GENETIC DIVERSITY AND ANTIGENIC VARIATION OF RABIES VIRUSES FROM DIFFERENT HOST SPECIES IN EASTERN AND WESTERN PARTS OF KENYA

EVALYNE NYAMBURA WAMBUGU

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE IN MASTER OF SCIENCE IN APPLIED MICROBIOLOGY OF THE UNIVERSITY OF EMBU

AUGUST, 2024

DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

Signature…… Date…….

Evalyne Nyambura Wambugu
Department of Biological Sciences
B526/1501/2021

This thesis has been submitted for examination with my (our) approval as the University supervisor(s)

Signature…… Date…….

Dr. Sarah N. Kituyi
Department of Biological Sciences
University of Embu

Signature…… Date…….

Dr. John N. Waitumbi
Basic Science Laboratory
Walter Reed Army Institute of Research-Africa

DEDICATION

I am deeply grateful to Dr. Sarah Kituyi and Dr. John Waitumbi for their exceptional mentorship and support throughout my MSc study. My sincere thanks go to Dr. Thumbi, Dr. Beth Mutai, and the dedicated staff of the Basic Science Lab for their invaluable contributions. I extend special appreciation to Mr. Clement Masakhwe and Mr. Kimita Gathii for their expert advice. I acknowledge the Uniformed Services University of the Health Sciences (USU) for funding and the University of Embu for the scholarship that made this journey possible. I am profoundly thankful to my family for their unwavering love and support, and to the Lord God Almighty for His guidance and provision. I am honored to dedicate this thesis to them.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to the individuals who have played pivotal roles in my journey, culminating in the completion of this thesis. First and foremost, I am grateful to my beloved grandparents “cucu” and “guka.” Your unwavering and unconditional love has been an ever-present force, steadfastly walking by my side throughout the mishaps in life. It is your love that has nurtured the roots from which my aspirations have blossomed, and for that, I am forever grateful. I am deeply grateful to my brother Patrick, whose presence has been a source of immense comfort. To my mother Njeri, I offer profound gratitude for the gift of life. Collectively, my family has shown me what true love is and I will forever be grateful for their presence in my life. I am indebted and grateful to my supervisor and mentor, Dr. John Waitumbi. His wise counsel, day-to-day guidance and belief in my abilities have shaped not only my research but personal growth. His invaluable insights, expertise, feedback, and mentorship has allowed me to surpass my own limitations and evolve into a more resilient individual. Lastly, am extremely grateful to Dr. Sarah Kituyi, her unwavering presence and steadfast guidance became a source of comfort when problems seemed insurmountable. I am forever indebted to your instrumental role in the successful completion of this thesis. This thesis is a testament to the collective efforts, resilience and unwavering beliefs, and stands as a tribute to the immeasurable contributions of all these remarkable individuals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ARAV	Aravan lyssavirus
ABLV	Australian bat lyssavirus
BIC	Bayesian Information Criterion
BBLV	Bokeloh lyssavirus
BLAST	Basic Local Alignment Search Tool
BWA	Burrow-wheeler aligner
bp	base pairs
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CNS	Central Nervous System
CVIL	Central Vertinary Investigational laboratories
CVS	Challenge Virus Standard strain
cDNA	Complementary DNA
Ct	Cycle threshold
dFAT	Direct-fluorescent antibody test
DNA	Deoxyribonucleic acid
DUVV	Duvenhage lyssavirus
ds	Double stranded
EBLV 1	European bat lyssavirus type 1
EBLV 2	European bat lyssavirus type 2
GARD	Genetic Algorithm for Recombination Detection
GBLV	Gannoruwa bat lyssavirus
G	Glycoprotein gene
HnRT-PCR	Heminested reverse transcriptase polymerase chain reaction

HS	High sensitivity
HTI	Hybridization buffer
ID	Identification number
IKOV	Ikoma lyssavirus
ICTV	International Committee of Taxonomy of Viruses
IRKV	Irkut lyssavirus
KHUV	Khujand lyssavirus
L	Large-structural protein
LBV	Lagos bat lyssavirus
LLEBV	Lleida bat lyssavirus
M	Matrix protein
MAK	Makueni
ML	Maximum-likelihood
MLCA	Maximum Likelihood Clade Assignment
MOKV	Mokola lyssavirus
NAOH	Sodium hydroxide
ng/μL	nanogram/microliter
nM	Nanomolar
NTD	Neglected Tropical Diseases
N	Nucleoprotein gene
OIE	Office International Des Epizooties
P	phosphoprotein
PV	Pasteur vaccine strain
PCR	Polymerase Chain Reaction

PEP	post-exposure prophylaxis
PM	Pit Moore Vaccine strain
pM	picomolar
PV	Pasteur Vaccine strain
qRT-PCR	quantitative Real-Time Reverse-transcription Polymerase Chain Reaction
RABV	Rabies virus
RNA	Ribonucleic acid
RDP4	Recombination Detection Program version 4
RNP	Ribonucleoprotein complex
RT-PCR	reverse-transcription PCR
SIA	Siaya
SISPA	Sequence Independent Single Primer Amplification
SHIBV	Shimoni bat lyssavirus
SNP	Single Nucleotide Polymorphism
NaOH	sodium hydroxide
TWBLV	Taiwan bat lyssavirus
µL	microliter
UFboot	ultrafast bootstraps
UV	Ultraviolet
vRNA	Viral Ribonucleic acid
V3	Version 3
VCF	Variant Calling File
VNA	Virus Neutralizing Antibody

WCBV	West Caucasian bat lyssavirus
WGS	Whole Genome Sequencing
WHO	World Health Organization
ZDU	Zoonotic Disease Unit

ABSTRACT

Rabies, a viral illness that causes deadly encephalitis, claims over 59,000 lives globally each year, with approximately 2,000 deaths happening in Kenya despite effective countermeasures for both humans and dogs. This study sought to improve the understanding of the genetic differences of the rabies virus (RABV) collected from the brain stem tissues of animals with suspected rabies in two geographic rabies hotspots: Makueni county, in the eastern region, and Siaya county, in western Kenya. Furthermore, the study investigated discrepancies among the antigenic sites of the RABV vaccines currently approved for use in Kenya and the study samples. Between July 2021 and September 2022, 164 brain stem tissues were collected from animals suspected of rabies after testing positive for RABV antigens using rapid diagnostic kits. Subsequently, in the laboratory, the samples were re-screened for rabies ribonucleic acid (RNA) by quantitative Real-Time Reverse-transcription Polymerase Chain Reaction (qRT-PCR) that targets the RABV Large-structural protein gene (L gene). Negative samples by the L gene qRT-PCR were analyzed by conventional PCR that targets the RABV Nucleoprotein gene (N gene). Phylogenetic analysis was carried out using whole genome sequences (WGS), as well as single genes (nucleoprotein (N) and glycoprotein (G). The amino acid changes in the antigenic regions of individual proteins (N and G) in the research sequences were compared to three RABV vaccine sequences: Pitman-Moore L503 (PM), Challenge Virus Standard (CVS), and Pasteur vaccine (PV). The L gene qRT-PCR revealed positive results for 156 of the 164 brain stem tissues. Eight samples failed to amplify the L-gene but amplified by N gene. On sequencing, 141 samples produced sequences suitable for analysis. Phylogenetic analysis using WGS and single genes confirmed that the RABV strains belonged to the Cosmopolitan clade that branched into two distinct phylogeographic subclades: sequences from eastern Kenya predominantly grouped within the Africa 1b subclade with only three sequences in the Africa 1a subclade. Conversely, the sequences from western Kenya formed clusters within the Africa 1a subclade while three were in the 1b subclade. In keeping with this phylogeographic clustering, the western Kenya Africa 1a subclade's closest ancestor was found in Sudan, while the eastern Kenya Africa 1b subclade had its origin in Tanzania. Within the western Kenya 1a subclade, 8 lineages were observed and 5 within the eastern Kenya 1b subclade. The amino acid homologies of the N gene between the study sequences and the vaccines for RABV were found to be approximately 97.6% for the PV vaccine strains, 98.5% for PM, and 97.8% for CVS. Similarly, the homologies with the G gene were at least 92.95% for PV, 92.19% for PM, and 93.3% for CVS. Our findings corroborate the geographic segregation of RABV between the eastern and western regions of Kenya. The data indicates that there is restricted viral movement, likely facilitated by the transportation of domestic dogs by humans. Good vaccine efficacy is predicted by the low amino acid variations in the antigenic sites of RABV vaccines and the study samples, suggesting that a lack of programmatic vaccination coverage is a major contributor to the endemicity of RABV in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Rabies, an extremely severe and fatal disease affects both humans and various mammalian species (Yousaf *et al*., 2012). The rabies-causing virus falls under the genus *Lyssavirus* within the *Rhabdoviridae* family. These lyssaviruses induce inflammation in the brain, leading to the characteristic neurological symptoms observed in affected individuals (Singh *et al*., 2017). Rabies virus (RABV) is one of the lyssaviruses, primarily responsible for causing rabies in humans (Kuzmin *et al.*, 2010). Rabies remains a significantly disregarded disease despite its ancient origins, particularly in less economically advanced countries. It holds the 12th position on the World Health Organization's (WHO) list of major lethal diseases, emphasizing the significant impact it has on global health (Bihon *et al*., 2020). In recognition of its importance, rabies was included in the Office International Des Epizooties (OIE) list of multiple species diseases in 2019, highlighting its relevance to both human and animal health (Organization, 2015; Smith *et al*., 2019). The global prevalence of rabies is a persistent risk to humans and animals alike, resulting in substantial long-term societal and financial burdens in many countries.

Rabies has a long history, of more than 4000 years, with its first recognition dating back to around 2300 BC in Egypt (Singh *et al*., 2017). Aristotle, in his book "History of Animals," described rabies as a fatal disease that affected not only dogs but also other animals bitten by rabid dogs. In the sixteenth century, an Italian physician, Girolamo Fracastoro, proposed that any breaks in the human skin resulting from a bite by a rabid animal were significant events for the development of rabies in humans (Ahmad *et al*., 2019). A significant milestone in the history of rabies came in the 1880s when the first successful rabies vaccine was developed by Louis Pasteur. The vaccine demonstrated its effectiveness in preventing rabies in Joseph Meister, a shepherd boy who had sustained a bite from a rabid wolf (Brightman, 2012). The spread of the virus followed patterns of human colonization, reaching Europe and subsequently Africa (Talbi *et al*., 2009). In 1768, rabies was first described in the New World (the American continent) in Boston, from where it spread to other states in the USA (Ramsey, 2017). The history of rabies demonstrates the progressive

understanding and discovery of the disease as well as the development of preventive methods to resist its destructive effects.

The pathogenic virus is largely concentrated in infected animals' saliva and is most usually spread by bites or scratches, while transmission through non-bite exposures has been recorded, albeit extremely rarely (Brunker & Mollentze, 2018). Non-bite exposures may include organ transplants or inhalation of airborne droplets containing the virus (Singh *et al*., 2017). To facilitate transmission, RABV has the ability to alter the behavior of the infected animal. Infected animals may lose fear, exhibit exceptional aggression, and attack other animals or humans without provocation (Schnell *et al.*, 2010). Once it infiltrates the body via bites or scratches, the virus migrates through peripheral nerves to the spinal cord and subsequently to the brain, where it replicates. During this migration phase, the virus is not readily detectable. Once the virus infiltrates the brain, it triggers encephalitis, resulting in inflammation and related neurological consequences, which coincides with the beginning of early signs of the illness (Singh *et al*., 2017). Subsequently, the virus travels along other nerves from the brain to the salivary glands, from where it is shed into the saliva. Bites from rabid dogs are the main route of infection, accounting for nearly all instances of rabies spread to humans, comprising up to 99% of such cases (Pantha *et al*., 2020). Dogs, bats, foxes, raccoons, and skunks are reservoir hosts for RABV, while other affected species, including humans, are considered dead-end hosts (Müller & Freuling, 2020).

In addition to RABV, there are other viruses that belong to the genus *Lyssavirus* (Shipley *et al*., 2021). They share serological and antigenic similarities with RABV and can cause neurological diseases that are indistinguishable from rabies (OIE, 2009). The Rabies-related viruses include: Aravan lyssavirus (ARAV), Australian bat lyssavirus (ABLV), Bokeloh lyssavirus (BBLV), Duvenhage lyssavirus (DUVV), European bat lyssavirus type 1 (EBLV 1) and type 2 (EBLV 2), Irkut lyssavirus (IRKV), Taiwan bat lyssavirus (TWBLV), Khujand lyssavirus (KHUV), Gannoruwa bat lyssavirus (GBLV), Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV), Shimoni bat lyssavirus (SHIBV), West Caucasian bat lyssavirus (WCBV), Ikoma lyssavirus (IKOV), and Lleida bat lyssavirus (LLEBV) (Shipley *et al*., 2021). These lyssaviruses, along with RABV have been classified into two

phylogroups based on their genetic relatedness (Marston *et al*., 2012 ; Ceballos *et al*., 2013). Phylogroup I includes RABV, ARAV, ABLV, BBLV, DUVV, EBLV 1, EBLV 2, IRKV, TWBLV, KHUV, and GBLV, while phylogroup II includes LBV, MOKV, and SHIBV. Some lyssaviruses like WCBV, IKOV, and LLEBV do not fit into either phylogroup as a result of genetic divergence and lack of cross-neutralization (Rupprecht *et al*., 2018).

The RABV genome codes for five different proteins: the glycoprotein (G), phosphoprotein (P), nucleoprotein (N), Large-structural protein (L) and matrix protein (M) (Liu *et al.,* 2014). The RABVs greatly differ in their antigenic makeup of their G and N genes. The antigenic disparities between the RABVs variants and the currently available vaccine strains have the possibility to alter the efficacy of vaccines (Zhang *et al*., 2013). Rabies vaccines currently in use have demonstrated a strong track record in effectively safeguarding against tested RABVs variants. However, there remains a potential risk if the RABVs undergo evolutionary changes that lead to notable alterations in antigenicity (Wang *et al*., 2019). Vigilantly tracking antigenic variants across diverse animal hosts and geographic regions is crucial for assessing the effectiveness of existing vaccine strains against emerging lyssavirus strains.

Rabies is widespread, affecting every continent around the world, with the exception of the Antarctica and certain insular islands. Each year, rabies tragically claims the lives of approximately 59,000 humans worldwide, with a startling 95% of these cases typically found in Asia and Africa. Canine rabies, which has been endemic in household dogs, is particularly prevalent in these regions (Hampson *et al*., 2015). Vulnerability to rabies is often associated with factors such as poverty and a lack of awareness about the disease (Davis *et al*., 2015). In rural areas, where there are high populations of stray dogs and limited consistency in mass dog vaccinations, approximately 80% of human rabies cases transpire. Alarmingly, individuals aged 15 years and below account for approximately 40% of these cases (Sararat *et al.*, 2022). Although some vaccines are efficient and beneficial in protecting humans against rabies, they are not easily accessible or available to those in need (Kuzmin *et al*., 2010). This lack of access to vaccines further exacerbates the burden of rabies in

affected regions, underscoring the need for improved availability and distribution of life-saving vaccines to vulnerable populations.

Rabies has a 100% fatality rate once clinical symptoms manifest (Singh *et al*., 2017). However, diagnosing rabies can be challenging as the disease presents with symptoms that resemble other encephalitic illnesses like cerebral malaria in humans and foot-and-mouth disease in animals. This similarity often leads to misdiagnosis (Dodet *et al*., 2015). The direct-fluorescent antibody test (dFAT) is widely regarded as a standard rabies diagnostic procedure. However, its precision is limited to fresh samples, making it difficult to work with decomposing samples or archival specimens. To address this issue, molecular assays, such as conventional gel-based reverse-transcription PCR (RT-PCR) assays with predetermined methods, were evaluated as confirmatory tests to validate dFAT results. These molecular methods detect the ribonucleic acid (RNA) of the RABV and are particularly useful in cases where the sample quality is compromised (Ceballos *et al*., 2013). The current study used molecular approaches to evaluate the diversity of RABV across several species in Kenya.

In Kenya, rabies poses significant challenges both in terms of public health and veterinary concerns, ranking among the five most prioritized zoonotic diseases (Ngugi *et al.*, 2018). Historical records show that a dog on the outskirts of Nairobi was diagnosed with the very first confirmed case of rabies in 1912, while the initial documented incidence of rabies in humans happened in 1928, afflicting a female native of the western Kenyan region near Lake Victoria (Bitek *et al*., 2018). Nevertheless, the exact scale of rabies burden in the country has been difficult to ascertain due to a weak surveillance system, which has impeded accurate data collection and analysis. Additionally, there is a dearth of available information concerning the genetic characterization of RABVs in Kenya. To bridge these gaps, the principal objective of the study was to contribute genetic data by investigating the genetic diversity and relationships of Kenyan RABV vis-a-vis other RABV sequences available in the public databases and to determine the antigenic variations of the G and N genes of the RABVs compared to the currently used vaccines. The genetic data and insights gained from the epidemiology of RABVs in Kenya will be critical in shaping the ongoing rabies vaccination and eradication programs. By

understanding the evolutionary relationships and antigenic variations of RABVs, stakeholders and policymakers can understand whether vaccine failure is a possibility or not and therefore implement appropriate actions to control and prevent the dissemination of rabies in Kenya. The study's findings will contribute to enhancing disease surveillance, improving vaccination efforts, and ultimately working towards the overarching objective of eliminating rabies as a public health threat in Kenya.

1.2 Statement of the problem

Since the initial reported case in 1912, rabies has maintained an endemic presence in Kenya, and it is still a substantial public health hazard with a projected 2,000 deaths a year for humans, despite being a vaccine-preventable disease with Makueni and Siaya counties being the most affected counties (Republic of Kenya, 2014; Bitek *et al*., 2018). A recent assessment conducted by the Kenyan government on zoonotic diseases identified rabies among the top five diseases of animals that are of concern to humans (Ngugi *et al.*, 2018). However, the full impact of the disease is likely underestimated, and its elimination has been hampered by a recurring cycle of neglect and inadequate information about the disease (Tiembré *et al.*, 2018). The situation is further aggravated by a lack of sufficient diagnostic centers, poor disease surveillance, and the complex nature of rabies reservoirs, which make control and prevention efforts challenging (Fisher *et al*., 2018). One of the inherent challenges in the control of rabies is the inadequate submission of suspect samples from various host species, other than dogs, to veterinary laboratories for testing. This lack of information on the disease in different host species, especially wildlife is a challenge to effective control efforts. This study, sought to address some of these challenges by detecting rabies in various host species from two geographically distinct hotspots and subsequently performing genetic diversity analysis coupled with analysis of antigenic variation at vaccine target sites.

1.3 Justification of the study

In this study, the evolutionary relationship of RABV was investigated by analyzing samples obtained from suspected rabid animals in different regions of western and eastern parts of Kenya, where there are notable high disease occurrences. The research also aimed to address the lack of genetic information on Kenyan RABVs, which is crucial for understanding the epidemiology of the disease. The outcomes

derived from this study provide valuable knowledge for informing policies and strategies aimed at effectively eliminating the disease. Additionally, the study examined the antigenic variations of the genes encoding the G and N proteins which are important targets for rabies vaccines and monoclonal antibody therapies. This analysis has provided insights into the suitability and effectiveness of the three rabies vaccine strains currently used in Kenya.

1.4 Research questions

1. What RABV strains are responsible for rabies in eastern and western Kenya?
2. What are the genetic differences in rabies viruses obtained from eastern and western regions of Kenya, and how do they compare to those available in global databases?
3. What antigenic variations exist in the G and N genes of the RABV strains that could reduce the effectiveness of the rabies vaccine or monoclonal antibodies used for post-exposure therapy?

1.5 Objectives

1.5.1 General objective

To determine the genetic diversity and antigenic variation of RABV from different host species in eastern and western parts of Kenya.

1.5.2 Specific objectives

1. To determine the strains of RABVs responsible for cases of rabies in different hosts in eastern and western Kenya.
2. To determine evolutionary relationships of the RABV strains in reference to global strains available in the public genetic sequence databases.
3. To determine the extent of antigenic variations in the gene encoding the G and N proteins.

CHAPTER TWO

LITERATURE REVIEW

2.1 The rabies virus genome

RABVs are negative-sense, non-segmented, single-stranded RNA viruses. The genome is distinctive in its bullet-shaped morphology, measuring approximately 180 nm in length and 75 nm in cross-sectional diameter. Two key components of the genome include the encircling cell envelope and the ribonucleoprotein complex (RNP) (Horie *et al.*, 2021). The RNP, located at the central core, comprises the nucleoprotein (N), the large structural protein (L), and the phosphoprotein (P). The N protein encapsulates the RNA genome, while the P and L proteins are essential for RNA synthesis and replication (Wunner & Conzelmann, 2020). Surrounding the RNP core is a lipid-bilayer protein envelope, with the matrix protein (M) positioned between the RNP core and the envelope. Glycoprotein (G) spikes are evenly distributed and anchored on the envelope surface, protruding outward (Wunner & Conzelmann, 2020).

