DETECTION OF ARENAVIRUSES IN RODENTS, SHREWS AND ELEPHANT SHREWS FROM SELECTED WILDLIFE-HUMAN INTERFACES IN TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGEREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

The present study was conducted to investigate the presence of Arenaviruses from rodents, shrews and elephant shrews captured in selected wildlife-human interfaces in Tanzania. The study involved six sites with high potential for contact between wildlife and humans namely; Ruaha, Kilombero, Mtwara, Mbeya, Mbinga and Mikumi. A total of 121 animals comprising 111 rodents, 3 shrews and 7 elephant shrews were screened for Arenaviruses using conventional Polymerase Chain Reaction (PCR). The genetic relatedness of Arenaviruses was evaluated by conducting phylogenetic analysis of partial sequences of the S gene. The association between age and sex with the presence of Arenaviruses was assessed. Of the 121 animals, 7 (5.8 %) were shedding Arenavirus. All positive animal samples were obtained from the Ruaha site at crop raiding and peridomestic interfaces. Eighty six percent of the infected animals were *Mastomys* sp. and 14 % were *Arvicanthis* sp. Age and sex of the animals were not significantly associated with occurrence of Arenaviruses in rodents, shrews and elephant shrews ($P > 0.05$). Additionally, Arenavirus detection in fecal specimens was not significantly different from the detection in oropharyngeal specimens ($P > 0.05$), clearly indicating that both specimens are useful for Arenavirus testing. Phylogenetic analysis showed that isolates obtained from this study were related to the Old World Arenaviruses, and include strains of Morogoro virus and a novel strain of Arenavirus. In conclusion, the present study has confirmed the presence of Arenaviruses closely related to other known Old World Arenaviruses in the Ruaha ecosystem.

DECLARATION

I, Ruth Maganga, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor is concurrently being submitted to any other institution.

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DEDICATION

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LIST OF SYMBOLS AND ABBRVIATIONS

ABI	Applied Biosystems Incorporated
ARS16V/ ARS1	Arenavirus polymerase chain reaction 1 primer set
ARS3V/ARS7C-mod	Arenavirus polymerase chain reaction 2 primer set
BLAST	local alignment search tool
bp	base pair
CA	California
CI	confidence interval
cDNA	complementary deoxynucleic acid
DNA	deoxyribonucleic acid
E	ear length
EDTA	ethylenediaminetetra-acetic acid
ELISAs	enzyme-linked immunosorbant assays
EXOSAP IT	exonuclease 1 and shrimp alkaline phosphate cocktail
FL	Florida
GP	glycoprotein
GPC	glycoprotein precursor
HB	Huntington Beach
HBL	head and body length
HF	hind foot
IDs	indentities
IgG	immunoglobulin G
IgM	immunoglobulin M
IGR	intergenic regions
JUNV	Junin Virus

Kb	kilobase
L RNA	long ribonucleic acid
L segement	long segment
LASV	Lassa virus
LCM	Lymphocytic Choriomengitis Virus
M13	filamentous phage M13
$MgCl_2$	magnesium chloride
min	minute
NCBI	National Center for Biotechnology Information
NP	nucleoprotein
NY	New York
OR	odds ratio
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S RNA	short ribonuceic acid
S segement	short segment
S.O.C	super optimal broth in catabolite repression
sp	specie
ssRNA	single stranded RNA
SUA	Sokoine University of Agriculture
T	tail length
TA	Taq-polymerase
TBE	Tris Borate EDTA
TOPO	Topoisomerase 1
qPCR	quantitative polymerase chain reaction.

UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	voltage
VTM	viral transport medium
Z protein	zinc binding protein
∞	infinity

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Arenaviruses are bisegmented ambisense single stranded RNA viruses, belonging to family Arenaviridae, responsible for viral hemorrhagic fevers in humans and animals (Jay *et al*., 2005). The viruses are transmitted primarily by rodents (Nitatpattana and Chauvancy, 2000). Murid rodents are the principal reservoirs of Arenaviruses, but host relationships have not been extensively studied (Jay *et al*., 2005). According to Gunther *et al*. (2009), each Arenavirus is associated with either one species of host or few closely related rodents which form part of the natural reservoir for the virus. The viruses can cause chronic infections in their respective principal rodent hosts, whereby transmission can occur from one generation to another (Buchmeier *et al*., 2007). This phenomenon is very important for long term maintenance of the virus in nature. Most infections in rodents are asymptomatic; the viruses do not appear to cause any obvious illness (Buchmeier *et al*., 2007).

Arenaviruses are divided into two groups: Old world and New World Viruses (Clegg, 2002). The differences between these groups are distinguished geographically and genetically (Delagado *et al*., 2008). When an Arenavirus is classified as “Old World”, this means it was found in the Eastern Hemisphere in places such as Europe, Asia, and Africa. Examples are Morogoro virus, Mopeia virus, Merino Walk virus, Luna virus, Ippy virus and Gbagroube virus (Delagado *et al*., 2008). When the virus is found in the Western Hemisphere, in places such as South and North America, it is classified as “New World”. Examples are Junin virus, Machupo virus, Chapare virus, Pichinde virus, Sabia virus and

Pirital virus (Delagado *et al*., 2008). Lymphocytic choriomeningitis (LCM) virus is the only Arenavirus that exist in both areas, but it is classified as an Old World virus (Nitatpattana and Chauvancy, 2000). Genetic analysis is currently used to investigate relatedness of Old World Arenaviruses and New World Arenaviruses, whereas serologic assays were used to separate Old from New World viruses in the past. Moreover, nucleoprotein and glycoprotein genetic analysis has made it easier to appropriately classify Arenaviruses (Fig. 1). There are three distinct evolutionary lineages that are known to have emerged within the New World Arenaviruses. Lineage A includes Flexal, Parana, Pichinde, Pirital, Tamiami, and Whitewater Arroyo. Lineage B contains Guanarito, Junin, Machupo, Sabia, Amapari and Tacaribe. Lineage C is a home to only two viruses, Latino and Oliveros. All four of the South American Arenaviral hemorrhagic fevers are members of B lineage, and this suggests that this phenotype was passed down by a common pathogenic ancestor (Nitatpattana and Chauvancy, 2000).

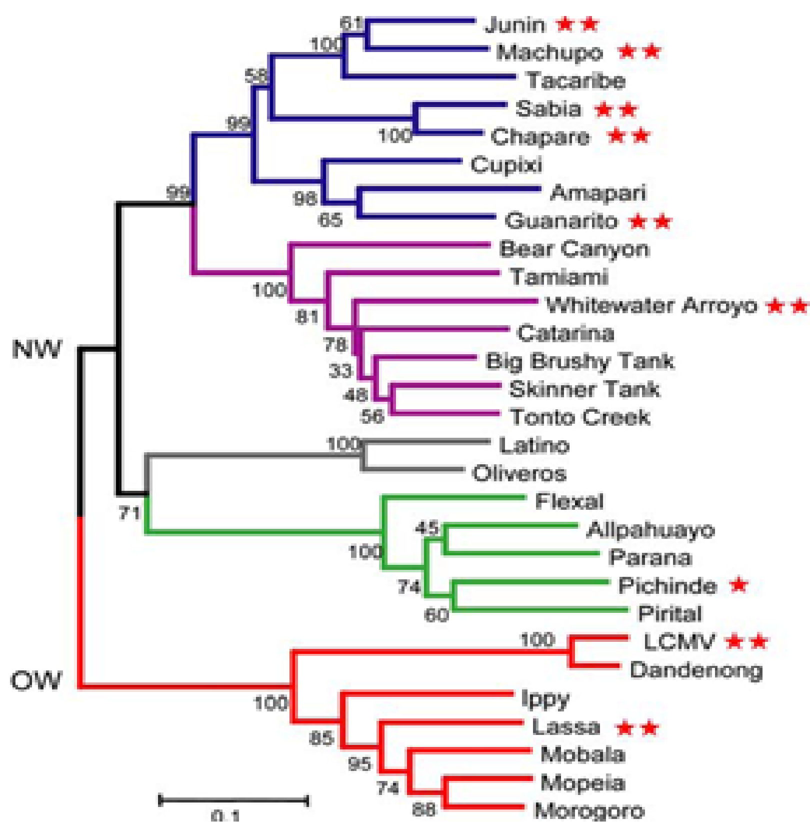

Figure 1: Phylogenetic relationships between Arenaviruses. The Old World (OW) virus lineage is depicted in red, and New World (NW) lineages are subdivided into clades: A (green), B (blue), C (gray) and a recombinant A-B lineage (purple). This phylogenetic tree is based on amino-acid comparisons of the GPC. Red stars that follow virus names indicate the ability to infect humans: ** denote lethal hemorrhagic fever viruses and lymphocytic choriomeningitis virus (LCMV), and * identifies infections with few or no clinical manifestations. This image is adapted from (de Bellocq *et al.,* 2010).

Some of the New World and Old World Arenaviruses cause life threatening hemorrhagic fevers, which can be transmitted between people. These viruses include Machupo virus, Guanarito virus, Junin virus, Lujo virus, Lassa virus, Sabia virus and Whitewater Arroyo virus (Gunther and Lenz, 2004; Delagado *et al*., 2008). However, the majority of the Old World Arenaviruses, with the exception of Lujo virus, Lassa virus, and Lymphocytic Choriomeningitis virus, are not known to cause human disease (Briese *et al*., 2009).

The geographic distribution of Arenaviruses is patchy and usually determined by the distribution of the rodent hosts, though the ranges of Arenaviruses are frequently smaller than the ranges of their host species. Cases of hemorrhagic fevers attributed to Arenaviruses have been recently reported in Africa and South America and Europe (Childs *et al*., 1991; Mills *et al*., 1991; Demby *et al*., 2001; Salazar-Bravo *et al*., 2004). Currently, six Arenaviruses have been reported in Africa: Lassa, Mobala, Ippy, Lujo, Mopeia and Morogoro viruses, while the only known Arenavirus in Europe is LCM virus carried by the house mouse, *Mus musculus* (Oldstone, 2002; Gowen *et al*., 2010). According to Günther *et al.* (2009), the seroprevalence of Arenaviruses in Tanzania was 20 % in *Arvicanthis* sp. (unstriped grass rats) from Iringa region and 17 - 18 % in *Mastomys natalensis* (multimammate mice) from Arusha and Morogoro. A prevalence of 23.7 % for Arenavirus infection in *M. natalensis* was found on the campus of the Sokoine University of Agriculture in Morogoro.

Arenavirus infections can pose a great threat to public health, especially in the geographic areas where the viruses are endemic, since they can be transmitted from their rodent reservoirs to humans. Most of the viral hemorrhagic fevers caused by Arenaviruses are characterized by nonspecific febrile illnesses, which may be misdiagnosed as other tropical diseases that present with fever such as typhoid, leptospirosis, rickettsia infection and malaria (Nitatpattana and Chauvancy, 2000). Some Arenavirus infections have up to a 30 % mortality rate, and are especially dangerous in pregnant women infected during the third trimester of pregnancy (Briese *et al*., 2009).

The detection of Arenaviruses is very challenging and highly dependent on disease outbreaks and rodent surveys in suspected endemic areas (Cajimat *et al*., 2007; Briese *et*

al., 2009). Diverse diagnostic methods are used to test for Arenaviruses. The most reliable method is virus isolation, but it presents a high exposure risk to laboratory technicians and should be performed in high level containment areas, specifically biosafety level 4 laboratories (Gunther and Lenz, 2004). These facilities are scarce in Tanzania and are very expensive to install, rendering this method almost impossible to utilize. Recently, new methods such as immuno-histochemical assays for detection of Arenavirus antigen, reverse transcriptase polymerase chain reaction assays for Arenavirus specific RNA and microarray for differential diagnosis of viral hemorrhagic fever viruses have been developed as useful adjuncts to virus isolation (Ksiazek *et al*., 1999). However, most of these detection methods are not well established in Tanzanian laboratories, hence causing poor surveillance systems. This limits awareness of occurrence and distribution of Arenaviruses in Tanzania.

To date, only two studies on Arenaviruses have been conducted in Tanzania using molecular diagnostic methods (Gunther *et al.*, 2009; de Belloq *et al*., 2010). These studies demonstrated that Arenaviruses which do not cause human infection are actively circulating in Tanzania and that the natural reservoirs of these Arenaviruses, the murid rodents, exist in large populations at wildlife-human interfaces. It is not yet known if pathogenic Arenaviruses are circulating in Tanzania. They could be present, but no study so far has identified them. Since a large proportion of people in Tanzania live in areas where there is extensive human and animal interaction, there is a high possibility that these viruses can be transmitted to humans. Therefore, the knowledge of circulation of pathogenic and nonpathogenic Arenaviruses in various species of rodents is of public health importance.

This study utilized a rapid and a sensitive diagnostic method, reverse transcription polymerase chain reaction (RT-PCR), to detect Arenaviruses in rodents sampled in six selected trapping sites in Tanzania. This technique has been shown to have an excellent analytical sensitivity and specificity in the diagnosis of Arenavirus infection in rodents (Olschlager *et al*., 2010; Ogawa *et al*., 2011). The study also compared the difference in detection of Arenaviruses in fecal and oropharyngeal specimens from rodents, shrews and elephant shrews. The information obtained is expected to provide relevant knowledge on the type of specimen to be used for Arenavirus testing. The study has also established the phylogenetic relationship between Arenaviruses, hence providing an opportunity for discovery of unknown Arenavirus pathogens circulating in Tanzania.

1.2 Problem Statement and Justification

Arenavirus-related illnesses in humans have been reported in many countries in the world which cause high mortality and morbidity rate to humans that consequently have great impact to public health and are of major economic importance to the community. Nevertheless, there are limited studies in Tanzania on occurrence of Arenaviruses, geographical distribution and their pathogenicity. Major outbreaks of Arenavirus illnesses, characterized by hemorrhagic fever, were reported in Nigeria in 1969 caused by Lassa virus (Sogoba *et al*., 2012). In 2008, illnesses due to Lujo virus were reported in Zambia (Ishii *et al*., 2011). The latter eventually spread to South Africa, a neighboring country. Recent increases in transborder trading activities, open borders, migration of people from one country to another, increasing agricultural activities and human-animal interactions place Tanzanians at risk of exposure to Arenaviruses.

This study provides baseline information on the occurrence of Arenaviruses at selected wildlife-human interfaces of Tanzania through screening of rodents in selected sites. This study also compares the detection rates of Arenaviruses in fecal and oropahryngeal

specimens from rodents, shrew and elephant shrews. The information is very important for public health professionals, as it helps to define the current distribution of the virus and the best samples for Arenavirus testing. Sharing this information will raise public awareness of the role played by different rodents in the transmission of Arenaviruses and contribute to developing potential mitigation strategies to block the transmission pathways. Furthermore, the data provides a platform for further investigations on the ecology of Arenaviruses in Tanzania.

1.3 Research Objectives

1.3.1 Main objective

To determine the presence of Arenaviruses, relationship of age and sex with the occurrence of Arenaviruses, comparison of detection between fecal and oropharyngeal samples, and genetic relatedness of Arenaviruses in rodents, shrews and elephant shrews captured from selected wildlife-human-livestock interfaces and sites in Tanzania.

