Molecular Characterization of Rift Valley Fever Virus during the East African Outbreak, 2006 – 2007

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This work is dedicated to all those who lost their lives, relatives and friends, material and financial wealth from Rift Valley fever outbreaks in Kenya, Tanzania and Somalia (2006-2007).

To my parents Charles and Naomi Nderitu for their distinguished and unwavering support, this was a great motivation for me to complete this work.

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LIST OF ABBREVIATIONS

ABI	Applied Biosystems
AW 1	Wash buffer 1
AW 2	Wash buffer 2
AVL	Lysis buffer
AVE	Elution buffer
CDC	Centers for Diseases Control and Prevention
cDNA	Complementary DNA
CFSPH	Center for Food Security and Public Health
CIDRAP	Center for Infectious Disease Research and Policy
CO_2	Carbon Dioxide gas
CPE	Cytopathic Effect
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
ddNTPs	Di-deoxyribonucleotide triphosphates
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunoassay
FBS	Fetal Bovine Serum
GDD	Global Disease Detection
IEIP	International Emerging Infections Programme
IgG	Immunoglobulin G (Gamma)
IgM	Immunoglobulin M (Mu)

ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
KFSSG	Kenya Food Security Steering Group
MEM	Minimum Essential Media
NCRs	Non-Coding Regions
N	Neutralizing
NCBI	National Center for Biotechnology Information
NEP	North Eastern Province
nt	Nucleotides
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
POP	Performance Optimized Polymer
Pmoles	Pico moles
QRT-PCR	Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic acid
RNP	RNase P
RPM	Revolutions per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RVF	Rift Valley Fever
RVFV	Rift Valley Fever Virus

SYBR SyberGreen

TAE Tris, acetate and EDTA buffer

Taq *Thermus aquaticus*

µl microlitre

WHO World Health Organization

ABSTRACT

Rift Valley Fever (RVF) is a disease caused by Rift Valley Fever Virus (RVFV). It belongs to the phlebovirus family. It causes a febrile illness in livestock and humans. RVF outbreaks follow heavy rainfall and flooding. The virus is transmitted to humans through mosquito bites or contact with infected animal products. RVF has periodically caused major outbreaks in Sub-Saharan Africa, and elsewhere in Africa and recently also in the Arabian Peninsula. From December 2006, RVF outbreak occurred sequentially in East Africa (Kenya, Somalia, and Tanzania); starting from Northeastern province (NEP) of Kenya, then in Coast province of Kenya and also in mid and Southern regions of Somalia in early January, 2007. In February 2007, the outbreak was reported in Rift Valley province of Kenya and in Tanga and Dodoma regions of Tanzania. Following these sequential outbreaks, the question was whether the outbreaks were caused by a viral strain that was spreading from region to region or viral strains that were dormant within the regions but were activated by the heavy rains and flooding. To address this question, full genome sequencing of RVF virus isolates from each of the four regions was done and compared with historic strains. The full genome of the 2006-2007 strains displayed minimal changes from the historic strains. The variation in all the segments was highest in the M segment (1.9%), followed by S segment (1.7%), and finally L segment (1.4%). An analysis done by region indicated the highest average divergence to be in the Tanzanian isolates (0.8%).

The Garissa isolates (Northeastern province, Kenya) had the next highest diversity (0.7%). Isolates from Baringo, (Rift Valley province, Kenya) and those from Kilifi and Malindi (Coast province, Kenya) were the least divergent (0.6%). These results indicated that strains were highly conserved in relation to historic outbreaks. However; detailed analysis of nucleotide and predicted amino acid sequences revealed that unique differences occurred between isolates of various regions; this was more evident in the Tanzania and Garissa strains. These results clearly indicate that virus strains from each region were uniquely different, representing de novo activation of dormant RVFV strains in these regions. A single formulation of vaccine could be used to protect the population at risk from different geographical regions and this should be taken into consideration. Continuous surveillance and characterization of the virus should also be carried out as the virus has been shown to be endemic to different regions and could cause outbreaks following heavy rains and flooding.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Historical Background

1.1.1 Discovery of Agents and Vectors of Rift Valley Fever (RVF)

Rift Valley Fever (RVF) is an arthropod borne viral disease that primarily causes epizootics of abortion and high mortality in domestic animals, during which humans become infected. RVF-like disease was first described by Montgomery and Stordy in 1912 – 1913 near Lake Naivasha (Red Gems - http://medias.cnrs.fr/redgems/article). However, it was not until the 1930 – 1931 epizootic near Lake Naivasha that detailed studies on the disease identified Rift Valley fever virus (RVFV) as the etiologic agent (Daubney *et al*., 1931). Apart from being associated with abortions and mortality in livestock, RVFV causes primarily an influenza–like illness in humans with occasional cases of encephalitis, retinitis and hemorrhagic syndrome (Daubney *et al*. 1931).

Initial scientific progress in studying RVF was rapid, quickly established and was based on:

- The pathology of the disease in susceptible domestic, wild and laboratory animals evidenced in lesions of hepatic necrosis of the liver.
- The transmission by a relatively large number of mosquito species

- Serologic evidence indicating the disease occurred in numerous sub–Saharan African countries.

The virus appeared to be maintained in enzootic and epizootic cycles that were poorly understood in the South and East Africa (Meegan and Bailey, 1989). The initial perception that the virus was limited to sub-Saharan Africa has changed and the virus is now known to cause disease in a wide range of ecological zones in Africa (Arthur *et al.*, 1993; Balkhy and Memish 2003; Diggoute and Peters, 1989), and threatens to spread to other continents (Gargan *et al.*, 1988). This was evidenced in 2000 when the virus caused an outbreak of RVF in Saudi Arabia and Yemen (CDC, 2000)

1.1.2 History of RVF epidemics

Until 1977, the history of RVF outbreaks in Africa was limited to sub-Saharan Africa. (Daubney *et al.,* 1931) Between 1950 and 1951, a RVF outbreak that occurred in South Africa killed approximately 100,000 sheep and cattle and resulted in over 20,000 human cases. Still in South Africa in 1953, 1956 and 1959, RVF outbreaks recurred in animal and human populations. (Easterday, 1965) For the first time in 1977-78, a RVF outbreak was reported north of the Sahara in Egypt where up to 200,000 human cases were reported resulting in 600 deaths. This is the most severe outbreak of RVF in humans ever reported. The impact of the Egyptian outbreak on livestock production was devastating because of the abortions and morbidity it caused in sheep, cattle and buffalo. Appearance of RVF in a place that was ecologically different from regions of previous outbreaks, with severe clinical picture, and higher morbidity and mortality rates was a cause for alarm. In 1987 RVF appeared in Mauritania, a country without prior history of RVF activity, resulting in 337 human cases and 49 deaths (Digoutte & Peters 1989; Sall *et al*., 1998; 1999). This was the first outbreak reported in West Africa (Walsh, 1988). In 1993, the disease reappeared in Egypt, where for the first time, ophthalmologic symptoms, an infrequent delayed symptom of the RVFV infection were reported in 41 human patients (Arthur *et al*., 1993; Laughlin *et al.,* 1979b; Meegan, 1979). In 1998, there were severe RVF outbreaks in Kenya, Mauritania and Somali. The virus was first reported outside continental Africa in Madagascar in 1979 (Morvan *et al.,* 1992). In September 2000, an outbreak of RVF was

reported in Saudi Arabia and Yemen, resulting in 882 human cases with 124 deaths in Saudi Arabia and 1,087 cases with 121 deaths in Yemen (CDC, 2000; Balkhy and Memish, 2003).

1.1.3 Social and economic impact of RVF

Outbreaks of RVF are characterized by high attack rates in domestic animals. Depending on the species, up to 30% of adult animals die, however, abortions are the most frequent clinical sign occurring in 80 to 100% of pregnant animals (Daubney *et al.,* 1931; Kaschula, 1957). The direct impact of widespread animal infection (sheep, cattle, goats and camels) is substantial economic losses and reduction in available animal protein among pastoralist communities. Indirectly, RVF outbreaks result in loss of foreign income due to restriction of exportation of animals from the affected countries and the expense of control measures (Meegan and Bailey, 1989)

In humans, a more direct social effect is the disability caused in the nutritionally deficient populations. Although, only a small percentage develop severe disease complications, in an extensive outbreak the total infections may lead to an appreciable number of people suffering from ocular or encephalitic complications or dying from hemorrhagic RVF (Meegan and Bailey, 1989).

1.2 Epidemiology of RVF

1.2.1 Geographic Distribution of RVF

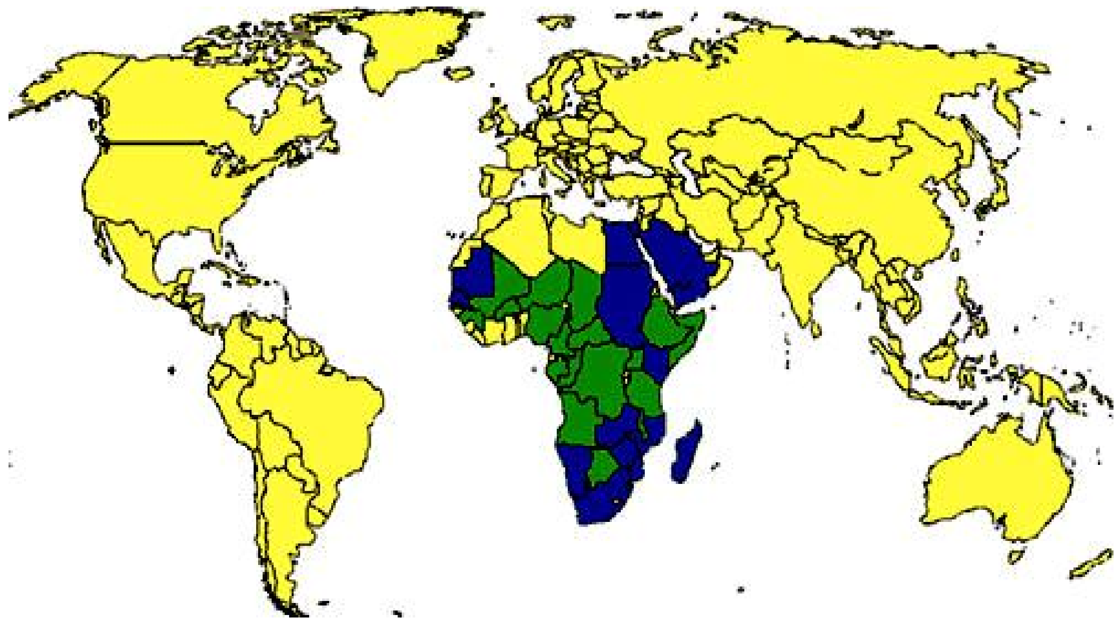


Fig 1: Geographic distribution of past RVF outbreaks

Source: http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/rvfmap (2008).

Blue - Countries with endemic disease and substantial outbreak

Green - Countries known to have some cases, periodic isolation of virus, or serologic evidence of RVF.

Yellow - Countries with no evidence of RVF disease

The distribution of RVF outbreaks has been limited to Sub-Saharan Africa, however in 2000 an outbreak of RVF occurred in Saudi Arabia and Yemen. Figure 1 shows the distribution of outbreaks of RVF with countries in blue being endemic

RVF zones, while those in green having experienced mild disease or evidence of antibodies against RVF being witnessed.

Although epizootics of Rift Valley Fever are most characteristic of sheep and cattle raising areas of east and southern Africa, the virus exists throughout sub Saharan Africa. Epizootics in sheep, goats and cattle along Senegal river basin has been associated with a water control project dam (Archie *et al*., 2007). In addition epizootics in the cattle raising areas of Madagascar have been reported (Morvan, *et al.,* 1992). This shows the ecological diversity of the virus (Peters and Meegan 1981). The distribution of RVF virus antibodies (humans and livestock) with recurrent outbreaks has shown the presence of the virus in most countries of sub-Saharan Africa.

1.2.2 Incidence of RVF

Studies have shown that RVFV infection in a flock of sheep results in 90-100% abortions in pregnant ewes within few days. Mortality in young lambs is up to 100% with 20-60% of older sheep dying (Daubney *et al.,* 1931; Easterday, 1965; Barnad and Botha, 1977; Shimsony and Barzilai, 1983). Outbreaks in cattle, goats and other ruminants can be less severe but similar statistics have been reported in sheep (Daubney *et al.,* 1931; Barnad and Botha., 1977; Shimsony and Barzilai, 1983).

In sub-Saharan Africa, human disease occurs during major epizootics but severe illness and death are rare (Van Velden *et al.,* 1977; Swanepoel *et al., 1979;* McIntosh *et al.,* 1980).

The Egyptian epidemic in 1977 that extended from October to December of the same year represented an intrusion of RVFV into a new area. These areas had no evidence of previous infection (antibodies), in both humans and livestock (Meegan, 1979). However, definitive incidence data were not established (Meegan *et al*., 1981). Official figures indicate an approximate 20,000 human clinical cases with 598 deaths for a case mortality of 3% (El-Akkad, 1978). Other data sources however show a variation in the range of morbidity, from 20,000 to greater than 200,000 (Johnson *et al*., 1978; Abdel-Aziz *et al*., 1980; Meegan, 1981). In the military, a mortality rate of 0.2% was reported by El-Gebaly (1978) and in hospitalized cases a mortality rate of 14% was cited (Madkour, 1978; El-Gebaly, 1978). There were differences observed in the Egyptian outbreak versus those seen earlier elsewhere. The Egyptian outbreak had more human involvement with more severe cases and Meegan (1979), came up with three postulates. Firstly *Culex pipiens* species of the mosquito were ubiquitous, secondly, the virulence of the virus might have changed, possibly through reassortment of the viral genome and thirdly, the human host could be more susceptible to the virus because of local endemic diseases (Hoogstraal *et al*, 1979; Meegan, 1979)

1.2.3 Seasonal Distribution of RVF

Most of the sub-Saharan RVF epizootics appear to follow periods of excessive rainfall. In South Africa, correlation between exceptionally rainy years and RVF outbreaks has been reported (McIntosh and Jupp, 1981). The rainy conditions display a 20 year cyclical pattern that alternates with 10 years of drought with epizootics clustering around the rainy seasons (McIntosh and Jupp, 1981).

In Northern Africa, these viral outbreaks have occurred in ecologically dry areas and appear centered near irrigated areas like the Nile River (Shimsony and Barzilai, 1983; Peters and Meegan, 1981). In Senegal, virus transmission appears to be clearly endemic with fluctuations based on the rainfall (Zeller *et al.,* 1997). RVF seroprevalence reached a peak of around 70% after the 1987 epizootic, dropped to 30.8% in 1988 and then decreased continuously until 1993. This drop in RVF prevalence corresponded to a period of low rainfall. During a period of heavy rainfall, (1993 – 95), RVF activity re-emerged as epizootics among herds of the lower Senegal River basin (Thonnon *et al.,* 1999)

In Eastern Africa, epizootics follow periods of unusually heavy rains (60 to 100 times heavier than normal) and flooding (CFSPH, 2007). The 1997-1998 RVF outbreak in East Africa followed a heavy spell of rainfall (From late October 1997 through January 1998) resulting in the worst flooding since 1961(CDC, 1998). The key factors in the association of rainfall and virus activity appears to be widespread rainfall which results in a value greater than the annual mean, together with its persistence as measured by the frequency and continuous of the rain over a

long period, however, some other factors may be involved due the fact that during some periods of heavy local rains, no epizootics have been observed (Davies *et al*., 1985)

1.2.4 Risk Factors Associated With RVF

During an outbreak of RVF close contact with animals and particularly their body fluids, either directly or via aerosols has been identified as the most significant risk factor. A study carried out in Senegal also showed no statistical association between seropositivity in compound members and reported febrile illness in the same group. Even though this population had previously demonstrated that Crimean Congo Hemorrhagic Fever (CCHF) virus infection was associated with tick bites, no relationship between reported tick exposure and RVFV was found. Potential risk factors not associated with past RVFV infection involved outdoor activities, exposure to vectors or infected hosts (sleeping outdoors or near animals), contact with blood of healthy humans (births, circumcision, tattooing), or exposure to animal blood (branding, slaughter, cooking meat). Drinking healthy or sick animal milk did not increase risk. However, nursing sick people, assisting animals during abortions/births and treating sick animals was highly predictive of past RVFV infection (Wilson *et al.,* 1994). After the 1997-1998 outbreak in northeastern Kenya, risk factors that were identified to be associated with RVF disease included an array of activities connected to animal exposures. The most

significant was contact with sheep (particularly sheep blood or other body fluids), being male, and housing animals indoors with household members. Children who were less than 15 years of age were significantly less likely to have had RVFV infection (Woods *et al.*, 2002), most likely because they were not involved in occupational risk factors like herding and slaughtering.

