SCREENING OF FILOVIRUS RIBONUCLEIC ACID IN HUMANS, WILD CAUGHT NON-HUMAN PRIMATES, BATS AND RODENTS IN LAIKIPIA NORTH SUB-COUNTY, KENYA.

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A Research Thesis Submitted in Partial Fulfillment of The Requirements for the Award of Degree of Doctor of Philosophy In Medical Virology in the School of Medicine, Kenyatta University.

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my beloved daughter, family members and friends.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

AGM:	African Green Monkey
AIDS:	Acquired Immunodeficiency Syndrome
BDBV:	Bundibugyo
BMOV:	Bombali Ebolavirus
CDC:	Center for Disease Control
cDNA:	Complimentary Deoxyribonucleic Acid
cPCR:	Consensus Polymerase Chain Reaction
CTD:	C Terminal Domain
D:	Delta
DDT:	Dithiothrenol
DNA:	Deoxyribonucleic acid
dNTP:	Deoxyribonucleotide Triphosphate
dsRNA:	Double Stranded Ribonucleic Acid
dsRNA:	Double Stranded RNA
EBOV:	Ebolavirus
EDTA:	Ethylene Diamine Tetra Acetic Acid
ELISA:	Enzyme Linked Immunosorbent Assay
Fas:	fas Cell Surface Death Receptor
FDA:	Food and Drug Administration
GP:	Glycoprotein
HF:	Haemorrhagic Fever
HIV:	Human Immunodeficiency Virus
HTS:	High throughput Sequencing
HUJV:	Huangjiao virus
ICTV:	International Committee on Taxonomy of Virus
IFNα:	Interferon Alpha
IFNβ:	Interferon Betta
IFNγ:	Interferon Gamma
IgG:	Immunoglobulin Gamma
IgM:	Immunoglobulin mu
IL 10:	Interleukin 10
IL 1β:	Interleukin 1 β
IL 6:	Interleukin 6
IL-1RA:	Interleukin 1 Receptor Agonist
INF:	Interferon
IR:	Intragenic
IRF 7:	Interferon Regulatory Factor 7
IRF:	Interferon Regulatory Factor
Jak:	Janus Kinase
L:	Large
LLOU :	*Lloviu Cuevavirus*
MARV:	Marburg Virus

$MgCl_2$:	Magnesium Chloride
MIP:	Macrophage Inflammatory Protein
mRNA:	Messenger Ribonucleic Acid
NHPs:	Non-Human Primates
NP:	Nucleoprotein
nt:	Nucleotides
NTD:	N Terminal Domain
OD:	Optical Density
ORF:	Open Reading Frame
RAVV:	Ravn Virus
RESTV:	Reston Virus
RNA:	Ribonucleic Acid
RNAi:	RNA Interference
RT LAMP:	Reverse Transcriptase Loop Mediated Isothermal Amplification
RT qPCR:	Reverse Transcriptase Real Time Polymerase Chain Reaction
RT-PCR:	Reverse Transcription Polymerase Chain Reaction
RVSV-ZEBOV:	Vesicular Stomatitis Virus Ebolavirus Vaccine
sGP:	Soluble Glycoproteins
sIL-6R:	Soluble Interleukin 6 Receptor
ssGP:	Small Soluble Glycoproteins
ssRNA:	Single Stranded Ribonucleic Acid
STAT 1:	Signal Transducer and Activator of Transcription 1
sTNF-R1:	Soluble Tumour Necrotic Factor Receptor 1
sTNF-R11:	Soluble Tumour Necrotic Factor Receptor 11
SUDV:	Sudan Virus
TAFV:	Tai Forest Virus
TLR:	Toll Like Receptor
TF:	Tissue Factor
TRAIL:	Tumor Necrotic Factor Related Apoptosis Inducing Ligand
UV:	Ultra Violet
VHFs:	Viral Haemorrhagic Fever
VP:	Viral Protein
WHO:	World Health Organization
XILV:	Xlang virus
ZEBOV:	Ebolavirus

ABSTRACT

In the recent decade, highly pathogenic human viruses have constantly emerged or re-emerged in different geographical locations worldwide approximately annually with the majority jumping from animals to humans. The major factors that have led to emergence of these viral diseases include urbanization, changes to local ecosystem and changes in human behaviours. Many of the emerging and re-emerging viruses are Ribonucleic acid (RNA) viruses with a high potential for re-assortment, recombination and mutation leading to new/different pathogenic viral strains. Despite the fact that non-human primates, bats and rodents have increasingly been implicated as potential sources of emerging zoonotic viral diseases, there is paucity of scientific data on *Filoviruses* circulating at the animal human interface in Laikipia North Sub-county, Kenya. The aim of this study was to detect and characterize filoviruses circulating in Laikipia, County Kenya. A cross sectional study design was adopted. Sampling technique employed in this study for human population was convenience sampling while for animal samples, capture release method using PREDICT 2 protocols was used. A total of 1092 samples of whole blood/blood swabs/blood clots (n=377), oral swabs (n=405) and rectal swabs/fecal (n=310) material were obtained from consenting individuals and the aforementioned animals. The samples were stored in TRizol® reagent (Thermo Fisher Scientific). The viral ribonucleic acid (RNA) was extracted. From the RNA, complimentary deoxyribonucleic acid (cDNA) was synthesized using Invitrogen superscript III first strand synthesis kit. The cDNA was amplified using PREDICT 2 filovirus universal primers in a consensus reverse transcriptase polymerase chain reaction (RT-PCR) targeting the Large (L) gene. In addition, immunoglobulin gamma enzyme linked immunosorbent assay (IgG ELISA) was conducted only on human and non-human primate serum samples. The IgG ELISA targeted IgG antibodies against Ebolaviruses and Marburg virus. Risk factors associated with zoonotic infections were assessed using surveys. All the samples tested negative for filoviruses using consensus RT-PCR and IgG ELISA. On risk factors analysis, bush meat was reported to be in circulation while less than 10% of the participants reported having interaction with wild animals in the last 6 months of the study. Despite the negative results in laboratory assays (consensus RT-PCR and IgG ELISA),cultural beliefs and practices, the circulation of bush meat and interaction of the community with wild life pre-disposes these population to the risk of contracting zoonotic viruses other than filoviruses. This study has identified potential risk factors associated with zoonotic disease transmission at the study area which are getting into contact with animals, sharing of water points with animals and use of untreated water for domestic use. These results suggest that circulation of filovirus was absent in humans, non-human primates, rodents and bats from Laikipia North Sub-county. Considering our findings, the study recommends continuous surveillance, systematic collection of samples from human population and animals for laboratory analysis for planning, implementation and evaluation of potential emerging filoviruses within the region and the use of both serological and molecular assays for detection of filoviruses.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

There is a great concern among biomedical scientists and public health professionals regarding cross-species transmission of pathogens between animals and humans. These concerns are mounting as humans are increasingly coming into closer proximity to wild animals for a variety of reasons (Rhyan & Spraker, 2010). The human-animal interface created by increased human animal interactions has created an opportunity for cross species zoonotic virus transmission.

The underlying factors that influence viral emergence or cross species transmission include genetic virus variation, individual host susceptibility, anthropogenic factors and climate changes (Geoghegan et al., 2016; Judson et al., 2016; Longdon et al., 2014; Streicker et al., 2012; Wolfe et al., 2005). The most frustrating fact is that most of these emerging and re-emerging zoonotic viruses are lethal to humans. They have been listed among potential agents of bioterrorism by the Centre of Disease Control (CDC, 2019; Ryan, 2008). Examples of these agents/diseases include filoviruses, arenaviruses, viral encephalitis and henipavirus e.g. Nipah virus (CDC, 2019).

Wildlife are known reservoir hosts of zoonotic diseases playing a major role in the transmission of emerging and re-emerging viruses in humans (Johnson et al., 1996; Kruse et al., 2004; Wolfe et al., 2005). Of importance are bats, non-human primates (NHPs) and rodents (Han et al., 2016; Smith & Wang, 2013). Over 60% of known emerging human

diseases identified between 1940 and 2000 are zoonotic. Out of these diseases, more than 70% originated from wildlife (Jones et al., 2008). From the 70%, the second highest emerging infectious diseases are from viruses. These dominate the list and are the most frequently emerging deadly human pathogens documented in the recent past (Woolhouse and Gaunt, 2007). Moreover, RNA viruses account for a third of all emerging and re-emerging human viral diseases (Howard & Fletcher, 2012). This study sought to describe RNA viruses of the family *Filoviridae* circulating in Laikipia North Sub County, Kenya.

Filoviruses are amongst zoonotic viruses of great public health importance with a case fatality rate of up to 90%, depending on the virus strain (Martina and Osterhaus, 2009). Filoviruses are widely distributed in Sub-Saharan Africa (Formenty et al., 1999). Currently, there is paucity of data on *Filoviruses* circulating in Kenya, though in 1987 there was an isolated fatal case of *Marburg* virus infection (Johnson et al., 1996). Since then, there has been no reported cases of *Marburg* transmission to humans in Kenya. In 2007, Marburg virus was isolated from Egyptian fruit bat or Egyptian rousette (*Rousettus aegyptiacus*) bat obtained from Kitum Cave, Kenya (Kuzmin et al., 2010a). At that time, there were no cases of human infections reported. The largest and most devastating outbreak of Filovirus infection reported in humans was the *Ebolavirus* (EBOV) outbreak which occurred in West Africa between 2014-2016 (WHO, 2017). Bat species were implicated as the source of this outbreak (Lo et al., 2017; Alexander et al., 2015). This was followed by other ebolavirus outbreaks which occurred in the year 2018-2020 and 2021 in Democratic Republic of Congo (DRC) and Guinea, respectively (WHO, 2021a).

With these unknown viruses harboured in reservoir hosts waiting to erupt at the least expected time, there is need for early detection, identification and control of these viruses before species jump into humans occurs. Therefore, this study was aimed at detection of filoviruses and identifying the risk factors associated with their transmission in Laikipia North Sub-County, Kenya with the purpose of giving an early indication of a looming filoviral outbreak.

1.2 Statement of the Problem and Justification

The health of humans and animals is largely interconnected, with 6 out of 10 emerging human infectious diseases originating from animals (Towner et al., 2009). Non-human primates (NHPs), bats and rodents have been known to harbour zoonotic viruses in nature which silently circulate in the hosts (O'Shea, 2014). Moreover, in Laikipia County Kenya, these animals live in close proximity to humans which facilitates easy contact. This would stir the jump of deadly unknown viruses from animals to humans. The deadliest diseases are known to be those which are newly introduced to human population from animal species (Andersen, 2020). Since the last human case of MARV in 1987, there is paucity of data on *Filoviruses* circulating in humans, NHPs, bats and rodents in Kenya.

In Laikipia North Sub-county, NHPs and rodents have been known to forage for food in human settlements and ranches thus increasing animal human interactions. This has resulted to an increased frequency of NHPs-human conflicts which predisposes man to zoonotic viruses. Ribonucleic acid (RNA) viruses have high mutation rates which may

facilitate the interspecies jump and establish themselves in human hosts (Elena & Sanjuán, 2005).

Climate change in Kenya has led to scarcity of water and food, especially in arid and semi-arid areas like Laikipia North, Sub-county. This has forced humans and animals including bats, rodents and NHPs to share the scarce resources (water points). The sharing of these water points has largely increased the interaction between animals and humans. This consequently has exposed humans to ongoing life cycles of potentially lethal zoonotic viruses such as *Filoviruses* whose transmission mechanism is not well understood (McKay & Hoye, 2016).

Since NHPs, bats and rodents have been implicated as possible reservoir hosts for *Filoviruses*, there is need for continued virus bio-surveillance in these animals. This bio-surveillance can act as an early indicator of a looming viral zoonotic epidemic in humans. A One Health approach for action against neglected tropical diseases which recognizes that human and animal health are interlinked should be emphasized (Moloo, 2022).

1.3 Research Questions

i. What are the Filoviruses circulating in humans, non-human primates, bats and rodents in Laikipia North Sub-County, Kenya?
ii. What are the molecular signatures of Filoviruses within species circulating in humans, non-human primates, bats and rodents in Laikipia North Sub-County, Kenya?

iii. What are the molecular signatures of Filoviruses between species circulating in humans, non-human primates, bats and rodents Laikipia North Sub-County, Kenya?

iv. What is the molecular epidemiology of Filoviruses circulating in humans, non-human primates, bats and rodents in Laikipia North Sub-County, Kenya?

v. What are the risk factors associated with Filovirus transmission in humans?

1.4 Objectives

1.4.1 General Objective

To determine molecular characteristics of *Filoviruses* circulating in humans, wild caught non-human primates, bats and rodents in Laikipia North Sub-County, in Kenya.

1.4.2 Specific Objectives

i. To detect and identify *Filoviruses* circulating in humans, NHPs, bats and rodents in Laikipia North Sub-County, Kenya.

ii. To compare *Filoviruses* within species circulating in humans, NHPs, bats and rodents in Laikipia North Sub-County, Kenya.

iii. To compare *Filoviruses* between species circulating in humans, NHPs, bats and rodents in Laikipia North Sub-County, Kenya.

iv. To determine the molecular epidemiology of *Filoviruses* circulating in humans, NHPs, bats and rodents in Laikipia North Sub-County, Kenya.

v. To identify risk factors associated with *Filovirus* transmission in humans from Laikipia North Sub-County, in Kenya.

1.5 Study Output

This study was aimed at detecting and characterizing filoviruses circulating in humans, non-human primates, bats and rodents with the purpose of acting as an early warning sign to a looming pandemic. The study has identified human behaviour that were potential risk factors associated with filovirus transmission in Laikipia North, Sub County, Kenya.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Filoviruses

Filoviruses are viruses that belong to the order *Mononegavirales* and family *Filoviridae* (ICTV, 2020). The term *Filo* in the word *Filoviridae* originates from a Latin word filum meaning "thread" like. Under an electron microscope, these viruses have a thread like appearance or appear to have filamentous structure (Goldsmith, 2014). There are 11 viral families, 7 sub-families, 89 genera and 422 species in the order *Mononegavirales* as of October, 2020 (ICTV, 2020). Within the order *Mononegavirales*, *Filoviruses* are closely related members of the families *Paramyxoviridae, Pneumoviridae* and *Sunviridae* (Kuhn et al., 2019). All these are unsegmented viruses (Afonso et al., 2016).

Based on their molecular properties, the family *Filoviridae* consists of six genera namely *Ebolavirus*, *Marburgvirus*, *Cuevavirus*, *Striavirus*, *Thamnovirus* and *Dianlovirus* with a total of 11 species of viruses. (Kuhn et al., 2019; Negredo et al., 2011; Shi et al., 2018). These viruses have a wide range of hosts, different geographical predispositions and different genome organization (Feldmann & Klenk, 1996a). Of public health importance are viruses in the genus *Ebolavirus* and *Marburgvirus*.

The genus *Ebolavirus* comprises of six species of ebola viruses namely *Bundibugyo ebolavirus* (BDBV), *Zaire ebolavirus* (EBOV), *Reston ebolavirus* (RESTV), *Sudan ebolavirus* (SUDV), *Taï Forest ebolavirus* (TAFV) and *Bombali ebola virus* (BOMV)

(Dudas & Rambaut, 2014; Goldstein et al., 2018). The latter was the most recently discovered species of ebolaviruses. Of the aforementioned ebolavirus species, BDBV, SUDV and EBOV have been associated with large human outbreaks (Basler, 2015). The genus *Marburg virus* has one species, *Marburg Marburgvirus* which has been divided into two, Marburg virus (MARV) and Ravn virus, RAVV (Rougeron et al., 2015). The *Cuevavirus* genus comprises of only one species namely *Lloviu cuevavirus* (LLOV) (Kuhn et al., 2019; Maruyama et al., 2014). *Striavirus* and *Thamnovirus* genus have one species each Xīlǎng virus (XILV) and Huángjiāo virus (HUJV), respectively (Kuhn et al., 2019). In humans, RESTV, has been associated with asymptomatic infections while *LLOV*, BOMV, XILV and HUJV filoviruses have not been reported to cause human infections and disease (Goldstein et al., 2018; Messaoudi et al., 2015; Miranda et al., 1999). In non-human species, ebolavirus infection is lethal (Miranda & Miranda, 2011). Lloviu cuevavirus has been reported to be lethal in bats (Negredo et al., 2011).

2.2 General structure of Filoviruses of the Genus Ebolavirus, Marburgvirus and Cuevavirus.

Filoviruses are pleomorphic. They appear either as long filamentous forms, or shorter U-shaped, 6 shaped or circular configurations as shown in figure 1 (Khataby et al., 2016; W. Shi et al., 2008). The most characterized filoviruses are the EBOV, SUDV and MARV viruses (Burk et al., 2016). Filovirus virions have great variation in length with a width of approximately 80nm. Infectious virions have been associated with filoviruses of approximately 665nm in *Marburgvirus* and 805nm in *Ebolavirus* (ICTV 9th Report,

2017). The virion is composed of a nucleoprotein (NP) complex which is surrounded by a lipid enveloped region (Bukreyev et al., 2014).

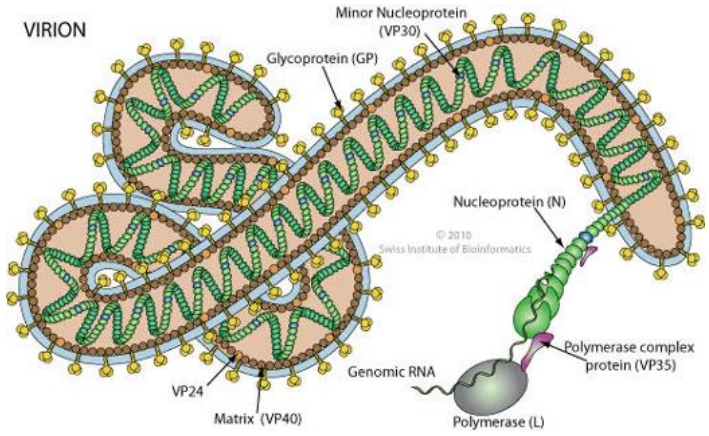

Figure 1: Schematic representation of filovirus (Shi et al., 2008).

2.2.1 Genome structure of Filovirus of the Genus Ebolaviruses, Marburgvirus and Cuevavirus

The genome organization of filoviruses is rather similar (Mühlberger, 2007). The ebolavirus genome is often used as a prototype because it is one of the well studied filovirus genomes (Feldmann et al., 2020). Filoviruses have a non-segmented (monopartite), single stranded linear negative-sense RNA (ssRNA) genome, measuring approximately 18.9-19 kilo base pairs long; ebolaviruses-19kb, Marburgvirus-18.9kb (Feldmann & Klenk, 1996a). The viral genomes have long non-coding regions (non-translated regions and highly transcriptional regions) at the 3’ end upstream gene, transcription stop and start signals and a 5′ non-translated regions of the down-stream gene of their messenger RNA (Alonso and Patterson, 2013). The viral genome encodes for 7 genes (Barrette et al., 2011). The gene order is characterized by: 3′ - Nucleoprotein (NP) - Viral protein35 (VP 35) - Viral protein 40 (VP 40) - Glycoprotein (GP) - Viral protein 30 (VP 30) - Viral protein 24 (VP24) - Large protein (L) - 5′ as shown in figure 2

(Emanuel et al., 2018). The RNA is enclosed by the NP with the viral proteins enclosed within the envelope region. The GP is the only viral protein exposed on the outer surface of the virus. EBOV GP gene is proteolytically translated into 2 sub-units (Wool-Lewis & Bates, 1999)

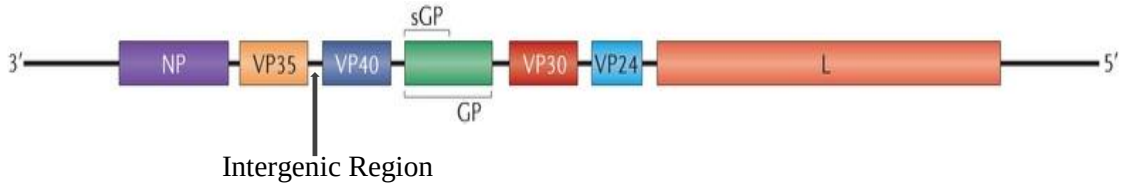

Figure 2: Genome organization of filoviruses (Shi et al., 2008).

Filovirus genes have individual open reading frames (ORF) flanked by long non-coding regions except for LLOV (Emanuel et al., 2018; Mühlberger, 2007). Lluvio virus has seven ORF encoded by six mRNA transcripts of which one is a dicistronic\bicistronic mRNA and contains both VP24 and L ORF. *Lloviu cuevavirus* (LLOV) has a unique start signal 3'-CUUCUU(A\G)UAAUU-5' (Negredo et al., 2011). Filoviral genes are separated by intergenic sequences/gene of different lengths, which are not conserved in all filoviruses (Brauburger et al., 2014; Jun et al., 2015). Nucleotide sequences at the 3'-terminus are complementary to similar regions on the 5' terminus. At the extreme 3′ (leader) and 5′ (trailer) ends of the genomes, there are extragenic regions/genes which are highly conserved compared to the intergenic regions (IR)/genes, (ICTV, Report, 2011). The extragenic regions carry the promoters for transcription and replication (Neumann et al., 2009). Each gene is flanked by conserved transcription start and stop codons (Shi et al., 2008).

Intergenic regions (IRs)/genes separate the viral genes and vary in size. In between genes NP and VP35, VP40 and GP there are approximately 4nt long IR sequences (Figure 2). A large IR sequence of approximately 144nt separates VP30 and VP24. Gene overlaps are found in between VP35 and VP40, GP and VP30 and VP and L genes (Sanchez et al., 1993). IR genes/sequences play a minor role in viral transcription regulation (Brauburger et al., 2015).

2.2.2 Filovirus Associated Proteins and Their Functions

Filoviruses are composed of genomic RNA molecule, four virion-associated structural proteins, three membrane associated proteins and non-structural proteins as shown on figure 1 (Shi et al., 2008). The structural proteins are nucleoprotein (NP), RNA-dependent RNA polymerase co-factor (VP35), transcriptional activator (VP30) and RNA-dependent RNA polymerase Large Protein (L). The membrane associated structural proteins are the spike surface protein, glycoprotein (GP) which is a full length type 1 transmembrane and class I fusion protein, VP40 and VP24 (primary and secondary matrix proteins which are non-glycosylated proteins) which are associated with the inner side of the virion membrane (ICTV, Report, 2011). The non- structural proteins of filoviruses are soluble glycoproteins (sGP), small soluble glycoproteins (ssGP), shed glycoproteins and delta peptides (δ peptides) in ebolaviruses (Cook & Lee, 2013; Mehedi et al., 2011). Other than the δ peptides, the three non-structural GP gene products are identical at the N terminal primary sequences of 295 amino acids with differences at the C terminal portions following the transcriptional editing site (Mehedi et al., 2011).

2.2.2.1 Nucleoprotein (NP)

The nucleoprotein is the first gene of filoviruses. It is the major part of filovirus nucleocapsid. The nucleoprotein gene is encoded by gene 1 in filoviruses. It is located at the 3' end of the genome and is preceded by a putative leader sequence. The gene starts with a transcriptional gene sequence 3'-UACUCCUUCUAAU and ends with the polyadenylation site sequence 3'-UAAUUCUUUUUU (Sanchez et al., 1989). The NP of EBOV has two distinct domains that is the hydrophobic N terminal half with approximately 1-400 amino acids and a hydrophilic C terminal half. The N and C terminals are separated by a long disorder linker of about 250 amino acids (Dziubańska et al., 2014). These two terminals makes EBOV viruses different from all the viruses in the order *Mononegavirales* (Dziubańska et al., 2014). The molecular architect of the C terminal domain in the NP from BDBV and TAFV proteins are highly similar to that of EBOV, while the MARV protein is significantly different from EBOV protein (Baker et al., 2016). Filovirus NP has several conserved regions within the genome (Darling et al., 2017; Ali & Islam, 2015).

The nucleoprotein oligomerizes to form helical filaments at the core of the virion. In addition, it encapsidates the viral genome hence providing protection of ssRNA. It also acts as a scaffold for other viral proteins within the viral nucleocapsid. Nucleoprotein is an important factor in viral replication (Watanabe et al., 2006). The viral nucleoprotein binds to L, VP24, VP30 and VP35. NP, VP24 and VP35 are needed for development of filamentous structures of the nucleocapsid (Shi et al., 2008; Huang et al., 2002).

Ebolavirus viral VP24 interacts with the NP to facilitate nucleocapsid assembly and genome packaging during viral replication cycle (Banadyga et al., 2017).

2.2.2.2. Viral Protein 35 (VP35)

Virion protein 35 (VP35) has multiple functions in both the virus and the host. It is an important component in viral RNA synthesis in virus replication and acts as a viral RNA polymerase complex (RNA dependent RNA co-factor). For virus replication to occur, the N terminal coiled domain is required for oligomerization and a C terminal dsRNA binding region (Reid et al., 2005). The lack of oligomerization will lead to failure of interaction with the L protein leading to a stop in virus replication.