1.3.2 Specific objectives

i. To determine the presence of Arenaviruses in various species of rodents, shrew and elephant shrews using molecular diagnostic techniques.
ii. To determine the proportion of Arenvirus positive samples in six selected sites (Ruaha, Kilombero, Mbeya, Mtwara, Mbinga and Mikumi) in Tanzania.
iii. To determine the relationship between age and sex with the occurrence of Arenaviruses in rodents, shrews and elephant shrew.
iv. To determine the difference in detection of Arenavirus between fecal and oropharyngeal specimens.
v. To determine phylogenetic relationships of Arenaviruses detected in selected sites in Tanzania.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background Information on Rodents, Shrew and Elephant Shrews

Rodents belong to the mammalian order Rodentia, which consists of almost 1,700 species (Fiedler, 1990). There are about 89 genera of rodents and 290 species of rodents in Africa, which are arranged into 14 families. East Africa contains about 62 genera and 161 species found in 12 of the 14 rodent families. The rodent fauna is diverse, ranging from larger porcupines (*Hystrix* sp.) weighing 20 kg to small African Pygmy mice (*Mus minutoides*) weighing only 5 - 7g (Fiedler, 1990). Elephant shrews belong to the order Macroscelidea. Recent evidence suggests that they may belong to a clade of African mammals, the Afrotheria, which also includes hyraxes, elephants, sea cows, aardvarks, golden moles, and tenrees. They are represented by a single family, Macroscelididae, which includes 4 genera and 19 species. They are adapted for leaping, with hind limbs much longer than forelimbs. Elephant shrews also have elongated snouts and large eyes and ears. They range from mouse-sized to size of a squirrel or large rat (Clancy *et al*., 2013). Shrews are classified in the order Soricomorpha and family Soricidae. There are over 300 species of shrews, making them the most diverse group in the order Insectivora (shrews, moles, and relatives). They are small, mouse-sized mammals with an elongated snout, a dense fur of uniform color, small eyes, and five clawed toes on each foot. Its skull, compared to that of rodents, is long, narrow, and lacks the zygomatic arch on the lateral side characteristic of rodents. The teeth are small, sharp, and commonly dark-tipped (Sasaki *et al*., 2015).

Rodents, shrew and elephant shrews are found in a variety of terrestrial habitats, including man-made environments. Among the rodents, shrew and elephant shrews, there are

species that are arboreal and live mostly in trees and species that are fossorial and spend most of their life underground, while others are semi-aquatic. Rodents and shrews can be herbivorous, feeding on grasses (graminivorous), roots, leaves, seeds (granivorous), or fruits (frugivorous). Many are omnivorous or capable of consuming a variety of foods, while a few are insectivorous and feed mostly on seeds. Elephant shrews, on the other hand, mainly feed on insects, spiders, centipedes, millipedes and earthworms (Fiedler, 1990).

Most rodents, shrews and elephant shrews do not adversely affect humans, but at least 35 species of rodents have been reported to cause damage to agricultural crops (Fiedler and Fall, 1994). Cereals such as wheat, sorghum, maize and barley are more susceptible to damage by rodents, shrews and elephant shrews, though root crops, vegetables, plantation crops and stored foods may also be damaged. Stored foods are commonly destroyed by rodents living in peridomestic environments such as multimammate rats (*Praomys natalensis*), African giant rats (*Cricetomys gambianus*), African dormice (*Graphiurus murinus),* and common spiny mice (*Acomys cahirinus*). Many of these rodents are involved in disease transmission to humans.

Important diseases in eastern Africa affecting humans and livestock involving rodents, shrews and elephant shrews include plague, leptospirosis, boutonneuse fever (African tick typhus), Murine typhus, and Q-fever (Fiedler and Fall, 1994). Recently, Arenavirus hemorrhagic fevers in humans have been reported to be transmitted by rodents. For example, there are studies which have reported Lassa fever, a western African Arenavirus hemorrhagic fever, is hosted by multimammate rats, including the pygmy mouse and black rats (Mbugua *et al*., 2002). Viruses closely related to Lassa virus have also been isolated

from *Praomys* sp. in Mozambique (Mbugua *et al*., 2002) and Zimbwabwe (Johson *et al*., 1981). In Tanzania, high seroprevalences of antibodies to Lassa virus have also been reported in *Praomys* sp. and *Arvicanthis* sp. (Mbugua *et al*., 2002). Other hemorrhagic fever viruses in eastern Africa such as Hantaviruses were reported in unidentified field rodents from Kenya and Uganda (Mbugua, 2002).

Some of the mentioned diseases have been circulating among rodent, shrews and elephant shrew populations for many years, but outbreaks have been reported to occur in humans only when contact between infected rodents or shrews and humans is prompted (de Thoisy *et al.*, 2014). Usually, contact is influenced by a number of factors, such as an increase in rodent, shrew and elephant shrew populations, survival of disease agents and human activities (de Thoisy *et al.*, 2014). Encroachment of humans in natural habitats of the rodents has also propagated the frequency of contact between humans and rodents. There are quite a number of interfaces (areas of interaction) associated with human-rodent interactions. Some of these interfaces include: hunting, high traffic, crop raiding and peridomestic interfaces (Fiedler, 1990).

Peridomestic interfaces are areas where wild animals, such as rodents, shrews and elephant shrews commonly interact with humans in and around human dwellings, villages, and work sites. Rodent species which prefer living in peridomestic areas include the black rat (*Rattus rattus*), the house mouse (*Mus musculus*), the Norway rat (*Rattus norvegicus*), the African giant rat (*Cricetomys gambianus*) and the African dormice (*Graphiurus murinus)* (Fiedler, 1990). Hunting or bushmeat interfaces are areas where wild animals, including rodents, shrew and elephant shrews are consumed after being hunted, killed, and slaughtered. Rodent species commonly consumed by humans are *Rattus exulans, Rattus*

norvegicus, Rattus flavipectus, Rhizomys sp., *Praomys* sp., and *Mastomys natalensis* (Wang *et al*., 2015). Crop raiding interfaces are areas where wild animals, including rodents, shrews and elephant shrews, interact with humans by feeding on crops in agricultural human fields or in fruit and vegetable markets. There are about 25 rodent species reported to dwell in agricultural fields causing losses in crops such as cereals, legumes, vegetables, root crops, cotton, and sugarcane. Some of these rodent species are *Mastomys natalensis*., *Praomys* sp. and *Arvicanthis* sp. (Fiedler and Fall, 1994). High traffic interfaces are areas around highway, such as train stops, where there are means of transport, including roads, railways, and buildings. Wild animals seek shelter in vehicles, trains or station buildings, and at the same time, humans use these areas for transport, relaxation and conducting small businesses. The majority of rodent species around these areas are *Mastomys natalensis* (Fiedler, 1990).

2.2 Determination of Age, Species and Sex of Rodents, Shrews and Elephant Shrews

There are several studies that have documented ways of identifying the age, sex and species of rodents, shrews and elephant shrews (Karaseva and Telitsina, 1996; Klevezal, 2007). Traditional methods of identifying ages of rodents, shrews and elephant shrews depend on the use of physical characteristic such as fur coverage, body size and weight. However, these approaches are known to have a lot of weaknesses. Characteristics such as body size and weight cannot entirely reflect the age of rodents, shrews and elephant shrews because, at a certain stage of life, different species of rodents, shrews and elephant shrews belonging to the same age group, may vary in weight and body size. For example, in some species the body size and weight of the adults can be similar to the weight and body size of sub-adults and juveniles in other species.

Species identification based on morphology alone is very complex especially when very closely related species have to be discriminated from each other (Patton and Da Silva,

1995). Morphological methods require input of taxonomic experts, who can be challenging to access. Currently, new molecular methods have been established. Molecular methods are very efficient since they can discriminate very closely related or identical species by analyzing their DNA and protein sequences. For example, Wetton *et al*. (2002) used molecular techniques in identifying rodent species. Studies combining morphological and molecular analysis have got a potential to identify a great diversity of rodents, shrew and elephant shrew species, resulting in identification of new species, and revalidation of previously described ones (Patton and Da Silva, 1995; Smith and Patton, 1999).

Many studies have determined the sex of rodents, shrews and elephant shrews by using simple methods. For example, the most popular approach is the observation of physical features which are obvious among males and females. Some of these features are presence of nipples in females and testicles in males. Others are measurement of the urogenital distance or observation of any pregnancy incase it is a suspected female (Gilboa *et al*., 2014).

2.3 Historical Background of Arenaviruses

Lymphocytic Choriomeningitis virus (LCMV) was the first Arenavirus to be discovered. It was isolated in 1933 during a study of an epidemic of St. Louis encephalitis (Armstrong *et al*., 1934), identified as the cause of aseptic (nonbacterial) meningitis. By the 1960s, similar viruses were discovered and classified into the new family of Arenaviridae. Since 1956 when Tacaribe was found (Downs *et al*., 1963), new Arenaviruses have been discovered after every one to three years. A number of hemorrhagic fever causing viruses have been isolated in rodents. Junin virus was the first of these to be recognized,

discovered in 1958 (Parodi *et al*., 1958). The virus was reported to cause Argentine hemorrhagic fever in a limited agricultural area of pampas in Argentina (Weissenbacher *et al*., 1987). Several years later in 1963, Machupo virus was isolated in the Benin province (Johnson *et al.,* 1965). The next to be isolated was Lassa virus, associated with human illness in Nigeria (Sogoba *et al*., 2012). The most recent additions to these human pathogenic viruses were Guanarito virus detected in Venezuela in 1989 (Salas *et al*., 1991), Sabia virus in Brazil in 1993 (Lisieux *et al*., 1994), Chapare virus in Bolivia in 2004 (Delagado *et al*., 2008), and Lujo virus in South Africa in 2008 (Briese *et al*., 2009).

2.4 Arenaviruses

Arenaviruses are zoonotic pathogens, which belong to family Arenaviridae. They are spherical to pleomorphic in shape, having a diameter of 110 to 130 nm. All are enveloped in a lipid (fat) membrane. When viewed by thin section microscopy, they look like sandy particles (Fig. 2) from which they acquire their name, Arenosus, which means sandy (Lee *et al*., 2000; Enria *et al*., 2011).

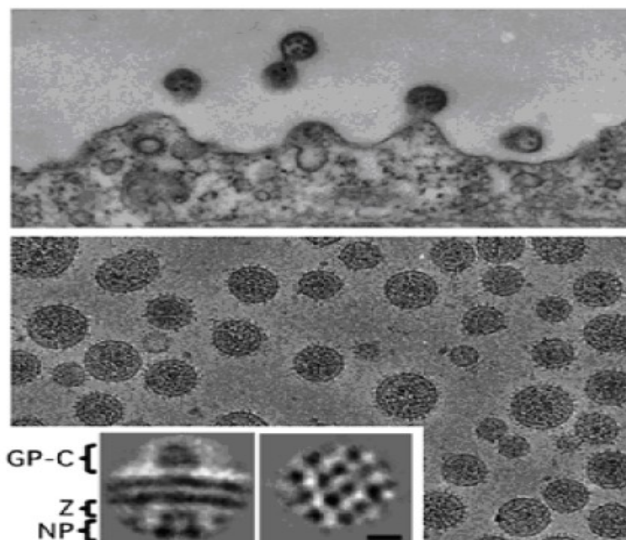

Figure 2: Electron microscopy of Arenaviruses (Emerson and Purcell*,* 2006)

2.5 Structure and Genome of Arenaviruses

2.5.1 External structure

The virions of Arenavirus are spherical in shape and contain an envelope nucleocapsid. The envelope has surface projections, which surrounds two nucleopcapsids. The nucleocapsids contain polymerase complex and nucleoprotein complex. The surface projections are club-shaped peplomers that are spaced widely apart and cover the surface only. They are embedded in a lipid bi-layer that comprises the surface glycoproteins (GP). The surface projections are made up of one type of protein and are 8 - 10 nm long (Perez *et al*., 2003).

2.5.2 Arenavirus genome

The virus contains a beaded nucleocapsid with bisegmented negative RNA strands encoding four virus proteins in ambisense manner. The ssRNA ambisense genome of Arenaviruses is predominantly negative-sense except for a part of the 5' ends of both fragments. A complete genome of Arenaviruses extends about 10 000 to 11 000 nucleotides. The genome has a circular viral coded terminal protein, which is not covalently closed. The nucleotide sequences at the 3' terminus are largely complementary to the semial regions on the 5' end. Due to this complementarity, it is believed that 3' and 5' end base pair to form panhandle structures causing a circular nature of Arenavirus (Fig. 3). Unlike the 5' ends, the 3' terminus in Arenaviruses does not have a cap (Perez *et al*., 2003).

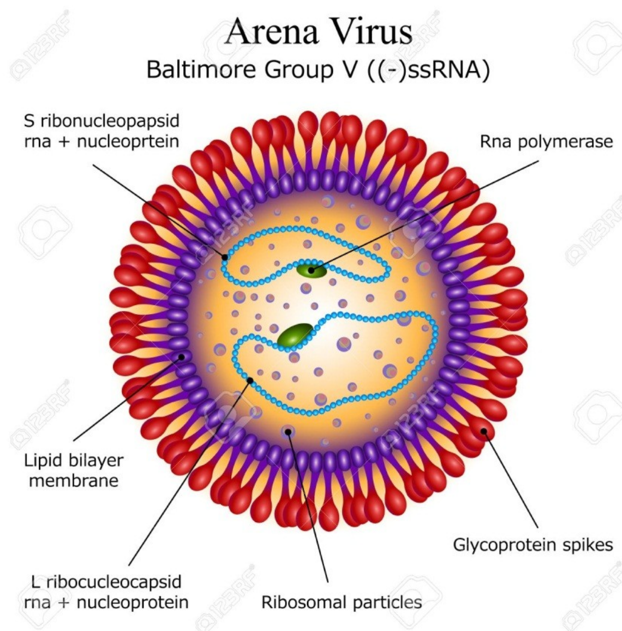

Figure 3: Diagram of Arenavirus particle structure (Emerson and Purcell, 2006)

The virus genome has two segments, the large segment called L and the small segment called S (Fig. 4). The small RNA segment S (~ 3.4 kb), encodes the glycoprotein precursor (GPC) and the nucleocapsid protein (NP), important immunogens of the virus. The large (L) segment (~ 7.2 Kb), encodes the small zinc-binding protein (Z) that functions as a matrix protein, interacting with L and NP and other host proteins, playing a role in viral transcription and replication (Eichler *et al.*, 2003; Shtanko *et al.*, 2010). The nucleoprotein gene is located on the 3’ end of the S RNA segment, while the polymerase gene is located at the 3’ end of the L RNA segment. On the 5’ ends of the S and L RNA segments exists the GPC and Z protein genes, respectively (Perez *et al.*, 2003). According to (Perez *et al.*, 2003), the nucleotide sequence at the 3’ terminus of the New and Old World Arenaviruses is conserved. This is important not only for its evolutionary implications, but also because the 3’ terminus of both S and L RNA segments are identical at 17 of their final 19 nucleotides.

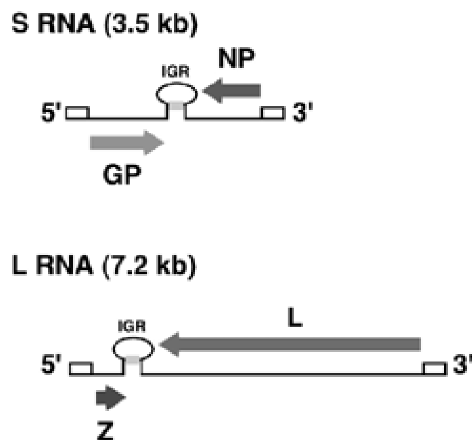

Figure 4: Diagram of the Arenavirus genome showing the S and L RNA segments encoding different viral genes. The arrows denote the coding strategy. Also shown are the non-coding intergenic regions (IGR) which form 1 - 3 stem-loop structures that play an important role in viral entry and transcription (Emerson and Purcell, 2006).

2.5.3 Arenavirus proteins

The Arenavirus genome encodes four proteins: NP, GPC, Z, and L. The most important structural protein in Arenavirions is the nucleoprotein (NP), which has a molecular size between 60 - 68 kd. The NP associates with virion RNA in an arrangement of bead like structures. It is also the predominant protein component of the nucleocapsid. It was shown in 1984 that the NP may become phosphorylated following an acute infection, and that this new form becomes increasingly prevalent in persistent infections (Bruns *et al*., 1984). Once a cell has become infected, NP has been shown to localize exclusively within the cell cytoplasm. The exception to this rule is that Pichinde NP contains a degradation fragment that localizes instead within the nucleus of infected cells. The precursor GPC protein, which is cleaved post-translationally into GP1 and GP2, is generally between 70 - 80 kd in size (Lenz *et al*., 2001; Beyer *et al*., 2003; Kunz *et al*., 2003). This cleavage

requires prior glycoslyation and must take place in the area surrounding the trans-Golgi network. The GP1 protein is cleaved from the amino-terminal side of the GPC while the GP2 protein is cleaved near an arginine-arginine motif which happens to be conserved in all Arenaviruses except for Tacaribe. No GPC proteins remain uncleaved and this explains why both glycoproteins are found to exist in virions in equimolar amounts.

The GP1 and GP2 are by no means, particularly similar to one another, however. GP1 is the peripheral membrane glycoprotein and it assembles into homotetrameric complexes held together through disulfide bonding. These GP1 complexes make up the globular head of glycoprotein spikes (Borrow *et al*., 1991). On the other hand, GP2 is the integral membrane of glycoprotein although, it does include a membrane spanning domain. Cross-linking studies have shown that highly charged residues at the carboxyl end of GP2 molecules interact with NP in the cytosol. Similar to the homotetrameric GP1 complexes, GP2 molecules form homotetramers and become the stalk portion of the glycoprotein spike. The GP1 and GP2 homotetramers bind one another through ionic interactions. The L protein is the largest protein by far encoded by the Arenavirus genome, with a molecular size between 180 - 250 kd (Djavani *et al*., 1997). The L gene site is responsible for encoding the viral polymerase, and the L protein is a component of the nucleocapsid. Alternatively, the Z protein is the smallest protein encoded by the Arenavirus genome. It has a molecular size of 11 kd. As mentioned earlier, the function of the Z protein is not entirely clear, however, it is believed to be a structural protein that is also a component of the nucleocapsid. Unlike NP which specifically localizes to the cytoplasm, Z protein has been shown to appear in the cytoplasm and the nucleus of LCMV-infected cells (Kyle., 2009).

2.6 Arenavirus Host Ranges

Several studies have investigated the transmission cycles of Arenaviruses (Lee *et al*., 2000; Enria *et al*., 2011; Ishii *et al*., 2011). According to Jay *et al*. (2005), humans are believed to be incidental hosts in the natural cycle of the virus. Rodents are the primary hosts of the virus, and play the major role in maintaining the virus. In addition to their natural rodent hosts, Arenaviruses can infect other animals, including rabbits, dogs, chickens, bats, and primates (Zapata and Salvato, 2013).

2.7 Arenavirus Transmission

2.7.1 Rodent-human transmission

There are 22 known Arenaviruses, but only 8 are known to cause human infection (Enria *et al*., 2011). Humans can be infected through mucosal exposure to aerosols, or by direct contact of abraded skin with urine, saliva, feces and nesting material from infected rodents. The incidental contact depends on the habits of both human and rodents. For example, when infected rodent species prefer a field habitat, human infection will likely be associated with agricultural work. In areas where the rodent species habitat includes human homes or buildings, infection commonly occurs in a domestic setting. Rodents frequently enter human dwellings in pursuit of food, shelter and warmth. Poor sanitation attracts rodents to invade peoples' dwellings, which increases the risk of acquiring Arenavirus infections. Pet trade can also be another source of transmission. In Europe, large outbreaks of Lymphcytic Choriomeningitis (LCMV) were linked to infected mice sold as pets (Pythoud *et al*., 2015). Climatic conditions can also influence the emergence and outbreak of Arenavirus infections. In Guinea, Liberia, Nigeria and Sierra Leone, the occurrence of Lassa fever increases during the late rainy or the early dry seasons, when the rodent population increases (mostly in February to April) (Jay *et al*., 2005).

2.7.2 Human-human transmission of Arenaviruses

Transmission of Arenaviruses between people may occur through direct contact with blood or other excretions from an infected individual (de Bellocq *et al*., 2010). Evidence shows that airborne transmission is also possible (Mendenhall *et al*., 2010). Increased migration can transfer Arenavirus infections from endemic areas to non-endemic areas. Recently, it was reported that organ transplant is another means of transmission of Arenavirus infections among humans (Lee *et al*., 2000; Fischer *et al*., 2006; Mendenhall *et al*., 2010).

Arenaviruses, such as Lassa and Machupo viruses, are associated with secondary person-to-person and nosocomial (health-care-setting) transmission. Contact with contaminated material such as medical equipment used in treating an infected patient can lead to transmission. Congenital infections have also been reported whereby the infant may present with a swollen baby syndrome (Childs *et al*., 1991; Lee *et al*., 2000; Briese *et al*., 2009). Lassa virus infection acquired during pregnancy is linked to abortion and high case fatality (Briese *et al*., 2009).

2.7.3 Rodent-rodent transmssion of Arenaviruses

The natural cycle of Arenaviruses occurs and is maintained within rodent populations, such that rodent species are chronically infected with the virus (Buchmeier *et al.*, 2007). Arenaviruses do not appear to cause any obvious illness in rodents. Some Arenaviruses are passed from mother rodents to their offspring during pregnancy, and hence remain in the rodent population generation after generation. Most of the infections transmitted between adult rodents are due to fighting, grooming, nasal secretion, milk from infected dams and inflicting bites. Viruses are shed into the environment through urine or droppings of infected hosts (Child *et al*., 1991; Nitatpattana and Chauvancy, 2000).

2.8 Symptoms of Arenavirus Infections in Humans

Illness due to Arenavirus infection in humans may be mild to severe or fatal. The incubation period is about 10 days. Many viral hemorrhagic fevers are clinically similar. Arenaviruses can cause acute hemorrhagic fevers associated with symptomatic fever, headache, general malaise, impaired cellular immunity, eventual neurologic involvement and hemostatic alterations that may ultimately lead to shock and death.

2.9 Pathogenesis of Arenaviruses

The first steps of every virus infection begin with interaction of a virus with its receptor molecules on the cell membrane and its subsequent entry into the host cell. These steps are important determinants of cellular tropism, host range and pathogenesis. To enter into the permissive host cell, Arenaviruses use the GP1 part of the glycoprotein spike for initial binding to the cell surface receptors (Borrow *et al*., 1991). Arenaviruses enter the cell by way of a large endocytic vesicle once they have attached to the cellular protein receptors.

According to Simone and Buchemeier (1995), viral nucleocapsids are delivered in the host cells by pH dependent fusion between virions and endosomal membranes. It is said to be pH dependent because GP1 and GP2 dissociate at an acidic pH and trigger fusion activity. After nucleocapsids enter the cytoplasm, viral replication (Fig. 5) is initiated by the L protein (the viral polymerase), forming the nucleoprotein mRNA which is sometimes seen as early as two hours after infection. NP typically makes its appearance sometime between 6 - 12 hours. The NP protein, L protein and genomic RNA combine to form nucleocapsids, which consequently arrange host cell ribosomes to virion partices. Viral receptor binding represents an important target for development of antiviral drugs.

The mechanism of entry of Old World Arenaviruses and the New World Arenaviruses is different. They use different primary receptors for attachment onto a host cell. The Old World Arenaviruses use alpha-dystroglycan receptor, while human New World Arenaviruses use cargo receptor transferrin receptor 1. According to a study conducted by Borrow and Oldstone in 1992, New World Arenaviruses Junin virus (JUNV) enters cells via a clathrin-dependent pathway, while the Old World Arenavirus LCMV uses a clathrin-independent pathway. Most recent studies suggest cell entry of LCMV and Lassa virus (LASV) uses a cholesteral dependent endocytotic pathway (Shah *et al.*, 2006; Vela *et al.*, 2007; Rojek *et al.*, 2008).

Generally, entry of the virus into the human body and a primary replication of the virus in the reticulo-endothelial system is usually followed by viremia. In severely affected individuals, endothelial cell damage causes erythrocytic and platelet dysfunction, which leads to increased vascular permeability, capillary leakage, and altered cardiac function. Cytokines and other soluble mediators probably contribute to the pathogenesis and dysfunction of the vascular endothelium. Death is believed to be due to hypovolemic shock and vascular leakage (Lee *et al*., 2000; Briese *et al*., 2009; Schattner *et al*., 2013).

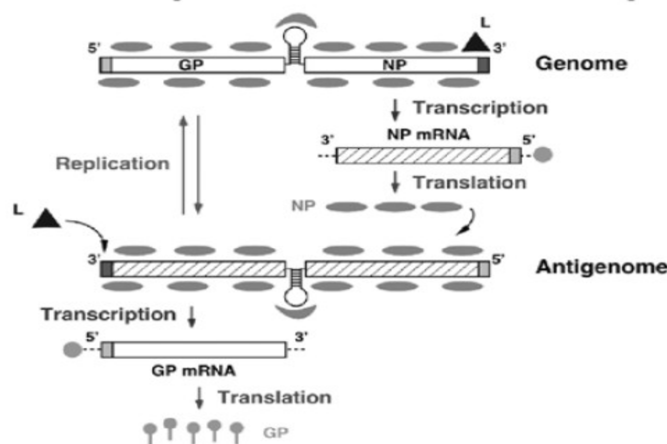

Figure 5: Basic Arenavirus replication and transcription for the S segment (Emerson *et al*., 2006)

2.10 Detection of Arenaviruses

Currently, there is no gold standard for Arenavirus detection. Serology-based assays, such as enzyme-linked immunosorbent assays (ELISAs) are often used to detect IgM or IgG. Immuno-flourencent assays are also used (Hideki *et al*., 2012). Drawbacks in these tests are cross reaction between Arenaviruses and long assay times. In contrast, virus neutralization tests are highly specific, but neutralizing antibodies appear too late to be useful in immediate diagnosis. For example, patients with Lassa fever do not usually develop neutralizing antibodies until after they become ill. Arenavirus antigens can be detected in the blood or tissues using antigen-capture ELISAs (Gunther *et al.*, 2009).

Virus isolation can be done through cell culture, particularly vero cells. The presence of Arenavirus can be confirmed with immuno-chemical assays or RT-PCR. Arenaviruses can also be isolated in laboratory rodents such as suckling mice, guinea pigs or newborn hamsters. However, virus isolation requires maximum containment laboratories that are not always available. Molecular assays such as reverse transcription polymerase chain reaction (RT-PCR) do not require cell culture and results are available more quickly, but they are beyond resources and capabilities of many laboratories in developing countries. RT-PCR can detect viral nucleic acids in the blood or cerebral spinal fluid in the case of lymphocytic choriomeningitis virus (de Bellocq *et al*., 2010). There are some PCR tests that detect a wide range of Arenaviruses while others are more specific.

2.11 Treatment

Currently, there are limited therapeutic options for the treatment of Arenavirus infections. The only licensed drug for the treatment of human Arenavirus infection is ribavirin (Lee *et al*., 2000). Ribavirin reduces the mortality rate of people infected with Lassa virus and

Junin virus when administered at an early stage, soon after the onset of clinical signs. However, ribavirin is associated with significant toxicity (Lee *et al*., 2000). Other supportive treatment used in the treatment of Arenaviruses is human immune plasma, used to treat argentine hemorrhagic fever caused by Junin Arenavirus. It can decrease the mortality rate from 20 - 30 % to 1 - 2 %. Immune plasma is effective in patients with Bolivian hemorrhagic fever caused by Machupo virus. Otherwise, supportive care related to blood pressure monitoring and careful attention to fluid and electrolyte balance can also be life saving.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The present study investigated the presence of Arenaviruses in six selected sites in Tanzania characterized by high wildlife-human interaction. These sites included Ruaha, Kilombero, Mtwara, Mbeya, Mbinga and Mikumi (Fig. 6). Study sites were selected based on the knowledge of human-animal contact, geographic characteristics and accessibility of the locations. Rodents were captured in areas associated with human-rodent interactions called interfaces. Samples were collected in five interfaces, which were; near homes (peridomestic interface), areas where wild animals are slaughtered, killed and consumed (hunting interface), areas around highway (high traffic interface), and in crop fields (crop raiding interface) (as described in the literature review). Primary interfaces were interfaces where sampling was done.

Ruaha is an area characterized by a landscape dominated by the great Ruaha river, which is located in an ecotone zone, where the northern Sudano-Sahelian vegetation communities merge with the southern Zambezian (miombo) communities. Ruaha lies within latitude 8° 54′ 0″ South of the Equator and longitudinally 36° 43′ 0″ East of Greenwich. It is normally a hot and dry area. Temperatures can go as high as 36 °C. The area is characterized by the presence of rivers, streams, crop farms, short grass and long grasses. The primary interfaces in this site were crop raiding interface and peridomestic.

Kilombero is located in a large flood-plain, between Kilombero river in the south-east and the Udzungwa-Mountains in the north-west. On the south-east part of Kilombero river,

Ulanga District is situated. Kilombero lies within latitude 83° 1′ 0″ South of Equator and longitudinally 35° 56′ 0″ East of Greenwich. The area is predominantly rural with the semi-urban district head-quarters. It experiences moderate seasonal rainfall. Majority of the people around this area are subsistence farmers of maize and rice. There are large plantations of teak wood in the Kilombero and the neighbouring Ulanga districts. In the north-west of the district, Illovo Sugar Company's sugar-cane plantations occupy most of the lowlying area. The primary interface in this site was crop raiding interface.

Mtwara Region is one of Tanzania's 30 administrative regions. The boundary with Mozambique to the south is formed by the Ruvuma river. To the west, Mtwara is bordered by Ruvuma Region, to the north by Lindi Region, and to the east is the Indian Ocean. Mtwara lies within latitude 10° 16′ 25 ″ South of Equator and longitudinally 40° 10′ 58″ East of Greenwich. On average, the temperatures are always high in Mtwara ranging from 32 °C to 34 °C. The area is mostly rural covered by short and long grasses. Majority of people in this area hunt, trap and consume rodents as a main source of protein in their diet especially in the Makonde community. The primary interface in this site was hunting interface.

Mbeya Region is located on the south-west of Tanzania mainland, commonly known as Southern highland. To the north, it shares borders with Tabora and Singida regions and on the east borders Iringa Region. To the south it shares borders with Ruvuma while on the west there is Zambia and Malawi countries. It is accessible by road and TAZARA railways from Dar es Salaam through Pwani, Morogoro and Iringa regions. Mbeya lies within latitude 08° 54′ 00″ South of Equator and longitudinally 33° 27′ 00″ East of Greenwich. It is generally tropical marked by seasonal and altitudinal temperature

variations and sharply defined dry and rainy seasons. Temperature averages range between 16 °C in the highlands and 25 °C in the lowland areas. It is characterized by areas with high rainfall and fertile soils, with a lot of agricultural production, animal husbandry and fishing activities. The primary interfaces in this site were peridomestic interface and crop raiding interface.

