

**INTER – EPIDEMIC TRANSMISSION OF RIFT VALLEY FEVER VIRUS IN
NGORONGORO DISTRICT, NORTHERN TANZANIA**

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**A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY
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EXTENDED ABSTRACT

Rift Valley fever virus (RVFV) causes an acute Rift Valley fever (RVF) disease in humans and domestic ruminants whose occurrence assumes an epidemic pattern in most cases. Understanding the maintenance of RVFV in susceptible hosts and potential vectors during inter-epidemic periods (IEP) is vital for better understanding of disease transmission dynamics. The aim of this study was to investigate the transmission dynamics of RVFV in Ngorongoro district, Northern Tanzania.

This study involved Rift Valley fever virus-Immunoglobulin G (RVFV-IgG) seropositive samples (n=160) from cattle, sheep, goats and humans that were collected from previous cross-sectional survey conducted in parallel with mosquito sampling in Malambo, Meshili, Osinoni, Endulen and Nainokanoka villages. The selected seropositive samples were tested for the presence of RVFV using the real- time Reverse Transcription polymerase chain reaction (qRT-PCR) and conventional RT-PCR.

Adult mosquitoes were collected outdoor using the Centres for Disease Control and Prevention (CDC) light traps baited with carbon dioxide. The traps were set in proximity to potential breeding sites and cattle kraals. The mosquitoes were identified using conventional morphological keys, in addition, *Anopheles gambiae* complex species were identified using Conventional PCR.

Viral RNA was extracted directly from serum samples and mosquitoes using a QIAamp Viral RNA Mini Kit (QIAGEN), the molecular detection of RVFV from serum samples was first performed using qRT- PCR, positive samples were then rescreened using conventional RT-PCR. A total of 96 Pools each containing 10 monospecific potential mosquito vectors were also tested for RVFV RNA using qRT-PCR.

The qRT-PCR detected 2 (5%), 4 (10%) and 1 (2.5%) positive samples in cattle, sheep and goat sera respectively. These 7 positive samples following screening using qRT-PCR were also found to be positive after retesting using conventional RT-PCR. No RVFV was found in human sera. The detection of RVFV was found in domestic ruminants of Meshili (6 cases) and Malambo (1 case) villages.

A total of 2,094 adult mosquitoes belonging to 3 genera and 9 species were collected. Most of them were collected in Meshili village (87.5%, n= 1832), followed by Malambo (8.2%) and Osinoni (4%) villages. No mosquitoes were caught in Nainokanoka and Endulen villages. The nine species collected were *C. pipiens* complex, *C. antennatus*, *C. tigripes*, *C. annulioris*, *C. cinereus*, *An. arabiensis*, *An. squamosus*, *An. pharoensis* and *Ma. uniformis*. With the exception of *C. cinereus*, all the other eight species have been implicated in RVFV transmission. None of the 96 mosquito pools tested was found to be positive for RVFV.

This study has demonstrated the maintenance of the RVF virus in domestic ruminants during the inter-epidemic period in the absence of reported cases in livestock or humans. There is a need of active surveillance system to monitor circulation of the virus especially during IEPs for better understanding of the mechanisms of disease transmission in these villages.

In addition, various mosquito species potentially competent for RVFV transmission were collected. This demonstrates the local mosquito abundance that could propagate RVFV in the study villages. These vectors were heterogeneously distributed suggesting possible differences in disease transmission between the villages. However, comprehensive entomological study has to be undertaken during epidemic so as to identify mosquito species that can maintain and transmit the virus by bite.

DECLARATION

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

Athanas Dustan Mhina
(MPhil Candidate)

Date

The above declaration is confirmed

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DEDICATION

This work is dedicated to my parents, the late Mr. Dustan Joram Mhina and my mother Mrs Elizabeth Mhina for their good efforts to raise me in a good manner that has led me be who I am today. I also dedicate this work to my siblings for their moral support and encouragement.

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LIST OF PUBLISHED PAPERS

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LIST OF ABBREVIATIONS

CDC	Centre for Disease Control and Prevention
CIDB	Centre for Infectious Disease and Biotechnology
Ct	Cycle Threshold
DNA	deoxyribonucleic acid
E	East
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
Gn	glycoprotein
IEP	inter epidemic period
IgG	immunoglobulin G
IgM	immunoglobulin M
L	large segment
M	medium segment
NSm	non structural protein
OIE	World Organisation for Animal Health
P.C.R	polymerase chain reaction
p.m	after noon
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RT-PCR	Real Time Polymerase Chain Reaction
S	South
TVLA	Tanzania Veterinary Laboratory Agency
USA	United States of America
UV	Ultra Violet light
WHO	World Health Organization

1.0 GENERAL INTRODUCTION

1.1 Background Information

Rift Valley fever virus (RVFV), classified to the *Phlebovirus* genus of the *Bunyaviridae* family, a zoonotic disease characterized by periodic and severe outbreaks in humans and animals (Peters *et al.*, 1994). The virus is transmitted to livestock and humans by the bite of infected mosquitoes or exposure to tissues or blood of infected animals (Linthicum *et al.*, 1999).

Rift Valley fever is an emerging mosquito-borne zoonosis that is expanding in its range in Africa and the Middle East. RVF can have catastrophic economic impact on meat and dairy producers, causing high morbidity and mortality among affected livestock herds (Daubney *et al.*, 1931; WHO Fact Sheet., 2000), and invoking 2-3 year World Organisation for Animal Health (OIE) – mandated international embargoes of livestock exports during epizootics.

Human RVF outbreaks are primarily characterized by mild, acute febrile illness with spontaneous recovery, although in a small proportion (< 8%) of cases the disease can be associated with severe jaundice, rhinitis, encephalitis, hemorrhagic manifestations and death (Davies *et al.*, 1980). The virus was first identified in 1930, during an outbreak of sudden deaths and abortions among sheep along the shores of Lake Naivasha in the greater Rift Valley of Kenya (Daubney *et al.*, 1931).

During epidemics, the virus devastates livestock including cattle, sheep, goats and camels with mortality rates reaching 30% and 100% in adult and young animals, respectively (Madani *et al.*, 2003). It is responsible for abortions in 100% of pregnant cattle, sheep and goats infected by the virus (Nichol *et al.*, 2001).

Over the years the geographic distribution of the virus has since spread all over Africa including Madagascar and beyond (Gerdes *et al.*, 2004).

The disease crossed the African border in 2000-2001 where it was first reported to be responsible for a large outbreak among livestock and humans in the Arabian Peninsula (Balkhy *et al.*, 2003).

Massive outbreak was also reported in the entire of East African region in late 2006 through early 2007, following a period of heavier than usual rainfall and widespread flooding (Breiman *et al.*, 2008). By the time the disease abated, more than 1000 people had been diagnosed with RVF and more than 300 people had died of the disease (Breiman *et al.*, 2008). From the 2006/2007 outbreak there was a considerable socio-economic loss due to extensive morbidity and mortality of livestock and disruption of livelihoods, markets, and the meat industry that resulted from a ban on livestock slaughter (Rich *et al.*, 2010).

