Serological survey for human West Nile virus exposure in febrile clients attending selected health facilities in Trans Nzoia District, Kenya

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DECLARATION

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

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DEDICATION

It is with great pleasure that I dedicate this work to two people who have and will forever inspire me; my beloved parents Esther Wayua Ngoi and the late Wilfred Wambua Ngoi, God rest his soul.

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LIST OF ABBREVIATIONS AND ACRONYMS

Cx   *Culex*

CDC   Centre for Disease Control

CMR   Centre for Microbiology Research

CPE   Cytopathic effect

ELISA   Enzyme Linked Immunosorbent Assay

EMEM   Eagles Minimum Essential Medium

FCS   Foetal Calf Serum

GM   Growth media

HRPO   Horse Radish Peroxidase

ICF   Infected Culture Fluid

IgM   Immunoglobulin M

IgG   Immunoglobulin G

IFN-α   Interferon alpha

JE   Japanese Encephalitis

JEVC   Japanese Encephalitis Virus Complex

KEMRI Kenya Medical Research Institute

MEM   Minimum Essential Media

MM   Maintenance Media

NS   Non-Structural proteins

NUITM   Nagasaki University of Infectious Diseases and Tropical Medicine

OPD   o-phenylenediamine

PBS   Phosphate Buffer Saline

PBS-F   FCS diluted in PBS

PBS-T   Tween 20 diluted in PBS

PFU   Plaque Forming Units

PRM   Pre-membrane proteins

PRNT   Plaque Reduction Neutralization Test

RT-PCR   Reverse Transcriptase-Polymerase Chain Reaction

RT-LAMP Reverse Transcriptase Loop Mediated Isothermal Amplification

SLEV	St. Louis encephalitis viruses
UTR	Untranslated region
WHO	World Health Organization
WNV	West Nile Virus

ABSTRACT

West Nile Virus (WNV) is considered to be one of the most wide spread group of arboviruses in Africa, Asia and America. The geographical range of the WNV has broadly extended over the years and the virus has transcended geographical barriers and become established even in non-endemic areas. This sero-survey study reports WNV activity in Trans Nzoia district, Kenya. The study cohort consisted of 1114 serum samples from febrile patients attending three different health facilities in the area. Antibodies to West Nile Virus (WNV) were detected in 105 (9.8%) of the patients by indirect IgG Enzyme Linked Immunosorbent Assay (ELISA). Of these, 12 (1.1%) infections were confirmed by Plaque Reduction Neutralization Test (PRNT) therefore the seroprevalence of WNV in the area. This suggests that there is low WNV activity in Trans Nzoia district, Kenya. The detection of low levels of WNV and the low intensity of the severity of the clinical symptoms suggest a less virulent strain is in circulation or previous exposure to other closely related flavivirus. The linear increase in WNV IgG and IgM seroprevalence rates with age suggests continuous exposure of this population to the virus. Patients of both genders were exposed with 69% female and 31% male positive for WNV, this disparity can be attributed to cultural practices and activities that increase the risk of exposure among the female subjects. This study provides baseline information for further research and strategic planning. Therefore despite the low levels of WNV, appropriate interventions should be established by the public health authorities in view of the fact that it is a public health threat.

CHAPTER ONE

1.0 INTRODUCTION

1.1   Background of the West Nile Virus

West Nile Virus (WNV) is an arthropod-borne virus which was first isolated from the blood of a febrile woman in the West Nile District of Uganda in the year 1937 (Smithburn *et al.,* 1940). Since then the geographical distribution of the virus has expanded over the years (Huang *et al.,* 2002; Lorono-Pino *et al.,* 2003; Malan *et al.,* 2004). This epizootic virus has been identified in Africa and other parts of the world with severe outbreaks in both humans and animals being reported (Anderson *et al.,* 2001; Burt *et al.,* 2002). Several outbreaks of febrile illness and neuroinvasive disease have been associated with the virus. The febrile illness is characterized by fever, headache, general malaise, lymphadenopathy, body aches and muscle pains, anorexia, nausea, diarrhoea, vomiting and a transient macular rash (Hubalek *et al.,* 1999; Anderson *et al.,* 2001). Neuroinvasive disease on the other hand is characterized by encephalitis, meningitis and acute flaccid paralysis (Hubalek *et al.,* 1999; Anderson *et al.,* 2001; Hayes *et al.,* 2005).

The most notable outbreaks in humans were in; Europe with the largest in Romania which had 393 serologically confirmed cases of West Nile fever (Hayes *et al.,* 2005). The 1954 through 1957 outbreaks in Israel which caused severe West Nile fever and meningoencephalitis in adults (Komar, 1999; Anderson *et al.,* 2001; