SEROPREVALENCE OF RIFT VALLEY FEVER VIRUS AND SEROCONVERSION AMONG CATTLE DURING INTER-EPIZOOTIC/EPIDEMIC PERIODS IN SELECTED DISTRICTS, TANZANIA

MIRENDE KICHUKI MATIKO

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ABSTRACT

Rift Valley fever (RVF) is an enzootic/endemic zoonotic disease affecting human and animals in Africa and caused epidemic in Saudi Arabia and Yemen. The RVF is caused by the Rift valley fever virus (RVFV) of the order *Bunyavirales*, family *Phenuiviridae*, genus Phlebovirus. Outbreaks of RVF have a devastating impact on human and animal health in Africa, with heavy economic loss due to the banning of livestock export and the loss of food meat, coupled with human infection. They are often characterised by large, sweeping abortion storms and cause significant mortality in young livestock. The aim of the current study was to determine the seroprevalence rate and inter-epizootic/epidemic RVFV seroconversions as evidence of infection by this virus in domestic ruminants and rodents in areas with no previous history of RVF outbreaks. Efforts to understand the mechanism of inter-epizootic/epidemic maintenance and transmission cycle of RVFV generated serological evidence in several African countries that support a role for domestic ruminants and rodents. However, in Tanzania, as well as other RVFV enzootic countries, evidence can be confounded by the detection of RVFV antibody in domestic ruminants that most likely reflected infection acquired during the 2006/07 epizootic/epidemic in Tanzania, Somalia and Kenya, or the serological results were not confirmed by the specific neutralizing test. The current study overcame this potential confounder by selecting cattle that were born after the 2006/07 outbreak and by employing the more specific neutralizing test to confirm the identification of the antibody as being induced by RVFV. The study was conducted in Kyela and Morogoro districts, Tanzania, from June 2014 to May 2018. Serum samples to determine RVFV seroprevalence rate in cattle *(Bos indicus* and their crossbreed with *Bos Taurus)*, sheep (*Ovis aries*), goats (*Capra hircus*),

rodents (Muridae) and shrews (Soricidae) were collected in a cross-sectional study, while sera samples for determining inter-epizootic/epidemic RVFV seroconversions in cattle, sheep and goats were collected in a longitudinal study. A total of 356 serum samples from cattle, 304 from sheep and goats and 581 serum samples from rodents and shrews were collected and analysed for RVFV antibody by competitive and IgM capture Enzyme-linked immunosorbent Assay and plaque reduction neutralisation test.

Overall RVFV antibody seroprevalence rate in cattle by cELISA in both districts was 29.2% (104 of 356) with seroprevalence rates of 33% (47/147) in the Kyela district and 27% (57/209) in the Morogoro district. In total, 8.4% (30/356) of all cattle sampled had RVFV IgM antibody, indicating current disease transmission. When segregated by districts, the RVFV IgM antibody seroprevalence rate was 2.0% (3/147) and 12.9% (27/209) in Kyela and Morogoro district respectively. Mikese ward hard the highest RVFV IgM antibody seroprevalence rate 36.8% (14/38), then Magadu 11.7 (12/103), Bujonde 2.9% (2/70), Katumba Songwe 2.5% (1/40). None of the cattle sera sample from Kajunjumele ward was positive for RVFV IgM antibody. When the 104 cELISA positive sera samples were analysed by $PRNT_{80}$ to confirm that RVFV-specific antibody were present, the majority (89%, 93/104) had RVFV neutralising antibody. On the other hand, the RVFV seroprevalence rate in sheep and goats was 10.5% (32/304) with 4.9% (15/304) of all sera samples from sheep and goats being positive for RVFV IgM antibody. The RVFV neutralising antibody in small mammals was 8.8% (51/581) *M. natalensis* had the highest seroprevalence 8.9% (49/552), *Crocidura* spp 8% (2/25) and none of the sera from *R. rattus* was positive for RVFV neutralising antibody. The overall RVFV seroconversion rate was 25.7% (45/175). Eight cows that aborted during this period were positive for RVFV IgM antibody. This study demonstrated widespread RVFV inter-

outbreak circulation among domestic ruminants in areas without previous reports of outbreaks. The study also demonstrated RVFV neutralizing antibody in rodents and shrews, thus indicating the potential role of these mammal species in the inter-outbreak maintenance of the virus. In conclusion, the RVFV seroconversions among ruminants and small mammals provided evidence of active transmission of RVFV in the Kyela and Morogoro, district of Tanzania, and therefore, may represent an enzootic/endemic maintenance and transmission of RVFV during the inter-outbreak periods for this virus.

DECLARATION

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

_________________________ _________________________

Mirende Kichuki Matiko Date

(PhD. Candidate)

The declaration above is confirmed by;

_________________________ _________________________

Prof. Shaladhuri I. Kimera Date

(Supervisor)

_________________________ _________________________

Prof. Christopher J. Kasanga Date

(Supervisor)

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DEDICATION

To my loving wife Advera, for her true love and encouragement, to my late father and mother Matiko and Ghati for their guidance and to my sons, Matiko, Blessed and Praise for their patience and understanding during the time of this study.

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LIST OF ABBREVIATIONS AND SYMBOLS

ΔFBS	Heat-inactivated fetal bovine serum
µl	microliter
BHK	Baby Hamster Kidney
BSL-2	Biosafety level 2
CDC	Centre for Disease Control and Prevention
cELISA	Competitive Enzyme-linked Immunosorbent Assay
CI	Confidence Interval
CPE	Cytopathic effects
d	Precision
DE	Design Effect
df	degree of freedom
DIVA	Differentiate Infection from Vaccinated Animals
DNA	Deoxynucleic acid
EA	East Africa
EBME	Eagle’s Basal Medium with Earle’s salts
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
et al	and others
Gc	carboxyterminal glycoprotein
Gn	amino-terminal glycoprotein
HBSS	Hanks’ Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid

i.e.	that is
IBM	International Business Machines
ID	Innovative Diagnostic
IEP	Interepidemic Period
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L	Litre
ml	Millilitre
MP-12	mutagenized RVF 12th mutagenesis passage
MVL	Molecular Virology Laboratory
n	Sample size
NC	Negative Control
NSm-del	MP-12 non-structural glycoprotein deletion mutant
OD	Optical Density
OIE	Office International des Epizooties
OR	Odds Ratio
p	probability
PC	Positive Control
PCR	Polymerase chain reaction
PRNT	Plaque Reduction Neutralization Test
PS	Penicillin-Streptomycin
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
RTD-PCR	Real-Time Detection Polymerase Chain Reaction

RT-LAMP — Reverse Transcription Loop-mediated Isothermal Amplification

RT PCR — Reverse Transcription Polymerase Chain Reaction

RVF — Rift Valley Fever

RVFV — Rift Valley Fever virus

SPSS — Statistical Package for the Social Sciences

SSA — Sub Saharan Africa

SUA — Sokoine University of Agriculture

TNTC — Too Numerous to Count

UAV — Unmanned Aerial Vehicle

USA — United States of America

VNA — Virus Neutralising Assay

WHO — World Health Organisation

Z — standard normal deviate

µl — microliter

µl — microliter

BHK — Baby Hamster Kidney

BHK — Baby Hamster Kidney

BSL-2 — Biosafety level 2

BSL-2 — Biosafety level 2

CDC — Centre for Disease Control and Prevention

CDC — Centre for Disease Control and Prevention

cELISA — Competitive Enzyme-linked Immunosorbent Assay

cELISA — Competitive Enzyme-linked Immunosorbent Assay

CI Confidence Interval

CPE Cytopathic effects

CI Confidence Interval

CPE Cytopathic effects

d Precision

d Precision

DE Design Effect

DE Design Effect

df degree of freedom

df degree of freedom

DIVA Differentiate Infection from Vaccinated Animals

DIVA Differentiate Infection from Vaccinated Animals

DNA Deoxynucleic acid

EA East Africa

DNA Deoxynucleic acid

EA East Africa

EBME Eagle’s Basal Medium with Earle’s salts

EBME Eagle’s Basal Medium with Earle’s salts

ELISA Enzyme-linked Immunosorbent Assay

ELISA Enzyme-linked Immunosorbent Assay

EMEM Eagle's Minimum Essential Medium

EMEM Eagle's Minimum Essential Medium

et al and others

et al and others

Gc carboxyterminal glycoprotein

Gc carboxyterminal glycoprotein

Gn amino-terminal glycoprotein

Gn amino-terminal glycoprotein

HBSS Hanks' Balanced Salt Solution

HBSS Hanks' Balanced Salt Solution

HEPES 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid

HEPES 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid

i.e. that is

i.e. that is

IBM International Business Machines

IBM International Business Machines

ID Innovative Diagnostic

ID Innovative Diagnostic

IEP Interepidemic Period

IEP Interepidemic Period

IgG Immunoglobulin G

IgG Immunoglobulin G

IgM Immunoglobulin M

IgM Immunoglobulin M

L Litre

L Litre

ml Millilitre

ml Millilitre

MP-12 mutagenized RVF 12th mutagenesis passage

MP-12 mutagenized RVF 12th mutagenesis passage

MVL Molecular Virology Laboratory

MVL Molecular Virology Laboratory

n Sample size

n Sample size

NC Negative Control

NC Negative Control

NSm-del MP-12 non-structural glycoprotein deletion mutant

NSm-del MP-12 non-structural glycoprotein deletion mutant

OD Optical Density

OIE Office International des Epizooties

OD Optical Density

OIE Office International des Epizooties

OR Odds Ratio

OR Odds Ratio

p probability

p probability

PC Positive Control

PC Positive Control

PCR Polymerase chain reaction

PCR Polymerase chain reaction

PRNT Plaque Reduction Neutralization Test

PRNT Plaque Reduction Neutralization Test

PS Penicillin-Streptomycin

PS Penicillin-Streptomycin

qRT-PCR Quantitative Reverse Transcription Polymerase Chain Reaction

qRT-PCR Quantitative Reverse Transcription Polymerase Chain Reaction

RNA Ribonucleic Acid

RNA Ribonucleic Acid

RT PCR Reverse Transcription Polymerase Chain Reaction

RT PCR Reverse Transcription Polymerase Chain Reaction

RTD-PCR Real-Time Detection Polymerase Chain Reaction

RTD-PCR Real-Time Detection Polymerase Chain Reaction

RT-LAMP Reverse Transcription Loop-mediated Isothermal Amplification

RT-LAMP Reverse Transcription Loop-mediated Isothermal Amplification

RVF Rift Valley Fever

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RVFV Rift Valley Fever virus

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SPSS Statistical Package for the Social Sciences

SPSS Statistical Package for the Social Sciences

SSA Sub Saharan Africa

SSA Sub Saharan Africa

SUA Sokoine University of Agriculture

SUA Sokoine University of Agriculture

TNTC Too Numerous to Count

TNTC Too Numerous to Count

UAV Unmanned Aerial Vehicle

UAV Unmanned Aerial Vehicle

USA United States of America

VNA Virus Neutralising Assay

USA United States of America

VNA Virus Neutralising Assay

WHO World Health Organisation

WHO World Health Organisation

Z standard normal deviate

Z standard normal deviate

ΔFBS Heat-inactivated fetal bovine serum

ΔFBS Heat-inactivated fetal bovine serum

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Rift Valley Fever (RVF) is a phleboviral zoonotic disease that causes stormy abortions in ruminants and has an adverse impact on international livestock trade from enzootic countries. Rift Valley fever imposes dual impact that exacerbates the poverty cycle in the most marginalized livestock-dependent communities by causing a substantial health burden in human and at the same time affecting their livelihoods through the poor health of their livestock. The RVF disease was first reported in early 1930's in the Eastern Rift Valley province of Kenya as the cause of high rates of abortion (stormy abortions) in infected sheep and febrile illness in humans (Daubney, Hudson, and Garnham, 1931). Since then, the etiological agent, the Rift Valley Fever virus (RVFV) has been associated with many periodic epizootics and epidemics. It is mainly found in Sub Saharan Africa (SSA) along the Great Rift Valley. However, outside this domain, it has been reported in Senegal, Mauritania and South Africa (Gerdes, 2004; Herve, 1997). For the first time, an RVF outbreak was reported during the year 2000 outside the African continent, in Saudi Arabia and Yemen, (Abdo-Salem *et al*., 2006; Madani *et al*., 2003). This northward spread of RVFV suggests the possibility of the virus being introduced into Europe and North America through several species of mosquitoes competent for its transmission (Turell *et al*., 2008).

In 2006/2007, there was a recurrence of a massive outbreak of RVF in Kenya, Somalia and Tanzania with 1000 people infected and over 300 patients confirmed dead (Breiman *et al*., 2008). Recent spatial and temporal analysis of RVF in Tanzania showed that RVF-

like disease was reported for the first time in 1930, concurrently with the outbreak in Kenya, with a further 10 outbreaks reported from 1947-2007 (Sindato *et al*., 2014). Before the last outbreak in 2006/2007, RVF outbreaks involved animals only and were mainly confined to the northern regions of Tanzania. In contrast, the 2006/2007 outbreak spread to more than 10 administrative regions in the northern, eastern and central parts of the country. Contrary to the previous outbreaks, apart from thousands of animals, a total of 264 human cases were reported in the country, including 109 deaths (case–fatality rate, 41%). Of the 264 cases, 154 (53%) were laboratory-confirmed (WHO, 2007).

Recently, an epidemiological study conducted in the non-epizootic/epidemic area in Kyela district, Mbeya region, in south-western Tanzania revealed a seroprevalence rate of 29.3% in humans (Heinrich *et al*., 2012). However, RVF has not been diagnosed clinically in this area, nor has any outbreaks been reported. This finding suggested the existence of RVFV in an area which has not been documented as RVF epizootic/epidemic in Tanzania. To date, the status of RVFVinfection in animals from Kyela has not been assessed.

It is possible that RVFV related abortions in animals and febrile illness in human may have been overlooked and febrile syndromes, encephalitis, retinitis and haemorrhagic fever in human misdiagnosed as malaria. The other possibility is that there is a low virulence strain of RVFV or other related pathogenic *phlebovirus* circulating in this area.

