

**MOLECULAR DETECTION AND CHARACTERIZATION OF CORONAVIRUS
INFECTION IN OLIVE BABOONS (*Papio anubis*), BATS AND RODENTS IN LAIKIPIA
COUNTY, KENYA**

BY

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DECLARATION

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

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DEDICATION

To the Almighty God and Father of our Lord and savior Jesus Christ, my late parents Chief and Mrs Manzhi Rimfa, my faithful wife Mrs. Mercy Nannyam Rimfa, and the boys; Nanchang D. Rimfa, Nandul J. Rimfa, Nanlir D. Rimfa, and Nandom J. Rimfa. I also dedicate this work to the entire Rimfa and Zitta's family for their persistent prayers and support while away on study leave, and also to my Uncle, rtd corporal (Chief) Nanven A. Rimfa who enrolled me into school. To Dr. Emmanuel Miri, Mr. Samuel Waplang, ACI Selchak Rimfa, and Baba Dandam who stood by me to victory.

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LIST OF ABBREVIATIONS

BLAST:	Basic Local Alignment Search Tool
bp :	Base pair
CDC:	Center for Disease Control
cDNA:	complementary DNA
CoV:	Coronavirus
CRT:	Capillary Refill Time
DNA :	Deoxyribonucleic acid
dNTP :	Deoxynucleotide triphosphates
EDTA :	Ethylenediaminetetraacetic acid
EIDs:	Emerging infectious diseases
ORF:	Open Reading Frame
PCR :	Polymerase Chain Reaction
PPE:	Personal Protective Equipment
qPCR:	Quantitative Polymerase Chain Reaction
RFS:	Ribosomal Frame Shift
rRNA :	Ribosomal ribonucleic acid
RT-PCR:	reverse transcriptase Polymerase Chain Reaction
SARS:	Severe acute respiratory syndrome
TB:	Tuberculosis
µl :	Microliter

ABSTRACT

Background: In the last few years, members of the subfamily coronavirinae of the family coronaviridae have been involved in human and animal epidemics causing high morbidity and mortality rates. The Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS) were linked to the alpha- and beta-coronaviruses with wildlife as sources of infections. Porcine epidemic diarrhea virus occurred and traced its origin from bats. The occurrence of these diseases has motivated interest to discover more zoonotic sources of coronaviruses and their transmission dynamics at the human-wildlife interfaces.

Objective: The general objective of this study was to determine and characterize the presence of coronaviruses in olive baboons, bats and rodents using reverse transcription PCR (RT-PCR) in Laikipia, County Kenya.

Methodology: 130 olive baboons (*Papio anubis*), 202 bats from 2 species (*Chaerephon* sp. and *scotophilus* sp.) and 161 rodents from eight species (*Acomys kempis*, *Acomys percivalli*, *Elephantulus rufescens*, *Gerbilliscus robustus*, *Aethomys hindei*, *Myomyscus brodernani*, *Grammonys dolichorus*, and *Saccostomus meamsi*) were humanely trapped and sampled in the dry season of September, 2017 and wet season of May, 2018. In total, 260 oral and rectal swabs were obtained from olive baboons, 404 from bats and 322 from rodents. These samples were screened for coronavirus (CoV) RNA using reverse transcription PCR. To increase the chances of viral detection, two cost-effective consensus PCR assays targeting RNA-dependent RNA polymerase gene to detect Coronaviruses (Watanabe assay targeting the polymerase gene at nucleotides 14,370-14,750 and Quan assay amplified at 17,480-17,820) were used.

Results: Eight positive samples were obtained from bats in the rainy season constituting 8% (n=100) and 3.96% (n=202) by Quan assay. BLAST and phylogenetic analysis showed all

sequenced isolates belonged to unclassified alpha-coronavirus genus in the genbank. Five of the isolates are distant relatives of Kenyan bat coronaviruses and are new strains of the virus. Three clustered with reference bats coronavirus sequences from Kenya in the database. Elsewhere in the world, no cluster relationship was identified with other bat coronaviruses.

Conclusion: This study demonstrated coronaviruses may be endemic in bat population in Kenya and with five new strains (384, 396, 383, 382, and 385) detected that require further genome analysis.

CHAPTER ONE

1.0 INTRODUCTION

Wildlife populations constitute large and often unknown reservoirs of infectious agents (Chomel *et al.*, 2007) and have played key roles in the emergence of new diseases by being reservoirs of these pathogens. Global travels, cultural practices, religious pilgrimages, deforestation, agricultural expansion, and urbanization are risk factors in emergence of new diseases (Daszak *et al.*, 2001).

Coronaviruses have caused illness in humans and animals (pigs, cattle, dogs, cats, domestic and wild birds, bats, and rodents) and therefore need to be given serious consideration due to their public health risks. Recent important coronavirus epidemics such as porcine epidemic diarrhea (PEDv), porcine delta coronavirus (PDCoV), swine acute diarrhea syndrome coronavirus (SADS-CoV), severe acute respiratory syndrome (SARS), and Middle East Respiratory syndrome (MERS) have links to bats as sources of infection (Guan *et al.*, 2003; Braun, 2008; Wacharapluesadee *et al.*, 2013; De Benedictis *et al.*, 2014; Zhou *et al.*, 2018) and are noteworthy. Initial report of the outbreak of SARS was in 2002 in Guangdong Province of Southern part of China. The disease was characterized by headache and fever followed by acute respiratory symptoms (cough, dyspnea and pneumonia) and caused 10% mortality. MERS occurred in Saudi Arabia about ten years (2012) later with a mortality rate of 35%. In animals, the porcine epidemic diarrhea for example has caused massive disease in piglets with high mortality in USA, Europe and China (Song *et al.*, 2015).

Interspecies spill-over of coronaviruses into new hosts occur frequently causing stress to public and livestock health (Guan *et al.*, 2003; Lau *et al.*, 2005; Ferguson and Van, 2014). With this in context, it is important that all coronaviruses circulating in wildlife especially at active human-

wildlife-livestock interfaces be determined as an important emergency preparedness step to emerging zoonotic episodes.

Data on wildlife species as reservoirs of coronaviruses in Kenya are very scanty though it is considered a “hotspot” for emerging zoonotic epidemics (Figure 2.6). With the population of Kenya growing at a geometrical rate (Mwangi *et al.*, 2016), the need to determine coronaviruses circulating in olive baboons, bats and rodents is significant. Laikipia County is rich in biodiversity and has the second largest concentration of wildlife in Kenya after Tsavo in Narok County. With the increased demand for space for agriculture, human settlement and search for green pasture for livestock, encroachments into wildlife habitats have increased in the County. This rapid human population growth coupled with changes in temperature and habitat fragmentation has altered wildlife distribution resulting in several interfaces being created (Kilpatrick, 2011). Therefore, to determine the presence of coronaviruses, understand the transmission pattern and the cultural practices of humans at the interfaces is crucial for the prevention of future human disease outbreaks. The semi-arid and arid nature of part of Laikipia County offers the opportunity to study the human-wildlife interactions and the potential consequence on human health through disease surveillance. The rich population of olive baboons, bats and rodents in this region may possibly be due to the presence of abundant water, food, resting sites, and few predators.

There is scarcity of data on coronaviruses in olive baboons, bats and rodents in Laikipia County. Therefore, the need to describe the diversity of coronaviruses in wildlife in this region in the context of possible emerging events is significant. This study was aimed at determining the presence of coronaviruses using molecular technique in olive baboons, bats and rodents in Laikipia County Kenya, and to compare with those existing elsewhere in the world.

1.2 Statement of the problem

There is a global rise in coronavirus outbreaks resulting in high morbidity and mortality rates among humans and animals. For example the SARS and MERS epidemics have shown that coronaviruses can cross species barrier to emerge rapidly in humans. The pig industry, has witnessed major setback due to porcine epidemic diarrhea virus (PEDV) (Jung and Saif, 2015). In Laikipia County, there is massive continuous encroachment into wildlife reserved areas (Kivai, 2010) and the practice of handling and eating of dead animals by the people has made Laikipia a high risk area for coronavirus disease transmission.

Olive baboons, bats and rodents are widely distributed throughout Laikipia County and given that they live near and or highly interact with humans, there could be great implication and hence the need for surveillance of coronaviruses in these taxa.

1.3 Justification

Most devastating infectious diseases in humans originate from wildlife (Wolfe *et al.*, 2007) and countries with high biodiversity. Land cover and land use have been predicted as “hotspots” for emergence and re-emergence of coronavirus infection (Keesing *et al.*, 2010). Also, due to increasing human population, climate change, and habitat alteration, human-wildlife-livestock interactions have increased (Thuku *et al.*, 2013; Mwangi *et al.*, 2016). There is a complex human-wildlife-livestock interaction in Laikipia County with a strong possible risk of spill-over events. The human-animal interactions, high-risk food practices (consumption of sick and dead bats and rodents), poor water quality practices, sharing of water points with wildlife, and cultural practices of the people in Laikipia County are pre-disposing factors to disease emergence (Kamau *et al.*, 2017. unpublished data). Since prediction and prevention of viral epidemics is a major concern in

the human health sector, understanding of reservoirs of coronaviruses can help in knowing where epidemics or pandemics may emerge (Haydon *et al.*, 2002; Greger, 2007; Jones *et al.*, 2008; Keesing *et al.*, 2010). Transmission and maintenance of coronaviruses in wildlife is favored by animals with close social interaction, high population densities and their large social network (Haydon *et al.*, 2002; Keesing *et al.*, 2010).

1.4 Research Hypothesis

Olive baboons, bats and rodents in Laikipia County, Kenya are reservoirs of coronaviruses.

1.5 Main Objective

The general objective of this study was to characterize coronaviruses in olive baboons, bats and rodents in Laikipia County, Kenya.

1.6 Specific Objectives

- i. To determine the presence of coronaviruses infecting olive baboons, bats and rodents in Laikipia County, Kenya.
- ii. To establish the genetic diversities of coronaviruses infecting the wildlife species above in relation to isolates from other regions of the world.

1.7 Significance of the study

New coronavirus diseases have emerged in the world that tends spread fast among humans and animals cause enormous public health distress, economic losses, and developmental shock. This study therefore added preparedness data on coronaviruses, the ecology, evolutionary divergence, knowledge of the reservoir hosts, cultural practices of the people at the human-wildlife interfaces that predispose them to disease outbreak, and the transmission cycle. This study has provided

information on coronaviruses circulating in baboons, bats and rodents, hence a database in Laikipia County. This is significant for policy makers in the health sector in terms of decision making on rapid prevention and control.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Coronaviruses

2.1.1 Taxonomy

In 1968, the name “coronavirus” was coined. It was described so due to the corona-like or the appearance of a crown when viewed by electron microscopy (*de Groot et al.*, 2012). The global Committee on viral nomenclature in 1975 established the Coronaviridae family and at the 10th world-wide Nidovirus symposium in June 2005 in Colorado Springs, a proposal was made to divide the coronaviridae into two subfamilies namely the coronavirinae and torovirinae (cause enteric diseases in cattle and possibly in humans). Members of the Coronaviridae, Arteriviridae, and Roniviridae families constitute the Nidovirales order.

2.1.2 Classification and Pathogenicity of coronaviruses

As a subfamily in the coronaviridae family, coronavirinae is classified into four genera; alphacoronaviruses, betacoronaviruses, deltacoronaviruses, and gammacoronaviruses. Evolutionarily, the coronaviridae family is made up of related non-segmented, single stranded, positive sense, and enveloped RNA viruses of mammals (humans inclusive) and birds. Coronaviruses have the largest genome of all RNA viruses ranging from 26-32kb. The N protein in the virion greatly enhances the infectivity of these viruses (*Yount et al.*, 2000; *Casais et al.*, 2001; *Grossoehme et al.*, 2009). Among the subfamily coronavirinae, the alpha- and beta coronaviruses have caused much disease in mammals including humans. The members of the alpha-coronavirus genus include BtCoV 512, BtCoV-HKU8, BtCoV-HKU2, alphacoronavirus 1 which causes transmissible gastroenteritis (TGEV), porcine epidemic diarrhea (PEDV), and bat coronavirus 1, human coronavirus HCoV-NL63, and HCoV-229E. Gamma-coronaviruses include

whale coronavirus SW1 and avian coronaviruses. Deltacoronavirus genus includes coronavirus HKU11, HKU12, and HKU13. From the beta-coronavirus genus, four lineages (A, B, C, and D) have been identified and each having a unique set of accessory genes. Lineage A includes the following betacoronavirus 1 (bovine coronavirus BCoV), human coronavirus (HCoV-OC43 and HCoV-HKU1), murine coronavirus (MHV); Lineage B includes Severe Acute Respiratory Syndrome (SARS-CoV) and other bat coronaviruses; Lineage C includes Pipistrellus bat coronavirus HKU5 (BtCoV-HKU5), Tylonycteris bat coronavirus HKU4 (BtCoV-HKU4), Middle East Respiratory Syndrome (MERS-CoV) which emerged in 2012 as a new member in lineage C, and is related closely to bat coronaviruses HKU4 and HKU5 (Zaki *et al.*, 2012; de Groot *et al.*, 2013; Drexler *et al.*, 2014). It is the first lineage C beta-coronavirus isolated from humans, Lineage D includes Rousettus bat coronavirus HKU9 (BtCoV-HKU9).

2.1.3 Physical structure

The subfamily coronavirinae are spherical in shape with large surface projections called glycoproteins. These surface proteins range from the spike glycoprotein (S), enveloped protein (E), membrane-bound protein (M) and a nucleocapsid (N). Members of the Betacoronavirus have in addition, a spike-like short protein called hemagglutinin esterase (HE) (de Groot *et al.*, 2011) whose role though poorly understood, is believed to be important in binding to host cell.

They also have a molecular diameter of about 125 nm (Barcena *et al.*, 2009). The nucleocapsid is contained within the envelope of the virion and appears helically symmetrical. This is an uncommon feature among positive-sense RNA viruses, but is common in negative-sense RNA viruses.

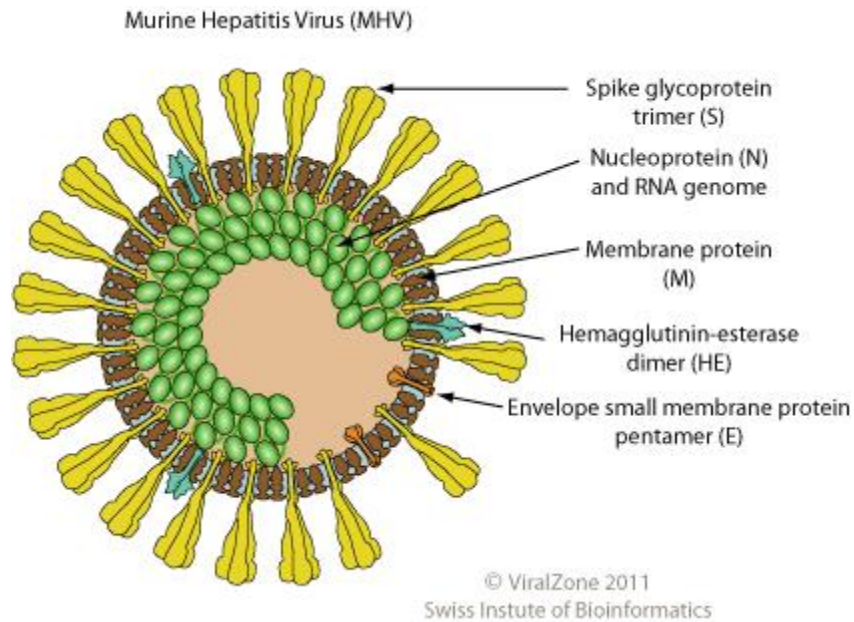


Figure 2.1: Structural features of a coronavirus (Hulo *et al.*, 2010)

2.1.4 Genome Structure

Coronaviruses are enveloped, positive-sense RNA virus (Fehr and Perlman, 2015) and have long genome of 30 kilobases (Fehr and Perlman, 2015) compare to other viruses. Non-structural proteins are encoded by twenty of these kilobases, while ten of the remaining kilobases encode for structural and accessory functions. The 5' cap and 3' poly-A tail are included in the coronavirus genome and allows the virus to act as mRNA molecule to translate replicase polyproteins (Fehr and Perlman, 2015). The activities of the accessory proteins are not well known, though it has been hypothesized that they are involved in viral pathogenesis (Fehr and Perlman, 2015). The genome has between 6 and 7 open reading frames (ORFs) in the gene order of 5' to 3' direction. The ORF1a and 1b constitute $2/3^{\text{rd}}$ of total genome structure and responsible for encoding the non-structural poly-proteins (nsp) while the remaining four others downstream encode the structural protein (sp) namely, the “spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N).” The betacoronaviruses have an additional structural protein; hemagglutinin-esterase

(HE) gene located between ORF1b and S (spike protein) and is important as a receptor binding site for the group. SARS-CoV and MERS-CoV have similar genome organization with other coronaviruses, but have unique structure due to evolutionary changes.

