GENERATION OF MONOCLONAL ANTIBODY AGAINST RIFT VALLEY FEVER VIRUS AND EVALUATION OF ITS DIAGNOSTIC POTENTIAL

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Generation of monoclonal antibody against Rift Valley fever virus and evaluation of its diagnostic potential

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DECLARATION

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

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DEDICATION

To my:

Late father, Mahfudh Ashur, Mother, Zahra Karama, siblings and the entire Shuwaleh family.

Husband Amer Ali and daughter Maryam Amer.

It is through your encouragement, love, care and ever ending support that I achieved this.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACUC	Animal Care and Use Committee
BALB/c	Bagg's Albino ("c" represents the gene coding for "color" in the mouse)
CBB	Coomasie brilliant blue
CDC	Centers for Disease Control and Prevention
CHKV	Chikungunya virus
CPE	Cytopathic effect
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
ELISA	Enzyme Linked Immunosorbent Assay
ENSO	El Niño/Southern Oscillation
ERC	Ethical Review Committee
FCS	Fetal calf serum
FRNT	Focus reduction neutralization test
GM	Growth medium
HAT	Hypoxanthine-aminopterin-thymidine
HGPRT	Hypoxanthine Guanine Phosphoribosyl Ttransferase
HRP	Horse Radish Peroxidase

HT	Hypoxanthine-Thymidine
ICF	Infected culture fluid
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KEMRI	Kenya Medical Research Institute
LC-MS-MS	Liquid chromatography-mass spectrophotometry mass spectrophotometry
L-Gln PS	L-Glutamine with Penicillin and Streptomycin
mAb	Monoclonal antibody
MM	Maintenance medium
MEM	Minimum Essential Medium
NEAA	Non-essential amino acids
OD	Optical Density
ORF	Open reading frame
pAb	Polyclonal antibody
PBS-T	Phosphate Buffered Saline with 0.05% Tween 20
PEG	Polyethylene Glycol
RdRp	RNA-dependent RNA polymerase
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription-polymerase chain reaction

RT-LAMP	Reverse transcription-loop mediated isothermal amplifictaion
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
SSC	Scientific Steering Committee
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus

ABSTRACT

Rift Valley fever virus (RVFV) belongs to the family *Bunyaviridae* and genus *Phlebovirus* and it causes Rift Valley fever (RVF) which is one of the major viral hemorrhagic fevers in Kenya and Africa as a continent. RVFV is transmitted by *Aedes* and *Culex* mosquito bites and contact with infected body fluids including blood and milk. Due to the presence of other Bunya viruses in Kenya that have the same symptoms as RVFV (like Crimean-Congo Hemorrhagic Fever Virus and Nairobi Sheep Disease Virus), there is a need for RVFV specific diagnostic tests. This may be achieved by use of monoclonal antibodies (mAbs) against RVFV to set up diagnostic systems, considering that mAbs are currently widely used not only for research purposes, but also for diagnostics and therapeutic purposes. This study was aimed at developing mAbs against RVFV and their diagnostic potential validated by use of the mAbs in ELISA systems. BALB/c mice were immunized with purified, inactivated RVFV (attenuated live vaccine strain: Smithburn) and the rise in IgG titers was tested by IgG indirect ELISA. When the IgG titers were high enough, the mouse spleen was harvested and splenocytes were fused with mouse myeloma SP2 cells. Four clones of anti-RVFV mAbs were successfully established. Among them, clone "P1E5" was further cultured in large scale and purified by protein G column. The purified mAb was characterized by western blot, focus reduction neutralization test (FRNT), and isotyping. For determining its diagnostic potential, the purified mAb was applied in antigen detection ELISA and antigen capture IgG indirect ELISA as the capture antibody. The antigen detection ELISA was used to test the cross reactivity of the anti-RVFV P1E5 mAb to other arboviruses; dengue virus serotypes 1-4 (DENV 1-4), yellow fever virus (YFV), West Nile virus (WNV) and chikungunya virus (CHIKV). At the same time, the antigen capture IgG indirect ELISA was also used to test 22 human serum samples. The results obtained were compared to two other similar ELISA systems that used mAb against the nucleocapsid (N) protein of RVFV and anti-RVFV polyclonal antibodies (pAbs) as the capture antibodies. When applied in western blot, the mAb could detect RVFV and

specifically reacted with L segment derived protein (250kDa). The mAb did not show any neutralizing activity in the FRNT. Upon isotyping, the mAb was found to be IgG1 and carrying the Kappa light chain. When the P1E5 mAb was applied in antigen detection ELISA it specifically detected RVFV but not DENV 1-4, YFV, WNV and CHIKV. In the antigen capture IgG indirect ELISA, the mAb could detect IgG from patient sera that were known to have anti-RVFV IgG. The results obtained were in agreement with two other ELISA systems that used mAb against the N protein of RVFV and anti-RVFV pAbs as the capture antibodies. The anti-RVFV mAb was successfully applied in antigen detection ELISA and antigen-capture IgG indirect ELISA. Results from this project demonstrate the potential for development of a platform for RVF diagnosis in Kenya, using immunoreagents that can be produced locally. At the same time, this project has contributed in setting up the technology of mAb development in a local laboratory; hence the technology can be transferred and applied for other infectious agents, thus enhancing diagnosis of infections in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background information

1.1.1 Arboviruses in Kenya

Arboviruses are made up of a heterogenous group of zoonotic viruses that are transmitted by arthropods which include mosquitoes, ticks and sand flies (Ochieng *et al.*, 2013). Majority of the Medical important arboviruses, among which include *Flaviviridae, Togaviridae* and *Bunyaviridae* (Powers *et al*., 2000) are endemic in Tropical Africa. Emerging zoonotic diseases threaten the health and security of human and animal populations throughout the world (Gubler, 2002). Since arboviruses can be spread by competent mosquito vectors across great distances, they pose substantial risk to other regions in which the disease is currently non endemic (Gubler, 2002). Often these arboviruses remain undetected by health care systems (La Beaud *et al.,* 2008).

Kenya has had multiple arboviruses outbreaks in the past two decades resulting in economic and public health distress. Some of these outbreaks include yellow fever in 1992-1993(Sanders *et al.,* 1998*)*, chikungunya fever in 2004 (Sergon *et al.,* 2008) and Rift Valley fever (RVF) in 1997-1998 (Woods *et al.,* 2002) and 2006-2007 (CDC, 2007).

Rift Valley fever virus (RVFV), which causes RVF, was first identified in Kenya in 1930, along the shores of Lake Naivasha in the Rift Valley region, when there was an outbreak of sudden deaths and abortions among sheep in that region (Daubney *et al.,* 1931; Daubney & Hudson, 1932; Pepin *et al.,*2010). RVF mainly affects ruminants and humans (Davies & Martin, 2006), and it causes abortion in pregnant susceptible ruminants and high mortality in newborn animals (Chevalier *et al.,* 2010). Outbreaks of RVF in humans have resulted in substantial human illness and livestock losses in Kenya (Woods *et al.,* 2002; CDC, 2007). The source of RVFV that is considered as the primary vector and which initiates disease outbreaks is the *Aedes* mosquitoes, while the

Culex mosquitoes are the secondary vectors, which are important in amplification of the virus during outbreaks (Turell *et al.*, 2008; Sang *et al.*, 2010).

1.1.2 Monoclonal and polyclonal antibodies

Antibodies, also known as immunoglobulins are Y-shaped proteins that are produced by plasma cells, and are used by the immune system to identify and neutralize any antigen by recognizing unique parts of the antigen known as epitopes (Boenisch *et al.*, 2009). Each antibody is composed of two identical heavy chains and two identical light chains. The heavy chains differ in antigenic and structural properties, and determine the class and subclass of the molecule. The two light chains are either of type kappa (κ) or lambda (λ). Distribution of κ and λ chains differs in all immunoglobulin classes and subclasses, as well as between different species. Immunoglobulins are classified into five classes i.e. IgG, IgM, IgA, IgD and IgE (Neil *et al.*, 2005).

Polyclonal antibodies (pAbs) are a heterogeneous mixture of antibodies directed against various epitopes of the same antigen (Figure 1.1 A). The antibodies are generated by different B-cell clones of the animal and as a consequence are immunochemically dissimilar. Monoclonal antibodies (mAbs) are a homogeneous population of immunoglobulin directed against a single epitope, (Figure 1.1 B). The antibodies are generated by plasma cells from a single B-cell clone from one animal and are therefore immunochemically similar (Boenisch *et al.*, 2009).

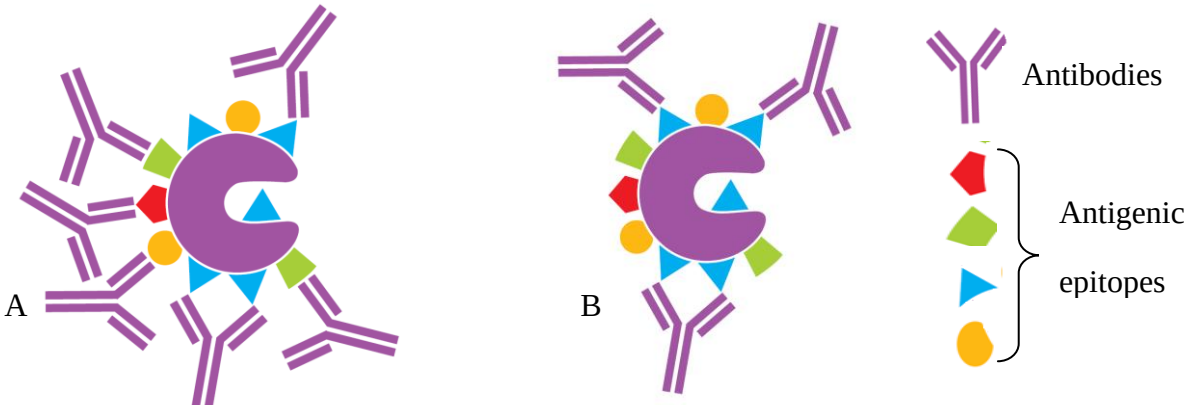


Figure 1.1: Reaction of pAb and mAb to antigenic epitopes (Boenisch *et al.*, 2009)

(A) pAbs bind to various epitopes on an antigen and (B) a single clone of mAb binds to a specific epitope on an antigen.

The pAbs and mAbs have their own advantages and disadvantages, as shown in Table 1.1, with the most important advantage of mAb being its mass production is much easier compared to the pAb, while its major disadvantage is that its development takes a long time and requires a lot of expertise. The major advantage of the pAb is that it takes a short time to produce but its main difficulty lies in its mass production, whereby a number of laboratory animals will be required.

Table 1.1: Advantages and disadvantages of polyclonal and monoclonal antibodies (Boenisch *et al.*, 2009)

	Advantages	**Disadvantages**
Polyclonal antibodies	• Development of pAbs takes a shorter period of time and less technical skill is required. • Better reactivity because they are produced by a large number of B cell clones which generate antibodies to different epitopes. • Due to multiple epitopes, a change in the structure of an epitope cannot affect the reactivity of the pAb.	• Mass production of pAbs requires the use of a number of laboratory animals. • Batch variation among immunized animals. • pAb is produced by more than one clone of B cells hence it has a possibility of cross-reactivity between closely related antigens.
Monoclonal antibodies	• Once the desired mAb has been developed, its mass production becomes relatively easy; it involves culture of the hybridoma cells only. • Each mAb has a homogeneous nature, hence the Abs produced have a similar reactivity. • Since mAb is produced by a single clone of B cells, it will be more specific for the particular antigen it is targeting.	• Development of mAbs takes a longer period of time and requires a lot of technical skills and expertise. • Lower reactivity because the mAb targets only one single epitope. • Due to a single epitope, small changes in the structure of the epitope can affect the reactivity of the mAb.

1.2 Problem statement

Epidemics and epizootics of RVF results in massive loss of livestock, consequent export impediments, and significant human morbidity and mortality which lead to a great

economic devastation in the affected areas (Laughlin *et al*., 1979; WHO, 2000; CDC, 2002). Currently, whenever an outbreak is suspected in a certain region within Kenya, samples have to be collected and transported to the reference laboratory at Kenya Medical Research Institute (KEMRI) for diagnosis before an outbreak is declared and corrective measures are put in place. For example during the 2006-2007 outbreak in East Africa, serum samples from suspected human cases collected from Kenya, Somalia and Tanzania were tested in KEMRI - Nairobi laboratories. The samples were tested using real-time reverse transcription polymerase chain reaction (real-time RT-PCR), IgM capture ELISA and virus isolation (Leonard *et al*., 2010). Transporting the samples to the reference laboratory and applying techniques that take long to produce results, like virus isolation, leads to deaths of livestock, loss of human lives and increased negative impacts of the RVF outbreak. There is a need to come up with RVFV specific diagnostic tools that will enable early diagnosis of RVF, and routine surveillance of RVF.

1.3 Justification

This study was aimed at developing mAbs against RVFV and applying them in antigen detection ELISA and antigen capture IgG indirect ELISA for the diagnosis of RVF. The antigen detection ELISA will facilitate timely and reliable diagnosis of RVF (Fafetine *et al.*, 2013) from blood, milk and mosquito samples, since the RVFV can be detected during the viremic period (Morvan *et al.*, 1992). This will greatly improve the implementation of control strategies using vaccinations in the early stages of an outbreak and will ultimately lead to a reduction in human and livestock deaths which will also reduce the economic losses associated with RVF outbreaks. The antigen capture IgG indirect ELISA will be a useful tool in surveillance studies since it can detect anti-RVFV IgG antibodies in human and animal sera. This data will enable the public health officials to assess and understand the accurate distribution of RVF in Kenya and as a result, they can plan ahead and allocate required resources in the event of RVF outbreak. Since anti-RVFV mAbs can offer RVF specific diagnostic platform, it is necessary to have them developed locally although there are others which have been developed in

other countries like Saudi Arabia (Zaki *et al*., 2006) and Spain (Raquel *et al*., 2010). The already developed mAbs have to be evaluated for their reactivity with the local strain of RVFV in Kenya. The mAbs developed locally in this study will be selected based on their reactivity with the local Kenyan RVFV strain; they will be readily available, cost effective and will help in formulation of more diagnostic systems for RVF in the country.

Setting up the mAb development technology in a local laboratory is important in that, once this technique has been set up in this study using RVFV, it can also be applied to other microbes. The mAb development technology will enhance research studies in the fields of diagnosis as well as vaccine development for various viruses and bacteria found in Kenya, hence improving the health status of Kenyans.

1.4 Research questions

1. What is the possibility of generating mAb against RVFV?
2. What is the possibility of applying the mAbs generated against RVFV in ELISA systems for diagnosis of RVF?

1.5 Hypothesis

1.5.1 Null hypothesis

Locally produced mAbs against RVFV cannot be used for the development of antigen detection ELISA and antigen capture IgG indirect ELISA for diagnosis of RVF.

1.6 General objective

To develop mAb against RVFV and evaluate its diagnostic potential.

1.7 Specific objectives

1. To generate mAb against RVFV by fusion of spleen from immunized BALB/c mice with Myeloma SP-2 cells.
2. To characterize the developed mAb against RVFV by FRNT, isotyping and western blot analysis.

3. To evaluate the diagnostic potential of the developed mAb against RVFV by developing an antigen detection ELISA for detection of RVFV in ICF.

4. To evaluate the diagnostic potential of the developed mAb against RVFV by developing an antigen capture IgG indirect ELISA for detection of anti-RVFV IgG in human sera using the anti-RVFV mAbs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Rift Valley fever virus

Rift Valley fever virus (RVFV) belongs to the family *Bunyaviridae* and genus *Phlebovirus* (Pepin *et al.,* 2010) and it has been isolated from more than 30 mosquito species (Gunn *et al.,* 2004). Structurally, the virus is enveloped, spherical and 80-120 nm in diameter and has short glycoprotein spikes (Gn and Gc) projecting through a bilayered lipid envelope (Figure 2.1) (Tolou *et al.,* 2009). The virus is readily inactivated by organic solvents and acid conditions below pH 6.0. The RNA genome of the virus is divided into three segments, large (L), medium (M) and small (S) segments. The L and M segments are of negative polarity, while the S segment is of ambisense polarity (Giorgi *et al*., 1991; Muller *et al*., 1994; Bouloy & Weber, 2010). The S segment of RVFV codes for the nucleocapsid (N) protein, in the negative sense, and it codes for a non-structural (NSs) protein in the positive sense (Giorgi *et al*., 1991). The N protein is closely associated with the RNA genome in the virion particle, and the NSs protein inhibits host gene transcription in the infected cells hence blocking interferon production (Billecocq *et al*., 2004; Le May *et al*., 2005).

The M segment encodes four proteins in a single open reading frame (ORF): the glycoproteins Gn (encoded by amino-terminal sequences) and Gc (encoded by carboxy-terminal sequences), and two other proteins called NSm1 (78kDa) and NSm2 (14kDa) (Won *et al.,* 2006; Gerrard *et al.,* 2007). The glycoproteins, Gn and Gc are located on the virion envelope and they play a role in virus attachment to initiate infection and they carry epitopes that elicit the production of neutralizing antibodies, which are important for protective immunity (Bird *et al*., 2011; Papin *et al.*, 2011; Piper *et al.,* 2011). On the other hand, the NSm2 protein functions to suppress virus induced apoptosis while the function of the NSm1 is not yet known (Anderson & Peters 1988; Won *et al*., 2007).

The L segment encodes the L protein, which is the viral RNA-dependent RNA polymerase (RdRp), which is packed together with the genomic RNA segments within the virus particles (Muller *et al*., 1994, Liu *et al.,* 2008). The three RNA segments are encapsidated by the N protein (Figure 2.1) to form ribonucleoprotein complexes, which serve as the functional templates for viral RNA synthesis (Ortı´n & Parra, 2006).

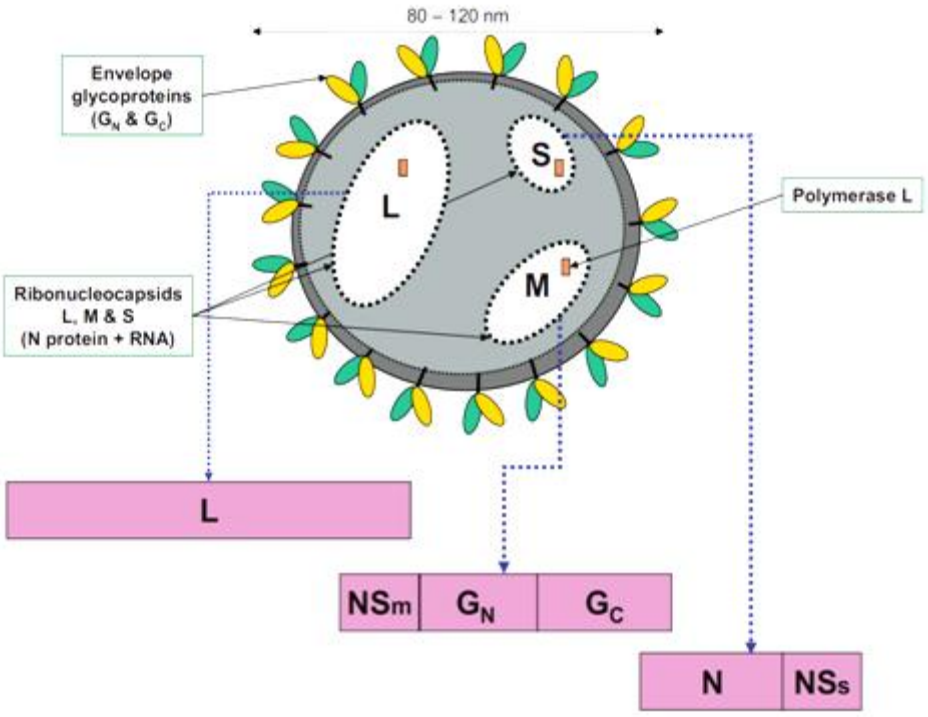


Figure 2.1: The structure of RVFV (Tolou *et al.,* 2009).

The RVFV L protein which is approximately 240 kDa, associates with the ribonucleoprotein to catalyse viral transcription and replication (Lopez *et al*., 1995). A study by Brennan *et al*. (2011) showed that L protein is a membrane associated protein and it could be detected in the perinuclear region of infected cells, which suggested that it is a representation of the late-stage RVFV replication complexes. The fact that the L protein is a membrane associated protein has also been proved in other studies carried out on bunyaviruses such as Bunyamwera virus (Fontana *et al*., 2008; Shi & Elliott,

2009), Tula hantavirus (Kukkonen *et al*., 2004) and Crimean–Congo hemorrhagic fever virus (Bergeron *et al*., 2010).