The RABV genome spans approximately 12,000 nucleotides, with the first 58 nucleotides at the 3' end forming the noncoding leader sequence. Encoded by this genome are five viral proteins in the order of 3'-N-P-M-G-L-5' (Zhang & Fu, 2012). The N protein, a highly conserved component of the RNP, is particularly noteworthy. Comprising 450 amino acids, it plays a crucial role in viral RNA replication and encapsulation. Variations in the N region are instrumental in genotyping, subtyping, and conducting phylogenetic analysis of RABVs (Ajorloo *et al*., 2018).

The G protein, a surface fusion protein spanning the envelope, is accountable for inducing protective virus-neutralizing antibodies. It encodes 524 amino acids and is a principal antigen in eliciting immune responses and providing immunity against RABV (Morimoto *et al.*, 1998). Conversely, the matrix protein, although small, is significant. It oligomerizes and binds to the exterior of the nucleocapsid, enhancing the structural stiffness of the virion. With 202 amino acids, it potentially serves as a link between the nucleocapsid and the G protein (Sinkie & Mekelle, 2017).

The L protein, the largest protein ranging from 2,127 to 2,142 amino acids, plays a pivotal role in RNA synthesis and capping processes throughout the virus life cycle.

Concurrently, the phosphoprotein, with 297 to 303 amino acids, interacts with the N protein, forming N-P complexes crucial for various functions. It assists in chaperoning newly synthesized N protein, preventing its polymerization, and facilitates the encapsulation of viral RNA by the N protein. Both proteins are integral to RABV replication, assembly, and pathogenicity (Asaye & Getachew, 2014).

2.2 The antigenic sites in the G and N genes of RABVs

The G and N genes are highly immunogenic and have antigenic sites that serve as targets for vaccines. Vaccination plays an essential role in controlling rabies by stimulating the production of neutralizing antibodies against the G and N antigens (Ajorloo *et al.*, 2018).The G antigenic sites are divided into regions I-IV (Figure 2.1) and a minor site (Evans *et al*., 2018). These sites have been mapped to specific amino acid positions: antigenic site I (263-264), antigenic site II a (198-200), antigenic site II b (34-42), antigenic site III (330-338), antigenic site IV (226-231), and antigenic site a (342-343; the minor site) (Sabeta, 2015). The occurrence of an arginine at position 333 within the ectodomain segment of the G substantially impacts the virus's pathogenicity, regardless of the infection site or viral dosage. Interestingly, substituting arginine at position 333 with glutamate renders RABV nonpathogenic. Reverse genetics technology has capitalized on this mutation to generate rabies vaccines (Yang *et al.*, 2013).

The N contains four antigenic sites (Figure 2.1): site 1 (358-367), site II (313-337), site III (374-383), and site IV (410-413) (Yang *et al.*, 1999; Goto *et al.*, 2000; Rahmahani *et al*., 2019). The genetic material of RABVs is highly prone to mutations due to replication errors, as the virus lacks a proofreading mechanism. These mutations give rise to new viral strains that are highly adaptable (Rahmahani & Rantam, 2018). Consequently, it is crucial to constantly monitor the antigenic correlation among different RABV strains and the current viral vaccine strains (Table 2.1) to assess the efficacy of the vaccines used, particularly in Kenya. This monitoring helps to understand the effectiveness of the current vaccines against the evolving strains of RABV (Wang *et al.*, 2019).

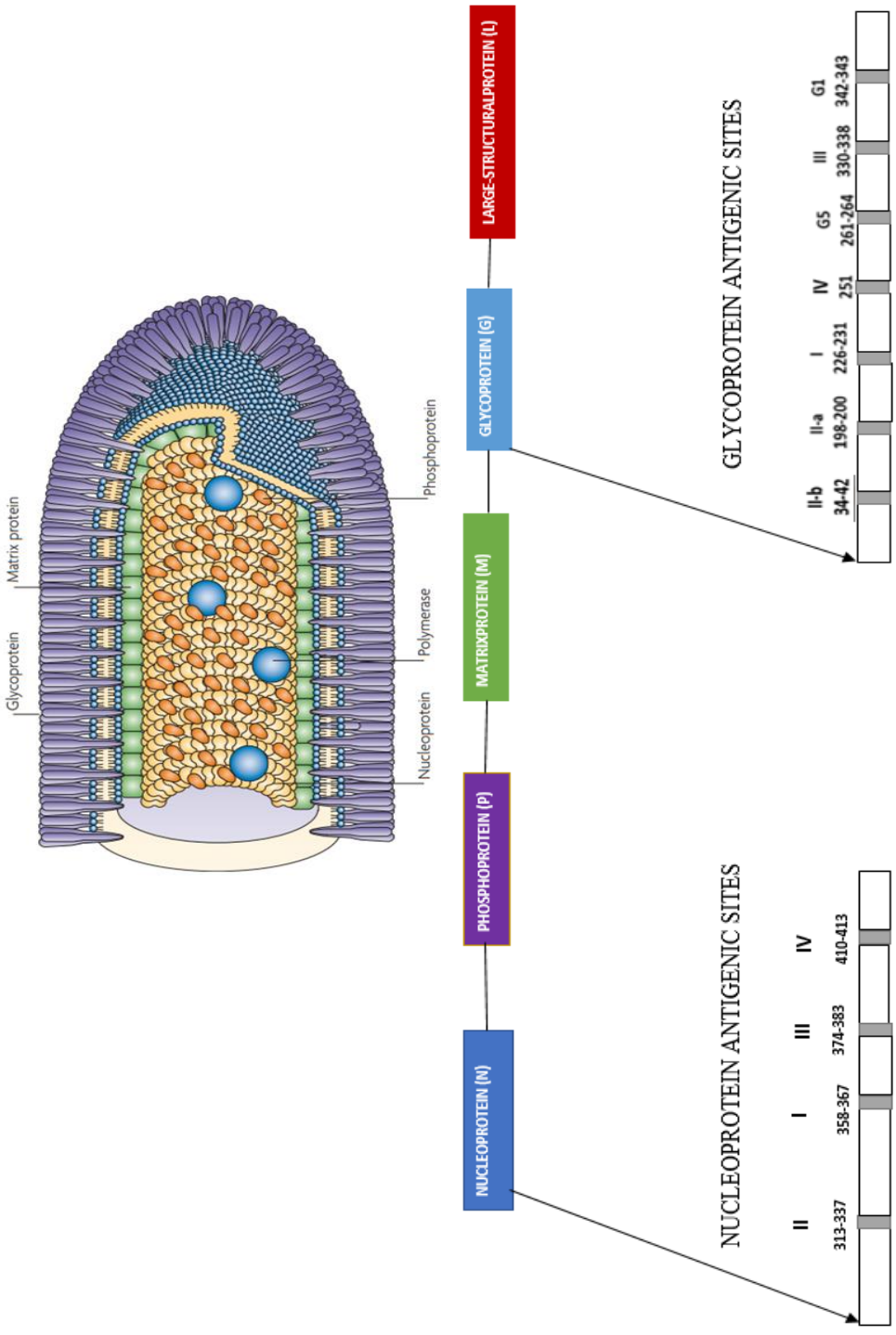


Figure 2.1: Diagrammatic representation of the RABV genome and the antigenic sites of the nucleoprotein and glycoprotein genes (Schnell *et al*., 2010).

Table 2.1: Common RABV vaccines used in Kenya and the viral vaccine strains used to manufacture the vaccines

	Brand name	Viral vaccine strain component
1	Verorab*	Pitmoore-1503 Strain
2	Indirab®*	Pitmoore-1503 Strain
3	Raksharab**	Challenge Virus Standard (CVS) Strain
4	Defensor® 3**	Pasteur Virus strain

* Human vaccine

** Veterinary vaccine

2.3 Molecular taxonomy

Lyssaviruses, including RABV, have been the subject of increased surveillance, leading to the discovery of several novel lyssaviruses in recent decades (Coertse *et al.*, 2021; Zandi *et al.*, 2021). Prior to these discoveries, RABV was considered the only lyssavirus until the identification of LBV in Nigeria in 1956, indicating the existence of rabies-related viruses (Coertse *et al.*, 2021). The term "rabies-related viruses" refers to lyssaviruses that are antigenically related to RABV (OIE, 2009; Kuzmin *et al.*, 2010). The Rabies-related viruses include: ARAV, ABLV, SHIBV, WCBV, IKOV, BBLV, DUVV, EBLV 1, EBLV 2, IRKV, TWBLV, KHUV, GBLV, LBV, MOKV, and LLEBV (Shipley *et al.*, 2021). Over time, the classification of lyssaviruses has undergone progressive change. At first, they were classified based on serological and antigenic cross-reactivity, but with advancements in molecular typing and sequencing, classification shifted to genotypes (Kuzmin *et al.*, 2010; Rupprecht *et al.*, 2018). However, the International Committee on Taxonomy of Viruses (ICTV) acknowledges viral species as the taxonomic entity for differentiation within the genus *Lyssavirus*, instead of using the term "genotype" (Kuzmin *et al.*, 2010).

Various criteria are considered to differentiate lyssaviruses, including genetic distance, nucleotide identity thresholds, variations in antigenic patterns, geographic spread, and host selection variability (Kuzmin *et al*., 2010). Demarcation is based on genetic distance with a quantitative threshold of 80-82% nucleotide identity for the entire N gene or concatenated coding regions of N+P+M+G+L genes (Kuzmin *et al*., 2010; Rupprecht *et al*., 2018). The classification also incorporates the segmentation of two major phylogroups based on genetic distances within the G protein ectodomain and serological cross-reactivity (Fisher *et al.*, 2018; Rupprecht *et al*., 2018). Members within the same phylogroup have a certain level of amino acid sequence similarity and the occurrence of cross-neutralization antibodies, while members in different phylogroups have lower sequence identity and lack cross-neutralization (Rupprecht *et al*., 2018).

Within RABV, there are two primary phylogenetic clusters: bat-related RABV and dog-related RABV. The bat-related group is mainly found in the New World (Americas), but the dog-related RABV has a global distribution (Zhou *et al.*, 2013; Caraballo *et al.*, 2021). Dog-related RABV is further divided into six distinct clades: Indian subcontinent, Asian, Africa 3, Arctic-related, Cosmopolitan, and Africa 2 (Bourhy *et al*., 2008; Pant *et al*., 2013). Three clades, Cosmopolitan clade (Africa 1a, Africa 1b, Africa 1c and Africa 4 subclades), Africa 2 clade, and Africa 3 clade, are specific to Africa (Talbi *et al.*, 2009) (Figure 2.2). Geographical obstacles influence the distribution of these clades, indicating limited gene flow (Brunker *et al*., 2015). Africa 1a subclade dominates eastern and northern Africa whereas Africa 1b subclade is predominant in central, southern and eastern Africa. Africa 1c subclade circulates in Madagascar. Africa 4 subclade was recently discovered in northern Africa (Sadeuh-Mba *et al*., 2017). Africa 2 clade predominantly circulates in west Africa. The Africa 3 clade is sustained by the mongoose carnivores of south Africa (Bourhy *et al.*, 2008; Hayman *et al.*, 2011) through a sylvatic epidemiological cycle (Sadeuh-Mba *et al*., 2017).

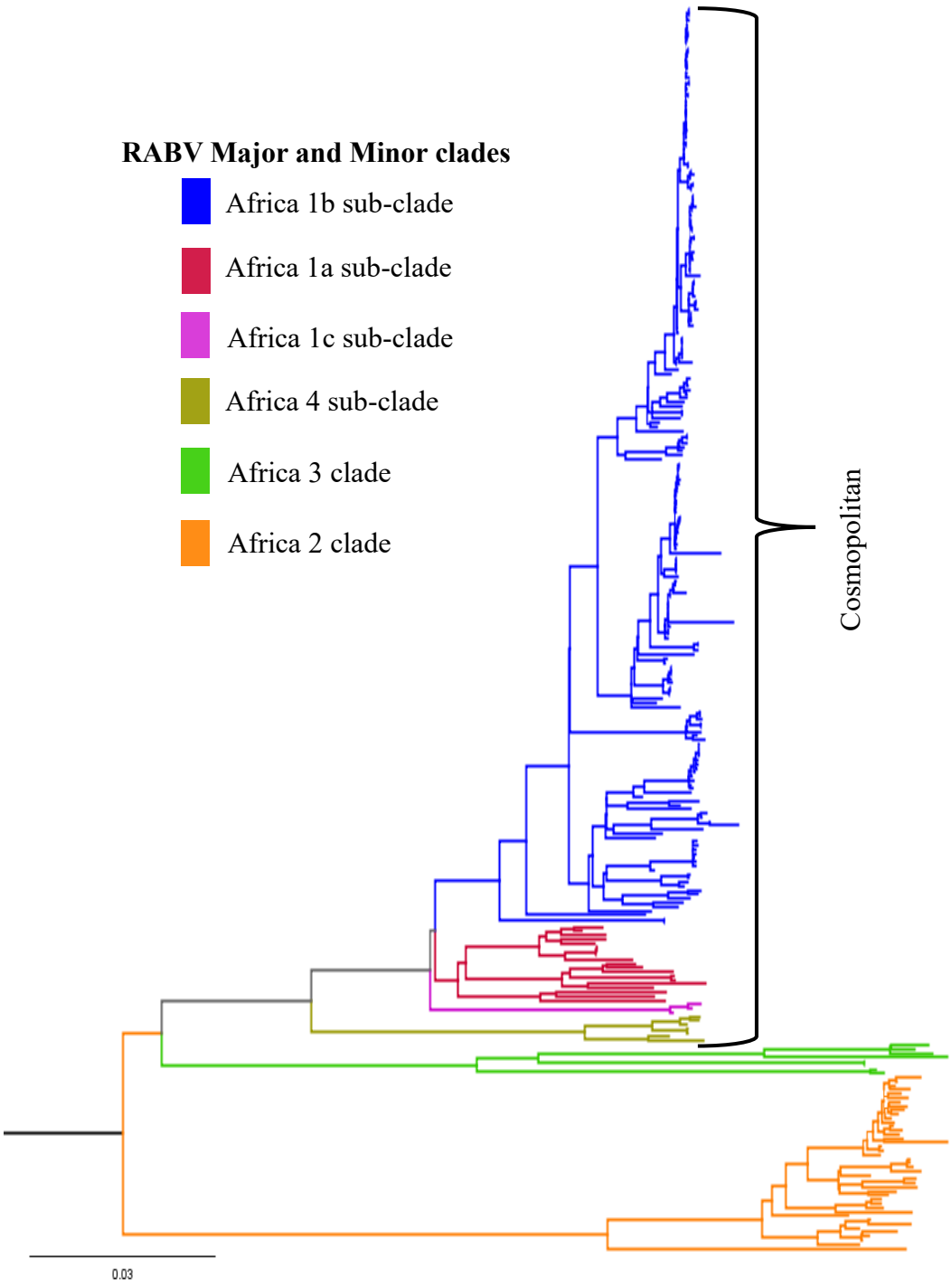


Figure 2.2: A phylogenetic representation of the geographical distribution of RABV in Africa

2.4 Distribution and host range

Rabies is regarded as the earliest documented case of a bat-associated spillover infection in humans, and it is believed that the first hosts were bats from which lyssaviruses originated (Klein *et al*., 2021). While bats carrying rabies were initially recognized in the Americas, the disease is currently present in bats on every continent excluding the Antarctica (Maki *et al*., 2017). Both human and animal health are still seriously threatened by the virus as it expands into new host species and geographical regions (Fisher *et al*., 2018). Apart from bats, carnivores also serve as primary reservoir hosts for different lyssaviruses, and spillover infections can occur to other mammals, including humans and cows, although humans are considered dead-end hosts (Müller & Freuling, 2020). Bats in the New World are known to transmit RABV, while bats in the Old World (Africa, Europe, and Asia) harbor a variety of other lyssaviruses but not RABV (Matsumoto *et al.*, 2017). RABV is the only lyssavirus that has established itself in terrestrial carnivores worldwide and in bats in the Americas (Ceballos *et al*., 2013). Rabies-related lyssaviruses generally have a more restricted geographical circulation and host range in comparison to RABV.

All the lyssaviruses classified in phylogroup I circulate in bats, with the exception of RABV, which has also adapted to persist in carnivores and other mammals (Kuzmin *et al.*, 2012). Among the members of phylogroup II, both LBV and SHIBV circulate in bats, while the reservoir host for MOKV remains unknown (Kuzmin *et al*., 2011). WCBV and LLEBV were isolated from bats, while IKOV was isolated from an African civet. However, the phylogenetic relatedness of IKOV to WCBV and LLEBV suggests that IKOV may be a spill-over infection in the African civet, and the actual reservoir host for IKOV is likely to be bats (Streicker *et al*., 2012). In Africa, six lyssaviruses are known to be in circulation: IKOV, LBV, MOKV, DUVV, SHIBV, and RABV. Among these, only RABV, DUVV, and MOKV have been associated with human deaths in Africa (Scott & Nel, 2021). RABV stands as the predominant lyssavirus reported in Africa, with domesticated dogs responsible for more than ninety-nine percent of all recorded human fatalities each year (Hampson *et al*., 2015).

2.5 Detection of rabies virus

Routine rabies diagnostic tests typically involve the examination of brain tissue, which is usually obtained postmortem. However, performing postmortem laboratory diagnosis on human patients is often challenging due to difficulties in obtaining permissions to take brain material, which can be influenced by religious, cultural beliefs, and consent issues (Coertse *et al.,* 2010). In such cases, ante-mortem diagnostic tests are recommended, which involve the detection of RABV RNA in skin biopsies and various body fluids such as cerebrospinal fluid, saliva and urine (Fooks *et al.*, 2014). Antemortem diagnostic tests are made possible by the virus's extensive spread throughout the neurological system.

World Organization for Animal Health and WHO suggest the use of direct fluorescent antibody test (dFAT), which was created in the late 1950s and is still considered the "gold standard" method for confirming rabies cases (Mayes & Rupprecht, 2015). dFAT provides reliable results on fresh specimens, but its sensitivity is compromised in cases where brain tissue is decomposed or archived (Aravindh Babu *et al.*, 2012). In instances where dFAT results are unclear or negative, WHO recommends doing another test (WHO Expert Consultation on Rabies: Third report, 2018).

Currently, confirmation of rabies diagnosis relies on molecular techniques such as real-time reverse-transcription polymerase chain reaction (qRT-PCR). Molecular techniques can detect the RNA of RABV even in poor-quality brain samples when dFAT yields negative results. In 2018, OIE accepted the use of molecular methodologies for rabies diagnosis (Marston *et al.,* 2019). qRT-PCR is the primary diagnostic assay as it allows for amplification and detection within a closed tube system, providing a quicker and timely detection of RABV RNA in questionable samples (Rupprecht *et al.*, 2018). Both ante-mortem and postmortem diagnosis of rabies can be performed using qRT-PCR in samples with low viral load (Goravey *et al.*, 2021). Additionally, heminested reverse transcriptase polymerase chain reaction (HnRT-PCR), which utilizes a cocktail of primers, can detect RABV and other lyssaviruses in decomposed or archived brain samples, and the amplified products can be used for sequencing (Araújo *et al*., 2008).

Surveillance in genomic data is increasingly recognized as a powerful tool for monitoring infectious diseases, offering significant insights into disease management. However, its implementation is still in its early stages in developing nations, where many emergent and endemic zoonotic diseases, such as rabies, prevail, and sequencing capabilities are limited (Brunker *et al.*, 2020). Despite these challenges, sequencing lyssaviruses, including RABV, can provide substantial benefits. By tracking the movement of the virus and analyzing its evolution over time, scientists can gain insights into its spread and distribution, aiding in mapping the geographical distribution and identifying high-risk areas where targeted intervention strategies are crucial (Gigante *et al.*, 2020).

Studying the host range of RABV also heavily relies on genome sequencing. By identifying different strains or variants, researchers can determine the potential transmission pathways and identify species that may be more susceptible to the virus. This knowledge is important for implementing effective control measures and preventing the transmission of rabies to vulnerable populations (Gigante *et al*., 2020). During outbreaks, rapid sequencing and analysis of viral genomes can provide real-time information on the source of the virus, transmission patterns, and potential reservoirs. This data allows for prompt decision-making and the implementation of focused control actions, such as planned vaccination programs and preventive measures to decrease human and animal exposure to the virus (Brunker *et al.*, 2018). Overall, the sequencing of RABV and the use of genomic surveillance are invaluable tools for monitoring and controlling rabies outbreaks, understanding the virus's spread and evolution, and implementing effective prevention and control strategies. While challenges exist, particularly in resource-limited settings, efforts are being made to enhance sequencing capabilities and promote the integration of genomics into infectious disease surveillance worldwide (Brunker *et al.*, 2020).