1.3 The Virus

1.3.1 Etiology of RVFV

The RVFV belongs to the phlebovirus genus and it is an arthropod borne virus that has been shown to infect humans through mosquito bites or contact with infected animal products.

Heavy rainfall creates pools of standing water or "dambos" that create a habitat for breeding of *Aedes sp* mosquito vectors (Linthicum *et al.,* 1999). Although the life cycle of RVFV has been linked to *Aedes sp* mosquitoes, other mosquito species can be infected, act as mechanical vectors and transmit the virus (Gad *et al.,* 1987; Gargan *et al*., 1988; Jupp & Cornel 1988). Survival of the virus during interepizootics is believed to depend on transovarial transmission of virus in flood water Aedes mosquitoes (Laughlin *et al.*, 1979b). No animal reservoirs are known to harbour the virus during these periods. However, a recent study demonstrated high prevalence of RVFV antibodies in Kenyan wildlife, including buffalos,

warthogs, giraffes, gerenuks and waterbucks, while absent in most carnivores. This may suggest the existence of a cryptic cycle involving wildlife (Evans *et al.,* 2008)

1.3.2 Host Range of RVFV

Both laboratory inoculations and natural infections show a wide range of animals that can be infected by RVFV, ranging from mice to the hippopotami (Easterday, 1965). A study by Evans *et al.,* (2008) also showed high levels of neutralizing antibodies in some wildlife species. This included African buffalo, elephants, warthogs, black rhino, common zebra, Thomson's gazelle, lesser kudu, impala and waterbuck. Whereas no virus or viral RNA was detected in the wild animals, the study suggested that, wild animals could get infected with RVFV (Evans *et al.,* 2008). The host range defined by serology is wider than that defined by the disease; antibodies are detected in wider range of host animals in comparison to hosts coming up with clinical disease.

1.3.3 Strain Variation of RVFV

Typical virologic assays including plaque neutralization do not demonstrate antigenic differences between RVFV strains collected over many years (Peters and Meegan., 1981). Wild type and laboratory derived strains appear antigenically identical. However genetic analyses show unique oligonucleotide profiles for many strains including those isolated from the same regions (Cash *et al.,* 1981).

Some of the strains isolated from Egypt were shown to be more pathogenic for certain laboratory rats than any of strains isolated from other regions (Peters and Anderson, 1981). Phylogenetic analysis by Sall *et al.,* (1999) suggested the existence of an endemic enzootic maintenance cycle of the virus in the different regions. There is also an alternate circulation of the virus in enzootic-endemic form through a maintenance cycle or epidemic-epizootic mode inferring a natural reassortment (Sall *et al*., 1999). However, after the Yemen/Saudi Arabia outbreak of 2000-2001, phylogenetic analysis indicated a close relationship with no evidence of genetic reassortment between Saudi Arabia/Yemen RVFV isolates and those circulating earlier in East Africa (Shoemaker *et al.,* 2002). The lineages developed by Bird (2007) indicated that RVFV strains with diverse geographic origins being found within the same lineage showing a close relationship between viruses isolated from different geographic locations (Bird *et al.,* 2007).

1.3.4 Genome Organization of RVFV

RVFV has a negative sense, single stranded tripartite RNA genome made up of three segments; the L (large), M (medium), and S (small) segments (Elliott *et al.,* 1992). The anti-sense L Segment (6404 nt) encodes the L protein, a viral RNA–dependent RNA polymerase. The M segment (3885 nt) encodes four proteins, including two major envelope glycoproteins Gn (or G1) and Gc (or G2) that bind to a viral receptor molecule to initiate cell infection and two non minor proteins. In

addition there is a 14 kDa non-structural protein (NSm), and a 78kDa protein that is reported to be a structural protein (Struthers *et al.,* 1984). The S segment (1690 nt) encodes a nucleocapsid (N) protein and another non-structural protein (NSs) (Bouloy 1991; Elliott *et al.,* 1991).

A study that compared 33 strains of RVF virus spanning a period of 56 years (1944-2000) found minimal diversity of 5% at the nucleotide level and 2% at amino acid level (Bird *et al.,* 2007). Even the M segment which codes for surface glycoproteins and expected to show considerable diversity due to pressure from the host defense mechanism had similar low levels of nucleotide and amino acid diversity (Bird *et al.,* 2007).

1.4 RVF Disease Associations

1.4.1 RVF in Humans

As at 1975, the knowledge of human RVF disease was limited to a small number of cases often as a result of laboratory accidents (Daubney *et al*., 1931; Francis and Magill, 1935; Smithburn *et al*., 1949.) The disease was characterized by a sudden onset of fever, with an occasional early recurrence (saddleback fever), severe myalgia, headache, retroorbital pain and anorexia. The disease lasted for 4 – 7 days followed by complete recovery. Loss of vision during the 1950 – 1951 South African RVF epizootic among a few patients was attributed to RVFV infection even though definitive etiologic diagnosis was not possible (Mundel and Gear, 1951; Freed, 1951; Schrire., 1951). Later in 1975, South Africa experienced yet another Rift Valley Fever epizootic where an expanding spectrum of clinical disease was described in 17 cases, 12 of which had previously undescribed encephalitis. Four of the patients died from hemorrhagic syndrome (Van Velden *et al.,* 1977). During the 1977 Egyptian epidemic, four clinical syndromes were repeatedly observed and documented as resulting from RVFV infection. The most common form of illness was acute febrile disease; however, some patients developed encephalitis, ocular or hemorrhagic complications after the febrile phase (Abdel-Wahab *et al*., 1978; Laughlin *et al*., 1979a; Meegan *et al*., 1981).

<u>Ocular complications</u>: Many patients with ocular complications were seen during the Egyptian outbreak. They had a typical RVF febrile illness, but 7 to 20 days

after the onset of symptoms, there was diminution of visual acuity. The most prevalent clinical signs were macular, paramacular or extramacular retinal lesions, frequently occurring bilaterally. Edema, hemorrhage and vasculitis were frequently observed. About half of the patients with more severe lesions had permanent loss of central vision. (Siam and Meegan, 1980; Siam, *et al.*, 1980.)

Meningoencephalitis: This was first reported in patients during the 1975 South African epizootic (Van Velden *et al.*, 1977). They were frequently seen in Egypt and another case was reported in Zimbabwe in 1978 (Maar *et al.*, 1979). The occurrence of encephalitic disease was similar to that of ocular disease where, the acute phase was followed by hallucination, disorientation and vertigo after 5 to 10 days (Meegan and Bailey, 1989). Long term neurological complications have been reported but their fatalities appear infrequent (Laughlin *et al.*, 1979b).

Hemorrhagic RVF syndrome: This was not documented until the 1975 epizootic in South Africa. The hemorrhagic manifestation was attributed to 598 fatalities in Egypt in 1977 (Meegan *et al.*, 1981). After 2 to 4 days following the acute febrile illness, jaundice and hemorrhagic manifestations occurred, and 3 to 6 days later, the patient either died or slowly convalesced and recovered.

When severe RVF disease occurs, it manifests with symptoms such as acute febrile illness, hepatitis, encephalitis, hemorrhagic fever and ocular sequelae with mortality rates of between 10 and 20% (Pettersson *et al.,* 1971; Obijeski *et al.,* 1976; Laughlin *et al.,* 1979a; Balkhy & Memish 2003).

Disease manifestations vary according to the animal species and also the age. Severe disease is associated with high viremia, which facilitates vector infection (Davies, 1975).

1.4.2 RVF in Domestic Animals and Wildlife

Rift Valley Fever in Africa appears limited to domestic ruminants with some evidence that exotic stock are more severely affected than indigenous species (Davies, 1975). Disease outbreaks are most obvious in sheep and cattle characterized by abortions and high fatal neonatal disease. The most susceptible animals are the lamb, calf and kid (Daubney *et al.,* 1931). For animals that are less than 7 days old, the incubation period is between 12 and 18 hours followed by death in 24 to 48 hours (Easterday *et al*., 1962a; Easterday *et al*., 1962b; Coackley *et al*., 1967).

The adult sheep has a less severe but similar clinical syndrome that includes temperature rise, rapid pulse, unsteady gait and sometimes nasal discharge, erosion of tongue and skin of the scrotum and sometime death. Although adult cattle and goats may display a similar disease, it is less severe than that of adult sheep (Easterday *et al*., 1962a; Coackley *et al*., 1967).

Exotic sheep and cattle are less resistant to RVF than indigenous African livestock but some indigenous African breeds may also be severely affected by the disease (Eisa *et al.,* 1977; Ksiazek *et al.,* 1989).

A high prevalence level was found in domesticated Asian water buffaloes during the Egypt epidemic (1977–78). Abortion and mortality rates of 12.1 and 7.2% respectively were recorded in buffalo on government farms; however these rates differ little from those usually observed and so it was not clear whether the losses were due to RVF only (Ghaffar, 1981). A low prevalence of antibody to RVF virus has been found in a few species of antelopes but no evidence of disease was recorded (Davies, 1975).

Swine, horses, domestic buffaloes and camels do not become ill even though abortions have been reported in camels and buffaloes (Easterday, 1965). Data from samples collected between 1999 and 2005 in Kenya indicates a number of wild animals that maybe permissive to RVFV infection. A high prevalence (>15%), of neutralizing antibodies against RVFV were detected in buffalo, lesser kudu, thomson's gazelle, impala, black rhino and waterbuck (Evans *et al.,* 2008)

1.4.3 Diagnostic Procedures for RVF detection

Circulation of the virus to a high titer is evident in most susceptible animals and its isolation is successful if virus is transported on wet ice. The most common diagnostic specimen at the height of an epidemic in the past epizootics is liver, placenta, and fetus. Ideally paired sera should be collected; in the acute phase and during convalescence, 14 – 28 hours after the onset of symptoms (Peters and Meegan, 1981; CIDRAP, 2004).

Suitable samples or specimens for antibody testing from live animals include heparinized blood and serum. Pathological specimens include liver, spleen, kidney, lymph nodes and heart blood. Brain tissue from aborted fetuses may also be tested (CIDRAP, 2004).

In the laboratory different methods have been used for diagnosis of RVF. Histopathology can reveal liver necrosis (the first disease indication) in all susceptible animals, the degree of pathology being related to species and age of animal involved (Meegan and Bailey, 1989).

A definitive diagnosis is accomplished by isolation and identification of the virus or by serological demonstration of a fourfold or greater rise in specific, neutralizing (N) antibody titer between acute and convalescent sera (Pini *et al.,* 1973; Meegan *et al.,* 1981).

1.4.3.1 Detection of viral antigens

Specific identification of RVFV antigen may be made rapidly by immunofluorescent staining of cover slip preparations or 8-chamber tissue culture slides. This provides a means of pleriminary identification. To each, a diagnosis before appearance of cytopathic effect in cell cultures can be made (El Mekki and Van der Groen., 1981).

A sandwich ELISA utilizing hyperimmune mouse serum as capture antibody was reported to detect $10^{3.5}$ plaque forming units/ml in viraemic rhesus monkeys (Niklasson *et al.,* 1983).

1.4.3.2 Detection of viral antibodies

Classical methods for detection of antibodies to RVFV include haemaglutination-inhibition, complement fixation, indirect immunofluorescence and virus neutralization tests (Swanepoel, *et al.,* 1986a; Paweska, *et al.,* 2005). ELISA has been shown to be sensitive in early detection of immunological responses to infection or vaccination with RVFV (Paweska *et al.,* 2003). Virus neutralization tests are highly specific but highly laborious, expensive and require several days for completion. It can only be performed when standardized stock of live virus and tissue cultures or mice are available (Tesh *et al.,* 1982).

1.4.3.3 Detection of the viral nucleic acid

Polymerase Chain Reaction (PCR) affords a means to amplify defined nucleic acids before detection by Nuclei Acid Hybridization (NAH) protocols (Mohamed and Imadeldin, 2006). Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR) is a genetic method that has an exceptional analytical sensitivity and specificity. It is an excellent tool for high throughput identification and monitoring of infectious agents of human, animal and environmental origin during an ongoing infection (Manojkumar and Mrudula 2006). Jonas *et al.,* (2008) also describe and evaluate a sensitive (SyberGreen) SYBR green-based method to quantify the copy numbers of RVFV RNA molecules in different samples of biological origin. Both methods offer a means of detection of RVFV RNA in the initial stages of infection before the onset of antibodies (WHO, 2000; OIE, 2008).

Peyrefitte *et al.,* (2008) described the development and validation of one-step, single-tube, real-time accelerated reverse-transcription loop-mediated isothermal amplification (RT-LAMP) for the detection of the L RNA segment of Rift Valley fever virus (RVFV). The assay described is highly sensitive with detection limit of approximately 10 RNA copies per assay and results generation in less than 30 minutes.

1.4.3.4 ***Invitro* cultivation of virus**

The virus can be grown in a number of cell lines including baby hamster kidney cells, Monkey kidney (Vero) cells, chicken embryo reticulum, and primary cultures from cattle or sheep. Hamsters, adult or suckling mice, embryonated chicken eggs or 2-day old lambs can be used (CFSPH, 2007).

1.5 Transmission Cycle of RVF

Following the discovery of Rift Valley Fever in Kenya, several hematophagous arthropods have been incriminated as possible vectors based on the isolation of the virus from these species usually during peak transmission periods but sometimes after the outbreak peaks (Smithburn *et al.,* 1948; Davies and Highton, 1980; Meegan *et al.,* 1980; McIntosh and Jupp, 1981). Meegan *et al.,* (1980) compiled a list of vectors that included 23 mosquito species plus a *Culicoides*, a *simulium* and a *Rhipicephalus* tick. Two cycles have been observed to occur in the transmission of RVFV; an epizootic and inter – epizootic cycle with the epizootic cycle usually occurring after heavy rains (Linthicum *et al.,* 1985). During an epizootic cycle, the virus is maintained between arthropod mosquito vectors and mammalian hosts (particularly cattle and sheep) that serve as amplifiers for the virus. Inter – epizootic maintenance exists by transovarial transmission of the virus in the eggs of *Aedes spp.* of the Neomelaniconium group. The virus is transmitted biologically in the eggs of these flood water mosquito species which may remain dormant for long periods in floodplains or grassland (Linthicum *et al,* 1985)

During the Egyptian epizootic of 1977-78, preliminary results showed an abundance of *Culex pipiens* and was thought to be the most likely vector. The high levels of viremia observed in domestic animals and in humans during epizootics makes mechanical transmission of the virus by haematophagous arthropods possible (Hoogstraal *et al.,* 1979)

A study carried out in Senegal (2002-2003), gave evidence of rains preceding abnormal populations of mosquitoes of several species (Mondet *et al.,* 2005), while egg harvests in Nebraska proved that the evolution of *A.vexans* population are correlated to rain patterns, peaks of activity appearing 10 days after rains in the beginning of the rainy season and 20 days at the end (Janousek and Kramer, 1999)

Transmission of the virus can also occur in laboratory workers. Eight cases who were involved in studying the disease were accidentally infected and blood that was drawn from them with subsequent inoculation in mice caused their deaths (Smithburn *et al.,* 1949). In Zimbabwe, infection of all members of Miller's field team was most probably by inhaling the virus (Miller *et al.,* 1963). The high virus titers in the blood, the unusual stability of RVFV in aerosols and the ability to infect experimental animals by inhalation (Miller *et al.,* 1963) implicated aerosol transmission.