VP35 together with NP and VP24 are essential for nucleocapsid formation (Hammou et al., 2016). In the host, VP35 plays a role in virus virulence through the facilitation of host immune evasion by antagonization and inhibition of interferon alpha (IFNα) and interferon beta (IFNβ) (Dilley et al., 2017; Hammou et al., 2016; Albariño et al., 2015). Different filoviruses employ different mechanisms in inhibition of IFNα and IFNβ (Edwards et al., 2016). Mutations in VP35 would hinder the filovirus replication and antagonist functions (Morwitzer et al., 2019). VP35 N and C regions are highly conserved among filoviruses (Zhu et al., 2017; Kirchdoerfer et al., 2015).

2.2.2.3 Virion Protein 30 (VP30)

Virion protein 30 (VP30) acts as a transcriptional activator and is important in virus replication (Biedenkopf et al., 2016). For transcription to occur, VP30 must be in a

dephosphorylated form which promotes mRNA synthesis and viral genome replication (Tigabu et al., 2018; Ilinykh et al., 2014; Modrof et al., 2002). In replication, the interaction between phosphorylated VP30 and NP forms VP30-NP complex (Kirchdoerfer et al., 2016). The VP30-NP complex modulates RNA synthesis in EBOV (Xu et al., 2017). Furthermore, the formation of ribonucleoprotein complex/proteins which is a combination of viral genome, L protein, VP35 and VP30 is involved in budding of mature virions from the infected cells (Spiegelberg et al., 2011).

2.2.2.4 Virion Protein 40 (VP40)

Virion protein 40 (VP40) is a primary matrix protein. It is the most abundant viral matrix protein expressed during infection with Ebolavirus (Madara et al., 2015). VP40 is located under the viral envelope where it maintains the structural integrity of the virus particle (Feldmann & Klenk, 1996b). It plays a major role in virus assembly and budding. In addition to this, the matrix protein interacts with the lipid bilayer (Dessen et al., 2000).

Viral protein 40 (VP40) interacts with GP to form the filaments of ebolaviruses (Noda et al., 2002). The well defined N terminal domain (NTD) and C terminal domain (CTD) gives VP40 the ability to transition to different conformational changes. The C terminal domain triggers oligomerization of VP40 into hexamers through plasma membrane interactions (Bornholdt et al., 2013). The conformational changes are important in membrane trafficking (a butterfly shaped dimer structure) through formation of NTD-NTD dimeric interphase. These acts as structural building blocks of cylindrical viral matrix filament (hexameric structure) through polymerization of hexamers and their

CTDs to control viral transcription (RNA binding octomeric ring structure) through RNA binding to NTD (Bhattarai et al., 2017; Bornholdt et al., 2013).

It has been hypothesized that VP40 plays a role in immune cell dysfunction (Oda et al., 2016). This occurs through packing of VP40 in exosomes. This in turn can be able to decrease the viability of immune cells through induction of bystander apoptosis of the immune cells (Pleet et al., 2017). Additionally, VP40 interacts with GP viral surface protein, lipid envelope and nucleocapsid complex which contains NP, VP35, L, VP30 and VP24 proteins. In so doing, it is therefore associated to the functions of the aforementioned proteins (Kolesnikova et al., 2017). VP40 follows L protein in terms of conserved regions (Sanchez & Rollin, 2005).

2.2.2.5 Virion Protein 24 (VP24)

Virion protein 24 (VP24) is a secondary matrix protein and a membrane protein (Hammou et al., 2016). It is a minor matrix protein that plays a role in assembly of viral nucleocapsid, genome packaging and virion budding during replication cycle of the virus (Banadyga et al., 2017; Hammou et al., 2016; Han et al., 2003). In addition, VP24 mediates nucleocapsid transport (Takamatsu et al., 2018). The VP24 specificity determining positions (T131S, M136L and Q139R) acts as interferon (IFN) antagonist by inhibiting IFN λ1 gene expression (He et al., 2017; Schwarz et al., 2017).

VP24 is the most conserved viral protein in ebolaviruses (Sanchez & Rollin, 2005).

2.2.2.6 Large protein (L)

Large protein (L) is also referred to as RNA dependent RNA polymerase, is the largest protein in filoviruses. The L protein is highly conserved in negative stranded RNA viruses after VP24 (Schmidt & Hoenen, 2017; Volchkov et al., 1999). For L protein to take part in transcription and replication, it forms a complex with other factors such as NP, VP30 and VP35 (Mühlberger, 2007; Trunschke et al., 2013). In addition, L protein contains all the catalytic/enzymatic functions necessary for transcription and replication (Boehmann et al., 2005). For replication to take place in Marburg virus, L proteins works together with nucleocapsid proteins, NP and VP35 while for ebolavirus, an additional VP30 is needed (Mühlberger et al., 1992).

2.2.2.7 Glycoprotein

Glycoprotein (GP) is the only known protein expressed on the surface of *Filoviruses* and is extremely immunogenic. It is the fourth of seven genes in filoviruses. It gives the trimeric spike form appearance on the surface of the virus. Glycoprotein gene in filoviruses encodes for full length (676 residue glycoprotein) GP_1 sub-unit and GP_2 subunit, small dimeric non-structural form secretary glycoprotein (sGP) 364 residue pre-secreted and small secreted glycoprotein (ssGP) 298 residue small secreted glycoprotein (de La Vega et al., 2015). The full length glycoprotein (GP_1 sub-unit, GP_2 subunit) are products of GP gene transcriptional editing which occurs in the golgi apparatus and endoplasmic reticulum. GP_1 sub-unit and GP_2 subunit ($GP_{1,2}$) form the trimeric structures located on the surface of the virus as virion spikes. GP_1 acts as a receptor binding sub-unit while GP_2 acts as a membrane fusion sub-unit. The main functions of $GP_{1,2}$ complex

is to bind on the surface of the host cell and catalyze membrane fusion during viral entry into the cell (Lee and Saphire, 2009). Glycoprotein$_{1,2}$ ($GP_{1,2}$) complex has a high ability to bind onto several cell surface molecules, it determines the broad cellular tropism of ebolaviruses (filoviruses) which contributes to the complex pathogenicity of ebolaviruses (Ning et al., 2017).

Secretary glycoprotein (sGP) is the primary product encoded by the GP gene through fusion of furin. It is in a dimer form found in the five ebolavirus species i.e BDBV, EBOV, RESTV, SUDV and TAFV (de La Vega et al., 2014; Volchkova et al., 1998). It has been suggested that sGP can act as a substitute of GP_1 hence giving it a structural protein function (Iwasa et al., 2011). In ebolavirus pathogenesis, sGP has anti-inflammatory properties to both neutrophils and macrophages (Bradley et al., 2018; Pallesen et al., 2016; Maruyama et al., 2014). This leads to immune activation and increased permeability thus increases ebolavirus pathogenicity (Escudero-Pérez et al., 2014). Additionally, sGP might play a role in apoptosis through the intrinsic pathway (Wauquier et al., 2010). Small soluble glycoproteins (ssGP) function remains unclear though it appears to have a similar structure to sGP (Lai et al., 2014).

The delta (Δ) peptide is the sGP cleavage product at amino acid 321-324 at the C-terminus of sGP (Volchkova et al., 1999). Its function in ebolavirus pathogenicity is not well understood though it is believed to act as a viroporin. During ebola virus infection, Δ peptides acting as viroporin, leads to an increase in ion permeability which causes

cytotoxicity (He et al., 2017). Furthermore, it is thought to have a role in ebolavirus and marburg virus cell entry during filovirus replicative cycle (Lee & Saphire, 2009).

2.3 Replication of Filoviruses

Filovirus replication generally occurs in the cytoplasm of infected cells. Filoviruses attach to the cell membrane with the aid of the surface glycoprotein (GP). The virus is subsequently taken up by micropinocytosis leading to formation of an endosome. The endosome acidifies leading to cleavage of GP by the cellular proteases (cathepsin B and cathepsin L). This allows the GP to interact with the host protein (Mühlberger, 2007).

Glycoproteins mediate fusion of the virus and the endosomal membrane thus releasing the viral ribonucleocapsid into the cytosol. At the cytosol, the viral RNA's dependent RNA polymerase un-coats the nucleocapsid and transcribes the genome into seven positive stranded mRNA's with the help of NP, VP35 and L proteins (Mühlberger 2007). The positive stranded mRNAs are then translated to structural and non-structural proteins (Figure 3).

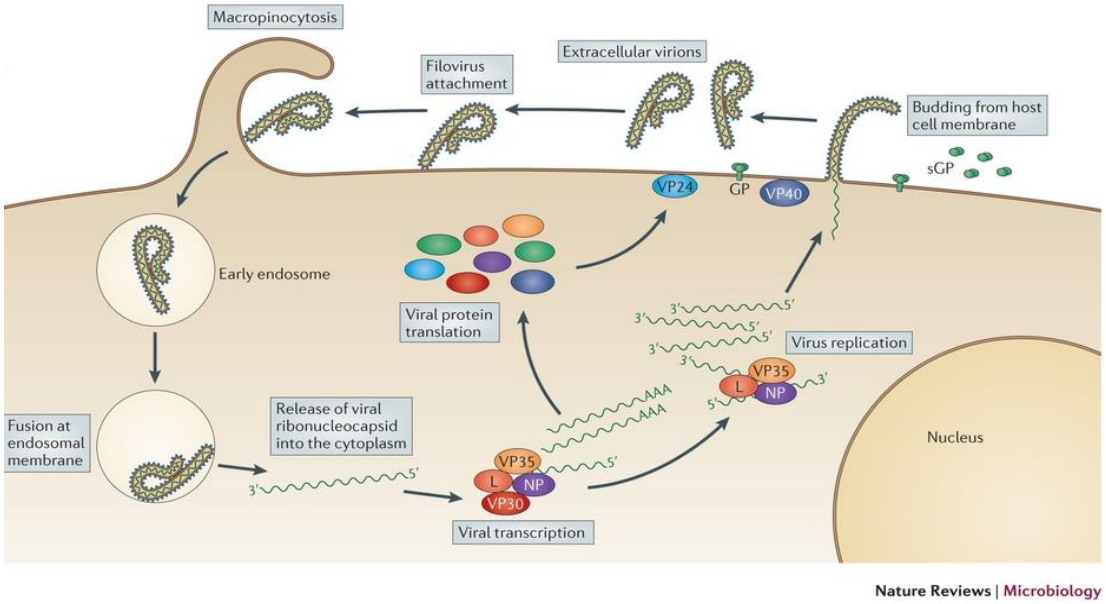

Figure 3: Replication of filoviruses (Messaudi et al., 2015).

2.4 Epidemiology of Filoviruses

Filovirus outbreaks have majorly occurred in the African continent, especially in West and East Africa (Abramowitz et al., 2018; Peterson et al., 2004). A few imported cases of filovirus infection have been reported in South Africa, Europe, United States of America and Asia affecting both animals and humans (Dovih et al., 2019; Weyer & Blumberg, 2014; McCarthy, 2014; Timen et al., 2009). In Russia, laboratory contamination infections of EBOV have also been reported (Kortepeter et al., 2008). This leaves filovirus problem not only for the African continent but the world due to the imported and laboratory contamination cases reported. The distribution of filoviruses worldwide is generally not well documented due to the few outbreaks that have occurred over the years in humans, swine, non-human primates and bats. There are other strains of filoviruses

that have been detected in animals with no known human infections or transmission (Cantoni et al., 2016).

2.4.1 Epidemiology of *Marburg marburgvirus*

The first documented outbreak of filovirus was the *Marburg virus*. This occurred simultaneously in Marburg and Frankfurt, Germany and in Belgrade, Serbia in 1967 (WHO, 2021b). The outbreak was believed to have originated from laboratory workers who handled experimental NHPs imported from Africa (Brauburger et al., 2012). Other outbreaks occurred successively in the world. The suspected reservoir host the fruit bat is majorly distributed in the tropics (Figure 4). In August 2021, West Africa (Guinea) confirmed its first ever human case of Marburg infection (CDC, 2021c).

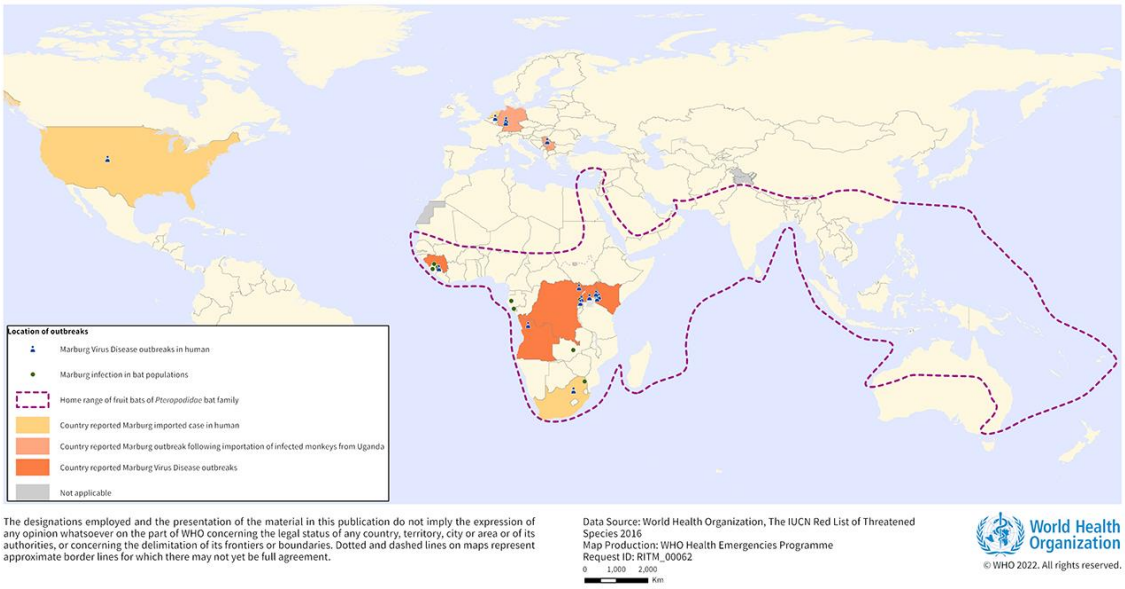

Figure 4: World map showing countries affected by Marburgvirus and distribution of fruit bats (WHO, 2022).

Since the first outbreak of marburg virus in 1967, the virus has continued to cause infections in Central and East Africa. In addition, imported case has been reported in United States of America and Netherlands. Below is Table 1 showing chronological list of marburg outbreaks reported in the past (WHO, 2021b).

Table 1: Cases of Marburg outbreaks in the world.

Year	**Country**	**Cases**	**Case Fatality Rate**
2017	Uganda	3	3(100%)
2014	Uganda	1	1(100%)
2012	Uganda	15	4(27%)
2008	Netherlands (Ex-Uganda)*	1	1(100%)
2008	United States of America, Colorado (Ex-Uganda)*	1	0(0%)
2007	Uganda	4	2 (50%)
2005	Angola	374	329 (88%)
1998-2000	Democratic Republic of the Congo	154	128 (87%)
1987	Kenya	1	1 (100%)
1980	Kenya	2	1 (50%)
1975	South Africa	3	1 (33%)
1967	Yugoslavia*	2	0 (0%)
1967	Germany*	29	7 (24%)

The majority of Marburg virus infections in humans have occurred in Africa. *Cases in Germany, Netherlands, Yugoslavia and United States of America were all imported from Africa (Amman et al., 2012, Fujita et al., 2009, Time 2009).

2.4.2 Epidemiology and Distribution of Ebolaviruses

The first EBOV outbreak was reported in South Sudan and Democratic Republic of Congo concurrently in 1976, which occurred in a rural setting. Subsequently, the outbreak was followed by several outbreaks in animals and humans in Africa, Philippines, Russia, Italy and United States of America (USA) as highlighted in Table 2 (CDC, 2017). Furthermore, fruit bats species which are suspected reservoir hosts for filoviruses are distributed in several countries that have had or not had ebola virus outbreaks (Figure 4 and 5). The most recent outbreak was in October 2021, where a human case of ebolavirus was confirmed in Congo, West Africa (CDC, 2021b).

Table 2: Cases of Ebolavirus infections in the world.

Year	**Country**	**Reported Cases**	**Case Fatality Rate**
2021	Guinea	12	6 (50%)
2020	DRC	130	55 (42.3%)
2018	DRC	54	33 (61%)
2017	DRC	8	4 (50%)
2014	DRC	66	49 (74%)
2014-2016	Guinea, Liberia and Sierra Leone	28,616	11,310 (40%)
2012- 2013	Uganda	6	3 (50%)
2012	DRC	36	13 (36.1%)
2012	Uganda	11	4 (36.4%)
	Uganda	1	1 (100%)
2008- 2009	DRC	32	15 (47%)

2008	Philippines	6	0 (0%)
2007- 2008	Uganda	149	37 (25%)
2007	DRC	264	187 (71%)
2004	Russia	1	1 (100%)
2004	Sudan (South Sudan)	17	7 (41%)
2003	DRC	35	29 (83%
2002- 2003	DRC	143	128 (89%)
2001- 2002	DRC	57	43 (75%)
2001- 2002	Gabon	65	53 (82%)
2000-2001	Uganda	425	224 (53%)
1996	Russia	1	1 (100%)
1996	South Africa	2	1 (50%)
1996-1997	Gabon	60	45 (74%)
1996	Gabon	37	21 (57%)
1995	DRC (formerly Zaire)	315	250 (81%)
1994	Côte d'Ivoire (Ivory Coast)	1	0 (0%)
1994	Gabon	52	31 (60%)
1992	Italy	0	0 (0%)
1989-1990	Philippines	3	0 (0%)
1990	USA	4	0 (0%)
1989	USA	0	0 (0%)
1979	Sudan (South Sudan)	34	22 (65%)
1977	Zaire	1	1 (100%)
1976	England	1	0 (0%)
1976	Sudan (South Sudan)	284	151 (53%)
1976	Zaire (DRC)	318	280 (88%)

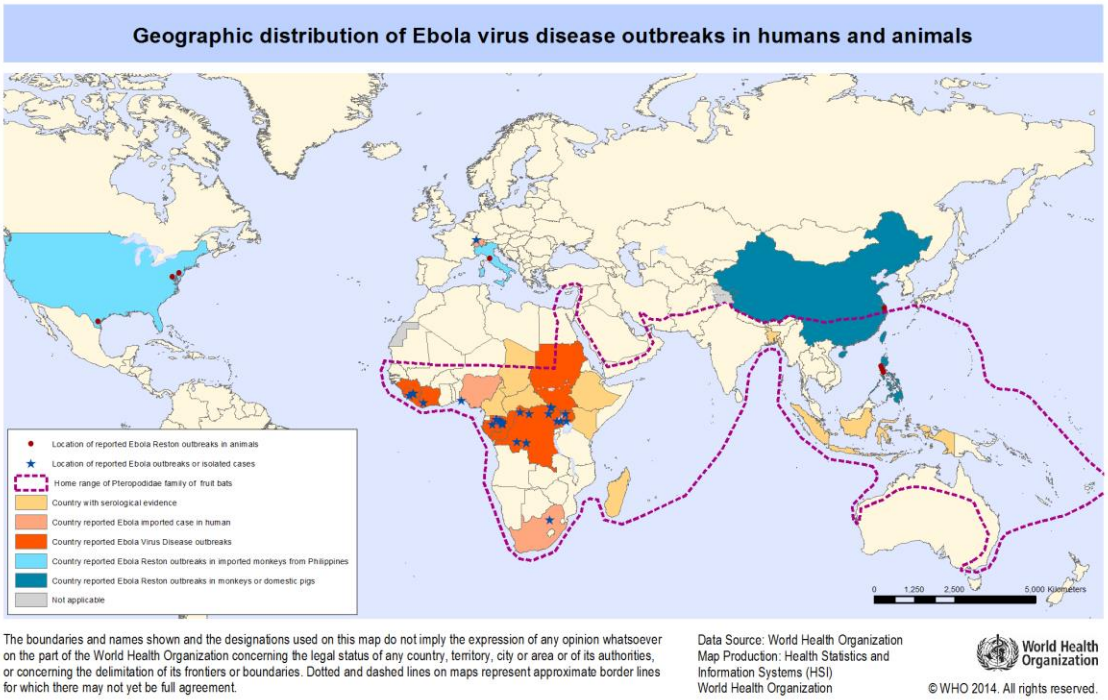

Figure 5: World map showing countries affected by Ebolavirus and distribution of fruit bats (WHO, 2014)**.**

2.4.3 Epidemiology of Other Filoviruses

In Asia (China), Ebolaviruses have been isolated from fruit bats while in Philippines, Ebola Reston virus has been isolated in pigs with 4 reported asymptomatic human cases since 1989 to 2018 (CDC, 2021a; He et al., 2015; Barrette et al., 2011). In Europe (Spain) Lloviu cuevavirus was isolated from a dead insectivorous bat (Negredo et al., 2011). Lloviu cuevavirus is endemic in France, Portugal and Spain which caused massive deaths in schreiber's bat in 2002 (Negredo et al., 2011). Xīlǎng virus and Huángjiāo virus have been isolated from stripped frog fish (Shi et al., 2018).

2.4.4 Reservoir hosts, amplification and accidental hosts

The natural hosts of Filoviruses are not well understood though fruit bats, insectivorous bats, rodents, swine, duikers and fish have been reported as natural reservoir hosts while NHPs, have been implicated as accidental hosts. (Mekibib & Ariën, 2016; Olival & Hayman, 2014; Swanepoel et al., 2007; Groseth et al., 2007; Towner et al., 2009; Calisher et al., 2006). Filovirus gamma globulin (ebolavirus and marburg virus) antibodies have been detected in several bat species (Pourrut et al., 2007; Pourrut et al., 2009). Furthermore, virus specific RNA of filoviruses has also been isolated from bats, NHPs, rodents, swine and fish (Shi et al., 2018; Towner et al., 2009; Peterson et al., 2004; Jahrling et al., 1990). With the several outbreaks that have occurred, it is still unclear as to what role the suspected reservoir hosts play in filovirus transmission cycles (Feldmann and Feldmann, 2013). In a majority of the cases, the source of human infections was not identified (Table 3).

Table 3: Sources of human ebolavirus infections.

Outbreak, location, year	**Country**	**Ebola Species**	**Index Case Source of infection**
Yambuku, 1976	DRC	EBOV-Z	Unknown
Nzara, 1976	Sudan	EBOV-S	Unknown
Tandala, 1977	DRC	EBOV-Z	Unknown
Nzara, 1979	Sudan	EBOV-S	Unknown
Cote d'Ivoire, 1994	Cote d'Ivoire	EBOV-IC	Chimpanzee
Mekouka, 1994	Gabon	EBOV-Z	Chimpanzee, gorilla
Kikwit, 1995	DRC	EBOV-Z	Unknown

Mayibout, 1996	Gabon	EBOV-Z	Chimpanzee
Booué, 1996	Gabon	EBOV-Z	Chimpanzee
Gulu, 2000	Uganda	EBOV-S	Unknown
Mekambo, 2001–2002	Gabon	EBOV-Z	Gorilla, chimpanzee, duiker
Mbomo Kelle, 2001–2002	RC	EBOV-Z	Gorilla, chimpanzee, monkey
Kelle, 2003	RC	EBOV-Z	Gorilla, duikers
Mbandza Mbomo, 2003	RC	EBOV-Z	Monkey, duikers
Yambio, 2004	Sudan	EBOV-S	Unknown
Etoumbi, 2005	RC	EBOV-Z	Unknown
Bundibugyo, 2007	Uganda	EBOV-B	Unknown
Kasai Occidental province 2008	DRC	EBOV-ZP	Unknown
Pangasinan site and Bulacan site 2008	Philippines	REBOV	Pigs
Luwero District, 2011	Uganda	EBOV-S	Unknown
Luwero, Jinja, Nakasongola And Kibaale Districts 2012	Uganda	EBOV-S	Unknown
Orientale Province, 2012	DRC	EBOV-B	Unknown
Équateur Province 2014	DRC		
2014	Guinea, Liberia, Sierra Leone , Senegal, Mali, Nigeria, Spain, USA, United Kingdom and Italy	EBOV-Z	Index case source unknown. Largely human to

			human transmission
Bas Uélé Province, 2017	DRC	EBOV-Z	Unknown
Équateur Province, 2017 and 2020	DRC	EBOV-Z	Suspected animal reservoir (2020).
North Kivu Province, 2021	DRC	EBOV-Z	Humans to humans transmission. Index case source unknown,

The document sources of ebolaviruses include chimpanzee, gorilla, duiker, monkey and pigs (Leroy et al., 2009).

2.5 Transmission Mechanisms (Intra and Inter Species Transmission)

Due to limited Filovirus outbreaks and related epidemiological studies documentation, the mode of transmission of filoviruses is purely based on the limited data available thus transmission is not well understood in both humans and animal cases. There are several documented modes of *Filoviral* transmission as demonstrated in Figure 6.

