SCREENING MOSQUITOES FOR RIFT VALLEY FEVER VIRUS AND BLOOD MEAL SOURCES DURING THE 2006/2007 OUTBREAK IN KENYA

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EGERTON UNIVERSITY

JUNE, 2011

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I hereby declare that this thesis is my original work and has not been submitted for award in any institution of learning to the best of my knowledge.

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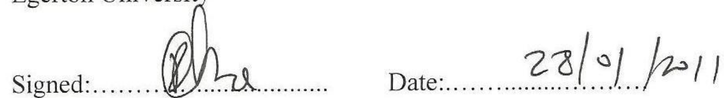

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DEDICATION

I dedicate this thesis to my father Phillip Ouma, my mother Rose Ouma, my brothers Bernard Onyango and Sylvester Otieno and sisters Peris Aoko and Santa Akinyi and to my grandmother Elizabeth Ouma for their unwavering support during my entire period of research.

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ABSTRACT

Rift Valley Fever (RVF) is a mosquito borne viral infection, first reported in the Rift Valley province of Kenya in 1912 and identified in 1931. Major outbreaks have been reported in Kenya in 1997/1998 and 2006/2007. Baringo, Garissa and Kilifi district of Kenya were hot spots in the last major RVF outbreak that occurred in the country in 2006/2007. Investigations were conducted during the outbreak to establish putative mosquito vectors and vertebrate host of RVF virus. Engorged female mosquitoes (*Aedes, Anopheles*, *Culex*, *Hodgesia* and *Mansonia,* genera) were selected from mosquitoes sampled outdoor in Baringo, Garissa and Kilifi districts using CO_2-baited CDC light traps, and singly cryopreserved. Heads and abdomens of the individual mosquito samples were screened for the virus by cell culture and RT-PCR. Putative vertebrate hosts of RVF were determined by amplification and sequencing cytochrome b (cytb) and cytochrome c oxidase I (COI) genes from DNA extracted from the blood meals obtained from the mosquito abdomens. The cyt b and COI sequences were annotated through bioinformatic pipeline suite comprising of 1) BioEdit 2) Basic Local Alignment Search Tool (BLAST) and 3) Barcode of Life Data Systems (BOLD) database. A total of 33, 162 and 21 engorged mosquitoes from Baringo, Garissa, and Kilifi districts respectively were analysed. From the mosquito samples, RVF virus was detected in the heads of five *Mansonia uniformis* (Baringo district), one *Aedes mcintoshi* (Garissa district) and four *Aedes ochraceous* (Garissa district). Among the infected mosquitoes, *Ae. mcintoshi* had blood meals from donkey(s) (*Equus asinus*), *Ae. ochraceous* from sheep (*Ovis aries*), human (*Homo sapiens*) and *Ma. uniformis* from sheep and goat (*Capra hircus*). However, an *Ae. ochraceous* from Garissa and *Hodgesia* spp from Baringo and *Ma. uniformis* (Baringo district) had the RVF virus in the blood meals putatively from goat and two sheep respectively, but not in their head tissues. Other blood meals detected in the mosquitoes included those from, Bird (*Milvago chimachima*), blue duiker (*Cephalophus monticola*), cattle (*Bos taurus*), rat (*Mus musculus*), frog (*Anura* and *Colostethus* sp.) or cat (*Felis catus*). These findings incriminate *Mansonia uniformis* as putative vectors of RVF in Baringo, and *Ae ochraceous* and/or *Ae. mcintoshi* in Garissa districts. This finding suggests possible involvement of Donkey, goat, human and sheep in RVF virus transmission/amplification/maintenance in Baringo and Garissa. These vectors and hosts may have played a role in to the epidemiology of RVF in Baringo and Garissa districts in between 2006 and 2007. Further investigations are required to understand on interactions between these vectors, host and the virus in order to shed light on their specific roles in the epidemiology of RVF.

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LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
BOLD	Barcode of Life Data Systems
bp	base pairs
BSL3	Bio Safety Laboratory Level Three
COI	Cytochrome C oxidase I gene
CPE	Cytopathic effects
CVR	Centre for Virology Research
Cyt B	Cytochrome B gene
dNTPs	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
mtDNA	Mitochondrial DNA
NCBI	National Centre for Biotechnology Information
PBS	Phosphate buffered saline
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RVFV	Rift Valley Fever Virus
TAE	Tris-Acetate Ethylenediaminetetra-Acetic Acid
Taq	*Thermus aquaticus*
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION

1.0 Background information

Rift Valley Fever (RVF) is an arthropod borne viral infection that causes abortion morbidity and mortality among infected livestock (Faye *et al.,* 2007). Medical significance of RVF outbreak in Kenya is characterized by fatal hemorrhagic syndrome, hepatitis and encephalitis among livestock (Njenga *et al.,* 2009). The manifestation of severe RVF disease in humans is variable and the range of clinical signs includes hepatitis, retinitis, delayed-onset encephalitis and, in the most severe cases, hemorrhagic disease (Madani *et al.,* 2003). The outbreak is episodic and correlates with climate variability (LaBeaud *et al.,* 2007). The largest outbreak in Africa in 1997/1998 affected East, West and North Africa was associated with El Niño/Southern oscillation phenomenon, which in turn was associated with rapid increase in local mosquito vector populations (Anyamba *et al*., 2008). In this outbreak, 0.09 million human were infected and 0.1 million domestic animal died from RVF infection (Anyamba *et al*., 2008). In 2006/2007 there was another RVF outbreak in Kenya, Somalia and Tanzania in which 300 people died (WHO, 2007).

The RVF virus is spread by at least 30 mosquito species in six genera (Kuno and Chang 2005). Among these mosquitoes, *Aedes* are the best characterized reservoirs/maintenance vectors (Turell *et al.,* 1996) with *Culex, Mansonia* and *Anopheles* potentiating horizontal transmission (Seufi and Galal, 2010). These vectors of RVFV, vertebrate hosts they feed on, the virus itself can be determined using a number of contemporary techniques that include serology (Staak *et al.,* 1981), enzyme linked immunosorbent assay (ELISA) (Chow *et al.,* 1993) and/or polymerase chain reaction (PCR) (Kent, 2009). In host preference, anthropophilic and anthropophagic tendencies in the vector has been attributed to demand for the isoleucine rich blood meal from humans and other animals required by the vector for vitellogenesis and reproductive fitness (Benedictis *et al.,* 2003). Host preferences studies of mosquito vector in relation to RVF transmission can also be determined by analyses of mosquito blood meals this critically important approach that has previously been employed in estimating efficiency of pathogen transmission, and assessing the relative human and domestic animal disease risks in other vector - pathogen models. The models include; ticks - Borrelia in Lyme disease (Pichon *et al*., 2003), sand flies - *Leishmania* in Leishmaniasis (Haouas *et al*., 2007), tsetse flies - trypanosomes in

trypnosomiasis (Steuber *et al.*, 2005), *Anopheles* mosquitoes - *Plasmodium* in malaria transmission (Scott *et al.*, 2006), *Culex* mosquitoes - West Nile virus in West Nile fever (Apperson *et al.*, 2002; Kilpatrick *et al.*, 2006) and *Culex* - Eastern Equine encephalitis virus in encephalitis (Hassan *et al.*, 2003). Additionally, efficient transmission of RVF virus depends on the convergence in time and space between the mosquito vectors, vertebrate host and virus (Kent 2009). The extent of contact between the mosquito vectors and the community of vertebrate hosts can either amplify or dilute RVF transmission depending on competence of each vertebrate species for RVF virus amplification, relative abundance of each host species present in the community, host susceptibility and host preference of the mosquito vector (Keesing *et al.*, 2006). Age of host, relative abundance, immune status, competence of reservoir, and infection rates collectively contribute to RVF virus transmission (Lord *et al.*, 1996).

As an emerging diseases for which very little is known about non-human reservoirs, identification of mosquito blood meals of RVF vectors can be valuable in identifying viral amplifying hosts or reservoirs of the virus (Kent 2009). This can be achieved by molecular biology approaches that employ reliable mitochondrial genes that identify vertebrate sources of arthropod blood meals (Castro *et al.* 1998). Some of the Mitochondrial genes such as cytochrome c oxidase I (Ivanova *et al.,* 2007) and cytochrome B (Cytb) (Boakye *et al.*, 1999; Sant'Anna *et al.,* 2008) have short DNA sequence fragment barcodes (Kerr *et al.,* 2007), that have found recent application as standardized, novel and more precise approach in species identifications, including elucidation of sources of blood meals in ticks (Ixodes ricinus L), (Ascari: Ixodidae) (Boakye *et al.,* 1999), mosquitoes (Cupp *et al.,* 2004; Alcaide *et al.,* 2009) and more recently in tsetse flies (Farikou *et al.,* 2010). Despite these novelties, the technique has not been evaluated in understanding interactions between the RVF virus and its putative hosts/reservoirs. The RVF virus-mosquito interaction studies can also be augmented by virus detection strategies including cell culture techniques (Mohamed and Imadeldin, 2006) coupled with Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) on RNA of the virus using RVF specific primers that amplify a portion of the virus genome (Ibrahim *et al.,* 1997). These techniques and approaches can be applied to establish putative vertebrate and mosquito hosts of RVF virus in Baringo, Garissa, and Kilifi districts of Kenya collected during the major RVF outbreak in 2006/2007. This information obtained can find application in forecasting risk of RVF infections and improvement response tools for use in preventing or controlling future outbreaks.

1.2. Statement of the problem

Rift Valley fever is a major public health and animal health challenge that causes severe losses during outbreak. However some of the key factors that lead to outbreak such as competent mosquito vectors of RVFV, there feeding pattern(s) and vertebrate hosts/reservoir of the virus in nature are poorly understood. This knowledge is important in understanding the epidemiology of RVF and development of effective integrated control strategies that targets the key component in RVF epidemiology.

1.3. Main objective

To determine the factors that are important in epidemiology RVFV in areas affected by the last major outbreak in 2006/2007 in order to develop strategies for management of the disease.

1.4. Specific objectives

1. To determine prevalence of RVF virus in engorged mosquitoes sampled from Baringo, Garissa or Kilifi districts of Kenya during RVF outbreak in 2006/2007.
2. To determine specific vertebrate blood meal source(s) in mosquito species sampled from Baringo, Garissa or Kilifi districts of Kenya during RVF outbreak in 2006/2007.

1.5. Hypotheses

1. There are no RVF viruses in mosquitoes, and their respective blood-meals, sampled from Baringo, Garissa or Kilifi districts of Kenya during RVF outbreak in 2006-2007.
2. Mosquito species sampled from Baringo, Garissa or Kilifi districts of Kenya during RVF outbreak in 2006-2007 do not posses blood meals from specific vertebrates.

1.6. Justification

Rift Valley Fever has not been well characterised in Kenya. Additionally, the vectors, hosts/reservoirs that transmit/amplify/maintain the viruses in diverse ecologies have not been fully determined yet this is central to understanding RVF epidemiology and formulating strategies for disease management. Information on vertebrate host species and RVF vectors is essential in understanding their respective roles in the disease transmission cycle. Identification of the host range of these vectors is also key in elucidation of the cyclic outbreaks in Kenya. Specifically, knowledge of the transmission cycle, including the

intermediate vectors/host and reservoirs of the virus in nature will assist in public health management by at least two ways. First, when the outbreaks are predicted, the efficient mosquito vector incriminated can be targeted in routine vector control either directly at larval breeding sites, or using strategies that prevent acquisition of blood meal from preferred hosts. Secondly, the next outbreak can be averted by an integrated approach that collectively addresses the risk factors presented by the various hosts and the virus. This study provides data that is necessary for developing an integrated approach to managing RVF

CHAPTER TWO
LITERATURE REVIEW

2.1. Introduction to Rift Valley Fever virus

Rift Valley Fever virus (RVFV) is a member of the *Phlebovirus* genus within the *Bunyaviridae* family. It is primarily transmitted by mosquitoes and causes a life threatening disease among both humans and animals (Daubney and Hudson, 1932). The virus was first identified in 1930, during an outbreak of sudden deaths and abortions among sheep in the greater Rift Valley of Kenya (Daubney *et al.,* 1931). The geographic distribution of the virus has since expanded significantly since RVFV has been identified in most countries of the African continent (Seufi and Galal, 2010) and Madagascar (Gerdes, 2004). Rift Valley fever outbreak emerged outside Africa in the Arabian Peninsula in 2000–2001 (Youssef *et al.,* 2008, Gaff *et al.,* 2007) and caused devastating effects in livestock and humans (Balkhy and Memish 2003). More recently, it has been detected for the first time in the Archipelago of Comores, located between Mozambique and Madagascar, on Island of Mayotte (Sissoko *et al.,* 2009). Due to the increasing range of high numbers of competent vector species present in currently RVF-free regions, such as Europe (Moutailler *et al.,* 2008) and the USA (Turell *et al.,* 2008a), international agencies have issued warnings about the risks of introduction of RVFV into RVF-free zones (Breiman *et al.,* 2008; Chevalier *et al.,* 2010; Zabransky, 2005) and thus coordinated efforts to better prepare for a possible emergence of RVFV are needed.

