SERO-EPIDEMIOLOGY OF SELECTED ARBOVIRUSES AMONG FEBRILE PATIENTS IN THE LAKE/RIVER BASINS OF LAKE BARINGO, LAKE NAIVASHA AND RIVER TANA, KENYA

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DECLARATION

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

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This work has been published as "Seroepidemiology of Selected Arboviruses in Febrile Patients Visiting Selected Health Facilities in the Lake/River Basin Areas of Lake Baringo, Lake Naivasha and Tana River, Kenya" in the Vector borne zoonotic diseases journal.

DEDICATION

This thesis is dedicated to my husband Joseph Tonui who has always encouraged me to work hard in my postgraduate studies and for the financial support he offered. I also dedicate it to my children Amon, Trevor and Nicole for their moral support. To my parents who educated me and taught me to give my best in all that I do in life however difficult.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AVID	Arbovirus Infection and Diversity
CCHFV	Crimean-Congo Hemorrhagic Fever virus
CDC	Centres for Disease Control and Prevention
CO_2	Carbon dioxide
CHKV	Chikungunya Virus
CI	Confidence Interval
CVR	Centre for Virus Research
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagle's Minimal Essential Medium
DMEM	Dulbecco's Modified Eagle Medium
ERC	Ethical Review Committee
HRP	Horse Radish Peroxide
ICIPE	International Centre of Insect Physiology and Ecoogy
ID	Identification
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
ml	millilitre
MOPH & S	Ministry of Public Health and Sanitation
nm	nanometre
OD	Optical Density
ODC-	Optical Density of the Negative Control
ODC+	Optical Density of the Positive Control
ODCO	Optical Density Cut-Off
C++	Positive Control
C-	Negative Control
ONNV	O'nyong nyong virus
OR	Odds Ratio

PBS	Phosphate Bovine Solution
PP	Percentage Positivity
PRNT	Plaque Reduction Neutralization Test
PFU	Plaque Forming Unit
rpm	revolutions per minute
RT–PCR	Reverse Transcriptase–Polymerase Chain Reaction
RVFV	Rift Valley Fever Virus
SOP	Standard Operating Procedures
SSC	Scientific Steering Committee
SFV	Semliki Forest Virus
TMB	Tetramethylbenzidine
WHO	World Health Organization
WNV	West Nile Virus
YFV	Yellow Fever Virus

ABSTRACT

Arboviruses cause emerging and re-emerging infections affecting humans and animals. They are spread primarily by blood-sucking insects such as mosquitoes, ticks, midges and sand flies. Changes in climate, ecology, demographics, land-use patterns and increasing global travel have been linked to an upsurge in arboviral disease. Outbreaks occur periodically followed by persistent low level circulation. This study aimed at determining the seroepidemiology of selected arboviruses among febrile patients in selected lake/river basins of Kenya. Using a hospital-based cross-sectional descriptive study design, febrile patients were recruited into the study retrospectively using simple random sampling from an ongoing study and actively from three health facilities namely; Marigat, Mai Mahiu and Kotile. Out of the 379 patients enrolled, 285 were retrospective samples and 94 were collected actively. A structured questionnaire was administered to collect socio-demographic and clinical data. The patient's venous blood was tested for IgM and IgG antibodies to determine exposure to selected arboviruses including Crimean Congo Haemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV), West Nile virus (WNV), and *Alphaviuses*. Samples positive for *Alphaviuses* and WNV were further subjected to plaque reduction neutralisation test (PRNT) to confirm the presence of the specific antibodies. PRNT was not performed for CCHFV and RVFV because they are highly pathogenic viruses that require high containment to manipulate in the laboratory which is not available in Kenya. Multiple logistic regression models were used to assess the risk factors associated with the exposure of humans to CCHFV, RVFV, WNV and *Alphaviuses*. Important socio-demographic and clinical characteristics associated with arboviral infections like age, site, contact and fever were identified. Of the 379 samples examined, only on sample was positive for Anti-CHIKV IgM antibodies and 46.4% (n=176, 95% CI: 41.4-51.5%) were IgG positive for at least one of these arboviruses. Virus specific prevalence for CCHFV, RVFV, WNV and *Alphaviuses* was 25.6%, 19.5%, 12.4% and 2.6%, respectively. These prevalences varied significantly ($p<0.001$) with geographical site, where Tana recorded the highest overall arboviral seropositivity of 60%, followed by Baringo (52%) and Naivasha (32%). PRNT results for *Alphaviruses* confirmed that the actual virus circulating in Baringo was Semliki Forest virus (SFV), in Naivasha was CHIKV, O'nyong nyong virus (ONNV), and SFV, and in Tana delta Sindbis virus (SINDV). Among the *Flaviviruses* tested, WNV was circulating in all the three sites. This study also determined that age, site, gender and contact with goats were risk factors for arboviral infections. The findings of this study suggest a high burden of febrile illness in humans due to CCHFV, RVFV, WNV and *Alphaviuses* infection in the river/lake

basin regions of Kenya, which should be brought to the attention of public health authorities in these areas.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information on Arboviruses

Arboviruses are now among the most important emerging infectious diseases facing the world. They are spread primarily by blood-sucking insects, like mosquitoes, ticks and sandflies (Gubler, 2002). Most arboviruses that are of public health importance have been classified into three families namely; *Flaviviridae*; *Togaviridae* and *Bunyaviridae* (Gubler *et al*., 1998). Transmission of disease is successful based on availability of reservoirs, amplifiers, susceptible vertebrate hosts and the virus ability to develop high titre viraemia for adequate duration of time to infect large number of vectors that will then infect available susceptible individuals (Calisher, 1994). The vectors get infected after ingesting an infective blood meal which goes through an extrinsic incubation period during which the virus replicates in the vector's tissues hence transmitting it through salivary secretions to susceptible vertebrate host during subsequent feeding causing viraemia and sometimes illness (Sang & Dunster, 2001). Most arboviruses circulate among wild animals, and may cause disease after spill over transmission to humans and agriculturally important domestic animals which serve as dead-end hosts (Weaver & Reisen, 2010).

Changes in climate, ecology, demographic**s**, land-use patterns and increasing global travel have been linked to an upsurge in arboviral disease (Gould & Higgs, 2009; Weaver & Reisen, 2010). Environmentally driven outbreaks like RVF in East Africa was closely associated with the heavy rainfall that occurred during the *El Niño* phenomenon in 1997/98 and 2006/07 and also by human activity e.g. irrigation in West Africa (Woods *et al.,* 2002; Nguku *et al*., 2010). Viruses such as CHIKV have undergone genetic transformation to cause more extensive outbreaks in Indian Ocean islands and India (Schuffenecker *et al*., 2006). Most recently outbreaks of CHIKV were reported in the Democratic republic of Congo (1999-2000), Gabon (2007), Indian Ocean islands (2005/6), India (2006/2007), Italy (2007), Thailand (2009), Sierra Leone (2012), Cambodia (2012) and the Caribbean (2013-2014) (WHO report, 2014). The absence of effective vaccines and therapeutic treatment for most arboviral infections underscores the need for active surveillance to monitor circulation in order to inform public health decision for early warning and response. The present study aimed at determining the seroepidemiology and associated risk factors of selected arboviruses among febrile patients in the lake/river basin areas of Baringo, Naivasha and Tana, in Kenya.

1.2 Statement of the Problem

Outbreaks occur periodically across Kenya e.g. RVFV outbreak in 1996/7 and 2007/2008, CHIKV in Lamu and DENV in Mandera in 2011 and Mombasa from 2012 to date, but limited surveillance takes place during inter-epidemic periods. The low level circulation that persists during these periods has never been quantified (Sutherland *et al.*, 2011). The public health importance of these diseases is mainly appreciated during an outbreak by governments due to the severity of symptoms caused. Most recently, Kenya has experienced multiple Dengue Fever virus (DENV) outbreaks since February 2013 to date in Mombasa and Mandera (MOH Quarterly bulletin, June 2013; KEMRI unpublished lab reports 2014). Lake and river basin regions are unique ecological sites that facilitate the occurrence of arboviral pathogens due abundance and high survival of vectors resulting in high hosts vector interacting within the ecological niche and subsequent virus transmission. Increased human activities and climatic changes have lead to increased frequency of outbreaks. The detection of arbovirus activity from vectors in the last ten years poses a public health concern in Kenya. Several arboviruses are endemic in Kenya and considerable arboviral activity with high diversity described in Kenya in the recent years (Crabtree *et al*., 2009). Important arboviral agents such as WNV and RVFV have been isolated from mosquito vectors in Kenya (LaBeaud,*et al*., 2011b; Sang *et al.,* 2011; Ochieng *et al*., 2013). Other entomological studies have demonstrated the same case (Sang *et al*., 2010) and hence suggest that there is a likelihood of transmission of the virus to humans. The true prevalence of arbovirus circulation in Kenya remains unknown and diagnosis of febrile illnesses remains a challenge to date. Some of the undiagnosed febrile illnesses in health facilities can be attributed to arboviral activity in the region. The burden of arbovirus infection to humans relating to morbidity and mortality lead to losses in manpower hours and economic activities and hence it is important to study the epidemiology of disease in the selected study sites. Effective surveillance in health facilities can help in answering some of these questions in the selected regions.

1.3 Justification

Monitoring of disease circulation is important in assessing disease risk in human populations and early detection of virus circulation and appropriate planning of disease control strategies. Arboviruses account for some of the febrile illnesses of unknown aetiology observed in the selected public health facilities in these areas and hence the study will help in understanding of disease epidemiology. The three health facilities were selected based on their proximity to

the lake/river basin areas as well as the number of reported unexplained febrile patients being seen at the clinics on a daily basis as reported by clinicians during site selection field visit made. This study assumed that all the febrile illnesses of unknown origin were caused by arboviruses. The selected arboviruses i.e. CCHFV and RVFV *(Bunyaviridae)*, WNV (*Flaviviridae*) and CHIKV (*Togaviridae*) represented three different families' giving a good basis for comparing different families of arboviruses circulating in the selected sites. The study has provided data that will be useful in assessing disease risk level of human and animal population in the lake basin regions and detect virus circulation which is a critical parameter in planning for emergency response and appropriate disease control measures during epidemics. The study aimed at determining the prevalence of arboviruses in the areas and the level of exposure of humans to these viruses in these diverse ecologies. This will inform the public health officials about the levels of risk and suggestions have been made on the necessary control measures to be put in place.

1.4 Research Questions

1. What are the socio-demographic and clinical characteristics of the febrile patients from the lake/river basin areas of Baringo, Naivasha and Tana, Kenya?
2. What is the seroprevalence of CCHFV, WNV, RVFV and *Alphaviuses* among febrile patients from the river/lake basin areas of Baringo, Naivasha and Tana, Kenya?
3. What are the risk factors associated with transmission of CCHFV, WNV, RVFV and *Alphaviuses* to humans from the river/lake basin regions in Baringo, Naivasha and Tana, Kenya?

1.5 Hypothesis

This was a laboratory-based cross-sectional descriptive study so no hypothesis was adopted because descriptive studies are also known as hypothesis generating studies (Grimes & Schulz, 2002).

1.6 Objectives

1.6.1 General Objective

To conduct a seroepidemiological study of selected arboviruses among febrile patients from the lake/river basin areas of Baringo, Naivasha and Tana, Kenya.

1.6.2 Specific Objectives

1. To determine the socio-demographic and clinical characteristics of the febrile patients from the lake basin areas of Baringo, Naivasha and Tana, Kenya.
2. To determine the seroprevalence of CCHFV, WNV, RVFV and *Alphaviuses* among febrile patients from the lake/river basin areas of Baringo, Naivasha and Tana, Kenya.
3. To determine the risk factors associated with transmission of CCHFV, WNV, RVFV and *Alphaviuses* from febrile patients in the lake/river basin areas of Baringo, Naivasha and Tana, Kenya.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of Arboviral Infections

Arboviral infections are a global health problem accounting for significant morbidity and mortality in human and animal populations. Arboviruses belong to the families *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, and *Rhabdoviridae*, and are transmitted to humans and animals by the bite of infected arthropods (Ansari & Shope, 1994). These viruses cause epidemics throughout Africa, middle east, America and Europe and they were not well known until recently (Gubler, 2002). Rodents and birds are significant vertebrate hosts, while humans are usually not involved in the maintenance and spread of most arboviruses but act as dead-end hosts. Ecologic changes and human behaviour are important in the spread of these infections. In the recent years several pathogenic arboviruses have apparently dispersed to new locations. West Nile virus (WNV) is one of the well-known viruses that have moved from Europe to the Americas where the infection was not known to occur (Kramer *et al.*, 2008). Majority of the recently recognised human viral diseases are zoonoses and due to the ecologic and human behaviour changes, they switch hosts and infect humans (Gubler, 2002). Circulation of zoonotic arboviruses is known to occur in sylvatic and peridomestic cycles in which wild animals and humans are involved. Humans and domestic animals are classified as "dead-end hosts" because they do not produce significant viremia to facilitate virus transmission, but can only develop clinical syndrome (Weaver & Reisen, 2010).

Arboviral infections in human population are determined by the probability of contacts between the vector, the human population and, for many viruses, the amplifying vertebrate host. The host reservoir for many viruses may vary from: birds for viruses causing encephalitis, monkeys for viruses like Yellow Fever Virus (YFV); horses for viruses like WNV; sheep for RVFV or rodents. The emergence of WNV in the Americas, Japanese Encephalitis Virus (JEV) in Australasia, the outbreaks of dengue in Tanzania, Somalia, Djibouti, Eritrea and Sudan (Sang, 2006, Network A. F. E., 2013), the recent outbreak of CHIKV in Northern Italy and the re-emergence of YFV in South America is an indication that arboviral infections are expanding geographically (Mackenzie *et al*., 2004; Petersen *et al.*, 2005). A number of arboviruses have emerged or re-emerged through dramatic spread and occurrence of epidemics driven by environmental factors e.g. the RVF outbreak in East Africa that was closely associated with the heavy rainfall that occurred during the Elnino phenomenon in 1998 and also by human activity e.g. irrigation in West Africa (Woods *et al.*,

2002). Irrigation creates breeding sites for vectors and significantly increases relative humidity favouring vector longevity.