Mikumi is located near Morogoro and nestled between the Uluguru mountains to the northeast, and the Ruebo mountains, Udzungwa mountains, and the Rift escarpment to the south west. The landscape of Mikumi to the north-west is characterized by the alluvial plain of the river basin Mkata. Mikumi lies within latitude 07° 24′ 26″ South of Equator and longitudinally 36° 58′ 20 ″ East of Greenwich. The average temperature in Mikumi is 24.0 °C and about 1132 mm of precipitation falls annually. The vegetation of this area consists of savannah dotted with acacia, baobab, tamarinds, and some rare palm. The southeast part of the park is less rich in wildlife, and not very accessible. The primary interface in this site was high traffic or transport interface.

Mbinga District is one of the five districts of the Ruvuma Region of Tanzania. It is bordered to the north by the Njombe Region, to the east by Songea Rural District and Songea Urban District, to the south by Mozambique and to the west by Lake Nyasa. Mbinga area lies within latitude 10° 49′ 60″ South of Equator and longitudinally 34° 49′ 60″ East of Greenwich. It is characterized by mild temperature averaging 23 °C depending on altitude and season. It experiences adequate rainfall annually. The vegetation consists of woodland, bushland thicket and grasslands. Common vegetation in the area comprises planted trees, bamboos and shrubs. However, much of the natural vegetation is currently being depleted through human activities such as coffee plantation, maize farms, firewood, timber, charcoal, tobacco curing and brick making as well as road construction. The primary interface in this site was hunting interface.

Figure 6: A map of Tanzania showing sites where rodent sampling was performed in this study. Rodents were sampled in Ruaha, Kilombero, Mtwara, Mbeya, Mbinga and Mikumi site.

Source : United Republic of Tanzania (2014). [http//www.lib.utexas.edu] site visited on 30 / 8 / 2015

3.2 Study Design

The design of the present study was cross sectional, and study animals were rodents, shrews and elephant shrews. The target sample size was estimated according to Borremans *et al*. (2011) using the following formula:

$n = (z\alpha / 2)^2 \times pq \div d^2$

Where: n = required sample size, α = probability of type 1 (0.05 sided), p = estimated prevalence or proportion of Arenavirus infection (8.4 %) (Borremans *et al*., 2011).

Q = 1 - p, d = Margin of error / allowable error in estimating prevalence for expected confidence level, z 0.05 / 2 = z 0.025 = 1.96 at 95 % confidence interval

$n = 1.96^2 \times (0.084\ (1 - 0.08)) / 0.05^2$

n = 118

Using this formula, the required sample size was 118 animals.

3.3 Rodent Trapping

Rodents, shrews and elephant shrews were trapped in the six selected sites (Fig. 6). At each site, Sherman box traps (H.B. Sherman, FL, USA) baited with a mix of peanut butter and maize flour were set near vegetation and houses (peridomestic interface), in crop fields (crop raiding interface), in areas where rodents, shrews and elephant shrews were commonly hunted for food (hunting interface), and near a main highway (high traffic interface). Traps were set in the evening and checked early in the morning to minimize heat stress experienced by the animals. Within each site, trapping was conducted at multiple locations to represent the diversity of human-wildlife contact interfaces. As we were not focusing on determining rodent, shrew and elephant shrew density and abundance, traps were set opportunistically in transects at each site. Trap placement was roughly 10 m apart in the trap lines targeting areas with vegetation cover. Approximately

100 traps were set at each site per trap night. Traps were set for up to three nights at each site.

Captured rodents, shrews and elephant shrews were anesthetized with isoflurane diffusion anesthesia (Abbott House, Vanwall Business Maidenhead, UK). Sterile, synthetic swabs (Hardwood Production Company, LP, Guilford, Maine) were used to collect oropharyngeal samples. Duplicate oropharyngeal swabs were stored in separate cryovials, one containing one milliliter of lysis buffer (Biovision incorporated, California, United States) and another containing one milliliter viral transport media (VTM) (Remel Europe Ltd, Dartford, UK). For animals that defecated during sampling, approximately 200 mg of fresh fecal material was collected and similarly stored in cryovials containing lysis buffer and VTM. If the animal did not defecate, two rectal swabs were collected with one swab stored in VTM another in lysis buffer. Samples were labelled with animal identification numbers and kept in cryovials, which were stored in liquid nitrogen dry shippers at - 196 °C in the field prior to long term storage at - 80 °C at the Sokoine University of Agriculture (SUA) wildlife viral diagnostic laboratory. Weight, tail length and other body measurements of the animals were taken to help with species identification. The species, age class (juvenile, sub adult, or adult), and sex of the rodents were also recorded. Afterwards, rodents, shrews and elephant shrews were released back in areas where there were originally trapped.

3.4 Determination of Age, Sex and Species of Rodents, Shrews and Elephant Shrews

Rodents, shrews and elephant shrews were grouped into three classes; juveniles, subadults and adults. To determine age classes (juvenile, subadult, or adult), animals from different taxonomic groups were considered separately. Classification was based on physical

appearance, morphometric measurements, and weight for each age group. For example, *Mastomys* sp. rodents were evaluated for age according to characteristics of their genus rather than comparing them to animals from other groups such as *Praomys* sp. (Olenev, 2009).

Sexes were identified based on morphological features namely; urogenital distance, nipples, and testicles. Urogenital distance is the distance between a rodent's genital area and its anus. Females have a genital area (the vagina or urethra) that is much closer to the anus (the opening directly under the tail) while males have a genital area (penis) that is much further away from the anus than in females. To validate this observations, females were further examined on the stomach area to confirm the presence of nipples. For the case of males observation was further made to determine the presence of testicles.

Captured animals were identified to the lowest taxonomic level possible using taxonomic nomenclature (Kingdon, 1974, 1997; Wilson and Reeder, 2005). For rodents, identification was completed to genus level for most animals. The common *Mastomys natalensis* were identified to species level. Shrews were also identified to the species level. Since the four-toed elephant shrews have very distinct features (Fig. 7) for physical identification, they were identified to species level: *Petrodromus tetradactylus* (Emmons and Feer, 1990; Richard *et al.*, 1999).

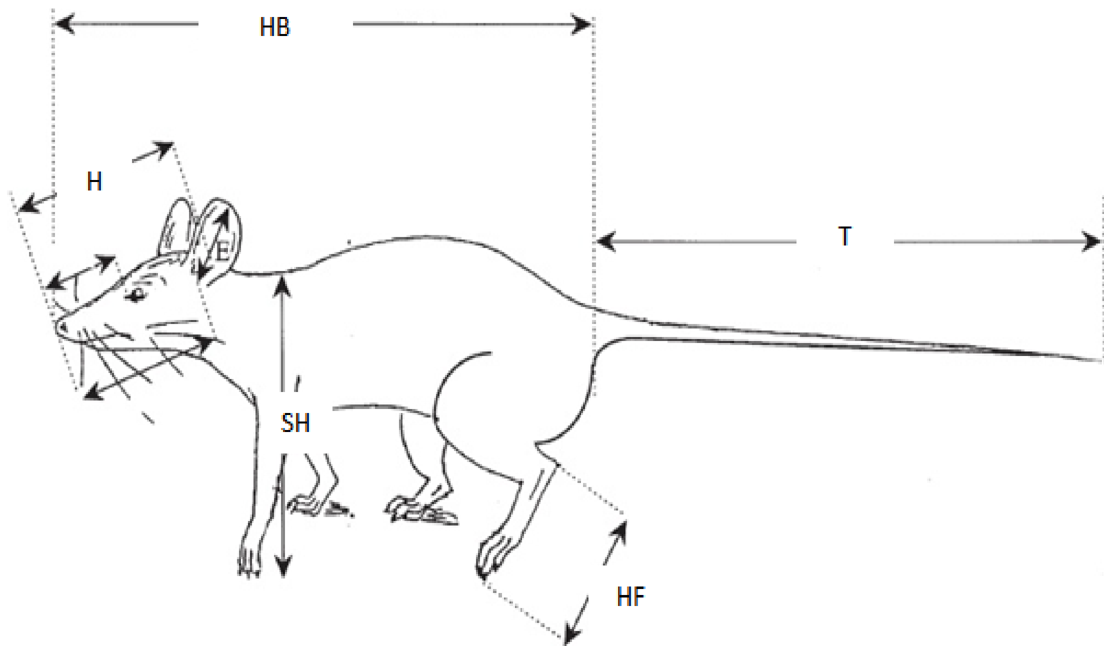

Figure 7: Anatomical features for identification of rodents, shrews and elephant shrews. The drawing shows measurements that were taken on rodents, shrews and elephant shrews to identify the species, as previously reported by Emmons and Feer (1990). HB is the head and body length, T is the tail length, HF is the hind foot length, and E is the ear length.

3.5 Laboratory Detection of Arenaviruses

Laboratory procedures were conducted at the PREDICT molecular laboratory at Sokoine University of Agriculture. The detection of Arenavirus RNA was done using standard PCR techniques as previously described by Lozano *et al*. (1997). Briefly, the procedures included the extraction of viral RNA from oropharyngeal and fecal specimens followed by cDNA synthesis. The obtained cDNA was used in the detection and genetic characterization of Arenaviruses using RT-PCR, sequencing and phylogenetic analysis. To screen for the presence of RNA in extracted oropharyngeal and fecal swabs, a beta actin PCR was performed. Moreover, to check whether contamination of positive samples occurred from the universal positive control, a contamination control PCR was performed.

3.5.1 RNA extraction and purification

RNA was extracted using the QIAmp viral mini kits (QIAGEN, Valencia, CA, USA), following the manufacturer's protocol (Appendix 1). The procedure involved lysis of cells in the samples using lysis buffers provided in the kit and purification of RNA using spin columns coated with silica membranes. The adherence of pure RNA on the walls of the silica membranes, coupled with a series of wash and centrifugation steps, enabled the separation of pure RNA from contaminants and lysed cell debris. Pure RNA was eluted in low salt buffer AVE and immediately stored at - 80 °C until use.

3.5.2 cDNA synthesis

Complementary DNA synthesis from fully spliced messenger RNA was performed in a reaction catalyzed by a reverse transcriptase (SuperScript® III) (Invitrogen, Carlsbad, CA). The optimized standard procedures used for cDNA synthesis are found in Appendix 2. Complementary DNA synthesis master mix was prepared in two steps, a master mix 1 containing random hexameric primers, dNTPs and the RNA template, and a second master mix (mix 2) containing reverse transcriptase, RNase inhibitor and buffers (Table 1 and 2). Mix 1 was first incubated at 65 °C for 5 min followed by chilling at 4 °C. Afterwards, mix 1 was mixed with mix 2 followed by cDNA synthesis using an amplification profile shown in Fig. 8.

Table 1: cDNA synthesis reaction mixture (Mix 1)

No.	Component	Volume (µl)
1.	50 ng / µl Random hexamer	1.0
2.	10 Mm dNTPs	1.0
3.	RNA template	8.0
	Total volume per reaction	10.0

Table 2: cDNAsynthesis reaction mixture (Mix 2)

No.	Component	Volume (µl)
1.	10 x RT buffer	2.0
2.	25 mM $MgCl_2$	4.0
3.	0.1 M DTT	2.0
4.	RNase OUT	1.0
5.	Superscript III reverse transcriptase	1.0
	Total volume per reaction	10.0

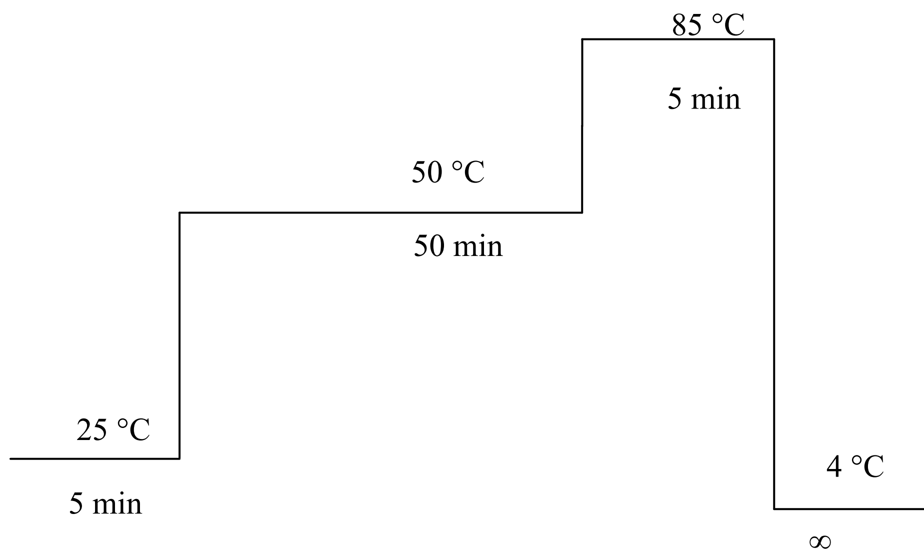

Figure 8: Cycling conditions for cDNA synthesis for mixture 2. Hexameric primers were annealed at 25 °C for 5 mins followed by reverse transcription at 50 °C for 50 mins. Afterwards, the reverse transcriptase was inactivated at 85 °C for 5 mins followed by chilling at 4 °C.

3.5.3 Beta actin PCR

Beta actin PCR was done to amplify beta actin genes present in the extracted RNA in order to verify successful extraction. Complementary DNA was used as a template in this PCR. Since beta actin genes are continuously transcribed into mRNA in animal cells, it was expected that all samples will be positive regardless of whether the specimens

contained Arenaviruses or not. Primers used were: Actin Forward (5’ - ACC TGA CWG ACT ACC TCA TGA AG - 3’) and Actin Reverse (5’ - GCT TGC TGA TCC ACA TCT GCT G - 3’), to initialize the amplification of beta actin genes. Master mix preparation was done according to Table 3. The proportion of each reagent in the master mix was measured according to a standardized protocol (Appendix 3) optimized by our laboratory in reference to the user manual that came with the kit. Cycling conditions are illustrated in Fig. 9. Afterwards, PCR products were stored at - 80 °C.