2.2. Genetic organization of RVFV

Rift Valley Fever virus is an enveloped RNA virus characterized by a genome composed of three segments designated S, M and L of negative or ambisense polarity (Bird *et al.,* 2008). The small segment (S) is 1600 bases in size and codes for virus nucleoprotein and non-structural proteins which are critical in virulence of the virus, the medium segment (M) is 3800 bases and codes for glycoproteins, while the large segment (L) is 6400 bases and codes for viral RNA-dependent RNA polymerase (Bird *et al.,*2007). Ultrastructural studies by electron microscopy and negative staining described RVFV particles measuring 90–110 nm in diameter (Ellis *et al.,* 1979). The envelope is composed of a lipid bilayer containing the G_n and G_c glycoproteins forming surface sub-units, 5–8 nm in length, regularly arranged on its surface (Von Bonsdorff and Pettersson, 1975). The viral ribonucleoproteins (RNP) corresponding to each of the three genomic segments, associated with numerous copies of the nucleoprotein N and the RNA dependent RNA polymerase L, are packaged into the virion. Recent studies by cryo-electron microscopy revealed that RVFV has an icosahedral

symmetry (Freiberg *et al.,* 2008) and the structure is highly ordered and the surface covered by a shell of 120–122 glycoprotein capsomers arranged in an icosahedral lattice (Huiskonen *et al.,* 2009). Three dimensional resolution revealed that the capsomers resemble hollow cylinders situated at five and six-coordinated positions. Inside the envelope, a layer of RNP is located proximal to the inner leaflet of the membrane, strongly suggesting an interaction between the cytosolic tail of the glycoproteins and the RNP which would compensate for the absence of matrix protein in the viruses of this family (Sherman *et al.,* 2009). Replication of the genome occurs in the cytoplasm of infected cells where virions mature by budding in the Golgi compartment (Nichol *et al.,* 2005). During the viral cycle, the glycoproteins play an essential role for the penetration of the virus and their proper processing is crucial for the maturation and budding of the virion. The glycoproteins, being the most exposed components of the virus during infection, are recognized by the immune system and induce the production of neutralizing antibodies, which play a predominant role in protection. The glycoproteins also mediate virus entry into many cell types through specific receptors which, in the case of RVFV and many other bunyaviruses, remain to be identified (Filone *et al.,* 2006). Entry is predicted to employ a class II fusion mechanism that is activated by low pH following endocytosis of the virion (Filone *et al.,* 2006). Little is known regarding the early phases of infection that precede the release of virus RNP into the cytosol. The information on RVFV genome has been useful for identification of its presence in suspect samples; this is done by amplification of a portion of the M segment or entire S and M segments utilizing specific RVF RT-PCR primers (Bird *et al.,* 2007). This method of detection can also be coupled with monitoring the activity of the virus in cell cultures such as monolayer Vero cell lines, the viral particles are then harvested from these cells by centrifugation (Mohamed and Imadeldin, 2006).

2.3. Rift Valley fever virus vectors and transmission cycles

Rift Valley fever virus has the potential to infect a variety of hematophagous vectors, including ticks (Labuda and Nuttall 2004) and flies (Fontielle *et al.,* 1998; Lee 1979; Linthicum *et al.,* 1985; Van Velden *et al.,* 1977). However, unlike the majority of arboviruses RVFV is adapted to a narrow range of vectors (Chevalier *et al.,* 2004; Jupp *et al.,* 2002). Vectors of RVFV are classified into two groups; the first group is reservoir/maintenance vectors which include Aedes species of the subgenera *Neomelaniconion* (Davies and Martin, 2006) and *Aedimorphus* (Ramos *et al.,* 1994) always associated with freshly flooded temporary or semi-permanent fresh-water bodies, hence the collective name flood water

Aedes (Fontielle *et al.,* 1994; Gear *et al.,* 1955) and the second group is the epidemic/amplifying vectors consisting of *Culex spp* and *Mansonia spp*. *Anopheles spp. Eretmapodites spp Coquillettidia spp.* associated with more permanent freshwater bodies, (McIntosh and Jupp 1981). Transmission paradigm in reservoir/maintenance vectors involves survival of RVFV in mosquito eggs through transovarial transmission from females to their progeny during periods of drought and this forms a vehicle for survival of RVFV over long periods of time in these vectors (Becker,1989; Gargan *et al.,* 1988). Epidemic/amplifying vectors play a secondary role of magnifying transmission rates by feeding on infected hosts after which they get infected and consequently transfer infection during subsequent feeding (Pepin *et al.,* 2010). During feeding, infected and well adapted mosquito for RVFV transmission pass the virus to the susceptible vertebrate host. Preference and selection of vertebrate host is determined by genetic factors which implicates anthropophilic behaviour seen in *Aedes aegypti* and *Anopheles gambiae* (Kuno and Chang 2005). Behavioural and ecological factors also set the limits within which vectors select vertebrate hosts depending on the densities of susceptible vertebrate hosts in a given locality (Kuno and Chang 2005). This transmission cycle which brings together RVFV, suitable vectors and vertebrate hosts still remain elusive and appear to correlate with climate variability (Anyamba *et al.,* 2008). In the midst of this cycle, the vector plays a central role and remains infective throughout its lifetime (Sang and Dunster, 2001). According to Marquardt *et al.,* 2005, members of RVFV transmission cycle bear the following characteristics; are capable of infecting and replicating in two phylogenetically disparate systems, cause a high titter viremia in natural host, should not be unduly virulent to kill host before new vector can be infected and virus must replicate in the invertebrate host to enable its biological transmission. Once the vector is infected with RVFV, the virus has to adapt itself to overcome different barriers within the vector. Such barriers are presented by midgut and salivary gland (Kramer *et al.,* 1981) which seem to be the determining factors of the vector competence for the various species (Beerntsen *et al.,* 2000). Ovarian escape barriers on the other hand facilitate trans-ovarian transmission to subsequent generation (Marquardt *et al.,* 2005). The transmission cycle of RVF virus can be illustrated by the schematic cycle in Figure 1.

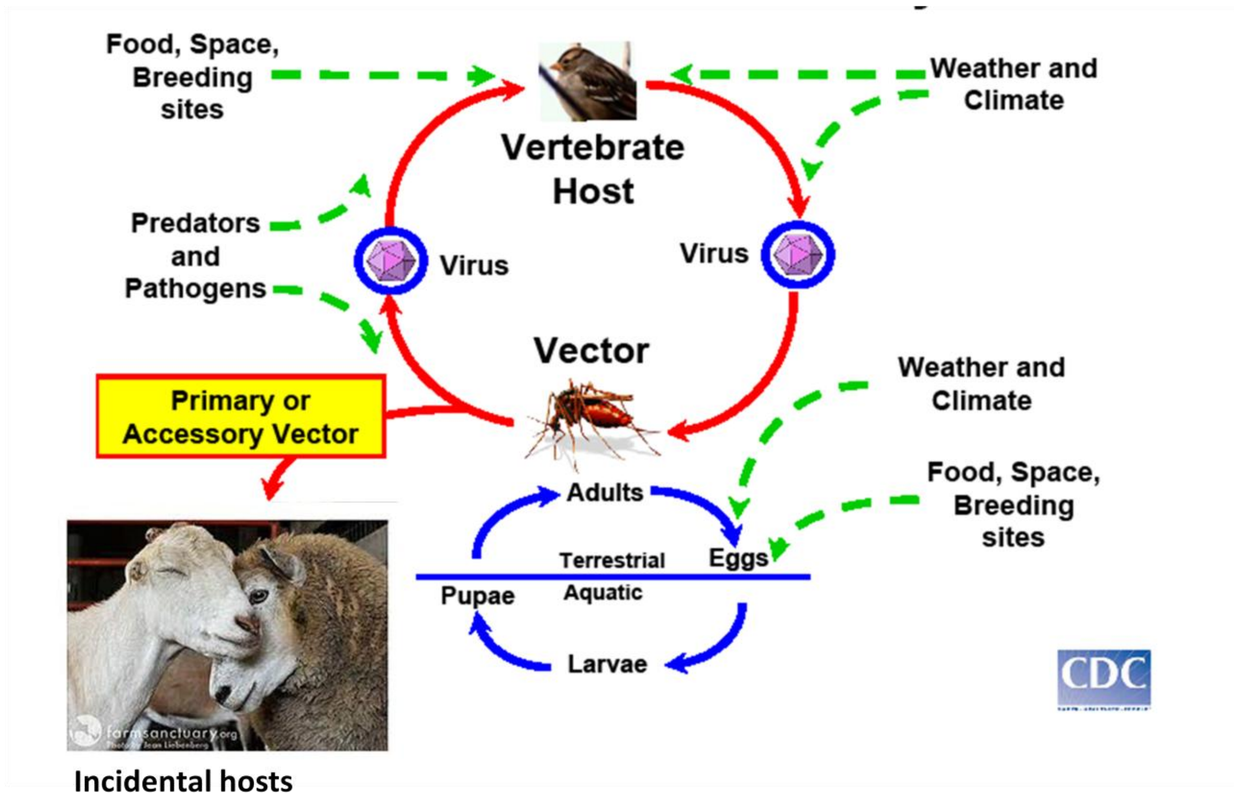


Figure 1. Transmission cycle of Rift Valley Fever virus

Source: http://www.cdc.gov/ncidod/dvbid/arbor/schemat.pdf; accessed: 30/11/2010 at 17:30)

2.4. Rift Valley fever infection in animals and humans

Rift Valley fever infection of animals and human occurs by the bite of an infected mosquito or by direct contact with infected animal tissues, bodily fluids and fomites, particularly if associated with abortions (Pepin *et al.,* 2010). Aborted foetal materials and placental membranes contain large numbers of virus particles which can either contaminate the local environment directly or infect animals in close contact (Craig *et al.,* 1967). The virus may then persist for relatively long periods in the environment as has been demonstrated during in vitro experiments (Theiler *et al.,* 1957). The relative contribution and importance of each mode of transmission varies according to the stage of the outbreak, in the first stage, the bites of infected mosquitoes are the predominant mode of transmission whereas direct contact of infected animal and tissues becomes predominant during the amplification stage of the epizootic (Zabransky, 2005). Transmission via infected mosquitoes remains important for the dissemination of RVFV between herds or flocks over short distances but also allows for the emergence and dissemination of the disease over longer distances, throughout a region or a country, but has to be preceded by the movement of infected animals (Chevalier *et al.,* 2004) Mosquito-borne transmission is also the most important mode during an enzootic cycle with an active circulation of the virus between susceptible animals. Aborted materials constitute another route of virus transmission, through direct contact with foetal envelopes, placenta and the foetus (Pepin *et al.,* 2010). Virus within these tissues may stay infectious over a long period as RVFV particles are rather resistant to inactivation when in a protein-rich environment (WHO, 2007). On the contrary, nasal and lacrymal secretions have been shown to be free from RVF in controlled laboratory studies (Walker *et al.,* 1970) though shedding of RVFV into milk has potentially large consequences for public health and has been implicated in an outbreak in Mayotte (Sissoko *et al.,* 2009). To date, the presence of RVFV in the feces or urine of infected animals has not been demonstrated. In the vast majority cases, infection with RVFV is asymptomatic. However, RVFV epidemics can involve hundreds or thousands of individuals (Madani *et al.,* 2003). The manifestation of severe RVF disease in humans is variable and the range of clinical signs includes hepatitis, retinitis, delayed-onset encephalitis and, in the most severe cases, hemorrhagic disease (Madani *et al.,* 2003). The overall case fatality ratio is estimated to be between 0.5% and 2%. Human cases with jaundice, neurological disease, or hemorrhagic complications are at increased risk of fatality (Morvan *et al.,* 1991). Rift Valley fever

infection and disease has been described in susceptible animals and particularly in ruminants, and efforts have been directed at identification of competent vectors of RVFV (Swanepoel and Coetzer 2004). However, detailed descriptions of the natural disease have not been conducted during more recent epizootics. Instead, the most recent descriptions of RVF epizootics have focused on the analytical and predictive epidemiology of the disease (Vignolles *et al.,* 2009). Susceptibility of RVF disease in young animals needs to be studied keenly and compared to adult animals (Zhao *et al.,* 2008). The classical hallmark of RVF infection which is extensive abortions among pregnant ruminants regardless of the stage of pregnancy also needs a keen and clear focus. Host preference of RVFV mosquito vectors is central to better understand RVF transmission cycle and epidemiology.