Arboviruses can be controlled mainly by employing vector control measures for vectors like mosquitoes and ticks to reduce transmission. Management of breeding habitats like drainage of swamps, stagnant pools of water, removal of outdoor water containers, plant pots, empty cans can eliminate breeding grounds for mosquitoes. Use of insecticides and larvicides in homes and outdoor environment is useful. People can avoid outdoor activities that expose them to vectors, use personal protective measures like sleeping under mosquito nets, apply mosquito repellants and wear protective clothing to reduce exposure to vectors (Gould & Higgs, 2009).

2.2 Crimean–Congo haemorrhagic fever virus

Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne disease that is distributed widely in the world i.e. in Africa, Asia, Eastern Europe and the Middle East (Chinikar *et al*., 2012). It is caused by CCHFV belonging to the family *Bunyaviridae* and genus *Nairovirus*. It is mainly transmitted by ticks of the genus *Hyalomma*. It circulates between ticks and vertebrates i.e. domestic and wild animals (Enzoonotic cycle) and small vertebrates like hares and hedgehogs act as amplifying hosts because they harbour immature ticks (nymps) (Ergonul & Whitehouse, 2007). Infections in humans occur through infected tick bites or by contact with CCHFV patient in the acute phase of infection. The case fatality rate of CCHF is up to 40% (Ergonul & Whitehouse, 2007). Livestock infection is generally subclinical with antibodies against CCHFV detected in the sera in regions of Europe, Asia and Africa (Hassanein *et al.,* 1997). Butchers and abattoir workers are at risk of infection from animal slaughtering and butchering. Previous studies have shown the CCHFV infection occurs in birds, like ostriches and guinea fowls (Hoogstral, 1979). CCHFV antibodies have been detected in donkeys, goats, cattle, horses, sheep and pigs in Europe, Asia and Africa (Shimshony, 2010). Very few serological studies have been conducted on CCHFV in Kenya with one human case detected in western Kenya in 2000 (Dunster *et al*., 2002). Most recent study in Ijara district, Kenya showed that 19% of human febrile patients tested positive for CCHFV IgG antibody (Lwande *et al.*, 2012). Some of the clinical manifestations of CCHFV infection include fever, myalgias, severe headache, joint pain, chills, nausea and haemorrhagic manifestations. These should be considered during differential diagnosis in

conjunction with risk factors like tick bites, exposure to infected animals or contact with suspected cases and outdoor activities in endemic places (Zavitsanou *et al*., 2009).

2.3 West Nile Virus

This is an RNA virus that is transmitted by mosquitoes and it is classified under the family *Flaviviridae* and genus *Flavivirus*. It is widely distributed throughout Africa, the Middle East, Asia, parts of Europe, Australia, North and South America, and the Caribbean (Venter *et al.,* 2009). Transmission of the virus occurs through an enzootic cycle involving birds as the vertebrate host and ornithophilic mosquitoes as the main vectors (Murgue *et al.,* 2002; Rappole & Hubalek, 2003). Most of the Human infections caused by WNV are asymptomatic, although it can cause symptoms that range from 'flu-like illness' to life-threatening neurological complications such as meningoencephalitis and flaccid paralysis (Hayes *et al*., 2005). WNV was first detected and isolated from a human case in 1934 in the West Nile district of Uganda (Smithburn *et al.,* 1940) and has been reported worldwide, including many African countries (Murgue, 2002; Blitvich, 2008). It is known to occur in much of East and South Africa with humans and other mammals e.g. horses are incidental hosts and do not play a role in the natural preservation of WNV (Lanciotti *et al.,* 2000). Seroepidemiologic studies suggest that asymptomatic infections frequently occur in horses (Nielsen *et al*., 2008) but neurologic infections result in a high case-fatality rate (30%–40%) (Ward *et al*., 2006). Most human infections in Kenya are self-limiting and subclinical, often misdiagnosed posing a challenge in diagnosis (Morrill, 1991; Sang & Dunster, 2001). The human case fatality rate of WNV ranges between 10 and 50% (Tsai *et al*., 1998; Nash *et al*., 2001; Chowers *et al*., 2001)**.**

2.4 Chikungunya virus

Chikungunya fever is a mosquito-borne viral disease first described during an outbreak in southern Tanzania in 1952 (Ross, 1956). CHIKV is an *Alphavirus* of the family *Togaviridae* (Johnston & Peters, 1996). The name 'chikungunya' derives from a root verb in the *Kimakonde* language, meaning, "to become contorted" and describes the stooped appearance of victims with joint pain. It is generally spread through bites from *Aedes aegypti* and *A. albopictus* mosquitoes (Pialoux *et al*., 2007). Chikungunya circulates in Africa, Asia and the Indian subcontinent. CHIKV has become the most prevalent *Alphavirus* infection in the world, particularly prevalent in Africa and Southeast Asia (Prasanna *et al*., 2006; AbuBakar *et al*., 2007). Human infections in Africa have been at relatively low levels for a number of

years, but in 1999-2000 there was a large outbreak in the Democratic Republic of the Congo, and in 2007 there was an outbreak in Gabon (Peyrefitte *et al*., 2007). East Africa among them Kenya and the Indian Ocean Islands (e.g. Comoros, Seychelles, Mauritius) have experienced a major outbreak between 2004 and 2005 (Njenga *et al*., 2008). CHIKV infection can cause an acute weakening illness, most often characterized by fever, severe joint pain and rash (Bonn, 2006). After an incubation period of two to twelve days, there is an abrupt onset of fever and arthralgia with chills, headache, photophobia, conjunctival injection, and abdominal pain. Migratory polyarthritis affects mainly the small joints and a rash may appear within two to three days of disease. Irregularly, the virus can cause haemorrhagic syndrome (Sergon *et al*., 2004).

2.5 Rift Valley Fever virus

Rift Valley fever is a viral zoonotic disease caused by RVF Virus from genus *Phlebovirus* of *Bunyaviridae* family (Linthicum *et al*., 1985). It is spread by important mosquito vectors in East Africa including *Aedes Ochracious, Aedes Mcintoshi, Culex pipiens* and Anopheles squamosus through an infected bite. Previous studies indicate that *Aedes Mcintoshi* is the primary vector of RVFV in Kenya (Sang *et al*., 2010). It mainly affects domestic animals especially young animals for example lamb and calves and can be transmitted to humans through direct contact with blood, tissue, abortus or amniotic fluid of infected animals or through infected mosquito bites causing fever (LaBeaud, 2008). The disease was reported for the first time in 1915 among livestock in Kenya, but the virus was isolated for the first time in 1930 (Daubney *et al*., 1931). Periodic RVF outbreaks occur across Sub-Saharan Africa with the last major outbreaks occurring in East Africa in 1997-1998 and 2006-2007 (Sang *et al.*, 2010). Major outbreaks leads to high numbers of infections in humans and livestock posing a threat to public health and a risk in livestock production in resource limited countries (WHO, 2000). These outbreaks cause more devastating effects on pastoral communities and local herders because of loss of adult animals and the next crop of newborn livestock affecting their economic status which is dependent on milk and meat production (LaBeaud, 2008). The clinical characteristics of infected patients may include; high fever, myalgia and malaise and in some cases, encephalitis, haemorrhagic fever and death (CDC, 2002).

2.6 Yellow fever virus

Yellow fever virus (YFV) is a mosquito-borne virus spread primarily by *Aedes (Stegomyia)* species of mosquito. YFV belongs to the family *Flaviviridae* and genus *Flavivirus*. It was

first isolated in West Africa in 1927 (Barrett & Higgs, 2007). The estimated annual YF incidence is 200,000 cases, and 30,000 deaths, in sub-Saharan Africa where over 500 million people are at risk with a case fatality rate of 50% (WHO, 2000). In East Africa, outbreaks of YF have been reported in Kenya in 1992-95 (Sanders *et al.,* 1998, Reiter *et al.,* 1998), Sudan in 2003, 2005 and 2010 (Onyango *et al.,* 2004, WHO, 2005) and Uganda in 2011 (WHO, 2011). Most recently outbreaks of the disease have been reported in countries bordering Kenya like Tanzania, Somalia, Djibouti, Eritrea and Sudan. The virus originated in sylvatic cycles, in Asia and Africa and it is maintained in non-human primates and forest-dwelling *Aedes* mosquitoes, with the potential to spill into the human population involving *Aedes aegypti* (Hanley *et al.,* 2013). This poses a serious public health concern in Kenya to due to possible spill over from these neighbouring countries. There is an effective licensed attenuated vaccine called 17D for yellow fever used mainly in the endemic areas (Gubler, 2002).

2.7 Dengue virus

Dengue virus (DENV) like YF is a mosquito-borne virus spread primarily by *Aedes (Stegomyia)* species of mosquito. It belongs to the family *Flaviviridae* and genus *Flavivirus*. Dengue virus has four serotypes (1,2,3,4). It circulates in both sylvatic and urban cycles, and is maintained in non-human primates as the host vertebrates. It is rapidly increasing in the tropics and occurs worldwide with an incidence of 50-100 million cases per year. Dengue outbreaks have also increased frequency in the region with reports in Tanzania, Somalia, Djibouti, Eritrea and Sudan (Sang, 2006, Network A. F. E., 2013). Dengue is the most important re-emerging arbovirus worldwide, it was first reported in Kenya during the 1982 outbreak (Johnson *et al.,* 1982). In 2011, the second dengue outbreak in Kenya occurred in Mandera in the North Eastern Kenya. Subsequently between 2013 and 2014, another dengue outbreak occurred in Mombasa, where Den-1, 2 and 3 were detected (Unpublished data). Dengue virus (DENV) infection causes a wide range of clinical symptoms, ranging from mild, non-specific symptoms to classic dengue fever, with high fevers and severe arthralgia. Subsequent infection can lead to dengue hemorrhagic fever (Mease *et al.,* 2011). The case fatality rate of dengue fever can be kept below 1 % if managed well.

2.8 Seroprevalence of arboviruses

Most populations in sub-Saharan Africa experience a great burden of disease due acute febrile illnesses (Breman, 2001; Campbell *et al.*, 2004). A greater percentage of these diseases are related to common diseases like malaria and typhoid fever, but recent studies have shown that they are misdiagnosed and a proportion of these illness could be caused by other pathogens not considered in the African clinical set up (Breman, 2001; Ndip *et al.*, 2004; Reyburn *et al.*, 2004). Detection of these viruses is hindered by poor diagnosis due to non-specific clinical presentation and limited surveillance that is being conducted in Africa (Sang & Dunster, 2001). In the 1980s, there was an increase of YFV cases in Africa (LaBeaud *et al.*, 2007, LaBeaud, 2008). CHIKV has caused outbreaks from the 1960s to the 1980s and after 20 years, an outbreak was reported in the Congo in 2000 and subsequently in Eastern Africa and India in 2005-2006 (Pastorino *et al.*, 2004). Africa has also experienced outbreaks of DENV, RVFV and ONNV (Haddow, 1960; Johnson *et al.*, 1982; Sang *et al.*, 2010)

Despite the fact that most studies have been conducted on prevalence of arboviruses in vectors, paucity of data exists on the seroprevalence of these arboviruses (Sanders *et al*., 1999; Chretien *et al*., 2007). There was a recent epidemic of CHIKV along the Indian Ocean Islands showing the ability of the virus to spread to new areas (Chretien *et al*., 2007). The epidemic started along the Indian Ocean region where it is speculated to have emerged from Kenya (Lamu & Mombasa; July, 2004), before reaching the Comoros in January, 2005 and Seychelles (March, 2005), followed by Mauritius. Systematic studies showed a prevalence of 75% in the population in the Kenya (Lamu) outbreak, 63% in the Comoros, and 26% in Mayotte (2006) (Bonn, 2006) (Chretien *et al*., 2007).

Outbreaks of DENV have occurred in Kenya (Johnson *et al*., 1982). Most recently, Kenya experienced a DENV outbreak in September 2011 with more cases being reported in December 2012 (MOH Quarterly bulletin, June 2013). Kenya has experienced RVFV outbreaks recently in 2006-2007 (Nguku *et al.*, 2010). Recent Sero survey conducted in selected sites in Kenya showed circulation of WNV (9.5 %), CHIKV (34 %), DENV (14.4%) and YFV (9.2 %) among rural Kenyan adults (Mease *et al*., 2011) and CCHFV seroprevalence of 19 % in Ijara district (Lwande *et al*., 2012), but much remains unknown about the true prevalence of arboviruses in the lake basin regions of Kenya.

2.9 Risk Factors Associated with Arboviral Infections

In recent decades there has been an increase in arbovirus outbreaks worldwide, especially in the sub-Saharan Africa. Arboviruses require blood sucking arthropods to spread from host to host (WHO, 1985). The worldwide increase in arbovirus activity is due to recent circumglobal/demographic changes in climate, human/societal activities, epidemiology and viral genetics (Weaver & Reisen, 2010). Majority of the recently recognised human viral diseases are zoonoses and due to the above changes, they switch the hosts and infect humans (Gubler, 2002). Some of the factors that cause sporadic outbreaks or clusters of human cases include environmental factors like human activities that enhance vector population densities (irrigation, heavy rains followed by floods, higher than usual temperatures, and formation of ecologic niches enabling the mass breeding of mosquitoes) allow the re-emergence of this mosquito-borne disease. For instance, global warming scenarios hypothesize warmer, more humid weather that may produce an increase in the distribution and abundance of mosquito vectors (Reeves *et al.*, 1994). Other risk factors associated with arboviruses e.g. CCHVF and RVFV include contact by trapping of animals, farming, slaughter, food preparation or veterinary work. Casual contacts for example neighbour keeping chickens or proximity to a slaughterhouse are also risk factors. In addition, eating of raw fowl products or drinking blood, butchering of sick animals, milking for pastoralists, handling of patients in acute phase of infection and tick or mosquito bites can expose one to infection (Chevalier *et al.*, 2010). Living in the lake basin areas also predisposes humans and animals to infection because of high vector abundance in the area. In the Kenyan Lake Victoria basin, very high *Alphavirus* seropositivity (>60%) was seen in 2004 (Coldren *et al*., 2005).