2.1.5 Functional receptor

The surface S protein of coronaviruses have two trimeric glycoproteins, which are the N-terminal S1 and the C-terminal S2 subunits. The S1 recognizes and attach to host cell receptor while the S2 helps in membrane fusion. In terms of genetic variation, S1 shows higher variability compared to S2 (Masters, 2006). This variability in the S protein accounts for coronavirus tissue tropism and wide host range (Gallagher and Buchmeier, 2001). For example it has been identified that angiotensin-converting enzyme 2 (ACE2) is the functional receptor of SARS-CoV (Li *et al.*, 2003; Hamming *et al.*, 2004; Ding *et al.*, 2004) and has 193 amino acid fragment (amino acid 318–510) of SARS-CoV S protein and binds efficiently to receptor site of host cell compared to the whole S1 domain which is considered as the receptor binding domain (Li *et al.*, 2005). Variation in the amino acids residues at the ACE2 could result in different binding efficiency among SARS-CoVs (Li *et al.*, 2005; Qu *et al.*, 2005; Li *et al.*, 2005). In MERS-CoV, dipeptidyl peptidase 4 (DPP4, also known as CD26) is the functional receptor (Raj *et al.*, 2013) and is conserved in mammals making it possible for them to replicate in cell lines belonging to human and non-human primate. Others are swine, bats, horse, goat, civet, rabbit, camel, hamster, ferret, dog, and cat. Virus does not grow on cell lines of mice (Raj *et al.*, 2013; de Wit *et al.*, 2013; Barlan *et al.*, 2014; Eckerie *et al.*, 2014; van Doremalen *et al.*, 2014). MERS-CoV can recognize the DPP4 from livestock and can support its replication (Barlan *et al.*, 2014; van Doremalen *et al.*, 2014). “The RBD of MERS-CoV consists of approximately amino acid 240 residues, located at amino acids 367–606, which fold into a structure consisting of two sub-domains, the core sub-domain and the external sub-

domain. The core sub-domain of MERS-CoV RBD is structurally similar to that of the SARS-CoV, but the external sub-domain (also named as RBM) is different to that of the SARS-CoV (Lu *et al.*, 2013; Wang *et al.*, 2013; Chen *et al.*, 2013).” Research has also shown that not all coronaviruses have exoribonuclease activity which therefore limits their ability to infect a host cell (Smith *et al.*, 2013). The rate of mutations can increase in those without exoribonuclease activity compare to those that exhibit it (Smith *et al.*, 2013). Those that do not have exoribonuclease function have less ability to infect host organisms (Smith *et al.*, 2013). Very critical for the virus replication, is the expression of exoribonuclease in the genome of coronaviruses. Therefore, in future vaccine production, the gene expressing exoribonuclease needs to be inhibited (Smith *et al.*, 2013).

2.1.6 Viral Replication

The replication of coronaviruses occur in the cytoplasm of host cell after infection. This occurs by binding to host receptor-sites (Delmas *et al.*, 1992; Li *et al.*, 2003 Hofmann *et al.*, 2005; Raj *et al.*, 2013) and depending on the virus strain and target cell, the virus gain entry into the cytoplasm by endocytotic process (Nash and Buchmeier, 1997; Wang *et al.*, 2008). On entry into the cytoplasm of the host cell, the virus un-coats and releases the RNA genome, which have a 5' methylated cap and a 3' poly-adenylated tail allowing it to attach to the virus ribosomes for translation. They also have a replicase protein that makes negative strand for use in making more copies of the mRNA. These negative strands are used for the replication of new positive strand RNA genomes. New viral proteins (N, M and S) are coded by mRNA. The N protein attaches to the genomic RNA while the M protein integrates with the endoplasmic reticular (ER) membrane. The S protein binds to host cell and assembles nucleo-capsids with helical twisted RNA, which buds into the ER lumen and are encased with membrane. The offspring of the virus are then transported by Golgi apparatus

to the cell membrane which is then released into extracellular space by exocytosis as seen in Figure 2 below:

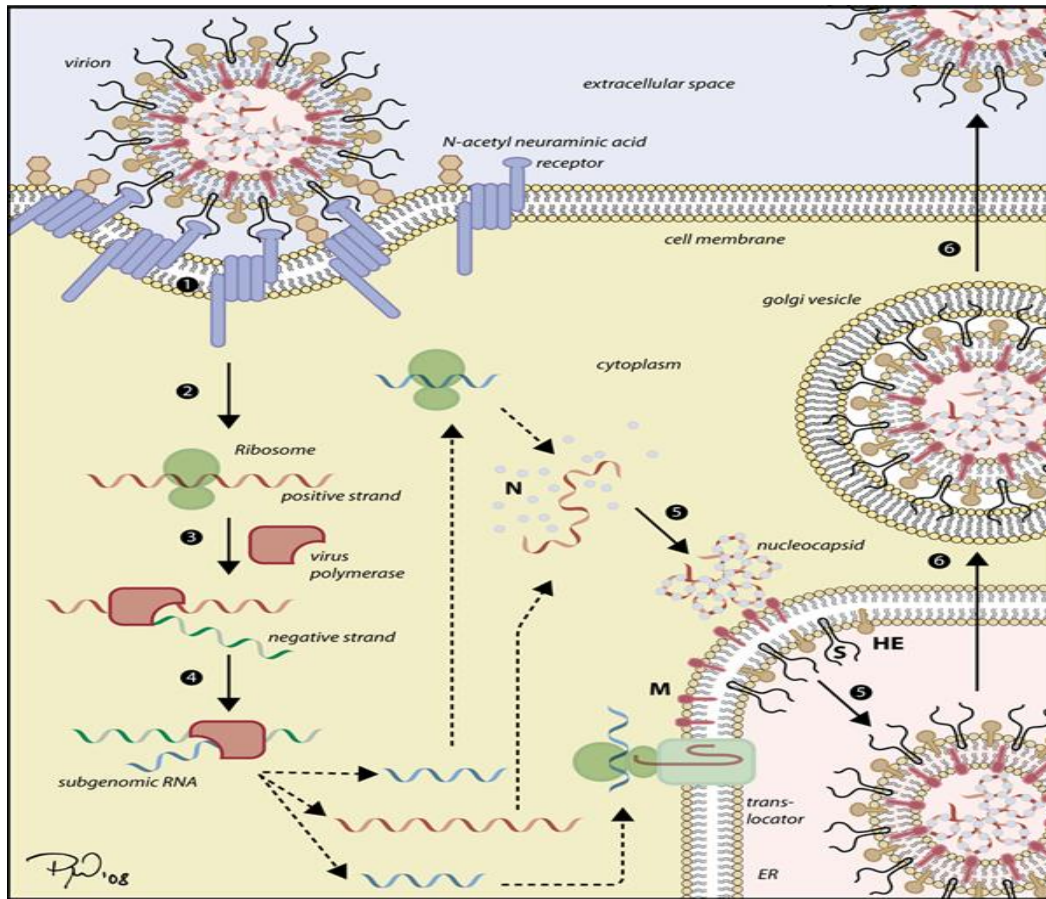


Figure 2.2: Replication cycle of coronavirus (Fehr and Perlman, 2015)

2.1.7 Ecology of coronavirus

The ecology of coronaviruses involves understanding their relationship with other mammals such as olive baboons, rodents, bats, livestock, and the environment (respiratory and gastrointestinal tracts) in which the virus thrive. Novel strains of coronavirus such as SARS-CoV and MERS-CoV are zoonotic in origin. They replicate in their reservoirs which are long term host at a stable rate (Vijaykrishna *et al.*, 2007). During the interspecies transmission, the virus can be triggered to replicate exponentially in the new host (Vijaykrishna *et al.*, 2007). In a phylogenetic and dating

study by Vijaykrishna and Smith (2007), it was confirmed that coronavirus undergo increase in population growth at constant rate in bats, but in a new host, the virus replicates in an epidemic-like fashion making researchers to establish that bats are natural host of coronaviruses (Vijaykrishna *et al.*, 2007).

The respiratory and gastrointestinal tracts are the physical environment where coronavirus replicate in their hosts making it suitable for a wide range of mild to mortal diseases (AGE) (Jevsnik *et al.*, 2016). In addition, report has it that coronaviruses were discovered in bats more in winter, moderate cases in spring and autumn but none discovered in summer. This finding indicates a correlation between changes in weather and the viral transmission (Jevsnik *et al.*, 2016).

2.1.8 Diseases cause by coronaviruses

Diseases of high impact on public and livestock health have been caused by coronaviruses (CoV), including porcine epidemic diarrhea virus (PEDV) and more recently, porcine delta-coronavirus (PDCoV) in pigs. Infectious bronchitis virus (IBV) and turkey coronavirus in poultry and Murine hepatitis virus (MHV) in rodents have been described (Marten *et al.*, 2001; Ignjatovic *et al.*, 2002; Cavanagh, 2005; Ma *et al.*, 2015; Lee, 2015). For example, porcine epidemic diarrhea occurred in 2013 in the US resulting in mortality rate of about 100% in affected piglets. Approximately, 10% of America's pig population was affected in just a year (Mole, 2013). The disease then spread across Europe following the devastating effect in USA and Asia (Hanke *et al.*, 2017). Bovine coronavirus, canine respiratory coronavirus, and dromedary camel coronavirus likely originated in bats (Vijgen *et al.*, 2005; Nathalie *et al.*, 2016; Lu *et al.*, 2017) while PDCoV interestingly is likely to have originated in birds (Guan *et al.*, 2003; Lau *et al.*, 2005; Ma *et al.*, 2015;). Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) was reported to have infected over 8,000 people from different continents of the world regardless of color and race with a mortality rate of

about 10% (Ksiazek *et al.*, 2003; Peiris *et al.*, 2003; Marra *et al.*, 2003; Rota *et al.*, 2003). In 2012, another coronavirus outbreak occurred, the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Saudi-Arabia infecting more than 1,700 people with a mortality rate of about 36% (Zaki *et al.*, 2012; de Groot *et al.*, 2013). Widespread respiratory, gastrointestinal and central nervous system diseases have also been recorded in humans and animals in general. Human health, transportation and trade in animal products have been loss (Enjuanes *et al.*, 2006; Perlman and Netland, 2009). Through mutation, recombination and spillover, coronaviruses have shown capability of adapting to new ecological areas with ease and hence, can alter their host range and tissue tropism (Graham and Baric, 2010). Coronaviruses therefore have posed constant health threats with long-term implication. It is therefore very vital to investigate the zoonotic sources of coronaviruses and to understand their transmission dynamics at the wildlife-human-livestock interfaces as this have important implications for global health and economic stability. The global distribution of cases of SARS and number of deaths from each country is shown in (Figure 2.3). Consequently, there is significant interest in assessing olive baboons, bats and rodents for coronaviruses considering their role in disease dissemination at the human-wildlife-livestock interfaces.

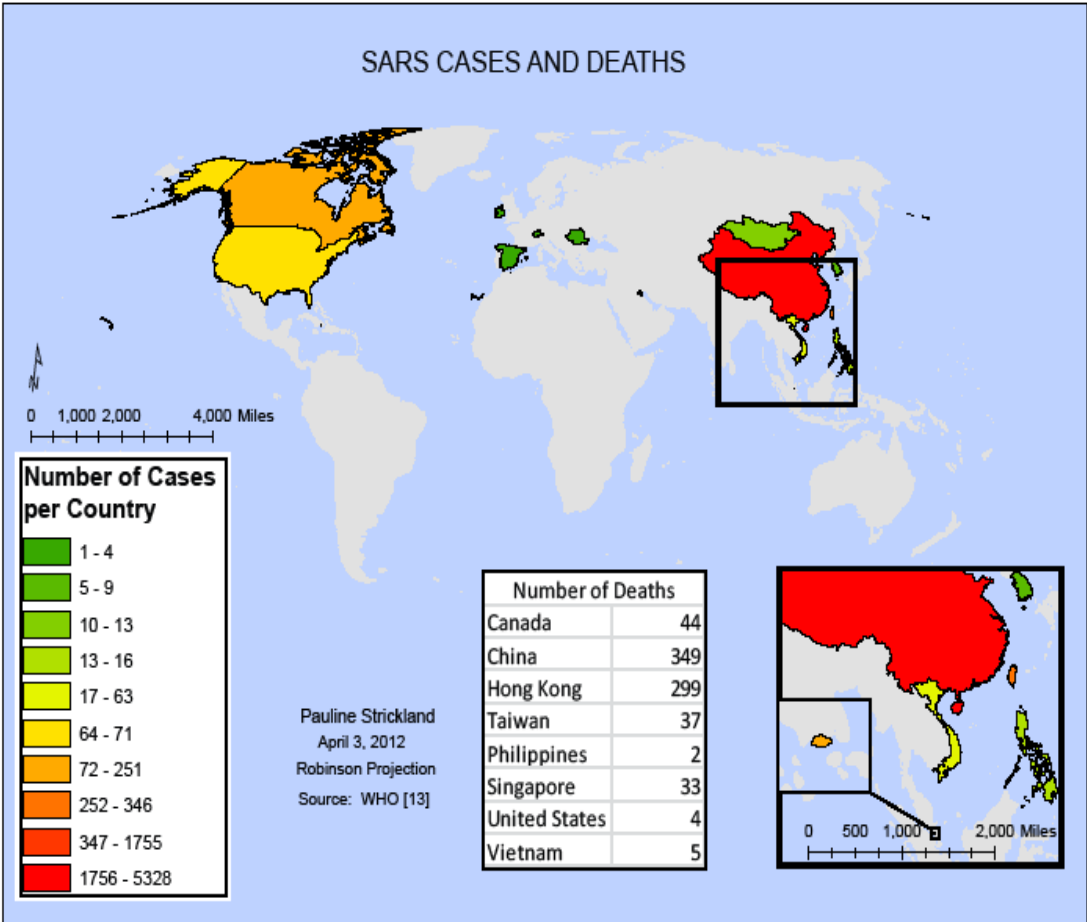


Figure 2.3: Global distribution of cases of SARS and number of deaths. Source: WHO (Jiang *et al.*, 2013).