2.2 Distribution of Rift Valley fever virus

Although RVFV was originally isolated in Kenya, the virus is also endemic in other countries of East and South Africa (CDC, 2002). Historically, RVF was restricted to sub-Saharan Africa but by 1977 it had spread to northern Africa (Meegan, 1979; Chevalier *et al.*, 2010), 1987 to West Africa (Jouan *et al*., 1988; Pepin *et al.*,2010), and to the Arabian Peninsula in 2000 (Shoemaker *et al*., 2002). Figure 2.2 shows countries where RVF is endemic, and the years in which RVF outbreaks have been reported, and countries that have evidence of RVF (either through virus isolation or serologic tests) have also been shown.

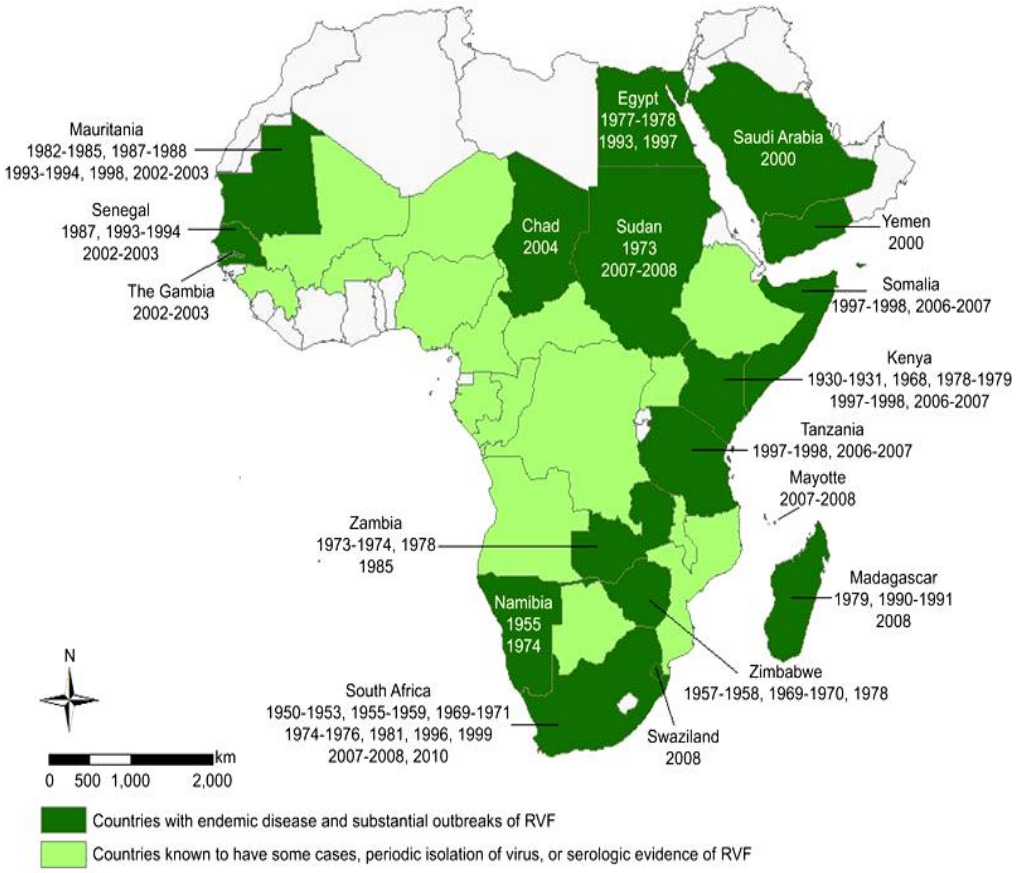


Figure 2.2: A figure showing the distribution of RVF (Pourrut *et al*., 2010)

The occurrence of the first confirmed outbreaks of RVF among humans and livestock outside Africa, occured in the Arabian Peninsula (Jupp *et al*., 2002). This carries the implication of further spread of infection into non-enedemic RVF areas since the causative agent, RVFV, is capable of utilizing a wide range of mosquito vectors (Turrel *et al*., 1998).

The two major outbreaks that have affected Kenya in 1997-1998 (CDC, 1998) and 2006-2007 (WHO, 2007) occurred following the rainfalls associated with the El Niño/Southern Oscillation (ENSO). The episodic RVF outbreaks are linked to the ENSO phenomena due to the episodic warming and cooling of sea surface temperatures in the Pacific Ocean and Indian Ocean (Linthicum *et al*., 1999). As a result there occurs persistent and widespread rainfall in the Horn of Africa which acts as the principal driving factor for RVF outbreaks (Nichol, 1993). These rainfalls results in a high growth of vegetation, creating ideal conditions for the flooding of dambos, which serve as mosquito habitats (Rolin *et al.*, 2013). The flooding of dambos induces hatching of transovarially infected *Aedes* mosquito eggs that were dormant in the soil, producing infected adult females in seven to ten days. It is these infected *Aedes* mosquitoes that transmit RVFV to domestic and wild animals, and humans (Linthicum *et al.*, 1984; Linthicum *et al.*, 1987; Linthicum *et al.*, 1999). The RVFV infected eggs can survive for several years when conditions are unsuitable for active transmission, i.e. during cold and dry periods; and the virus is reinitiated in the event of a heavy rainfall (Mondet *et al.*, 2005).

The largest human outbreak of RVF was reported in East Africa affecting Kenya, Tanzania and Somalia between late 1997, and early 1998 following ENSO rainfall (CDC, 1998). Approximately 27,500 cases and 170 deaths due to hemorrhagic fever were reported during the 1997-1998 outbreak (LaBeaud *et al*., 2008). The latest Kenyan RVF outbreak also occurred in association with ENSO rains between November 2006 - April 2007, affecting Kenya, Tanzania and Somalia (CDC, 2007). During the outbreak

period, a total of 1,062 human cases and 315 fatalities were reported throughout the region, and dramatic losses to livestock production also occurred (CDC, 2007).

2.3 Life cycle of Rift Valley fever virus

The life cycle of RVFV has distinct endemic and epidemic cycles, as shown in previous research (Linthicum *et al.*, 1985; Assaf *et al.*, 2010). During the endemic cycle, which occurs during the dry season or inter-epizootic periods, the RVFV persists through vertical transmission in *Aedes* mosquito eggs, and the vertically infected *Aedes* mosquitoes introduce RVFV into domestic animal populations when the mosquito habitats flood (Figure 2.3). The epidemic or epizootic cycle is brought about by the subsequent increase in *Culex* mosquito populations, which serve as secondary vectors if the immature mosquito habitats remain flooded long enough (Logan *et al.*, 1992; Pepin *et al.*, 2010).

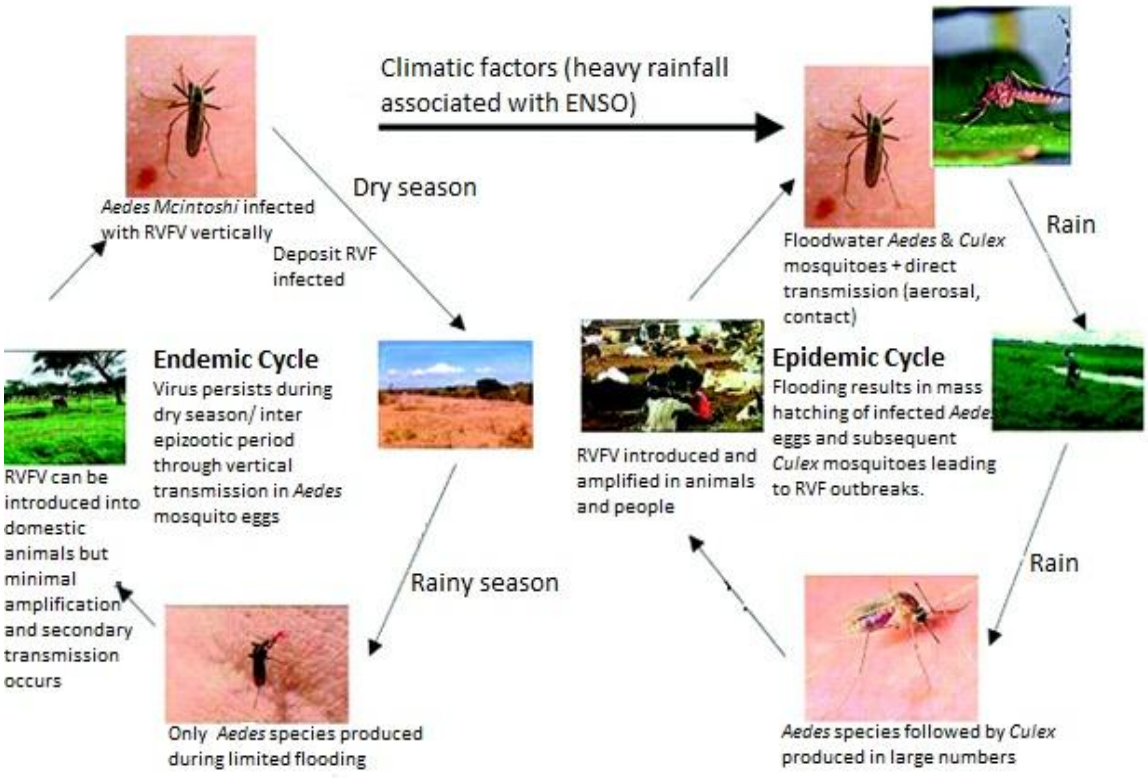


Figure 2.3: Transmission cycle of Rift Valley fever virus (Assaf *et al*., 2010)

2.4 Transmission of Rift Valley fever virus

RVFV is transmitted to livestock, wildlife and humans by bites from infected species of *Aedes* and *Culex* mosquitoes which are considered as the primary vectors and secondary vectors, respectively (Davies & Highton, 1980). The female *Aedes* mosquitoes transmit the RVFV to their progeny transovarially during periods of drought (Gargan *et al.*, 1988). These *Aedes* eggs can survive in dry conditions and they will only hatch after a period of dehydration; potentially making them the ideal vehicle for survival of RVFV over long periods of time. When such habitats flood after rainfall, biological transmission occurs via infected mosquito saliva to domestic herbivores through mosquito bites.

The virus infects domestic animals like cattle, goats, sheep and camels (Daubney *et al*., 1931; Bouloy & Weber, 2010), and also humans (Faye *et al*., 2007). Infected animals develop high levels of viremia within a period ranging averagely from six to eighteen hours or to eight days. When the larval habitats remain flooded for more than 2 to 3 weeks, the floodwater *Aedes* are succeeded by *Culex* mosquitoes whose eggs cannot withstand desiccation but they hatch and lead to a population explosion of *Culex* mosquitoes (Linthicum *et al.*, 1983). It is during the viremic period that infection of *Culex* mosquitoes as secondary arthropod vectors occurs, during blood meal leading to subsequent infection of other animals and humans (Logan *et al*., 1991; Pepin *et al*., 2010).

Rift Valley fever can also be acquired through exposure to tissue, blood, milk or body fluids of infected animals (Jup *et al*., 2002; Flick & Bouloy, 2005) and direct contact with infected animals, during handling for slaughter or veterinary and obstetric procedures (Chambers & Swanopoel, 1980; McIntosh *et al*., 1980; Brown *et al*., 1981). Inhalation of infectious aerosols generated during processing of RVF samples in the laboratories, can be a route of infection (Smithburn *et al*., 1949; Anderson *et al*., 1991).

2.5 Symptoms of Rift Valley fever in human infections

The incubation period of Rift Valley fever is two to six days following infection and can cause several different disease syndromes (Woods *et al.*, 2002). Most commonly, people with RVF have either no symptoms or a mild illness associated with fever and liver abnormalities. Patients who become ill usually experience fever, generalized body weakness, back pain, and dizziness at the onset of the illness. Typically, patients recover within two days to one week after onset of illness (Swanepoel & Paweska, 2011). The glycoproteins Gn and Gc play a role in virus attachment to initiate infection and have been shown to carry epitopes that elicit the production of neutralizing antibodies, a correlate of protective immunity (Besselaar & Blackburn, 1994).

However, 8-10% of this may develop much more severe symptoms, which may include ocular disease, encephalitis and hemorrahagic fever (Ringot *et al*., 2004). The severe RVFV pathogenesis is associated with strong proinflammatory cytokine and chemokine responses in terminal stages of the disease (Gray *et al.*, 2012).

Patients with ocular disease, which sometimes accompanies the mild symptoms described above, develop lesions on the eyes 1-3 weeks after onset of initial symptoms. These patients also experience blurred and decreased vision, and the lesions usually disappear after 10-12 weeks. However, for those patients with lesions occurring in the macula, 50% of them will have permanent vision loss (Elwan *et al*., 1997; Madani *et al*., 2003; Al-Hazmi *et al*., 2005).

Encephalitis normally leads to headaches, coma, or seizures; this occurs in less than 1% of patients and this may occur after one to four weeks after the appearance of first symptoms. However, encephalitis rarely causes death, although the patients sometimes may experience severe neurological deficits which could persist depending on the severity of the symptoms (Van Velden *et al*., 1977; Laughlin *et al*., 1979; Alrajhi, *et al.*, 2003).

Hemorrhagic fever occurs in less than 1% of overall RVF patients, but fatality for those who develop the hemorrhagic fever is around 50%. Symptoms of hemorrhagic fever may begin with jaundice and other signs of liver impairment, followed by vomiting blood, bloody stool, or bleeding from gums, skin, nose, and injection sites. These symptoms appear for two to four days and death usually occurs three to six days after (Peters, 2000; Al-Hazmi *et al.,* 2003).

2.6 Immune responses in Rift Valley fever virus infection

For the control of the initial phase of virus dissemination and eventual animal survival, a robust innate immune response is critical. A vigorous adaptive immune response is developed rapidly following RVFV infection, with the production of detectable neutralizing antibodies which are primarily directed against the viral glycoproteins, Gn and Gc (Paweska *et al.,* 2005). The neutralizing antibodies are accompanied by the production of IgM and IgG antibodies raised against N and NSs proteins (McElroy *et al.,* 2009).

Viremia occurs at the start of the infection, with IgM appearing in circulation three to five days after disease onset; this period is also associated with cessation of viremia. The IgM may persist for up to five months in some cases (Morvan *et al.,* 1992). At 10-14 days after the onset of infection, IgG antibodies appear, and they may persist for life (Figure 2.4).

Neutralizing antibodies are the key factor for the initial and persistent protection of infected animals (Peters *et al.,* 1986) and are, consequently a good correlate of the protection induced by vaccines against RVFV. If vaccinated animals produce a high level of neutralizing antibodies, they will very likely be protected against experimental challenge or subsequent natural viral infection under field conditions (Eddy *et al.,* 1981). The convalescent immunity after a natural infection lasts for a long period of time and offspring of immune mothers may passively acquire maternal immunity for the first three to four months of their lives (FAO, 2003).

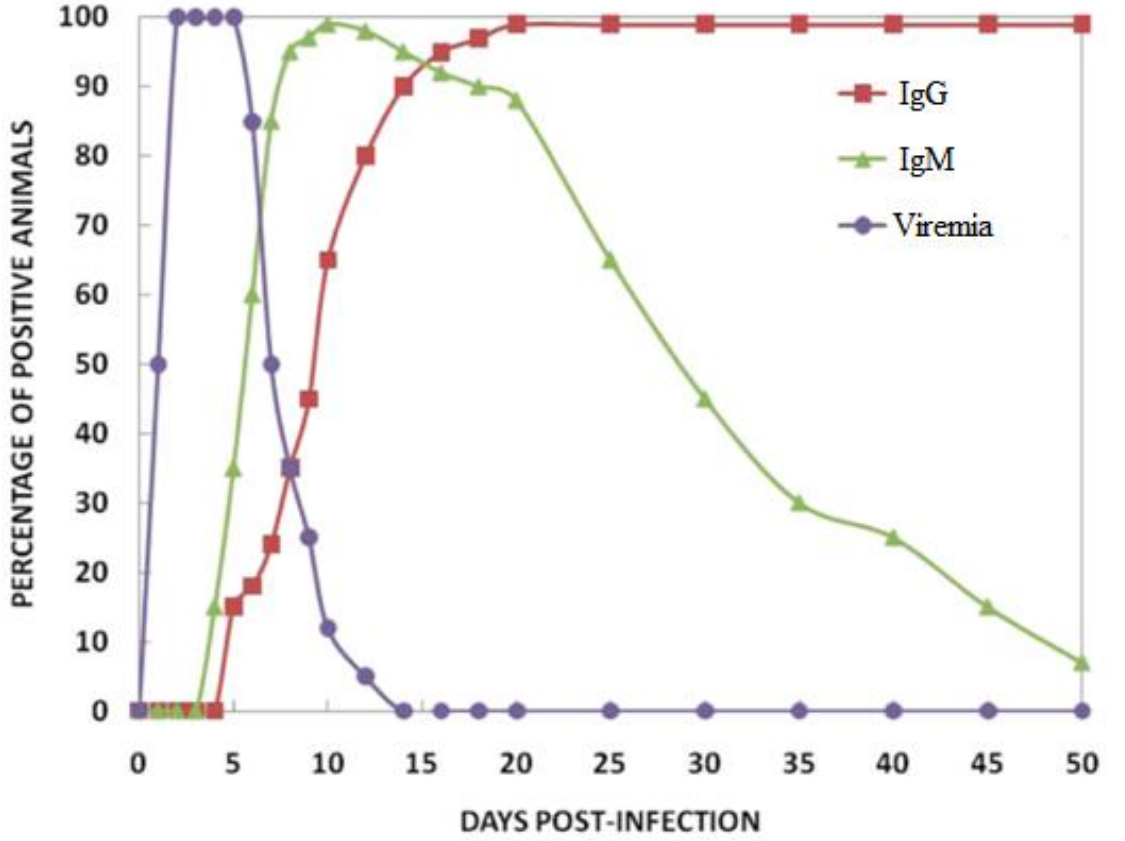


Figure 2.4: Schematic representation of time course of viremia and antibody responses against RVFV (Morvan *et al.*, 1992).

2.7 Diagnosis of Rift Valley fever

The spread of RVFV beyond its endemic boundary with lack of a suitable human vaccine (Lihoradova *et al*., 2012), has facilitated the international demands of coming up with validated diagnostic tests for the rapid diagnosis of RVF (Pepin *et al*., 2010).

The gold standard of RVF diagnosis is virus isolation, which can be achieved in cell cultures using a variety of cell lines including, mosquito cell lines, Vero, and BHK21 (Amwayi *et al.*, 2007). This technique is lengthy and expensive and at the same time, virus isolation requires biosafety level-three laboratory which might not be readily available in the most commonly affected regions (Pepin *et al*., 2010). The RVFV can be identified in cell cultures by the appearance of CPE and confirmed by virus

neutralization tests, immunofloresence assays, RT-PCR (Garcia *et al.,* 2001; Sall *et al.,* 2002), real-time RT-PCR (Bird *et al*., 2007), genome sequencing and antigen detection ELISA (Nikalsson *et al*., 1983). Reverse-transcription loop mediated isothermal amplification assay (RT-LAMP) has also been evaluated for the detection of RVFV isolates and clinical specimen (Peyrefitte *et al*., 2008; Le Roux *et al*., 2009). While RT-PCR, real time RT-PCR, RT-LAMP and genome sequencing are reproducible and highly sensitive techniques of RVF diagnosis, they are still considered as research tools in many laboratories. Antigen detection ELISA has been successfully used for the detection of viruses and viral antigen and it has been found to be a rapid, sensitive, and specific method (Nikalsson *et al.,* 1983).

Antibodies against RVFV can be detected by serological methods which include virus neutralization tests i.e. focus reduction neutralization test, plaque reduction neutralization test (Xu *et al*., 2007) and ELISAs, i.e IgM capture ELISA and IgG indirect ELISA (Paweska *et al*., 1995). Several serological techniques are used for RVF diagnosis especially in epizootic situations. However, in addition to possibilities of serological cross reactions between RVFV and other phleboviruses (Shope *et al*., 1981), specific antibodies against RVFV may not be readily detectable during the acute phase of an infection.

Considering that viremia caused by RVFV reaches very high titers (10^4 to 10^9 PFU/ml) for several days after infection in many species (Meegan, 1979; Peters & Meegan, 1981), antigen detection based assay would therefore be the most suitable for disease diagnosis. Other antigen detection techniques like RT-PCR and RT-LAMP can then be used as confirmatory tests.