2.6 Epidemiology of rabies in Kenya

Rabies has a long-standing history in Kenya, presenting a significant public health concern. Approximately 2,000 individuals succumb to rabies in Kenya every year, with the highest incidence reported in parts of western (Siaya, Kisumu, and Homa Bay) and eastern (Machakos, Kitui, Makueni counties) regions (Ngugi *et al*., 2018; Oketch, 2022). Children below the age of 15 years are particularly affected by the

disease. Rabies is classified as a disease that mandates reporting as per the provisions of the Disease Control Act and Rabies Act of Kenya (Muriuki, 2016).

The major reservoir and transmitter of rabies in Kenya are domestic dogs. They account for 93% of bites, with 78% of these being stray dogs. It is projected that 1,000 to 2,000 human bites occur every year in Kenya (Ngugi *et al*., 2018). Since the 1980s, more than 85% of Kenyan counties have regularly reported having confirmed instances of rabies (Bitek *et al*., 2018). Based on verified cases analyzed at Kabete's Central Veterinary Investigation Laboratories (CVIL), the disease prevalence is primarily observed in dogs, with subsequent occurrences reported in cattle, cats, goats, sheep, pigs, wildlife, and humans (Muriuki, 2016; Bitek *et al*., 2018).

The most common lyssavirus found in Kenya is the RABV, while other lyssaviruses include LBV, MOKV, DUVV, and SHIBV (Muriuki, 2016). The SHIBV was isolated in bats at the Shimoni caves in Kwale county in 2009, and the LBV has been isolated in bats in Mt Elgon caves (Kuzmin *et al*., 2010). A 34-year-old Dutch female doctor was claimed to have contracted rabies at a campsite located within Tsavo West National Park as a result of the DUVV (Thiel *et al*., 2009). According to the WHO, the canine biotype of RABV is accountable for the significant number of human rabies cases. In Kenya, only the dog-related Cosmopolitan clade has been identified (Gigante *et al.*, 2020).

WHO set a worldwide goal in 2015 to eliminate human fatalities caused by rabies transmitted by dogs by the year 2030 (Ma *et al*., 2019). The theme of the 2022 world rabies day emphasized the One Health approach and the "Zero by 2030" goal, raising awareness regarding the prevention of rabies and emphasizing the urgency of its elimination. To effectively combat rabies, the Ministry of Health and Ministry of Agriculture, Livestock and Fisheries in Kenya devised a "Strategic Plan for the Elimination of Human Rabies in Kenya (2014 – 2030)." This comprehensive plan sets forth the country's objectives including widespread vaccination for dogs, administration of post-exposure prophylaxis (PEP) following potential exposure and public education initiatives, all aimed at achieving a rabies-free status by 2030 (Republic of Kenya, 2014).

However, there is still a lack of definitive conclusions regarding the distribution of RABVs, their subclades, and genetic diversity in Kenya. The goal of the current

investigation was to assess the current dissemination of RABV in high-risk regions, examine the genetic diversity and evaluate the efficacy of available vaccines for implementation in Kenya, in alignment with the strategic plan established by the Ministry of Health and Ministry of Agriculture, Livestock and Fisheries. The objectives were to determine the strains responsible for rabies cases in different hosts, understand the evolutionary relationships of RABV strains compared to global strains, and assess antigenic variations in the genes encoding the G and N, which are targets for rabies vaccines and monoclonal antibody therapies. The findings from this study are crucial for planning and optimizing control efforts and responses to rabies cases. The study provides high-resolution information to aid in the creation of intervention tactics for future rabies control, aligning with the objective of eliminating rabies by the year 2030.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Ethical statement

The RABV collection study received approval from the Scientific and Ethical Research Unit of the Kenya Medical Research Institute (Approval number: KEMRI/SERU/CGHR 046/3268) (Appendix 1).

3.1 Study design

The study conducted a laboratory-based prospective surveillance. The laboratory setting was necessary to achieve the study's objectives effectively and to provide detailed insights into RABV characteristics.

3.1.1 Sample collection, handling and transportation

Brain stem samples were collected from animals suspected of having rabies. To ensure sample integrity, the samples were immediately shipped in liquid nitrogen dry shippers to the Walter Reed Basic Science Laboratory-Kenya Medical Research Institute located in Kisumu. Upon arrival, the samples were immediately transferred to a -80 ºC freezer until they were required for further analysis.

3.1.2 Study areas

Samples were obtained from two specific areas in Kenya that have recorded the highest occurrences of rabies cases in humans. These areas included Siaya county located in the western region, along with Makueni and Machakos counties located in the eastern region, with one sample uniquely collected from Mwala Sub-county in Machakos (Figure 3.1). Separated by a distance of 540 miles, these regions are characterized by intricate natural barriers such as Lake Victoria in the West, the Rift Valley in the central region with multiple lakes along its floor, and the Aberdare and Nandi escarpment to the east. The presence of these natural barriers creates distinct ecological environments that could potentially influence the distribution and dynamics of rabies virus transmission in each region. By selecting study locations that represent different geographical regions within Kenya, this research aimed to capture the diverse and distinctive characteristics of rabies transmission in these respective areas.

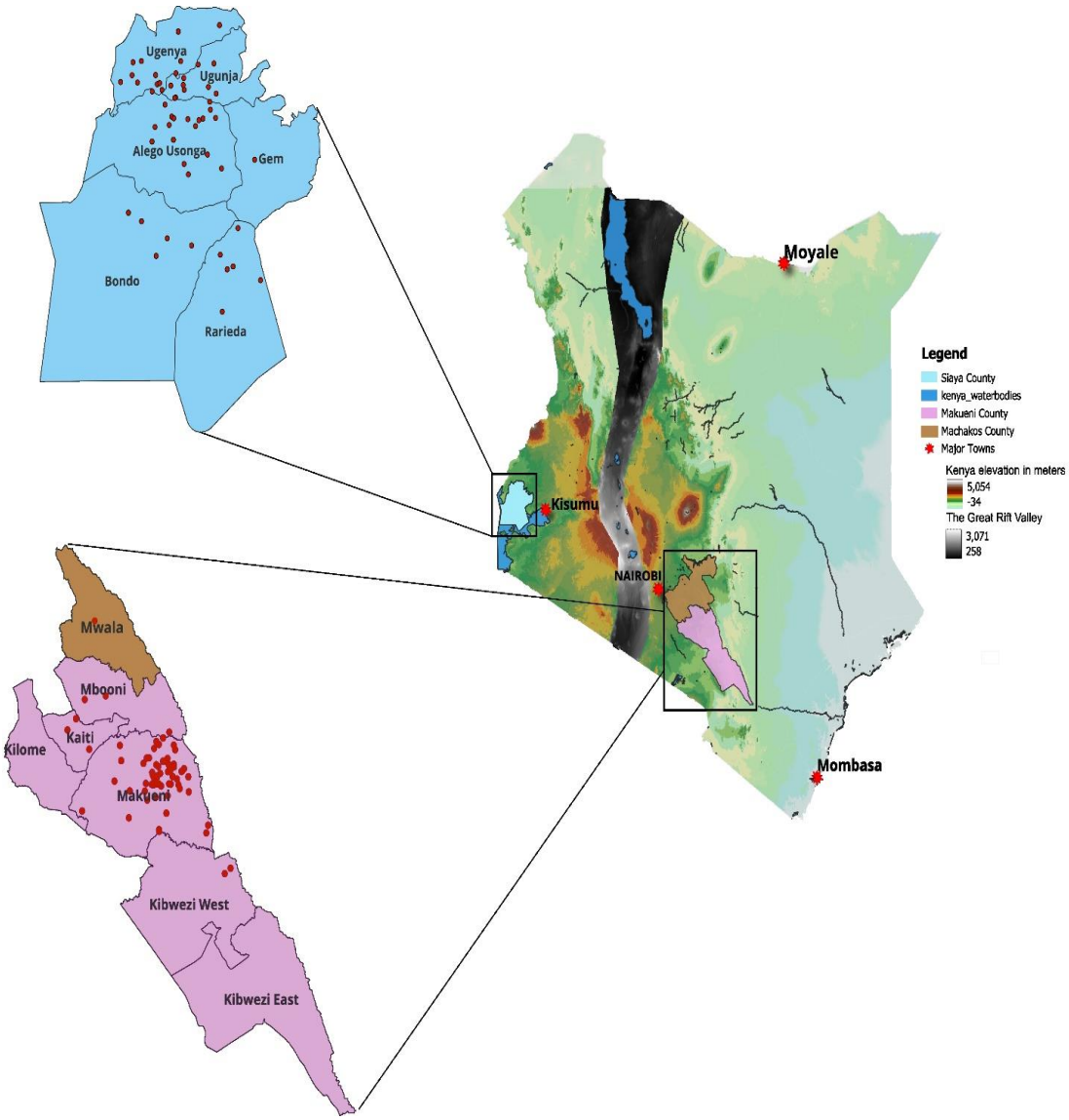


Figure 3.1: Kenyan map showing the study sample collection sites in Siaya and Makueni counties in western and eastern Kenya respectively.

The two counties are separated by 540 miles with complex natural barriers such as the floor of the Great Rift Valley, six lakes distributed along the valley's floor, the Aberdare and Nandi escarpment lies to the east and the Lake Victoria is situated to the west. The map was created using ArcGIS software (https://www.arcgis.com/index.html).

3.1.2 Study samples and sampling design

A total of 164 brain stems were collected, with 82 collected from Makueni county and 82 from Siaya county for the study. Between July 2021 and September 2022, brain tissues were collected from rabid animals. The samples consisted of the

following: 48 domestic dogs, 2 cats, 7 cows, 1 honey badger, and 24 goats from Makueni county, and 59 domestic dogs, 1 sheep, and 22 cows from Siaya county. The sampling approach used in this study was purposive, involving the deliberate selection of samples from animals exhibiting signs and symptoms associated with rabies, confirmed by a positive rabies detection using a standard rapid diagnostic test (Bio Note, Republic of Korea).

3.2 Objective One

To determine the strains of RABV responsible for cases of rabies in different hosts in eastern and western Kenya.

3.2.1 Extraction of RABV RNA

Viral RNA extraction from the 164 brain stem tissues was performed using the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, USA). The extraction process followed the manufacturer's instructions. Briefly, 20 mg of tissue was transferred to a 2 mL tube, and 500 µL of TRIzol reagent along with 2 PYREX^{TM} solid glass beads were introduced to the sample. The sample was then disrupted using a Fisher Scientific^{TM} Bead Mill 24 Homogenizer from ThermoFisher Scientific, operating at 6 m/s for 45 seconds. After disruption, the mixture was centrifuged at 1,000 × g for 1 minute, and 200 µL of the supernatant was loaded into the KingFisher^{TM} Flex automated extraction system from ThermoFisher Scientific. The RNA extraction was performed in the machine following the manufacturer's recommendations. Subsequently, the concentration and quality of the extracted RNA were determined using the Qubit ds HS Assay kit from (Invitrogen, USA).

3.2.2 Screening for RABV

To confirm the presence of RABV RNA in 164 samples that were positive for RABV antigens using the rapid test kits (BioNote, Republic of Korea), two screening approaches were adopted. The primary method used was quantitative Reverse transcriptase Real-Time Polymerase Chain Reaction (qRT-PCR), specifically targeting the L gene. The L gene is a well-established marker for detecting the African 1b and 1a RABV strains (Faye *et al*., 2017). In this method, RNA obtained from the brain samples was amplified by qRT-PCR targeting the L gene, using forward and reverse primers and a probe indicated in Table 3.1. The L-gene RT-qPCR reaction mixture comprised of 4 µL of nucleic acids, 10 µL of 2x Sensifast master mix

(Meridian Bioscience, OH, USA), 0.8 µL of forward and reverse primer mix, each at a concentration of 10 µM, 0.4 µL of TaqMan QSY probe (Applied Biosystems, USA) at a concentration of 10 µM, 0.4 µL of RiboSafe RNase inhibitor, and 0.2 µL of reverse transcriptase and 3.4 µL of nuclease-free water. Amplification was carried out in an ABI 7500 (Applied Biosystems, MA, USA) as follows: a reverse transcriptase cycle at 45 °C for 10 min and 95 °C for 3 min to inactivate the reverse transcriptase, followed by 40 cycles of 95 °C for 30 sec and 60 °C for 1 min. For quality control, each reaction included a positive control and a non-template (negative) control. The cycle threshold (Ct) was set at 40. All reactions with Ct values below 40 were considered positive.

Table 3.1: The specific primers and probe used to detect the L gene of RABV by qRT-PCR

Primer	**Sequence (5`3`)**
10 µM Forward primer	GGTTTCCGGDGCYGTDCCTC
10 µM Reverse primer	CCTAGGGGAGACYTTGCCRT
10 µM Probe	6FAM-CCCGTCAYATAGGGTCRGCTCARGGGC-BBQ

Samples that tested negative by L gene qRT-PCR were screened by heminested PCR targeting the N gene using a cocktail of primers shown in Table 3.2, as outlined by Heaton et al., 1997, with minor adjustments. The N gene is more conserved (Meng *et al.*, 2007) making it a broader target for the detection of RABV that may be missed by the L gene. For the N gene PCR, the RNA was first converted to complementary deoxyribonucleic acid (cDNA) (see section 3.3.1.2) and used for the primary amplification in a final volume of 25 µL, comprising 2.5 µL of the cDNA, 12.5 µL of MyTaq enzyme (Meridian Bioscience), 0.4 µL of forward (JW12) and reverse (cocktail of primers mix (JW6 1+2+3) at a concentration of 10 µM each, and 8.4 µL of PCR water. The amplification was performed in a 7500 Thermal Cycler (Applied Biosystems) using the following conditions: initial heating at 95°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes.

Subsequently, HnRT-PCR was conducted using 2.5 µL of the primary amplicons as a template, along with 12.5 µL of MyTaq enzyme (Meridian Bioscience), 0.4 µL of forward (JW12) and reverse cocktail of primers mix (JW10 1+2+3) at a concentration of 10 µM each, and 8.4 µL of PCR water, in a final volume of 25 µL. The thermal cycling conditions remained the same as for the primary amplification. A RABV positive control was used to confirm that the PCR reaction was working correctly. A non-template control (PCR water) was used as a negative control to monitor for any potential contamination. PCR products were resolved in a 1.5% agarose gel stained by Gel Red® (Biotium, Hayward, CA, USA) and visualized under Ultraviolet (UV) light after electrophoresis (80 V, 1 hour).

Table 3.2: The primers used for heminested amplification of the N gene for detection of RABV RNA

Primer	**Sequence (5`3`)**
JW12 (Forward primer)	ATGTAACACCYCTACAATTG
JW6 (1) (Reverse primer)	CAATTCGCACACATTTTGTG
JW6 (2) (Reverse primer)	CAGTTGGCACACATCTTGTG
JW6 (3) (Reverse primer)	CAGTTAGCGCACATCTTATG
JW10 (1) (Reverse primer)	GCTAGGGTTTTCCCAGTCACGACGTTGTCATCAAAGTGTCTGCTC
JW10 (2) (Reverse primer)	GCTAGGGTTTTCCCAGTCACGACGTTGTCATCAATGTGTGRTGTTC
JW10 (3) (Reverse primer)	GCTAGGGTTTTCCCAGTCACGACGTTGTCATTAGAGTATGGTGTTC

3.3 Objective two

To determine evolutionary relationships of the RABV strains in reference to global strains available in the public sequence databases.

3.3.1 Whole genome sequencing (WGS)

3.3.1.1 DNAse digestion

To remove the host genomic DNA, 30 µL of the extracted RNA from samples positive at screening was mixed with 1.5 µL of Turbo DNAse and 3.5 µL of Turbo DNAse buffer (Thermofisher Scientific, USA). The mixture was then incubated at a temperature of 37 ºC for 30 minutes.

3.3.1.2 Sequence Independent Single Primer Amplification (SISPA)

The first cDNA strand was reverse transcribed using the JH17N8 5`-GTTTCCCAGTAGGTCTCNNNNNNNN-3` primer that contained a degenerate 8-mer sequence at the 3' end using the LunaScript RT SuperMix kit (New England Biolabs, MA, USA) with the following thermal cycling regimen: 1 cycle of 25 °C for 2 min, 55 °C for 10 min, 95 °C for 1 min followed by cooling to 4 °C. The second cDNA strand was generated by amplification using NEB Next Ultra II Q5 master mix (New England BioLabs) with 10 µM of primers JH17N8 and JHP21 (5` GTTTCCCAGTAGGTCTC 3`) with the following thermal cycling regimen: 1 cycle of 94 °C for 3 min, 25 °C for 30 sec, 72 °C for 1 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min. A final extension step of 72 °C for 1 min was included, followed by cooling to 4 °C.

3.3.1.3 Library preparation

The produced dsDNA was cleaned using Agencourt AMPure XP beads from Beckman Coulter (CA, USA) according to the manufacturer's instructions and used to generate sequence libraries with the Invitrogen Colibri ES DNA library prep kit (ThermoFisher Scientific, USA). The technique involved fragmenting and detailing the cDNA with 5 µL of 5X fragmentation enzyme. Indexing was carried out with unique dual index primers to allow each sample to be identified. Size selection was then performed by adding size-specific beads. To amplify the library, 20 µL of double-stranded DNA, 25 µL of 2X library amplification master mix, and 5 µL of primer mix were used in a thermocycler. The initial step was at 98°C for 30 seconds, followed by 12 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Following that, the reaction was extended at 72°C for 1 minute before being held at 4°C. After amplification, a cleanup was performed by mixing 50 µl of the DNA library with 40 µl of DNA cleanup beads.

3.3.1.4 Library quantification, normalization and pooling

Library quantification was performed using the Qubit ds HS Assay kit (Invitrogen, Thermoscientific) using procedures outlined in the kit protocol. The average library size was measured on the 4200 Tape Station System (Agilent Technologies, CA, USA), following the manufacturer's procedure. Briefly, the reagents were thawed at room temperature for 30 minutes and then vortexed before a brief spin. In the first plate, 2 µL of high sensitivity ladder was loaded, followed by dispensing 2 µL of each library into individual wells. Next, 2 µL of the high sensitivity buffer was added to each well and vortexed for 1 minute, followed by a brief spin. The 96-well plate was placed into the Agilent 2200 tape station, and high sensitivity screen tape and tips were loaded into the machine. Normalization of the library was performed using the formula nM = (A × 1,000,000) / (660 × B), where A represents the concentration of the library in ng/µL, B is the average base pair length of the library obtained from the tape station, and 660 represents the average molecular weight of a nucleotide.

3.3.1.5 Library denaturing and miseq sample loading

After adjusting the pooled library's concentration to 4 nM, it was further diluted to 2 pM and denatured for 5 minutes using 0.2 N NaOH. To monitor sequencing quality, five percent of the PhiX control library (Illumina, CA, USA) was spiked into the pooled library. The library was then denatured at 96°C in an incubator for 2 minutes. Finally, the 9.5 pM library was loaded onto the MiSeq reagent cartridge kit V3 (Illumina, CA, USA) on the MiSeq platform, where it was sequenced using the 600 cycles v3 and P3 reagents.

3.3.2 Targeted sequencing of the N gene amplicons

Amplicons with noticeable bands after heminested PCR were purified using AmpureXP beads (Beckman Coulter, CA, USA) and used to create sequence libraries with the Invitrogen Colibri ES DNA library prep kit (ThermoFisher Scientific, USA), as detailed in section 3.3.1.3. The 9.5 pM library was sequenced on the MiSeq platform using 600 v.3 paired-end chemistry (Illumina, CA, USA), as detailed in section 3.3.1.5.

3.3.3 Sequence assembly and phylogenetics

Demultiplexed sequence reads for both shotgun RNA sequencing (whole genome sequencing) and targeted sequencing were retrieved from the sequencing platforms.

The quality of the fastq files was evaluated using FastQC v0.12.1 and processed with the ngs_mapper pipeline (https://github.com/VDBWRAIR/ngs_mapper). In brief, the pipeline filtered out low-quality reads (Q < 20), failed reads, sequencing adapters, and short reads (< 50 nucleotides) using Trimmomatic v0.35 and Cutadapt v1.9.1. The filtered reads were then mapped to a RABV genome from Tanzania (GenBank accession: KY210291), which had the closest homology to our sample sequences, using BWA v0.7.12. Samtools v0.1.19 was used to create pileups from the read alignments. Several Python scripts within the pipeline generated the consensus genome, a VCF file, and coverage visualizations.

Sequence alignments of individual RABV N and G genes were also extracted from the whole genome sequences for single gene analysis. RABV-GLUE (http://rabv-glue.cvr.gla.ac.uk/) was employed to assign the RABV to major and minor clades for the whole genome, N and G sequences. Further classification of the whole genomes into lineages was performed using MADDOG (Campbell *et al*., 2022).