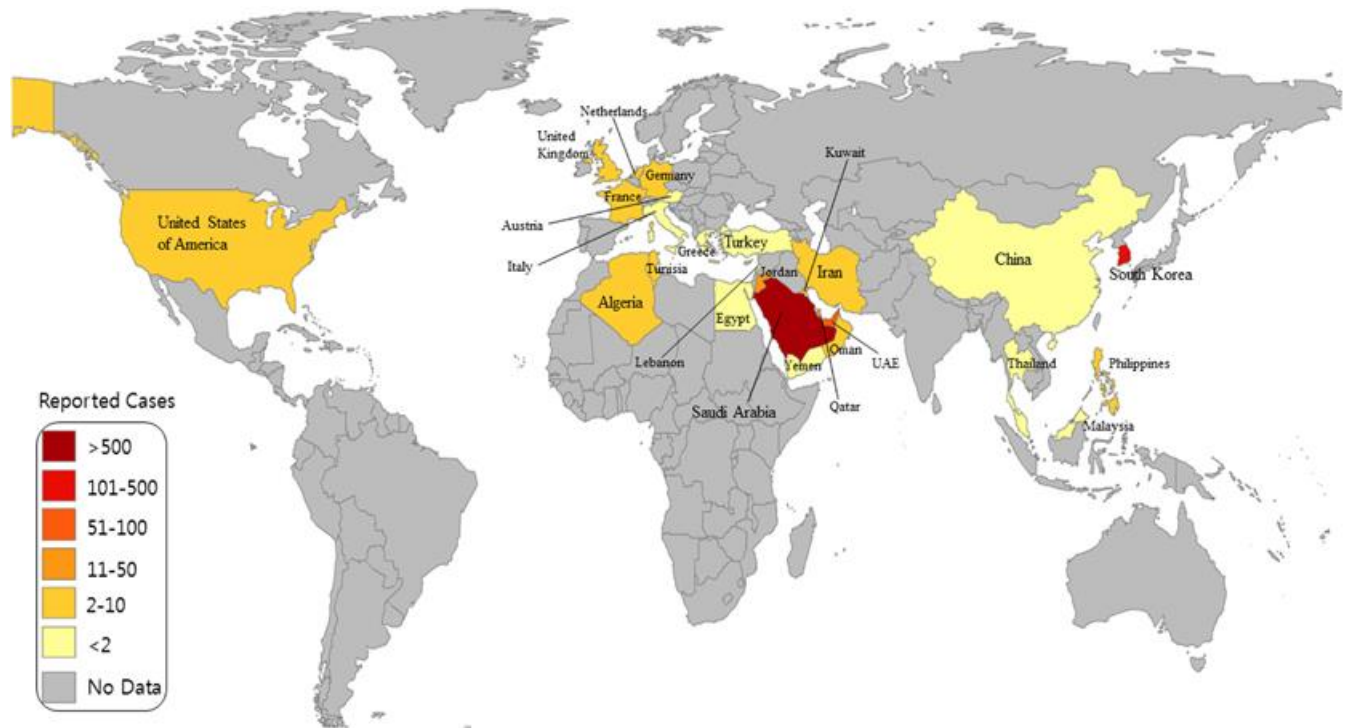


Figure 2. 4: The graphic information was based on MERS-CoV updates of 7 July 2015 by World Health Organization. (WHO, 2015).

2.1.9 Coronaviruses in Kenya.

In Laikipia County, camels have shown a sero-prevalence of 46.9% to MERS-CoV antibodies (Deem *et al.*, 2015) and there are now confirmed coronaviruses circulating in humans in Kenya playing important role in respiratory illness among children (Sipulwa *et al.*, 2016). Samples from humans in Tana River County have tested positive to MERS-CoV antibodies and both victims were livestock farmers though no clinical symptoms were observed (Liljander *et al.*, 2016). Alpha- and beta-coronaviruses have been described in bats in parts of Kenya (Waruhiu *et al.*, 2017) and also, relatives of HCoV (NL63 and 229E) and recombination history have been described. This has given new strong insight into CoV evolution and evidence that HCoV-NL63 has a zoonotic recombinant origin (Tao *et al.*, 2017). Coronavirus groups 1 and 2 have also been identified in Kenya including SARS-like CoVs (Tong *et al.*, 2009). This confirmed that bats are principal

reservoirs and also likely sources of coronaviruses for many species, including humans and Kenya has been described as “hotspot” for emergence and re-emergence of zoonotic diseases. Possible transmission pattern from wildlife to humans and livestock has been described in (Figure 2.5)

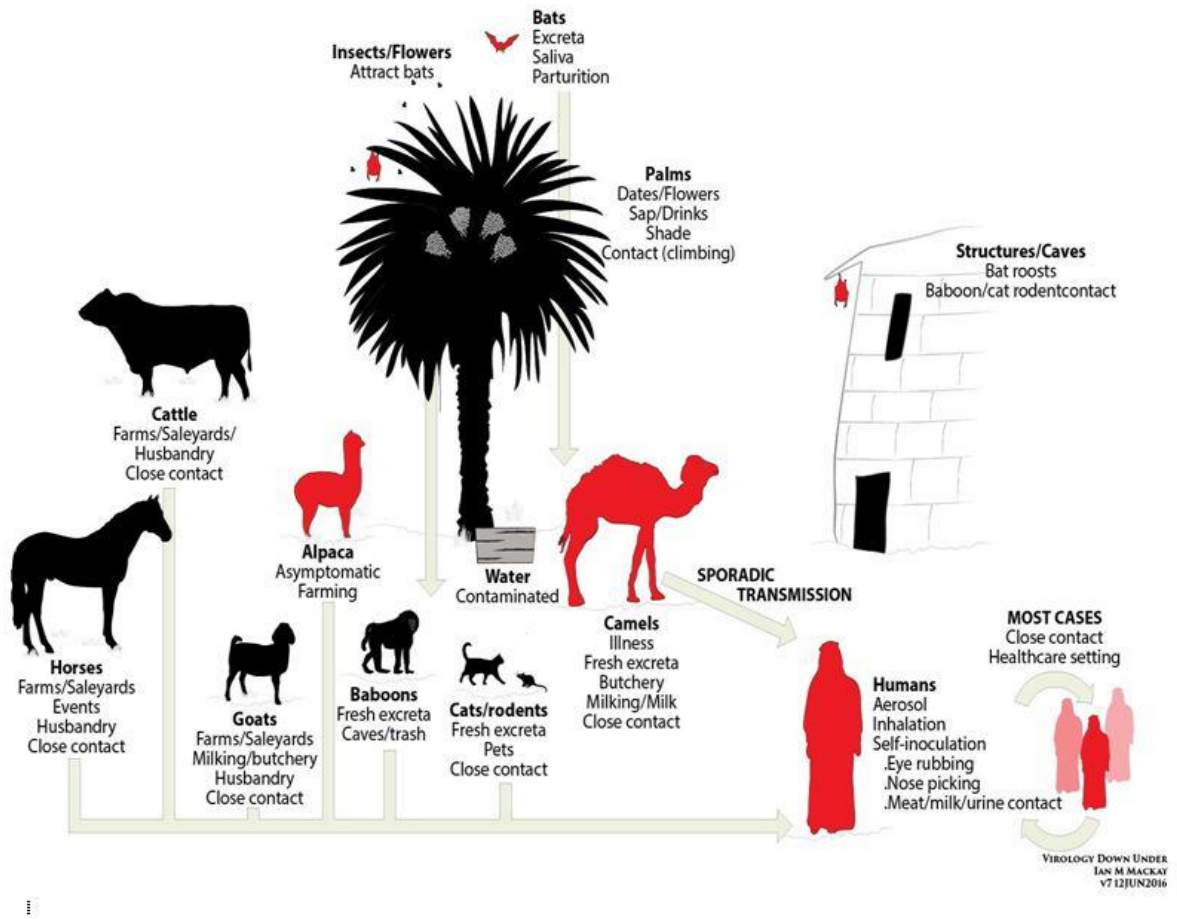


Figure 2.5: Model of transmission pattern of coronaviruses at the human-wildlife-livestock interface (Mackay, 2015).

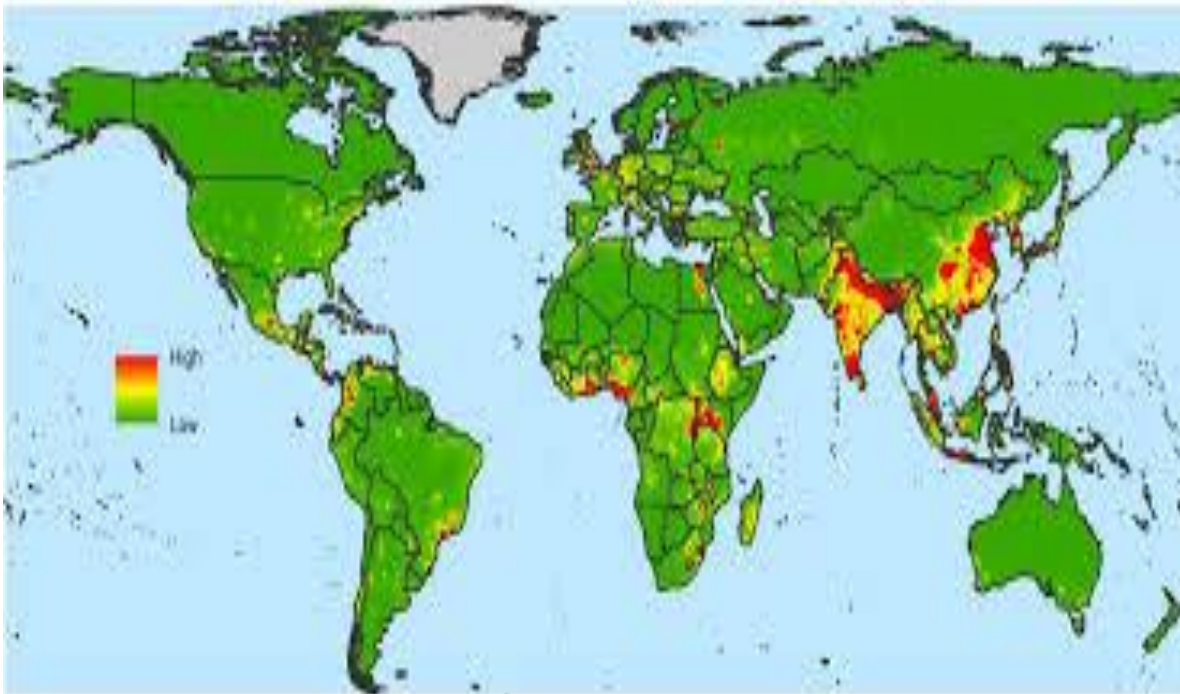


Figure 2.6: Predicted heat map showing relative risk of zoonotic EID events. Red is the highest, yellow mid-level risk, green indicates lowest risk. Kenya falls among regions of high risk EID events (Jones *et al.*, 2008).

2.2 Molecular Diagnosis of coronaviruses

Different techniques have been applied for viral detection and identification. Electron microscopy and cell culture techniques are examples. Notwithstanding, for viruses in small amount or those that cannot be cultivated, only molecular methods are required to amplify more copies for use in RT-PCR (Bexfield and Kellam, 2011). Also, molecular method offers the likelihood of identifying divergent viruses. Due to the capacity of these agents to easily mutate and cross from animals to humans, there is need for coronavirus identification and characterization.

2.3 PCR-based methods

The PCR methods can be used to detect and characterize viruses. Amplicons are obtained by using primers specific for the group of viruses under study. Consequently, the conserved sequences for the group of viruses and previous knowledge of those sequences are compulsory requirements (Bexfield and Kellam, 2011). Molecular-based techniques therefore are highly sensitive and allow a wide range of applications. The specificity of the primers used in the PCR can be modulated to allow further classification of the group of viruses; that is coronavirus serotyping (genotyping). Quantitative PCR (qPCR) is another modality of PCR, which allows the quantification (and detection) of virus particles in the sample.

2.4 Limitation of RT-PCR method

PCR-based methods have limitations besides the detection of previously known sequences. For instance, chemical inhibitors may be present in samples and may hinder the performance of the PCR (Costantini *et al.*, 2010). In addition, there could be mutations in the primer site which might compromise the efficiency of detection; this problem is common in viruses which have high mutation rate. Though the multiple steps required by the RT-PCR method (nucleic acid extraction, barcoding, amplification, electrophoresis, gel purification, cloning, and sequencing) is cumbersome, its use in surveillance has been advocated (WHO, 2018; Kelly-Cirino, 2019).

2.5 Sample populations

2.5.1 Olive Baboons (*Papio anubis*)

Anubis baboons have been considered to have the largest range of all baboons. They are widely spread in over 25 African countries. These animals adapt easily to any habitat and are found in savannah areas and plain grassland to even evergreen tropical rain forests. Anubis baboons live in

families called troops, of between 20 and 50 members per troop though can sometimes exceed 100 baboons. The troop size is usually determined by environmental conditions and availability of sustaining resources such as water and food. These animals have high genetic similarities with humans and share common diseases. Morphologically, they have multiple colored hair coats ranging from greenish-grey rings and yellowish brown (Rowe, 1996; Groves, 2001). The young ones are born with black natal coat, which change to the adult coloration as they age. All Male adults have characteristic mane on their heads spreading down to the shoulders and terminating at the back. (Groves, 2001). The ischial callosities, ears and faces appear dark grey to black. Unlike other primates that have flat faces, olive baboons have long and pointed muzzles. Their long tails, which are about 38-58cm are held up and have a broken appearance (Groves, 2001). They have specialized cheek pouches, which are sacs used for temporal food storage. Olive baboons exhibit sexual dimorphism, with the males twice larger than the females. The male weighs about 24 kg on average while the female is about 14.7 kg. They are highly flexible, adaptable, and opportunistic animals. Their habitat range is very wide and they have variable feeding habits. Like other animals, anubis baboons are affected by many ecological factors, though they are more adaptable and colonized a wide variety of niches across Africa. Due to their ecological flexibility, they do quite well when living alongside humans and consume a wide variety of foods.

Olive baboons are diurnal, omnivorous, and semi-terrestrial monkeys found in bush-lands, woodlands and grasslands of Laikipia (Palombit *et al.*, 2013). They eat a variety of food items including leaves, flowers, seeds, pods, fruits, gum, grasses, herbs, and often small animals within their reach (Barton *et al.*, 1992). The abundance of water, food and secure sleeping sites and refuge explains why troops of olive baboons are in abundance in Laikipia.

2.5.2 Bats species and ecology

2.5.2.1 *Chaerephon pumilus*

This species are small in size and belong to the family Molossidae and order Chiroptera. The chaerephon bats are also called little free-tailed bats and are the smallest species in the genus *Chaerephon*. The body length is between 54 to 102 mm (Kingdon, 1974; Smithers, 1983) with the ventral fur lighter in color than the dorsal part. The fur at the dorsal part is short and blackish-brown (Bouchard, 1998) but at the ventral surface where the wings connect to the flank, the hairs observed are pale or white. The species from north-eastern Africa have white wing while those from southern Africa have dark wing (Kingdon, 1974; Smithers, 1983; Happold, 1987; Jacobs *et al.*, 2004). *Chaerephon* species have large round ears, which are larger than the size of their heads (Bouchard, 1998). They have asymmetrically bi-lobed tragus and covered by large antitragus (Smithers, 1983). A forehead tuft is found in the males making their outline recognizable during flight (Bouchard, 1998). There are physical changes in the wing and pelage colors observed in this species (Goodman and Ratrimomanarivo, 2007).

2.5.2.2 Habitats and ecology

Chaerephon bats are found across different habitats from semi-arid savannah to forested areas (Happold, 1987). They are found in Zimbabwe within dry mopane woodland habitats (Skinner and Chimimba, 2005), under roofs, corrugated roofs or other crevices in buildings provide suitable roosting sites for little free-tailed bats within built-up areas though, natural roosting sites include cracks and crevices in rocks and trees (Skinner and Chimimba, 2005; Mickleburgh *et al.*, 2008; Monadjem *et al.*, 2010).

2.5.2.3 Diet of Chaerephon bats

Chaerephon bats are insectivorous. They feed on a wide range of small insects. Appendix 2 shows the wide geographical range of this species of bats across Africa.

2.5.3 *Scotophilus dingamii*

2.5.3.1 Description

The *Scotophilus dingamii* is African yellow house bats. They are smaller in size compare to the giant yellow house bat but larger to the lesser Asiatic yellow house bat. One can weigh about 27 grams with an average body length of 130 mm. It has a dog-like facial look and with shades of olive, grey or red wings. *Scotophilus dingamii* have short fur with hint of brown covering the back while the abdomen has a bright yellow fur covering. A brown and transparent inter-femoral membrane color is very remarkable. *Scotophilus dingamii* is a medium-sized bat with a brown to dark-brown pelage above and bright-yellow or orange-yellow below. The pelage is short and sleek with individual hairs uni-colored. The wings are relatively long and dark-brown. The face is plain, without any nose-leafs and the ears are moderately sized, with characteristically shaped long, narrow tragus.