As evidenced in the 2006-2007 Rift Valley Fever outbreak of eastern Africa and from previous reports, epizootics occur simultaneously over geographic regions that are separated by several hundred kilometers following unusually heavy rainfall and hence large numbers of mosquitoes. The most plausible reason for this being the endemic nature of the virus in different geographic regions (Davies, 1975; McIntosh and Jupp, 1981; Davies *et al.,* 1985).

1.6 Prevention and Control of RVF

Measures taken in the prevention of RVF include mosquito control and the protection of human beings during epizootics. This is particularly by the prophylactic immunization of veterinary surgeons, laboratory workers and those whose duties expose them to infection.

The most important measure is to prevent infection of domestic animals which serve as amplifiers of the virus during outbreaks of the disease. Prevention particularly to cattle and sheep involves prophylactic immunization.

1.6.1 Immunization against RVF

Immunization of susceptible animals is the most effective means of control of RVF (Meegan and Bailey, 1989). Two types of vaccines are currently used to immunize sheep and cattle in Africa (Shope *et al.*, 1982; Assad *et al.,* 1983). The attenuated live virus vaccine (Smithburn strain) is highly effective, but causes a number of sheep to abort after immunization (Sall *et al.,* 1998).

Formalin – inactivated vaccines have been used in South Africa, Egypt and Israel (Assad *et al.,* 1983). Even though they are safe and effective, they require multiple inoculations. The live vaccine is not recommended for non-endemic areas due to the potential for reversion of vaccine to virulence, and hence the formalin-inactivated vaccine is recommended for non – endemic areas (Meegan and Bailey, 1989).

A safe, effective formalin–inactivated vaccine is available for those at-risk, laboratory personnel and field researchers (Eddy *et al.,* 1981).

1.6.2 Vector Control

Elimination of adult mosquitoes involves spraying of walls with long-acting insecticides and barricading of doors and windows with wire mesh screens (Gear, 1988). In Egypt between 1977 and 1978, insecticides were used both inside and outside human and animal shelters to reduce the adult populations of potential vectors. However, the use of these chemicals has not been shown to actually decrease the incidence of the disease (Meegan and Bailey, 1989).

Breeding grounds of mosquitoes should be eliminated by eliminating water collection points in the vicinity of houses. Treatment of stagnant water with larvicides to prevent mosquitoes breeding is also important (Gear, 1988). In sub-Saharan Africa where many potential vectors occur each with their own unique life cycles, the use of chemicals for adult vector control has not been proven effective. Further more, the disease is closely linked with flood water mosquitoes and biological transmission in the 'dambos', suggesting the importance of an encapsulated formulation of insecticide to control larval stages could be better before the emergence of adult mosquitoes (Meegan and Bailey, 1989).

1.6.3 Protection of human beings

Sleeping under mosquito nets provides some protection when mosquitoes are prevalent. Human infections however have occurred where the patients acquire the infection while handling animals that are infected. In the first recognized epizootic of South Africa in 1951, all the practicing veterinary surgeons in the Orange Free State and several in Transvaal contracted the disease from not wearing protective gear while performing post-mortem studies on cattle and sheep (Gear *et al.,* 1951). The minimum gear that should be worn includes mask, gloves, gown, waterproof apron, and spectacles or goggles as the eyes may be contaminated by droplets (Gear, 1988).

1.7 Background of the 2006-2007 RVF Outbreak in East Africa

In mid-December 2006, several unexplained fatalities associated with fever and generalized bleeding were reported to the Kenya Ministry of Health (KMOH) from Garissa District in North Eastern Province (NEP). By 20th December, a total of 11 deaths had been reported. Serum samples were collected and sent to KEMRI/CDC laboratories in Nairobi. From the first 19 patients' serum samples, Rift Valley fever (RVF) virus RNA or immunoglobulin M (IgM) antibodies against RVF virus were found in samples from 10 patients; all serum specimens were negative for yellow fever, Ebola, Crimean-Congo hemorrhagic fever, and dengue viruses. The outbreak was confirmed by isolation of RVF virus from six of the specimens (CDC, 2007).

1.7.1 Garissa-Northeastern Province

Garissa, the first location to report the RVF outbreak, is a town located in the north eastern province and serves as the provincial headquarters (Fig 2). Garissa borders Wajir district to the north, Tana River to the west, Ijara to the south, Isiolo to the north west and Somali to the east. More than 50% of the district's population are pastoralists (KFSSG, 2008).

1.7.2 Kilifi-Coast Province

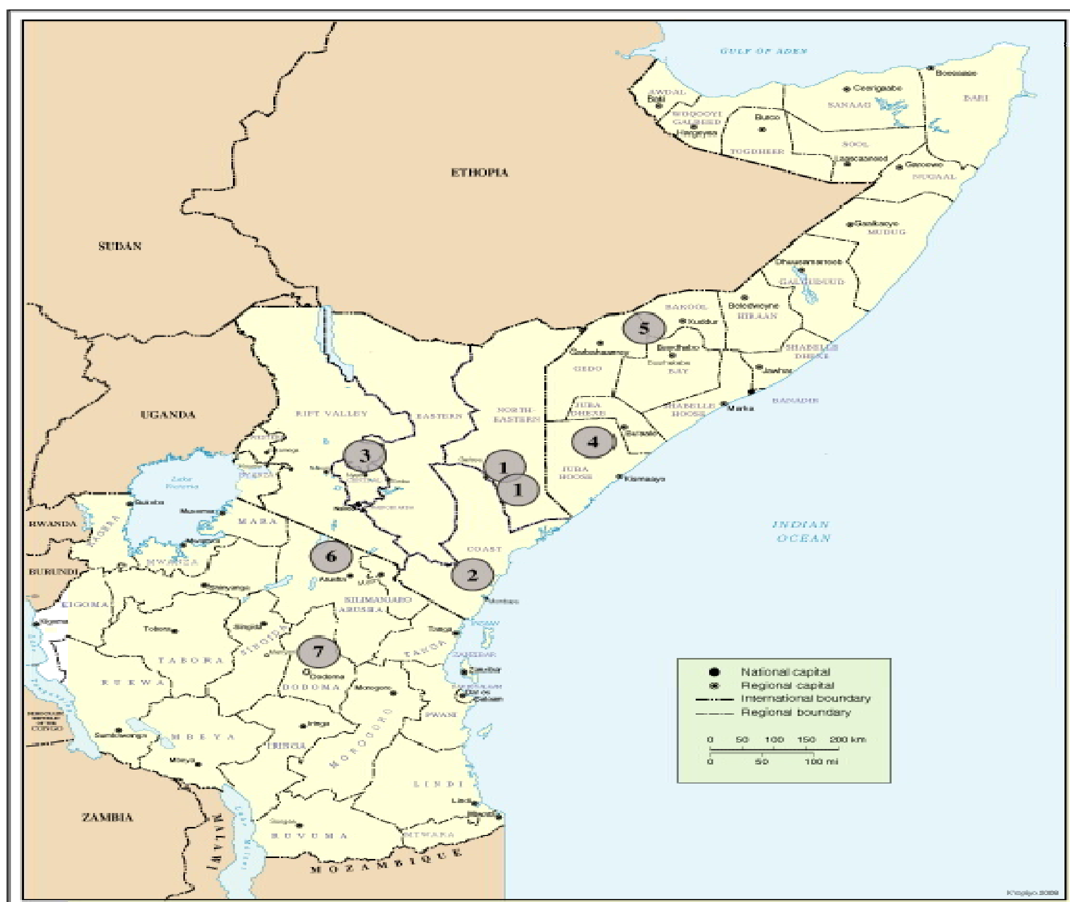
After Garissa, the cases of RVF were reported in Kilifi and Malindi of the coast province of Kenya (Fig 2). Kilifi is a town along the Kenyan coast in the coast province. The major occupation in this district is cash cropping and dairy farming.

There is a bimodal rainfall pattern in the district. Short rains are experienced between October and December with the long rain between March and July (KFSSG report, 2008).

1.7.3 Baringo-Rift Valley Province

Baringo, the third region to report RVF disease, is district in the Rift Valley Province whose residents like those in the neighbouring districts lead an agro – pastoralist way of life (Fig 2).

By 12th March 2007, a total of 684 cases including 155 deaths (case fatality ratio = 23%) of RVF had been reported in the country. 333 cases were from North Eastern province, 183 in Rift Valley Province, 141 in the Coast Province, 14 in Central Province, and 13 in the Eastern Province. Of these 684 cases, 234 (34%) were laboratory confirmed (WHO, 2007).



Source: Njenga, 2008.

Fig 2: East Africa map showing sequential outbreak of the RVF disease in 2006-07.

1. - Garissa in North Eastern Province, Kenya

2. - Kilifi, Malindi in Coast Province, Kenya

3. - Baringo in Rift Valley Province, Kenya

4. – Juba, Somalia

5. – Gedo, Somalia

6. - Arusha, Tanzania

7. - Dodoma, Tanzania

1.7.4 United Republic of Tanzania

By 13th January Tanzania started reporting cases up to 3rd May (Fig 2). A total of 264 cases with 109 deaths (case-fatality ratio 41%) were reported in 10 out of the 21 regions of Tanzania. Twelve cases were reported in Arusha, 1 in Dar es Salaam, 156 in Dodoma, 4 in Iringa, 6 from Manyara, 50 in Morogoro, 5 in Mwanza, 5 in the Pwani, 24 in Singida, and 1 in Tanga regions. Of the 264 cases, 154 (60%) were laboratory confirmed (WHO, 2007)

1.7.5 Somalia

From 19th December 2006 to 20th February 2007, a total of 114 cases including 51 deaths (case fatality ratio = 45%) of RVF were reported in Somalia. Seventy three cases were reported in lower Juba area, 26 in Gedo region, 7 in Hiran, 2 in middle Juba region, 4 cases in middle Shabelle region, and 1 case in lower Shabelle region. Of the 114 cases that were reported, 3 (3%) were laboratory confirmed (WHO, 2007).

1.8 Justification for the Study

The 2006 – 2007 an RVF outbreak caused considerable morbidity and mortality in both human and animal populations. The outbreak began in early December 2006 in Garissa district of north eastern province of Kenya. In early January 2007, RVF was detected in Kilifi district, Coast province and in mid January 2007 in Baringo district in the Rift Valley Province of Kenya. The case fatality rate associated with the Kenyan outbreak was 23% (WHO, 2007). The initial reports of RVF cases in Tanzania were from early February 2007, and they continued reporting cases up to mid June 2007. The outbreak in Tanzania led to a case fatality rate of 41% (WHO, 2007). The cases that were reported in Tanzania also had a high rate of encephalopathy (89%) while in Kenya, this was not observed. It was not clear whether the strains causing these sequential outbreaks (i.e. from early December 2006 in Garissa, through January 2007 in parts of Coast and Rift valley Provinces up to mid June 2007 in Tanzania) were the same and this posed the question as to whether this was a virus that was resident in these regions and only got activated after the heavy rains and floods. Further, the high case fatality in Tanzania (41%), in relation to that in Kenya (23%) led to questions as to whether the virus had changed and thus the big difference in the case fatalities in the two countries while the outbreaks were only two months apart. The high encephalopathy rate in the Tanzania cases compared to the Kenyan cases also questioned the similarity of the strains involved in these outbreaks. The M segment which codes for regions targeted by neutralizing antibodies (i.e. the 2 glycoproteins) would be the most

probable to show some diversity from the most recent outbreaks (i.e. that in Kenya in 1997 – 1998 and that in Saudi in 2000) if there were any mutation in their nucleotide and amino acid sequences. This formed the basis of this work.

1.9 Null Hypothesis

The 2006-2007 East African RVF outbreak was caused by different RVFV strains that were dormant in the different geographical regions and were only activated by the onset of the heavy rains in each of these regions.

1.10 Alternate Hypothesis

The 2006 – 2007 East African RVF outbreak was caused by the same RVFV strain that had its origin in Garissa and later on spread to the other regions

1.11 Objectives of the Study

1.11.1 General Objective

To determine the molecular epidemiology of 2006 – 2007 RVFV outbreak by genetically characterizing the RVFV that was responsible for this outbreak from the different affected regions.

1.11.2 Specific objectives

1. To determine the full genome sequences of selected RVFV isolates recovered during the 2006 – 07 RVF outbreak.

2. To analyze the nucleotide and amino acid sequences by comparing them with each other and with other historical RVFV strains.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Specimen collection

In this study, 8 human serum samples that were positive during diagnosis of Rift Valley Fever were used (Table 1). The selection criteria for the samples were based on;

- Area of origin of the samples. This was considered so as to have a representation of the areas that were involved in the active circulation of the virus.
- The Cycle Threshold (ct) value derived from Real Time PCR assays. From NEP, four samples were isolated, Coast province two, Rift Valley 3, Tanzania 3 and Somalia none. Based on the ct values (cut off value of 25), only 2 samples from each of these regions met the criteria.

These samples had been kept at –80ºC at the Centers for Disease Control and Prevention Biosafety Level 3 (BSL3) laboratory within the Kenya Medical Research Institute (KEMRI) headquarters, Nairobi.

2.2 Historical strains

Two strains were used as reference sequences were for comparison purposes. These were obtained from the National Centre for Biotechnology Information (NCBI) website. These were Kenya 9800523, (GenBank accession number DQ380196.1) whose origin is Kenya after the outbreak of 1997-1998. The other referral strain was from Saudi Arabia after the outbreak in 2000, Saudi 2000-10911, (GenBank accession number DQ380197.1).

Table 1: Strains of 2006-2007 RVFV isolates sequenced and analyzed

Virus Strain	**Origin**	**RT-PCR**	**Sequence**
Gar-004	Garissa	+	Full Genome
Gar-008	Garissa	+	Full Genome
Kil-006	Kilifi	+	Full Genome
Mal-032	Malindi	+	Full Genome
Bar-032	Baringo	+	Full Genome
Bar-035	Baringo	+	Full Genome
Tan-001	Tanga-Tanzania	+	Full Genome
Tan-002	Dodoma-Tanzania	+	Full Genome

2.3 Virus Isolation

Virus isolation from human sera was carried out as described earlier (Gould and Clegg, 1985). Vero cells-a cell line derived from the kidney of a normal adult African green monkey was used (Yasamura and Kawakita, 1963). So as to attain the optimum number of T-25 ($25cm^2$) Vero cells were sub cultured prior to

inoculation (Appendix 2). The cells were grown to about 90% confluence in minimum essential medium (MEM) containing 10% fetal bovine serum, 2% L-glutamine, and 1% antibiotics in ten T-25 tissue culture flasks (Appendix 1). These were then washed twice with 5mls each of Phosphate Buffered Saline (PBS) equilibrated at 37ºC in a water bath. PBS was finally aspirated and 8 flasks inoculated with a mixture of 100ul serum sample and 300ul of Maintenance Media (Minimum Essential Media supplemented with 2% fetal bovine serum, 2% L-glutamine, and 2% antibiotics) (Appendix 1). The other 2 flasks were inoculated with 400ul of Maintenance Media (MM) to serve as negative controls. The inoculums and the cells were incubated for 1 hour at 37ºC in a CO^2 incubator and were rocked after every 15 minutes (Appendix 3). After the incubation, 4mls of MM were added to all the 10 flasks. The inoculated cells were incubated at 37°C and 5% carbon dioxide for up to 12 days. The cultures were monitored daily for development of cytopathic effects (CPE) which included cell rounding and disruption of the monolayer (lytic CPE). Upon development of CPE in over 90% of the cells, the flasks were frozen at -80°C and freeze-thawed 5 times to facilitate Vero cell lysis and virus release. The flasks were then thawed, the contents scraped and then transferred into a 15 ml falcon tube (centrifuge tube). Centrifugation was performed at 2,300 X g for 10 minutes and the supernatant filtered through 0.22 um Millipore syringe filter. Filtered virus-containing supernatant was aliquoted into cryovials and stored at -80°C.