2.10 Diagnosis of Arboviral Infections

Several methods can be used for diagnosis of arboviruses from a variety of samples including vectors, wildlife, human and livestock. Serological tests, such as enzyme-linked immunosorbent assays (ELISA), may confirm the presence of IgM and IgG antibodies (Alison *et al*., 2000) specific to the virus in circulation. IgM antibody levels are highest within three to five weeks after the onset of illness and persist for about two months (Johnson *et al.*, 2000). The virus may be isolated from the blood during the first few days of infection through cell culture. Molecular methods such as reverse transcriptase–polymerase chain reaction (RT–PCR) may be used to detect the infecting viruses. RT–PCR products from clinical samples may also be used for genotyping the virus, allowing comparisons across virus samples from various geographical sources (Kuno, 2000). Other methods that can be

used for arboviruses diagnosis include Haemagglutination-Inhibition test used in qualitative and quantitative detection of IgG antibody, Haemagglutination test used in the antigen titration and Plaque Reduction Neutralization Test (PRNT), a gold standard for virus diagnosis to rule out cross reactivity within a virus genus (Shope, 1980).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The study utilised archived samples from a hospital-based cross-sectional descriptive survey and retrospective data from an ongoing study that started in September 2009 to December 2012. To augment the already collected samples and retrospective data, samples and data of febrile patients from identified clinical centres were collected with the assistance of clinical officers in the three respective study districts. The sample size varied from district to district depending on the population size of the district, as explained in Section 3.4.

3.2 Study Sites

This study was conducted in three sites namely; Baringo, Naivasha and the Tana (Figure 3.1). These sites were selected because Baringo and Tana are known hotspots for RVFV outbreaks and Naivasha selected for comparison because it is an endemic site for RVFV infection. Previous studies have also isolated other arboviruses from vectors collected from these sites and there is a likelihood of transmission to humans. The lakes and river basins in these areas are also breeding sites for the vectors and a habitat for many virus reservoirs.

3.2.1 Baringo

Baringo county has a population of 173, 815 (Kenya National Bureau of Statistics, 2009) and is about 1000m above sea level, latitude of 0°28'N and a longitude of 35°58'E. The area has harsh physical and climatic conditions and hence is very sparsely populated with an average of 31people/$km^{2.}$ The research area covered the basin between Lake Baringo and Lake Bogoria. It is a critical habitat and refuge for more than 500 species of birds with migratory water birds that are significant regionally and globally, as well as large mammals and invertebrate community. Fishing is important for local, social and economic development. Lake Bogoria and the animals are a source of attraction to tourists in the area. The local inhabitants are classified as agro pastoralists practising limited crop production, fishing and livestock rearing as a source of income. Lake Baringo is the main source of water for both livestock and domestic use. The health facility selected in this area was Marigat district hospital, belonging to the ministry of health. It is found in Marigat town which is the main town near Lake Baringo. It serves smaller settlements around the area including Kampi ya Samaki and Loruk. The lake habitat allows for interaction of animals, vectors and human

population including new visitors through tourism that can allow transglobal movement of infections.

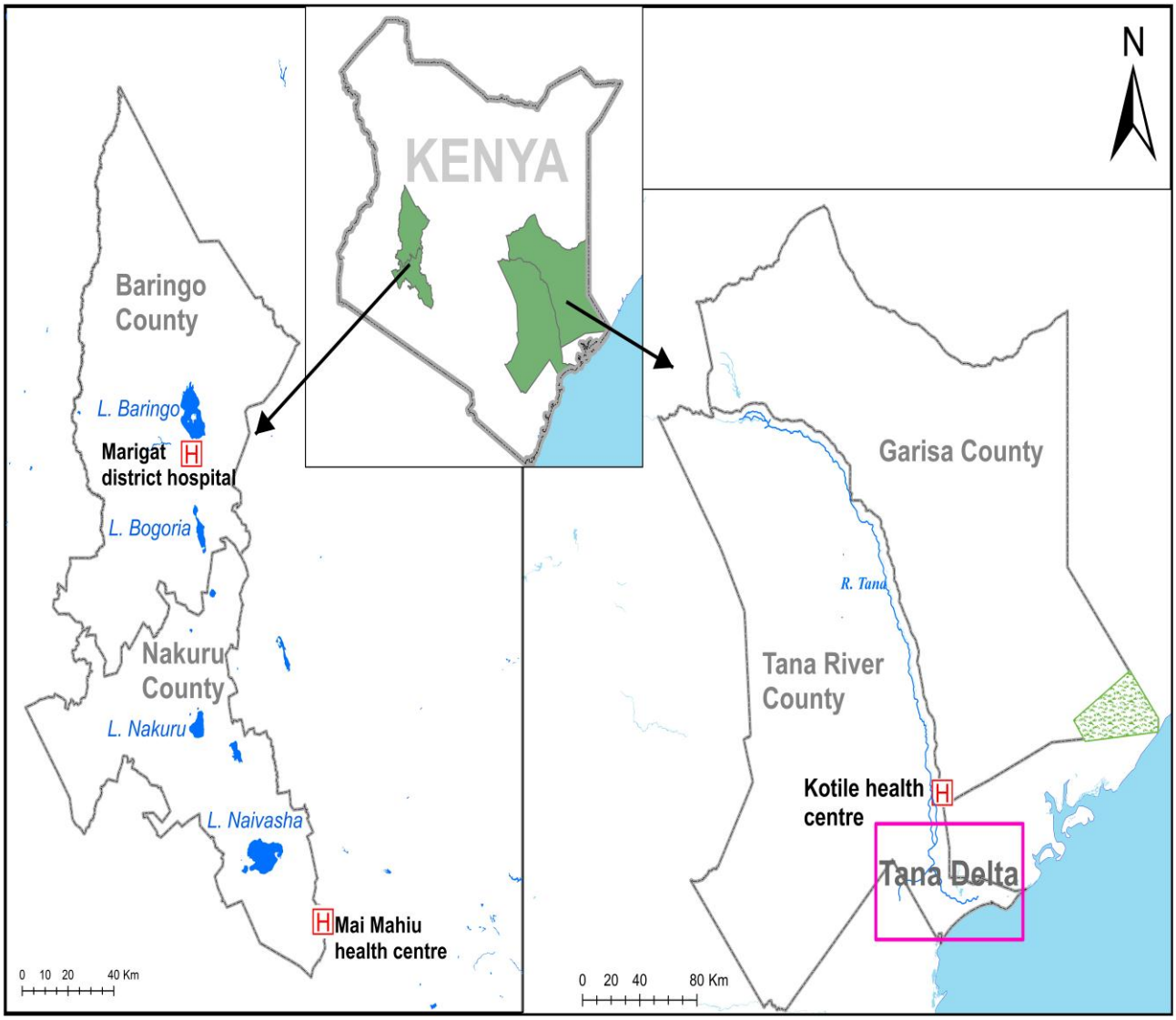

Figure 3.1: Map of Kenya showing location of Lakes Baringo and Bogoria,Lake Naivasha and River Tana. Source: International Centre of Insect Physiology and Ecology (ICIPE) Geospatial Information Unit, generated for the study.

3.2.2 Naivasha

Naivasha is found in Nakuru county with a population of 225, 547 (Kenya National Bureau of Statistics, 2009). The main livelihood of people living in this area is pastoralism by the Maasai and agriculture in Naivasha area. Large flower and horticulture farms along the shores of Lake Naivasha are the highest economic activity and source of employment in the area. Naivasha is also a popular tourist destination with visitors touring the lake Naivasha to

observe bird life and hippopotamus behaviour and other wild animals. The lake shores are very good breeding sites of vectors and hence its interactions with livestock, birds, wild animals and human activity may lead to infection transmission. The study health facility selected in this area was Maai Mahiu health centre located near Lake Naivasha. It serves people living in Naivasha district.

3.2.3 Tana

Kotile is one of the seven administrative divisions of Ijara district in Garissa County. The division is next to Tana River which is a source of water for the community in the locality. Ijara district lies approximately between latitude 1° 7`S and 2° 3`S and longitude 40° 4`E and 41° 32`E. The district is sparsely populated with a population of 164,115 consisting of 11,445 households with an average size of 5 members. Most parts of Kotile are semi-arid rangeland. Rain falls in two seasons, the long rains in March to April and the short rains in October to December. The rainfall is unreliable with some short periodic torrential down pours and hence frequent droughts due to low altitudes and temperature variations (20 to 38°C). The main economic livelihood of inhabitants is livestock keeping (90%) with pastoralism as the main enterprise. The species of birds found here include both the game birds (plain birds) and the water fowls mostly along the Tana River. Several wild animals are found in the region too. The selected health facility in the area was Kotile health centre. It is found in Ijara district, Kotile division and serves the population that leave around the Tana region.

3.3 Study Population

The study population comprised all persons (male and female) aged 5 years and above with a clinical case definition of acute febrile illness characterised by fever of temperature greater than or equal to 38ºC upon arrival to the study health facility.

3.3.1 Inclusion criteria

All persons (male and female) aged 5 years and above, presenting to the clinic with high fever ≥ 38ºC, who gave consent (≥18 years) or ascent (12 to 17 years) to participate in the study were included in the study. Consent was obtained from the parents or guardians for those children under 12 years of age. Screening for malaria was not a requirement to participate in the study. All minors were included in the study only if their parent/guardian provided consent and or if the minor assented to participate, if old enough to do so.

3.3.2 Exclusion criteria

The patient was excluded from the study if he or she was less than 5 years of age or refused to give consent/ascent for himself/herself or for the minors (aged < 12 years) whose parents or guardians declined to give consent for their participation in the study. Children <5 years of age were excluded from the study because of the technical difficulties of drawing blood samples and obtaining their guardians' consents.

3.4 Sample size determination

The minimum sample size for the study was calculated using the formula developed by Cochran (Cochran, 1963). The prevalence of CHIKV of 34% was used based on a serosurvey study results by Mease *et al.*, 2011. This was the most recent serosurvey for arboviral infection in Kenya where they obtained a prevalence of 14.4% for DENV, 9.5% for WNV, 9.2% for YFV, 0.7% for RVFV and 34.0% for CHIKV. Previous inter epidemic serosurveys for RVFV have documented prevalence ranging from 1% to 19% during the inter-epidemic period and 32% in high risk areas during epidemics (Labeaud *et al*., 2008). Thus, the sample size estimated using CHIKV's prevalence of 34% was used because (1) it was the most recent prevalence; and (2) it gave the biggest sample size than the ones obtained using any of the other prevalences. Naing *et al.,* 2006 advised that in situations where different parameters and objectives lead to different sample size estimates for the same study, the biggest sample size obtained out of all calculations should be taken as the sample size because it would accommodate the rest. The sample size used in this study was dependent on the desired degree of precision and the anticipated prevalence, and was derived from the formula:

$$n = \frac{Z^2 p(1-p)}{d^2}$$

Where:

n= Desired sample size

p= The anticipated prevalence in the target population with the characteristic being measured

Z= 1.96 standard error (95% confidence level of the standard deviation from the mean)

d= The desired precision, taken to be 0.05 (Naing *et al.,* 2006).

After substituting appropriately, the base sample size is calculated as follows;

$$n = \frac{1.96^2 * 0.34(1 - 0.34)}{0.05^2} = 345 \text{ samples}$$

The base sample size was further increased by 10% to account for contingencies, thus giving a final sample size of 379 people. Samples distribution in the three project sites was apportioned according to the population sizes of the respective districts (Probability Proportional to Size), such that the district with the highest population was allocated more samples and vice-versa. A total of 117 (30.8%) patients were recruited from Baringo district with a population of 173,815 (Kenya National Bureau of Statistics, 2009), 152 (40%) patients from Naivasha with a population of 225,547 (Kenya National Bureau of Statistics, 2009) and 110 (29.1%) from the Tana with a population of 164,115. The total Population in all the three sites was 563,477 (Kenya National Bureau of Statistics, 2009).

3.5 Questionnaire administration

A structured questionnaire was administered to collect socio-demographic and clinical data. Upon recruitment of an eligible patient, the clinician documented the gender and age of the patient. In addition to that, the clinician asked questions on the current residence, duration of stay in the residence, travel history, occupation, contact and mode of contact with animals and/or birds, if there were any sick animals or people at home, the reason for coming to hospital, clinical manifestations of the patient, signs of bleeding, date of onset of disease and if the patient had missed school or work depending on the age (Appendix 6). The clinician would then make a clinical diagnosis based on the symptoms presented by the patient.

3.6 Sampling procedure

This study was nested in an ongoing project entitled; An Integrated Response System for Emerging Infectious Diseases in East Africa (SSC Number; 1560) (Appendix 8), also known as Arbovirus Infection and Diversity (AVID) project. The project was initiated in December 2009 and hence some of the patients had been enrolled into the study. A total of 1231 samples had been collected and stored in the freezer for duration of one and a half years. Out of the stored serum samples, we randomly selected 285 serum samples from a total of 1231 serum samples using simple random sampling (SRS) and sampling with probability proportional to size (PPS) from the three projects sites. The remaining 94 samples were also distributed among the 3 sites using probability proportional to size (PPS) and collected actively until 31st of December 2012. A total volume of 5 ml of blood was collected from all

the study participants using red-capped vacutainer tubes with a clot activator. Serum was processed by centrifugation at 1500 rpm for 10 minutes. The serum was aliquoted into 1 ml volumes placed in sterile bar-coded cryovials and stored in liquid nitrogen at the health facilities until collection and transportation in dry ice to KEMRI/CVR laboratories where they were kept at -70°C, processed and tested as soon as possible.

3.7 Laboratory testing procedures

These tests were conducted using commercial kits based on manufacturer's instructions (Appendix 7) and sample processing SOPs in the KEMRI/CVR laboratory.

3.7.1 Enzyme-Linked Immunosorbent assay (ELISA)

All the Serum samples brought to the laboratory were screened for exposure to Crimean–Congo hemorrhagic fever virus (CCHFV), West Nile virus (WNV), Rift Valley Fever Virus (RVFV) and Chikungunya virus (CHIKV). This was done using commercial kits namely; WNV IgM capture ELISA kit (Panbio), WNV IgG Indirect ELISA kit (panbio), Vectocrimean-CHF-IgM Kit (Vector Best), Vectocrimean-CHF-IgG Kit (Vector Best), CHIKV IgM μ-capture ELISA Kit (NovaTec GMBH), CHIKV IgG capture ELISA Kit (NovaTec GMBH), RVF IgM ELISA kit (Biological Diagnostic Supplies Limited) and RVF recN IgG ELISA kit (Biological Diagnostic Supplies Limited) (Appendix 7).