To establish the phylogenetic relationships of Kenyan RABV within an African context, a comprehensive subset of curated, annotated, and published RABV datasets from Africa was collected from the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (https://www.bv-brc.org/). The alignments of the complete RABV polyprotein, the entire N protein, and the entire G protein of the study genomes and context samples were conducted in CLC Genomics workbench v8.5.1 using the Muscle plugin (QIAGEN, Hilden, Germany). The aligned sequences were run in the Genetic Algorithm for Recombination Detection (GARD), a software implemented in the Hyphy package available on the Data Monkey server (https://www.datamonkey.org) to avoid using sequences from recombination. Phylogenetic inference was done using Maximum-likelihood (ML) methods implemented in IQ-TREE v2.2.0 using nucleotide substitution models as determined by Bayesian Information Criterion (BIC) tests in the inbuilt Model Finder in IQ-TREE. Node support was evaluated with a combination of approximate likelihood tests (SH-alrt) and ultrafast bootstraps (UFboot) with 1000 replicates each computed in IQ-TREE (Minh *et al.*, 2020). The resulting phylogenetic trees were then visualized and annotated using Figtree v1.4.2.

3.4 Objective three

To determine the extent of antigenic variations in the gene encoding the glycoprotein and nucleoprotein

3.4.1 Analysis of antigenic variations

To identify amino acid variations at the N and G vaccine target sites of the study genomes and the vaccine strains, sequence alignments of the G (n=144) and N (n=134) proteins were performed using CLC Genomics Main Workbench. Study samples' amino acid sequences were aligned to three commonly used RABV vaccine strains, namely Pitman Moore (PM) (accession number: DQ099525), Pasteur vaccine (PV) (accession number: M13215), and Challenge Virus Standard (CVS) (accession number: AF406696 for N and AF406694 for G gene).

CHAPTER FOUR

RESULTS

4.1 Study samples and the associated metadata

The metadata of study samples: ID, animal species, date of collection, PCR results, genome size, clade/subclade, and lineages are shown in **Appendix 2.**

4.2 Objective 1: To determine the strains of RABV responsible for cases of rabies in different hosts in eastern and western Kenya.

4.2.1 Majority of the RABV were of the cosmopolitan African 1a and African 1b

Out of the 164 brain samples that had been confirmed positive at the initial screening with the rapid diagnostic kit, 156 (77 from Siaya and 79 from Makueni) had RABV by qRT-PCR that targeted the L gene of RABV, indicating that these samples were of Cosmopolitan African 1a and African 1b lineages. A representative of the qRT-PCR amplification plot is shown in **Figure 4.1**. The cycle threshold (Ct) for the 156 RABV positive samples ranged from 10.97 to 28.37 (shown in **Appendix 2)**.

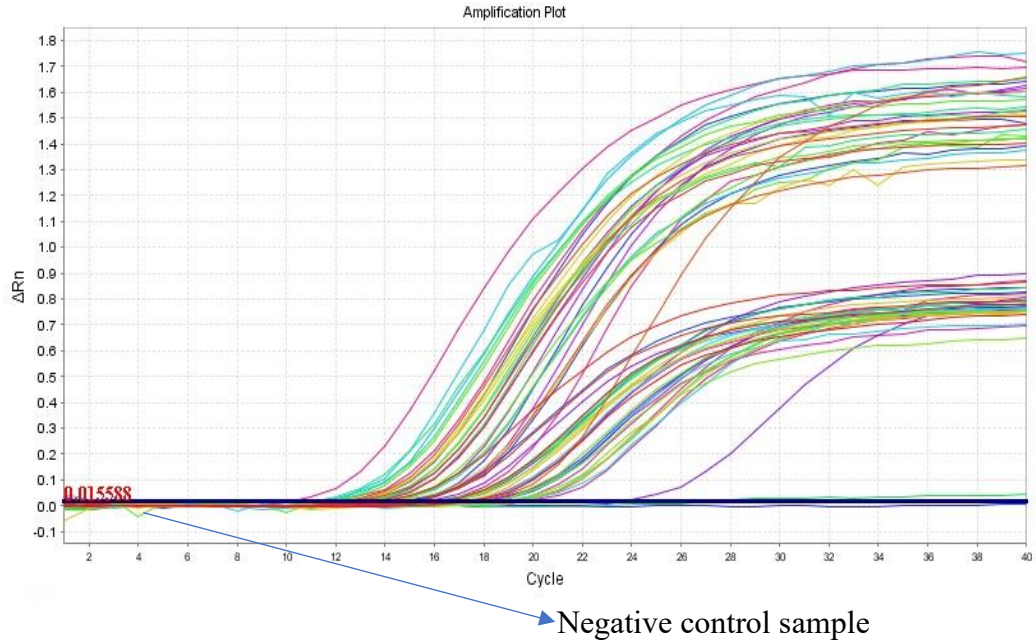


Figure 4.1: A representative qRT-PCR amplification plot showing varying Ct values, defined as the amplification cycle at which positive samples crossed the threshold set at 0.015588.

Of the 164 brain samples that had been confirmed positive at the initial screening with the rapid diagnostic kit, 8 (5 from Siaya and 3 from Makueni) failed to amplify by qRT-PCR targeting the L gene, but amplified with the N gene heminested PCR approach. An expected fragment size of 600 bp was observed in all samples (**Plate 4.1**, shown with an arrow). A second spurious band of about 400 bp was observed (**Plate 4.1**, shown with an arrowhead), but on sequencing, it was determined not to have RABV sequences. The correct lineage could only be ascertained after sequencing.

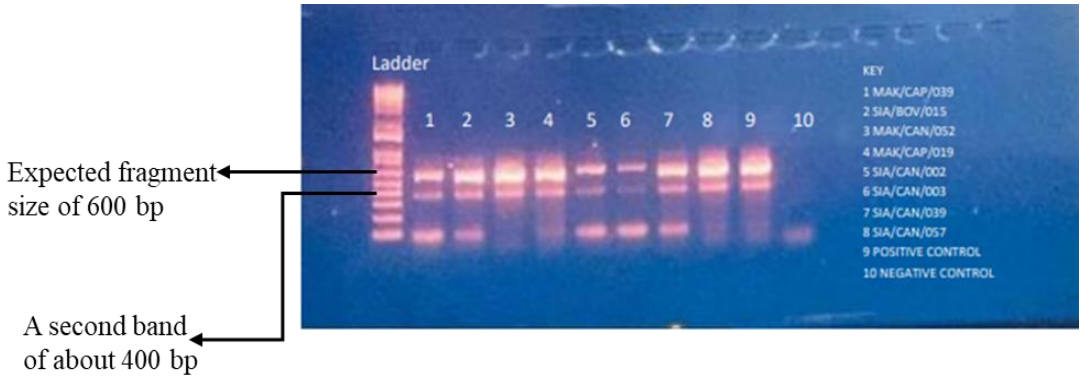


Plate 4.1: 1.5% agarose gel showing fragment amplification sizes of the N gene by heminested PCR.

The correct band size of the N gene was 600 bp (arrow). A second spurious band of about 400 bp was observed (arrowhead), but on sequencing, it was determined not to have RABV sequences.

4.2.2 Rabies virus-host species

As shown in **Figure 4.2**, Domestic dogs contributed most of the RABV-positive samples (65%), followed by cows (18%), and goats (14%). The remaining species accounted for only 1%.

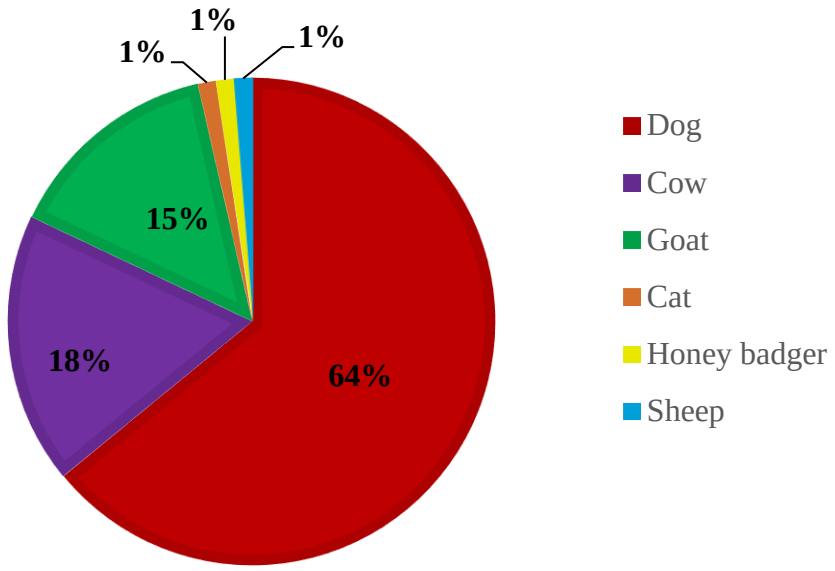


Figure 4.2: A pie chart illustrating the overall positivity rate of RABV among the host species.

4.3 Objective 2: To determine evolutionary relationships of the RABV strains in reference to global strains available in the public sequence databases

Of the 156 brain biopsies positive for RABV by qRT-PCR of the L gene, 148 samples with genome lengths ranging from 10,024 to 11,923 base pairs (average genome coverage of 97%) were used for whole genome analysis (**Appendix 2**). From the whole genome sequences, we also extracted 134 N genes (average coverage of above 90%) and 144 G genes (average coverage of above 90%) for single gene analysis. To the N gene analysis, we also added 8 N genes obtained by targeted sequencing. The raw sequence data from this study has been deposited in the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) Sequence Read Archived under Bio Project numbers OR256801 and OR270967–1061.

4.3.1 Phylogenetic analysis

4.3.1.1 Phylogenetic analysis trees drawn from WGS

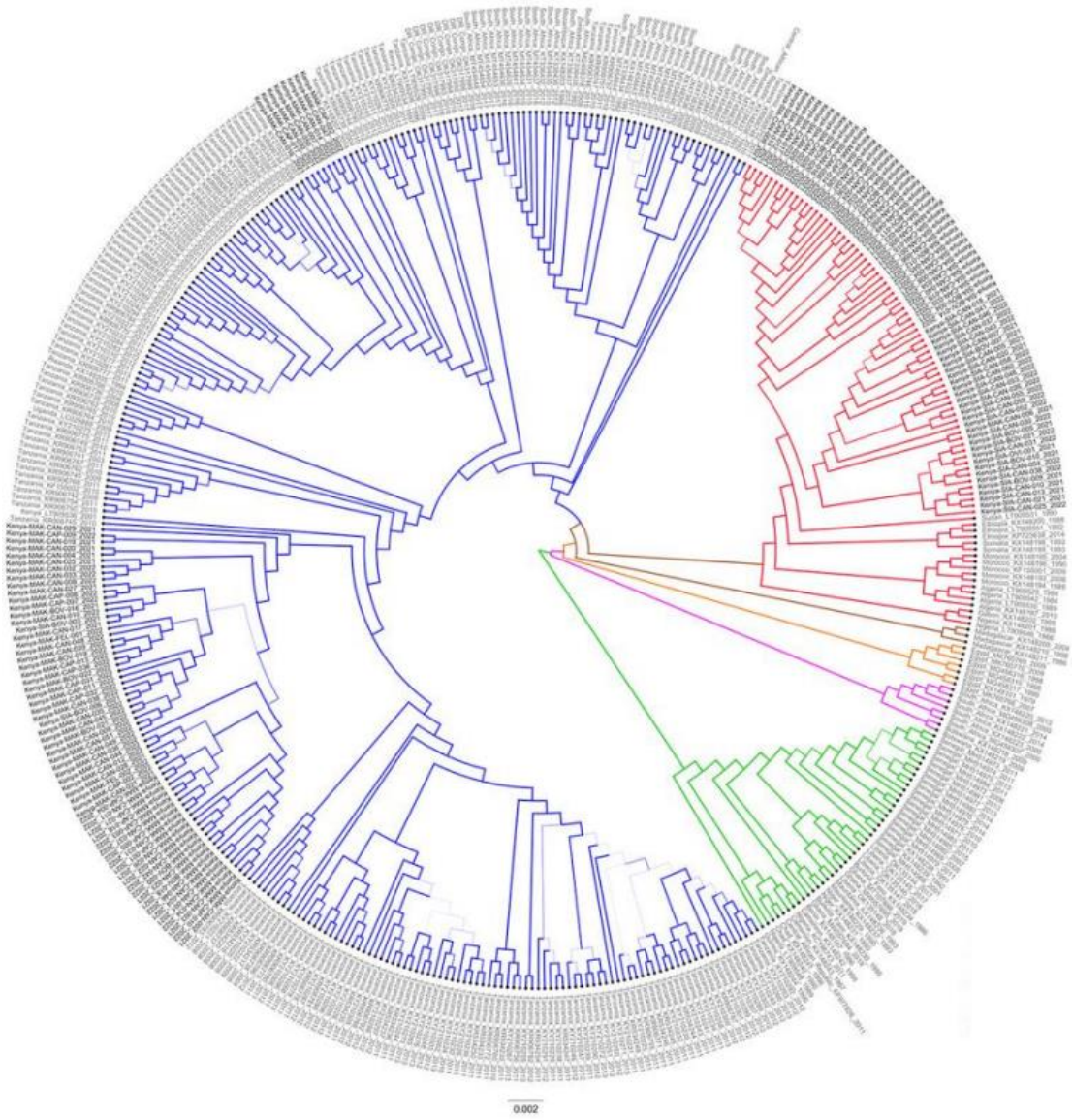
Of the 148 RABV genomes, 145 passed the IQ-TREE and were used to construct WGS Maximum likelihood phylogenetics, together with those sampled from Africa (n=300), available in the Bacterial and Viral Bioinformatics Resource Center (BV-BRC). Individual N and G gene sequences (142 for the N gene and 144 for the G gene) and together with those deposited in the BV-BRC (n=322 for the N gene and

n=140 for the G gene), were used to construct individual gene phylogenetic trees. The over-representation of N sequences (n=322) in the BV-BRC database compared to G (n=140) is because the N gene has been the method of choice for phylogenetic analysis evaluation before the advent of WGS.

Maximum likelihood phylogenetic trees drawn from the WGS are shown in **Figure 4.3**. **Panel A** shows major and minor clades of the RABV whole genomes from the study samples and those available in BV-BRC databases. All the study genomes (shaded black) clustered with Cosmopolitan clade, either the Africa 1b subclade (shown in blue lines) that dominates eastern and northern Africa or the Africa 1a subclade (shown in red lines) that is predominant in central, southern and eastern Africa.

Branch sections of the Africa 1b subclade and Africa 1a subclade that clustered with the study genomes are shown in **Figure 4.3**, Panels B and C respectively. The Makueni (eastern Kenya) genomes (**Panel B**) overwhelmingly (n=73 out of 76) clustered with the Africa 1b subclade with only three genomes (Kenya/MAK/CAP-014/2022, Kenya/MAK/CAN-005/2021 and Kenya/MAK/CAN/006/2021) clustering with Africa 1a subclade (Panel C, shown as blue stars). In contrast, the Siaya (western Kenya) genomes overwhelmingly (n=67 out of 69) clustered with Africa 1a subclade, (**Panel C**,) with only two genomes from rabid cows (Kenya/SIA/BOV-003/2021 and Kenya/SIA/BOV-006/2022), clustering with the Africa 1b (Panel B, shown in red stars). Within the Africa 1b study genomes, two clusters are discernable: Panel B highlighted in grey and with the prefix "Kenya-MAK" (shown as I), which was the major cluster containing 65 genomes and a minor cluster comprising 8 genomes shown as II. The latter group clustered with Kenyan genomes that had been collected previously from Kericho and Nakuru. Both clusters I and II were closely related to sequences from Tanzania. The Africa 1a subclade from the study samples was more homogeneous and all genomes formed one cluster (**Panel C**), highlighted in grey and with the prefix "Kenya-SIA").

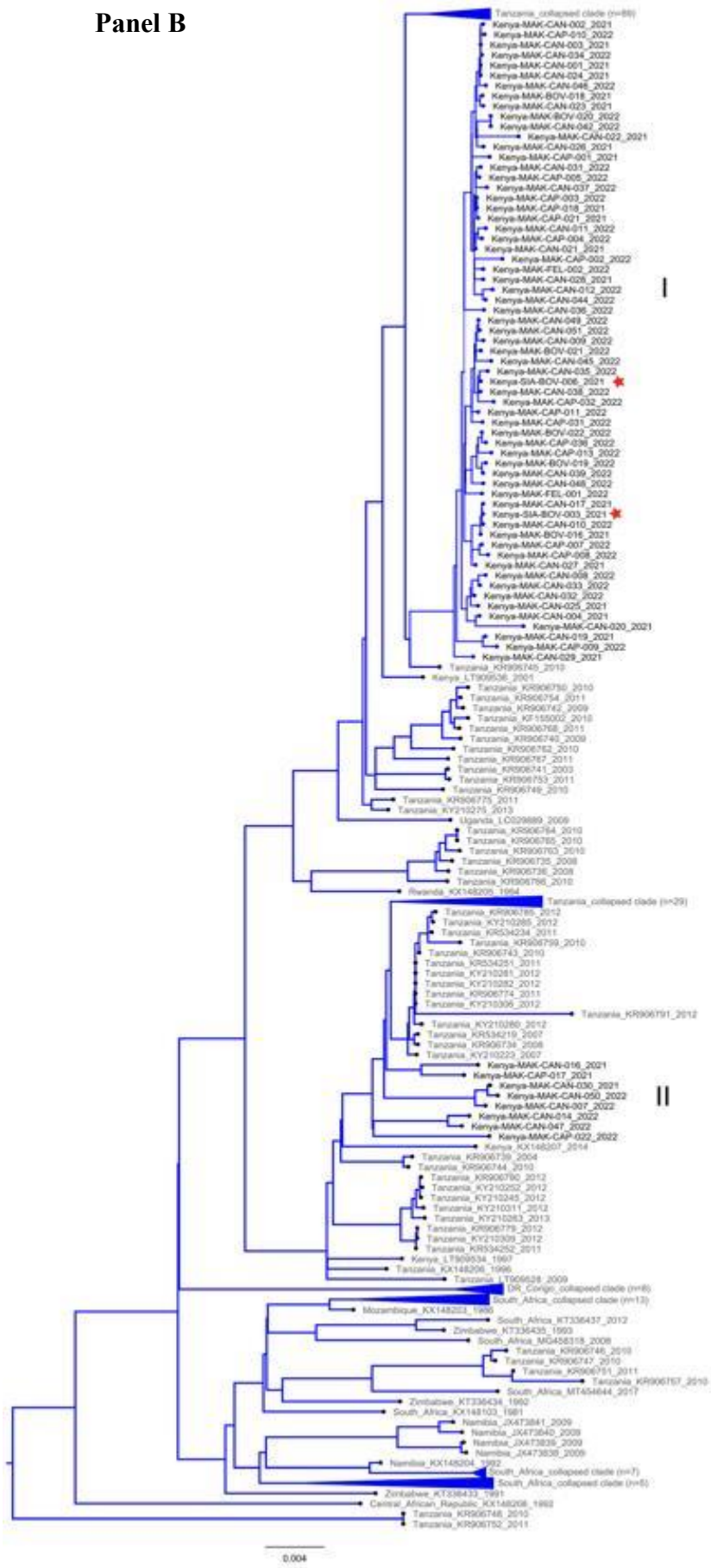
Panel A



Key: Branching lines are color-coded to indicate major clades of rabies virus

Cosmopolitan A1a subclade
Cosmopolitan A1b subclade
Africa 2 clade
Cosmopolitan A1c subclade
Cosmopolitan AF4 subclade
Africa 3 clade

Panel B



Panel C

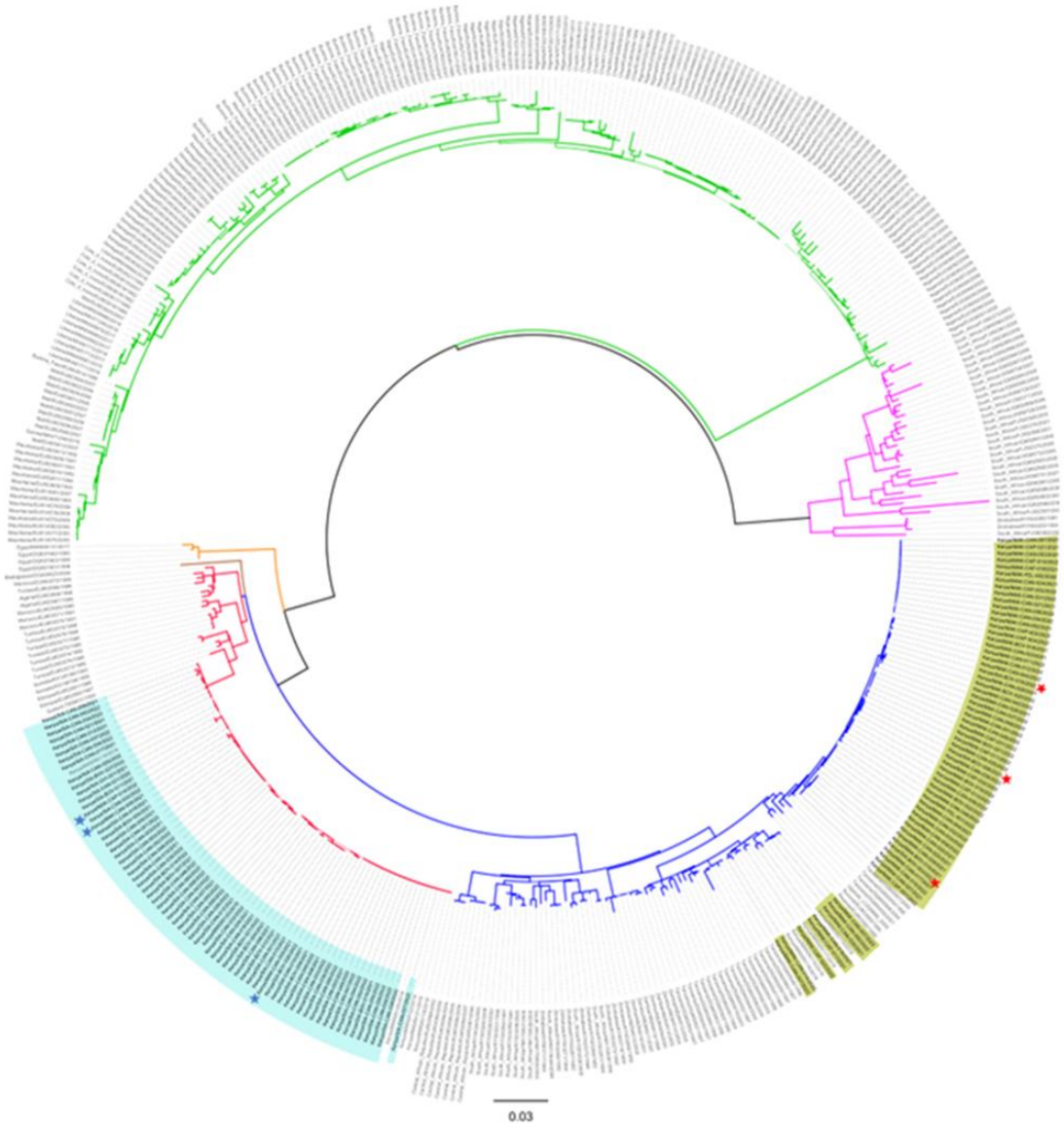
Figure 4.3: Phylogenetic tree showing sub-clade classification of study genomes.