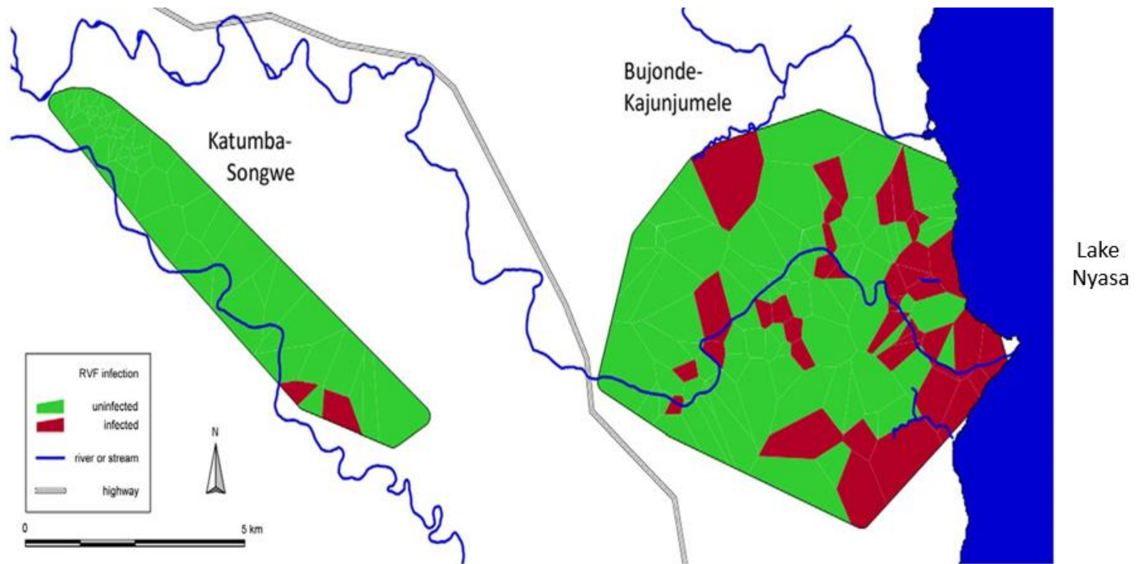

Figure 1: Location of households with RVF IgG antibody positive participants. Courtesy (Heinrich *et al.,* 2012)

Since sero-surveillance or virus isolation studies have not been conducted in southern Tanzania, it is difficult to conclusively understand the cause of the observed RVFV seroprevalence in this area. It is, therefore, important to carry out investigations to determine the status of disease in animals and to identify and characterise the virus(es) that could be circulating in Kyela district, including possible maintenance hosts of RVFV in nature.

1.2 Problem Statement and Justification

Evidence of RVFV circulating during the inter-epizootic/epidemic period has been documented by the detection of RVFV-antibody in various livestock species in areas with the history of RVF outbreaks (Sumaye *et al*., 2013) Additional sero-surveys in areas without a previous history of RVF outbreak or clinical cases in humans (Heinrich *et al*., 2012; Kifaro *et al*., 2014) detected evidence of previous RVFV infections in sheep and goats and humans.. The detection of antibody in areas where no clinical disease has been

reported (Heinrich *et al*., 2012; Kifaro *et al*., 2014) raises the question of whether the disease is overlooked due to a lack of effective surveillance systems, or whether there are strains of RVFV that are not recognized because of low pathogenicity. Continuous monitoring of the antibody prevalence in susceptible species is, therefore, highly recommended in enzootic areas. In addition, the role of mammals as maintenance hosts for RVFV remains largely unknown (Olive *et al*., 2012). Despite evidence in favour of rodents acting as maintenance hosts of RVFV, this role is not fully understood and published data are conflicting (Olive *et al*., 2012). It is therefore important to carry out further studies in rodents to determine their possible role in the maintenance of the RVFV.

The aim of this study was to determine the seroprevalence rate of RVFV in non-vaccinated domestic ruminants (cattle, sheep and goats) and rodents during the inter-epizootic/epidemic period. Additionally, the study was conducted to determine if there was active RVFV transmission among domestic ruminants and their possible role in the inter-epizootic/epidemic maintenance and transmission cycle of the virus in areas with no history of RVF outbreak in Tanzania.

The data obtained from this study will serve as a valuable basis in developing appropriate and effective counter-measures to manage RVFV infection both in livestock and humans. The current control measures of RVF are based on banning of livestock movements and ring vaccination to prevent the spread of the virus. However, such control measures may not be effective because the outbreaks may be due to reemergence of enzootic viruses in each outbreak foci, probably via spontaneous hatching of infected *Aedes* mosquito eggs or expansion of an enzootic RVFV that is being maintained through low-level cycling among vertebrates and possibly humans (Nderitu *et al*., 2011). This is further complicated by the

ability of RVFV to infect many arthropod species and prevalence of RVFV antibody in many animal species (Evans *et al*., 2008). It is, therefore, crucial to understanding the mechanisms driving viral ecology including the enzootic transmission cycles.

Relatively high RVFV antibody prevalence rates were detected in humans in Kyela district (Heinrich *et al*., 2012), but the status of possible disease in livestock in this district has not been determined. The seroepidemiological data in humans from Kyela district clearly points out the existence of RVFV in this area. Since no symptomatic disease in human or livestock has been described in the Kyela and Morogoro districts, an appropriate hypothesis is the occurrence of an enzootic low-level transmission cycle of RVFV. However, the mechanisms involved in the maintenance and transmission cycle of RVFV remains unknown. It is possible that the low-level transmission cycle is a result of the circulation of RVFV strains that are well adapted to their vectors and vertebrate hosts, including humans and, therefore, do not cause severe disease. These well-adapted strains may contribute to the enzootic maintenance of RVFV and may act as a source of a more pathogenic emerging strain of virus when transmitted to non-immune livestock and human populations. Alternatively, the low-level transmission cycle may be a result of the circulation of avirulent strains of RVFV.

The overall goal of this study was to determine the seroprevalence of the RVFV antibody in domestic ruminants (cattle, sheep and goats) and rodents to shed light on their potential involvement in maintenance and transmission of the virus in Kyela and Morogoro districts. Another goal of this study was to monitor RVFV seroconversion(s) in selected herds of cattle, sheep and goats. Monitoring seroconversion for new infections would

detect ongoing RVFV activity, and thus used for identifying and mapping hot spot-areas with the low level of virus circulation.

1.3 Hypothesis

There is undetected low-level RVFV circulating in the Kyela and Morogoro districts of Tanzania which does not cause clinical disease in infected livestock and wild rodents. Alternatively, clinical cases are not detected due to the lack of specific monitoring systems or they are misdiagnosed for other febrile illnesses.

1.4 Research Questions

1. What is the RVFV infection status of domestic ruminants in Kyela and Morogoro districts during the inter-epizootic/epidemic period?
2. What is the RVFV infection status of small wild mammals during the inter-epizootic/epidemic period?
3. What is the seroconversion rate in domestic ruminants to RVFV infection during the inter-epizootic/epidemic period?

1.5 Objectives:

1.5.1 Overall objective

The overall objective of this study was to determine the RVFV seroprevalence rates during an inter-epizootic/epidemic among domestic ruminants and feral rodent in selected areas of Kyela and Morogoro districts of Tanzania.

1.5.2 Specific objectives

(i) To determine the seroprevalence rate of RVFV IgM and IgG antibody in cattle, sheep and goats.

(ii) To examine the RVFV neutralizing antibody in rodents and shrews during an inter-epizootic/epidemic period.

(iii) To determine the RVFV seroconversion rates in domestic ruminants during the inter-epizootic/epidemic periods.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of the Disease

The Rift Valley Fever (RVF) disease was first reported in early 1930's after massive abortions (stormy abortions) in exotic sheep in the Rift Valley Province of Kenya (Daubney *et al*., 1931). Since then, the RVFV has been associated with many periodic epizootics/ epidemics in many countries of Sub-Saharan Africa (SSA). The disease has also been associated with devastating outbreaks outside the SSA region including West Africa (Mauritania, Senegal and Gambia), North Africa (Egypt and Sudan). The disease has also crossed the Indian ocean to Mayotte and Madagascar. The first RVF was reported outbreak outside the African continent during 2000 in Yemen and Saud Arabia (*Madani et al*., 2003).

Recent spatial and temporal analysis of RVF in Tanzania showed that an RVF-like disease was reported for the first time in 1930, and 10 outbreaks have since been reported between 1947 and 2007 (Sindato *et al*., 2014). In 2006/2007, there was a recurrence of a massive RVF outbreak in East Africa (EA), with more than 1000 suspected cases in humans resulting in 350 deaths across the EA countries, as well as thousands of livestock abortions and deaths (WHO, 2007).

In Tanzania, the 2006/2007 RVF outbreak was more widely spread than the previous ones affecting more than 10 regions in the northern, eastern-central and southern parts of the country (Sindato *et al*., 2014). The geographical coverage and the severity of the disease is increasing with time. For example, prior to 2006/2007, no RVF human cases were

reported in Tanzania. In addition, the previous outbreaks were associated with less morbidity and mortality in livestock. In contrast, the 2006/2007 RVF outbreak was associated with high case fatality rate of more than 30% in human, affected more than 10 regions in the north, east, central and southwestern parts of Tanzania and caused high morbidity and mortality in livestock (Sindato *et al*., 2014).

2.2 Distribution

Rift valley fever is an emerging zoonotic disease, as it has crossed significant natural geographic barriers such as the Indian Ocean, the Sahara Desert and the Red Sea to reach naive and different ecological locations. Since then, the disease has been associated with periodic epidemics/epizootics in Eastern Africa (Tanzania, Kenya and Somalia) as well as other African countries including Zimbabwe, South Africa, Egypt, Mauritania, Senegal, Gambia, and Madagascar (Gerdes, 2004; Zeller *et al*., 1997). In 2006-2007 there was a recurrence of a massive outbreak in Kenya, Somalia and Tanzania with 1000 people diagnosed infected and over 300 patients confirmed dead (Breiman *et al*., 2008). In Tanzania alone, apart from thousands of animals, 264 humans were clinically affected, of whom 109 died (WHO, 2007). RVFV emerged outside of the borders of the African continent for the first time in 2000, affecting ruminants and humans in Saudi Arabia and Yemen (Abdo-Salem *et al*., 2006; Al-Hazmi *et al*., 2003). Furthermore, there is the possibility that the virus will be introduced into Europe and North America since several species of mosquitoes have been shown to be competent vectors of this virus (Turell *et al*., 2008).

From 1930 when RVF-like disease was reported for the first time in Tanzania, further outbreaks were reported in 1947, 1957, 1960, 1963, 1968, 1977/1978, 1989, 1997/1998

and 2006/2007 (Sindato *et al*., 2014), with a total of 10 outbreaks involving a total of 194,750 and 309 cases in ruminants and human, respectively. RVF cases were not documented among humans prior to the 2006 outbreak.

The spatial distribution of the RVF cases in Tanzania shows that before 1980 the outbreaks affected domestic ruminants in four districts of northern Tanzania (Sindato *et al*., 2014). The four districts included Ngorongoro, Simanjiro, Monduli and Hai were involved in the outbreaks from 1930 to 1978 and were persistently involved in subsequent outbreaks from 1997 to 2007. From 1980 to 2007, there were the spatial progression of the spread of cases from north to east, central and southern parts of the country (Figure 2) with an increase in the number of villages that reported cases from 2 villages in 1930 to 175 villages in 2006/2007 (Sindato *et al*., 2014).

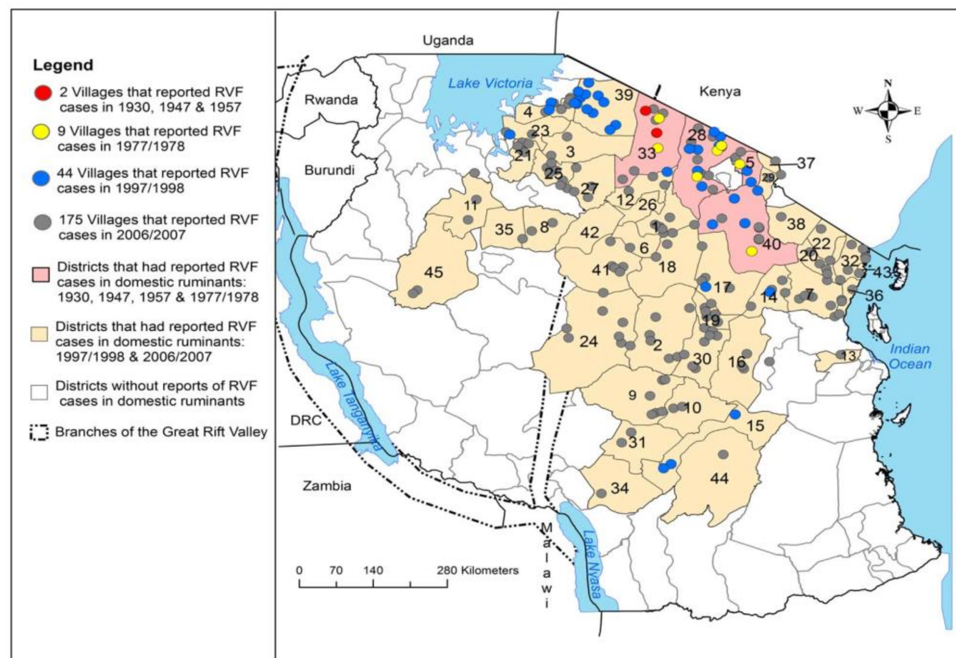

Figure 2: The space-time progression of Rift Valley fever outbreaks by district and villages; 1930 to 2007. Courtesy (Sindato *et al.,* 2014)

2.3 The Aetiology

Rift Valley Fever is a zoonotic disease which is caused by the RVFV of the family *Bunyaviridae,* genus *Phlebovirus.* The RVFV is serologically related to other *phleboviruses*, such as sandfly fever Naples and Sicilian viruses. It is an enveloped RNA virus with a tripartite genome consisting of a Large (L), Medium (M), and Small (S) segments. The L-segment encodes an RNA-dependent RNA polymerase (L protein). The M segment encodes for two glycoproteins (Gn and Gc) used for viral attachment and a non-structural protein Nsm, while the S segment encodes for a protein (N protein) and nonstructural protein NSs.