During the last outbreak, the first reported case of RVF among humans was in January 2007 in Arusha, northern part of Tanzania (Mohamed *et al.*, 2010). By the end of the outbreak in June 2007, a total of 511 suspected RVF cases had been recorded from 10 of the 21 regions of Tanzania, with Laboratory confirmation of 186 cases and another 123 probable cases (Mohamed *et al.*, 2010). Between February and June 2007, other regions of Tanzania including Manyara, Tanga, Dodoma, Morogoro, Dar es Salaam, Pwani, Iringa, Mwanza and Singida had reported suspected cases of RVF (Mohamed *et al.*, 2010). RVF seems to be of major public health problem and it is responsible for not only economic losses but also in claiming human life.

The virus has a trisegmented, single stranded RNA genome in which two segments (L and M) have a negative polarity, while the third (S segment) is ambisense (Giorgi *et al.*, 1991). It has been proposed that similar to other Bunyaviruses, RVFV has three segments with a circular “panhandled” secondary structure (Hewlett *et al.*, 1977) formed by RNA sequences on the ends of each segment (Ronnholm *et al.*, 1987). While the L segment encodes the viral RNA polymerase used for replication and messenger RNA transcription, the M segment encodes two glycoproteins (Gn and Gc) and a non-structural protein that can be expressed by itself (Nsm1) or in fusion with Gn (Nsm2) (Gerrard *et al.*, 2007). In its antisense orientation, the S segment expresses the nucleoprotein (N protein), while its complementary orientation encodes the non-structural protein NSs.

Rift Valley fever virus has the potential to infect a remarkable array of vectors, including ticks and a variety of flies (Daubney *et al.*, 1933), unlike the majority arboviruses which tend to be adapted to a narrow range of vectors (Chevalier *et al.*, 2004).

Vectors of RVFV can be classified into “reservoir/maintenance” vectors, including certain *Aedes* species mosquitoes (Diptera: Culicidae) associated with freshly flooded temporary (Fontenille *et al.*, 1994 and 1995) or semi-permanent fresh water bodies (Gear *et al.*, 1955), and “epidemic/amplifying” vectors consisting of *Culex* spp. associated with more – permanent fresh water bodies (McIntosh *et al.*, 1981).

The vertebrate hosts are typically only viraemic for 2-7 days (Davies *et al.*, 1981; Easterday *et al.*, 1962; McIntosh *et al.*, 1973; Olayeye *et al.*, 1996), implying that the chronic infection of the invertebrate vector is more important for survival of RVFV from season to season, the vector apparently serves as the reservoir host. Provided the larval habitats remain flooded for more than 2 or 3 weeks, the floodwater *Aedes* are succeeded

by *Culex* spp, which oviposit in small egg-rafts on the surface of the water. These eggs are unable to withstand desiccation; however the egg rafts lead to a population explosion of *Culex* spp. mosquitoes which become infected upon feeding on viraemic vertebrate hosts. Whereas the floodwater *Aedes* spp. tend to remain in the immediate vicinity of the larval habitats and only feed at dusk and dawn, the more nocturnal *Culex* spp., e.g., *Culex (Culex) theileri*, are more likely to disperse to find vertebrate hosts to feed on, leading to extensive dissemination of virus and the appearance of epidemics (Pepin *et al.*, 2010).

The epidemiology of RVF consists of both epizootic and interepizootic cycles (Meegan and Bailey, 1988). However, epizootic outbreaks do not occur at random, but instead are strongly linked to excessive rainfall and local flooding events. During an epizootic event, virus circulates among infected arthropod vectors and mammalian hosts, particularly cattle and sheep, which represent the most significant livestock amplifiers of RVFV (Linthicum *et al.*, 1985).

The inter-epizootic survival of RVFV is believed to depend on transovarial transmission of the virus in floodwater through *Aedes* mosquitoes (Linthicum *et al.*, 1985). Virus can persist in mosquito's eggs until the next period of heavy rainfall when they hatch and yield RVFV infected mosquitoes. Depending on factors such as availability of sufficient numbers of competent mosquito vectors, presence of susceptible vertebrates, appropriate environmental conditions, infected mosquitoes have the potential to infect a relatively small number of vertebrate hosts or to initiate a widespread RVF epizootic.

The aim of this study was to determine the transmission of RVFV during inter-epidemic period in Ngorongoro district, an area that has repeatedly faced a number of disease epidemics.

1.2 Objectives of the Study

1.2.1 General Objective

The general objective of this study was to determine the inter-epidemic transmission of RVFV in Ngorongoro District, Northern Tanzania.

1.2.2 Specific Objectives

The specific objectives were:

- i. To investigate the inter-epidemic viral activity in humans and domestic ruminants in the study area, and
- ii. To determine the abundance, composition and inter-epidemic viral activity in potential mosquito vectors responsible for RVFV transmission.

2.0 GENERAL MATERIALS AND METHODS

2.1 Study Design

This study involved RVFV-IgG seropositive samples (n=160) from cattle, sheep, goats and humans that were collected from a cross-sectional survey conducted in parallel with mosquito sampling. These seropositive serum samples were further analysed using a qRT-PCR and one step conventional RT-PCR for the viral RNA detection and amplification. Adults mosquitoes were collected using the CDC light traps baited with carbon dioxide, the traps were set to homes where animals (sheep, cattle and goats) are kept. Pools of potential mosquito vectors responsible for RVFV transmission were subjected to qRT-PCR for virus detection.

2.2 Study Area

The study was carried out in five villages of Ngorongoro district located in northern Tanzania. The district has repeatedly faced a number of RVF outbreaks, the last epidemic was recorded in 2007. The villages that participated in this study were Malambo, Meshili, Osinoni, Endulen and Nainokanoka.

3.0 CONCLUSIONS AND RECOMMENDATIONS

3.1 Conclusions

This study has demonstrated the maintenance of the RVF virus in domestic ruminants during the inter-epidemic period in the absence of the reported cases in livestock or humans.

It has been observed that RVFV potential mosquito vectors are heterogeneously distributed in the villages within Ngorongoro District an area that has repeatedly faced a number of RVF epidemics. This highlights the potential for the re-emergence of the disease in the study area and should inform the design of appropriate control measures.

3.2 Recommendations

- i. There is a need of active surveillance system to monitor circulation of the virus in susceptible hosts and vectors especially during IEPs for better understanding of the mechanisms of disease transmission in these villages.
- ii. There is a need of investigating the molecular characteristics of virus isolates collected from different parts of Tanzania in order to define their genetic and antigenic features that could be associated with overt pathogenicity and future epidemics.
- iii. Comprehensive entomological investigation should be carried out during the rainy season or during period of active RVF outbreak to improve understanding of transmission potential of RVF mosquito vectors.

- iv. There is a need of investigating factors responsible for the survival of the virus in *Aedes* eggs during dry period without the virus being denatured.
- v. Further studies need to be undertaken to investigate the ecological factors that influence the distribution of potential mosquito vectors in the area.

