.4) patients had a confirmatory laboratory test performed for malaria diagnosis. Of these 43 laboratory diagnostic tests, only 8 (18.6%) were positive. Fourty-one patients had a presumptive malaria diagnosis based on the clinical symptoms and the clinician's judgement.

6.4 DISCUSSION

In this chapter, we determine the prevalence of four orthobunyaviruses, BUNV, NRIV, Ilesha and Germiston viruses, in febrile patients attending health facilities in three different regions of Kenya. The overall seroprevalence for either of BUNV, NRIV, Ilesha virus and Germiston virus was 25.8%, indicative of significant circulation of these viruses among the Kenyan population. The lowest seroprevalence was against Germiston virus which suggests that human exposure is low and may be attributed to the feeding preference of the implicated mosquito vector, *Culex rubinotus*, which feeds primarily on rodents, to a moderate degree on domestic ungulates and rarely on humans (Jupp et al. 1976).

It is interesting to note that majority of infections occurred in persons greater than 15 year old indicating that this may represent exposure to these viruses during the last RVF outbreak in 1997/98 in which NRIV was isolated from hemorrhagic fever patients and antibodies detected in others (Briese et al. 2006). The prevalence of antibodies against BUNV, NRIV, Ilesha virus and Germiston virus increased with age suggesting endemicity of these viruses in Sangailu and Kotile districts. Seroprevalence was highest in persons aged ≥50 years for BUNV and Ilesha virus whereas NRIV and Germiston virus seroprevalence was higher in persons ≥30 years. About 27% of patients with hemorrhagic fever investigated in the 1997/1998 outbreak had evidence of acute NRIV infection (Briese et al. 2006). Thus, it is possible that some of the cases in the present study may be persons with IgG antibodies acquired in previous exposure during the previous outbreak.

There was a high degree of cross-neutralization between the BUNV and Ilesha virus assays which could not be distinguished by comparative PRNT. This was unexpected given there is little cross-neutralization between the two viruses (Hunt and Calisher 1979, Karabatsos 1985). Additionally, 12 patients had neutralizing antibodies to BUNV and NRIV and likewise could not be distinguished by comparative PRNT suggesting that these may represent persons doubly infected many years ago and as such, trace amounts of antibody remaining were insufficient to yield a 4-fold or greater titer between the implicated viruses. Alternatively, in the absence of virus isolation, there could be other yet to be identified orthobunyaviruses circulating within the same region. Although we did not screen for Shokwe virus, previously isolated in Kenya, antibodies against the virus do not neutralize any of the viruses tested in the current study (Hunt and Calisher 1979, Nashed et al. 1993). Additionally, it is possible that some of the patients seropositive for NRIV may have been infected with Batai virus (Nashed et al. 1993) although this virus has not been isolated in Kenya. We also did not have a virus from a different serogroup as a control for our neutralization assays.

The failure of public health system to identify those infected with these viruses may be attributed to the non-specific nature of their clinical presentation making it difficult to differentiate them from other diseases that present with febrile illness including malaria, typhoid and brucellosis. Additionally, this may be compounded by the unavailability of laboratory assays that can be used to detect these viruses in such remote and poor settings and this can explain why about half the patients seropositive for atleast one *Orthobunyavirus* had a presumptive malaria diagnosis. In many settings, malaria is generally ascribed to all febrile illnesses unless confirmation is made by laboratory diagnosis. However, malaria-like symptoms are also observed for arbovirus infections and as such, presumptive malaria diagnosis by the clinicians may be inappropriate. Additionally, only approximately 18% of patients tested had confirmed malaria infection indicating that there are likely other causes of febrile illness than malaria alone. More importantly, once a smear is positive to malaria, public health laboratories rarely test for alternative causes of disease. While all patients in this study sought care for unspecified fever, we could not determine whether any of these febrile

illnesses resulted from infection by either of the four viruses because there was no follow up to obtain convalescent sample for determination of rise in antibody titer.

The seroprevalence of BUNV, NRIV, Ilesha and Germiston viruses in Sangailu district was significantly higher than that in Kotile and lowest in Naivasha while Germiston seroprevalence was higher in Kotile. The difference in arbovirus prevalence between different locations has been reported in other arboviral prevalence studies within Kenya (Mease et al. 2011, Lwande et al. 2012) and can be explained by the different climatic conditions within these regions that influence economic activities of residents. The nomadic nature of inhabitants of North Eastern Kenya may result in spread of the viruses due to their close interaction with domestic animals and possible interaction of their animals with wild animals, which may be reservoirs of these viruses, during grazing (Lwande et al. 2012).

Although not statistically significant, herdsmen were at higher risk of infection by all orthobunyaviruses than housewives, farmers, students and businessmen. This may be so because of their close proximity to animal herds that may expose them to bites from mosquitoes and or ticks that have previously fed on infected animals as determined for RVF virus (Anyangu et al. 2010). Housewives were the second most affected, possibly through taking care of small livestock like sheep and goats in addition to milking (Lwande et al. 2012).

In conclusion, the present study demonstrates serological evidence of *Orthobunyavirus* activity in Sangailu and Kotile districts of Garissa County in northern Kenya. Age and location are all risk factors for BUNV and NRIV exposure. Orthobunyaviruses, among other arboviruses, should be considered when investigating etiologies of fever illnesses in North Eastern Kenya especially during seasons of high mosquito abundance.

Chapter 7

Concluding Remarks

BUNV is the prototype virus of the *Bunyaviridae* family and *Orthobunyavirus* genus. These viruses with segmented RNA genomes have the potential for genetic reassotment and and/or drift. While these viruses can cause human disease, they were not associated with hemorrhagic fever symptoms until NRIV, a natural reassortant virus of BUNV and BATV, was isolated from hemorrhagic fever patients and antibodies detected in several others during the 1997/1998 RVF outbreak in East Africa and retrospectively in the 1987/1988 outbreak in Sudan. While genetic drift and reassortment are known to occur frequently in the *Bunyaviridae* family, the epidemiological consequences of these evolutionary events are poorly understood and further hampered by lack of complete genome sequences. Additionally, BUNV and NRIV have been isolated in different mosquito species in surveillance exercise in northern Kenya but the role of these vectors in the maintenance and transmission of these viruses are poorly known. Moreover, actual disease burden of these viruses in Kenya remains to be determined since diagnostic laboratories seldom test for these infections. Thus, in this study we investigated the dynamics of these viruses in selected transmission foci in order to understand their circulation in an effort to provide knowledge that can be applied by public health authorities to bring these infections under control and also to predict their emergence.

In Chapter 2, we obtain complete gene sequences of two BUNV and three NRIV isolates from recent surveys in Kenya and investigate their genetic diversity with respect to time, location and vector species of origin. The Kenyan BUNV and NRIV isolates clustered distinctly from other BUNV and NRIV isolates from different geographic regions with insignificant differences with respect to time, region and vector species of isolation. However, the Kenyan isolates sequenced in this study were closest to the NRIV isolates from the 1997/1998 Kenya-Somalia hemorrhagic fever outbreak. Phylogenetic analyses reveal a rough geographic/temporal association of NRIV isolates,

but no clustering by source of the isolate. Our data did not support any evolutionary clustering of viral isolates based upon the source of isolation hence does not support the hypothesis of distinct virus isolates circulating in different host species.

In chapter 3, we presented genotypic and phenotypic characteristics of BUNV and NRIV isolates. In this chapter, we compare growth characteristics of purified plaque-isolated biological substrains of the two viruses in cell culture and evaluated pathogenesis in suckling mice. The LP substrains grew to a higher titer on Vero cells than the SP phenotype. However, the SP phenotype of BUNV was more neurovirulent than the LP phenotype contrary to our expectation. We attributed the observed phenotype to a non-synonymous mutation in the predicted active site of the L protein and propose further mutagenesis studies to confirm this observation.

In chapter 4, we determined the vector competence of, *Aedes aegypti*, *Culex quinquefaciatus* and *Anopheles gambiae* for transmission of BUNV and NRIV. Our results indicated that *Aedes aegypti* was moderately susceptible to BUNV infection but was incompetent for NRIV. However, *Anopheles gambiae* was moderately susceptible to both BUNV and NRIV viruses, while *Culex quinquefaciatus* was refractory to both viruses. In chapter 5, we investigated the role of vertical transmission in the maintenance of BUNV in nature. We determined that both *Aedes aegypti* and *Anopheles gambiae* transovarially transmitted BUNV to their progeny and that the prevalence of vertical transmission was related to ovarian cycle. We thus underscore the need to continually monitor emergent arboviral genotypes circulating within particular regions as well as vectors mediating these transmissions in order to preempt and prevent their adverse effects. We recommend that the genetic mechanism for species specificity/vector competence due to reassortment needs further investigation.

Finally, in chapter 6, we report findings from a retrospective serosurvey of febrile patients attending three health facilities located in Sangailu, Kotile (in Garissa, North Eastern Kenya) and Naivasha (in Rift Valley) of Kenya. BUNV and NRIV specific antibodies were detected in 84 (24.3%) of 345

persons tested; Prevalences were 11.9% for BUNV and 15.9% for NRIV. We determined that age and location were risk factors for BUNV and NRIV infections. We propose that in future these infections should be considered when investigating etiologies of febrile illness in patients reporting to health facilities in such endemic areas especially during seasons of high mosquito abundance.

While public health efforts have focused on well characterized viruses such as RVF, WNV, chikungunya and dengue viruses, the emergences of orthobunyaviruses such as NRIV and Schmallenberg virus as human and veterinary pathogens emphasize the need for in-depth characterization of these viruses and determination of their true public health impact. Additionally, we propose re-analysis of all three segments of available *Orthobunyavirus* isolates for correct identification of circulating viruses. The findings of this research thesis also underscore the need to continually monitor emergent arboviral genotypes circulating within particular regions as well as vectors mediating their transmissions to enable appropriate and timely intervention by public health authorities in the event of an outbreak. Finally, our work provides evidence of exposure of residents of North Eastern province of Kenya to Orthobunyaviruses and underscores the need to design and validate testing tools for use in febrile patients within such regions in order to diagnose and effectively treat these infections.

Chapter 8

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Appendices

Appendix 1; Nucleotide and amino acid sequences determined in this study aligned against selected Bunyamwera and Ngari virus isolates from GenBank. Viral complementary strand sequences are presented in the 5'-3' direction.

Appendix 1A: Ngari virus S segment nucleotide sequence alignment

```
NRIV_DakArD28542_(JX857316)  AGTAGTGTACTCCACACTACAAACTTGCTATTGTTGAAAATCGCTGTGCTATCAAATCTAACAGAAGGTCATTAAAGGCTCTTTAATGATTGAGTTAGAATTTCATGATG 110
NRIV_9800521_(JX857325)      ..........................................................T.......................................... 110
NRIV_9800535_(JX857328)      ..........................................................T.......................................... 110
NRIV_SUD-HKV141_(JX857322)   ..................................T.....................A.......................................... 110
NRIV_SUD-HKV66_(JX857319)    ..................................T.....................A.......................................... 110
GSA/TS7/5170_WT              ..........................................................T.....................A.......................... 110
ISL/TS2/5242/WT              ..........................................................T.....................A.......................... 110
TND/S1/19801/WT              ..........................................................T.....................A.......................... 110

NRIV_DakArD28542_(JX857316)  TCGCTGCTAACACCAGCAGTACTTTTGACCCAGAGGTCGCATACGCTAACTTTAAGCGTGTCTACACCACTGGGCTTAGTTATGACCACATACGAATCTTCTACATTAAA 220
NRIV_9800521_(JX857325)      ..........................................................C.......................................... 220
NRIV_9800535_(JX857328)      ..........................................................C.......................................... 220
NRIV_SUD-HKV141_(JX857322)   ..........................................................C.......................................... 220
NRIV_SUD-HKV66_(JX857319)    ..........................................................C.......................................... 220
GSA/TS7/5170_WT              ..........................................................C.......................................... 220
ISL/TS2/5242/WT              ..........................................................C.......................................... 220
TND/S1/19801/WT              ..........................................................C.......................................... 220

NRIV_DakArD28542_(JX857316)  GGACGCGAGATTAAAACTAGTCTCGCAAAAAGAAGTGAATGGGAAGTTACACTTAACCTTGGGGGCTGGAAGATTACTGTATATAATACGAATTTTCCTGGCAACCGGAA 330
NRIV_9800521_(JX857325)      .............................................................................................................. 330
NRIV_9800535_(JX857328)      .............................................................................................................. 330
NRIV_SUD-HKV141_(JX857322)   ..........................................................G..................................................... 330
NRIV_SUD-HKV66_(JX857319)    ..........................................................G..................................................... 330
GSA/TS7/5170_WT              .............................................................................................................. 330
ISL/TS2/5242/WT              .............................................................................................................. 330
TND/S1/19801/WT              .............................................................................................................. 330

NRIV_DakArD28542_(JX857316)  CAACCCAGTTCCTGACGATGGTCTTACCCTCCACCGCCTCAGTGGATTCCTTGCCAGGTACCTACTTGAGAAGATGCTAAAAGTCAGTGAACCAGAAAAATTGATCATCA 440
NRIV_9800521_(JX857325)      .............................................................................................................. 440
NRIV_9800535_(JX857328)      .............................................................................................................. 440
NRIV_SUD-HKV141_(JX857322)   .............................................................................................................. 440
NRIV_SUD-HKV66_(JX857319)    .............................................................................................................. 440
GSA/TS7/5170_WT              ..............................................................................................................C.......... 440
ISL/TS2/5242/WT              ..............................................................................................................C.......... 440
TND/S1/19801/WT              ..............................................................................................................C.......... 440

NRIV_DakArD28542_(JX857316)  AATCAAAAATAATCAATCCATTGGCTGAAAAAAACGGGATCACTTGGAATGATGGGGAGGAAGTTTACCTCTCCTTTTTTCCAGGATCAGAAATGTTCCTAGGAACTTTC 550
NRIV_9800521_(JX857325)      ................C.............................................................................................. 550
```

```
NRIV_9800535_(JX857328)          ..................................................................................................... 550
NRIV_SUD-HKV141_(JX857322)      .....................................................................................T.............. 550
NRIV_SUD-HKV66_(JX857319)       ..................................................................T.................................. 550
GSA/TS7/5170_WT                 .....................................................................................T.............. 550
ISL/TS2/5242/WT                 .....................................................................................T.............. 550
TND/S1/19801/WT                 .....................................................................................T.............. 550

NRIV_DakArD28542_(JX857316)     AGATTCTATCCCTTGGCAATTGGGATCTACAAAGTCCAGCGCAAGGAAATGGAACCAAAATACCTTGAAAAGACAATGCGACAGAGGTATATGGGACTAGAAGCAGCAAC 660
NRIV_9800521_(JX857325)          ..................................................................................................... 660
NRIV_9800535_(JX857328)          ..................................................................................................... 660
NRIV_SUD-HKV141_(JX857322)      ..........................T.....................................................A..................... 660
NRIV_SUD-HKV66_(JX857319)       ..........................T.....................................................A..................... 660
GSA/TS7/5170_WT                 .......................T.............................................................................. 660
ISL/TS2/5242/WT                 .......................T.............................................................................. 660
TND/S1/19801/WT                 .......................T.............................................................................. 660

NRIV_DakArD28542_(JX857316)     TTGGACTGTTAGTAAATTGACAGAAGTTCAGTCTGCATTAACAGTTGTCTCCAGCTTGGGTTGGAAGAAAACTAATGTTAGTGCAGCTGCCAGGGACTTCCTTGCTAAAT 770
NRIV_9800521_(JX857325)          .....................................................................................A............... 770
NRIV_9800535_(JX857328)          .....................................................................................A............... 770
NRIV_SUD-HKV141_(JX857322)      ..................................................................................................... 770
NRIV_SUD-HKV66_(JX857319)       ........C............................................................................................. 770
GSA/TS7/5170_WT                 ..............................C.G.................................................................... 770
ISL/TS2/5242/WT                 ..............................C.G.................................................................... 770
TND/S1/19801/WT                 ..............................C.G.................................................................... 770

NRIV_DakArD28542_(JX857316)     TTGGAATCAACATGTAAGCAGGGAAGCATTTTAAATCGGGCTAAAATCATCAGTTTTAATTTGGCTAAAAGGGTTGTTTCAACCCACAAAATAACAGCTGTTTGGGTGGG 880
NRIV_9800521_(JX857325)          ..................................................................................................... 880
NRIV_9800535_(JX857328)          ..................................................................................................... 880
NRIV_SUD-HKV141_(JX857322)      ............................G.......T.................................................................. 880
NRIV_SUD-HKV66_(JX857319)       ............................G.......T.................................................................. 880
GSA/TS7/5170_WT                 ..................................................................................................... 880
ISL/TS2/5242/WT                 ..................................................................................................... 880
TND/S1/19801/WT                 ..................................................................................................... 880

NRIV_DakArD28542_(JX857316)     TGGTTGGGGACAGAAAGACAGCGGACTAAATTAACATTACATTATTGATGGTATTTTAAGTTTTAGGTGGAGCACACTACT 961
NRIV_9800521_(JX857325)          ..................................................A.............................. 961
NRIV_9800535_(JX857328)          ..................................................A.............................. 961
NRIV_SUD-HKV141_(JX857322)      ..................................................A.............................. 961
NRIV_SUD-HKV66_(JX857319)       ..................................................A.............................. 961
GSA/TS7/5170_WT                 ..................................................A.............................. 961
ISL/TS2/5242/WT                 ..................................................A.............................. 961
TND/S1/19801/WT                 ..................................................A.............................. 961
```

Appendix 1B: Bunyamwera virus S segment nucleotide sequence alignment

```
BUNV_(NC_001927)                 AGTAGTGTACTCCACACTACAAACTTGCTATTGTTGAAAATCGCTGTGCTATTAAATCCAACAGAAGGTCATTAAAGGCTCTTTAATGATTGAGTTGGAATTTCATGATG 110
BUNV_BLCNNS_(D00353)             ..................................................................................................... 110
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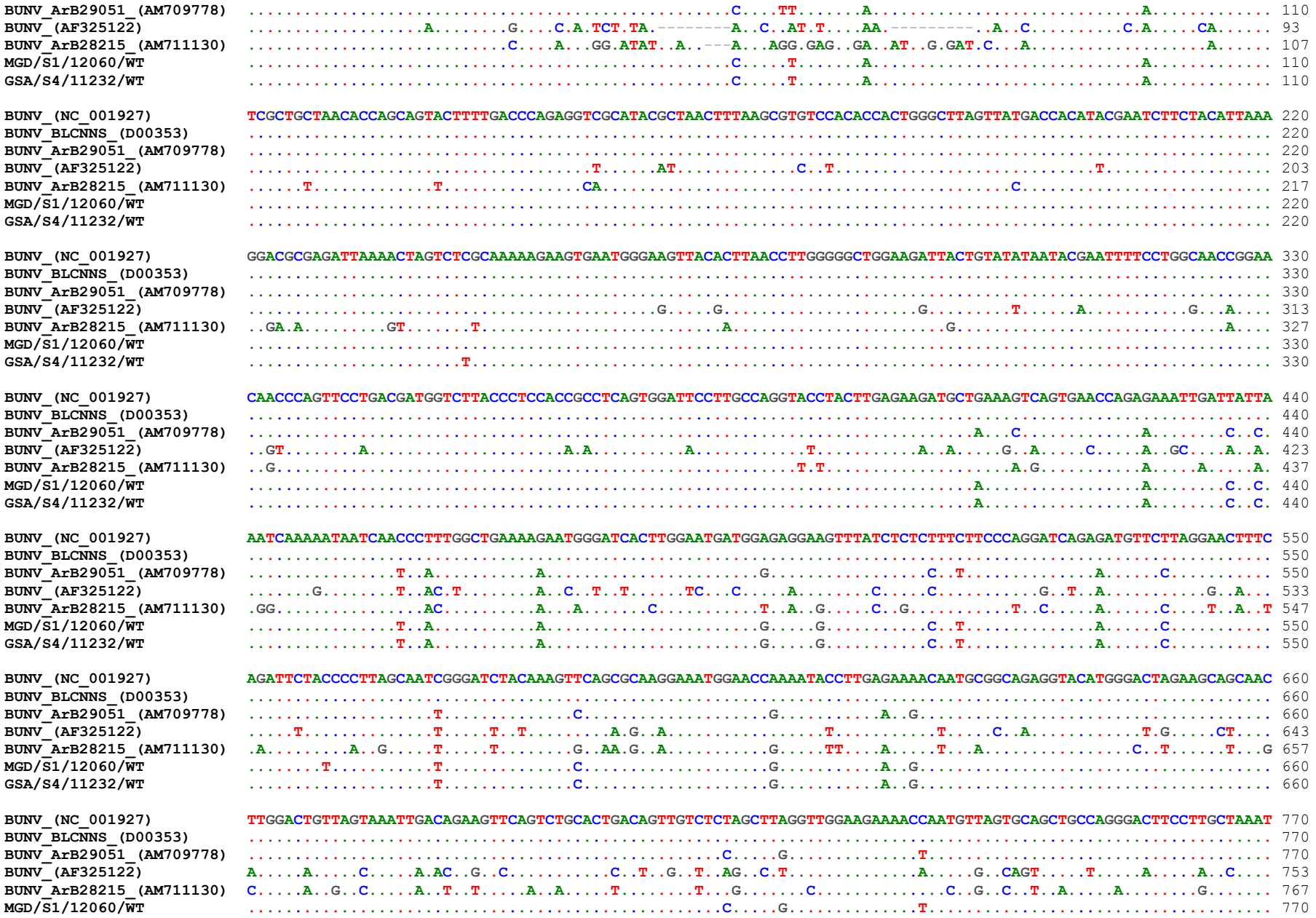
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BUNV_(AF325122) 93
BUNV_ArB28215_(AM711130) 107
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GSA/S4/11232/WT 110
BUNV_(NC_001927) TCGCTGCTAACACCAGCAGTACTTTTGACCCAGAGGTCGCATACGCTAACTTTAAGCGTGTCCACACCACTGGGCTTAGTTATGACCACATACGAATCTTCTACATTAAA 220
BUNV_BLCNNS_(D00353) 220
BUNV_ArB29051_(AM709778) 220
BUNV_(AF325122) 203
BUNV_ArB28215_(AM711130) 217
MGD/S1/12060/WT 220
GSA/S4/11232/WT 220
BUNV_(NC_001927) GGACGCGAGATTAAAACTAGTCTCGCAAAAAGAAGTGAATGGGAAGTTACACTTAACCTTGGGGGCTGGAAGATTACTGTATATAATACGAATTTTCCTGGCAACCGGAA 330
BUNV_BLCNNS_(D00353) 330
BUNV_ArB29051_(AM709778) 330
BUNV_(AF325122) 313
BUNV_ArB28215_(AM711130) 327
MGD/S1/12060/WT 330
GSA/S4/11232/WT 330
BUNV_(NC_001927) CAACCCAGTTCCTGACGATGGTCTTACCCTCCACCGCCTCAGTGGATTCCTTGCCAGGTACCTACTTGAGAAGATGCTGAAAGTCAGTGAACCAGAGAAATTGATTATTA 440
BUNV_BLCNNS_(D00353) 440
BUNV_ArB29051_(AM709778) 440
BUNV_(AF325122) 423
BUNV_ArB28215_(AM711130) 437
MGD/S1/12060/WT 440
GSA/S4/11232/WT 440
BUNV_(NC_001927) AATCAAAAATAATCAACCCTTTGGCTGAAAAGAATGGGATCACTTGGAATGATGGAGAGGAAGTTTATCTCTCTTTCTTCCCAGGATCAGAGATGTTCTTAGGAACTTTC 550
BUNV_BLCNNS_(D00353) 550
BUNV_ArB29051_(AM709778) 550
BUNV_(AF325122) 533
BUNV_ArB28215_(AM711130) 547
MGD/S1/12060/WT 550
GSA/S4/11232/WT 550
BUNV_(NC_001927) AGATTCTACCCCTTAGCAATCGGGATCTACAAAGTTCAGCGCAAGGAAATGGAACCAAAATACCTTGAGAAAACAATGCGGCAGAGGTACATGGGACTAGAAGCAGCAAC 660
BUNV_BLCNNS_(D00353) 660
BUNV_ArB29051_(AM709778) 660
BUNV_(AF325122) 643
BUNV_ArB28215_(AM711130) 657
MGD/S1/12060/WT 660
GSA/S4/11232/WT 660
BUNV_(NC_001927) TTGGACTGTTAGTAAATTGACAGAAGTTCAGTCTGCACTGACAGTTGTCTCTAGCTTAGGTTGGAAGAAAACCAATGTTAGTGCAGCTGCCAGGGACTTCCTTGCTAAAT 770
BUNV_BLCNNS_(D00353) 770
BUNV_ArB29051_(AM709778) 770
BUNV_(AF325122) 753
BUNV_ArB28215_(AM711130) 767
MGD/S1/12060/WT 770

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GSA/S4/11232/WT          ..................................................C.....G.............T.................................. 770

BUNV_(NC_001927)         TCGGAATCAACATGTAAGCAGGGATGCATTTTTAATCGGGCTAAAGTCATCTGTTTTAATTTGGCTAAAAGGGTTGTTTCAACCCACAAAAT-AACAGCTGCTTGGGTGG 879
BUNV_BLCNNS_(D00353)     ..................................................................................................-..... 879
BUNV_ArB29051_(AM709778) .T.....T.................A......C.............A.....A.....................................-........T........ 879
BUNV_(AF325122)          .T.....T.G.......ATGATC.GT..GAAC..GATTT.GC...A.TTAAGA.A.................A..CC.AGG.CT.A.....TCA.......TG........ 863
BUNV_ArB28215_(AM711130) .T..C.....T......TTT..T.ATTTGAA......T...A..CA.TT.AAAA......CA.......T.T.GGT...A.............-......AT......... 876
MGD/S1/12060/WT          .T.....T.................A.......A............A.....A.....................................-........T........ 879
GSA/S4/11232/WT          .T.....T.................A.....................A.....A.....................................-........T........ 879

BUNV_(NC_001927)         GTGGTTGGGGACAGAAAGACAGCGGGCTAAATCAACATTATATTGTTAATGGTATTTTAAGTTTTAGGTGGAGCACACTACT 961
BUNV_BLCNNS_(D00353)     ..................................................................................... 961
BUNV_ArB29051_(AM709778) ........................A......T........C...A.....A..................................... 961
BUNV_(AF325122)          ...................ATA.A.C---.T.T.TTC..GA..CA..GCA.T.C.............................. 943
BUNV_ArB28215_(AM711130) .......C....CA...A..CATT..---..G.TC.TTCA.C...C.G.T.T.GTA.A.......................... 956
MGD/S1/12060/WT          ........................A......T.......C...A.............................. 961
GSA/S4/11232/WT          ........................A......T..T....C...A.............................. 961
```