2.5. Screening of engorged mosquito blood meals for vertebrate host identification

Earlier analytical techniques for blood meals identification of hosts have focused on serological methods and between 1920s-1940s, precipitin test was predominantly used, while in 1970s, McKinney developed fluorescent antibody technique (McKinney *et al.,* 1972). In 1980s-1990s, enzyme linked immunosorbent assay (ELISA) was used in host identification (Ngo and Kramer, 2003). Even though the serological methods have proved valuable in host identification, they are faced with problem of production of high quality antisera against all potential hosts followed by elimination of antibodies that can result to cross reactivity of serum protein from phylogenetically related species (Steuber *et al*., 2005)

A more recent and convenient method of screening blood meal for host identification is provided by mitochondrial DNA (mtDNA), because of its high copy number in a cell (Woods *et al.,* 2009) and a conserved 350bp sequence of cytochrome B (cytb) can be used successfully for host identification (Pierce *et al*., 2008). Another valuable portion of mtDNA is a 648bp fragment of cytochrome C oxidase I (COI) gene vastly used in identification of species and regarded as a standard barcode (Ratnasingham and Hebert, 2007; Muturi *et al.,* 2011). Both exhibit sufficient interspecific variation to discriminate mammalian host samples (Boakye *et al.,* 1999). Both sequences can be amplified by broad range of primers and thus provide a promising target of host identification (Stoeckle, 2003). The order of nucleotide in each amplicon can be determined through DNA sequencing and the identity of the species known by querying bioinformatic databases.

2.6. DNA sequencing

DNA sequencing involves the determination of the order of nucleotides in a sample DNA strand. The benefit of sequencing DNA coupled with PCR is that sequence from a

minute quantity of samples (Tablet and Bouvet, 1991) like mammalian hair (Morin *et al.,* 1992) can be determined. Two methods are available for determination of DNA sequence; Maxam and Gilbert in 1977 devised a method that is based on chemical cleavage reactions specific to individual bases. The method is not popularly used despite its advantage of permitting direct sequencing of small fragments; the other method is by Sanger *et al.,* 1997 which relies on controlled interruption of *in vitro* DNA replication. This method is also referred as the dideoxynucleotide chain termination method because the synthetic nucleotides lack the hydroxyl group at the 3'carbon atom therefore during synthesis, a dideoxynucleotide can be added to the growing DNA strand causing the chain elongation to stop due to lack of a free 3'-hydroxyl group needed to form phosphodiester bond needed for the polymerization process. The ratio of deoxynucleotides to didioxynucleotide is high thus enhancing several nucleotides to be added to the growing DNA strand before insertion of dideoxynucleotide which stops the replication process. The individual fragments are then separated by sequence length on a gel giving individual bands. Each of the four dideoxynucleotides fluoresces with a different colour when illuminated by a laser beam and an automatic scanner provides a print out of the sequence usually referred to as electropherogram chromatograms which consist of forward sequence and a complementary sequence. Editing of the two sequences results in a consensus sequence which can be used to query GenBank and Barcode of Life Data System (BOLD).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Determination of prevalence of RVF virus in mosquitoes, and their respective blood-meals

3.1.1. Study areas

This Study was undertaken in three ecologically distinct districts in Eastern, Rift Valley and Coastal provinces in Kenya where RVF outbreak was reported in human and livestock. Baringo District (latitude 0°28’N, longitude 35°58’E) is located in the Rift Valley Province of Kenya, 250 km northwest of Nairobi. The low lying arid part of Baringo consists of northern Acacia-Commiphora bushlands and thickets and has experienced severe land degradation caused by uncontrolled grazing. Harsh physical and climatic conditions have led to a sparsely populated district (average of 31 people/km^2) where the local inhabitants, classified as agropastoralists, subsist mainly on limited crop production and livestock rearing. The collection of mosquitoes was conducted at a site near Lake Baringo where the annual rainfall ranges from 300 to 700 mm, and the daily temperature varies between 16 and 42°C (Ryan *et al.,* 2006).

Garissa district (latitude 0°26’S, longitude 39°40’E), which is located in the North Eastern Province of Kenya bordered by the Tana River to the west and Somalia to the east (Fig.2). The district has an area of 44,952 km^2 and characterized as an arid area with *Acacia-Commiphora* bushlands and thickets (Statoids, 2010). Rainfall is sporadic, averaging approximately 200–500 mm per year, with occasional torrential storms causing extensive flooding. The average temperatures range from 20 to 38°C and the altitude varies from 70 to 400 m above sea level. The soil is generally sandy with scattered areas of dark clay that tend to retain water after the rains and serve as watering holes and grazing land for livestock and wild animals. The sparse population (~7 people/km^2) of the district is found concentrated around the water sources and also around small market centers. The people are largely nomadic, moving between districts with their large herds of livestock in search of water and pasture land.

Kilifi town (latitude 3°36’S, longitude 39°51’E), located in Kilifi district, Coast Province, is 318 km south of Garissa. The district contains a moderately dense population (~114 people/km^2) (Statoids, 2010) and the vegetation is characterized by a mix of East African mangroves and coastal forests that are comprised of dense woods, swamps, dry scrub, and commercial plantations. Annual rainfall for the district ranges from 750 to 1,200 mm, while the average temperature maximum is 30°C. The soils are fertile and subsistence farming of corn, coconut, goats, chicken, and cattle is widespread. The collection of mosquitoes was conducted on 12 January 2007 at homesteads that were associated with confirmed or suspected RVF cases (Sang *et al.,* 2010).

Figure 2 Mosquito sampling areas in the three 2006-2007 RVF hot spots in Kenya

3.1.2. Sampling design and procedure

In each of the three districts, sampling sites were selected based on RVF case reports in humans or livestock. Mosquitoes were sampled using CO_2-baited CDC light traps placed at outdoor sites in the three districts; Logumgum (Baringo District), El-humow (Garissa District) and Gongoni (Kilifi District). The traps were laid at 6pm and collected at 7am the next day (Sang *et al.,* 2010). After collection, mosquitoes were anesthetized using triethylamine and identified on ice to species level based on their morphological features such as body size and colour, sexual organs, thoracic features, wing venation, mouth parts, scutellum and legs using taxonomic keys (Gillett, 1972). Immediately after identification, engorged mosquitoes were stored singly in 1.5mL epperndoff tubes while non engorged mosquitoes were sorted out for a separate study. The samples were then transported in a liquid nitrogen shipper to the laboratory where they frozen at -80°C in a freezer.

3.1.3. Vero cell line growth and RVF virus propagation.

Dissection and processing of mosquito head and abdomen was conducted in a bio-safety laboratory level 3 (BSL3) of Kenya Medical Research Institute (KEMRI) by methods of (Coleman *et al.,* 2007). Briefly, the abdomens of fully engorged frozen mosquitoes were carefully separated from the rest of the body using sterile fine forceps and scalpel blades. Individual abdomen were placed in a 1.5 mL micro-centrifuge tube containing one 4.5 mm copper bead and 300 µL minimum essential medium (MEM) supplemented with 15% fetal bovine serum, 2% L-glutamine and 2% antibiotic mixture (fungizone, 100 U/mL penicillin, and 100U/mL streptomycin), and vortexed to completely homogenized abdomen. The homogenates were centrifugation at 4°C and 12,000 rpm for 10 minutes, and the resulting supernatants stored at -80°C for subsequent viral and DNA assays.

Screening of blood meals and mosquito heads for RVF virus was conducted in accordance with standard protocol (O'guinn and Turell, 2002). Fifty micro liter blood meal or head supernatant was inoculated in a confluent monolayer of Vero cell line in order to facilitate viral expansion through replication. Prior to the inoculation, Vero cells were grown in Minimum Essential Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine and antibiotic mixture (fungizone, 100 U/mL penicillin, and 100 U/mL streptomycin). Cells were grown in 25 and 75 cm^2 cell culture flasks (Corning Incorporated, Corning, NY 14831) and incubated at 37°C in a humidified incubator with 5% CO_2. Upon attaining a confluent monolayer, the cells were trypnised and 1 mL used to seed

every well in a 24 well cell culture plates. This was incubated overnight for attachment of the cells to take place. To each well, 50 μL of the abdomen or the head supernatant was seeded in duplicates including positive and negative controls of RVF, incubated at 37°C for attachment to occur then 1 mL Minimum Essential Eagle's Medium (MEM) supplemented with 2% fetal bovine serum (FBS), 2% L-glutamine and antibiotic mixture (fungizone, 100 U/mL penicillin, and 100 U/mL streptomycin) was added to each well. The inoculated cell culture plates were incubated at 37°C in a humidified incubator with 5% CO_2 in BSL 3 facility. They were observed daily through 7 days for cytopathic effects (CPE) which signifies viral growth. Wells showing cytopathic effects were noted and the virus harvested by centrifugation at 3000 rpm for 15 minutes after attaining 75% CPE. All negative cultures were blind passaged once and observed daily for 4 days for any CPE. Any homogenates that remained negative (no CPE) after the passage were considered negative and discarded. Two hundred and fifty microlitre aliquot of the positive virus culture was used for RVF specific RT-PCR and remainder stored in liquid nitrogen for future use.

3.1.3. Viral RNA extraction

Viral RNA was extracted from positive CPE cultures using Trizol LS reagent according to manufactures instructions and as modified by O'Guinn and colleagues. Briefly, 250 μL aliquot of positive cultures was transferred into a 1.5 mL micro centrifuge tube containing 750 μL of Trizol LS reagent and allowed to stand at room temperature for 10 minutes in a BSL 3 facility to allow for inactivation of the virus. The rest of the procedure was carried out in a Bio safety level 2 facility. Two hundred micro litres of chloroform (Reagent Grade) was added to the sample and mixed by vortexing. The sample was allowed to incubate at room temperature for 10 minutes followed by centrifuging sample at 12000 rpm for 10 minutes at 4°C. Five hundred micro litres of the clear aqueous phase solution was put in clean RNASE free 1.5 mL snap cap micro centrifuge tube containing 1μL glycogen, this was followed by addition of 500 μL isopropanol (Reagent Grade). The sample was vortexed and incubated at room temperature for 10 minutes, centrifuged at 12000 rpm for 10 minutes at 4°C.

The liquid was emptied into a discard container leaving the pellet. Five hundred micro litres of 75% ethanol was added to wash the pellet. The tube containing the pellets was centrifuged for 2 minutes at 12000 rpm room temperature. The residual liquid was emptied into a discard container taking care not to remove the pellet. Twelve micro litres of nuclease free water was added to re-suspend the pellet then let stand at room temperature for 5 minutes

followed by vortexing to mix. The RNA sample was used immediately for reverse transcription.