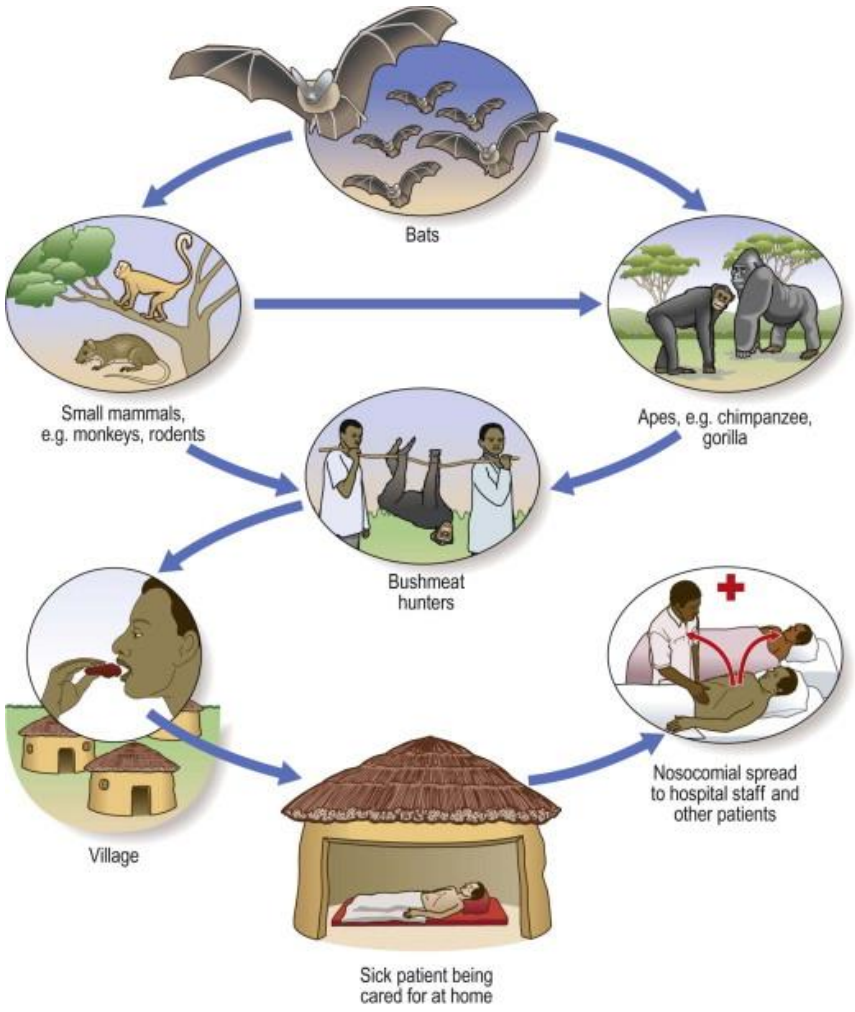

Figure 6: Transmission cycles of filoviruses (Subrahmanyeswari et al., 2019)**.**

The main mode of human to human transmission is through direct contact with bodily fluids of an infected persons/patient. Ebolavirus particles have been isolated from blood, saliva, tears/conjunctiva, aqueous humor, breast milk, semen, vaginal fluid, urine, stool, sweat, amniotic fluid and cerebral spinal fluid (Vetter et al., 2016; Mate et al., 2015). Infections have occurred to caregivers who washed, fed, medicated and even worked closely with infected persons (Selvaraj et al., 2018). In addition, insufficient or improper use of personal protective equipment during patient care, transportation, cleaning and

environmental disposal or disinfection activities increases the risks of viral transmission through contact of contaminated bodily fluids or items. Exposure of care givers to patients with unidentified filovirus infection is a risk factor (Selvaraj et al., 2018).

Animal to animal mode of transmission has not been well studied though in experimental animals, aerosol mode of transmission has been recorded. Infectious virus particles have also been isolated from skin, body fluids and nasal secretions of experimentally infected non-human primates (Mekibib and Ariën 2016). Spill-over of filoviruses from animals to humans is believed to occur through humans coming into contact with contaminated bodily fluids and fecal material of infected animals (Osterholm et al., 2015).

2.6 Risk Factors Associated With Filovirus Transmission

Several risk factors associated with filovirus transmission from animals to humans have been documented. The major risk factor associated with animal to human transmission include getting into contact with contaminated animal products which include bodily fluids and blood from infected animals. These will normally occur during hunting expeditions and preparing of game meat as a protein supplement. In addition, getting into contact with contaminated animal fecal materials during tourism activities, research activities and children playing in areas known to be inhabited with suspected reservoir hosts (Changula et al., 2014).

Risk factors associated with human to human transmission includes touching ill patients, getting into direct contact with bodily fluids including semen of infected individuals. In

case of an outbreak, safe burial procedures should be practiced and also avoiding contact with the corpse (WHO, 2020).

2.7 Pathogenesis of Filovirus

Pathogenesis of Ebola virus has been elucidated by the use of non-human primates as disease models. Non-human primates (NHPs) are the gold standard animal model for the study of filoviruses in particular ebola viruses (Siragam et al., 2018). Zaire ebolavirus has been used in many laboratory studies as it produces similar symptoms in NHPs as is presented in humans. The other filoviruses known to infect humans produce similar results with low case fatalities. 10-30% of infected patients can survive through mobilization of a strong adaptive immune response (Bray, 2005; Bray & Geisbert, 2005). Filoviruses employ majorly two pathogenic mechanisms of infections. First, the mechanisms in which host cells results in direct damage to tissues and second, the mechanisms in which tissue injury is brought about through interactions of the virus and innate and adaptive immune system (Falasca et al., 2015).

The virus targets the liver, adrenal cortex, spleen and lymphatic tissues, skin, gastro-intestinal tract, testis, heart and central nervous system (Martines et al., 2015; Messaoudi et al., 2015). Liver spleen and lymphnodes are the early target organs of filoviruses (Simmons et al., 2003). The main cells that are infected by EBOV include fibroblasts especially fibroblastic reticular cells, endothelial cells, epithelial cells, Kupffer cells in the liver, hepatocytes, adrenal gland tissue cells, monocytes, macrophages and dendritic cells with a bias to macrophages and dendritic cells (Bray & Geisbert, 2005; Martines et

al., 2015). For ebolavirus to be able to kill a variety of infected cells, they bind to a wide range of cell lectins which results to necrosis (Takada et al., 2004).

The early cell targets for filovirus infection are the macrophages, Kupffer cells, the dendritic cells and monocytes (Olejnik et al., 2011). These cells compose both the innate and adaptive immune system (Charles et al., 2001). Monocytes/macrophage lineage cells once infected, are believed to disseminate the virus to other cells and multiple target organs through lymphatics and via blood. These occur as either free virus or virus within the cell (Falasca et al., 2015). This takes place through recruitment of other immune cells which leads to the production of pro-inflammatory cytokines and chemokines such as interleukin 1 betta (IL-1β), IL-6, interleukin 1 receptor agonist (IL-1RA), soluble interleukin 6 receptor (sIL-6R), IL-10, tumor necrotic factor alpha (TNF α), soluble tumor necrotic factor receptor 1 (sTNF-R1), sTNF-R11, macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β (Bixler & Goff, 2015; Fernando et al., 2015a; Baize et al., 2002). Other than chemokines and cytokines produced by the cells, other mediators such as nitric oxide, leukotrienes and reactive oxygen molecules are produced. The effects of production of pro-inflammatory cytokines, chemokines and other mediators leads to vasodilation of local blood vessels, initiation of coagulation, increase in the permeability of the endothelial lining, upregulation of the expression of endothelial surface-adhesion molecules, activation of the immune cells and triggering the release of additional cytokines (Bray & Mahanty, 2003; Francesconi et al., 2003).

During infection of the monocytes/macrophages with EBOV, the cells over produce tissue factors. Tissue factor (TF) is important in the development of coagulopathy observed in EBOV infections in both humans and NHPs (Geisbert et al., 2003). In addition IL6 (pro-inflammatory cytokine) up-regulates the production of TF in monocytes which may lead to coagulation irregularities (Grignani & Maiolo, 2000). Monocyte chemoattractant molecule, macrophage inflammatory protein (MIP) 1α can be released from splenocyte activated by TF thus leading to coagulopathy (Bokarewa et al., 2002).

Dendritic cells are the most affected compared to monocytes or macrophages (Waterman, 1999). The connective tissue surrounding the endothelial cells become infected subsequently (Mahanty and Bray, 2004). Generally, the epithelial cells become infected only if they come into contact with other infected cells that amplify the virus.

Interferon (IFN) is the major innate defense against viral infections (Hoffmann et al., 2015). Filovirus VP24 and VP35 aid filoviruses to evade the host immune system through inhibition of nuclear translocation of transcription factor, blocks the signaling pathways that leads to the expression of type 1 INFs and type 1 IFN induced genes, RNA silencing and inhibition of dsRNA dependent protein kinase translation (Schwarz et al., 2017). In addition VP 35 impairs the maturation of dendritic cells which are key to filovirus pathogenesis (Kühl & Pöhlmann, 2012).

In ebolavirus pathogenesis, lymphocytes are not infected by the virus. In contrast, lymphocytes have been reported to undergo apoptosis in human and animal models during infection (Claire et al., 2017). Considering that monocyte and macrophages infected by filoviruses do not undergo apoptosis, lymphocyte apoptosis has been linked to activation of fs-7 associated surface antigen (FAS) pathway and tumor necrosis factor (Tumor necrotic factor- related apoptosis inducing ligand, TRAIL) pathways. In addition, the binding of the virus glycoprotein to dendritic cells activates the TLR4 (Toll like receptor 4) pathway which triggers apoptosis (Iampietro et al., 2017). Apoptosis majorly occurs in the late stage of infection and contributes to immunosuppression (Mahanty & Bray, 2004).

2.7.1 Immune Evasion Mechanism

Ebolavirus employs several mechanisms to evade the immune system. The viral protein VP35 is capable of capping dsRNA and interacts with interferon regulatory factor 7 (IRF7) to prevent detection of the virus by immune cells (Messaoudi *et. al.,* 2015). The main role of the soluble glycoprotein (sGP) is not well understood though it is capable of subverting the anti-$GP_{1,2}$ antibody response. The $GP_{1,2}$ protein has shown anti-tetherin activity and the ability to hide cell-surface proteins. The VP24 interferes with the production of interferons (IFNs) and with IFN signaling in infected cells (Audet and Kobinger, 2015). This has clearly shown the mechanisms employed by EBOV to evade the immune system.

Viral protein 24 (VP24) is responsible for inhibition of anti-viral transcriptional genes by blocking the nuclear translocation of phosphor STAT 1 thus IFN mediated signaling. Marburg virus VP40 inhibits jak (janus kinase) and STAT phosphorylation leading to a compromised immune system. Ebolavirus VP40 and VP30 counter RNAi (RNA interference) pathway (Ramanan et al., 2011).

2.8 Clinical Manifestation of Filovirus Infection

2.8.1 Humans

In humans, filoviral infections are lethal and are associated with multiple hemorrhagic manifestations. The incubation period of filoviruses ranges between 2-21 days in humans (Fowler et al., 2014). Ten to thirty percent of patients infected with filovirus will recover with a fever for about 5 to 9 days. In cases resulting to death, clinical signs develop early, with death occurring between 6-16 days following infection (Feldmann and Klenk, 1996). The time that patients take to report to hospital from the onset of signs and symptoms is critical. The patients who report between 1-10 days are more likely to survive while those reporting from 6-16 days are more likely to die (Qin et al., 2015). In addition, age is a critical factor in filovirus (EBOV) survivorship with patients who are less than 30 years of age tend to survive more compared to patients who are more than 30 years of age (Qin et al., 2015).

The signs and symptoms of filovirus are non-specific and overlap between ebolavirus and marburg (Fernando et al., 2015b; Knust et al., 2015; Lado et al., 2015). Disease onset is sudden with fever, chills, myalgia (muscle pain), headache and lack of appetite. These

symptoms may be followed by abdominal pain, nausea, anorexia or/and vomiting, diarrhoea, sore throat, cough, arthralgia (joint pain) pharyngeal and conjunctival vasodilatation including retro-orbital pain (Dallatomasina et al., 2015; Lado et al., 2015). Patients are normally dehydrated and present with mental symptoms like confusion/disorientation, irritability, nervousness, restlessness, screaming and insomnia (Qin et al., 2015). They may develop non-pruritic, maculo-papular centripetal rash associated with varying degrees of erythema, which peels off by day 5 to 7 of illness. Haemorrhagic manifestations develop at the peak of illness. Other signs and symptoms may include extreme fatigue, weakness, loss of appetite or anorexia, chest pain, difficulty in breathing, hiccups, conjunctivitis, uveitis and the patient may fall into a coma (Dallatomasina et al., 2015; Lado et al., 2015; Varkey et al., 2015). Laboratory parameters are less characteristic (Feldmann and Klenk, 1996).

2.8.2 Non-Human Primates

In experimental studies, the signs and symptoms of filovirus (Ebolavirus and Marburg virus) infections in NHPs are similar to humans with a few differences. Non-human primate species exhibit slightly different signs and symptoms in relation to blood clotting. In African green monkeys generalized fibrin thrombosis is seen and in baboons prominent haemorrhages are seen (Ryabchikova et al., 1999). In cynomolgus macaques, the animals show febrile illness, anorexia, diarrhoea, skin rash and haemorrhage. The infection spreads to the liver, adrenal glands and endothelial cell just as seen in humans (Siragam et al., 2018; Ignatiev et al., 2000).

2.8.3 Rodents

Rodents have been used as disease models for both wild type Ebola viruses and Marburg viruses. In wildtype ebolavirus and marburg virus experimental mouse model, immunocompetent mice were resistant to inoculated wildtype ebolavirus and marburg (Bray, 2001; Qiu et al., 2014). When ebolavirus was inoculated in immunocompromised mice, the mice developed some signs and symptoms of filovirus infections (Siragam et al., 2018).

In experimental hamsters inoculated with marburg virus, the animals showed similar immunological signs seen in humans. The hamsters developed marburg haemorrhagic fever (Marzi et al., 2016).

2.8.4 Bats

Experimental Egyptian rousette bat models infected with ebolavirus (reston, Sudan, Bundibugyo, Taï forest) and marburg virus; did not show any sign and symptoms of filovirus infections (Jones et al., 2015). Sudan ebolavirus used for inoculation showed slightly wide spread compared to marburg virus which was widely disseminated. The bats did not shed or show any signs of viremia with Sudan ebolavirus compared to marburg virus which showed viremia, oral and rectal shedding. The antigens (Sudan, Ebola, Bundibugyo, Taï Forest, Reston and Marburg Virus antigens) were also found in the liver and spleen (Jones et al., 2015).

2.9 Diagnosis of filoviruses

An early detection of filovirus disease would greatly improve disease outcome. Diagnosis of filoviruses starts with signs and symptoms, any reported contact or exposure to an infected individual, travel to or from affected regions and confirmation with laboratory assays (Bebell & Riley, 2015). Currently a number of methods have been developed for detection of filoviruses in specimens. These methods include both serological and molecular assays (Martin et al., 2015).

Serological assays such as enzyme linked immunosorbent (ELISA) have been used to detect immunoglobulin gamma (IgG) and immunoglobulin mu (IgM) antibodies against filovirus (Bower & Glynn, 2017). Immunoglobulin gamma (IgG) detection is suitable for surveillance studies. Anti-filovirus (Ebolavirus) IgM presence can be used for the early detection and shows current ebolavirus disease status of an individual or animal (Dokubo et al., 2018; Ansari, 2014). Enzyme linked immunosorbent assays (ELISA's) have also been used in the detection of ebolavirus antigens (Niikura et al., 2001). Some ELISA's use specific viral antigens, whole lysate viral antigens, recombinant proteins and synthetic peptides (Babirye et al., 2018; Ayouba et al., 2017; Ikegami et al., 2003).

For detection and diagnosis of filoviral genes, reverse transcriptase polymerase chain reaction (RT PCR) targeting different regions of filoviral genome have been used. The RT-PCR is the gold standard for filovirus diagnosis due to its high specificity (Cherpillod et al., 2016). Reverse transcriptase real time polymerase chain reactions (RT qPCR) have been developed though they are more expensive compared to RT PCR (Jääskeläinen et al., 2015). Other tests like reverse transcriptase loop mediated isothermal amplification

(RT-LAMP) have been developed (Kurosaki et al., 2016). Tests such as virus isolation using VERO or VERO 6 cell lines, Immunoblot, Immuno-fluorescence microscopy, histopathology and immunohistochemistry have been employed in the past (Fischer et al., 2018; Muehlenbachs et al., 2017; Moyen et al., 2015; Saijo et al., 2006) .

Newer tools for diagnosis and detection of filoviruses are continuously being developed including Luminex based assays, rapid diagnostic point of care tools (immunochromatographic test or lateral flow immunoassays) both for genome or protein detection and antibody detection (Babirye et al., 2018; Biava et al., 2018; Brangel et al., 2018a; Ayouba et al., 2017).

2.10 Management and Prevention of Filovirus Infections in Humans

Currently there is no world health organization (WHO) approved treatment for filovirus disease (Sridhar 2015; (Mire et al., 2015). These diseases are treated symptomatically. Patients receive intravenous fluids and electrolytes, mechanical oxygen for ventilation, continuous renal replacement therapy, antibiotics, other medications (for blood pressure, cough, diarrhoea, vomiting, pain, fever and pain) and investigational therapies i.e two experimental interventions (Sprecher et al., 2017; Uyeki et al., 2016; WHO, 2015). Many research groups using animal models are working towards development of filovirus treatment regimen or drugs for humans (Mire et al., 2017). Currently research using different molecules is being conducted on different animal models. The following agents have been shown to be effective against filovirus infections antibodies (human monoclonal antibodies, neutralizing antibody and IgG), small molecules (synthetic adenosine analogue, anti-oxidant molecule), antisense RNA molecules

(phosphorodiamidate morpholino oligomers) and clotting factors (fibrinogen, vitamin K, blood transfusion, anti-coagulants, pro-clotting factors) (Anthony & Bradfute, 2015).

Even though there is a lot of research on vaccine studies for filoviruses, there are no WHO approved vaccines for prevention of filoviruses infections (Sridhar 2015; (Mire et al., 2015). Only one vaccine (being the first) the rVSV-ZEBOV (Ervebo ™), developed by NewLink USA was approved in December 19, 2019 by the U.S. food and drug administration, FDA (Center for Disease Control and Prevention, 2020). rVSV-ZEBOV is a vesicular stomatitis virus based vaccine expressing the glycoprotein of ZEBOV (Lévy et al., 2018).

Currently, there are other ongoing research on filoviral vaccines in different research groups and are at different stages of clinical trials (Matz et al., 2019). These include recombinant human adenovirus serotype 5 expressing the EBOV glycoprotein (Ad5-EBOV), GamEvac-combi vaccine which combines VSV and Ad5-EBOV, chimpanzee adenovirus 3 (ChaAd3) plus modified vaccinia Ankara vaccine MVA (ChAd3+MVA), recombinant human adenovirus serotype 26 (Ad26-EBOV) plus MVA vaccine and EBOV DNA vaccine (Matz et al., 2019).

With the lack of a variety of vaccines, potential to cause an epidemic/pandemic, inadequate information on distribution and reservoir hosts of *filoviruses,* this study sought out to detect and identify emerging *filoviruses* circulating in humans, wild caught non-human primates, bats and rodents in Kenya. This was with the aim of acting as warning

sign to a looming epidemic or pandemic and shedding light on the distribution of filoviruses in Kenya.

2.11 Risk Factors of Transmission of Filovirus

2.11.1 Risk Factors Associated With Filovirus Transmission in Humans

The risk factors associated with the transmission of filoviruses from animals to human population include getting into direct contact with infected animals through butchering of bush meat (blood and bodily fluids), exposure to fecal material from infected animals, population growth leading to humans encroaching into forested areas and travel from areas where filovirus outbreaks have been reported (CDC, 2021b). The risk factors associated with filovirus infections between humans to humans include direct contact (through broken skin and mucous membrane) with bodily fluids of infected individuals or persons who died from filovirus infections, including blood, feces, vomit semen; and through contaminated fomite (WHO, 2020).

2.11.2 Risk Factors Associated With Filovirus Transmission Among Non-human Primates, Bats and Rodents

The risk factors associated with filovirus transmission in animals is not well documented. However, it has been documented that during death of a primate (Chimpanzee) the animals respond in the same way humans do thus exposing them to the agent that caused demise of the animal. The responses include shaking, dragging and beating of the body (Stewart et al., 2012; Teleki, 1973). In 1994 EBOV outbreak was reported in a community of chimpanzees. The following include the risk factors associated with the outbreak

chimpanzees had consumed meat from an infected red colombus monkey (high risk), getting into close contact with infected individuals through sexual contact (low risk) or during infant breast feeding (high risk) (Formenty et al., 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Sites

This study was conducted in Laikipia North sub-county, Laikipia County, Kenya. Laikipia North has a human population size of 36,184 as per the 2019 Kenya population and housing census (Kenya National Bureau of Statistic, 2019). Laikipia county is ranked as the second in wildlife population with a wide range of animals (both domestic and wild animals) that freely transverse the county (Evans & Adams, 2016; Georgiadis et al., 2007). The county lies between latitudes 0^0 18″ and 0^0 51″ North, longitude 36^0 11″ and 37^0 24″ East (Figure 7) and covers a total area of 4,650.50 Km^2.

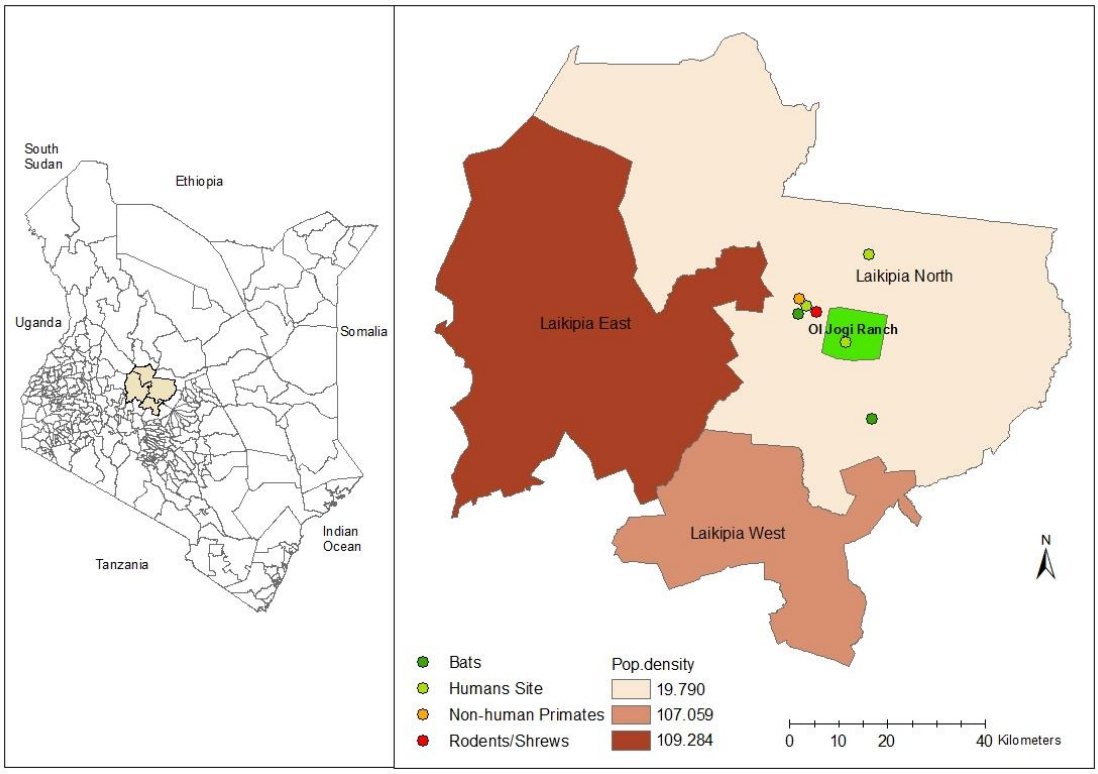

Figure 7: Map of Kenya showing position of Laikipia, County (Created by ArcGIS Desktop 10.7; Esri, Redlands CA. USA).

The landscape of Laikipia North is majorly covered by savanna grass land, community settlements, private ranches and conservancies. This region is classified as arid and semi-arid.

The study site was chosen due to its high numbers of wildlife living in close proximity to humans and the low annual rainfall recordings ranging from 150mm-750mm with compounding effects of frequent dry spells. Due to scarcity of water, animals and humans are forced to share the scanty water resources thus increasing human-animal interaction (Chepkwony et al., 2018). In addition, the main livelihood of the communities living in Laikipia North, Sub-county is pastoralism (Ameso et al., 2018). This in turn escalates the interaction between wild animals, domestic animals and humans thus putting them at a risk of zoonotic viruses' jump through inter-species transmission, cross species transmission or transmission to humans (DePuy et al., 2014; Lwande et al., 2015).

The samples were collected from are Oljogi and Ilmotiok villages (Figure 8). These villages were selected because the communities live in open villages thus increasing interaction between humans, wild and domestic animals. In addition, these villages had no access to piped water meaning, they have to share the scarce watering points with both domestic and wild animals. Bats were sampled from Mobile clinic (latitude 0.3738 and longitude 36.9052) and Naibor (latitude 0.163965 and longitude 37.022873). Rodents were sampled from Ranch house (latitude 0.32406 and longitude 36.90435) and non-human primates (NHPs) were sampled from Mpala Bridge site (Latitude 0.18072 and Longitude 36.54447) as shown in Figure 8.