2.4 Transmission

The RVFV is spread to animals primarily by the bite of infected mosquitoes, mainly the *Aedes* species, which can acquire the virus from feeding on infected animals. Vertical transmission via the infected mosquito eggs also transmits the virus directly to the mosquito offspring leading to new generations of infected mosquitoes. Humans are mainly infected by close contact with fluids or organs of infected animals, such as through handling of animal tissue during slaughtering, assisting with animal births, conducting veterinary diagnostic procedures, as well as by contact with carcasses or aborted fetuses. Certain occupational groups such as herders, farmers, slaughterhouse workers and veterinarians are, therefore, at higher risk of infection (Swai and Schoonman, 2009). Aerosol transmission to humans is possible due to high virus levels body fluids of infected animals during viraemia.

2.5 Host Range

Contrary to other phlebovirus which are host specific, RVFV can infect many host species. Sheep and goats are more susceptible with high mortality in lambs and kids under

two weeks of age approaching 100%. In adult sheep, mortality may reach 30% with abortions approaching 100% in pregnant ewes. Clinical signs in adult sheep and goats are not consistent but may include a rise in body temperature, mucopurulent nasal discharge, unsteady gait and high abortion rate up to 100% amongst pregnant ewes (Davies and Martin, 2006; Glancey, Anyamba, and Linthicum, 2015).

Cattle are less susceptible than sheep, some are subclinical and mortality averages 5% with some abortions. Clinical signs in adult cattle include high temperature, abortion, salivation, anorexia, fetid diarrhoea, and a rapid decrease in milk production. Abortion may be the only marked sign in cattle and mortality in adult cattle is usually less than 10%. In addition, RVFV infection has been associated with congenital abnormalities (stillbirths, mummified fetuses, defects of the central nervous system, musculoskeletal problems, hydrocephaly, and cerebellar hypoplasia) in fetal or neonatal ruminants (Javanbakht, Mardjanmehr, Tavasoly, and Nazemshirazi, 2014).

Dogs and cats are susceptible and although they develop viremia, they do not show clinical signs of RVF. Infection of pigs is also asymptomatic while birds are refractory (Olive *et al*., 2012). Several wildlife species including African buffalo, black rhino, lesser kudu, impala, African elephant, kongoni, and waterbuck have also been shown to be serologically positive (Evans *et al.,* 2008).

2.6 Disease in Humans

RVFV infection in human mostly inapparent or cause self-limited moderate influenza/malaria-like illness that may progress to severe and fatal, illness. The incubation period from the time of infection to the development of signs of the disease range from 2

to 6 days. The infection may cause mild-flu like symptoms that include fever, headache, muscle and joint pain, nausea and vomiting (Madani *et al.*, 2003). The majority of mild cases recover in 4 to 7 days. Alternatively, RVFV infection may develop into a severe disease in 1% of infected humans and is characterized by retinitis, haemorrhages, and encephalitis with the case fatality rate of the hemorrhagic syndrome form can be as high as 50% (Kahlon *et al.,* 2010). Only a few patients develop ocular lesions, encephalitis or severe hepatic and renal disease with haemorrhagic manifestations or fatal haemorrhagic form of the disease (Meegan, 1979).

2.7 Disease in Animals

In livestock, RVFV infection is characterized by high rates of abortion and neonatal mortality primarily in sheep, goats and cattle. Other clinical signs and symptoms include anorexia, high temperature (up to 40^0 C), blood-stained nasal and lachrymal discharges, a high respiration rate, respiratory distress and death (Davies and Martin, 2003). At post-mortem, the general features include liver enlargement and necrosis, which is initially focal later becoming generalized, hepatitis, haemorrhages, necrotic and marked hepatomegaly. The viral burden becomes high resulting into petechial and ecchymotic haemorrhages throughout the carcass; severe haemorrhagic gastroenteritis; lymphadenitis, pulmonary oedema, emphysema and chocolate-brown digested blood in abomasum (Davies and Martin, 2003).

2.8 Diagnosis

Early detection of RVF suspected cases is crucial to ensure that timely control measures are implemented to reduce the spread of the disease. The sudden onset of large numbers of abortions (abortion stormy) and mortalities among young animals in affected livestock,

together with the appearance of the disease in humans, is considered characteristic of an RVF epizootic/epidemic (Gerdes, 2004). The onset of acute haemorrhagic fever cases in humans should lead to suspicion of RVF outbreak in enzootic regions.

2.8.1 Histopathology

The histopathology performed on diagnostic tissues (e.g. liver) preserved in formaldehyde in the field is beneficial particularly in remote areas with no cold chain facilities. Alternatively, diagnostic tissues can be kept in cool boxes with ice packs for short transport to the laboratory. The histopathological examination of the liver of affected animals and humans will reveal hepatic lesions characteristic of RVFV infection and immunostaining may allow the specific identification of RVF viral antigen in tissue (Coetzer, 1982; OIE, 2017).

2.8.2 Virus isolation

Rift Valley Fever virus can be isolated from whole blood or serum collected during the acute febrile stage of the disease (Anderson *et al.*, 1989). At post-mortem, the virus can also be isolated from the brain, liver, spleen and organs of the aborted foetus, while the in-vivo isolation can be performed in suckling mice. However, RVFV isolation should be performed in biosafety level 3 or 4 laboratories. Various cell cultures can be employed for in-vitro isolation of the virus including African green monkey kidney cells (Vero), baby hamster kidney (BHK) cells and AP61 mosquito cells. Immunostaining or reverse-transcription polymerase chain reaction (RT-PCR) can be used to confirm the identity of the virus.

2.8.3 Serology

Serological diagnosis, usually by virus neutralisation assay (VNA) or enzyme-linked immunosorbent assay (ELISA), is commonly used to confirm RVFV infection in an

affected individual (animal or human). Several assays are available for the detection of RVFV antibody in a variety of animal species. The VNA is highly specific, but the ELISA IgG antibody are cross-reactive with other Phlebovirus antibodies (Pepin, Bouloy, Bird, Kemp, and Paweska, 2010b).

The VNA is the gold standard serological assay, generally used for vaccine potency determination and is the World Organisation for Animal Health (OIE) prescribed test for international trade (OIE, 2017). The test is highly specific and, unlike some ELISA based assays, can be used to test serum for RVFV antibody obtained from a wide range of host species. However, VNA can only be performed in appropriate biosafety and biosecurity facilities as this test involves the manipulation of live virus.

ELISAs can be employed to confirm the presence of either specific IgM antibody, which appears transiently from fay 4 to about day 8 after infection or at the time when IgG antibody appears after infection and may persist for several years (Paweska *et al*., 2005; Williams *et al*., 2011). Several ELISA assays have been developed using either whole cell lysate derived from infected cells or purified nucleocapsid protein as antigen. However, the commercially available ELISAs (IgG and IgM) are based upon recombinant RVFV nucleocapsid protein, despite the potential for high background issues with this antigen (Faburay *et al.*, 2013). The detection of IgM antibody indicates a current or recent infection, while IgG-based ELISAs cannot distinguish between past and current infection unless paired serum samples are analysed (acute and convalescent) and a four-fold increase in antibody titre is observed (OIE, 2017).

2.8.4 Molecular methods

Molecular techniques are used to detect RVFV Ribonucleic Acid (RNA) during the acute febrile phase of the disease when high levels of viremia occur in both humans and

animals. A range of highly sensitive nucleic acid-based molecular tests have been developed for RVFVRNA including nested RT-PCR methods (Sall *et al.*, 2002), quantitative real-time PCR (Busquets *et al.*, 2010; Garcia *et al.*, 2001; Odendaal, Fosgate, Romito, Coetzer, and Clift, 2014), multiplex PCR-based microarray assay and RT Loop-mediated isothermal amplification (RT-LAMP) (Roux *et al.*, 2009).

Whilst molecular techniques have proven useful during RVF outbreaks (Gerdes, 2004), the short duration of viremia (3–4 days (Ikegami and Makino, 2011; Smith et al., 2010), and the dependence on specialised equipment and skills, still pose challenges to their widespread applicability for detecting and monitoring the spread of the virus in the field. However, molecular approaches allow for the rapid identification of the genetic lineages and hence the source and pathogenicity of the viral strains involved in the RVF outbreak (Bird *et al.*, 2007). Sensitive and specific molecular assays may also be used for the early detection of RVFV RNA in mosquito pools thereby contributing to disease surveillance, vector species identification and disease control (Lutomiah *et al.*, 2014; Mwaengo *et al.*, 2012)

2.9 Economic Impact

The adverse economic effects of RVF have attributed to abortions and perinatal mortalities as well as the collapse of domestic and international livestock markets; the latter due to the imposition of quarantine and prohibition of movement of livestock and their products from affected areas. Reduced consumption of animal products because of fear and anxiety amongst consumers also contributes significantly to economic losses. Rift Valley Fever also causes immense losses due to human morbidities and mortalities. Therefore, RVFV represents a significant health burden for people living in poor countries where malnutrition is frequent and health care is of low standards (Orinde, 2013).

2.10 Prevention and Control

Zoonotic diseases like RVF are major obstacles in the pathway out of poverty for over one billion poor livestock keepers in Africa and Asia. No drugs for the treatment of the disease exist, thus the prevention and control of RVF rely on vaccination of animals and control of the insect vectors.

No vaccines are currently available for human protection. The initial RVF epizootic cycle involves domestic ruminants, and most human infections are acquired by contact with viraemic animals, thus, vaccination of ruminants is the favoured as an indirect method of preventing human disease. However, there is no fully licensed vaccine for veterinary or human use available in non-enzootic countries, and in enzootic countries, there is no clear policy or practice of routine/strategic livestock vaccinations as a preventive or mitigating strategy against potential RVF disease (Faburay, LaBeaud, McVey, Wilson, and Richt, 2017). The earliest vaccines such as the live-attenuated Smithburn vaccine (Smithburn, 1949) have contributed significantly to the control of RVF in enzootic countries in Africa. However, the production and use of this vaccine have been associated with health risk as they lack important attributes, such as the differentiation of infected and vaccinated animals (DIVA) capability. Efforts to develop efficacious and safe vaccines have resulted in the development of many next-generation candidates, including live attenuated, inactivated, recombinant, and DNA vaccine candidates (Faburay *et al.*, 2017; Ikegami and Makino, 2009). Experiments to test the efficacy and safety of the more promising vaccines, such as MP-12ΔNSm (Morrill *et al.,* 2013) in indigenous ruminant breeds in Tanzania is near completion, and the results indicate that the vaccine is safe and protective for sheep, goats and calves (Watts *et al.*, 2018, person comm.). Also, the MP-12ΔNSm has the potential of differentiating naturally infected from vaccinated animals (DIVA).

Decreasing the contact with infected materials such as blood, body fluids, aborted foetuses or tissues of infected animals can prevent RVFV infection in humans. Protecting against mosquito bites and other bloodsucking insects by using bed nets and mosquito repellents are equally effective prevention methods. Wearing protective equipment to avoid exposure to blood or tissues of infected animals is an important protective measure particularly for veterinarians and other persons dealing with animal handling or helping animals during birth. Proper disposal of aborted foetuses and the placenta could prevent further spread of the virus.

Vector control: Strategic larvicidal treatment of mosquito breeding habitats is recommended. However, it is difficult to apply the larvicides in places with extensive mosquito breeding sites such as wetlands and flood basins. This underscores the critical need to explore novel technologies that overcome the above limiting factors and develop effective next-generation vector control strategies to prevent RVF.

Among the more attractive approaches for application of larvicides, is the use of unmanned aerial vehicles (UAVs), or “drones”, for surveillance and control of medically important arthropods (Knapp, Macdonald, Malone, Hamon, and Richardson, 2015; Vitek, Gutierrez, and Dirrigl, 2014). The potential value of drones is that they offer the option of overcoming the disadvantages associated with traditional methods used to reduce larval mosquito populations such as difficulty in identifying, mapping, accessing and treating mosquito breeding sites.

Quarantines restrict movement of animals from enzootic/epizootic areas where RVFV transmission is occurring and it prevents viraemic animals from introducing the virus to

the uninfected area. For this reason, all export of livestock and their products from infected areas should be banned during RVF epizootic periods. Nevertheless, if there were large numbers of competent mosquito vectors present, capable of RVFV transmission, the possibility of the introduction of RVFV would still be high.

2.11 Inter-epidemic Virus Circulation

In RVFV enzootic regions, outbreaks occur within a cycle of 3 to 17 year (Pepin *et al.*, 2010), which is an average inter-epizootic/epidemic interval of 7.9 years. The RVFV maintenance between the long inter-epizootic/epidemic periods is not fully understood. It has been widely hypothesized that the virus is maintained via transovarial infected *Aedes* mosquito eggs in east Africa (Linthicum *et al.*, 2016; Pepin *et al.*, 2010). Serological evidence suggests that the virus could as well be maintained through inter-epizootic/epidemic circulation in domestic ruminants, wild animals and humans (Heinrich *et al.*, 2012; Lichoti *et al.*, 2014; Sumaye *et al.*, 2013, 2015).

RVF outbreaks occur at irregular intervals of about 5 to 15 years or longer (Davies, Linthicum, and James, 1985). However, the natural reservoir of the virus during this long inter-epizootic/epidemic period is still a matter of debate and the postulated enzootic cycle remains poorly understood (Evans *et al.*, 2008). Several mosquito species have been implicated for in the involvement in the transmission of RVFV. The vectors of RVFV are classified either as ''enzootic/maintenance'' or ''epizootic/amplifying'' (Pepin *et al.*, 2010). According to the current understanding, RVFV is transmitted transovarially by floodwater *Aedes* mosquitoes. The infected mosquito eggs hatch after heavy rainfall and flooding to produce RVFV-infected mosquitoes (Davies *et al.*, 1985). This result in high

population densities of RVFV-infected mosquitoes that could trigger an outbreak in domestic ruminant and spill-over to cause infection among humans.

2.12 The Role of Mammals in the Maintenance of RVFV

The role of mammals in the maintenance and their function as the reservoir for RVFV remains largely unknown (Olive *et al*., 2012). In enzootic areas, severe RVF recurrent epizootics/epidemics may be caused by the amplification of the RVFV in different ecological zones; the virus being maintained either as a result of persistence in eggs of floodwater *Aedes* mosquito species or via low-level cycling among the mosquito vectors and vertebrates. Alternatively, the virus may be introduced in a naive ecological zone via animal or vector movements.