2.4 Isolation of Ribonucleic Acid (RNA)

RNA was extracted from infected Vero cells. A modified isolation method of acid guanidinium thiocyanate-phenol-chloroform extraction was used (Chomczynski and Sacchi, 1987; Sambrook *et al.*, 2001).

RNA extraction was performed using QIAamp Viral RNA mini kit (Qiagen Inc, Amsterdam, Netherlands), which combines both the selective binding properties of a silica-gel-based membrane with the high-speed microspin or vacuum technology. Vero cell supernatant from previous cell culture was equilibrated to room temperature (15-25°C). 560µl of viral lysis (AVL) buffer containing carrier RNA was pipetted into 1.5ml micro-centrifuge tubes. Cell-culture supernatant (140µl) was added to the buffer AVL/Carrier RNA in the tube. This was mixed by pulse-vortexing for 15sec. To allow for complete lysis, the samples were incubated at room temperature (15-25°C) for 10min. The 1.5ml tubes were briefly centrifuged to remove drops from the inside of the lid. To precipitate RNA absolute ethanol (560µl) was added to the samples and mixed by pulse-vortexing for 15sec. After mixing, the microfuge tubes were briefly centrifuged to remove drops from the lid. The mixture (630µl) was carefully pipetted into the QIAamp spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and centrifuged at 6000 X g for 1min. The QIAamp spin column was placed into a clean 2ml collection tube, and the tube containing the filtrate discarded. The QIAamp spin column was carefully opened and the remaining mixture of sample-lysis buffer added until the entire sample was loaded. For the purpose of obtaining pure RNA,

other unbound components such as proteins and genomic DNA, a two step wash process was performed. The QIAamp spin column was carefully opened and 500µl of buffer AW1 added. The cap was closed and centrifuged at 6000 X g for 1min. The QIAamp spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate discarded. The QIAamp spin column was carefully opened and 500µl of buffer AW2 added. The tubes were capped and centrifuged at full speed (20,000 X g) for 3min. The QIAamp spin column was placed in a new 2ml collection tube and the old collection tube with filtrate discarded. This was centrifuged at full speed (20,000 X g) for 1min. The QIAamp spin column was placed in a clean 1.5ml micro-centrifuge tube. The collection tube containing filtrate was discarded. The QIAamp spin column was carefully opened and 60µl of buffer AVE equilibrated to room temperature added. The cap was closed and incubated at room temperate for 10 minutes. This was centrifuged at 6000 X g for 1min.

2.5 Primer Design

RT-PCR primer sets were designed using previously published primer sets (Bird *et al.,* 2007) of RVFV. Internal primers for sequencing were designed from nucleotide sequences of RVFV analyzed in this study.

The primer sequences were verified by use of the NCBI blast site in the internet and the DNAStar software (DNASTAR Inc., MadisonWI).

In the NCBI blast site, primer sequences were pasted and the use of Basic Local Alignment Search Tool (BLAST) employed. After a few minutes of the tool trying to locate the closest march to the primer sequence in the gene bank, results would show the closest relative of the primer sequence that was pasted. In this case, the primers sequences were related to the RVFV sequences already in the genebank and to their respective segments. The results from the search also confirmed whether the primer was a forward or a reverse primer by giving the plus/plus designation for forward primers and plus/minus for reverse primers.

As a back up to the BLAST search, sequences already in the genebank were input into the DNASTAR software as well as the primer sequences via one of the DNASTAR modules, Editseq. By the use of Megalign module of the same DNASTAR software, the sequences from the genebank were aligned against the primer sequences. The results of the alignment gave the exact position of the primer sequences in comparison with the full genome sequences from the genebank as well as their exact march and their orientation (i.e. confirmation as to whether the primer sequence is a forward or a reverse primer).

2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The one step RT – PCR was optimized using Invitrogen's Superscript III one step RT – PCR system with Platinum® *Taq* DNA polymerase (Invitrogen, San Diego,

CA) for the study. Both cDNA synthesis and PCR amplification were performed in the same tube using gene specific primers and the target RNA.

Forward and Reverse primer sets (Table 2) were used to amplify the whole segments of S (1690nt) and M (3885nt) while the L segment (6404nt) was amplified using two primer sets that resulted in two overlapping fragments. Each primer set was used to amplify RNA extracts from 1st passage Vero cell supernatant of each of the 8 isolates.

Table 2: Primer sets for RT-PCR

Primer	**Sequence**	**Size**	**Segment**
RVFS-AFwd	ACACAAAGCTCCCTAGAGATAC	22	S
RVFS-ARev	ACACAAAGACCCCCTAGTG	19	S
RVFM-AFwd	ACACAAAGACGGTGC	15	M
RVFM-ARev	ACACAAAGACCGGTGC	16	M
RVFL-AFwd	ACACAAAGGCGCCCAATC	18	L
RVFL-3482Rev	GGAAGCATATAGCTGCGG	18	L
RVFL-2845Fwd	GAGACAATAGCCAGGTC	17	L
RVFL-ARev	ACACAAAGACCGCCCAATATTG	22	L

Key

S - Small L - Large Fwd - Forward

M - Medium Rev - Reverse

2.7 RT-PCR reaction mix

Into each 0.2ml MicroAmp, nuclease free, thin walled PCR reaction tube, the following reagents were added: 25µl of 2X reaction mix, 1.5µl of forward primer (50pmols), 1.5µl of reverse primer (50pmols), 2µl of SuperScript™ III RT/Platinum® *Taq* mix and 10µl autoclaved distilled water. To this mix, 10µl of the template RNA was added for a final reaction volume of 50µl. This mix was then gently mixed by inverting the tube several times, then centrifuged briefly to ensure that all the components were at the bottom of the amplification tube.

2.7.1 Thermocycling parameters

The reaction tube with the mix was placed in a pre-heated ABI 9700 thermo cycler and the reaction volume set to 50µl. The thermocycling process was different for the different segments because of the different lengths of segments to be amplified.

The optimized cycle parameters for the S segment were; an incubation at 51ºC for 30 minutes followed by a rapid thermal ramp at 94ºC for 2 minutes. The *Taq* polymerase employed in this process is inactive at ambient temperature and was activated at this temperature.

This activation of the *Taq* polymerase was followed by 40 cycles that included; denaturation of the double stranded DNA at 94ºC for 15 seconds, annealing of the primers at 56ºC for 30 seconds after which *Taq* polymerase was allowed to elongate the primers at 68ºC for 2 minutes.

A final extension was performed at 68ºC for 5 minutes. Finally there was a rapid thermal ramp to 4ºC and held until ready purify. These final two steps were non cyclic steps.

For the M and L segments, the parameters were; an incubation at 51ºC for 30 minutes, followed by a rapid thermal ramp for *Taq* polymerase activation at 94ºC for 2 minutes. This was followed by 40 cycles of DNA denaturation at 94ºC for 15 seconds, annealing of the primers at 56ºC for 30 seconds, and a primer extension at 68ºC for 4 minutes. There was a final extension at 68ºC for 10 minutes followed by rapid thermal ramp to 4ºC and held until ready purify. Again the final two steps were non-cyclic steps.

2.8 Gel electrophoresis

Electrophoretic analysis of the PCR products was performed using Agarose gels.

Preparation of the Agarose gel incorporated; mixing 100mls of 1X TAE buffer (0.04M Tris Base, 0.04 acetate and 0.001M EDTA) with 1.5 grams of electrophoresis grade Agarose. The mixture was boiled by heating in a microwave oven until the solution became clear (i.e. no particles of Agarose were observed). The solution was then cooled under running water to about 50°C, after which 5µl of Ethidium Bromide (an intercalating agent that complexes with DNA and emits a fluorescence when exposed to Ultra Violet light) was added. This mixture was swirled gently and then poured into the gel tray. The gel was then allowed to set

(harden) for about 30 minutes. The hardened gel was placed in an electrophoresis tank (with a positive and a negative electrode), and the 1X TAE buffer poured to cover the whole gel up to the top surface. The end with the comb was placed towards the cathode end and the comb removed. Using a clean sample loading tray, 2 µl of loading dye (Bromophenol Blue), was added to the tray and mixed with 5µl of the PCR product and loaded in to the wells in the gel. A molecular weight size marker – 123 ladder, was also prepared by mixing 2µl of Bromophenol Blue (BPB), 2µl of the 123 ladder and 8µl of PCR grade water and also loaded.

The electrophoretic tank was covered, the electrodes connected to a power supply and were run at 100 volts for 1 hour. The dye aided in tracking the migration of the PCR products across the gel. The bands were then viewed under Ultra Violet (UV) light at 312 nm wavelength. Using a handheld 0.7X electrophoresis hood with Wratten 22A filter and a lens of f = 103nm, photographs of the gel were taken with the UV light on using Polaroid 667 (ASA 3000) film.

2.9 DNA Fragment recovery and purification

RT-PCR products which were the template for downstream sequencing reactions had left over primers, unincorporated nucleotides and non-specific products. For these reasons the PCR products were purified by gel extraction procedure. After analysis of amplicons from RT-PCR by gel electrophoresis, the samples that

fluoresced under UV illumination at the expected sizes (based on the PCR primer pairs used), were loaded again onto a 1% agarose gel for the purposes of purification. After an electrophoresis of approximately 45 minutes, the fragments were excised and purified using QIAquick Gel Extraction Kit (Qiagen, Inc, Amsterdam, Netherlands). DNA fragments were excised using a clean scalpel. Any extra agarose gel was sliced off so as to have minimal amounts of the Agarose. The gel slice was weighed in a colorless 1.5ml tube and 3 volumes of buffer QG added to 1 volume of the gel (100mg ~ 100µl) i.e. 300µl of buffer QG were added to 100mg of gel slice. The gel slice in buffer QG was incubated in a thermo mixer at 50°C for 10 minutes. After the 10 minutes incubation with intermediate vortexing every 2-3 minutes, the colour of the resulting solution was confirmed to be yellow after which 1 gel volume of isopropanol was added. The tube was mixed by pipetting up and down several times. To bind the DNA, the solution was pipetted into a QIAquick column inserted in a collection tube and centrifuged for 1 minute at 10, 000 × g. The flow-through was discarded and the QIAquick column inserted into a clean collection tube. 0.5mls of buffer QG were added to the QIAquick column so as to remove all traces of Agarose and centrifuged for 1 minute at 10, 000 × g. The flow-through was discarded and the column placed in a clean collection tube. To wash, 0.75mls of buffer PE were added to the QIAquick column, let to stand for 5 minutes then centrifuged for 1 minute at 10, 000 × g. To remove the residual ethanol that had been added to buffer PE, the flow-through was discarded, the QIAquick column placed in a clean

collection tube and centrifuged for an additional 1 minute at 10, 000 × g. The column was placed in clean sterile 1.5ml tube. To elute the DNA, 30µl of PCR grade water was added to the centre of the QIAquick column and let to stand for 10 minutes, then a centrifugation of 10, 000 × g was performed for a minute. This was stored at temperatures below -20°C until ready for sequencing reactions.

2.10 Full Genome Sequencing

Genomic sequencing was done using the dideoxy-synthesis termination method (Sanger *et al.,* 1977) whose principle is explained in appendix 4. To determine the order of nucleotides in the different isolates, a premixed ready reaction mix was used. The ready reaction mix included deoxynucleoside triphosphates dNTPs, labeled dideoxyNucleoside Triphophates (ddNTPs), an enzyme Amplitaq DNA polymerase, magnesium chloride, and a buffer. These reagents were suitable for performing fluorescence based cycle sequencing reactions on single stranded DNA templates, PCR fragments and on long templates, (ABI Prism 310 Genetic Analyzer, Perkin-Elmer/Applied Biosystems)

The mixture for performing sequencing PCR included 4.0µl of Big-dye terminator ready reaction mix, 2µl of 5X sequencing buffer, 1.5 µl of either forward or reverse primer (50picomoles), 5µl of template double stranded DNA and the volume was topped up to 20µl using PCR grade water. The tubes were sealed, vortexed and spun briefly.

The samples were placed in a thermocycler, the reaction volume set to 20µl and subjected to 25 cycles of amplification cycles as; rapid thermal ramp to 96^0C, 96^0C for 10 seconds (Denaturation of double strands). Next was a rapid thermal ramp to 50^0C and holding for 5 seconds where primers are allowed to anneal followed by rapid thermal ramp to 60^0C that allowed for elongation/termination synthesis for 4 minutes. Finally there was a rapid thermal ramp to 4^0C and this step which was non cyclic was held until ready to purify.

2.11 Centri-Sep spin column purification of labeled DNA

CENTRI-SEP spin columns technology was employed for the purposes of recovery of DNA while excluding small molecules (nucleotides, excess dideoxynucleotides, buffer salts) from DNA sequencing reactions,. This column system is based on the description by Sambrook of gel filtration for the purification of DNA from nick translation reactions (Sambrook *et al.,* 2001). Each unit comprised of special fritted microfuge tube, dry gel, wash tube and sample collection tube. The gel provided excellent recovery of DNA fragments larger than 16 base pairs while removing more than 98% of salts, Nucleoside Triphosphates (NTP's) and other low molecular weight compounds.

2.11.1 Sample processing of sequencing PCR products

Before processing the sample, the gel (see section 2.11) in the column was rehydrated with 0.80 mls of reagent grade water, let to stand and excess interstitial fluid removed.

The sequencing PCR product was transferred onto the top center of the gel bed without touching the walls of the column. This was necessary so as to ensure an efficient purification. After placing the column in 1.5ml eppendorf tube, the sample was collected by spinning at 750 X g for 2 minutes. The spin column was then discarded and, the sample dried in a vacuum centrifuge ready for resuspension.

2.11.2 Resuspension of sequencing PCR products

The end product of drying (see section 2.11.1), a pellet, was resuspended in 20µl of HI-DI formamide. This was then vortexed and centrifuged briefly. The 20µl of the resuspended sample were then loaded onto the 96 well plate and incubated for 2 mins at 95°C. These were flash cooled by placing on ice for 2 minutes, loaded onto the sequencer (ABI 3100 GA) and ran for 2 and a half hours.

2.11.3 Electrophoretic separation of cycle sequencing fragments

Electrophoretic analysis (as in figure 3) gave a distribution of the dyes each showing the sequence of the particular nucleotides in respect to the label. DNA (from RT-PCR) was analyzed following an automated ABI prism® Big Dye Terminator™ v3.1 Cycle Sequencing kit for Capillary Sequencers ABI 3100 Genetic Analyzer (ABI Prism 310 Genetic Analyzer, Perkin-Elmer/Applied Biosystems).