Appendix 1C: Ngari virus M segment nucleotide sequence alignment

```
NRIV_DakArD28542_(JX857317)  AGTAGTGTACTACCGATACAAATCACAACTATAATTGCAAGATGTTGCTACTTCTTGTCTTATTAATCCCTTGCTATGTATCAGCCTCTCCTGTAGTAACAAGATGCTTC 110
NRIV_9800521_(JX857326)      ..................................................................C.................................. 110
NRIV_9800535_(AY593725)      ..................................................................C.................................. 110
NRIV_SUD-HKV141_(JX857323)   ..................................................................C.................................. 110
NRIV_SUD-HKV66_(JX857320)    ..................................................................C.................................. 110
GSA/TS7/5170_WT             ..............................................................T...C.................................. 110
ISL/TS2/5242_WT             ..............................................................T...C.................................. 110
TND/S1/19801_WT             ..............................................................T...C.................................. 110

NRIV_DakArD28542_(JX857317)  CATGGAGGGCAATTGATAGCAGAGAAGAAATCCCAAACAGCTGTATCAGAATTTTGCCTGAAAGATGATGTCTCTACTATTAAGTCAGAAATAACATATGAGAAGAACAA 220
NRIV_9800521_(JX857326)      ..................................................................................................... 220
NRIV_9800535_(AY593725)      ..................................................................................................... 220
NRIV_SUD-HKV141_(JX857323)   ..........................................................C.......................................... 220
NRIV_SUD-HKV66_(JX857320)    ..................................................................................................... 220
GSA/TS7/5170_WT             .....................................................................................G.......... 220
ISL/TS2/5242_WT             .....................................................................................G.......... 220
TND/S1/19801_WT             .....................................................................................G.......... 220

NRIV_DakArD28542_(JX857317)  CACTGGTTTATTTGCCCATAGCAAAGTTTTACGAAATTGGATCATAAAAGACTGGAAAAATTGCAACCCTGTTCCCACAGCAGGGGGCAGCATAAATGTTATAGAAGTCA 330
NRIV_9800521_(JX857326)      ..................................................................................................... 330
NRIV_9800535_(AY593725)      ..........................................................T.......................................... 330
NRIV_SUD-HKV141_(JX857323)   ..................................................................................................... 330
NRIV_SUD-HKV66_(JX857320)    ..................................................................................................... 330
GSA/TS7/5170_WT             ..................................................................................................... 330
ISL/TS2/5242_WT             ..................................................................................................... 330
TND/S1/19801_WT             ..................................................................................................... 330

NRIV_DakArD28542_(JX857317)  ATACAGATTTGAGCTTGACCACAAAAACATATGTGTGTAGCAGAGATTGCACAATTACAGTTGATAAAGAGGATGCTCAGATAATTTTCCAAACCGAGAAGTTGAACCAT 440
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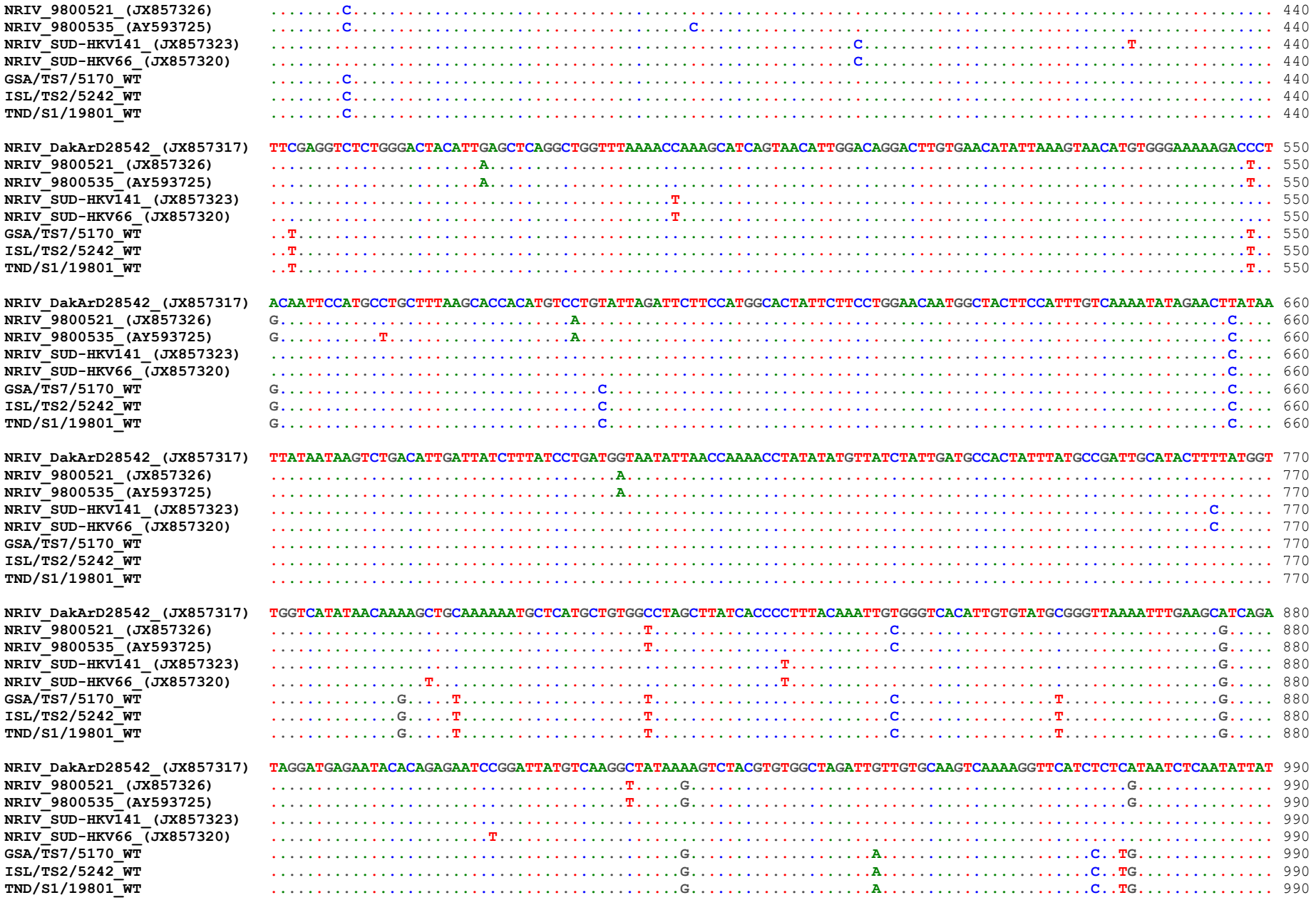
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GSA/TS7/5170_WT 440
ISL/TS2/5242_WT 440
TND/S1/19801_WT 440
NRIV_DakArD28542_(JX857317) TTCGAGGTCTCTGGGACTACATTGAGCTCAGGCTGGTTTAAAACCAAAGCATCAGTAACATTGGACAGGACTTGTGAACATATTAAAGTAACATGTGGGAAAAAGACCCT 550
NRIV_9800521_(JX857326) 550
NRIV_9800535_(AY593725) 550
NRIV_SUD-HKV141_(JX857323) 550
NRIV_SUD-HKV66_(JX857320) 550
GSA/TS7/5170_WT 550
ISL/TS2/5242_WT 550
TND/S1/19801_WT 550
NRIV_DakArD28542_(JX857317) ACAATTCCATGCCTGCTTTAAGCACCACATGTCCTGTATTAGATTCTTCCATGGCACTATTCTTCCTGGAACAATGGCTACTTCCATTTGTCAAAATATAGAACTTATAA 660
NRIV_9800521_(JX857326) 660
NRIV_9800535_(AY593725) 660
NRIV_SUD-HKV141_(JX857323) 660
NRIV_SUD-HKV66_(JX857320) 660
GSA/TS7/5170_WT 660
ISL/TS2/5242_WT 660
TND/S1/19801_WT 660
NRIV_DakArD28542_(JX857317) TTATAATAAGTCTGACATTGATTATCTTTATCCTGATGGTAATATTAACCAAAACCTATATATGTTATCTATTGATGCCACTATTTATGCCGATTGCATACTTTTATGGT 770
NRIV_9800521_(JX857326) 770
NRIV_9800535_(AY593725) 770
NRIV_SUD-HKV141_(JX857323) 770
NRIV_SUD-HKV66_(JX857320) 770
GSA/TS7/5170_WT 770
ISL/TS2/5242_WT 770
TND/S1/19801_WT 770
NRIV_DakArD28542_(JX857317) TGGTCATATAACAAAAGCTGCAAAAAATGCTCATGCTGTGGCCTAGCTTATCACCCCTTTACAAATTGTGGGTCACATTGTGTATGCGGGTTAAAATTTGAAGCATCAGA 880
NRIV_9800521_(JX857326) 880
NRIV_9800535_(AY593725) 880
NRIV_SUD-HKV141_(JX857323) 880
NRIV_SUD-HKV66_(JX857320) 880
GSA/TS7/5170_WT 880
ISL/TS2/5242_WT 880
TND/S1/19801_WT 880
NRIV_DakArD28542_(JX857317) TAGGATGAGAATACACAGAGAATCCGGATTATGTCAAGGCTATAAAAGTCTACGTGTGGCTAGATTGTTGTGCAAGTCAAAAGGTTCATCTCTCATAATCTCAATATTAT 990
NRIV_9800521_(JX857326) 990
NRIV_9800535_(AY593725) 990
NRIV_SUD-HKV141_(JX857323) 990
NRIV_SUD-HKV66_(JX857320) 990
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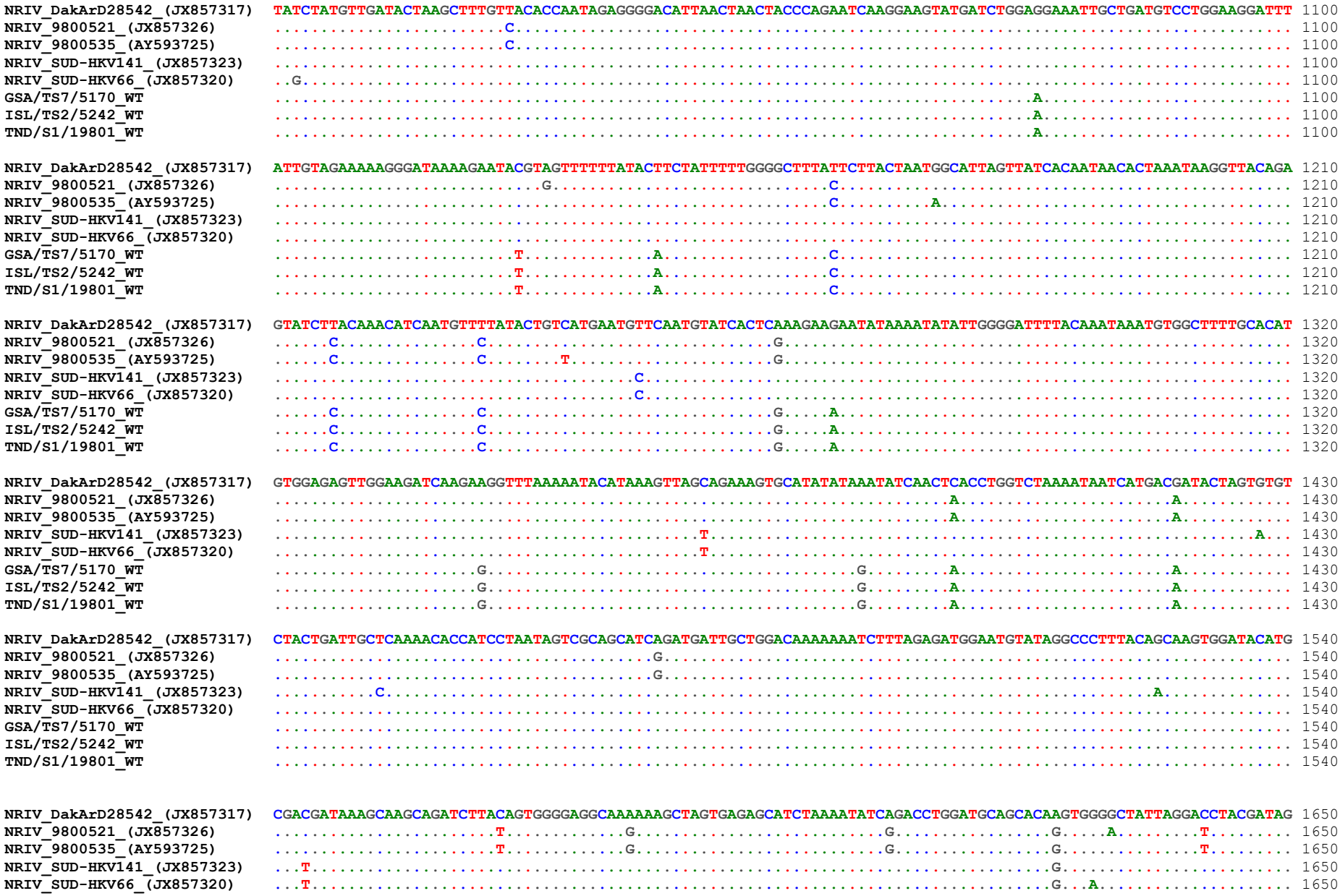
ISL/TS2/5242_WT 990
TND/S1/19801_WT 990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NRIV_9800521_(JX857326) 1100
NRIV_9800535_(AY593725) 1100
NRIV_SUD-HKV141_(JX857323) 1100
NRIV_SUD-HKV66_(JX857320) 1100
GSA/TS7/5170_WT 1100
ISL/TS2/5242_WT 1100
TND/S1/19801_WT 1100
NRIV_DakArD28542_(JX857317) ATTGTAGAAAAAGGGATAAAAGAATACGTAGTTTTTTATACTTCTATTTTTGGGGCTTTATTCTTACTAATGGCATTAGTTATCACAATAACACTAAATAAGGTTACAGA 1210
NRIV_9800521_(JX857326) 1210
NRIV_9800535_(AY593725) 1210
NRIV_SUD-HKV141_(JX857323) 1210
NRIV_SUD-HKV66_(JX857320) 1210
GSA/TS7/5170_WT 1210
ISL/TS2/5242_WT 1210
TND/S1/19801_WT 1210
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NRIV_SUD-HKV66_(JX857320) 1320
GSA/TS7/5170_WT 1320
ISL/TS2/5242_WT 1320
TND/S1/19801_WT 1320
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NRIV_9800535_(AY593725) 1430
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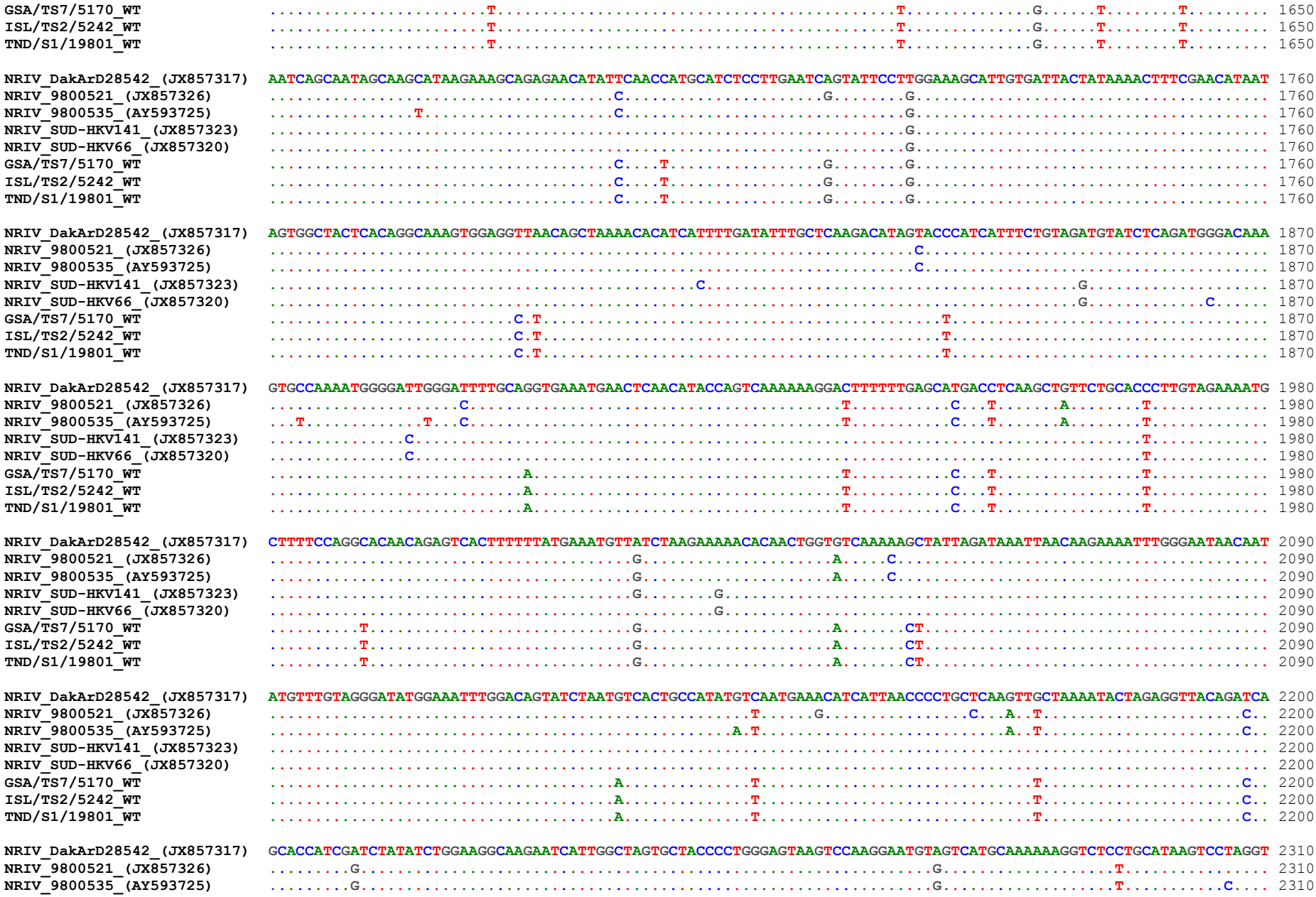

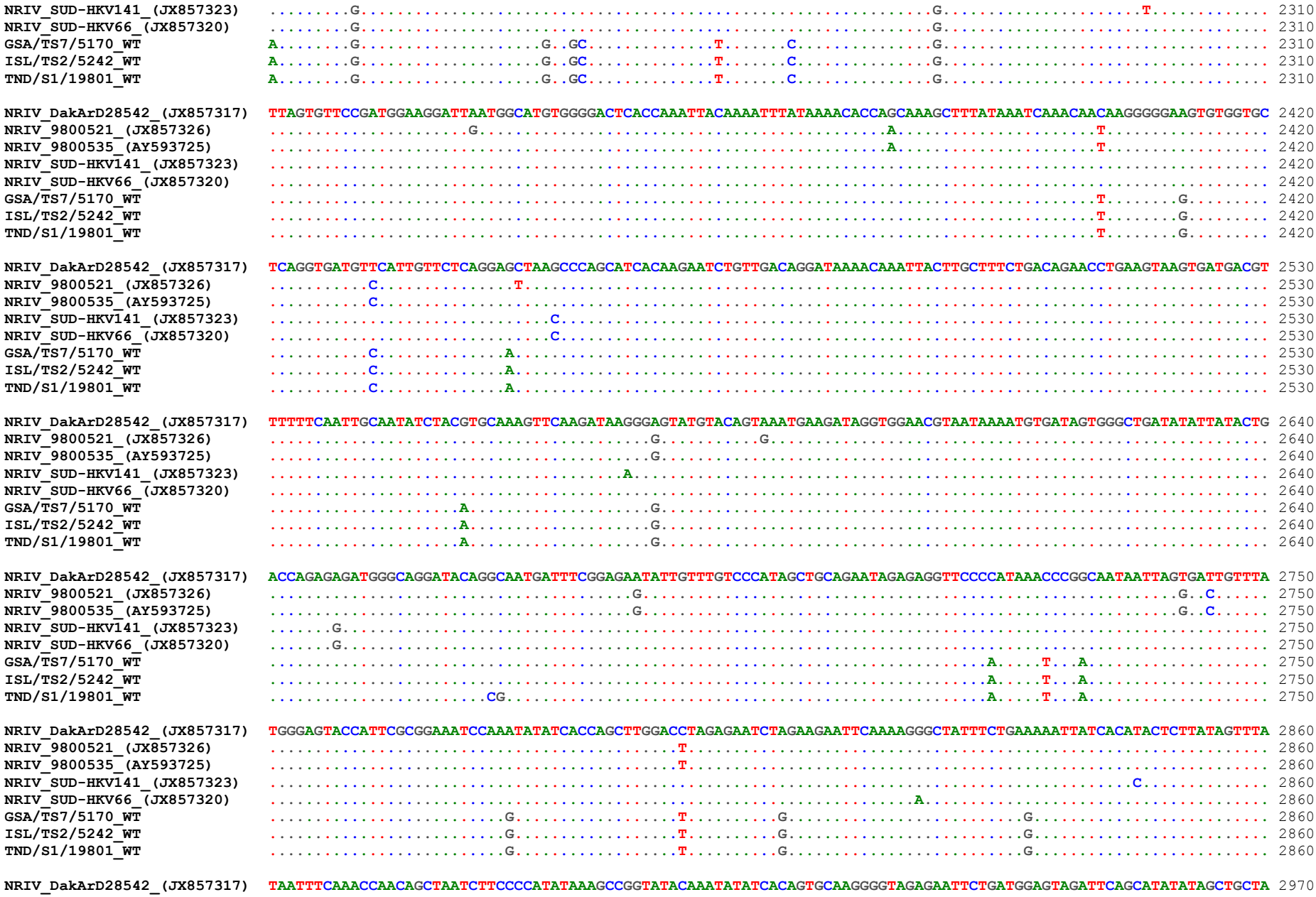
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NRIV_9800535_(AY593725) 2420
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NRIV_SUD-HKV66_(JX857320) 2420
GSA/TS7/5170_WT 2420
ISL/TS2/5242_WT 2420
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NRIV_9800535_(AY593725) 2530
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NRIV_SUD-HKV66_(JX857320) 2530
GSA/TS7/5170_WT 2530
ISL/TS2/5242_WT 2530
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NRIV_9800535_(AY593725) 2640
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TND/S1/19801_WT 2640
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NRIV_9800535_(AY593725) 2750
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NRIV_SUD-HKV66_(JX857320) 2750
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ISL/TS2/5242_WT 2750
TND/S1/19801_WT 2750
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NRIV_9800535_(AY593725) 2860
NRIV_SUD-HKV141_(JX857323) 2860
NRIV_SUD-HKV66_(JX857320) 2860
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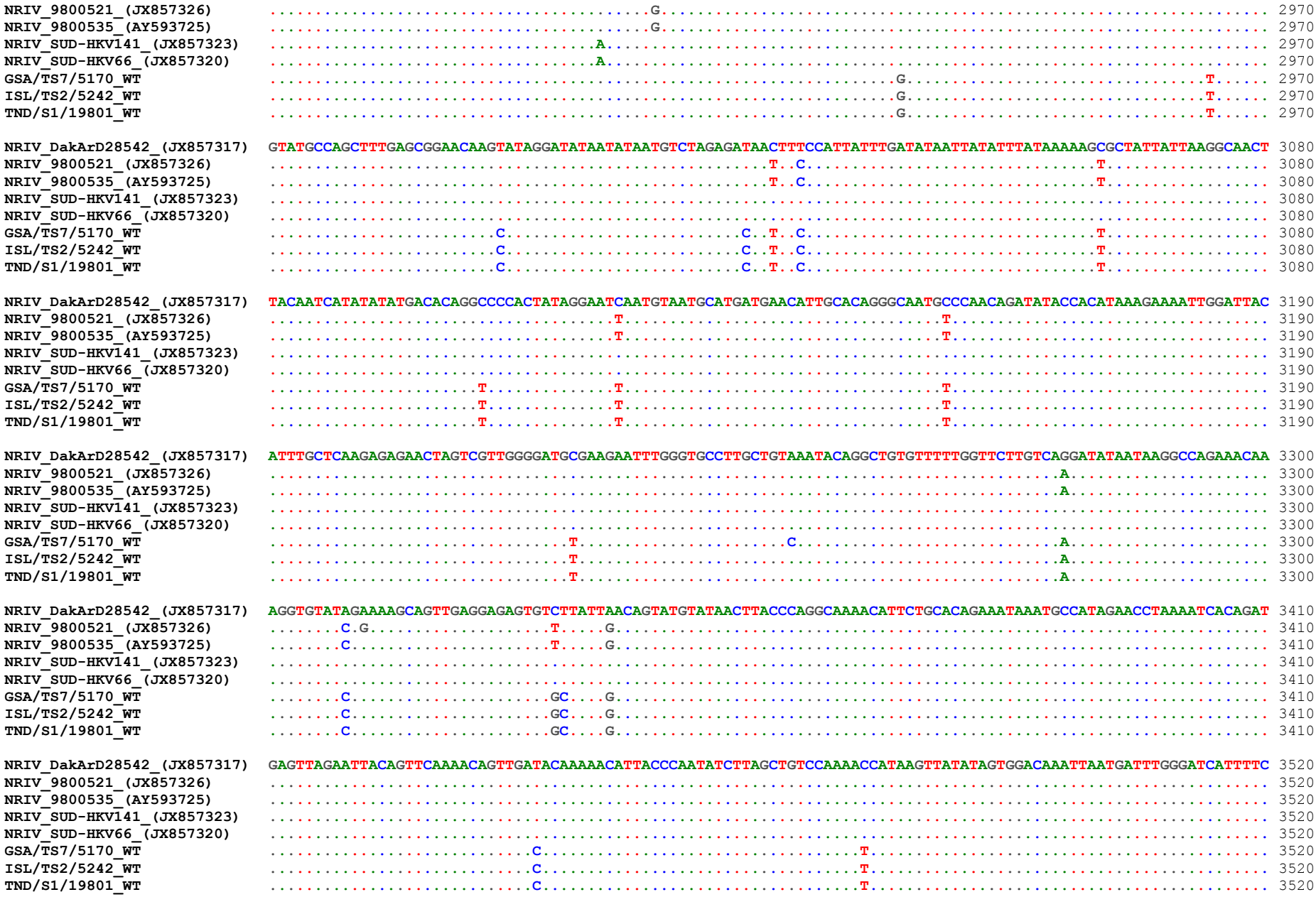
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ISL/TS2/5242_WT 2970
TND/S1/19801_WT 2970
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ISL/TS2/5242_WT 3080
TND/S1/19801_WT 3080
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NRIV_SUD-HKV141_(JX857323) 3190
NRIV_SUD-HKV66_(JX857320) 3190
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ISL/TS2/5242_WT 3190
TND/S1/19801_WT 3190
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NRIV_9800535_(AY593725) 3300
NRIV_SUD-HKV141_(JX857323) 3300
NRIV_SUD-HKV66_(JX857320) 3300
GSA/TS7/5170_WT 3300
ISL/TS2/5242_WT 3300
TND/S1/19801_WT 3300
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NRIV_9800535_(AY593725) 3410
NRIV_SUD-HKV141_(JX857323) 3410
NRIV_SUD-HKV66_(JX857320) 3410
GSA/TS7/5170_WT 3410
ISL/TS2/5242_WT 3410
TND/S1/19801_WT 3410
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NRIV_9800535_(AY593725) 3520
NRIV_SUD-HKV141_(JX857323) 3520
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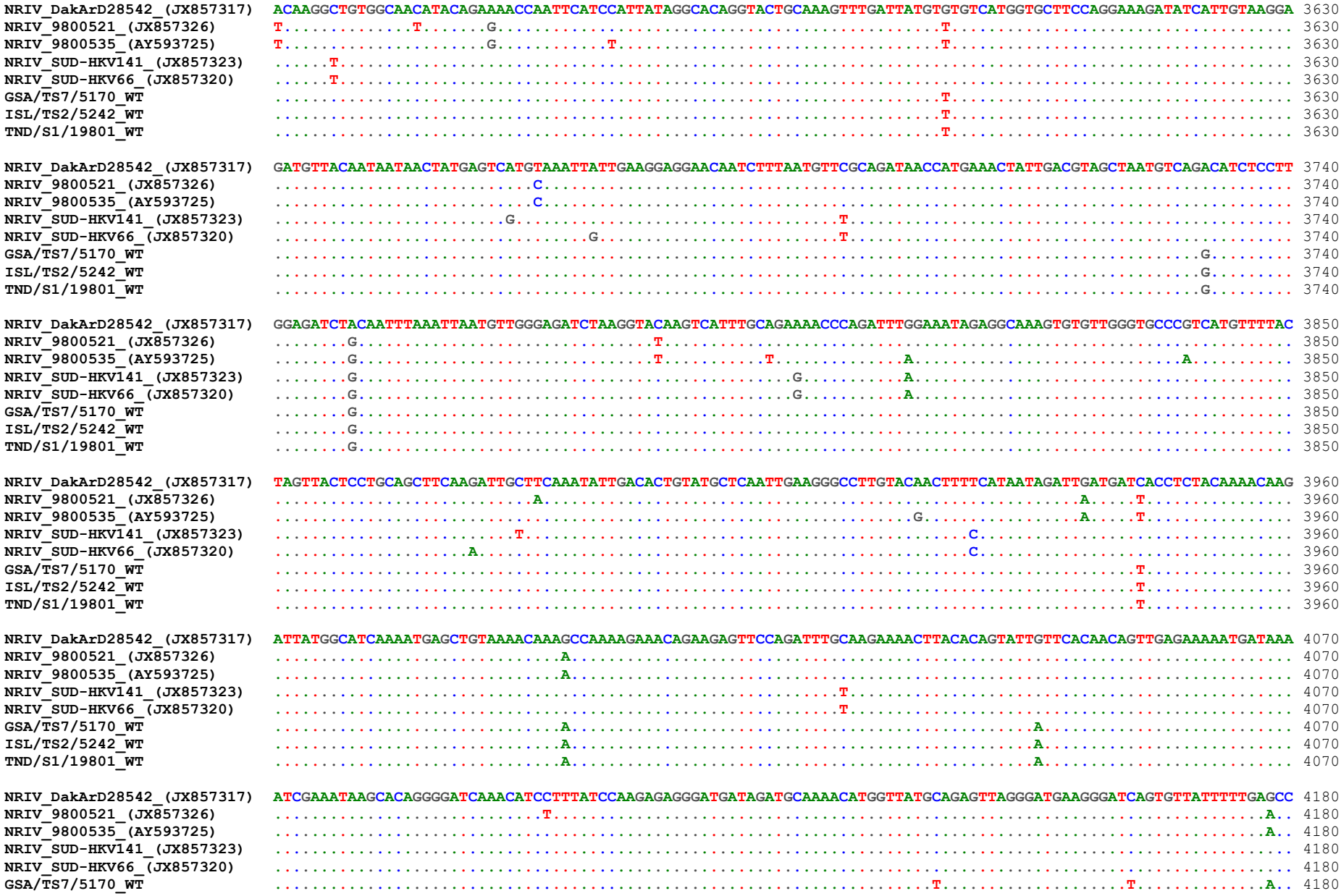
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NRIV_9800535_(AY593725) T.....G.....T.....T..... 3630
NRIV_SUD-HKV141_(JX857323) .....T..... 3630
NRIV_SUD-HKV66_(JX857320) .....T..... 3630
GSA/TS7/5170_WT .....T..... 3630
ISL/TS2/5242_WT .....T..... 3630
TND/S1/19801_WT .....T..... 3630
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NRIV_9800535_(AY593725) .....C..... 3740
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NRIV_SUD-HKV66_(JX857320) .....G.....T..... 3740
GSA/TS7/5170_WT .....G..... 3740
ISL/TS2/5242_WT .....G..... 3740
TND/S1/19801_WT .....G..... 3740
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NRIV_SUD-HKV141_(JX857323) .....G.....G.....A..... 3850
NRIV_SUD-HKV66_(JX857320) .....G.....G.....A..... 3850
GSA/TS7/5170_WT .....G..... 3850
ISL/TS2/5242_WT .....G..... 3850
TND/S1/19801_WT .....G..... 3850
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NRIV_9800535_(AY593725) .....G.....A.....T..... 3960
NRIV_SUD-HKV141_(JX857323) .....T.....C..... 3960
NRIV_SUD-HKV66_(JX857320) .....A.....C..... 3960
GSA/TS7/5170_WT .....T..... 3960
ISL/TS2/5242_WT .....T..... 3960
TND/S1/19801_WT .....T..... 3960
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NRIV_9800535_(AY593725) .....A..... 4070
NRIV_SUD-HKV141_(JX857323) .....T..... 4070
NRIV_SUD-HKV66_(JX857320) .....T..... 4070
GSA/TS7/5170_WT .....A.....A..... 4070
ISL/TS2/5242_WT .....A.....A..... 4070
TND/S1/19801_WT .....A.....A..... 4070
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NRIV_9800535_(AY593725) .....A.. 4180
NRIV_SUD-HKV141_(JX857323) 4180
NRIV_SUD-HKV66_(JX857320) 4180
GSA/TS7/5170_WT .....T.....T.....A.. 4180

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ISL/TS2/5242_WT            .......................................................................T....................T..............A.. 4180
TND/S1/19801_WT            .......................................................................T....................T..............A.. 4180

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NRIV_9800535_(AY593725)      .................................................A............................................................ 4290
NRIV_SUD-HKV141_(JX857323)   ..........................................................................................A................... 4290
NRIV_SUD-HKV66_(JX857320)    ..........................................................................................A................... 4290
GSA/TS7/5170_WT              ................................................A....T........................................................ 4290
ISL/TS2/5242_WT              ................................................A....T........................................................ 4290
TND/S1/19801_WT              ................................................A....T........................................................ 4290

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NRIV_9800535_(AY593725)      .................................................T..A.A...TTC.------------------------........................ 4376
NRIV_SUD-HKV141_(JX857323)   ..........................T...........................A.......------------------------........................ 4376
NRIV_SUD-HKV66_(JX857320)    ..........................T...........................A.......------------------------........................ 4376
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ISL/TS2/5242_WT              .................................................T....A...TT..------------------------......................A. 4376
TND/S1/19801_WT              .................................................T....A...TT..------------------------......................A. 4376

NRIV_DakArD28542_(JX857317)  AAGATAAATTTTATATATTAGCCTTAGGGCAAATTAGCTGTTATTATATCGGTAGCACACTACT 4464
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NRIV_9800535_(AY593725)      ...--........................................................... 4438
NRIV_SUD-HKV141_(JX857323)   ................................................................ 4440
NRIV_SUD-HKV66_(JX857320)    ................................................................ 4440
GSA/TS7/5170_WT              -----...........G............................................... 4435
ISL/TS2/5242_WT              -----...........G............................................... 4435
TND/S1/19801_WT              -----...........G............................................... 4435