Different RVFV lineage from different foci were identified to be responsible for the sequential RVF outbreaks in Kenya, Tanzania and Somalia in 2006/2007 (Nderitu *et al.*, 2011). This could probably be via spontaneous hatching of infected mosquito eggs or expansion of a virus that was maintained through low-level cycling among vector mosquitoes, vertebrates and possibly humans (Nderitu *et al.*, 2011). However, the inter-epizootic/epidemic periods (IEP) mechanisms for local maintenance or persistence of the virus remain uncertain.

Most arthropod-borne viruses persist in nature because they are maintained in an arthropod – vertebrate host cycle, including both feral animals and humans, but a predominant animal reservoir for RVFV has not been identified. Rodents have been implicated as RVFV reservoir and the likely candidate species include the African genera *Arvicanthis* and *Micaelamys* and the widely introduced roof rat, *Rattus rattus* (Olive *et al*.,

2012). Evidence supporting a role for rodents include the detection of RVFV antibody in several rodent species (Gora *et al*., 2000; Herve, 1997). Also, the virus was isolated from the brain of the *Rattus rattus* during the 1977-1978 epizootic in Egypt (Imam, El Karamany, and Darwish, 1979).

In Eastern Africa, it is believed that RVFV is maintained during the inter-epizootic/epidemic periods in floodwater *Aedes* mosquitoes (Linthicum, Davies, Kairo, and Bailey, 1985). This is contrary to what is happening in western and central Africa where the virus could be maintained by horizontal transmission between ruminants and mosquitoes and by vertical transmission during the dry season (Chevalier, Thiongane and Lancelot, 2009). In Gabon, serological evidence of RVFV in people has been demonstrated in areas where cattle herds are rare (Pourrut *et al*., 2010).

Evidence of inter-epizootic/epidemic circulation of RVFV in areas with a history of RVF outbreaks has been reported (Herve, 1997; Sumaye *et al*., 2013; Swai and Schoonman, 2009). However, RVFV antibodies have also been detected in areas where no clinical disease has been reported (Fafetine *et al*., 2013; Heinrich *et al*., 2012), thus raising the question of whether the disease is overlooked due to a lack of effective surveillance systems, or whether there are strains of RVFV with low pathogenicity.

Another possibility could be that there are non-pathogenic Paleoviruses (should this be Phleboviruses??) or other viruses that are genetically related to RVFV. Analysis of RVFV isolates revealed the concurrent circulation of viruses belonging to different lineages and viruses that resulted from gene segment reassortment (Bird *et al*., 2007; Grobbelaar *et al*., 2011; Nderitu *et al*., 2011).

In this study, surveillance was conducted in Kyela and Morogoro districts to determine if RVFV and/or RVFV antibody could be detected in domestic ruminants (cattle, sheep and goats) and in wild rodents as evidence of active RVFV transmission. Evidence of RVFVs circulation during inter-epizootic/epidemic periods has been reported based on the detection of RVFV antibody in ruminant species (cattle = 11.03%; sheep = 11.86 and goats = 11.37%) in the Kilombero river valley (Robert D. Sumaye *et al.*, 2013). The RVF outbreak during 2006/07 affected domestic ruminant and humans in the Kilombero river valley. Additional surveys in areas without any previous history of RVF outbreaks (Kifaro *et al.*, 2014) or clinical cases in humans (Heinrich *et al.*, 2012) detected serological evidence of RVFV infection in sheep and goats, and human.

Continuous surveillance for RVFV antibody in susceptible animal species is highly recommended in enzootic areas. In addition, the role of mammals as maintenance hosts for RVFV remains largely unknown (Olive *et al.*, 2012). Furthermore, the detection of antibodies in areas where no clinical disease has been reported (Heinrich *et al.*, 2012; Kifaro *et al.*, 2014) raises the question of whether the disease is overlooked due to lack of effective surveillance systems, or whether there are strains of the RVFV with low pathogenicity. This study also determined RVFV seroconversion rates in non-vaccinated domestic ruminants during the inter-epizootic/epidemic period.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Areas

This study was conducted from June 2014 to May 2018 in the Kyela and Morogoro districts, Tanzania. During the 2006/2007 RVF outbreak in Tanzania, ten regions were affected (WHO, 2007). The Morogoro region was one of the ten regions affected by the last RVF outbreak in Tanzania, Kenya and Somalia, but only two districts of Kilombero and Ulanga were affected, while the Morogoro district was not affected (Sindato *et al.*, 2014). The Mbeya region, where the Kyela district is located, was not one of the affected regions during the 2006/2007 RVF outbreak in Tanzania (Sindato *et al*., 2014; WHO, 2007).

Mbeya region is located in the south-western part of the country. Most of the Kyela district is lowland situated in the Great Rift Valley at 505 m above sea level, in the floodplains of Lake Nyasa. It receives heavy rains, of about 2000-3000 mm per annum and floods are common in March through May. The district has a warm and humid climate, with a mean daily temperature of 23℃. Together with Lake Nyasa, the district also has four large rivers, (Songwe, Mbaka, Lufilyo, and Kiwira), and many streams (Mkalizi, Kampala, Mgaya, Chiji, Kandete, Masukila, Njisi, and Kubanga). Agriculture dominates livelihoods and economic activities of the Kyela district. In addition to rain-fed paddy farming, other crops include banana and cocoa cultivation. Other livelihood activities include livestock farming and fishing. Because of the water logging condition, few sheep and goats are kept in the district. Few (1-5) cattle are kept per household by

tethering in communal grazing areas during the day and on the doorsteps of their houses at night for fear of theft, providing an animal reservoir of RVFV in the proximity of humans. The Morogoro district is located within the Morogoro region, 200 km east of Dar es Salaam. The annual average rainfall for Morogoro ranges between 500 and 1800 mm with temperatures between 18°C to 28°C. The main occupation of the inhabitants include crop cultivation and livestock keeping and a number of livestock species are kept including cattle, goats, sheep, pigs, camels, donkeys and horses. The livestock production in Morogoro is organised under commercial and traditional sectors. The livestock production systems are pastoralism, agro-pastoralism and small-scale intensive system which is becoming popular as land shortage force many livestock keepers to intensify their production. In the latter system, mainly crossbred animals are kept, and cut and carry system of feeding is practised.

3.2 Selection of Districts and Wards

The sampling process involved a two-stage purposive selection of districts and wards based on the findings of the past studies (2) reporting status of RVF outbreaks in Tanzania. The number of wards was not based on statistical considerations, but on logistic and resource availability. Based on the above, the Kyela and Morogoro districts with no previous history of RVF outbreaks were selected. In both districts, all veterinary officers were consulted to identify wards within each district considered to be at highest risk of RVF occurrence. Criteria used included areas subject to regular flooding, ecological features suitable for mosquito breeding, the relatively high concentration of domestic ruminants, proximity to rivers, ponds and lakes. The wards within the districts that were identified with most of these epidemiological characteristics were selected for the study.

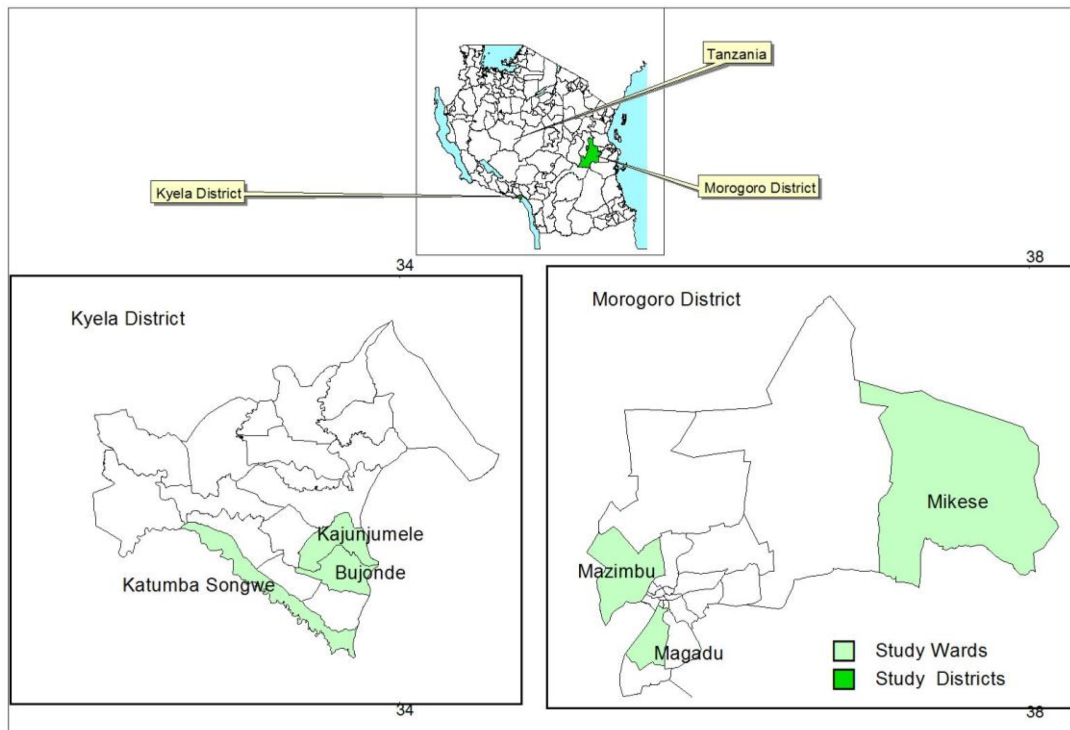

Figure 3: Selected study wards in Kyela and Morogoro districts, Tanzania

Within the selected wards, all households keeping cattle, sheep and goats and not having a history of vaccination against RVF were identified using local official veterinary records. The spatial and temporal patterns of RVF outbreaks in Tanzania; 1930 to 2007 (Sindato *et al.*, 2014b), showed that no previous outbreak had occurred in the two study districts. It was further confirmed during the study where, in each district, the veterinary offices were asked for any occurrence of RVF disease and/or outbreak, and history of livestock RVFV vaccination. Furthermore, information on the animal movements into the selected wards was retrieved from the veterinary officials, household heads and herdsmen. Additional information on the sources of replacement heifers was also requested. Wards without inward migration of animals from other areas were selected for the study.

3.3 Design, Sampling and Data Collection

Samples to determine RVFV seroprevalence in ruminants and small mammals were collected in a cross-sectional study, while those samples for determining inter-

epizootic/epidemic RVFV seroconversions were collected in a longitudinal study. In Kyela district, selection of wards and villages was purposively done based on the previous report of high RVFV seroprevalence in human (Heinrich *et al*., 2012). Based on that report, the wards of Kajunjumele, Bujonde and Katumba Songwe were found to have high RVFV seroprevalence and were included in this study (Fig. 1).

In Morogoro district, easily accessible farms in Magadu, Mazimbu and Mikese wards, were purposively selected for inclusion in the study.

3.3.1 Sample collection from ruminants

Blood samples were collected from three livestock populations, namely cattle, goats and sheep from the selected wards and villages. Assuming the prevalence of RVF antibody to be 10%, a total of 348 cattle (*Boss indicus* and the crosses between *Boss indicus* and exotic breed *Boss taurus*), local breeds of sheep (*Ovis aries*) and goats (*Capri hircus*) were sampled. The goats consisted of crosses between local breeds and exotic breeds of Toggenburg and Norwegian dairy goats. The sample size was determined using the formula below (Dohoo, Martin, and Stryhn, 2009).

$$n = \frac{Z^2_{(1-\alpha)} p(1-p)}{d^2} DE \quad \text{...(1)}$$

Where; n = sample size

DE = 2 (Design effect)

$Z_{1-\alpha}$ = $Z_{0.95}$ = is standard normal deviate (1. 96 corresponding to 95% confidence interval)

p = 10%, Estimated prevalence

d = 5%, Precision

The inclusion criteria included a history of non- vaccinated status against RVFV, animals born after the 2006/2007 outbreaks, calves of above 6 months of age and owners consent to using the animals for study. The exclusion criteria included those animals that had been vaccinated against RVFV, present during the last RVFV outbreak in Tanzania, animals less than 6 months and if owners consent was not granted.

3.3.2 Sample collection from cattle for serological testing

The local breed of zebu cattle (*Bos indicus*) and the crosses with the exotic breed (*Bos taurus*) were sampled by collecting 5 ml of blood from the jugular vein into plain vacutainer tubes. The criteria for selection of animals included a history of non-vaccinated status against RVFV, animals born after the 2006/2007 outbreak, calves above 6 months of age, and owners consent to using the animals for study. Herd and individual animal epidemiological data were obtained from the household head and herders as well as through clinical examination. The data collected included the breed, sex and age and feeding practices. In addition, a history of animal movements into the herd and whether the animals were born within the herd or introduced (moved) into the herd from another district was recorded. Sampling was based on only those herds with restricted animals without the history of movements to high RVF risk areas.

The blood samples were collected in vacutainer tubes without an anticoagulant, labelled and stored in a cooler box with ice packs while in the field. Before blood collection, animals were restrained into the crush or by use of ropes and halters. The blood was allowed to coagulate before serum was separated into a 1.5 ml cryovial tube, labelled and stored in a cool box with ice packs until transfer to the laboratory for analysis. Serum samples were stored at -80°C until analysis.

Animals were restrained using cattle crushes, ropes and halters and blood samples (5 ml) were collected from the jugular vein into 6 ml vacutainer tubes. The blood samples were kept in a cool box with ice packs and allowed to clot in the field before being transported to the Kyela veterinary laboratory and virology laboratory at Sokoine University of Agriculture (SUA) respectively. Serum was separated and stored at -80^0 C until tested for RVF IgG/IgM antibody.

3.3.3 Sample collection from sheep and goats for serological testing

In Kyela district, households with flocks of sheep (*Ovis aries*) and goats (*Capra hircus*) inwards previously shown to have high RVF seroprevalence in human (Heinrich *et al.*, 2012) were selected for the serosurvey. Sampling was a two-stage, procedure involving villages and households as the first and second stages respectively. In each village, all households with small ruminants were recorded to form the sampling frame and then randomly picked for inclusion in the study. Because of the low number of small ruminants per household in the district, all animals in the household were sampled. In the Morogoro district, five farms were purposively sampled because of the easy access and having large number of animals for sampling.