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Molecular detection of Rift Valley fever Virus inter-epidemic activity in domestic ruminants and humans in Ngorongoro District, Northern Tanzania.

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ABSTRACT

Background: Rift Valley fever virus (RVFV) causes an acute Rift Valley fever (RVF) disease in humans and domestic ruminants whose occurrence assumes an epidemic pattern in most cases. Understanding the maintenance of the virus during inter-epidemic period (IEP) is vital for better elucidation of the disease transmission dynamics. The aim of this study was to investigate RVFV inter-epidemic activity in humans and domestic ruminants in Ngorongoro District, Northern Tanzania.

Methods: Rift Valley fever virus Immunoglobulin G seropositive serum samples (n=160) from cattle, sheep, goats and humans collected from a previous cross-sectional survey

were analysed using real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and conventional RT-PCR .

Viral RNA was extracted directly from serum samples using a QIAamp Viral RNeasy Mini Kit (QIAGEN). The molecular detection of the virus was first performed using the rRT- PCR followed by the one -step conventional RT-PCR targeting the S segment of RVF virus.

Results: The rRT- PCR detected 2 (5%), 4 (10%) and 1 (2.5%) RVFV genomes in cattle, sheep and goats sera respectively. These 7 positive samples for rRT-PCR were also positive by the conventional RT-PCR. No positive results for either viral antigen or genome were obtained in human sera. RVFV was found in Meshili (6 cases) and Malambo (1 case) villages from domestic ruminants.

Conclusion: These findings have demonstrated the maintenance of the RVF virus in domestic ruminants during the inter-epidemic period in the absence of reported cases in livestock or humans. There is a need of active surveillance system to monitor circulation of the virus especially during IEPs for better understanding of the mechanisms of disease transmission in these hotspots.

Keywords: Rift Valley fever, domestic ruminants, humans, transmission, Tanzania

1.0 INTRODUCTION

Rift Valley fever is a mosquito borne viral zoonotic disease that affects domestic ruminants, wild ruminants and humans (LaBeaud *et al.*, 2011; Evans *et al.*, 2008), it is caused by Rift Valley fever virus (RVFV) belonging to the genus Phlebovirus of the family Bunyaviridae

The transmission of RVFV in domestic animals is either through bites from different species of infected mosquitoes, mainly *Aedes* and *Culex* genera or by direct contact with infected animal tissues and body fluids, particularly if associated with abortion (Davies & Martin, 2006). Transmission of the virus to humans is thought to occur by arthropod vectors, aerosols of blood or amniotic fluid, or other direct contact with infected animals (Wood *et al.*, 2002).

Rift Valley fever is accompanied by a wave of abortion and perinatal mortality in sheep, goats, cattle and camel (Fyumagwa *et al.*, 2011), the clinical signs include anorexia, high temperature, blood stained nasal and lachrymal discharges, respiratory distress and death (Davies & Martin, 2003). In humans it is a febrile or influenza like illness that can be associated with , severe headaches, muscle and joints pains, anorexia, high respiration rate, vomiting, diarrhoea, jaundice and sometimes severe fatal haemorrhagic syndrome (Daubney *et al.*, 1931; Harper, 2004; Mohamed *et al.*, 2010).

Periodic severe RVF outbreaks involving livestock and humans have occurred in Africa following heavy rainfall and flooding (Munyua *et al.*, 2010). Large, severe RVF outbreaks include the 1977-1979 outbreak in Egypt that affected over 200 000 people and resulted in over 600 deaths, and the 1997-1998 outbreak in East Africa (Kenya, Somalia and Tanzania) that affected over 100 000 people (Woods *et al.*, 2002; El Akkad *et al.*, 1978).

Outbreaks have also occurred in Mauritania, Senegal, Sudan, Madagascar, South Africa and in the Middle Eastern countries of Saudi Arabia and Yemen (Zeller *et al.*, 1997; Al-Afaleq *et al.*, 2003; Shoemaker *et al.*, 2002).

In Tanzania, the epidemics occurred in 1930s, 1947, 1957, 1977, 1997 and 2006-2007 (FAO, 1999; NASA, 2002; WHO, 2007), the most notable one was in 2006-2007 (Fyumagwa *et al.*, 2011).

Detection of Immunoglobulin G (IgG) antibodies against RVF by Enzyme Linked Immunosorbent Assay (ELISA) indicates previous exposure to the virus while detection of Immunoglobulin M (IgM) indicates recent infection (Paweska *et al.*, 2005), confirmation of RVF can be done using molecular techniques (qRT-PCR and nucleic acid sequencing) (Garcia *et al.*, 2001; Sall *et al.*, 2002).

In Tanzania, Ngorongoro district has repeatedly reported a number of RVF epidemics. The objective of this study was to investigate the virus inter-epidemic activity in humans and domestic ruminants for better understanding of the disease transmission dynamics.

MATERIALS AND METHODS

Study site

Ngorongoro district lies between Longitude 35.5° E and Latitude -2.75° S. It is a semi-arid rangeland area in the Rift Valley just to the south of the Kenyan-Tanzanian border. Predominantly it consists of Maasai ethnic groups and a large minority of Watemi. The vegetation mainly consists of various shrubs and *Acacia* bushes, and livestock species kept are primarily cattle, goats, sheep and donkeys.

This area normally experiences two rainy seasons: a short rainy season between October and December, and a long rainy season between March and May. Typically, the annual precipitation averages between 500 and 1,000 mm.

The study was carried out in five villages of Ngorongoro District located in northern Tanzania. The district has repeatedly faced a number of RVF outbreaks and the last epidemic was recorded in 2007. These villages were randomly selected from seventeen villages of Ngorongoro District that reported RVF epidemics in the past. The villages that participated in this study were Malambo, Meshili, Osinoni, Endulen and Nainokanoka (Fig. 1).