Table 3: Beta actin PCR reaction mixture

No.	Component	Volume (µl)
1.	10 x PCR buffer	2.50
2.	50 mM $MgCl_2$	0.75
3.	10 mM dNTPs	0.50
4.	10 µM Actin Forward Primer	0.50
5.	10 µM Actin Reverse Primer	0.50
6.	Platinum *Taq* DNA Polymerase	0.10
7.	Nuclease free water	19.15
8.	cDNA template	1.00
	Total Volume per reaction	25.00

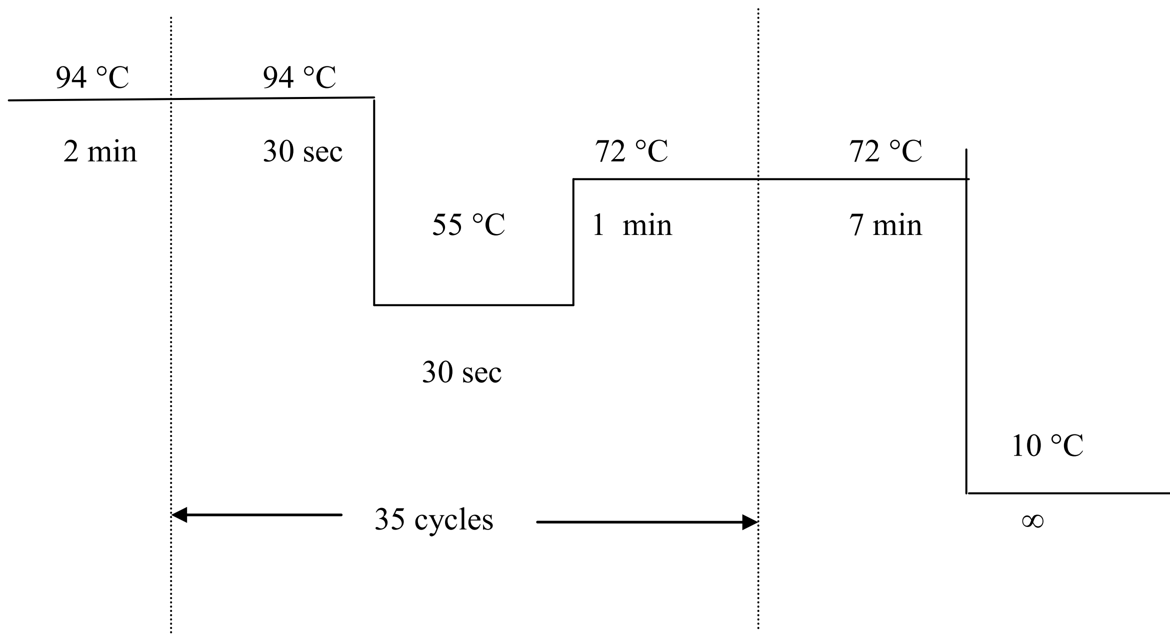

Figure 9: Cycling conditions for amplification of beta actin genes in collected fecal and oropharyngeal specimen. The PCR included an initial denaturation step at 94 °C for 2 mins followed by 35 cyles of denaturation (94 °C, 30 sec) annealing (55 °C, 30 sec) and extention (72 °C, 1 min). Final extention at 72 °C for 7 mins was followed by chilling at 10 °C.

3.5.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to distinguish and separate DNA fragments of different sizes. PCR products were separated on a 1.5 % agarose gel in 0.5 % TBE buffer (SERVA Electrophoresis, Heidelberg, German) stained with gel red (a nucleic acid stain) (Phonex Research Products, Candler, USA). Each well was loaded with 10 µl of the PCR product and 2 µl of blue-orange 6 x DNA loading dye (Promega, Madison, USA). Samples were separated along with a 2000 bp DNA ladder (Bionexus, Oakland, CA) at 100 V for 90 mins. Separated PCR products were visualized using a gel documentation system (Uppland, CA, USA).

3.5.5 Arenavirus detection using Arenavirus specific polymerase chain reaction

The screening for Arenaviruses was done at the family level in order to detect known and unknown viruses. Platnum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, US) was used for amplification. The primers were custom made by Invitrogen to amplify the S gene of the virus genome. The PCR involved two separate (non-nested) reactions (Lozano *et al.*, 1997), whereby the first reaction involved a primer set, ARS16V and ARS1, whose sequences were 5' - GGC ATW GAN CCA AAC TGA TT - 3' and 5' - CGC ACC GGG GAT CCT AGG C - 3', respectively. The second reaction involved the use of primers, ARS3V and ARS7C-Mod whose sequences were 5' - CAT GAC KMT GAA TTY TGT GAC A - 3' and 5' - ATR TGY CKR TGW GTT GG - 3', respectively. Two products were expected at the end of the PCRs and positive samples from reaction 1 were expected to have a band size of 640 bp. In the second reaction, positive samples were expected to have a band size of 460 bp. The reactions were carried at a total volume of 25 µl in each reaction tube, containing 1 µl of cDNA (Appendix 4). The universal control used was a plasmid containing a DNA sequence of approximately 420 bp. This universal positive control (1µl diluted to 1000 µl of nuclease free water) was designed with a tag of nucleotides that correspond to an amino acid sequence spelling P-R-E-D-I-C-T. A sample blank containing PCR reagents and nuclease free water was used as a negative control. Samples were electrophoresed at 100 V for 90 mins in agarose gel followed by gel documentation. Table 4 illustrates the contents of Arenavirus reaction 1 and 2 mixtures. The PCR cycles for reaction 1 reaction 2 were performed in a Techne TC 4000 thermocycler (Applied Biosystem 850, California, US). Cycling conditions are illustrated in Fig. 10.

Table 4: Arenavirus PCR reaction mixture

		Volume (µl)	
No.	Component	Reaction mixture 1	Reaction mixture 2
1.	10 x PCR buffer	2.50	2.50
2.	50 mM $MgCl_2$	0.75	0.75
3.	10 mM dNTPs	0.50	0.50
4.	10 µM ARS16V primer	1.00	-
5.	10 µM ARS1 primer	1.00	-
6	10 µM ARS3V primer	-	1.00
7	10 µM ARS7C primer	-	1.00
8	Platinum *Taq* DNA Polymerase	0.10	0.10
9	cDNA template	1.00	1.00
10	Nuclease free water	18.15	18.15
	Total volume per reaction	25	25

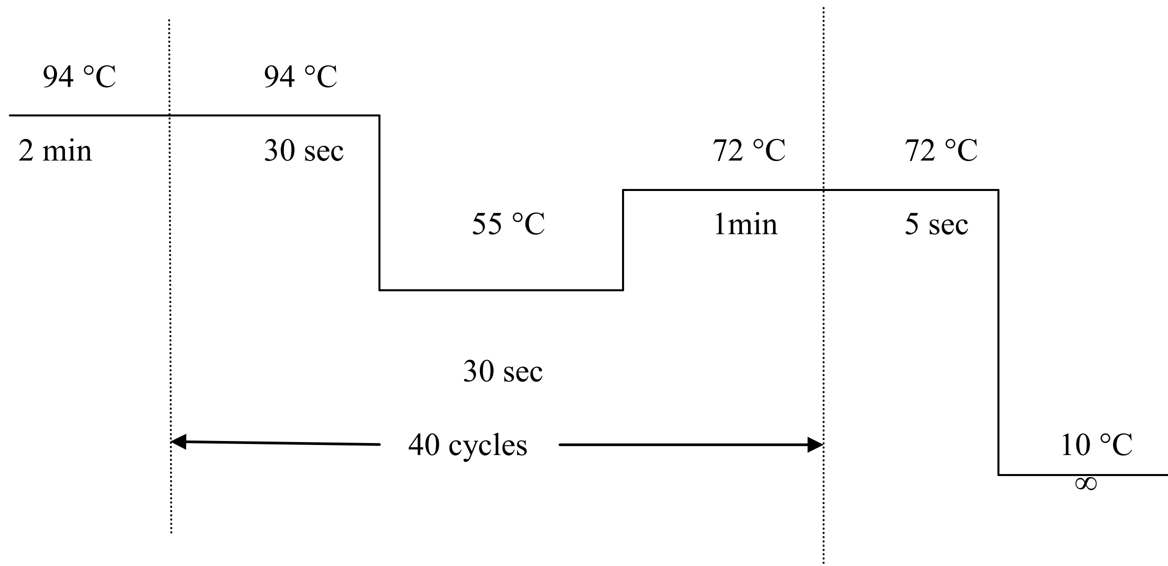

Figure 10: Cycling conditions for amplification of Arenavirus virus genes in oropharyngeal and fecal specimen. The PCR included an initial denaturation step at 94 °C for 2 mins followed by 40 cyles of denaturation (94 °C, 30 sec) annealing (55 °C, 30 sec) and extention (72 °C, 1 min). Final extention at 72 °C for 7 mins was followed by chilling at 10 °C.

3.5.6 Contamination control PCR

Primers specific to the universal control were used in the contamination control PCR to determine if suspect positives resulted from contamination by the universal positive control. The primers used were PREDICT forward primer (5' - GGG CCT AGA GAA GAT ATT TGT ACT - 3′) and PREDICT reverse primer (5' - CGC CAT TGA CAT CCT CGA AG - 3′). The polymerase chain reaction was performed using invitrogen platinum Taq PCR kit (Life Technologies, Grand Island, USA) was used to provide reagents needed for the PCR. All presumptive positive samples were diluted at a ratio of 1 : 5 in RNase free water before they were added to the PCR mixture. Contents of the contamination control PCR reaction mixture and cycling condition are shown in Table 5 and Fig. 11, respectively. Afterwards, PCR products were electrophoresed at 100 V for 90 mins, on a

1.5 % agarose gel stained with gel red, and visualized using gel documentation system (Uppland, CA, USA).

Table 5: Contamination control PCR reaction mixture

No	Component	Volume (µl)
1.	10 x PCR buffer	2.50
2.	50 mM $Mgcl_2$	0.75
3.	10 mM dNTPs	0.50
4.	10 µM PREDICT Forward Primer	1.00
5.	10 µM PREDICT Reverse Primer	1.00
6.	Platinum *Taq* DNA Polymerase	0.10
7.	Arenavirus positive sample	1.00
8.	Nuclease Free water	18.15
	Total volume per reaction	25

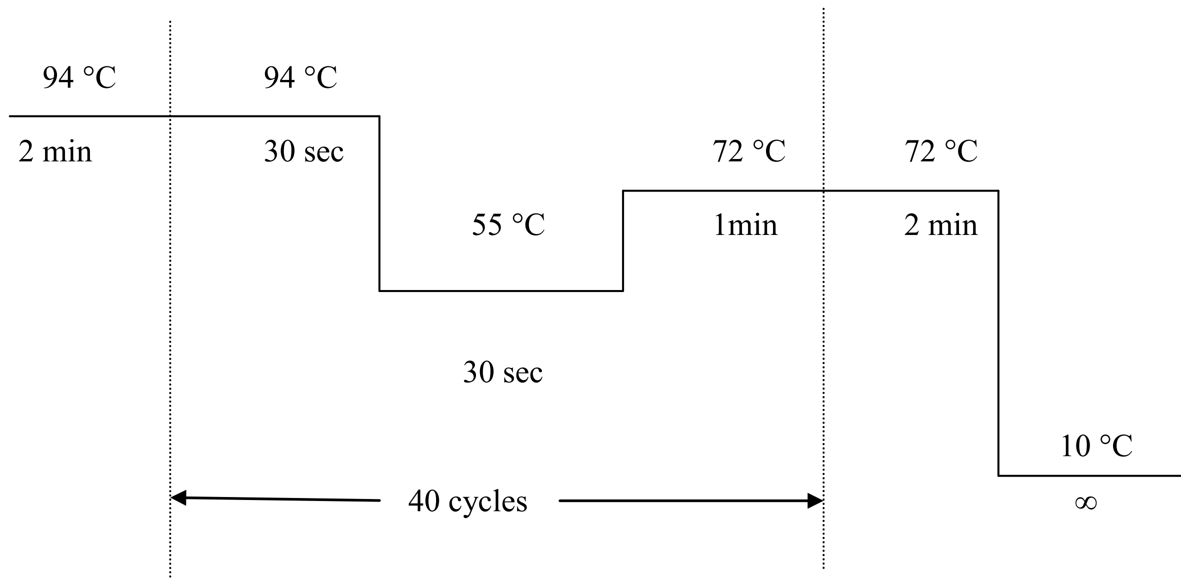

Figure 11: Contamination control PCR conditions. The PCR included an initial denaturation step at 94 °C for 2 mins followed by 40 cyles of denaturation (94 °C, 30 sec) annealing (55 °C, 30 sec) and extention (72 °C, 1 min). Final extention at 72 °C for 7 mins was followed by chilling at 10 °C.

3.5.7 Extraction of the desired DNA fragment from Arenavirus agarose gel

Presumptive positive samples, which were not contaminated with the universal control, were sliced out of the Arenavirus PCR gel, and DNA extraction was done using the Millipore extraction kit (Millipore Corporation, 80 Ashly Road, Bedford, USA). The excised gel samples were each placed in a pre-assembled device containing a filter device, agarose gel nebulizer, a micro centrifuge tube and a modified Tris Acetate buffer. Samples were centrifuged at 5,000 x g for 10 mins. The extracted DNA was then stored in the capped filtrate vial in at - 80 °C.

3.5.8 Transformation of desired DNA fragments into competent cells and cloning

Cloning was done using the TOPO TA cloning kit (Life Technologies, Grand Island, USA). A TOPO cloning reaction was set up by mixing DNA extracted from the gel and a

TOPO vector. Salt ($MgCl_2$) was included in the mixture to increase the number of transformants by 2 - 3 folds. The reaction was incubated at room temperature for 20 mins and then placed on ice prior to transformation into one shot competent cells.

The transformation of the TOPO cloning reaction into one shot competent cells involved addition of 2 µl of the TOPO reaction mixture into a vial of one shot chemically competent *E. coli*, followed by incubation on ice for 20 mins and heat shock for 30 seconds at 42 °C without shaking. Tubes were immediately transferred on ice. Two hundred and fifty microlitre of super optimal broth with catabolite repression (S.O.C) medium was added into the reaction mixture at room temperature and the tube was tightly capped and shaken horizontally at 200 revolutions per minute for an hour at 37 °C. One hundred microlitres of the transformation mixture was spread on a pre-warmed selective plate and plates were incubated at 37 °C. After 8 hours, colonies were observed. At least 10 colonies were picked from each plate for analysis.

3.5.9 Analyzing positive transformants by M13 PCR

Primers used in this reaction were 2 µM M13 forward primer 5' - GTA AAA CGA CGG CCA G - 3' and M13 reverse primer 5′- CAG GAA ACA GCT ATG AC - 3′. A high fidelity master mix was used as indicated in the TOPO TA kit manual. The procedures involved aliquoting 48 µl of PCR super master mix into a 0.5 ml micro-centrifuge tube and adding 1 µl of the forward and reverse primers. Ten colonies were suspended individually in 50 µl of the PCR reagent mix and incubated for 10 mins at 94 °C to lyse the cells and inactivate the nucleus. This was followed by annealing at 50 °C for 40 Sec, elongation at 72 °C for 90 sec conducted for 30 cycles. The final extension involved incubation at 72 °C for 10 mins and stored at 4 °C. Visualization was done by gel electrophoresis.

3.5.10 ExoSAP IT PCR product clean up and DNA sequencing

Polymerase chain reaction products obtained from amplification of DNA fragments cloned onto M13 vectors were purified using exonuclease I and shrimp alkaline phosphatase (EXOSAP-IT). The enzymatic digestion of unused primers and dNTPs was performed at 37 °C for 5 mins. The enzymatic digestion was stopped by inactivating the enzyme at 80 °C for 15 mins. Afterwards, a sequencing PCR was performed using M13 primers using a Big Dye Terminator kit V 3.0 (Applied Biosystems, Foster City, CA). Sequencing PCR products were separated in an ABI 3730 Genetic Analyser (ABI, Foster city, CA).

3.6 Data Analysis

Fishers exact'stest was used to compare the detection of Arenaviruses among different age groups and sex of rodents, shrews and elephant shrews. Similarly, chi-square was used in determining the difference in detection between oropharyngeal and fecal specimen at a critical probability of $P < 0.05$. Comparison of similarity of sequences obtained in this study with sequences available at the National Center for Biotechnology Information (NCBI) database was performed using BLASTn. Multiple alignment of selected Arenavirus S gene sequences obtained from NCBI with sequences of Arenavirus obtained in this study was done using MEGA 6.0. A phylogenetic tree was constructed using the maximum likelihood model. Bootstrapping simulation was employed to analyze the strength of support for each clade or monophyletic group. Seven sequences retrieved from GenBank (Table 6) and those identified in this study, were used in constructing the phylogenetic tree (Table 6).