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Appendix 1D: Bunyamwera virus M segment nucleotide sequence alignment

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BUNV_(NC_001926)   AGTAGTGTACTACCGATACATCACAAACCTTTCAGAGACACATCTTTATTTCCAAGATGAGAATTCTAATACTGCTTTTAGCAGTCACTCAACTGGCTGTGAGTAGCCCA 110
BUNV_(M11852)      .............................................................................................................. 110
BUNV_(JF961341)    -------------------------------------------------------------------------------------------------------------- 1
GSA/S4/11232_WT    ...................................................T.........G.......................T...A...........G........ 110
MGD/S1/12060_WT    ...................................................T.........G.......................T...A...........G........ 110

BUNV_(NC_001926)   GTTATCACTAGATGCTTTCATGGTGGGCAACTGATTGCAGAAAGGAAATCCCAAACATCGATTTCAGAATTCTGCATTAAAGATGACGTTTCTATGTTAAAATCAGAGAT 220
BUNV_(M11852)      .............................................................................................................. 220
BUNV_(JF961341)    -------------------------------------------------------------------------------------------------------------- 1
GSA/S4/11232_WT    ...G.......................................................A..C............................................... 220
MGD/S1/12060_WT    ...G.......................................................A..C............................................... 220

BUNV_(NC_001926)   TGTCTACACAAAAAATGATACTGGGATTTTTGGCCACAGTAAAGTGTTTCGTCACTGGACGATCACAGACTGGAAAGCATGCAACCCTGTTGTTACGGCCGGTGGTAGTA 330
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BUNV_(JF961341)    --------.....................................T..............A.......................T...........A............. 102
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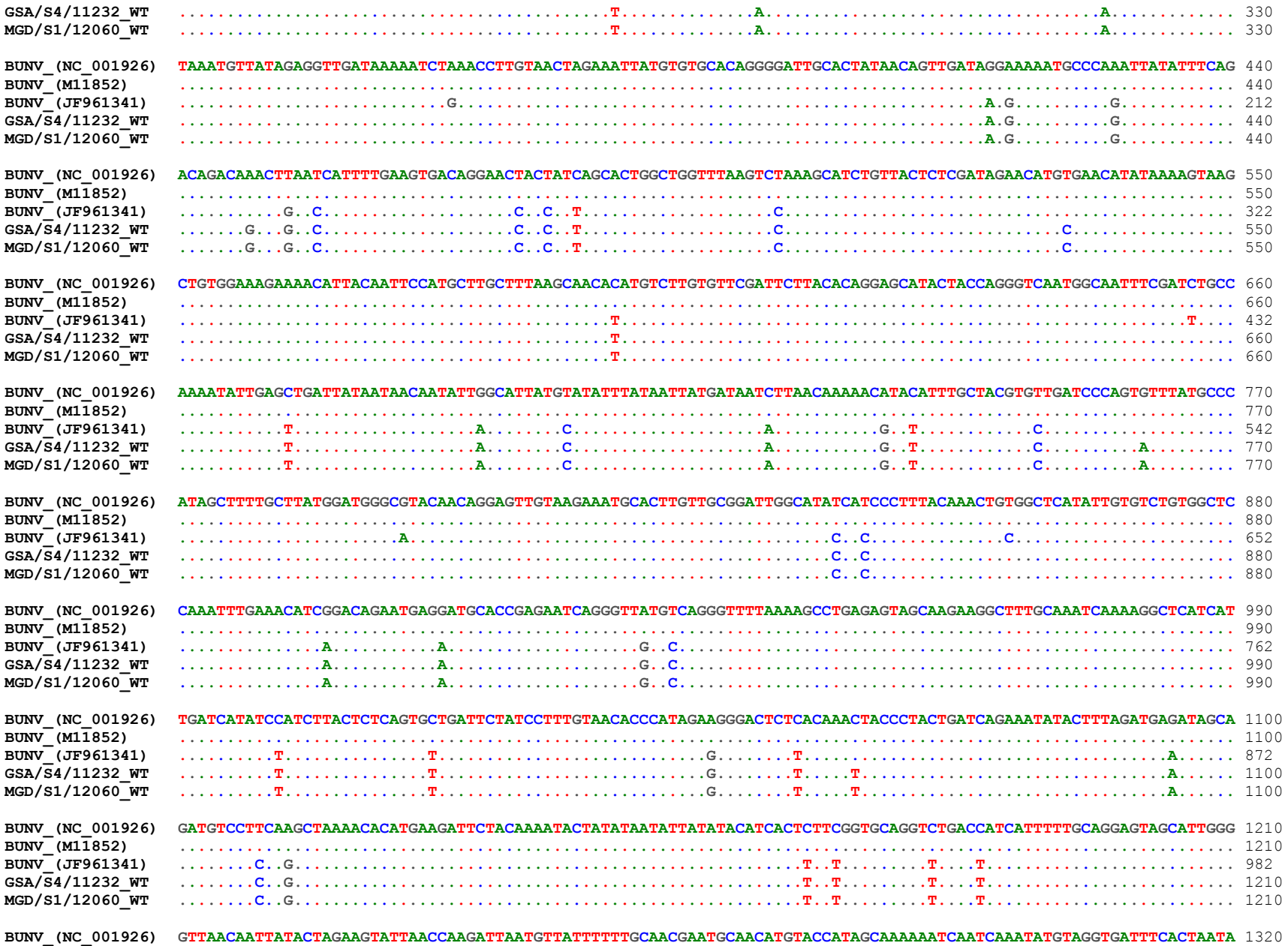
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MGD/S1/12060_WT 440
BUNV_(NC_001926) 550
BUNV_(M11852) 550
BUNV_(JF961341) 322
GSA/S4/11232_WT 550
MGD/S1/12060_WT 550
BUNV_(NC_001926) 660
BUNV_(M11852) 660
BUNV_(JF961341) 432
GSA/S4/11232_WT 660
MGD/S1/12060_WT 660
BUNV_(NC_001926) 770
BUNV_(M11852) 770
BUNV_(JF961341) 542
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MGD/S1/12060_WT 770
BUNV_(NC_001926) 880
BUNV_(M11852) 880
BUNV_(JF961341) 652
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MGD/S1/12060_WT 880
BUNV_(NC_001926) 990
BUNV_(M11852) 990
BUNV_(JF961341) 762
GSA/S4/11232_WT 990
MGD/S1/12060_WT 990
BUNV_(NC_001926) 1100
BUNV_(M11852) 1100
BUNV_(JF961341) 872
GSA/S4/11232_WT 1100
MGD/S1/12060_WT 1100
BUNV_(NC_001926) 1210
BUNV_(M11852) 1210
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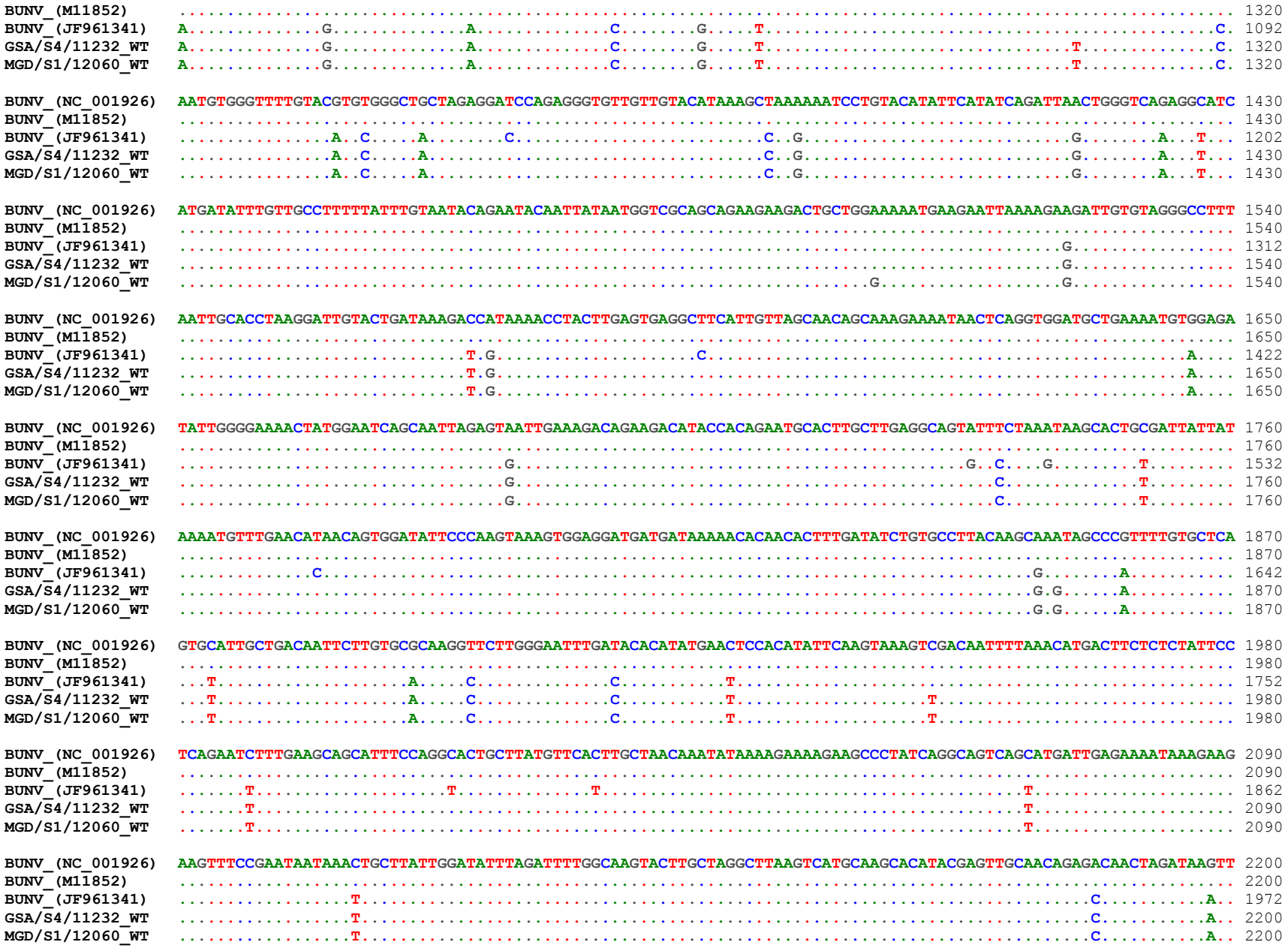
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BUNV_(JF961341) 1422
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MGD/S1/12060_WT 1650
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BUNV_(M11852) 1760
BUNV_(JF961341) 1532
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MGD/S1/12060_WT 1760
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BUNV_(M11852) 1870
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MGD/S1/12060_WT 1870
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BUNV_(M11852) 1980
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GSA/S4/11232_WT 1980
MGD/S1/12060_WT 1980
BUNV_(NC_001926) 2090
BUNV_(M11852) 2090
BUNV_(JF961341) 1862
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MGD/S1/12060_WT 2090
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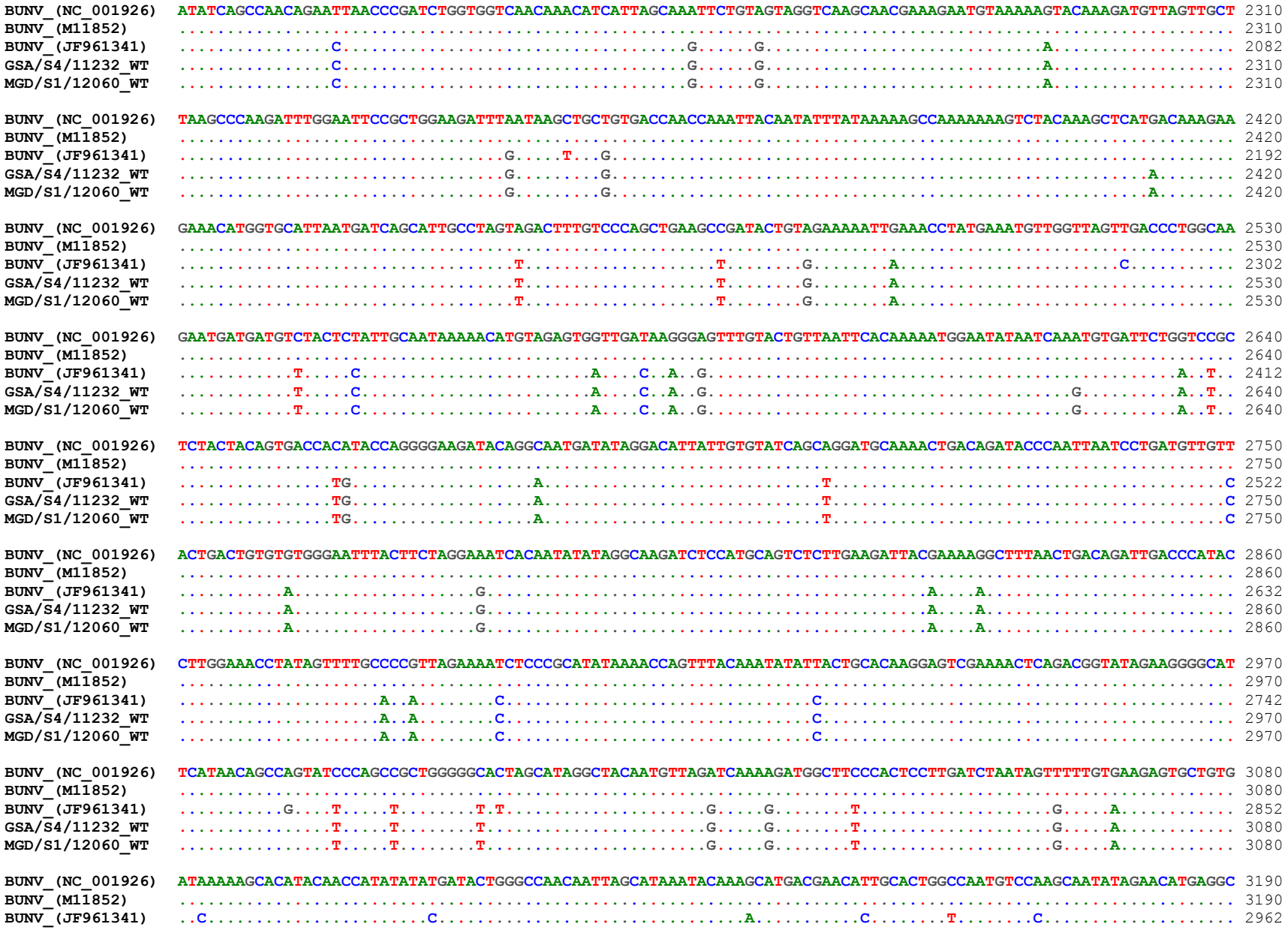
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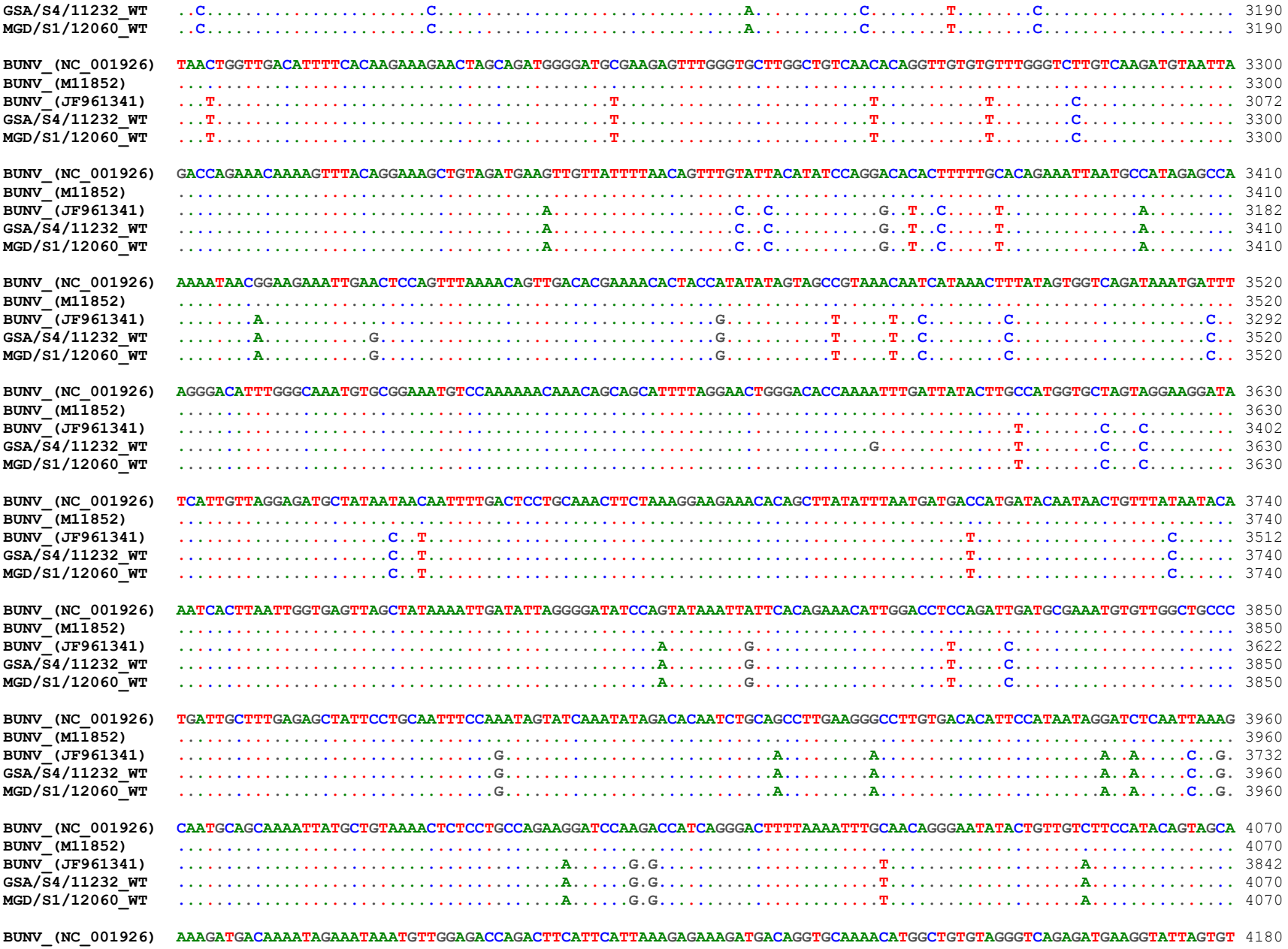
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BUNV_(M11852) 3300
BUNV_(JF961341) ...T.....T.....T.....T.....C..... 3072
GSA/S4/11232_WT ...T.....T.....T.....T.....C..... 3300
MGD/S1/12060_WT ...T.....T.....T.....T.....C..... 3300
BUNV_(NC_001926) GACCAGAAACAAAAGTTTACAGGAAAGCTGTAGATGAAGTTGTTATTTTAACAGTTTGTATTACATATCCAGGACACACTTTTTGCACAGAAATTAATGCCATAGAGCCA 3410
BUNV_(M11852) 3410
BUNV_(JF961341) .....A.....C..C.....G..T..C.....T.....A..... 3182
GSA/S4/11232_WT .....A.....C..C.....G..T..C.....T.....A..... 3410
MGD/S1/12060_WT .....A.....C..C.....G..T..C.....T.....A..... 3410
BUNV_(NC_001926) AAAATAACGGAAGAAATTGAACTCCAGTTTAAAACAGTTGACACGAAAACACTACCATATATAGTAGCCGTAAACAATCATAAACTTTATAGTGGTCAGATAAATGATTT 3520
BUNV_(M11852) 3520
BUNV_(JF961341) .....A.....G.....T.....T..C.....C.....C.. 3292
GSA/S4/11232_WT .....A.....G.....G.....T.....T..C.....C.....C.. 3520
MGD/S1/12060_WT .....A.....G.....G.....T.....T..C.....C.....C.. 3520
BUNV_(NC_001926) AGGGACATTTGGGCAAATGTGCGGAAATGTCCAAAAAACAAACAGCAGCATTTTAGGAACTGGGACACCAAAATTTGATTATACTTGCCATGGTGCTAGTAGGAAGGATA 3630
BUNV_(M11852) 3630
BUNV_(JF961341) .....T.....C...C..... 3402
GSA/S4/11232_WT .....G.....T.....C...C..... 3630
MGD/S1/12060_WT .....T.....C...C..... 3630
BUNV_(NC_001926) TCATTGTTAGGAGATGCTATAATAACAATTTTGACTCCTGCAAACTTCTAAAGGAAGAAACACAGCTTATATTTAATGATGACCATGATACAATAACTGTTTATAATACA 3740
BUNV_(M11852) 3740
BUNV_(JF961341) .....C..T.....T.....C..... 3512
GSA/S4/11232_WT .....C..T.....T.....C..... 3740
MGD/S1/12060_WT .....C..T.....T.....C..... 3740
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BUNV_(M11852) 3850
BUNV_(JF961341) .....A.....G.....T.....C..... 3622
GSA/S4/11232_WT .....A.....G.....T.....C..... 3850
MGD/S1/12060_WT .....A.....G.....T.....C..... 3850
BUNV_(NC_001926) TGATTGCTTTGAGAGCTATTCCTGCAATTTCCAAATAGTATCAAATATAGACACAATCTGCAGCCTTGAAGGGCCTTGTGACACATTCCATAATAGGATCTCAATTAAAG 3960
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GSA/S4/11232_WT .....G.....A.....A.....A..A.....C..G. 3960
MGD/S1/12060_WT .....G.....A.....A.....A..A.....C..G. 3960
BUNV_(NC_001926) CAATGCAGCAAAATTATGCTGTAAAACTCTCCTGCCAGAAGGATCCAAGACCATCAGGGACTTTTAAAATTTGCAACAGGGAATATACTGTTGTCTTCCATACAGTAGCA 4070
BUNV_(M11852) 4070
BUNV_(JF961341) .....A.....G.G.....T.....A..... 3842
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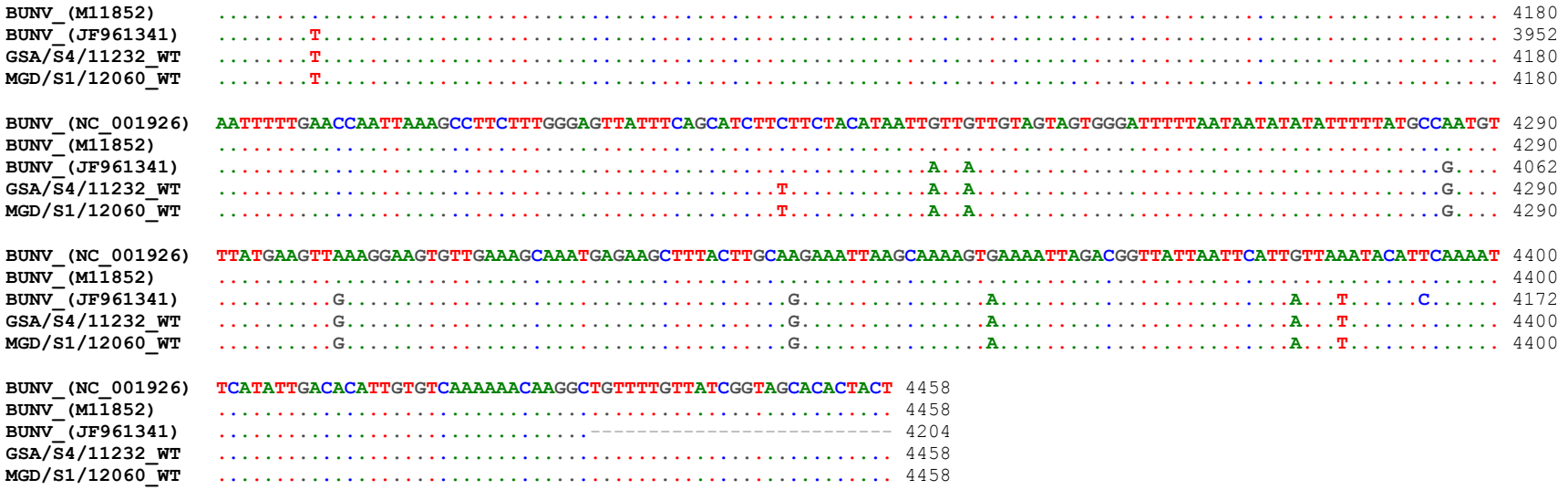
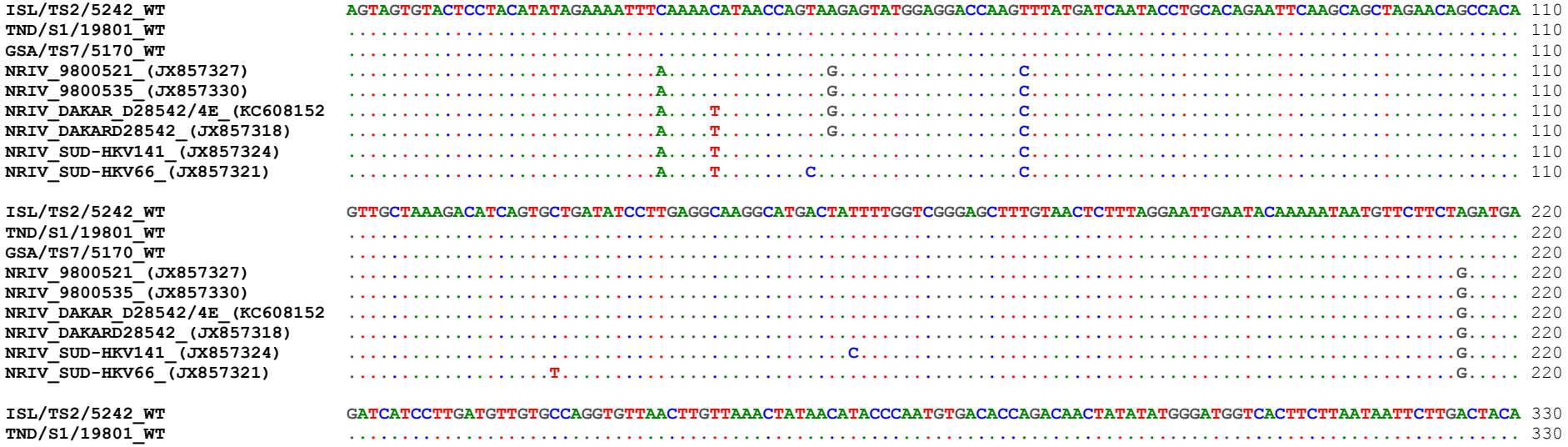


Appendix 1E: Ngari virus L segment nucleotide sequence alignment



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GSA/TS7/5170_WT                .............................................................................................................. 330
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NRIV_DAKAR_D28542/4E_(KC608152 .............................................................................................G................ 330
NRIV_DAKARD28542_(JX857318)    .............................................................................................G................ 330
NRIV_SUD-HKV141_(JX857324)     ................A............................................................................G................ 330
NRIV_SUD-HKV66_(JX857321)      ..............................T..............................................................G................ 330

ISL/TS2/5242_WT                AAGTCTCAGTTGGGAATGATAGTAGTGAAATCACATATAAAAAATACACCAGTTTGATTCTCCCGGTGATGTCTGAATTGGGTATAGATACAGAAATAACTATTATTAGG 440
TND/S1/19801_WT                .............................................................................................................. 440
GSA/TS7/5170_WT                .............................................................................................................. 440
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NRIV_9800535_(JX857330)        ........................................G.........................................................G........... 440
NRIV_DAKAR_D28542/4E_(KC608152 ...............................T........G..G......................................................G........... 440
NRIV_DAKARD28542_(JX857318)    ...............................T........G..G......................................................G........... 440
NRIV_SUD-HKV141_(JX857324)     ........................................G.........................................................G........... 440
NRIV_SUD-HKV66_(JX857321)      ........................................G.........................................................G........... 440

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TND/S1/19801_WT                .............................................................................................................. 550
GSA/TS7/5170_WT                .............................................................................................................. 550
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GSA/TS7/5170_WT                .............................................................................................................. 660
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NRIV_SUD-HKV141_(JX857324)     ................................................G........................................................G.... 660
NRIV_SUD-HKV66_(JX857321)      ...G............................................G........................................................G.... 660

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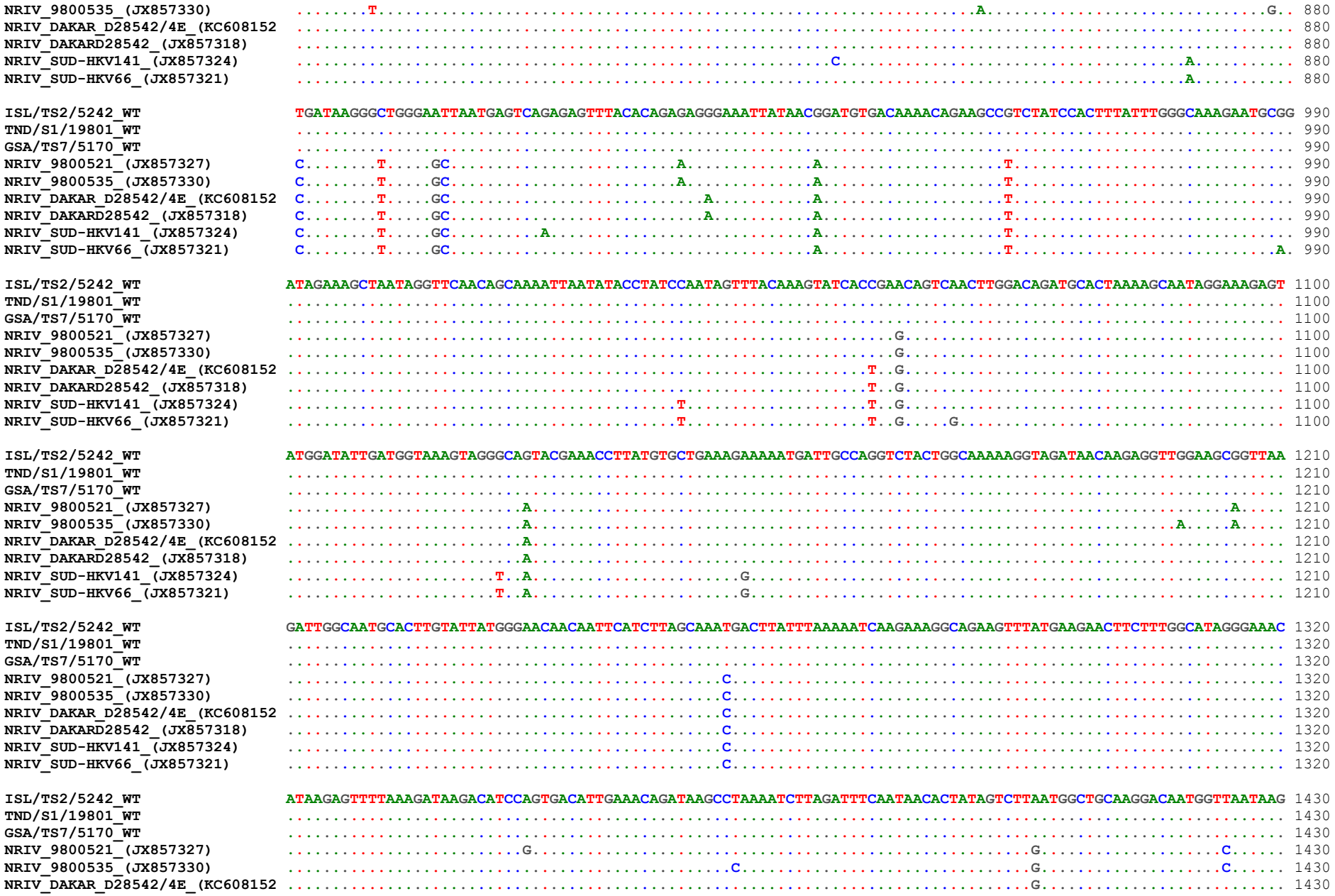
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NRIV_DAKARD28542_(JX857318) 990
NRIV_SUD-HKV141_(JX857324) 990
NRIV_SUD-HKV66_(JX857321) 990
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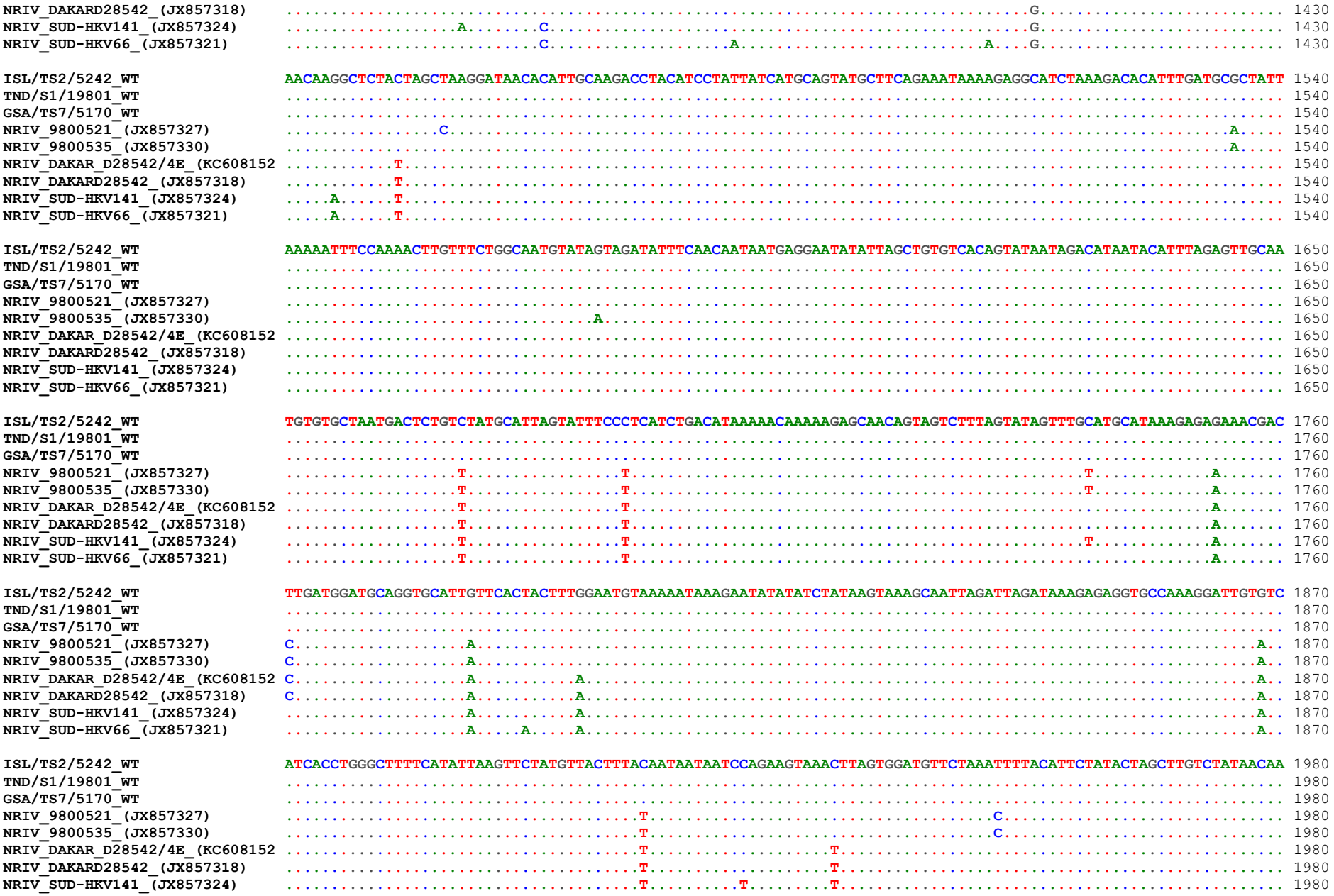
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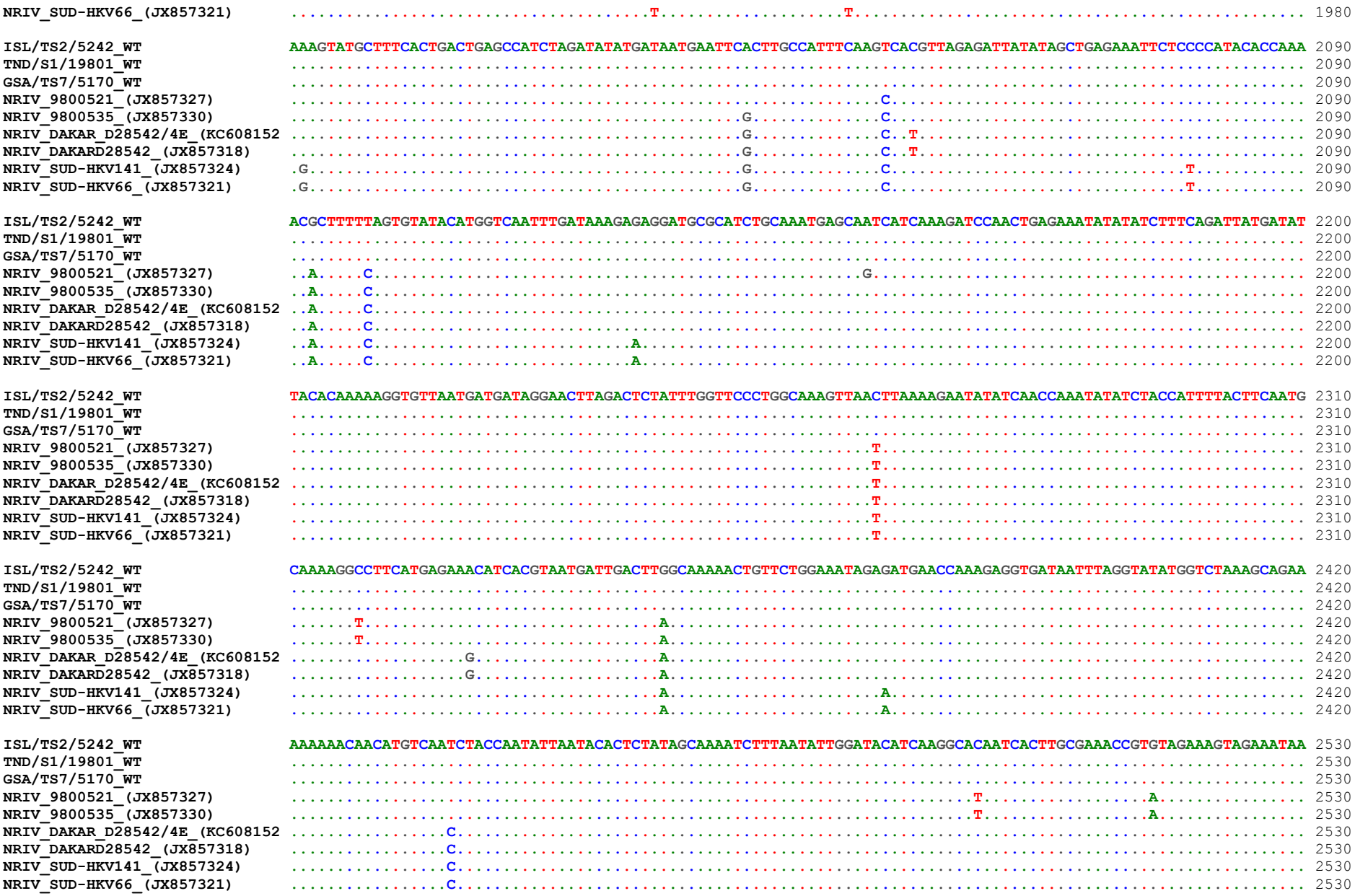
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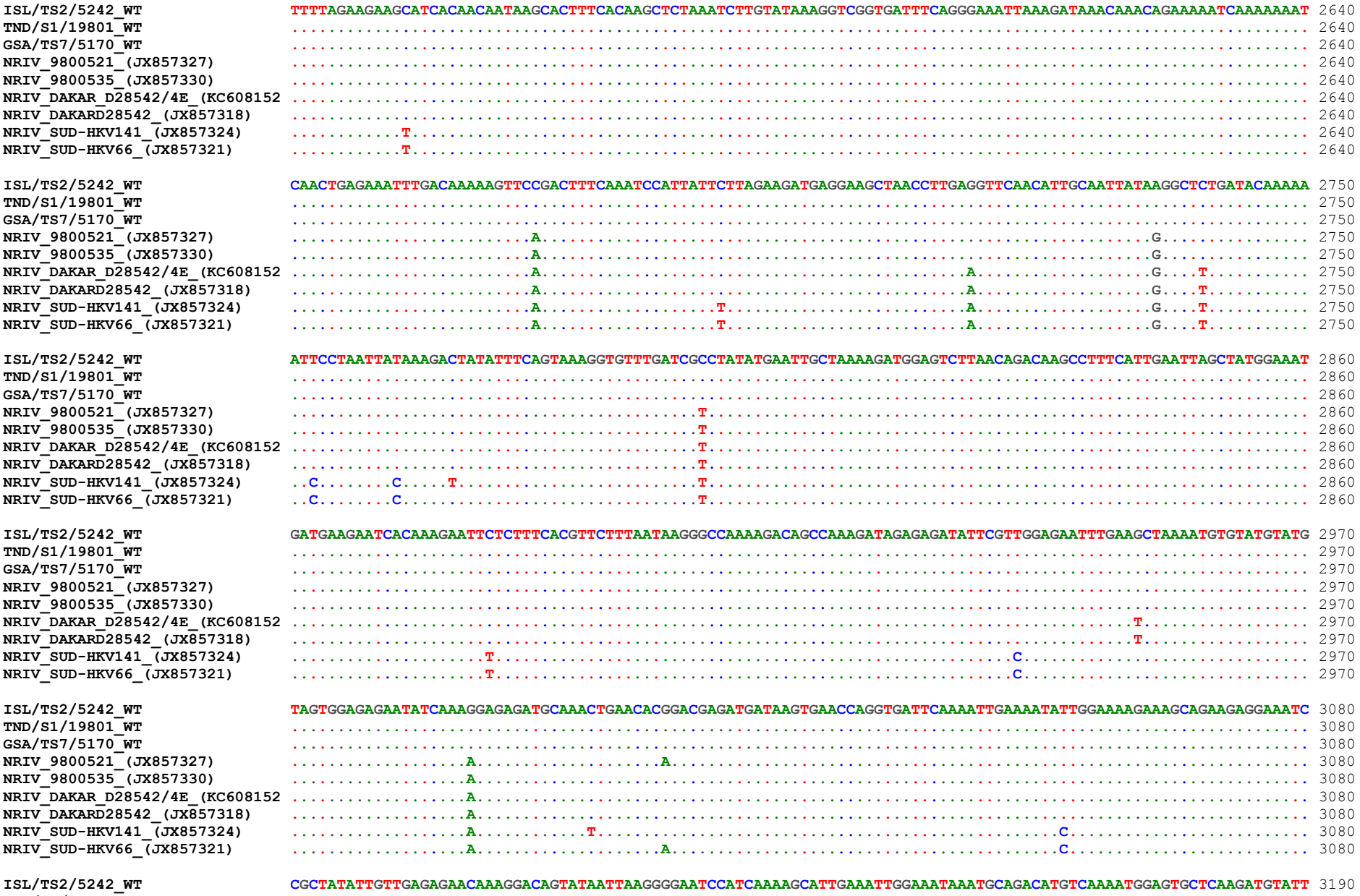
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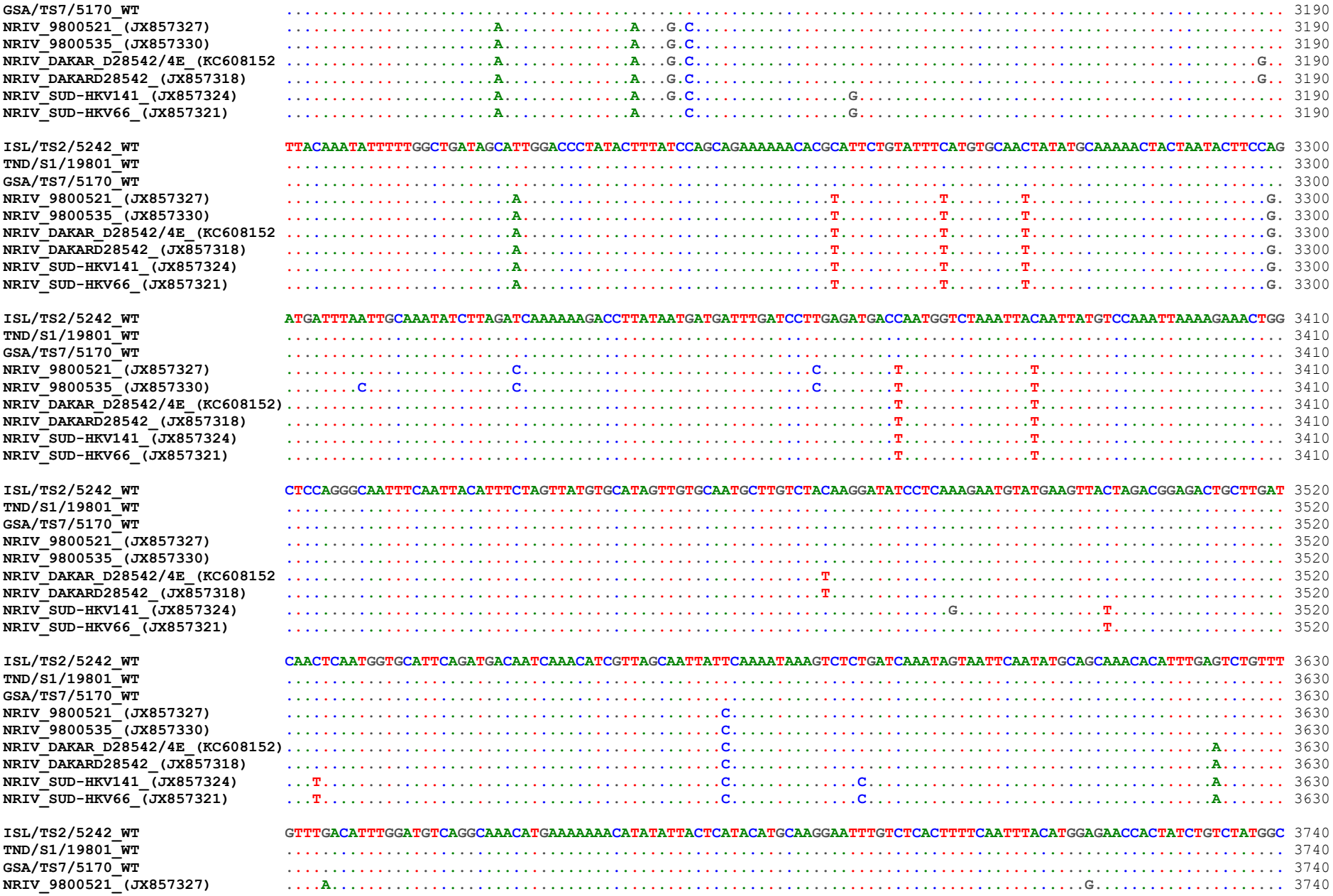
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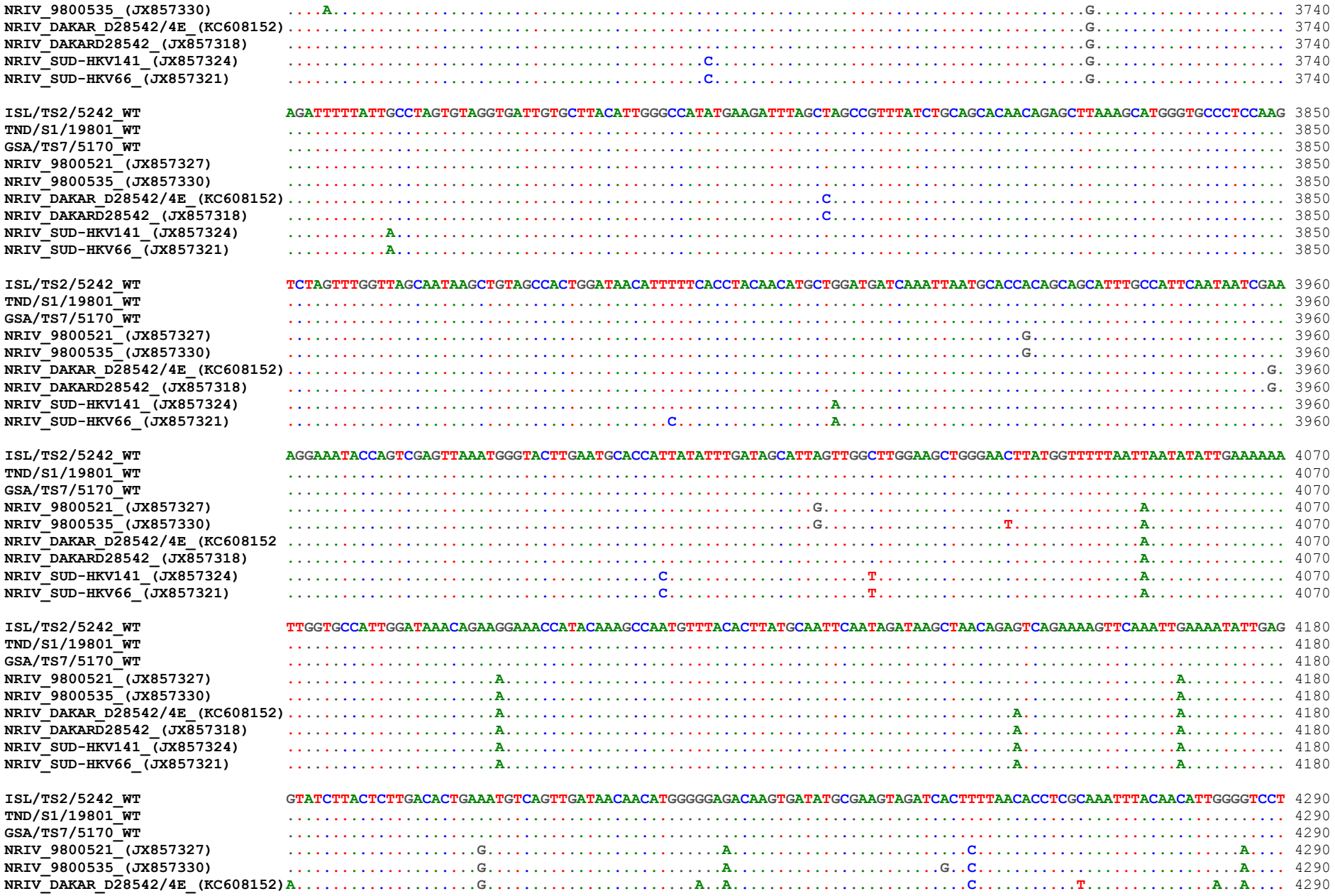
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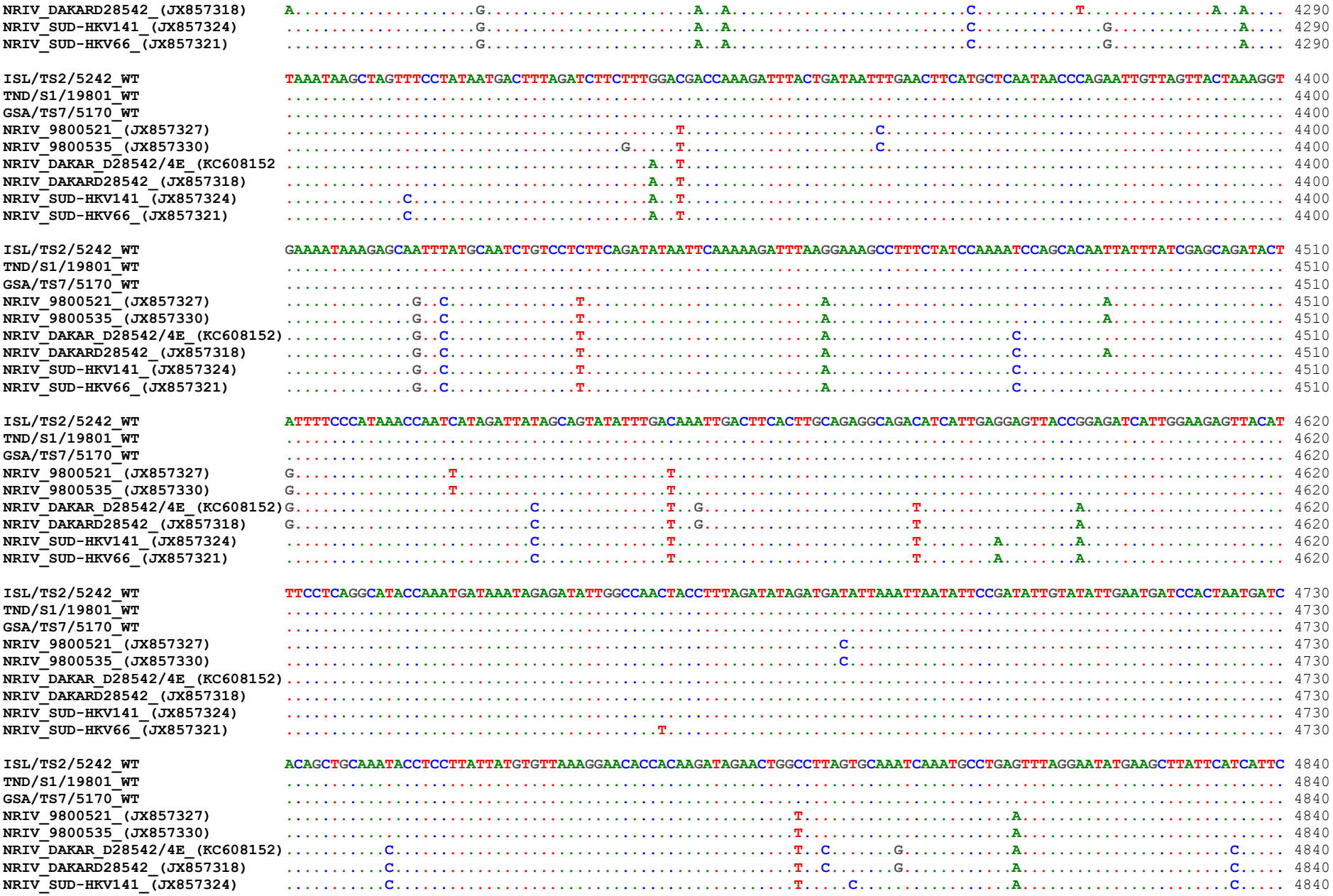
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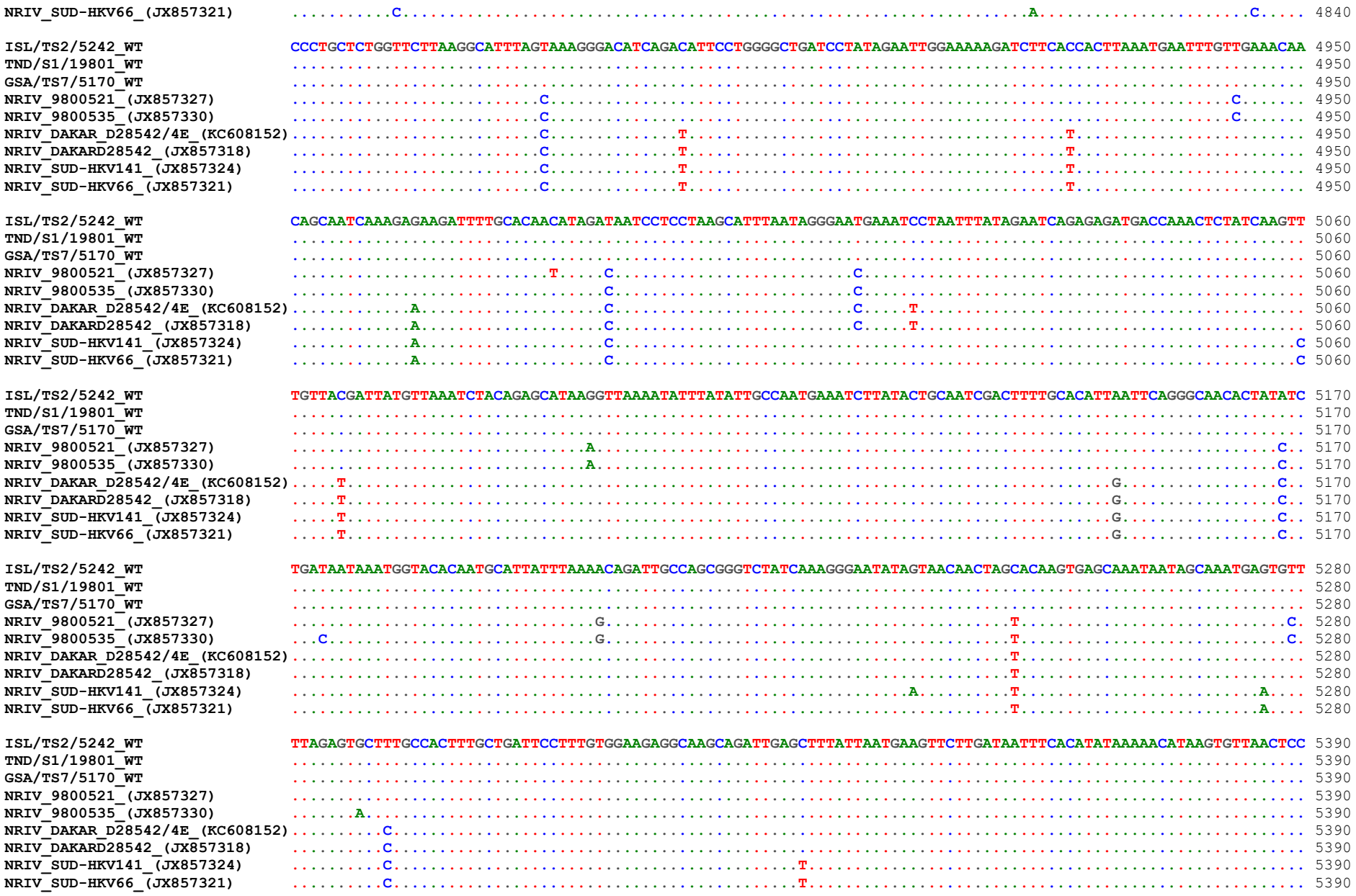
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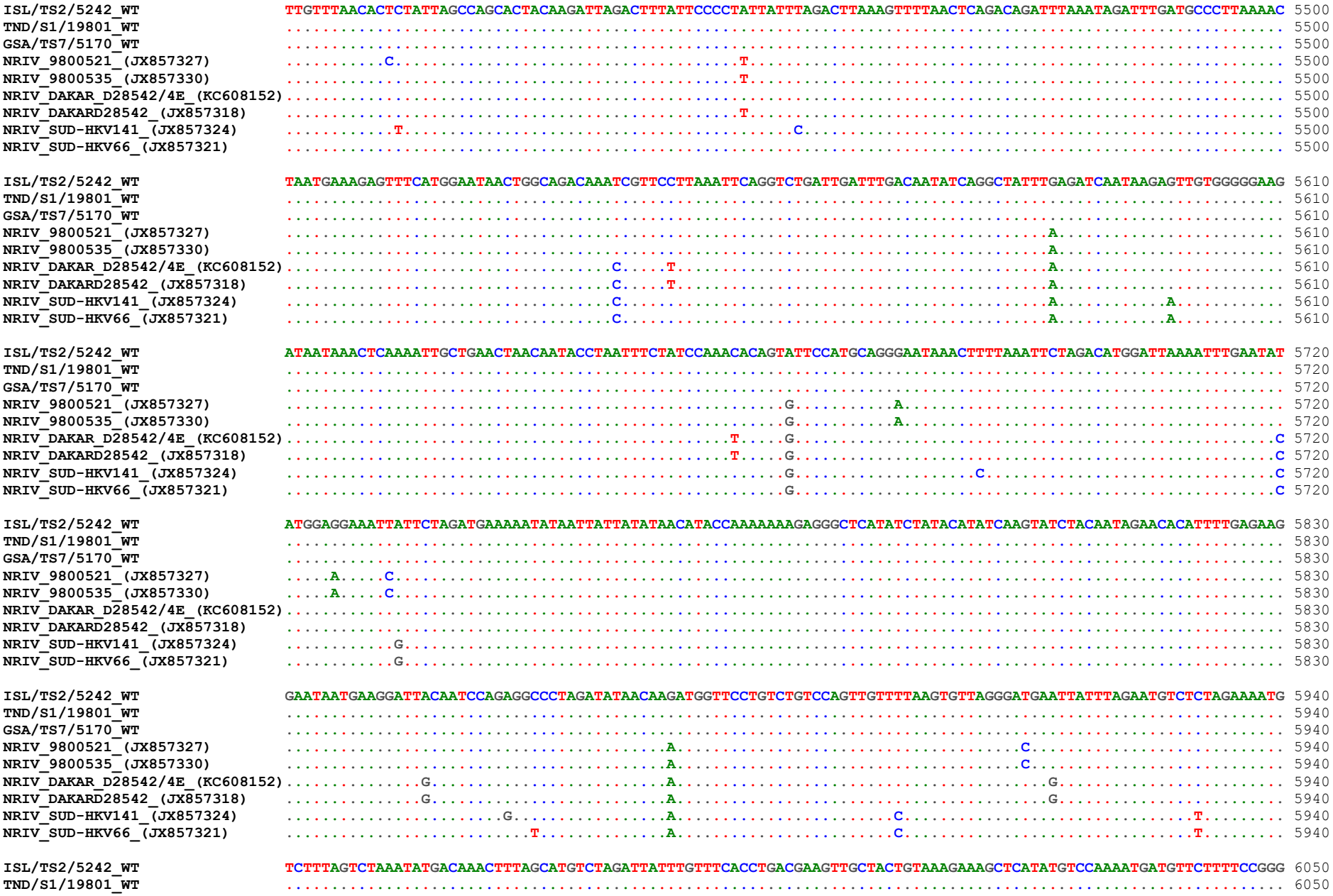
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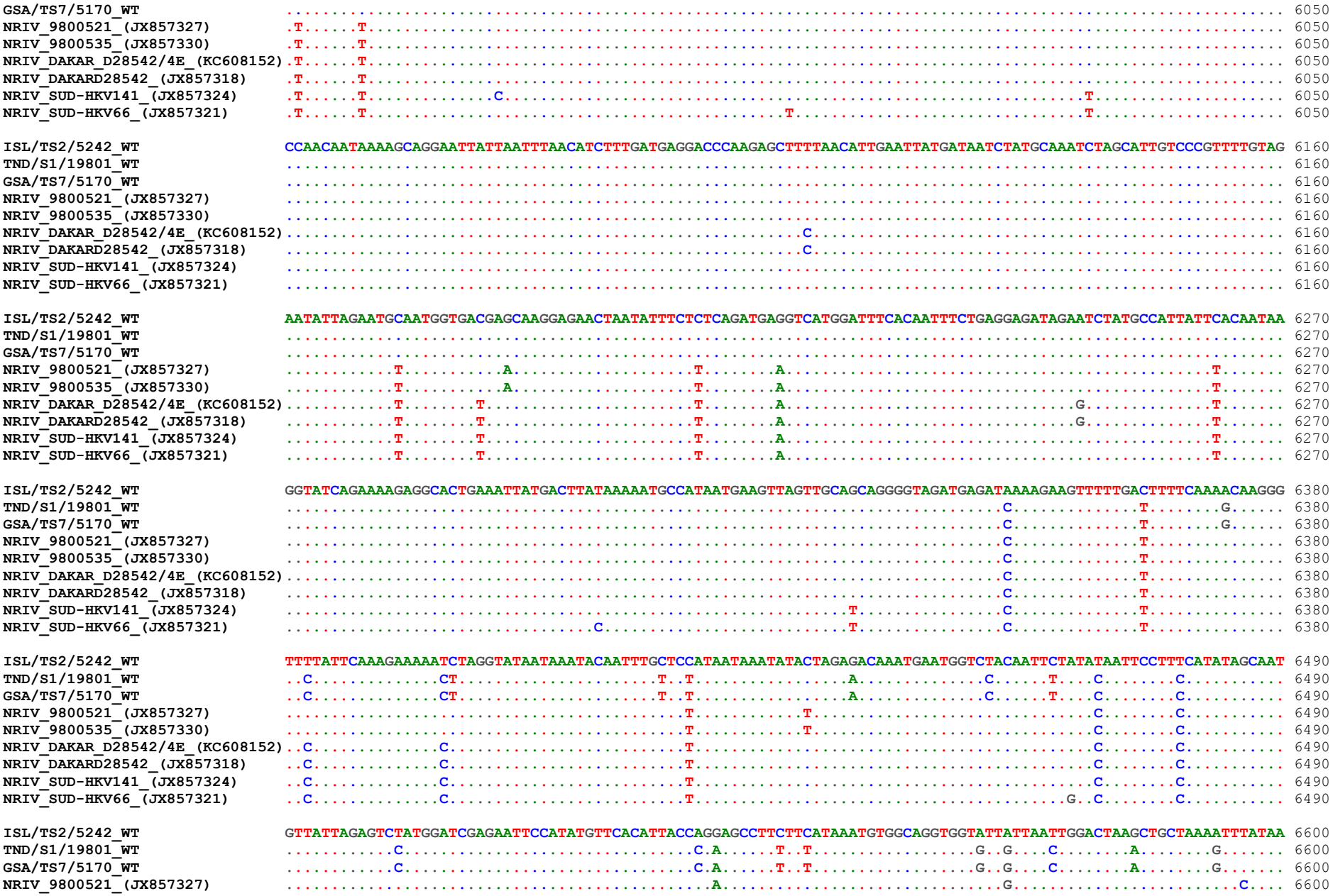
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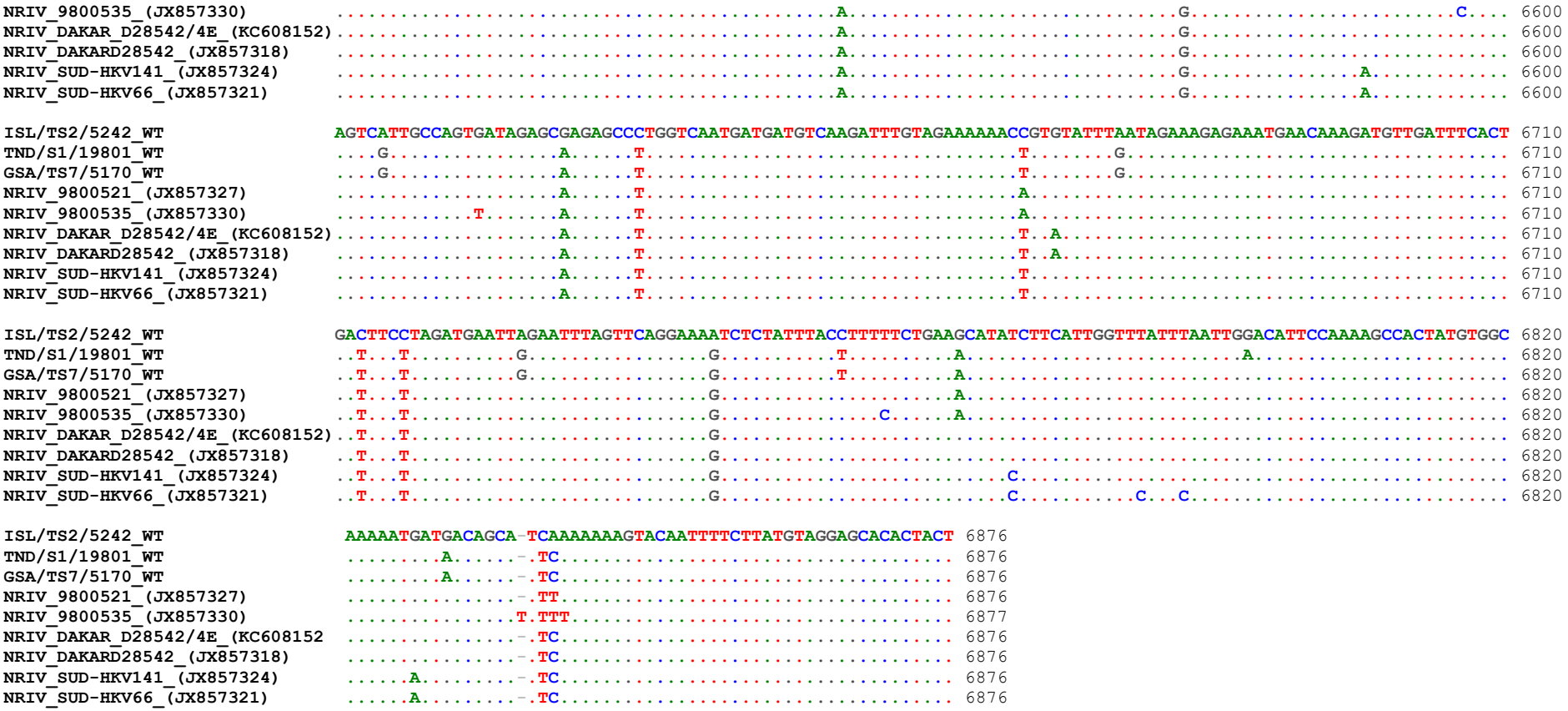
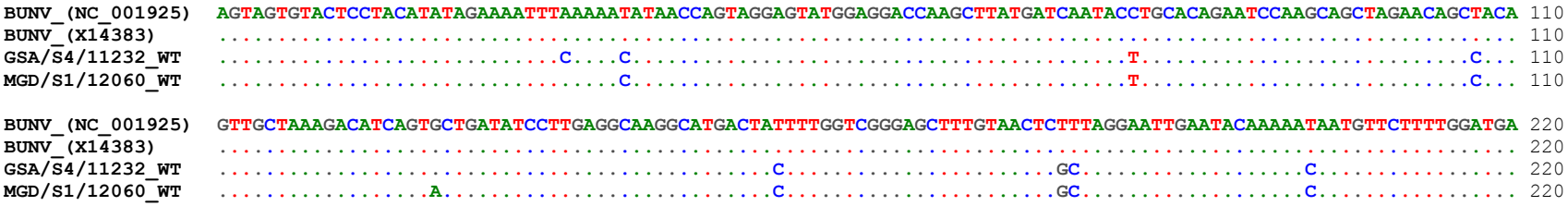


Appendix 1F: Bunyamwera virus L segment nucleotide sequence alignment



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BUNV_(X14383)     ............................................................................................................. 330
GSA/S4/11232_WT  G...........................................T.............................C..T..................... 330
MGD/S1/12060_WT  G...........................................T.............................C..T..................... 330

BUNV_(NC_001925)  AAGTCTCAGTTGGGAATGATAGTAGTGAAATCACATATAAGAAATACACCAGTTTGATTCTCCCAGTGATGTCTGAATTGGGTATAGATACAGAAATAGCTATTATTAGG 440
BUNV_(X14383)     ............................................................................................................. 440
GSA/S4/11232_WT  ....................................A..........C.....................................................C..... 440
MGD/S1/12060_WT  ....................................A..........C.....................................................C..... 440

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MGD/S1/12060_WT  ..........................................................T........C..................................... 550

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BUNV_(X14383)     ............................................................................................................. 660
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MGD/S1/12060_WT  ..............................T.........................C.................................A.....A.... 660

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BUNV_(X14383)     ............................................................................................................. 770
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MGD/S1/12060_WT  ..........................................................T........C..................................... 770

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MGD/S1/12060_WT  ....A.....................................C.....G.............................G..................... 880

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BUNV_(X14383)     ............................................................................................................. 990
GSA/S4/11232_WT  T...........................................G.....................................C....C.........G. 990
MGD/S1/12060_WT  T...........................................G.....................................C....C.........G. 990