3.7.1.1 IgM capture ELISA

The test serum samples were inactivated by heating at 56ºC for 30 minutes. The samples were further diluted in the ratio of 1:10 (10 μl of the sample and 90 μl of the serum dilution buffer) with the serum dilution buffer (Tris Buffered Saline) and mixed thoroughly to eliminate the complement interference or prozone effect observed in the case of high antibody titers hence improving the sensitivity of the test. A hundred microlitres of the diluted samples was transferred into a Microtiter well plate pre-coated with anti-human IgM antibodies except for the control wells and incubated for 60 minutes at 37ºC. Control sera (Positive and Negative control) supplied with each of the kits were added to the control wells based on the kits instructions (Appendix 7). After the incubation period, the plates were washed 3 to 6 times manually depending on the kit and excess liquid removed by tapping

onto a pile of folded filter paper. A hundred micro litres of conjugate solution (made of conjugate concentrate, virus antigen and conjugate dilution buffer) was then added onto each well except from the first well with serum dilution buffer and incubated for 90 minutes at 37 ºC. Washing of the wells was repeated 5 times and 100 µl of working Tetramethylbenzidine (TMB) solution (Made of TMB concentrate and Substrate buffer) added into each well then incubated in a dark place for 25 minutes at 18 to 25ºC. The reaction was stopped by adding 100 µl of stop solution to the wells. The optical density of each well was read with a microplate reader set at 450 nm.

3.7.1.2 Indirect IgG Capture ELISA

The test serum samples were heat inactivated at 56ºC for 30 minutes. A hundred micro litres of the serum dilution buffer (SD) were pipetted into the first well to serve as the absorption control of TMB solution. The test samples were further diluted in the ratio of 1:10 (10 µl of the sample and 90 µl of the solution) with serum dilution buffer (Tris buffered saline) and mixed thoroughly to eliminate the complement interference or prozone effect observed in the case of high antibody titers hence improving the sensitivity of the test. A hundred microlitres of the diluted samples were transferred into a Microtitre well plate pre-coated with anti-human IgG antibodies except for the control wells, covered with a film and incubated for 60 minutes at 37 ºC. Control sera (Positive and Negative control) supplied with each of the kits were added to the control wells based on the kits instructions (Appendix 7). After the incubation period, the plates were washed 5 times and excess liquid removed by tapping onto a pile of folded filter paper. A hundred microlitres of conjugate solution (made of conjugate concentrate, virus antigen and conjugate dilution buffer) were then added onto each well except from the first well with serum dilution buffer and incubated for 90 minutes at 37 ºC. Washing of the wells was repeated 5 times and 100 µl of working TMB solution (Made of TMB concentrate and Substrate buffer) added into each well then incubated in a dark place for 25 minutes at 18 to 25 ºC. The reaction was stopped by adding 100 µl of stop solution (Sulphuric acid) to the wells. The optical density of each well was read with a micro plate reader set at 450 nm.

3.7.1.3 Interpretation of Results of both IgM and IgG ELISA

Interpretation of results was done as per the kit instructions (Appendix 7). For CCHFV, the mean value of optical density of the negative control sample (ODC–) was expected not

exceed 0.250 Optical Density (OD) units. The mean value of ODC+ was supposed to be lower than 1.0 OD units. The OD of the TMB solution absorption control should not exceed 0.10 OD units. The cut-off OD value (ODCO) was ODC– + 0.2. The results of the analysis were considered negative if OD for the corresponding well OD of sample ≤ 0.9xODCO, the results of the analysis were considered Intermediate if 0.9 x ODCO< OD of sample≤1.1xODCO and hence the test was repeated. The results of the analysis were considered positive if OD of sample > 1.1xODCO. The presence of IgM indicated current or acute infection and the presence of IgG to CCHF virus reflected a current or previously occurred infection. For WNV, the kit was supplied with a calibrator. The average absorbance of the triplicates of the calibrator was calculated and this figure multiplied by the calibration factor supplied on the specification sheet. Panbio units were then calculated by dividing the sample absorbance by the mean cut-off value obtained and multiplying by 10. Panbio units of <9 were be interpreted as negative, 9-11 as equivocal and >11 were interpreted as positive sample. For CHIKV, the cut-off was taken as the mean absorbance value of the cut-off control determinations. Samples were considered positive if the absorbance value is higher than 10% over the cut-off. Samples found with an absorbance value of 10% above the cut-off were considered as positive and samples with absorbance value lower than 10% below the cut-off were considered negative. For RVFV, the amount of colour produced was proportional to the amount of anti-RVFV IgG or IgM. The positive control OD values should fall between 0.81 (Lower control limit) to 1.7 (Upper Control Limit) for the test to be valid. Two intermediate OD values of the positive control were used for the calculation the net mean OD value of the positive control (C++). The value was then used in the calculation of percentage positivity(PP) for the negative control (C-) and test serum as follows:

$$PP=\frac{\text{Net OD serum (C-, or Test serum)}}{\text{Net mean OD C++}}$$

Threshold PP value: Sera producing Percentage Positivity (PP) values ≥ 29 were considered to be positive and those less than these values were considered as negative.

3.7.2 Plaque reduction neutralization test (PRNT)

Samples that gave positive results for WNV and CHIKV by IgG ELISA were further tested using Plaque Reduction Neutralization Test (PRNT) to confirm the actual virus infecting the

patient by confirming the presence of specific neutralising antibodies of CHIKV and WNV so as to rule out other *Alphaviruses* and *Flaviviruses* known to be endemic, specifically SFV, SINDV, ONNV, DENV, Uganda S virus (UGSV) and YFV**.** Each virus isolate was diluted to a standard concentration that produced approximately 20-50 plaques. Serum samples were pre-heated at 56^{o}C for 30 minutes. Each sample was serially diluted in a sterile 96 well plate to determine the endpoint titre or highest dilution that neutralizes at least 99% of the virus ($PRNT_{99}$) at 1:20 to 1:640 concentration in maintenance media (minimum essential media (Sigma) with Earle's salts, 2% foetal bovine serum, 2% glutamine, 100U/ml penicillin, 100µg/ml streptomycin, and 1µl/ml amphotericin B). A constant amount of diluted virus was added into each well of the 96 well plate containing serially diluted serum samples and incubated for 1hour at 37^{0}C. The virus–antibody mixture was then transferred to a 24-well plate with confluent Vero cell monolayer and incubated in CO_2 for 1hour for virus adsorption after which an overlay of 1.25% methylcellulose was added and incubated for 5 to 7 days. The plates were retrieved from the incubator and stained with 0.25% crystal violet in absolute ethanol. $PRNT_{99}$ antibody titre for each virus was required to be 4-fold greater than the other viruses in the family tested to make an etiological diagnosis.

3.8 Ethical Considerations

The study was approved by Kenya Medical Research Institute's Scientific Review (SSC No.2350) (Appendix 1) and Ethical Review Committees (ERC Ref: KEMRI/RES/7/3/1) (Appendix 2). Consent was sought from all potential candidates and those meeting all inclusion criteria and willing to participate were recruited after signing a written informed consent. All information obtained in this study was kept confidential under lock and key in the principal investigators office. Questionnaire data and laboratory results were stored separately under unique study identity number only i.e. the bar-code numbers for sample identification and easy tracking.

3.9 Data Management

Field data captured in the study questionnaires were stored securely and accessed only through a password. This data were linked to the lab results of the tests conducted on the human samples. Hard copies of the results and data were kept in locked cupboards. No patient names were used. Unique ID numbers (i.e., bar-code numbers) were used for sample identification in the laboratory. The bar-code numbers were printed in triplicates for the sample, questionnaire and consent form for easy sample traceability in case of a mix-up or in

case of any patient follow-up required. Bar-coding also helped in avoiding transcription errors in the study which may happen when writing down the sample numbers on the sample tubes and the forms.

3.10 Data Analysis

The analyses were performed using STATA version 10.1 (StataCorp, College Station, TX). The main endpoints of interest were IgM and IgG antibodies against CCHFV, CHIKV, WNV, and RVFV and neutralisation antibodies for CHIKV and WNV. The IgM, IgG or PRNT status was dichotomized as either positive or negative based on the optical density (OD) values or presence of neutralising antibodies for PRNT as described earlier. Data on counts/proportions were compared using Chi Square or Fisher's exact test. For each outcome, a multiple logistic regression model was fitted to study the association with the risk factors while controlling for other variables, and Odds ratio (OR) used to measure the association. The factors considered included age of the patients, gender (female=0, male=1), contact with animals and/or birds (no=0, yes=1), whether or not a subject had tick-bites (no=0, yes=1), and occupation. Site was also controlled for by including site variable in the model. All tests were performed at 5% level of significance.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic and Clinical Characteristics of the Study Participants

In total, 379 human serum samples from febrile patients matching the inclusion criteria were screened by ELISA from Naivasha (152), Baringo (117) and Tana (110) health facilities. The mean age for the participants was 24.4, the median age was 22 years and the range was 5 to 80 years; while 10 individuals did not give their age details for unknown reasons. The ages were further classified into five age groups of size 10 except for those below 10 years and above 40 years. The results indicated that most of the subjects were in the age group between 20-29 years (n=109; 29%), followed by those aged 10-19 years (n=91; 24%), and only 14% (n=52) were at least 40 years old (Figure 4.1). 224 (59%) of them were females and 155 (41%) males (Table 4.1).

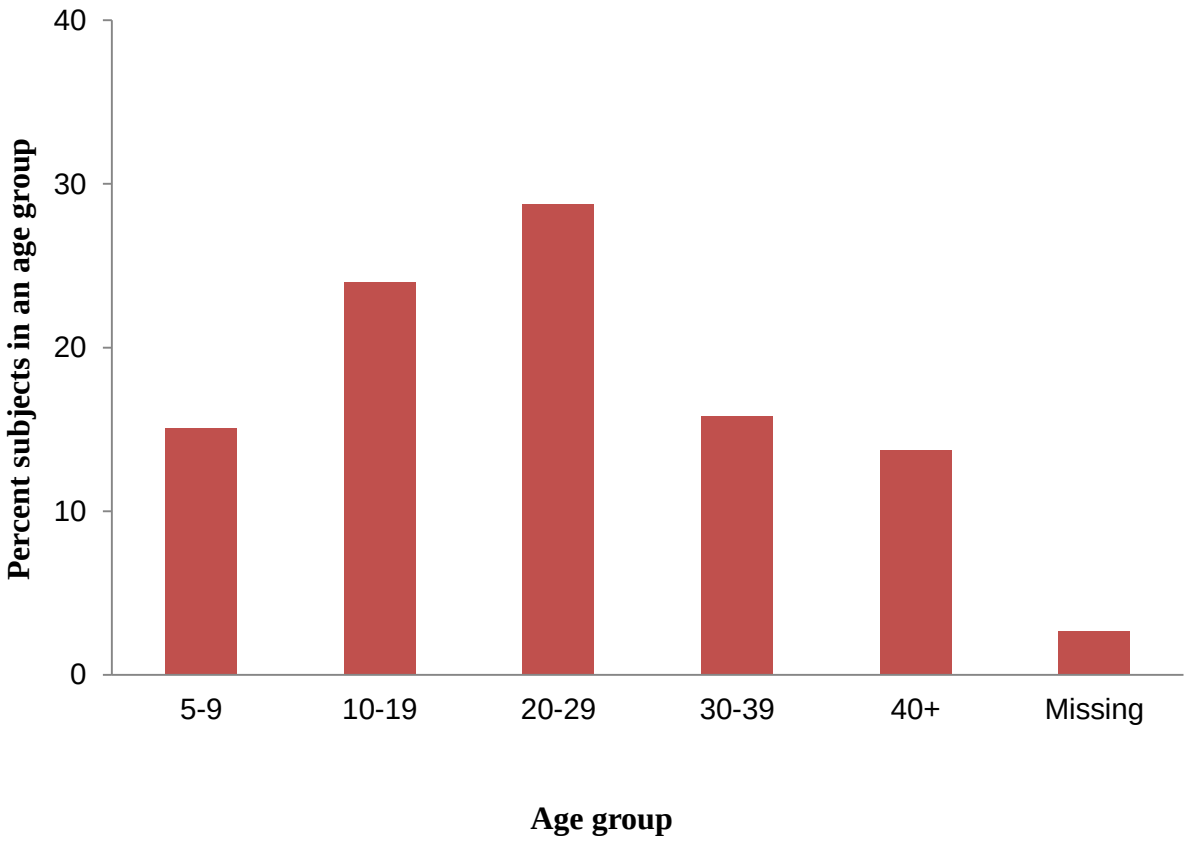

Figure 4.1: Age distribution of study participants sampled for arboviral infections from three different sites in Kenya. On the X-axis is age group and Y-axis, the percent of participants in an age group.

Table 4.1: Demographic characteristics of study participants sampled for arboviral infections from three different sites in Kenya*.

Characteristic		Site			
		Naivasha	**Baringo**	**Tana**	**Total**
		n= 152	**n = 117**	**n =110**	**N= 379**
Sex					
	Female	88 (39)	71(32)	65(29)	224 (59)
	Male	64 (41)	46 (30)	45 (29)	155 (41)
Age group					
	5-9	4 (7)	43 (75)	10 (18)	57 (15)
	10-19	29 (32)	40 (44)	22 (24)	91 (24)
	20-29	66 (60)	18 (17)	25 (23)	109 (29)
	30-39	28 (47)	5 (8)	27 (45)	60 (15)
	40+	20 (38)	6 (12)	26 (50)	52 (14)
	Missing	5 (50)	5 (50)	0 (0)	10 (3)

* Values are n(%) per site; n, number per site; N, total number of study participants

Table 4.2: Clinical characteristics of study participants sampled for arboviral infections in three different sites in Kenya

Clinical symptom	% subjects	
	No	Yes
Fever	0.00	100.00
Headache	8.97	91.03
Joint Aches	24.01	75.99
Muscle Aches	30.07	69.92
Chills	35.36	64.64
Abdominal pain	42.48	57.52
Cough	54.09	45.91
Sore throat	63.85	36.15
Vomit	64.91	35.09
Diarrhoea	69.40	30.60
Breathing difficulty	79.16	20.84
Rash	90.50	9.50
Dark Urine	90.77	9.23
Blood stool	96.04	3.96
Siezures	96.83	3.17
Bleeding	97.89	2.11
Periorbital pain	98.15	1.85
Jaundice	98.42	1.58
Bruises	98.94	1.06

% of study participants presenting with or without listed clinical characteristics

The study participant's clinical temperatures were ranging between 38^{o}C and 41^{o}C, with an average of 38.3^{o}C. Clinical characteristics of the subjects are summarized in Table 4.2. It shows that fever (100 %) was the most common symptom reported, followed by headache (91.03%), joint aches (75.99%), muscle aches (69.93%), chills (64.64%) and the least observed symptoms were periorbital pain (1.85%), Jaundice (1.58%) and bruises (1.06%) (Table 4.2).