2.5.3.2 Habitat and Distribution

This bat is widely distributed in the in sub-Saharan Africa starting from Senegal, the Gambia, Ethiopia, South Africa, Lesotho, and Swaziland. Record of the population of *Scotophilus dingamii* is scarce (Monadjem and Griffin, 2016). They have a wide habitat range from woodland savannas, forest and mountains and can also live with humans both in rural and urban areas. *Scotophilus dingamii* has been found in dried leaves of palm trees, dark caves, tree trunks, old abandoned houses, cracks in houses and crevices and also between overlapping corrugated iron sheets. Their name was derived from the ease of adaptation to humane presence. In natural and man-made

constructions, *Scotophilus dingamii* has been found to tuck into dark narrow clefts. Yellow house bats can tolerate extreme weather conditions (Hutson *et al.*, 2008; Sinaga and Maryanto, 2008; Bates *et al.*, 2008; Monadjem and Griffin, 2016).

2.5.3.3 Diet of *Scotophilus dingamii*

The major diets of yellow bats are small insects though this depends on the environment where they live and the available food preys. Majorly, flying insects (hymenopterans and dipterans) are preferred and these insects are found around riparian forest at night's time and under the canopies of tall trees. They prey also on moths, beetles, bees, and wasps.

2.5.3.4 Behavior and ecology

Depending on space, yellow house bat can roost singly although they are grouping mammals. A large cave can hold between 12 and 30 groups of bats. To avoid predation, yellow bats often have more than one roosting sites in the forest. They particularly go out for food at sun set and feed for more than 2 hours to get before resting at day time (Kruger National Park, 2009).

2.5.3.5 Geographic distribution

The yellow house bats are found in most sub-Saharan countries as seen in Appendix 3.

2.6 Rodents

2.6.1 *Acomys kemp*

(*Acomys kemp*) is often called Kemp's spiny mouse and belongs to the family Muridae. They are largely found in Tanzania, Somalia and Kenya. Their natural habitats preferably are rocky areas and dry savanna. "Acomys" was derived from the Greek word 'acme' meaning pointy referring to the shape of the snout (Haughton *et al.*, 2016). 'Spiny mouse' is the common name and is used to describe the thick spine-like hairs along its dorsum which provides protection against predation. *Acomys kemp* have large protruding black eyes, scaly tails with scattered short hairs, and large

ears. Other species for example, *A. cahirinus* have black and white, golden brown peppered color, (*A. percivali* and *A. cineraceus*) have grayish to rusty orange color. They also have creamy white ventral coat. *Acomys* have very complex social structure (Porter *et al.*, 1983; Porter *et al.*, 1986) including communal breeding (Makin and Porter, 1984) and paternal care (Frynta *et al.*, 2011). Eighteen distinct species have been recognized by the International Union for the Conservation of Nature (IUCN) though the taxonomy within genus still remains controversial (Barome *et al.*, 2001).

2.6.2 Geographical distribution.

The African continent, Middle East and South-west Asia are home to the *Acomys* and have a wide range of habitat. A wide range of species are found in Kenya, Tanzania, Uganda, Sudan, Ethiopia, South Africa, and Egypt. There are also a number of species found in Jordan, Israel, and the Arabian Peninsula. *Acomys cahirinus* and *Acomys dimidiatus* group have particularly branched out to the Mediterranean islands of Cyprus as *Acomys nesiotes* and crete has *Acomys minous*. The genus has not yet been found in the wild in southern Europe. *Acomys* has been reported in Iran and Pakistan (Jeremy and Bates, 1994; Nowak and Walker, 1999; Mendelssonhn and Yom-Tov, 1999). They are found mainly in dry semi-arid, rocky desert and short grassland. In Kenya, they have been described as dwellers of the rocky canyons, rock formation called kopjes and the cliff bases. These animals can live commensally with man, inhabiting the crevices of human dwellings (Haughton *et al.*, 2016), palm groves, gardens and fruit orchards. They do not build nests, dig burrows but take refuge in whatever shelter the terrain offers.

They have been so named according to the location where they are found for example, Egyptian spiny mouse (*Acomys cahirinus*), Cretan spiny mouse (*A. minous*), Cyprus spiny mouse (*A. nesiotes*), Arabian spiny mouse (*A. dimidiatus*) or Turkish Spiny Mouse (*A. cilicicus*). Others were

named based on the color of their coats such as the grey spiny mouse (*A. cineraceus*) and the golden spiny mouse (*A. russatus*) while others go exclusively by their scientific names, as *A. kempi*, *A. percivali* and *A. wilsoni*. Appendix 4 shows the distribution of the *Acomys* rodents globally.

2.7 *Elephantulus rufescens*

These rodents belong to the family Macroscelididae and are commonly called rufous elephant shrew, rufous sengi or East African long-eared elephant-shrew. They are found in Kenya, Somalia, Ethiopia, Tanzania, and South Sudan inhabiting the tropical or sub-tropical, savanna dry shrub land (Rathbun, 2015). *Elephantulus rufescens* have long and very soft proboscis, dark-brown tail with length reaching the head. They do not exhibit sexual dimorphism and adults and young ones have similar coat's color. The fur at the dorsal part of the body has fine texture and colored brown, reddish-brown or buff whereas the fur at the ventral body part is white. The colors of the soil in which *E. rufescens* live contribute or influence the coloration of the dorsal fur (Koontz and Roeper, 1983). However, Juveniles have brown feet while adults have white feet. They have large eyes surrounded by white rings and dark patch extending to the rear of the animal. The large ears have no fur on them and both male and female have sternal gland which is indicated by short fringed white hairs. The males are cryptochids while females have three pair of teats. Appendix 4 shows the map of the distribution of *E. rufescens*.

2.7.1 Ecology, diet, and behavior

Elephantulus rufescens are typically very active at day time and peaks at dusk and dawn while resting at midday. Trails are built beneath leaf litter by mating male and female which provide protection as this animal does not burrow, build or use shelters. Several spots for resting are found

throughout the trails and serve also as scent-marking and sunbathing areas (Rathbun and Redford, 1981). These trails are constructed and maintained by pushing aside leaf litter and loose debris as the forefoot is moved laterally (Koontz and Roeper, 1983). Cleaning foraging trails is done by the male where all activities are performed. The trails are also used as escaped routes from predators. Insect form the major food resource of their diet in the dry season, but during the rainy season, seeds are largely consumed (Neal, 1984). To allow for quick escape from predators *E. rufescens* keep their feet under their body during resting periods along the trails. They take flight with the slightest noise heard (Koontz and Roeper, 1983). Monogamy is a common practice among this species though the monogamous pair do not spend most of their time together. Usually the female dominates the male in their society (Lumpkin and Fred, 1986).

2.8 Sanger Sequencing

If characterizing a new species is the objective, sequencing is applied after PCR methods to identify the amplified products. In some cases, primers can be used to amplify conserved regions of the virus genome. Those fragments are then sequenced (Sanger sequencing) and assembled to reveal the identity of the virus. Sanger sequencing can also be applied in genotyping after the amplification of a region that has the purpose for that, either structural or non-structural regions of the gene of interest for example, RdRp gene.

Identification of viruses using sequencing methods has some constraints. The input RNA for sequencing must exist in a considerable amount otherwise, a cloning step to enrich for fragments might be necessary before sequencing (Bexfield and Kellam, 2011).

The first step in Sanger sequencing requires the application of RT-PCR which involves the sample being subjected to nucleic acid extraction of the viral RNA particles. The capsid of the virus is disrupted by chemical means and the RNA genome is released. RNA, corresponding to the whole

genome or a region of it, is later converted to cDNA by Reverse Transcription. A PCR reaction creates multiple copies of the cDNA that can be detected and purified in a gel electrophoresis. Purified DNA is sequenced by chain-termination method (Sanger sequencing). Reads are then computationally aligned and compared to available references in order to reveal the genotype of the virus (BLAST). In this case, similarities between the references and the sequences obtained from the study are a crucial step. Figure 2.7 shows the flow chart of the laboratory analysis.

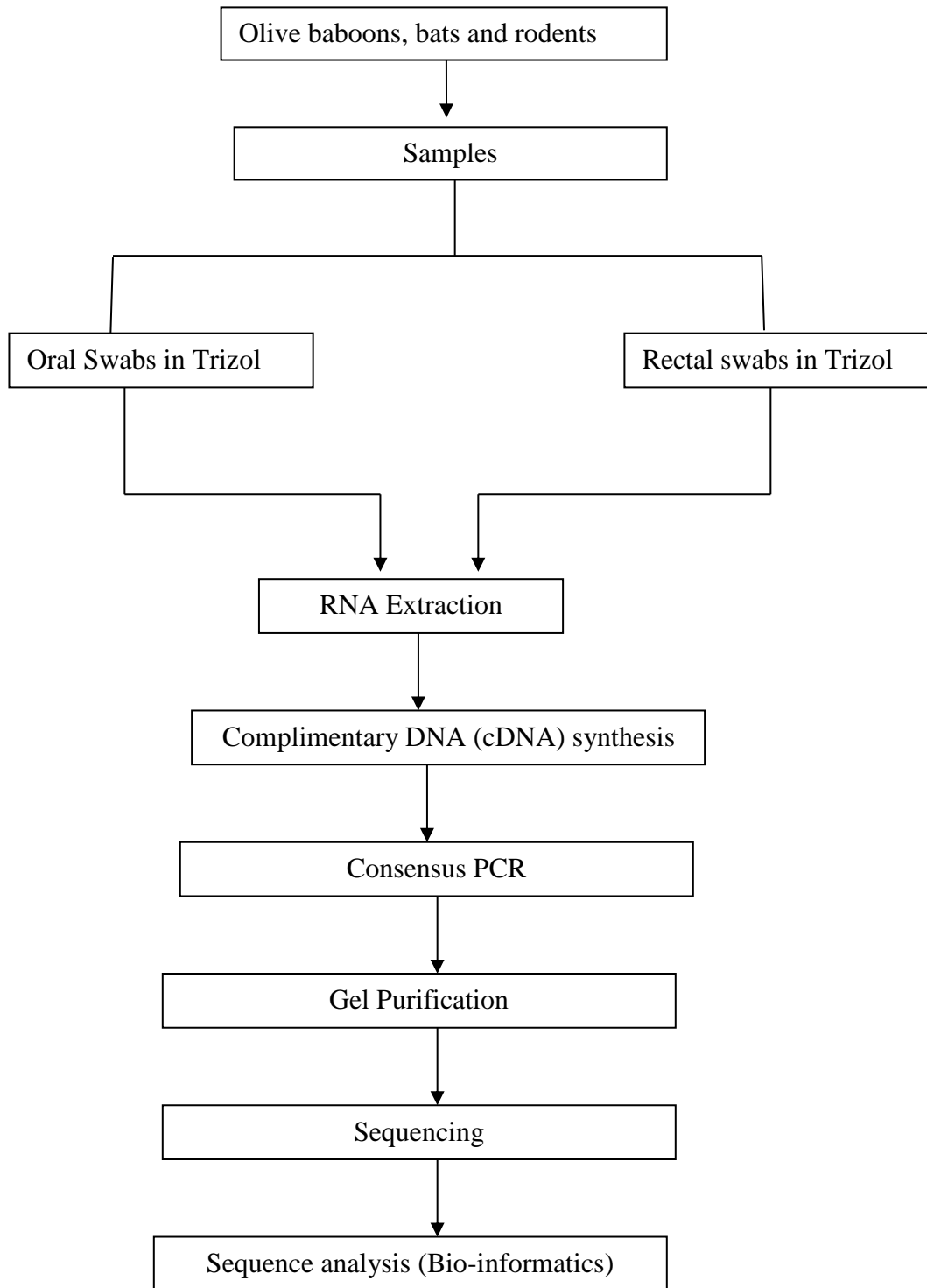


Figure 2. 7: Flow chart of sample collection to laboratory analysis

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was carried out in Laikipia County located in Central Kenya in the Rift Valley (Figure 3.1) at the foot of Mount Kenya (Co-ordinates 0⁰5'N 36⁰40'E). It has a total land area of about 9,700 km². Laikipia County experiences an annual mean rainfall of about 400mm in north and 1200mm in south-west and also a mean annual temperature of about 26°C on low ground and 16°C on high altitude. It has a rich vegetation type made up of *Acacia commiphora* bush-land and thicket, afro-montane and semi-desert grassland and shrub-land conducive for olive baboons and rodents to thrive. The ecotones and mosaics vegetation types accounts for the rich biological diversity of Laikipia. A growing human population of about 400,000 has been recorded with 76% living in rural areas and interacts closely with rodents and olive baboons. By 2030, it is expected that an increase of about 600,000 will be recorded. Recently, the human population density has increased to about 42 people per km² to 300 people per km². Approximately, 90% of Laikipia is unfavourable for rain-fed crop cultivation due to persistent dryness. Only 2% has high potential for agriculture, 65% (5820 km²) as wildlife habitat, and 38% (3650km²) natural habitat.



Figure 3. 1: Map of Kenya showing Laikipia County.

The people of Laikipia County are primarily livestock farmers. Statistically, 80% live on livestock farming. Large-scale ranching constitutes about 37% of the land use, 32% by pastoralists, 21% by small-holder farmers, and 5% exclusively for wildlife-based tourism. Apart from Maasai Mara National Reserve, Laikipia maintains very high population densities of large wild mammals. It has about 21 species of carnivores, 8 species of primates and 28 species of ungulates among others. The number of bats species is very high, and they live around and in human dwellings, food stores, clinics and restaurants.

3.2 Study Design and Sampling frequency.

Olive baboons, rodents and bats were purposively sampled through a cross sectional study. The purposive selection technique was deliberately used to select conservancies for reason of being "typical" of the population of the animals and the presence of human-wildlife-livestock interaction. Sampling was seasonally based, one in dry season and the second in the rainy season after locating the animals' activity sites. The seasonal sampling was to enable understanding of possible effect of season on the viral shedding and transmission dynamics within and between species.

3.3 Sample size

Due to logistical considerations, 130 olive baboons, 202 bats and 161 rodents were humanely trapped and sampled. The sampling was carried out in two seasons; dry of September, 2017 and wet season of May, 2018.

3.4 Capture and sampling techniques

The samples were collected at very high-risk interfaces where direct or indirect contact by these animals with humans might promote spillover of viral zoonoses (Anthony *et al.*, 2017). The selected sampling areas included human residential areas, clinics, water points, research center, occupational exposure areas, and ecotourism locations. All animals sampled were identified morphologically and classified as Adult, sub-adult and juvenile by a team of biologists and veterinarians.

3.5 Field trapping of olive baboons (*Papio anubis*) and anesthesia

The first step was to locate where the olive baboons were based in the conservancy and a complete study of their activities carried out to give a picture of the troops feeding, drinking, resting, and

sleeping sites. With full understanding of the troops, a baiting and trapping site was selected. The baiting was done using shelled corn and green corn.

Large collapsible cages of 1.9m high with a base of 0.9m square base and a weighted door were used. Olive baboons were habituated to the traps through regular feeding for three days at the selected site with fresh shelled and green corn. They were allowed to feed freely around and inside the cages with doors left opened. When the baboons were used to the feeding regime, the traps were then set. Many traps were erected than there were animals in the troop to optimize the operation's success. The traps were set in the evening while the baboons were at their sleeping site, to prevent premature entrance into the cages and affecting the operation and also to prevent them from observing the setting process. Trapping procedures were carried out based on the Institute of Primate Research-Standard Operating Procedures (IPR, 2009). When setting the trap, a cob of corn was tied to the extreme upper part of the cage using a thin thread while the other end of the thread was attached to the door via the back and top of the cage. When the animal entered the cage and took the cob, the thread broke and the door dropped trapping the animal. They were then anesthetized by intramuscular injection of a combination of 3.5mg/kg ketamine (Kyron Laboratories, Johannesburg) and 0.035mg/kg medetomidine (Domitor; Pfizer, Berlin, Germany). The anesthetic combination was administered by hand injection using 19G needle and anesthetized animals were then wheeled to a sampling tent erected about 100m from the trapping site. Adequate monitoring of vital parameters was carried out to prevent complications of hyper- and or hypothermia while they were weighed, aged, sexed, and reproductive stage collected. Oral and rectal swabs were collected and atipamezole (Dexdomitor, Pfizer, Germany) was administered intra-muscularly at 0.0175mg/kg to revive each animal at a saved shady location away from possible predators and con-specifics. Appendix 6 shows the stages of habituation and trapping.