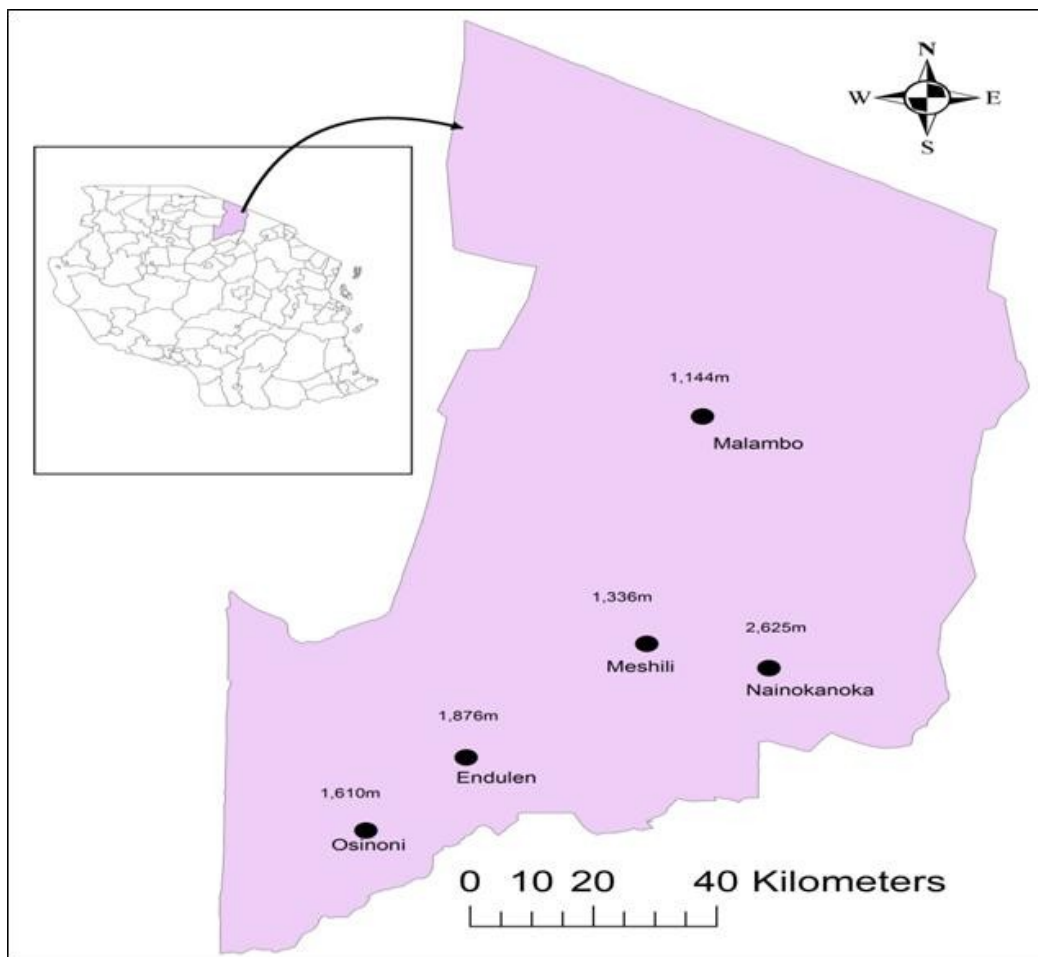


Figure 1: Map of Ngorongoro showing location of the study villages and their elevation in metres above sea level (m). The insert is the map of Tanzania

Study design

This study involved RVFV-IgG seropositive samples (n=160) from cattle, sheep, goats and humans that were collected from a previous cross-sectional survey conducted in May 2013 in the study villages.

The Rift Valley fever virus Immunoglobulin G seropositive serum samples (n = 160) included in this study for polymerase chain reaction (PCR) tests were sourced from previous cross-sectional study in randomly selected domestic ruminants (Sindato *et al.*, 2015) and humans (Sindato *et al.*, unpublished data) during the IEP from the study villages. The serum samples from domestic ruminants were obtained from animals born after the last RVF outbreak in 2006/2007 and had no history of being vaccinated against RVFV. A total of 160 serum samples were randomly selected from cattle, sheep, goat and humans (Table 1). The selected serum samples were tested for the presence of RVF viral RNA qRT-PCR and the one step conventional RT-PCR for the viral RNA detection and amplification.

Table 1: Number of serum samples selected randomly for RT- PCR tests from a pool of RVFV-IgG positive samples from domestic ruminants and humans

Village	Goats		Sheep		Cattle		Humans	
	No. IgG +ve	No. selected for PCR	No. IgG +ve	No. selected for PCR	No. IgG +ve	No. selected for PCR	No. IgG +ve	No. selected for PCR
Meshili	30	20	25	20	85	30	25	15
Malambo	26	13	21	12	55	20	22	15
Osinoni	ND	-	ND	-	ND	-	4	4
Endulen	ND	-	ND	-	ND	-	15	7
Nainokanoka	ND	-	ND	-	ND	-	4	4

Legend:

ND = not detected

Total RNA extraction

Ribonucleic acid (RNA) was extracted from the serum samples using a column purification kit (QIAGEN, Valencia, CA, USA), according to the manufacture's instructions.

Real- time Reverse Transcriptase Polymerase Chain Reaction

The molecular detection of the virus was first performed using the real time RT-Polymerase chain reaction system (Applied Biosystems 7500 Fast Real – Time PCR System).

The primers used were, Forward: S432 (5'ATGATGACATTAGAAGGGA 3') and Reverse: NS3m (5'GATGCTGGGAAGTG ATGAG 3') and the TaqMan probe CRSSAr (5' ATTGACCTGTGCCTGTTGCC 3') targeting 298 base pair fragment of the S-segment of the RVF virus. The results of the qRT-PCR were assessed by the Ct value, the negative Ct value for any test and control sample was considered at Ct of ≥ 50.0

Conventional RT-PCR

The one -step conventional RT-PCR amplification was carried out using a one-step Access RT-PCR system (AgPath-ID™ one-step RT-PCR kit, Applied Biosystems, USA). All PCR amplifications were carried out at a 25µl volume per tube. Thermal profiles were performed on a GeneAmp PCR system 9700 (Applied Biosystems, USA). A set of primers, Forward: S432 (5'ATGATGACATTAGAAGGGA 3') and Reverse: NS3m (5'GATGCTGGGAAGTGATGAG 3') targeting 298 base pair of the S –segment of RVF virus was used. The amplified products were loaded into a gel of 2%, the gel was stained with gel red and the PCR products were visualized under the U.V light.

RESULTS

Real – Time RT- PCR and Conventional RT- PCR

The molecular detection indicated that 4.4% of samples (n =7) were positive for RVF virus genome by real-time RT-PCR. Positive results were obtained in domestic ruminants from two villages (Meshili and Malambo). Of the positive samples, 86% (n=6) were obtained from Meshili village where as 14% (n=1) was obtained in Malambo village. All human samples were negative for RVF virus.

The conventional RT- PCR was only performed in the 7 samples that tested positive for RVF virus using qRT-PCR, the assay revealed the same positive results in the 7 samples (Fig. 2).

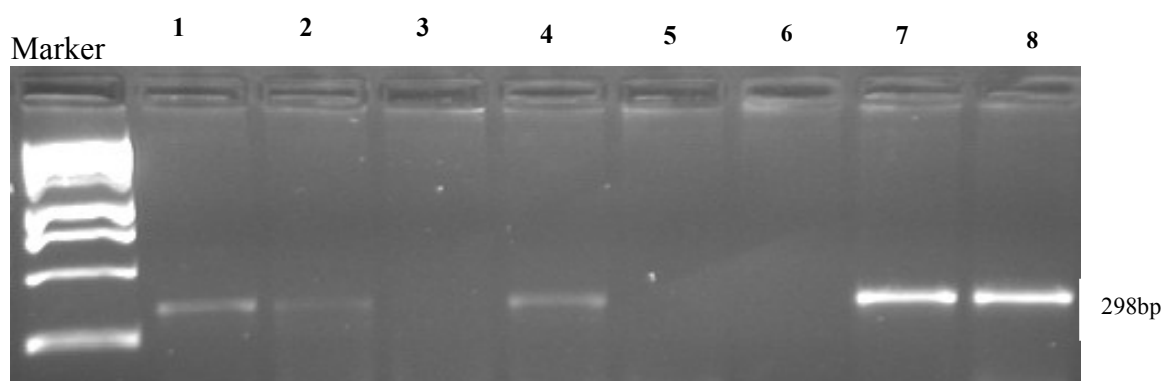


Figure 2: Agarose gel electrophoresis showing RT-PCR products of RVFV in serum samples

Lane 1 is 1kb DNA ladder, expected size about 298-bp S RNA segment gene of RVFV.