Blood samples were collected from the jugular vein using an 18 gauge needle attached to a 5 ml vacutainer tubes and kept in a cool box with ice packs. The blood samples were transported to Kyela district veterinary and Sokoine University of Agriculture (SUA) laboratories, respectively and kept at 4°C in a refrigerator overnight. The expressed serum was then aliquoted into two sterile vials per each sample and stored at 4°C in Kyela until transported to SUA laboratory. At SUA, the serum samples were stored at -80^0 C until tested for RVFV antibody.

3.3.4 Age and breed determination

Individual animal age was estimated from epidemiological data collected from household heads and herders, and where possible, by review of available records on the date of birth and dentition. Records were available from farms keeping crossbred dairy cattle. Dentition was used in determining the age of cattle divided into young or adult, depending on the eruption of the permanent incisors (Kikule, 1953). All cattle that had at least a permanent middle incisor were categorised as adult, while those without were categorised as young. Normally, the permanent incisors in cattle erupt at about 18 months of age and by 24 months they are fully developed. To exclude sampling young animals less than six months old, age was estimated by asking the head of the household, herd boys and other members of the household for the month, season and year of birth. Also, we performed physical observation of animal size and asked if they still were suckling. To avoid sampling animals present during the 2006/2007 RVF outbreak, animals that had initial wear on their incisor teeth (5 to 6 years old) and those which had noticeable wear (7 to 8 years old) were excluded from the study.

Breed types were recorded as local or cross-breed, depending on the body colouration, the presence of a hump and horns. Local breeds were shorthorn humped zebu with various body colouration. Sex of the animal and test results were provided in the same data set.

The age of individual animals was estimated based on available history and when possible, by reviewing available records on the date of birth and dentition. Records were available from farm owner on crossbred dairy cattle and goats in Morogoro. Dentition was used in determining the age of cattle as young or adult depending on the eruption of the first permanent incisors. All cattle that had at least a permanent middle incisors were categorised as adult while those without were categorised as young animals. Normally, the

permanent incisors in cattle erupt about 18 months and by 24 months, they are fully developed. Sheep and goats were classified as young when they had only deciduous teeth or adult when at least the middle pair of deciduous incisors has been replaced by permanent incisors. To exclude sampling young animals less than six months old, age was estimated by asking the head of the household, herd boys and other members of the household for the animal's month/season and/or year of birth.

3.3.5 Sample collection from rodents and shrews

Trapping of rodents and shrews was conducted concurrently with blood sample collection from cattle and small ruminants from June 2014 to October 2015. Trapping was done in selected habitats near the animal farms and villages using Sherman® live traps, (Sherman Traps, Inc., Tallahassee), (Fig. 4). In the fallow lands, 100 baited Sherman® traps were set per site for three consecutive nights. The traps were inspected every morning and captured animals were identified to genus or species according to (Kingdon, 1997).

Figure 4: Live Sherman trap set at Magadu ward, Morogoro, Tanzania Animal handling and processing

Captured animals were anaesthetized using diethyl ether, and 20 µl of blood drawn from the supraorbital veins of captured animals using glass capillary tubes. Blood samples were centrifuged at 1200XG for 10 minutes. to obtain sera which were stored at -80° C until tested for RVFV and/or RVFV antibody.

3.4 Assessment of Inter-epizootic/Epidemic RVFV Seroconversion in Cattle, Sheep and Goats

For detection of new cases of RVFV infection, two herds of cattle and two flocks of sheep and goats found to have high seroprevalence during the cross-sectional study in Magadu ward, Morogoro were monitored from January to May 2018. A total of 282 animals (145 cattle, 99 goats and 37 sheep) above 6 months old were individually identified using numbered ear-tags. Initially, serum samples were collected from individual animals and analysed for RVFV neutralising antibody by PRNT80 method. Among the 281 animals, 79 animals were positive for RVFV neutralising antibody. The animals which tested positive were excluded from the subsequent follow-up study thus remaing with a total of 202 animals. These animals were monitored from January to May 2018. Serum samples were collected in March, April and May and were analysed for RVFV neutralising antibody by PRNT80. An animal was considered to have seroconverted when a previously negative PRNT titre (<1:10) turned positive (⩾ 1:10) in subsequent follow up visit. The RVFV incidence was determined by calculating the rate of new seroconversion cases. In addition to monitoring the seroconversion, different potential RVFV risk factors including the farm, age, breed, sex and species were identified.

3.5 Serological Testing

3.5.1 Multi-species Competition Enzyme-Linked Immunosorbent assay (cELISA)

Each serum sample was analysed with the commercial Innovative Diagnostic (IDvet) Screen® RVF competition multispecies ELISA (cELISA) (IDvet, Montpelier, France). The commercial cELISA is based on the recombinant RVFV nucleoprotein and detects both RVFV IgM and IgG antibodies. The cELISA was carried out according to the manufacturer's instruction (see appendix 1 for protocol)

3.5.2 RVF IgM capture ELISA

The IgM ELISA test was employed for cELISA positive samples only. These samples were analysed with the commercial Innovative Diagnostic RVF IgM Capture kit (IDvet, Montpelier, France) according to the manufacturer's instruction (see appendix 2 for protocol)

The optical densities (ODs) obtained from the samples at 450 nm were validated in accordance with the manufacturer's instructions as follows:

The net OD was calculated: net OD = $OD_{\text{even well}}$-$OD_{\text{odd well}}$……………………….(2)

The plate was valid if the mean value of the net positive control OD was greater than 0.35 and the ratio of the mean values of the net positive and negative control (absolute value of ODs) is greater than 3 (net OD_{PC}/net $OD_{NC > 3}$

3.5.2.1 Interpretation of antibody detection results

For each sample, the percentage of the ratio of sample and positive control (s/p%) was calculated.

S/P% = net OD_{sample}/net $OD_{\text{postive control}}$……………………………….……………(3)

Samples presenting a S/P percentage (S/P%):

(i) Less than or equal to 40% were negative

(ii) Between 40% and 50% were doubtful

(iii)Greater than or equal to 50% were positive

3.5.3 Plaque reduction neutralization test

All samples that were positive for RVFV antibodies by the cELISA kit were analysed by $PRNT_{80}$. The $PRNT_{80}$ protocol used was adopted as previously described *(J. C. Morrill et al.*, 1991). The RVFV MP-12 vaccine strain, propagated in Vero-E6 cells, was used in the PRNT assay.

Each PRNT assay included the test sera, and a known RVFV antibody positive serum sample and an RVFV antibody-negative serum sample from cattle. Each serum sample was diluted in Hanks' Balanced Salt Solution (HBSS) supplemented with one % each of HEPES, penicillin and streptomycin and heat-inactivated fetal bovine serum (FBS). The dilutions of sera samples were made in 96 well plates beginning with a 1:5 dilution in the first wells followed by 4-fold serial dilutions of 1:20, 1:80, 1:320, 1:1280, and 1:5120 in each of subsequent wells (see appendix 3 for protocol)

3.7 Data Analysis

All data collected during this serosurvey of cattle were entered on a Microsoft Excel spreadsheet and were imported into IBM® SPSS® Statistics version 20. Descriptive statistics were calculated and univariable analysis was performed to assess the association between potential risk factors and RVFV seropositivity and the likelihood ratio statistic at p-value ≤ 0.05 used to determine statistical significance.

Data for sheep and goats were compiled in Microsoft Office-Excel 2007 (Microsoft, USA). Statistical analyses were processed in Rstudio software at a confidence interval of 95%. The Chi-square test was used to determine statistical significance difference between different groups. The individual serological status was the binomial response and the variables (ward, age, sex, and type of grazing) were the explicative factors.

Data for rodents and shrews were compiled in Microsoft Office-Excel 2007 (Microsoft, USA). Statistical analyses were processed in Rstudio software and different descriptive statistics were calculated. Univariable analysis was carried out to assess the association between potential risk factors and RVFV seropositivity (p-value $\leq$ 0.05) during the longitudinal study.

In the longitudinal study, the incidence rate (I), which is the number of new cases of the disease in a population per animal-time during a given period was calculated using the formula:

$$I = \frac{Number\ of\ new\ cases\ of\ a\ disease\ at\ a\ defined\ time\ period}{number\ of\ animal\ time\ at\ risk\ during\ the\ time\ period} \ldots\ldots(4)$$

The "animal-time" which is the denominator was calculated as follows:

(i) Let Dt period of follow-up.

(ii) Animal-time for animals that remained healthy for the whole period, $T1 = (N1) \times (Dt)$

(iii) Animal-time animals that develop the disease, $T2 = (N2) \times (½Dt)$, assuming that the disease occurred at the mid of the visit.

(iv) Sum of animal-time = $T1 + T2$

CHAPTER FOUR

4.0 RESULTS

4.1 RVFV Seroprevalence Rate in Cattle

A total of 356 cattle serum samples were analysed for RVFV antibody, of which 147 samples were from the Kyela district and 209 samples were from the Morogoro district. The overall seropositivity by cELISA was 29.2% (104/356) and a seroprevalence rate of 32% (47/147) and 27% (57/209) were recorded among animals in Kyela and Morogoro districts, respectively. Animals older than 2 years were more likely to be seropositive than animals younger than 2 years (OR = 0.19; *p* = 0.000); (Table 1). Likewise, zebu cattle were more likely to be seropositive than crosses, (OR = 2.5; *p* = 0.000). There were no significant differences between the districts (OR = 0.98; *p* = 1.0) and the type of holding (OR = 1.25; *p* = 0.34).

Table 1: Potential risk factors related to RVFV seroprevalence in cattle in Kyela and Morogoro districts

Risk Factor	Level	%Positive (n)	*OR*	95%(CI)	*P-value*
District	Kyela	32.0(47)	0.8	0.50-1.27	0.34
	Morogoro	27.3(57)			
Age	Adult	38.9(91)	0.19	0.1-0.35	<0.001*
	Young	10.7(13)			
Breed	Cross	20.0(36)	2.5	1.57-4.05	<0.001*
	Zebu	38.6(68)			
Sex	Female	29.3(80)	0.98	0.57-1.69	0.95
	Male	28.9(24)			
Holding	Farm	27.3(57)	1.25	0.79-1.99	0.34
	Open	32.0(47)			

When positive samples by cELISA were tested for RVFV IgM antibody by a capture ELISA, 8.4% (30/356) of the samples were positive for IgM antibody. When segregated by districts, IgM antibody seroprevalence rate was 2% (3/147) and 12.9% (27/209) in Kyela and Morogoro district, respectively (Table 2). In Morogoro district, seroprevalence rates for RVFV IgM antibody were 36.8% (14/38), 11.7% (12/103) and 1.5% (1/68) for Mikese, Magadu and Mazimbu wards, respectively, while in Kyela district, the IgM seroprevalence rates were 2.9% (2/70) and 2.5% (1/40) in Bujonde and Katumba Songwe wards, respectively. The RVFV IgM antibody was not detected in cattle serum samples collected in the Kajunjumele ward. The distribution of IgM antibody in districts and wards is shown in Table 2.

Table 2: Distribution of RVFV IgM and neutralising antibody in cattle in Kyela and Morogoro district

District	Ward	Total Samples (n)	IgM Positives (n)	%IgM positives	$\%PRNT_{80}$ positive of cELISA positives (n)
Kyela		147	3	2.0	100 (n=47)
Morogoro		209	27	12.9	80.7 (n=57)
	Bujonde	70	2	2.9	100 (n=25)
Kyela	Kajunjumele	37	0	-	100 (n=15
	Katumba Songwe	40	1	2.5	100 (n=7)
	Magadu	103	12	11.7	85 (n=33)
Morogoro	Mazimbu	68	1	1.5	0 (n=3)
	Mikese	38	14	36.8	86 (n=21)

Positive cattle sera samples by c-ELISA were also tested for RVFV neutralising antibody by PRNT and overall, 89% of the samples were positive. All the c-ELISA antibody-positive serum samples from Kyela district were positive for RVFV neutralising antibody, while 80.7% of the samples from Morogoro district were positive for RVFV neutralising antibody (Table 2).

4.2 RVFV Seroprevalence Rate in Sheep and Goats

A total of 304 sera samples, including 25 sheep and 279 goats, were analysed for RVFV antibody by c-ELISA. The distribution of the analysed samples according to different variables is shown in Table 3.

Table 3: Potential risk factors associated with RVFV antibody seroprevalence in sheep and goats in Kyela and Morogoro districts

Variable	Level	Total samples (n)	c-Elisa positives (n)	% c-Elisa positive	Chi-square	df	*P-value*
District	Kyela	25	1	4.0	0.51	1	0.48
	Morogoro	279	31	11.1			
Age	Adult	282	30	10.6	<0.1	1	1
	Young	22	2	9.1			
Breed	Cross	61	3	4.9	1.86	1	0.17
	Local	243	29	11.9			
Species	Sheep	25	6	24.0	3.81	1	0.05
	Goats	279	26	9.3			
Sex	Female	283	27	9.5	3.87	1	0.05
	Male	21	5	23.8			
Management	Extensive	98	11	11.2	0.45	1	0.5
	Semi-intensive	206	21	10.2			

The overall RVFV seroprevalence rate in sheep and goats was 10.5% (32/304)). Kajunjumele ward in Kyela district had a seroprevalence rate of 4% (1/25) and the rates for Magadu and Mikese wards were 10.2% (21/206) and 13.5% (10/74) in the Morogoro district, respectively (Table 4). When positive serum samples by cELISA were retested for RVFV IgM antibody, the overall seroprevalence rate in sheep and goats was 4.9 (15/304). Mikese ward had the highest RVFV IgM seroprevalence rate of 8.1(6/74), followed by Magadu ward with 4.4% (9/206)), while IgM antibody was not detected in the sheep and goat serum samples collected from Kajunjumele ward (Table 4).

Table 4: Inter-epizootic/epidemic RVFV IgG and IgM antibody prevalence rate in sheep and goats in different wards in the Kyela and Morogoro district

Ward	Total sample (n)	c-Elisa positives (n)	% c-Elisa positive	IgM positives (n)	%IgM positives
Kajunjumele	24	1	4.2	0	-
Magadu	206	21	10.2	9	4.4
Mikese	74	10	13.5	6	8.1
Total	**304**	**32**	**10.5**	**15**	**4.9**

4.3 RVFV Neutralising Antibody in Rodents and Shrews

A total of 581 small mammals were captured, consisting of two species of rodents (*Mastomys natalensis and Rattus rattus* and un-identified shrew (C*rocidura)* species. Among these, *M. natalensis* (n = 552) was the best represented (95%) followed by *Crocidura* spp 4.3%, (n = 25 and *R rattus* had the least capture rate, 0.7%, (n = 4), Table 5. With regards to the capture rate by the site of trapping, Magadu and Mikese wards had the highest capture rates of 46% and 44% respectively. Bujonde ward in Kyela district had the lowest capture rate (10%); (Table 5).