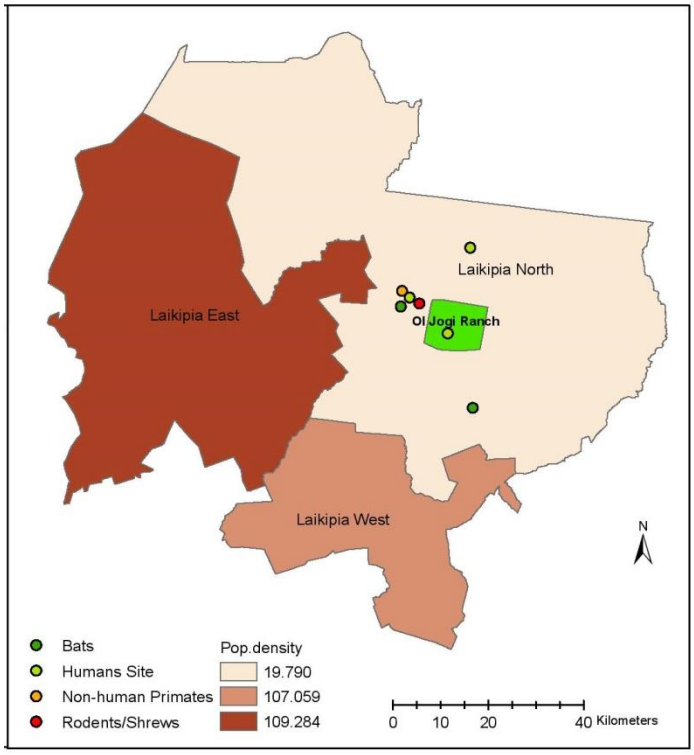

Figure 8: Map of Laikipia North Sub-county showing sampling sites for humans, bats, rodents and non-human primates (Created by ArcGIS Desktop 10.7; Esri, Redlands CA. USA).

3.2 Study Population

3.2.1 Recruitment of Human Subjects

The study human population comprised of children, adult males and females who are residents of Oljogi or Ilmotiok villages. Children were defined as any person between 2 years and 17 years. Laikipia North is sparsely populated with an average density of 13 person's per KM^2 (County statistics office, 2018).

All the villages in the study had a Community Health Volunteers (CHVs). The CHVs helped with the recruitment of study subjects and they also acted as the study's liaison

persons. They recruited participants by organizing community meetings where they introduced and described the purpose of the study. The participants were given the dates and location of sample collection. At the sample collection site, a medical camp was organized. As the participants came for sample collection, general medical check-up was conducted followed by administration of questionnaires.

3.2.1.1 Inclusion Criteria

i. Only residents (both healthy and diseased) who had lived in Oljogi or Illmotiok villages for a period of 6 months and above were included in the study.
ii. Only community members (both healthy and diseased) who had consented to take part in the study were included.
iii. Only community members (both healthy and diseased) who presented themselves at the sample collection centre that is the clinic and tents during sampling were included in the study.
iv. Any community member (both healthy and diseased) over 2 years of age was included in the study.
v. Both males and females (both healthy and diseased) were included in the study.

3.2.1.2 Exclusion Criteria

i. All visitors who had stayed for less than 6 months in Oljogi or Illmotiok villages were excluded in the study.
ii. Children less than 2 years of age were excluded from the study.

3.2.2 Animal subjects

The study animal subjects included bats, rodents and NHPs species at different development stages trapped in cages from Oljogi, Illmitiok, Pyramid or Mpala villages were used. Animals were identified using different morphological features.

3.2.2.1 Inclusion Criteria

i. Both sick, healthy and injured animals were included in the study.
ii. All genders were included in the study.
iii. All ages were included in the study.

3.3 Study Design

This was a cross sectional study where we collected samples from a sub-set of the population at one point in time without follow-up. The study was carried out between 4^{th}-11^{th} May, 2018 with an aim of detecting and characterizing filoviruses circulating in humans, bats, rodents and NHPs in Laikipia North Sub-County, Laikipia County, Kenya.

3.4 Sample Size Determination

The minimum sample size was estimated using Daniel, 2001 method where the prevalence of ebolavirus being the most prevalent filovirus was used (Naing et al., 2006; Daniel, 2001). In this study, we did not manage to collect the calculated sample sizes as estimated by Daniel, 2001 due to the study population being difficult to access and due to limited resources. However, for animal sampling we sampled to saturation where all the trapped animals were used in the study.

The prevalence of infection for sample size calculation was estimated using documented and published prevalence for humans, bats and non-human primates. The following formulae was used to determine an optimum or adequate sample size.

$$N = \frac{Z^2 P(1-P)}{D^2}$$

Where:

N= Minimum sample size required

Z= Confidence level of 90%, 95% or 99%

P= Postulated prevalence.

D= precision (Confidence interval)

3.4.1 Sample Size Calculation for Humans

To arrive to an estimate sample size, the following assumptions were made. The prevalence for filoviruses for human samples from other studies was between 2.7-19.4% (Becquart et al., 2010). To obtain an estimate sample size, we used a prevalence (P) of 19.4%, confidence level of 95% (Z) of 1.96 and a D of 5%.

$$N = \frac{1.96^2 \times 0.194(1-0.194)}{0.05^2}$$

$$N = 236.4$$

The calculated minimum human sample size was 237 samples. Considering that the calculated sample size is ≥5% of the total population i.e

$$\frac{236.4}{36184} \times 100 = 0.7\%$$

The study therefore used the finite correction sample size calculation formulae (Daniel, 2001).

$$n' = \frac{NZ^2P(1-P)}{d^2(N-1) + Z^2P(1-P)}$$

n' = Sample Size with a Finite Population Correction

N = Population Size

Z = Z statistic for a level of confidence (95%)

P = Expected Proportion (19.4%)

d = Precision (In proportion of 1)

Therefore:

$$n' = \frac{36{,}184 \times 1.96^2 \times 0.194(1-0.194)}{0.05^2(36{,}184-1) + 1.96^2\ 0.194(1-0.194)} = \mathbf{238.12}$$

The calculated sample size was **238** individuals.

3.4.2 Sample Size Calculation for Bats

The postulated prevalence used for bats was 3.5% (Steffen et al., 2019). To avoid the problem of a small sample size due to a prevalence (P) of less than 10% i.e 3.5%, the study made the sample size suitable by dividing the P by 2 and using it as our precision (D) (Naing et al., 2006). Therefore, P was divided (P=3.5%) by 2 prevalence giving us a precision (D) of 0.00175. Due to financial limitation, the study reduced the confidence level to 90% which gave us a Z value of 1.645

Therefore:

$$N = \frac{1.645^2 \times 0.035(1 - 0.035)}{0.0175^2}$$

$$N = 304.7 \text{ samples}$$

Therefore the calculated minimum sample size for bats was **305** samples.

3.4.3 Sample Size Calculation for Non-human Primates

Postulated prevalence of filoviruses infection in non-human primates is 18.7% (Mulangu et al., 2016). The study used a Z value of 1.645 (confidence interval of 90%) and a D value of 5%.

$$N = \frac{1.645^2 \text{ x } 0.19(1\text{-}0.19)}{0.05^2}$$

N= 166.83 samples

Therefore the calculated minimum non-human primate sample size was **167** samples.

3.4.4 Sample Size Calculation for Rodents

Due to the unavailability of filoviral prevalence in rodents, the study used an estimated prevalence of 50% to approximate the appropriate sample size (Naing et al., 2006). For the confidence interval Z we used 90% due to financial limitation.

$$N = \frac{1.645^2 \text{ x } 0.5(1\text{-}0.5)}{0.05^2} = 270.6 \text{ samples}$$

N= 271 samples

Therefore the calculated minimum rodents sample size was **271** samples.

3.5 Sampling Techniques

The study employed two sampling techniques. For humans simple convenience sampling technique was used while for animals capture mark release sampling technique was used.

3.5.1 Human Subjects Sampling Technique

Simple convenience sampling technique was used to recruit subjects/participants. The participants came from individuals who lived in close proximity to ranches. A pilot study was not performed since different research groups had conducted studies in these regions (Graham et al., 2019). The study subjects (humans) were recruited from Oljogi and Illmotiok, in Laikipia North, Sub-county. The subjects were recruited through word of mouth which was taken to the villages through the liaison project persons, who are county volunteer community-based health workers (Hoye et al., 2016). Each community-based health worker was given details on the project objectives, the sampling dates, time and the medical camp date and site. Every individual who turned up for the medical camp at the health facility and who signed the consent form was sampled. The minors consents were signed by the guardians.

The preference of human participants to come to the health facility for sampling was encouraged due to the fact that villages were located far apart from each other. The harsh terrain and financial constraints made it impossible for us to reach the villages (Butynski & de Jong, 2015). All consenting human participants were sampled for whole blood, oral swabs and fecal material. United States agency for international development (USAID)

PREDICT 2 protocols were followed to the latter (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.). The samples were given numbers (sample identification number, ID) in ascending order starting from 1. The type of sample was recorded e.g. blood, oral or fecal material below the sample identification number and the date included on the vial after the type of sample. The samples that were collected in the same site were all stored in one bag. The bag was labelled the sampling site and date. The records on the vials were also transferred on a hard copy.

3.5.2 Animal Sampling Techniques

Convenience sampling technique with capture mark release method was employed for animal species (Arnold & Ortiz-Pelaez, 2014; Britton et al., 2019). With the aid of experienced veterinarians, traps were set at different times during the day and at random locations including bats flying paths, watering points for different NHPs troops and different feeding grounds. The animals had an equal opportunity to be trapped and sampled. All the animals (NHPs, bats and rodents) trapped were sampled for whole blood/blood swabs/blood clots, oral swabs and rectal swabs/fecal material. United states agency for international development (USAID) PREDICT 2 protocols were strictly followed (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

The samples were given numbers (sample identification number, ID) in ascending order taking into consideration the taxa of the animals e.g. rodents rod001, bats bat001 and non-human primates NHP001. The type of sample was recorded for example blood, oral or fecal material below the sample identification number and the date included on the vial

after the type of sample. The samples that were collected in the same site and from the same taxa were all stored in one bag. The bag was labelled the sampling site and date. The records on the vials were also transferred on a hard copy.

3.6 Sample Collection

During sampling, there was minimal risk of injury to animals, humans and laboratory team. The sampling team was taken through vigorous training on personal protective equipment, capturing and handling of animal and human samples, respectively. Rabies vaccine was given to all veterinarians who were sampling animals. Hepatitis B vaccine was given to all scientists handling human samples. Ethical principles for both animals and human participants were followed to the latter ensuring minimal known physical and psychological harm to human participants and animals. Humane methods of sample collection were used which included collection of oral and rectal swabs. Non-human primates were sedated before sample collection. For bats and rodents, due to their small size in nature blood clots/swabs were collected. Once the samples had been collected, the animals were marked and released (Brainard et al., 2016).

Whole blood/blood clots/blood swabs, oral swabs and rectal swabs/fecal material were collected humanely and kept in 2ml cryovials containing 2 ml of TRizol® reagent. TRizol® reagent was used to maintain RNA integrity during tissue homogenization and for breaking down of cells.

3.7 Human Sampling Procedure

Before the administration of questionnaires, the participants were taken through the objectives of the study and the questions in the questionnaires. All consenting adults and children between 12-17 years of age whose guardians had consented took part in the study. Questions were read to the participant in their preferred language. This was followed by participants signing the consent forms, Appendix XI. Questionnaires were administered to the participants after which samples (oral swabs, rectal swabs and blood) were collected from each. Samples were collected from only consenting adults while for children (2 to 18 years), consent was sought from parents or the guardians Appendix XI (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.7.1 Collection of Blood Sample

Venipuncture was used to collect blood samples by qualified medical practitioners. A suitable venipuncture site on antecubital fossa where three veins (cubital, cephalic and basilica vein) pass was identified and selected. Any of the vein was selected depending on the visibility and if it was palpable. A tourniquet was placed 3-4 inches away from the selected site. A vein was selected and the area cleaned with 70% alcohol. The area was allowed to dry. The subjects were requested to make a fist. A needle was swiftly inserted through the skin into the lumen of the vein. This was followed by blood collection. After removal of the needle/blood collection, the selected area was covered with a dry swab under pressure to stop ongoing bleeding. All needles were disposed into a sharps collection box. The samples were divided into three aliquots. The first was stored in 2mls screw top cryotubes containing 500µl of TRizol® reagent and the second was stored in

Ethylene-diamine-tetra-acetic acid (EDTA) tubes for serum extraction. The third aliquote of 5mls blood was stored in EDTA tubes for serum. (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.7.2 Oral Swab Collection

The subjects were instructed to sit upright facing a light source and requested to open their mouths wide. Using a sterile polyester swab, all areas of the mouth were swabbed including the cheeks, tongue, areas between the cheeks and the gum and inside the lips. The samples were stored in 2mls screw top cryotubes containing 500µl of TRizol® reagent (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.7.3 Collection of Fecal Material

The subjects were requested to provide fecal sample. From the fecal sample, approximately 2gms was collected and put in 2mls screw top cryotubes containing 500µl of TRizol® reagent (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.). No rectal swabs were collected due to cultural restrictions.

3.8 Bat Handling and Sampling Procedure

Harp nets were used to capture the bats. With the help of an experienced veterinarian, the bats were identified into species using their morphology. The bat species were identified using different key morphological features as listed in Table 4 (Kingdom, 2015; Kingdon et al., 2013). The bats were given both scientific and common English names.

Table 4: Key morphological features for sampled bat species.

Bat species	Common Name	Species Characteristic feature
Chaerephon pumilus	Little free-tailed bat	Dorsal Pelage: Black/brown/greyish-brown Wing membrane: White or blackish-brown Ventral pelage: Pale tan/white Rounded ears, bigger than head
Scotophilus dinganii	Yellow-bellied House Bat	Dorsal pelage: Sepia brown, greenish-brown, greyish-brown or reddish-brown
		Ventral pelage: Pale yellow, bright yellow or orange-yellow

Each bat was placed in a porous cotton bag with a draw string mouth. They were kept in a cool dry place until sampling. When sampling time reached, the bats were removed from the bags and the following samples were collected: blood swab, oral swab and rectal swab using PREDICT 2 Protocols (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.8.1 Collection of Blood Swab

Bats were manually restrained between the thumb and palm of the non-preferred hand. The wing was extended until the forearm and upper arm formed a 90° angle. The bleed site that is the brachial or propatagial vein, was prepared by swabbing with a 70 percent ethanol swab. A 25 gauge needle was used to puncture either the brachial, the propatagial, cephalic and saphenous veins. Venous blood, on the surface of the skin was sampled

using a sterile tip. To stop bleeding, pressure was put on the site using sterile dry cotton wool (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.8.2 Collection of Oral Samples Using Swabs

The bats mouths were opened wide using hands while facing a light source. Using a sterile polyester swab, all areas of the mouth were swabbed including the cheeks, tongue, areas between the cheeks and the gum and inside the lips. The samples were stored in 2mls screw top cryotubes containing 500µl of TRizol® reagent (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.8.3 Rectal Swab Collection

A sterile polyester swab was inserted into the rectal region of the bat avoiding contamination from other regions and the environment. The swab was slowly rotated for 10 seconds and transferred into a 2mls cryotube vial containing 500µl of TRizol ® (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.9 Rodent Handling and Sampling Procedure

Prior to sampling, the rodents were anaesthetized using isoflurane. Zero point four (0.4ml) of isoflurane was applied to a cotton ball. The cotton ball was put into a metallic tea ball. The tea ball was placed in a clear plastic holding bag with the rodent. The animals were watched closely until anesthetized (Breathing rate increase then slow as the animal progressed further under anaesthesia. When the withdrawal reflex was suppressed after pinching the toe, the rodent was removed from the bag for processing.

An additional cotton ball soaked with 0.2 ml isoflurane kept in a 50 ml screw cap tube was kept for emergency that is when the animals begins to wake up during blood collection. If the animals began to wake up during blood collection, the 50 ml tube was unscrewed and the animal's nose was positioned over the tube to re-anaesthetize the animals (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

The rodents were identified using their key morphological features as listed in Table 5 (Kingdom, 2015; Kingdon et al., 2013).

Table 5: Key morphologiccal features used to identify sampled rodents.

Rodent species	**Common Name**	**Species Characteristic feature**
Acomys kempi	Kemp's spiny mouse	- stiff and coarse pelage - spines from mid-back to base of tail and on rump Dorsal pelage: orange-buff, darker on mid-dorsal line and not speckled Hairs: soft, whitish grey at base, orange at tip Flanks and Cheeks: dull orange lips, chin, throat and chest: White Ventral pelage: pure White sharply delineated from flanks Tail - about 94% of head body length - scaly, almost naked - brownish-grey above with black bristles - paler below with whitish bristles
Acomys percivali	Percival's spiny mouse	Coarse and stiff pelage with strong spines from mid back to base of tail Dorsal pelage: grey to greyish-brown

		Hairs/spines: pale grey at base, darker grey at tip Flanks: grey, usually paler than dorsal pelage (occasionally with some cinnamon on flanks) Ventral pelage: white, usually not stiff or spiny, clearly delineated from color of flanks Tail: about 76 % of head body length almost naked with scattered short bristles
Aethomys hindei	Hinde's veld rat	Dorsal pelage: Medium brown Dorsal Hairs: grey at base with pale brown tips Flanks: Paler with few black hairs Ventral pelage: greyish-white Ventral hairs: pale grey on basal half, whitish on terminal half Tail: 70-110% of head body length, Hind limbs: with scattered white hairs on upper surface
Gerbilliscus robustus	Fringe-tailed gerbil	Dorsal pelage: dark brown, flecked with black. Dorsal Hairs: grey at base, with cinnamon, dark brown or black tip Flanks: paler, orange-brown, with fewer black-tipped hairs Ventral pelage: pure white Tail: 115% of head body, thickly covered with small short hairs; color varied –brown with varying amounts of black to nearly all black, usually with pencil of hairs on terminal third; paler below; upper and lower colors usually clearly delineated

		Hind limbs: Soles of hind feet naked, darkly pigmented
Grammomys *species.*	Thicket Rats	Dorsal pelage: grey, brown or rufous without any patterning Hairs: short and soft Ventral pelage: pure white or cream Tail: very long semi-prehensile tail, 120–160% of head body Hind limbs: relatively small hind feet
Grammomys *dolichurus*	Woodland Thicket Rat	Small arboreal mouse Dorsal pelage: gingery-brown or cinnamon-brown suffused with grey and black. Dorsal Hairs: dark grey at base, gingery-brown on terminal, often with back tip Ventral pelage: Ventral pelage usually pure white, clearly delineated from dorsal pelage, often with thin band of pale orange between dorsal and ventral pelage Tail: extremely long, 150–180% of head body, scaly, with short brown or black bristles; long black hairs form terminal pencil at tip Hind limbs: Hind feet relatively short
Musca *species*	Old World Mice and Pygmy Mice	Dorsal pelage: greyish or brownish-grey Dorsal Hairs: dark grey at base, pale grey or brown at terminal end, sometimes with black tip Flanks: paler

		Ventral pelage: buffy-brown, pale grey, or white, merging into coloration of flanks Tail: 90–100% of head body Hind limbs: thin, almost naked, slightly darker above than below
Saccostomus *mearnsi*	Mearns' Pouched Mouse	Dorsal pelage: pale to dark grey, dark brown or brownish-grey Dorsal Hairs: hairs medium grey at base, grey or brownish-grey at tip Flanks: Flanks slightly paler Ventral pelage: grey Ventral hairs: sometimes tipped with white to give frosted appearance Tail: short, 50% of head body length, thick at base, without scales, well covered with grey to brownish-grey hairs above, white below Fore and hind limbs: Fore- and hindlimbs dark grey, short and stocky; four digits on forefoot, five digits on hindfoot Chin and base of muzzle whitish-grey Well-developed cheek pouches

This was followed by immediately by sample collection.

3.9.1 Blood Sample

Lateral tail vein blood or ventral tail vein blood was used for blood sampling.

In lateral tail vein blood collection, a tourniquet was used. The superficial veins were located on either side of the tail. A needle 27G was used to enter at a shallow angle about one third down the length of the tail. Blood swabs or clots were collected. Once the sample was collected, the animals were removed from the restrainer (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

For ventral tail vein blood collection, a tourniquet was used. The vein is located at both the central and ventral part of the tail. The ventral tail vein in mice is deeper than the lateral tail vein. As with the lateral tail vein bleeding, start one third of the way down the tail. Blood clots/blood swabs were collected in 2mls screw capped cryovials containing 500µl of TRizol® reagent (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.9.2 Oral Swab Collection

The rodent's mouths were safely opened wide using a pair of sterilized forceps while facing a light source. Using a sterile swab, all areas of the mouth were gently swabbed including the cheeks, tongue, areas between the cheeks and the gum and inside the lips. The samples were stored in 500µl of TRizol® reagent. The shaft of the swab was cut with ethanol wiped pair of scissors (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.9.3 Rectal Swab Collection

A sterile polyester swab was moistened with saline. Depending on the size of the rodent the swab (for smaller animal's paediatric swabs were used) was inserted into the anal region of the rodent avoiding contamination from other regions. The swab was slowly

rotated for 10 seconds. Using ethanol wiped, flame-sterilized scissors, the swab shaft was cut and transferred into a vial containing 500µl of TRizol® reagent. (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.10 Non-human Primate Sampling Procedure

Before sampling of non-human primates (NHPs) started, they were identified using the morphological/physical features in Table 6 (Magden et al., 2015; Smith, 2012; Higham et al., 2008).

Table 6: Key morphological features used to identify non-human primates

Papio anubis	***Chlorocebus aethiops***
Greenish-grey(olive) coat	Yellow to greenish brown coat
Skin on the face is dark grey covered with fine fur	Face hands and feet are black
Large in size	Smaller compared to baboons
Dark gray to black face color with coarser fur	White undersides and fur on their brows and cheeks
Long pointed muzzles	Relatively flat muzzles compared to those of the baboon
Bare abdomens that are reddish in females	Bluish skin on their abdomen
Males are larger than females with hair forming a mane from the top of their head	Males have bright scrotal areas that are blue in color
Females experience sexual swellings where their perineal skin turns red in color	No swelling in females

3.10.1 Blood Samples

Blood samples were collected from the femoral vein of NHPs. The animals were anesthetized using ketamine (5-10mg/Kg) which was followed by the placement of the animals in a dorsal position with the hind limbs extended. The skin over the femoral triangle was sterilized with 70% alcohol. The femoral vein was identified by placing a finger in the femoral triangle and locating the pulse. A needle was inserted at 45 to 60^0 angle with the bevel up. Slight negative pressure was applied to the syringe barrel as the needle went deeper into the identified area with a palpable pulse. The needle was advanced until a flash of blood occurred in the syringe. The blood sample was aspirated. Once the required amount was achieved (5mls), the needle was removed and a firm

pressure using sterile gauze was applied to the punctured site to stop bleed. The samples were divided into two aliquots. The first was stored in 2mls screw top cryotubes containing 500µl of TRizol® reagent and the second was stored in EDTA tubes for serum extraction (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.10.2 Oral Swab Collection

The animals were sedated with a mixture of ketamine hydrochloride and xylazine at a dose of 10mg/kg ketamine-hydrochloride and a dose of 0.5mg/ml xylazine administered intramuscularly. Under sedation, the animal mouths were opened facing a light source. Using a sterile polyester swab, all areas of the mouth were swabbed including the cheeks, tongue, areas between the cheeks and the gum and inside the lips. The samples were stored in 2mls screw top cryotubes containing 500µl TRizol® reagent (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.10.3 Rectal Swab Collection

Under sedation, a sterile polyester swab was inserted into the rectal region of the NHPs avoiding contamination from other regions. The swab was slowly rotated for 10 seconds and transferred into a vial containing 500µl of TRizol® reagent. All animals were released at the same site they were trapped after sample collection.

3.11 Sample transportation

Once sample collection was completed, the samples were kept in liquid nitrogen tanks (-195.8º C to -210º C. The samples were transported to the Institute of Primate Research Molecular Biology laboratory where they were transferred into -80º C freezers. (*PREDICT+2+SOP+Book_v3.+Aug2020.Pdf*, n.d.).

3.12 Laboratory Analysis

To meet study objectives number one to three, reverse transcriptase polymerase chain reaction (RT-PCR) was conducted on blood, rectal swabs and oral swabs and enzyme linked immunosorbent assay (ELISA) on serum samples from humans and non-human primates only.

3.12.1 Molecular Detection of Filoviruses

3.12.1.1 Ribonucleic acid (RNA) Extraction

The Direct-zol RNA extraction kit (Zymo research, USA) was used to extract total ribonucleic acid (RNA) from blood clots/blood swabs, fecal material/rectal swabs and oral swabs from bats, NHPs, rodents and humans. Good laboratory practices were practiced in this study (Kuslich et al., 2019).

To protect RNA degradation the samples were stored in TRizol solution and transported using liquid nitrogen (dry ice). Once at the institute the samples were transferred into a -80º C freezer. During RNA extraction, all steps were maintained on ice. The extraction

was done in a minimum number of samples to avoid long waiting time during sample processing.