1-5 = serum samples, 6 = negative control

7 = positive control 1, 8 = positive control 2

DISCUSSION

This study has demonstrated evidence of RVF virus circulation in domestic ruminants during an inter-epidemic period (IEP) with acute virus infections being detected among

the domestic ruminants in the study area. The survey was conducted 6 years after the last RVF virus outbreak in 2006/2007. The last RVF outbreak affected both humans and livestock (Linthicum *et al.*, 1999).

During the course of this study, there were no reports of human RVF or reports of increased abortion or suspected RVF outbreak by the veterinary and / public health authorities in the study area.

Inter epidemic detection of RVF virus antigen has also been previously reported in goats in Kigoma, Tanzania in an area with no history of RVF epidemic (Kifaro *et al.*, 2014). A cross sectional study that was conducted in six regions of Tanzania during inter- epidemic period namely Arusha, Dodoma, Mara, Mbeya, Kigoma and Kagera revealed seroprevalence among the domestic ruminants that were born after the last epidemic of 2007 with the RVFV IgG circulating antibodies indicating transmission during the IEP without any clinical disease symptoms (Sindato *et al.*, 2015).

Human inter-epidemic detection of IgG circulating antibodies has been reported in Mbeya, Tanzania in 2007/08 when there were no previous reports of RVF virus outbreak in the region (Heinrich *et al.*, 2012). A sero- survey conducted during inter-epidemic period in the Kilombero valley in Tanzania among humans and domestic ruminants revealed sero-positivity with the IgM circulating antibodies indicating an active/recent infection with no reports of epidemic or clinical disease in the area (Sumaye *et al.*, 2013; Sumaye *et al.*, 2015).

This phenomenon has been explained as a result of high herd immunity following the RVF virus outbreak as demonstrated by high prevalence in animals that were present during the

previous epidemic in the area or a circulation of non-virulent strain of the RVF virus (Sumaye *et al.*, 2013).

There have been reports across African countries that have demonstrated evidence of inter-epidemic transmission of the RVF virus among humans and domestic ruminants without any clinical disease presentation (LaBeaud *et al.*, 2008; Lichoti *et al.*, 2014). An entomological investigation conducted during the same period revealed higher abundance and diversity of RVF virus potential mosquito vectors in Meshili village, these vectors have been incriminated for the transmission of the disease in Kenya, Sudan, Mauritania and Madagascar (Mhina *et al.*, 2015).

However, the same entomological investigation could not detect the virus activity in the mosquito pools tested (Mhina *et al.*, 2015).

These two villages were among the villages in Ngorongoro District that were hit by the RVF epidemic in 2006/07. Considering the fact that the virus is actively circulating in some domestic ruminants and the presence of potential mosquito vectors in the area, the risks of disease emergence is high. Little is still known on the mechanism by which the virus is maintained in susceptible hosts and their role in transmission of the disease during inter-epidemic period.

CONCLUSION

These findings have demonstrated the maintenance of the RVF virus in domestic ruminants during the inter-epidemic period in the absence of the reported cases in livestock or humans. There is a need for active surveillance system to monitor circulation

of the virus in susceptible hosts and vectors especially during IEPs for better understanding of the mechanisms of disease transmission in the area.

ACKNOWLEDGEMENTS

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Ethical consideration

The ethical approval for the previous survey that collected the samples from human was obtained from the Medical Research Coordinating committee of the National Institute for Medical Research (NIMR), Tanzania. (Protocol No. NIMR/HQ/R.8a/Vol.IX/1296)

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Rift Valley fever potential mosquito vectors and their infection status in Ngorongoro District in northern Tanzania

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Abstract

Background: Rift Valley fever (RVF) is a mosquito-borne viral zoonotic disease. Rift Valley fever virus (RVFV) has been isolated from more than 40 species of mosquitoes from eight genera. This study was conducted to determine the abundance of potential mosquito vectors and their RVFV infection status in Ngorongoro Conservation Area of northern Tanzania.

Methods: Adult mosquitoes were collected outdoors using the CDC light traps baited with carbon dioxide in five randomly selected villages namely, Meshili, Malambo, Osinoni, Endulen and Nainokanoka. The study was carried out towards the end of rainy season in May 2013. The traps were set in proximity to potential breeding sites and cattle kraals. The collected mosquitoes were identified to genus and species using morphological keys. They were tested for RVFV RNA using real time reverse transcription-polymerase chain reaction (rRT-PCR).

Results: A total of 2,094 adult mosquitoes belonging to three genera and nine species were collected. Most of them (87.5%) were collected in Meshili, followed by Malambo (8.2%) and Osinoni (4%) villages. No single mosquito was collected in Nainokanoka or Endulen. The nine species collected were *Culex pipiens* complex, *Cx. antennatus*, *Cx. tigripes*, *Cx. annulioris*, *Cx. cinereus*, *Anopheles arabiensis*, *An. squamosus*, *An. pharoensis* and *Mansonia uniformis*. No RVFV RNA was detected in the mosquito specimens.

Conclusion: Various RVFV potential mosquito species were collected from the study villages. These mosquito vectors were heterogeneously distributed in the district suggesting a variation in RVF transmission risk in the study area.

Keywords: Rift Valley fever, mosquito, virus infectivity, transmission, Tanzania

Introduction

Rift Valley fever (RVF) is an acute febrile arthropod-borne viral zoonotic disease of mainly human and ruminants caused by a member of *Phlebovirus* genus of the family *Bunyaviridae*. Rift Valley fever outbreaks have been reported in Kenya, Tanzania, South Africa (WHO, 2007; WHO, 2010; Archer *et al.*, 2011), Mauritania (Ahmed *et al.*, 2011), Senegal (Jocelyn *et al.*, 1999), Sudan (Adam *et al.*, 2010) and Madagascar (Jeanmaire *et al.*, 2011). In 2001-2002 RVF outbreaks were reported beyond Africa in Saudi Arabia and Yemen (Shoemaker *et al.*, 2002). The disease affects cattle, sheep, goats and camels with mortality rate reaching 30% and 100% in adult and young animals, respectively (Madani *et al.*, 2003). RVF is characterised by abortions in cattle, sheep and goats (Nichol *et al.*, 2001) and by mild, acute febrile illness with spontaneous recovery in humans. In small proportion of cases the disease in human it can be associated with severe jaundice, rhinitis, encephalitis and haemorrhagic manifestations and death (Davies, 1980).