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BUNV_(X14383)     ............................................................................................................. 1100
GSA/S4/11232_WT  ..............................................C.....................A.....................................C 1100
MGD/S1/12060_WT  ..............................................C.....................A.....................................C 1100

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MGD/S1/12060_WT  ....................G.....................................................................G..A.......... 1210

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MGD/S1/12060_WT  ................C.....................................C.....................T.....G.....................A. 1320

BUNV_(NC_001925)  ATAAGAGTTTTAAAGATAAGACATCTAGCGACATTGAAACGGATAAGCCTAAAATCTTAGATTTCAATAATACTATAGTCCTGATGGCTGCAAGAACAATGGTTAATAAA 1430
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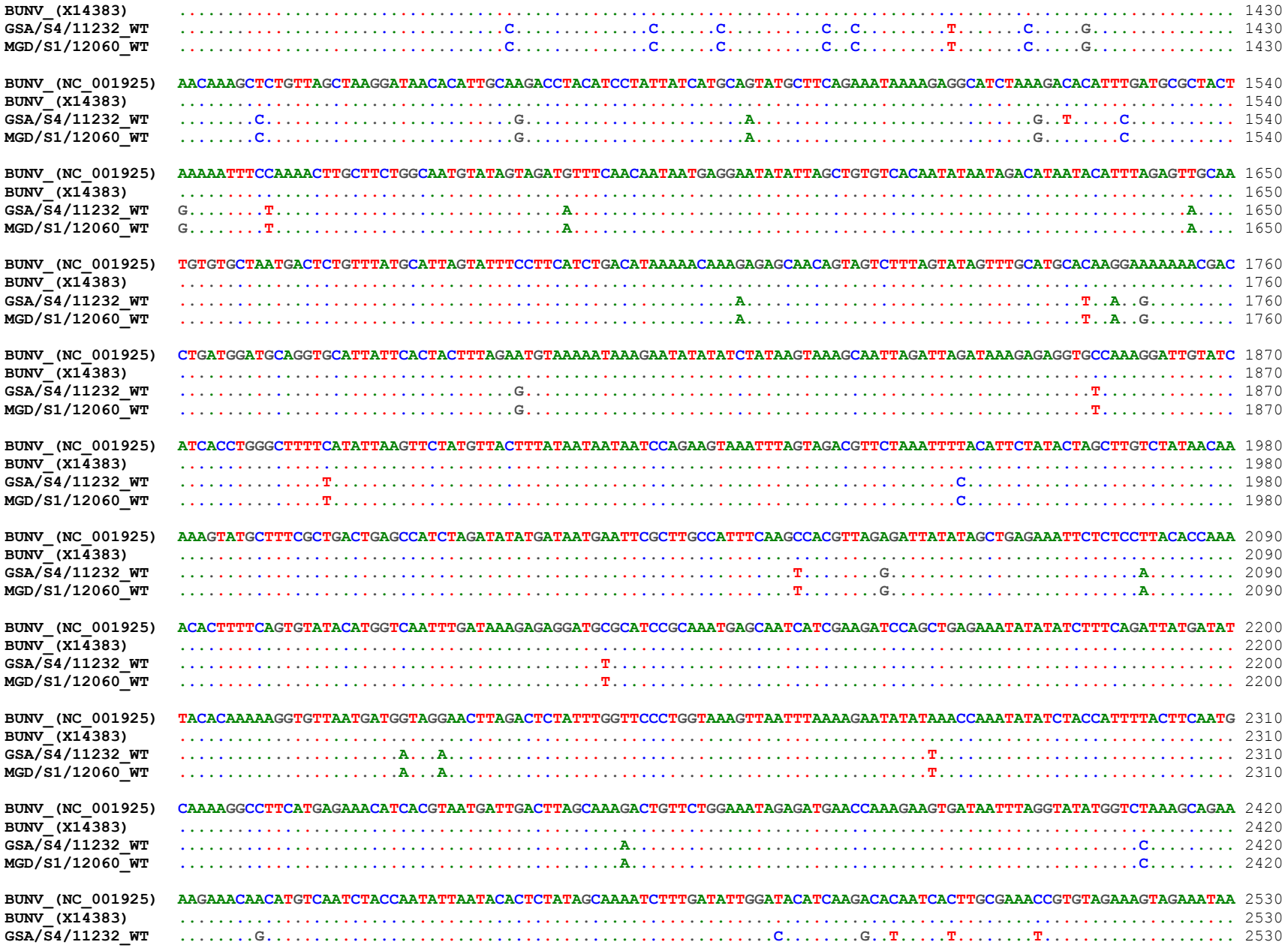
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BUNV_(NC_001925) AACAAAGCTCTGTTAGCTAAGGATAACACATTGCAAGACCTACATCCTATTATCATGCAGTATGCTTCAGAAATAAAAGAGGCATCTAAAGACACATTTGATGCGCTACT 1540
BUNV_(X14383) 1540
GSA/S4/11232_WT 1540
MGD/S1/12060_WT 1540
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BUNV_(X14383) 1650
GSA/S4/11232_WT 1650
MGD/S1/12060_WT 1650
BUNV_(NC_001925) TGTGTGCTAATGACTCTGTTTATGCATTAGTATTTCCTTCATCTGACATAAAAACAAAGAGAGCAACAGTAGTCTTTAGTATAGTTTGCATGCACAAGGAAAAAAACGAC 1760
BUNV_(X14383) 1760
GSA/S4/11232_WT 1760
MGD/S1/12060_WT 1760
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BUNV_(X14383) 1870
GSA/S4/11232_WT 1870
MGD/S1/12060_WT 1870
BUNV_(NC_001925) ATCACCTGGGCTTTTCATATTAAGTTCTATGTTACTTTATAATAATAATCCAGAAGTAAATTTAGTAGACGTTCTAAATTTTACATTCTATACTAGCTTGTCTATAACAA 1980
BUNV_(X14383) 1980
GSA/S4/11232_WT 1980
MGD/S1/12060_WT 1980
BUNV_(NC_001925) AAAGTATGCTTTCGCTGACTGAGCCATCTAGATATATGATAATGAATTCGCTTGCCATTTCAAGCCACGTTAGAGATTATATAGCTGAGAAATTCTCTCCTTACACCAAA 2090
BUNV_(X14383) 2090
GSA/S4/11232_WT 2090
MGD/S1/12060_WT 2090
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BUNV_(X14383) 2200
GSA/S4/11232_WT 2200
MGD/S1/12060_WT 2200
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BUNV_(X14383) 2310
GSA/S4/11232_WT 2310
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BUNV_(X14383) 2420
GSA/S4/11232_WT 2420
MGD/S1/12060_WT 2420
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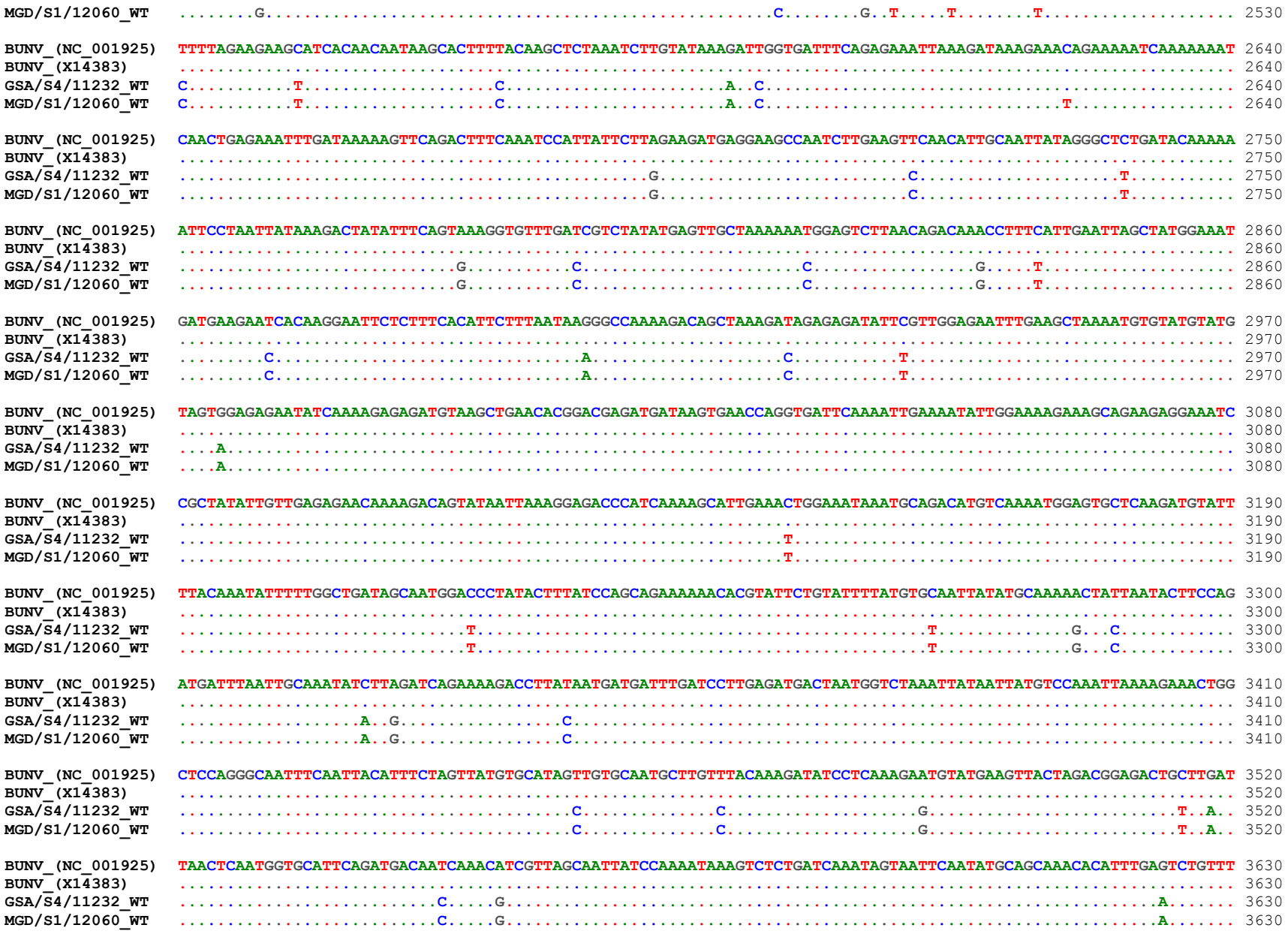
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BUNV_(X14383) 2640
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MGD/S1/12060_WT 2640
BUNV_(NC_001925) 2750
BUNV_(X14383) 2750
GSA/S4/11232_WT 2750
MGD/S1/12060_WT 2750
BUNV_(NC_001925) 2860
BUNV_(X14383) 2860
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BUNV_(X14383) 2970
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MGD/S1/12060_WT 2970
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MGD/S1/12060_WT 3080
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BUNV_(X14383) 3190
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MGD/S1/12060_WT 3190
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BUNV_(X14383) 3300
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MGD/S1/12060_WT 3300
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BUNV_(X14383) 3410
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MGD/S1/12060_WT 3410
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GSA/S4/11232_WT 3520
MGD/S1/12060_WT 3520
BUNV_(NC_001925) 3630
BUNV_(X14383) 3630
GSA/S4/11232_WT 3630
MGD/S1/12060_WT 3630

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BUNV_(X14383)     ............................................................................................................. 3740
GSA/S4/11232_WT   .....................................................................................................A.... 3740
MGD/S1/12060_WT   .....................................................................................................A.... 3740

BUNV_(NC_001925)  AGATTTTTATTGCCTAGTGTAGGTGATTGTGCTTACATTGGGCCATATGAAGATTTAGCCAGCCGTTTATCTGCAGCACAACAGAGCTTAAAGCATGGGTGCCCTCCAAG 3850
BUNV_(X14383)     ............................................................................................................. 3850
GSA/S4/11232_WT   ..................................T..............G.....................................G..................... 3850
MGD/S1/12060_WT   ..................................T..............G.....................................G..................... 3850

BUNV_(NC_001925)  TCTAGTTTGGTTAGCAATAAGCTGTAGCCACTGGATAACATTTTTCACTTACAACATGCTGGATGATCAAATTAATGCACCACAGCAGCATTTGCCATTCAATAATCGGA 3960
BUNV_(X14383)     ............................................................................................................. 3960
GSA/S4/11232_WT   ............G........T.....................................T..........C.....................A.............. 3960
MGD/S1/12060_WT   ............G........T.....................................T..........C.....................A.............. 3960

BUNV_(NC_001925)  AGGAAATACCAGTTGAGTTAAATGGGTACTTGAATGCACCATTATATTTGATAGCATTAGTCGGCTTGGAAGCTGGGAACTTATGGTTTTTAATAAATATATTGAAAAAA 4070
BUNV_(X14383)     ............................................................................................................. 4070
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MGD/S1/12060_WT   ..........................................................................C.........................A..G... 4070

BUNV_(NC_001925)  TTGGTGCCATTGGATAAACAGAAAGAAACTATACAAAGCCAATGTTTACACTTATGCAATTCAATTGATAAGCTGACAGAATCAGAAAAGTTCAAATTAAAAATATTGAG 4180
BUNV_(X14383)     ............................................................................................................. 4180
GSA/S4/11232_WT   ................G..........C.............C..................................................................... 4180
MGD/S1/12060_WT   ................G..........C.............C..................................................................... 4180

BUNV_(NC_001925)  ATATCTTACTCTTGACACTGAGATGTCAGTTGATAACAACATGGGAGAAACAAGTGATATGCGAAGTAGATCACTCTTAACGCCTCGCAAATTTACAACATTGGGATCCT 4290
BUNV_(X14383)     ............................................................................................................. 4290
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MGD/S1/12060_WT   ..............T.....A.............T.....................................G..G...C....A.....T.....................T. 4290

BUNV_(NC_001925)  TAAATAAACTAGTTTCCTATAATGACTTTAGATCTTCTTTAGACGACCAGAGATTTACTGATAATTTGAACTTCATGCTTAATAACCCAGAATTGTTAGTTACTAAAGGT 4400
BUNV_(X14383)     ............................................................................................................. 4400
GSA/S4/11232_WT   ....C.............................C...........A.................................C..T..T...........C........C 4400
MGD/S1/12060_WT   ....C.............................C...........A.................................C..T..T...........C........C 4400

BUNV_(NC_001925)  GAAAATAAAGAGCAGTTCATGCAATCTGTCCTTTTCAGATATAATTCAAAAAGATTTAAAGAAAGCCTTTCCATCCAAAACCCAGCACAATTATTTATTGAGCAGATACT 4510
BUNV_(X14383)     ............................................................................................................. 4510
GSA/S4/11232_WT   ..................................T...........T.................T.....T.......................C........... 4510
MGD/S1/12060_WT   ..................................T...........T.................T.....T.......................C........... 4510

BUNV_(NC_001925)  GTTTTCCCATAAACCAATCATAGATTACAGCAGTATATTTGATAAATTAACCTCACTTGCAGAAGCGGATATCATTGAAGAGCTACCAGAGATCATTGGAAGAGTTACAT 4620
BUNV_(X14383)     ............................................................................................................. 4620
GSA/S4/11232_WT   ..................................................G..T.............A.............T........................... 4620
MGD/S1/12060_WT   ..................................................G..T.............A.............T.....................C.... 4620

BUNV_(NC_001925)  TTCCTCAGGCATACCAGATGATAAATAGAGATATTGGCCAACTACCTTTAGATATAGATGATATTAAGTTAATATTCCGGTATTGTATATTGAATGATCCACTAATGATC 4730
BUNV_(X14383)     ............................................................................................................. 4730
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MGD/S1/12060_WT   .C.....A........A.....................................................................T..A.......................... 4730

BUNV_(NC_001925)  ACAGCTGCAAACACTTCCTTATTATGTGTTAAAGGAACACCACAAGATAGAACTGGCCTCAGTGCAAGTCAAATGCCTGAATTTAGAAATATGAAACTTATTCACCATTC 4840
BUNV_(X14383)     ............................................................................................................. 4840
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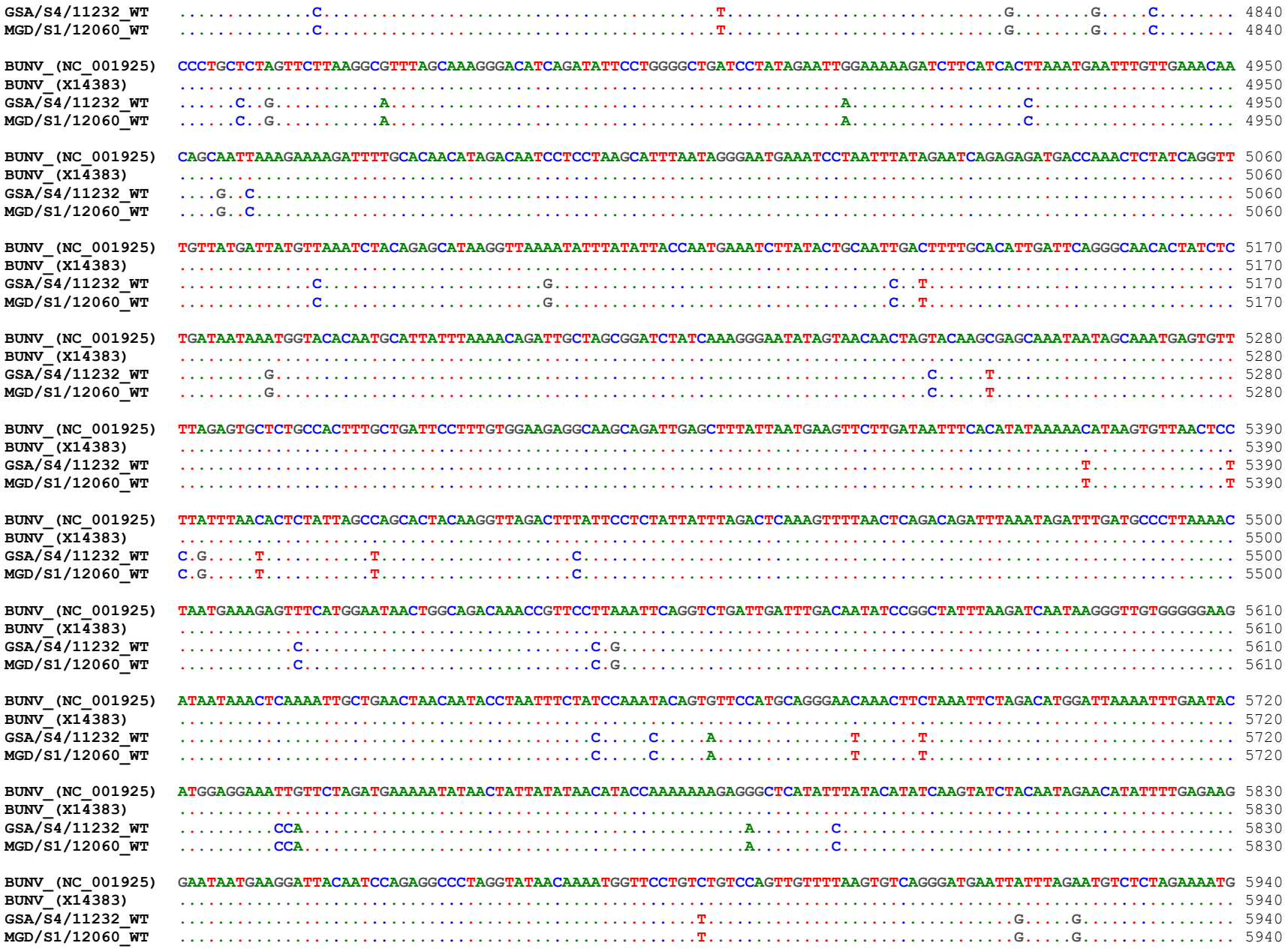
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MGD/S1/12060_WT 4840
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BUNV_(X14383) 4950
GSA/S4/11232_WT 4950
MGD/S1/12060_WT 4950
BUNV_(NC_001925) CAGCAATTAAAGAAAAGATTTTGCACAACATAGACAATCCTCCTAAGCATTTAATAGGGAATGAAATCCTAATTTATAGAATCAGAGAGATGACCAAACTCTATCAGGTT 5060
BUNV_(X14383) 5060
GSA/S4/11232_WT 5060
MGD/S1/12060_WT 5060
BUNV_(NC_001925) TGTTATGATTATGTTAAATCTACAGAGCATAAGGTTAAAATATTTATATTACCAATGAAATCTTATACTGCAATTGACTTTTGCACATTGATTCAGGGCAACACTATCTC 5170
BUNV_(X14383) 5170
GSA/S4/11232_WT 5170
MGD/S1/12060_WT 5170
BUNV_(NC_001925) TGATAATAAATGGTACACAATGCATTATTTAAAACAGATTGCTAGCGGATCTATCAAAGGGAATATAGTAACAACTAGTACAAGCGAGCAAATAATAGCAAATGAGTGTT 5280
BUNV_(X14383) 5280
GSA/S4/11232_WT 5280
MGD/S1/12060_WT 5280
BUNV_(NC_001925) TTAGAGTGCTCTGCCACTTTGCTGATTCCTTTGTGGAAGAGGCAAGCAGATTGAGCTTTATTAATGAAGTTCTTGATAATTTCACATATAAAAACATAAGTGTTAACTCC 5390
BUNV_(X14383) 5390
GSA/S4/11232_WT 5390
MGD/S1/12060_WT 5390
BUNV_(NC_001925) TTATTTAACACTCTATTAGCCAGCACTACAAGGTTAGACTTTATTCCTCTATTATTTAGACTCAAAGTTTTAACTCAGACAGATTTAAATAGATTTGATGCCCTTAAAAC 5500
BUNV_(X14383) 5500
GSA/S4/11232_WT 5500
MGD/S1/12060_WT 5500
BUNV_(NC_001925) TAATGAAAGAGTTTCATGGAATAACTGGCAGACAAACCGTTCCTTAAATTCAGGTCTGATTGATTTGACAATATCCGGCTATTTAAGATCAATAAGGGTTGTGGGGGAAG 5610
BUNV_(X14383) 5610
GSA/S4/11232_WT 5610
MGD/S1/12060_WT 5610
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BUNV_(X14383) 5720
GSA/S4/11232_WT 5720
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BUNV_(X14383) 5830
GSA/S4/11232_WT 5830
MGD/S1/12060_WT 5830
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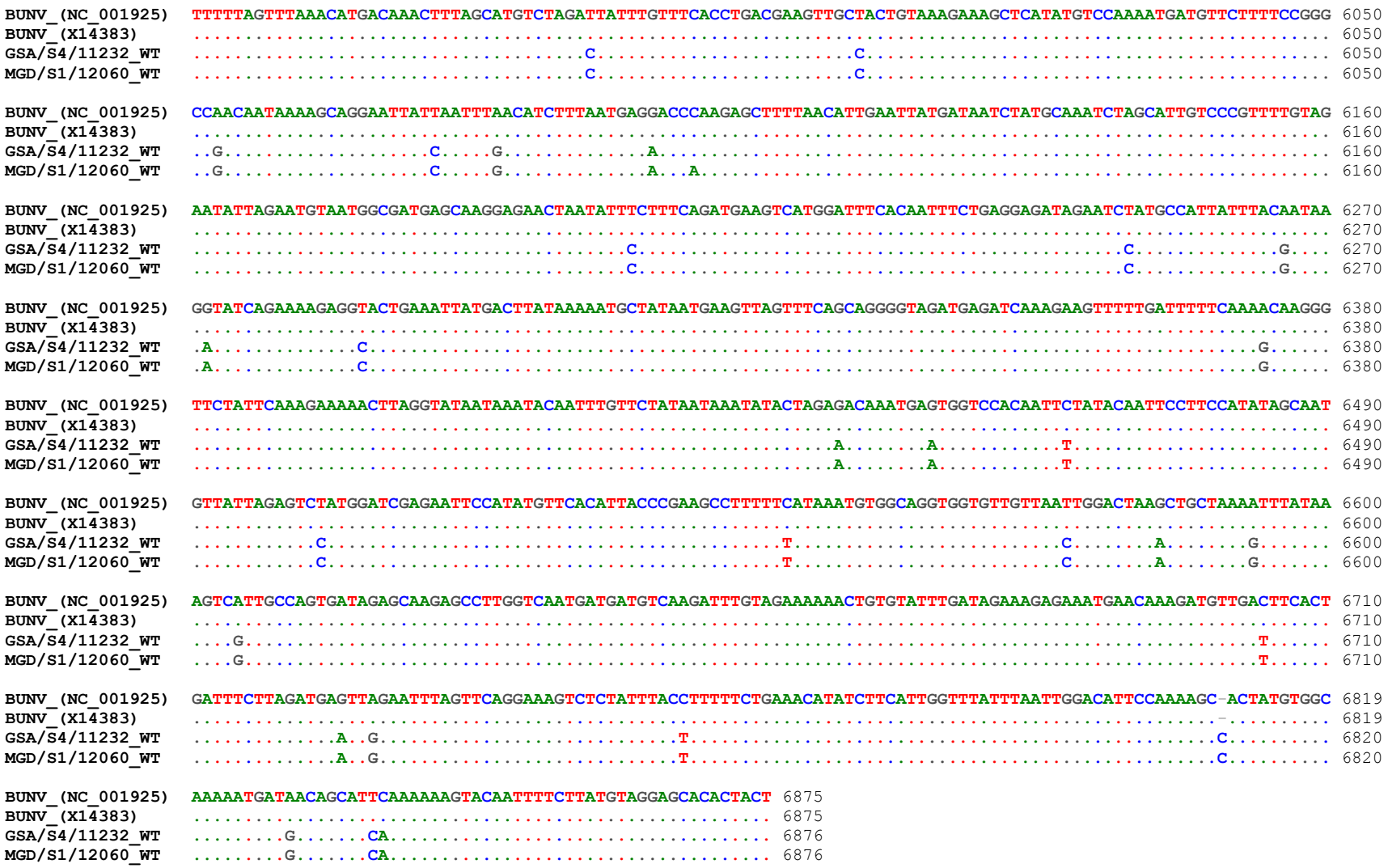
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BUNV_(X14383) 6050
GSA/S4/11232_WT 6050
MGD/S1/12060_WT 6050
BUNV_(NC_001925) CCAACAATAAAAGCAGGAATTATTAATTTAACATCTTTAATGAGGACCCAAGAGCTTTTAACATTGAATTATGATAATCTATGCAAATCTAGCATTGTCCCGTTTTGTAG 6160
BUNV_(X14383) 6160
GSA/S4/11232_WT 6160
MGD/S1/12060_WT 6160
BUNV_(NC_001925) AATATTAGAATGTAATGGCGATGAGCAAGGAGAACTAATATTTCTTTCAGATGAAGTCATGGATTTCACAATTTCTGAGGAGATAGAATCTATGCCATTATTTACAATAA 6270
BUNV_(X14383) 6270
GSA/S4/11232_WT 6270
MGD/S1/12060_WT 6270
BUNV_(NC_001925) GGTATCAGAAAAGAGGTACTGAAATTATGACTTATAAAAATGCTATAATGAAGTTAGTTTCAGCAGGGGTAGATGAGATCAAAGAAGTTTTTGATTTTTCAAAACAAGGG 6380
BUNV_(X14383) 6380
GSA/S4/11232_WT 6380
MGD/S1/12060_WT 6380
BUNV_(NC_001925) TTCTATTCAAAGAAAAACTTAGGTATAATAAATACAATTTGTTCTATAATAAATATACTAGAGACAAATGAGTGGTCCACAATTCTATACAATTCCTTCCATATAGCAAT 6490
BUNV_(X14383) 6490
GSA/S4/11232_WT 6490
MGD/S1/12060_WT 6490
BUNV_(NC_001925) GTTATTAGAGTCTATGGATCGAGAATTCCATATGTTCACATTACCCGAAGCCTTTTTCATAAATGTGGCAGGTGGTGTTGTTAATTGGACTAAGCTGCTAAAATTTATAA 6600
BUNV_(X14383) 6600
GSA/S4/11232_WT 6600
MGD/S1/12060_WT 6600
BUNV_(NC_001925) AGTCATTGCCAGTGATAGAGCAAGAGCCTTGGTCAATGATGATGTCAAGATTTGTAGAAAAAACTGTGTATTTGATAGAAAGAGAAATGAACAAAGATGTTGACTTCACT 6710
BUNV_(X14383) 6710
GSA/S4/11232_WT 6710
MGD/S1/12060_WT 6710
BUNV_(NC_001925) GATTTCTTAGATGAGTTAGAATTTAGTTCAGGAAAGTCTCTATTTACCTTTTTCTGAAACATATCTTCATTGGTTTATTTAATTGGACATTCCAAAAGC-ACTATGTGGC 6819
BUNV_(X14383) 6819
GSA/S4/11232_WT 6820
MGD/S1/12060_WT 6820
BUNV_(NC_001925) AAAAATGATAACAGCATTCAAAAAAGTACAATTTTCTTATGTAGGAGCACACTACT 6875
BUNV_(X14383) 6875
GSA/S4/11232_WT 6876
MGD/S1/12060_WT 6876

Appendix 1G: Ngari virus NSs protein amino acid sequence alignment

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NRIV_DakArD28542_(JX857316)  MMSLLTPAVLLTQRSHTLTLSVSTPLGLVMTTYESSTLKDARLKLVSQKEVNGKLHLTLGAGRLLYIIRIFLATGTTQFLTMVLPSTASVDSLPGTYLRRC 101
NRIV_SUD-HKV141_(JX857322)   ..........................................................................................................  101
NRIV_SUD-HKV66_(JX857319)    ..........................................................................................................  101
NRIV_9800521_(JX857325)      ..........................................................................................................  101