Ribonucleic acid (RNA) extraction was conducted in a biological safety cabinet level/class 2 to avoid contamination. The swabs were thawed on ice. On thawing, the samples were vortexed to homogenize. To remove particulates, the mixture was centrifuged at 12,000 x g for 1 minute. From the supernatant, 250µl was withdrawn and dispensed into an RNase-free Eppendorf tube. Onto the 250µl supernatant, 250µl of 100% ethanol was added and vortexed to mix. The mixture was transferred into a zymo-spin column in a collection tube. This was centrifuged for 1 min at 12,000 x g. The collection tube with the flow through was discarded and the Zymo-spin column transferred into a new collection tube. The Zymo-spin column now contained the RNA which is bound to the membrane. Then 400µl of Direct-zol RNA Pre-Wash was added to the column and centrifuged for 1 min at 12,000 x g and the flow through was discarded. This was to remove excess ethanol from the column. To the column, 700µl of RNA wash buffer was added and centrifuged at 12,000 x g to remove excess ethanol. The collection tube plus flow through were then discarded. To ensure complete removal of the wash buffer, the column was centrifuged for 2 minutes at 12,000 x g. The column was transferred into an RNase free tube. To elute RNA from blood samples, 50µl of DNase/RNase free water was directly added into the spin column and 30µl of DNase/RNase free water was added for swab samples elution to concentrate the RNA. This was immediately followed by complementary deoxyribonucleic acid (cDNA) synthesis to avoid the degeneration of extracted RNA (Alves et al., 2016).

3.12.1.2 cDNA Synthesis

Immediately after RNA extraction, the RNA was kept on ice in preparation for cDNA synthesis. cDNA synthesis was conducted under a biological safety cabinet level/class 2 using the Invitrogen superscript III first strand synthesis kit (Cat# 18080-400), following manufacturer's instructions. The components of the kit including random hexomers, deoxyribonucleotide (dNTPs), 10X reverse transcriptase (RT) buffer, 25mM $MgCl_2$ and 0.1M dithiothrenol (DDT) were first allowed to thaw on ice.

In a single microfuge tube, a master mix was prepared containing 1 µl of random hexamer and 1 µl dNTPs per sample (Appendix IV). Polymerase chain reaction (PCR) tubes were labelled according to the samples that is cDNA and sample name. The work station was set up including clean pipettes, tips and a small container to hold the used tips. The random hexamer/dNTP mixture was vortexed and span briefly. A master mix was prepared by adding 2 µl of the random hexamer primers and dNTPs mix (as calculated in Appendix IV) into the labelled PCR tubes. Then 8 µl of the sample was added into each tube and incubated at 65ºC for 5 minutes in the thermocyclers/PCR machines. This was followed by a minute incubation on ice. This was then briefly vortexed and span after the incubation on ice (Doran, 2021).

In a single 1.5ml micro centrifuge tube, master mix 2 was prepared using the following reagents; 2 µl of 10X RT buffer, 4 µl of 25mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNase OUT and Superscript III reverse transcriptase (RT) as shown in Appendix IV. This was

calculated depending on the number of samples with an extra sample to account for pipetting error (Appendix IV). A 10 µl of this master mix was added into each of the PCR tubes (master mix 1 reaction tube). This was homogenized by pipetting up and down and then briefly span followed by incubation at 25° C for 10 minutes, 50° C for 50 minutes, 85° C for 5 minutes and chilled at 4° C in a thermocycler/PCR machine. After the incubation, 1 µl of RNase H was added into each tube. The tubes were returned into the thermocycler/PCR machine and incubated at 37° C for 20 minutes. This resulted to approximately 21 µl of cDNA which was divided into two aliquots of approximately 10.5 µl each and labelled. One aliquot was stored at -80° C freezer while the other at -20° C freezer, respectively. The samples at -20° C freezer were for immediate use while at -80° C freezer were for future use (Doran, 2021).

3.12.1.3 Quality Control of Extracted RNA

The quality of RNA extracted was checked by subjecting the cDNA to barcoding PCR targeting mitochondrial cytochrome b oxidase 1 was required (Townzen et al., 2008) . Mitochondrial genes lack introns, limited recombination and exhibit haploid mode of inheritance hence that are preferred over nuclear genes. All cells contain numerous mitochondria DNA (mtDNA) copies hence evidence of good quality mtDNA represents good quality of extracted RNA (Gray et al., 1999). The following primer sequences were used (Townzen et al., 2008).

CytB_F: 5’ – GAGGMCAAATATCATTCTGAGG – 3’

CytB_R: 5’ – TAGGGCVAGGACTCCTCCTAGT – 3’

Invitrogen Platinum Taq Kit (cat#: 10966-026) was used for mitochondrial cytochrome b PCR. PCR tubes were labelled according to the templates. For a 25 µL reaction and depending on the number of templates, the following reagents were used per template with an extra template calculated for pipetting error; 2.5µL of 10X PCR buffer, 0.75 µL $MgCl_2$ (50mM), 0.5 µL dNTP (10mM), 0.1 µL Platinum Taq DNA Polymerase, 18.15 µL molecular grade water, 1 µL Forward primer at 10µm, 1 µL of reverse primer at 10µm and 1 µL template of the cDNA as described on Appendix V. Other than Platinum Taq DNA Polymerase which was kept at -20° C and added last. All the aforementioned reagents were kept on ice to thaw.

To prepare the master mix, all the reagents other than the template were added onto RNase free PCR tube as described on Appendix V. Then 24 µL of the master mix was transferred from the RNase free PCR tube into each labeled PCR tubes (labels were done according to the sample that is the template). This was followed by adding 1 µL of the template/sample into the respective labelled tube.

The tubes were vortexed and slightly centrifuged. The tubes were placed in the thermocycler/PCR machine which was set with the following conditions; 94° C for 2 minutes; 50 cycles - 94° C for 30 seconds (denaturation), 52° C for 50 seconds (annealing), 72° C for 60 seconds (elongation); 72° C for 7 minutes (final elongation) and 10° C cooling (Appendix VI). All the steps from sample preparation to PCR step were done under ice or cold conditions to maintain the integrity of the procedure. The resulting PCR products

were visualized on a 1.5% agarose gel by loading the agarose gel with a mixture of 1X 3µL loading dye (6X loading dye, Thermo Scientific Lot 000619174) with 10 µL of the amplicons. The expected band size for barcoding mitochondrial cytochrome b band size was at approximately 457bp (Townzen et al., 2008).

3.12.1.4 Preparation and Casting of Agarose Gel

The gel/electrophoresis tank, gel tray and combs were rinsed with distilled water and air dried before casting the gel. This was followed by fitting in the gel tray into the gel/electrophoresis tank ensuring that the casting dams were well fit and tight. The gel tray and combs were cleaned using 70% ethanol after which the combs were inserted into the gel tray. To prepare 1.5% agarose gel in 100ml Tris acetate EDTA buffer (TAE Buffer) Appendix VII, 1.5g of agarose powder was added into 100ml TAE buffer working buffer.

This mixture was heated in a microwave for 1 minute to bring to boil with minimum evaporation in order to allow the agarose powder to dissolve. Once the agarose powder was dissolved, it was cooled on running water until it was approximately 50° C. The conical flask was swirled constantly without creation of bubbles. To this, 10µl of 0.5 µg/ml of ethidium bromide was added into the agarose and swirled until it mixed well. The working agarose was poured slowly into the gel tank avoiding the formation of bubbles. In case of bubbles, these were removed using a pipette tip before the gel started to polymerize. The comb was inserted immediately into the gel tank. The working agarose was allowed to set at room temperature for 45 minutes without movement until it

polymerized completely. Once the gel polymerized, the comb was removed. This created separate individual wells on the gel (Maniatis et al., 1982).

3.12.1.5 Loading the Gel

The wells created with the comb were used to load the samples. The gel was placed into the electrophoresis tank that contained 1X TAE buffer which completely covered the gel. Approximately 3 µL of 1x loading dye for each sample was pipetted onto a parafilm and subsequently 10 µL of each sample was added to each loading dye and mixed thoroughly. The mixture was loaded onto each corresponding well. A 100bp DNA ladder was added to the first and the last wells of the gel. The gel tank was connected to electrodes on the power pack which was turned on at 100 volts. The DNA was left to migrate for 1 hour or until it reached the middle of the gel.

The power was turned off after the DNA had reached half way of the gel and the gel was removed from electrophoresis tank for visualization on the gel doc (GDS-1365 Enduro™ GDS gel documentation system) using ultra-violet (UV) transluminator (Maniatis et al., 1982). To check the quality of RNA, the expected band size for barcoding mitochondrial cytochrome b was at approximately 457bp. For secondary amplification of filoviral gene the expected genomic size after the secondary amplification was at 630bp.

3.12.2 Nested Reverse Transcriptase Polymerase Chain Reaction (Nested rtPCR)

Once the quality of the extracted RNA was confirmed through the barcoding cytochrome b PCR, the cDNA was subjected to a nested reverse transcriptase polymerase chain

reaction (RT-PCR) using modified *filovirus* degenerate primers targeting the L gene (RNA polymerase) (630bp) which is highly conserved in filoviruses (Zhai et al., 2007). The primers were modified at base 5, 8, 9,11, 17 and 20 with I, Y, H, V, T, I, I and Y respectively, to improve their use for pathogen discovery. A nested PCR step was also introduced to increase sensitivity. The PCR primer sequences and PCR conditions as described in Table 7.

Table 7: Primer sets and PCR conditions for primary amplification of filovirus genome.

Primers:	**Forward:** TITTYTCHVTICAAAAICAYTGGG	
	Reverse: ACCATCATRTTRCTIGGRAAKGCTTT	
PCR Conditions		
94° C for 5 minutes		
40 cycles	94° C for 1 minute	
	52° C for 1 minute	
	72° C for 1 minute	
72^0C	7 minutes	
(final extension)		
10° C	1 minute	

After primary amplification, a secondary amplification was conducted. One microliter of the primary amplicon was used as a template for the secondary PCR. The primer sets and primer conditions in Table 8 were used.

Table 8: Primer set and PCR conditions for secondary amplification of filovirus genome

Primers:	Forward: TITTYTCHVTICAAAAICAYTGGG
	Reverse: GCYTCISMIAIIGTTTGIACATT
PCR Conditions	
94^0C for 5 minutes	
40 cycles	94^0C for 1 minute
	52^0C for 1 minute
	72^0C for 1 minute
7^0C for 7 minutes (final extension)	
Cool at 10^0C for 1 minute	

All amplicons, each 10 µL was run on 1.5% gel electrophoresis for visualization using GDS-1365 Enduro™ GDS gel documentation system with a 365 nm UV transluminator. The expected genomic size after the secondary amplification was at 630bp.

3.12.3 Quality Control Check in Molecular Laboratory

For positive control, Filoreston at 5pg/µl was used. Filoreston positive control was prepared from a segment of Reston ebola virus L gene. This gene is conserved in all known filoviruses. The expected band size was at 630bp. For negative control RNase free water was used. If a band was observed with the negative control, the whole procedure was repeated with a fresh batch of RNase free water and PCR reagents. For RNA quality check, the cDNA was taken through PCR using the cytochrome b mitochondrial gene Appendix VI. Samples with bright bands indicated high quality of RNA, while for

samples without bands indicated low RNA quality or no RNA was extracted. Samples with no bands observed indicated low quality or no RNA was extracted. Ribonucleic acid extraction was repeated for these samples. Good laboratory procedures were adhered doing in all laboratory steps.

3.12.3 Enzyme Linked Immunosorbent Assay

The Enzyme Linked Immunosorbent assay (ELISA) was conducted on human and non-human serum samples only. Due to the small nature of rodents and bats, the study was not able to collect enough blood (approximately 1ml) for serological assays without having detrimental effects to the small mammals. Owing to lack of enough blood samples from bats and rodents (< 1ml), the study was unable to process serum samples.

The recombinant glycoproteins (GP) of Mayinga strain (ZEBOV) ebolavirus, Boniface strain (SEBOV), Cote d'Ivoire strain (CIEBOV), Bundibugyo ebolavirus strain (BEBOV), reston ebolavirus strain (Pennsylvania strain) and Angola strain (MARV) were used to detect antibodies against ebolaviruses. Each recombinant GP represents one species of ebolaviruses and MARV (Nidom et al., 2012a).

3.12.3.1 Serum Preparation for IgG Antibody ELISA

Serum was separated from blood by centrifugation at 2000 X g for 10 minutes in a swing out rotor. The supernatant was collected using a pasteur pipette, ensuring the red blood cells were not picked. The serum was kept in 2mls vials at -20°C until use (Tuck et al., 2009).

3.12.3.2 Serological Detection of Filovirus IgG Antibodies by ELISA

Filovirus glycoprotein (GP) based ELISA was performed as previously described (Nidom et al., 2012b).

3.12.3.3 ELISA Protocol for Detection of Anti-filovirus Antibodies.

Flat bottomed 96 well ELISA plates (Nunc Maxisorp) were coated with 50µl/well of recombinant glycoproteins (filoviral GP antigens) and control antigens (1µg/ml in Phosphate buffer saline Appendix VIII, PBS Appendix VIII). The plates were incubated at room temperature for 2 hours. After incubation, the unbound antigen was discarded. A 100µl of bovine serum albumin 10 (BSA 10), blocking buffer (Appendix IX) was added onto the plate. This was followed by incubation at room temperature for 2 hours. After incubation, BSA 10 was washed once with phosphate buffer saline with 0.05% TWEEN20 (PBST) (Appendix X). Into each well, (except the controls) 50µl of test serum samples (primary antibody) diluted in 1:100 dilutions (into 1X PBST) was added to the plate and incubated at 4°C overnight. The controls (negative) were also added at 50µl into each respective control well.

After overnight incubation, the sample dilutions were discarded and the plates washed 4 times with PBST. Secondary antibody (anti-monkey horse radish conjugated antibodies) at 50µl was added per well and incubated at room temperature for 1 hour (hr). After the 1hr incubation, the secondary antibody was discarded and the plates washed 5 times with PBST. Substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution (Sigma

Cat# T0440) at 50µl was added into each well. The plate was incubated for 1hr in the dark at room temperature for 30 minutes. After 30 minutes incubation in the dark, 50µl stop solution (1M sulphuric acid) was added. The absorbance was measured at 450nm using the ELISA reader. The specific optical density (OD) values were calculated by subtracting non-specific binding (values given by GP antigens OD minus values given by control antigen OD), Appendix XII. Any sample that had an outlier reading of above 1.000OD at a wavelength of 450nm was to be deemed as a possible positive. The study lacked a positive control serum sample for the ELISA test. The study utilized negative control serum samples from Institute of Primate Research colony born *Papio anubis* (Olive Baboons) and *Chlorocebus aethiops* (African Green Monkeys).

3.13 Data analysis

3.13.1 Molecular Detection of Filoviruses (Phylogenetic Analysis)

It was expected that the nucleotide sequences obtained from ABI3730 Sanger sequencer were to be aligned using multiple alignment done by Clustal-W program. Distances between pairs of sequences would have been estimated using the DNADIST program of the PHYLIP package (version 3.4) (Felsenstein, 1991). Phylogenetic trees would be constructed using MEGA. Phylogenetic relationships for the nucleotide sequences of the L gene would have been determined. The robustness of phylograms would have been evaluated by 1,000 bootstrap resampling. Phylogenetic analyses of the isolates would be carried out in every case using maximum likelihood (the highest likehood) and neigbourhood joining.

3.13.2 Serological Detection of Filoviruses

The study did not have a positive control for the anti-filovirus IgG antibodies. The study adapted the Rout method to detect multiple outliers using GraphPad Prism version 9.1.1. The Rout outlier test is a statistical test based on the False Discovery Rate (FDR). Alpha or co-efficient Q is specified which is the maximum desired FDR. Rout assumes the data has Gaussian or normal distribution. This test detects outliers with non-linear regression. The alpha or co-efficient Q in this study was set at 0.01 (1%) thus 0.99 (99%) identified outliers were true outliers (Motulsky & Brown, 2006).

Rout outlier test was conducted on one plate at a time, so as to avoid any discrepancy on optical density (OD) on the plates, since plates have different OD from each other when tested without the sample or reagents. All outliers above 0.8000 were deemed as possible positive samples (Nidom et al., 2012b).

3.13.3 Questionnaire data analysis

To meet objective number four, the study conducted a survey using questionnaires for all human participants (Appendix XI). The questionnaire targeted human animal interactions and sanitation in the last 6 months before the month of May, 2018. The main aim of this survey was to establish high risk behaviour in relation to contracting filoviral infections (zoonotic).

The questionnaire was adapted from an existing one with some modifications (Hagström & Eckerdal, 2017). Questionnaire reliability analysis was carried out on the perceived

task values scale comprising 56 items. The acceptable reliability conducted by Cronbach's alpha showed the questionnaire had reached the acceptable reliability, $\alpha = 0.8$ (Heo et al., 2015). In addition, the questionnaire was pre-tested using 30 participants who were not taking part in the study. Their responses and understanding of the questions allowed for revision of the questions to a point that the questions were easy to understand and to answer. Any participant less than 17 years was termed as a child. Children between 2 to 17 years were included with consent from the guardians.

Data from the questionnaires was weighed and analysed using R version 3.5.2 with $P<0.005$ was considered statistically significant. Results were expressed as proportions (%). Frequency was used to describe the demographics of participants and contact with domestic and wild animals. Pearson Chi square was used to express the relationship between the participants who had come in contact with animals from those who had not. For sanitation, Fisher exact test was used to express the relationship between participants who treated water from those who did not treat water from open water sources.

3.14 Ethical Consideration and Research Permit

Ethical approval for human studies was obtained from Kenya Medical Research Ethical Review Committee Ref. No.: KEMRI/RES/7/3/1 (Appendix I). For animal studies ethical approval was obtained from the Animal Care and Use Committee (ACUC) from the Institute of Primate Research Ethical Review Committee Ref. No.: IERC/08/16 (Appendix II). The research permit was obtained from National Council of Science and Technology (NACOSTI) permit number: NACOSTI/P/19/48092/2643 (Appendix III).

The survey tool and research protocol were reviewed and approved by institutional research board of University of California Davis # 804522-32) and Kenya Medical Research Ethical Committee Ref. No. : KEMRI/RES/7/3/1.

The questionnaires were read out loud to the study participants. This was followed by the participants signing individual consent form. The questions on the questionnaires were read out one by one to the individuals in a closed office where they answered accordingly. This was followed by sample collection. Due to cultural barriers, participants were allowed to give the samples that they were comfortable with. Assent was sought orally from children below 17 years which was followed by a signed consent from the guardians (Dockett & Perry, 2011). For age 6-13 a simple description of the child involvement in the research was given in the presence of a guardian. For ages between13-17, a complete oral description of the study was given. A verbal assent was required which was documented on the consent form (University of Nebraska, 2022).

To maintain anonymity and confidentiality of the study participants, each individual was given a unique code. On a separate document the names were matched with the unique codes and kept separately. In addition, the collected data was transferred from hard copies to soft copies. All computer based data was encrypted with only two data entry individuals having the passwords to the files. The hard copies were stored in an office cabinet under lock and key. During laboratory analysis, the unique identifiers were used for all study participants (Reid & Brief, 2009).

3.14.1 Ethical Challenges During the Sampling

In humans the major ethical challenge the study encountered during sampling was the determination of age that is who is an adult or child. This was because minors under the age of 18 were married with a family. Due to age (under 18 years) we clustered them as minors though in the community they were deemed as “adults”. In addition, obtaining rectal swabs was a challenge. This led us to request for fecal material which again due to cultural practices we had to convenience them to give us the fecal material.

CHAPTER FOUR

4.0 RESULTS

4.1 Sample Description

A total of 1,092 (405 oral swabs, 310 rectal swabs/fecal material, 377 whole blood/blood swabs/clots) samples from humans, bats, rodents and non-human primates were collected and tested using reverse transcriptase polymerase chain reaction (rtPCR) for the presence of *Filoviruses* (Table 9).

Table 9: Types of samples collected per taxa.

	No. of samples collected			
	Oral	**Rectal**	**Blood**	**Total**
Bats	100	100	96	296
Rodents	98	98	95	291
NHPs	62	62	56	180
Humans	145	50	130	325
Total	**405**	**310**	**377**	**1092**

The samples collected were from 145 humans, 100 bats, 98 rodent and 62 non human primates.

4.2 Diversity of study subjects

A total of 12 animal species were sampled. Two bats species (n=100) and humans (n=145) were involved in the study (Table 10). Human age, gender and educational background were recorded.

Table 10: Types and number of bats sampled per species.

Bats Species	Number of Bats	Common Name
Chaerephon pumilus	80	Little free tailed bat
Scotophilus dinganii	20	African yellow house bat/Yellow bellied house bat/Dingan's bat

Eight (8) species of rodents (n=98) were sampled in the study (Table 11). Of all the rodents sampled *Acomys kempi* was the most abundantly trapped (29.5%). The least sampled was *Saccostomus mearnsi* where only 1 was sampled, 1% (Table 11).

Table 11: Types of rodent species and number of rodents sampled per species.

Species	**No. of rodents**
Acomys kempi (Spiny mouse)	29
Aethomys hindei (Hinde's rock rat)	7
Acomys percivali (Percival's spiny mouse)	5
Gerbilliscus robustus (Fringe tailed gerbil)	25
Grammomys species. (Eastern rainforest rast)	11
Musca Species (House mouse)	5
Grammomys dolichurus (Woodland thicket rat)	15
Saccostomus mearnsi (East African pouched mouse)	1

Two non-human primate species were sampled (n=62) (Table 12). The most sampled non-human primate was *Papio anubis* where 53 individuals were captured while the rest (9) were *Chlorocebus pygerythus* (Table 12).

Table 12: Types of non-human primate species and number of non-human primates sampled per species.

Non-human Primates

Species	**Number of primates**	**Common Name**
Chlorocebus pygerythus	9	Vervet monkeys
Papio anubis	53	Olive baboons

Different blood/blood clots/blood swabs, oral swabs and rectal swabs were collected from each animal species (Table 13).

Table 13: Types and number of samples collected per species.

Bats Species	**Blood**	**Oral**	**Rectal**
Cherophon pumilus	78	80	80
Scotophilus dinganii	18	20	20
Rodents Species	**Blood**	**Oral**	**Rectal**
Mus spp	5	5	5
Grammomys dolichoros	14	15	15
Saccostomus mearnsi	1	1	1
Acomys kompi	28	29	29
Aetomys hindei	7	7	7
Acomys percivali	5	5	5
Gerbilliscus robustus	24	25	25
Grammomys spp	11	11	11

Non-human primates Species	**Blood**	**Oral**	**Rectal**
Chlorocebus pygerythrus	9	9	9
Papio Anubis	47	53	53

4.3 Detection of Filoviruses by PCR

4.3.1 Quality check results for RNA extraction

Quality check was performed on all extracted RNA samples by amplifying the mitochondrion cytochrome b gene region. The expected band size was ~457 bp (Figure 9). The brighter the band the better the quality of the RNA extracted. No band meant the quality of RNA extracted was poor and a repeat was done to the sample.

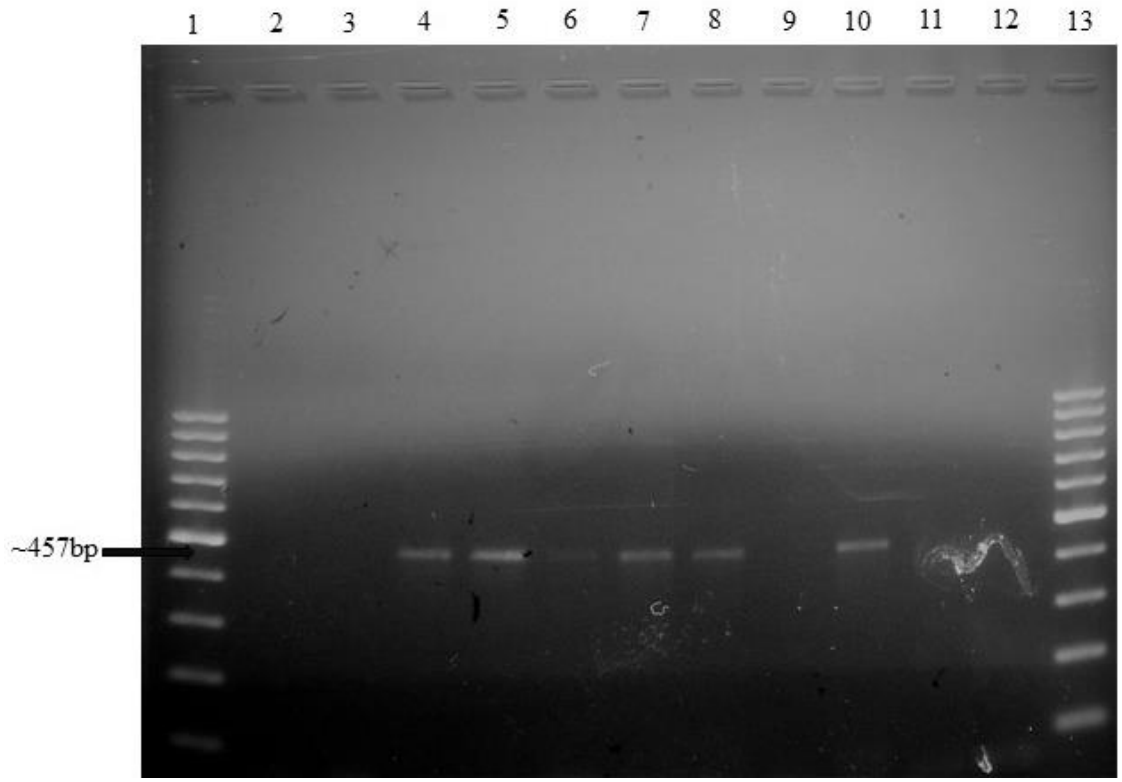

Figure 9: Representative gel image for RNA quality control check for humans, non - human primates, bats and rodents blood samples.