3.6 Restraint and handling.

Olive baboons can cause severe bite wounds regardless of their size to handlers. They can grasp with both hands and feet therefore heavy-duty leather gloves were worn to protect against such bites. Hand restraint was avoided as a primary means of restraint. Strictly, chemical restraint was employed but, in a situation where hand restraint was used, it was done humanely to avoid stressing the animal especially infants or severely weak individuals. Baboons are social animals and very protective of each other. Care was taken during capture process to protect personnel against defensive attacks from adult males that were not trapped. Experienced personnel were fully employed to watch for aggressive approaches from defensive baboons.

3.7 Anesthetic drugs and other chemicals

The capture exercise placed personnel at risk of injury and therefore immobilization was done under the supervision of a well-trained veterinarian during physical and chemical restraint of wild animals. Precautions were taken to minimize the probability of human injury from animal attacks, capture equipment and anesthetic drugs. Reconstituting the anesthetic drugs was done by the team and gloves and glasses were worn and splash boxes used to reduce risk of accidental exposure when mixing the drugs in syringes. Also, squirting, spilling or spraying of the drugs was avoided as exposure to α -2 agonist (medetomidine) is life threatening and current post exposure management is nonspecific and unreliable. Induction time of all immobilized baboons was recorded and animals were taken to sampling tent some few meters away from trapping location.

3.8 Monitoring of Anesthesia

In the tent, immobilized animals were placed a long wooden table covered with a plastic covering and on lateral recumbency with the tongue allowed to fall to the side so that saliva can drain freely from the mouth. This also allowed the animal to breathe freely. The eyes were covered with a blindfold to prevent damage to the retina and cornea. Measures were also adequately taken to prevent hyper- or hypothermia through monitoring of anesthetic induction time, temperature (thermometer), respiratory rate, oxygen saturation (pulse-oximeter), mucus membrane color, capillary refill time (CRT), palpebral reflex, and depth of anesthesia. The anesthetic ratings were scored and recorded as, excellent, good, fair, or poor.

3.9 Reversal of Anesthesia

After all samples were taken, the baboons were conveyed in a truck to a shady place some distance from the sampling site and atipamezole administered intra-muscularly at 0.0175mg/kg to reverse the effects of medetomidine to enhance quick recovery from ketamine. Sampled animals were marked to avoid re-capture and sampling. They were monitored for recovery and released only when they fully recovered from anesthesia by observing them in safe areas, away from hazards and potential predators or aggressive con-specifics. Those animals that suffered injuries in the course of capture were treated appropriately before the release.

3.10 Bat Capture, Handling, and Sampling

Two bats sampling sites were identified at Mpala ranch hospital, community and Naibor clinic. Capture technique used was mist nets and hand capture.

3.10.1 Handling Procedures

All Bats trapped were carefully and humanely removed and placed into porous cotton bags (with draw-string mouth) and hung (Appendix 9) on a sturdy line in a cool dry place until sampling time. Prior to specimen collections (oral and rectal swabs), each bats was weighed (in grams) in bags using a Pesola hanging scale. The bag was weighed first and then bat and bag weighed together. The weight of bag was subtracted from total weight (bag + bat) to get the weight of bat. The bats were removed from the bag and sampled. Bags used after previous samplings were washed and disinfected. Bats were not held longer than six hours and before each bat was released, 100% fruit juice was given orally using syringe to boost energy.

3.10.2 Rodents capture, handling and sampling

Rodents were trapped using H.B.Shermans (Collapsible Traps, Carolina Biological Supply Company) traps, which were set in the morning and evening close to human settlements and at the Mpala research center base on the species of rodents at these locations. Traps were set open at sunset, checked at sunrise and often twice a day to avoid predation. All traps were placed in areas protected from direct sunshine and covered with vegetation to prevent consequent heat stress. Captured rodents were handled humanely to avoid excessive stress leading to hypothermia. Trapped rodents were removed from traps by placing a clear plastic handling bag over the trap. The rodent was then coaxed into the bag by gently rocking the trap with door opened. Before sampling, each rodent was weighed by using Pesola scale. The animal was first weighed plus the bag and after the animal was anesthetized and removed, the empty bag was reweighed and subtracted from the total weight obtained (Bag + rodent).

The anesthetized animal was held by pinching the skin between the thumb and fore-finger at the point where the rodent's spine meets the head. Therefore, rodent's body was positioned firmly

across the hand by extending the fore-finger and thumb back as far as possible while maintaining a firm grip on the scruff. The tail was placed between the fingers of the same hand. Care was adequately taken to avoid grasping too much skin to obstruct the airways. Care was also taken to monitor the breathing rate, color of the ears, nose, and oral cavity for stress and cyanosis.

3.10.3 Rodent Anesthesia

Rodents were each anesthetized with isoflurane (Isoflurane 250ml UK, 100% liquid inhalation vapor). Factors taken before anesthetizing each animal were age, sex, and reproductive stage, which can affect drug potency and in turn cause abrupt respiratory depression - especially in older individuals. Depending on the weight of animal, 0.4 ml of isoflurane was applied to a cotton ball and put in a plastic tube (nose cone; 0.4 ml is the appropriate dose for a 20g mouse, adjust the dose as needed for larger rodents) and rodent's nose was placed into the tube. The animal was monitored closely until it attained plane anesthesia.

When fully anesthetized, the rodent was removed from the plastic tube for processing. As a precautionary measure, an additional cotton ball soaked with 0.2 ml isoflurane was placed in a 50 ml tube and capped to avoid escape of the anesthetic gas. This was to be used to re-anesthetize the animal if it begins to wake up during sampling.

3.11 Animal Care and Safety during Capture

Olive baboons, Bats and Rodents can sustain injuries or develop pathologic conditions or suffer stress during capture that could put their lives in danger or decrease their chances of survival in the wild. Therefore PREDICT guidelines were followed to minimize injury or harm to these animals during the capture and handling exercises.

3.12 General Zoonoses and Biosafety Precautions

The risk of exposure to infectious material containing zoonotic pathogens is high when handling wildlife. Therefore hand washing was observed strictly to reduce the risk of pathogen transmission before and after the use of PPE. Absolute care was taken to avoid exposure to blood, saliva and feces which may contain pathogens including Herpes B, Ebola, Marburg, Influenza, Coronaviruses and tuberculosis (TB).

3.13 Use and disposal of Personal Protective Equipment (PPE)

All PREDICT guidelines were followed when wearing and removing components of PPE. At the end of the sampling exercise, all PPE were removed and kept in infectious waste bags and incinerated at the Institute for Primate Research.

3.14 Use and disposal of sharp objects.

All sharp objects such as needles, syringes and scalpel blades were carefully kept in sharp waste container and properly disposed at the Institute of Primate Research. No injuries associated with these objects were recorded during the period of sampling.

3.15 Sample collection

Oral and rectal samples were collected. In the sampling process, three people were assigned per sampling unit: one positioned the animal, the second collected the samples and the third managed the tubes (lid opening, collection of sample from the sample taker, screwing back the lid tightly, labeling, and keeping in liquid nitrogen).

3.15.1 Oral swabs

In collection of oral swabs, a mouth speculum was used to keep the mouth opened. Polyester-tipped swabs were used to gently rub the back of the animal's throat thoroughly until it was

saturated with saliva. The swabs were put in 500µl Trizol and excess plastic shaft was cut off using a scissors and the sample closed tightly. All samples were labeled and kept in liquid nitrogen and transported to the Institute of Primate Research stored at -80°C and analyzed later.

3.15.2 Rectal swabs

Before inserting the rectal swab into the rectum of the animal, the swab was dipped in viral transport medium (VTM) and used to lubricate the anus to decrease the risk of trauma during insertion. The swab was then gently inserted into the rectum and swabbed against the rectal mucosa. The swabs collected were placed into a cryovial with 500µl Trizol, labeled and stored in liquid nitrogen.

3.16 Packaging and transportation of samples

This study was carried out in the phase II of the PREDICT/USAID approved grant for the surveillance of infectious zoonotic viruses in biodiversity rich countries. Both oral and rectal swabs were collected with sterile swabs, stored in trizol and preserved in liquid nitrogen in the field before transporting to – 80°C at the Institute of Primate Research in Nairobi.

3.17 RNA extraction

A total of 260 oral and rectal specimens obtained from olive baboons, 322 from rodents and 404 from bats were extracted to obtain the RNA. During the extraction process, samples were thawed on ice and RNA isolation was carried out from the oral and rectal swabs preserved in 500µl Trizol using Zymo Direct-zol (www.zymoresearch.com) following the manufacturer's protocol. The isolated viral RNA was eluted in 50ul of elution buffer and 8ul of it was used as template for RT-PCR (cDNA synthesis). Reverse-transcription was performed using Superscript III kit (Invitrogen, San Diego, CA, USA) protocol and all cDNA were stored at – 20°C pending used.

3.18 cDNA analysis

The products obtained from RT-PCR were analyzed on 1.5% (w/v) of agarose (Sigma, USA) gel in 1 x TAE (40mM Trisbase, 40 mM acetic acid, 1mM EDTA) buffer. A 1.5% TAE/ agarose was prepared by heating 1.5g agarose in a 100ml of 1 x TAE buffer to boiling. The hot agarose solution was then cooled to about 50 or 60°C and 5µl of EtBr added and swirled to mix. The solution was then poured into a gel casting chamber with appropriate combs placed in position. The polymerized gels were then transferred into electrophoresis tanks covered with 1 X TAE buffer (running buffer). A 6 x blue DNA loading dye (Fermentas, USA) was premixed with 10µl of the samples and loaded into each well in the gel. After loading the samples, the positive control and a no-template-control (NTC) were then loaded and an empty well left between them and the samples (avoid contamination). A 1 kb DNA ladder (fermentas, USA) 3µl was loaded at both ends of the gel to give a 100bp molecular marker. The samples were electrophoresed at 100V for 2 hours and bands were visualized under a UV transilluminator (Herolab E.A.S.Y 442K, Germany).

3.19 RNA quality check (barcoding)

1µl of the synthesized cDNA was used as template in a 25µl reaction to check for the RNA quality (Townzen *et al.*, 2008). The reaction mixture contained Invitrogen Platinum Taq kit, 2.5µl 10x PCR buffer, 0.75µl MgCl₂ (50mM), 0.5µl dNTP (10mM), 0.1µl Platinum Taq DNA polymerase, 18.15µl Molecular grade water, 1µl of Forward and Reverse primers at 10µM, and 1µl of the template. This was run at the PCR reaction conditions of: 94°C for 2min, a 50 cycles of 94°C for 30 sec (denaturation), 52°C for 50 sec (annealing), and 72°C for 60 sec (elongation). 72°C for 7 min (Final elongation) and 10°C for cooling. The primer sequences used were: CytB_F: 5' - GAGGMCAAATATCATTCTGAGG -3' and

CytB_R: 5' - TAGGGCVAGGACTCCTCCTAGT -3'. Mitochondrial cytochrome b was target gene with a molecular size of approximately 457 bp. The product was visualized using 10µl of the PCR product on a 1.5% agarose to check for the RNA quality.

3.20 PCR and DNA sequencing

To target the non-overlapping fragments of the orfab, two broadly reactive consensus PCR assays (Anthony *et al.*, 2017) were used to detect both none and unknown coronaviruses (Quan *et al.*, 2010; Watanabe *et al.*, 2010). The Watanabe and Quan assay approach were used to increase the chances of viral discovery in resource limited countries that are rich in biodiversity and “hotspots” for possible emerging epidemics. Positive amplicons were cut and gel purified. Products of the purified samples were subjected to secondary amplification of the PCR reaction to confirm the presence of the amplicons eluted for sequencing. All confirmed amplicons were sent to MacroGen (The Netherlands) and sequenced (traditional Sanger dideoxy sequencing) according to standard protocols.

3.20.1 ‘Watanabe assay’ RT-PCR for Coronavirus

This assay amplifies roughly 434 bp fragment of the RNA-dependent RNA polymerase (RdRp) corresponding to nucleotides 14,370-14,750 in the human coronavirus genome (*Strain229E*). This assay was modified from the original publication (Watanabe *et al.*, 2010) as primer sequences to increase the ability of the assay to detect widely variant coronaviruses. A second hemi-nested step was also carried to increase sensitivity. The 434 bp fragment of the RNA-dependent RNA polymerase gene was amplified using the following set of conserved primers: Primers for primary amplification were:

CoV-FWD3: 5' - GGTGGGAYTAYCCHAARTGTGA -3' and CoV-RVS3:
5'-CCATCATCASWYRAATCATCATA-3'.

Primers used for secondary amplification were:

CoV-FWD4: 5' - GAYTAYCCHAARTGTGAYAGAGC -3' and CoV-RVS3: 5' -
CCATCATCASWYRAATCATCATA -3'.

A 25µl reaction mixture of cDNA, PCR buffer (10µM), 50µM of each dNTPs and 0.1µl Taq polymerase (Boehringer Mannheim, Germany), 18.15µl molecular grade water, 1µl template was obtained. Universal Control 1 (DNA version) at a concentration of 10pg/µl was used as positive control in the reaction. The mixtures was amplified in 94°C for 2 min pre-heating, 35 cycles of 94°C for 20 seconds denature, 50°C for 30 seconds annealing, and 72°C for 30 seconds elongation, 72°C for 5 min final elongation, and 10°C for cooling in an automated thermal cycler. The primary amplification targeted RNA-dependent RNA polymerase (RdRp) gene with a molecular base pair (bp) of 440. In the secondary reaction, a 1:100 dilution of the primary universal control was used. Secondary PCR products were also visualized using 1.5% agarose gel. The lower base pair band of 434 was considered as the correct size in a positive reaction.

3.20.2 'Quan assay' RT-PCR for coronavirus

Quan assay amplified 328 bp fragments of a different peptide downstream of the RdRp, corresponding to the nucleotides 17,480-17,820 on the human coronavirus genome (*Strain 229E*) (Quan *et al.*, 2010). In the primary reaction, the following primers were used:

CoV-FWD1: 5' -CGTTGGIACWAAAYBTVCWYTICARBTRGG -3' and CoV-RVS1:
5'-GGTCATKATAGCRTCAVMASWWGCNACATG -3'.

For secondary reaction, the following primers were used: CoV-FWD2:

5' -GGCWCCWCCHGGNGRCAATT -3' and, CoV-RVS2:

5' -GGWAWCCCCAYTG YTGWAYRTC -3'. Both primary and secondary PCR reaction were carried out in a 25µl reaction with 2.5µl of 10x PCR buffer, 0.75µl MgCl₂ (50nM), 0.5µl dNTP (10nM), 0.1µl Platinum Taq DNA polymerase, 18.15µl molecular grade water, 1µl forward and reverse primers at 10µm and 1µl of the template. The primary reaction condition were 95°C for 5 min (pre-heating), a 15 cycles of 95°C for 30 sec (denaturation), 65°C for 30 sec (annealing), 72°C for 45 sec (elongation). Another 40 cycles of 94°C for 30 sec (denaturation), 50°C for 30 sec (annealing), and 72°C for 45 sec (elongation), 72°C for 5 min (final elongation) and 10°C for cooling. PCR products were visualized on a 1.5% agarose gel and approximately 520bp was considered as positive using universal control 1 (DNA version). The same protocol was also used for the secondary reaction except for 35 cycles instead of 40 and 328bp amplicons were considered positives when visualized on a 1.5% agarose gel.

3.21 Contamination check of the positive amplicons

All positive PCR products obtained were checked against possible contamination using the universal control 1 contamination check PCR. The amplified product targeted a PREDICT tag of nucleotides that corresponded to the amino acid sequence P-R-E-D-I-C-T after sequencing and translation.