NRIV_9800535_(JX857328)      ..........................................................................................................  101
ISL/TS2/5242/WT(KM507342)    ..........................................................................................................  101
GSA/TS7/5170_WT(KM507341)    ..........................................................................................................  101
TND/S1/19801/WT(KM507343)    ..........................................................................................................  101
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Appendix 1H: Ngari virus N protein amino acid sequence alignment

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NRIV_DakArD28542_(JX857316)  MIELEFHDVAANTSSTFDPEVAYANFKRVYTTGLSYDHIRIFYIKGREIKTSLAKRSEWEVTLNLGGWKITVYNTNFPGNRNNPVPDDGLTLHRLSGFLA 100
NRIV_SUD-HKV141_(JX857322)   ............................H.......................................V..................... 100
NRIV_SUD-HKV66_(JX857319)    ............................H.......................................V..................... 100
NRIV_9800521_(JX857325)      ............................H.......................................................... 100
NRIV_9800535_(JX857328)      ............................H.......................................................... 100
GSA/TS7/5170_WT(KM507341)    ............................H.......................................................... 100
ISL/TS2/5242/WT(KM507342)    ............................H.......................................................... 100
TND/S1/19801/WT(KM507343)    ............................H.......................................................... 100

NRIV_DakArD28542_(JX857316)  RYLLEKMLKVSEPEKLIIKSKIINPLAEKNGITWNDGEEVYLSFFPGSEMFLGTFRFYPLAIGIYKVQRKEMEPKYLEKTMRQRYMGLEAATWTVSKLTE 200
NRIV_SUD-HKV141_(JX857322)   .......................................................................................................... 200
NRIV_SUD-HKV66_(JX857319)   .......................................................................................................... 200
NRIV_9800521_(JX857325)     .......................................................................................................... 200
NRIV_9800535_(JX857328)     .......................................................................................................... 200
GSA/TS7/5170_WT(KM507341)   .......................................................................................................... 200
ISL/TS2/5242/WT(KM507342)   .......................................................................................................... 200
TND/S1/19801/WT(KM507343)   .......................................................................................................... 200

NRIV_DakArD28542_(JX857316)  VQSALTVVSSLGWKKTNVSAAARDFLAKFGINM 233
NRIV_SUD-HKV141_(JX857322)   ................................. 233
NRIV_SUD-HKV66_(JX857319)    ................................. 233
NRIV_9800521_(JX857325)      ................................. 233
NRIV_9800535_(JX857328)      ................................. 233
GSA/TS7/5170_WT(KM507341)    ................................. 233
ISL/TS2/5242/WT(KM507342)    ................................. 233
TND/S1/19801/WT(KM507343)    ................................. 233
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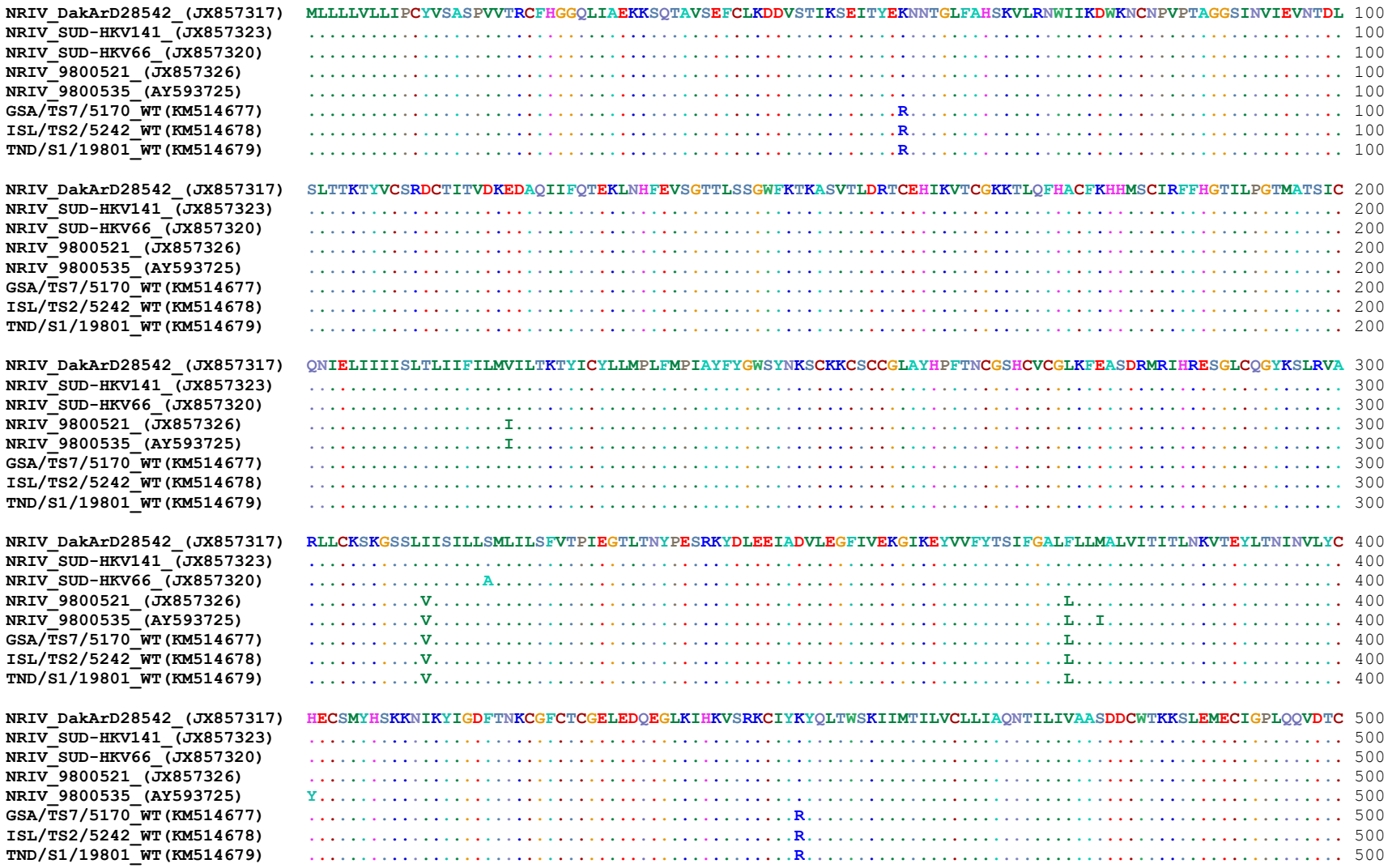
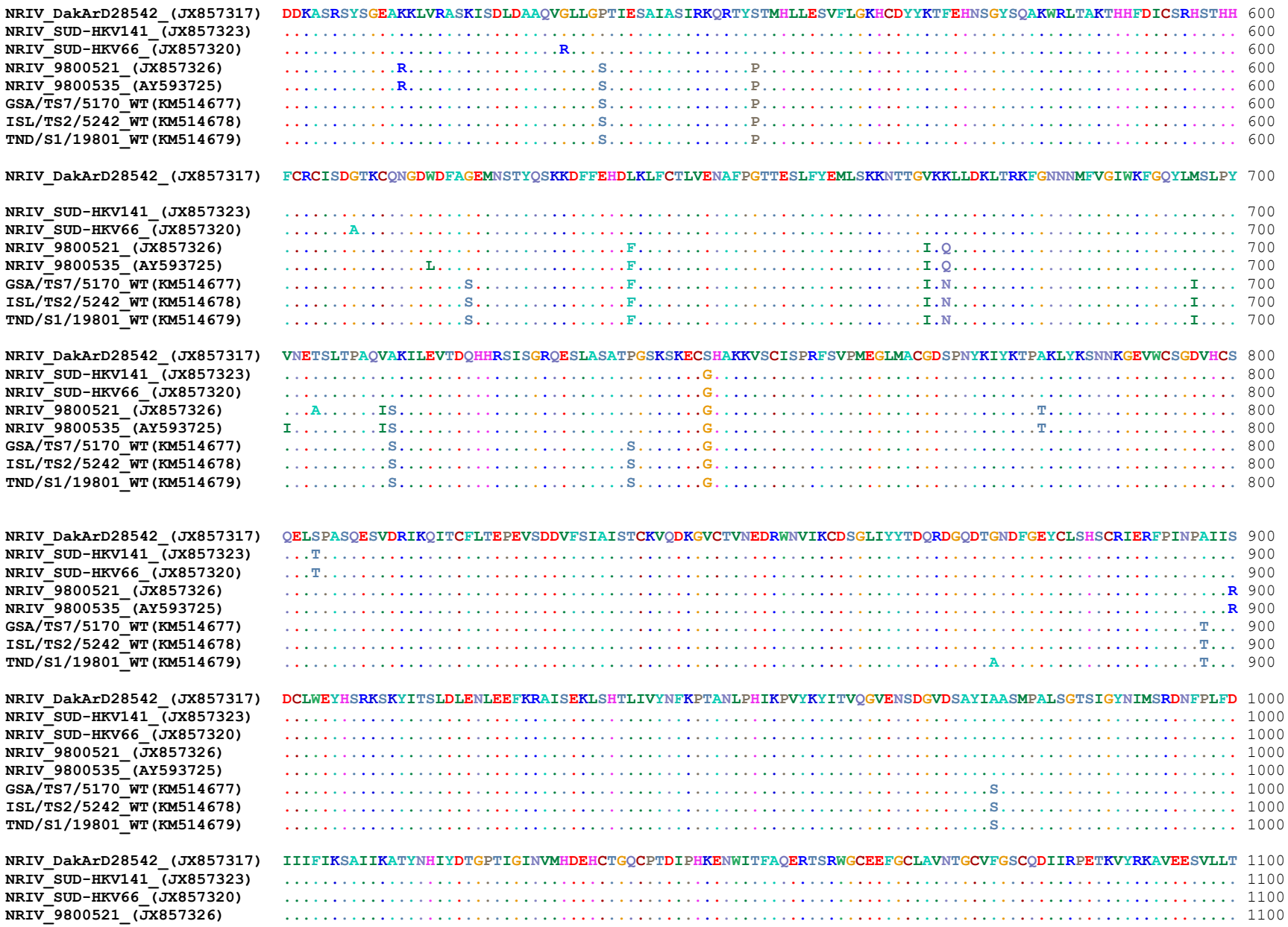
Appendix 1I: Ngari virus M protein amino acid sequence alignment

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NRIV_SUD-HKV141_(JX857323) 600
NRIV_SUD-HKV66_(JX857320) 600
NRIV_9800521_(JX857326) 600
NRIV_9800535_(AY593725) 600
GSA/TS7/5170_WT(KM514677) 600
ISL/TS2/5242_WT(KM514678) 600
TND/S1/19801_WT(KM514679) 600
NRIV_DakArD28542_(JX857317) FCRCISDGTKCQNGDWDFAGEMNSTYQSKKDFFEHDLKLFCTLVENAFPGTTESLFYEMLSKKNTTGVKKLLDKLTRKFGNNNMFVGIWKFGQYLMSLPY 700
NRIV_SUD-HKV141_(JX857323) 700
NRIV_SUD-HKV66_(JX857320) 700
NRIV_9800521_(JX857326) 700
NRIV_9800535_(AY593725) 700
GSA/TS7/5170_WT(KM514677) 700
ISL/TS2/5242_WT(KM514678) 700
TND/S1/19801_WT(KM514679) 700
NRIV_DakArD28542_(JX857317) VNETSLTPAQVAKILEVTDQHHRSISGRQESLASATPGSKSKECSHAKKVSCISPRFSVPMEGLMACGDSPNYKIYKTPAKLYKSNNKGEVWCSGDVHCS 800
NRIV_SUD-HKV141_(JX857323) 800
NRIV_SUD-HKV66_(JX857320) 800
NRIV_9800521_(JX857326) 800
NRIV_9800535_(AY593725) 800
GSA/TS7/5170_WT(KM514677) 800
ISL/TS2/5242_WT(KM514678) 800
TND/S1/19801_WT(KM514679) 800
NRIV_DakArD28542_(JX857317) QELSPASQESVDRIKQITCFLTEPEVSDDVFSIAISTCKVQDKGVCTVNEDRWNVIKCDSGLIYYTDQRDGQDTGNDFGEYCLSHSCRIERFPINPAIIS 900
NRIV_SUD-HKV141_(JX857323) 900
NRIV_SUD-HKV66_(JX857320) 900
NRIV_9800521_(JX857326) 900
NRIV_9800535_(AY593725) 900
GSA/TS7/5170_WT(KM514677) 900
ISL/TS2/5242_WT(KM514678) 900
TND/S1/19801_WT(KM514679) 900
NRIV_DakArD28542_(JX857317) DCLWEYHSRKSKYITSLDLENLEEFKRAISEKLSHTLIVYNFKPTANLPHIKPVYKYITVQGVENSDGVDSAYIAASMPALSGTSIGYNIMSRDNFPLFD 1000
NRIV_SUD-HKV141_(JX857323) 1000
NRIV_SUD-HKV66_(JX857320) 1000
NRIV_9800521_(JX857326) 1000
NRIV_9800535_(AY593725) 1000
GSA/TS7/5170_WT(KM514677) 1000
ISL/TS2/5242_WT(KM514678) 1000
TND/S1/19801_WT(KM514679) 1000
NRIV_DakArD28542_(JX857317) IIIFIKSAIIKATYNHIYDTGPTIGINVMHDEHCTGQCPTDIPHKENWITFAQERTSRWGCEEFGCLAVNTGCVFGSCQDIIRPETKVYRKAVEESVLLT 1100
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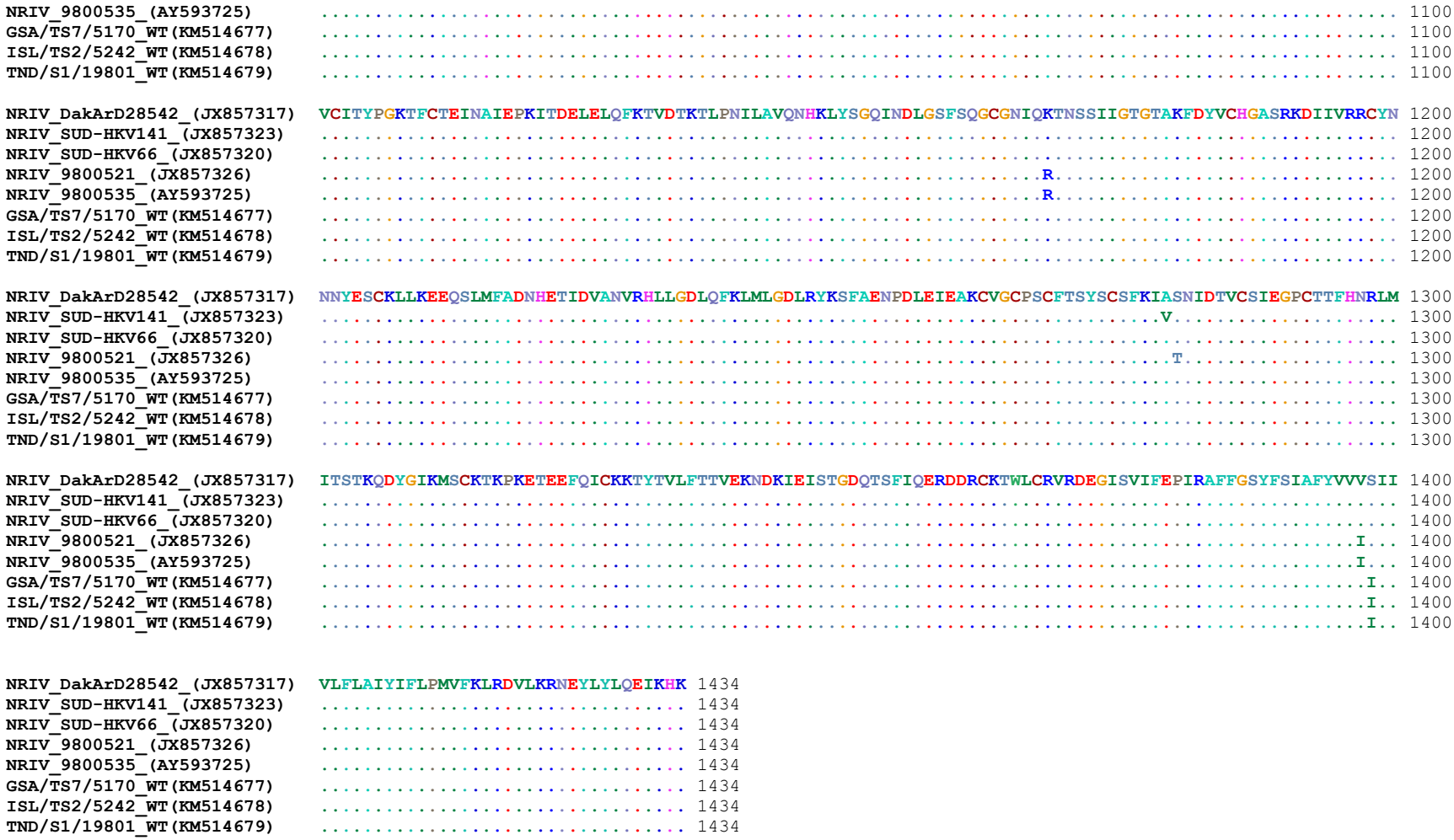
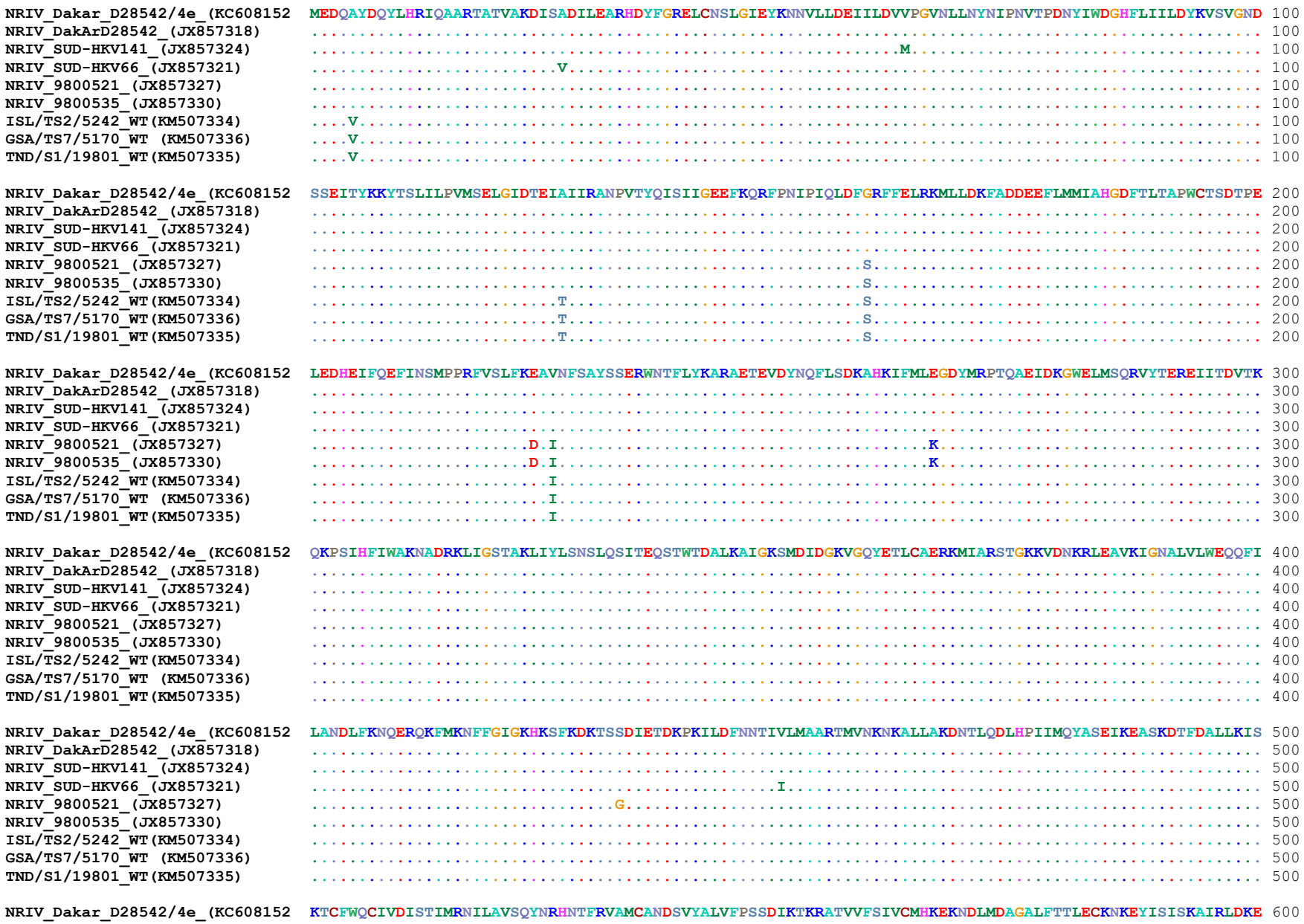


Appendix 1J: Ngari virus L protein amino acid sequence alignment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NRIV_DakArD28542_(JX857318) 100
NRIV_SUD-HKV141_(JX857324) 100
NRIV_SUD-HKV66_(JX857321) 100
NRIV_9800521_(JX857327) 100
NRIV_9800535_(JX857330) 100
ISL/TS2/5242_WT(KM507334) 100
GSA/TS7/5170_WT (KM507336) 100
TND/S1/19801_WT(KM507335) 100
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NRIV_DakArD28542_(JX857318) 200
NRIV_SUD-HKV141_(JX857324) 200
NRIV_SUD-HKV66_(JX857321) 200
NRIV_9800521_(JX857327) 200
NRIV_9800535_(JX857330) 200
ISL/TS2/5242_WT(KM507334) 200
GSA/TS7/5170_WT(KM507336) 200
TND/S1/19801_WT(KM507335) 200
NRIV_Dakar_D28542/4e_(KC608152 LEDHEIFQEFINSMPPRFVSLFKEAVNFSAYSSERWNTFLYKARAETEVDYNQFLSDKAHKIFMLEGDYMRPTQAEIDKGWELMSQRVYTEREIITDVTK 300
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NRIV_SUD-HKV141_(JX857324) 300
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NRIV_9800521_(JX857327) 300
NRIV_9800535_(JX857330) 300
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TND/S1/19801_WT(KM507335) 300
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NRIV_DakArD28542_(JX857318) 400
NRIV_SUD-HKV141_(JX857324) 400
NRIV_SUD-HKV66_(JX857321) 400
NRIV_9800521_(JX857327) 400
NRIV_9800535_(JX857330) 400
ISL/TS2/5242_WT(KM507334) 400
GSA/TS7/5170_WT (KM507336) 400
TND/S1/19801_WT(KM507335) 400
NRIV_Dakar_D28542/4e_(KC608152 LANDLFKNQERQKFMKNFFGIGKHKSFKDKTSSDIETDKPKILDFNNTIVLMAARTMVNKNKALLAKDNTLQDLHPIIMQYASEIKEASKDTFDALLKIS 500
NRIV_DakArD28542_(JX857318) 500
NRIV_SUD-HKV141_(JX857324) 500
NRIV_SUD-HKV66_(JX857321) 500
NRIV_9800521_(JX857327) 500
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ISL/TS2/5242_WT(KM507334) 500
GSA/TS7/5170_WT (KM507336) 500
TND/S1/19801_WT(KM507335) 500
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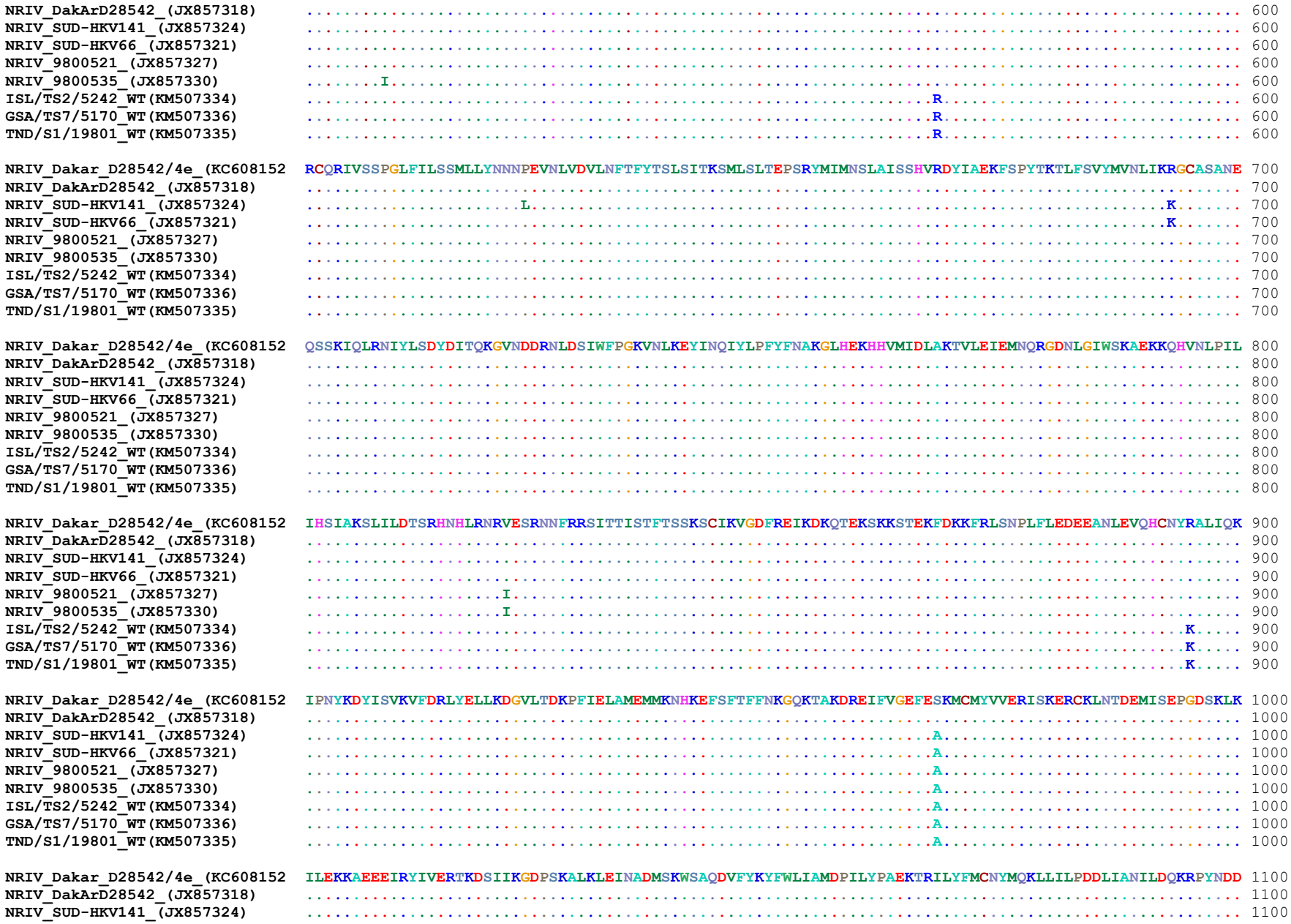
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NRIV_SUD-HKV141_(JX857324)
NRIV_SUD-HKV66_(JX857321)
NRIV_9800521_(JX857327)
NRIV_9800535_(JX857330)
ISL/TS2/5242_WT(KM507334)
GSA/TS7/5170_WT(KM507336)
TND/S1/19801_WT(KM507335)
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IHSIAKSLILDTSRHNHLRNRVESRNNFRRSITTISTFTSSKSCIKVGDFREIKDKQTEKSKKSTEKFDKKFRLSNPLFLEDEEANLEVQHCNYRALIQK
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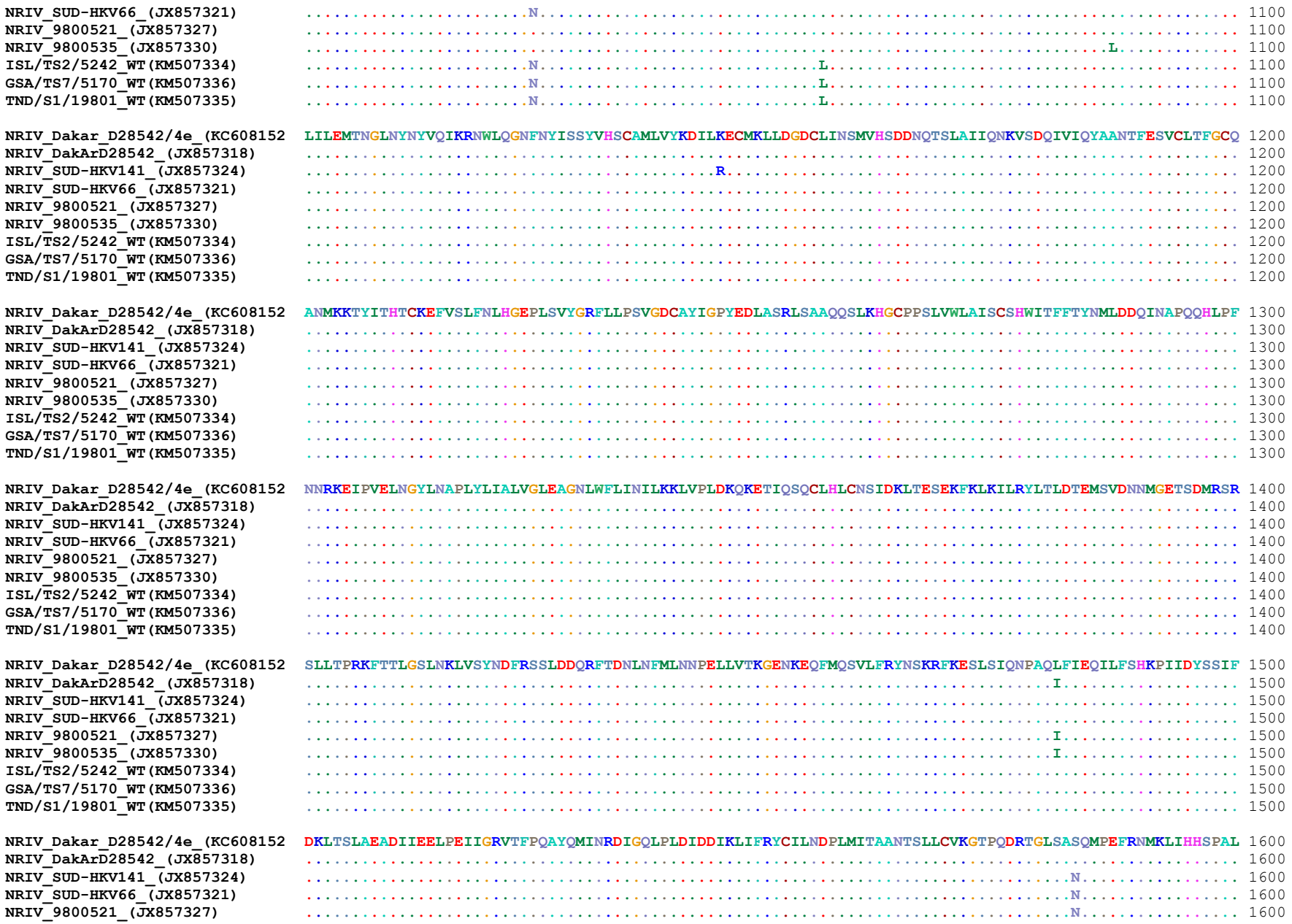
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NRIV_9800521_(JX857327) 1100
NRIV_9800535_(JX857330) 1100
ISL/TS2/5242_WT(KM507334) 1100
GSA/TS7/5170_WT(KM507336) 1100
TND/S1/19801_WT(KM507335) 1100
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NRIV_SUD-HKV66_(JX857321) 1200
NRIV_9800521_(JX857327) 1200
NRIV_9800535_(JX857330) 1200
ISL/TS2/5242_WT(KM507334) 1200
GSA/TS7/5170_WT(KM507336) 1200
TND/S1/19801_WT(KM507335) 1200
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ISL/TS2/5242_WT(KM507334) 1300
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NRIV_SUD-HKV141_(JX857324) 1400
NRIV_SUD-HKV66_(JX857321) 1400
NRIV_9800521_(JX857327) 1400
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ISL/TS2/5242_WT(KM507334) 1400
GSA/TS7/5170_WT(KM507336) 1400
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NRIV_DakArD28542_(JX857318) 1500
NRIV_SUD-HKV141_(JX857324) 1500
NRIV_SUD-HKV66_(JX857321) 1500
NRIV_9800521_(JX857327) 1500
NRIV_9800535_(JX857330) 1500
ISL/TS2/5242_WT(KM507334) 1500
GSA/TS7/5170_WT(KM507336) 1500
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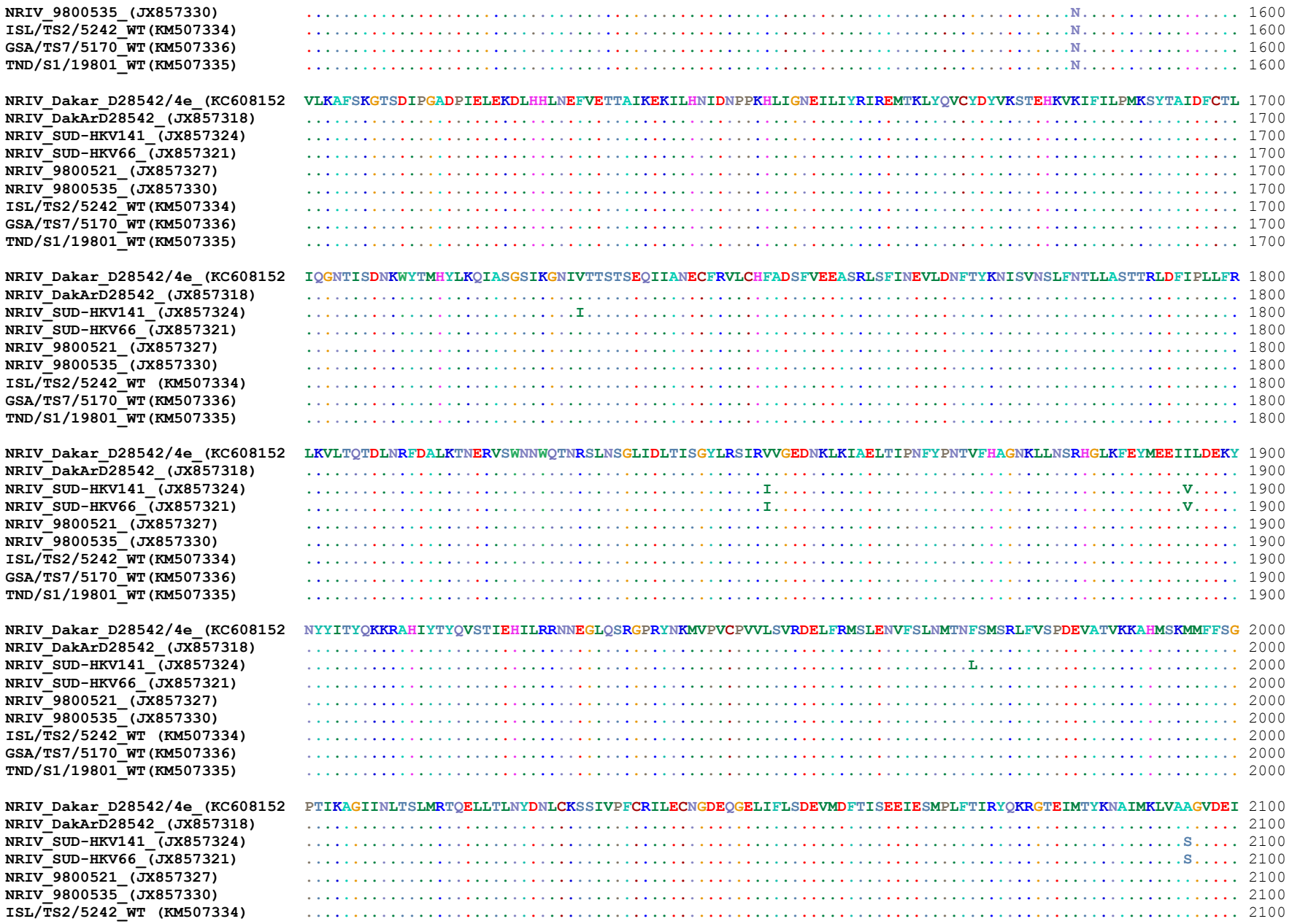
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NRIV_DakArD28542_(JX857318) 1700
NRIV_SUD-HKV141_(JX857324) 1700
NRIV_SUD-HKV66_(JX857321) 1700
NRIV_9800521_(JX857327) 1700
NRIV_9800535_(JX857330) 1700
ISL/TS2/5242_WT(KM507334) 1700
GSA/TS7/5170_WT(KM507336) 1700
TND/S1/19801_WT(KM507335) 1700
NRIV_Dakar_D28542/4e_(KC608152 IQGNTISDNKWYTMHYLKQIASGSIKGNIVTTSTSEQIIANECFRVLCHFADSFVEEASRLSFINEVLDNFTYKNISVNSLFNTLLASTTRLDFIPLLFR 1800
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NRIV_SUD-HKV141_(JX857324) 1800
NRIV_SUD-HKV66_(JX857321) 1800
NRIV_9800521_(JX857327) 1800
NRIV_9800535_(JX857330) 1800
ISL/TS2/5242_WT (KM507334) 1800
GSA/TS7/5170_WT(KM507336) 1800
TND/S1/19801_WT(KM507335) 1800
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NRIV_DakArD28542_(JX857318) 1900
NRIV_SUD-HKV141_(JX857324) 1900
NRIV_SUD-HKV66_(JX857321) 1900
NRIV_9800521_(JX857327) 1900
NRIV_9800535_(JX857330) 1900
ISL/TS2/5242_WT(KM507334) 1900
GSA/TS7/5170_WT(KM507336) 1900
TND/S1/19801_WT(KM507335) 1900
NRIV_Dakar_D28542/4e_(KC608152 NYYITYQKKRAHIYTYQVSTIEHILRRNNEGLQSRGPRYNKMVPVCPVVLSVRDELFRMSLENVFSLNMTNFSMSRLFVSPDEVATVKKAHMSKMMFFSG 2000
NRIV_DakArD28542_(JX857318) 2000
NRIV_SUD-HKV141_(JX857324) 2000
NRIV_SUD-HKV66_(JX857321) 2000
NRIV_9800521_(JX857327) 2000
NRIV_9800535_(JX857330) 2000
ISL/TS2/5242_WT (KM507334) 2000
GSA/TS7/5170_WT(KM507336) 2000
TND/S1/19801_WT(KM507335) 2000
NRIV_Dakar_D28542/4e_(KC608152 PTIKAGIINLTSLMRTQELLTLNYDNLCKSSIVPFCRILECNGDEQGELIFLSDEVMDFTISEEIESMPLFTIRYQKRGTEIMTYKNAIMKLVAAGVDEI 2100
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NRIV_SUD-HKV141_(JX857324) 2100
NRIV_SUD-HKV66_(JX857321) 2100
NRIV_9800521_(JX857327) 2100
NRIV_9800535_(JX857330) 2100
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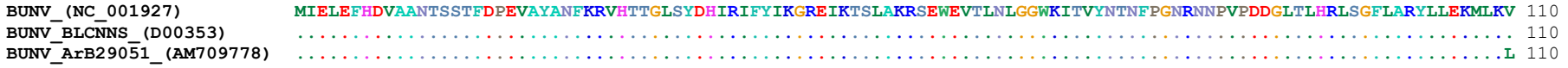
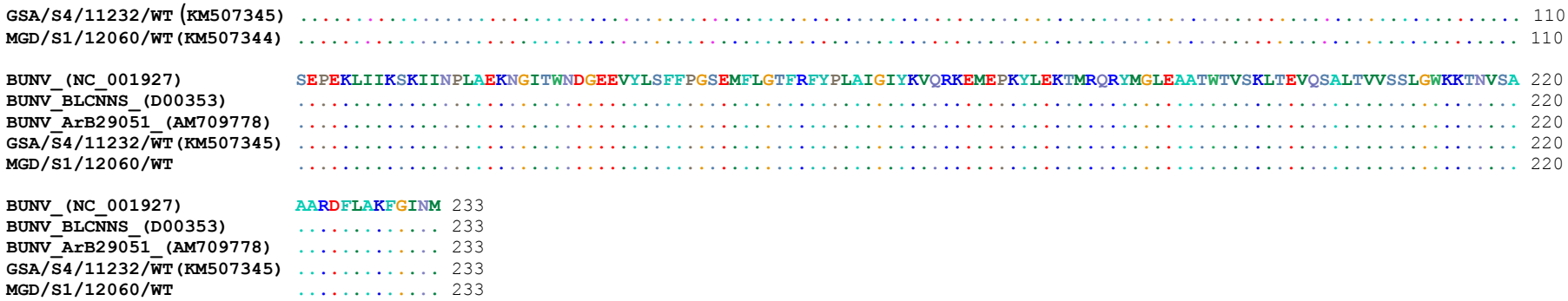
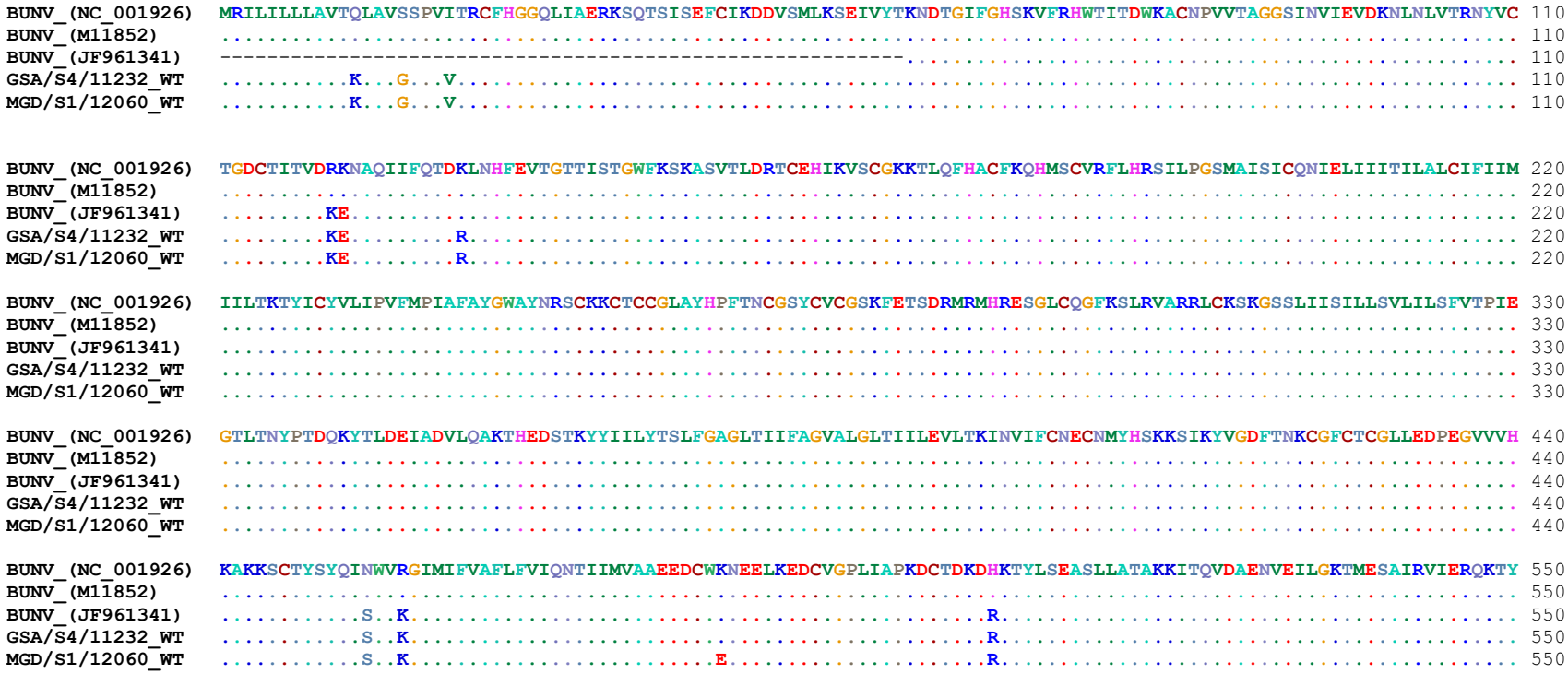
Appendix 1K: Bunyamwera virus NSs protein amino acid sequence alignment

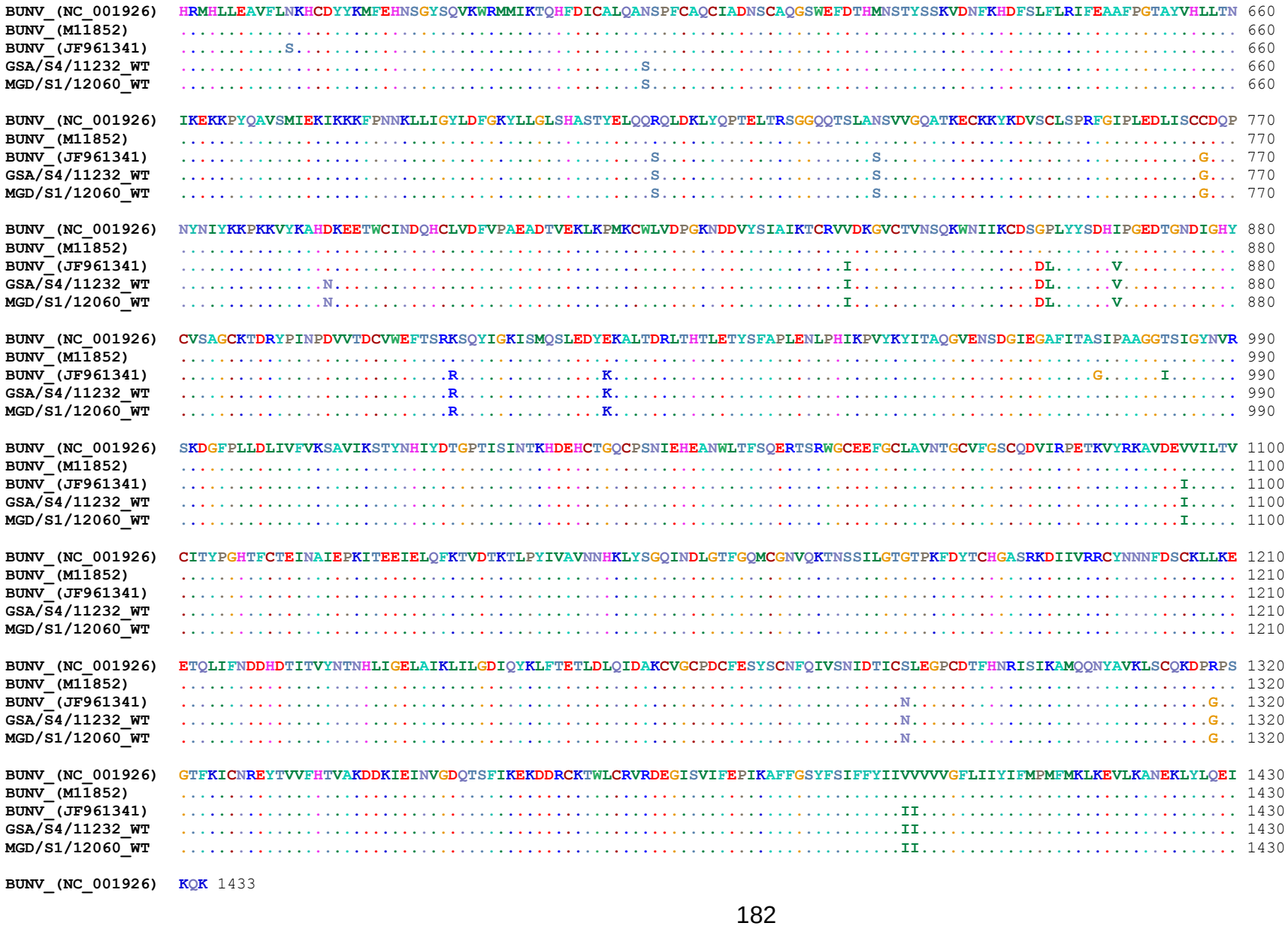
Appendix 1L: Bunyamwera virus N protein amino acid sequence alignment





Appendix 1M: Bunyamwera virus M protein amino acid sequence alignment

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BUNV_(M11852) 660
BUNV_(JF961341) 660
GSA/S4/11232_WT 660
MGD/S1/12060_WT 660
BUNV_(NC_001926) IKEKKPYQAVSMIEKIKKKFPNNKLLIGYLDFGKYLLGLSHASTYELQQRQLDKLYQPTELTRSGGQQTSLANSVVGQATKECKKYKDVSCLSPRFGIPLEDLISCCDQP 770
BUNV_(M11852) 770
BUNV_(JF961341) 770
GSA/S4/11232_WT 770
MGD/S1/12060_WT 770
BUNV_(NC_001926) NYNIYKKPKKVYKAHDKEETWCINDQHCLVDFVPAEADTVEKLKPMKCWLVDPGKNDDVYSIAIKTCRVVDKGVCTVNSQKWNIIKCDSGPLYYSDHIPGEDTGNDIGHY 880
BUNV_(M11852) 880
BUNV_(JF961341) 880
GSA/S4/11232_WT 880
MGD/S1/12060_WT 880
BUNV_(NC_001926) CVSAGCKTDRYPINPDVVTDCVWEFTSRKSQYIGKISMQSLEDYEKALTDRLTHTLETYSFAPLENLPHIKPVYKYITAQGVENSDGIEGAFITASIPAAGGTSIGYNVR 990
BUNV_(M11852) 990
BUNV_(JF961341) 990
GSA/S4/11232_WT 990
MGD/S1/12060_WT 990
BUNV_(NC_001926) SKDGFPLLDLIVFVKSAVIKSTYNHIYDTGPTISINTKHDEHCTGQCPSNIEHEANWLTFSQERTSRWGCEEFGCLAVNTGCVFGSCQDVIRPETKVYRKAVDEVVILTV 1100
BUNV_(M11852) 1100
BUNV_(JF961341) 1100
GSA/S4/11232_WT 1100
MGD/S1/12060_WT 1100
BUNV_(NC_001926) CITYPGHTFCTEINAIEPKITEEIELQFKTVDTKTLPYIVAVNNHKLYSGQINDLGTFGQMCGNVQKTNSSILGTGTPKFDYTCHGASRKDIIVRRCYNNNFDSCKLLKE 1210
BUNV_(M11852) 1210
BUNV_(JF961341) 1210
GSA/S4/11232_WT 1210
MGD/S1/12060_WT 1210
BUNV_(NC_001926) ETQLIFNDDHDTITVYNTNHLIGELAIKLILGDIQYKLFTETLDLQIDAKCVGCPDCFESYSCNFQIVSNIDTICSLEGPCDTFHNRISIKAMQQNYAVKLSCQKDPRPS 1320
BUNV_(M11852) 1320
BUNV_(JF961341) 1320
GSA/S4/11232_WT 1320
MGD/S1/12060_WT 1320
BUNV_(NC_001926) GTFKICNREYTVVFHTVAKDDKIEINVGDQTSFIKEKDDRCKTWLCRVRDEGISVIFEPIKAFFGSYFSIFFYIIVVVVVGFLIIYIFMPMFMKLKEVLKANEKLYLQEI 1430
BUNV_(M11852) 1430
BUNV_(JF961341) 1430
GSA/S4/11232_WT 1430
MGD/S1/12060_WT 1430
BUNV_(NC_001926) KQK 1433

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BUNV_(M11852)      ... 1433
BUNV_(JF961341)    ... 1376
GSA/S4/11232_WT    ... 1433
MGD/S1/12060_WT    ... 1433
```