Genome sequencing resulted in many fragments of different lengths depending on the point of termination of synthesis of a new strand (Appendix 5). The fragments were separated electrophoretically by the automated ABI 3100 genetic analyzer. Since each ddNTP was labeled with a specific dye, a Charge Coupled Device camera detected each dye and gave an output to the analyzing software where the information was processed and the template nucleotide sequence generated and data stored automatically.

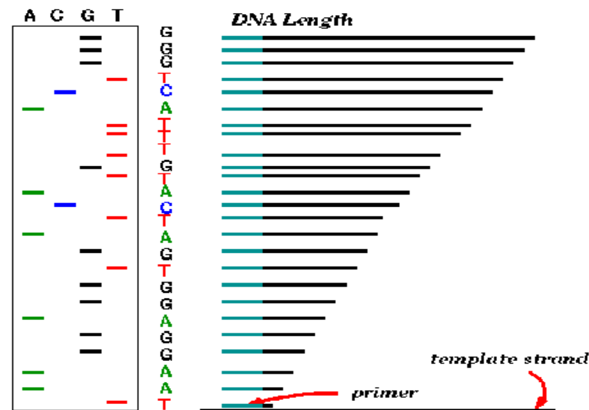


Fig 3: An illustration of electrophoresis of the cycle sequencing products. **Source:** www-math.mit.edu/~lippert/18.417/ (2008).

The data was then retrieved and edited with the help of the International Union of Pure and Applied Chemistry diagram which guided in areas where the machine was unable to determine the nucleotide. By viewing an already generated electropherogram, the highest peak between the two nucleotides qualified for the nucleotide in sequence.

2.12 Data Analysis

The RVFV sequences were analyzed with DNASTAR computer program, (DNASTAR Inc., MadisonWI*)*. DNASTAR provides tools for sequence assembly, editing, alignment and analysis. It consists of Lasergene, comprehensive software for DNA and protein sequence analysis, contig assembly and sequence project management. DNASTAR Lasergene software consists of an integrated suite of seven modules. The modules of Lasergene are: SeqBuilder - visualization and sequence editing Video*,* SeqMan Pro - sequence assembly and single nucleotide polymorphism discovery, MegAlign - sequence alignment, PrimerSelect - oligo primer design, Protean - protein structure analysis & prediction, GeneQuest - gene finding, EditSeq - utility for importing unusual file types.

The Data Manager enables data integration between the Lasergene modules so that edits, additions and deletions made to a sequence in one module will synchronize and automatically update when opened in most other modules. RVFV study employed the use of PrimerSelect, SeqMan Pro, MegAlign and EditSeq.

SeqMan Pro enabled the assembly of sequence data from Sanger sequencing. A few fragments to genome assemblies can be done at the push of a button. SeqMan Pro provides two different assembly methods; the Classic Assembler, and the Pro Assembler for assemblies of 100 sequences or more. SeqMan Pro removes unreliable data, including poor quality ends, sub-minimal length reads, and vector and contaminating host sequences in a single pass, then assembles the trimmed data and calls the consensus.

SeqMan Pro uses DNASTAR's unique trace quality evaluation method to call the most accurate consensus sequence possible. This method reduces the depth of coverage needed for accurate sequence determination, saving time. SeqMan Pro also uses dual-end sequence data when available to group and order contigs, allowing you to fill gaps quickly with minimal additional sequencing efforts.

To evaluate the assembly, a single alignment window allowed viewing key aspects including; two consensus sequences for the same contig, called by different methods, multiple sequences and their underlying trace data, candidate SNPs and sequence and consensus conflicts.

EditSeq interfaces with other programs including GenBank, FASTA, MacVector, GCG®, Text, ABI®, and word processing. Dynamic links between sequence and annotations exhibited automatic updating of feature coordinates with sequence editing and inclusion of features with copying/pasting of sequences. Available functions included reverse complement, invert, translate, back-translate, and open reading frame identification, (DNASTAR Inc., MadisonWI).

Pure sequences were analyzed using Basic Local Alignment Sequence Tool (BLAST) to confirm their identity (Altschul *et al*. 1997). Once contigs had been obtained from the SeqMan Pro, next was to simply BLAST* them against National Center for Biotechnology Information (NCBI's) data to gather information already known about related sequences. The NCBI BLAST finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical

significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

MegAlign offered choice of four pairwise and four multiple sequence alignment methods for aligning nucleic acid or polypeptide sequences. Own sequences or public data directly from NCBI* was entered. To find more related sequences for alignment, a BLAST query was ran, and then the sequences wanted from the list of matches dropped in. Views of alignments were easily customized to highlight the similarities or differences of the sequences. Differences in chemical, structural or functional characteristics between sequences were also displayed as well as groupings or consensus. MegAlign also enabled construction of phylogenetic trees, generation of detailed numerical reports or export data of sequence comparisons, (DNASTAR Inc., MadisonWI*).*

CHAPTER 3

3.0 RESULTS

3.1 RVF isolates

Over twenty (N = 20) RVF virus strains were recovered following Vero cell inoculation. The eight strains sequenced and analyzed for this study are listed in Table 1.

3.2 RT-PCR of RVFV isolates

The entire S and M segments yielded amplicons that had sizes ranging from 1690 nucleotides (S segment), 3885 nucleotides (M segment). L segment yielded two overlapping fragments of sizes; $L_{(A)}$ 3482 and $L_{(B)}$ 3559 nucleotides long.

The following gel pictures show the positive samples that had been isolated in Vero cells and a conventional RT-PCR performed. Even no positive and negative controls are shown in these plates, the initial diagnostic real time RT-PCR assays (appendix 8) included both negative and positive controls. This appendix shows the raw data from the real time machines. Appendix 9 shows the report generated after analysis, indicating sample names and their corresponding ct values for positive samples. Undetermined means samples were negative.

In plate 1 are the PCR products from amplification of the M segment of the RVF genome.

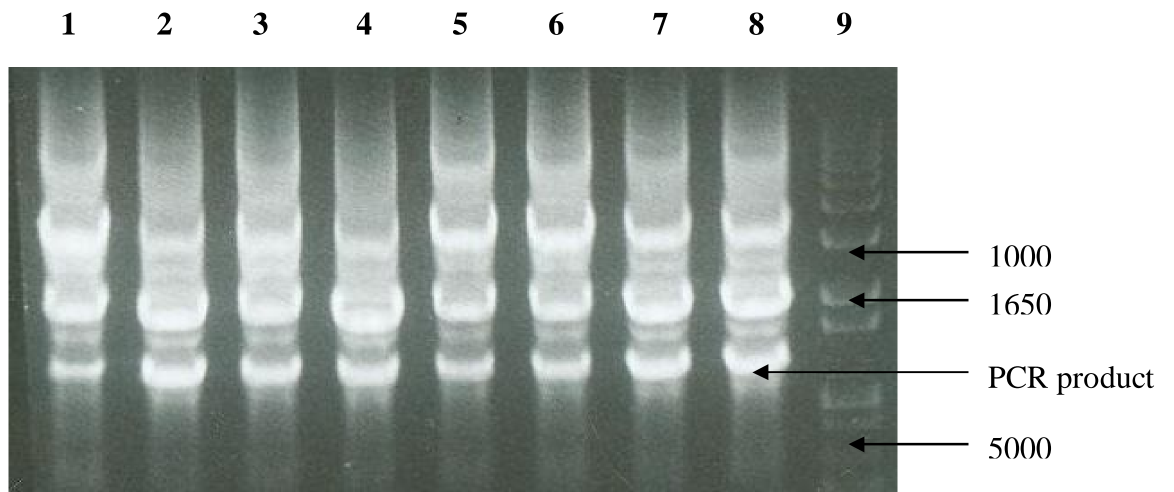


Plate 1: 1.5 Agarose gel stained with ethidium bromide of the M segment of RVFV.

In lane 9, a 1 kb DNA marker; lane 1-Gar 004, lane 2-Gar 008, lane 3-Kil 006, lane 4-Mal 032, lane 5-Bar 032, lane 6-Bar 035, lane 7-TZ 001, lane 8-TZ 002.

In plate 2, the first part of the L segment was loaded and the gel picture shows the amplicons (PCR products) from the PCR.

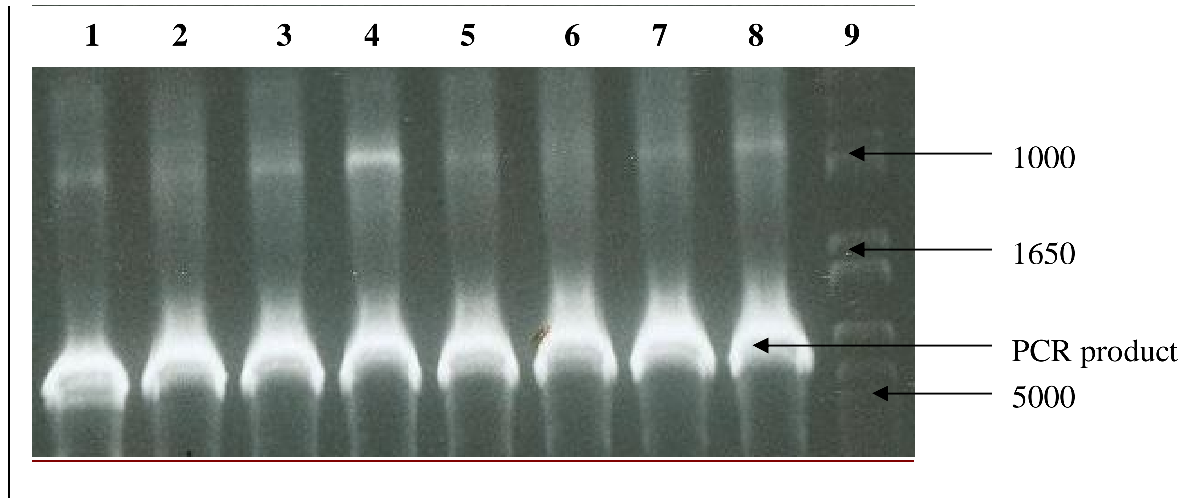


Plate 2: 1.5 Agarose gel stained with ethidium bromide of the L $_{(A)}$ segment of RVFV.

In lane 9 a 1 kb DNA marker; lane 1-Gar 004, lane 2-Gar 008, lane 3-Kil 006, lane 4-Mal 032, lane 5-Bar 032, lane 6-Bar 035, lane 7-TZ 001, lane 8-TZ 002.

Plate 3 shows the PCR products from the latter part of the L segment of the RVFV genome.

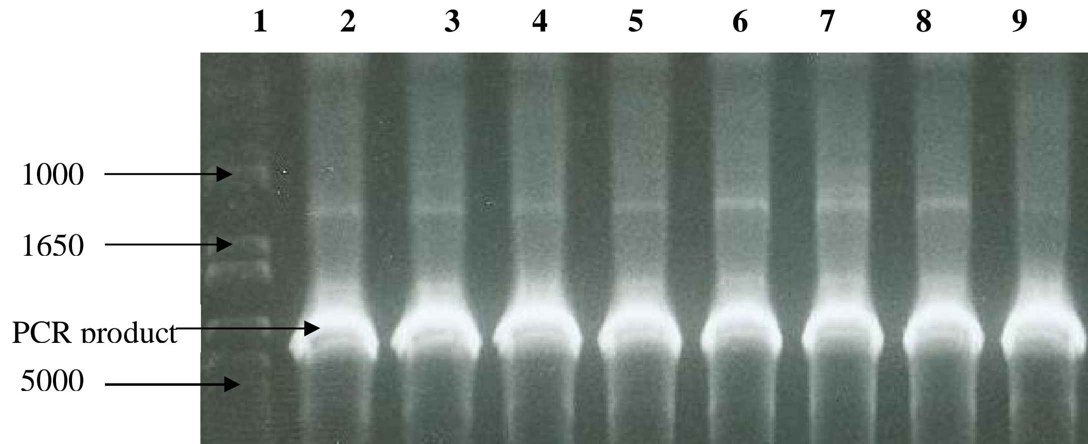


Plate 3: 1.5 Agarose gel stained with ethidium bromide of the $L_{(B)}$ segment of RVFV.

In lane 1 a 1 kb DNA marker; lane 2-Gar 004, lane 3-Gar 008, lane 4-Kil 006, lane 5-Mal 032, lane 6-Bar 032, lane 7-Bar 035, lane 8-TZ 001, lane 9-TZ 002.

3.2.1 RVFV Genome Sequencing and analysis

Previously, one human sequence from the Kenya 1998, Kenya 9800523, (GenBank accession number DQ380196.1) outbreak had been reported and also one from the Saudi 2000 outbreak, Saudi 2000-10911, (GenBank accession number DQ380197.1). We sequenced eight additional human isolates from the earliest detected RVFV after the Garissa epidemic in North Eastern province of Kenya to the last outbreak in Tanzania by primer walking. The isolates included two from Garissa (Gar 004 and Gar 008), two from the Kenyan coast (from Kilifi, Kil 006, and from Malindi, Mal 032), two from Baringo (Bar 032 and Bar 035), and two from Tanzania (TZ 001 and TZ 002). Thus, full length sequence for eight isolates has been generated. The sequences generated from this study will be submitted to the genebank (National Center for Biotechnology Information-NCBI).

The 3' and the 5' genome Non Coding Region (NCRs) ends of the RVFV genomes were highly conserved with minimal changes from the consensus. For each segment, the consensus sequence consists of seven or more sequences sharing the same nucleotide at a given position and areas with no clear agreement are indicated with the nucleotide in red. The first 11 and last 11 nucleotides were identical except for single nucleotide differences between S segments of Baringo 035 (positions 5 and 8) and Tanzania 001 (at positions 9,10 and 11) (Fig. 4).

L segment reports single nucleotide changes in Garissa 008 (position 10) and Baringo 035 (position 6) (Fig 4).

At the 5' prime end on the other hand, the S and M segments were completely identical. The divergence occurred in the L segment. At positions 6396, 6397, 6398 and 6401, there were differences from the consensus in Malindi 032, Kilifi 006 and Baringo 035 (Fig 5).

Comparison of each of the genes among the isolates indicated a sequence identity that was over 98%. The sequence variation across all the segments was highest (1.9%) in M segment (Table 6), followed by S segment 1.7% (Table 3), and the least (1.4%) divergent was in L segment (Table 9). The average divergence in analysis carried out by region again displayed low diversity. Tanzanian strains which had the highest diversity displayed an average divergence of 0.8%, Garissa strains 0.7%, Baringo 0.6% and those from Kilifi and Malindi 0.6% (Tables 3, 6 and 9).

All the sequence distances and similarities (tables 3, 4, 6, 7, 9 and 10) were calculated using ClustalW in the Megalign module of the DNASTAR software.