Table 6: Arenavirus strains retrieved from the GenBank used in the phylogenetic tree construction

Strain Type	Origin and year	Accession number	Reference
Morogoro virus strain 3017/2004	Tanzania, 2008	DQ3288777	Gunther *et al*., 2009
Mopeia virus strain AN 21366-BN1	Mozambique, 1991	M33879	Gunther *et al*., 2009
Mobala virus strain ACAR 3080 MRC5 P2	Ethiopia, 2006	AY342390	Emonet *et al*., 2006
Luna virus strain LSK-2	Zambia, 2011	AB693148	Ishii *et al*., 2011
Lassa virus strain LASV 253-NIG-2011	Nigeria, 2011	KM822045	Sogoba *et al*., 2012
Ippy virus strain Dak An B 188 d	Central African republic, 2006	DQ328877	Emonet *et al*., 2006
Gbagroube virus strain CIV 674	Cote d'lvoire, 2011	GU83085	Coulibaly-N'Golo *et al*., 2011
Lymphocytic Choriomeningitis Virus	Spain, 2012	JN8724951	Perez-Ruiz *et al*, 2012
Wenzhou virus	China, 2014	KM051422	Li *et al.*, 2014
Gairo virus	Tanzania, 2015	KJ85661	Gryseels *et al*., 2015

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence of Arenaviruses in Rodent, Shrew and Elephant Shrew Species

4.1.1 Molecular detection of Arenaviruses in rodent, shrew and elephant shrews

A total of 121 fecal specimens and 121 oropharyngeal specimens obtained from rodents, shrews and elephant shrews were screened for the presence of beta actin RNA. All samples were found positive (Fig. 12). Twelve specimens (7 fecal and 5 oropharyngeal specimens) from Arenavirus PCR conducted using ARS16V and ARS 1 primers, were found positive (Fig. 13). There were no Arenavirus positive specimens identified in Arenavirus PCR conducted using ARS3V and ARS7C-Mod primer. Twelve Arenavirus positive specimens identified in Arenavirus PCR reaction ARS16V / ARS1, were tested for universal positive control contamination. None of the samples were found to be contaminated. Sequencing results based on partial sequences of the S gene (glycoprotein precursor gene and nucleoprotein gene), confirmed the 12 specimen were Arenavirus positive. Sequences of Arenavirus S gene obtained from this study are indicated in Appendix 5.

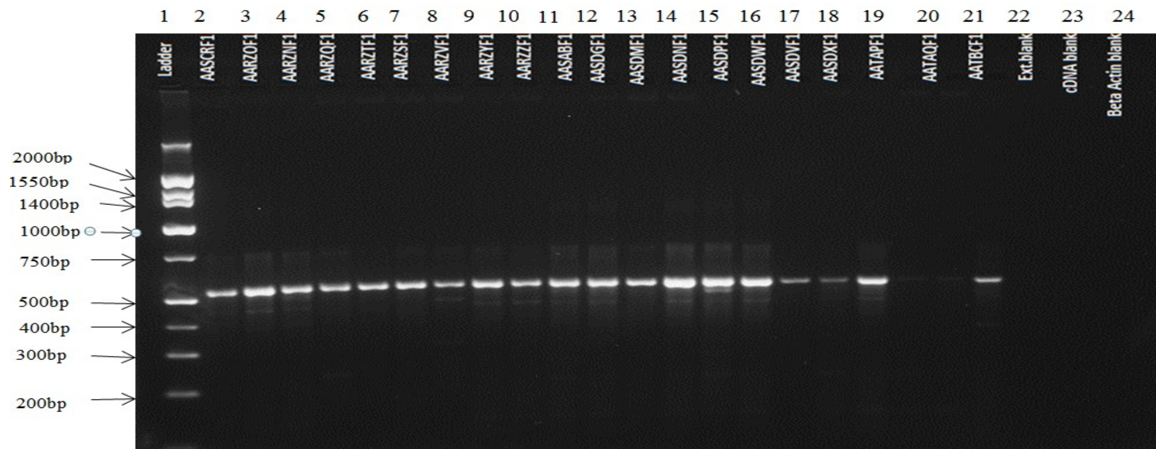

Figure 12: Detection of beta actin house keeping RNA in fecal and oropharyngeal swabs obtained from rodents, shrews and elephant shrews. A gel picture showing beta actin gene amplicons with band sizes of approximately 528 bp visualized under a UV trans-illuminator. The first lane contains a DNA marker, lane 2 - 21 contain samples, while lane 22 - 24 contain negative controls used in beta actin PCR, extraction and cDNA synthesis, respectively as labelled on the gel picture.

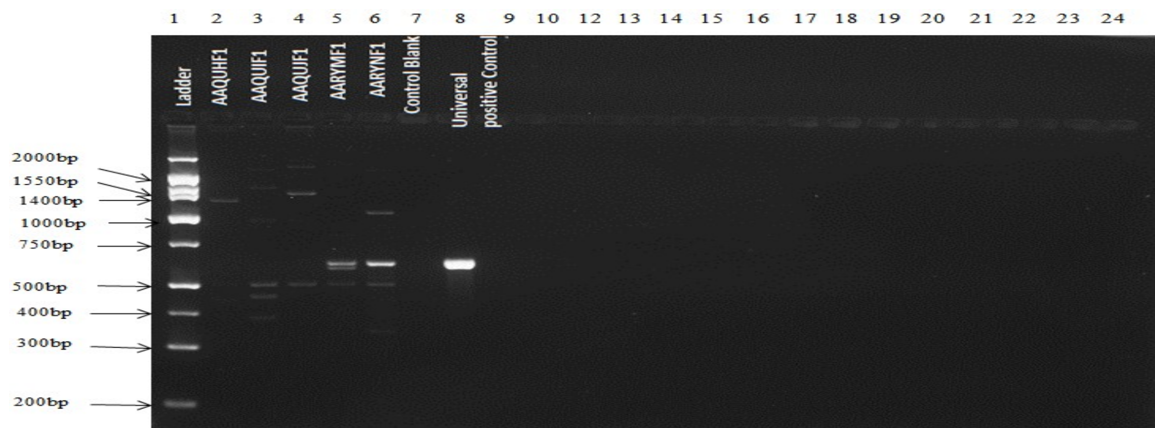

Figure 13: Detection of Arenavirus in fecal and oropharyngeal swabs from rodents, shrews and elephant shrews. A gel picture showing Arenavirus PCR products after a PCR using ARS16V / ARS1 primers, visualized under a UV trans-illuminator with two samples showing band sizes of approximately 640 bp, where lane 1 contains a DNA marker, lane 2 - 6 contains samples, lane 7 contains a negative control and lane 8 contains the universal positive control.

4.1.2 Arenaviruses in rodent species

From 121 rodents, elephant shrews and shrews captured, nine different species were identified, with the majority being *Mastomys natalensis* (85 %) followed by *Petrodromus tetradactylus* (6 %) and six other groups, which were *Pelomys* sp., *Praomys* sp., *Myomys* sp., *Crocidura monax, Arvicanthis* sp. and *Mus* sp., comprising (9 %) of all captured animals. Out of 103 *Mastomys natalensis,* 6 (5.8 %) were Arenavirus positive, and 1 out of 2 *Arvicanthis* sp. were positive. There was no significant difference in Arenavirus detection among the species ($P > 0.05$). Eighty six percent of the positive samples were from *Mastomys natalensis* and 14 % were *Arvicanthis* sp., while there were no Arenaviruses detected in other species (*Pelomys* sp., *Praomys* sp., *Myomys* sp., *Crocidura monax, Mus* sp. *and Petrodromus tetradactylus*).

4.2 Arenaviruses in the Selected Sites

A total of 121 rodents, elephant shrews and shrews were captured in six different sites; 32 animals were captured from Kilombero crop raiding interface, 11 from Mbeya peridomestic and crop raiding interfaces, 9 from Mbinga hunting interface, 10 from Mikumi high traffic interface, 14 from the Mtwara hunting interface, and 45 from Ruaha crop raiding and peridomestic interfaces. There were four varieties of rodent species captured in Ruaha and Kilombero, other sites had either one or two varieties of small mammal species. Table 7 shows number of rodent species trapped per site. Out of 121 rodents, 7 (5.8 %) were Arenavirus positive (Table 8). All Arenavirus positive rodents were obtained from the Ruaha in crop raiding and peridomestic interface in Mbolimboli.

Table 7: Species of rodent, shrew, and elephant shrew trapped in selected sites (N = 121)

Trapping sites	Number of captured rodent species in different trapping sites								Total
	Mastomys natalensis.	*Petrodromus* sp.	*Pelomys* sp.	*Praomys* sp.	*Myomys* sp.	*Crocidura monax*.	*Arvicanthis s*p.	*Mus* sp.	
Ruaha	35	7	0	0	0	0	2	1	45
Kilombero	28	0	2	1	1	0	0	0	32
Mbinga	8	0	0	1	0	0	0	0	9
Mbeya	11	0	0	0	0	0	0	0	11
Mtwara	12	0	0	0	0	2	0	0	14
Mikumi	9	0	0	0	0	1	0	0	10
Total	103	7	2	2	1	3	2	1	121

Table 8: Proportion of Arenavirus positive samples in selected sites

Trapping sites	Proportion (%) of Arenavirus positive samples	
	Positives	Total
Ruaha	7 (38)	45
Kilombero	0 (0)	32
Mbinga	0 (0)	9
Mbeya	0 (0)	11
Mtwara	0 (0)	14
Mikumi	0 (0)	10
Grand Total	7 (5.8)	121

4.3 Relationship Between Age, Sex and Presence of Arenaviruses in Rodent, Shrews and Elephant Shrews

Out of 121 rodents, shrews and elephant shrews, 53 % were females and 47 % were males. The results show 6.3 % (4 / 64) of the females were Arenavirus positive while 5.3 % (3 / 57) of the males were Arenavirus positive. No significant difference in detection of Arenavirus was found between males and females ($p > 0.05$) (Table 9). Sex did not have any association with occurrence of Arenaviruses in rodents (OR = 0.83, 95 % CI = 0.18 - 3.89). Majority of the captured rodents 80 % (97 / 121) were adults. Arenaviruses were only detected in adult rodents, hence no test was carried out to compare detection across age groups. For a similar reason, determination of the relationship between age and the presence of Arenavirus was also not conducted.

Table 9: Comparison of Arenavirus detection among age groups and sex of rodent, shrews and elephant Shrews

Factor	Factor category	Number of samples (n)	Proportion (%) of Arenavirus positives	Proportion (%) of negatives	Fischer's exact test P values
Age	Juveniles	10	0.0	100	
	Subadults	14	0.0	100	
	Adults	97	7.2	92.8	
Sex	Male	57	5.3[a]	94.7	1
	Female	64	6.3[a]	93.8	

Proportions followed by the same letters are not significantly different at P < 0.05

4.4 Comparison of Arenavirus Detection Between Fecal and Oropharyngeal Specimens

Results show that 7 (5.7 %) of the fecal specimens were Arenavirus positive and 5 (4.1%) of the oropharyngeal specimens were positive. There was no significant difference in Arenavirus detection between fecal and oropharyngeal specimens (P > 0.05), Table 10.

Table 10: Comparison of Arenavirus detection between oropharyngeal and fecal specimens of rodent, shrews and elephant Shrews

Factor	Factor category	Number of specimen (n)	Proportion (%) of Arenavirus positives	Proportion (%) of negatives	Chi square test P values
Type of specimen	Feces / rectal swabs	121	5.7	94.3	0.77
	Oropharyngeal swabs	121	4.1	95.9	

4.5 Phylogenetic Analysis

A total of 12 Arenavirus S gene nucleotide sequences were obtained in the present study. The identity of these sequences relative to other Arenavirus sequences at GenBank was determined using BLASTn. Sequence AARZBF1 was 72 % identical to Ippy virus (Accession number DQ328877), while the other 11 sequences had varying nucleotide identities between 86 - 88 % with Morogoro virus (Accesion number EU914103) (Table 11). The 12 Arenavirus nucleotide sequences obtained from the present study were translated into amino acid sequences using MEGA 6.0. Alignmment of translated amino acid sequences showed that sequence AARZBF1 was different from the rest of Arenavirus sequences obtained in this study (Fig. 14).

Table 11: BLAST results showing homologous gene sequences at the NCBI database

Sample sequence ID (Query sequence)	Strain sequences obtained from the database similar to sample query sequences	Accession numbers for sequences obtained from the database	% Similarity	Query cover	Reference for sequences obtained from the database
AARZBF1	Ippy virus strain	DQ328877	72 %	68 %	Emonet *et al*, 2006
AAQTJO1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009
AAQTJF1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009
AAQTSO1	Morogoro virus strain	EU914103	88 %	99 %	Gunther *et al*., 2009
AAQTSF1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009
AARYNO1	Morogoro virus strain	EU914103	86 %	99 %	Gunther *et al*., 2009
AARYNF1	Morogoro virus strain	EU914103	86 %	99 %	Gunther *et al*., 2009
AAQTKO1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009
AAQTKF1	Morogoro virus strain	EU914103	88 %	99 %	Gunther *et al*., 2009
AAQUAO1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009
AAQUAF1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009
AARYMF1	Morogoro virus strain	EU914103	87 %	99 %	Gunther *et al*., 2009

Figure 14: An aligned portion of the translated nucleotide protein matrix. Stars at the top correspond to conserved regions in each column. The first column on the left side provides a list of the sample identities for each sequence.

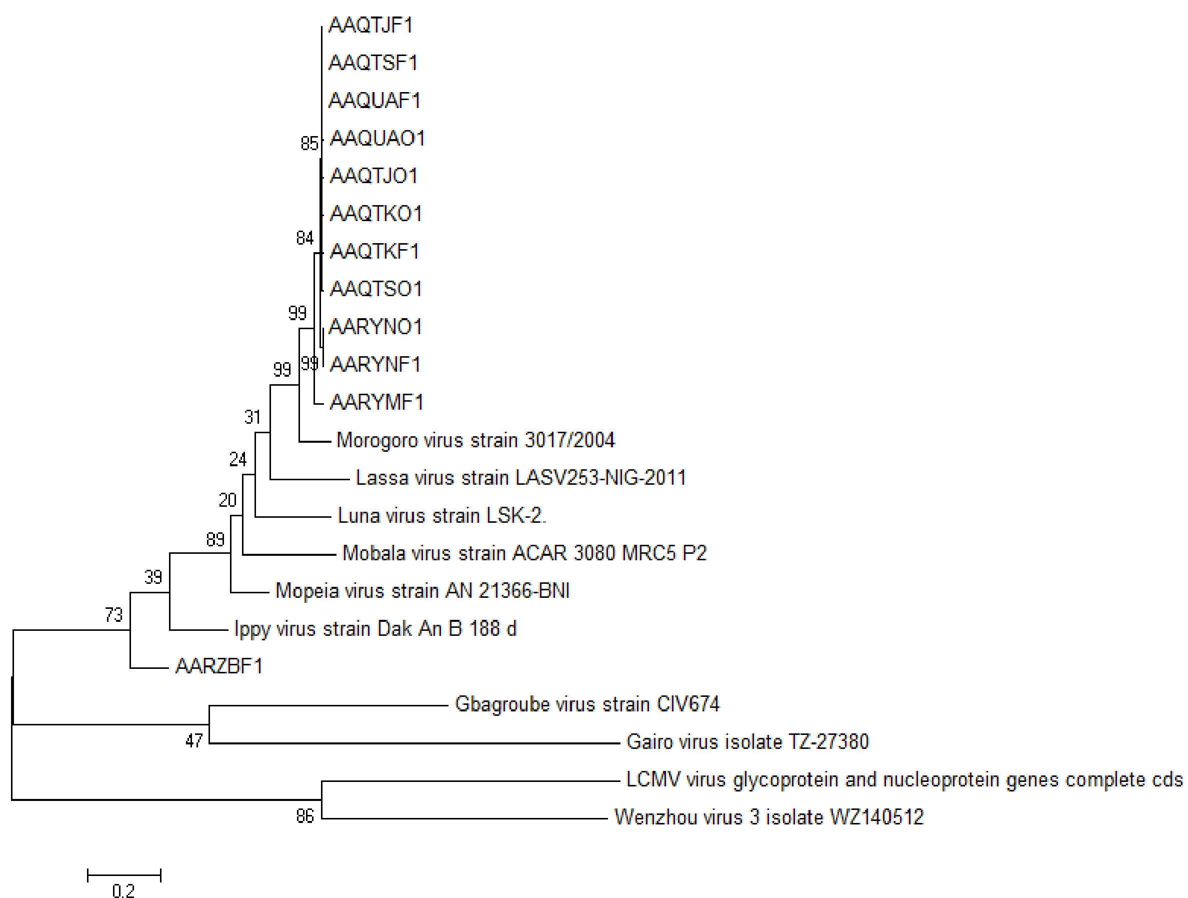

Figure 15: Phylogenetic analysis of Arenaviruses. The phylogenetic tree was constructed based on the glycoprotein precursor and nucleoprotein gene of the S sequence using the Maximum Likelihood method in MEGA 6 software. The phylogeny was inferred following 1000 bootstrap replications.