Appendix 1N: Bunyamwera virus L protein amino acid sequence alignment

```
BUNV_(NC_001925)  MEDQAYDQYLHRIQAARTATVAKDISADILEARHDYFGRELCNSLGIEYKNNVLLDEIILDVVPGVNLLNYNIPNVTPDNYIWDGHFLIILDYKVSVGNDSSEITYKKYT 110
BUNV_(X14383)     ..........................................................................................................  110
GSA/S4/11232_WT   ..........................................................................................................  110
MGD/S1/12060_WT   ......................T...........................................................................................  110

BUNV_(NC_001925)  SLILPVMSELGIDTEIAIIRANPVTYQISIIGEEFKQRFPNIPIQLDFGRFFELRKMLLDKFADDEEFLMMIAHGDFTLTAPWCTSDTPELEEHEIFQEFINSMPPRFVS 220
BUNV_(X14383)     ..........................................................................................................  220
GSA/S4/11232_WT   ..........................................................................................................  220
MGD/S1/12060_WT   ..........................................................................................................  220

BUNV_(NC_001925)  LFKEAVNFSAYSSERWNTFLYRARAETEVDYNQFLSDKAHKIFMLEGDYMRPTQAEIDKGWELMSQRVYTEREIITDVTKQKPSIHFIWVKNADRKLIGSTAKLIYLSNS 330
BUNV_(X14383)     ..........................................................................................................  330
GSA/S4/11232_WT   ....................K.....................................................A.....................  330
MGD/S1/12060_WT   ....................K.....................................................A.....................  330

BUNV_(NC_001925)  LQSITEQSTWTDALKAIGKSMDIDGKVGQYETLCAERKMIARSTGKKVDNKRLEAVKIGNALVLWEQQFILANDLFKNQERQKFMKNFFGIGKHKSFKDKTSSDIETDKP 440
BUNV_(X14383)     ..........................................................................................................  440
GSA/S4/11232_WT   ..........................................................................................................  440
MGD/S1/12060_WT   ..........................................................................................................  440

BUNV_(NC_001925)  KILDFNNTIVLMAARTMVNKNKALLAKDNTLQDLHPIIMQYASEIKEASKDTFDALLKISKTCFWQCIVDVSTIMRNILAVSQYNRHNTFRVAMCANDSVYALVFPSSDI 550
BUNV_(X14383)     ..........................................................................................................  550
GSA/S4/11232_WT   ..........................................................I...............................................  550
MGD/S1/12060_WT   ..........................................................I...............................................  550

BUNV_(NC_001925)  KTKRATVVFSIVCMHKEKNDLMDAGALFTTLECKNKEYISISKAIRLDKERCQRIVSSPGLFILSSMLLYNNNPEVNLVDVLNFTFYTSLSITKSMLSLTEPSRYMIMNS 660
BUNV_(X14383)     ..........................................................................................................  660
GSA/S4/11232_WT   ..........................................................................................................  660
MGD/S1/12060_WT   ..........................................................................................................  660

BUNV_(NC_001925)  LAISSHVRDYIAEKFSPYTKTLFSVYMVNLIKRGCASANEQSSKIQLRNIYLSDYDITQKGVNDGRNLDSIWFPGKVNLKEYINQIYLPFYFNAKGLHEKHHVMIDLAKT 770
BUNV_(X14383)     ..........................................................................................................  770
GSA/S4/11232_WT   ..........................................................D...............................................  770
MGD/S1/12060_WT   ..........................................................D...............................................  770

BUNV_(NC_001925)  VLEIEMNQRSDNLGIWSKAEKKQHVNLPILIHSIAKSLILDTSRHNHLRNRVESRNNFRRSITTISTFTSSKSCIKIGDFREIKDKETEKSKKSTEKFDKKFRLSNPLFL 880
BUNV_(X14383)     ..........................................................................................................  880
GSA/S4/11232_WT   ..........................................................................................................  880
MGD/S1/12060_WT   ..........................................................................I.............................  880
```