Panel A illustrates major and minor clades of the study genomes and other African genomes in the BV-BRC database. All study genomes (shaded black) form clusters within the Cosmopolitan clade, specifically either the Africa 1b subclade (depicted by blue lines) or the Africa 1a subclade (depicted by red lines). Panel B, focusing on the Africa 1b subclade, Makueni RABV genomes overwhelmingly cluster within it, except for three genomes (Kenya/MAK/CAP-014/2022, Kenya/MAK/CAN-005/2021, and Kenya/MAK/CAN/006/2021) which align with the Africa 1a subclade (indicated by blue stars in Panel C). Panel C, highlighting study genomes from Siaya, western Kenya, reveals that the majority (67 out of 69) cluster with the Africa 1a subclade, while only two genomes (Kenya/SIA/BOV-003/2021 and Kenya/SIA/BOV-006/2022) cluster with the Africa 1b subclade (depicted as red stars in Panel A).

4.3.1.2 Phylogenetic analysis trees drawn from individual N and G genes

To enable the comparison of data generated by the newly introduced WGS approach, N and G single genes phylogenetic trees were also generated (**Figure 4.4**, Panels A and B) and accrued data compared to the WGS tree. The single gene trees, derived from analyzing the individual N gene (Panel A) and G gene (Panels B) demonstrated a similar geographic division of the Kenya RABV sub-clades compared to the phylogenetic tree obtained through WGS.

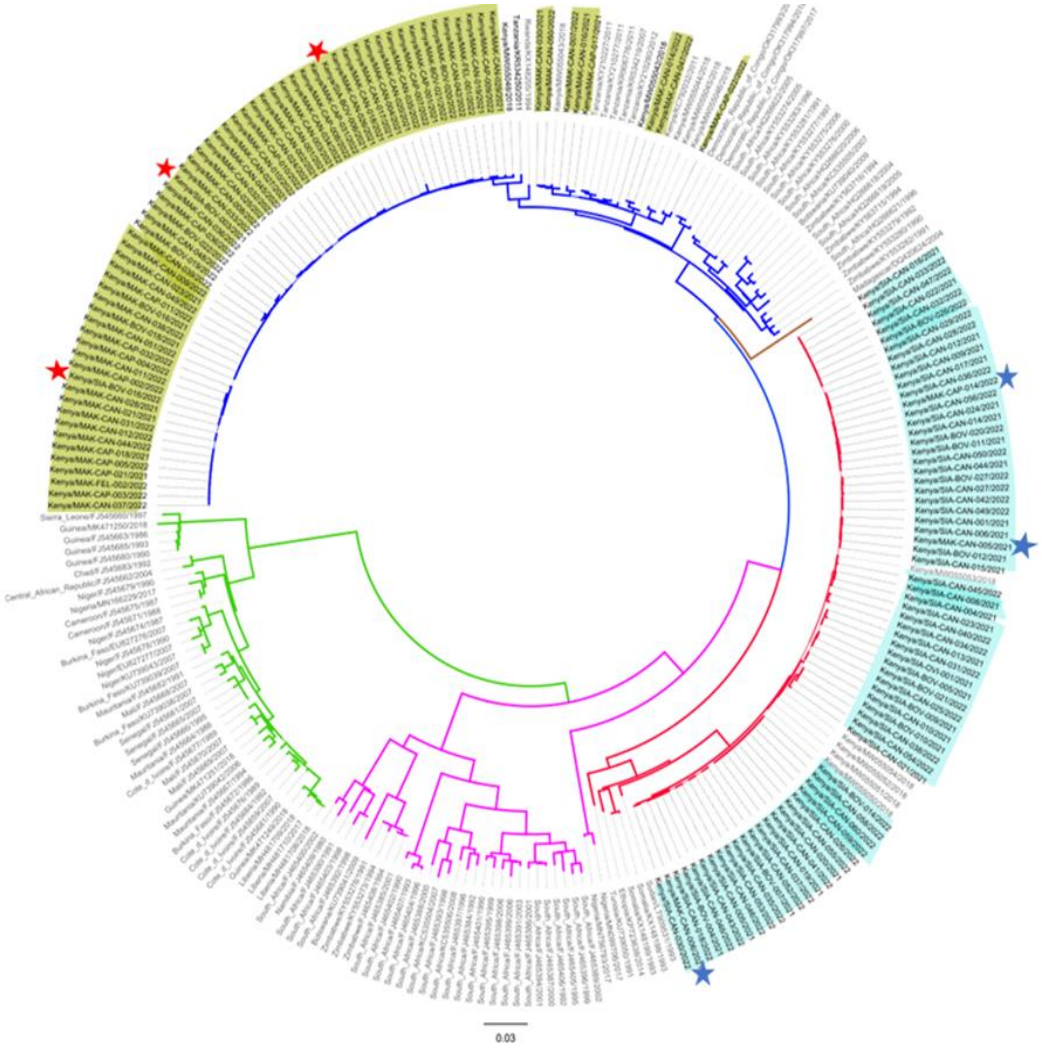
Panel A



Key: Branching lines are color coded to indicate major clades of rabies virus

Cosmopolitan A1a subclade
Cosmopolitan A1b subclade
Africa 2 clade
Cosmopolitan A1c subclade
Cosmopolitan AF4 subclade
Africa 3 clade

Panel B



Key: Branching lines are color-coded to indicate major clades of rabies virus

Cosmopolitan A1a subclade
Cosmopolitan A1b subclade
Africa 2 clade
Cosmopolitan A1c subclade
Cosmopolitan AF4 subclade
Africa 3 clade

Figure 4.4: N and G genes phylogenetic trees.

The N (Panel A) and G (Panel B) genes show phylogenetic trees drawn from the study genomes (highlighted in blue for Siaya, western Kenya genomes and highlighted in yellow for Makueni, eastern Kenya genomes) and other African genomes retrieved from the BV-BRC database. Similar to the WGS phylogenetic tree, all the study genomes clustered with Cosmopolitan clade, with Siaya genomes

clustering with Africa 1a subclade (shown in red lines) and Makueni genomes clustering with Africa 1b subclade (shown in blue lines). Genomes are color coded by sub-clade and clade. The scale bar represents genetic distance.

4.3.1.3 Derivation of Kenya's RABV lineages

Further finer resolution of the Kenyan genomes subclades performed using standardized universal lineage definition of whole genome sequences identified 13 distinct lineages, 8 in the western Kenya, 5 in the eastern Kenya, and one unclassified lineage from eastern Kenya (**Figure 4.5**). The N and G single genes lacked the discriminatory power for lineage assignments.

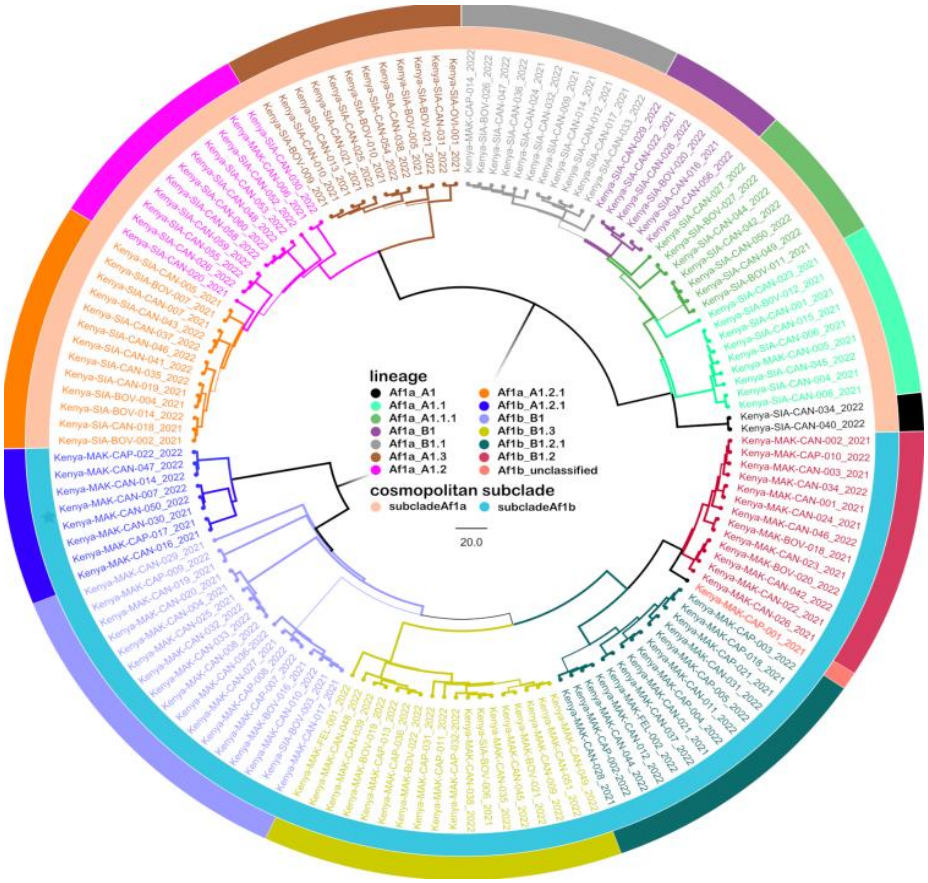


Figure 4.5: Phylogenetic tree showing lineage classification of whole genome sequences.

4.3.1.4 Mutation analysis reveals primer-binding variations responsible for false-negative qRT-PCR results

To determine why the L gene primers/probe failed to amplify 8 brain stem samples, a mutation analysis of the L gene at the primer/probe binding sites was performed

using the Basic Local Alignment Search Tool (BLAST). As shown in **Table 4.1**, multiple mutations at key positions within the L primer binding site were observed. For the forward primer, transitions were detected in samples SIA/CAN/057, MAK/CAP/019, and MAK/CAN/052, whereas sample MAK/CAP/033 exhibited transversions. In contrast, for the reverse primer, transversions were identified in samples MAK/CAP/019 and SIA/CAN/057, while transitions were found in samples MAK/CAN/052 and MAK/CAP/033.

Panel A

	1								10									20
Forward primer	G	G	T	T	T	C	C	G	G	D	G	C	Y	G	T	D	C	C	T	C
SIA/CAN/057	A	G	T	T	T	C	C	G	G	T	G	T	T	T	T	G	T	C	C	T
MAK/CAP/019	A	G	T	T	T	C	C	G	G	T	G	T	T	T	T	G	T	C	C	C
MAK/CAN/052	G	G	T	T	T	C	T	G	G	C	G	C	C	G	T	G	C	C	T	C
MAK/CAP/033	A	G	T	T	T	C	G	G	G	G	G	G	C	A	A	T	C	C	T	T

Panel B

	1								10									20
Reverse primer	C	C	T	A	G	G	G	G	A	G	A	C	Y	T	T	G	C	C	R	T
SIA/CAN/057	C	C	A	A	G	G	A	G	A	G	G	C	T	T	T	A	C	C	T	T
MAK/CAP/019	T	C	T	C	G	A	G	G	G	A	G	C	T	T	T	A	C	C	G	A
MAK/CAN/052	C	C	T	A	G	G	G	G	G	A	G	T	C	T	T	G	C	T	G	T
MAK/CAP/033	C	C	T	A	G	G	A	G	G	A	G	C	C	T	T	A	C	T	G	T

Table 4.1: Mutations in the Large-structural protein gene that could affect primer binding sites among initially negative samples. Panel A: Mutations at the binding site of the forward primer. Panel B: Mutations found within the binding site of the reverse primer.

4.4 Objective 3: To determine the extent of antigenic variations in the genes encoding the glycoprotein and nucleoprotein.

4.4.1 Analysis of the glycoprotein gene antigenic sites

The amino acid sequences of the G gene antigenic sites located between amino acids 20-439 for the RABV vaccine strains (PM, PV, and CVS) commonly available in

Kenya are shown in **Table 4.2**. The sequences were 100% homologous to the G genes of the study genomes.

Table 4.2: Amino acid sequences of the G gene antigenic sites for the RABV vaccine strains

	Site II-b 34-42	Site II-a 198-200	Site I 226-231	Site IV 251	Site G5 261-264	Site III 338-338	Site G1 342-343
1. PASTEUR-G-Vacc-str	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
2. PITMOORE-G-Vacc-str	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
3. CVS-G-Vacc-str	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
4. SIA-BOV-010	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
5. SIA-BOV-009	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
6. SIA-oVI-001	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
7. SIA-CAN-025	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
8. SIA-bOV-005	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
9. SIA-CAN-043	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
10. SIA-CAN-048	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
11. SIA-CAN-037	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
12. SIA-CAN-041	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
13. SIA-CAN-060	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
14. SIA-CAN-059	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
15. SIA-CAN-046	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
16. SIA-BOV-014	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
17. SIA-CAN-026	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
18. SIA-CAN-018	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
19. SIA-CAN-019	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
20. SIA-CAN-055	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
21. SIA-CAN-058	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
22. SIA-CAN-052	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
23. SIA-CAN-044	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
24. SIA-CAN-035	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
25. SIA-CAN-050	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
26. SIA-CAN-033	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
27. SIA-CAN-047	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
28. SIA-BOV-026	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
29. SIA-CAN-016	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
30. SIA-CAN-032	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
31. SIA-CAN-034	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
32. MAK-CAN-005	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
33. SIA-BOV-012	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
34. SIA-CAN-042	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
35. SIA-CAN-049	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
36. SIA-CAN-022	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
37. SIA-CAN-028	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
38. SIA-CAN-029	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
39. SIA-CAN-008	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
40. SIA-CAN-040	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
41. MAK-cAN-030	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
42. MAK-CAP-012	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
43. MAK-CAN-007	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
44. MAK-CAN-050	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
45. MAK-CAN-014	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
46. MAK-CAN-052	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
47. MAK-CAP-017	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
48. MAK-CAP-033	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
49. MAK-BOV-021	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
50. MAK-CAN-009	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
51. MAK-CAN-045	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
52. MAK-CAN-049	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
53. MAK-CAN-051	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
54. MAK-BOV-022	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
55. MAK-CAN-033	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
56. MAK-CAN-025	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
57. MAK-BOV-016	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
58. SIA-BOV-003	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
59. MAK-CAN-038	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
60. MAK-CAP-011	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
61. MAK-CAN-023	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
62. MAK-CAN-037	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
63. MAK-BOV-018	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
64. MAK-CAP-021	SPIDIHHLX	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
65. MAK-CAN-019	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
66. MAK-CAN-001	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
67. Mak-Can-002	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
68. Mak-Can-024	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
69. MAK-CAP-010	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
70. MAK-CAN-044	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
71. Mak-Cap-022	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
72. MAK-FEL-002	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
73. MAK-CAP-005	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
74. MAK-CAP-008	SPIDIHHLS	MPE	CGFVDE	G	AXOT	FGKAYTIF	ME
75. MAK-CAN-011	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
76. Mak-Can-004	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
77. Mak-Fel-001	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
78. MAK-CAN-048	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
79. MAK-CAN-028	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
80. MAK-CAN-042	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
81. MAK-BOV-020	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME
82. MAK-CAN-029	SPIDIHHLS	MPE	CGFVDE	G	AMOT	FGKAYTIF	ME

However, differences in sequence homology outside the antigenic sites were identified in other parts of the G gene, including the signal peptide (amino acids 1-19), transmembrane region (amino acids 440-461), cytoplasmic domains (amino acids 462-505) and other positions within the ectodomain area outside the antigenic sites. Notably, the amino acid residues that are significant for pathogenicity: aa37,

aa194, aa242, aa255, aa265, aa268, aa330lys, and aa333arg, were conserved among the study isolates. On pairwise comparison, the full G gene sequences of the study genomes showed a minimum of 90% similarity with the three vaccine strains.

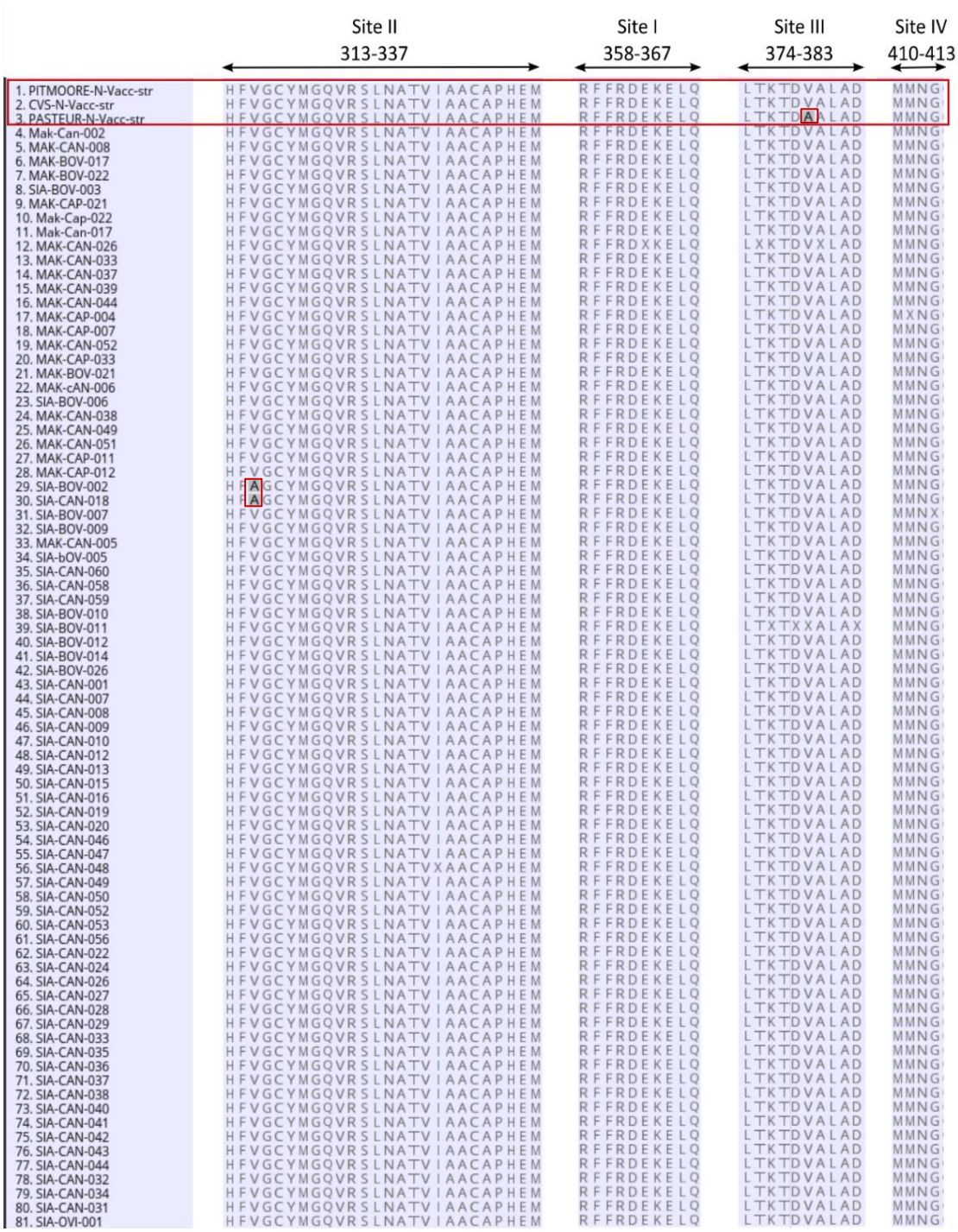
Table 4.3: The glycoprotein gene of wild RABV show >90% homology to the vaccine strains

Percentage Pairwise identity of the Glycoprotein gene from our study samples to the RABV Vaccine strains		
Pasteur Vaccine strain (PV)	Challenge Virus Standard (CVS) Strain	Pitmoore-1503 Vaccine strain (PM)
≥93.0%	≥93.3%	≥92.2%

4.4.2 Analysis of the nucleoprotein gene antigenic sites

In contrast to the G gene amino acid sequences that were 100% homologous to the vaccine antigenic sites, the study N genes were variant at antigenic sites II in two study samples (Kenya/SIA-CAN-018/2021 and Kenya/SIA-BOV-007/202) where alanine was substituted by valine at position 315 (A315V), while the three vaccine strains and all the other study samples had alanine. In addition, while all the study samples, PM and CVS had valine at position 379 of antigenic site III, the PV was variant with V379A.