The primer sequence used were, PREDICT-Fwd: 5' -GGGCCTAGAGAAGATATTTGTA CT-3 and REDICT-Rvs: 5' - CGCCATTGACATCCTCGSSG -3'. The Platinum Tag Kit (cat#: 10966-026) protocol was used for the PCR at the thermocycler condition of 94°C for 2 min. 40 cycles of 94°C for 30 sec denature, 55°C for 30 sec annealing, 72°C for 1 min elongation, 72°C for 7 min final elongation, and 10°C for cooling. This amplified a 412 bp fragment of the universal 1 plasmid

which was the target gene. The result was visualized by running a 10ul of the PCR product on a 1.5% agarose gel and then viewed in a Vilber Lourmat Vilber Lourmat Transilluminator (Z654469)

3.22 Gel Purification of positive amplicons

Gel purification of positive fragments were done by cutting out the bands of interest from the gel and purified by using Thermo Scientific GeneJet Gel Extraction Kit (USA) following the manufacturers guide. The PCR of the purified product was run to confirm the availability of the products in the elution buffer before sending to Macrogen (The Netherlands) for sequencing.

3.23 Sequencing of positive amplicons

Eight PCR amplicons of the samples amplified using primers targeting 328 bp fragments of the RNA dependent RNA polymerase gene were sequenced with both the forward and reverse primers. The Sanger sequencing was performed using Sequencing Kit (BigDye® Terminator v3.1 Cycle Sequencing Kits; Applied Biosystems) and Sequencer (ABI 3730xl DNA Analyzer; 96 capillary type), PCR machine (Eppendorf Master Cycler pro 384). The manufacturer's sequencing protocol was followed: The reactions of the sequences were performed in the Master Cycler pro 384 (Eppendorf) using the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using universal primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with the BigDye XTerminator® Purification Kit (Applied Biosystems). The samples were injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems). The amplicons were sequenced and analyzed for Basic Local Alignment Search Tool (BLAST) and compared with reference sequences of the pol-genes of coronaviruses in the GenBank database. The alignment was done using ClustW multiple alignment

method on BioEdit® software package version 7.2.5.0 (<http://www.mbio.ncsu.edu/BioEdit.html>). The construction of the phylogenetic tree was done by the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with bootstrap confidence based on 1000 repetitions. The tree was drawn to scale, with branch lengths measured in the number of substitution per site. Evolutionary analysis were conducted using MEGA version 6 (Tamura *et al.*, 2013) software.

Data analysis

Data entry for statistical analysis was done using the Microsoft excels 2013. All samples positive for coronaviruses in relation to RdRp genes detected were estimated. Molecular data were analyzed by bioinformatics tools using BLASTn, sequence alignments using CLUSTALW (<http://www.genome.jp/tools/clustalw/>), and phylogenetic analysis using MEGA version 6 software (Tamura *et al.*, 2013) employing Tamura-Nei (Tamura and Nei, 1993) model.

CHAPTER FOUR

4.0 RESULTS

All the 260 oral and rectal swabs obtained from olive baboons and 322 from rodents tested negative for coronaviruses. Eight positive samples were obtained from bats in the wet season samples of May 2018 constituting 8% (n=100) of samples from the wet season and 3.96% (n=404) of total bats sampled from both seasons. Of the eight positive samples, two were from rectal swabs (0.99%) and six from oral swabs (2.97%). Six out of the eight bats infected by coronavirus were from *Chaerephon pumilus* and two from *Scotophilus dingamii*. The summary of results was based on sex, age, lactating, gravid, and type of samples (Tables 4.1 and 4.2).

Table 4. 1: Summary of animal species screened for coronaviruses in Laikipia County, Kenya.

Animals	Species screened	Number	Pos	F	A	SA	Ju	L	G	OS	RS
1.Olive baboons	<i>Papio anubis</i>	130	0	67	86	16	27	0	3	130	130
2.Bats	<i>Chaerephon pumilus</i>	188	6	111	173	24	7	0	1	188	188
	<i>Scotophilus dingami</i>	14	2	8	10	1	2	0	0	14	14
3.Rodents	<i>Acomys kempfi</i>	30	0	10	30	0	0	0	2	30	30
	<i>Acomys percivalli</i>	29	0	9	27	1	1	0	0	29	29
	<i>Elephantulus rufescens</i>	6	0	0	4	0	2	0	0	6	6
	<i>Gerbilliscus robustus</i>	20	0	11	17	3	0	0	0	20	20
	<i>Aethomys hindei</i>	60	0	31	56	1	2	0	2	60	60
	<i>Myomyscus brodimani</i>	1	0	0	1	0	0	0	0	1	1
	<i>Grammomys dolichorus</i>	7	0	5	7	0	0	0	1	7	7
	<i>Saccostomus meamsi</i>	8	0	6	8	0	1	0	1	8	8

Table 4. 2: Summary of bats' results tested for coronaviruses in Laikipia County, Kenya

Animal	No. of	Pos	F	A	SA	Ju	L	G	OS	RS
Species	bats									
Chaerephon sp.	188	6	111	173	24	7	0	3	188	188
Scotophilus sp.	14	2	8	10	1	2	0	0	14	14

Abbreviations for tables 1 and 2 above: Sp= Species; Pos= Positive; F= Female; A= Adult; SA= Sub-adult; Ju= Juvenile; L= Lactating; G= Gravid; OS= Oral swab; RS= Rectal swab.

4.1 PCR detection of coronavirus

Coronavirus detection was done on oral and rectal swabs of bats by nested Quan PCR assay targeting the polymerase gene on the human coronavirus genome (*strain 229E*). This yielded specific bands of approximately 328 bp as shown in Figure 4.1 indicating the presence of the virus in the samples analyzed.

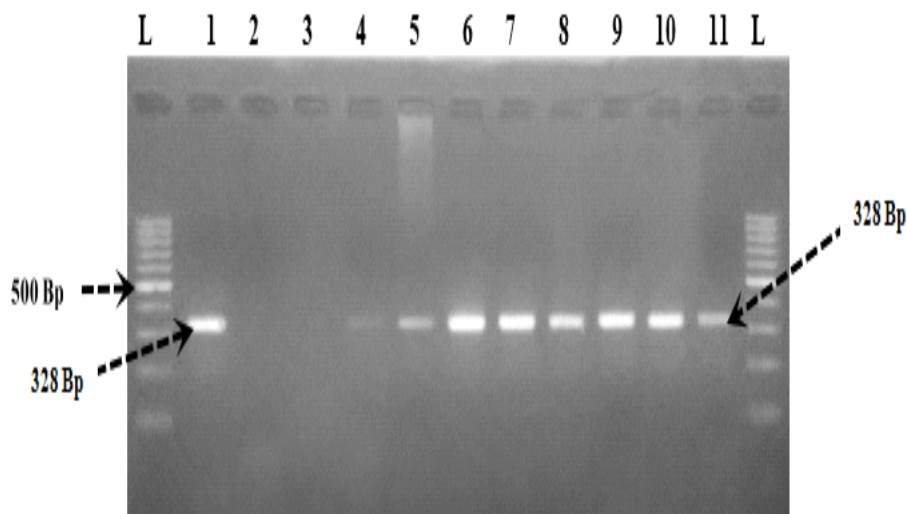


Figure 4. 1: PCR amplicons for RNA-dependent RNA polymerase gene using family-specific PCR primer. (From left to right) Lane M: molecular marker of 100bp, lane 1, 2, 3: universal control 1, NTC, empty well respectively. Lanes 4-11: samples 361 & 389 (rectal swabs); 365, 382, 383, 384, 385, 396 (oral swabs). Samples were collected from bats.

4.2 Nucleotides sequence alignment

All partial nucleotide sequences generated from the 328bp PCR products were obtained and analyzed for Basic Local Alignment Search Tool (BLAST) search program of the National Center for Biotechnology Information (NCBI) website. The result obtained from the BLAST exercise showed sample isolates have 98-100% homology with those retrieved from the genbank. The sequences of eight Kenyan strains and six strains from Germany were downloaded from the genbank. The alignment was done using Clustal W multiple alignment method on Bioedit® software package version 7.2.5.0 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Molecular phylogenetic and evolutionary analyses of nucleotide sequences were performed using MEGA version 6 (Tamura *et al.*, 2013). The phylogenetic tree was constructed by the maximum likelihood method using bootstrap analysis with 1000 replicates. Results of the multiple sequence alignment are shown in Appendices 9 and 10. The multiple sequence alignment was conserved except for sample 8, which showed region of variability.

4.3 Phylogenetic analysis

Phylogenetic analysis was carried out based on a 328 bp fragment of the RNA-dependent RNA-polymerase (RdRp) gene region to determine the genetic diversity of the eight (8) coronavirus sequences, and also to understand the genetic relationship with previously characterized isolates. All eight (8) sequences belonged to the unclassified alpha-coronavirus genus (α CoV) in the database. Three (3) of the isolates (361, 365 and 389) were identical and clustered in cluster I with reference batCoVs detected in Kenya in the genbank (Figure 4.2). Isolates 384 (oral swab), 396 (oral swab), 383 (oral swab), 382 (oral swab), and 385 (oral swab) are distant relative of Kenyan bat coronaviruses in the genbank and clustered in cluster III. When compared with sequences of bat coronaviruses from Germany (Figure 4.2), the isolate bat sequences discovered in Kenya did

not cluster with sequences detected from Germany. The eight (8) positive isolates were detected from two (2) species of bats (2 from *Scotophilus dingamii* and 6 from *Chaerephon pumilus*). Of the eight positive samples obtained, two (2) were from rectal swabs and six (6) from oral swabs and all positive isolates were obtained from the wet season of May, 2018 sampling exercise. In terms of location, all positive results were from Mpala mobile clinic, ranch house and school.

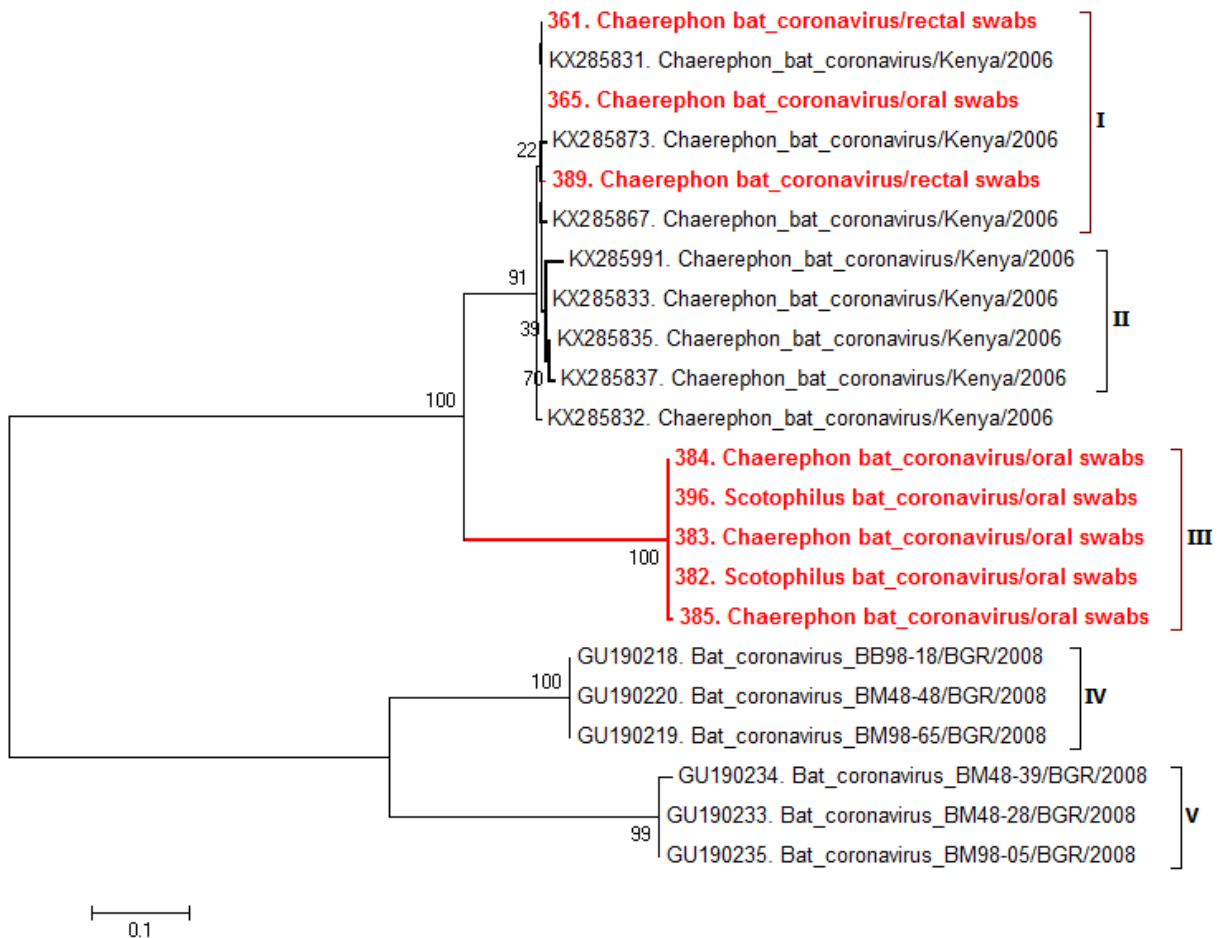


Figure 4. 2: Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with bootstrap confidence based on 1,000 repetitions. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis was done using 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). Samples in red are isolates from the study.

CHAPTER FIVE

5.0 DISCUSSION

This study demonstrated absence of coronaviruses in the population of olive baboons sampled. It may be at this point difficult to say if baboons are reservoirs of the virus. It may therefore, likely mean that they are not typical source or reservoirs of coronaviruses or maybe like humans, are also considered at risk of exposure or may be known to suffer from the same disease. Baboons have been known to prey on bats and rodents (Palombit, 2013) or eaten fruits contaminated by fecal or saliva from bats, which could constitute an opportunity for virus spillover. Although bats are known reservoirs of coronaviruses, spillover events may not have occurred in this case. In a recent study by Olarinmoye *et al.*, (2017), serum antibodies to HCoV_s (22%) in free-ranging commensal baboons has been reported in Ta'if in Saudi Arabia with other studies reporting 50% seroprevalence in caged macaques and baboons (Kummer *et al.*, 1981). However, molecular technique used in this study did not detect active coronaviruses infection. Though all samples from olive baboons in this showed absence of coronaviruses, a final conclusion may not yet be established that they are not reservoirs until further studies are conducted in other counties in the country.

About 3.96% of bats samples tested positive for coronavirus, which confirmed that coronaviruses are common in this group of mammals. All the positive samples were collected in the wet season. This study also demonstrated that samples from the respiratory and gastrointestinal tract contained the virus. Interesting finding was that the isolates clustered with bat coronaviruses from Kenya in the database, indicating that they are possibly confined to bats in Kenya. This study showed that bat coronaviruses are not only restricted to the “5-former provincial regions of Kenya” (Waruhiu *et al.*, 2017) but also to Laikipia County. The results of this study also showed that large collection of bats sp. nest in clinics, human dwellings (roofs, cracks in building, between roofing sheets, and

ceilings), farm houses, laboratory, and buttresses in Laikipia County. Nests and bat feces have been found by doors, food stores and restaurants in the study area and 0.99% of the positive samples are from rectal swabs. Some of these bats may be from the sample area, migrated from other locations and or have mingled with migratory bats. The fear of a coronavirus spillover is possible as observed by a preliminary study by Kamau *et al.*, (2017), which revealed existence of highly complex human-wildlife-livestock interface in Laikipia County, Kenya and risk of disease transmission due to high human-wildlife interaction. Also reported were the high-risk food practices, such as consumption of sick animals or collection of animals found dead, the presence of animal feces found around food stores and sharing of waterholes with olive baboons (Kamau *et al.*, 2017).