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BUNV_(NC_001925)  EDEEANLEVQHCNYRALIQKIPNYKDYISVKVFDRLYELLKNGVLTDKPFIELAMEMMKNHKEFSFTFFNKGQKTAKDREIFVGEFEAKMCMYVVERISKERCKLNTDEM  990
BUNV_(X14383)     ........................................................................................................................  990
GSA/S4/11232_WT   ........................................................................................................................  990
MGD/S1/12060_WT   ........................................................................................................................  990

BUNV_(NC_001925)  ISEPGDSKLKILEKKAEEEIRYIVERTKDSIIKGDPSKALKLEINADMSKWSAQDVFYKYFWLIAMDPILYPAEKTRILYFMCNYMQKLLILPDDLIANILDQKRPYNDD 1100
BUNV_(X14383)     ........................................................................................................................ 1100
GSA/S4/11232_WT  ........................................................................................................................ 1100
MGD/S1/12060_WT  ........................................................................................................................ 1100

BUNV_(NC_001925)  LILEMTNGLNYNYVQIKRNWLQGNFNYISSYVHSCAMLVYKDILKECMKLLDGDCLINSMVHSDDNQTSLAIIQNKVSDQIVIQYAANTFESVCLTFGCQANMKKTYITH 1210
BUNV_(X14383)     ........................................................................................................................ 1210
GSA/S4/11232_WT  ........................................................................................................................ 1210