2.8 Hybridoma technology

Hybridoma technology was discovered in 1975 by two scientists, Georges Kohler and Cesar Milstein (Kohler & Milstein, 1975). It involves the development of cells that are engineered to produce desired monoclonal antibodies continuously (Bretton *et al.,* 1994). It is achieved through the use of plasma cells which are generated from the spleen

of mice that have been challenged with the relevant antigen. The splenocytes are fused with myeloma cells (Figure 2.5) that are of the same origin as the mice used. The fusion is carried out using polyethylene glycol (PEG) to make the cell membranes of both cells more permeable and allow them to fuse and result into a single cell, the hybridoma cell (Yang & Shen, 2006).

After the fusion of splenocytes and myeloma cells, the fused cells are grown in selection medium, containing Hypoxanthine Aminopterine Thymidine (HAT) solution. HAT selection is based on the fact that under normal circumstances, cells synthesize nucleotides by the de novo pathway. However, the presence of aminopterin blocks the de novo pathway and the cells utilize the salvage pathway instead. The salvage pathway is only possible for cells that have the enzyme hypoxanthine-guanine phosphoribosyl tranferase (HGPRT). The myeloma cells lack HGPRT, hence the myeloma cells that fuse with other myeloma cells or do not fuse at all end up dying under HAT media condition. As for the splenocytes, since they have HGPRT, the splenocytes that fuse with other splenocytes or do not fuse at all survive under HAT media condition for a few weeks, however, they eventually die because they do not have the ability to divide indefinitely. Only the hybridomas between splenocytes and myeloma cells survive, since the splenocytes have HGPRT and the myeloma cells confer the ability of indefinite growth (Christine & Deborah 1995). The culture fluid of the surviving hybridoma cells is checked by various assays for example ELISA (Christian *et al*., 2008) or immunoflorescence assay (Xu *et al*., 2007; Zaki *et al*., 2006). The positive clones are cloned by limiting dilution (Steven *et al.,* 2001) until a single hybrid cell is obtained. The single hybridoma cell is gradually upscaled in cultures and the culture fluid is pooled until a certain volume is attained, then finally the culture fluid is purified to obtain the purified mAbs (Zaki *et al*., 2006).

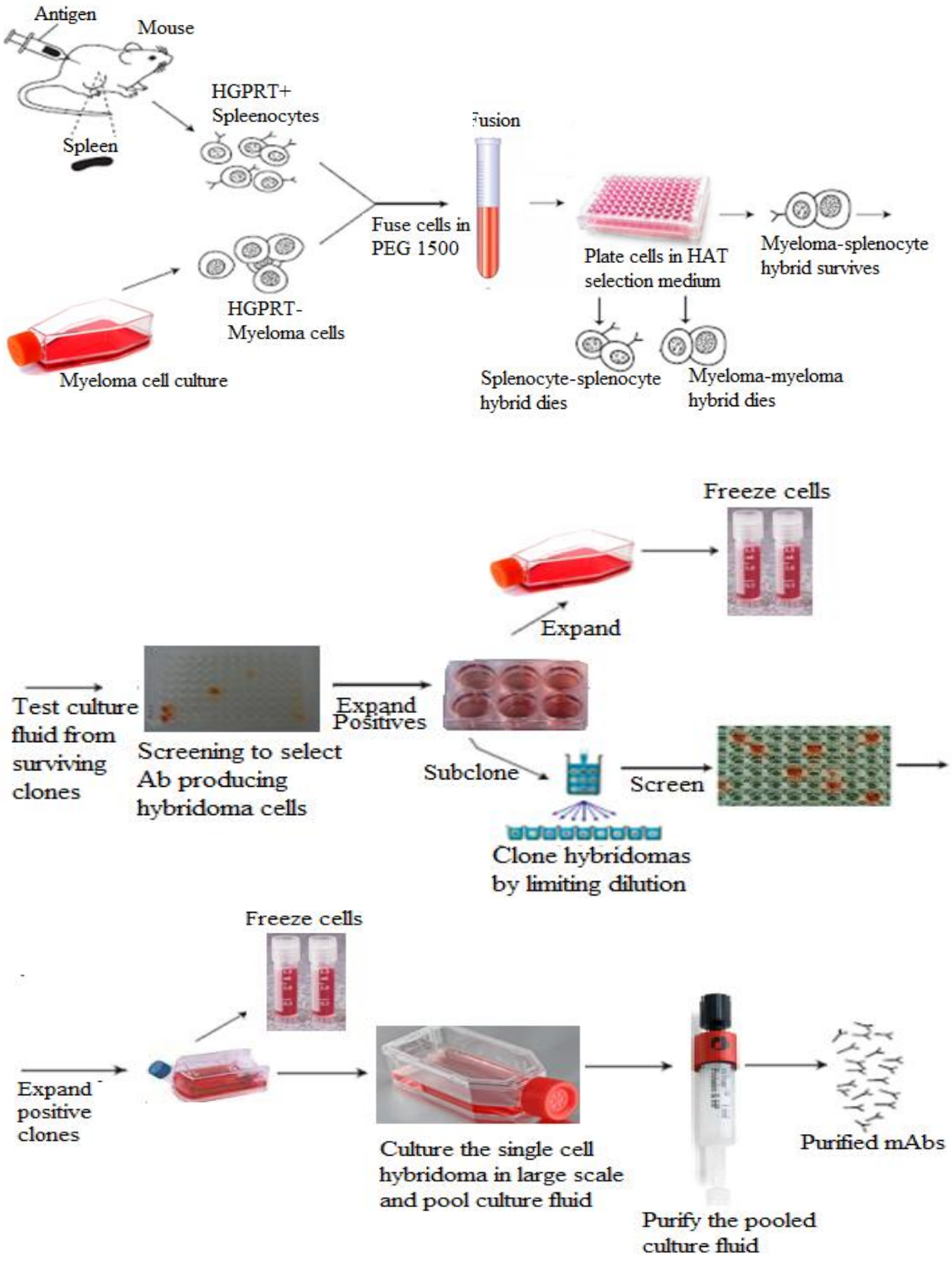


Figure 2.5: A flow diagram representing the major steps involved in the hybridoma technology. (Diagram modified from Greenfield, 2014)

2.9 Monoclonal antibodies and their applications

The mAbs are antibodies produced by a population of cells derived from a single ancestral B cells, giving them a monospecificity and homogenous nature. Additionally, the mAb has a potential for long term production, enabling the development of standardized, secure and reproducible immunoassay systems (Nelson *et al*., 2000). They are considered to be essential tools in many molecular immunology investigations and have become key components in a wide range of clinical laboratory diagnosis and treatment (Nelson *et al.*, 1997). Once a mAb has been developed against a certain antigen, it can be used to detect the presence of this antigen. Examples of diagnostic techniques that apply mAbs include ELISA (Hornbeck, 1991), enzyme-linked immunospot assay (ELISPOT) (Klinman & Nutman, 1994), immunofluorescence and immunohistochemistry (Harlow & Lane 1999), and western blot (Gallagher *et al*., 1998). Therapeutic mAbs function through a number of mechanisms such as modulating signaling pathways, inducing apoptosis of cells which express the target and blocking of targeted molecule function, this occurs by the mAbs destroying the malignant tumor cells and preventing tumor growth by blocking the specific receptors (Breedveld, 2000; Sanjay & Sean, 2006).

2.10 Prevention and treatment of Rift Valley fever

The spread of RVFV can be prevented by effective vaccination of livestock and humans (Ikegami & Makino, 2009). At the moment, there is no approved human vaccine for mass vaccination programs (Murakami *et al*., 2014). The first RVFV vaccine to be developed is the Smithburn strain, developed by Smithburn through serial intracerebral inoculation of mice (Smithburn, 1949). The vaccine had only partially lost its virulence and it caused abortions and teratogenesis in ewes, cows and goats (Botros *et al*., 2006). The MP-12 strain of RVFV, which was developed by serial passage of RVFV wild strain ZH548 in the presence of the mutagen 5-fluorouracil, is attenuated and at the same time, it retains its immunogenicity (Morrill *et al*., 1987; Hubbard *et al*., 1991; Morrill *et al*., 1991; Baskerville *et al*., 1992; Morrill*et al*., 1997; Morrill & Peters, 2003). This forms a promising live vaccine for both humans and animals, however, it can invade the

central nervous system and undergo efficient replication in immunocompromised animals, and may potentially do so in immunocompromised humans as well (Papin *et al.*, 2011). As a result, it was necessary to develop an RVFV vaccine which will be highly immunogenic and with reduced or no neurovirulence. In a study carried out by Murakami *et al*., 2014, a novel, single-cycle replicable MP-12 (scMP-12) was generated. The scMP-12 did not cause any systemic infection in the immunized host, and it did not show any sign of neurovirulence after intracranial inoculation into suckling mice; this demonstrated its safety. To test its immunogenicity, scMP-12 elicited neutralizing antibodies in immunized mice, which protected the mice from wild strain RVFV challenge. Data from this study shows that scMP-12 has a very high potential of being developed into a safe RVFV vaccine.

Most commonly implemented control measures right after an outbreak of RVF are restrictions on animal movements, avoidance or control of the slaughter of ruminants and animal vaccination in the affected areas. The main purpose of vaccination is to limit the negative impact of the outbreak and prevent further spread of the RVFV (OIE, 2014). The use of insect repellents and bed nets during outbreaks and creating awareness are also effective counter measures (Chevalier *et al*., 2010).

For treatment of RVF, antiviral drug therapies are limited only when administered shortly after the viral exposure. For example, ribavirin was used to treat patients during the RVF outbreak in Saudi Arabia in the year 2000. Treatment with this drug was only able to limit the hemorrhagic symptoms, but did not prevent the neurological symptoms (Balkhy & Memish, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a laboratory based experimental study.

3.2 Study site

This study was carried out in the KEMRI - Production Department facilities.

3.3 Materials

3.3.1 Vero cell line

This is a mammalian cell line derived from the African green monkey kidney cell. This cell line was maintained in growth medium, Eagle's minimum essential medium (EMEM) (Life Technologies, New York, USA), supplemented with 10% fetal calf serum (Life Technologies, New York, USA), non-essential amino acids (Life Technologies, New York, USA), L-glutamine penicillin-streptomycin (Life Technologies, New York, USA) in 75cm^2 tissue culture flasks. The cells were passaged once or twice a week (depending on the growth) using trypsin-EDTA for detachment from the surface of the flask. Splitting ensured the cells did not overgrow and it maintained their viability so that they could have a good response when used for large scale propagation of the RVFV.

3.3.2 Myeloma cell line (SP-2)

Myeloma cell line, SP-2, which are of mouse BALB/c origin, was used for making the hybridoma through fusion with splenocytes from the immunized mice. This myeloma cell line was obtained from Nagasaki University, Japan, and they were revived one week prior to fusion. The cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan), supplemented with fetal calf serum, L-glutamine penicillin-streptomycin, non-essential amino acids and sodium bicarbonate after autoclaving.

3.3.3 Rift Valley fever virus

RVFV live attenuated vaccine strain (Smithburn), was obtained from Kenya Veterinary Vaccines Production Institute (KEVEVAPI). The virus was purified and formalin inactivated, then used for the immunizations of mice and rabbits.

3.3.4 Rabbits

Two New Zealand white rabbits were purchased from Ngong rabbit breeders' farm and their health condition was monitored for at least one week before the immunizations commenced. One rabbit was used for the immunizations while the other served as a control.

3.3.5 BALB/c female Mice

Ten BALB/c female mice, six to eight weeks old were purchased from International Livestock Research Institute (ILRI) and their health condition was monitored for at least one week before the immunization commenced. Eight mice were injected with the RVFV immunogen while the other two served as controls.

3.4 Methods

3.4.1 Propagation and purification of Rift Valley fever virus

3.4.1.1 Cytodex solution preparation

Cytodex (GE healthcare, Buckinghamshire, UK) is a microcarrier for cell culture having a surface area of 4.40cm^2/g under culture conditions; it was prepared by washing with normal saline. Ten grams of cytodex was added to 500ml of normal saline in a bottle; the mixture was shaken gently and left to stand until the cytodex settled at the bottom of the bottle. The supernatant was poured out, and 300ml to 400ml of normal saline was added for a second wash; washing was repeated three times. Finally, 500ml of normal saline was added to the washed cytodex and autoclaved at 121°C for 15 minutes, allowed to cool and stored at room temperature.

3.4.1.2 Cell culture

Vero cells were scaled up into 150cm^2 tissue culture flasks with growth medium (GM: MEM with 10% FCS). When the cells had formed a confluent monolayer, four 150cm^2 tissue culture flasks were inoculated with 10ml per flask of RVFV at a dilution of 1:9 in maintenance medium (MM: MEM with 2% FCS). The cells were observed daily for any signs of cytopathic effect (CPE) and recorded. Thereafter, the infected culture fluid (ICF) and cells were harvested and inoculated into the spinner flasks. One hundred milliliters of the cytodex solution was added into a one liter spinner bottle (Corning, New York, USA). The uninfected cells from two 150cm^2 tissue culture flasks were detached using trypsin-EDTA, the suspension was transferred into the spinner bottle having the cytodex solution and 500ml GM was added. The cells were cultured at 37°C for three days, during which time they were stirred using a low speed magnetic stirrer (As one, Osaka, Japan). One milliliter of cell suspension was collected and observed under the microscope to assess the concentration of cells attached to the cytodex (preferred cell coverage is 50% to 100%).

When the cell coverage of cytodex was about 80-90%, the magnetic stirrer was stopped for a while to allow the cytodex with attached cells to settle down. The spent medium was discarded gently ensuring the cytodex was not poured out then, 50ml to 100ml of RVFV ICF and infected cells were poured into the spinner bottle. The spinner bottle was incubated at 37°C for two hours with gentle mixing every 20 minutes, after which 800ml of MM was added. RVFV infected cells were cultured for six days at 37°C while stirring and the spinner flasks were observed daily for any contamination by observing the formation of a cloudy or white layer in the medium.

3.4.1.3 Harvesting the virus

On the sixth day, the magnetic stirrer was stopped, and the spinner bottle transferred into a biosafety cabinet. The ICF in the spinner bottle was filtered using a large funnel and No.1 Whatman filter paper (Whatman, Maidstone, UK) so as to get rid of cells and the cytodex. The filtered ICF was centrifuged at 6,800×g for 30 minutes at 4°C in an Avanti

J-26 XP high-speed centrifuge (Beckman, California, USA). The supernatant was pooled into a sterile two liters conical flask containing a magnetic stirrer and 22.2g of sodium chloride (Wako, Osaka, Japan) and 60g of polyethylene glycol 6000 (PEG6000) (Wako, Osaka, Japan) added into the conical flask for every one liter of ICF. The mixture was stirred slowly overnight at 4°C and the following day, the ICF centrifuged at 6,800×g for 30 minutes at 4°C, and the supernatant discarded. The precipitate on the surface of the centrifuge tubes was resuspended in sodium chloride Tris-EDTA (STE) buffer containing 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, at pH of 7.4, then pooled, and centrifuged at 10,700×g for 20 minutes at 4°C. The supernatant was then harvested and the precipitate resuspended with STE buffer and applied in the sucrose gradient ultracentrifugation (Morita and Igarashi, 1989).

3.4.1.4 Sucrose density gradient ultracentrifugation

Two centrifuge tubes (Ultraclear tubes, Beckman: 40 ml capacity) of sucrose gradient were prepared in STE buffer. The stepwise sucrose was prepared manually as follows: 3ml of 50% sucrose in STE followed by gradually decreasing concentrations with 4ml of 45%, 4ml of 40%, 4ml of 35%, 3ml of 30% and 2ml of 15%. Finally 18ml of the sample was layered on the sucrose gradient, one tube contained the supernatant derived virus solution and the other contained the precipitate derived virus solution. These centrifuge tubes were centrifuged at 42,600×g at 4°C for 16 hours in a Beckman Coulter Optima L-90 XP ultracentrifuge (Beckman, California, USA) using a SW32 Ti rotor. After 16 hours, the tubes were carefully removed and the virus antigen (usually contained in a visible white band) recovered using a density gradient fractionator (Instrument Specialties Co. Nebraska, USA) at 254nm wavelength. All the fractions indicated at the start of the peak absorbance to the end were all pooled together.

3.4.1.5 Determining the virus concentration

The concentration of the purified antigen was determined by an ultraviolet spectrophotometer, GeneQuant (Amersham, UK) at 280nm and 260nm wavelengths, by applying the formula:

Protein Concentration (mg/ml) = (1.45 X OD_{280} – 0.74 X OD_{260}) × Dilution Factor.

The purified antigen was aliquoted into 1.5ml cryovials, and labeled with all details including date and concentration, and stored in -80°C deep freezer for future use.

3.4.2 Inactivation of Rift Valley fever virus

One hundred microliters of 10% formalin (Wako, Osaka, Japan) was added into 900µl of RVFV solution and the mixture was incubated at 4°C for 48 hours to allow inactivation. The inactivated RVFV solution was stored at -80°C and later tested for complete inactivation by focus assay.

3.4.3 Test for complete inactivation of the purified Rift Valley fever virus

Vero cells were seeded in a sterile 96-wells Nunc tissue culture plate at a concentration of 2×10^5 cells/ml and the plate incubated at 37°C in 5 % CO_2. The purified inactivated RVFV was 10 fold serially diluted, i.e. 1×10^{-1} to 1×10^{-10} in MM and 0.1ml of each dilution was inoculated into four wells of each column in the plate except the last column (control wells) where only MM was added. To compare the virus titer, undiluted to 1×10^{-11} dilutions of purified RVFV, without inactivation was also inoculated in four wells for each dilution. The plate was incubated at 37°C incubator in 5 % CO_2 for two hours, and then 150µl of 1.25% methylcellulose MEM with 2% FCS was overlayed in each well. The virus infected cells were cultured at 37°C incubator with 5 % CO_2 for 18 hours, after which fixation was done by adding 250 µl of 10% formalin per well and incubated overnight, at room temperature. Next, permeabilization was done by removing the formalin and adding 200 µl /well of 1% NP40 in phosphate-buffered saline ($PBS^{(-)}$) and incubated at room temperature. After twenty minutes, the plate was washed three times with $PBS^{(-)}$, allowing three minutes incubation between the washes. One hundred microliters of original concentration blockace (Yukijirushi, Sapporo, Japan) was added per well and the plate was incubated at room temperature for 30 minutes, and then washed as previously described. One hundred microliters of anti-RVFV IgG high titer rabbit serum at a dilution of 1,000× was added per well, the plate incubated for one hour at 37°C, and then washed as previously described. Then, 100µl

of 5,000× diluted horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (American Qualex, California, USA) was added and incubated for one hour at 37°C then washed as previously described. Lastly, 100µl of the substrate solution, which was prepared using 3,3'-diaminobenzidine tetrahydrochloride (DAB) 10mg tablet (Wako, Osaka, Japan) dissolved in 10ml of $PBS^{(-)}$ with 0.03% of H_2O_2 was added per well and the reaction incubated for 30 minutes at room temperature. The plate was then washed with distilled water, allowed to dry and the foci were counted by observing under a stereo microscope (NIKON, Tokyo, Japan).

3.4.4 Immunogen preparation

The purified, inactivated RVFV was diluted in STE buffer to attain a concentration of 200µg/ml for mice immunization and 250µg/ml for rabbit immunization. The diluted antigens were divided into 1ml aliquots in 1.5ml cryovials and stored at -80˚C.

Working in a biosafety cabinet, 1ml of the inactivated virus was picked using a glass syringe and a 23gauge needle. Using another glass syringe, 1ml of Freund's complete adjuvant containing heat-killed and dried Mycobacterium tuberculosis, paraffin oil and mannide monooleate (MP Biomedicals, California, USA) was picked. The two syringes were connected to a three-point connector, and the open end of the connector closed. The inactivated virus and the adjuvant were then mixed thoroughly to constitute the immunogen. During the booster immunizations, the immunogen was prepared using the same procedure, but the inactivated virus was mixed with Freund's incomplete adjuvant containing only paraffin oil and mannide monooleate (MP Biomedicals, California, USA).