S segment 3' Non Coding Region (nt 1-80)

```
                                  1                                                                            80
Consensus        3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Gar 004          3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Gar 008          3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Kil 006          3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Mal 032          3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Bar 032          3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Bar 035          3'   ACAAAAGGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
TZ 01            3'   ACACAAAAGACCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
TZ 02            3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Kenya 98         3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
Saudi 2000       3'   ACACAAAGACCCCCTAGTGCTTATCAAGTATATCATGGATTACTTTCCTGTGATATCTGTTGATTTGCAGAGTGGTCGT   5'
```

M segment 3' Non Coding Region (nt 1-80)

```
                              1                                                                            80
Consensus        3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
Gar 004          3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTTGGGTGTGTGAAGCGGTTATTAGAGTG   5'
Gar 008          3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
Kil 006          3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
Mal 032          3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
Bar 032          3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
Bar 035          3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
TZ 01            3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
TZ 02            3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
Saudi 2000       3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCAGTTATTAGAGTA   5'
Kenya 98         3'   ACACAAAGACGGTGCATTAAATGTATGTTTTATTGACAATTCTAATCTCGGTTCTGGTGTGTGAAGCGGTTATTAGAGTG   5'
```

L segment 3' Non-Coding Region (nt 1-80)

```
                  1                                                                            80
Consensus        3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Gar 004          3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Gar 008          3'   ACACAAAGGGGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Mal 032          3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Kil 006          3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Bar 032          3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Bar 035          3'   ACACACAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
TZ 01            3'   ACACAAAGGCGGCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGTTTTGTTAGAGTGCCAATCAA   5'
TZ 02            3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGCTTTGTTAGAGTGCCAATCAA   5'
Saudi 2000       3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGTTTTGTTAGAGTGCCAATCAA   5'
Kenya 98         3'   ACACAAAGGCGCCCAATCATGGATTCTATATTATCAAAACAGCTGGTTGACAAGACTGGTTTTGTTAGAGTGCCAATCAA   5'
```

Fig 4: Alignment of part of the 3' non coding region of S, M and L genome segments of the RVFV.

S segment 5′ Non Coding Region (nt 1613-1693)

```
                               1613                                                                            1693
Consensus             3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Gar 004               3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Gar 008               3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Kil 006               3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Mal 032               3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Bar 032               3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Bar 035               3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
TZ 01                 3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
TZ 02                 3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Saudi 2000            3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
Kenya 98              3′       TGAGCAGCAAACTGGATCGCAAGCTCTTGATAGTTGTCCATTATTGTAATAGTGTTTGTATCTCTAGGGAGCTTTGTGT    5′
```

M segment 5′ Non-Coding Region (nt 3841-3885)

```
                               3841                                        3885
Consensus             3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Gar 004               3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Gar 008               3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Kil 006               3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Mal 032               3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Bar 032               3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Bar 035               3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
TZ 01                 3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
TZ 02                 3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Saudi 2000            3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
Kenya 98              3′       GTTGGGAATTAAACTAACTCTTTGAAGTTGCACCGGTCTTTGTGT                5′
```

L segment 5′ Non-Coding Region (nt 6321-6404)

```
                               6321                                                                                 6404
Consensus             3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGTCTTTGTGT 5′
Gar 004               3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGTCTTTGTGT 5′
Gar 008               3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGTCTTTGTGT 5′
Mal 032               3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGGGGGTCTTTTGT 5′
Kil 006               3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGGTCTTTTGT 5′
Bar 032               3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGGGGTCTTTGTGT 5′
Bar 035               3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGGTCTTTTGT 5′
TZ 01                 3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGTCTTTGTGT 5′
TZ 02                 3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGGCTTTGTGT 5′
Saudi 2000            3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGTCTTTGTGT 5′
Kenya 98              3′       AGATTTAGGGATACTATGCTAGTATTGGAATCCATGTGGGTTCTGATACTAGCATAGTGCTACAATATTGGGCGGTCTTTGTGT 5′
```

Fig 5: Alignment of part of the 5′ non coding region of S, M and L genome segments of the RVFV.

3.2.2 Phylogenetic analysis of RVFV genome based on the S segment

The S segment (1690 nt) encodes a nucleocapsid protein N in the anti-viral sense and a non-structural protein NS in the viral sense (Bouloy 1991; Elliott *et al.,* 1991). Analysis for the 8 isolates and the two reference sequences from the gene bank was done based both on the whole nucleotide sequence, and the translated amino acid sequences

Comparison between whole nucleotide sequences of isolates from the different regions (2006 – 2007 RVFV outbreak) showed strains that were highly similar. The nucleotide sequence similarity ranged between 98.8% and 99.8% with a divergence that was as high as 1.7%. When the reference sequences from the genebank were included in the analysis, they showed a slight increase in their divergence from the 2006 -2007 outbreak. Their similarities decreased to as low as 98.8% while their divergence rose to a highest 1.7% (Table 3)

Table 3: Whole length Sequence distances of the S segment nucleotides

Strain No.	Strain	Gar 004	Gar 008	Kil 006	Mal 032	Bar 032	Bar 035	TZ 001	TZ 002	Saudi 2000	Kenya 98
1	Gar 004	***	99.1	99.1	98.8	99.1	98.8	98.5	99.1	98.3	98.9
2	Gar 008	0.9	***	99.4	99.1	99.4	99.1	98.3	99.4	98.4	99
3	Kil 006	0.9	0.6	***	99.6	99.8	99.7	98.6	99.9	98.8	99.3
4	Mal 032	1.2	0.9	0.4	***	99.5	99.3	98.3	99.6	98.5	99.1
5	Bar 032	1	0.7	0.2	0.5	***	99.5	98.6	99.8	98.7	99.3
6	Bar 035	1.2	0.9	0.3	0.7	0.5	***	98.3	99.6	98.5	99.1
7	TZ 001	1.6	1.7	1.4	1.7	1.4	1.7	***	98.6	98.6	99.1
8	TZ 002	0.9	0.6	0.1	0.4	0.2	0.4	1.4	***	98.8	99.3
9	Saudi 2000	1.7	1.6	1.3	1.6	1.3	1.5	1.4	1.3	***	99.4
10	Kenya 98	1.1	1	0.7	1	0.7	0.9	1	0.7	0.6	***

3.2.3 Analysis of the RVFV genome based on NSs protein

Pairwise comparison of the NSs protein of the 2 strains from Garissa revealed 7 differences at nucleotide level while those from the Kenyan coast (Kilifi and Malindi), were completely identical at the nucleotide level. The two strains from Baringo displayed a single nucleotide difference and those from Tanzania, 9 nucleotide differences amongst themselves.

There was a similarity range of between 98.4-100% and 98.1-100% at nucleotide and amino acid levels respectively (Table 4)

3.2.4 Analysis based on the N protein of the RVFV genome

Pairwise comparison of the N protein of the two strains from Garissa revealed four differences at nucleotide level while those from the Kenyan coast (Kilifi and Malindi), revealed two differences at the nucleotide level. Comparison of the two strains from Baringo came up with four nucleotide differences and those from Tanzania eight nucleotide differences amongst themselves. This comparative analysis was done on the regions that code for the N protein.

There was a sequence similarity range of between 98.4 – 99.7% and 98 – 100% at the levels of nucleotide and amino acids respectively (Table 4)

Table 4: Nucleotide and amino acid identity (%) of the RVFV genomic segment S and their predicted protein products.

S segment/ N protein		Gar 004	Gar 008	Kil 006	Mal 032	Bar 032	Bar 035	TZ 001	TZ 002	Saudi 2000	Kenya 98	
Strain												
Gar 004		***	99.2	98.4	98.4	98.4	97.6	98.4	98	98.4	98.4	
Gar 008		99.5	***	99.2	99.2	99.2	98.4	99.2	98.8	99.2	99.2	
Kil 006		99.2	99.5	***	100	100	99.2	100	99.6	100	100	
Mal 032		99.2	99.5	99.7	***	100	99.2	100	99.6	100	100	
Bar 032	Nucleotide	99.2	99.5	99.7	99.7	***	99.2	100	99.6	100	100	Amino Acid
Bar 035		98.9	99.2	99.7	99.5	99.5	***	99.2	98.8	99.2	99.2	
TZ 001		98.4	98.6	98.9	98.9	98.9	98.6	***	99.6	100	100	
TZ 002		99.2	99.5	99.7	99.7	99.7	99.5	98.9	***	99.6	99.6	
Saudi 2000		98.4	98.6	98.9	98.9	98.9	98.6	98.9	98.9	***	100	
Kenya 98		98.6	98.9	99.2	99.2	99.2	98.9	99.2	99.2	99.7	***	
S segment/ NSs protein												
Strain												
Gar 004		***	98.9	98.5	98.5	98.1	98.5	99.2	98.5	97.7	98.9	
Gar 008		99.1	***	99.6	99.6	99.2	99.6	99.6	99.6	98.9	100	
Kil 006		99	99.9	***	100	99.6	100	99.2	100	98.5	99.6	
Mal 032		99	99.9	100	***	99.6	100	99.2	100	98.5	99.6	
Bar 032	Nucleotide	98.9	99.7	99.9	99.9	***	99.6	98.9	99.6	98.1	99.2	Amino Acid
Bar 035		99	99.9	100	100	99.9	***	99.2	100	98.5	99.6	
TZ 001		99.1	99	98.9	98.9	98.7	98.9	***	99.2	98.5	99.6	
TZ 002		99	99.9	100	100	99.9	100	98.9	***	98.5	99.6	
Saudi 2000		98.4	98.7	98.6	98.6	98.5	98.6	98.5	98.6	***	98.9	
Kenya 98		99.2	99.6	99.5	99.5	99.4	99.5	99.4	99.5	99.1	***	

From the predicted amino acid residues shown in Table 5 below, the Garissa strains have similar deviations from the rest of the strains at positions 237 and 238. They display threonine and serine at position 237 and 238 respectively, whereas the rest of the strains display alanine in the N gene. In the NSs gene, at position 202, both the Garissa strains and one Tanzanian strain have lysine while all the rest have arginine at the same position (Table 5).

Table 5: Amino Acid residue differences between the 2006 – 07 RVF outbreak strains and the Kenya 98 outbreak strain in the S genes.

Gene	Amino Acid Position	Kenya 9800523	Gar 004	Gar-008	Kil-006	Mal-032	Bar-032	Bar-035	TZ 001	TZ 002
N	45	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	**His**
	138	Pro	Pro	Pro	Pro	Pro	Pro	**His**	Pro	Pro
	147	Pro	**Ser**	Pro	Pro	Pro	Pro	Pro	Pro	Pro
	188	Thr	Thr	Thr	Thr	Thr	Thr	**Ser**	Thr	Thr
	237	Ala	**Thr**	**Thr**	Ala	Ala	Ala	Ala	Ala	Ala
	238	Ala	**Ser**	**Ser**	Ala	Ala	Ala	Ala	Ala	Ala
	242	Lys	**Gln**	Lys	Lys	Lys	Lys	Lys	Lys	Lys
NSs	153	Asn	Asn	Asn	Asn	Asn	**Lys**	Asn	Asn	Asn
	186	Met	**Val**	Met	Met	Met	Met	Met	**Val**	Met
	202	**Lys**	**Lys**	**Lys**	Arg	Arg	Arg	Arg	**Lys**	Arg
	206	Ser	**Ala**	Ser	Ser	Ser	Ser	Ser	Ser	Ser
	208	Ala	**Pro**	Ala	Ala	Ala	Ala	Ala	Ala	Ala

3.2.5 Analysis of the M segment of the RVFV genome

The M segment (3885 nt) encodes for four proteins. Two major envelope glycoproteins, Gn (or G1) and Gc (or G2), which bind to a viral receptor molecule to initiate virus infection. It also encodes two non minor proteins, the 14 kDa non structural NSm protein and the 78kDa protein that is reported to be a structural protein (Struthers *et al.,* 1984).

The Open Reading Frame (ORF) of the M segment is 3594 nucleotides long. Based on this region, the strains from Garissa had 22 nucleotide differences between them. Those from the Kenyan coast revealed 6 nucleotide differences while those from Baringo revealed 10 nucleotide differences. The Tanzanian strains were almost completely identical except for 1 nucleotide difference between them.

Analysis was carried out based on the nucleotide alignment of the whole segment which resulted in divergence range of between 0.1 – 1.9% (Table 6). Basing the alignment on the open reading frame, a similarity range of 98.2 – 100% and 98.3 – 100% at the nucleotide and amino acid levels respectively was recorded for all isolates (Table 7).

At amino acid positions 172 and 173, strains from Malindi, and the two from Baringo show a difference by having arginine and leucine respectively as the amino acid while all the other strains have methionine and threonine. At amino acid positions 235 and 238, the strains from the Kenyan coast (Malindi and Kilifi)

and those from Baringo, have amino acids; aspartic acid and lysine respectively while the strains have histidine (position 235) and proline (position 238) (Table 8). The two strains from Tanzania are identical at amino acid positions 534, 572, 573, 574, 576, 577 and 579. This similarity is not shared by the other strains (Table 8).

Table 6: Whole segment sequence distances of the M segment nucleotides

Strain No.	**Strain**	Gar 004	Gar 008	Kil 006	Mal 032	Bar 032	Bar 035	TZ 001	TZ 002	Saudi 2000	Kenya 98
1	Gar 004	***	99.4	99.4	99.2	99.2	99.2	99.1	99.3	98.2	98.4
2	Gar 008	0.6	***	99.6	99.4	99.4	99.4	99.4	99.5	98.5	98.7
3	Kil 006	0.6	0.4	***	99.6	99.7	99.6	99.3	99.5	98.5	98.7
4	Mal 032	0.9	0.6	0.4	***	99.6	99.7	99.1	99.2	98.2	98.5
5	Bar 032	0.8	0.6	0.3	0.4	***	99.6	99.1	99.3	98.2	98.5
6	Bar 035	0.9	0.6	0.4	0.3	0.4	***	99.2	99.3	98.3	98.6
7	TZ 001	0.9	0.6	0.7	0.9	0.9	0.8	***	99.9	98.3	98.6
8	TZ 002	0.8	0.5	0.5	0.8	0.8	0.7	0.1	***	98.3	98.5
9	Saudi 2000	1.9	1.6	1.6	1.8	1.8	1.7	1.7	1.7	***	99.6
10	Kenya 98	1.6	1.3	1.3	1.5	1.5	1.5	1.5	1.5	0.4	***

Table 7: Nucleotide and amino acid identity (%) of the RVFV genomic segment M and their predicted protein products.