Table 5: Small mammal species captured at different trapping sites and RVFV neutralising antibody prevalence rates in Morogoro and Kyela districts

Species	Bujonde	Magadu	Mikese	Total	PRNT80 positives (n)	% Positives	%Capture
Crocidura spp	2	16	7	25	2	8	4.3
Mastomys natalensis	55	250	247	552	49	8.9	95
Rattus rattus	1	1	2	4	0	-	0.7
Total	**58**	**267**	**256**	**581**	**51**	**8.8**	

The overall RVFV sero-prevalence rate in rodents and *Crocidura* species was 8.8% (51/581). *M natalensis* had the highest prevalence 8.7% (49/552), followed by *Crocidura* species 8% (2/25) and none of the sera from *R. rattus* was found to have RVFV

neutralising antibodies (Table 5). With regards to the RVFV neutralising antibody seroprevalence rate by site of trapping, Magadu wards had the highest seroprevalence rate of 13.1% (35/267) followed by Mikese 5.5% (14/256) and Bujonde ward in Kyela district had the least seroprevalence rate of 3.4 (2/58) (Table 6)

Table 6: Inter-epizootic/epidemic RVFV neutralising antibody prevalence rate in rodents and shrews in selected wards of the Kyela and Morogoro district

Trapping site	Total capture (n)	Negative (n)	Positives (n)	%Positives
Bujonde	58	56	2	3.4
Magadu	267	232	35	13.1
Mikese	256	242	14	5.5
Total	581	530	51	8.8

The RVFV neutralising antibody titres in rodents and shrews ranged from 10 to 10240 (Table 7).

Table 7: Distribution of 80% plaque reduction neutralization endpoints in rodent and shrew species trapped in Kyela and Morogoro districts

Family	**Species**	**PRNT80 positives (n)**	**PRNT 80 end titres**
Muridae	*M.natalensis*	10	1:10
	M.natalensis	13	1:160
	M.natalensis	6	1:2560
	M.natalensis	10	1:40
	M.natalensis	10	1:640
Soricidae	*Crocidura spp*	1	1:160
	Crocidura spp	1	1:2560

4.4 Inter-epizootic/Epidemic RVFV Seroconversion in Cattle, Sheep and Goats

In the inter-epizootic/epidemic RVFV seroconversion study, at the beginning of the study, 28% (79/282) of the animals had RVFV neutralising antibody and were excluded from the follow-up study. A total of 28 animals were lost to follow in the period from January to March 2018, thus remaining with a total of 175 animals. The number of animals positive on the RVFV neutralising antibody in the longitudinal study is summarized in Table 8. Overall, RVFV seroconversions were detected in 25.7% (45/175), of the animals (Table 8), between January and May 2018. The initial sampling was carried out in January 2018 during the drought period. The subsequent samplings were carried out from March to May 2018, the period which coincided with the long-rainfall. During the second screening in March 2018, a total of 16 new cases of RVFV were shown by the detection of neutralising antibody.

The third screening was carried out in April 2018 and a total of 14 out of 159 remaining animals were found to seroconvert to RVFV infection (Table 8). Furthermore, a total of 15 animals were found to seroconvert during the fourth screening in May 2018.

Table 8: Number of cattle, sheep and goats seroconverted to RVFV infection during the inter-epizootic/epidemic period in Morogoro district, January-May 2018

Farm	Animal type	Jan-18	Number of new RVFV cases detected from January to May 2018			
			Mar-18	Apr-18	May-18	Total animals seroconverted
Farm 1	Cattle (n)	25	3	3	5	11
	Sheep (n)	3	2	3	1	6
	Goats (n)	25	4	1	3	8
Farm 2	Cattle (n)	16	2	2	4	8
	Sheep (n)	3	1	1	1	3
	Goats (n)	7	4	4	1	9
	Total (n)	**79**	**16**	**14**	**15**	**45**

During the period of the longitudinal study from January to May 2018, a total of 8 cows had an abortion and all eight cows were positive for RVFV IgM antibody. The 8 abortion cases involved cows that were negative during the initial screening for RVFV neutralising antibody in January 2018. When RVFV seroconversion from January to May was assessed with respect to various risk factors (herd, species, age, breed and sex), there was no statistically significant difference between the animals that seroconverted and those that did not seroconvert. Overall the incidence rate was 1.9 new cases of RVF disease per 1,000 animals per year.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Rift Valley Fever Virus Seroprevalence in Cattle

The overall seroprevalence rate of RVFV antibody in cattle by cELISA was 29.2% (104). This figure is high compared to the seroprevalence of 11.3%, and 13.1% previously reported in Tanzania and Kenya (Owange *et al*., 2014; Sindato *et al*., 2015; Sumaye *et al*., 2013). However, it is lower than the RVF seroprevalence of 38.7% of cattle reported for cattle (Chengula, Kasanga, Mdegela, Sallu, and Yongolo, 2014). The variation could be ascribed to the differences in time when the samples were collected, the methods used to analyse the samples as well as the agro-ecological conditions of the study sites. For example, the RVF seroprevalence rate found by Chengula and others was high probably because samples were collected during the 2006/2007 RVF outbreak. Furthermore, the samples for this study were collected from areas with no previous history of RVF outbreaks as opposed to that by Chengula and others. Thus, the results reported in this study indicate that domestic cattle from Kyela and Morogoro districts were infected by RVFV and could be contributing to the maintenance cycle of the virus. The two study districts had no previous history of RVF outbreak.

The cELISA method used to analyse cattle serum samples detects both IgG and IgM RVFV antibody. The RVFV IgG antibody persist in animals for life following infection, and therefore, detection provides a reliable index of previous infection by RVFV (Munyua et al., 2010; Thiongane, Gonzalez, Fati, and Akakpo, 1991) but does not indicate when the animals were infected. However, the detection of RVFV IgM antibody indicated that the virus was actively circulating sub-clinically in the two districts of Kyela and Morogoro during the time of sampling. This is supported by the fact that RVFV IgM antibody persist

for only 6 to 8 weeks after initial infection (Davies and Martin, 2006), and disappear in 50% of infected animals after 45 days, and are absent in almost 100% of infected animals by 120 days post-infection (J. T. Paweska, Burt, and Swanepoel, 2005). Mikese ward had the highest IgM seroprevalence (66.7%) followed by Magadu (36.4%) and Mazimbu had the lowest prevalence (33.3%). These results agree with those from other studies that detected RVFV activity in human in Kyela district and in sheep and goats in Kigoma region where the disease has never been reported before (Heinrich *et al*., 2012; Kifaro *et al*., 2014; Pourrut *et al*., 2010).

To summarize, RVFV IgM antibody was detected in all study wards except Kajunjumele, with Morogoro district having a relatively high IgM seroprevalence compared to Kyela. These findings indicated the presence of active RVFV infection at the time of sampling, during the dry season, at least in the wards examined. Despite the small number of wards and animals tested for IgM, these findings clearly demonstrated the circulation of RVFV during IE periods in non-outbreak areas. It is not clear why the circulating RVFV in these areas did not lead to clinical disease, and the possible mechanisms for virus maintenance remain to be elucidated. However, possible explanations could be circulation of non-virulent strains of RVFV in these areas or misdiagnosis excluding RVF for other febrile conditions with similar clinical features of fever and abortions. A limitation of the present study was that unfortunately did not attempt to isolate RVFV from the IgM-positive animals, due to biosafety issues. RVFV is classified as a biosafety level-3 agent and demands biosecurity measures not available during the study. Furthermore, we did not perform any RT-PCR analysis to detect virus RNA.

The differences in RVFV seroprevalence rates could also be attributed to the activities that facilitate animal movements such as livestock trade, lending animals among the

community members and payment of dowry. These animal movements could introduce RVFV into new areas.

In this study, RVF IgM antibody was detected in all study wards, with Morogoro district having the highest seroprevalence rate of 12.9% (n = 27) compared to 2% (n = 3) for Kyela. These findings indicated active transmission of RVFV at the time of sampling, (during the dry season), at least in the wards examined. Despite the small number of wards and animals tested for IgM antibody, these findings clearly demonstrated the circulation of RVFV in non-outbreak areas. It is not clear why the circulating RVFV in these areas do not cause disease, and the possible mechanisms for virus maintenance remain to be elucidated. However, the possible explanation could be the circulation of the non-virulent strain of RVFV in these areas, or that cases of RVF are misdiagnosed for other febrile illnesses among livestock that present similar clinical features of RVF disease.

The risk factors associated with RVFV transmission in areas with a history of outbreaks have been identified. However, further work is required to determine the possible risk factors associated with RVFV transmission in areas with no history of the outbreak. The observed RVFV transmission hotspots in Magadu and Mikese point to locally existing factors playing a major role in RVFV maintenance and transmission dynamics. Although entomological surveys were not conducted, the existence of suitable mosquito breeding habitats was evident.

The presence of water in farms throughout the year provides suitable habitats for the breeding of RVFV mosquito vectors. Persistent water in aquaculture ponds and waste

lagoons close to barns and grazing places for animals at Magadu farm may serve as important breeding habitats for the *Aedes* mosquito species. The presence of old machinery like tractors, discarded combined harvesters, old automobile tires and water storage containers may serve as water holding places thus providing harbourage and breeding habitats for mosquitoes and continuous low-level transmission of RVFV to vertebrate hosts.

Older cattle (>2 years old) were found to be at a higher risk of having RVFV antibodies than younger cattle (OR = 0.19, 95% CI (0.1-0.35). These findings agree with reports from studies which found higher seroprevalence rates in older animals (where) (Fontenille *et al*., 1998; Jeanmaire *et al*., 2011; Sumaye *et al*., 2013; Umuhoza *et al*., 2017).

In this study, indigenous zebu breed appeared more likely to be RVFV seropositive than crosses (OR = 2.5, 95% CI (1.57-4.05). This could be because the sampled crossbred animals were from dairy farms where there is frequent use of acaricides to control tick infestation. These acaricides contain insect repellents that could also protect cattle from mosquito bites, (Mnyone, 2018, personal communication) thus reducing the RVFV transmission to these animals. No significant difference between sex or the type of holding was observed.

5.2 Seroprevalence of RVFV in Sheep and Goats

The current study demonstrated that the overall RVFV antibody seroprevalence rate was 4.9% in sheep and goats in Kyela and Morogoro districts. The current RVFV antibody prevalence rate is lower as compared to the seroprevalence rate of 11% in sheep and goats reported from Kilombero flood valley, Tanzania by Sumaye *et al*. (2013). The difference

could probably be due to the fact the previous study was conducted in a location with a history of RVF outbreak where flooding favoured the abundance of mosquito vectors of RVFV. However, this result is comparable to an RVFV seroprevalence rate of 5.4% in sheep and goats in the Kigoma region with no previous history of RVF outbreak (Kifaro *et al*., 2014).

Recent RVFV infection in sheep and goats was supported by the detection of RVFV IgM antibody in 4.9% of the sheep and goat samples tested. This proportion is significantly higher than 12% reported in small ruminants in Mozambique (Fafetine *et al*., 2013) and Kenya (Lichoti *et al*., 2014).

Other studies in Tanzania reported 9 out of 190 samples of unspecified ruminant species to be IgM positive (Sumaye *et al*., 2013). Another study did not detect RVFV IgM antibody despite that the study was conducted in areas which experienced RVF disease during the last (2006/2007) outbreak in Tanzania (Sindato *et al.*, 2015).

The RVFV IgM seroprevalence rate was higher in goats (53.8%) than in sheep (16.7%). The detection of IgM antibody provided evidence of RVFV circulation during the inter-epizootic/epidemic in Kyela and Morogoro district where RVF outbreaks have never been reported. There were no reports of human RVF cases and no reports of increased abortion in sheep and goats by the livestock health authorities in these areas. However, evidence of inter-epizootic/epidemic active circulation of RVFV in areas with a history of RVF outbreaks has been reported (Sumaye *et al*., 2013; Swai and Schoonman, 2009b; Wensman *et al*., 2015).

RVFV antibodies have also been detected in areas where no clinical disease has been reported in human (Heinrich *et al.*, 2012) and in sheep and goats (Kifaro *et al.*, 2014). Virus circulation occurring among apparently healthy animals has been observed in Somalia (Soumare *et al.*, 2007), Uganda (Magona, Galiwango, Walubengo, and Mukiibi, 2013), Mayotte (Lernout et al., 2013), and Madagascar (Veronique Chevalier *et al.*, 2011). Thus, the RVFV IgM antibody detected in this study in areas where the disease has never been reported further confirms virus circulation in small ruminants without clinical disease manifestation.

The RVFV seroprevalence rate in sheep, 24% (6/24) and goats, 9.3% (26/279) were statistically different ($p = 0.05$). In one study in Tanzania, the prevalence rate was 12.5% and 4.7% for sheep and goats, respectively (Kifaro *et al.*, 2014). The results also corroborate with other studies that observed higher seroprevalence rates of RVFV in sheep than goats (Jeanmaire *et al.*, 2011; Rostal et al., 2010), while one study did not find a significant difference (Sumaye *et al.*, 2013). The reason for the observed difference in RVFV seroprevalence rate in sheep and goats is not known; however, it could be that one species is more susceptible than the other. The possible difference in susceptibility could be due to a number of factors such as host genetic background and differences in animal management, but more studies are needed to elucidate this.

Out of 304 small ruminant samples tested, 25 were from sheep, all from the Morogoro district. For this reason, the present results should be interpreted based on a small number of sheep serum samples tested. The relatively fewer sheep sera analyzed reflects the cultural preference of rearing more goats than sheep, such that the population of sheep in Tanzania is about five times lower than that of goats (Tanzania Bureau of Statistics,

2012). The absence of sheep in the study wards in Kyela was further influenced by the fact that the wards are found in the low-land that frequently experiences flooding and therefore, limits keeping sheep in these areas because of foot rot.