3.1.4. First Strand cDNA synthesis from viral RNA by RT-PCR

Ten microlitres of the RNA of interest was pipetted together with 2 µL random hexamer (100 nmoles) into 200 µL thin walled PCR reaction tube and placed in a thermal cycler programmed as follows; 70°C for 10 minutes to denature RNA secondary structures then cooled to 4°C for 5 minutes. The following was added to the reaction tube; 4 µL of 5x first strand reaction buffer, 2 µL of 0.1M DTT, 0.25 µL of RNase inhibitor (40U/µL) 1 µL of Superscript III reverse transcriptase (Invitrogen, Inc.) and 1 µL of 10mM dNTP. The sample was spun down in a mini-centrifuge and heated to 25°C for 15 minutes, 42°C for 50 minutes and then followed by 70°C for 15 minutes then at 4°C. The cDNA was stored at -20°C for later use.

3.1.5. Amplification of RVF cDNA by Polymerase Chain Reaction

The PCR amplification of targeted viral sequences was accomplished as follows. Two micro litre of the cDNA was used in conventional PCR using 50 pmoles of RVF specific primers (Ibrahim *et al.,* 1997). The forward RVF1 FWD 5'-GACTACCAGTCAGCTCATTACC-3' and reverse primer sequence RVF2 REV 5'-TGTGAACAATAGGCATTGG -3' amplify a portion of RVF virus M-segment genome with amplicons of 551bp. Thermal cycling profile was as follows; first denaturation at 95°C for 2 minutes, followed by 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds followed by elongation at 72°C for 45 seconds. Final extension was achieved at 72°C for 7 minutes. Amplicons were resolved in 2% Ethidium Bromide stained agarose gel matrix. Resolved amplicons was documented using a gel imaging system.

3.1.6. Purification and sequencing of amplicons

The main reason for cleaning PCR products was to free it to from primer dimer were purified directly using promega kit for PCR clean-up (Promega Corporation) following manufactures instruction. Briefly, 20 µL of PCR product was placed in a 200 µL thin walled PCR tube followed by addition of equal volume of membrane binding solution. The mixture was vortexed and transferred to SV mini column, spun at 12000 rpm for 1 minute. The column was washed twice with wash buffer and spun at 12000 rpm for 3 minutes to remove residual wash solution. The column was transferred to a clean 1.5 mL micro centrifuge tube and DNA eluted in 30 µL nuclease free water. Three micro litres was resolved in a 1% EtBr

stained agarose gel matrix to confirm the recovery the purified DNA. The remaining purified product was sent to ILRI for sequencing.

3.2. Determination of specific vertebrate blood meal source (s) in mosquito species

Dissection and processing of mosquito abdomen was conducted in a bio-safety laboratory level 3 (BSL3) of Kenya Medical Research Institute (KEMRI) by methods of (Coleman *et al.,* 2007). Briefly, the abdomens of fully engorged frozen mosquitoes were carefully separated from the rest of the body using sterile fine forceps and scalpel blades. Individual abdomen were placed in a 1.5 mL micro-centrifuge tube containing one 4.5 mm copper bead and 300 µL minimum essential medium (MEM) supplemented with 15% fetal bovine serum, 2% L-glutamine and 2% antibiotic mixture (fungizone, 100 U/mL penicillin, and 100U/mL streptomycin), and vortexed to completely homogenized abdomen. The homogenates were centrifugation at 4°C and 12,000 rpm for 10 minutes, and the resulting supernatants stored at -80°C for subsequent extraction of DNA.

3.2.1. Extraction of genomic DNA from mosquito abdomen

The supernatant (100 µL) from dissected and processed abdomen were placed in 100 µL Trizol-LS (Invitrogen Inc., Carlsbad, CA) in a 1.5 mL micro-centrifuge tube to stabilise the inherent genomic DNA and potentially inactivate RVF virus present in the sample. The Genomic DNA was extracted from the samples by QIAGEN DNeasy extraction kit (Qiagen Inc., Maryland, CA, USA), following manufacturers instruction. Briefly 20 µL of proteinase K was added then 200 µL of lysis buffer AL. The mixture was vortexed briefly for 30 seconds then incubated at 56°C for 10 minutes in a water bath. After incubation, 200 µL of absolute ethanol was added and vortexed thoroughly for 1 minute. Using a pipette, the mixture was transferred into a DNeasy Mini spin column in a 2 mL collection tube followed by centrifugation at 8000 rpm for 1minute. The flow-through was discarded. The spin column was placed in a new 2 ml collection tube. Five hundred micro litres of wash buffer AW1 buffer was added and sample centrifuged at 8000 rpm for 1 minute. The flow through was discarded and spin column removed carefully so that it does not come in contact with the flow-through and placed in a new collection tube. Five hundred micro litres of wash buffer AW2 was added to the column and centrifuged at 14000 rpm for 3minutes. The column was removed carefully and placed in a new 2 mL micro centrifuge tube and 25 µL of AE buffer was added for elution. Elution was done by centrifugation at 8000 rpm for 1 minute.

3.2.2. Polymerase Chain Reaction of Blood meal DNA

Polymerase Chain Reaction was conducted on the mosquito blood meal DNA with taxon specific vertebrate primers for cytochrome C oxidase I (COI) (VF1d_t1 Forward 5'-TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYG G-3', VR1d_dt reverse 5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYC A-3') and cytochrome B (Cyt B) (L14841Forward 5'-CCATCCAACATCTCAGCATGATGAAA-3', H15941 reverse 5'-GCCCCTCAGAATGATATTTGTCCTCA-3') genes by methods of Ivanova *et al.,* (2007) and Kocher *et al.,* (1989) respectively. Briefly, 1.5 µl DNA product was amplified with 0.02 U/µl of Phusion DNA polymerase (Finnzymes OY, Finland) in the buffer (provided by the manufacturer) and 0.5 µM primer, 200 µM dNTPs in the presence of the specific primers for cytochrome C oxidase I (COI) or cytochrome B (Cyt B). A concentration of 7.5 mM $MgCl_2$ was used in each reaction, to provide optimum yield and specificity. Reactions were carried out in PTC 100 thermocycler. The first cycle included 30 seconds at 98°C, 40 seconds at 56°C, and 45 seconds minute at 72°C. Subsequent cycles involved 10 seconds at 98°C, 30 seconds at 56°C, and 40 seconds at 72 °C for 35 cycles for COI. Similar conditions were applied for Cyt B amplification except that annealing temperature and duration, and extension duration and were 61°C and 20 and 30 seconds respectively. Final primer extension was conducted at 72°C for seven minutes. A no-sample negative control was used.

3.2.3. Purification and documentation and of COI and Cyt b PCR products

The PCR products mixed with 3µL loading dye (15% ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA) were loaded onto Ethidium Bromide 1.5 % agarose gels in a TAE buffer (Samrook *et al.,* 1989). On every gel, a 100bp DNA ladder molecular weight marker (Fermatas, Hanover, MD) was run (80 V for an hour) to confirm expected molecular weights of the amplification products.

Putative COI (730bp) and Cyt b (350bp) bands in the gel were excised using a sterile scalpel, placed in 1.5mL micro centrifuge and extracted using Quickclean DNA gel extraction kit (GenScript Corporation) following manufacturer's instructions. Briefly, appropriate binding solution was added to the gel slice and incubated at 50°C for 10 minutes with occasional vortexing until the gel was solubilised. One volume of isopropanol was then added and the mixture vortexed before being transferred to a quickclean column and centrifuged at 12000 rpm for 1 minute. The flow through was discarded and column loaded with 500 µL of wash

solution and centrifuged at 12000 rpm for 1 minute. Additional centrifugation was performed at 12000 rpm for 1 minute to remove residual wash solution. The column was transferred to a clean 1.5 mL micro centrifuge tube and 20 µL of elution buffer added to the centre of the column and incubated at room temperature for 3 minutes. The column was centrifuged at 12000 rpm for 1 minute to elute DNA. From the recovered DNA, 3 µL was resolved in a 1% EtBr stained agarose gel matrix to confirm the recovery of the purified DNA (Eastman Kodak Company, USA)

3.2.4. DNA sequencing and analyses

Each purified DNA product with a concentration of 30ng/µL was sequenced at International Livestock Research Institute (ILRI). Sequencing was done using the Big Dye® terminator version 3.1 cycle sequencing kit (Applied Biosystems USA) which is based on the dideoxynucleotide chain termination method (Sanger *et al.,* 1997). The reaction was analyzed using ABI 3730 DNA analyzer. The sequencing primers were the respective forward and reverse primers used for COI and CytB amplification. The nucleotide sequences obtained were edited using BioEdit sequence alignment editor version 7.0.9 to generate a consensus sequence. BOLD identification engine was used to query the BOLD database and match of closely related sequences was used to identify the species fed on by the mosquito. A BLAST search was also carried out to identify reference sequences present in GenBank nr database at National Centre for Biotechnology Information (NCBI).

3.3. Data analyses

DNA sequences obtained from the sequencing procedure were edited using BioEdit, version7.0.9 software to remove primers and to check for segregating sites within the forward and the reverse sequences. Edited nucleotide sequences (consensus sequence) for cytochrome C oxidase I was queried in Barcode of life data system (BOLD) to enable the identity of homologous sequence in the database and thus vertebrate species. Basic local alignment search tool (BLAST) was used to probe GenBank databases nr for identification of edited sequences of cytochrome B. Information on species of vertebrate hosts generated by bioinformatic searches and prevalence of RVF virus among individual mosquitoes and vertebrate was tabulated. Rift Valley Fever virus infections on individual mosquito's species was also recorded in tables as positives and negatives and percentage prevalence calculated

as a quotient of number of individual mosquito species positive for RVF over the total number of the species expressed as percentages.

CHAPTER FOUR
RESULTS

4.1. Prevalence of RVF virus in mosquitoes, and their respective blood-meals

4.1.1. Mosquito samples collected in Baringo, Garissa and Kilifi district study sites

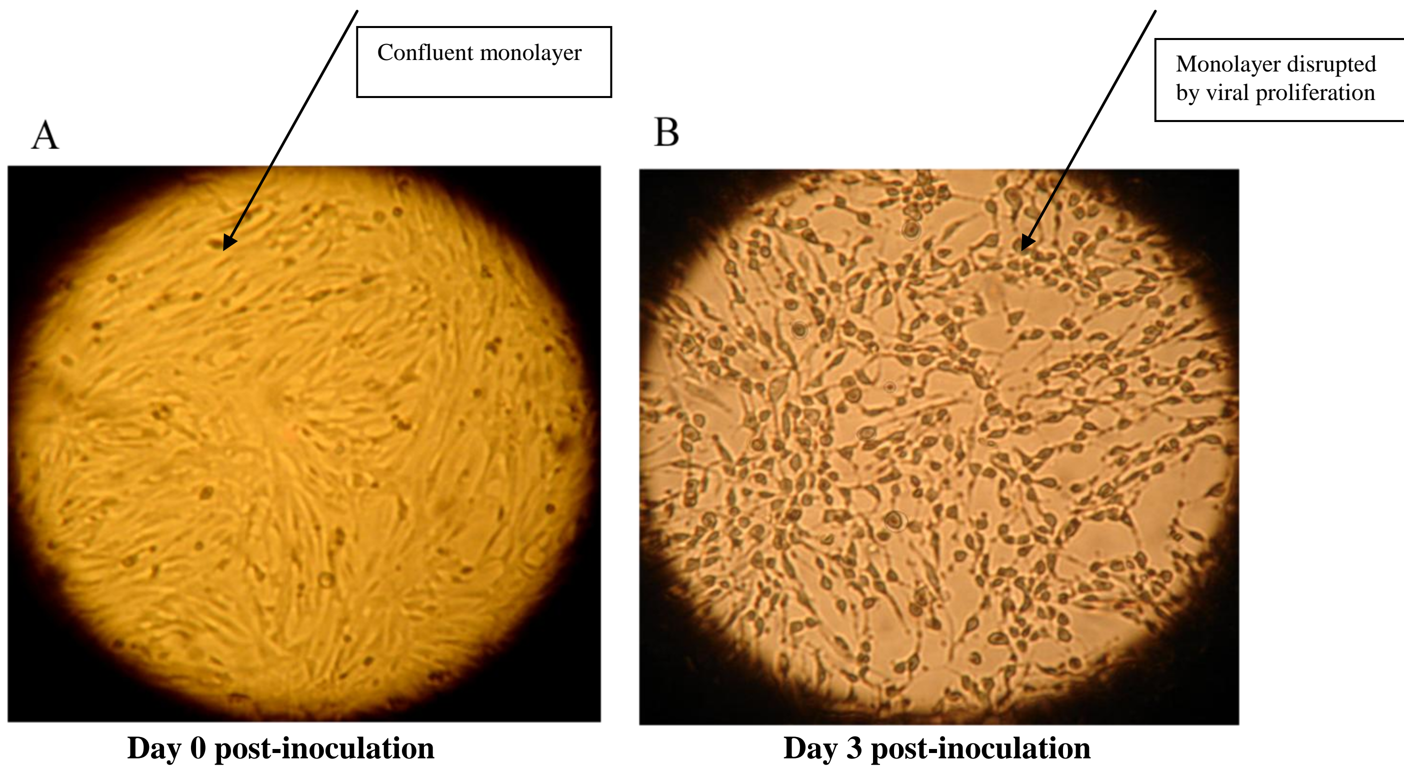
Overall, 216 mosquito were sampled in the study areas, among which 33, 162 and 21 mosquitoes were sampled in Baringo, Garissa, and Kilifi districts respectively. The samples consisted of three, five and 11 species in Baringo, Garissa, and Kilifi districts respectively. Most (72.73%) of the mosquitoes sampled in Baringo were *Mansonia uniformis* while the rest were *Hodgesia spp* (21.21%) and *Mansonia africana* (6.06%). *Aedes ochraceous* were the predominant (67.28%) in Garissa districts followed by *Aedes mcintoshi (*27.78%). The others were *Aedes Sudanensis* (3.09%) *Culex poicilipes* (1.23%) and *Anopheles caustani* (0.62%). In Kilifi district, *Anopheles gambiae* predominated *(*28.57%) among the samples followed by *Anopheles squamosus* (14.29 %). Other mosquitoes presents were *Culex poicilipes Anopheles caustani, Culex bitaeniorhynchus* each comprising (9.52%), the samples*,* while *Aedes pembaensis or Culex pipiens* comprised 4.76% of the same.