M segment/ M polyprotein		Gar 004	Gar 008	Kil 006	Mal 032	Bar 032	Bar 035	TZ 001	TZ 002	Saudi 2000	Kenya 98	
Strain												
Gar 004		***	98.8	98.9	98.8	98.7	98.7	98.9	98.9	98.3	98.4	
Gar 008		99.4	***	99.2	99.2	98.9	99.1	98.9	98.9	98.7	98.7	
Kil 006		99.3	99.6	***	99.7	99.5	99.7	99.2	99.2	98.9	99	
Mal 032		99.3	99.6	99.8	***	99.7	99.9	99.1	99.1	98.8	98.9	
Bar 032	Nucleotide	99.2	99.4	99.7	99.8	***	99.7	98.8	98.8	98.6	98.7	Amino Acid
Bar 035		99.2	99.5	99.7	99.9	99.7	***	99	99	98.7	98.8	
TZ 001		99.2	99.4	99.4	99.3	99.2	99.3	***	100	98.6	98.7	
TZ 002		99.2	99.5	99.4	99.4	99.2	99.3	100	***	98.6	98.7	
Saudi 2000		98.2	98.5	98.5	98.4	98.3	98.3	98.3	98.3	***	99.6	
Kenya 98		98.5	98.8	98.8	98.7	98.6	98.6	98.6	98.6	99.6	***	

Table 8: Amino Acid residue differences between the 2006 – 07 RVF outbreak strains and the Kenya 98 outbreak strain in the M genes

Gene	Amino Acid Position	Kenya 9800523	Ken/Gar-004/06	Ken/Gar-008/06	Ken/Kil-006/07	Ken/Mal-032/07	Ken/Bar-032/07	Ken/Bar-035/07	Tan/Tan-001/07	Tan/Tan-002/07
NS-M	12	Leu	**Trp**	Leu	Leu	Leu	Leu	Leu	Leu	Leu
	114	Ile	Ile	Ile	**Val**	Ile	Ile	Ile	Ile	Ile
	123	**Thr**	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
G1 (Gn)	172	Met	Met	Met	Met	**Arg**	**Arg**	**Arg**	Met	Met
	173	Thr	Thr	Thr	Thr	**Leu**	**Leu**	**Leu**	Thr	Thr
	235	His	His	His	**Asp**	**Asp**	**Asp**	**Asp**	His	His
	238	Pro	Pro	Pro	**Lys**	**Lys**	**Lys**	**Lys**	Pro	Pro
	352	Lys	**Gln**	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	410	Ser	**Arg**	Ser	Ser	Ser	Ser	Ser	**Arg**	**Arg**
	413	Cys	**Arg**	Cys	Cys	Cys	Cys	Cys	Cys	Cys
	436	His	**Arg**	His	His	His	His	His	His	His
	449	Gly	**Arg**	**Arg**	Gly	Gly	Gly	Gly	Gly	Gly
	534	Gly	Gly	Gly	Gly	Gly	**Val**	Gly	Gly	Gly
	572	His	His	His	His	His	His	His	**Gln**	**Gln**
	573	Gly	Gly	Gly	Gly	Gly	Gly	Gly	**Ala**	**Ala**
	574	Leu	Leu	Leu	Leu	Leu	Leu	Leu	**Trp**	**Trp**
	576	Asn	Asn	Asn	Asn	Asn	Asn	Asn	**Ile**	**Ile**
	577	Tyr	**Asn**	Tyr	Tyr	Tyr	Tyr	Tyr	**Ser**	**Ser**
	579	Cys	**Ser**	Cys	Cys	Cys	Cys	Cys	**Trp**	**Trp**
	581	Thr	**Ser**	Thr	Thr	Thr	Thr	Thr	Thr	Thr
	589	**Val**	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
	595	**Ile**	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
	602	**Ile**	Val	Val	Val	Val	Val	Val	Val	Val
	659	**Val**	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala

G2 (Gc)	717	Gly	Gly	Gly	Gly	Gly	**His**	Gly	Gly	Gly
	724	Gly	Gly	Gly	Gly	Gly	Gly	**Arg**	Gly	Gly
	942	**Asn**	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
	954	**Ile**	Val	Val	Val	Val	Val	Val	Val	Val
	1014	Met	**Arg**	Met	Met	Met	**Arg**	Met	Met	Met
	1018	Phe	Phe	**Leu**	Phe	Phe	Phe	Phe	Phe	Phe
	1019	Glu	Glu	**Lys**	Glu	Glu	Glu	Glu	Glu	Glu
	1066	**Glu**	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp
	1094	Glu	Glu	**Lys**	Glu	Glu	Glu	Glu	Glu	Glu
	1107	Pro	Pro	**Ala**	Pro	Pro	Pro	Pro	Pro	Pro
	1110	Val	Val	**Met**	Val	Val	Val	Val	Val	Val

3.2.6 Analysis of the RVFV genome based on the L segment

L Segment (6404 nt) encodes the L protein, a viral RNA – dependent RNA polymerase (Struthers *et al.,* 1984). The ORF of the L segment is 6279 nucleotides long.

Based on this region, the Garissa strains had 29 nucleotide differences between them, Kilifi and Malindi had 6 differences, Baringo strains 11 differences and the strains from Tanzania 68 differences at nucleotide level.

The whole segment analysis generated similarities that ranged between 98.6 and 99.9% with a divergence that was as high as 1.3% (Table 9). An analysis of the region that codes for the viral RNA – dependent RNA polymerase revealed homology ranges of 98.7 – 99.9% and 99.2 – 99.9% at nucleotide and amino acids levels respectively. (Table 10)

Table 9: Whole segment sequence distances of the L segment nucleotides

Strain No.	**Strain**	Gar 004	Gar 008	Kil 006	Mal 032	Bar 032	Bar 035	TZ 001	TZ 002	Saudi 2000	Kenya 98
1	Gar 004	***	99.5	99.7	99.7	99.7	99.6	98.8	99.7	98.7	98.9
2	Gar 008	0.5	***	99.6	99.6	99.6	99.6	98.7	99.6	98.6	98.8
3	Kil 006	0.3	0.4	***	99.9	99.8	99.8	98.9	99.8	98.8	99
4	Mal 032	0.3	0.4	0.1	***	99.7	99.8	98.9	99.8	98.8	98.9
5	Bar 032	0.3	0.4	0.2	0.3	***	99.7	98.8	99.8	98.7	98.9
6	Bar 035	0.4	0.4	0.2	0.2	0.3	***	98.8	99.8	98.7	98.9
7	TZ 001	1.2	1.3	1.1	1.2	1.2	1.2	***	98.9	99	99.1
8	TZ 002	0.3	0.4	0.2	0.2	0.2	0.2	1.1	***	98.8	99
9	Saudi 2000	1.3	1.4	1.2	1.3	1.3	1.3	1.1	1.2	***	99.7
10	Kenya 98	1.2	1.2	1.1	1.1	1.1	1.1	0.9	1	0.3	***

Table 10: Nucleotide and amino acid identity (%) of the RVFV genomic segment L and their predicted protein products.

L segment/ L polyprotein		Gar 004	Gar 008	Kil 006	Mal 032	Bar 032	Bar 035	TZ 001	TZ 002	Saudi 2000	Kenya 98	
Strain												
Gar 004		***	99.4	99.7	99.7	99.7	99.6	99.5	99.6	99.3	99.5	
Gar 008		99.5	***	99.6	99.6	99.6	99.5	99.4	99.5	99.2	99.4	
Kil 006		99.7	99.7	***	99.9	99.9	99.8	99.7	99.8	99.5	99.7	
Mal 032		99.8	99.7	99.9	***	99.9	99.8	99.7	99.8	99.5	99.7	
Bar 032	Nucleotide	99.7	99.6	99.8	99.8	***	99.8	99.6	99.8	99.4	99.6	Amino Acid
Bar 035		99.7	99.6	99.8	99.9	99.8	***	99.6	99.8	99.4	99.6	
TZ 001		98.8	98.7	98.9	99	98.9	98.9	***	99.6	99.7	99.8	
TZ 002		99.7	99.7	99.9	99.9	99.8	99.9	98.9	***	99.4	99.6	
Saudi 2000		98.7	98.6	98.8	98.8	98.7	98.8	98.9	98.8	***	99.8	
Kenya 98		98.9	98.8	99	99	98.9	99	99.1	99	99.7	***	

CHAPTER 4

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

RVFV were sequenced for two reasons. First, to determine the genetic diversity of the RVFV isolates from the different geographical regions of the 2006 – 07 outbreak. Second, to determine whether the sequential outbreaks that occurred were caused by the same virus, or they were different strains, resident in these regions and only came into activation after the heavy rains and flooding.

This study revealed that there was a high level of sequence homology at both nucleotide and amino acid level among the RVFV isolates and also with the reference strains (Tables 4, 7 and 10). In comparison to the reference strains, the translated number of amino acids was equal among the strains from the 2006-07 RVF outbreak from the different geographical regions. There was no significant variation in length in all the segments when compared to the historic strains. Again, there were no variations at the deduced amino acid length of both the NP (245 amino acids), or NSs (264 amino acids) of the S segment.

Our findings were in agreement with a study conducted by Bird *et al.,* (2007) that also found a high level of conservation at both nucleotide and amino acid level, the

highest sequence disparity being 5% at nucleotide level and 2% at amino acid level. These findings were also in line with an earlier study which found a divergence ranging from 0 to 9.6% at the nucleotide level which corresponded to 0 to 9.5 % at the amino acid level (Sall *et al.,* 1997). It is important to note that the study by Bird *et al.,* (2007) encompassed 33 samples collected from 1944 to 2000 from throughout Africa including strains differing in pathogenecity in disease models. The study by Sall *et al.,* (1997) was on samples collected over a period of 38 years from diverse localities, from different hosts and the analysis was based on the NSs coding region of the S segment. The finding of relative conservation of the RVFV genome over 75 years since the virus was first isolated defy the expectation that selective pressure would have molded the evolutionary patterns of the virus, particularly in the G1 and G2 glycoproteins that are the targets for neutralizing antibodies.

There were differences among isolates from the same region but, at the same time there were similarities in the strains that were not observed in all the other strains. In the S segment, the strains from Garissa were different at four positions but similar at two positions, a similarity that was absent in all the other strains. In the M segment, the strains from Baringo showed 4 amino acid similarities, which were lacking in all the other strains except for Mal 032. At positions 172, 173, 235 and 238 there was an amino acid similarity among the strains from Baringo and the Malindi strain but this similarity extended to Kil 006 at positions 235 and 238.

The isolates from Tanzania were similar to Gar 004 at amino acid positions 410. At position 572, 573, 574, 576, 577 and 579 the strains from Tanzania were completely identical (Table 8); however, at these same positions they were completely different from all the other strains. At positions 352, 413, 436, 577, 579 and 581 of the M segment, the Garissa 004 was completely different from all the other isolates in the amino acid make up (Table 8). The comparative analysis of the ends (both the 3' and 5') of the RVFV genome displayed highly conserved ends as is shown in figures 6 and 7.

There was evidence of the mentioned difference above from the sequence distances and similarities at both nucleotide and amino acid levels. The evidence displayed by similarity and divergence figures in strains from the same region versus those from different regions was similar distances. For example, at nucleotide level, there was an average similarity of 99.2% between Garissa isolates and Baringo isolates while the same Garissa isolates had an average similarity of 98.1% when compared to Tanzania. In general, viruses isolated at about the same time from different persons within any local outbreak have identical or very similar oligonucleotide maps whereas viruses from earlier or later outbreaks exhibit increasing divergence (Holland *et al.,* 1982). In a virus isolated from one outbreak and with very low historic levels of divergence (highly conserved), the expectation would be close to 100% identities between all the strains sequenced if the same strain caused the outbreaks. However, there were

changes in the nucleotide and amino acid sequences that were unique to isolates from the same region. This indicated that the isolates were not of the same origin.

These differences are also evidenced in the sequence distances. A single isolate from one region would display an almost equal sequence homology to both isolates from another region, e.g. In the N protein of the S segment, Tanzania 001 reveals a sequence homology of 98.4 and 98.6 to Gar 004 and Gar 008 respectively at nucleotide level.

Further comparison was done between the 2006-2007 outbreak strains and historical strains that caused outbreaks in Mauritania and 1987 and in Egypt in 1977-1978. These were naive RVF ecologies which had never experienced a RVF outbreak prior to these time lines. The Egyptian 1977-1978 strains had a diversity of 0.34% in the S segment, 0.21 in the M segment, and 0.2 in the L segment. The strains from Mauritania had a lower diversity with of 0.1, 0.17 and 0.08 in S, M, and L segments respectively. The 2006-2007 outbreak strains were more divergent with distances of 0.87 in the S segment, 0.61 in M segment and 0.52 in the L segment. The strains that caused the 2006-2007 outbreak displayed differences amongst themselves; greater differences that were not evident in strains that caused the Egyptian and Mauritanian. When the analysis was done to include strains from Egypt and Mauritania, the differences that came up (highest divergences of 0.34 and 0.1 respectively) were not as high as those displayed by the 2006-2007 outbreak isolates. The Egyptian outbreak had a greater disease margin but less divergent in the isolated strains. The Mauritania strains were also

less divergent in comparison to the 2006-2007 outbreak. These two historic outbreaks give evidence to a virus that is conserved in one single outbreak unlike the 2006-2007 outbreak which was showed a great difference in the strains isolated. This goes on to indicate a virus that was not similar even though occurring in the same period (2006-2007) supporting de novo activation of an RVFV strain that was resident in the different regions.

4.2 Conclusion

The results generated from this study show clearly that the RVFV isolates from the outbreak in different locales were not the same genetically. There were differences that occurred in the nucleotide and amino acid sequences. These were changes that were unique and specific to isolates from the same region and were not observed in the isolates from other regions. If the same virus had spread across all the regions, then the expected outcome was similar changes in the sequences that were analysed which was not the case. These findings confirm that RVFV is endemic in certain regions and that preventative measures immediately after RVF risk forecasting is probably the best approach to either pre-empting an outbreak or reducing the effects of an outbreak. RVF outbreaks can be forecasted with significant accuracy using global weather patterns, and this information can be released as early as 2 – 4 months before an outbreak. This time may be used to institute various control measures including vaccination of livestock and high-risk humans, vector control, and public education.

The different case fatalities observed in these regions may be attributed to the unique amino acid changes that were detected. Even though this study was not designed in a manner to determine the virulence of the different strains, the amino acid changes may have resulted also in the encephalopathy syndrome observed in the Tanzanian cases.

The low case fatalities in Kenya could also be as a result of a passive surveillance system that is conducted in the country. As a result of this, it could be possible that

the surveillance system was able to pick up RVF cases in Kenya early in the outbreak as compared to the other countries that were affected. As a result, resources were mobilized early in the outbreak and thus leading to lower case fatalities.

4.3 Recommendations

The finding in this and other studies that RVFV is highly conserved suggest that vaccination of livestock and humans would be an effective control measure. Any safe and efficacious vaccine is likely to work for years and in diverse geographical regions given that the virus does not change significantly.

Two modes of transmission by the mosquito having been quoted (ovarial and mechanical transmission). Therefore protection of humans from mosquitoes should be emphasized.

Monitoring of rainfall patterns and abundance of mosquito populations are able to aid in predicting the circulation of the virus. Therefore surveillance activities and virus characterization should be continuously conducted.

The virus is resident in different regions and with the evidence provided in this work, the virus does not spread but rather depends on the activation of the dormant virus after heavy rains, and therefore surveillance, vaccination and vertebrate protection from arthropods should all go hand in hand towards protection and control of the disease.

Even though these viruses segregate according to regions (Appendix 7), the role of transmission routes other than mosquito vectors, once an epizootic has occurred should not be downplayed and public health issues and dissemination about the disease should be emphasized.

Analytical studies should be carried out between the different arthropod and vertebrate hosts that are implicated during an epizootic to determine whether the

viral strains involved are the same or different. These studies should span a longer time period, geographical range and a wider variety of species.

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APPENDICES

Appendix 1: Culture Medium for Vero Cells

The medium used was the modified minimum essential medium developed in 1957 by H. Eagle from Sigma Aldrich co. The cells were grown in Growth Medium (GM), a high nutrient medium, which constituted 500mls of minimum essential medium supplemented with 10% fetal bovine serum – 50mls, 2% L-glutamine – 10mls, 1% antibiotic/antimycotic – 5mls. A low nutrient medium (Maintenance Medium) was also prepared to maintain the cells without overgrowing. The maintenance medium (MM) was made up of 500mls of minimum essential medium supplemented with 2% fetal bovine serum – 10mls, 2% L-glutamine – 10mls, 2% antibiotic/antimycotic – 10mls.

Appendix 2: Vero Cells Sub-culturing

A fully confluent flask of T150 ($150cm^2$), was passaged by decanting the medium, then washing 2-3 times with 3mls of sterile phosphate buffered saline (PBS), pre-warmed to 37°C in a water bath.

For detaching the cells from the substratum/monolayer, 0.5mls of pre-warmed trypsin (37°C) was added to the flask, gently rocked to ensure that it covered the whole monolayer surface, then incubating it for 3 minutes in a 5% CO^2 incubator at 37°C. By sharply tapping the surface of the flask with the monolayer, the cells detached and when viewed under an inverted light microscope, the cells could be

seen floating. Since over exposure of the cells to trypsin could be detrimental, 5mls of growth media were added after 30 seconds to stop the action of trypsin. Purging was performed by pipetting up and down the contents of the flask. This ensured complete breakup of cell wall to cell wall contact and cell wall to flask surface contact. For passaging in a ratio of 1:4, GM was topped up to 20mls then pipetting 5mls of cell suspension into 4 T25 ($25cm^2$) flasks. These were labeled with the cell type, date, and the passage number. These vented flasks were tightly closed, and incubated at 37°C in a 5% CO^2 incubator. The cells were observed daily and when the monolayer was about 90% confluent, they were inoculated with the patient serum. All the above procedures were carried out in a level 3 biosafety cabinet to ensure the safety of the laboratory personnel and to maintain aseptic conditions.