There was a significant difference in RVFV seroprevalence rate in females (9.5%) as compared to male animals (23.8%), $p = 0.05$. A higher seroprevalence rate was reported in female than male animals in a slaughterhouse in Chad (Ringot, Durand, Tolou, Boutin, and Davoust, 2004), while a serosurvey in Madagascar reported higher seroprevalence in male animals (Jeanmaire *et al*., 2011). The observed higher seroprevalence rate in male sheep and goats was not expected due to the fact that normally female animals are kept longer in a herd than male animals for reproduction purposes. The longer they stay in the herd, the greater the risk of exposure to the bites by infected mosquitoes thus, infection by RVFV. However, the present results should be interpreted based on a small number of serum samples from males tested (21), while a total of 283 serum samples from females were tested. The different timing and agroecological conditions between our study sites and the Madagascar survey site, i.e. 7 years versus 3 months post-outbreak, might explain the different result on the risk associated with sex in sheep and goats. Age-wise, 10.6% (n = 30) of the adults compared to 10.0% (n = 2) of the young sheep and goats were seropositive. However, there was no significant difference between adult and young sheep and goats, ($p = 1$). The detection of IgM antibody is suggestive of a recent infection, without reported clinical cases, posing questions as to whether the disease is overlooked due to lack of surveillance systems, or whether there are RVFV strains with low pathogenicity that do not lead to clinical disease. Since, the cELISA positive samples were confirmed by PRNT, which is the gold standard serological method with high sensitivity and specificity, the possibility of cross-reaction with related Phleboviruses or

other viruses that are genetically related to RVFV in the Kyela district is very unlikely. Since no virus isolates from Kyela or Morogoro are available it is hard to explain the nature of this seroprevalence in absence of clinical infection. In addition, these findings suggested the persistence of low-level RVFV circulation involving a cycle of the virus in domestic ruminants and mosquitoes and therefore offers another mechanism besides the possibility that the virus is maintained in mosquito eggs between outbreaks.

The short-lived IgM antibody detected in a few animals demonstrated evidence of recent RVFV infection. This observation further supports the increasing body of evidence pertaining to RVFV s transmission during the inter-epizootic/epidemic periods in sheep and goats. Inter-epizootic/epidemic transmission is likely to be difficult to detect even where active disease surveillance is in place because most of the infections are either subclinical or mistaken for other diseases (Heinrich *et al*., 2012; Pourrut *et al*., 2010). Despite the seroconversions observed, there have been no previous reports of the epizootic/epidemic or clinical disease report in Morogoro district. The explanation for this could be the circulation of the non-virulent strain of RVFV in these areas. Other reports of seroconversion with no previous epidemic/epizootic or clinical disease reports have hypothesized the circulation of non-virulent RVFV strains during the inter-epidemic periods (Heinrich *et al*., 2012; Pourrut *et al*., 2010). But also, cases of RVF could easily be confused with other diseases with similar clinical features like abortions thus overlooked and under-reported.

Given the potential of infection by RVFV and the movement of exotic breeds of goats across districts for dairy projects, there are chances of introducing the virus to new areas. Thus, there is a need to screen animals for RVF before they are shipped out.

5.3 Rift Valley Fever Virus Neutralizing Antibody in Rodents and Shrews

In this study both rodent (Muridae) and *Crocidura* spp (Soricidae), were shown to have RVFVs neutralising antibody. This is the first report of RVFV antibody in rodents and shrew in Tanzania. The rodent and shrews serum samples tested were collected from Kyela and Morogoro, the districts with no previous history of RVF outbreaks. Rodents and shrews could be involved in the RVFV maintenance in nature and may act as a source of virus for infecting the mosquito vectors and subsequent transmission to susceptible vertebrate hosts. These results agree with other reports that demonstrated antibodies in *A. abyssinicus* (Pretorius, Oelofsen, Smith, and van der Ryst, 1997). Also, viremia was experimentally demonstrated through inoculation of rodent species with RVFV (Weinbren and Mason, 1957), thus suggesting that rodents could act as maintenance hosts for this virus.

RVF outbreaks in East Africa are associated with unusually heavy rainfall and abundant mosquito populations and the disease occur over wide geographic areas. In this study, the RVFV neutralising antibody was demonstrated in rodents and *Crocidura* spp sera samples collected from June 2014 to October 2015, the time coinciding with low rainfall. These findings demonstrated continuous activity of RVFV even in periods of low rainfall, probably due to the existence of local environmental factors that favour the multiplication of mosquito vectors that transmit the virus to rodents and shrews. The rodents and *Crocidura* spp could thus be acting as cryptic carriers during interepizootic periods (Pretorius *et al.*, 1997). The detection of RVFV neutralising antibody in this study corroborate with reports from other studies which found serological evidence of RVFV antibody in several rodent species (Gora *et al.*, 2000; Pretorius *et al.*, 1998; Saluzzo *et al.*, 1987; Youssef and Donia, 2001; Zeller *et al.*, 1997).

RVFV neutralising antibody was demonstrated in sera samples collected from rodents and shrews. The seroprevalence rate varied with species) and location. *M. natalensis* had the highest seroprevalence rate (8.7%), followed by *Crocidura* spp (8%) and none of the 4 serum samples from *R. rattus* tested positive. The seroprevalence rate of RVFV neutralising antibody demonstrated in this study is higher than that found by another study which found RVFV neutralising antibody in one of 1260 sera of murids (Swanepoel, Blackburn, Efstratiou, and Condy, 1978). Also, another study failed to detect RVFV neutralising antibody in rodents (Scott and Heisch, 1959). The highest rate of RVFV neutralising antibody was found in the Magadu ward (12.7%), Mikese (5.5%) in Morogoro district and Bujonde (3.5%) in Kyela district. The detection of RVFV neutralising antibody among rodents and shrews captured at each trapping site indicated wide circulation of the virus in the Magadu, Mikese and Bujonde wards. This could also reflect wide circulation of RVFV in rodents, shrews and other small mammalian species in other endemic areas.

Since trapping of small mammals was conducted in areas with no previous history of RVF outbreak, the existence of RVFV strains with reduced pathogenicity is highly probable.
High RVFV antibody prevalence rates (including IgM) were demonstrated in sera collected from clinically healthy humans in Bujonde and Kajunjumele wards of the Kyela district (Heinrich *et al*., 2012). Furthermore, RVFV antibody was demonstrated in cattle sera collected concurrently with the trapping of rodents and shrews in Kyela and Morogoro districts. Since there were no clinical cases in cattle or human reported from these areas, it is hypothesized that RVFV strains exist which are well adapted to their vector-insect as well as mammalian hosts and do not cause clinical disease. This hypothesis is supported by reports from other studies that found RVFV antibody in

humans and domestic ruminants during inter-epizootic/epidemic periods without apparent clinical cases (Durand *et al*., 2003; LaBeaud *et al*., 2008; Pourrut *et al*., 2010; Sumaye *et al*., 2015; Swai and Schoonman, 2009).

5.4 Inter-epizootic/Epidemic RVFV Seroconversion among Domestic Ruminants

This study demonstrated RVFV seroconversions among domestic ruminants (cattle, sheep and goats) during the inter-epizootic/epidemic period (between January 2018 and May 2018). During the initial screening, 28% (79/281) of the animals were found to have RVFV neutralising antibody. However, none of these animals was found to have RVFV IgM antibody. This implies enzootic circulation of the RVFV in these herds since there was no active circulation during the time of initial screening.

The initial screening was carried out in January 2018, the time when it was dry. The high amount of rainfall has been shown to be related to the high activity of the RVFV because of the increase in mosquito populations. Heavy rains that result in prolonged flooding increase mosquito habitat suitability that ensures the proliferation of vector populations, thus influencing the risk of disease emergence, transmission and spread (Davies *et al*., 1985).

Sixteen animals of those initially negative for RVFV neutralising antibody seroconverted within the period between January and March 2018. Similarly, 14 and 15 animals also seroconverted to RVFV infection during the period between March and April and April to May 2018 respectively. None of the risk factors tested was found to be s significant with RVFV inter-epizootic/epidemic seroconversions.

The detection of RVFV seroconversions among the domestic ruminants confirms the ongoing RVFV infection in these two herds. Further evidence of low-level active RVFV circulation was demonstrated by the detection of IgM antibody among eight animals, which aborted during the observation period. Although there was no previous history of RVF outbreaks in Morogoro, high inter-epizootic/epidemic RVFV seroprevalence rates were previously demonstrated among these two herds during 2014 and 2015. No reports of clinical cases or outbreaks among animals and human populations were reported during this period. Thus, detection of RVFV neutralising antibody two years later confirms the continuation of virus infection among these herds. It is also possible that RVFV is wide-spread among other herds, thus there is a need to conduct sero-surveys over wider areas to include more herds.

Previous studies have detected RVFV seropositivity among humans and domestic animals during the long interepizootic/epidemic periods in Tanzania (Heinrich *et al*., 2012; Sumaye *et al*., 2013, 2015; Swai and Schoonman, 2009; Wensman *et al*., 2015). However, most of these were cross-sectional studies for detection of RVFV IgG antibody. Given the persistence of IgG antibody over several years, the actual date of infection cannot be deduced from cross-sectional studies. In cross-sectional studies, only serological findings are available for analysis, while the determination of specific RVF-related clinical signs and sequelae to assess clinical reflection of serological findings is not possible. Moreover, the risk factors at the time of infection may have differed from the time of seroprevalence assessment, leading to conclusions that RVFV infection during the inter-epizootic/epidemic period occurs without manifestation of the clinical symptoms.

In this study, monitoring for RVFV seroconversions allowed assessment of specific RVFV infection-related clinical signs, sequelae and real-time risk factors. Based on the results from seroprevalence studies, it has been hypothesized that there are non-virulent RVFV strains (Heinrich *et al*., 2012; Pourrut *et al*., 2010; Sumaye *et al*., 2013). However, contrary to this hypothesis, the occurrence of abortions in eight cows during the study period demonstrated that many clinical cases associated with RVFV infection during the inter-epizootic/epidemic period are underreported or misdiagnosed with other diseases presenting similar clinical signs. The eight cows that aborted were found to be positive for RVFV IgM antibody, thus confirming the recent infection.

Cases of RVFV infection during inter-epizootic/epidemic periods may not be detected because under pastoral livestock management system routine pregnancy diagnosis is not performed and close monitoring of the animals is lacking. The lost foetuses are easily eaten by scavenging dogs and other predators without the knowledge of the livestock keepers. Abortions due to RVFV infection could be one of the factors that result in long inter-calving intervals in animals kept under pastoral management system (Kanuya *et al*., 2006; Matiko, Kanuya, Waldmann, Ropstad, and Reksen, 2008). Therefore, there is a need for proper follow-up of cases and improved diagnostic capacity for RVFV activity among livestock and human populations. This calls for a concerted national and regional effort to develop and implement control strategies involving interdisciplinary experts from medical, veterinary and allied sciences in disease research. Control and/or prevention of RVF require a common approach between medical and veterinary personnel as well as an integrated regional approach that operates based on 'One Medicine' philosophy.

The findings of this study support other reports that suggest that average rainfall is important in the maintenance of low-level RVFV transmission during the inter-epizootic/epidemic period (A. Desirée LaBeaud, Cross, Getz, Glinka, and King, 2011).

The samples which were collected during the dry period in January 2018 were all negative when tested for short lived RVFV IgM antibody, meaning that there was no recent infection. Within a period of 80 days, 9.1% of the animals seroconverted and two among these animals had IgM antibody probably due to the long rains which started in March 2018.

Contact between infected vectors and naive vertebrate hosts is vital for successful RVFV transmission (Weaver and Barrett, 2004). Using the CDC light traps, different mosquito species including *Aedes* and *Culex* species, the known vectors for RVFV were trapped around the two study herds (data not shown). Thus, the study area for RVFV seroconversions appears to have both the vectors and hosts required to maintain inter—epizootic/epidemic transmission among domestic animals. Furthermore, wild rodents implicated as the potential maintenance hosts for RVFV during the inter-epizootic/epidemic periods trapped near the two study herds were found to have RVFV neutralising antibody. This demonstrates their possible involvement in the maintenance and transmission cycles of the virus without necessarily requiring transovarial transmission via virus-laden dormant mosquito eggs. To confirm this, a longitudinal study with concurrent collection of mosquitoes and sera from ruminants and wild rodents is needed from this area to determine their status regarding RVFV infection. In addition, studying the population dynamics of mosquitoes, ruminants and wild rodents in relation to changes of RVFV infections from these species over time will elucidate their role in virus maintenance during the inter-epizootic/epidemic periods.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study demonstrated serological evidence of previous widespread RVFV inter-epizootic/epidemic circulation among domestic ruminants in areas without previous reports of outbreaks. This study also detected RVFV neutralizing antibody in rodents and shrews in Tanzania, thus implicating a role for these mammalian species in the inter-epidemic maintenance of the virus. The RVFV neutralizing antibody rate was high in *M. natalensis*, which is a commensal rodent species and potentially a source of RVFV virus infection for humans and livestock. Therefore, public health and animal health workers should be made aware of the possibility that rodents and shrews may be a source of RVFVs infection for humans and livestock.

Demonstration of RVFV seroconversion in ruminants provided evidence of ongoing active inter-epizootic/epidemic transmission of RVFV in Morogoro district. This observation provided a better understanding of the involvement of domestic ruminants in the inter-epizootic/epidemic maintenance and transmission of RVFV. Furthermore, the occurrence of abortions in cows demonstrated that RVFV infection during inter-epizootic/epidemic periods was associated with clinical symptoms (abortion), thus a close follow-up of animals for cases detection is needed. Given that most rural inhabitants of the sampled districts live in close contact with the animals and do experience frequent mosquito bites, human infection with RVFV could occur regularly, and be an important, but overlooked cause of febrile conditions frequently treated as malaria cases.

Although several RVFV epizootic/epidemics have occurred in Tanzania, no epizootic/epidemics have been reported in Kyela or Morogoro districts. The detection of

RVFV antibodies in sera collected from cattle, sheep and goats in Kyela and Morogoro districts supported RVFV among livestock populations in these districts. The detection of RVFV IgM antibody in some animals supported active foci of recent transmission. However, the mechanism of virus maintenance during inter-epizootic/epidemic periods is unclear. In East Africa, it is speculated that RVFV is maintained in the eggs of Aedes species mosquito as a result of transovarial transmission and that epizootics/epidemics occur following an increase in the mosquito population after abnormally heavy rains.