4.2 IgM and IgG Seroprevalence results for CCHFV, CHIKV, WNV, and RVFV

None of the study participants was positive for anti-CCHFV, anti-WNV and anti-RVF IgM. An acute case of anti-CHIKV IgM was detected in Tana (1/379). A total of 176 (46.44%, 95%CI 41.39-51.48%) patients had IgG antibodies against at least one of the four arboviruses. The prevalence of each virus calculated separately and for all the four viruses combined by site are presented in Figure 4.2 and Table 4.3.

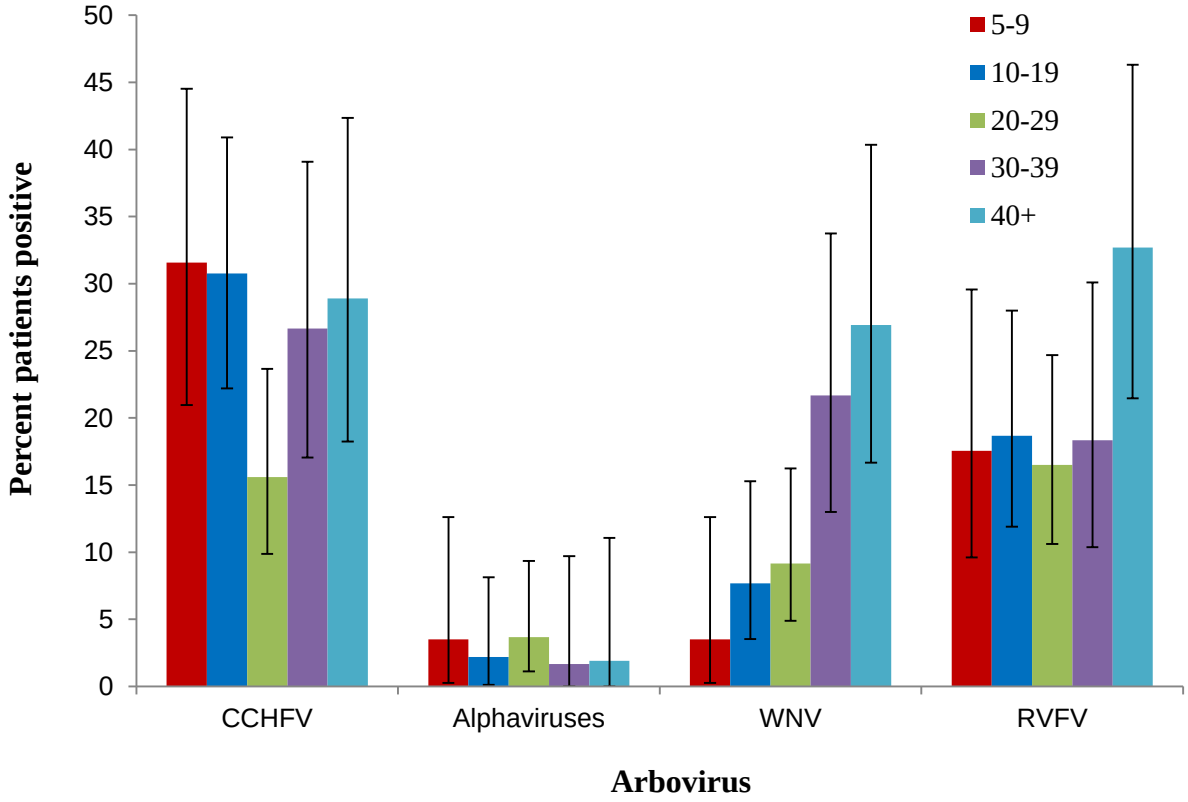

Figure 4.2: IgG antibody prevalence of CCHF, *Alphaviruses*, WNV and RVF viruses by age group by ELISA method for study participants sampled for arboviral infections in three different sites in Kenya. On the X-axis is age group; Y-axis, Prevalence of CCHFV, Crimean Congo Haemorrhagic Fever; *Alphaviruses*; WNV, West Nile Virus; RVFV, Rift Valley Fever Virus.

Figure 4.2 shows the prevalence of each of the arboviruses by age group. In general, apart from *Alphaviruses*, there was an increase of infection with age for all the arboviruses except for those that were aged between 5-19 years for CCHFV and RVFV. The prevalence for WNV increased with age.

Table 4.3: Anti-arboviral IgG antibodies prevalence (number positive) for each virus separately and combined of study participants sampled for arboviral infections in three different sites in Kenya.

Variable	CCHFV %(n)	*Alphavirus* %(n)	WNV %(n)	RVFV %(n)	All Viruses combined
All sites	25.59 (97)	2.64 (10)	12.40 (47)	19.53 (74)	46.44 (176)
Site					
Naivasha	17.76 (27)	1.97 (3)	2.63 (4)	13.82 (21)	32.24 (49)
Baringo	35.04 (41)	2.56 (3)	5.13 (6)	21.37 (25)	52.14 (61)
Tana	26.36 (29)	3.64 (4)	33.64 (37)	25.45 (28)	60.00 (66)
P value	0.005	0.667	<0.001	0.053	<0.001

Values are % positive(n); CCHFV, Crimean Congo Haemorrhagic Fever; CHIKV, *Alphavirus*; WNV, West Nile Virus; RVFV, Rift Valley Fever Virus

The anti-CCHFV IgG prevalence was 25.6% (95%CI 21.2-30.0). The prevalence of CCHFV varied by site ($p<0.001$) and it was the most prevalent virus in all the three sites. The prevalence was highest among those aged 5-19 and 40+ years old at 31.08% and 28.85%, respectively. The prevalence among those who had contact with animals (did not have contact with animals) was 28.47% (16.48%).

The IgG prevalence for WNV was 12.4% (95%CI 9.10-15.70%). The prevalence of WNV varied by site ($p<0.001$) and increased with age. Those aged 40 years and above were more likely to be exposed than the younger populations (Figure 4.2 and Table 4.3). All of the samples positive for WNV by ELISA were confirmed to be WNV by PRNT recording an overall prevalence of 12.4% (Table 4.4).

The overall IgG prevalence for RVFV was 19.5% (95%CI 15.5-23.5%). There was a borderline significant association between RVFV prevalence and site ($p=0.053$). Its prevalence increased with age (Table 4.3).

The overall IgG prevalence for *alphaviruses* was 2.6% (95%CI 1.0-4.3%). There was no statistically significant difference in *alphavirus* prevalence among the sites (p=0.667). Of the 10 samples positive for *alphaviruses* by ELISA, 1(10%) neutralised with CHIKV, 5(50%) with Semliki forest virus (SFV), 3(30%) neutralised with ONNV, and 1(10%) with Sindbis virus. The overall prevalence of CHIKV and SINDV confirmed by PRNT were both 0.26%, while the prevalence of SFV was 1.3% and ONNV was 0.8% (Table 4.4).

Table 4.4: Plaque Reduction Neutralization Test (PRNT) results for *Alphavirus* and *Flavivirus* genera for study participants sampled for arboviral infections in three different sites in Kenya.

Site	***Alphavirus Genus***				
	SFV	**CHIKV**	**SINV**	**ONNV**	**Total**
Naivasha	0 (0.0)	1 (0.7)	0(0.0)	0(0.0)	n=152
Baringo	2 (1.7)	0 (0.0)	0(0.0)	3(2.6)	n=117
Tana	3 (2.7)	0 (0.0)	1(0.9)	0(0.0)	n=110
All sites	5(1.3)	1(0.26)	1(0.26)	3(0.8)	N=379
	Flavivirus Genus				
Site	**UGSV**	**DENV**	**WNV**	**YFV**	**Total**
Naivasha	0 (0.0)	0 (0.0)	4(2.6)	0(0.0)	n=152
Baringo	0 (0.0)	0 (0.0)	6(5.1)	0(0.0)	n=117
Tana	0 (0.0)	0 (0.0)	37(33.6)	0(0.0)	n=110
All sites	0 (0.0)	0 (0.0)	47(12.4)	0 (0.0)	N=379

Values are n(%); SFV, Semliki Forest virus; CHIKV, Chikungunya virus; SINV, Sindbis virus; ONNV, O'nyong nyong virus; UGSV, Uganda S virus; DENV, Dengue virus; WNV, West Nile virus; YFV, Yellow Fever virus; N, total participants

4.3 Risk factors associated with CCHFV, CHIKV, WNV and RVFV infection

Sixty six percent of the patients reported to have had contact with goats (66%; n=250) mainly through farming. The most common occupation among the study participants was teaching/schooling (39%; n=148), followed by housewives (19%; n=75), farmers (14%; n=54), business (13%; n=48), and herdsmen at 6% (n=21) (Table 4.5).

Table 4.5: Potential risk factors for arbovirus infection by site for study participants sampled in three different sites in Kenya.

Risk Factors	Site			
	Naivasha	**Baringo**	**Tana**	**Total**
	n= 152	**n = 117**	**n =110**	**N = 379**
Tick bite				
No	139(46)	52(17)	110(37)	301(79)
Yes	13(17)	65(83)	0(0)	78(21)
Contact with donkey				
No	68(25)	111(41)	93(34)	272(72)
Yes	84(79)	6(6)	17(16)	107(28)
Contact with goats				
No	55(43)	9(7)	65(50)	129(34)
Yes	97(48)	108(43)	45(18)	250(66)
Contact with cows				
No	55(31)	41(23)	79(45)	175(46)
Yes	97(48)	76(37)	31(15)	204(54)
Contact with ducks				
No	132(37)	117(33)	109(30)	358(94)
Yes	20(95)	0(0)	1(5)	21(6)
Made contact through farming				
No	70(31)	48(21)	108(48)	226(60)
Yes	82(54)	69(45)	2(1)	153(40)
Made contact through slaughter				
No	138(38)	117(32)	110(30)	365(96)
Yes	14(100)	0(0)	0(0)	14(4)
Occupation				
Teaching/schooling	35(24)	87(59)	26(18)	148(39)
Business	40(83)	3(6)	5(10)	48(13)
Casual worker	17(52)	7(21)	9(27)	33(9)
Farmer	29(54)	7(13)	18(33)	54(14)
Herdsman	9(43)	4(19)	8(38)	21(6)
House Wife	22(29)	9(12)	44(59)	75(20)

Values are n(%) of participants; N, total number of participants enrolled in the study

The logistic regression analysis results for each virus are summarised in Table 4.6. Those aged 19 years and below were combined when fitting the logistic regression model to avoid computational problems due small numbers.. Compared to Naivasha and after controlling for the other factors (i.e., gender, age, whether a patient had tick-bites, and contact with goats), the results showed that the odds of CCHFV infection were similar in the three sites. The odds of infection was however higher in females than in males but not statistically significant. The results further indicated that those who had contact with goats were three times more likely to be infected with CCHFV than those who didn't (OR=3.38, 95%CI 1.68-6.80).

Occupation was not significant for CCHFV as well as the other three viruses. There was no difference in odds of being infected with *alphavirus* by site. Infection was seen to be higher among males. Compared to the first age group, the odds of being infected were higher among those aged between 20-29 years but not statistically significant (OR=1.66 95%CI 0.36-7.64).

Compared to Naivasha, the odds of infection with WNV were higher in Tana (OR=18.26, 95%CI 5.94-56.12). The results also showed that the risk of infection by WNV increased significantly with age and was higher in males compared to females. Relative to 5-19 years and after adjusting for other factors (i.e., site, gender, and contact with ducks), those in the age groups 20-29 years were almost twice (OR=2.11, 95%CI 0.75-5.97), 30-39 years were thrice (OR=3.55, 95%CI 1.26-10.00) and 40+ years were four times (OR=4.12, 95%CI 1.47-11.51) highly likely to be infected by WNV.

The odds of infection with RVFV were higher in Baringo (OR=2.11, 95%CI 1.00-4.45) and Tana (OR=2.47, 95%CI 1.11-5.52) as compared to Naivasha. These odds were also higher in females than males, but the difference was not statistically significant (OR=0.72 95%CI 0.42-1.25). Those aged 40+ years were highly likely to be infected by RVFV (OR=2.63, 95%CI 1.18-5.85) than those aged 5-19 years.

Those who made contact with animals and/or birds through farming and slaughtering had 44% (OR=1.44, 95%CI 0.73-2.84) and 5% (OR=1.05, 95%CI 0.21-5.33), respectively, higher (but not statistically significant) odds of being infected by RVFV than those who did not make any contact with animals and/or birds through farming and slaughtering (Table 4.6).

Table 4.6: Multiple logistic regression model results for each of the endpoints*

Variable	OR (95%CI)			
	CCHFV	***Alphaviruses***	**WNV**	**RVF**
Site				
Naivasha	1.00	1.00	1.00	1.00
Baringo	1.57 (0.76-3.23)	1.46 (0.25-8.57)	2.83 (0.68-11.81)	**2.11 (1.00-4.45)**
Tana	1.84 (0.96-3.51)	2.27(0.47-10.96)	**18.26 (5.94-56.12)**	**2.47 (1.11-5.52)**
Gender:Male	0.97 (0.59-1.59)	2.31 (0.63-8.43)	1.66 (0.81-3.42)	0.72 (0.42-1.25)
Age-group (years)				
5-19	1.00	1.00	1.00	1.00
20-29	**0.48 (0.24-0.96)**	1.66 (0.36-7.64)	2.11 (0.75-5.97)	1.17 (0.57-2.42)
30-39	1.07 (0.50-2.26)	0.62 (0.06-6.29)	**3.55 (1.26-10.00)**	1.21 (0.51-2.84)
40+	1.14 (0.53-2.46)	0.61 (0.06-6.11)	**4.12 (1.47-11.51)**	**2.63 (1.18-5.85)**
Tick bite	1.00 (0.47-1.89)			
Contact with goat	**3.38 (1.68-6.80)**			
Contact with ducks			2.10 (0.16-27.87)	
Type of contact made				
Farming				1.44 (0.73-2.84)
Slaughter				1.05 (0.21-5.33)

* CCHFV, Crimean Congo Hemorrhagic Fever; WNV, West Nile virus; RVFV, Rift Valley Fever virus; Blank-Risk factor excluded from model; OR,Odds Ratio; Bolded values are statistically significant i.e. doesn't include the null value.