4.1.2. Detection of RVF virus in head homogenates of the mosquito samples and their respective abdominal blood-meals

Representative result of Vero cell lines culture of RVF virus in the head homogenates and abdominal blood meals of the mosquito samples is presented in Fig 3. There was a transformation of the confluent monolayer Vero cell line from the characteristic spindle shaped morphology (Fig 3, Panel A) to fibrillar rods with disruption of the monolayer (Fig 3, Panel B), characteristic RVF virus induced cytopathology in the cell line. The cells rounded up and detached from the surface of culture vessel. Additionally, color of the phenol red indicator changed from red to orange/yellow, indicative of pH change due to active viral metabolism in the cell culture.

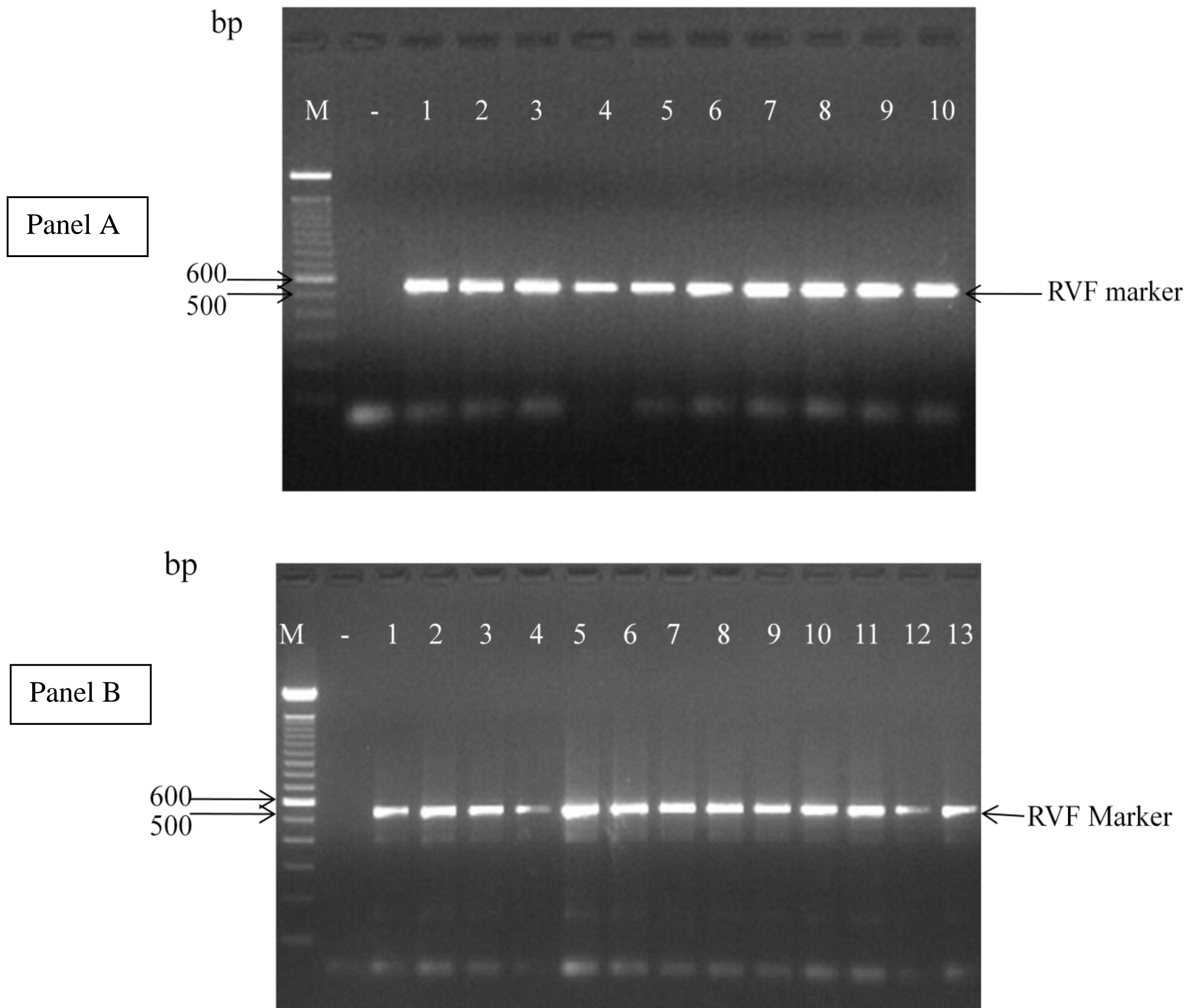
Among the samples, RVF virus was successfully cultured from head homogenates and abdominal blood meals of five *Ma. uniformis* mosquitoes sampled from Baringo*,* four *Ae. ochraceous* one *Ae. mcintoshi* mosquitoes sampled from Garissa districts (Table 1). However, the virus was successfully cultured from the abdominal blood meals but not from head homogenates from a *Ma. uniformis* and a *Hodgesia spp* sampled from Baringo and one *Ae. ochraceous* sampled from Garissa districts. Screening of mosquitoes from Kilifi district did not have positives for RVF virus. Presence of the RVF viruses in the Vero cell lines cultures successfully inoculated by head homogenates and/or abdominal blood meals from

respective mosquito samples were individually confirmed by RT-PCR results (Fig. 4). Additional *in silico* analyses of the sequences of the RT-PCR products indicted that all the viruses were homologous to RVF virus strain, based on segment M glycoprotein gene.



Individual head homogenates of RVF virus infected mosquito were cultured in Vero cell lines and monitored for cytopathic activities at 37°C and 5% CO_2 incubator for three days. Panel A); Morphology of the monolayer of the cell line at the onset of inoculation with head homogenate samples. Panel B) cytopathology of the monolayer of the cell line three days post inoculation with head homogenate sample infected with RVF virus. Cytopathology is indicative of presence of the virus activity.

Figure 3. Culture of RVF virus in the head homogenate of virus infected mosquito



Head homogenates and abdominal blood meals from mosquito samples were separately cultured in Vero cell line cultures. Cultures that established putative RVF virus infections were harvested at the peak of virus proliferation (3 dpi). The viral RNA was extracted and reverse transcribed to cDNA, which was amplified via PCR, resolved on 2% agarose gel and visualized through ethidium bromide staining. Panel A represents PCR products from cell cultures putatively infected with RVF virus in homogenates from heads of mosquitoes. Panel B represents PCR products from cell cultures putatively infected with RVF virus in abdominal blood meals in the mosquito samples. The lanes denote the following cDNA PCR products; lanes M is 100bp marker, 1-5*Ma. uniformis;* 6-9 *Ae. ochraceous;* 10 *Ae. mcintoshi;* 11. *Ma uniformis*, 12 *Hodgesia spp* and 13 *Ae. ochraceous*

Figure 4. Detection of RVF virus RNA in Vero cell line cultures successfully inoculated putative RVF virus in mosquito sample head homogenates and abdominal blood meals

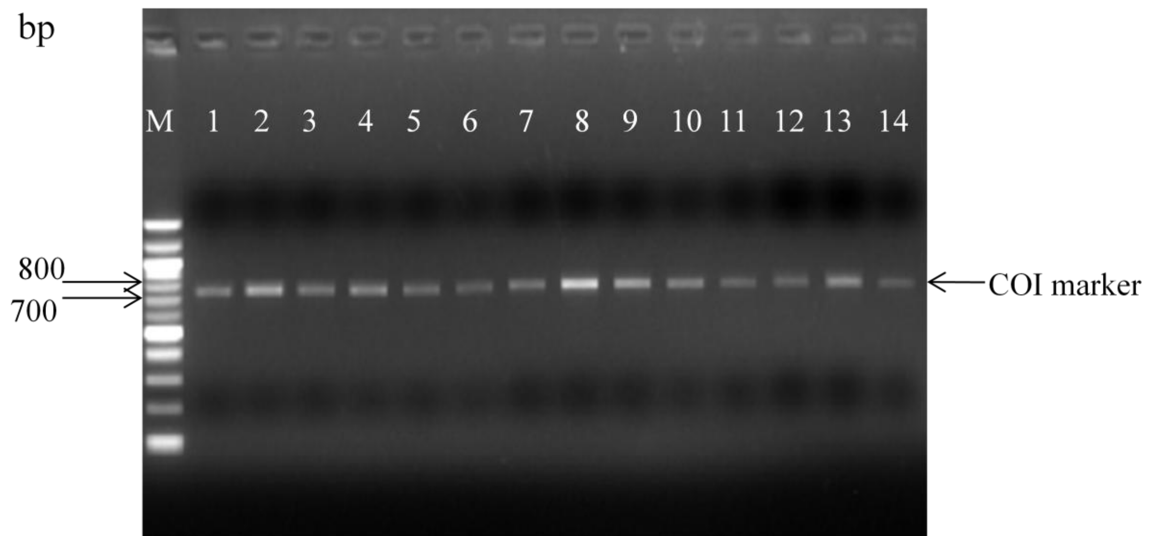
4.2. Specific vertebrate blood meal source(s) in mosquito species

4.2.1. Sequence analysis

Among the 216 individual mosquito blood meal homogenate putative sources of 145 (67.13%) blood meals were successfully determined and traced to specific and relevant vertebrate host. Others (11.03%) had matches in lower eukaryotes in the BOLD database. The rest could not be determined due to incomplete COI fragment or lack of COI/ cyt b amplification. Domesticated animals predominated the variety of putative vertebrate blood meal sources as revealed by COI (Fig 5) and cyt b DNA sequences. The sequences exhibited high sequence similarities (92-100%) to their homologous in the BOLD database, indicative of a positive match.

4.2.2. Blood meal identification from mosquito sampled in Baringo district

Summary of putative sources of blood meals in the mosquitoes sampled in Baringo district, as determined by GenBank/BOLD, are presented in Table 1.The bulk of blood meals in *Ma. uniformis* were Sheep. Additionally, *Ma. uniformis* had blood from most of the vertebrates host evaluated while *Ma. africana* had the least. Humans were among the least preferred hosts. However source(s) of blood meal in one and seven of the *Hodgesia spp* and *Ma. uniformis* could not be determined.