3.4.5 Polyclonal antibody propagation in rabbits

One milliliter of the immunogen with adjuvant prepared at a concentration of 125µg/ml was injected into the rabbit subcutaneously in the hind limbs, using a 23 gauge needle. The needle was inserted under the skin; and aspirated lightly to ensure that it is not placed within a blood vessel. If blood appeared in the syringe, the needle was repositioned slightly (Suckow, *et al.*, 2012). After the first immunization, six booster

shots were administered to the rabbits. The boosters were administered when the antibody titers reached a plateau or began to decline. Bleeding was done every week, during which the rabbit was immobilized in a wooden box restrainer that restrained the whole body and allowed easy access to the rabbit's ears. Using a 23gauge needle and 5ml syringe that contained about 200µl of heparin, 2ml to 5ml of blood was extracted from the rabbit's ear veins.

3.4.6 Immunization of mice

The mice were immunized intraperitoneally in the lower right quadrant of the abdomen with 100µl of the immunogen with equal volume of adjuvant prepared at a concentration of 100µg/ml. The mouse was restrained by holding it with its head tilted lower than the body to avoid injury to internal organs. After swabbing the lower right quadrant with 70% ethanol, a 1 ml tuberculin syringe with a 27 gauge needle (BD, New Jersey, USA) was introduced slowly through the skin, subcutaneous tissue and peritoneum. After inserting, the needle was aspirated lightly to ensure that it is not in the bladder or intestines, and if nothing was drawn, the immunogen with adjuvant was slowly injected, otherwise, the needle was repositioned (Tan, 2004). After the first immunization, seven booster shots were administered to the mice in two weeks' interval. Boosters were administered so that the bleeding was done every two weeks, where 100-200µl of blood was drawn from the tail vein into a heparin-coated capillary tube.

3.4.7 Assessment of antibody titer

Blood extracted from the rabbits and mice was separated by centrifuging at 700×g for 10 minutes. The sera were collected and stored in 1.5ml cryovials, labeled and kept at -30°C for subsequent analysis. The IgG titers of the sera from mice and rabbits were assayed individually by IgG indirect ELISA and booster injections were administered at two weeks' interval until the desired antibody titer was attained.

The mice and rabbits were all bled prior to immunization and the pre-immunization serum used as a baseline control in assessing the rise in antibody titer.

3.4.8 IgG indirect ELISA

A ninety six wells ELISA plates (NUNC Maxisorp, Roskilde, Denmark) was coated with purified RVFV at a concentration of 250ng/100µl/well, except the blank wells and the plate was incubated at 4°C overnight or 37°C for one hour. One hundred microliters of blockace was added into all the wells except the blank wells, and the plate was incubated at room temperature for one hour, after which the coating antigen and the blockace were discarded, and the plate was washed with PBS-T three times allowing three minutes incubation in between the washes. Next, 100µl of 1,000× diluted standard serum samples at a twofold serial dilution of $2^0 \times 10^3$ to $2^{13} \times 10^3$ (dilutions were done in PBS-T with 10% blockace) were added into the wells to draw a standard curve. Then, 100µl of the serum samples were also added at a dilution of ×1,000 in PBS-T with 10% blockace and for the blank wells, only PBS-T was added. The plate was incubated at 37°C for one hour, after which the plate was washed as previously described. Next, 100µl of HRP conjugated anti-mouse IgG (American Qualex, California, USA), at a dilution of ×5,000 was added into all the wells except the blank wells to which only PBS-T was added, and the plate was incubated at 37°C for one hour. Then, the plate was washed as previously described, and 100µl of the substrate was added into all the wells except the blank wells, to which only the substrate buffer was added. The substrate was composed of: *o*-phenylenediamine dihydrochloride (OPD) 5mg tablet (Sigma Chemical, St. Louis, USA), 10ml substrate buffer (0.05 M Citrate phosphate buffer at pH 5.0) and 10 µl of 30% hydrogen peroxide. The plate was incubated in the dark, at room temperature for 20 to 30 minutes. One hundred microliters of 1N sulphuric acid was added to stop the reaction in all the wells, including the blank. The plate OD was measured at wavelength 492nm, using a Multiscan EX reader (Thermo Scientific, Massachusetts, USA) which applies Ascent software version 2.6 (Thermo Scientific, Massachusetts, USA).

3.4.9 Harvesting of spleen from the mouse

The mouse with the highest IgG titer was selected for the final booster; this was done intravenously through the tail vein, with inactivated antigen only, without any adjuvant.

Three days after the final booster, the mouse was euthanized using diethyl ether. The mouse's body was disinfected with 70% ethanol and allowed to dry, and then it was pinned onto a dissecting board. Using the appropriate equipment from a sterile dissecting kit, a notch was made around the abdomen region and the skin was pulled back to expose the internal organs. The spleen was located and removed, and then placed in a sterile petri dish containing 5ml of RPMI 1640 medium with 10% FCS (complete RPMI medium) at room temperature and was immediately transported to the laboratory. The mouse carcass was put in a red biohazard-labeled bag, autoclaved and then incinerated.

3.4.10 Preparation of splenocytes from the spleen

In the laboratory, manipulation of the spleen was done inside a biosafety cabinet. Fatty tissues on the spleen were carefully trimmed off and the spleen was immobilized using a sterile forceps and punctured carefully and gently using 23G needle at various points so as to enable the release of splenocytes. Complete RPMI medium was gently injected into the spleen and the cell suspension was gently drawn and released through a 40 µm cell strainer (BD Falcon, New Jersey, USA), connected to a 50ml centrifuge tube. Three milliliters of complete RPMI medium was added to the syringe and flushed again into the spleen; the suspension was drawn and pooled into the 50ml centrifuge tube through the cell strainer.

This process was repeated until the spleen remained as a thin membrane, at this point, the cell strainer was removed and placed on the Petri dish then the remains of the spleen were put in the strainer and using the inner plunger of a syringe, it was slowly crushed as complete RPMI medium was added into the strainer.

The 50ml centrifuge tube containing the cell suspension was centrifuged at 260×g for five minutes to pellet the cells. The supernatant was poured off and the pellet was resuspended in 10ml of 1% ammonium chloride solution and incubated at 37°C for ten minutes to get rid of the red blood cells from the splenocytes suspension. After ten minutes, 20ml of RPMI-1640 medium without FCS (incomplete RPMI medium) was added into the centrifuge tube and centrifuged at 340×g for 10 minutes at room

temperature. The supernatant was discarded and from this point, the splenocytes and myeloma cells were washed three times in separate tubes by centrifuging at 340×g for ten minutes at room temperature in 20ml of incomplete RPMI medium.

After the final wash, the splenocytes and myeloma cells were resuspended in incomplete RPMI medium, and part of the cells were diluted in trypan blue (100µl cell suspension and 100 µl trypan blue). The diluted cells were counted under the inverted microscope.

3.4.11 Fusion

After counting, and calculating the total concentration of the cells, the splenocytes and myeloma cells in incomplete RPMI medium were combined in a 50ml centrifuge tube in a ratio of 5:1. The mixture of splenocytes and myeloma cells were centrifuged twice at 130×g for ten minutes at room temperature. The supernatant was carefully removed using a transfer pipette and after the final wash, the pellet was centrifuged without resuspending it in medium, for one minute to remove any additional supernatant. The pellet was then disrupted by tapping the bottom of the tube and the tube was placed in a beaker with water at 37°C inside the biosafety cabinet.

The six minutes fusion process was carried out as follows:

- Minute 1: 1.0ml of PEG 1500 (Roche Life Science, Indiana, USA) was added drop wise while swirling the tube
- Minute 2: The PEG and cells were mixed by swirling
- Minute 3: 1.0ml of incomplete RPMI medium was added slowly
- Minute 4: Another 1.0ml of incomplete RPMI medium was added slowly
- Minute 5: 8.0ml of incomplete RPMI medium was added with gentle swirling
- Minute 6: 10.0ml of FCS was slowly added at the base of the tube

After the six minutes fusion process, the cells were pelleted at 70×g for ten minutes and the supernatant was discarded. Five milliliters of complete RPMI medium was added to the pellet without resuspending and incubated at 37°C incubator for ten minutes. Then 40ml of complete RPMI supplemented with 1× conc. HAT solution containing 5 mM

sodium hypoxanthine, 20 µM aminopterin and 0.8 mM thymidine as final concentration (Life Technologies, New York, USA) was added into the tube and the pellet was resuspended.

Twenty milliliters of fusion solution was mixed with 20ml of warm complete RPMI containing 1× conc. HAT solution in a sterile trough. All the 50ml fusion solution was transferred, 200µl per well into sterile 96-wells tissue culture plates and the cultures incubated at 37°C in 5% CO_2 and observed daily.

For the wells that were observed to have a colony growth, the medium in that particular well was changed by removing 100µl of the spent medium and adding 100µl of fresh complete RPMI medium with 1× conc. HAT solution. After seven days, spent medium was changed in all the wells; ensuring that each well had its own tip. The wells with colony growth had their culture fluids tested three days after change of medium by IgG indirect ELISA. The positive clones underwent limiting dilution in complete RPMI medium with 1× conc. HAT solution to get single clones in each well.

3.4.12 Limiting dilution

The hybridoma cells were counted and diluted in 5.0ml of complete RPMI medium with 1× conc. HAT solution to achieve a density of 1×10^3 cells/ml. From this stock, 100µl was added to row A, in plate one, representing 100 cells/well. Next, 1.8ml of complete RPMI medium with 1× conc. HAT solution and 0.2ml of the cell suspension of 1×10^3 cells per ml was added into a 15ml centrifuge tube, making a 2ml stock of 10× dilution. This cell suspension was mixed and 100µl was added to row B, in plate one, representing 10 cells/well.

Then, 7.2ml of complete RPMI medium with 1× conc. HAT solution was added to the remaining 0.8ml in the 15ml centrifuge tube to make a second 10× dilution which represented one cell/well. This cell suspension was mixed and 100µl was added to row C and D, in plate one. Lastly, 9.5ml of complete RPMI medium with 1× conc. HAT solution was added to the remaining 5.6ml in the reservoir and mixed thoroughly, 100µl

was added to the rest of the plate one and all of plate two. This represented 0.3 cells/well.

The sub cloned plates were placed in a 37°C incubator with 5% CO_2 and the rest of the cells were up scaled in 24 wells plate to a 25cm^2flask containing 10ml of complete RPMI medium with 1× conc. HAT solution. At least two batches of each clone were stored at -80°C with cell stock media.

Seven days later, 150µl of spent medium was removed from the each well of the sub cloned plates and 200µl of complete RPMI medium with 1× conc. HAT solution was added. The plates were observed under the microscope and the wells that had a single colony growing had their culture fluid tested for production of anti-RVFV antibodies by IgG indirect ELISA. The clones that tested positive were grown in complete RPMI medium supplemented with 1× conc. HT solution containing 10 mM sodium hypoxanthine and 1.6 mM thymidine as final concentration (Life Technologies, New York, USA) and gradually up scaled until they were stable before being transferred into serum free medium, i.e. Hybridoma SFM (Life Technologies, New York, USA).

3.4.13 Monoclonal antibody purification

The single clones that had tested positive in IgG indirect ELISA were cultured in Hybridoma SFM and the culture fluid was pooled to obtain a total volume of 500ml. IgG monoclonal antibodies were purified from this culture fluid using mAb trap kit (GE Healthcare, Uppsala, Sweden) that contains a HiTrap column pre packed with protein G Sepharose, following the manufacturer's instructions. The concentration of the purified anti-RVFV mAb was determined by spectrophotometry.

3.4.14 Characterization of the monoclonal antibodies

3.4.14.1 Isotyping

The class, subclass and light chain type of the mAb was determined using an "IsoStrip Mouse Monoclonal Antibody Isotyping Kit" (Roche Life Science, Indiana, USA), following the manufacturer's instructions. Briefly, the sample was pipetted into the

provided development tube, and agitated so that the coloured latex beads are completely resuspended and the isotyping strip was placed in the tube. The results were then read within one to five minutes.

3.4.14.2 Focus reduction neutralization test

The hybridoma culture fluid was heat inactivated at 56°C for 30 minutes, and then diluted serially in two folds and mixed with an equal volume of RVFV (30 FFU/0.1ml in mixed solution) in MEM 2% FCS and kept at 37°C with 5% CO_2 for one hour. One hundred microliters of the mixture was inoculated into each well of Vero cell in a 96 wells plate and incubated at 37°C for one hour in a 5% CO_2 incubator. The wells were then overlaid with 1.25% methylcellulose with MEM and incubated for 18 hours before overnight fixation with 10% formalin. The formalin was removed and the plate was washed once with $PBS^{(-)}$, then the cells were permeabilized using 1% NP-40 in $PBS^{(-)}$ at room temperature for 20 minutes. The plate was then washed three times using $PBS^{(-)}$ allowing three minutes incubation between the washes and blocking was done using original blockace at room temperature for 30 minutes. After washing the plate with $PBS^{(-)}$ three times,100µl of anti-RVFV rabbit serum at 1000× dilution was added to each well and the plate was incubated at 37°C for one hour. The plate was again washed three times, and then 100µl of HRP-conjugated anti-rabbit IgG (H&L) (American Qualex, California, USA) at 5,000× dilution was added to each well and incubated at 37°C for one hour, then washed three times.

One hundred microliters of substrate solution, which was prepared using 10mg DAB tablet (Wako, Osaka, Japan) in 10ml of $PBS^{(-)}$ with 0.03% of H_2O_2 was added into each well and the plate was incubated at room temperature for 10-30 minutes. The DAB stained cells were observed under the stereo microscope and the stained foci were noted, then the DAB was discarded and the plate was washed with distilled water. The numbers of foci were counted under a stereo microscope and the 50% focus reduction neutralization antibody titer ($FRNT_{50\%}$) of serum sample was calculated.

3.4.14.3 Testing cross-reactivity of the mAb with other arboviruses

An antigen detection ELISA was set up and used to test the anti-RVFV mAb reactivity with RVFV and other arboviruses which include DENV-1, DENV-2, DENV-3, DENV-4, YFV, WNV and CHIKV. All these viruses were titrated using focus assay and their concentrations adjusted to 5.0×10^4 FFU/ml then applied in the antigen detection ELISA (Refer to 3.4.15.2) at the antigen culture fluid step.

3.4.14.4 Western blot

The purified RVFV antigen that was used to react the anti-RVFV mAb was separated on two different concentration of polyacrylamide gels, one was the stacking gel constituted using 6.8ml water, 2.1ml of 40% polyacrylamide mix (Fischer Scientific, Leicestershire, UK), 3ml of 1.5M Tris-HCl pH 8.8 (BioRad, California, USA), 120µl of 10% SDS, 9µl of tetramethylethylenediamine (TEMED) (Thermoscientific, Massachusetts, USA) and 60µl of 10% ammonium persulphate (APS) (BioRad, California, USA). TEMED and APS are used to catalyze acrylamide polymerization when preparing gels for electrophoresis.

The second one was the separating gel, constituted using 3.3ml water, 375µl of 40% polyacrylamide mix, 1.25ml of 0.5M Tris-HCl pH 6.8 (BioRad, California, USA), 50µl of 10% SDS, 4µl of TEMED and 30µl of 10% APS. The purified RVFV sample was diluted in NuPAGE 4× conc. LDS sample buffer (Novex Life Technologies, California, USA) to make it 1× conc. and sonicated, 100mM dithiothreitol (DTT) (BioRad, California, USA) was then added to a final concentration of 50mM. The sample mixture was heated at 90°C for five minutes and immediately put on ice. Twenty microliters of the sample was then loaded into the gels with a dual colour 250 KDa protein marker (Biorad, California, USA). The gel was immersed in a 1× SDS-PAGE running buffer (14.4g glycine, 3.02g Tris base, 1g SDS and 900ml double distilled water) for 45 minutes.

One of the gels was stained in a staining solution containing 40% methanol, 10% acetic acid and 0.25% Coomassie R-250 for one hour and then destained overnight in a

destaining solution containing 7.5% acetic acid and 10% methanol. The gel was then visualized using ImageQuant LAS 500 machine (GE Healthcare, Uppsala, Sweden) using the colorimetric option. This CBB staining was important to reveal the protein separation and to enable us to visualize the band to which the anti-RVFV mAb would target.

The other two gels were treated for western blot analysis, one was reacted with the P1E5 mAb and the second one was used as a negative control. All the steps were the same in the two gels except at the application of the primary antibody; the negative control membrane was reacted with mouse pre-serum which did not contain any anti-RVFV antibodies while the other membrane was reacted with P1E5 mAb.

The bands on the gel were transblotted to a polyvinylidene difluoride (PVDF) membrane (ATTO, Tokyo, Japan) using a Bio-Rad blotting device. The PVDF membrane, eight pieces of absorbent papers and the gel were equilibrated in transblot buffer (60ml of ×1 Tris-CAPS, 45ml of 15% methanol and water to top up to 300ml). The PVDF membrane was then blocked in 5ml of BlockingOne (Nacalai Tesque, Kyoto, Japan) at 4°C overnight. The following day, the membrane was washed once in Tris buffered saline with 0.1% tween 20 (TBS-T) buffer for ten minutes and reacted with mAbs (300× dilution) at room temperature for two hours, followed by three times washing with ten minutes incubation. After which the membrane was incubated with 10,000× diluted HRP conjugated anti-mouse IgG (American Qualex, California, USA) at room temperature for one hour, followed by three times washing for ten minutes. The reaction was visualized using 2ml of Amersham ECL prime western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) which was allowed to react with the membrane for five minutes then the membrane was dried and the image was captured by chemiluminescence using the LAS-500 machine (GE Healthcare, Uppsala, Sweden)

3.4.15 Evaluation of the anti-RVFV mAb's diagnostic potential

3.4.15.1 Antigen capture IgG indirect ELISA

An antigen capture IgG indirect ELISA was set up and used to test 22 human samples. Maxisorp 96 wells ELISA plate (NUNC) was coated at 4°C overnight with anti-RVFV purified mAb diluted in coating buffer at a concentration of 20µg/ml. The following day, blocking was done at room temperature for one hour using original conc. blockace. The plate was then washed with PBS-T three times, allowing three minutes incubation between the washes. RVFV ICF titrated as 120 ELISA units was added into all the wells, except blank wells where only PBS-T was added and the plate incubated at 37°C for one hour. The plate was washed as previously described and 100× diluted human serum samples, positive control and negative control samples were added into the wells, and the plate was incubated at 37°C for one hour. After washing the plate, 100µl of 5,000× diluted HRP-conjugated anti-human IgG (American Qualex, California, USA) was added to each well and incubated at 37°C for one hour, and washed again as previously described. One hundred microliters of OPD substrate was added in each well and the plate was incubated at room temperature for 30-45 minutes before stopping the reaction by adding 100µl of 1N H_2SO_4. The optical densities were measured at 492nm and the IgG titers were calculated using a standard curve, and any sample that had IgG titers $\geq$ 1:3,000 was considered as a positive.

The sensitivity and specificity of the anti-RVFV purified mAb was tested by comparison of this test to the gold standard which used anti-RVFV pAb as the capture antibodiy. The ELISA system using the anti-RVFV purified mAb was further compared to a similar ELISA system that used anti-RVFV N protein mAb as the capture antibody.

3.4.15.2 Antigen detection ELISA

Maxisorp 96 wells ELISA plate (NUNC) was coated at 4°C overnight, with 100µl of anti-RVFV purified mAb diluted to 20µg/ml in coating buffer. The following day, blocking was done at room temperature for one hour, using original conc. blockace. The plate was then washed with PBS-T three times, allowing three minutes incubation

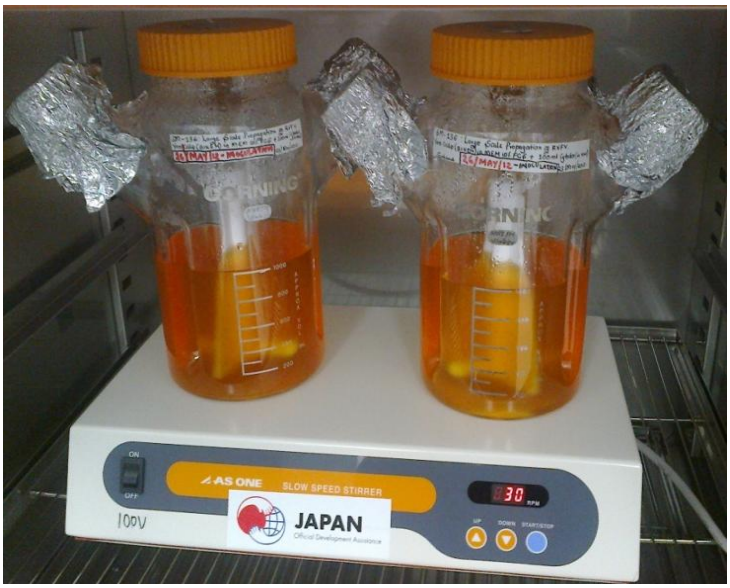
between the washes. One hundred microliters of the 5.0×10^4 FFU/ml RVFV ICF and a twofold serially diluted positive standard was added into adjacent wells and PBS-T was added into the blank wells. The positive and negative controls were added into the plate at this step. The plate was incubated at 37°C for one hour, after which it was washed as previously described. Next, high titer anti-RVFV rabbit pAb at 10,000× dilution was added into all the wells except the blank wells and the plate was incubated at 37°C for one hour, after which it was washed as previously described. After washing the plate, 100µl of 5,000×HRP-conjugated anti-rabbit IgG (H&L) (American Qualex, California, USA) was added to each well and incubated at 37°C for one hour, then washed again. One hundred microliters of OPD substrate was added to each well and the plate was incubated at room temperature for 30-45 minutes. Then, 100µl of 1N H_2SO_4 was added in all the wells to stop the reaction. The optical densities were measured at 492nm and the OD_{492} value of the negative control was used to calculate the cut off points of the negatives and positives. This was done by doubling the OD_{492} value of the negative control and any OD_{492} value above this was considered as a positive. In this case, the OD_{492} value of the negative control was 0.74, hence any ICF sample that had an OD_{492} value >1.48 was considered as a positive.