According to the evolutionary relationship portrayed in the phylogenetic tree (Fig. 15), study sequences corresponded to the Old World Arenaviruses. Sequences belonging to samples: AAQTSF1, AAQUAF1, AAQTJF1, AAQTJO1, AAQTSO1, AAQUAO1, AAQTKO1, AAQTKF1, AARYNO1, AARYNF1, AARYMF1 clustered at 99 % bootstrap replication with the Morogoro virus. Sequence AARZBF1 clustered with sequences from the present study, as well as Lassa virus, Luna virus, Mobala virus, Mopeia virus, Morogoro and Ippy virus at a 73 % bootstrap replication. Although

sequence AARZBF1 seems to be closely related to Ippy virus than the rest of the sequences obtained in the present study, it seems to diverge to its own branch as seen on the tree.

At the top of the tree, samples identified in this study were further grouped into smaller monophyletic groups showing closer relationships between sequences AAQTSF1, AAQUAF1, AAQTJF1, AAQTSO1, AAQUAO1, AAQTKO1 and AAQTKF1 while sample AARYMFI diverged to its own sub-clade. Samples AARYNO1 and AARYNF1 were shown to be more closely related to each other by 99 % bootstrap replications compared to other sequences.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Molecular Detection of Arenaviruses in Rodent, Shrew and Elephant Shrew Species

This study employed a combination of molecular techniques in detecting Arenaviruses. Conventional approaches such as PCR used in this study, have assisted in the detection of Arenaviruses, suggesting that molecular methods are useful tools for detection of Arenaviruses not only in outbreaks, but also in non-outbreak screening. Detection of Arenavirus based on the S gene has recently gained popularity as a tool for detection Arenavirus at the family level (Lavergne *et al*., 2014). This study focused on identifying Arenaviruses based on the S gene using a PCR method with selective primers (ARS16V / ARS and ARS3V / ARS7C), where 5.8 % of the captured rodents, shrews and elephant shrews were found to be Arenavirus positive.

Eighty six percent of the infected rodents were *Mastomys natalensis* suggesting that this specie is a major and important Arenavirus rodent reservoir in Ruaha. However, given the few sites that were included in this study, other species may play an important role in Arenavirus transmission in other areas of Tanzania. Previous studies done by Mbise *et al*. (1995) and Van Hooft *et al*. (2008) reported that *Mastomys natalensis* are widely distributed in the whole of Africa and reservoirs of many important zoonoses, including plague. For this reason, management of these species will likely impact transmission of Arenaviruses and other rodent-borne zoonotic pathogens in Tanzania and Africa as a whole.

5.2 Arenaviruses in the Selected Trapping Sites and Interfaces

A relatively large proportion of rodents were trapped in Ruaha followed by Kilombero. Rodent populations in these two sites were more diverse compared to other sites. As mentioned, the dominant species were *Mastomys natalensis* although a variety of other rodent species were also seen. The distribution of these rodents was probably influenced by geographical characteristics in those areas. For example, in a previous study conducted by Trimble *et al*. (2011), it was reported that the distribution of rodent species, mainly *Mastomys natalensis*, was highly influenced by altitude and climatic conditions. According to that study, *Mastomys natalensis* preferably occurred in low altitude and high rainfall areas in the eastern coastal region, as well as the northeastern part of South Africa. Moreover, *Mastomys natalensis* have also been reported to be distributed in habitats such as savannas, woodlands, forest clearances, houses and cultivated fields (Kingdon, 1997). This observation was consistent with observations witnessed in this study, since a large proportion of rodents were captured in Ruaha crop raiding interface, which was chiefly dominated by cultivated fields.

The majority of study animals were obtained from crop raiding interfaces. Rodents especially *Mastomys natalensis* may be particularly numerous at this interface as agricultural fields offer a readily available food source. Studies by Leirs *et al*. (1997) and Odhiambo *et al*. (2008), reported that rodents have a wide variety of food items in their diets. They feed more on seeds, arthropods and grasses during wet seasons and other plant materials during the dry season. Most of these food materials are found in agricultural fields. Although not all rodents invade crop fields, majority of rodents were highly located in crop raiding interface than other interfaces.

Rodents shedding Arenavirus at crop raiding interfaces in the present study suggests that humans could be exposed at this interface due to contact with the infected rodents. People could also be at risk of exposure to Arenaviruses in other interfaces such as peridomestic interface, where 14 % of Arenavirus positive rodents were identified. The small sample size in this study limits the ability to distinguish among interfaces in terms of risk. Arenavirus strains identified in this study did not match with any pathogenic strains in humans. However, it cannot be guaranteed how many strains of Arenaviruses are circulating in that area. Therefore, further investigations are required in the Ruaha crop raiding interface to improve understanding of the ecology and epidemiology of these viruses in this system. Human samples should also be included to reveal the pattern of circulation of Arenaviruses between human beings and rodents.

5.3 The Relationship Between Age and Sex to Presence of Arenaviruses

The majority of captured rodents were adults. Higher infection rates were observed among the adults, but this observation was not significant ($P > 0.05$) when compared to other age groups. This observation was probably due to the fact that majority of successfully captured animals were adults compared to other age groups, hence no significant conclusions were drawn. However, according to a study conducted by Borremans *et al.* (2011), higher infection rates were observed among the juveniles. Juveniles have been reported to be more susceptible to infection due to weak immunity, higher intrinsic physiologic susceptibility and higher contact rates than older animals (Peters and Zaki, 1999).

Arenavirus infection was not significantly different between males and females ($P > 0.05$). This meant that both males and females were equally likely to be shedding Arenavirus.

These results were consistent with another study conducted by Mills *et al*. (1991), but sometimes infection rates between males and females can differ. In a study conducted by Calisher *et al*. (1999) in Colorado on Hantaviruses (a close relative of Arenaviruses) using serological tests, it was shown that there were periodical differences in sero-conversion between males and females. This was attributed to mechanisms of transmission during the two periods. Transmission during the breeding season was caused by antagonistic encounters between males. Thus, causing higher infection rates in males while winter communal nesting led to more infection rates in females. Therefore, more studies are required to examine how infection rates differ between males and females depending on seasons.

5.4 Comparison of Arenavirus Detection between Fecal and Oropharyngeal Specimens

Detection of Arenaviruses was not significantly different between oropharyngeal and fecal specimens ($P > 0.05$). The implication is that each of these specimens is equally useful for detection of Arenaviruses, and both saliva and fecal samples are useful for screening small mammals for Arenaviruses in field settings. However, it should be noted that the type of contact between humans and rodent reservoirs also plays a role in deciding which specimen to be used for testing. At present, there are no studies which have been done to compare the shedding of Arenaviruses in feces and oropharyngeal swabs.

Quantification of viruses in feces and oropharyngeal swabs is important since it helps in evaluating the chances for human exposure and, potentially, the chances for severe disease (Mills *et al*. 1996). This study provides a baseline for the shedding pattern of Arenaviruses

in feces compared to oropharyngeal specimens. However, since it was not the scope of this study, the present study did not quantify the shedding levels of Arenaviruses in feces and oropharyngeal swabs. Therefore, further investigations are required to determine level of shedding of Arenaviruses in feces and oropharyngeal swabs using quantitative molecular techniques such as qPCR.

5.5 Phylogenetic Analysis

The evolutionary relationship portrayed in the phylogenetic tree showed that, DNA sequences identified in this study corresponded with the non-pathogenic Old World Arenaviruses. Sample AARZBF1 was the only novel Arenavirus, while the rest of the samples were strains of Morogoro virus. In addition, sample AARZBF1 was carried by an *Arvicanthis* rodent specie while the rest of the samples were hosted by *Mastomys natalensis*. This finding agrees with other studies, which reported that *Mastomys* species, specifically *Mastomys natalensis,* are known to be natural hosts of the Morogoro virus (Borremans *et al*., 2011).

It should be noted that DNA sequences of Arenaviruses obtained in this study, which were closely related to Morogoro virus, were obtained from Ruaha. Ruaha is a different geographical area from where Morogoro virus was previously reported. According to Gunther *et al*. (2009), Morogoro virus was previously identified in Morogoro. Therefore, results from this study suggest that there is a possibility that Morogoro viruses might be widespread in Tanzania. Since the present study only focused on investigating partial sequences of the S gene, it does not clearly verify if these sequences, which are closely related to Morogoro virus, are actually Morogoro viruses. Further invetigations based on

complete genomes of the S and L genes are required to conduct additional characterization of these species.

The relationship portrayed on the phylogenetic tree between sample AARZBF1 and Ippy virus is very interesting. The two species fall in the same monophyletic group but their interfaces and geographic locations were different. However, their rodent reservoirs were the same. Ippy virus was previously reported in central Africa (Emonet *et al.,* 2006) in a rodent host namely *Arvicanthis niloticus* that was trapped in a field, while in the present study, sample AARZBF1 was also identified in an *Arvicanthis* sp. trapped at a peridomestic interface in Ruaha, Tanzania. However, DNA sequence alignment of sample AARZBF1 and Ippy virus revealed there is only 72 % similarity between sample AARZBF1 and Ippy virus at a coverage of 68 %, indicating that the two strains are not completely similar. The differences between sample AARZBF1 and Ippy virus are probably attributed to different geographical locations. Studies conducted previously (Domingo *et al*., 1997; Fulhorst *et al*., 2001; Weaver *et al*., 2001), revealed that geographic and climatic differences have an impact on strain variation over time or can isolate them into distinct species. Therefore, further investigations will help to clarify the relationship between sample AARZBF1 and Ippy virus by observing the divergence of these two strains from each other focusing on whole genomes. However, the low coverage and percentage similarity of sample AARZBF1 to published sequences, suggests that it is a new species.

Non-pathogenic African Old World Arenaviruses have frequently been reported in literature, but the natural history and clinical importance of these viruses remains unclear

(Tesh *et al*., 1993; Lisieux *et al*., 1994; Fulhorst *et al*., 2001). Arenaviruses identified in the present study are considered non-pathogenic viruses, but their role and pathogenicity need to be further investigated. Additional molecular studies of Arenaviruses in wild rodent species will help to clarify the relationships among these viruses.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study has confirmed the presence of Arenaviruses in Ruaha, Tanzania. Arenaviruses identified in this study were closely related to known Old World Arenaviruses. Humans could be exposed to Arenaviruses at the Ruaha crop raiding interface, and potentially at other interfaces in this area. Pathogenicity of these Arenaviruses needs to be investigated. This study has also shown that both oropharyngeal and fecal specimens are equally useful for detection of Arenaviruses.

Infected rodents maybe a danger to humans, especially in areas where there is close contact between humans and rodents such as crop raiding and peridomestic interfaces. Therefore, there is a need of controlling rodents around households and farm areas. The present study has also revealed that there is a possibility that Old World Arenaviruses closely related to Morogoro virus and novel Arenaviruses are endemic in Tanzania.

6.2 Recommendations

Further investigations are required in Ruaha to determine the pattern of circulation of Arenaviruses between humans and rodents. Arenavirus positive DNA sequences obtained in the present study were similar with known Old World Arenaviruses, but further investigations need to be conducted to determine if they are pathogenic or not. Although this study was the first to compare the detection of Arenaviruses in oropharyngeal swabs and fecal specimen, only few samples were Arenavirus positive. Therefore, further

research is recommended, especially when there are more positive samples inorder for meaningful comparison of Arenavirus detection between oropharyngeal swabs and fecal specimen to be conducted. Finally, there is a need of controlling rodents in households and farms as they have a potential of carrying Arenaviruses.

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APPENDICES

Appendix 1: RNA extraction protocol

RNA extraction protocol using the QiampViral RNA kit.

Qiagen Cat # 52904

Things which need to be ready;

Spray RNase Away (Molecular Bio-products cat : 7002) on all work surfaces and pipettes

- Thaw samples on ice.
- Buffer AVE should be at room temperature.
- Buffer AW1 is applied as a concentrate and 100 % Ethanol must be added before being used.
    - Add 25 ml of Ethanol to 19ml of concentrated AW1 giving final volume of 44 ml. Check off the box on the lid and write initials and date.
- Buffer AW2 is applied as a concentrate and 100 % Ethanol must be added before being used.
    - Add 30 ml of 100 % Ethanol to the 13 ml of concentrated AW2 giving final volume of 43 ml. Check off the box on the lid and write initials and date.
- Reconstitute carrier RNA to buffer AVE and later mix with buffer AVL.
    - Dissolve 310 µl Buffer AVE to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg / µl divide into conveniently sized aliquots and store - 20 °C. This is done only once for each kit. Do no freeze thaw the aliquots of carrier RNA more than 3 times.

- Check buffer AVL for precipitate, and if necessary incubate at 80 °C until precipitate is dissolved. Calculate the volume of Buffer AVL-carrier RNA mix needed per batch of samples by selecting the number of samples to be simultaneously processed. For example, if processing 10 samples you will need 5.60 ml of buffer AVL and 56 µl of the above carrier RNA-AVE mix.

Procedure:

1. Pipette 560 µl of prepared buffer AVL containing carrier RNA into 1.5 ml micro-centrifuge tube.
2. Add 140 µl VTM, blood, plasma, serum, urine, cell-culture supernatant or cell free body fluid to the buffer AVL carrier RNA in the micro-centrifuge tube. Mix by pulse vortexing for 15 s.
3. Incubate at room temperature (15 - 25) °C for 10 min.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560 µl of ethanol (96 - 100 %) to the sample and mix by pulse vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from the inside of the lid.
6. Carefully apply 630 µl of the solution from step 5 to the Qiamp Mini column into a clean 2 ml collection tube and discard the tube containing the filtrate.
7. Carefully open the QIAmp Mini column and repeat step 6
8. Carefully open the QIAmp mini column and add 500 µl of buffer AW1. Close the cap and centrifuge at 6000 × g (8000 rpm) for 1min. Place the Qiamp Mini column in a clean 2 ml collection tube (provided) and discard the tube containing the filtrate.