Table 4.4: Amino acid substitutions of nucleoprotein encoding gene from the isolated samples compared to vaccine strains



On pairwise comparison, the complete N gene of the genomes under study showed a minimum of 90% homology with the three vaccine strains

Table 4.5: The nucleoprotein of wild RABV show >90% homology to the vaccine strains

Percentage Pairwise identity of the Nucleoprotein gene from our study samples to the RABV Vaccine strains		
Pasteur Vaccine strain (PV)	Challenge Virus Standard (CVS) Strain	Pitmoore-1503 Vaccine strain (PM)
≥97.6%	≥97.8%	≥98.5%

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Rabies continues to be a substantial hazard to public health owing to a rise of increasingly aggressive strains, their adaptability within new hosts, and their spread to different geographical areas (Okonko *et al*., 2010). This study used whole genome sequencing to investigate the diversity of RABVs in two geographically isolated regions of Kenya, specifically Siaya county in the western region and Makueni county in the eastern region **(Figure 3.1)**, and to explore whether there are mutations in antigenic sites of the wild RABV strains that would negatively impact efficacy of the currently used RABV vaccines. These two counties were selected because they consistently report the highest incidence of human rabies deaths among the projected 2,000 mortalities reported annually in Kenya (Republic of Kenya, 2014). Furthermore, both counties are actively working towards achieving the global objective of zero human rabies cases by 2030 (Consortium, 2019).

A cohort of 164 samples was tested for RABV RNA using qRT-PCR targeting the L gene. Out of these samples, 156 tested positive (see **Appendix 2** for detailed results). However, eight samples did not amplify for the L gene. As these samples originated from animals displaying indicative symptoms of rabies and had tested positive in the RABV antigen test, further screening was conducted using heminested PCR targeting the N gene. All eight samples tested positive in the N gene heminested PCR. The N gene, known for its higher conservation (Meng *et al.*, 2007), serves as a broader target for RABV detection, complementing the L gene assay. Notably, multiple mutations were identified in the primer binding sites of the L gene **(Table 4.1)**, providing a plausible explanation for the initially negative L gene qRT-PCR results. These genetic variations highlight the importance of targeting multiple genes in RABV diagnostics by PCR.

Out of the 164 samples collected for the study, 107 were from dogs (see **Figure 4.2** and **Appendix 2**). It is noteworthy that the remaining 54 samples, obtained from other domestic animal hosts, all had a documented history of being bitten by a rabid domestic dog. This observation underscores the crucial role played by domestic dogs

in rabies transmission. Compared to other hosts such as bats and raccoon-skunk-maintained RABV, domestic canine RABV is the most widespread and a major contributor to the approximately 59,000 human deaths reported annually worldwide (Hampson *et al*., 2015). In Kenya, as well as in many other regions of Africa and Asia, domestic dogs serve as the primary reservoir and vector for rabies transmission, accounting for over 99 percent of the deaths reported annually (Brunker *et al*., 2015; Campbell *et al.*, 2022). This observation underscores the urgent need for comprehensive measures aimed at controlling and preventing the transmission of domestic canine RABV. Such measures could include widespread, well-coordinated mass vaccination campaigns targeting the canine population, promoting responsible pet ownership practices, community education on rabies prevention, and improved surveillance systems to detect and respond to emerging rabies cases. Additionally, besides domestic dogs, which accounted for 65% of the infected hosts (107/164), cattle and goats represented 18% and 14% respectively, aligning with findings reported by Bitek *et al*. in 2018. It is crucial to raise awareness among livestock owners about the potential risks associated with dog-to-ruminant transmission and advocate for the vaccination of dogs to minimize RABV transmission

Previous phylogenetic studies performed using single genes of RABV such as N and G have shown that there are multiple geographical groupings of RABV in Africa, with viruses from eastern Africa having a different genetic makeup than those from western, central, and southern regions of the continent (Brunker *et al*., 2012). The present study utilized whole genomes and single genes to characterize the diversity of the RABVs in western and eastern Kenya. Of the three major clades of RABV found in Africa, namely, Africa 2, Africa 3, and Cosmopolitan, only the Africa 1a and 1b sub-clades in the Cosmopolitan clade were detected (**Figure 4.3** and **Figure 4.4**). Overwhelmingly, the RABV from Makueni, eastern region clustered with the Africa 1b sub-clade whereas RABV from Siaya, western Kenya clustered with the Africa 1a sub-clade. Our study confirms the inferences of previous research that shows an apparent geographical isolation between the RABV strains in eastern and western Kenya (Gigante *et al*., 2020). This geographical isolation is probably due to the multiple landscape features **(Figure 3.1)** such as Lake Victoria, lakes along the Great Rift Valley, escarpments on either side of the Rift Valley, mountains ranges such as the Aberdare, and the Nandi hills that restrict free movement of animals

between the regions, thus promoting localized viral evolution. This restriction has led to the development of unique viral strains in each region, demonstrating how geographical isolation drives genetic divergence and influences the evolutionary trajectory of the virus. Similar geographical isolation affecting genetic drift has also been observed in raccoon rabies in the eastern United States (Wheeler & Waller, 2008).

Outlier Africa 1b genomes that were in western Kenya (**Figure 4.3, Panel C and Figure 4.4,** blue stars) and Africa 1a in eastern Kenya (**Figure 4.3, Panel B** and **Figure 4.4,** red stars) were however observed. This observation indicates that the restriction in viral migration is not absolute. National roads and bridges, which traverse geographical barriers, can serve as pathways for the movement of rabid animals or reservoirs of the RABV (Denduangboripant *et al.*, 2005). It is crucial to highlight that this movement is intricately linked to human mobility, occurring as individuals travel from one region to another. In light of these findings, there is a clear emphasis on the importance of implementing regulated and controlled movement of animals. This strategic approach becomes essential to mitigate the risk of disease transmission, recognizing the dynamic nature of viral migration and the potential for outliers in different geographic regions.

An interesting finding was the dissimilarity between the closely related strains of the RABV in western Kenya and other Africa 1a sub-clade members in neighboring countries. For, example, the closest member of the western Kenya Africa 1a sub-clade members was a sequence from Sudan, and even so, the strain is very distinct (**Figure 4.3, Panel C**). These observations suggest that the members of the Africa 1a circulating in Kenya have evolved separately since introduction, most likely from northern, central, and western Africa regions. The other likely explanation for this dissimilarity is the under-sampling of Africa 1a genomes. For example, while our study samples were collected in 2021 and 2022, genomes from the other clade members were collected between 1986 and 2015.

Unlike the western Kenya Africa 1a genomes, the eastern Kenya Africa 1b genomes were less homogeneous and branched into two groups, a major group of 65 genomes that clustered together and a minor group of 8 genomes that clustered with genomes that had been collected previously from Kericho and Nakuru, both in Kenya (**Figure**

4.3, Panel B). The presence of the Africa 1b subclade in locations near western Kenya, such as Nakuru and Kericho, indicates an ongoing encroachment of the 1b subclade into western Kenya. This observation challenges the notion of absolute geographical isolation between eastern and western Kenya, highlighting the dynamic nature of viral distribution and the potential for genetic interchange between these regions. Notably, the genomes from Makueni county, eastern Kenya showed closest relation to RABV genomes from Tanzania.

The phylogeographic distribution of RABV observed in this study, such as the presence of Africa 1b in Makueni county, eastern Kenya, with its closest relatives in Tanzania, (**Figure 4.3, Panel B)** and **Figure 4.4)** and Africa 1a in Siaya county, western Kenya, with closest relatives in genomes previously obtained from Sudan (**Figure 4.3, Panel C** and **Figure 4.4)**, signifies a cross-border exchange of RABV strains between Kenya and its neighboring nations. This aligns with historical records that have documented instances of cross-border rabies transmissions, as seen in the 1980s from Tanzania to Kenya (Zoonotic Disease Unit report, Republic of Kenya, 2014). Human activities, including the movement of animals, some of which may be reservoirs of RABV, likely facilitate these cross-border transmissions. Similar cross-border RABV migrations have been documented in other African and Asian countries (Brunker *et al*., 2015). These findings underscore the urgent need for collaborative efforts that extend beyond Kenya's borders to effectively eliminate rabies. Collaborating with neighboring nations becomes crucial, as rabies control cannot be achieved solely through the efforts of a single country, given the likelihood of reintroduction from surrounding endemic countries (Gongal & Wright, 2011). To reach the global target of eliminating all cases of RABV by 2030, Kenya's rabies control efforts must encompass collaboration with neighboring countries.

By employing finer resolution using standardized universal lineage definition approaches as proposed by (Campbell *et al*., 2022), the study of whole genome sequences further resolved into 13 distinct lineages (8 in western Kenya and 5 in eastern Kenya) and one unclassified lineage from eastern Kenya (**Figure 4.5**). The N and G single genes lacked the discriminatory power for lineage assignments. The identification of multiple lineages within the sub-clades indicates ongoing genetic diversification within the RABV population. Each lineage represents a unique set of

mutations that have accumulated during the transmission process. The study does not definitively establish whether lineage diversification in RABV correlates with virulence, host range, or transmissibility, leaving these aspects to be explored in future research. Nonetheless, these findings underscore the critical importance of genetic surveillance to comprehend the epidemiological implications associated with the derived lineages. The comprehensive understanding provided by whole genome sequences highlights the significance of employing advanced methodologies for more accurate insights into the genetic dynamics of RABV populations.

The presence of geographically distinct subclades and lineages of the RABV raises the question of whether there are functionally important amino acid changes between these variants that could impact immune response to the currently employed vaccines in Kenya. The G protein has a crucial role in the neurotropism of the RABV and is a primary target for antibodies that neutralize the virus (Wang *et al*., 2019). The nucleoprotein is also a target for RABV-neutralizing antibodies (Kgaladi *et al.*, 2013; Ajorloo *et al*., 2018). In this study, we investigated whether the wild RABV strains observed in the current study contain substitutions in the antigenic sites of N and G genes that could affect the efficacy of the currently used rabies vaccines. On aligning the study's G genes to the same gene in the PV, CVS, and PM vaccine strains, a 100% homology was found (**Table 4.2).** A similar alignment and comparison using the N gene of study genomes revealed a variance at antigenic sites II in two study samples where valine was substituted to alanine at position 315 (A315V), while the three vaccine strains and all the other study samples had alanine (**Table 4.4**). In addition, while all the study samples, PM and CVS had valine at position 379 of antigenic site III, the PV was variant with A379V. Although these replacements are few to impact vaccine efficacy, they raise concerns about potential cumulative changes that could eventually impact vaccine efficacy and underscore the need for continued monitoring of such changes. Monitoring for potential mutations in the antigenic vaccine sites helps assess their potential implications for vaccine effectiveness (Wang *et al.*, 2019). By keeping track of changes in antigenic sites, public health officials can identify any emerging strains that may require updates to the vaccine strains or modifications in vaccination strategies.

The high level of homology observed between the antigenic sites of the study wild-type RABV and the vaccine strains indicates that these vaccines are predicted to be effective in controlling the disease (**Table 4.3** and **Table 4.5**). This suggests that the endemicity of RABV in Siaya and Makueni is more likely to be due to the lack of vaccine utilization rather than the ineffectiveness of the vaccines. The findings strongly suggest that implementing well-coordinated vaccination programs could effectively control the disease in the studied areas. For example, the estimated dog population in Kenya is five million, with 70% of the dogs having an owner (Ngugi *et al.*, 2018). However, in 2013, only 125,000 doses of animal vaccines were procured by the Directorate of Veterinary Services (Republic of Kenya, 2014), indicating that the number of doses procured does not correlate with the dog population. This indicates a potential discrepancy between the actual vaccination needs and the procurement strategy. While there are no official statistics on rabies vaccine coverage, inadequate vaccines, expensive costs, diminished awareness about the extent of the rabies problem, and insufficient inter-sectoral collaboration are identified as contributing factors to low vaccine coverage (Republic of Kenya, 2014). In Kenya, government-sponsored vaccination campaigns are conducted once a year, where the vaccine is provided for free. However, on other days, dog owners have to pay for the vaccination, which can be expensive, ranging from Kenya Sh5,000 to Sh7,500. This cost makes it challenging for many dog owners to afford regular vaccinations. Insufficient or inconsistent vaccination rates among the intended population can have a negative impact and contribute to the ongoing presence of rabies within the country.

While the study provides important insights into rabies in Kenya, some limitations are worth highlighting. One, the study focused on specific regions of Kenya, namely Makueni county, in eastern region of Kenya, and Siaya county, in western part of Kenya. While genomes from these regions provided valuable information needed for understanding the local dynamics of rabies, the findings may not be representative of the entire country or other geographic areas that may have different ecological or epidemiological characteristics. More broad studies will be required to shed light on RABV genetic diversity across the country. Two, the study used samples largely from domestic dogs that were already rabid and did not investigate the source of dog rabies.

Thus, while there is evidence of transmission from dog to other domestic livestock, there is zero information on where the dogs` rabies came from.

5.2 Conclusions

This study provides significant insights into the genetic variability of RABVs in counties located in both the western and eastern regions of Kenya, as well as their antigenic variations.

1. Rabies in Makueni County in the eastern region and Siaya County in the western region is attributed to RABVs from the cosmopolitan clade, specifically within the African 1a and African 1b sub-clades.
2. While a diverse range of animal species can be infected with rabies, domestic dogs were identified as the key reservoirs and transmitters of the virus, contributing to spillover infections in livestock animals.
3. Our findings demonstrated a clear geographical isolation between the RABV populations in eastern and western Kenya. The RABVs from the eastern region clustered predominantly with the African 1b sub-clade, while those from the western region clustered with the African 1a sub-clade. This phylogeographical isolation is attributable to the geographical features that restrict animal movement, thus allowing localized viral evolution.
4. Nevertheless, limited viral migration was observed, possibly mediated by human transportation of asymptomatic infected animals across county borders.
5. The study found limited antigenic variations between the currently used vaccines and the wild-type RABV strains, as well as a high degree of homology. The high homology suggests that the immune response elicited by the vaccines should be effective against the wild-type viruses.

5.3 Recommendations

The findings of this study have informed the following recommendations.

1. For PCR diagnostics, targeting multiple genes is essential to circumvent amplification failure due to genetic variations in one gene hence reducing the risk of false negatives, which occur when a test fails to detect a pathogen despite its presence.

2. There is a need for effective and widespread vaccination of domestic dogs, the primary transmitter of RABV in Kenya. Ensuring that domestic dogs receive proper and timely rabies vaccinations will significantly reduce the risk of rabies transmission to other animals.
3. There is need for enhanced genomic surveillance beyond Makueni and Siaya counties which are the hot spots of rabies in Kenya. This way, it can be ascertained whether the phylogeographic isolation of RABV observed in this study holds true for the whole country. Such information is crucial in understanding transmission patterns and therefore helps in rabies control programs and elimination strategies by providing valuable insights into the transmission dynamics, virus evolution, and effectiveness of control measures. By monitoring virus evolution, scientists can anticipate the emergence of novel viral variants and adopt control strategies accordingly. Genomic surveillance also helps assess the effectiveness of rabies vaccines by comparing genetic sequences of circulating RABV with vaccine strains.
4. The government needs to enforce controlled animal movements between different regions to prevent the introduction of viruses into new areas. Unregulated animal transport can lead to outbreaks in regions where the disease was previously absent, potentially causing significant health and ecological impacts. By controlling these movements, the government can mitigate the risk of new virus introductions and manage the spread of infectious diseases more effectively.
5. To understand the hidden circle of RABV transmission in domestic dogs, it will be important to conduct detailed epidemiological investigations in order to understand potential transmission patterns between domestic canines, domestic animals and wild animals.
6. Continuous surveillance of potential mutations in antigenic sites is recommended to assess their cumulative impact over time. This proactive approach will help ensure that any emerging changes are detected early, allowing for timely updates to vaccine formulations if necessary, thereby maintaining their effectiveness in protecting against rabies.

REFERENCES

Ahmad, E., Iqbal, M. S., & Qasim, M. (2019). Rabies virus: A mini review. *Life Science Journal of Pakistan*, *1*(1), 40–43.

Ajorloo, M., Mirzaei, H., Sadeghi, Y., Tarban, N., Soltani, S., Mohammadi, F. S., Davarinejad, P., Roudy, M. A., Jahantigh, H. R., & Abouhamzeh, K. (2018). Evaluation and phylogenetic analysis of regular rabies virus vaccine strains. *Archives of Iranian Medicine*, *21*(3), 101–110.

Araújo, D. B., Langoni, H., Almeida, M. F., & Megid, J. (2008). Heminested reverse-transcriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. *BMC Research Notes*, *1*(1), 1–6.

Aravindh Babu, R. P., Manoharan, S., Ramadass, P., & Chandran, N. D. J. (2012). Evaluation of RT-PCR assay for routine laboratory diagnosis of rabies in post mortem brain samples from different species of animals. *Indian Journal of Virology*, *23*(3), 392–396.

Asaye, M., & Getachew, N. (2014). Rabies virus proteins and their mechanism of pathogenecity. *Indian Journal of Drugs*, *2*(3), 89–95.

Bihon, A., Meresa, D., & Tesfaw, A. (2020). Rabies: knowledge, attitude and practices in and around South Gondar, North West Ethiopia. *Diseases*, *8*(1), 5.

Bitek, A. O., Osoro, E., Munyua, P. M., Nanyingi, M., Muthiani, Y., Kiambi, S., Muturi, M., Mwatondo, A., Muriithi, R., & Cleaveland, S. (2018). A hundred years of rabies in Kenya and the strategy for eliminating dog-mediated rabies by 2030. *AAS Open Research*, *1*.

Bourhy, H., Reynes, J.-M., Dunham, E. J., Dacheux, L., Larrous, F., Huong, V. T. Q., Xu, G., Yan, J., Miranda, M. E. G., & Holmes, E. C. (2008). The origin and phylogeography of dog rabies virus. *The Journal of General Virology*, *89*(Pt 11), 2673.

Brightman, C. (2012). Rabies: an acute viral infection. *Trends in Urology & Men's Health*, *3*(5), 31–33.

Brunker, K., Hampson, K., Horton, D. L., & Biek, R. (2012). Integrating the landscape epidemiology and genetics of RNA viruses: rabies in domestic dogs as a model. *Parasitology*, *139*(14), 1899–1913.

Brunker, K., Jaswant, G., Thumbi, S. M., Lushasi, K., Lugelo, A., Czupryna, A. M., Ade, F., Wambura, G., Chuchu, V., & Steenson, R. (2020). Rapid in-country sequencing of whole virus genomes to inform rabies elimination programmes. *Wellcome Open Research*, *5*.