CHAPTER FOUR

RESULTS

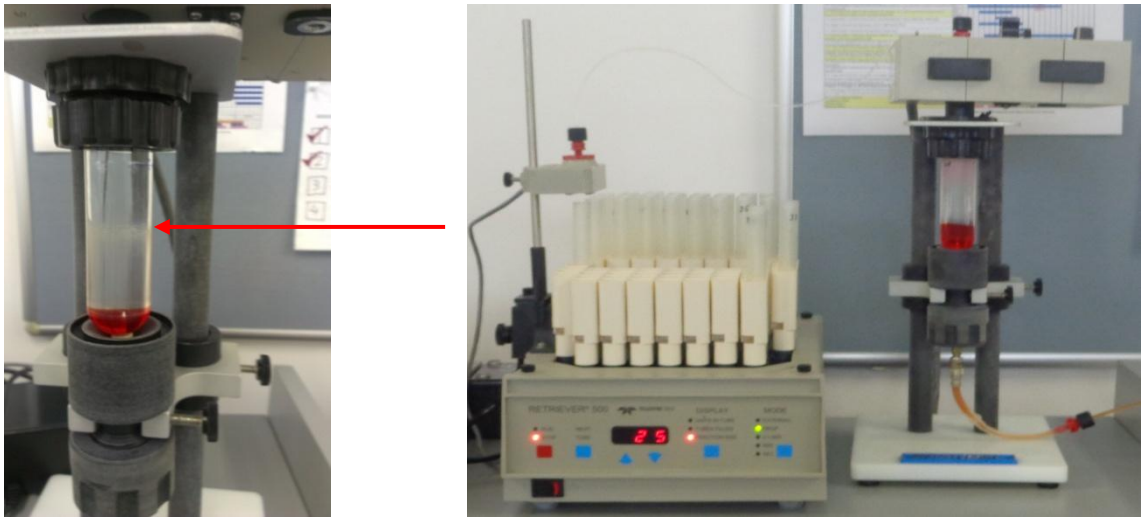
4.1 Propagation and purification of Rift Valley fever virus

The purified RVFV was prepared in two batches and a total of 2.03 mg/ml and 2.81 mg/ml of the purified RVFV were obtained. Figures 4.1 A and 4.1 B show the RVFV that was cultured in large scale culture and its precipitation, while Figure 4.1C shows the white band containing the purified RVFV and Figure 4.1 D shows the fraction collection tubes where the purified RVFV was collected into.



A

B

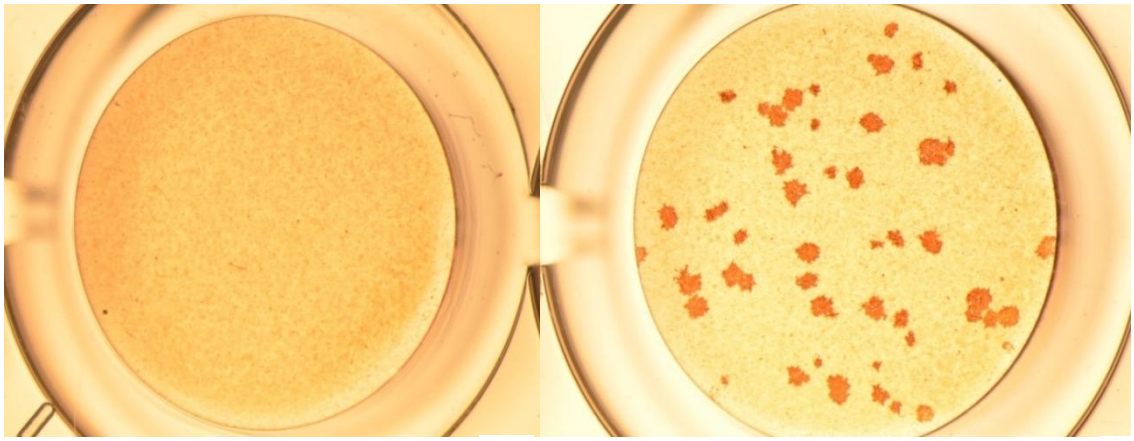


C D

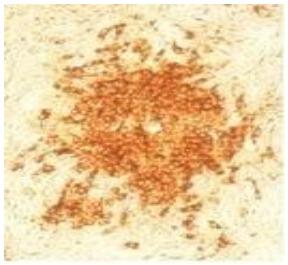
Fig 4.1: Large scale cultured and purified RVFV. (A) Cultured RVFV in one liter spinner bottles. (B) PEG precipitation of the virus at 4°C. (C) Purified RVFV band indicated by the arrow. (D) The purified RVFV collected in tubes

4.2 Inactivation of the purified Rift Valley fever virus

The purified formalin inactivated RVFV when tested for complete inactivation by focus assay showed that the inactive virus did not infect the Vero cells, therefore no foci were observed (Figure 4.2 A). Whereas the RVFV infected the Vero cells and some foci were observed (Figure 4.2 B) with the DAB stained Vero cells clearly observed (Figure 4.2 C).



A B



C

Figure 4.2: Foci images as seen under a stereo microscope. (A) No focus was observed in the 96 wells plate with inactivated RVFV (20× magnification). (B) Clear foci were observed in 96 wells plate with RVFV (20× magnification). (C) Higher magnification of RVFV focus in Vero cells (50 × magnifications).

4.3 Anti-Rift Valley fever virus titers in immunized rabbits

The anti-RVFV IgG titers in the immunized rabbit increased gradually to about 1:40,000 after which the titer decreased slightly and then shot up to 1:250,000 (Figure 4.3). The arrows indicate the timings when the first immunization and booster shots were done, and it is evident that the titers went up every time a booster shot was administered. The titer increased to a maximum value of 1:300,000, whereby it became constant and then started to decline and finally it became constant.

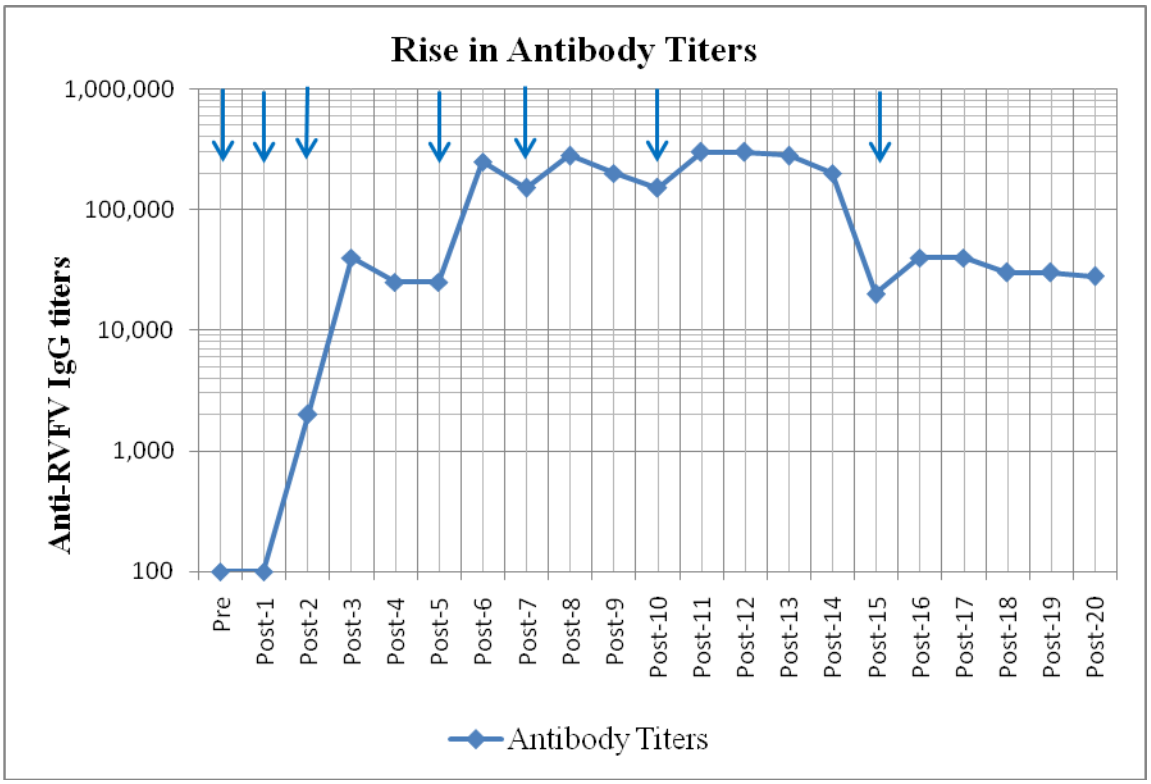


Figure 4.3: Anti-RVFV IgG titers in the immunized rabbit sera over a period of time. "Pre" represents the bleeding done before immunizations started. "Post" represents the bleeding done every two weeks. Arrows indicate the first immunization and subsequent booster shots.

4.4 Anti-Rift Valley fever virus titers in immunized mice

Testing of the mice sera using IgG indirect ELISA showed that there was no rise in anti-RVFV IgG titers in the control mice No. 9 and No.10, their titers were at a baseline, i.e. 1:1,000 as seen in Figure 4.4. The highest titer attained was observed in mouse 6, which was 1:60,000, while mouse 1 and mouse 8 followed with titers of 1:20,000 and mice 3, 4, 5 and 7 had titers of 1:6,000.

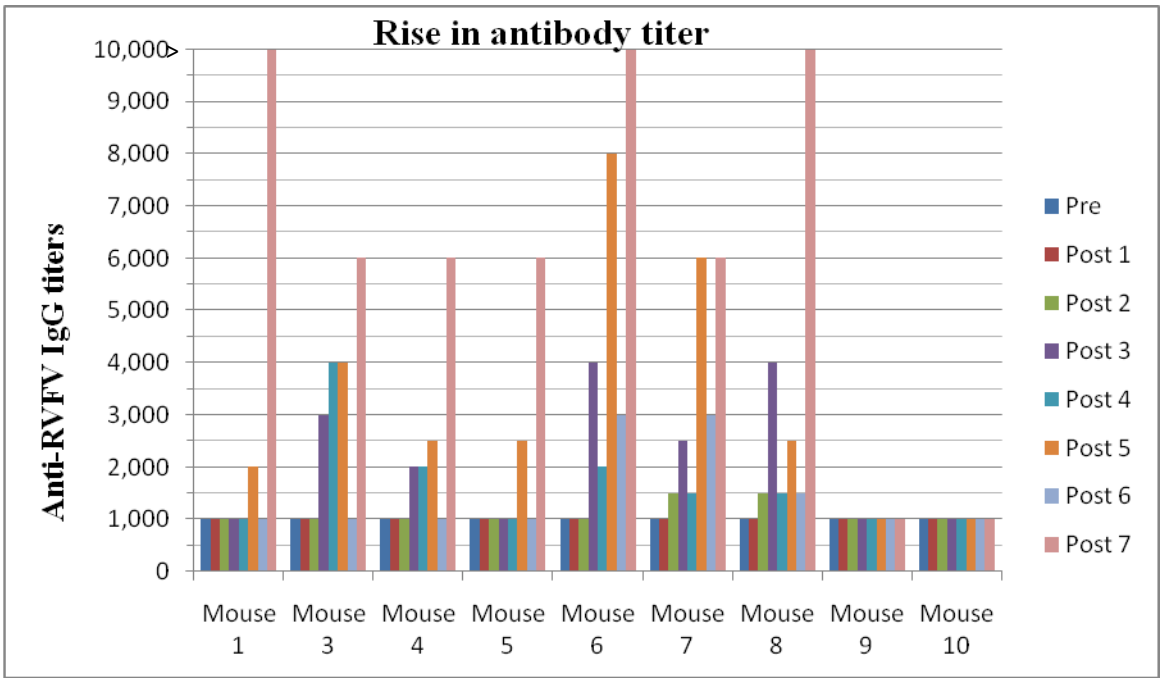


Figure 4.4: Anti-RVFV IgG titers in the immunized mice from pre-serum to the seventh post immunization. "Pre" represents the bleeding done before immunizations started. "Post" represents the bleeding done every two weeks.

Each mouse that was immunized with the virus, responded differently, although there was a general similar pattern that was observed in all the mice. For example, there was no increase in the anti-RVFV IgG titers with the first immunization in all the mice. This was expected, since the first phase of response which occurs after the animal's initial encounter with an antigen is considered as a low-level primary response that is predominantly of the IgM class (Carey *et al.*, 1995), and this assay only tested for IgG. The anti-RVFV IgG titers declined between post 5 and 6 because there was a delay in the administration of the sixth booster shot, whereas all the other boosters were administered in two weeks' interval, this was done on the fourth week. The mice's response to the RVFV varied, the mice started the production of anti-RVFV IgG at different times. Mice 7 and 8 started after the second booster shot, mice 3, 4 and 6 after the second booster shot while mice 5 and 1 after the fourth booster shot.

4.5 Generation of hybridomas and cloning

The culture fluid from the hybridoma clones were tested by IgG indirect ELISA. The hybridoma culture fluid in the well E5 of the ELISA plate (Figure 4.5, Table 4.1) gave an OD value of 2.469 at 492nm in the IgG indirect ELISA, indicating it was positive. There were a few weak positives at C8, E12, F12, G12 and H12 which gave OD values ranging from 0.123 to 0.326 at 492nm (Table 4.1). Some of the clones that gave the weak positive results from the IgG indirect ELISA, stopped the production of anti-RVFV IgG antibodies within a short time after subcloning while others did not grow at all during the subcloning.

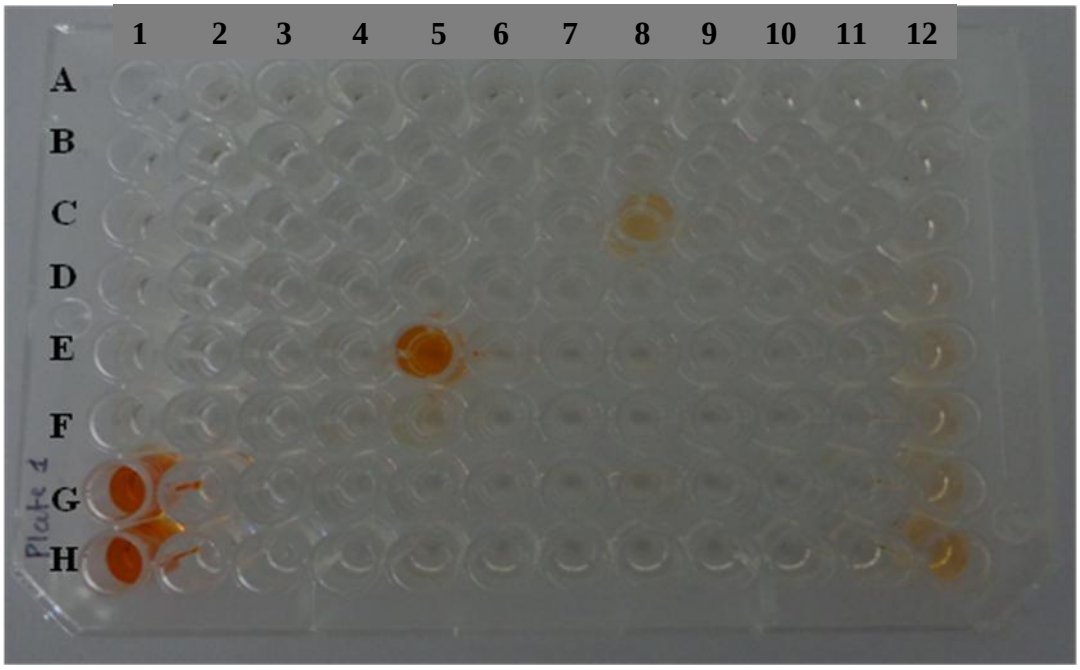


Figure 4.5: IgG indirect ELISA plate showing the positive clone P1E5. Wells G1 and H1 are positive control. Wells C1 and D1 are the negative control.Well E5 is the positive hybridoma clone. Wells C8, E12, F12, G12 and H12 are weak positives.

Table 4.1: Results of screening plate one by ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.002	0.014	0.016	0.029	0.015	0.025	0.015	0.022	0.020	0.020	0.022	0.028
B	0.001	0.009	0.014	0.014	0.012	0.008	0.015	0.046	0.014	0.014	0.014	0.016
C	0.025	0.017	0.009	0.011	0.011	0.008	0.012	0.326	0.022	0.021	0.012	0.042
D	0.021	0.007	0.012	0.011	0.027	0.009	0.013	0.011	0.013	0.008	0.045	0.077
E	0.019	0.011	0.013	0.020	2.469	0.029	0.016	0.033	0.014	0.009	0.012	0.123
F	0.029	0.014	0.016	0.044	0.095	0.024	0.011	0.009	0.014	0.012	0.014	0.129
G	3.327	0.012	0.012	0.008	0.014	0.008	0.009	0.048	0.009	0.007	0.008	0.159
H	3.382	0.025	0.014	0.022	0.034	0.022	0.021	0.016	0.024	0.028	0.040	0.313

Wells G1 and H1 show the OD of the positive control. Wells C1 and D1 show the OD of the negative control. Well E5 shows the OD of the positive hybridoma clone. Wells C8, E12, F12, G12 and H12 show the OD of the weak positives.

The positive clones underwent cloning by limiting dilution until a single cell clone was obtained as shown in the Figure 4.6. A total of four single cell clones were obtained, and named as P1E5, P3E7, P3C10 and P3D6. Out of these four clones, the P1E5 clone was successfully purified, characterized and its diagnostic potential was tested by applying it in antigen detection ELISA and antigen capture IgG indirect ELISA.

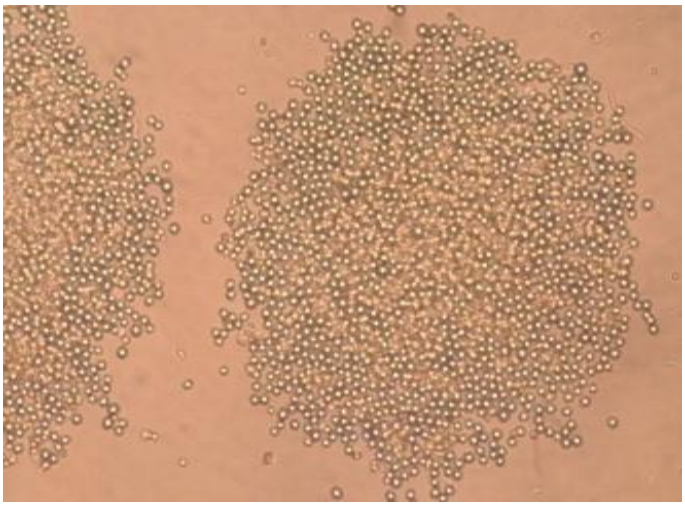


Figure 4.6: Single cell hybridoma clones attained after limiting dilution. Represents the mAb since it originates from a single cell, the mAb has been separated from the pAbs present in the fused cells.

4.6 Monoclonal antibody purification

Purification of the 500ml culture fluid from the single cell hybridoma clone P1E5 using mAb Trap kit produced a total of 4.84 mg. From the Figure 4.7, tubes No. 11 and 12 contained the purified mAb of interest, i.e. 2.17 mg/ml and 2.67 mg/ml, and these were pooled together.