The gene of interest is at approximately 457bp.Well 1 and 13 are the molecular weight size marker/ladder of 100bp, well 2 is the negative control, well 3 is the blank, wells 4 and 5 represents human blood samples, wells 6 and 7 represents blood samples from rodents, well 8 and 9 represents bats blood samples and wells 10 to 12 are representatives of blood samples from non-human primates blood samples. The samples with a band at ~457bp represent good quality of RNA extracted while the ones with no bands represent poor quality of RNA and a repeat extraction and barcoding PCR was undertaken.

4.3.2 Detection of Filoviruses in Human Samples

Out of the 325 samples (oral 145, rectal 50, blood 130) from humans, all tested negative for Filovirus RNA. Below is a representative gel for the secondary amplicons (Figure 10).

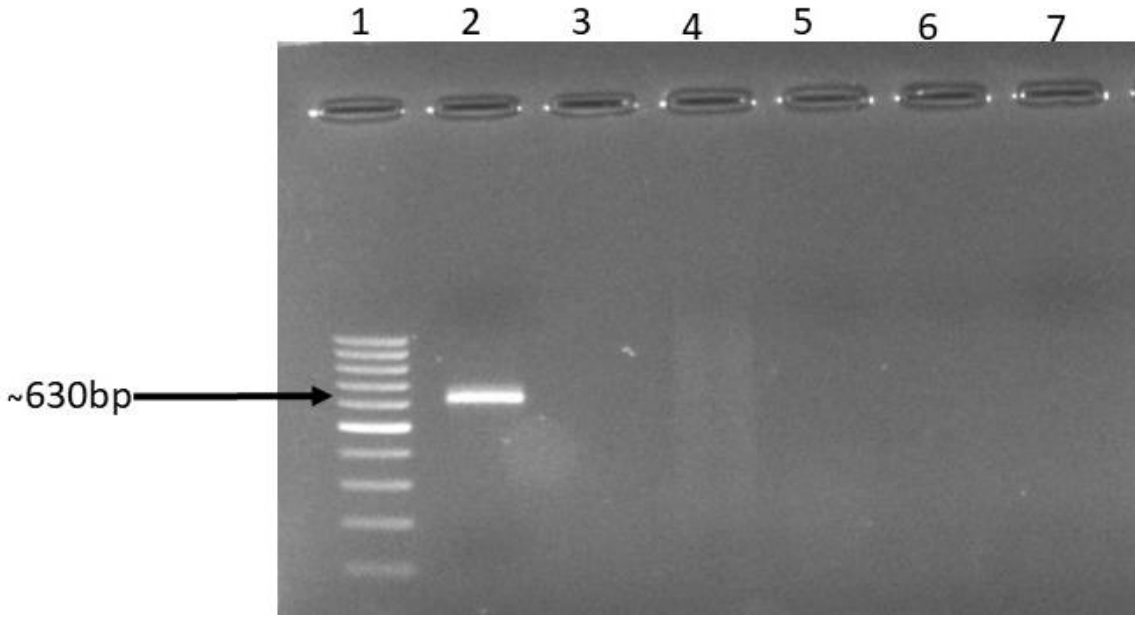

Figure 10: Representative gel image for human blood sampls amplicons after secondary amplification.

Well 1 is the molecular weight size marker/ladder of 100bp, well 2 is the positive control, well 3 is the negative control and wells 4 to 7 are representative samples. All the sample representatives did not show any bands and therefore they were all negative for filovirus RNA.

4.3.3 Detection of Filoviruses from Bat Samples

Of the total 296 (100 oral swabs, 100 rectal swabs and 96 blood swabs) originating from bats (*Chaerephon pumilus* and *Scotophilus dinganii*), all tested negative for filovirus RNA by RT-PCR. Figure 11 is a representative gel image of the bats results.

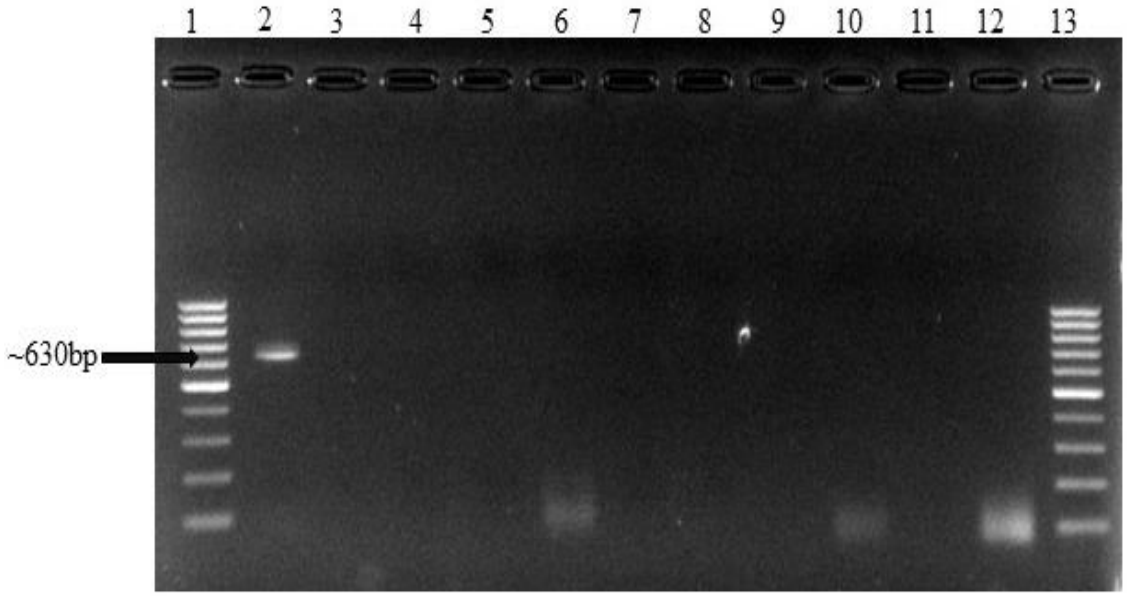

Figure 11: Representative gel image for bats blood samples after secondary amplification.

Well 1 and 13 are the molecular weight size marker/ladder of 100bp, well 2 is the positive control, well 3 is the negative control and wells 4 to 12 are representative samples. All the sample representatives did not show any bands and therefore they were all negative for filovirus RNA.

4.3.4 Detection of Filovirus from Rodent Samples

Of the 291 (98 oral swabs, 98 rectal swabs and 95 blood samples) samples tested from rodents (*Acomys kempi, Aethomys hindei, Acomys percivali, Gerbilliscus robustus, Grammomys spp., Grammomys dolichurus, Mus Spp.* and *Saccostomus mearnsi),* all were negative for *Filoviruses* RNA. Figure 12 is a gel representation of these results.

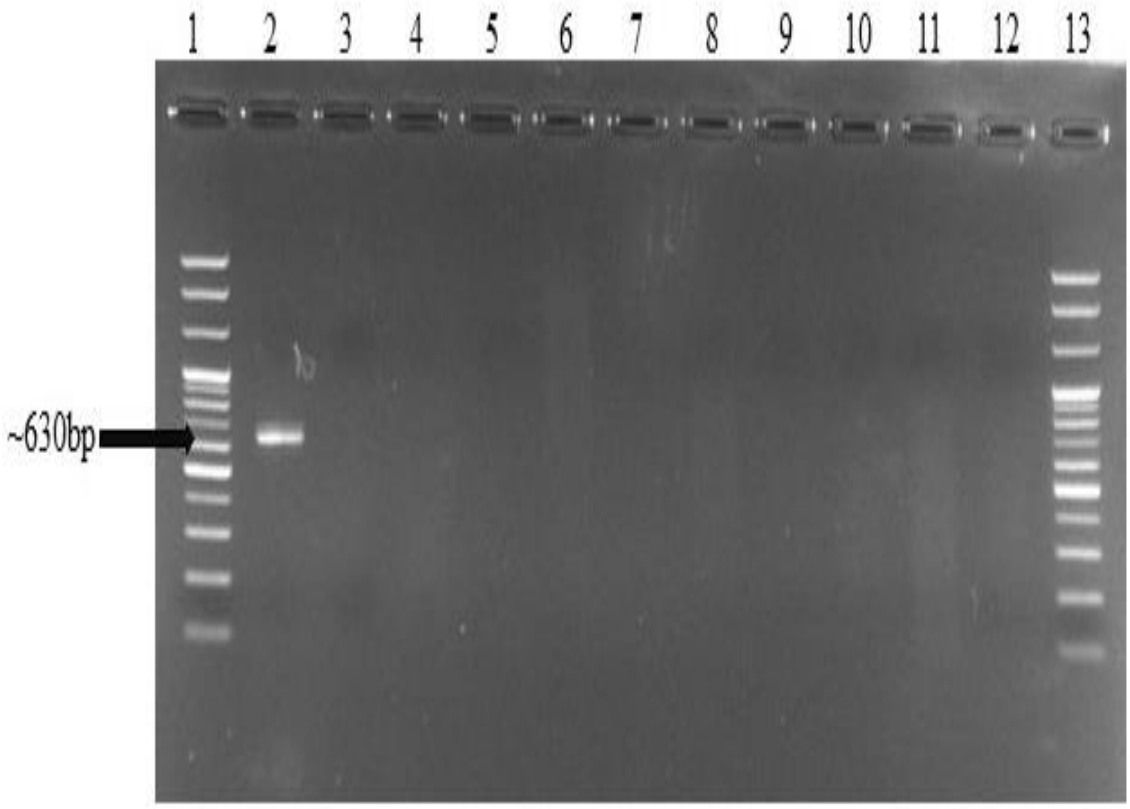

Figure 12: Representative gel image for rodents blood samples amplicons after secondary amplification.

Well 1 and 13 the molecular weight size marker/ladder of 100bp, well 2 is the positive control, well 3 is the negative control and wells 4 to 12 are representative samples. All

the sample representatives did not show any bands and therefore they were all negative for filovirus genome.

4.3.5 Detection of Filovirus from Non-Human Primate Samples

From a total of 180 samples from non-human primates sample (62 oral swabs, 62 rectal swabs and 56 blood swabs), all tested negative for Filovirus RNA by RT-PCR. Figure 13 is a gel representation of the results.

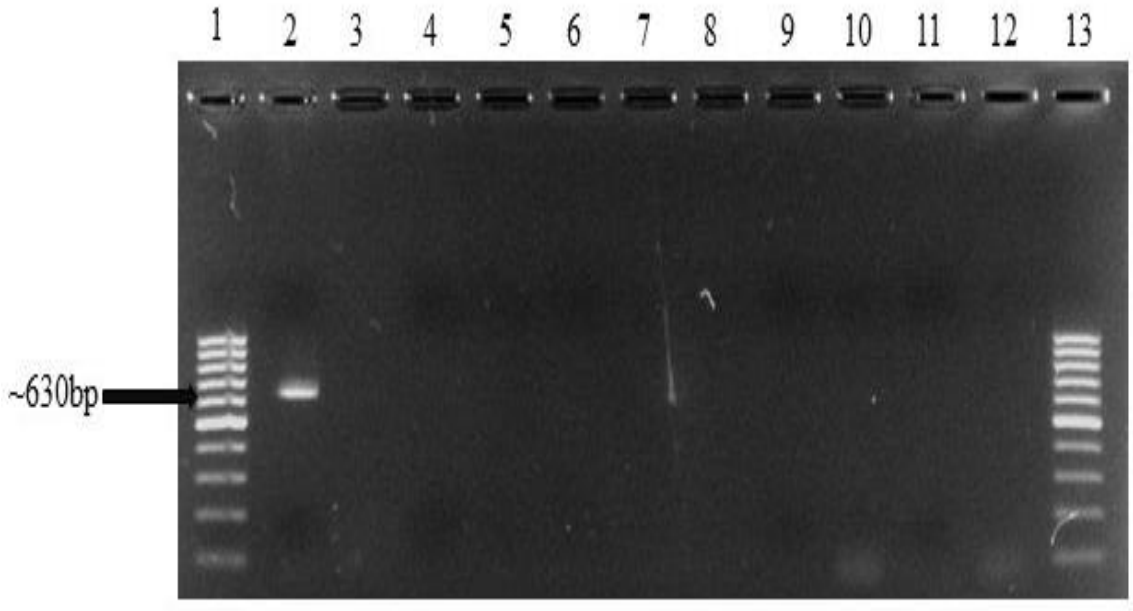

Figure 13: Representative gel image for non-human blood samples amplicons after secondary amplification.

Well 1 and 13 are the molecular weight size marker/ladder of 100bp, well 2 is the positive control, well 3 is the negative control and wells 4 to 12 are representative samples.

All the samples tested negative for consensus PCR thus it was least probable to test the hypothesis.

4.4 Serological Detection of Filoviruses from Human and Non-Human Primates

The study tested a total of 186 serum samples from humans and NHPs with an aim of detecting IgG antibodies against recombinant glycoproteins (GP) of Zaire Ebolavirus (Mayinga strain), Sudan ebolavirus (Boniface strain), Tai forest ebolavirus (Cote d'Ivoire strain), Bundibugyo ebolavirus (Bundibugyo strain), reston ebolavirus strain (Pennsylvania strain) and Marburgvirus (Angola strain). All ELISA plates were analysed using ROUT outlier test.

The samples were tested in duplicates. The averages of the OD readings were calculated using excel workbook. The averages were used in Rout outlier test.

4.4.1 Human ELISA IgG Results

A total of n=130 serum samples from humans were tested for anti filoviral antibodies. One outlier was detected for Sudan Ebolavirus, 2 outliers for Marburg virus and 3 outliers for Bundibugyo Ebolavirus and Tai forest Ebolaviruses, respectively (Table 14).

Table 14: Samples with outliers in human.

Filovirus strain	**Outliers detected**	**Optical Density**
Zaire Ebolavirus	0	0
Sudan Ebolavirus	1	0.4505
Bundibugyo ebolavirus	1	0.5275
	2	0.50335 0.38005
Reston Ebola Virus	0	0
Tai Forest	1	0.53965
	1	0.2798
	1	0.3869
Marburg virus (Angola Strain)	1	0.3869
	1	0.55275

The outliers were detected for each individual ELISA plate (Figure 14, Figure 15, Figure 16, Figure 17 and Figure 18). Each plate had a different OD due to the plate background.

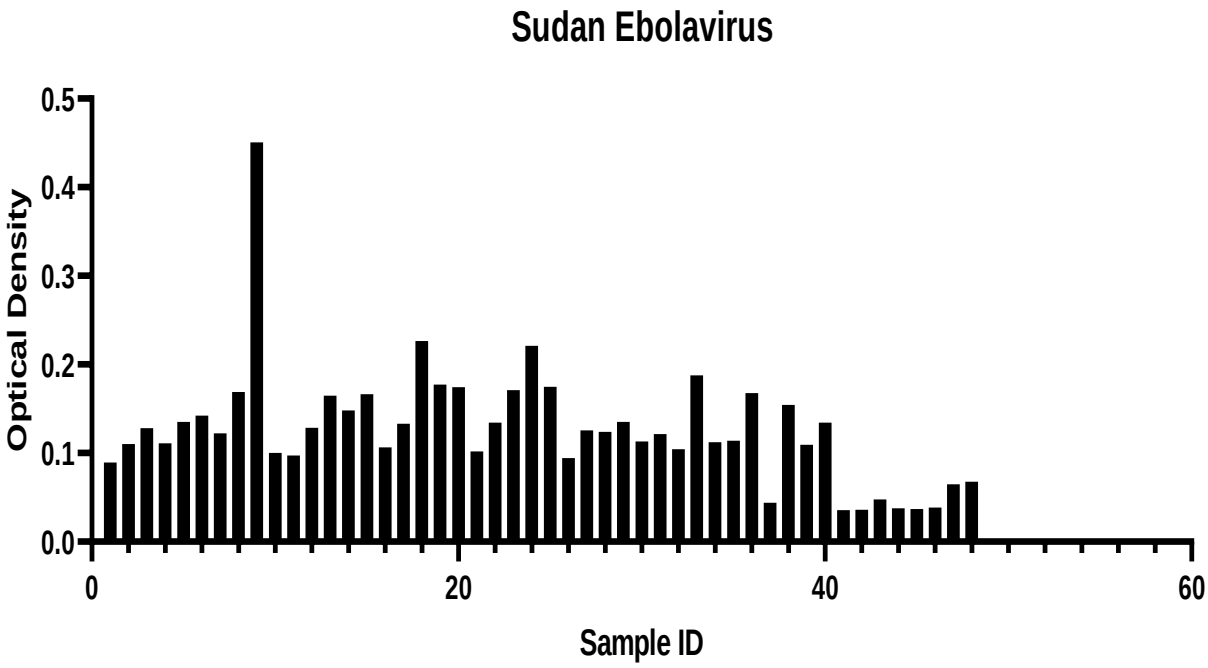

Figure 14: Representation of outliers detected against Sudan Ebolavirus in human serum samples.

Figure 14 represents outliers detected against Sudan Ebolavirus in human serum sampes using Rout outlier test. The optical density was 0.4505 at a wavelength of 450nm on plate 1. The outlier is sample number 9. The negative control serum is sample number 46 (Oloive baboons) and 47 (African Green monkey).

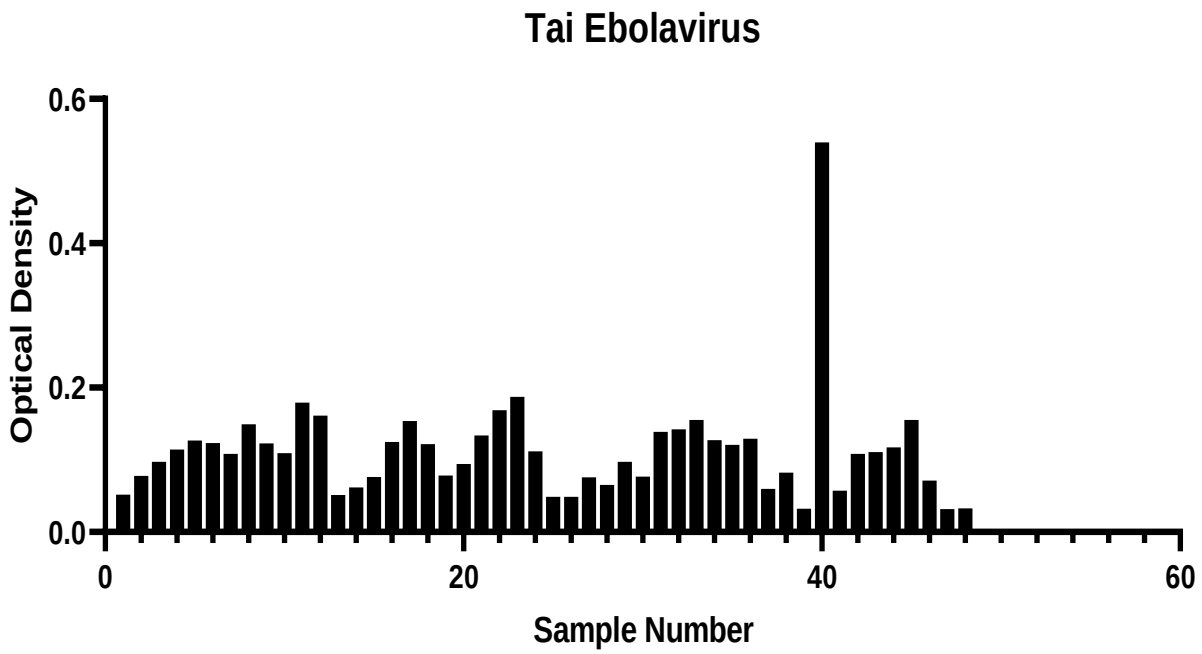

Figure 15: Representation of outliers detected against Tai forest Ebolavirus in human serum samples.

Figure 15 represents outliers detected against Tai forest Ebolavirus in human samples. An optical density of 0.5397 at a wavelength of 450nm on plate 1 was detected as an outlier. The outlier sample is sample number 40. The negative control serum sample is sample number 47 (Olive baboons) and 48 (African green monkey).

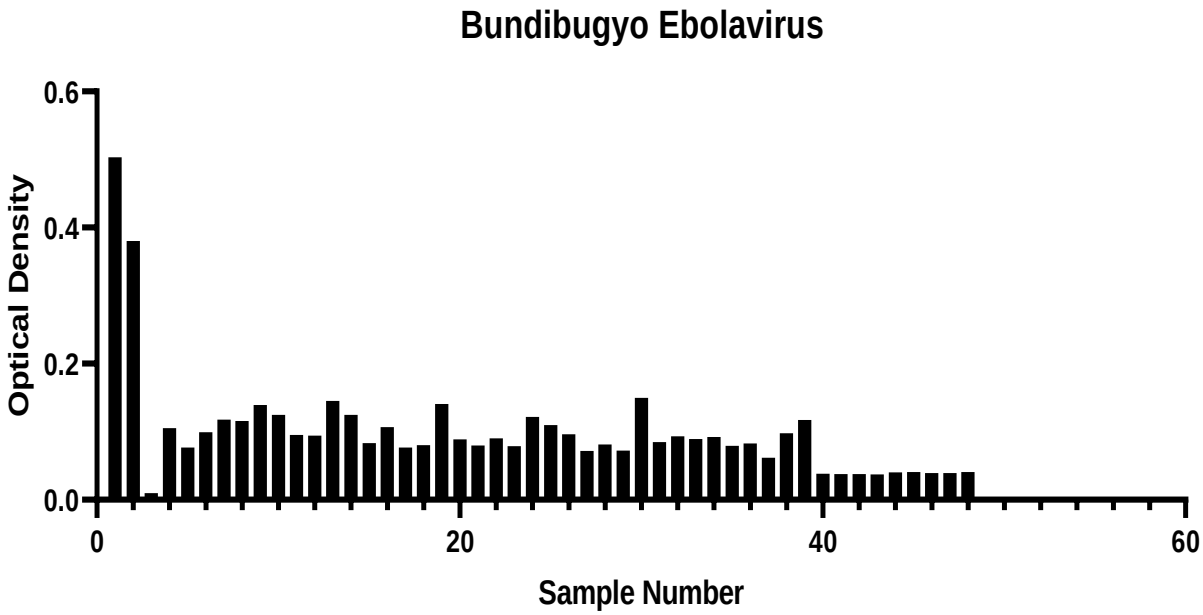

Figure 16: Representation of outliers detected against Bundibugyo Ebolavirus in human serum samples.

Figure 16 is a representation of outliers detected against Bundibugyo Ebolavirus in human serum samples. An optical density of 0.5034 and 0.3801 was detected as an outlier at a wavelength of 450nm on plate 2. Sample 1 and 2 were the outliers (Figure 16). The negative controls were 48 (AGM) and 49 (Olive baboons).

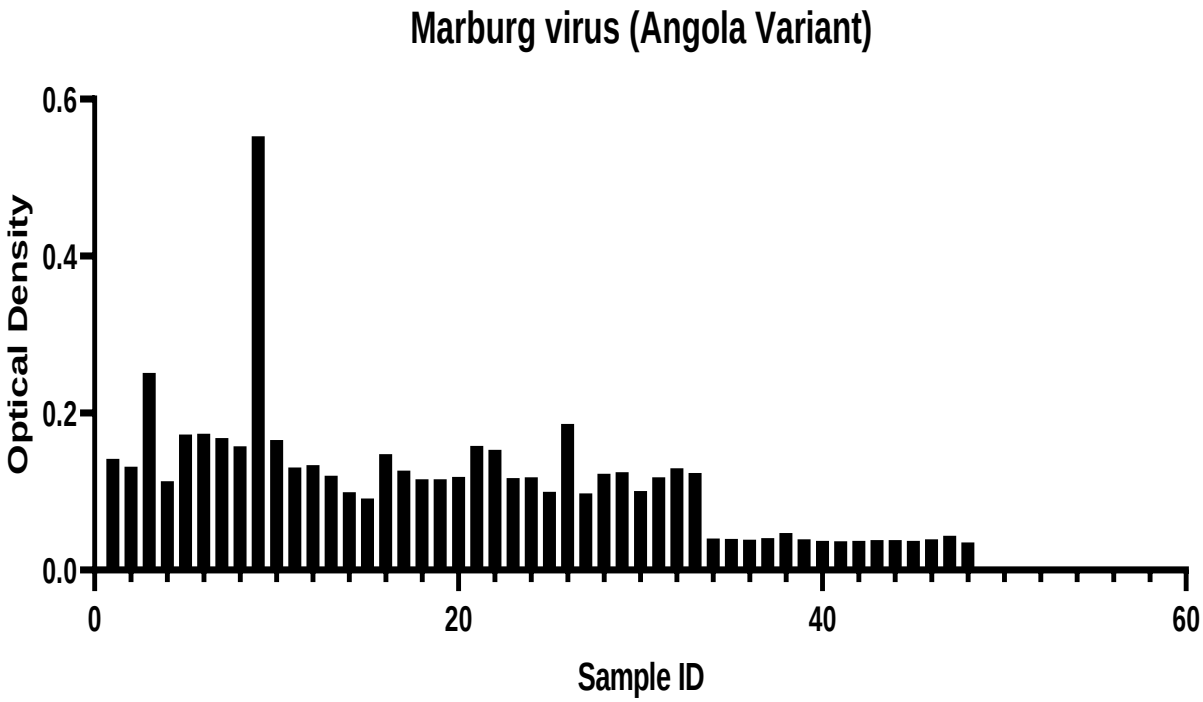

Figure 17: Representation of outliers detected against Marburg virus in human samples.