CHAPTER FIVE

5.0 DISCUSSION

This study focused on habitats with similar ecological, environmental and biological factors to quantify the burden of arboviral diseases among human populations whose main source of livelihood is pastoralism. The overall prevalence of arboviral infection varied across the sites ($p<0.001$), with Tana recording the highest prevalence of 60%. The odds of infection with WNV and RVFV was also higher in Tana compared to Naivasha. The high risk in Tana can be attributed to environmental modification by the river with flood plains formed during rains and increased temperatures that favour breeding of mosquitoes, which transmit arboviruses. The convergence of wildlife, migratory birds and livestock for water and pasture, especially during the dry seasons leads to exposure to the vectors. Tana is a home for many birds species (e.g. *Passeriformes*) that have been implicated as a preferred avian host for SINV and WNV (Komar *et al*., 2003; Buckley *et al.,* 2003). This finding has public health implications because prevention programs should be site specific and not country wide.

The study showed that CCHFV was the most prevalent virus in all the sites with an overall prevalence of 25.6%. This is higher than the 19% prevalence obtained in a study by Lwande *et al* (2012) in Ijara District, Kenya. Circulation of the virus in humans is expected given that this virus has been isolated previously from *Hyalomma spp.* of ticks in Kenya due to possibility of tick bites or exposure to viremic animals infested with ticks (Sang *et al.,* 2011). Being a female and being in contact with goats increased the risk of exposure to CCHFV. Higher risk of CCHFV among those who had contact with goats can be attributed to the fact that goats are heavily infested by ticks, which can bite humans when handling the goats. This finding supports the findings of Sargianou *et al* (2013) where contact goats was seen to be a risk factor of infection with CCHFV. Prevalence of CCHFV was highest among those aged 5-19 and 40+ years old which partly compares well with the findings by Lwande *et al* (2012) where CCHFV was most prevalent among those aged between 40 and 49 years. This could be attributed to the age group being in close and frequent contact with animals during herding, high risk occupations like butchers, physicians and veterinarians or during trade exposing them to infection. The age group 5-19 years and females could get exposed during lambing or milking within the homestead where they spend most of their time. The middle age group 20-

29 years were at a significantly lower risk of infection compared to the younger age group (i.e. 5-19). This is an interesting finding because other studies have shown an increase in infection with age (Fisher-Hoch *et al*., 1992; Lwande *et al*., 2012). This finding needs further investigation of the risk factors associated with infection among the younger age group not investigated in this study. This observation can, however, be attributed to the middle aged persons being most active in the society at school, colleges or engaged in formal employment away from home and hence less exposure to tick bites and infected livestock.

The odds of infection with CCHFV in Baringo were higher compared to Naivasha probably because of geographic, environmental and cultural differences in the two sites. Baringo is more arid compared to Naivasha with most of its population practicing nomadic pastoralism. It experiences warmer temperatures and is close to two big lakes namely, Lake Baringo and Lake Bogoria providing humidity and arid conditions suitable for the *Hyallomma* ticks, the primary vector for CCHFV. There are more livestock (e.g. goats) in the region compared to Naivasha and hence high risk of exposure to ticks and viraemic animals by the population through contact by herding, slaughtering and tick bites. There is a big livestock market in Baringo as well as an active slaughterhouse where exposure may occur.

Alphavirus infections were shown to occur in low levels in all the three study sites. To rule out cross reactivity in the *Alphavirus* family, PRNT, a gold standard for antibody testing was used to confirm the actual circulating antibodies in the 10 samples positive for CHIKV. This increases the power of the study because PRNT is a confirmatory test for cross reacting antibodies. It was confirmed that the 10 samples that had tested positive for anti-CHIKV IgG ELISA neutralised with CHIKV (1/10) from Naivasha, SFV (5/10) 2 from Baringo and 3 from Tana, ONNV (3/10) all from Baringo, and SINDV (1/10) from Tana by PRNT. This can be attributed to the antigenic similarity among *alphaviruses* that results in cross reactivity posing a challenge in diagnosis of arboviral infection (Sutherland *et al*., 2011). One acute febrile patient tested positive for anti CHIKV IgM. This was later confirmed by PRNT to be a SFV infection. The patient was a male farmer from Tana aged 36 years. He presented with the following signs; headache, fever (38.3^{o}C), sore throat, abdominal pains, joint aches, muscle ache, itchy body and with a clinical diagnosis of typhoid and malaria. The outcome of the patient is not known because there was no follow-up done on patients recruited in this cross-sectional survey. The virus is widely distributed in Africa and with an outbreak of 22 cases being reported in Central Africa republic in 1987 (Mathiot *et al*., 1990). Besides,

multiple isolations of SFV from mosquitoes and ticks have been documented in parts of Kenya supporting the probability of transmission of the virus to the reported case in Tana area (Crabtree *et al.*, 2009; Ochieng *et al.*, 2013; Lwande *et al.*, 2013).

The prevalence of WNV increased with age with those aged 40 years and above being at a higher risk of infection than the younger populations. This finding is similar to the findings by Mease *et al* (2011) where seropositivity of WNV increased with age. This suggests a possibility of exposure to infection overtime giving a cumulative increase in IgG seropositivity with age as documented by Sutherland *et al* (2011). Males were also at higher risk of infection than females. This can be attributed to men being outdoor most of the time hence exposure to WNV mosquito vectors like *Culex univittatus* and C.*pipiens*. An interesting finding is that, the odds of infection were higher in Baringo and Tana compared to Naivasha. This can be ascribed to the similarity in environmental characteristics of the first two sites being semi arid, higher temperatures and humidity conducive for survival of competent vectors for WNV (*Culex* quinquefasciatus, *Cx. univittatus* and Cx. *pipiens*) isolated previously from these sites (Labeaud *et al.*, 2011b).

The prevalence of RVFV infection also varied with site and gender, with females being at a higher risk than males (OR=0.72, 95%CI 0.42-1.25). This observation contradicts previous findings where the risk of infection with RVFV varied with gender with males being at a higher risk than females due to their direct contact with animals during herding (Labeaud, 2008, 2011a). A possible explanation for these results is that more women than men in these areas do participate in high risk activities like taking care of livestock and small stocks like sheep and goats, by milking and by giving hand during slaughtering. This finding, however, compares with findings from a study conducted in Lokichoggio, Kenya were women were at greater risk of infection with RVFV than men 22.4% versus 14.7 % ($p \leq 0.025$) (Labeaud *et al.*, 2007). Although not statistically significant, being in contact with animals and/or birds through farming and slaughtering increased the risk of RVFV infection. This agrees with other studies that have revealed that domestic mammals (such as camels, donkeys, horses and cattle), wild animals and birds are potential reservoirs of these viruses (Pak et al., 1975; Komar *et al.*, 2003). The logistic regression model results (Table 4.6) showed that the risk of RVFV infection increased with age as expected and was more pronounced in the oldest age group. This qualifies the fact that the younger age group do not make direct contact with infected animals and that there is a possibility of the elderly having been exposed over time in the previous outbreaks in 1996/97 and 2006/07 and hence persisting antibodies detected

(Labeaud *et al.,* 2011a). The high prevalence of infection by RVFV in Tana and Baringo could be due to widespread flooding during rainy season and extended standing of water, and hence hatching of transovarially infected flood water *Aedes* mosquitoes e.g. *Ae. mcintoshi* (Woods *et al.,* 2002). This is a common phenomenon in Baringo and Tana, which is mostly semi-arid, with soil that has high water retention capacity. The high temperatures and relative humidity in these areas also provide favorable breeding sites for mosquitoes (Labeaud *et al.,* 2007).

Cross reactivity was observed in the *Alphavirus* genus with samples previously positive for CHIKV virus by ELISA being confirmed to have antibodies for SFV, ONNV and SINDV. This is similar with the findings of a study done in Cameroon where cross-reactivity was observed in the *Alphavirus* genus especially for CHIKV and ONNV (Kuniholm *et al.,* 2006). One sample neutralized by both *Alphavirus* (SINDV) and *Flavivirus* (WNV) by PRNT. Co-infection with more than one virus was observed in 19% of the patients by ELISA and in one sample by PRNT. This was also reported in a study conducted in Nigeria where 92% of the patients were co-infected with more than one arbovirus, typhoid and/or Malaria (Baba *et al.,* 2013). The occurrence of co-infections can be due to current or past exposure to vectors transmitting the virus. Lack of sensitive diagnostic methods, as observed by other studies, can lead to underestimation of the burden arboviral prevalence in the population (Sutherland *et al.,* 2011).

The highest prevalence recorded was from CCHFV (25.6%) transmitted by ticks. The most common mosquito-borne arbovirus documented in the three sites was RVFV (19.5%) followed by WNV (12.4%) and *Alphaviruses* were least prevalent (2.6 %). These viruses have been shown to be circulating during the inter-epidemic periods in these areas (Labeaud, 2008, Mease *et al*., 2011).There was only one acute *Alphavirus* case detected in the study. Previous research has shown that IgM antibodies diminish within 45 days of exposure (Madani *et al.,* 2003). The low IgM prevalence is possible due to delayed health seeking behavior by febrile patients due to limited resources for health care and very far distances of travel to arrive at a medical facility. Future studies can explore the use of more sensitive methods like PCR not performed in this study to detect acute febrile cases.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

- The study identified important socio-demographic and clinical characteristics associated with arboviruses infection e.g. age, site, contact and fever. These parameters should be put into consideration when treating patients with febrile illness for proper diagnosis.
- This study confirms occurrence and exposure of human population to CCHFV, RVFV, WNV and *Alphaviruses* in the river/lake basin areas of Baringo, Naivasha and Tana. These infections are often treated as malaria or typhoid or classified as fevers of unknown origin, hence the study has shown that there is a lot of under-reporting of arboviral infection posing a serious public health concern in the study area. The presence of multiple co-infections underscores the need to design and validate testing tools for use in febrile patients within such regions in order to diagnose and effectively treat these infections.
- This study determined that age, site and contact with goats were risk factors for arboviral infections. This is an important finding in designing disease control strategies and awareness creations.

6.2 RECOMMENDATIONS

- In order to accurately diagnose infections among febrile patients, clinicians should use clear case definitions based on the most common socio demographic and clinical characteristics documented by this study such as high fever, travel history, contact pattern, residence and bleeding while attending to patients presenting to the health facilities. Hospitals should be equipped well and staff empowered to be able to deal with these infections.
- The high prevalence varying significantly by site recorded in the study sites warrants the need for health care providers to be informed of the high risk of arboviral infections in the river/lake basin areas of Baringo, Naivasha and Tana. Efforts to recognize and identify cases should be made through awareness creation among

clinicians in the affected areas. There is need for active surveillance and use of improved diagnostic tools to monitor circulation in order to inform public health decision for early warning and response. These infections should be considered when investigating etiologies of febrile illness in patients reporting to health facilities in such endemic areas especially during seasons of high mosquito abundance in future.

- Infection control measures targeting the significant risk factors should be put in place to alleviate the burden of disease in these areas. The community should also be educated on the risks factors associated with these infections and possible prevention and control measures should be employed to stop transmission. Disease control and prevention strategies should be virus and site specific for effectiveness.

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APPENDICES

Appendix 1

Kenya Medical Research Institute Scientific Steering committee approval letter

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/100787 **31st August, 2012**

Caroline Tigoi

Thro'
Director, CVR
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

REF: SSC No. 2350 (Revised) – Seroepidemiology of selected arboviruses among febrile patients on the lake/river basin areas of Baringo, Naivasha and Tana, Kenya.

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its **193rd** meeting held on **6th August 2012** and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the revised protocol within 2 weeks from the date of this letter i.e, 14th September, 2012 to SSC for onward transmission to ERC office.

We advise that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

03 SEP 2012
P. O. Box 54628 - NAIROBI

Appendix 2

Kenya Medical Research Institute Ethical Review approval letter

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 **March 11, 2013**

TO: **CAROLINE CHEPNGENO TIGOI**
PRINCIPAL INVESTIGATOR

THROUGH **DR. FRED OKOTH**
THE DIRECTOR, CVR
NAIROBI

Forwarded
DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI
18/3/13

Dear Madam,

RE: **SSC NO. 2350- (REV): SEROEPIDEMIOLOGY OF SELECTED ARBOVIRUSES AMONG FEBRILE PATIENTS IN THE LAKE/RIVER BASIN AREAS OF BARINGO, NAIVASHA AND TANA, KENYA.**

This is to inform you that during the 212th meeting of the KEMRI/ERC held on 26th February 2013, the above referenced study was reviewed.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective the **26th February 2013**

Please note that the authorization to conduct this study will automatically expire on **25th February 2014.** If you plan to continue with the study beyond this date please submit an application for continuation approval to the ERC secretariat by **15th January 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 SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Yours sincerely,

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

CENTRE FOR VIRUS RESEARCH
18 MAR 2013
P.O. Box 54628 - NAIROBI

Appendix 3

Board of Postgraduate approval of research proposal letter

0722562348

OFFICE OF THE GRADUATE
RECEIVED
04 SEP 2013
PROGRAM COORDINATOR
TROMID

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY

DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000
NAIROBI – 00200
KENYA
Email: director@bps.jkuat.ac.ke

TEL: 254-067-52711/52181-4
FAX: 254-067-52164/52030

REF: BPS/ TM303-1069/2011 **8th August, 2013**

Ms. Caroline Chepngeno Tigoi
c/o COHES
JKUAT

Dear Ms. Tigoi

RE: APPROVAL OF RESEARCH PROPOSAL AND SUPERVISORS

Kindly note that your research proposal entitled: **"Seroepidemiology of selected arboviruses among febrile patients in the Lake/River basin areas of Baringo, Naivasha and Tana, Kenya"** has been approved. The following are your approved supervisors:-

1. Dr. Rosemary Sang
2. Dr. Juliette R. Ongus

Yours sincerely

PROF. BERNARD OTOKI MOIRONGO
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Ag. Principal, COHES

/pw

Appendix 4

Informed Consent Form

Request: My Name is …… I am a student at KEMRI INTROMID and I would like to request for your participation in this study.