In this study, coronavirus was not detected during the dry season of September, 2017 indicating that weather condition and/ or seasonality may have effect on replication and transmission dynamics of the virus which support findings by Lau *et al.*, (2005) and Jevsnik *et al.*, (2016). They have observed that more coronaviruses were discovered in winter, few cases in spring and autumn but none were discovered in the summer period which indicates a relationship involving changes in weather condition and the biology of the virus. The positive results obtained from samples collected in the wet season of May 2018 in Laikipia County may therefore be due to favorable weather condition for viral multiplication and transmission. This period also coincides with the breeding season of bats (virus survives longer in milk thereby increasing the transmission from lactating bats to juveniles), rodents, olive baboons, vectors such as blood sucking insects, and ticks. The wet season also influences influx of tourists and researchers to Mpala conservancy and environs and increase in number of livestock and other wildlife in search of green pasture. Opportunity for human-wildlife-livestock interaction therefore, at this period is high and may

result in viral spillover events. The six positive samples described from oral swabs constituting 2.97% of all the results may indicate the respiratory tract as favorable route for the shedding of the virus compared to the rectal (gastrointestinal tract) route (0.99%). This finding was supported by Cauchemez *et al.*, (2016) who reported 20% cases of MERS were due to primary infections and mostly the juveniles are more susceptible and “shed large amount of the virus from the upper respiratory tract (Adney *et al.*, 2014).” In a report by Zhou *et al.*, (2017), intestinal route of transmission was described as an alternative route. The primary route of infection and transmission of coronaviruses therefore is the respiratory droplets and saliva in direct contact with susceptible population (Durai *et al.*, 2015). This study also described the presence of coronaviruses in both species of bat sampled (*Chaerephon pumilus* and *Scotophilu dingamii*) confirming these bats species may be principal reservoirs.

Eight (8) species of rodents were sampled in this study (*Acomys kemp*, *Acomys percivalli*, *Elephantulus rufescens*, *Gerbilliscus robustus*, *Aethomys hindei*, *Myomyscus brodernani*, *Grammonys dolichorus*, and *Saccostomus meamsi*) and all tested negative for coronaviruses in both dry and wet season. This is in contrast to other findings around the world where coronavirus nucleic acid were detected in rodents. A possible explanation may be that sufficient interaction between bats and rodents may not have been established despite species abundance to facilitate spillover events. New coronaviruses have been discovered in rodents by molecular techniques in the UK and Poland (Tsoleridis *et al.*, 2016). Also in France, *Apodemus flavicollis* and *Myodes glareolus* have tested positive for beta- and alpha-CoVs (Monchatre-Leroy *et al.*, 2017) though within the same region, *Myodes glareolus*, *Microtus agrestis* and *Apodemus sylvaticus* tested negative. Other research findings in China have also confirmed the presence of alpha- and beta-coronaviruses in three species of rodents namely *Eothenomys fidelis*, *Apodemus ilex* and *Apodemus*

chevrieri (Ge *et al.*, 2017). In another study, *Rattus norvegicus* in China tested positive for coronavirus (Lau *et al.*, 2015). This study therefore, is in-conclusive that rodents are not reservoirs of coronaviruses in Kenya. More investigation needs to be carried out in other parts of Kenya especially the arid and semiarid parts where large numbers of rodents are found to exist and interacts well with humans.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The following conclusions were made:

1. The study detected the presence of coronaviruses in bats in Laikipia County, Kenya.
2. Three bat isolates from this study clustered with unclassified alpha-coronavirus genus from Kenya in the GenBank. Five formed a monophyletic group and are also from alpha coronavirus genus. In comparison with bat coronaviruses from Germany in the database, isolates from this study were genetically different and showed no cluster relationship.
3. Positive results were described more in oral swabs than rectal swabs.

6.2 Recommendations

1. Larger-scale surveillance is needed to fully conclude whether olive baboons and rodents are not reservoirs of coronavirus in Laikipia County, Kenya.
2. A full genome analysis is recommended to characterize the new coronaviruses detected in the bat samples in this study and to confirm their implication on human health.
3. For future studies, sampling should target oral swabs rather than rectal swabs, and the wet season rather than the dry season.

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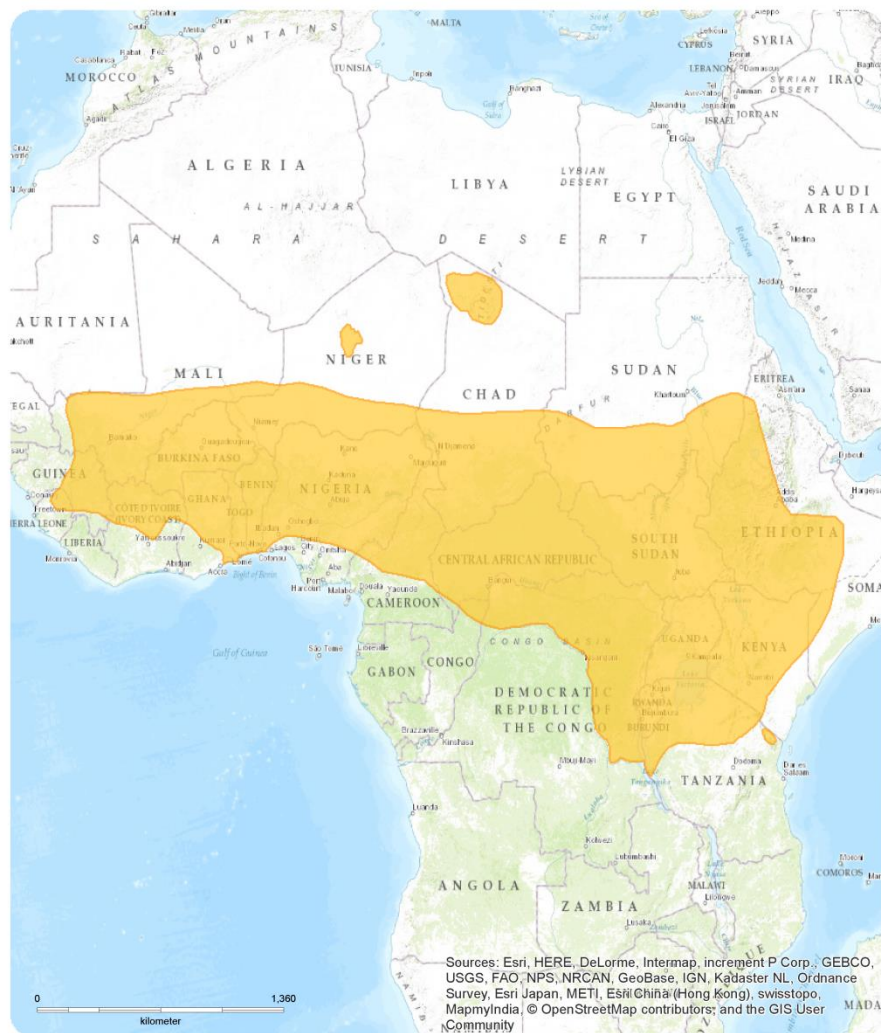
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APPENDICES

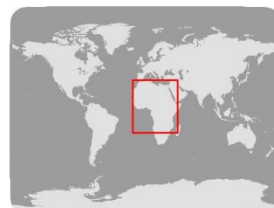


Papio anubis

Range

■ Extant (resident)

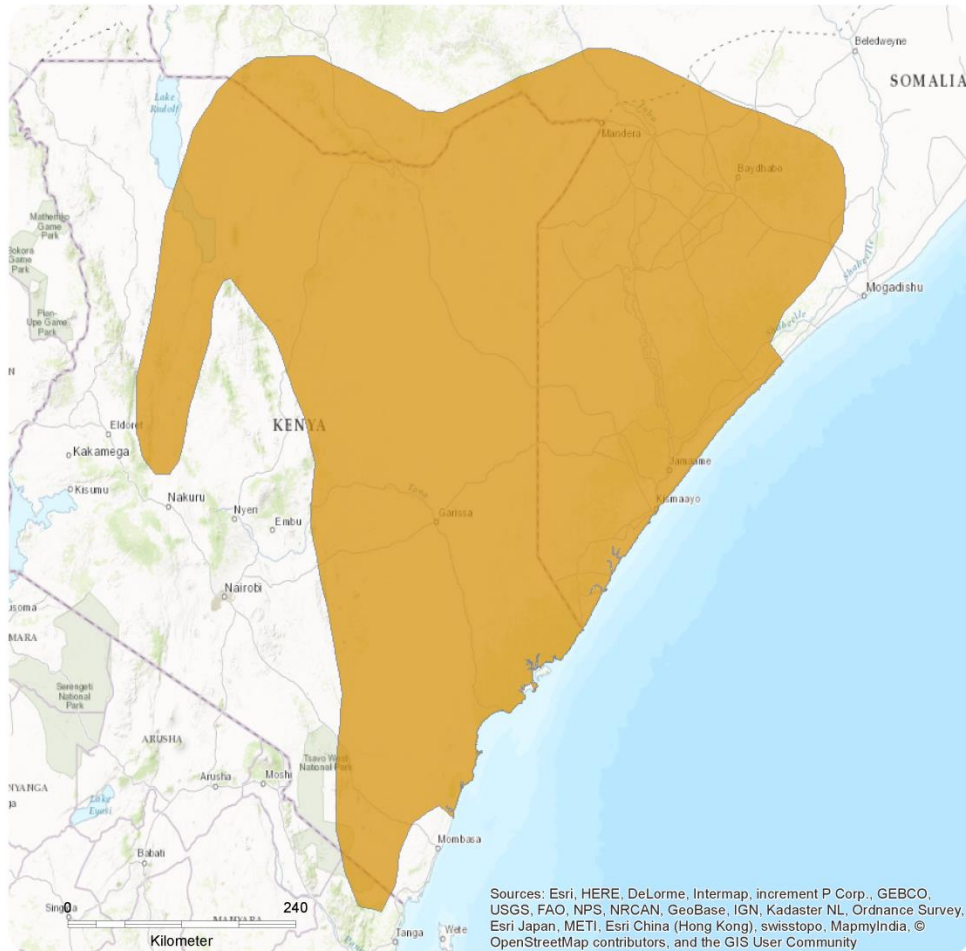
Compiled by:
IUCN (International Union for
Conservation of Nature)




The boundaries and names shown and the designations used on this map do not imply any official endorsement, acceptance or opinion by IUCN.



Appendix 1: Geographical distribution of olive baboons (*Papio anubis*) (Kingdon *et al.*, 2008).



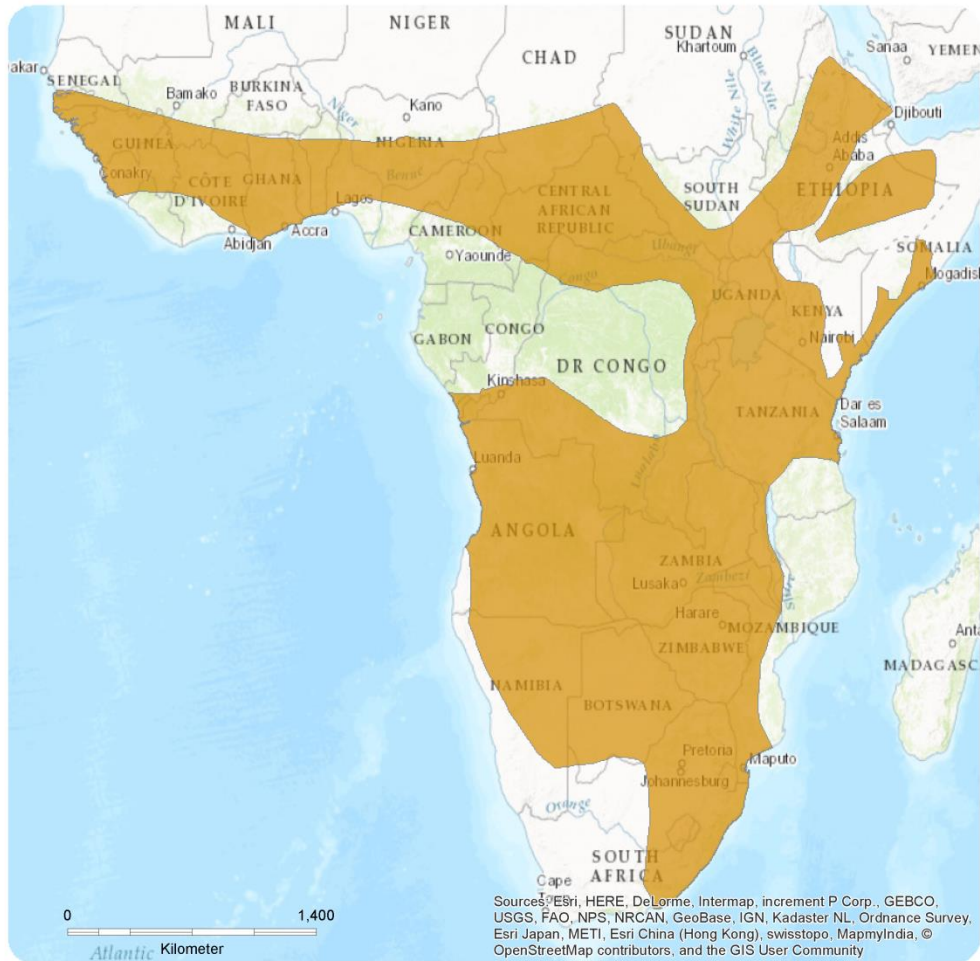
Range
 Extant (resident)


Compiled by:
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Appendix 2: Geographical distribution of *Acomys Kempis* (Cassola, 2016)



Range
 Extant (resident)

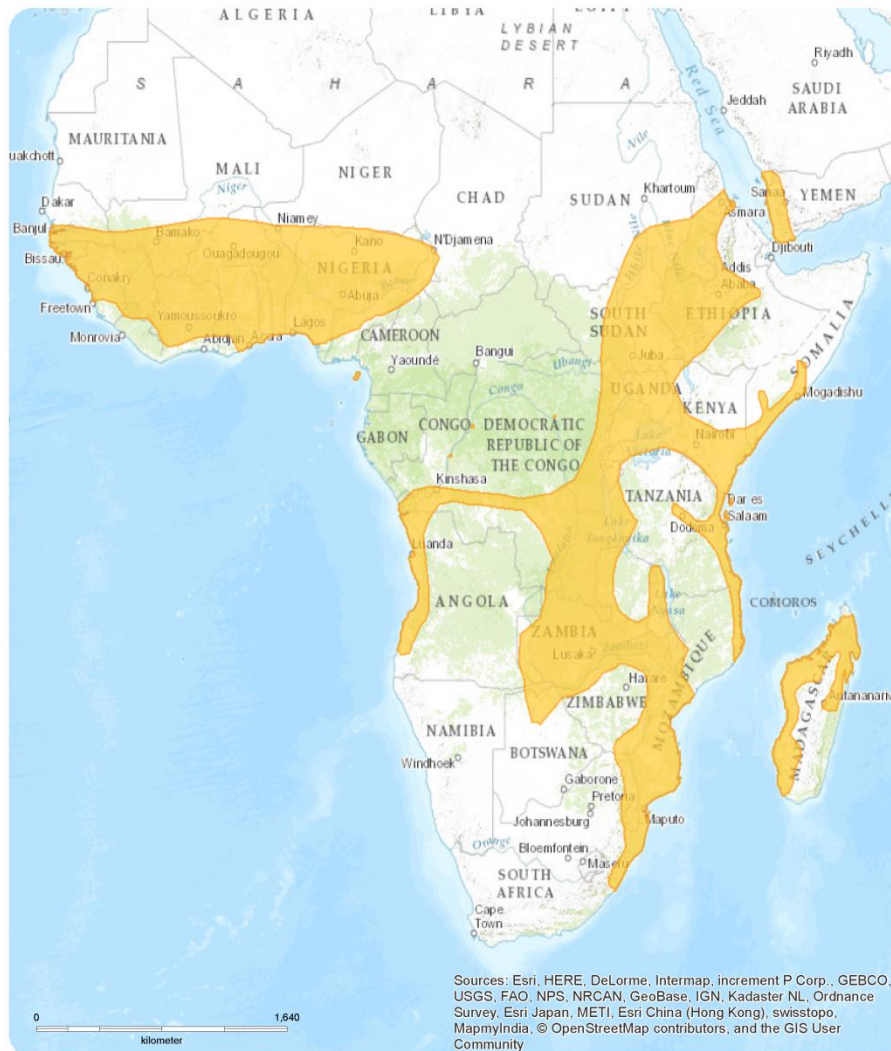
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Appendix 3: Geographical distribution of *Scotophilus* sp (Monadjen and Griffin, 2017).