Rift Valley fever virus is transmitted between animals and humans by mosquitoes, particularly those belonging to the *Aedes*, *Culex* and *Anopheles* genera (Easterday *et al.*, 1962; Laughlin *et al.*, 1979). Humans also acquire infection through direct contact with blood or

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aborted materials from infected animals (Swanepoel & Coetzer, 2004). It is believed that during the inter-epidemic period (IEP) the RVFV is maintained in the eggs of *Aedes* mosquitoes (primary vector) which breed in isolated depressions called *dambos* during periods of extensive rainfalls and floods (Linthicum *et al.*, 1985; Chevalier *et al.*, 2005; Mohamed *et al.*, 2010). When *Aedes* mosquito infect animals with RVFV, virus amplification occurs in these vertebrate hosts, and then *Culex* and *Anopheles* species (secondary vectors) transmit the virus further to a wider area beyond the area of the original outbreak (McIntosh, 1972).

Rift Valley fever virus has been isolated from more than 40 species of mosquitoes from eight genera (Ratovonjato *et al.*, 2011). Laboratory studies indicate that numerous species of mosquitoes and sand flies are susceptible to oral infection, some of which are able to transmit RVFV by bite (Sang *et al.*, 2010). In Tanzania, RVF outbreaks have been reported in Arusha, Dodoma, Iringa, Kilimanjaro, Manyara, Mara, Morogoro, Mwanza, Pwani, Shinyanga, Singida, Tabora and Tanga regions (Sindato *et al.*, 2014). Besides the long persistence of RVF in Ngorongoro district in Arusha Region (Sindato *et al.*, 2014, 2015), little is known about the distribution of potential mosquito vectors and their RVFV infection status. Limited number of studies have reported presence of potential mosquito vectors of RVF in Ngorongoro district (Mweya *et al.*, 2013; 2015). The objective of this study was therefore to determine the abundance and RVFV infection status of potential mosquito vectors during the IEP in Ngorongoro Conservation Area (NCA) of northern Tanzania.

Materials and Methods

Study site

This study was carried out in Ngorongoro Conservation Area (NCA) in Ngorongoro district in northern Tanzania. NCA is one of the three divisions of Ngorongoro District and covers an area of 8,292m². NCA was established in 1959 as a multiple land use area, designated to promote the conservation of natural resources, safeguard the interests of the indigenous residents and promote tourism. The main features of the NCA include the Ngorongoro Crater, The Serengeti Plains and the catchment forest (<http://www.ngorongorocrater.org/welcome.html>). This area is largely of hilly terrain interspersed with broad U-shaped valley. The vegetation consists mainly of various shrubs and *Acacia* bushes.

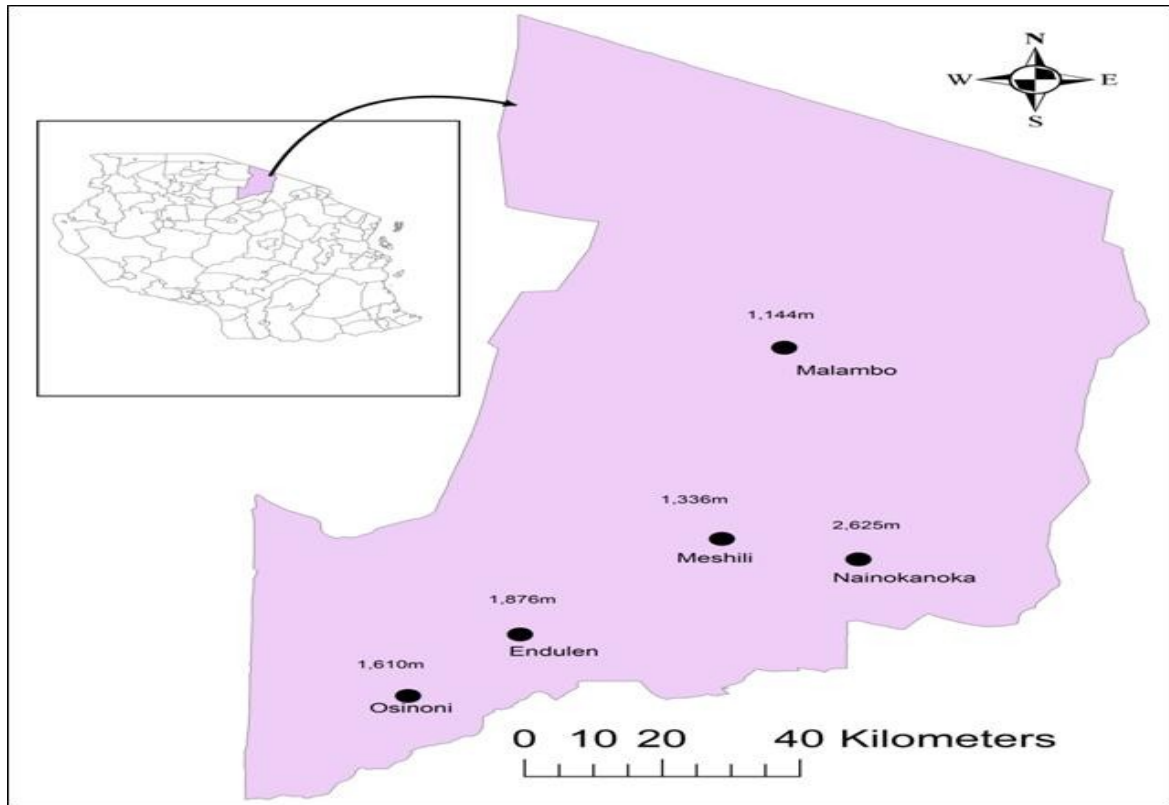


Figure 1: Map of Ngorongoro district showing location of the study villages

NCA normally experiences two rainy seasons: a short rainy season between October and December, and a long rainy season between February and May. Typically, the annual precipitation averages between 500 and 1,000 mm. The area is occupied predominantly by Maasai pastoralists. Other minority ethnic groups living in this area are Hadzabe, Ndorobo and Sonjo. The livestock species kept in the area are primarily cattle, goats, sheep and donkeys. The study villages were Malambo (1,144m), Meshili (1,336m), Osinoni (1,610m), Endulen (1,876m) and Nainokanoka (2,545m). These study villages were randomly selected from a sampling frame of 17 villages of Ngorongoro Conservation Area.

Mosquito collection and identification

Adult mosquitoes were collected in May 2013 using CDC light traps baited with carbon dioxide. Three traps were set approximately 1.5m above the ground in proximity to potential breeding sites and cattle kraals. The traps were set at 17:30 hrs in the evening and retrieved the following morning at 06:00 hours. Mosquitoes were killed by freezing; sorted into sex and abdominal status (fed, unfed, semi gravid, gravid), and were stored in 1.5 mL labelled Eppendorf tubes. Mosquitoes were identified to genera and species levels using conventional taxonomic keys (Edwards, 1941; Gillies & De Meillon, 1968; Service, 1990). They were then preserved and transported in a liquid nitrogen gas to the laboratory at Amani Medical Research Centre in Tanga. The *Anopheles gambiae* s.l. was identified further to species level using the standard polymerase chain reaction (PCR) technique (Scott *et al.*, 1993).