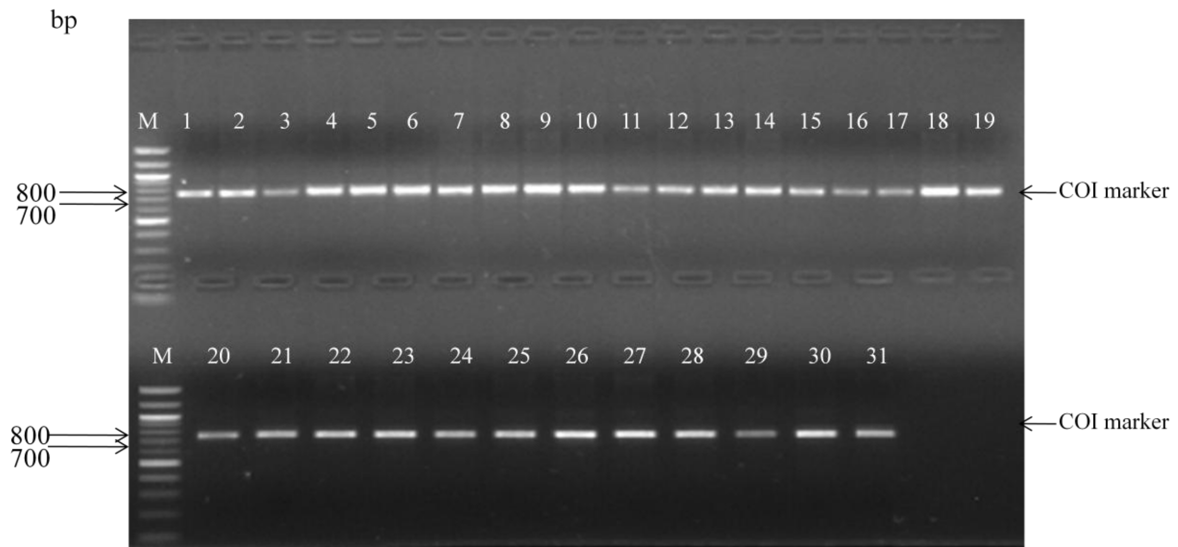


Abdominal blood meal homogenates from mosquito sampled in Baringo district. A fragment of cytochrome c oxidase was amplified via PCR and resolved on 2% agarose gel and visualized through ethidium bromide staining. This gel photo represents PCR products from 14 *Ma uniformis* abdominal blood meal homogenates. M- 100bp plus DNA ladder

Figure 5. Cytochrome c oxidase I DNA fragment amplification from abdominal blood meal homogenates of *Ma. uniformis* sampled in Baringo district in the 2006/2007 RVF outbreak

4.2.3. Blood meal Identification from mosquito sampled in Garissa district

Putative sources of blood meals in the mosquitoes sampled in Garissa district during 2006/2007 RVF outbreak as determined by matches/hits in GenBank/BOLD databases are presented in Table 2. Cytochrome c oxidase I markers were successfully amplified (Fig. 6) in blood meal homogenates from 60 *Ae. ochraceous*, 37 *Ae. mcintoshi* and 4 *Ae. sudanensis* 2 *Cx. poicilipes* and 1 *An. caustani* mosquito species (Table 1). *In silico* analyses of sequences from the amplicons of these sequences revealed that putative sources of blood meals in the mosquitoes were diverse, goats and donkey predominated in *Ae. ochraceous* and *Ae. mcintoshi* respectively. These two species also predominated in variety of blood meals among the mosquito sampled, though source(s) of blood meals in a significant proportion of them could not be determined.

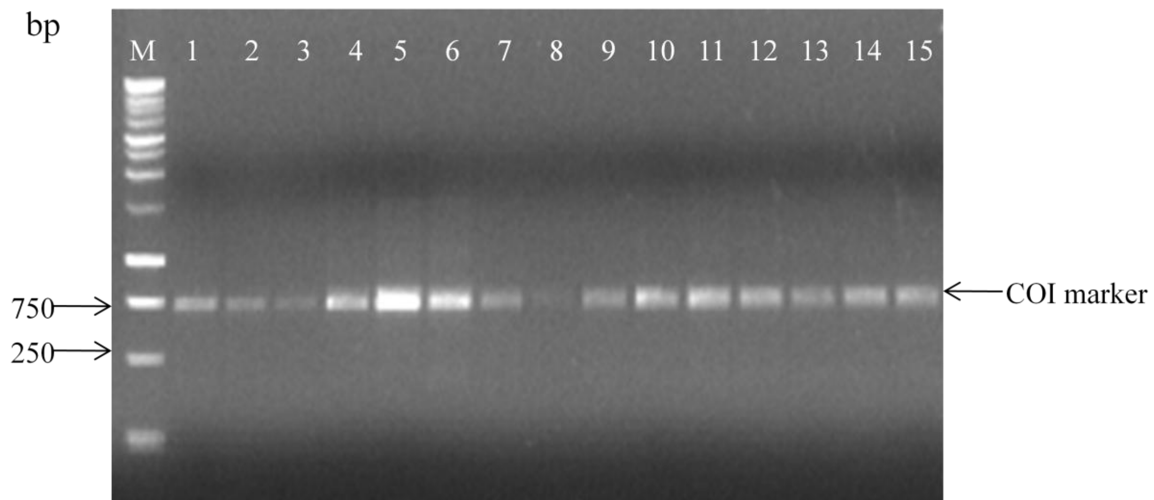


Abdominal blood meal homogenates of selected mosquitoes sampled in Garissa district. A fragment of cytochrome c oxidase was amplified via PCR and resolved on 2% agarose gel and visualized through ethidium bromide staining. This gel photo represents PCR products from 31 *Ae. ochraceous* abdominal blood meal homogenates. M- 100bp plus DNA ladder (Fermentas)

Figure 6 Cytochrome c oxidase I DNA fragment amplification from abdominal blood meal homogenates of *Ae.ochraceous* sampled in Garissa district in the 2006/2007 RVF outbreak.

4.2.4. Blood meal Identification from mosquito sampled in Kilifi district

Table 1 presents a summary of putative vertebrate host of mosquitoes sampled in Kilifi district during the 2006/2007 outbreak. Cytochrome c oxidase I sequences were successfully amplified (Fig. 7) obtained from 15 blood meal homogenates. Sequencing and searches of the COI sequence against GenBank and BOLD databases revealed that humans were the most predominant putative source blood meals for the mosquitoes in this region, with cats being the least. However source(s) of blood meals of an Ae. *pembaensis, Cx. pipiens, Cx. poicilipes, Cx. univittatus* and two *Cx. bitaeniorhynchus* could not be determined.



Abdominal blood meal COI amplification of mosquitoes sampled in Kilifi district. A fragment of cytochrome c oxidase was amplified via PCR and resolved on 2% agarose gel and visualized through ethidium bromide staining. This gel photo represents PCR products of the 15 samples that succefully purified and sequenced. The lanes 1-2 *An. caustani*; 3-8 *An. gambiae* 9-11 *An. squamosus* 12 *Cx poicilipes* and 13-15 *Ae. mcintoshi*. M-1kb plus DNA ladder (Fermentas)

Figure 7. Cytochrome c oxidase I DNA fragment amplification from abdominal blood meal homogenates of selected engorged mosquitoes sampled in Kilifi district in the 2006/2007 RVF outbreak

TABLE 1

Summary of engorged female mosquito screening data and Rift Valley fever virus (RVFV) reverse transcription-polymerase chain reaction (RT-PCR) results from three districts in Kenya

				Blood Meal Analysis				Mosquito infection/dissemination		
Study Area	Mosquitoes Species	Vertebrate Host	n	CPE	pi-hd	RT-PCR		CPE	pi-hd	RT-PCR
Garissa	*Aedes ochraceous*	Goat (*Capra hircus*)	25	-	7			-	7	
		Goat (*Capra hircus*)	1	+	5			+	7	+
		Donkey (*Equus asinus*)	6	-	7			-	7	
		Cow (*Bos taurus*)	13	-	7			-	7	
		Sheep (*Ovis aries*)	9	-	7			-	7	
		Sheep (*Ovis aries*)	3	3+	7	3+		3+	7	3+
		Bird (*Milvago chimachima*)	1	-	7			-	7	
		Human (*Homo sapiens*)	1	+	5	+		-	7	
		Human (*Homo sapiens*)	2	-	7			-	7	
		¤	48	-	7			-	7	
	Aedes mcintoshi	Donkey (*Equus asinus*)	14	-	7			-	7	
		Donkey (*Equus asinus*)	1	+	5	+		+	7	+
		Sheep (*Ovis aries*)	7	-	7			-	7	
		Goat (*Capra hircus*)	9	-	7			-	7	
		Cow (*Bos taurus*)	6	-	7			-	7	
		¤	8	-	7			-	7	
	Aedes Sudanensis	Cow (*Bos taurus*)	4	-	7			-	7	
		¤	1							
	Culex poicilipes	Donkey (*Equus asinus*)	1	-	7			-	7	
		Goat (*Capra hircus*)	1	-	7			-	7	
	Anopheles caustani	Cow (*Bos taurus*)	1	-	7			-	7	
Baringo	*Mansonia uniformis*	Sheep (*Ovis aries*)	5	-	7			-	7	
		Sheep (*Ovis aries*)	4	4+	<7	4+		4+	7	4+
		Cephalophus monticola	2	-	7			-	7	
		Rat (*Mus musculus*)	1	-	7			-	7	
		Cow (*Bos taurus*)	1	-	7			-	7	
		Goat (*Capra hircus*)	1	+	5	+		-	6	
		Frog (*Colostethus sp*)	1	-	7			-	7	
		Frog (*Anura sp.*)	2	-	7			-	7	
		¤	1	+	5	+		+	7	+
		¤	6	-	7			-	7	

CPE = cytopathic effect n = number of vertebrate hosts ¤ = vertebrate host not determined, pi-hd = post inoculation harvest day

TABLE 1

Summary of engorged female mosquito screening data and Rift Valley fever virus (RVFV) reverse transcription-polymerase chain reaction (RT-PCR) results from three districts in Kenya

				Blood Meal Analysis				Mosquito infection/dissemination		
Study Area	Mosquitoes Species	Vertebrate Host	n	CPE	pi-hd	RT-PCR		CPE	pi-hd	RT-PCR
	Hodgesia spp	Sheep *(Ovis aries)*	1	+	5	+		-	7	
		Cow *(Bos taurus)*	1	-	7			-	7	
		Human (*Homo sapiens)*	1	-	7			-	7	
		Goat (*Capra hircus)*	1	-	7			-	7	
		Rat (*Mus musculus)*	1	-	7			-	7	
		Frog (*Anura sp.)*	1	-	7			-	7	
		¤	1	-	7			-	7	
	Mansonia africana	Sheep *(Ovis aries)*	2	-	7			-	7	
Kilifi	*Aedes pembaensis*	¤	1	-	7			-	7	
	Anopheles caustani	Human (Homo sapiens)	2	-	7			-	7	
	Anopheles gambiae	Cat (*Felis catus)*	1	-	7			-	7	
		Human (*Homo sapiens)*	5	-	7			-	7	
	Anopheles squamosus	Human (*Homo sapiens)*	3	-	7					
	Culex bitaeniorhynchus	¤	2	-	7			-	7	
	Culex. pipiens	¤	1	-	7			-	7	
	Culex poicilipes	¤	1							
	Culex poicilipes	Goat (Capra hircus)	1	-	7			-	7	
	Aedes mcintoshi	Goat (Capra hircus)	3	-	7			-	7	
	Culex univittatus	¤	1	-	7			-	7	
	Total		**216**							

CPE = cytopathic effect n = number of vertebrate hosts ¤ = vertebrate host not determined, pi-hd = post inoculation harvest day