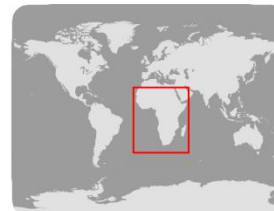


Chaerephon pumilus

Range

Extant (resident)

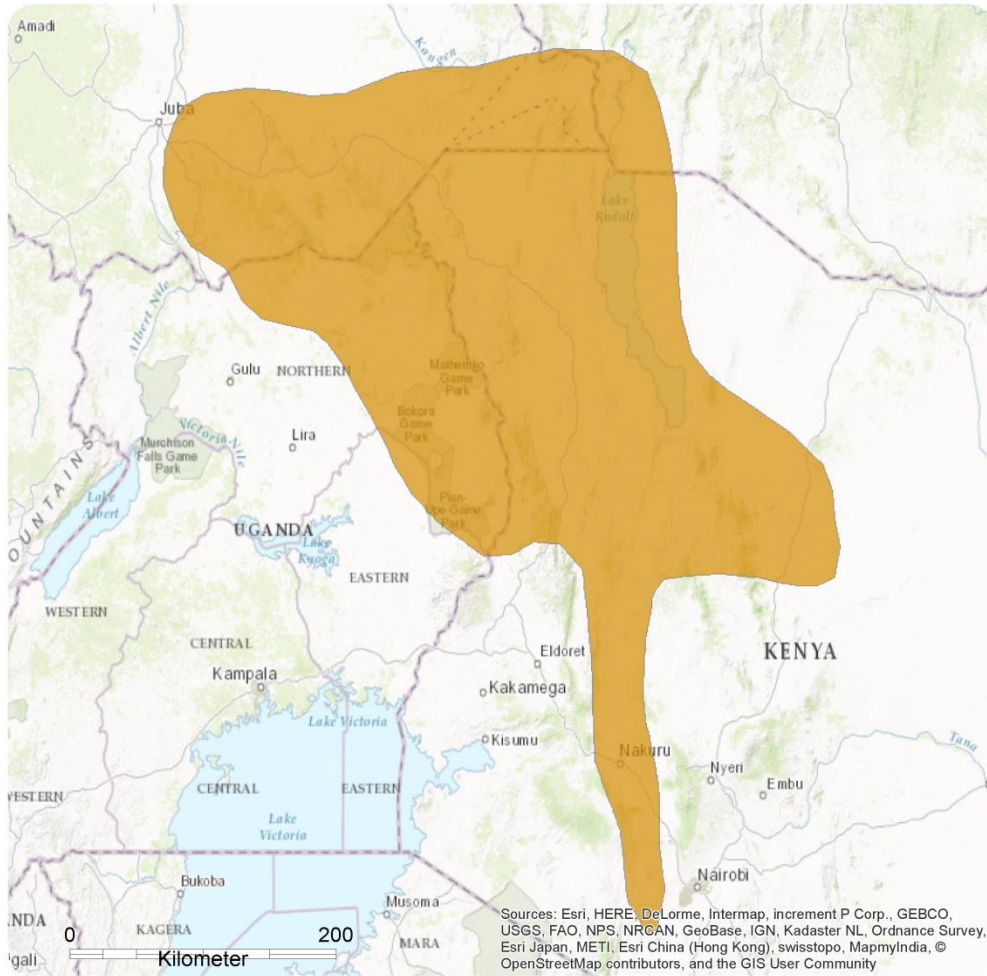
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Appendix 4: Geographical distribution of *Chaerephon pumilus* (Mickleburgh et al., 2014).



Range
 Extant (resident)

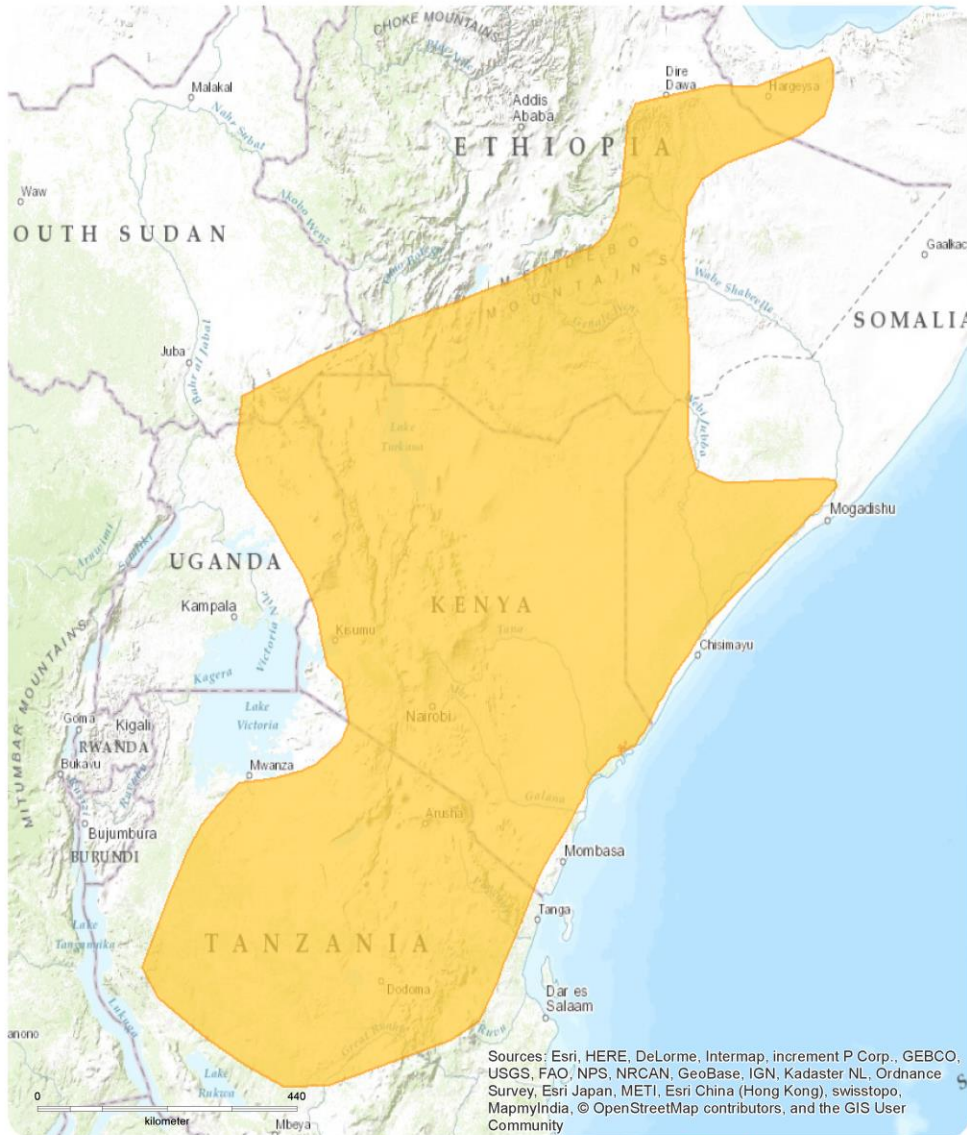
Compiled by:
 IUCN (International Union of Conservation of Nature)



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Appendix 5: Geographic distribution of *Acomys percivalli* (Cassola, 2016)

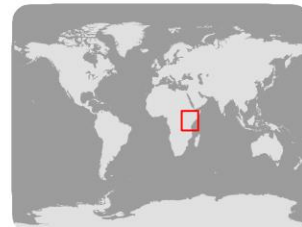


Elephantulus rufescens

Range

Extant (resident)

Compiled by:
G.B. Rathbun, California
Academy of
Sciences, www.sengis.org



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Appendix 6: Geographical Distribution of *Elephantulus rufescens* (Rathbun, 2015)



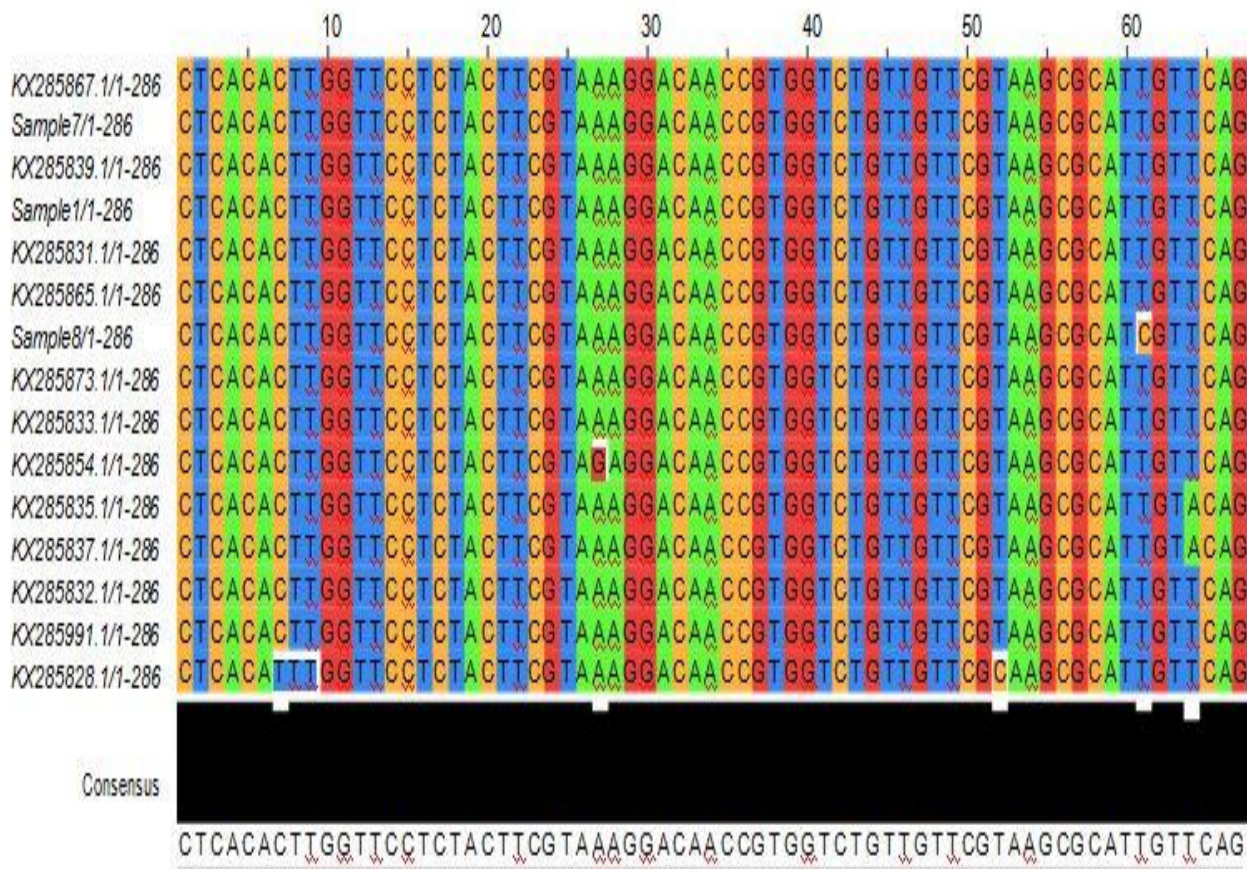
Appendix 7: Team ready for the sampling in full PPE



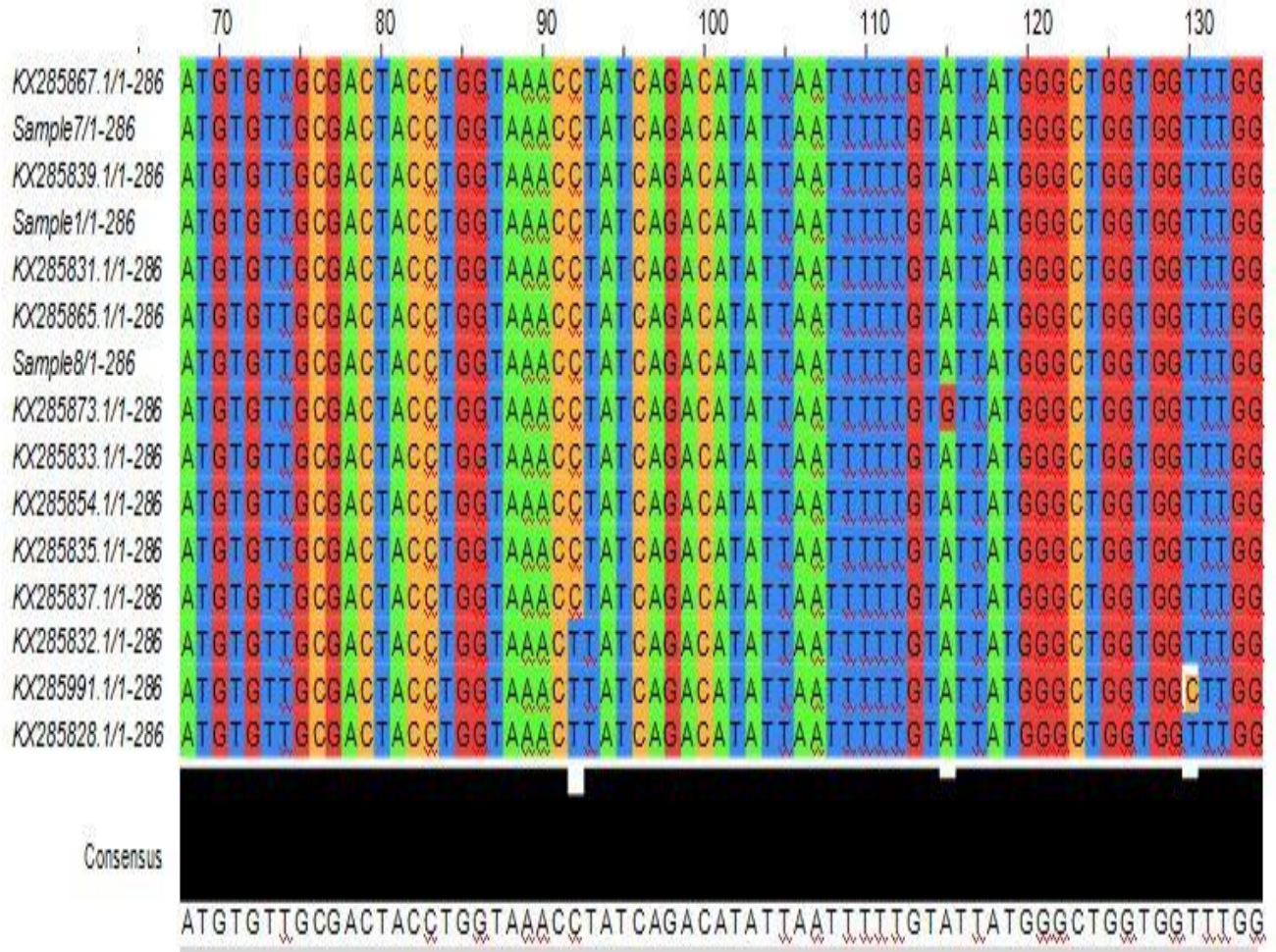
Appendix 8: Stages of the habituation and trapping of olive baboons. 1: locate where they are based and study their activities (troops feeding, drinking, resting, and sleeping sites); 2: Baiting and trapping site was selected. The baiting was done using shelled corn and green corn; 3: Trap setting; 4: Baboons trapped; 5: Feeding without realizing it has been trapped.



Appendix 9: Shows bat trapped in mist net and in porous bags hung on wooden line.



Appendix 10: Multiple alignment of the ‘query’ nucleotide sequences revealed that all the sequences of samples from the bats were conserved except for isolate sample 8 showing a region of variability represented by nucleotide (c). The conserved regions are represented by (CTCACACTTGGTTCCTCTACTTCGTAAAGGACAACCGTGGTCTGTTGTTTCGTAAGCGCATTGTT CAG) while the variable areas are C, G, A, A, T, and C.



Appendix 11: A multiple sequence alignment of RdRp gene fragments comparing sample sequence to those retrieved from the database. The sequences are homologous except for region of variability on sequence KX285832.1, KX28599.1, KX285828.1, and KX285873.1 from the database.