One leg from each *An. gambiae* s.l. mosquito was placed in a 1.5 ml Eppendorf tube, 30µl of 1X Tris – EDTA buffer solution was added in each tube. The leg was grounded thoroughly using a sterile pestle, the mixture was then short-span (5 seconds) to catch the DNA template down the tube. The PCR procedure included an initial cycle of denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 94°C, 72°C and 50°C for 30 seconds, and a final extra extension step at 72°C for 10 minutes using a Hybrid thermocycler. The resulting amplified Deoxyribonucleic Acid (DNA) was run on an ethidium bromide stained 2.5%

agarose gel and photographed under ultraviolet light illumination as described by Scott *et al.* (1993).

Viral RNA extraction

RNA was extracted from female and male mosquitoes using a column purification kit (QIAGEN, Valencia, CA, USA). Pools of mosquitoes were put into a microcentrifuge tube containing 150µL of RNeasy lysis buffer and finally ground with a disposable RNase free pestle. Each pool had 10 mosquitoes. After homogenization, samples were processed according to established protocol (Qiagen RNeasy Mini kit). The molecular detection of the virus was performed using the real time RT-PCR (Applied Biosystems 7500 Fast Real – Time PCR System). The primers Forward: S432 (5'ATGATGACATTAGAAGGGA 3') and Reverse: NS3m (5'GATGCTGGGAAGTG ATGAG 3') and the TaqMan probe CRSSAr (5' ATTGACCTGTGCCTGTTGCC 3') targeting 298 base pair fragment of the S- segment of the RVF virus.

Data analysis

Data was entered in Microsoft Excel spread sheet and imported into STATA version 12 (Statacorp, College Station, TX, USA) for analysis. Mosquito abundance (calculated as the number of mosquitoes collected per village) was compared between the study villages using chi-squared test. However, when the number of mosquitoes was below 5, the Fisher's exact test was applied.

Results

A total of 2,094 adult mosquitoes were collected and identified to nine species belonging to three genera. Mosquito abundance and diversity differed significantly between the study villages ($p < 0.0001$). All of the morphologically identified *An. gambiae* s.l. were genotyped as *An. Arabiensis*. Overall, *Cx. pipiens* complex was the predominant mosquito species (46.8%) followed by *An. pharoensis* and *An. arabiensis* (14.4%) (Table 1).

Table 1: Overall mosquito species composition in the study villages

Species	Number	%
<i>Anopheles arabiensis</i>	302	14.4
<i>Anopheles pharoensis</i>	408	19.5
<i>Anopheles squamosus</i>	269	12.8
<i>Culex pipiens</i> complex	981	46.8
<i>Culex cinereus</i>	53	2.5
<i>Culex antenattus</i>	24	1.1
<i>Culex tigripes</i>	19	0.9
<i>Culex annulioris</i>	17	0.8
<i>Mansonia uniformis</i>	21	1.0

Meshili accounted for the majority (87.5%) of the mosquito collected in NCA, followed by Malambo (8.2%) and Osinoni (4.0%) villages. No mosquitoes were collected in Endulen and Nainokanoka Villages during the study period (Table 2). In Malambo, *An. arabiensis* was the most abundant species (48.5%) followed by *Cx. pipiens* complex (43.3%) and *An. pharoensis* (8.2%). On the other hand, *Cx. pipiens* complex was the most abundant species (48.7%) in Meshili, followed by *An. pharoensis* (19.7%), *An. squamosus* (13.3%), *An. arabiensis* (10.9%), *Cx. cinereus* (2.9%), *Cx. antennatus* (1.4%), *Mansonia uniformis* (1.2%), *Cx. tigripes* (1%) and *Cx. annulioris* (0.9%). In Osinoni, *Anopheles pharoensis* predominated (37.4%), followed by *An. squamosus* (28.5%), *An. arabiensis* (19.8%) and *Cx. pipiens* complex (14.3%) (Table 2).

Table 2: Total number of mosquito vectors collected in each village

Species	Malambo N (%)	Meshili N(%)	Osinoni N(%)	Enduleni N(%)	Nainokanoka N(%)
<i>An. arabiensis</i>	83(48.5%)	201(10.9%)	18(19.7%)	0	0
<i>An. pharoensis</i>	14(8.1%)	360(19.6%)	34(37.3%)	0	0
<i>An. squamosus</i>	0	243(13.2%)	26(28.5%)	0	0
<i>Cx. pipiens</i>	74(43.2%)	894(48.7%)	13(14.2%)	0	0
<i>Cx. cinereus</i>	0	53(2.8%)	0	0	0
<i>Cx. antennatus</i>	0	24(1.3%)	0	0	0
<i>Cx. tigripes</i>	0	19(1%)	0	0	0
<i>Cx. annulioris</i>	0	17(0.9%)	0	0	0
<i>Ma. uniformis</i>	0	21(1.1%)	0	0	0
Total	171	1,832	91	0	0

A total of 960 of these were sorted into 96 pools according to their site of collection, species, and sex and were tested for RVFV RNA. The RVFV RNA was not detected in any of 96 tested mosquito pools.

Discussion

Assessment of abundance and diversity of RVF vectors, and their infectivity to RVFV provides important entomological features for the identification of potential high risk areas for RVF occurrence, which can provide guidance in the design of appropriate prevention and control measures. The findings of this study have shown that the abundance and diversity of potential RVF mosquito vectors vary between the study villages, suggesting the spatial variations in the risk of RVF occurrence in animals and humans within NCA. The high abundance and diversity of mosquitoes in Meshili suggests the potential suitability of environments in this village during the study period for the mosquito species collected. Although Malambo and Meshili are both located at lower altitudes than other study villages, the presence of seasonal pond at Meshili (which was filled with water during the study period) is likely to partly explain for the higher catches of mosquitoes in this village. This seasonal pond was also observed to be one of the obvious potential breeding sites in a malariometric survey conducted in the area about a decade ago (Mboera *et al.*, 2005). It is worth noting further that compared with Osinoni and Nainokanoka villages; Meshili, Malambo and Endulen have been persistently affected by past RVF outbreaks which were reported mainly during the period of prolonged heavy rainfall (Sindato *et al.*, 2014).

The potential role of prolonged rainfall and mass emergence of mosquitoes have been reported as risk factors for RVF epidemics (Ngulu *et al.*, 2010). Although it is not very clear what causes the differential abundance and diversity of mosquito vectors between the study villages, it is likely that high altitude provide less suitable habitat for mosquito breeding and survival compared with lower lying areas which are likely to be more susceptible to water stagnation during the rainy season (Bodker *et al.*, 2003). This observation and the sampling season may partly explain why no single mosquito was collected in Endulen and Nainokanoka villages during the study period.