What is the project called: Seroepidemiology of selected arboviruses among febrile patients in the lake/river basin areas of Baringo, Naivasha and Tana, Kenya.

What is this study about: The study is interested in finding out causes of illness in children and adults who have fever and/or malaria like illness. We would like to test your blood for germs that cause fever and Malaria like illness. If you agree to participate, we will collect a small amount of blood from vein on your arm and test it in the lab to see if we can find the germ that is causing your illness.

Who is running the study: The study is being run by Caroline Tigoi, Dr. Juliette Ongus and Dr. Rosemary Sang.

Do I have to participate: It is not a must for you to participate in the study. You are free to make a voluntary decision to participation or not to participate. There is no penalty for refusing to participate. In case you agree to participate and later change your mind about it, then you are free to withdraw from the study at any point.

What will happen to me if I participate in the study: If you agree to participate in the study, we will ask you a few questions about where you live, your illness and any medications you may have taken recently. We will then draw 5ml of blood from a vein in your arm. The blood will be transported to the KEMRI labs for testing of germs that may be causing your current illness. We will not test for HIV.

Are there any risks if I participate in the study: Asking of questions will take some of your time. You will possibly feel some mild discomfort, pain and bruising at the site of sample collection. It is very rare for infection to occur at the site where the blood is taken. In case of any injury of yourself (your child), we will provide medication at our cost.

Are there any benefits from the study: There is no direct benefit for participation in the study but it will lead to a better understanding of the causes of acute febrile illnesses in Kenya and improve the medical care in Kenya. The MoH and supporting medical community

can benefit from the knowledge of the identification of new or emerging diseases as the cause of acute febrile illnesses so that they know how to care for you and others in the future. Epidemics can be more readily identified, allowing the MoH to respond in a timely manner hence reducing the number of people who get affected.

Will there be any compensation for being in the study: There is no compensation to volunteers for their participation.

How long does the study last: This study requires only completion of a short questionnaire and one blood draw. There is no follow-up or further information needed. The questionnaire and blood draw will take about 30 minutes.

Who can participate in this study: Anyone can participate in the study if you have a fever without a source after evaluation by the clinician. If there is an obvious source of infection causing the fever, like an abscess or pneumonia, you need not (your child should not) participate.

Who will be able to see my information or lab results: Any information about you (your child's) will be kept very confidential. Only the people directly involved in the study will be able to see your information. We will keep all files in locked cabinets when they are not in use, and all blood stored in locked freezers. Your (your child's) name will not be used in any report resulting from this study. Any report from this study will refer to you/your child only by a study identification number and not by a name. All blood samples collected will be labelled with a study identification number; no names will be used.

What will happen to my blood: Your (your child's) blood will be tested for things that could cause fever. Your blood will NOT be tested for HIV. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. These samples will be labelled with only your study number. They will be secured in freezers at KEMRI laboratory and only study investigators and their authorized staff will have access. All safeguards ensuring privacy and confidentiality that are in place during this study period will also continue to be in place for the long-term storage of samples and if samples are sent outside of Kenya, no personal identifiers will be included.

If we do need to use the stored blood in the future we will first get permission from the Kenya National Ethical Review Committee.

Who can I contact about the study or my rights as a volunteer in this research study: If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact either:

Caroline Tigoi

Msc Student in Medical virology

ITROMID

PO Box 54628,

NAIROBI

Tel: 0722562348

Dr. Rosemary Sang

Centre for Virus Research

KEMRI

PO Box 54628,

NAIROBI

Tel. 0722 759492

Who should I contact if I have questions on my rights as a volunteer in this research study: If you have any question on your rights as a volunteer, you or your parent should contact**.**

The Secretary, National Ethical Review Committee

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

Subject Name _____

Subject's or Parent/Guardian's Signature: _____Date: _____

Physical Address/Home Description: _____

_____

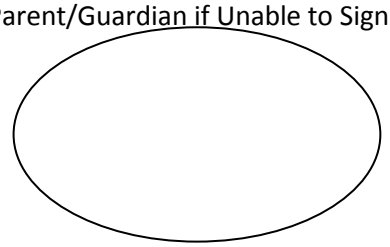
Family Name/Homestead Name: _____

Witness's Name: _____

Witness's Signature: _____ Date: _____

Study Number: _____

Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to Sign



Person Administering Consent:

Name:_____

Signature: _____

Appendix 5

Assent form for individuals 12 through 17 years of age

Request: My Name is …… I am a student at KEMRI INTROMID and I would like to request for your participation in this study.

What is the project called: Seroepidemiology of selected arboviruses among febrile patients in the lake/river basin areas of Baringo, Naivasha and Tana, Kenya.

What is this study about: The study is interested in finding out causes of illness in children and adults who have fever and/or malaria like illness. We would like to test your blood for germs that cause fever and Malaria like illness. If you agree to participate, we will collect a small amount of blood from vein on your arm and test it in the lab to see if we can find the germ that is causing your illness.

Who is running the study: The study is being run by Caroline Tigoi, Dr. Juliette Ongus and Dr. Rosemary Sang.

Do I have to participate: It is not a must for you to participate in the study. You are free to make a voluntary decision to participation or not to participate. There is no penalty for refusing to participate. In case you agree to participate and later change your mind about it, then you are free to withdraw from the study at any point.

What will happen to me if I participate in the study: If you agree to participate in the study, we will ask you a few questions about where you live, your illness and any medications you may have taken recently. We will then draw 5ml of blood from a vein in your arm. The blood will be transported to the KEMRI labs for testing of germs that may be causing your current illness. We will not test for HIV.

Are there any risks if I participate in the study: Asking of questions will take some of your time. You will possibly feel some mild discomfort, pain and bruising at the site of sample collection. It is very rare for infection to occur at the site where the blood is taken. In case of any injury of yourself (your child), we will provide medication at our cost.

Are there any benefits from the study: There is no direct benefit for participation in the study but it will lead to a better understanding of the causes of acute febrile illnesses in Kenya and improve the medical care in Kenya. The MoH and supporting medical community

can benefit from the knowledge of the identification of new or emerging diseases as the cause of acute febrile illnesses so that they know how to care for you and others in the future. Epidemics can be more readily identified, allowing the MoH to respond in a timely manner hence reducing the number of people who get affected.

Will there be any compensation for being in the study: There is no compensation to volunteers for their participation.

How long does the study last: This study requires only completion of a short questionnaire and one blood draw. There is no follow-up or further information needed. The questionnaire and blood draw will take about 30 minutes.

Who can participate in this study: Anyone can participate in the study if you have a fever without a source after evaluation by the clinician. If there is an obvious source of infection causing the fever, like an abscess or pneumonia, you need not (your child should not) participate.

Who will be able to see my information or lab results: Any information about you (your child's) will be kept very confidential. Only the people directly involved in the study will be able to see your information. We will keep all files in locked cabinets when they are not in use, and all blood stored in locked freezers. Your (your child's) name will not be used in any report resulting from this study. Any report from this study will refer to you/your child only by a study identification number and not by a name. All blood samples collected will be labelled with a study identification number; no names will be used.

What will happen to my blood: Your (your child's) blood will be tested for things that could cause fever. Your blood will NOT be tested for HIV. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. These samples will be labelled with only your study number. They will be secured in freezers at KEMRI laboratory and only study investigators and their authorized staff will have access. All safeguards ensuring privacy and confidentiality that are in place during this study period will also continue to be in place for the long-term storage of samples and if samples are sent outside of Kenya, no personal identifiers will be included.

If we do need to use the stored blood in the future we will first get permission from the Kenya National Ethical Review Committee.

Who can I contact about the study or my rights as a volunteer in this research study: If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact either:

Caroline Tigoi

Msc Student in Medical virology

ITROMID

PO Box 54628,

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Tel: 0722562348

Dr. Rosemary Sang

Centre for Virus Research

KEMRI

PO Box 54628,

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Tel. 0722 759492

Who should I contact if I have questions on my rights as a volunteer in this research study: If you have any question on your rights as a volunteer, you or your parent should contact**.**

The Secretary, National Ethical Review Committee

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

Subject's or Guardian's Signature: _____Date: _____

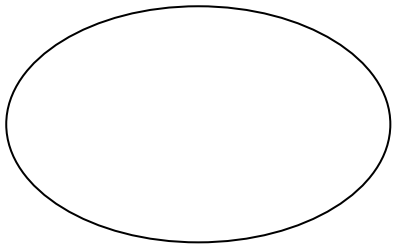
Permanent Address: _____

Witness's Name: _____

Witness's Signature: _____ Date: _____

Study Number: _____

Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to Sign



Person Administering Consent:

Name:_____

Signature: _____

Appendix 6

The research questionnaire

Interviewer's Name:	Stick Barcode Label here

Date of Collection: _____ (dd/month/yr)

Sex: 1. Male 2. Female ☐ Age: _____years

Where is your (your child's) current residence:

Village: _____ **Sub-county**: _____

County: _____

How long have you (your child) been living in this **Sub-county**?

_____years

_____months

During the past five days, where have you (your child) been mostly (check one)?

☐ Village of Residence

☐ In the country, but not in residence, Where? _____

☐ Out of the country, Where? _____

How many times have you (your child) travelled outside of your **county** in the last two months: _____

How long ago: <2 weeks 2-4 weeks 1-2 months Where:_____

Have you (your child) ever received a yellow fever vaccine?

1. Yes 2. No 3. Unknown ☐

Date of vaccination (if known) _____(dd/mm/yy)

If adult: What is your occupation: _____

If child: Where do you go to school: _____

Do you have contact with any of the following species of animals?

1. Bats
2. Geese
3. Ducks
4. Chickens
5. Other- Specify_____

Indicate which birds using the codes above (1-5):_____

1. Goats
2. Cows
3. Donkeys
4. Camels
5. Monkeys

Indicate which animals using the codes above (6-10): _____

Tick Bites: Yes/No _____

Others bites

For each species checked above:

List the species using the given codes

_____

2) Describe the contact, e.g., trapping, farming, slaughter, food preparation, veterinary work, casual contact (e.g., a neighbour keeps chickens, there is a slaughterhouse nearby), eating raw fowl products or drinking blood

_____

3. Were the animals showing signs of illness? Yes No

If ***Yes*** above, Specify the signs. ☐ ☐

YOUR CURRENT ILLNESS:

Why did you (your child) come to the hospital:

_____

Did you (your child) have any of the following?

	Yes	No	Uncertain			Yes	No	Uncertain
Fever					Abdominal Pain			
Chills					Vomiting			
Cough					Diarrha			
Difficulty Breathing					Blood in stools			
Sputum production					Bleeding			
Sore Throat					Bruising			
Headache					Rash			
Runny Nose					Joint Aches			
Eye Pain					Muscle Aches			
Seizures					Dark Urine			
					Jaundice			

Do you (your child) have any symptoms we did not mention: _____

If bleeding, where: 1. N/A ☐ 2.Gums ☐ 3. Nose ☐ 4. Injection Sites ☐

5. Other, Specify _____

How many days have you (your child) been sick: _____ Date of Onset: ___

How many days of school or work have you (your child) missed:_____

Does anyone you know have a similar illness? Yes ☐ No ☐

Who:_____ When did they become ill _____

Who:_____ When did they become ill _____

Who:_____ When did they become ill _____

What was your temperature in the clinic: _____ ^{0}C

DIAGNOSES MADE BY MOH PROVIDER:

1. _____
2. _____

TREATMENT PRESCRIBED BY MOH PROVIDER:

1. _____
2. _____
3. _____

Appendix 7

ELISA Manufacturers' instructions

1. CCHFV Antibody testing

Detection of all CCHF virus specific IgM and IgG antibodies will utilize the indirect ELISA format described by Harun, (2010). This will be done using a commercial kit supplied with its own internal negative and positive control (VectoCrimean-CHF-IgG and IgM ELISA test kits,Vector-Best, Novosibirsk, Russia).

Vectocrimean-CHF-IgM ELISA kit

1. All samples were heat inactivated at 56ºC for 30 minutes. Inactivated lysates of tissue culture cells infected with the CCHF virus and a similarly diluted mock antigen made from uninfected cells was used as positive and negative antigens respectively.
2. A CCHF virus positive and negative human serum by ELISA for CCHF virus-specific antibodies supplied with the kit served as positive control and negative control respectively (Vector-Best, Novosibirsk, Russia).
3. Horse Radish Peroxide (HRP) conjugated antihuman IgM, Fc specific diluted in Serum dilution buffer was used as a conjugate and ABTS peroxidase (Vector-Best, Novosibirsk, Russia) Parts A and B combined 1:1 as a substrate.
4. One half of the plate was coated with 100µl per well of positive antigen diluted at 1:1000. The second half of the plate was coated with 100µl per well of mock control antigen diluted at 1:1000.
5. The plates was then be incubated in a moist environment overnight at 2ºC to 8ºC and then washed 3 times with 300µl of wash buffer (Phosphate Buffer Saline (PBS) by Vector-Best, Novosibirsk, Russia).
6. One hundred microlitre of sample serum (as well as positive and negative controls) was added to both positive and negative wells at 1:100 dilution (hence each sample was tested against positive and negative antigens), incubated for 1 hour in moist condition at 35ºC to 37ºC and then washed 3 times with 300 µl per well of wash buffer.
7. One hundred microlitre of diluted HRP labelled with anti-human IgM antibody was added and the plates incubated for 1 hour at 35ºC to 37ºC.
8. One hundred microlitre of the ABTS peroxidase substrate was added to each well.

9. The plates was incubated for 30 minutes at 35ºC to 37ºC and then read spectrophotometrically at 405 nm on the ELISA reader (ELx808 IU, Biotek, USA).