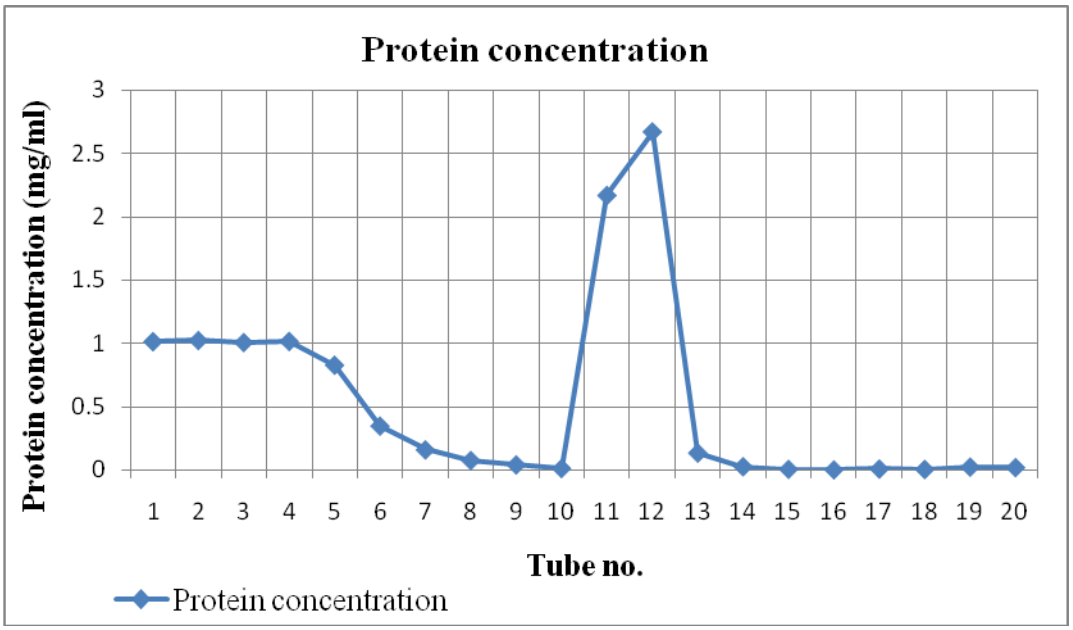


Figure 4.7: Purification of P1E5 mAb. Tubes no. 11 and 12 contained the purified mAb since their protein concentrations were the highest (2.17 mg/ml and 2.67 mg/ml).

4.7 Rift Valley fever virus antigen detection ELISA

Using the antigen detection ELISA that was set up in this study, the cross-reactivity of P1E5 mAb was tested against other arboviruses (DENV 1, DENV 2, DENV 3, DENV 4, YFV, WNV and CHIKV). The results showed that the anti-RVFV mAb could only pick RVFV and not any of the other arboviruses tested (Table 4.2).

Table 4.2: Results of the cross-reactivity test of P1E5 mAb using antigen detection ELISA

	NC	PC	DENV-1	DENV-2	DENV-3	DENV-4	YFV	WNV	CHIKV	RVFV
O.D	0.74	1.97	0.77	0.69	0.65	0.67	0.70	0.78	0.62	2.26
+/-	-	+	-	-	-	-	-	-	-	+

Any of the culture fluid sample that had an OD value > 1.48, was considered as a positive. NC is the Negative Control and PC is the Positive Control.

4.8 Antigen capture IgG indirect ELISA

Out of the 22 samples that were tested using antigen capture IgG indirect ELISA, four samples tested positive (Table 4.3). The same samples were tested with a similar ELISA set up, but with different capture antibodies; anti-RVFV N protein mAb and anti-RVFV pAb, and the results for the three tests were in agreement (Table 4.3).

Table 4.3: Comparison of three antigen capture IgG indirect ELISA with different capture antibodies using 22 human serum samples.

Capture Ab	**1*** Anti-RVFV pAb	**2*** Anti-RVFV N protein mAb (3D11)	**3*** Anti-RVFV L protein mAb (P1E5)
Sample no. 1	+	+	+
Sample no. 2	+	+	+
Sample no. 3	+	+	+
Sample no. 4	+	+	+
Sample no. 5	-	-	-
Sample no. 6	-	-	-
Sample no. 7	-	-	-
Sample no. 8	-	-	-
Sample no. 9	-	-	-
Sample no. 10	-	-	-
Sample no. 11	-	-	-
Sample no. 12	-	-	-
Sample no. 13	-	-	-
Sample no. 14	-	-	-
Sample no. 15	-	-	-
Sample no. 16	-	-	-
Sample no. 17	-	-	-
Sample no. 18	-	-	-
Sample no. 19	-	-	-
Sample no. 20	-	-	-
Sample no. 21	-	-	-
Sample no. 22	-	-	-

Numbers 1-22 represent the human serum samples tested. 1*, 2* and 3* are the different antibodies that were applied in the antigen capture IgG indirect ELISA as the capture antibodies. 1* was the gold standard test, 2* was an ELISA set up using Anti-RVFV N protein mAb and 3* was the ELISA set up using the developed anti-RVFV L protein mAb.

The cut-off values in the antigen capture IgG indirect ELISA systems were determined differently depending on the laboratory protocols where the Abs were developed. These were as follows:

1* The positives were samples that had an adjusted OD value of ≥ 0.2; the adjusted value was obtained by subtracting the OD value of the sample from the negative control value.

2* The positives were samples whose ratio had a value of ≥ 2.0; the ratio was determined by dividing the sample OD by the negative control OD of that specific sample.

3* The positives were samples that had IgG titers ≥ 1:3,000.

Table 4.4: Sensitivity and specificity of P1E5 mAb in relation to the gold standard

		Antigen capture IgG indirect ELISA using Anti-RVFV pAb as capture antibody		
		Positive	Negative	Total
* Antigen capture IgG indirect ELISA using Anti-RVFV L protein mAb (P1E5) as capture antibody	Positive	4	0	4
	Negative	0	18	18
	Total	4	18	22

The sensitivity and specificity of the P1E5 mAb (anti-L protein mAb) was calculated by comparison to the gold standard and the Sensitivity and specificity of the P1E5 mAb were both found to be 100%.

Table 4.5: Sensitivity and specificity of P1E5 mAb in relation to anti-RVFV N protein

		Antigen capture IgG indirect ELISA using Anti-RVFV N protein mAb (3D11) as capture antibody		
		Positive	Negative	Total
* Antigen capture IgG indirect ELISA using Anti-RVFV L protein mAb (P1E5) as capture antibody	Positive	4	0	4
	Negative	0	18	18
	Total	4	18	22

The P1E5 mAb (anti-L protein mAb) was compared to anti-N protein mAb (3D11) in an antigen capture IgG indirect ELISA. The sensitivity and specificity of the P1E5 mAb were both found to be 100%.

4.9 Focus reduction neutralization test

On testing the P1E5 mAb for neutralizing activity by focus reduction neutralization test, the 50% focus reduction neutralization antibody titer was found to be less than ten ($FRNT_{50\%} = < 10$). This indicates that the mAb did not have any neutralizing activity.

4.10 Isotyping

Isotyping of the purified P1E5 mAb using an isotyping kit showed that its isotype was IgG1 as shown in Figure 4.8 A and the light chain was kappa, as seen in Figure 4.8 B. Both Figures 4.8 A and 4.8 B indicate the positive control regions of the strip marked as (+) to give an indication of the validity of the strip.

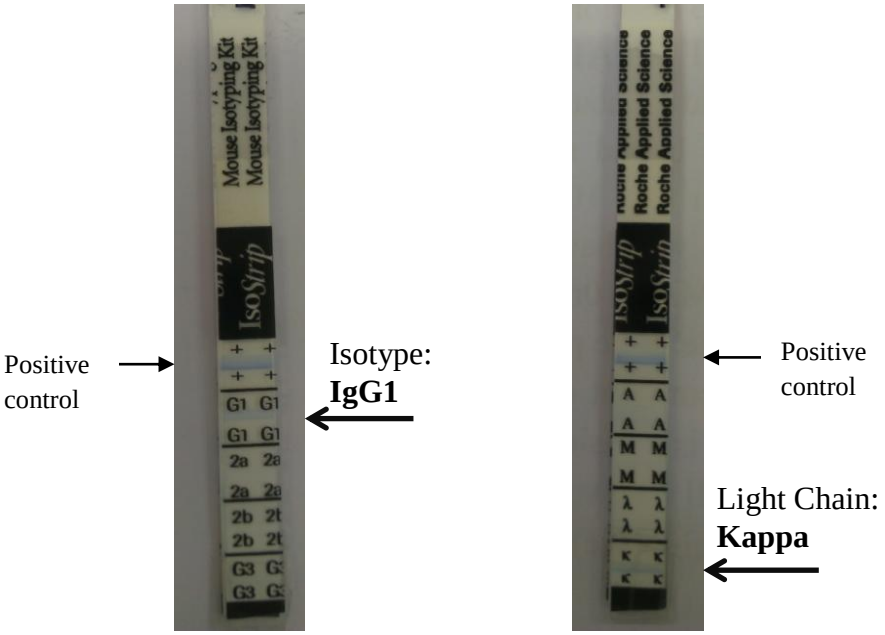


Figure 4.8: Isotyping results of P1E5 mAb. (A) Isotype result indicated as IgG1. (B) Light chain result indicated as Kappa. Each strip shows a positive control (+).

4.11 RVFV western blot analysis

The purified RVFV that was separated on a 7% SDS polyacrylamide gel and stained in CBB stain showed the presence of three bands with different molecular weights, with approximate weights of L protein, Gc protein and N protein bands appeared (Figure 4.9 A).

The other gel which had its bands transferred onto a nitrocellulose membrane and immunostained with the P1E5 mAb as the primary antibody, showed only the L protein region of the RVFV (Figure 4.9 B). It suggested that the mAb P1E5 targets the L protein of RVFV.

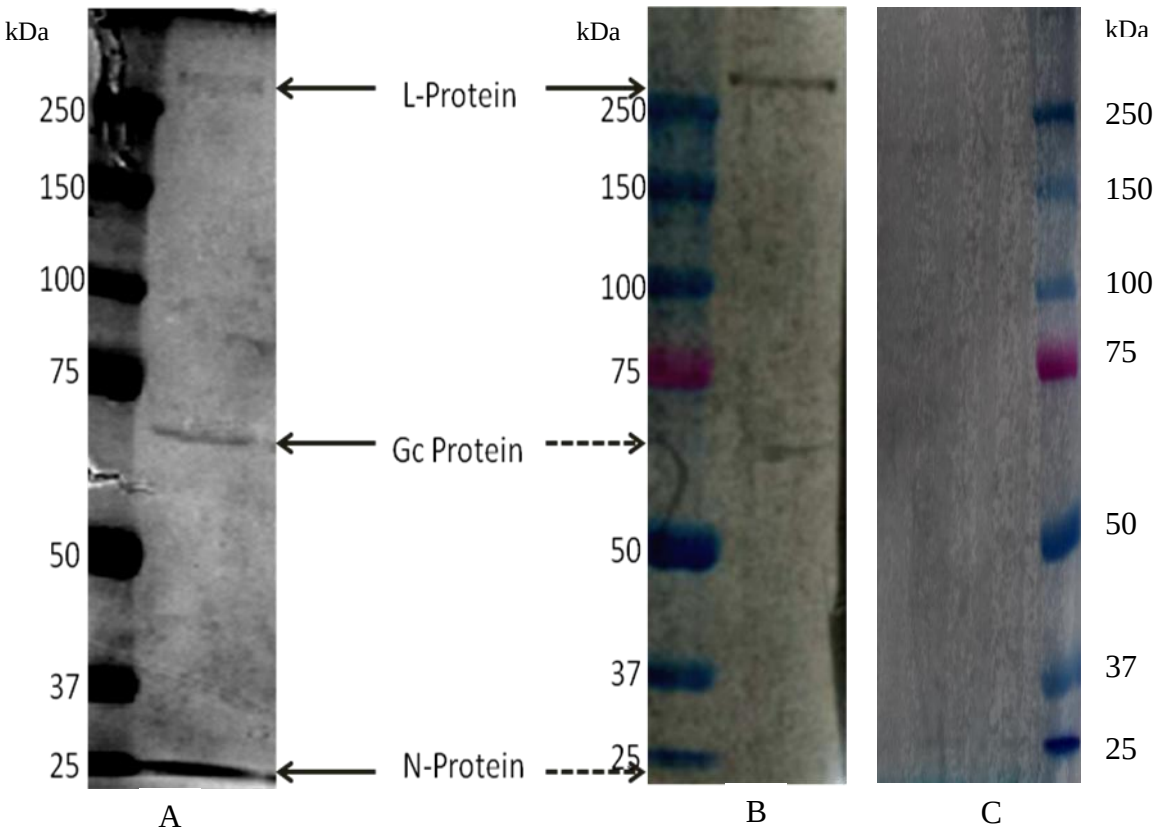


Figure 4.9: Images for the CBB stained gel and western blot analysis membrane. (A) CBB image showing the three proteins present in the purified RVFV. (B) Western blot image showing P1E5 mAb stained L protein. (C) Western blot image showing the negative control.

CHAPTER FIVE

DISCUSSION

5.1 Generation of mAb against Rift Valley fever virus

Both pAb and mAb were successfully developed in this study, and they were both applied in setting up of ELISA systems. This was the first time that mAb was being developed at the KEMRI-Production Department and the procedure proved to be challenging due to the various optimization processes that needed to be carried out. Several trials of the protocol were carried out before the anti-RVFV hybridoma cells were successfully obtained.

Some of the most crucial steps were found to be in the immunization of the mice and the fusion process. Following the mice immunizations, IgG titers were checked by IgG indirect ELISA, and according to a protocol by Fuller *et al.,* 1992, the mouse spleen can be used for fusion when the mouse has developed sufficient antibody titers. In this study, four out of seven mice had their highest titer as 1:6,000, while two of the mice had their highest titer as 1:20,000 and one of the mice had its highest titer as 1:60,000. The level of IgG titer that was considered sufficient before the mouse could be sacrificed and the spleen used in fusion was 1:6,000, this is because the splenocytes derived from mice with this titer were able to yield positive hybridoma clones.

On the other hand, in the entire procedure of harvesting the spleen, preparation of splenocytes and fusion, the most important step was the fusion where the PEG 1500 was added to the mixture of splenocytes and myeloma SP-2 cells. Some of the important factors that were considered were the ratio of splenocytes and myeloma cells which is normally 5:1 (McCullough, *et al.,* 2009), but this ratio can be increased to as high 10:1. The high ratio of the splenocytes yielded high fusion efficiency and at the same time, the excess of the splenocytes functioned as feeder cells, which supplied cell growth factors that promote the growth of the hybridoma cells. The medium used in the fusion process was at 37°C and the entire fusion process was carried out at 37°C. This temperature is crucial, since it favored the activity of PEG 1500 that allows fusion of the plasma

membranes of adjacent splenocytes and myeloma cells, resulting into a single cell (Greenfield, 2014). The speed of centrifugation after the six minutes fusion process is also very important and it should not exceed 70×g since the cell membranes of both splenocytes and myeloma cells have been weakened by the action of PEG 1500, therefore a high centrifugation speed might puncture these cells.

The mAb obtained was against a 250kDa protein, which is the RVFV L protein; this was an unexpected result, since there is no reported study that has shown the generation of mAbs against the L protein. The developed mAbs against RVFV commonly reported were against the structural proteins, i.e. the N, Gn, Gc proteins. The N protein is the most abundant and highly immunogenic, whereas the Gn and Gc are surface proteins that carry epitopes which elicit the production of neutralizing antibodies, a correlate of protective immunity (Raquel *et al*., 2010). The RVFV induces a potent antibody response against the N protein, but to a much lesser extent to both mature Gn and Gc proteins. A study by Zaki *et al*., (2006) reported the use of formalin inactivated RVFV in development of mAbs, and the study yielded mAbs against the N, Gn and Gc proteins. However, the methodology that they used differed from that applied in this study in that the RVFV was cultured in suckling mice by injecting through the intracerebral route, after which the brain was harvested and homogenized, then inactivated by formalin and used in immunization. Whereas in this study, the RVFV was propagated in cell culture and purified by sucrose density gradient ultracentrifugation, inactivated by formalin and used in mice immunization. Despite the fact that both studies utilized formalin inactivated whole RVFV, the different methodologies applied in these two studies could have resulted in the varying targets of the developed mAbs.

One possibility as to why the mAb developed in this study was not against the common structural proteins, could be that the 1% formalin inactivation of the purified RVFV masked the structural proteins leaving only the L protein intact. Formalin inactivation has been reported to mask tissue antigens due to cross-linking of protein amino acids residues (Ezaki, 2000).

Development of mAbs against varying epitopes is crucial in the establishment of a panel of mAbs that can be applied in diagnosis. Some possible methods that can be implemented so as to develop mAbs against N, Gn and Gc proteins, include lowering the formalin concentration that is used in the inactivation of the RVFV or carry out the RVFV inactivation using ß-propiolactone. Another method is to develop recombinant N, Gn and Gc proteins and applying these recombinant proteins in the immunizations of mice; this has been carried out in various studies. For example, a study by Jackel *et al.* (2014), developed mAbs against RVFV N, Gn and Gc proteins by immunization of the mice using recombinant N, Gn and Gc proteins and were evaluated for their use in a competitive ELISA for detection of IgG against RVFV. In another study, mAbs were developed against the RVFV N protein, but the mice were immunized using N protein DNA and the developed mAbs were tested for their applications in antibody detection competitive ELISA and antigen detection ELISA (Raquel *et al*., 2010).

5.2 Characterization of the developed mAb P1E5

Isotyping of P1E5 mAb revealed that the mAb was IgG1 and it was carrying the Kappa light chain. This was an expected result, since mice have 95% kappa chains, and majority of mAbs from mice usually have the kappa light chains (Farmilo & Stead, 2001).

The anti-RVFV P1E5 mAb did not show any RVFV neutralizing activity when tested by FRNT, this shows that the mAb can only function as a detecting antibody and not a neutralizing antibody. Neutralizing antibodies are primarily against the viral glycoproteins Gc and Gn (McElroy *et al.,* 2009), antibodies against L protein are not expected to have neutralizing activity.

The three bands visualized in the CBB analysis concurred with molecular weights that have been previously reported, i.e. Gc protein; 60 kDa (Gerrard & Nichol, 2007), N protein; 27 kDa (Donald *et al.,* 2010) and L protein; 238 kDa (Lopez *et al.,*1995). In the western blot analysis, it was seen that the epitope to which the P1E5 mAb was binding to, was the L protein. L protein is the RNA dependent RNA polymerase (RdRp) (Muller

et al., 1994) and since RVFV has a negative sense, single stranded RNA (Wilson, 2006), RdRp has to be present for it to carry out its function in cap-snatching which initiates mRNA synthesis and the transcription termination (Pepin *et al.*, 2010). The presence of the L protein (RdRp) during transcription and replication of the virus, gives the possibility of L protein being able to exist in the purified RVFV band. As a result, it was possible to develop antibodies against the L protein in this study, even though the L protein is considered to be a non-structural protein.

The fact that the developed mAb was targeting the RVFV L protein brings about the most unique and important aspect of this study. Brennan *et al.,* 2011, reported that despite the importance of RVFV L protein, little is known about the intracellular distribution of the polymerase or its other roles during infection and detailed studies of the RVFV L protein has been impeded due to lack of antibodies that can detect the L protein in infected cells. Hence, the mAbs developed in this study can enable detailed investigation of L protein synthesis, distribution and interactions by using a variety of experimental techniques.

However, more experiments need to be carried out so as to confirm whether the 250kDa protein that reacted with the mAb (P1E5) is really the RVFV L protein, as there could be a possibility that the 250kDa protein is a dimer, trimer, tetramer, pentamer or hexamer of other structural or non-structural RVFV proteins. This can be done using Liquid Chromatography–Mass Spectrophotometry- Mass Spectrophotometry (LC-MS-MS) technique whereby the amino acid sequence of the 250kDa protein will be analyzed. The LC-MS-MS will enable amino acid fingerprinting, and the amino acid sequence will be blasted in an available database such as Mascot (Okamoto *et al.,* 2010). It is not possible to analyze the 250kDa protein by DNA sequencing because the protein does not contain any viral genome. The viral RNA was lost during the processing of the RVFV for LC-MS-MS analysis. This eliminated the nucleic acids and only the proteins were left. In addition, during the SDS-PAGE, the purified protein was fragmented by sonication and the sample heated at 90°C and immediately put on ice.