DISCUSSION

In this study, RVFV virus was successfully screened in wild populations of single female mosquitoes (head tissues and blood meals). From a total of 33 engorged mosquitoes in Baringo district, *Ma. uniformis* was putatively revealed in this study as an important vector in RVFV transmission in Baringo district, in agreement with a previous report following RVF outbreaks (Sang *et al.,* 2010). Similar findings have been reported during 1997/1998 RVF outbreak in Mauritania and Senegal (Diallo *et al.,* 2005), Tanzania (Ogoma *et al.,* 2010) and much earlier in Kenya (Linthicum *et al.,* 1985). In a separate study that screened unfed mosquito pools sampled during the outbreak, *Ma. uniformis* pools were found to have higher infection rate (0.89) compared to *Ma. africana* (0.71) and *Cx. quinquefasciatus* (0.33) (Sang et al., 2010). Head tissues of five *Ma. uniformis* were positive for RVF implying a disseminated infection thus the four vectors were transmitting the virus. Additionally, one *Ma. uniformis* was positive for RVF in the blood meal from goat but not head tissues, this indicate that the mosquito had acquired the virus from goat and had not started transmitting the virus. In Garissa district, a total of 109 engorged *Ae. ochraceous* mosquitoes were screened for RVFV, four were positive for RVF virus. This translates to percentage prevalence of 3%. This also conforms to present data which puts the prevalence rate to 2-8% (Kokernot *et al.,* 1957; Turell *et al.,* 2008). This finding reveals the significance of *Ae. ochraceous* in RVF transmission in Garissa since the four had disseminated infection. One *Ae. ochraceous* had acquired RVF positive blood meals from human but the virus was not detected in the head tissues. In the same district, one *Aedes mcintoshi* which is a well known reservoir/maintenance vector (Davies and Martin, 2006) yielded one RVFV positive from a total of 45 that were screened. This translates to a percentage prevalence of 2.2%. This single case of *Ae. mcintoshi* was a disseminated infection and thus this mosquito was transmitting RVF during the outbreak. According to Linthicum *et al.,* 1985 two in a hundred mosquitoes will be infected with RVF during RVF outbreak. The percentage prevalence and female mosquito species that harbor RVFV support the epidemiological importance of *Aedes* family as competent vectors for RVFV (Huang, 1987). Understanding the number of single mosquito infected with RVF gives a better clue of the numbers of infected species per population as opposed to assaying mosquito pools which does not give a clear picture. However, screening for virus in single mosquitoes and estimating percentage prevalence alone are not always sufficient to determine vector capability. Abundance, longevity, distribution and feeding behavior are all important facets of what constitutes a good vector, as

are inherent capabilities such as threshold susceptibility to infective virus. The result presented on mosquito infections as well as high abundance of *Mansonia* mosquito in Baringo and *Aedes* in Garissa during the outbreak gives a clue the two genera played an important role in the epidemic/epizootic transmission of RVFV during this outbreak, and may have also contributed to the transmission of this virus during the 1997/1998 outbreak. In West Africa, *Ae. ochraceus* has been established as vector of RVFV along with *Ae. vexans arabiensis* and *Ae. dalzie* (Fontenille *et al.,* 1998; Zeller *et al.,* 1997). *Aedes mcintoshi* is members of the *Neomelaniconion* and is documented as reservoir and vector of RVFV in Kenya, in a study that detected RVFV in *Ae. mcintoshi* reared from field-collected larvae (Linthicum *et al.,* 1985). According to findings of this study, more focus need to be concentrated in establishing the ability of *Ma. uniformis* and *Ae. ochraceus* to transovarially transmit RVFV, investigations on the role of these two species in the maintenance of the virus between epidemics in Garissa are critically important.

Specific vertebrate host identification implicated sheep to a greater extent and goat as putative vertebrate hosts/reservoirs/amplifiers of RVFV in Baringo district. Four of the RVF positive blood meals from Baringo were from sheep and one from goat. This conforms to the present knowledge that the major casualties RVF are sheep and the first isolated case reported was in a sheep disease outbreak along the shores of Lake Baringo (Daubney *et al.,* 1931). It has also been extensively documented that the gravid and young one of sheep and goats die extensively during an outbreak (WHO, 2007). This present study also incriminates Donkey, goat, human and sheep as the putative hosts/reservoirs/amplifiers of RVF in Garissa district. It is important to note that donkeys are asymptomatic hosts of RVF. Studies need to be conducted to determine if this beast of burden is a reservoir host of RVFV. Rift Valley fever being an emerging disease, donkey could be one of the most aggressive animals migrating RVFV. The routes of dispersal of RVFV detected so far seem to be in parallel with the great migration routes of camels/donkeys. Therefore, there is some good evidence that viremic, but non-symptomatic infected camels and donkeys transported the virus to Egypt and possibly also to the Arabian Peninsula (Hoogstral *et al.,* 1979) after which the outbreak will then be facilitated by presence of competent vectors and amplifying hosts like human, goat and sheep. The RVF positive human blood meal in *Ae. ochraceous* sampled in Garissa district confirms that indeed RVF can be transmitted to human through mosquito bite and not contact with RVF infected tissues and blood. Other host sequences identified such as frogs and rats could also be important host in RVF transmission and therefore further studies could be directed towards understanding their roles as hosts.

This study has applied mitochondrial molecular markers as a method to accurately identify blood meals of mosquitoes and to understand mosquito–host interactions as well as RVF virus transmission. For identification purposes, both COI and Cytb genes were chosen. Amplification of COI gene was achieved by primers described by Ivanova *et al.,* (2007) designed to amplify a locus for mammals, birds and reptiles. Cytochrome c oxidase I is a 648-bp region at the 5' end of this gene and has been selected as the molecular target for DNA Barcoding (Ratnasingham and Hebert 2007; Wugh 2007). The study to a lesser extent has exploited amplification of cytochrome b gene using vertebrate universal primers (Kocher *et al.,* 1989).The generic primers were chosen since the target group would compose of mammals that are to a greater extent the casualty of RVF. Amplification with these primers worked well for most samples. The host target was broad considering that mosquitoes could feed on any vertebrate, universal vertebrate primers were thus used in the study. Therefore, it was important that these specific conserved primers first be validated on a known host of vertebrate before use to avoid misinterpretation of results (Kent and Norris 2005). PCR optimization conditions were necessary to ensure that the amplified DNA was from blood meal and not from mosquito or lower organisms. BLAST searches of the COI and sequences of the entire samples for the study and cytochrome b were done against the NCBI database as well as BOLD database for COI. For sequence identity to be revealed, the query sequence must have a minimum of 300bp from the barcode region of COI (Ratnasingham and Hebert 2007). Most sequences used to query the BOLD identification engine had lengths of 658bp. In many cases, BLAST searches of COI sequences also gave results from BOLD database.

CHAPTER FIVE

CONCLUSION

This study incriminates *Ma. uniformis* in RVF virus transmission in Baringo district and *Ae. ochraceous* and *Ae mcintoshi* in RVF virus transmission in Garissa district. Therefore, distribution and abundance of *Ma. uniformis, Ae. ochraceous and Ae. mcintoshi* that serves as vectors of RVF and suspected reservoirs/amplifying/maintenance hosts such donkeys, goats, sheep and human that participate in virus transmission needs to be mapped out in Kenya and other epidemic prone countries in the region if relevant and effective control measure are to be formulated aimed at combating the spread RVFV. Monitoring of the El Nino/ Southern Oscillation (ENSO) events will also help in predicting above normal rainfall in East Africa and may help relevant authorities to more effectively prioritize and optimize their resources by targeting control efforts at appropriate vector habitats ahead of outbreaks in an effort to minimize livestock and human exposure in the future.

RECOMMENDATIONS

Extensive studies need to be carried out to ascertain the ability of *Ma. uniformis* and *Ae. ochraceous* to transovarially transmit RVFV, investigations on the role of this species in the maintenance of the virus between epidemics in Baringo and Garissa are critically important. The significance of sheep as a host needs to be critically determined; more studies need to be undertaken to understand the role of sheep and RVF virus transmission. Additionally, donkey's role RVF transmission also needs to be a subject of focus as they could be reservoirs of RVFV, their migration with the pastoralist community could lead to emergence of RVFV in other RVFV free zones.

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APPENDICES

Appendix 1: DNeasy extraction kit contains the following reagents:

Kit Contents	250 preps
DNeasy mini spin columns (colorless) in 2 mL collection tubes	250
Collection tubes (2 mL)	500
Buffer ATL	50 mL
Buffer AL	54 mL
Buffer AW1 (concentrate)	95 mL
Buffer AW2 (concentrate)	66 mL
Buffer AE	2 @ 60mL
Proteinase K	6 mL

Appendix 2: QuickClean 5M Gel extraction kit contains:

Kit Contents	Kit Contents
Binding Solution II	2 @110 mL
Wash Solution	55 mL
Elution Buffer	25 mL
QuickClean Columns	250
2 mL collection tubes	250

Appendix 3

Buffers

a) 1X TAE buffer

Prepare 50X: 242 g of Tris base

- 57.1 mL glacial acetic acid
- 100mL 0.5M EDTA (pH 8.0)
- Dilute to 1X TAE working solution with dH_2O

b) 1X TBE buffer

Prepare 5X: 54g Tris base

- 27.5g boric acid
- 20mL 0.5M EDTA (pH 8.0)
- Dilute to 1X TBE working solution with dH_2O

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GU135856.1	Rift Valley fever virus strain FI-2 segment M glycoprotein Gn gene, pa	876	876	99%	0.0	100%	
GQ443256.1	Rift Valley fever virus strain 2034-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443253.1	Rift Valley fever virus strain 1730-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443250.1	Rift Valley fever virus strain 1586-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443249.1	Rift Valley fever virus strain 1585-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443248.1	Rift Valley fever virus strain 1464-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443247.1	Rift Valley fever virus strain 0897-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443246.1	Rift Valley fever virus strain 0895-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443245.1	Rift Valley fever virus strain 0892-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443244.1	Rift Valley fever virus strain 0889-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443241.1	Rift Valley fever virus strain 0863-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443240.1	Rift Valley fever virus strain 0855-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443239.1	Rift Valley fever virus strain 0854-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443238.1	Rift Valley fever virus strain 0853-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443237.1	Rift Valley fever virus strain 0852-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443236.1	Rift Valley fever virus strain 0851-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443235.1	Rift Valley fever virus strain 0850-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443234.1	Rift Valley fever virus strain 0849-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443233.1	Rift Valley fever virus strain 0848-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443232.1	Rift Valley fever virus strain 0847-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443231.1	Rift Valley fever virus strain 0846-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443230.1	Rift Valley fever virus strain 0845-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443229.1	Rift Valley fever virus strain 0776-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443228.1	Rift Valley fever virus strain 0693-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443224.1	Rift Valley fever virus strain 0619-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443223.1	Rift Valley fever virus strain 0448-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443222.1	Rift Valley fever virus strain 0428-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
GQ443221.1	Rift Valley fever virus strain 0427-08 segment M glycoprotein G2 gen	876	876	99%	0.0	100%	
EU574056.1	Rift Valley fever virus strain 2007000080 segment M, complete seque	876	876	99%	0.0	100%	
EU574054.1	Rift Valley fever virus strain 2007000222 segment M, complete seque	876	876	99%	0.0	100%	
EU574053.1	Rift Valley fever virus strain 2007000223 segment M, complete seque	876	876	99%	0.0	100%	
EU574052.1	Rift Valley fever virus strain 2007000224 segment M, complete seque	876	876	99%	0.0	100%	
EU574051.1	Rift Valley fever virus strain 2007000225 segment M, complete seque	876	876	99%	0.0	100%	
EU574050.1	Rift Valley fever virus strain 2007000473 segment M, complete seque	876	876	99%	0.0	100%	
EU574048.1	Rift Valley fever virus strain 2007000611 segment M, complete seque	876	876	99%	0.0	100%	
EU574047.1	Rift Valley fever virus strain 2007001107 segment M, complete seque	876	876	99%	0.0	100%	
EU574046.1	Rift Valley fever virus strain 2007001292 segment M, complete seque	876	876	99%	0.0	100%	
EU574045.1	Rift Valley fever virus strain 2007001443 segment M, complete seque	876	876	99%	0.0	100%	
EU574043.1	Rift Valley fever virus strain 2007001602 segment M, complete seque	876	876	99%	0.0	100%	
EU574041.1	Rift Valley fever virus strain 2007001809 segment M, complete seque	876	876	99%	0.0	100%	
EU574039.1	Rift Valley fever virus strain 2007002060 segment M, complete seque	876	876	99%	0.0	100%	
EU574038.1	Rift Valley fever virus strain 2007002444 segment M, complete seque	876	876	99%	0.0	100%	
EU574037.1	Rift Valley fever virus strain 2007002445 segment M, complete seque	876	876	99%	0.0	100%	
EU574036.1	Rift Valley fever virus strain 2007002476 segment M, complete seque	876	876	99%	0.0	100%	
EU574035.1	Rift Valley fever virus strain 2007002482 segment M, complete seque	876	876	99%	0.0	100%	
EU574034.1	Rift Valley fever virus strain 2007003081 segment M, complete seque	876	876	99%	0.0	100%	
EU574033.1	Rift Valley fever virus strain 2007003644 segment M, complete seque	876	876	99%	0.0	100%	
EU574032.1	Rift Valley fever virus strain 2007004193 segment M, complete seque	876	876	99%	0.0	100%	
EF467177.1	Rift Valley fever virus strain KEN07-KLF112 segment M, complete seq	876	876	99%	0.0	100%	
EF160116.1	Rift Valley fever virus strain H1 MAU 03 G2 protein (G2) gene, partial	876	876	99%	0.0	100%	