Since surveys for RVFV activity are not regularly carried out, the results from this study clearly indicated that the disease is underreported in many districts in Tanzania. There are indications that RVF is endemic in certain parts of the country and that subclinical infection occurs in cattle, sheep, and goats during the inter-epizootic/epidemic period. A Niche-based Modelling approach that assessed the influence of climate on the variability of habitat suitability and shifts for RVF vectors in relation to RVFV outbreaks in Tanzania identified agroecological conditions for the maintenance of the disease. Inter-epizootic/epidemic transmission of FVFV is difficult to detect even if active disease surveillance is in place in the livestock and/or human populations because most of the inter-epizootic/epidemic infections are subclinical or mistaken for other diseases.

6.2 Recommendations

Based on the findings of the present study, the following are recommended:

(i) It is being proposed that a more comprehensive and inclusive surveillance study be conducted to identify and characterize the RVFV inter-epizootic/epidemic maintenance hosts and its transmission cycles. Longitudinal investigations will lead to a better understanding of ongoing RVFV circulation thus, a better

understanding of the inter-epizootic/epidemic virus maintenance and transmission cycle.

(ii) The presences of RVFV antibodies in ruminants represent evidence of inter-epizootic/epidemic circulation of RVFV that cause mild or subclinical manifestation. Additional studies including the isolation of RVFV from humans, ruminants and mosquito vectors a is critical for confirming the existence of an enzootic RVFV maintenance and to determine whether or not there are any strain variations.

(iii) It has been reported that RVFV is passed from generation to generation of mosquito vectors through transovarial transmission, accounting for the continued presence of the virus in enzootic areas. In addition to this mechanism, the results demonstrate that domestic ruminants and rodents may be involved in the inter-epizootic/epidemic maintenance of the virus as well. Therefore, a more detailed surveillance study should be done to determine potential reservoirs and characterize the viral ecology in both mosquitoes and mammalian populations in different ecosystems in Tanzania and other countries.

(iv) Utilize the data generated by this study to design and conduct a comprehensive longitudinal study in an area such as Morogoro where the RVFV IgM antibody rate was high in domestic ruminants, especially in an area with evidence of clinical manifestation in domestic ruminants, such as cattle. The design should include a cohort of RVFV antibody negative animals followed over time with blood sampling at about 4 months intervals for at least 12 months or more to increase the likely-hood of detecting both RVFV and IgM antibody. Also, at the same time in close proximity to the cohort of animals, collect mosquitoes and sandflies once every 2 weeks and test for the virus in Vero cells using Cytopathic effects (CPE)

as an indicator of a possible virus isolate. Such a study will provide a better understanding of possible RVFV infection in domestic animals based on the isolation of RVFV and/or detection of RVFV IgM antibody. With the availability of RVFV, studies can be conducted to address the question as to whether or not the virus has reduced virulence. Also, by including studies on the mosquito and possibly sandfly populations, the findings will provide an understanding of the mosquito species seasonal succession, and link abundance and distribution of mosquito species with possible RVFV infection in domestic ruminants. This will facilitate a better understanding of the role of mosquitoes and domestic ruminants in the RVFV inter-epizootic/epidemic maintenance and transmission cycle and provide information required to establish effective surveillance and control programs.

(v) A better understanding of the inter-epizootic/epidemic cycles will allow better outbreak prediction and rapid response to prevent the RVFV infection of human and livestock populations.

(vi) Educate and disseminate information to public and animal health authorities to improve awareness and achieve more effective control measures to limit the spread of RVFV infection.

(vii) Develop simple and sustainable tool for surveillance of RVF disease and RVFV in enzootic countries. In addition, perform capacity building in diagnostics and transfer simple technology that can be used in the field to detect RVF and RVFV. The results of the field-based diagnostic test can later be confirmed with laboratory assays such as ELISA, virus neutralisation test, and molecular-based techniques.

(viii) This is the first study to demonstrate RVFVneutralizing antibody in rodents and shrews in Tanzania. It is recommended that additional studies be designed and

conducted in other areas with and without the history of RVF outbreaks since it is likely that a silent RVFV transmission cycle exists in rodents and shrews.

(ix) Small mammals should be included in RVFV control programs. (need to describe, will it be feasible for most surveillance and control programs to include small mammals, when many countries cannot afford routine surveillance and control programs for domestic ruminants.

(x) Attempt to isolate the RVFV from infected hosts followed by in-depth molecular characterization of the isolates are highly recommended so that the appropriate and tailored RVF control methods could be developed and implemented in the region.

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APPENDICES

Appendix 1 Multi-species Competition Enzyme Linked Immunosorbent assay

Serological testing

Multi-species Competition Enzyme-Linked Immunosorbent assay (cELISA)

Each serum sample was analysed with the commercial Innovative Diagnostic (IDvet) Screen® RVF competition multispecies ELISA (cELISA) (IDvet, Montpelier, France). The commercial cELISA is based on the recombinant RVFV nucleoprotein and detects both RVFV IgM and IgG antibodies. The cELISA was carried out according to the manufacturer's instruction. Briefly, 50 µl of the dilution buffer was dispensed into each well of a labelled ELISA plate pre-coated with recombinant RVFV nucleoprotein. Then, 50 µl of the internal positive (freeze-dried RVFV IgG positive bovine serum supplied by the manufacturer) and the internal negative control (supplied by the manufacturer) were added in duplicates. To the remaining wells, 50 µl of each sample was added. After mixing samples and controls with the TST dilution buffer (50 mM Tris/150 mM NaCl/0.1% Tween 20, pH 8.0), we incubated at 37°C for 1 hour. The wells were washed three times with washing buffer using a well plate washer (Thermo Scientific™ Wellwash™ Microplate Washer, Waltham, MA USA). Next, 100 µl of anti-nucleoprotein peroxidase (HRP) conjugate was added to the wells and the contents of the plate were incubated at room temperature for 30 min, followed by washing three times with 300 µl of wash solution as before to remove excess conjugate. Then, 100 µl of substrate solution 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and the plate was incubated at room temperature for 15 min in the dark. To terminate the reaction 100 µl of 2n Sulphuric acid ($2NH_2SO_4$) stop solution was added to each well. The presence of antibodies to RVFV was detected by lack of a colour change, whereas the absence of antibodies to RVFV was detected by a change in substrate colour to blue. The contents of

the wells of the microplate were read at a wavelength of 450 nm by a microplate absorbance reader (Molecular Devices, CA, USA).

For each cELISA experiment duplicate, internal controls were incorporated. The optical densities (ODs) of the control were detected at 450 nm. To verify the reliability and validity of the results obtained from each cELISA test, the average of the ODs of the two negative controls (NCs) was > 0.7 while the average of the two positive controls divided by the average OD of the NCs was > 0.3. For each sample, the competition percentage was calculated by dividing the OD of the sample by the average OD of the negative control multiplied by 100 ([ODsample/ODNC] x 100). A sample was considered positive if the value obtained from the formula was ≤ 40%. Any sample with a value of > 50% was considered to be negative, whereas values ranging from 40-50% were considered to be doubtful.

Appendix 2: RVF IgM capture ELISA

The IgM ELISA test was employed for cELISA positive samples only. These samples were analysed with the commercial Innovative Diagnostic RVF IgM Capture kit (IDvet, Montpelier, France) according to the manufacturer's instruction. Briefly, 40 µl of the diluent buffer was dispensed into each well of a labelled microwell plate pre-coated with anti-bovine-ovine-caprine IgM polyclonal antibodies. Then, 10 µl of the internal positive control (freeze-dried anti-RVFV recombinant NP bovine serum supplied by the manufacturer) and the internal negative control (supplied by the manufacturer) were added in duplicates. To the remaining wells, serum samples were added in duplicate and the plate with all samples was incubated at 37°C for 1 hour. The microplate wells were then washed three times with 300 µl by a microplate washer as above. Next, 50 µl of RVFV nucleoprotein or diluent buffer was added and incubated at 37°C for 1 hour. The wells were washed three times followed by the addition of 50 µl of anti-RVFV nucleoprotein horseradish peroxidase (HRP) conjugate solution to each well and incubation for 1 hour at 37°C. Again, the wells were washed three times as above and 100 µl of the substrate solution, TMB, was added to each well and then incubated for 15 min at room temperature in the dark. Then, 100 µl of stop solution was added to terminate the reaction. The presence of IgM antibodies to RVFV was detected by the appearance of blue colouration, which became yellow after the addition of the stop solution. The contents of the wells of the microplate were analysed at 450 nm by a microplate absorbance reader (Molecular Devices, CA, USA).

For each IgM antibody capture ELISA experiment duplicate internal controls were incorporated. The optical densities (ODs) obtained from the samples at 450 nm were validated in accordance with the manufacturer’s instructions as follows:

The net OD was calculated: net OD = $OD_{\text{even well}}$-$OD_{\text{odd well}}$

The plate was valid if the mean value of the net positive control OD was greater than 0.35 and the ratio of the mean values of the net positive and negative control (absolute value of ODs) is greater than 3 (net OD_{PC}/net $OD_{NC > 3}$

Appendix 3: Plaque Reduction Neutralization Test

For each sample, the percentage of the ratio of sample and positive control (s/p%) was calculated.

S/P% = net OD_{sample}/net $OD_{postive\ control}$

Samples presenting a S/P percentage (S/P%):

(i) Less than or equal to 40% were negative

(ii) Between 40% and 50% were doubtful

(iii)Greater than or equal to 50% were positive

Plaque reduction neutralization test

All samples that were positive for RVFV antibodies by the cELISA kit were analysed by $PRNT_{80}$. The $PRNT_{80}$ protocol used was adopted as previously described (Morrill *et al.*, 1991). The RVFV MP-12 vaccine strain, propagated in Vero-E6 cells, was used in the PRNT assay.

Each PRNT assay included the test sera, and a known RVFV antibody positive serum sample and an RVFV antibody-negative serum sample from cattle. Each serum sample was diluted in Hanks' Balanced Salt Solution (HBSS) supplemented with one % each of HEPES, penicillin and streptomycin and heat-inactivated fetal bovine serum (FBS). The dilutions of sera samples were made in 96 well plates beginning with a 1:5 dilution in the first wells followed by 4-fold serial dilutions of 1:20, 1:80, 1:320, 1:1280, and 1:5120 in each of subsequent wells. Each diluted serum sample was then mixed with an equal volume of 60-80 plaque-forming units (PFU) of MP-12 vaccine virus to final dilutions shown in figure 5. The quantification of PFU was confirmed by a plaque assay based on testing a mixture of equal volumes of the 60-80 PFU and HBSS to confirm that the final virus dose ranged from 30-40 PFUs. The antibody positive control consisted of a mixture of an equal volume of 60-80 PFU and a 1:10 dilution of antibody-positive cattle serum.

The antibody negative control consisted of a mixture of an equal volume of 60-80 PFU and a 1:10 dilution of RVFV antibody negative cattle serum. The virus/serum dilution mixtures were incubated at 37°C in the absence of CO_2 for one hour. Next, 50 µl of the virus/serum dilution mixtures were inoculated onto each of two Vero E6 cell monolayer cultures propagated in 24-well tissue culture plates and incubated for one hour at 37°C and 5% CO_2. Virus mixed with the antibody-positive control serum was inoculated onto twenty separate Vero E6 cultures. Virus mixed with antibody-negative control serum mixture was inoculated onto four Vero E6 cultures. After incubation for one hour at 37°C with 5% CO_2, each cell culture was overlaid with 0.5 ml of a Seakem agarose (1%) with an equal volume of 2X Eagle's Basal Medium with Earle's salts (EBME) supplemented with 8% FBS and one % penicillin/streptomycin, and Glutamine+8g/l HEPES. After two more days of incubation at 37°C with 5% CO_2, each culture was overlaid with 0.5 ml of a mixture of an equal volume of agarose (1%) and 2X EBME supplemented with 5% neutral red, 8% FBS, and penicillin and streptomycin (1%) and Glutamine+8g/l HEPES and incubated overnight at 37°C with 5% CO_2. The PFUs were counted and recorded for both the controls and cattle serum samples. An 80% reduction in the number of PFUs was used as the endpoint for antibody virus-neutralization titers (PRNT80). Wells with too high number of PFUs, that were impossible to count at that dilution, were recorded as TNTC (too numerous to count).

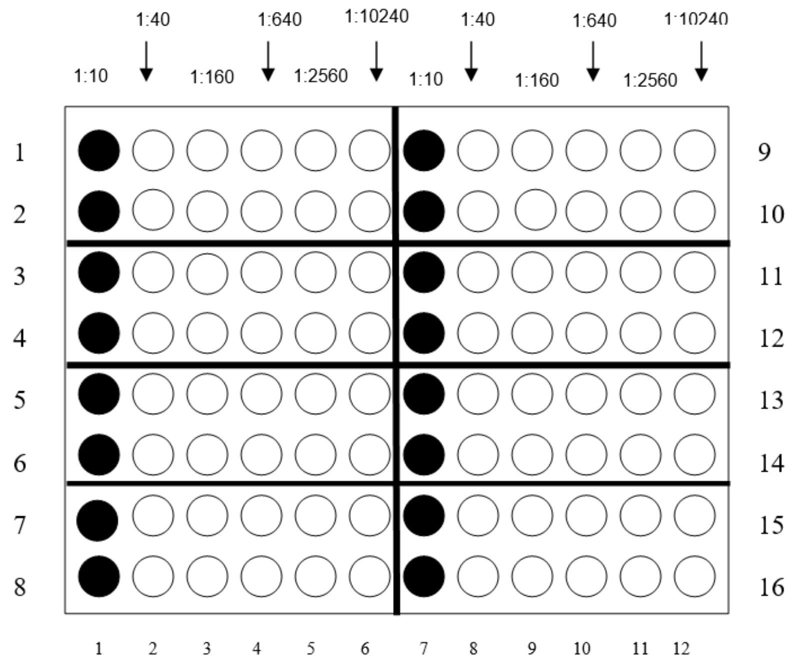

Figure 5: 96-well plate format with final dilutions indicated