9. Carefully open the QIamp Mini column and add 500 µl of buffer AW2. Close the cap and centrifuge at full speed (20 000 × g; 14 000 rpm for 3 min).
10. Recommend: Place the QIamp Mini column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAmp mini column in a clean 1.5 ml micro-centrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open Qiamp Mini column and add 20 µl of buffer AVE equilibrated to room temperature. Be sure to add the 20 µl of AVE to the center of the membrane without touching the membrane with the pipette tip. Close the cap and incubate at room temperature for 1 min. Centrifuge at 6000 × g (8000 rpm) for 1 min.
12. Carefully open Qiamp Mini column and add another 20 µl of buffer AVE equilibrated to room temperature. Be sure to add the 20 µl of AVE to the center of the membrane without touching the membrane with the pipette tip. Close the cap and incubate at room temperature for 1 min. Centrifuge at 6000 × g (8000 rpm) for 1 min. This will yield a final volume of 40 µl of RNA.
13. Remove Qiam Mini column. Transfer 20 µl of RNA to new clean 1.5 ml micro-centrifuge tube (not provided). Both tubes should be labeled the same and both should contain 20 µl or RNA.

Appendix 2: cDNA synthesis protocol

Invitrogen SuperScript III First-Strand cat # 18080 - 051

1. Spray 10 % bleach and wipe down work station.
2. Thaw your RNA on ice. Random hexamers, dNTPs, 10 × RT Buffer, 25 mM $MgCl_2$ and 0.1 M DTT should be thawed on ice. **Enzymes (RNase OUT and SuperScript III RT) should be maintained on ice or in the freezer until needed.**
3. Make calculations according to the number of samples you have and include an extra 10 % to each of your calculations.
4. The negative and positive controls should also be included in your calculations.
5. Label PCR tubes with cDNA, sample ID, and date.
6. Have clean pipettes and tips on your working bench, including a small disposal container for your tips.
7. Vortex and spin Random Hexomers and dNTPs.
8. Make mix 1 by adding Primer and DNTPs to a micro-centrifuge tube according to table below.

A. MIX 1

Component	1 × RN (µl)	N + 10 %
1) Random Hexomer	1	
2) dNTPs	1	
Total	2	

9. Aliquot 2 µl of mix 1 into labeled PCR tubes, then add 8 µl RNA in each tube.
10. Heat the above mixture to 65 °C for 5 min in the PCR machine and incubate on ice for at least 1 minute.

11. Briefly vortex and spin the reagents (table below).
12. Make mix 2 in a single micro-centrifuge tube according to the table and maintain on ice after adding enzymes.

B. MIX 2

Component	1 × RN (µL)	N + 10 %
1) 10 × RT Buffer	2	
2) 25 mM Mgcl2	4	
3) 0.1 MDTT	2	
4) RNase OUT	1	
5) SuperScript III RT	1	
Total	10	

13. Then add 10 µl of mix 2 into each PCR tube.
14. Mix by pipetting up and down and spin.
15. Find the program in the PCR machine and check that it is correct.
16. Incubation conditions should be;

25 °C for 5 min

50 °C for 50 min

85 °C for 5 min

Cool at 4 °C

17. Remove samples from the machine when the process is complete.
18. Add 1µl of RNase H into each tube.
19. Return to the machine and incubate at 37 °C for 20 min then 10 °C forever.
20. Remove the samples from the machine and store them at - 80 °C.

Appendix 3: Beta Actin PCR protocol

1. Thaw 10 x Buffer, $MgCl_2$, dNTP's, and Beta Actin primers (Forward and Reverse).
2. Turn on the PCR machine.
3. Do calculations according to the number of samples you have and include an extra 10 % to your calculations
4. The negative and positive control should also be included in your calculations.
5. The enzyme (Invitrogen Platinum Taq. Cat #: 10966 - 026) needs to be ready, but should be placed on ice or maintained in the freezer until when needed.
6. Label PCR tubes
7. Have clean pre-PCR pipettes and tips in PCR working station.
8. All reagents and materials should be placed in the PCR working station.
   Absolutely no template in PCR hood, no cDNA, no RNA and no DNA.
9. Begin by vortexing and spinning all the reagents.
10. Make the master mix in a single PCR tube in the following order.

MASTER MIX

Components	1 × Rn (μl)	N + 10 % (μl)
Buffer	2.5	
$Mgcl_2$	0.75	
dNTP's	0.5	
Actin F	0.5	
Actin R	0.5	
Taq	0.1	
H_20	19.15	
Total	24	

11. Aliquot 24 µl of the master mix into each labeled PCR tube
12. Return all the reagents to the freezer and immediately shift to the template addition area with the aliquots.
13. Remove your cDNA samples from the freezer and let them to thaw.
14. Set the pipettes and tips ready for use together with a small disposal container for the tips
15. Vortex and spin the samples.
16. Add 1µl of sample into each respective tube according to the labeling to make a total of 25 µl.
17. Find the program on PCR machine and check to make sure it's correct.

Cycle conditions:

a) 94 °C for 24 min
b) 94 °C for 30 sec
c) 55 °C for 30 sec
d) 72 °C for 1 min

(steps b–d: 35×)

e) 72 °C for 7min
f) Cool at 10 °C

18. Start the program, put your samples into the machine and let the reaction begin.

Appendix 4: Arena virus long PCR protocol

(Kit used: Invitrogen Platinum Taq DNA Polymerase. Cat # 10966 - 026)

Two primer pairs (AR16V and ARS1 for reaction 1, ARS3V and ARS7C-Mod for reaction 2) are used in this protocol to augment detection of Arenaviruses in wildlife samples. There is no nested PCR step in this protocol, both primer pairs are used for each samples in separate PCR reactions (Reaction 1 and 2 in Table 1 below) with the same PCR cycling conditions.

1. Clean PCR work station with bleach and 70 % Ethanol before use
2. Thaw 10 x Buffer, $MgCl_2$, DNTP's (Invitrogen Platinum Taq. Cat # 10297 - 018), and primers (primer pair 1: AR16V and ARS1, primer pair 2: ARS3V and ARS7C-Mod) on ice.
3. Turn on the PCR machine.
4. Do calculations according to the number of samples you have and include an extra 10 % to your calculations – according to table 1 below.
5. The negative and positive control should also be included in your calculations.
6. The enzyme (Invitrogen Platinum Taq. Cat #: 10966 - 026) needs to be ready but should be placed on ice or maintained in the freezer until when needed.
7. Label PCR tubes (PCR reaction name, Animal ID, samples type, date)
8. Have clean pre-PCR pipettes and tips in the PCR working station.
9. All reagents and materials should be placed in the PCR working station. **Absolutely no template in PCR hood, no cDNA, no RNA and no DNA.**
10. Begin by vortexing and spinning all the reagents.
11. Make the master mix in a single PCR tube in the following order.

MASTER MIX

Component	Reaction 1 1 × Rn (µl)	Reaction 2 1 × Rn (µl)	N + 10 % (µl)
Buffer	2.5	2.5	
$Mgcl_2$	0.75	0.75	
dNTP's	0.5	0.5	
ARS16V (F)	1.0	-	
ARS1 (R)	1.0	-	
ARS3V (F)	-	1.0	
ARS7C-Mod (R)	-	1.0	
Taq	0.1	0.1	
H_20	18.15	18.15	
Total	24	24	

12. Aliquot 24 µl of the master mix into each labeled PCR tube
13. Return all the reagents to the freezer and immediately shift to the template addition area with the aliquots.
14. Remove your cDNA samples from the freezer and let them to thaw.
15. Set the pipettes and tips ready for use together with a small disposal container for the tips
16. Vortex and spin the samples.
17. Add 1µl of sample into each respective PCR tube containing the master mix according to the labeling to make a total of 25 µl.
18. Find the program on PCR machine and check to make sure it's correct.

Cycle conditions:

g) 92 °C for 2 min

h) 92 °C for 30 sec ⎫

i) 55 °Cfor 30 sec ⎬ 40 ×

j) 72 °C for 1 min ⎭

k) 72 °Cfor 5min

l) Cool at 10 ° C

19. Start the program, put your samples into the machine and let the reaction to begin.
20. When the process is done remove the samples for the machine and store them at - 20 °C for short term storage or - 80 °C for long term storage.
21. Run PCR products on a 1.5 % agarose gel
22. The target size of the amplicons

    Reaction 1 = ˜ 640 bp (list primer pair)

    Reaction 1 = ˜ 460 bp (list primer pair)

Appendix 5: Arenavirus positive specimen sequences

AARZBF1 sequence:

GGCATTGAGCCAAACTGATTGTTTAAAAGTGAAGAATCCTTGACATCCCATAC
ACGGACCACTCCATCCGCTCCAGGTCTCATGTTGTTCCTTGGGCTAGACATTCC
GACCATATCTAGAAGCCTGCGCCTCTGGTCGAGTTGCTGAGCTGTTAGATTGC
CCATGTAGATCCCGGTGGTCTGAGGTCTTTCCGTCCTTATCACTTTGGACTTCA
GTCTGTCAAGATCAGCAGCCAAGACTAGCAAGTCATCAGAGGTCAGCTTACCG
ACCCTGAGAATGTTCCTTTGCTGGGTAGACTTTAGCTCCACTAGATTGAATAC
AGCCTGATTCAAGTCTCTCAATCGTTTCAGATCAGAGTCAGTTCGTTTGTCCTT
TCTCATGAGCCTTTGAACATTGGACACCTCAGCAAAGTCAATGCTGTGGAGAA
TAGCCTGAGCATCCTTTATGACAGACACCTTGACATTGCCACAGTATTGACCT
AGTTCTCTCCTGAGAGCTTGGGTCCATAGAAATGACTTTACTTCCTTGGAGTTT
GCCATTTCAGGTTTTGTACAACAGAGTGAATTAAGAGTTGACAATAAAATGCC
TAGGATCCCCGGTGCG

AAQTJO1 sequence:

GGCATAGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT
TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC
AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC
CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG
CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA
CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA
GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAATCGTCCCTCTTTTCCTTTC
TCATTAACCTTTGCACACTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA

GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAG

CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG

ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA

AAGCCTAGGATCCCCGGTGCG

AAQTJF1 sequence:

GGCATTGAACCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT

TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC

AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA

CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA

GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAATCGTCCCTCTTTTCCTTTC

TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA

GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAG

CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG

ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA

AAGCCTAGGATCCCCGGTGCG

AAQTSO1 sequence:

GGCATTGATCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGATT

CTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC

AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA
CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA
GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAATCGTCCCTCTTTTCCTTCC
TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA
GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAG

AAQTSF1 sequence:

GGCATTGAGCCAAACTGATTATTGAGCRGTTCTGGGTTTCTGACATCCCAGAT
TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC
AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC
CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG
CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA
CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA
GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAATCGTCCCTCTTTTCCTTTC
TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA
GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAG
CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG
ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA
AAGCCTAGGATCCCCGGTGCG

AARYNO1 sequence:

GGCATWGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT
TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC
AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGTGGCCTCTCACTTCTGATGACCTTRGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA

CTCTAAGGACATTCTTTTGTTGAGTGGATTTCAGTTCAACAAGGTTGTTGACAG

CCTGATTCAGATCCCTTAATCTCTTTAGGTCGGAATCGTCCCTCTTTTCCTTTCT

CATTAACCTTTGCACATTGCTAACCYCTGAGAAGTCCAGCCCGTGAAGGAGAG

CTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAACCTGAGAGC

TCTCTCCTCAGACTCTGAGTCCAAAGAAAGGACTTTACCTCCTTTGAGTTGGAC

ATACTCACAGTTGGTTCACAAGCTGTTTGATCTACAATTGGCGCAGTCAAAAA

GCCTAGGATCCCCGGTGCG

AARYNF1 sequence:

GGCATTGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT

TCTCACAATACCATCCCTAGATGGTCCTCTACTTAGAGGGCCACCTCCCATCCC

AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGTGGCCTCTCACTTCTGATGACCTTGGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA

CTCTAAGGACATTCTTTTGTTGAGTGGATTTCAGTTCAACAAGGTTGTTGACAG

CCTGATTCAGATCCCTTAATCTCTTTAGGTCGGAATCGTCCCTCTTTTCCTTTCT

CATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGAG

CTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAACCTGAGAGC

TCTCTCCTCAGACTCTGAGTCCAAAGAAAGGACTTTACCTCCTTTGAGTTGGAC

ATACTCACAGTTGGTTCACAAGCTGTTTGATCTACAATTGGCGCAGTCAAAAA

GCCTAGGATCCCCGGTGCG

AAQTKO1 sequence:

GGCATTGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT
TCTCACAATACCATCCTTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC
AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC
CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG
CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA
CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA
GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAATCGTCCCTCTTTTCCTTTC
TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA
GCTTGAGCATCCTTGATGACCTGAACCTTCACACTTGTGCAGAAGCCTGAGAG
CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG
ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA
AAGCCTAGGATCCCCGGTGCG

AAQTKF1 sequence:

GGCATAGATCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT
TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC
AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC
CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG
CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA
CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA
GCCTGATTCAGATCTCTTAATCTTTTTAGATCAGAATCGTCCCTCTTTTCCTTTC
TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA
GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAG

CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG

ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA

AAGCCTAGGATCCCCGGTGCG

AAQUAO1 sequence:

GGCATAGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT

TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC

AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA

CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA

GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAAACGTCCCTCTTTTCCTTTC

TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA

GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAG

CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG

ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA

AAGCCTAGGATCCCCGGTGCG

AAQUAF1 sequence:

GGCATAGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAGAT

TCTCACAATACCATCCCTAGATGGTCCTCTACCTAGAGGGCCACCTCCCATCCC

AACCATCTGTAGCAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGCGGCCTCTCACTTCTGATGACCTTGGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGAACAAGTAAGTCATCAGAAGTTAGTGTTCCAA

CCCTAAGGACATTCTTTTGTTGAGTGGATTTAAGTTCAACAAGGTTGTTGACA

GCCTGATTCAGATCTCTTAATCTCTTTAGATCAGAATCGTCCCTCTTTTCCTTTC

TCATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAGCCCGTGAAGGAGA

GCTTGAGCATCCTTGATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAG

CTCTCTCCTCAAACTCTGAGTCCAAAGAAAGGACTTCACCTCCTTTGAGTTGG

ACATACTCACAGTTGGTTCACAAGCTGTTTGATCCACAAATGACGCAGTCAAA

AAGCCTAGGATCCCCGGTGCG

AARYMF1 sequence:

GGCATTGAGCCAAACTGATTATTGAGCAGTTCTGGGTTTCTGACATCCCAAAT

TCTCACAACACCATCTCTAGATGGTCCTCTACCCAGAGGGCCACTTCCCATCCC

AACCATCTGTAACAAAGCCTTCCTCTGCTCCAGTTGTTGTGCTGTTAGATTCCC

CATGTACACACCTGCTGCCAGTGGCCTCTCACTTCTGATGACCTTGGCCTTGAG

CCTGTCTAAGTCGGCTGCAAGGACAAGCAAGTCATCAGAAGTTAGTGTTCCAA

CCTTAAGGACATTCTTTTGTTGAGTGGACTTCAGCTCAACAAGATTGTTCACAG

CCTGATTCAGATCTCTTAATCTCTTTAGATCGGAATCGTCCCTCTTTTCCTTTCT

CATTAACCTTTGCACATTGCTAACCTCTGAGAAGTCCAACCCATGAAGGAGAG

CTTGAGCATCCTTAATGACCTGAACCTTCACATTTGTGCAGAAGCCTGAGAGC

TCTCTCCTCAGACTCTGAGTCCAGAGAAAGGACTTTACCTCCTTTGAGTTGGAC

ATACTCACAATCGGTTCACAAGCTGTTTGATCTACAATTGGCGCAGTCAAAAA

GCCTAGGATCCCCGGTGCG