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU229279.1	Capra hircus isolate B-LK31 mitochondrion, complete genome	555	555	98%	8e-155	97%
GU229278.1	Capra hircus isolate A-MG25 mitochondrion, complete genome	555	555	98%	8e-155	97%
GU068049.1	Capra hircus breed Inner Mongolia White Cashmere mitochondrion, co	555	555	98%	8e-155	97%
GQ141263.1	Capra hircus isolate YCW1 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
GQ141261.1	Capra hircus isolate HTM cytochrome b (cytb) gene, complete cds; n	555	555	98%	8e-155	97%
FJ556564.1	Capra hircus isolate Goat01 cytochrome b (cytb) gene, partial cds; n	555	555	98%	8e-155	97%
EU130776.1	Capra hircus isolate HM18 cytochrome b (CYTB) gene, complete cds;	555	555	98%	8e-155	97%
EU130775.1	Capra hircus isolate HM13 cytochrome b (CYTB) gene, complete cds;	555	555	98%	8e-155	97%
EU350133.1	Capra hircus isolate GZB25 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350132.1	Capra hircus isolate GZB24 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350131.1	Capra hircus isolate GZB23 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350130.1	Capra hircus isolate GZB22 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350129.1	Capra hircus isolate GZB21 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350128.1	Capra hircus isolate GZB20 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350127.1	Capra hircus isolate GZB17 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350126.1	Capra hircus isolate GZB16 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350125.1	Capra hircus isolate GZB15 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350124.1	Capra hircus isolate GZB14 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350123.1	Capra hircus isolate GZB12 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350122.1	Capra hircus isolate GZB5 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350121.1	Capra hircus isolate GZB4 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350120.1	Capra hircus isolate GZB3 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
EU350118.1	Capra hircus isolate GZB1 cytochrome b (cytb) gene, complete cds;	555	555	98%	8e-155	97%
DQ514544.1	Capra hircus haplotype ChGr642 cytochrome b gene, complete cds; r	555	555	98%	8e-155	97%
DQ093614.1	Capra hircus breed black Bengal cytochrome b gene, complete cds; n	555	555	98%	8e-155	97%
DQ073048.1	Capra hircus breed Black Bengal cytochrome b gene, complete cds; n	555	555	98%	8e-155	97%
DQ089479.1	Capra hircus voucher YP ylb95 cytochrome b gene, complete cds; mi	555	555	98%	8e-155	97%
DQ089477.1	Capra hircus voucher YP 3221 cytochrome b gene, complete cds; mit	555	555	98%	8e-155	97%
AB044307.1	Capra hircus mitochondrial cytb gene for cytochrome b, complete cds	555	555	98%	8e-155	97%
AB004073.1	Capra hircus mitochondrial DNA for cytochrome b, complete cds	555	555	98%	8e-155	97%
AB004071.1	Capra hircus mitochondrial DNA for cytochrome b, complete cds	555	555	98%	8e-155	97%
AB004069.1	Capra aegagrus mitochondrial DNA for cytochrome b, complete cds	555	555	98%	8e-155	97%
D84204.1	Capra aegagrus mitochondrial DNA for cytochrome b, complete cds	555	555	98%	8e-155	97%
FM205715.1	Capra hircus mitochondrial partial cytb gene for cytochrome b, breed	553	553	97%	3e-154	97%
GU295658.1	Capra hircus isolate V07-146 mitochondrion, complete genome	549	549	98%	4e-153	96%
GQ141265.1	Capra hircus isolate YCW3 cytochrome b (cytb) gene, complete cds;	549	549	98%	4e-153	96%
FJ556557.1	Capra hircus isolate Goat03 cytochrome b (cytb) gene, partial cds; n	549	549	98%	4e-153	96%
EU130779.1	Capra hircus isolate HM5 cytochrome b (CYTB) gene, complete cds;	549	549	98%	4e-153	96%
EU130778.1	Capra hircus isolate HM3 cytochrome b (CYTB) gene, complete cds;	549	549	98%	4e-153	96%
EU130777.1	Capra hircus isolate HM2 cytochrome b (CYTB) gene, complete cds;	549	549	98%	4e-153	96%
EU130774.1	Capra hircus isolate G45 cytochrome b (CYTB) gene, complete cds; r	549	549	98%	4e-153	96%
EU350119.1	Capra hircus isolate GZB2 cytochrome b (cytb) gene, complete cds;	549	549	98%	4e-153	96%
DQ514546.1	Capra hircus haplotype ChMy57 cytochrome b gene, complete cds; m	549	549	98%	4e-153	96%
DQ514545.1	Capra hircus haplotype ChMy50 cytochrome b gene, complete cds; m	549	549	98%	4e-153	96%
AF533441.1	Capra hircus mitochondrion, complete genome	549	549	98%	4e-153	96%
DQ089476.1	Capra hircus voucher YP 2941 cytochrome b gene, complete cds; mit	549	549	98%	4e-153	96%
DQ089475.1	Capra hircus voucher YP 2914 cytochrome b gene, complete cds; mit	549	549	98%	4e-153	96%
AB044308.1	Capra hircus mitochondrial cytb gene for cytochrome b, complete cds	549	549	98%	4e-153	96%
AB004075.1	Capra hircus mitochondrial DNA for cytochrome b, complete cds	549	549	98%	4e-153	96%
AB004074.1	Capra hircus mitochondrial DNA for cytochrome b, complete cds	549	549	98%	4e-153	96%
AB004072.1	Capra hircus mitochondrial DNA for cytochrome b, complete cds	549	549	98%	4e-153	96%
AB004070.1	Capra hircus mitochondrial DNA for cytochrome b, complete cds	549	549	98%	4e-153	96%
GQ141264.1	Capra hircus isolate YCW2 cytochrome b (cytb) gene, complete cds;	544	544	98%	2e-151	96%

BOLDSYSTEMS v2.5 | Management & Analysis

Specimen Identification Request

Search Request:

Type : COI SPECIES DATABASE

Search Result:

Identification Summary :

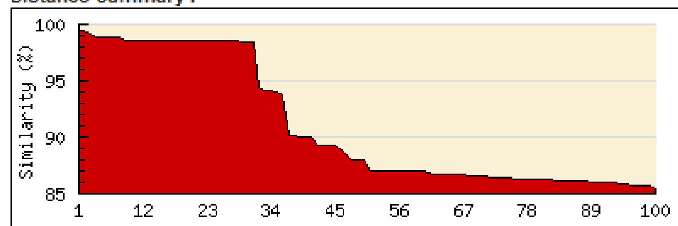
Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Mammalia	100
order	Artiodactyla	100
family	Bovidae	100
genus	Ovis	100
species	Ovis aries	99.5

A species level match could not be made, the queried specimen is likely to be one of the following :

-Ovis aries
-Ovis musimon

Tree Based Identification

Distance Summary :



Similarity scores of the top 100 matches

TOP 20 Matches :

Display option: default

Phylum	Class	Order	Family	Genus	Species	Specimen Similarity (%)
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.47
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.4
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.16
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.9
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.9
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.9
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*musimon*	98.9
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.84
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	98.62

BOLDSYSTEMS v2.5 | Management & Analysis

Specimen Identification Request

Search Request:

Type : COI SPECIES DATABASE

Search Result:

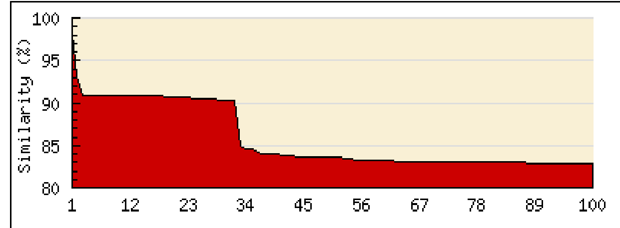
Identification Summary :

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Mammalia	100
order	Perissodactyla	100
family	Equidae	100
genus	Equus	98.4

A species level match could not be made, The nearest neighbor is Equus asinus.

Tree Based Identification

Distance Summary :



Similarity scores of the top 100 matches

TOP 20 Matches :

Display option: default

Phylum	Class	Order	Family	Genus	Species	Specimen Similarity (%)
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*asinus*	98.38
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*grevyi*	93.14
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.95
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.9
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.86
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.79
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.71
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*caballus*	90.71

BOLDSYSTEMS v2.5 | Management & Analysis

Specimen Identification Request

Search Request:

Type : COI SPECIES DATABASE

Search Result:

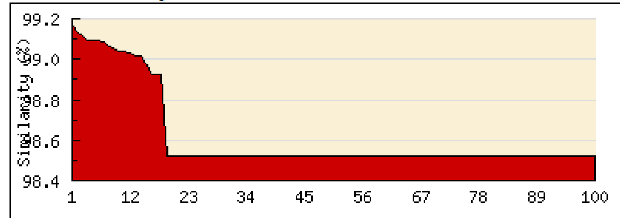
Identification Summary :

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Mammalia	100
order	Artiodactyla	100
family	Bovidae	100
genus	Bos	100
species	Bos taurus	99.2

A species level match has been made. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

Tree Based Identification | Species Page

Distance Summary :



Similarity scores of the top 100 matches

TOP 20 Matches :

Display option: default

Phylum	Class	Order	Family	Genus	Species	Specimen Similarity (%)
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.17
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.14
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.12
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.09
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.09
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.09
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.08
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.06
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.06
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.04
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.04
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.03
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.01
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	99.01
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	98.98
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	98.92
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	98.92
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	98.92
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	98.53
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*taurus*	98.53

BOLDSYSTEMS v2.5 | Management & Analysis

Specimen Identification Request

Search Request:

Type : COI SPECIES DATABASE

Search Result:

Identification Summary :

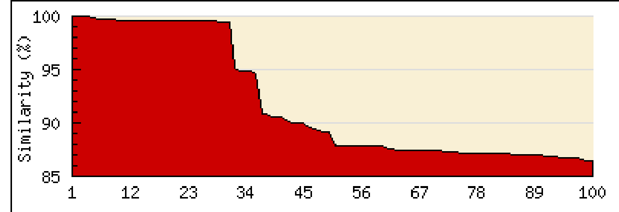
Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Mammalia	100
order	Artiodactyla	100
family	Bovidae	100
genus	Ovis	100
species	Ovis aries	100

A species level match could not be made, the queried specimen is likely to be one of the following :

-Ovis aries
-Ovis musimon

Tree Based Identification

Distance Summary :



Similarity scores of the top 100 matches

TOP 20 Matches :

Display option: default

Phylum	Class	Order	Family	Genus	Species	Specimen Similarity (%)
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	100
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	100
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	100
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	100
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.84
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.69
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.69
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.69
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*musimon*	99.69
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*aries*	99.54