Figure 17 is a representation of outliers detected against Marburg virus in human samples at optical density of 0.5528 at a wavelength of 450nm on plate 2. Sample 9 was the outlier. The negative controls were 48 (AGM) and 49 (Olive baboons).

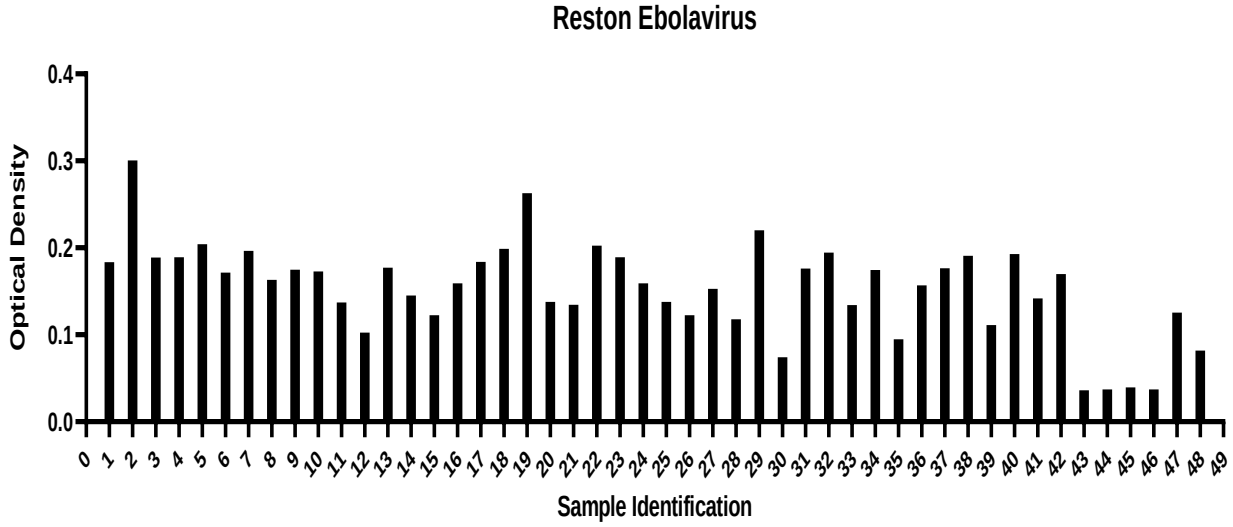

Figure 18: Representative graph for Reston ebolavirus in humans with no outliers.

Figure 18 represents a graph for Reston ebolavirus in human serum with no outliers detected. It illustrates the absence of outliers on an ELISA IgG plate test using ROUT outlier test. Sample number 1-47 are test samples while samples 48 (Olive baboons) and 49 (African Green monkey) were negative controls.

The outlier samples had an OD reading of between 0.2798 and 0.55275 (Table 14). Four samples had an OD of above 0.5 while 5 samples had an OD below 0.5. All the outlier samples with less than 0.8000OD were deemed as negative for anti-filoviral IgG antibodies (Nakayama et al., 2010).

All the human samples tested negative for ELISA consequently the hypothesis was not tested.

4.4.2 Non Human Primate ELISA IgG Results

The total number of serum samples tested were n=56. *Papio anubis* (Olive baboons) were 83.9% (47) while *Chlorocebus aethiops* (African green monkeys) were 16.1% (9). There were no outliers detected in non-human primates serum samples.

4.5 Risk factors for Filovirus Infection

Quantitative analysis of the behavioural questionnaire responses using R 3.5.2. Answers of "don't know" given in response to a behavioural question were coded as "NO" for analysis. All behaviour variables were binary "yes or no" responses. Fisher's exact test with a Holp p-value adjustment were conducted using the fmsb package to compare frequencies of each of the behavioural variables that related to animal contact in the villages.

4.5.1 Demographics

The youngest participant was 2 years old while the oldest participant was 78 years. The mean age of the participants was 24.4 years. Total number of participants was 146 males 79 (54.1%), females 65 (44.5%) and 2 (1.4%) bisexual (Figure 19).

Table 15: Gender distribution of participants.

Sex	**N**	**percent**
Males	79	54.1%
Females	65	44.5%
Bi-sexual	2	1.4%
Total	146	100.0%

The level of education of participants was tested. The highest level of education was tertiary with only 2.8% (4/144). Participants with primary level of education was 25% (36/144) and 6.9% (10/144) had secondary education level training.

4.5.2 Contact with animals

Contact with animals was analysed using Pearson Chi-square. A total of 118 (P value-<0.001) participants reported having contact with animals in the last 6 months before the study. In addition, 11-20% (<0.001) had reported to have or either, consumed a dead animal, handled dead animal bodily fluids/tissue, sold a dead animal, ate food contaminated with animal feaces or touched by animals, sick animal or uncooked meat (Table 16). There was a significance (p<0.001) in the number of participants who had contact with animals during the last 6 months before the study.

Table 15: Table of persons who had contact with animals in the last 6 months before the study.

	Contact/Use		
Source of Contamination	**NO N = 648[1]**	**YES N = 360[1]**	**p-value[2]**
Key			<0.001
Dead Animal Consumption	96 (15%)	48 (13%)	
Dead Animal Meat Fluid Handling	73 (11%)	71 (20%)	
Dead Animal Sale	120 (19%)	24 (6.7%)	
Faeces Food Contamination	82 (13%)	62 (17%)	
Food Touched by Animal	89 (14%)	55 (15%)	
Sick Animal Consumption	95 (15%)	49 (14%)	

Source of Contamination	Contact/Use		
	NO N = 648[1]	YES N = 360[1]	p-value[2]
Uncooked Meat	93 (14%)	51 (14%)	

[1]n (%)

[2]Pearson's Chi-squared test

Legend: NO are participants who answered NO to the source of contamination questions. YES are the respondents who answered yes to the source of contamination questions. N is the total number of animal contacts the participant had in the last 6 months before the study.

4.5.3 Water Hygiene

On water treatment the study compared people who used open water (un-uncovered well/pond/river) without treatment to those who treated water before use using Fisher's test. A total of N=134 participants reported using open source water with n=114 treating the water for domestic used (Table 16). There was a significance (p=0.063) in the use of untreated water by participants which depicts the level of sanitation.

Table 16: Treatment of water for domestic use among study participants.

Water Source	Overall, N = 144[1]	Water Treatment		
		NO N = 120[1]	YES N = 24[1]	p-value[2]
Uncovered Well	0 (NA%)	0 (NA%)	0 (NA%)	
Pond	134 (100%)	114 (85%)	20 (15%)	0.063
Covered Well	0 (NA%)	0 (NA%)	0 (NA%)	
River	134 (100%)	114 (85%)	20 (15%)	0.063

[1]n (%)
[2]Fisher's exact test

Legend: No are participants who did not treat water. YES are participants who treated water. NA are the participants who did not respond to this question on water treatment on the questionnaire.

Animal contact was reported in the study. About 35.4% (51) respondents reported having gotten into contact with domestic animals while less than 11.8% (17) of the respondents reported having contact with wild animals. The rest 53% (76) did not give a response (Figure 19).

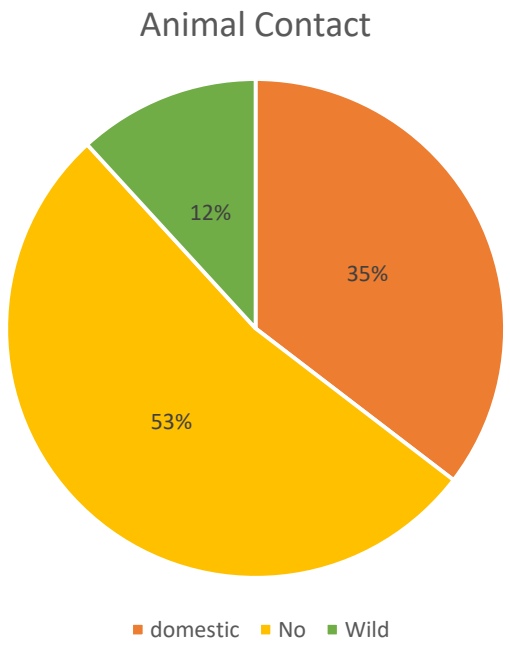

Figure 19: Proportion of participants who got into contact with domestic and wild animals.

The pie chart represents the percentages of participants who were in contact with wild and domestic animals. In addition it illustrates the number of participants who did not get into contact with animals.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The main aim of this study was to detect and molecularly characterize filovirus circulating in humans, non-human primates, bats and rodents. Filovirus ecology, reservoir hosts and spill over events to humans continue to remain a mystery (Feldmann et al., 2004). The suspected reservoir hosts are elusive and may be a source of infection to humans, if not well identified. Bats have been linked through index cases to be potential source of filovirus outbreaks (both Marburg and Ebola viruses) in Kenya and Uganda, respectively (Kuzmin et al., 2010; Towner et al., 2009; Wolfe et al., 1998).

Filoviruses have been detected if not isolated from non-human primates, bats and in experimental rodents (Kajihara et al., 2019; Caron et al., 2018a; Jayme et al., 2015; de Wit et al., 2011; Kuzmin et al., 2010b; Wittmann et al., 2007; Walsh et al., 2005; Leroy et al., 2004). However, the role played by these animals as reservoir hosts and in natural ecology is not well understood, while filovirus infections in humans continues being a threat (Peterson et al., 2004). By detection of known and novel animal pathogens before or just as they emerge in humans, may make it possible to mitigate against worldwide spread. To detect and identify animal species harbouring filoviruses in Laikipia North County, Kenya, over 1,000 samples from humans, different animal species (bats, rodents and non-human primates) and different sample types (blood/blood swabs, oral swabs and rectal swabs/fecal material) were tested. Data on humans, bats, rodents and non-human

primates is provided adding to the existing data on surveillance of filoviruses circulating in Kenya.

5.1.1 Molecular characteristic of filovirus between and within species (Bats, Non-human primates, rodents and humans)

Filovirus RNA detection using reverse transcriptase polymerase chain reaction (RT-PCR) is rare compared to serology. To date, a few studies have been able to detect the viral RNA in experimental bats and in nature (De Nys et al., 2018). The gold standard for detection of filovirus genome is the RT-PCR (Magro et al., 2017).

The first ever case of filovirus infection (Marburg virus) in humans occurred in 1987 in Germany and in Belgrade Serbia, former Yugoslavia, simultaneously (Ksiazek, 2014). This virus was isolated from individuals who were handling African green monkeys (*Chlorocebus aethiops*) imported from Uganda. The current study collected a total of 180 samples (56-blood, 62-oral swabs and 62-rectal swabs) from 62 (9 vervet monkeys and 53 olive baboons) non-human primates with an aim of amplifying filovirus L gene segment.

Vervet monkeys (*Chlorocebus pygerythus*) are closely related to the African green monkey (*Chlorocebus aethiopes*). Non-human primates over the years have been known to harbour a variety of zoonotic viruses not limited to viruses of the family *Filoviridae* (Burgos-Rodriguez, 2011). Filoviruses infections in non-human primates is a concern to human health as there is a link between filovirus infected non-human primates and human

outbreaks (Rouquet et al., 2005). In this study, the quality control rans for RT-PCR passed. However, the present study was not able to detect the L gene of filoviruses in non-human primates. The more likely reason as to why filoviruses were not detected in the current study is because primates exposed to these viruses are capable of mounting an immune response and clearing the virus while in a study by Bermejo, filoviral infections caused massive deaths in primates (Bermejo et al., 2006; Leroy et al., 2004). This study has generated a hypothesis on absence of filoviruses circulating in non-human primates in Laikipia County, Kenya.

Live bats have been implicated as the primary source of human exposure to filoviruses (ebolaviruses). In experimental studies where Egyptian rousette bats (*Rousette aegyptica*) were inoculated with Ebolaviruses (Sudan, Reston, Bundibugyo and Tai Forest Ebolaviruses) and marburg virus, the bats did not shed ebolavirus while marburg virus was shed through the oral and rectal route (Jones et al., 2015). In an experimental study to determine the shedding routes of filovirus inoculated bats, the virus was not isolated from oral and rectal swabs. These findings are comparable to the current study findings where filovirus genome was not isolated from rectal and oral swabs (Paweska et al., 2016). In this study, samples from *Chaerephon pumilus* and *Scotophilus dinganii* were collected and have been listed as a potential reservoir hosts of filovirus (Amman et al., 2017).

Rodents are suspected to be reservoir hosts due to their susceptibility to filoviruses (ebolavirus) infection in the laboratory. Filovirus has not been detected or isolated in

natural conditions from rodents (Caron et al., 2018a) However, in a couple of experimental studies, ebolavirus has been isolated from rodent organs and after several serial passages, rodents were able to adapt to the virus (Claire et al., 2017; Morvan et al., 1999). Furthermore, due to few mutations at several regions of ebolavirus genome, there is ease of these viruses adapting to new hosts which may result to novel pathogenic human ebolaviruses (Pappalardo et al., 2017). The current study sampled for oral, rectal and blood swabs unlike in the Morvan et al 1999 study where sampling was done in rodent organs. The current study findings are similar to studies conducted by Splengler and co-workers who were not able to detect viral RNA from oral swabs from ante-mortem animals while they were able to detect it in post-mortem animals. In post-mortem animals there might have been chronic viremia or high viral load (Splengler et al., 2015). In the current study, the rodents all tested negative and this may be attributed to low viral load in the samples. Considering the chance that filoviruses have not been naturally isolated from rodents, there is a possibility that rodents might have cleared the infections considering this study never tested for filovirus exposure (Serology IgG ELISA) in rodents. In rodents, filoviruses are better isolated in post mortem animals where the viral load is high enough to be detected by RT-PCR.

There are fears of cross species transmission of zoonotic viral infections which can lead to epidemics and pandemics. These animals act as “mixing vessels” for some of the zoonotic viruses which include filoviruses. In addition, with the increase in human population growth in the 2019 population census, compared to 2009, there is a high possibility of human encroachment into areas known to be inhabited with animals thus

exposing man to zoonotic viruses (Government of Kenya, 2019; Friant et al., 2015; Paige et al., 2014; Government of Kenya, 2009). Population growth leads to an increase in anthropogenic activities which might perturb disease dynamics in multi-host disease systems via impacts on cross species transmission rates or by allowing exposure of novel hosts to a rich pool of pathogen diversity as described by Murray and Daszak, 2013 (Murray & Daszak, 2013).

In as much as negatives results were obtained in all the samples, the possibility of these viruses circulating in bats and rodents from Laikipia North County, Kenya cannot be ruled out. This is because we did not conduct serological assays on these samples. Serological assay (detection of antibodies) would have ruled out the possibility of the bats and rodents having been exposed to filoviruses. The negative results might have been due to the bats and rodents clearing the virus naturally or/and the bats and rodents were not infected with filoviruses (Lacroix et al., 2021; Banerjee et al., 2020; Kuzmin et al., 2017).

The negative RT-PCR results do not necessarily mean that there is no exposure of animals and humans to filoviruses, in Laikipia North County, Kenya. There is a possibility of novel filoviruses circulating because in 2019 a novel Bombali Ebolavirus was detected in Taita Taveta County where no outbreak of filoviruses had ever been reported (Forbes et al., 2019). In addition, in 2014 Kenya was listed as one of the countries at high risk of imported filoviruses (ebolaviruses) due to its porous border with Uganda and being a busy international travel hub despite the fact that there has not been any reported human infections (Forbes et al., 2019; Pigott et al., 2016).

5.1.2 Serology (Anti-Filovirus Antibodies ELISA)

To rule out the possibility of humans and non-human primate exposure to filoviruses in humans and non-human primate populations, we conducted a serological assay (ELISA) to detect anti-filovirus IgG antibodies in serum. A total of 186 (130 humans and 56 non-human primates) serum samples were tested for anti-filovirus IgG antibody. Human samples showed a lower response to IgG antibody responses against filoviruses. This may be due to exposure to non-pathogenic filoviruses such as reston ebolavirus or individuals who were able to clear the virus after infection sometime in their life (Martell et al., 2019; Goldstein et al., 2018; Brangel et al., 2018b). The low IgG responses were interpreted as negative results due to the confirmatory tests by RT-PCR.

All the serum samples from non-human primates tested negative for anti-filovirus IgG antibodies. Coupled with the RT-PCR results in non-human primates which were negative, these results suggest that there was no filovirus circulating among the non-human primates in Laikipia North County, Kenya during the month of May. Despite the negative results on filoviruses in Laikipia North, County, there is a diversity of both wild animals and domesticated animals living in close proximity to humans. This posed a public health risk associated with zoonotic virus spill overs (Johnson et al., 2020).

5.1.3 Risk Factors Associated with Filovirus Transmission

Filovirus spill over event is a very rare event (European Centre for Disease Prevention and Control, 2021). However, if such spill overs do occur, the public health consequences

are high due to high morbidity and mortality. All human samples (faecal material, oral swabs and blood) tested negative for filoviruses by both RT-PCR and IgG ELISA. In addition to laboratory assays, risk factors in relation to filovirus transmission were analysed. Only 10 participants reported interactions with wild animals. Wild animals within Laikipia County include bats, non-human primates, rodents and duikers which are suspected reservoir hosts of filoviruses.

The major transmission mode of filoviral infection reported from the index human cases is through contact with infected reservoir hosts (bats) and intermediate/amplifying hosts faecal material and bodily fluids. In this study, the majority of the study population reported getting into contact with both domestic and wild animals, bush meat was in circulation in the villages and shared water points with wild animals which are all documented risk factors to filoviral transmission (Caron et al., 2018b). Additionally, consumption of untreated water, sharing of water sources with both wild and domestic animals and sleeping with domestic animals in the same rooms are all risk factors to contracting zoonotic viruses (Mossoun et al., 2015).

Even though the study results were negative for filoviruses, the risk factors observed, predisposes the study population to other zoonotic viruses spill over at the human animal interface. These risk factors include sharing of water points with animals and domestic use of untreated water from open water sources (river, uncovered wells and ponds), consumption of sick or dead animals, consumption of food contaminated by animal feces,

consumption of food touched with animals and coming into contact with wild/domestic/pets animals.

In as much as all samples tested negative, this study has added knowledge to the distribution and risk factors associated with transmission of filoviruses. The risk factor identified is getting into contact with suspected/infected animals such as bats.

5.2 Assumptions of the Study

The study was based on several assumptions derived from our current knowledge of filovirus reservoir hosts which include mammals. Bats in the order *Chiroptera*, non-human primates and rodents were targeted. These have been documented as possible or potential reservoir/amplification hosts of filoviruses. The second assumption made in this study was that humans who live in close proximity to wildlife have a high chance of infection from disease spill overs from animals (Kreuder et al., 2015). This led us to sample villages that were close to animals who tend to have contacts/multiple contacts with either/both wild and domesticated animals or animal products thus exposing them to zoonotic viral diseases.

This study opted for RT-PCR due to its high sensitivity compared to serological assays (antibody enzyme linked immunosorbent assays-ELISA) which may have cross reactivity with other viruses between immunoglobulin gamma, IgG (MacNeil et al., 2011; Towner et al., 2004). In addition, our primer set sequences could detect a variety of filoviruses.

5.3 Limitations of the Study

5.3.1 Sampling Biases

In this study we used harp nets to capture bats. Harp nets are mostly used to capture insectivorous bats and it has been shown that different species forage at different heights within a tropical forest (Hodgkison et al., 2004). Therefore the height of net will influence the species of bat captured. Nets may capture weaker animals that are unable to manoeuvre away from a net, or careless individuals that are less wary of obstacles. Therefore different traps and nets should be used for sampling to avoid sampling bias.

5.3.2 Laboratory Techniques

Reverse transcriptase polymerase chain reaction (RT-PCR) was the only method used for detection of filoviruses in bats and rodents only. This test only detects viral genome which might be cleared by individual subjects. Obtaining adequate blood from bats and rodents for serum preparation due to their small size was not viable thus the study did not have samples to conduct antibody, immunoglobulin gamma (IgG) capture enzyme linked immunosorbent assay (ELISA). ELISA was only performed on human and NHPs serum samples. Antibody (IgG) detection is of importance in that it provides evidence of virus exposure. Antibody detection (IgG) is a marker for epidemiological surveillance and after exposure IgG will always be detected in survivors and probably last indefinitely (Coarsey et al., 2017).

5.4 Conclusions

i. Based on the study findings, the possibility of filoviruses circulating in Laikipia, North County cannot be ruled out. This is because the study did not conduct serological assays in all the samples (bats and rodents) to detect previous exposure to filoviruses.

ii. Risk factors associated to filovirus transmission were identified in this study.

5.5 Recommendations

i. The study recommends continuous surveillance studies to be conducted at the animal human interface in the whole country. This will act as an early warning sign if a virus with zoonotic potential is detected. The role of bats, rodents and non-human primates in filoviral zoonosis still remains unclear due to the fact that viral RNA detection is very rare. Moreover, other than the aforementioned mammals being implicated as reservoir hosts for filoviruses, they are also known as reservoir hosts to many emerging and re-emerging human pathogens.

ii. Laboratory surveillance using serological (IgG and IgM antibody assays) assays should be incorporated in detection of filoviruses. This is because it is difficult to detect filoviruses using RT-PCR while serological assays point to exposure of study participants to the virus.

iii. Based on our findings, we recommend a longitudinal study should be conducted. This is because our study only sampled at a given time point with no follow up on the population.

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APPENDICES

Appendix I: Ethical Approval certificate KEMRI

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1

April 28, 2017

TO: ALLAN OLE KWALLAH,
PRINCIPAL INVESTIGATOR

THROUGH: THE DIRECTOR, CIPDCR,
BUSIA

Corrigendum
4/05/2017

Dear Sir,

RE: KEMRI/SERU/CIPDCR/017/3445 (*RESUBMISSION OF INITIAL SUBMISSION*): SURVEILLANCE FOR EMERGING ZOONOTIC DISEASE THREATS AND BEHAVIOURAL RISK CHARACTERISATION IN HIGH-RISK COMMUNITIES IN KENYA.

Reference is made to your letter dated April 21, 2017. The KEMRI Scientific and Ethics Review Unit (KEMRI/SERU) acknowledges receipt of the revised study documents on April 25, 2017.

This is to inform you that during the 261st Ethics Review Committee (ERC) held on March 21, 2017, the above referenced application was discussed.

This is to inform you that the Committee notes that the issues raised during the 261st of the Ethics Review Committee (ERC) held on March 21, 2017 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day **April 28, 2017** for a period of one year. Please note that authorization to conduct this study will automatically expire on **April 27, 2018.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **March 17, 2018.**

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

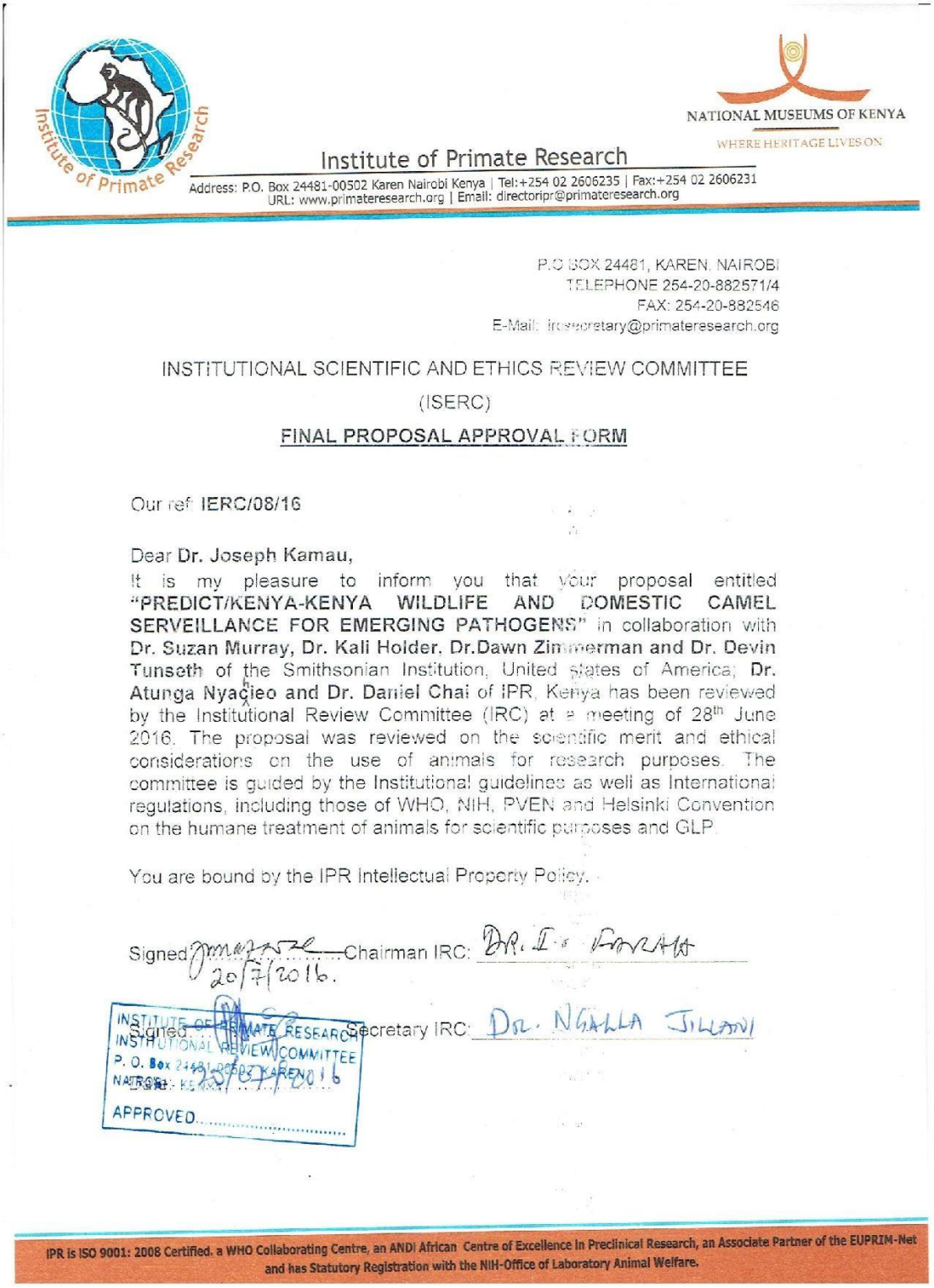
You may embark on the study.