Cx. pipiens complex, *An. arabiensis* and *An. pharoensis* were the common mosquito species collected in Malambo, Meshili and Osinoni villages, and all these species have been reported as potential vectors of RVF virus in Kenya (Sang *et al.*, 2010) and Mauritania (Nabeth *et al.*, 2007). In a recent study in Ngorongoro district Mweya and others (2013) found that *Cx.*

pipiens complex was the most abundant mosquito species followed by *Aedes aegypti*. *An. arabiensis* and *An. pharoensis* have been reported as vectors of RVFV in Sudan and Mauritania (Digoutte & Peters, 1989; Faye et al., 2003; Nabeth et al., 2007; Seufi & Galal, 2010). *Cx. antennatus*, *Cx. annulioris*, *Cx. tigripes* and *Ma. uniformis* which were collected in Meshili have been associated with the transmission of RVFV in Madagascar (Ratovonjatoet al., 2011; Balenghien et al., 2013), Nigeria and Kenya (Easterday et al., 1962; Sang et al., 2010). The vector competence of *Cx. antennatus* has also been demonstrated in laboratory studies (Easterday et al., 1962). While *Cx. tigripes* was reported as a potential vector of RVFV in Marigat and Ijara districts in Kenya (Tchouassi et al., 2012) *Ma. uniformis* was found infected with RVFV in Baringo District in the same country (Sang et al., 2010). *An. squamosus* was only collected in Meshili and Osinoni villages; this species has also been implicated with the transmission of RVFV in a study in Garisa District in Kenya (Sang et al., 2010) and Madagascar (Ratovonjatoet al., 2011).

Despite the fact that RVFV RNA was not detected in the collected mosquitoes in our study, RVFV RNA has been detected in cattle, sheep and goats sampled during the same period from Meshili and Malambo villages (A. Mhina et al., 2015 unpubl). Similar to our findings, a recent study in Ngorongoro district by Mweya et al. (2013) did not detect RVFV activity in the potential mosquito vectors collected during the IEP. While the salient reasons for this observation are not known, it should be noted that compared with IEP; during the outbreak phase infected animals develop high levels of viraemia, making it easy for mosquitoes that bite these animals to become infected with RVFV. An entomological study that was conducted during the last RVF outbreak in Kenya in 2006/2007 detected RVFV in *Ae. mcintoshi*, *Ae. ochraceus*, *Ae. pembaensis*, *Ma. uniformis*, *Cx. poicilipes*, *Cx. bitaeniorhynchus*, *An. squamosus*, *Ma. africana*, *Cx. quinquefasciatus* and *Cx. univittatus* (Sang et al., 2010).

It is important to note that the findings of our study are likely to have been affected by sampling technique, choice of sampling sites, duration of our study, season of sampling and sample size. Further studies targeting the periods of high mosquito activity with range of sampling techniques and sampling sites would improve our understanding on the abundance and diversity of the RVF potential mosquito vectors in the study area.

This study reports the presence of potential mosquito vectors for RVF within Ngorongoro Conservation Areas. The abundance and diversity of these potential vectors were significantly higher in Meshili village, suggesting that the risk of RVF occurrence is higher in this village than other study villages. These findings should inform the design of appropriate prevention and control measures.

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References

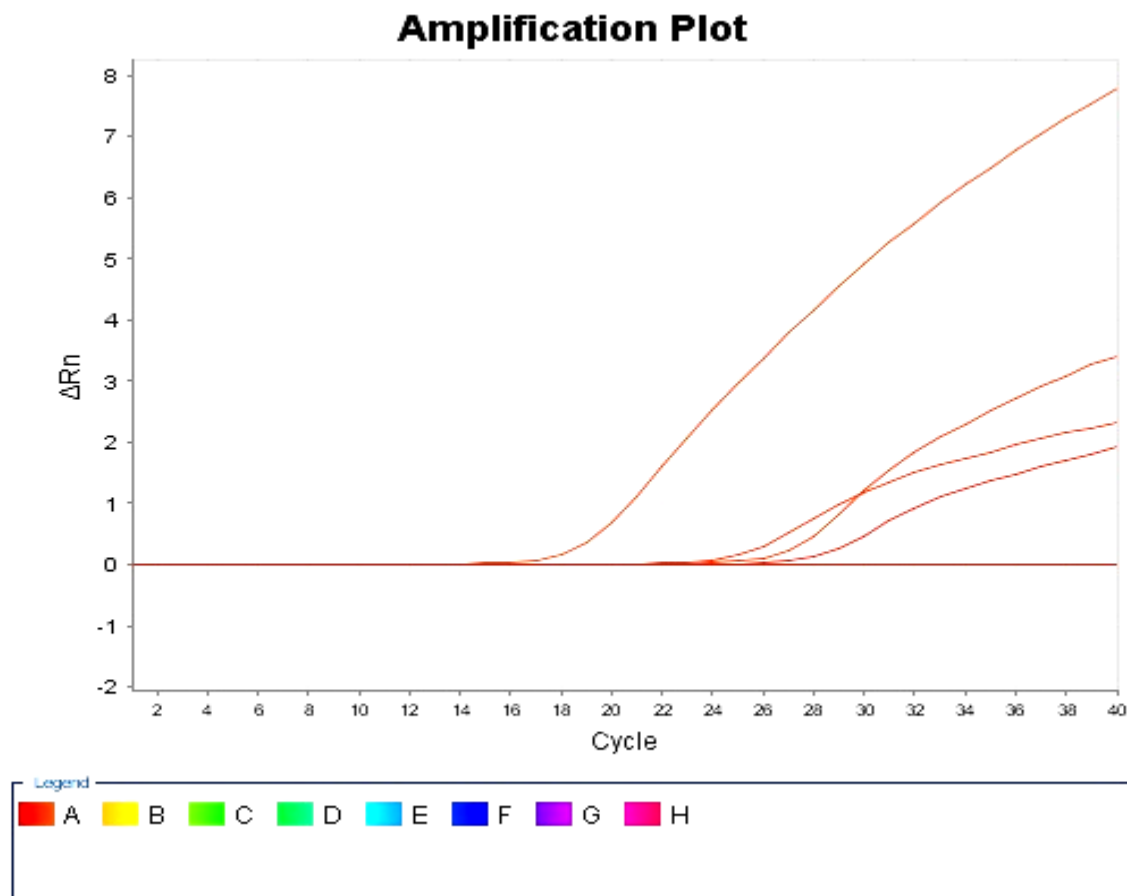
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APPENDICES

Appendix 1: Amplification plot for rRT-PCR showing positive serum samples for RVFV

Ct 16 is the positive control and the rest are positive serum samples (Ct 24, 25 and 26)

Appendix 2: Clearance Certificate for Conducting Medical Research in Tanzania

THE UNITED REPUBLIC OF
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23rd February 2012

Dr Calvin Sindato
Sokoine University of Agriculture,
Department of Veterinary Medicine and Public Health
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CLEARANCE CERTIFICATE FOR CONDUCTING
MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Spatial and temporal assessment of the risk of Rift Valley Fever occurrence in Ngorongoro District, Tanzania (Sindato C *et al*), has been granted ethics clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine. NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Approval is for one year: 23rd February 2012 to 22nd February 2013.

Name: Dr Mwelecele N Malecela

Signature

CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

CC: RMO
BMO

Name: Dr Dawa Muthando

Signature

ACTING CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, SOCIAL
WELFARE