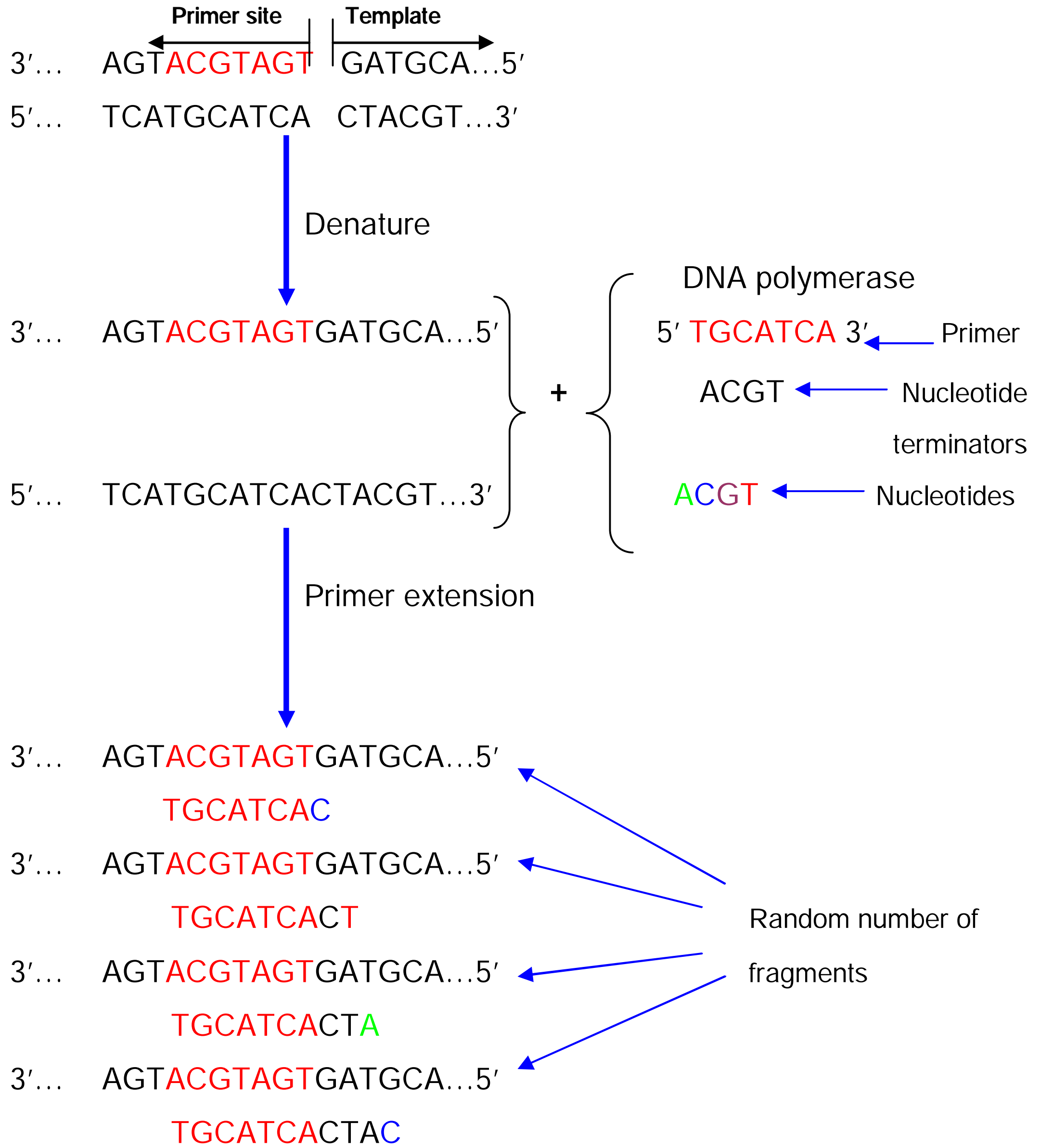
Appendix 3: Inoculation of Vero cells with patient serum

Inoculation of patient serum was done when the Vero cells were about 90% confluent. The medium was first decanted and the cells washed twice with 2-3mls of sterile PBS that had been pre-warmed to 37°C. The inoculums constituents were 100ul serum sample and 300ul of Maintenance Media (Minimum Essential Media). The inoculum and the cells were incubated at 37ºC in a CO^2 incubator and were rocked after every 15 minutes for 1 hour (adsorption phase). This was to allow the virus to attach to the cells.

Appendix 4: Sanger's principle

The method makes use of the 2′,3′-dideoxy and arabinonucleoside analogs of the normal deoxynucleoside triphosphates, which act as specific chain-terminating inhibitors of DNA polymerase. Because the ddT contains no 3′-hydroxyl group, the chain cannot be extended further, so that termination occurs specifically at position where dT should be incorporated. If a primer and template are incubated with DNA polymerase in the presence of a mixture of ddTTPs and dTTPs, as well as the other three deoxyribonucleoside triphosphates, a mixture of fragments all having the same 5′ and with ddT residues at the 3′ ends is obtained. When this mixture is fractionated by electrophoresis on denaturing acrylamide gels the pattern of bands shows the distribution of dTs in the newly synthesized DNA. By using analogous terminators for the other nucleotides in separate incubators and running the samples in parallel on the gel, a pattern of bands is obtained from which the sequence can be read off, (Atkinson *et al*., 1969; Sanger *et al.,* 1977).

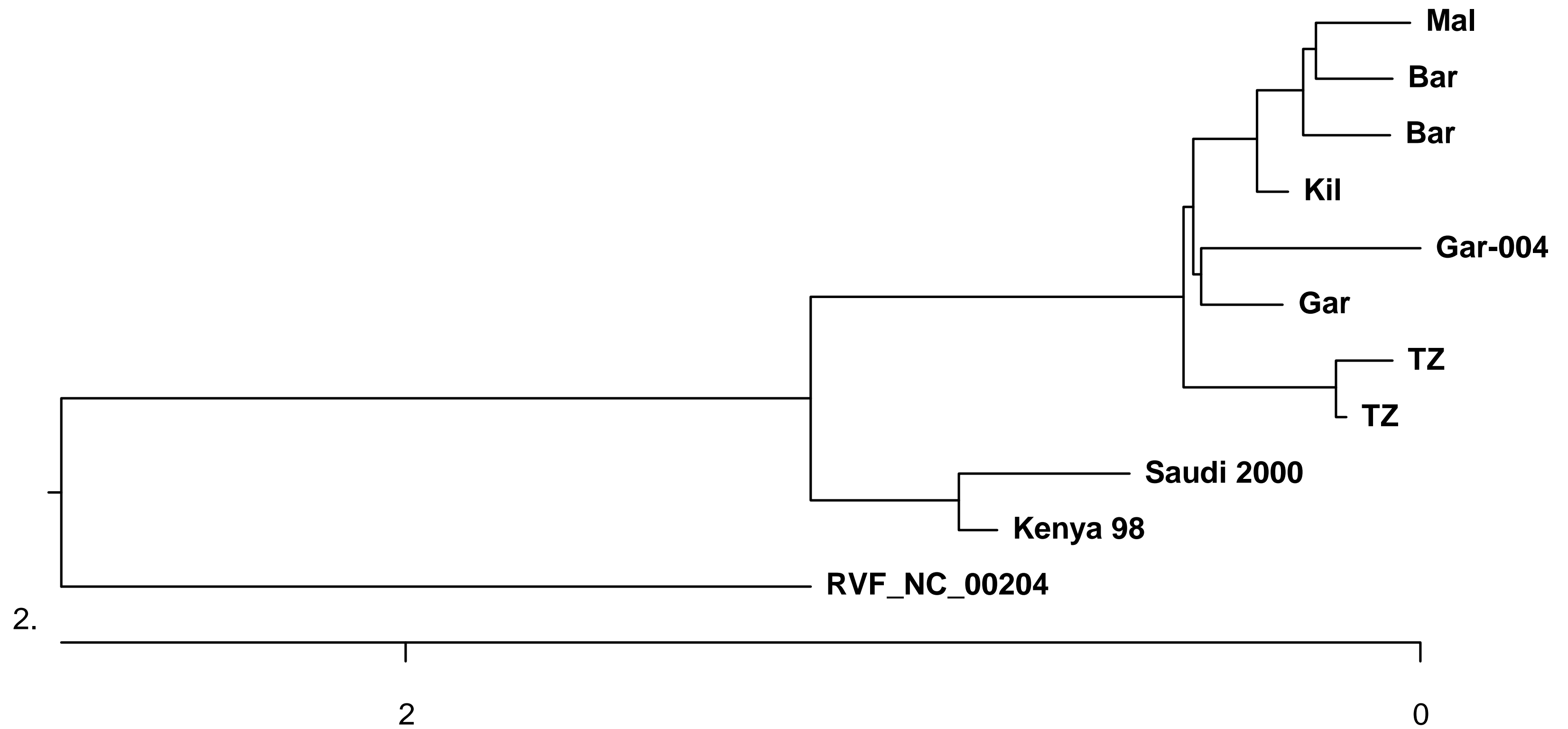
Appendix 5: Illustration of Sanger Method



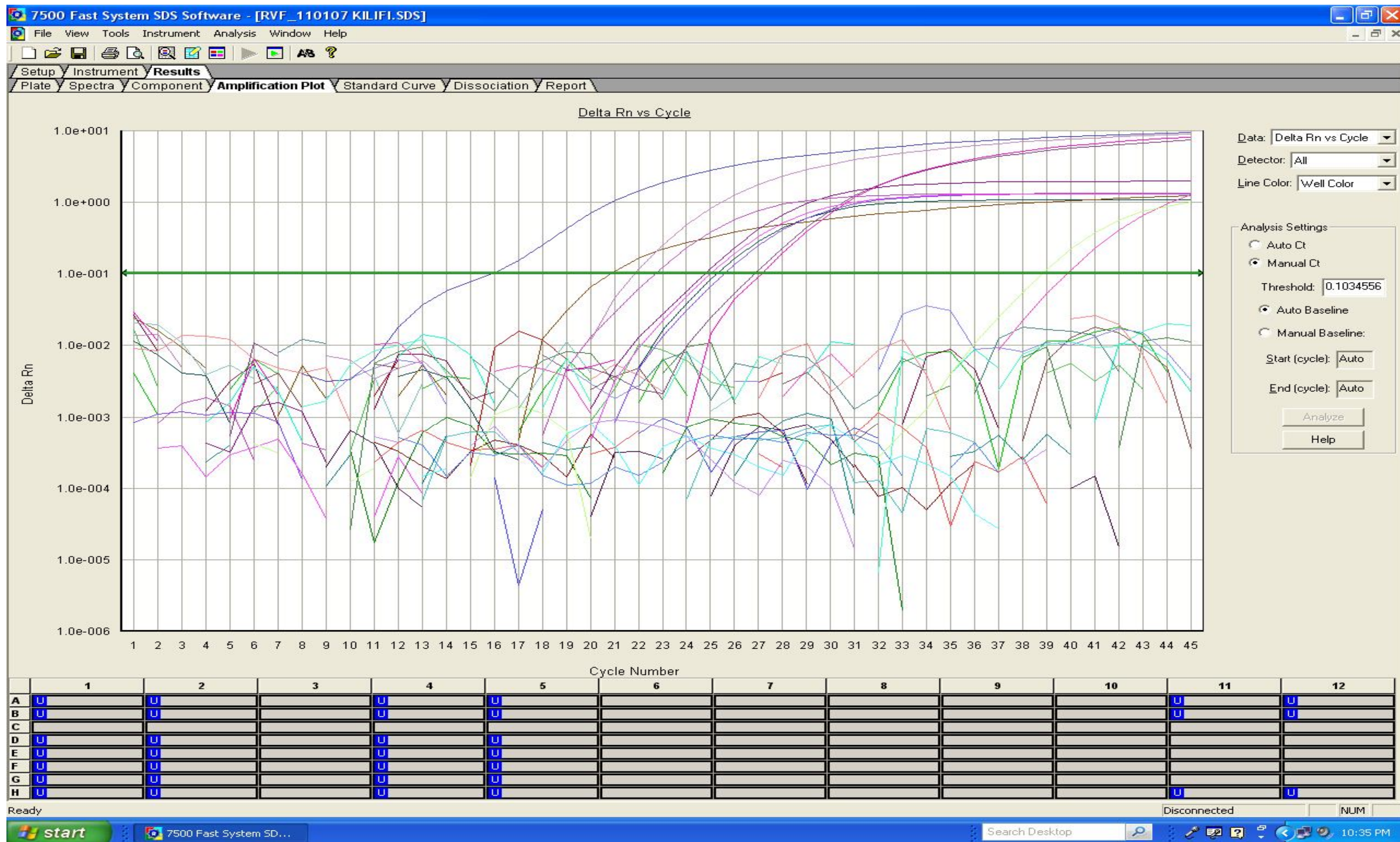
Appendix 6: Amino acid codes.

Amino acid	Three letter code	One letter code
Alanine	ala	A
arginine	arg	R
asparagine	asn	N
Aspartic acid	asp	D
asparagine or aspartic acid	asx	B
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glutamine or glutamic acid	glx	Z
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S
threonine	thr	T
tryptophan	try	W
tyrosine	tyr	Y
valine	val	V

Appendix 7: Phylogenetic tree of the M segment based on nucleotide sequences



Appendix 8: Raw real time RT-PCR data from Coast province samples, Kenya.



Appendix 9: Real Time PCR report from Coast province, Kenya.

Well	Sample Name	Detector	Task	Ct Value
A1	1	FAM	Unknown	Undetermined
A2	2	FAM	Unknown	Undetermined
A4	3	FAM	Unknown	Undetermined
A5	4	FAM	Unknown	Undetermined
A11	RVF -ve ctrl	FAM	Unknown	Undetermined
A12	RVF +ve ctrl	FAM	Unknown	15.99
B1	5	FAM	Unknown	Undetermined
B2	6	FAM	Unknown	Undetermined
B4	7	FAM	Unknown	25.22
B5	8	FAM	Unknown	Undetermined
B11	RVF -ve ctrl	FAM	Unknown	Undetermined
B12	RVF +ve ctrl	FAM	Unknown	20.84
D1	9	FAM	Unknown	Undetermined
D2	10	FAM	Unknown	Undetermined
D4	11	FAM	Unknown	Undetermined
D5	12	FAM	Unknown	Undetermined
E1	13	FAM	Unknown	Undetermined
E2	14	FAM	Unknown	26.81
E4	15	FAM	Unknown	Undetermined
E5	16	FAM	Unknown	24.73
F1	17	FAM	Unknown	Undetermined
F2	18	FAM	Unknown	21.81
F4	19	FAM	Unknown	Undetermined
F5	20	FAM	Unknown	22.65
G1	21	FAM	Unknown	Undetermined
G2	22	FAM	Unknown	27.09
G4	23	FAM	Unknown	Undetermined
G5	24	FAM	Unknown	25.51
H1	25	FAM	Unknown	Undetermined
H2	26	FAM	Unknown	39.85
H4	27	FAM	Unknown	Undetermined
H5	28	FAM	Unknown	25.01
H11	RNP -ve ctrl	FAM	Unknown	Undetermined
H12	RNP +ve ctrl	FAM	Unknown	38.8