Brunker, K., Marston, D. A., Horton, D. L., Cleaveland, S., Fooks, A. R., Kazwala, R., Ngeleja, C., Lembo, T., Sambo, M., & Mtema, Z. J. (2015). Elucidating the phylodynamics of endemic rabies virus in eastern Africa using whole-genome sequencing. *Virus Evolution*, *1*(1), vev011.

Brunker, K., & Mollentze, N. (2018). Rabies virus. *Trends in Microbiology*, *26*(10), 886–887.

Brunker, K., Nadin-Davis, S., & Biek, R. (2018). Genomic sequencing, evolution and molecular epidemiology of rabies virus. *Revue Scientifique et Technique (International Office of Epizootics)*, *37*(2), 401–408.

Campbell, K., Gifford, R. J., Singer, J., Hill, V., O'toole, A., Rambaut, A., Hampson, K., & Brunker, K. (2022). Making genomic surveillance deliver: A lineage classification and nomenclature system to inform rabies elimination. *PLoS Pathogens*, *18*(5), e1010023.

Caraballo, D. A., Lema, C., Novaro, L., Gury-Dohmen, F., Russo, S., Beltrán, F. J.,

Palacios, G., & Cisterna, D. M. (2021). A Novel Terrestrial Rabies Virus Lineage Occurring in South America: Origin, Diversification, and Evidence of Contact between Wild and Domestic Cycles. *Viruses*, *13*(12), 2484.

Ceballos, N. A., Morón, S. V., Berciano, J. M., Nicolás, O., López, C. A., Juste, J., Nevado, C. R., Setién, Á. A., & Echevarría, J. E. (2013). Novel lyssavirus in bat, Spain. *Emerging Infectious Diseases*, *19*(5), 793.

Coertse, J., Geldenhuys, M., le Roux, K., & Markotter, W. (2021). Lagos Bat Virus, an Under-Reported Rabies-Related Lyssavirus. *Viruses*, *13*(4), 576.

Coertse, J., Weyer, J., Nel, L. H., & Markotter, W. (2010). Improved PCR methods for detection of African rabies and rabies-related lyssaviruses. *Journal of Clinical Microbiology*, *48*(11), 3949–3955.

Consortium, W. H. O. R. M. (2019). Zero human deaths from dog-mediated rabies by 2030: perspectives from quantitative and mathematical modelling. *Gates Open Research*, *3*.

Davis, B. M., Rall, G. F., & Schnell, M. J. (2015). Everything you always wanted to know about rabies virus (but were afraid to ask). *Annual Review of Virology*, *2*, 451–471.

Denduangboripant, J., Wacharapluesadee, S., Lumlertdacha, B., Ruankaew, N., Hoonsuwan, W., Puanghat, A., & Hemachudha, T. (2005). Transmission dynamics of rabies virus in Thailand: implications for disease control. *BMC Infectious Diseases*, *5*(1), 1–11.

Dodet, B., Tejiokem, M. C., Aguemon, A.-R., & Bourhy, H. (2015). Human rabies deaths in Africa: breaking the cycle of indifference. *International Health*, *7*(1), 4–6.

Evans, J. S., Selden, D., Wu, G., Wright, E., Horton, D. L., Fooks, A. R., & Banyard, A. C. (2018). Antigenic site changes in the rabies virus glycoprotein dictates functionality and neutralizing capability against divergent lyssaviruses. *Journal of General Virology*, *99*(2), 169–180.

Faye, M., Dacheux, L., Weidmann, M., Diop, S. A., Loucoubar, C., Bourhy, H., Sall, A. A., & Faye, O. (2017). Development and validation of sensitive real-time RT-PCR assay for broad detection of rabies virus. *Journal of Virological Methods*, *243*(2017), 120–130. https://doi.org/10.1016/j.jviromet.2016.12.019

Fisher, C. R., Streicker, D. G., & Schnell, M. J. (2018). The spread and evolution of rabies virus: conquering new frontiers. *Nature Reviews Microbiology*, *16*(4), 241–255.

Fooks, A. R., Banyard, A. C., Horton, D. L., Johnson, N., McElhinney, L. M., & Jackson, A. C. (2014). Current status of rabies and prospects for elimination. *The Lancet*, *384*(9951), 1389–1399.

Gigante, C. M., Yale, G., Condori, R. E., Costa, N. C., Long, N. Van, Minh, P. Q., Chuong, V. D., Tho, N. D., Thanh, N. T., & Thin, N. X. (2020). Portable rabies virus sequencing in canine rabies endemic countries using the Oxford Nanopore MinION. *Viruses*, *12*(11), 1255.

Gongal, G., & Wright, A. E. (2011). Human rabies in the WHO Southeast Asia Region: forward steps for elimination. *Advances in Preventive Medicine*, *2011*.

Goravey, W., Husain, A., Ali, G. A., Al Maslamani, M. A., & Ziglam, H. (2021). Antemortem diagnosis of human rabies: A case report. *Clinical Case Reports*, *9*(2), 711–713.

Goto, H., Minamoto, N., Ito, H., Ito, N., Sugiyama, M., Kinjo, T., & Kawai, A. (2000). Mapping of epitopes and structural analysis of antigenic sites in the

nucleoprotein of rabies virus. *Journal of General Virology*, *81*(1), 119–127.
Hampson, K., Coudeville, L., Lembo, T., Sambo, M., Kieffer, A., Attlan, M., Barrat, J., Blanton, J. D., Briggs, D. J., Cleaveland, S., Costa, P., Freuling, C. M., Hiby, E., Knopf, L., Leanes, F., Meslin, F. X., Metlin, A., Miranda, M. E., Müller, T., Nel, L. H., Recuenco, S., Rupprecht, C. E., Schumacher, C., Taylor, L., Vigilato, M. A., Zinsstag, J., & Dushoff, J. (2015). *Estimating the global burden of endemic canine rabies*. PLOS Neglected Tropical Diseases, 9(4), e0003709. https://doi.org/10.1371/journal.pntd.0003709
Hayman, D. T. S., Johnson, N., Horton, D. L., Hedge, J., Wakeley, P. R., Banyard, A. C., Zhang, S., Alhassan, A., & Fooks, A. R. (2011). Evolutionary history of rabies in Ghana. *PLoS Neglected Tropical Diseases*, *5*(4), e1001.
Horie, M., Akashi, H., Kawata, M., & Tomonaga, K. (2021). Identification of a reptile lyssavirus in Anolis allogus provided novel insights into lyssavirus evolution. *Virus Genes*, *57*(1), 40–49.
Kgaladi, J., Nel, L. H., & Markotter, W. (2013). Comparison of pathogenic domains of rabies and African rabies-related lyssaviruses and pathogenicity observed in mice. *Onderstepoort Journal of Veterinary Research*, *80*(1), 1–13.
Klein, A., Calvelage, S., Schlottau, K., Hoffmann, B., Eggerbauer, E., Müller, T., & Freuling, C. M. (2021). Retrospective enhanced bat lyssavirus surveillance in Germany between 2018–2020. *Viruses*, *13*(8), 1538.
Kuzmin, I. V, Bozick, B., Guagliardo, S. A., Kunkel, R., Shak, J. R., Tong, S., & Rupprecht, C. E. (2011). Bats, emerging infectious diseases, and the rabies paradigm revisited. *Emerging Health Threats Journal*, *4*(1), 7159.
Kuzmin, I. V, Mayer, A. E., Niezgoda, M., Markotter, W., Agwanda, B., Breiman, R. F., & Rupprecht, C. E. (2010). Shimoni bat virus, a new representative of the Lyssavirus genus. *Virus Research*, *149*(2), 197–210.
Kuzmin, I. V, Shi, M., Orciari, L. A., Yager, P. A., Velasco-Villa, A., Kuzmina, N. A., Streicker, D. G., Bergman, D. L., & Rupprecht, C. E. (2012). Molecular inferences suggest multiple host shifts of rabies viruses from bats to mesocarnivores in Arizona during 2001–2009. *PLoS Pathogens*, *8*(6), e1002786.
Liu, X., Yang, Y., Sun, Z., Chen, J., Ai, J., Dun, C., Fu, Z. F., Niu, X., & Guo, X. (2014). A recombinant rabies virus encoding two copies of the glycoprotein gene confers protection in dogs against a virulent challenge. *PloS One*, *9*(2), e87105.
Ma, J., Li, S., Yang, Y., Wang, Q., & Huo, Y. (2019). Epidemiological and phylogenetic analysis of rabies virus isolated from humans in Henan province, China. *Archives of Virology*, *164*(11), 2811–2817.
Maki, J., Guiot, A.-L., Aubert, M., Brochier, B., Cliquet, F., Hanlon, C. A., King, R., Oertli, E. H., Rupprecht, C. E., & Schumacher, C. (2017). Oral vaccination of wildlife using a vaccinia–rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): a global review. *Veterinary Research*, *48*(1), 1–26.
Marston, D. A., Jennings, D. L., MacLaren, N. C., Dorey-Robinson, D., Fooks, A. R., Banyard, A. C., & McElhinney, L. M. (2019). Pan-lyssavirus real time RT-PCR for rabies diagnosis. *JoVE (Journal of Visualized Experiments)*, *149*, e59709.
Matsumoto, T., Nanayakkara, S., Perera, D., Ushijima, S., Wimalaratne, O., Nishizono, A., & Ahmed, K. (2017). Terrestrial animal-derived rabies virus in a juvenile Indian flying fox in Sri Lanka. *Japanese Journal of Infectious Diseases*,

70(6), 693–695.

Mayes, B., & Rupprecht, C. E. (2015). Direct fluorescent antibody test for rabies diagnosis. In *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention, Volume 2* (pp. 83–92). Elsevier.

Meng, S.-L., Yan, J.-X., Xu, G.-L., Nadin-Davis, S. A., Ming, P.-G., Liu, S.-Y., Wu, J., Ming, H.-T., Zhu, F.-C., & Zhou, D.-J. (2007). A molecular epidemiological study targeting the glycoprotein gene of rabies virus isolates from China. *Virus Research*, *124*(1–2), 125–138.

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., Von Haeseler, A., Lanfear, R., & Teeling, E. (2020). IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*, *37*(5), 1530–1534. https://doi.org/10.1093/molbev/msaa015

Morimoto, K., Hooper, D. C., Carbaugh, H., Fu, Z. F., Koprowski, H., & Dietzschold, B. (1998). Rabies virus quasispecies: implications for pathogenesis. *Proceedings of the National Academy of Sciences*, *95*(6), 3152–3156.

Müller, T., & Freuling, C. M. (2020). Rabies in terrestrial animals. In *Rabies* (pp. 195–230). Elsevier.

Muriuki, J. B. (2016). *Knowledge, Attitude and Practices on Rabies in Kisumu and Siaya Counties, Kenya*. University of Nairobi.

Ngugi, J. N., Maza, A. K., Omolo, O. J., & Obonyo, M. (2018). Epidemiology and surveillance of human animal-bite injuries and rabies post-exposure prophylaxis, in selected counties in Kenya, 2011–2016. *BMC Public Health*, *18*(1), 1–9.

OIE. (2009). *Rabies - Hydrophobia, Lyssa*. *RABS_2009*(October 2009), 1–9.

Oketch, A. (2022). Nairobi, Nakuru and six other counties register cases of rabies. *Daily Nation Newspaper*, 1–56. https://nation.africa/kenya/health/nairobi-nakuru-and-six-other-counties-register-cases-of-rabies-4008886

Okonko, I. O., Adedeji, O. B., Babalola, E. T., Fajobi, E. A., Fowotade, A., & Adewale, O. G. (2010). Why is there still rabies in the world?-an emerging microbial and global health threat. *Global Veterinaria*, *4*(1), 34–50.

World Health Organization. (2015). *The control of neglected zoonotic diseases: from advocacy to action: report of the fourth international meeting held at WHO Headquarters, Geneva, Switzerland, 19-20 November 2014*.

World Health Organization. (2018). *WHO expert consultation on rabies: third report* (Vol. 1012). World Health Organization.

Pant, G. R., Lavenir, R., Wong, F. Y. K., Certoma, A., Larrous, F., Bhatta, D. R., Bourhy, H., Stevens, V., & Dacheux, L. (2013). Recent emergence and spread of an Arctic-related phylogenetic lineage of rabies virus in Nepal. *PLoS Neglected Tropical Diseases*, *7*(11), e2560.

Pantha, S., Subedi, D., Poudel, U., Subedi, S., Kaphle, K., & Dhakal, S. (2020). Review of rabies in Nepal. *One Health*, *10*, 100155.

Rahmahani, J., & Rantam, F. A. (2018). Antigenic Site Of Glycoprotein Encoding Gene In Rabies Virus Isolate From Indonesia. *Philippine Journal of Veterinary Medicine*, *55*, 9–16.

Rahmahani, J., Suwarno, S., Yuniarti, W. M., & Rantam, F. A. (2019). Antigenic site of nucleoprotein gene from Indonesian rabies virus isolates. *Veterinary World*, *12*(5), 724.

Ramsey, E. C. (2017). *Rabid Response: Unpacking the history of the rabies virus to*

examine resource allocation.

REPUBLIC OF KENYA. (2014). *Ministry of Health and Ministry of Agriculture, Livestock and Fisheries Strategic Plan for the Elimination of Human Rabies in Kenya 2014-2030 REPUBLIC OF KENYA*. 1–52.

Rupprecht, C. E., Fooks, A. R., Abela-Ridder, B., & World Health Organization. (2018). *Laboratory techniques in rabies, volume 1*. World Health Organization.Sabeta, C. (2015). *Role of the glycoprotein G in lyssavirus pathogenicity*. *10*, 1177–1184.

Sadeuh-Mba, S. A., Momo, J. B., Besong, L., Loul, S., & Njouom, R. (2017). Molecular characterization and phylogenetic relatedness of dog-derived Rabies Viruses circulating in Cameroon between 2010 and 2016. *PLoS Neglected Tropical Diseases*, *11*(10), e0006041.

Sararat, C., Changruenngam, S., Chumkaeo, A., Wiratsudakul, A., Pan-Ngum, W., & Modchang, C. (2022). The effects of geographical distributions of buildings and roads on the spatiotemporal spread of canine rabies: An individual-based modeling study. *PLOS Neglected Tropical Diseases*, *16*(5), e0010397.

Schnell, M. J., McGettigan, J. P., Wirblich, C., & Papaneri, A. (2010). The cell biology of rabies virus: using stealth to reach the brain. *Nature Reviews Microbiology*, *8*(1), 51–61.

Scott, T. P., & Nel, L. H. (2021). Lyssaviruses and the fatal encephalitic disease rabies. *Frontiers in Immunology*, 5201.

Shipley, R., Wright, E., Lean, F. Z. X., Selden, D., Horton, D. L., Fooks, A. R., & Banyard, A. C. (2021). Assessing rabies vaccine protection against a novel lyssavirus, Kotalahti bat lyssavirus. *Viruses*, *13*(5), 947.

Singh, R., Singh, K. P., Cherian, S., Saminathan, M., Kapoor, S., Manjunatha Reddy, G. B., Panda, S., & Dhama, K. (2017). Rabies–epidemiology, pathogenesis, public health concerns and advances in diagnosis and control: a comprehensive review. *Veterinary Quarterly*, *37*(1), 212–251.

Sinkie, Y., & Mekelle, E. (2017). *Review On Rabies Infection And Pathogenesis*. Mekelle University.

Streicker, D. G., Altizer, S. M., Velasco-Villa, A., & Rupprecht, C. E. (2012). Variable evolutionary routes to host establishment across repeated rabies virus host shifts among bats. *Proceedings of the National Academy of Sciences*, *109*(48), 19715–19720.

Talbi, C., Holmes, E. C., De Benedictis, P., Faye, O., Nakoune, E., Gamatie, D., Diarra, A., Elmamy, B. O., Sow, A., & Adjogoua, E. V. (2009). Evolutionary history and dynamics of dog rabies virus in western and central Africa. *Journal of General Virology*, *90*(4), 783–791.

Tiembré, I., Broban, A., Bénié, J., Tetchi, M., Druelles, S., & L'Azou, M. (2018). Human rabies in Côte d'Ivoire 2014-2016: Results following reinforcements to rabies surveillance. *PLoS Neglected Tropical Diseases*, *12*(9), e0006649.

van7, P.-P. A. M., de Bie, R. M. A., Eftimov, F., Tepaske, R., Zaaijer, H. L., van Doornum, G. J. J., Schutten, M., Osterhaus, A. D. M. E., Majoie, C. B. L. M., & Aronica, E. (2009). Fatal human rabies due to Duvenhage virus from a bat in Kenya: failure of treatment with coma-induction, ketamine, and antiviral drugs. *PLoS Neglected Tropical Diseases*, *3*(7), e428.

Wang, W., Ma, J., Nie, J., Li, J., Cao, S., Wang, L., Yu, C., Huang, W., Li, Y., & Yu, Y. (2019). Antigenic variations of recent street rabies virus. *Emerging Microbes & Infections*, *8*(1), 1584–1592.

Wheeler, D. C., & Waller, L. A. (2008). Mountains, valleys, and rivers: the transmission of raccoon rabies over a heterogeneous landscape. *Journal of Agricultural, Biological, and Environmental Statistics*, *13*(4), 388–406.

Wilder-Smith, A., Ooi, E. E., Horstick, O., & Wills, B. (2019). Dengue. *The Lancet*, *393*(10169), 350–363. https://doi.org/10.1016/S0140-6736(18)32560-1

World Health Organization. (2018). WHO position paper on rabies vaccines. *Weekly Epidemiological Record*, *93*(16), 201–220.

Wunner, W. H., & Conzelmann, K.-K. (2020). Rabies virus. In A. C. Jackson & W. H. Wunner (Eds.), *Rabies* (pp. 43–81). Elsevier.

Yang, D.-K., Kim, H.-H., Lee, K.-W., & Song, J.-Y. (2013). The present and future of rabies vaccine in animals. *Clinical and Experimental Vaccine Research*, *2*(1), 19–25.

Yang, J., Koprowski, H., Dietzschold, B., & Fu, Z. F. (1999). Phosphorylation of rabies virus nucleoprotein regulates viral RNA transcription and replication by modulating leader RNA encapsidation. *Journal of Virology*, *73*(2), 1661–1664.

Yousaf, M. Z., Qasim, M., Zia, S., Ashfaq, U. A., & Khan, S. (2012). Rabies molecular virology, diagnosis, prevention and treatment. *Virology Journal*, *9*(1), 1–5.

Zandi, F., Goshadrou, F., Meyfour, A., & Vaziri, B. (2021). Rabies infection: an overview of lyssavirus-host protein interactions. *Iranian Biomedical Journal*, *25*(4), 226.

Zhang, G., & Fu, Z. F. (2012). *Complete genome sequence of a street rabies virus from Mexico*. Am Soc Microbiol.

Zhang, G., Wang, H., Mahmood, F., & Fu, Z. F. (2013). Rabies virus glycoprotein is an important determinant for the induction of innate immune responses and the pathogenic mechanisms. *Veterinary Microbiology*, *162*(2–4), 601–613.

Zhou, M., Zhou, Z., Kia, G. S. N., Gnanadurai, C. W., Leyson, C. M., Umoh, J. U., Kwaga, J. P., Kazeem, H. M., & Fu, Z. F. (2013). Complete genome sequence of a street rabies virus isolated from a dog in Nigeria. *Genome Announcements*, *1*(1), e00214-12.

APPENDICES

Appendix 1: Ethical approval for the study

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 October 25, 2019

TO: **DR. THUMBI MWANGI**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGHR**
KISUMU

28/10/2019

Dear Sir,

RE: **SERU PROTOCOL NO. 3268 *(REQUEST FOR ANNUAL RENEWAL AND PROTOCOL DEVIATION)*: APPLYING EPIDEMIOLOGICAL TECHNIQUES TO IMPROVE THE VISIBILITY OF NEGLECTED ZOONOTIC DISEASES: THE CASE OF RABIES ELIMINATION IN KENYA**

Thank you for the continuing review report for the period **August 03, 2017** to **October 04, 2019.** The Committee noted that a protocol deviation form has been submitted as the request for annual renewal was done after the expiration date of the last approval.

This is to inform you that the expedited review team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **October 25, 2019** through to **October 24, 2020**. Please note that authorization to conduct this study will automatically expire on **October 24, 2020**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU by **September 12, 2020.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation. You may continue with the study.