MGD/S1/12060_WT  ........................................................................................................................ 1210

BUNV_(NC_001925)  TCKEFVSLFNLHGEPLSVFGRFLLPSVGDCAYIGPYEDLASRLSAAQQSLKHGCPPSLVWLAISCSHWITFFTYNMLDDQINAPQQHLPFNNRKEIPVELNGYLNAPLYL 1320
BUNV_(X14383)     ........................................................................................................................ 1320
GSA/S4/11232_WT  ................Y....................................................................................................... 1320
MGD/S1/12060_WT  ................Y....................................................................................................... 1320

BUNV_(NC_001925)  IALVGLEAGNLWFLINILKKLVPLDKQKETIQSQCLHLCNSIDKLTESEKFKLKILRYLTLDTEMSVDNNMGETSDMRSRSLLTPRKFTTLGSLNKLVSYNDFRSSLDDQ 1430
BUNV_(X14383)     ........................................................................................................................ 1430
GSA/S4/11232_WT  ........................................................................................................................ 1430
MGD/S1/12060_WT  ........................................................................................................................ 1430

BUNV_(NC_001925)  RFTDNLNFMLNNPELLVTKGENKEQFMQSVLFRYNSKRFKESLSIQNPAQLFIEQILFSHKPIIDYSSIFDKLTSLAEADIIEELPEIIGRVTFPQAYQMINRDIGQLPL 1540
BUNV_(X14383)     ........................................................................................................................ 1540
GSA/S4/11232_WT  ........................................................................................................................ 1540
MGD/S1/12060_WT  ........................................................................................................................ 1540

BUNV_(NC_001925)  DIDDIKLIFRYCILNDPLMITAANTSLLCVKGTPQDRTGLSASQMPEFRNMKLIHHSPALVLKAFSKGTSDIPGADPIELEKDLHHLNEFVETTAIKEKILHNIDNPPKH 1650
BUNV_(X14383)     ........................................................................................................................ 1650
GSA/S4/11232_WT  ........................................................................................................................ 1650
MGD/S1/12060_WT  ........................................................................................................................ 1650

BUNV_(NC_001925)  LIGNEILIYRIREMTKLYQVCYDYVKSTEHKVKIFILPMKSYTAIDFCTLIQGNTISDNKWYTMHYLKQIASGSIKGNIVTTSTSEQIIANECFRVLCHFADSFVEEASR 1760
BUNV_(X14383)     ........................................................................................................................ 1760
GSA/S4/11232_WT  ........................................................................................................................ 1760
MGD/S1/12060_WT  ........................................................................................................................ 1760

BUNV_(NC_001925)  LSFINEVLDNFTYKNISVNSLFNTLLASTTRLDFIPLLFRLKVLTQTDLNRFDALKTNERVSWNNWQTNRSLNSGLIDLTISGYLRSIRVVGEDNKLKIAELTIPNFYPN 1870
BUNV_(X14383)     ........................................................................................................................ 1870
GSA/S4/11232_WT  ........................................................................................................................ 1870
MGD/S1/12060_WT  ........................................................................................................................ 1870

BUNV_(NC_001925)  TVFHAGNKLLNSRHGLKFEYMEEIVLDEKYNYYITYQKKRAHIYTYQVSTIEHILRRNNEGLQSRGPRYNKMVPVCPVVLSVRDELFRMSLENVFSLNMTNFSMSRLFVS 1980
BUNV_(X14383)     ........................................................................................................................ 1980
GSA/S4/11232_WT  ....................TI.............................................................................................. 1980
MGD/S1/12060_WT  ....................TI.............................................................................................. 1980

BUNV_(NC_001925)  PDEVATVKKAHMSKMMFFSGPTIKAGIINLTSLMRTQELLTLNYDNLCKSSIVPFCRILECNGDEQGELIFLSDEVMDFTISEEIESMPLFTIRYQKRGTEIMTYKNAIM 2090
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BUNV_(X14383)      ..........................................................................................  2090
GSA/S4/11232_WT    ..........................................................................................  2090
MGD/S1/12060_WT    ..........................K...............................................................  2090

BUNV_(NC_001925)   KLVSAGVDEIKEVFDFSKQGFYSKKNLGIINTICSIINILETNEWSTILYNSFHIAMLLESMDREFHMFTLPEAFFINVAGGVVNWTKLLKFIKSLPVIEQEPWSMMMSR  2200
BUNV_(X14383)      ..........................................................................................  2200
GSA/S4/11232_WT    ..........................................................................................  2200
MGD/S1/12060_WT    ..........................................................................................  2200

BUNV_(NC_001925)   FVEKTVYLIEREMNKDVDFTDFLDELEFSSGKSLFTFF  2238
BUNV_(X14383)      ..............................  2238
GSA/S4/11232_WT    ..............................  2238
MGD/S1/12060_WT    ..............................  2238
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Appendix 2: Primers used in sequencing of Kenyan Bunyamwera and Ngari virus isolates

Table 19: ***Primers used in sequencing of Kenyan Bunyamwera and Ngari virus isolates***.

Target virus	Target gene/protein	Primer sequence pair (5'-3')	Coordinate nucleotides	Reference
Bunyamwera	S segment	BUNS1 (AGTAGTGTACTCCACACTACAAACT) and BUNS9 (AGGAATCCACTGAGGCGGTGGAGG)	1-25 to 358-381	Yandoko et al, 2007
		BUNS4 (CTGGCAACCGGAACAACCCAGTT) and BUNS5 (GAGACAACTGTCAGTGCAGACTGAA)	318-340 to 687-711	Yandoko et al, 2007
		BUNS10 (TCAGTCTGCACTGACAGTTGTCTC) and BUNS2 (AGTAGTGTGCTCCACCTAAAACTTA)	688-711 to 937-961	Yandoko et al, 2007
Bunyamwera	**Polyprotein M segment**	Bunya M14C (CGGAATTCAGTAGTGTACTACC) and Bunya M619 (GACATATGYTGATTGAAGCAAGCATG)	1-14 to 586-576	Yanase et al, 2006)
		BUNM11F (TCAGCACTGGCTGGTTTAAG) and BUNM11R (ACCTGCACCGAAGAGTGATG)	481-500 to 1159-1178	This study
		BunM12F (GAGATAGCAGATGTCCTTCAAGC) and BunM12R (CAAGCAAGTGCATTCTGTGG)	1092-1114 to 1706-1725	This study
		Bun M3F (TTCACTAATAAATGTGGGTTTTG) and Bun M3R (ATGCTGACTGCCTGATAGGG)	1311-1333 to 2052-2071	This study
		Bun M4F (TCCAGGCACTGCTTATGTTC) and Bun M4R (TCTTCCCCTGGTATGTGGTC)	2003-2022 to 2652-2671	This study
		Bun M5F (TGTGATTCTGGTCCGCTCTAC) and Bun M5R (GCATCCCCATCTGCTAGTTC)	2625-2645 to 3217-3236	This study
		Bun M6F (CAAAGCATGACGAACATTGC) and Bun M6R (TCAAGGCTGCAGATTGTGTC)	3136-3155 to 3900-3919	This study
		Bunc7MF (CCAGATTGATGCGAAATGTG) BAT 3' end R (GAATTCAGTAGTGTGCTACC)	3821-3840 to 4445-4458	This study
Bunyamwera	**L Protein**	M13 BunL 1C Yanase (TGTAAAACGACGGCCAGTAGTGTACTCCT) and BunL605R Yunase (RGTGAARTCNCCATGTGC)	1-14 to 614-597	Yanase et al, 2006)
		Bun2LF (GTTGCTGGACAAGTTTGCTG) and Bun2LR (TTGCCAATCTTAACCGCTTC)	551-570 to 1200-1219	This study
		Bun3LF (AAAATGATTGCCAGGTCAAC) and Bun3LR (TATGAAAAGCCCAGGTGATG)	1152-1171 to 1870 - 1889	This study
		Bun5LF (AAGAAACAACATGTCAATCTACC) and Bun5LR (TAGCGGATTTCCTCTTCTGC)	2421-2443 to 3066-3085	This study
		Bunya L For JV (CAATATAATAGACATAATACATTTAGAGT) Bunya L Rev JV (CTCCATTTDGACATRTCTGCA)	1617-1645 to 3175-3154	This study

Appendix 3A

		BUNL6F (GCTGAACACGGACGAGATG) and BUNL6R (GGCCCAATGTAAGCACAATC)	3003-3020 to 3765-3784	This study
		BUNL7F (GGAGAACCACTATCTGTCTTTGG) and BUNL7R (ATTGTGCTGGGTTTTGGATG)	3717-3739 to 4472-4491	This study
		BUNL8F (GCAGTTCATGCAATCTGTCC) and BUNL8R (TGTTGCCCTGAATCAATGTG)	4412-4431 to 5144-5163	This study
		BUNL9F (TCACAGCTGCAAACACTTCC) and BUNL9R (TCAATCTGCTTGCCTCTTCC)	4729-4748 to 5315-5334	This study
		BUNL10F (AAGCGAGCAAATAATAGCAAATG) and BUNL10R (TACCTAGGGCCTCTGGATTG)	5256-5274 to 5866-5847	This study
		BUNL11F (CCGGCTATTTAAGATCAATAAGG) and BUNL11R (TGCTCATCGCCATTACATTC)	5575-5597 to 6168-6187	This study
		BUNL12F (TCTAGCATTGTCCCGTTTTG) and BUNL12R (TTTTGCCACATAGTGCTTTTG)	6138-6157 to 6803-6823	This study
		BUNL12F (TCTAGCATTGTCCCGTTTTG) And Bun 3'end LR (GTAAAACGACGGCCAGTAGTGTGCTCC)	6138–6157 to 6863–6875	This study
Ngari virus	**Polyprotein M segment**	BunyaM14C (CGGAATTCAGTAGTGTACTACC) and TrialR2 (TGACCCGCAATTTGTAAAGG)		This study
		BATM13F (CCAAACCGAGAAGTTGAACC) and BATM13R (AATCCTTCCAGGACATCAGC)	419-438 to 1080-1099	This study
		BATM14F (TGCTCATGCTGTGGTCTAGC) and BATM14R (ACCTCCACTTTGCCTGTGAG)	798-817 to 1769-1788	This study
		BATAIM3F CCTGGGGAAGCATTGTGATTACT BATAIM3R CTAGCCAGCGACTCTTGCCTTCC	1,704–1,726 to 2,206–2,228	Jost et al, 2011
		BATAIM4F GTCGCTGGCTAGTGCTACCTCTGG BATAIM4R CTGATTATTGTCGGATTTATTGGGAACCT	2,217–2,240 to 2,698–2,726	Jost et al, 2011
		BATAIM5F AAAGGTTCCCAATAAATCCGACAA BATAIM5R CAAATTCTTCACATCCCCAACGACTA	2,696–2,719 to 3,195–3,220	Jost et al, 2011
		BATAIM6F AGAATTTGGGTGCCTTGCTGTCA BATAIM6R AGATGTTTGGTCCCCTGTGCTTATTT	3,213–3,235 to 4,061–4,086	Jost et al, 2011
		BAT 3'end F (TGTTCGCAGATAACCATGAAAC) and BAT 3'end R (GAATTCAGTAGTGTGCTACC)	3688-3709 to 4425-4438	This study
	L segment	NgariL6F (TTTTGAGAAGGAATAATGAAGG) and NgariL6R (CCCCTGCTGCAACTAACTTC)	5821-5842 to 6320-6339	This study

Informed Consent Agreement

Request: I wish to request for your participation in the following research project:

Project title: Tick-Borne Virus Prevalence and Diversity in Pastoral Eco-Zone of Ijara District, North Eastern Province of Kenya

Study information: We are interested in finding out causes of illness in adults and children who have fever and/or malaria like illness. We want to use new methods that can detect any germs even those that are difficult to detect usually or have not been detected before. We want to draw a small amount of blood and test it in the lab to see if you have been exposed to germs that are carried by ticks.

Expertise in the study: The study is being run by Olivia Wesula Lwande a PhD student in Medical Virology at the Department of Medical Virology, University of Pretoria (Student number:11346192) , Dr. Rosemary Sang and a team of doctors and scientists from Kenya Medical Research Institute (KEMRI), Ministry of Public Health and Sanitation (MPH&S), International Centre of Insect Physiology and Ecology (ICIPE), International Livestock Research Institute (ILRI) and Ministry of Livestock/Department of Veterinary Services (MOL/DVS).

Participation: Participation in this study is voluntary. There is no penalty for refusing to participate. We will need only one sample from you but if need arises, we may come back to you to take a second sample to confirm our finding. If you start the study you (or your child) may discontinue your (or your child's) participation at any time. The principal investigatorsand co- investigators from KEMRI and MOP&S may decide to withdraw you (or your child) from the study if we are unable to obtain a blood sample from you (or your child).

Study procedure: You will be asked some questions about where you live, your illness and any medications you may have taken recently. Then about a tablespoon of blood will be taken from a vein in your arm. The blood will be put into small tubes so we can test for germs that may be causing your illness. We will not test for Human Immunodeficiency Virus (HIV).

Risks involved in the study: There is the possibility of mild discomfort, bruising and very rarely infection at the site where the blood is taken. But, should you (or your child) be injured as a direct result of participating in this research project, you (or your child) will be provided medical care, at no cost to you (or your child), for that injury. You (or your child) will not receive any injury compensation, only medical care. You (your child) should also understand that this is not a waiver or release of your (your child's) legal rights. If you wish, you should discuss this issue thoroughly with the principal investigator before you (your child) enroll in this study.

Benefits from the study: The study can lead to a better understanding of the causes of acute febrile illnesses in Kenya and improve the medical care in Kenya by improving detection methods and identifying some of the hidden germs that may be causing disease. The Ministry of Health (MoH) and supporting medical community can benefit from the knowledge of the identification of new or emerging diseases as the cause of acute febrile illnesses so that they know how to care for you and others in the future. Epidemics can be more readily identified, allowing the MoH to respond in a timely manner hence reducing the number of people who get affected.

Compensation for being in the study: There is no compensation to volunteers for their participation.

Duration: This study requires only completion of a short questionnaire and one blood draw. There is no follow-up or further information needed. The questionnaire and blood draw will take about 15 minutes.

Study participants: Anyone can participate in the study if you have a fever without a source after evaluation by the clinician. If there is an obvious source of infection causing the fever, like an abscess or pneumonia, you need not (your child should not) participate.

Confidentiality: Any information about you (your child's) will remain confidential. Only the people involved in the study will be able to see your information. We will keep all files in locked cabinets when they are not in use, and all blood stored in locked freezers. Your (your child's) name will not be used in any report resulting from this study. Any report from this study will refer to you/your child

only by a study identification number and not by a name. All blood samples collected will be labeled with a study identification number; no names will be used. Your (your child's) blood will be tested for things that could cause fever. Your blood will not be tested for HIV. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. These samples will be labeled with only your study number. They will be secured in freezers at KEMRI, ILRI or ICIPE facilities and only study investigators and their authorized staff will have access. All safeguards ensuring privacy and confidentiality that are in place during this study period will also continue to be in place for the long-term storage of samples and if samples are sent outside of Kenya, no personal identifiers will be included.

If we do need to use the stored blood in the future we will first get permission from the Kenya National Ethical Review Committee.

contact on information about the study or my rights as a volunteer in this research study: If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact either: **Mr Collins Otieno Odhiambo, International Centre for Insect Physiology and Ecology, P.O Box 30772-00100, NAIROBI Tel: 0725984059 or Dr. Rosemary Sang, Centre for Virus Research, KEMRI PO Box 54628, NAIROBI Tel. 0722 759492.**

Who should I contact if I have questions on my rights as a volunteer in this research study: If you have any question on your rights as a volunteer, you or your parent should contact**: The Secretary, National Ethical Review Committee, Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya Tel. 254-20-2722541.**

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

Subject Name _____

Subject's or Parent/Guardian's Signature:_____ _Date:_____

Physical Address/Home Description: _____

Family Name/Homestead Name: _____

Witness's Name: _____

Witness's Signature: _____

Date: _____

Study Number: _____

Thumbprint of of Volunteer or Volunteer's Parent/Guardian if Unable to Sign

Person Administering Consent:

Name: _____ Signature_____

Interviewer's Name:

Stick Barcode Label here

Appendix 3B

Assent Form for Individuals above 5 through 17 Years of Age

NOTE: This form should be signed by children above 5 years of age through 17 years of age who should give their assent when a parent or guardian has given permission to enroll.

What is the study called: Circulation Reassortment and Transmission of Bunyamwera and Ngari viruses in northern Kenya

Who is running the study: The study is being run by Mr Collins Otieno Odhiambo and Dr Rosemary Sang, and a team of doctors and scientists from Kenya Medical Research Institute (KEMRI), Ministry of Public Health and Sanitation (MPH&S), International Centre of Insect Physiology and Ecology (ICIPE), International Livestock Research Institute (ILRI) and Ministry of Livestock/Department of Veterinary Services (MOL/DVS).

Introduction: We are interested in finding out what germs cause fever and/or malaria like illness. We want to draw a small amount of blood and test it in the lab to see if we can find the germ that is causing your illness. We are asking you to be in this research study because you may have signs and symptoms that may have come due to this germ.

Purpose: The purpose of this study is to find out what germs cause fever and/or malaria like illness.

Procedures: You will be asked some questions about where you live and your illness. You may participate in this study by giving only blood. About a tablespoon of blood will be taken from a vein in your arm. The blood will be put into small tubes so we that can test for germs that may be causing your illness. These samples are only for this study.

Long Term Storage of Specimens

After the tests have been performed, we will store samples that are left in a confidential manner for future testing. After this study is over, we may do new tests for germs that might have caused your illness. We will not report the results of these tests to your doctor or to you. We will not do human genetic testing or test for evidence of HIV (human immunodeficiency virus) infection of the samples that you provide. If at a later date you change your mind, you may ask to remove these samples from long term storage and destroy them. If you choose to do so, please contact: **Dr. Rosemary Sang**, **Centre for Virus Research,** K**enya Medical Research Institute, PO Box 54628, NAIROBI**, **Tel. 0722 759492 or Dr. Charles Nzioka**, **Division of Disease surveillance and response (DDSR)**, **Ministry of Public Health and Sanitation P. O. Box 30016-00100, NAIROBI Tel: 0721234904.**

Risks and Discomforts

Your doctor will take blood from your arm using a needle. Drawing the blood may hurt a little. It may also cause some bruising, bleeding, and slight soreness at the puncture site. There is a small chance you could get germs in the spot where the blood was taken and become infected. If the area around the spot gets red and sore, you would need to go to the clinic.

Benefits: You will not directly benefit from the study. There is a benefit to society in general, through finding the cause of germs that may be causing your illness.

Confidentiality: We will keep the data collection, informed consent/permission and assent forms in a locked filing cabinet. Only study staff will be allowed to look at them. We will keep the forms private as much as legally possible. To protect your privacy, we will keep records and samples under code numbers rather than by name. However, we will maintain a link between code numbers and the forms that we keep in locked files. Your name or other facts that might point to you will not appear when we present this study or publish its results.

Costs/Compensation: Your parent/guardian will be responsible for the routine medical costs from your visit. These are costs that you would have if you were not in the research study. You will have

no charge for collection of blood samples. You will not pay for the research tests that we will do on these samples.

Right to Refuse or Withdraw: You do not have to be in this study. We will give you the usual care for your condition whether or not you are in the study, or if you leave the study later. You may leave the study at any time.

Persons to Contact: By signing this consent form and agreeing to be in this study, you are not giving up any of your rights. If you believe that you have been harmed, please contact: **Dr. Rosemary Sang**, **Centre for Virus Research,** K**enya Medical Research Institute, PO Box 54628, NAIROBI**, **Tel. 0722 759492 or Dr. Charles Nzioka**, **Division of Disease surveillance and response (DDSR)**, **Ministry of Public Health and Sanitation P. O. Box 30016-00100, NAIROBI Tel: 0721234904.**

If you have any question on your rights as a volunteer, you or your parent should contact.

The Secretary, National Ethical Review Committee

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE ASK STUDY TEAM BEFORE SIGNING.

Subjects Name**:** _____

Subject's or Guardian's Signature: _____

Permanent Address: _____Date:_____

Witness's Name: _____

Witness's Signature: _____

Study Number: _____Date_____

Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to Sign

Person Administering Consent:

Name:_____ Signature:_____

Date:

Stick Barcode Label here

Appendix 3C

Parent/Guardian Permission Form for Individuals above 5 through 17 Years of Age

NOTE: This form should be signed by patients 18 years of age or older who are able to give their legal consent. Minor children ages 5 to17 should sign this form to give their assent when a parent or guardian has given permission to enroll.

What is the study called: Circulation Reassortment and Transmission of Bunyamwera and Ngari viruses in northern Kenya.

Who is running the study: The study is being run by Mr Collins Otieno Odhiambo and Dr Rosemary Sang and a team of doctors and scientists from Kenya Medical Research Institute (KEMRI), Ministry of Public Health and Sanitation (MPH&S), International Centre of Insect Physiology and Ecology (ICIPE), International Livestock Research Institute (ILRI) and Ministry of Livestock/Department of Veterinary Services (MOL/DVS).

Introduction: We are interested in finding out what germs cause fever and/or malaria like illness. We want to draw a small amount of blood and test it in the lab to see if we can find the germ that is causing your illness. We are asking you/your child to be in this research study because you/your child may have illness that may have come due to this germ.

Purpose: The purpose of this study is to find out what germs cause fever and/or malaria like illness.

Procedures: You/your child will be asked some questions about where you live, your illness and any medications you may have taken recently. You/your child may participate in this study by giving only blood. About a tablespoon of blood will be taken from a vein in your arm. The blood will be put into small tubes so we can test for germs that may be causing your illness. These samples are only for this study.

Long Term Storage of Specimens

After the tests have been performed, we will store samples that are left in a confidential manner for future testing. After this study is over, we may do new tests for germs that might have caused your/your child's rash as these tests become available. We will not report the results of these tests to your doctor or to you. We will not do human genetic testing or test for evidence of HIV (human immunodeficiency virus) infection of the samples that you or your child provide. If at a later date you change your mind, you may ask to remove these samples from long term storage and destroy them. If you choose to do so, please contact: **Dr. Rosemary Sang**, **Centre for Virus Research,** K**enya Medical Research Institute, PO Box 54628, NAIROBI**, **Tel. 0722759492 or Dr. Charles Nzioka**, **Division of Disease surveillance and response (DDSR)**, **Ministry of Public Health and Sanitation P. O. Box 30016-00100, NAIROBI Tel: 0721234904.**

Risks and Discomforts

Your doctor will take blood from your/your child's arm using a needle. Drawing the blood may hurt a little. It may also cause some bruising, bleeding, and slight soreness at the puncture site. There is a small chance you/your child could get germs in the spot where the blood was taken and become infected. If the area around the spot gets red and sore, you/your child would need to go to the clinic.

Benefits: You/your child will not directly benefit from the study. There is a benefit to society in general, through finding the cause of germs that may be causing your illness.

Confidentiality: we will keep the data collection, informed consent/permission and assent forms in a locked file. Only study staff will be allowed to look at them. We will keep the forms private as much as legally possible. To protect your/your child's privacy, we will keep records and samples under numbers rather than by name. However, we will maintain a link between code numbers and the forms that we keep in locked files. Your/your child's name or other facts that might point to you/your child will not appear when we present this study or publish its results.

Costs/Compensation: You will be responsible for the routine medical costs from your/your child's visit. These are costs that you would have if you/your child were not in the research study. You will have no charge for collection of blood samples. You will not pay for the research tests that we will do on these samples.

Right to Refuse or Withdraw: You/your child does not have to be in this study. We will give you/your child the usual care for your/your child's condition whether or not you/your child are in the study, or if you/your child leave the study later. To leave the study, please contact your doctor. You/your child may leave the study at any time.

Persons to Contact: By signing this consent form and agreeing to be in this study, you are not giving up any of your rights. If you believe that you/your child have been harmed, please contact: **Dr. Rosemary Sang**, **Centre for Virus Research,** K**enya Medical Research Institute, PO Box 54628, NAIROBI**, **Tel. 0722 759492 or Dr. Charles Nzioka**, **Division of Disease surveillance and response (DDSR)**, **Ministry of Public Health and Sanitation P. O. Box 30016-00100, NAIROBI Tel: 0721234904.**

If you have any question on your rights as a volunteer, you or your parent should contact.

The Secretary, National Ethical Review Committee

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE ASK STUDY TEAM BEFORE SIGNING.

Subjects Name**:** _____

Subject's or Guardian's Signature: _____

Permanent Address:_____

Date:_____

Witness's Name: _____

Witness's Signature: _____

Study Number: _____Date:_____

Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to sign

Person Administering Consent:

Name: _____Signature: _____

Stick Barcode Label here

Appendix 4

QUESTIONNAIRE ON HUMAN EXPOSURE TO CRIMEAN-CONGO HEMORRHAGIC FEVER

Patient details:

Date of collection: _____(dd/month/yr)

Sex: 1. Male 2. Female Age:_____ years

Where is you (your child's) current residence?

Village _____District_____

Province: _____

How long have you (your child) been living in this district?

_____years

_____months

During the past five days, where have you (your child) been mostly (check one)?

☐ Village of residence

☐ In the country, but not in residence, Where? _____

☐ Out of the country, Where? _____

How many times have you (your child) traveled outside of your district in the last two months:

_____

How long ago: <2 weeks 2-4 weeks 1-2 months

Where: _____

How long ago: <2 weeks 2-4 weeks 1-2 months

Where: _____

How long ago: <2 weeks 2-4 weeks 1-2 months

Where: _____

Have you (your child) ever received a yellow fever vaccine?

1. Yes 2. No 3. Unknown ☐

Date of vaccination (if known) _(day/month/year)

If adult: What is your occupation: _____

If child: Where do you go to school: _____

Do you have contact with any of the following species of animals?

1. Bats

2. Geese

3. Ducks

4. Chickens

5. Other- Specify _____

Indicate which birds using the codes above (1-5): _____

6. Goats

7. Cows

8. Donkeys

9. Camels

10. Monkeys

Indicate which animals using the codes above (6-10):_____

Tick Bites: Yes/No _____

Others bites

For each species checked above:

List the species using the given codes_____

2) Describe the contact, e.g., trapping, farming, slaughter, food preparation, veterinary work, casual contact (e.g., a neighbor keeps chickens, there is a slaughterhouse nearby), eating raw fowl products or drinking blood_

3. Were the animals showing signs of illness? ☐ Yes ☐ No

If ***Yes*** above, Specify the signs.

YOUR CURRENT ILLNESS:

Why did you (your child) come to the hospital:_____

Did you (your child) have any of the following:

Signs/Symptoms	Yes	No	Uncertain
Fever/Chills			
Rash			
Abdominal pain			
Muscle pain			
Vomiting			
Headache			

Do you (your child) have any symptoms we did not mention: If bleeding, where:

1. N/A ☐ 2. Gums ☐ 3. Nose ☐ 4. Injection sites ☐

5. Other, Specify_____

How many days have you (your child) been sick: _____

Date of Onset:_____

How many days of school or work have you (your child) missed:_____

Does anyone you know have a similar illness? Yes ☐ No ☐

Who: _____When did they become ill _____

Who:_____When did they become ill _____

Who:_____When did they become ill_____

What was your temperature in the clinic:_____ ^{o}C

DIAGNOSES MADE BY MINISTRY OF HEALTH PROVIDER:

1._____

2._____

TREATMENT PRESCRIBED BY MINISTRY OF HEALTH PROVIDER:

1._____
2._____
3._____
4.

Appendix 5: Permission to uses figures

From: Richard Elliott <Richard.Elliott@glasgow.ac.uk>
Sent: 13 January 2015 17:16
To: Odhiambo, Collins Otieno
Subject: RE: Permission to use Bunyaviridae genomic illustrations

Dear Collins
You are free to use any published diagrams in a thesis provided you acknowledge their source.
Best regards,
Richard Elliott

Richard M. Elliott, FRSE
Bill Jarrett Professor of Infectious Diseases and
Wellcome Trust Senior Investigator

MRC - University of Glasgow Centre for Virus Research
Henry Wellcome Building
464 Bearsden Road
Glasgow G61 1QH
Scotland (UK)

Tel: +44 (0)141-330 2876
Fax: +44 (0)141-330 4874

_____

From: Odhiambo, Collins Otieno [codhiambo@icipe.org]
Sent: Tuesday, January 13, 2015 12:55 PM
To: Richard Elliott
Subject: Permission to use Bunyaviridae genomic illustrations
Dear Prof Elliot,
I am a PHD student working on the circulation, reassortment and transmission of Orthobunyaviruses. Thanks for your great work in the arboviral field. You have contributed greatly to our understanding of viruses of the Bunyaviridae family. I am putting up together a thesis on the same and I would like to request your permission to use figures illustrating the structure of the genomic component of these viruses.
Thank you.
Faithfully,
Collins Odhiambo

From: Patrick Masson via RT <viralzone@isb-sib.ch>
Sent: 22 January 2015 16:44
To: Odhiambo, Collins Otieno
Subject: [help #98285] [Viralzone] Request to use illustration

Dear Collins Odhiambo,

I hereby grant you permission to use the Bunyvirus viron picture you requested for your thesis.
Please cite the source "ViralZone, SIB Swiss Institute of Bioinformatics".

Best regards and good luck for your thesis, Patrick Masson.

From: Brett Ellis <brettellis@mac.com>
Sent: 13 January 2015 16:37
To: Odhiambo, Collins Otieno
Subject: Re: Permission to use figure in your publication

Hi Collins, you have my permission to use the figure. I may also have a powerpoint with the original figures from the paper if you need. Just let me know. Regards, Brett.

On Jan 13, 2015, at 8:48 AM, Odhiambo, Collins Otieno <codhiambo@icipe.org> wrote:

Dear Ellis,
I am a PHD student working on the circulation, reassortment and transmission of Orthobunyaviruses. I am putting up together a thesis on the same and I would like to request your permission to use figure one on your article titled, The ecological dimensions of vector-borne disease research and control.
Thank you.
Faithfully,
Collins Odhiambo