Yours faithfully,

for DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix II: Ethical Approval Institute of Primate Research



Institute of Primate Research

NATIONAL MUSEUMS OF KENYA
WHERE HERITAGE LIVES ON

Address: P.O. Box 24481-00502 Karen Nairobi Kenya | Tel:+254 02 2606235 | Fax:+254 02 2606231
URL: www.primateresearch.org | Email: directoripr@primateresearch.org

P.O BOX 24481, KAREN, NAIROBI
TELEPHONE 254-20-882571/4
FAX: 254-20-882546
E-Mail: irosecretary@primateresearch.org

INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE

(ISERC)

FINAL PROPOSAL APPROVAL FORM

Our ref: IERC/08/16

Dear Dr. Joseph Kamau,

It is my pleasure to inform you that your proposal entitled **"PREDICT/KENYA-KENYA WILDLIFE AND DOMESTIC CAMEL SERVEILLANCE FOR EMERGING PATHOGENS"** in collaboration with **Dr. Suzan Murray, Dr. Kali Holder, Dr.Dawn Zimmerman and Dr. Devin Tunseth** of the Smithsonian Institution, United states of America; **Dr. Atunga Nyaoieo and Dr. Daniel Chai** of IPR, Kenya has been reviewed by the Institutional Review Committee (IRC) at a meeting of 28th June 2016. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

You are bound by the IPR Intellectual Property Policy.

Signed..... Chairman IRC: DR. I. [illegible]
20/7/2016.

Signed..... Secretary IRC: DR. NGALLA JILLANI

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI, KENYA
Date: 25/07/2016
APPROVED

IPR is ISO 9001: 2008 Certified. a WHO Collaborating Centre, an ANDI African Centre of Excellence in Preclinical Research, an Associate Partner of the EUPRIM-Net and has Statutory Registration with the NIH-Office of Laboratory Animal Welfare.

Appendix III: Research Permit National Council of Science Technology (NACOSTI)

Permit No : NACOSTI/P/19/48092/26431

Date Of Issue : 3rd July,2019

Fee Recieved :Ksh 2000

THIS IS TO CERTIFY THAT:

MS. PERIS AUMA AMBALA

of KENYATTA UNIVERSITY, 0-100

Nairobi,has been permitted to conduct research in Laikipia County

on the topic: DETERMINATION OF MOLECULAR CHARACTERISTICS OF FILOVIRUSES CIRCULATING IN HUMANS, WILD CAUGHT NON-HUMAN PRIMATES, BATS AND RODENTS IN KENYA.

for the period ending:

3rd July,2020

.....

Applicant's Signature

.....

Director General

National Commission for Science, Technology & Innovation

Appendix IV: cDNA Synthesis Using Super-Script III First Strand Synthesis Kit

Master Mix Preparation

Master Mix 1		Number of samples	Total amount needed (µL)
Template	8.0 µL	10	80
Random Hexamer (Primer)	1.0 µL	10	10
dNTPs	1.0 µL	10	10

PCR Conditions for master mix 1

Incubate for 5 minutes at 65^0C. Chill on ice.

Master Mix 2	Amount	X Number of Samples e.g 10
5x Reaction Buffer	2 µL	20 µL
25mM $MgCl_2$	4 µL	40 µL
0.1M DDT	2 µL	20 µL
RNase OUT	1 µL	10 µL
Superscript III Reverse Transcriptase	1 µL	10 µL

PCR Conditions For Master Mix 2

5 minutes 25^0C

60 minutes 42^0C

Appendix V: Invitrogen Platinum Taq Kit (Cat #: 10966-026) For 25µL PCR Reaction

2.5 µL 10X PCR Buffer

0.75 µL $MgCl_2$ (50mM)

0.5 µL dNTP (10mM)

0.1 µL platinum Taq DNA Polymerase

18.15 µL Molecular grade water

1 µL Forward Primer @10 µm

1 µL Reverse Primer @ 10 µm

1 µL template

Appendix VI: Barcoding -Cytochrome b RT-PCR Protocol

PCR Reaction Conditions:

94^0C for 2 minutes		
50 cycles		94^0C for 30 seconds denaturation 52^0C for 50 Seconds 72^0C for 60 seconds elongation
10^0C for cooling		

Target: Mitochondrial Cytochrome b

Size Approximately 457 bp

Visualizing Results: Run 10 µL of the PCR amplicon on a 1.5% agarose gel

Appendix VII: Preparation of Tris Acetate EDTA (TAE) Buffer 50x Buffer Stock Solution

- 50ml of 0.5ml of 0.5 EDTA.
- 121.5g Tris Base.
- 28.5ml glacial Acetic acid.
- Which was added to 500ml of water.

1. To prepare 1X TAE working solution, 1:50 dilution of the 50X TAE stock solution into Millipore water was used.
2. The electrophoresis tank was filled with 1X TAE till the fill line.

Appendix VIII: Preparation of Phosphate Buffer Saline (10 X)

To make 1 litre in distilled water

$NaCl_2$ 80g

KCl 2.0g

Na_2HPO_4 14.4g

KH2PO4 2.4g

i. Dissolve all the above in 800ml of dH2O and adjust the volume to 1 litre.
ii. Ensure that the pH is 7.4.

Preparation of 1X Phosphate Buffer Saline From 10X

Formulae

Concentration 1 x Volume 1 = Concentration 2 x Volume 2

Concentration 1=Known concentration of stock solution (10X)

Volume 1 = Unknown volume to pick from stock.

Concentration 2 = Concentration needed.

Volume 2 = Volume you want to make.

Appendix IX: Preparation of blocking Buffer

Bovine serum albumin (BSA) in PBS (10mg/ml)

Calculate the total of wells needed e.g 48 wells and the amount per well which is 100µl per well.

Therefore for 50 wells (2 wells for pipetting error) = 50 x 100µl = 5000µl=5mls

10mg in 1ml

? mg in 5mls

$$= \frac{5\text{mls} \times 10\text{mg}}{1\text{mls}}$$

= **50mg BSA in 5 mls of PBS**

Note: The blocking buffer should be kept on ice or $+4^{0}$C at all times.

Appendix X: Preparation of Phosphate Buffer Saline (1 X) with 0.05% Tween20

Formulae

Concentration 1(C1) x Volume 1 (V1) = Concentration 2 (C2) x Volume 2 (C2)

Concentration 1 (C1) =Known concentration of stock solution (10X)

Volume 1 (V1) = Unknown volume to pick from stock.

Concentration 2 (C2) = Concentration needed.

Volume 2 (V2) = Volume you want to make.

C1 x V1 = C2 x V2

C1 = 0.05%

V1 = 1000mls

C2 = ?

V2 = 100mls

$$\frac{C1 \times V1}{V1} = C2$$

$\frac{0.05 \times 1000mls}{100mls}$ = **0.5mls** of Tween20 needed into 1000mls.

Appendix XI: Consent Form and Questionnaire Used for the Survey

Human Questionnaire Form

EIDITH

Directions for selecting modules for the interview

Select module(s) identified in Question 2 (if any).

Conduct the following QUICK CHECKS.

1. Add the primary work activity module (Question 25)
2. Add the Temporary Settlement Module if Temporary Settlement is NOT selected in Question 2 and dwellings is NOT permanent (Question 15)
3. Add Hunter Module if hunter/trapper/fisher is selected in Question 25 or "yes" to Question 55.

Livelihood Module Table (based on response to Question 25)
Complete the module that corresponds with the livelihood chosen as follows:

extraction of minerals, gas, oil, timber – extractive industry module
crop production – crop production module
wildlife restaurant business – wildlife restaurant module
wild/exotic animal trade business – market and value chain module
rancher/farmer animal production business - animal production module
meat processing, slaughterhouse, abattoir - animal production module
zoo/sanctuary animal health care – zoos & sanctuaries module
hunter/trapper/fisher – hunter module
nurse, doctor, traditional healer, community health worker - hospital or clinic health professional module

If no additional modules are selected, the interview is complete.

Human Questionnaire Form ID Instructions

Enter the Site and Event Form ID barcode number to the grid located at the top of page 1 of the Human Questionnaire Form.

Enter the Human Questionnaire Form ID barcode number to grid located at the top of each associated module.

The barcode is located at the bottom right hand corner of each page of the Human Questionnaire section and the Site and Event Characterization Form.

Use the number after the dash (-) and fill the grid with the numbers from top to bottom.

176916-50708

Human Questionnaire Form

EIDITH V 1.2

Add Site and Event Form ID:

Site name and date:

_____

(For reference only)

1. Consent Form Administered & Signed ☐ yes ☐ no Participant ID: _____

2. Description of Interview Location - Select all that apply.
(To be completed by interviewer prior to administrative questionnaire.
Prepare and download modules in advance.)

- ☐ Animal Production or Abattoir Site
- ☐ Crop Production Site
- ☐ Extractive Industry Site
- ☐ Market or Value Chain Site
- ☐ Temporary Settlement Site
- ☐ Tourism Site
- ☐ Wildlife Restaurant
- ☐ Zoos or Sanctuaries
- ☐ Hospital or Clinic - Health Professional
- ☐ Hospital or Clinic - Patient
- ☐ Natural Areas (eg. forest, urban park/garden)
- ☐ Other: _____

3. Date of interview _____

4. Begin time of interview _____
(Example: 17:50)

5. End time of interview _____
(Example: 19:20)

6. Where are you conducting this interview?

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____
Interviewer: Please collect GPS coordinates if administering using paper and pen.

7. Interviewer Observed Gender
- ☐ male
- ☐ female
- ☐ other

INTERVIEW/QUESTIONNAIRE BEGINS

Demographics Section (include observation question 7)

8. How old are you? _____
If the exact age is unknown, enter the respondent's estimated age.

9. Where do you live?

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____
Interviewer: Probe for landmarks or nearest known site if area unknown.
GPS coordinates to be identified and entered after completion of interview.

176917-50708

Human Questionnaire Form

10. How long have you lived there?
Select one option.
- ☐ <1 month
- ☐ 1 month - 1 year
- ☐ >1 - 5 years
- ☐ >5 - 10 years
- ☐ >10 years

11. How many other people live in the dwelling where you live? _____
Skip to question 14 if answer is 0.

12. How many in the dwelling are children less than 5 years old? _____

13. How many in the dwelling are male? _____

14. How many rooms are there in the dwelling where you live? _____
(Do not include bathroom or kitchen)

15. Is the dwelling a permanent structure (that cannot be moved)?
Interviewer: If answer is no, complete temporary settlement questionnaire.
- ☐ yes
- ☐ no

16. Do you get water from:
Select all that apply.
- ☐ piped in water/water taps
- ☐ covered well
- ☐ uncovered well/pond/river
- ☐ water truck/rainwater harvest
- ☐ other: _____

17. Do you treat your drinking water?
- ☐ yes
- ☐ no

18. If yes, how do you treat your water?
Select all that apply.
- ☐ boil
- ☐ filter
- ☐ add chlorine or bleach
- ☐ solar disinfection
- ☐ other: _____

19. Is your source for drinking water ever used by animals?
- ☐ yes
- ☐ no

20. In your dwelling is there a dedicated location for human solid waste/excreta?
(Example: toilet, latrine, designated area)
- ☐ yes
- ☐ no

21. Do you have containers for storing food for the household?
Select all that apply.
- ☐ yes, with covers
- ☐ yes, without covers
- ☐ no

Human Questionnaire Form Participant ID _____
(For reference only)

Livelihood Section

In this section, I'd like to ask you about education and the kinds of work activities that you have done since this time last year.

22. What is the highest level of education you have completed?
Select one option. (Skip for Cameroon.)
- ○ primary school
- ○ secondary school
- ○ college/university/professional
- ○ none

23. What is the highest level of education that your mother completed?
Select one option. (Skip for Cameroon.)
- ○ primary school
- ○ secondary school
- ○ college/university/professional
- ○ none

24. Since this time last year what are the activities you have done to earn your livelihood?
Select all that apply.
- ○ extraction of minerals, gas, oil, timber
- ○ crop production
- ○ wildlife restaurant business
- ○ wild/exotic animal trade/market business
- ○ rancher/farmer animal production business
- ○ meat processing, slaughterhouse, abattoir
- ○ zoo/sanctuary animal health care
- ○ protected area worker
- ○ hunter/trapper/fisher
- ○ forager/gatherer/non-timber forest product collector
- ○ migrant laborer
- ○ nurse, doctor, traditional healer, community health worker
- ○ construction
- ○ other: _____

25. If more than one activity was selected, what is the activity on which you spent the most time since this time last year?*
Select one option.
- ○ extraction of minerals, gas, oil, timber
- ○ crop production
- ○ wildlife restaurant business
- ○ wild/exotic animal trade/market business
- ○ rancher/farmer animal production business
- ○ meat processing, slaughterhouse, abattoir
- ○ zoo/sanctuary animal health care
- ○ protected area worker
- ○ hunter/trapper/fisher
- ○ forager/gatherer/non-timber forest product collector
- ○ migrant laborer
- ○ nurse, doctor, traditional healer, community health worker
- ○ construction
- ○ other: _____

26. Which best describes your job position?
Select one option.
- ○ manager/owner/foreman
- ○ worker
- ○ live and work at home independently (If chosen, skip to question 28)
- ○ professional
- ○ other: _____

27. Where do you work?

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Interviewer: Probe for landmarks or nearest known site if area unknown.
GPS coordinates to be identified and entered after completion of interview.

176919-50708

Human Questionnaire Form Participant ID _____ (For reference only)

Medical History Section

In this section, I'm going to ask you about any illness or sickness that is not known or recognized in the community, including by medical or treatment providers.

28. Where do you usually get treatment for medical problems? Select all that apply.
- ☐ clinic/health center
- ☐ hospital
- ☐ mobile clinic
- ☐ community health worker
- ☐ traditional healer
- ☐ dispensary or pharmacy

29. Have you ever had an unusual illness with any of the following symptoms: Select all that apply. (READ ONLY SYMPTOMS)
- ☐ fever with headache and severe fatigue or weakness (encephalitis)
- ☐ fever with bleeding or bruising not related to injury (hemorrhagic fever)
- ☐ fever with cough and shortness of breath or difficulty breathing (SARI)
- ☐ fever with muscle aches, cough, or sore throat (ILI)
- ☐ fever with diarrhea or vomiting
- ☐ fever with rash
- ☐ persistent rash or sores on skin
- ☐ no (Skip to question 33)
- ☐ yes but, none of these symptoms-describe: _____

30. Since this time last year, have you had any of these symptoms?
- ☐ yes
- ☐ no (Skip to question 33)

31. If yes, which ones? Select all that apply.
- ☐ fever with headache and severe fatigue or weakness (encephalitis)
- ☐ fever with bleeding or bruising not related to injury (hemorrhagic fever)
- ☐ fever with cough and shortness of breath or difficulty breathing (SARI)
- ☐ fever with muscle aches, cough, or sore throat (ILI)
- ☐ fever with diarrhea or vomiting
- ☐ fever with rash
- ☐ persistent rash or sores on skin
- ☐ yes but, none of these symptoms-describe: _____

32. In your opinion, when you were sick, what caused this sickness? Select all that apply.
- ☐ contact with sick people
- ☐ contact with wild animals
- ☐ contact with other animals
- ☐ bad food or water
- ☐ bad spirits/witchcraft
- ☐ wound or injury
- ☐ I don't know
- ☐ other: _____

33. Since this time last year, have any of the people you lived with had any of these symptoms?
- ☐ yes
- ☐ no (Skip to question 36)

34. If yes, which ones? Select all that apply.
- ☐ fever with headache and severe fatigue or weakness (encephalitis)
- ☐ fever with bleeding or bruising not related to injury (hemorrhagic fever)
- ☐ fever with cough and shortness of breath or difficulty breathing (SARI)
- ☐ fever with muscle aches, cough, or sore throat (ILI)
- ☐ fever with diarrhea or vomiting
- ☐ fever with rash
- ☐ persistent rash or sores on skin
- ☐ yes but, none of these symptoms-describe: _____

35. Since this time last year, did anyone you lived with die from this illness?
- ☐ yes
- ☐ no

Human Questionnaire Form

Participant ID _____
(For reference only)

Movement Section

In this section, I'm going to ask you about any travel you have done since this time last year.

36. Have you traveled since this time last year?
If answer is no, skip to the next section.
- ☐ yes
- ☐ no

37. Where have you traveled since this time last year? Anywhere else?

Provide details, such as name of town, nearest (or most frequent) well known place if unknown by interviewer (to be linked to GPS coordinates later)
Collect up to 6 locations.

Interviewer: Probe for landmarks or nearest known site if area unknown. GPS coordinates to be identified and entered after completion of interview.

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Notes: _____

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Notes: _____

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Notes: _____

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Notes: _____

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Notes: _____

Village/Town/City _____ District _____ Province/State _____

Latitude _____ Longitude _____

Notes: _____

If there are more than six locations check here. ☐
Do not collect additional location information.

38. Why have you traveled?
Select all that apply.
- ☐ work
- ☐ visit family
- ☐ moved
- ☐ religious reasons
- ☐ holiday/vacation
- ☐ go to hospital/seek medical care
- ☐ go to market
- ☐ other: _____

176921-50708

Human Questionnaire Form Participant ID _____ (For reference only)

Animal Contact Section

In this section, I'm going to ask you about the animals in your life.

If answered "no" under the "In your lifetime" column, then no answer is required under the "Since this time last year" column.

	In your lifetime...	Since this time last year...
39. Has an animal lived as a pet in or near your dwelling?	○ yes ○ no	○ yes ○ no
40. Have you handled live animals?	○ yes ○ no	○ yes ○ no
41. Have you raised live animals?	○ yes ○ no	○ yes ○ no
42. Have you shared a water source with animals for washing?	○ yes ○ no ○ don't know	○ yes ○ no ○ don't know
43. Have you seen animal feces in or near food before you have eaten it?	○ yes ○ no	○ yes ○ no
44. Have you eaten food after an animal has touched or damaged it? (Example: chew marks or	○ yes ○ no ○ don't know	○ yes ○ no ○ don't know
45. Do any animals come inside the dwelling where you live?	○ yes ○ no	○ yes ○ no
46. Have you cooked or handled meat, organs or blood from a recently killed animal?	○ yes ○ no	○ yes ○ no
47. Have you eaten raw or undercooked meat or organs or blood?	○ yes ○ no	○ yes ○ no
48. Have you eaten an animal that you knew was not well/sick?	○ yes ○ no ○ don't know	○ yes ○ no ○ don't know
49. Have you found a dead animal and collected it to eat or share?	○ yes ○ no	○ yes ○ no
50. Have you found a dead animal and collected it to sell it?	○ yes ○ no	○ yes ○ no
51. Have you been scratched or bitten by an animal?	○ yes ○ no	○ yes ○ no

52. The last time you were scratched, bitten or cut yourself while butchering or slaughtering, what did you do?
Select all that apply.

- ○ let someone else take over
- ○ wash wound with soap and water
- ○ rinse wound with water
- ○ bandage wound
- ○ visit doctor
- ○ nothing - kept working
- ○ never butcher or slaughter

Human Questionnaire Form

Animal Contact Section

53. Are there any risks associated with slaughtering or butchering when you have an open wound?

Interviewer: Do not read responses.

- ☐ no
- ☐ yes, but I don't know what they are
- ☐ yes, it can make you sick
- ☐ yes, it can poison you
- ☐ yes, it can infect you with a disease
- ☐ don't know
- ☐ other: _____

	In your lifetime...	Since this time last year...
54. Have you slaughtered an animal?	☐ yes ☐ no	☐ yes ☐ no
55. Have you hunted or trapped an animal? (If answered "yes" to "Since this time last year" also ask hunter questionnaire)	☐ yes ☐ no	☐ yes ☐ no

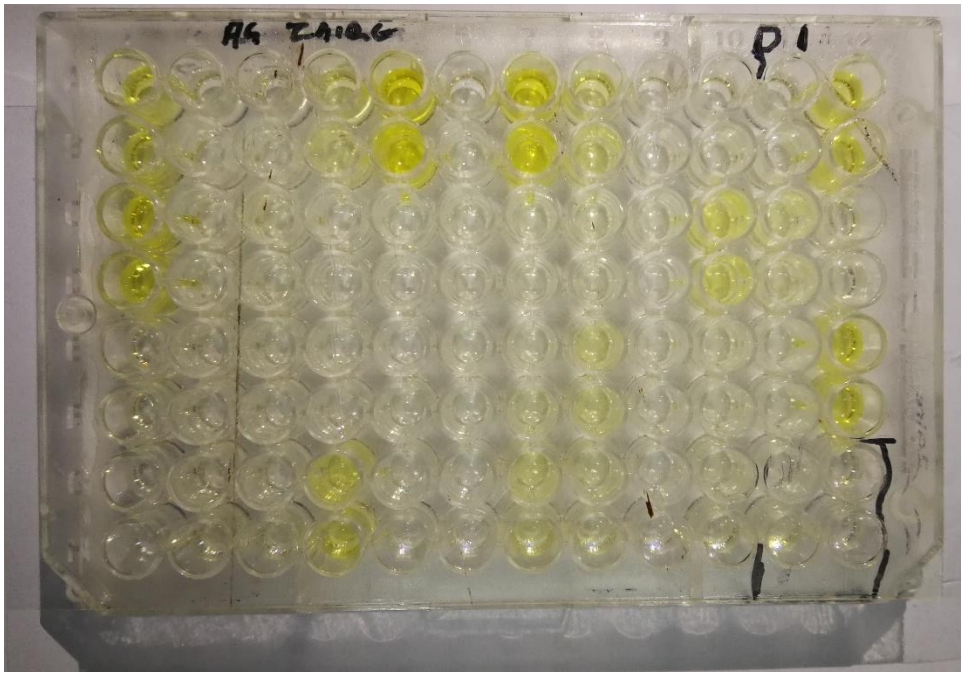
56. Interviewer: Circle all headings where "yes" was answered in the "Since this time last year" questions above.
Then ask which animals/mammals for each "yes" category.
Select all that apply.

	pet (39)	handled (40)	raised (41)	feces in or near food (43)	in house (45)	cooked/ handled (46)	eaten raw/ under cooked (47)	eaten sick (48)	found dead (49/50)	scratched/b itten (51)	slaugh-tered (54)	hunted/ trapped (55)
rodents/shrews	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
bats	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
non-human primates	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
birds	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
carnivores	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
ungulates	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
pangolins	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
poultry/other fowl	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
goats/sheep	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
camels	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
swine	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
cattle/buffalo	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
dogs	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
cats	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

57. Are you worried about diseases or disease outbreaks in live animals in your local market?
- ☐ yes
- ☐ no

END OF MAIN QUESTIONNAIRE

Appendix XII: ELISA IgG Sample Plate



Zaire Ebolavirus Antigen was used to coat the plate. Wells G11, G12, H11 and H12 are the controls. The samples with the yellow colour all read less than 1.0000OD at 450nm. Meaning all were negative.

ZAIRE HUMAN 15/6/2020 P1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.4276	0.0691	0.0742	0.2528	1.0182	0.0694	0.8938	0.3011	0.0753	0.0823	0.0888	0.4691
B	0.3852	0.0799	0.0719	0.226	1.0396	0.0633	0.8854	0.2945	0.0747	0.106	0.0847	0.5026
C	0.7638	0.0936	0.1251	0.0645	0.079	0.0823	0.1112	0.1366	0.1047	0.4286	0.2403	0.0953
D	0.7944	0.0888	0.1076	0.0634	0.0603	0.0781	0.106	0.1389	0.0806	0.4462	0.2025	0.1078
E	0.0758	0.0669	0.0678	0.0523	0.036	0.0671	0.0906	0.2633	0.088	0.1267	0.0796	0.5697
F	0.083	0.0637	0.0667	0.0563	0.0796	0.0718	0.1572	0.2459	0.0825	0.1058	0.0866	0.5034
G	0.0414	0.0903	0.0598	0.4277	0.0821	0.06	0.2584	0.1699	0.06	0.1309	0.0644	0.0719
H	0.0388	0.114	0.096	0.412	0.0746	0.0604	0.2112	0.1434	0.0629	0.1216	0.078	0.0767

KEY:

The samples are in duplicates. E.g Wells A1 and B1 = 1 .

Negative controls are in well G11 and H11 and G12 and H12

Samples on well A5 and B5 were repeated and were negative on a different plate.