Vectocrimean-CHF-IgG ELISA kit

1. The test serum samples were heat inactivated at 56ºC for 30 minutes.
2. Inactivated lysates of tissue culture cells infected with the CCHFV and a diluted mock antigen made from uninfected cells by the manufacturer was used as positive and negative antigens respectively (Vector-Best, Novosibirsk, Russia).
3. A positive anti-CCHF IgG human serum supplied with the kit served as positive control sera and serum negative by ELISA for CCHFV IgG specific antibodies served as a negative control(Vector-Best, Novosibirsk, Russia).
4. HRP conjugated anti-human IgG, Fc specific diluted in Serum dilution buffer was used as a conjugate and ABTS peroxidise parts A and B combined 1:1 as a substrate.
5. One half of the plate was coated with 100µl per well of positive antigen diluted at 1:1000. The second half of the plate was coated with 100µl per well of mock control antigen diluted at 1:1000.
6. The plates was then be incubated in a moist environment overnight at 2ºC to 8ºC then washed 3 times with 300µl of wash buffer Phosphate buffer saline (PBS), (Vector-Best, Novosibirsk, Russia).
7. One hundred microlitre of sample serum plus positive and negative controls was added to both the positive and negative wells at 1:100 dilutions.
8. This was incubated for 1 hour in moist condition at 35ºC to 37ºC and then washed 3 times with 300 µl per well of wash buffer.
9. One hundred microlitre of diluted HRP labelled with anti-human IgG antibody was added and the plates incubated for 1 hour at 35ºC to 37ºC.
10. One hundred microlitre of the ABTS peroxidase substrate was added to each well.
11. The optical density was read with a microplate reader set at 450 nm (main filer) and 620-650 nm (reference filter) using ELISA reader ELx808 IU (Biotek, USA).

Interpretation of Results of both CCHFV IgM and IgG ELISA

The adjusted OD will be calculated by subtracting the OD of the negative antigen coated wells from the positive antigen coated wells. The OD cut-off was calculated as the mean of the adjusted OD of the negative control sera plus three times the standard deviations. A

serum sample was considered positive if the adjusted OD value was greater than or equal to the assay cut-off or 0.2, which ever value is higher.

2. WNV Antigen Testing

Detection of all WNV virus specific IgM and IgG antibodies utilized the indirect ELISA format described by Malan, *et al.* (2003). This was done using a commercial supplied with its own internal negative and positive control (Panbio diagnostics).

WNV IgM capture ELISA

The IgM capture ELISA was performed following the manufacturer's instructions.

1. Serum samples was diluted in the ratio of 1:100 in sample diluent and added to microwells coated with anti-human IgM antibodies.
2. After 60 minutes incubation at 37°C, wells were washed six times and 100µl of HRP-conjugated anti-WNV monoclonal antibody-reconstituted WNV antigen solution supplied with the kit will be added to each well and incubated for 60 minutes at 37°C.
3. A six times washing step was follow and then 100 µl TMB substrate (Panbio diagnostics) was added and incubated for 10 minutes at Room Temperature.
4. This was followed by adding a stopping solution (100 µl of 1 M phosphoric acid) and the absorbance of each well determined spectrophotometrically at 450 nm (reference filter 600-650 nm) using ELx808 IU (Biotek, USA).

WNV IgG Indirect ELISA

The indirect IgG capture ELISA was done following the manufactures instructions (Panbio diagnostics).

1. Serum samples was diluted in the ratio of 1:100 in sample diluent and 100 µl added to microwells coated with WNV antigen and incubated at 37°C for 30 minutes.
2. After the wells were washed six times using Phosphate Buffer Saline, 100 µl of HRP-conjugated sheep anti-human IgG was added to each well and the wells were incubated at 37°C for 30 minutes.
3. The wells was washed six times a second time with wash buffer.
4. This was followed by adding 100 µl of TMB substrate and incubated in the wells for 10 minutes at Room Temperature.
5. A 100 µl Phosphoric acid stopping was added to stop the enzymatic reaction, and the resulting absorbance of each well was determined spectrophotometrically at 450 nm using ELx808 IU (Biotek, USA).

Interpretation of results of both WNV IgM and IgG ELISA

Since the kit is supplied with a calibrator, the average absorbance of the triplicates of the calibrator was calculated and this figure multiplied by the calibration factor supplied on the specification sheet. Panbio units were then calculated by dividing the sample absorbance by the mean cut-off value obtained and multiplying by 10. Panbio units of <9 were interpreted as negative, 9-11 were equivocal and samples were repeated and >11 were positive sample.

3. CHIKV Antigen Testing

CHIKV IgM µ-capture ELISA

This was a qualitative immunoenzymatic determination of IgM-class antibodies against Chikungunya using ELISA as per the Manufacturer's instructions.

1. Serum samples were diluted in the ratio of 1:100 with the Sample diluent supplied by the manufacture and thoroughly mixed with a Vortex. Positive and negative controls were ready to use and hence will not be diluted.
2. The serum samples (50µl) and controls were dispensed into the respective wells, covered with a foil supplied with the Kit and incubated for 1 hour at 37 °C.
3. After incubation, each well was washed three times with 300µl of washing solution and this was followed by dispensing 50 µl Chikungunya antigen Solution 1 into all wells except for the blank well and covered with foil, then incubated for 30 minutes at room temperature.
4. Washing step was repeated again followed by adding 50 µl Chikungunya Solution 2 (biotinylated Chikungunya antibody, stabilizers, pr eservatives and an inert blue dye) into all wells except for the blank well. The plate was then covered with the foil and incubated for 30 min at room temperature.
5. The washing step was repeated again and then 50 µl Streptavidin peroxidase conjugate was added into all wells except for the blank and covered with a foil and incubated for 30 min at room temperature.
6. Washing was repeated again and 100 µl TMB solution added into all wells. Incubation followed by for exact 15 min in the dark.
7. After incubation, 100µl Stop Solution (0.2 M sulphuric acid solution) was added into all wells in the same order and at the same rate as for the TMB substrate.
8. The absorbance of the specimen was measured at 450/620nm within 30 min after addition of the Stop Solution.

CHIKV IgG Capture ELISA

A qualitative immunoenzymatic determination of IgG-class antibodies to Chikungunya was performed based on the manufactures instructions (NovaTec Immundiagnostica).

1. All samples were diluted in the ratio of 1:100 using the sample Diluent and 50µl dispensed into the microtiter strip wells precoated with anti human IgG to bind corresponding antibodies of the specimen. Also 50µl of positive and negative controls and substrate blank were also dispensed to the marked wells. The wells were then covered using a foil supplied in the kit and incubated for 1 hour ± 5 min at 37±1°C.
2. After washing the wells three times with 300µl of washing solution to remove all unbound sample and control material, 50µl Chikungunya antigen solution 1 was added except for the blank well. The plate was then covered with foil and incubated for 30 minutes at room temperature.
3. After a further washing step as two above, 50 µl biotinylated Chikungunya antibody solution 2 was pipetted into the wells except for the blank well. The plate was covered with foil and incubated for 30 minutes at room temperature.
4. After washing again, 50µl Streptavidin peroxidase conjugate was dispensed into all wells except for the blank to bind to the captured Chikungunya-specific immune complex. The plate was covered with foil and incubated for 30 minutes at room temperature without exposing it to direct sunlight.
5. This immune complex was visualized by adding 100 µl TMB solution into all wells giving a blue reaction product. The plate was incubated for exact 15 minutes in the dark.
6. This was followed by adding 100µl Sulphuric acid (Stop Solution) to stop the reaction. Absorbance at 450 nm was read using an ELISA microwell plate reader, ELx808 IU (Biotek, USA) ELx808 IU (Biotek, USA).

Interpretation of Results for both CHIKV IgM and IgG ELISA

The cut-off was the mean absorbance value of the Cut-off control determinations. Samples were considered positive if the absorbance value was higher than 10% over the cut-off. Samples found with an absorbance value of 10% above or below the cut-off were not considered. Samples were considered negative if the absorbance value is lower than 10% below the cut-off.

4. RVFV Antigen Testing

RVF capture IgM ELISA

1. All volumes used were 100 µl/well, and all washes were performed 3 times for 15 seconds using 300 µl buffer per well.
2. The plates were coated with 100µl goat anti-human IgM diluted 1:500 in PBS and incubated plates covered with lids at 4°C overnight and plates washed thereafter.
3. 200µl /well blocking buffer was added after washing and incubated for 1h in moist chamber at 37°C, followed by washing the plates 3 times for 15s using 300µl of wash buffer per well.
4. 100µl of test and control sera diluted 1:400 in diluent buffer was added into all the wells except the control wells and incubated for 1h in moist chamber at 37°C. The plates were washed 6 times.
5. 100µl /well of RVFV Ag and Control Ag diluted 1:400 in diluents buffer rows A-D 1-12 and rows e-H 1-12, respectively and incubated for 1 hr in moist chamber at 37°C and followed by washing the plates 3 times.
6. 100µl of anti-mouse IgG Horseradish peroxidise (HRPO) conjugate diluted 1:6000 in diluents buffer and incubated for 1 hr in moist chamber then washed 6 times.
7. 100µl ABTS/well was added. The plates were left for 30 minutes at room temperature (22-25°C) in dark. 100µl of 1x concentrated SDS stop solution was added and the plates read at 405nm optical density.

Results, data expression, acceptance criteria and diagnostic interpretation

The amount of colour developed was proportional to the amount of anti-RVFV IgM antibody that has been captured. Net optical density (OD) values are first recorded for each serum as value determined with RVFV Ag minus the value determined with control Ag. Three levels of microplate acceptance were applied. The results on a test plate fulfilled the first level of internal quality control (IQC) acceptance if at least three of the OD values recorded for C++ fell within the range 0.75 (lower control limit) to 1.65 (upper control limit); if the results of two or more of the four replicates of C++ fell outside IQC limits then the plate was rejected and repeated. If the plate was accepted, then the two intermediate net OD values of C++ were used for the calculation of the net mean OD value of C++. This value was then used in subsequent calculations of percentage positivity (PP) of C+, C- and test sera as follows:

Net OD serum (C+, or C , or test serum)

Net mean OD C++

The results obtained on a test plate fulfilled the second level of IQC acceptance if the coefficient of variation {CV = (standard deviation of replicates/mean x 100) for PP values of two replicates of C++ (calculated from intermediate OD values) were less than 15% and two replicates of C+ were less than 14 %}]. Using the thresholds PP values provided below, the two intermediates of the C+ and C- control sera should have fallen within the same interpretive group, i.e. positive or negative (third level of IQC acceptance). The same principle was applied for the acceptance of individual test sera if they were assayed in duplicate. Threshold PP value: sera producing PP values ≥7.1 were considered to be positive and less than these values were considered to be negative.

RVF recN IgG ELISA

Recombinant Antigen based Indirect ELISA for detection of Anti-RVF IgG antibody in human sera

8. All volumes used were 100 µl/well, and all washes are performed 3 times for 15 seconds using 300 µl buffer per well.
9. The plates were coated with 100µl RVFV recN antigen diluted 1:2000 in Carbonate-bicarbonate buffer and incubated plates covered with lids at 4°C overnight. Thereafter, the plates were washed 3 times for 15s using 300µl of wash buffer per well.
10. 200µl /well blocking buffer was added after washing and incubated for 1h in moist chamber at 37°C. This was followed by washing the plates 3 times for 15s using 300µl of wash buffer per well.
11. 100µl of test and control sera diluted 1:400 in diluent buffer was added into wells and incubated for 1h in moist chamber at 37°C. The conjugate control wells consisted of only diluent buffer with no serum added. The plates were washed as in 2 above.
12. 100µl /well anti-human IgG HRPO conjugate diluted 1:20 000 in diluent buffer were added and incubated for 1h in moist chamber at 37°C. This was followed by washing the plates as decribed in 3 above.
13. 100µl of ABTS/well was added. The plates were left for 30 minutes at room temperature (22-25°C) in dark. 100µl of 1x concentrated SDS stop solution was added and the plates read at 405nm optical density.

Results, data expression, acceptance criteria and diagnostic interpretation

The amount of colour developed was proportional to the amount of anti-RVFV IgG antibody that bound to RVFV recN and was available to react with the detection system. Three levels of microplate acceptance were applied. The results on a test plate fulfilled the first level of internal quality control (IQC) acceptance if at least three of the OD values recorded for C++ fell within the range 0.81 (lower control limit) to 1.7 (upper control limit); if the results of two or more of the four replicates of C++ fell outside IQC limits then the plate was rejected and repeated. If the plate was accepted, then the two intermediate net OD values of C++ were used for the calculation of the net mean OD value of C++. This value was then used in subsequent calculations of percentage positivity (PP) of C+, C- and test sera as follows:

$$\frac{\text{Net OD serum (C+, or C-, or Test serum)}}{\text{Net mean OD C++}}$$

The results obtained on a test plate fulfilled the second level of IQC acceptance if the coefficient of variation {CV = (standard deviation of replicates/mean x 100) for PP values of two replicates of C++ (calculated from intermediate OD values) were less than 10% and two replicates of C+ were less than 14 %}]. Using the thresholds PP values provided below, the two intermediates of the C+ and C- control sera should have fallen within the same interpretive group, i.e. positive or negative (third level of IQC acceptance). The same principle was applied for the acceptance of individual test sera if they were assayed in duplicate. Threshold PP value: sera producing PP values ≥29 were considered to be positive and less than these values were considered to be negative.

Appendix 8

Kenya Medical Research Institute Scientific Steering committee approval letter AVID project (protocol 1560)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
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KEMRI/RES/7/3/1 **27th April 2009**

TO: **DR. ROSEMARY SANG (CO-PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. F. OKOTH,**
THE DIRECTOR, CVR
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
NAIROBI

RE: **SSC PROTOCOL No. 1560 (*2ND REVISION*): AN INTEGRATED RESPONSE SYSTEM FOR EMERGING INFECTIOUS DISEASES IN EAST AFRICA**

Dear Dr. Sang,

Reference is made to your letter dated 24th April 2009.

The Committee acknowledges the receipt of the following documents:
a. The Study Protocol.
b. The Informed Consent Document in English; in Somali (for the community in Ijara); in Kikuyu (for the community in Mai-Mahiu/Naivasha); in Kalenjin (for the community in Marigat).

The Committee notes that the contact information for both the study personnel and the Secretary of the KEMRI/National ERC are provided in the Consent Documents and is satisfied that the issues raised at the 164th meeting of 17th March 2009 have been adequately addressed. The study is hereby granted approval for implementation for a period of twelve (12) months effective the **27th day of April 2009.**

Please note that authorization to conduct this study will automatically expire on **29th March 2010.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat on **15th February 2010.**

You are required to submit any amendments to this protocol and other information pertinent human participation in this study to the SSC and ERC for review prior to initiation.

You may embark on the study.

Sincerely,

C. WASUNNA,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
28 APR 2009
P.O. Box 54628 - NAIROBI