Yours faithfully,

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix 2: The metadata of study samples

	Makueni county samples								
	Sample ID	Animal	Date of Collection	PCR Results	Genome coverage in nucleotides			Classification	
					Whole genome	N-gene	G-gene	Subclade	Lineage
1	MAK/FEL/001	Cat	12/Mar/22	+VE	11658	1318	1575	Africa 1b	AF1b_B1.3
2	MAK/FEL/002	Cat	09-May-22	+VE	11599	1318	1575	Africa 1b	AF1b_B1.2.1
3	MAK/CAN/001	Dog	29/Oct/21	+VE	11766	1318	1575	Africa 1b	AF1b_B1.2
4	MAK/CAN/002	Dog	5/Nov/21	+VE	11665	1353	1575	Africa 1b	AF1b_B1.2
5	MAK/CAN/003	Dog	15/Nov/21	+VE	11657	1312	1575	Africa 1b	AF1b_B1.2
6	MAK/CAN/004	Dog	18/Nov/21	+VE	11472	1318	1575	Africa 1b	AF1b_B1
7	MAK/CAN/005	Dog	4/Dec/21	+VE	11915	1353	1575	Africa 1a	AF1a_A1.1
8	MAK/CAN/006	Dog	30/Dec/21	+VE	11900	1353	1575	Africa 1a	AF1b_B1.3
9	MAK/CAN/007	Dog	27/Jan/22	+VE	11496	1312	1575	Africa 1b	AF1b_A1.2.1
10	MAK/CAN/008	Dog	31/Jan/22	+VE	11412	1353	1546	Africa 1b	AF1b_B1
11	MAK/CAN/009	Dog	14/Feb/22	+VE	11586	1318	1575	Africa 1b	AF1b_B1.3
12	MAK/CAN/010	Dog	25/Feb/22	+VE	10589	1318	1428	Africa 1b	AF1b_B1
13	MAK/CAN/011	Dog	25/Feb/22	+VE	11417	1312	1575	Africa 1b	AF1b_B1.2.1
14	MAK/CAN/012	Dog	10/Mar/22	+VE	10966	1318	1575	Africa 1b	AF1b_B1.2.1
15	MAK/CAN/014	Dog	11/Mar/22	+VE	11723	1318	1575	Africa 1b	AF1b_A1.2.1
16	MAK/CAN/016	Dog	26/Jul/21	+VE	11582	1312	1575	Africa 1b	AF1b_A1.2.1
17	MAK/CAN/017	Dog	10/Aug/21	+VE	11468	1353	1561	Africa 1b	AF1b_B1
18	MAK/CAN/018	Dog	11/Aug/21	+VE	11245	1313	1575	Africa 1b	AF1b_B1
19	MAK/CAN/019	Dog	17/Aug/21	+VE	11349	1238	1575	Africa 1b	AF1b_B1
20	MAK/CAN/020	Dog	1/Sep/21	+VE	11260	1318	1556	Africa 1b	AF1b_B1
21	MAK/CAN/021	Dog	5/Aug/21	+VE	11067	1345	1575	Africa 1b	AF1b_B1.2.1
22	MAK/CAN/022	Dog	3/Sep/21	+VE	10162	1281	1283	Africa 1b	AF1b_B1.2
23	MAK/CAN/023	Dog	27/Sep/21	+VE	11185	1318	1575	Africa 1b	AF1b_B1.2
24	MAK/CAN/024	Dog	27/Sep/21	+VE	11766	1318	1575	Africa 1b	AF1b_B1.2
25	MAK/CAN/025	Dog	24/Sep/21	+VE	11766	1318	1575	Africa 1b	AF1b_B1
26	MAK/CAN/026	Dog	9/Aug/21	+VE	11600	1353	1545	Africa 1b	AF1b_B1.2
27	MAK/CAN/027	Dog	24/Sep/21	+VE	11246	1318	1575	Africa 1b	AF1b_B1
28	MAK/CAN/028	Dog	1/Oct/21	+VE	11582	1318	1575	Africa 1b	AF1b_B1.2.1
29	MAK/CAN/029	Dog	14/Oct/21	+VE	11627	1318	1575	Africa 1b	AF1b_B1
30	MAK/CAN/030	Dog	18/Oct/21	+VE	11791	1342	1575	Africa 1b	AF1b_A1.2.1
31	MAK/CAN/031	Dog	22-Mar-22	+VE	11254	1318	1544	Africa 1b	AF1b_B1.2.1
32	MAK/CAN/034	Dog	23-Apr-22	+VE	11620	1353	1570	Africa 1b	AF1b_B1.2
33	MAK/CAN/033	Dog	20-Apr-22	+VE	11764	1353	1575	Africa 1b	AF1b_B1
34	MAK/CAN/032	Dog	13-Apr-22	+VE	11729	1318	1538	Africa 1b	AF1b_B1
35	MAK/CAN/035	Dog	10-May-22	+VE	10769	1353	1533	Africa 1b	AF1b_B1.3
36	MAK/CAN/036	Dog	17-May-22	+VE	10514	1079	1545	Africa 1b	AF1b_B1
37	MAK/CAN/037	Dog	04-Jun-22	+VE	11686	1353	1575	Africa 1b	AF1b_B1.2.1
38	MAK/CAN/039	Dog	15-Jun-22	+VE	11883	1353	1575	Africa 1b	AF1b_B1.3
39	MAK/CAN/038	Dog	13-Jun-22	+VE	11627	1353	1575	Africa 1b	AF1b_B1.3
40	MAK/CAN/044	Dog	17-Jun-22	+VE	11494	1353	1575	Africa 1b	AF1b_B1.2.1

41	MAK/CAN/043	Dog	21-Jun-22	+VE	9335	not used (less 10,000 nucleotides)			
42	MAK/CAN/042	Dog	15-Jun-22	+VE	11763	1318	1575	Africa 1b	AF1b_B1.2
43	MAK/CAN/045	Dog	26-Jun-22	+VE	10389	1292	1575	Africa 1b	AF1b_B1.3
44	MAK/CAN/049	Dog	05-Aug-22	+VE	11812	1353	1575	Africa 1b	AF1b_B1.3
45	MAK/CAN/048	Dog	22-Aug-22	+VE	11642	1318	1575	Africa 1b	AF1b_B1.3
46	MAK/CAN/047	Dog	28-Jul-22	+VE	11636	1353	1575	Africa 1b	AF1b_A1.2.1
47	MAK/CAN/046	Dog	26-Jul-22	+VE	11294	1351	1328	Africa 1b	AF1b_B1.2
48	MAK/CAN/051	Dog	25-Aug-22	+VE	11859	1353	1575	Africa 1b	AF1b_B1.3
49	MAK/CAN/050	Dog	16-Aug-22	+VE	11602	1312	1575	Africa 1b	AF1b_A1.2.1
50	MAK/CAN/052	Dog	17-Sep-22	-VE	Not sequenced				
51	MAK/WA/001	Honey badger	13-May-22	+VE	0	not used (less 10,000 nucleotides)			
52	MAK/CAP/001	Goat	2/Jul/21	+VE	10182	1206	1442	Africa 1b	Unclassified
53	MAK/CAP/002	Goat	23/Feb/22	+VE	10101	1318	1575	Africa 1b	AF1b_B1.2.1
54	MAK/CAP/003	Goat	1/Mar/22	+VE	11811	1353	1575	Africa 1b	AF1b_B1.2.1
55	MAK/CAP/005	Goat	18-Mar-22	+VE	11758	1264	1561	Africa 1b	AF1b_B1.2.1
56	MAK/CAP/004	Goat	19-Mar-22	+VE	11551	1353	1561	Africa 1b	AF1b_B1.2.1
57	MAK/CAP/008	Goat	12-Apr-22	+VE	11676	1271	1575	Africa 1b	AF1b_B1
58	MAK/CAP/007	Goat	30-Mar-22	+VE	11829	1353	1562	Africa 1b	AF1b_B1
59	MAK/CAP/009	Goat	03-May-22	+VE	11264	1134	1575	Africa 1b	AF1b_B1
60	MAK/CAP/010	Goat	17-Jun-22	+VE	11413	1317	1575	Africa 1b	AF1b_B1.2
61	MAK/CAP/013	Goat	12-Jul-22	+VE	11118	1318	1398	Africa 1b	AF1b_B1.3
62	MAK/CAP/012	Goat	08-Jul-22	+VE	9047	not used (less 10,000 nucleotides)			
63	MAK/CAP/011	Goat	05-Jul-22	+VE	11515	1353	1575	Africa 1b	AF1b_B1.3
64	MAK/CAP/015	Goat	08-Aug-22	+VE	1194	not used (less 10,000 nucleotides)			
65	MAK/CAP/014	Goat	02-Aug-22	+VE	11028	1312	1466	Africa 1a	AF1a_B1.1
66	MAK/CAP/016	Goat	19/Aug/21	+VE	6158	not used (less 10,000 nucleotides)			
67	MAK/CAP/017	Goat	28/Aug/21	+VE	11291	1312	1575	Africa 1b	AF1b_A1.2.1
68	MAK/CAP/018	Goat	2/Sep/21	+VE	11811	1353	1575	Africa 1b	AF1b_B1.2.1
69	MAK/CAP/019	Goat	4/Dec/21	-VE	Not sequenced				
70	MAK/CAP/021	Goat	29/Dec/21	+VE	11821	1353	1575	Africa 1b	AF1b_B1.2.1
71	MAK/CAP/022	Goat	14/Feb/22	+VE	11791	1353	1575	Africa 1b	AF1b_B1.2.1
72	MAK/CAP/033	Goat	01-Sep-22	-VE	Not sequenced				
73	MAK/CAP/032	Goat	25-Aug-22	+VE	10308	1308	1575	Africa 1b	AF1b_B1.3
74	MAK/CAP/031	Goat	12-Aug-22	+VE	10985	1318	1527	Africa 1b	AF1b_B1.3
75	MAK/CAP/036	Goat	15-Sep-22	+VE	10786	1295	1488	Africa 1b	AF1b_B1.3
76	MAK/BOV/016	Cow	18/Jul/21	+VE	11788	1340	1575	Africa 1b	AF1b_B1
77	MAK/BOV/017	Cow	13/Sep/21	+VE	9849	not used (less 10,000 nucleotides)			
78	MAK/BOV/018	Cow	21/Nov/21	+VE	11678	1318	1575	Africa 1b	AF1b_B1.2
79	MAK/BOV/019	Cow	03-Jun-22	+VE	11027	1318	1461	Africa 1b	AF1b_B1.3
80	MAK/BOV/020	Cow	15-Jun-22	+VE	11596	1328	1575	Africa 1b	AF1b_B1.2
81	MAK/BOV/021	Cow	05-Sep-22	+VE	11871	1353	1575	Africa 1b	AF1b_B1.3
82	MAK/BOV/022	Cow	15-Sep-22	+VE	11813	1353	1575	Africa 1b	AF1b_B1.3

	Siaya county samples								
	Sample ID	**Animal**	**Date of Collection**	**PCR Results**	**Genome coverage in nucleotides**			**Classification**	
					Whole genome	**N-gene**	**G-gene**	**Subclade**	**Lineage**
1	SIA/CAN/001	Dog	11/Aug/21	+VE	11876	1353	1575	Africa 1a	AF1a_A1.1
2	SIA/CAN/002	Dog	16/Aug/21	-VE	Not sequenced				
3	SIA/CAN/003	Dog	16/Aug/21	-VE	Not sequenced				
4	SIA/CAN/004	Dog	2/Aug/21	+VE	11300	1120	1569	Africa 1a	AF1a_A1.1
5	SIA/CAN/005	Dog	5/Sep/21	+VE	11159	1353	1575	Africa 1a	AF1a_A1.2.1
6	SIA/CAN/006	Dog	5/Sep/21	+VE	10892	1353	1543	Africa 1a	AF1a_A1.1
7	SIA/CAN/007	Dog	5/Sep/21	+VE	11354	1353	1405	Africa 1a	AF1a_A1.2.1
8	SIA/CAN/008	Dog	5/Sep/21	+VE	11922	1353	1575	Africa 1a	AF1a_A1.1
9	SIA/CAN/009	Dog	11/Sep/21	+VE	11922	1353	1575	Africa 1a	AF1a_B1.1
10	SIA/CAN/010	Dog	21/Sep/21	+VE	11700	1353	1575	Africa 1a	AF1a_A1.3
11	SIA/CAN/011	Dog	26/Sep/21	+VE	0	not used (less 10,000 nucleotides)			
12	SIA/CAN/012	Dog	14/Oct/21	+VE	11697	1353	1575	Africa 1a	AF1a_B1.1
13	SIA/CAN/013	Dog	14/Oct/21	+VE	11772	1353	1575	Africa 1a	AF1a_A1.3
14	SIA/CAN/014	Dog	27/Oct/21	+VE	11397	1283	1575	Africa 1a	AF1a_B1.1
15	SIA/CAN/015	Dog	3/Nov/21	+VE	11836	1353	1488	Africa 1a	AF1a_A1.1
16	SIA/CAN/016	Dog	3/Nov/21	+VE	11793	1353	1575	Africa 1a	AF1a_B1
17	SIA/CAN/017	Dog	3/Nov/21	+VE	11629	1318	1575	Africa 1a	AF1a_B1.1
18	SIA/CAN/018	Dog	4/Nov/21	+VE	11879	1353	1575	Africa 1a	AF1a_A1.2.1
19	SIA/CAN/019	Dog	6/Nov/21	+VE	11922	1353	1575	Africa 1a	AF1a_A1.2.1
20	SIA/CAN/020	Dog	25/Nov/21	+VE	11893	1353	1575	Africa 1a	AF1a_A1.2
21	SIA/CAN/021	Dog	9/Dec/21	+VE	11696	1347	1575	Africa 1a	AF1a_A1.3
22	SIA/CAN/022	Dog	20/Dec/21	+VE	11921	1353	1575	Africa 1a	AF1a_B1
23	SIA/CAN/023	Dog	22/Dec/21	+VE	11711	1276	1575	Africa 1a	AF1a_A1.1
24	SIA/CAN/024	Dog	27/Dec/21	+VE	11620	1353	1575	Africa 1a	AF1a_B1.1
25	SIA/CAN/025	Dog	1/Jan/22	+VE	11355	1318	1575	Africa 1a	AF1a_A1.3
26	SIA/CAN/026	Dog	14/Jan/22	+VE	11879	1353	1575	Africa 1a	AF1a_A1.2
27	SIA/CAN/027	Dog	18/Jan/22	+VE	11836	1353	1557	Africa 1a	AF1a_A1.1.1
28	SIA/CAN/028	Dog	31/Jan/22	+VE	11921	1353	1575	Africa 1a	AF1a_B1
29	SIA/CAN/029	Dog	5/Feb/22	+VE	11854	1353	1575	Africa 1a	AF1a_B1
30	SIA/CAN/030	Dog	10/Feb/22	+VE	10751	1353	1575	Africa 1a	AF1a_A1.2
31	SIA/CAN/031	Dog	13/Feb/22	+VE	11746	1353	1575	Africa 1a	AF1a_A1.3
32	SIA/CAN/032	Dog	26/Feb/22	+VE	11789	1353	1575	Africa 1a	AF1a_B1.1
33	SIA/CAN/033	Dog	3/Mar/22	+VE	11855	1353	1575	Africa 1a	AF1a_B1.1
34	SIA/CAN/034	Dog	6/Mar/22	+VE	11923	1353	1575	Africa 1a	AF1a_A1
35	SIA/CAN/035	Dog	13/Mar/22	+VE	11922	1353	1575	Africa 1a	AF1a_A1.2.1
36	SIA/CAN/038	Dog	20-Mar-22	+VE	11715	1353	1575	Africa 1a	AF1a_A1.3
37	SIA/CAN/037	Dog	18-Mar-22	+VE	11921	1353	1575	Africa 1a	AF1a_A1.2.1
38	SIA/CAN/036	Dog	16-Mar-22	+VE	11528	1353	1505	Africa 1a	AF1a_B1.1
39	SIA/CAN/042	Dog	13-Apr-22	+VE	11831	1353	1575	Africa 1a	AF1a_A1.1.1
40	SIA/CAN/041	Dog	02-Apr-22	+VE	11922	1353	1575	Africa 1a	AF1a_A1.2.1

41	SIA/CAN/040	Dog	01-Apr-22	+VE	11914	1353	1575	Africa 1a	AF1a_A1
42	SIA/CAN/039	Dog	29-Mar-22	-VE	Not sequenced				
43	SIA/CAN/045	Dog	09-May-22	+VE	11337	1343	1425	Africa 1a	AF1a_A1.1
44	SIA/CAN/044	Dog	01-May-22	+VE	11922	1353	1575	Africa 1a	AF1a_A1.1.1
45	SIA/CAN/043	Dog	27-Apr-22	+VE	11922	1353	1575	Africa 1a	AF1a_A1.2.1
46	SIA/CAN/047	Dog	26-May-22	+VE	11922	1353	1575	Africa 1a	AF1a_B1.1
47	SIA/CAN/046	Dog	23-May-22	+VE	11862	1353	1575	Africa 1a	AF1a_A1.2.1
48	SIA/CAN/050	Dog	16-Jun-22	+VE	11921	1353	1575	Africa 1a	AF1a_A1.1.1
49	SIA/CAN/049	Dog	09-Jun-22	+VE	11921	1353	1575	Africa 1a	AF1a_A1.1.1
50	SIA/CAN/048	Dog	03-Jun-22	+VE	11920	1353	1575	Africa 1a	AF1a_A1.2
51	SIA/CAN/059	Dog	28-Jul-22	+VE	11770	1353	1575	Africa 1a	AF1a_A1.2
52	SIA/CAN/058	Dog	21-Jul-22	+VE	11921	1353	1575	Africa 1a	AF1a_A1.2
53	SIA/CAN/057	Dog	14-Jul-22	-VE	Not sequenced				
54	SIA/CAN/056	Dog	13-Jul-22	+VE	11260	1353	1553	Africa 1a	AF1a_B1
55	SIA/CAN/055	Dog	12-Jul-22	+VE	11016	1335	1575	Africa 1a	AF1a_A1.2
56	SIA/CAN/054	Dog	29-Jun-22	+VE	11374	1343	1575	Africa 1a	AF1a_A1.3
57	SIA/CAN/053	Dog	27-Jun-22	+VE	11441	1353	1575	Africa 1a	AF1a_A1.2
58	SIA/CAN/052	Dog	26-Jun-22	+VE	11922	1353	1575	Africa 1a	AF1a_A1
59	SIA/CAN/060	Dog	19-Sep-22	+VE	11874	1353	1575	Africa 1a	AF1a_A1.2
60	SIA/BOV/001	Cow	20/Aug/21	+VE	0	not used (less 10,000 nucleotides)			
61	SIA/BOV/002	Cow	31/Aug/21	+VE	6579	not used (less 10,000 nucleotides)			
62	SIA/BOV/003	Cow	2/Sep/21	+VE	11762	1353	1575	Africa 1b	AF1b_B1
63	SIA/BOV/004	Cow	10/Sep/21	+VE	10755	1353	1498	Africa 1a	AF1a_A1.2.1
64	SIA/BOV/005	Cow	1/Oct/21	+VE	11878	1353	1575	Africa 1a	AF1a_A1.3
65	SIA/BOV/006	Cow	5/Oct/21	+VE	11807	1353	1569	Africa 1b	AF1b_B1.3
66	SIA/BOV/007	Cow	15/Oct/21	+VE	11406	1353	1575	Africa 1a	AF1a_A1.2.1
67	SIA/BOV/009	Cow	17/Oct/21	+VE	11920	1353	1575	Africa 1a	AF1a_A1.3
68	SIA/BOV/010	Cow	18/Oct/21	+VE	11864	1353	1575	Africa 1a	AF1a_A1.3
69	SIA/BOV/011	Cow	11/Nov/21	+VE	11424	1353	1504	Africa 1a	AF1a_A1.1.1
70	SIA/BOV/012	Cow	2/Dec/21	+VE	11854	1353	1575	Africa 1a	AF1a_A1.1
71	SIA/BOV/014	Cow	10/Feb/22	+VE	11912	1353	1575	Africa 1a	AF1a_A1.2.1
72	SIA/BOV/015	Cow	20/Feb/22	-VE	Not sequenced				
73	SIA/BOV/017	Cow	23-Mar-22	+VE	0	not used (less 10,000 nucleotides)			
74	SIA/BOV/019	COW	20-Apr-22	+VE	8881	not used (less 10,000 nucleotides)			
75	SIA/BOV/021	Cow	18-May-22	+VE	11580	1353	1575	Africa 1a	AF1a_A1.3
76	SIA/BOV/020	Cow	10-May-22	+VE	10024	1167	1575	Africa 1a	AF1a_B1
77	SIA/BOV/022	Cow	28-May-22	+VE	8892	not used (less 10,000 nucleotides)			
78	SIA/BOV/025	Cow	29-Aug-22	+VE	8094	not used (less 10,000 nucleotides)			
79	SIA/BOV/027	Cow	22-Jun-22	+VE	10424	1343	1575	Africa 1a	AF1a_A1.1.1
80	SIA/BOV/026	Cow	21-Jun-22	+VE	11749	1353	1575	Africa 1a	AF1a_B1.1
81	SIA/OVI/001	Sheep	23/Nov/21	+VE	11527	1353	1575	Africa 1a	AF1a_A1.3
82	SIA/BOV/016	cow	20-Feb-22	+VE	10509	1318	1545	Africa 1b	UNKNOWN