Another technique that can possibly be applied is the two-dimensional gel electrophoresis based western blot. The two-dimensional gel electrophoresis will reveal whether the 250kDa protein exists on its own or it is a mixture of two or more proteins and the western blot will reveal to which of the proteins the mAb bind to.

5.3 Antigen detection ELISA

The purified anti-RVFV mAb P1E5 developed in this study was applied as the catching antibody in an antigen detection ELISA. Various studies have shown the application of mAbs as capture antibodies for detection of different important pathogens in ELISAs due to the high specificities of mAbs (Saijo *et al*., 2007; Qiu *et al*., 2009; Liu *et al*., 2010). This developed ELISA system can further be tested for its potential in detection of RVFV in various samples, like human and animal sera, mosquito homogenates and also milk since the developed system was able to detect RVFV in ICF.

Using this ELISA system, the cross reactivity of the developed P1E5 mAb was tested with other arboviruses DENV 1-4, YFV, WNV and CHIKV that were from different families, and the mAb did not show any cross reactivity. This makes the developed anti-RVFV mAb to have a higher potential of efficient diagnosis since there is no possibility of it cross reacting with arboviruses from other families. However, there is need to test the mAb for cross reactivity with viruses that belong to the same family of *Bunyaviridae* as the RVFV such as the Bunyamwera virus, Kupe virus, Dugbe virus, among others. This will enable us to determine whether that mAb is virus specific or family and/or genus consensus.

5.4 Antigen capture IgG indirect ELISA

The antigen capture IgG indirect ELISA was used to detect anti-RVFV IgG in 22 human sera, using the anti-RVFV L protein mAb P1E5 as the capture antibody of RVFV in ICF assay antigen. The results were similar to other two antigen capture IgG indirect ELISAs that used anti-RVFV N protein mAb and anti-RVFV pAb as the capture antibodies. The antigen capture IgG indirect ELISA that used anti-RVFV pAb is the gold standard that was used to compare to the developed assay. This shows that the

developed anti-RVFV L protein has a potential of being applied in diagnosis of anti-RVFV IgG since both its sensitivity and specificity, in comparison to the gold standard was found to be 100%.

At the same time, it was necessary to compare the developed assay using an already existing antigen capture IgG indirect ELISA that uses mAbs. Hence the antigen capture IgG indirect ELISA that was developed using anti-L protein was compared to a similar set up that uses anti-N protein. Both sensitivity and specificity, were found to be 100%.

One major advantage of the antigen capture IgG indirect ELISA over the ordinary IgG indirect ELISA, is that in the antigen capture IgG indirect ELISA, the antigen applied is the RVFV ICF while in the IgG indirect ELISA, ultracentrifuge purified RVFV antigen is required as the assay antigen. The challenge is that the RVFV antigen purification process takes a long period of time and requires a capacity of large scale cell culture systems and ultracentrifugation in the laboratory. Hence this study has offered an alternative ELISA that can be applied in detection of anti-RVFV IgG antibodies, when there is an unavailability of purified RVFV antigen.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- It was possible to develop both pAb and mAb against RVFV, locally at the KEMRI Production Department facility.
- The study showed that the anti-RVFV P1E5 mAb has a diagnostic potential since it could detect RVFV antigen in the antigen detection ELISA, and it could also detect anti-RVFV IgG antibodies in the antigen capture IgG indirect ELISA, giving similar results when compared to the same test using different capture antibodies, i.e. anti-RVFV N protein mAb and anti-RVFV pAb.
- Currently, there is no report on the development of mAbs against the L protein of RVFV, and this forms a new basis of research in that the anti-L mAbs can be used in further characterization of the RVFV L protein and understanding the mechanism of RVFV assembly.
- With the availability of the hybridoma cells producing anti-RVFV mAbs, the cells can be cultured and the culture fluid harvested when the antibodies are required. This has created an economical and readily available supply of the anti RVFV antibodies locally, thus enhancing diagnosis of RVF in Kenya.

6.2 Recommendations

The following points are recommended for further work so as to maximize the benefits from this study:

- The 250kDa protein should undergo further analysis using LC-MS-MS and two-dimensional gel electrophoresis based western blot. This will confirm whether the protein is RVFV L protein or not and it will give an indication of any other structural feature that the protein could be having other than the polymerase activity.

- The P1E5 mAb should be tested for cross reactivity against other Bunyavirues which are closely related to RVFV such as the Bunyamwera virus, Kupe virus, Dugbe virus, among others.
- Since the development of mAbs is a new technique that has been set up in the local setting, it should be applied to other microbes so that new diagnostic and therapeutic tools can be developed.

6.3 Limitations of the Study

- In the mAb development, the immunogen used was the whole virion and not a recombinant protein of a certain epitope because the recombinant technique has not yet been successfully established in the KEMRI-Production Department facilities.
- Due to unavailability of a closely related virus belonging to the family *Bunyaviridae*, the developed anti-L protein mAb could not be tested for cross-reactivity with closely related viruses in the *Bunyaviridae* family.
- Limited number of human serum samples containing anti-RVFV antibody available for use in the antigen capture IgG indirect ELISA.
- The set up antigen detection ELISA was tested using ICF and not clinical samples, this was due to lack of serum samples containing RVFV.

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APPENDICES

Appendix I: ACUC approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O. Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 03.08.12 **21ST August 2012,**

Salame Mahfudh Ashur,
TM 302-1065/2011

Salame,

RE: KEMRI ACUC approval for "Generation of Monoclonal antibodies against Rift Valley Fever virus and evaluation of their diagnostic potential" Protocol

Following the resubmission of the above mentioned proposal to the ACUC addressing the issues raised earlier, the committee recommends that you proceed with your work after obtaining all the other necessary approvals.

The committee expects you to adhere to all the animal handling procedures on the rabbits and mice to be used as described in your proposal.

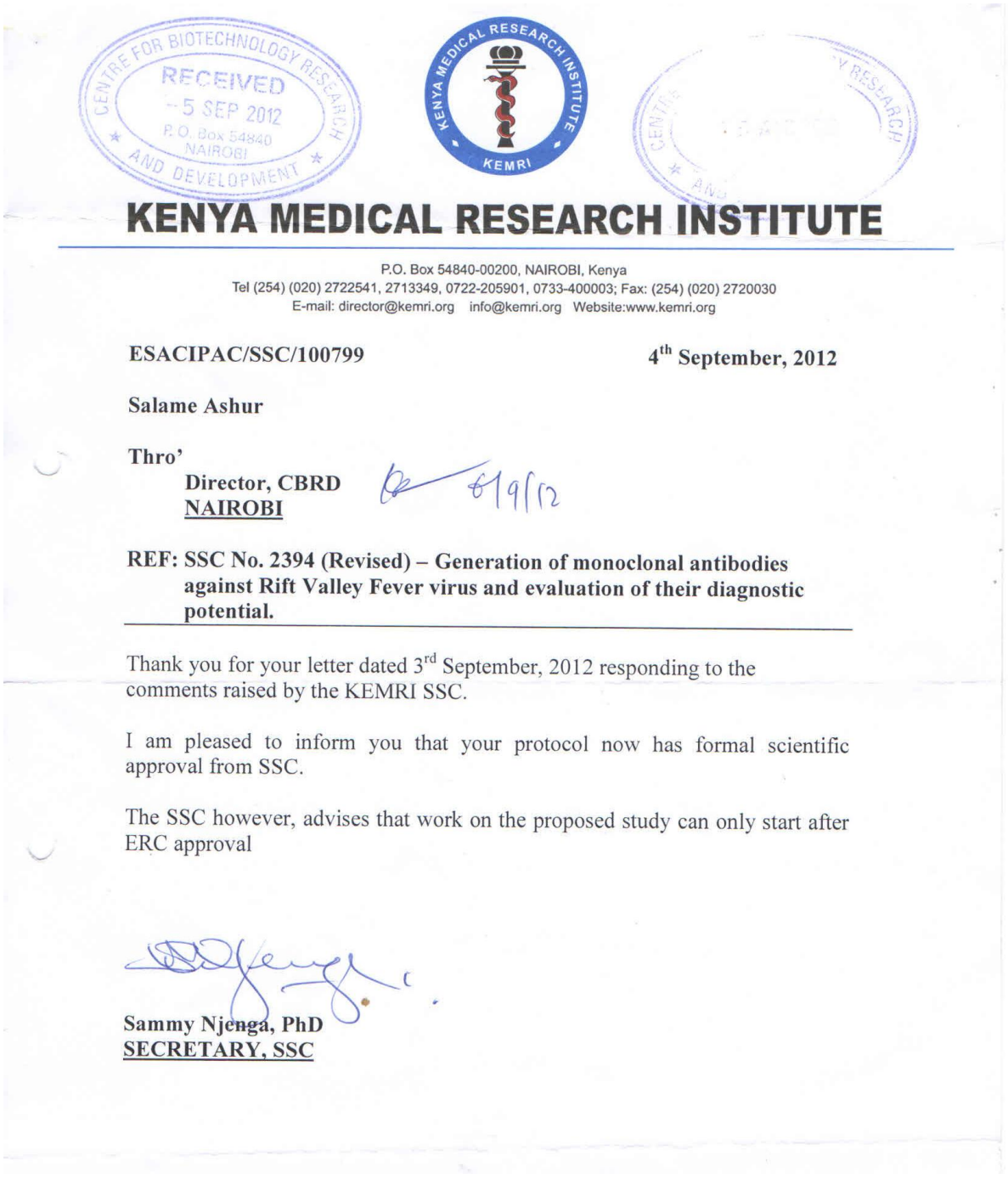
The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

c.c
Animal House

Appendix II: SSC approval of protocol (SSC No.2394)



CENTRE FOR BIOTECHNOLOGY RESEARCH AND DEVELOPMENT
RECEIVED
5 SEP 2012
P.O. Box 54840
NAIROBI

KENYA MEDICAL RESEARCH INSTITUTE
KEMRI

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/100799 **4th September, 2012**

Salame Ashur

Thro'
Director, CBRD
NAIROBI

6/9/12

REF: SSC No. 2394 (Revised) – Generation of monoclonal antibodies against Rift Valley Fever virus and evaluation of their diagnostic potential.

Thank you for your letter dated 3rd September, 2012 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval

Sammy Njenga, PhD
SECRETARY, SSC

Appendix III: ERCapproval of protocol (SSC No.2394)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **August 27, 2013**

TO: **SALAME M. ASHUR (PRINCIPAL INVESTIGATOR)**

THROUGH : **DR. KIMANI GACHUHI**
THE DIRECTOR, CBRD
NAIROBI

2/9/13

Dear Madam,

RE: **SSC PROTOCOL No. 2394 - REVISION 4 (*RESUBMISSION 3*): GENERATION OF MONOCLONAL ANTIBODIES AGAINST RIFT VALLEY FEVER VIRUS AND EVALUATION OF THEIR DIAGNOSTIC POTENTIAL**

Reference is made to your letter dated 12th August, 2013. The ERC Secretariat acknowledges receipt of the revised proposal on August 13, 2013.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document listed above and is satisfied that the issues raised at the initial meeting have been adequately addressed.

The study is granted approval for implementation effective this **27th day of August 2013**. Please note that authorization to conduct this study will automatically expire on **August 26, 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **July 15, 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

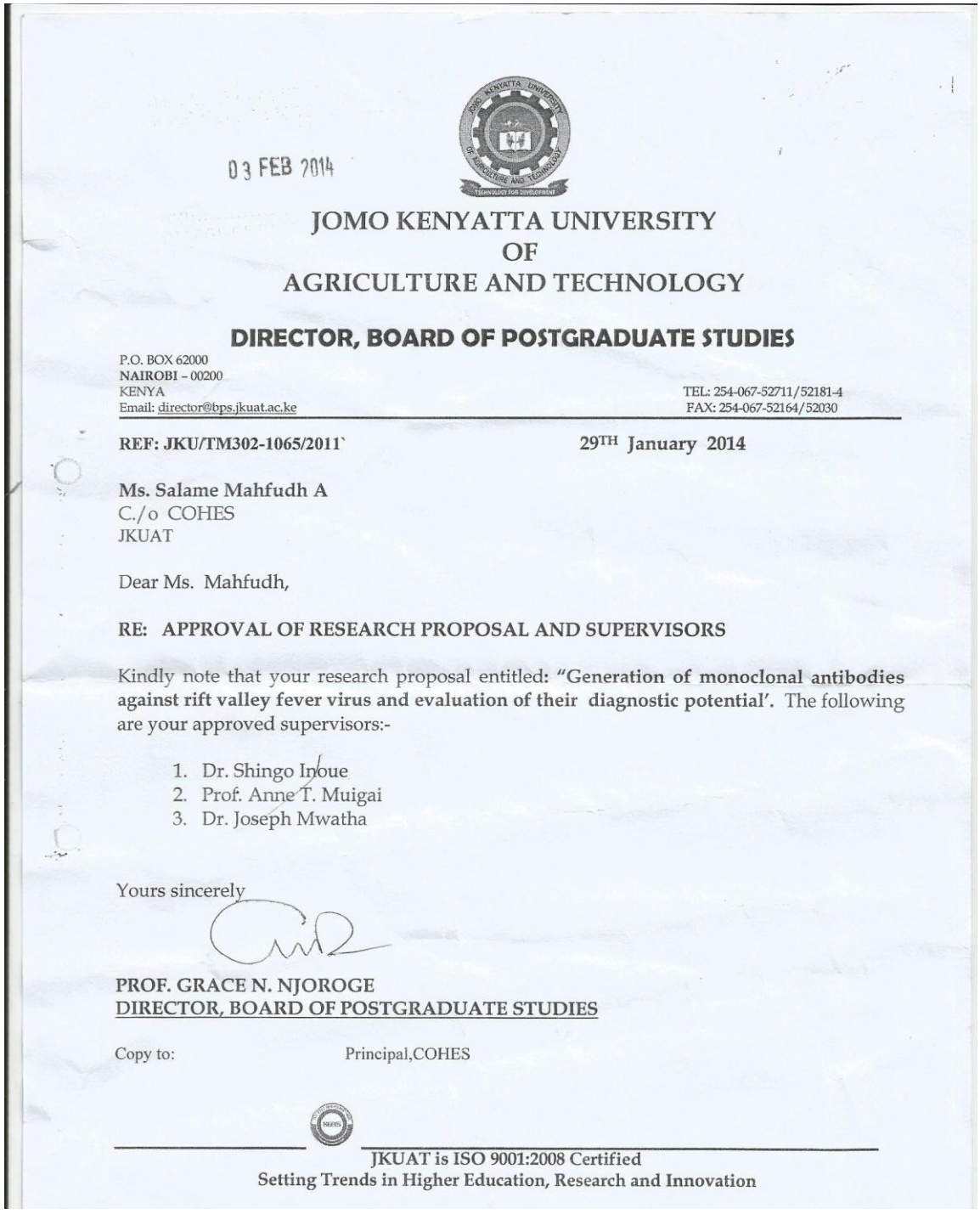
You may embark on the study.

Yours faithfully,

EAB

Dr. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

Appendix IV: JKUAT approval of proposal and supervisors



03 FEB 2014

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY

DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000
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KENYA
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REF: JKU/TM302-1065/2011` **29TH January 2014**

Ms. Salame Mahfudh A
C./o COHES
JKUAT

Dear Ms. Mahfudh,

RE: APPROVAL OF RESEARCH PROPOSAL AND SUPERVISORS

Kindly note that your research proposal entitled: **"Generation of monoclonal antibodies against rift valley fever virus and evaluation of their diagnostic potential'.** The following are your approved supervisors:-

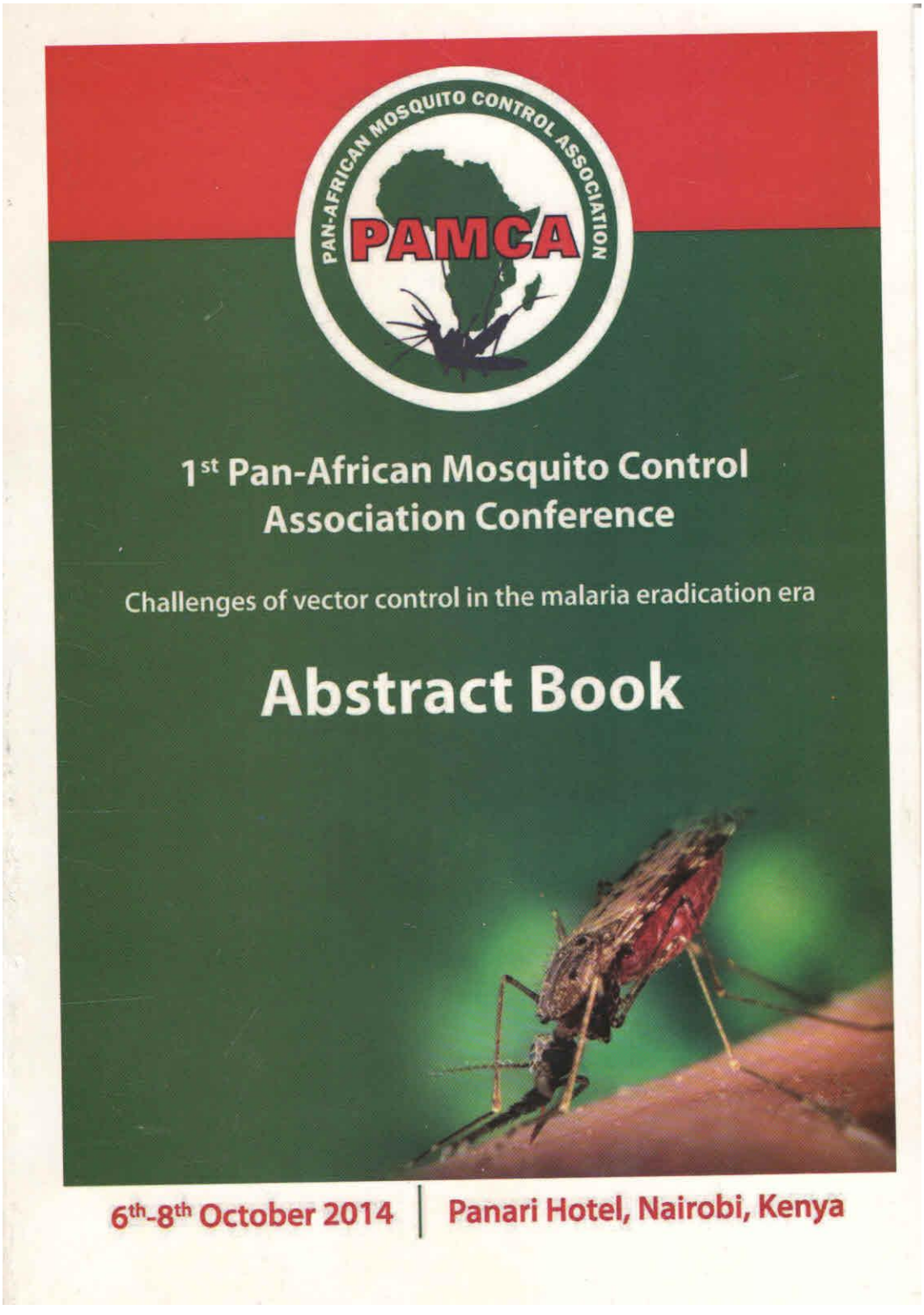
1. Dr. Shingo Inoue
2. Prof. Anne T. Muigai
3. Dr. Joseph Mwatha

Yours sincerely

PROF. GRACE N. NJOROGE
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Principal,COHES

Appendix V: Abstracts presented at conferences



Abstract 08

Development of rabbit polyclonal antibody based tests for analysis of rift valley fever virus infection

Salame Ashur[1], Shingo Inoue[2], Allan ole Kwallah[1,3], Nicholas Ragot[3], James Kimotho[3], Rosemary Sang[4], Matilu Mwau[1,5], Kouichi Morita[2], Anne Muigai[1], Joseph Mwatha[6]

[1]Institute of Tropical Medicine and Infectious Diseases, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya [2]Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan [3]Production Department, Kenya Medical Research Institute, Nairobi, Kenya [4]Centre for Virus Research, Kenya Medical Research Institute, Nairobi, Kenya [5]Centre for Infectious and Parasitic Diseases Control Research, Kenya Medical Research Institute, Busia, Kenya [6]Centre for Biotechnology and Research Development, Kenya Medical Research Institute, Nairobi, Kenya.

Background: Kenya has experienced large outbreaks of Rift Valley fever (RVF) among human and livestock in 1997-1998 and 2006-2007. However, the laboratory diagnosis depends on either supplies from foreign countries or by sending samples abroad. This study aims to develop laboratory diagnostic tests locally by using rabbit polyclonal antibody (pAb).

Methods: RVFV (attenuated live vaccine strain: Smithburn) was propagated using Vero cells and purified by ultracentrifugation. The purified RVFV was inactivated at 1% formalin and complete inactivation was confirmed by focus assay. New Zealand white rabbits were immunized with inactivated RVFV by intramuscular injections of 125µg/shot with boosters at two weeks interval until the IgG titers reached to high levels. Rabbit pAb against RVFV was purified from pooled sera by the combination of saturated ammonium sulfate precipitation method and protein G affinity column method. Antigen detection ELISA, focus reduction neutralization test (FRNT) and western blot analysis were performed using the rabbit sera.

Results: Anti RVFV rabbit hyper-immunized sera reached 1:100,000 IgG titer when tested using IgG ELISA. FRNT could confirm the presence of antibody against RVFV. The antigen detection ELISA could detect RVFV specifically. Furthermore, pAb was able to detect the L segment derived protein, nucleocapsid protein and glycoprotein of RVFV in western blot analysis.

Conclusion: In-house prepared rabbit pAb against RVFV was applicable for detection of viral antigen, and this has enabled a platform for diagnosis of RVF in Kenya.

Keywords: Rift Valley fever virus, polyclonal antibody, antigen detection ELISA, focus reduction neutralization test, western blotting assay

21ST MEDICAL LABORATORY SCIENTIFIC AND EXHIBITION CONFERENCE FOR THE ASSOCIATION OF KENYA MEDICAL LABORATORY SCIENTIFIC OFFICERS (AKMLSO)

PROGRAMME & BOOK OF ABSTRACTS

DATE: 7TH – 10TH OCTOBER 2014
VENUE: REEF HOTEL – MOMBASA

THEME:
INTEGRATION OF LABORATORY MEDICINE INNOVATIONS IN CLINICAL SYSTEMS FOR IMPROVED HEALTH CARE OUTCOMES

Vision: Ensuring Excellence in Medical Laboratory Practice in Kenya and Beyond

diarrhoeal disease and severe rotavirus associated disease are of critical importance in determining the impact of future vaccine introduction.

VI009: 10.00AM – 10.10 AM

Comparison of the performance of a commercial diagnostic Kit - Fast Track -and an in-house Multiplex PCR method in respiratory virus detection in Kilifi

Anne Bett[1], Caroline Gitahi[1], Susan Morpeth[1,3] and D. James Nokes[1,2]

1. KEMRI-Wellcome Trust Collaborative Research Programme, Kenya; 2. School of Life Sciences, University Warwick, UK; 3. London School of Hygiene and Tropical Medicine, UK

Background

Multiplex PCR assays have become the platform of choice in detection of respiratory pathogens with a number of commercial kits available; however few studies have assessed the performance of these kits in detection of viruses from clinical samples compared to in-house developed methods.

Methods

We compare the performance of Fast Tract Diagnostics (FTD) commercial kit to an in-house multiplex PCR in detecting 14 common respiratory viruses and a fungus. A total of 252 nasopharyngeal and oropharyngeal swabs collected in universal transport media were tested using a 33 target FTD kit and an in-house PCR assay (15targets).Viruses overlapping in the two assays were; Influenza A and B, Parainfluenza I, 2,3 and 4, RSV A and B, Adenovirus, Corona viruses (229E, OC43 and NL63) HMPVA/B and Rhinovirus.

Results

Of the 252 samples a respiratory pathogen was found in 113 samples (44.8%) this was picked by both methods. 177 samples were positive by either of the methods.FTD picked more Para-influenza viruses with 88.9% sensitivity vs 36.1% by MPX. On the other hand MPX picked more Corona viruses; 77.8% versus 61.1% by FTD.

Conclusion

Generally there was good agreement of above 95% observed with the two assays in detecting RSV, Flu, Adenovirus and HMPV with the highest analytical agreement of above 98% for RSV and Flu detection. However, notably FTD was able to detect more PIV viruses with 88.9% sensitivity as compared to 36.1% for the in house assay. This could have been attributed by the difference in primer design and/or the cost vs benefit of optimizing the triplex. MPX detected more Corona virus though there were few samples positives, a bigger sample size is recommended to draw a better conclusion. This suggests that assessing Multiplex PCR assay performance is feasible in deciding the best assay for a study.

VI010: 10.15AM – 10.25AM

Application of Monoclonal Antibodies against the L-Protein of Rift Valley Fever Virus in Diagnosis

Salame Ashur[1], Shingo inoue[2], Anne Muigai[1], Joseph Mwatha[1,3]

1. Institute of Tropical Medicine and Infectious Diseases – JKUAT

2. NAGASAKI UNIVERSITY/Kenya Medical Research Institute

3. Kenya Medical Research Institute

Background:

Rift Valley Fever Virus (RVFV) is an important mosquito borne pathogen, causing Rift Valley fever (RVF). It is responsible for human deaths and massive loss of livestock in Africa, and recently in the Arabian Peninsula. Since there is no disease specific treatment for RVF, appropriate diagnostic strategies coupled with measures to prevent transmission are essential for disease control. Therefore, the need for development of a field deployable diagnostic technique remains a priority.

The objective of this study was to set up antigen detection ELISA and antigen capture IgG indirect ELISA using monoclonal antibodies (mAb) against the L-Protein of RVFV.

Methods:

The hybridoma cells from a single clone were cultured large scale in serum free medium and the harvested culture fluid was purified using mAb Trap kit. The purified mAb was used as the catching antibody at a concentration of 200 g/ml in the antigen detection ELISA and antigen capture IgG indirect ELISA. The antigen capture IgG indirect ELISA was used to test 22 human samples and this was compared to the ordinary IgG indirect ELISA.

The antigen detection ELISA was used to test for cross reactivity of the anti-RVFV mAb with other arboviruses (Dengue Virus serotypes 1-4, yellow fever virus, West Nile virus and chikungunya virus). The culture fluids of these viruses were titrated by focus assay and standardized to 5.0×10^4 FFU/ml.

Results:
The anti-RVFV mAb did not show any cross reactivity with the other arboviruses (Dengue Virus serotypes 1-4, yellow fever virus, West Nile virus and chikungunya virus), only the RVFV culture fluid was detected. The human samples that were tested using antigen capture IgG indirect ELISA and IgG indirect ELISA gave similar results in both, although the IgG indirect ELISA had a higher background.

Conclusion:
The developed anti-RVFV mAb can be applied in detection of RVFV antigen and anti-RVFV IgG in human sera and this will provide a cheaper and readily available diagnostic platform.

VI011: 10.30AM – 10.40 AM

Opportunistic Infections in HIV/Aids Patients

Author: Judith Niver ,CMLC, Homa Bay

Abstract

Diarrhea is one of the major complications in patients living with HIV/AIDS in developing world. Cystoisospora belli (Isospora belli) is one of the common causes for watery diarrhea in immunocompromised patients.

Many patients with HIV are diagnosed at late stage of the disease.Indicator infection like isospora belli will help in early diagnosis and treatment of HIV and will definitely improve quality of life in young patients.

-The diagnosis of Cystoisospora belli is done by the examination of stool.Amongst the intestinal coccidian parasites Cystoisosporiasis can effectively be treated with trimethoprim-sulfamethoxazole.

-Cystoisospora belli is potentially treatable infectious agent in patients with HIV/AIDS.

Introduction

Diarrhea leads to high morbidity and mortality in HIV infected patients, Cryptosporidium, Cystoisospora, Cyclospora and Microsporidia are common opportunistic enteric parasites encountered in these patients.

-Proper and early identification of these opportunistic parasites is important in view of HIV prevalence in Homa Bay County which is about 25% the highest in the whole country.

-Cystoisospora belli was first described by Virchow in 1860 in villi of intestinal mucosa at autopsy. It is an AIDS defining illness if infection persists >4 weeks. Infection is acquired through fecal contaminated food or water and generally diagnosed by examination of stool and /or duodenal biopsy specimens with acid fast staining.

Findings

In Homa Bay county most of the facilities do not perform concentration method which is more sensitive compared to wet preparation which is routinely performed. Out of the 9 diagnostic laboratories surveyed, none of the laboratories can perform concentration method if requested for on the spot including the county referral hospital.

Concentration method it is not done routinely and therefore most of the clients being diagnosed for causes of diarrhea in the OPD and CCC are missed on is osporabelli because all of the laboratories are using the wet preparation as a routine.

Discussion

Irrespective of wide spread awareness about HIV, Large number of people are diagnosed at very late stage of the disease. Isosporiasis is one of the indicators of immunocompromised status of patients; pathogenesis of cystoisosporiasis is characterized by invasion of epithelial cells of distal duodenum and proximal jejunum with resulting cell damage.Extra intestinal forms are rare.

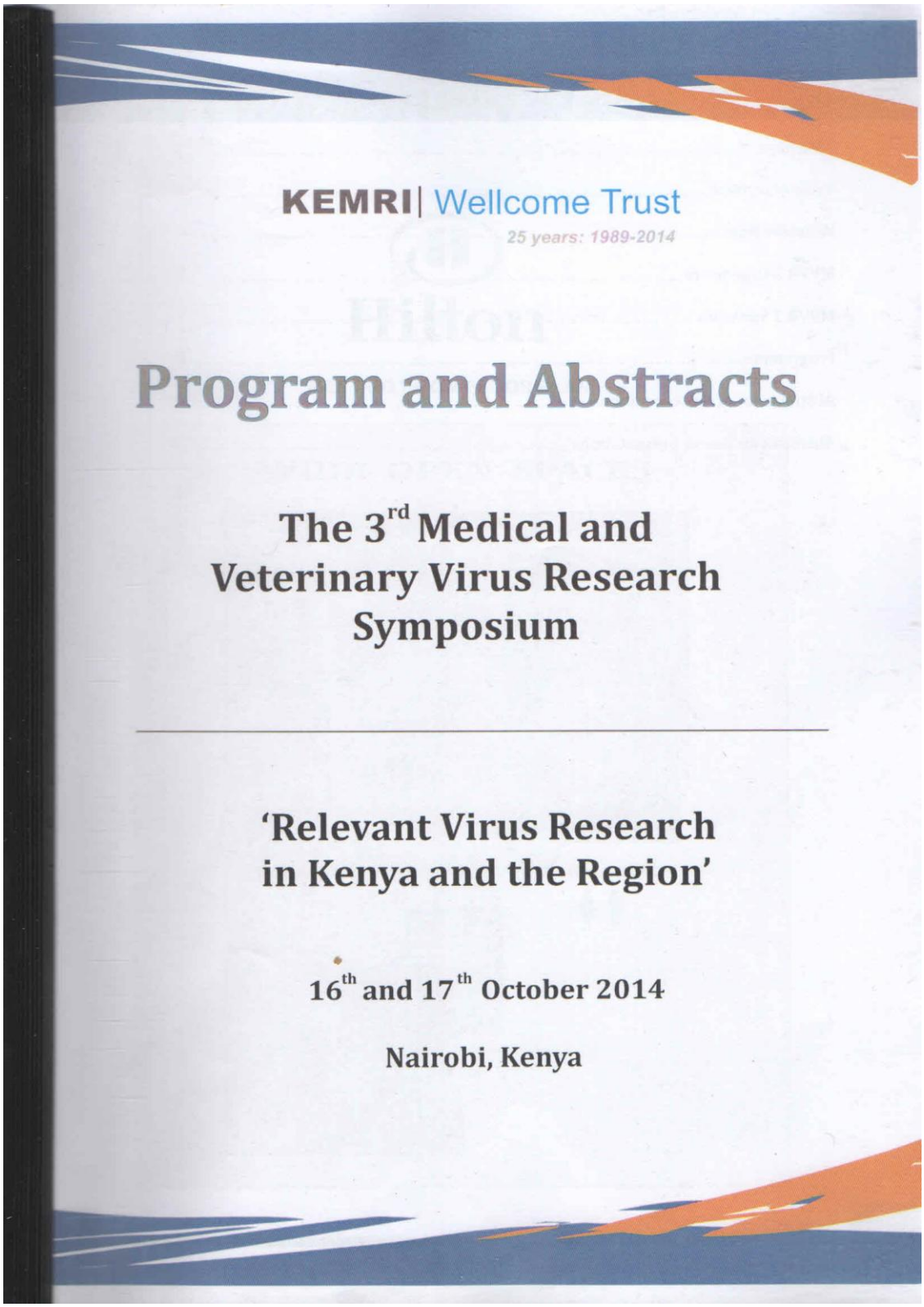
Cystoisospora belli is diagnosed by detection of the oocysts in stool or rarely bile samples. Oocysts can be observed in wet preparations, iodine stain preparation or acid fast stained smears of concentrated stool specimens.Most laboratories use wet preparations as opposed to concentrated method and this may lead to missed diagnosis of Cystoisospora belli.

Stool smears can also be stained with auramine – rhodamine, heamatoxyline and eosin and giemsa stain. Careful examination of stool sample and use of concentration techniques increases rate of detection of Cystoisospora belli cyst.

Hence to conclude routine screening of the stool samples of HIV seropositive patients with diarrhea should be done for prompt patient care, to prevent the fulminant form of the disease. As most of the opportunistic parasitic infections occur through the feacal oral route, they can be prevented by using safe drinking water and food by monitoring personal hygiene and by avoiding walking bare foot.

The laboratory should be encouraged to perform stool concentration method as a routine which will go along way in reducing missed diagnosis of Cystoisospora belli.

More research needs to be done to establish the prevalence of CYSTOISOSPORA BELLI IN Kenya and in Homa Bay County.


KEMRI| Wellcome Trust
25 years: 1989-2014
Program and Abstracts
The 3rd Medical and Veterinary Virus Research Symposium
'Relevant Virus Research in Kenya and the Region'
16th and 17th October 2014
Nairobi, Kenya

Development and characterizatoin of monoclonal antibodies against rift valley fever virus.

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Background: Rift Valley fever virus (RVFV) causes Rift Valley fever (RVF) which is one of the major viral hemorrhagic fevers in Kenya and East Africa. Due to its nature as a zoonotic and mosquito borne viral disease, it can infect domestic animals, wild animals and humans by mosquito bite and direct contact with blood and contaminated body fluid.

Objective: This study was designed to develop and characterize the monoclonal antibodies (mAbs) against RVFV.

Methodology: RVFV Smithburn live attenuated vaccine strain was purified using ultracentrifuge and sucrose gradient. The purified RVFV was used to immunize BABL/C mice for monoclonal antibody development. The hyper-immunized mice spleens were harvested from the mice and fused with myeloma cells (SP-2) using Polyethylene Glycol 1500. The hybridoma cells were screened for production of anti RVFV antibodies by IgG indirect ELISA and the positives clones underwent cloning by limiting dilution to obtain single clones. The mAb was characterized by western blot (WB), focus reduction neutralization test (FRNT), antigen detection ELISA and IgG isotyping.

Results: Four clones were obtained as anti-RVFV mAb So far. Two mAbs were isotyped as IgG1 Kappa chain type antibodies, the other two have not been determined, yet. These isotyped mAbs were further characterized as antibody against L segment derived protein of the RVFV by WB. These two mAbs did not show any neutralization activity against RVFV. However, it showed specific reaction with RVFV among various arboviruses by antigen detection ELISA.

Conclusions: Two mAbs were successfully developed and characterized as anti-RVFV mAbs.

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PROGRAM AND ABSTRACT BOOK

	Diseases and Department of Microbiology, University of Georgia, 30602 *Athens, GA, United States* **Introduction/background:** Initial evaluation of the Point of Contact Cathodic Circulating Antigen (POC-CCA) in multiple schistosomiasis endemic settings in Africa have suggested good sensitivity for detection of *S. mansoni* infections; however there is not enough data on variability and reliability of the POC-CCA test across different test days. The objective of this study was to investigate if there were any variations in the POC-CCA scores across the 5 collection days. **Methods:** 73 school children residing in a 15-30% schistosomiasis prevalence area were recruited into the study. The children provided 5 consecutive urine samples and 3 consecutive stool samples for testing. The urine samples were tested by POC-CCA rapid test and the stool samples by Kato-Katz (microscopy) technique (2 slides per stool sample). A "gold standard" which consisted of 8 tests (5 POC-CCA tests and 3 Kato-Katz tests) was used to calculate the positive predictive value (PPV) of the POC-CCA. **Results:** The PPV for the POC-CCA results across the 5 days was 100 %(94.1-100). When an individual tested positive by Kato-Katz on any of the 3 days, then at least 1 out of the 5 POC-CCA tests was also positive. Individuals who had moderate or high egg counts (100-399 egg per gram) also had positive POC-CCA test results. 75% of the participants in the study had consistent POC-CCA results compared to only 49% who had consistent Kato-Katz results. **Discussion/conclusion**: POC-CCA test is consistent compared to Kato-Katz method of testing making it an ideal tool for schistosomiasis screening especially for use in mapping surveys.
NTD8-A4:	**Testing the diagnostic potential of in-house developed monoclonal antibodies against rift valley fever virus.** Salame Ashur[1], Shingo Inoue[2], Allan ole Kwallah[1,3], Nicholas Ragot[3], Rosemary Sang[4], Kouichi Morita[2], Joseph Mwatha[5], Anne Muigai[1]. *1. Institute of Tropical Medicine and Infectious Diseases, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya. 2. Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan. 3. Production Department, Kenya Medical Research Institute, Nairobi, Kenya 4. Centre for Virus Research, Kenya Medical Research Institute, Nairobi, Kenya 5. Centre for Biotechnology and Research Development, Kenya Medical Research Institute, Nairobi, Kenya* **Background:** Monoclonal antibody (mAb) is currently widely used not only for research purposes but also for diagnostic purpose as well as therapeutic purposes. In this study, we aimed to develop mAb against Rift Valley fever virus (RVFV) and apply to the diagnostic tests to increase the accuracy of the results. RVFV belongs

	to Family *Bunyaviridae* and it causes Rift Valley fever (RVF) which is one of the major viral hemorrhagic fevers in Kenya and African continent. RVFV is transmitted by *Aedes* and *Culex* mosquito bites and contact with infected body fluids including blood and milk. Due to the presence of other Bunya viruses (e.g. Crimean-Congo hemorrhagic fever virus and Nairobi sheep disease virus) in Kenya, RVFV specific mAb based diagnostic tests are highly needed. **Methods:** BALB/C mice were immunized with purified, inactivated RVFV (attenuated live vaccine strain: Smithburn) and when the IgG titers were high enough, the mouse spleen was harvested and splenocytes were fused with mouse myeloma SP2 cells. Four clones of anti-RVFV mAb were successfully established. Among them, Clone "P1E5" was further cultured in large scale and purified by protein G column. The purified mAb was applied for antigen detection ELISA, antigen-capture IgG indirect ELISA and western blot. **Results:** The mAb (P1E5) was applied for antigen detection ELISA and specifically detected only RVFV but not dengue virus serotypes 1-4, yellow fever virus, West Nile virus and chikungunya virus. Antigen capture IgG indirect ELISA could detect IgG from patient sera that were known to have anti-RVFV IgG. Western blot using this mAb could detect RVFV and it specifically reacted with L segment derived protein (250kDa). **Conclusion:** Although further analysis using other Bunya viruses is required, these three tests could detect RVFV and anti-RVFV IgG from clinical specimen. **Keywords:** Rift Valley fever virus, monoclonal antibody, Antigen capture IgG indirect ELISA, Western blot
NTD8-A5	**Performance of Kato-Katz, Mini-Parasep and Mini-FLOTAC techniques in detection of intestinal helminth ova in Mbita district, Homabay county, Kenya** Annette Ngetich[1,3], Kimberley Won[2], Walter GZO Jura[3], Pauline NM Mwinzi[1], Maurice R Odiere[1] [1]*Neglected Tropical Diseases Branch, Centre for Global Health Research, Kenya Medical Research Institute, P. O. Box 1578-40100, Kisumu, Kenya* [2]*Centers for Disease Control and Prevention, Division of Parasitic Diseases and Malaria, Atlanta, GA, USA* [3]*Department of Zoology, Maseno University, P. O. Box 333-40105, Maseno, Kenya.* **Background:** Lack of knowledge about the true extent of multiparasitism is usually attributed to unavailability of a single diagnostic method which is able to combine robustness, simplicity, and good sensitivity in detecting all parasite species. Widely used diagnostic approaches such as the Kato-Katz technique lack sensitivity particularly in low endemicity areas, and hence reported prevalence rates considerably underestimate the true number of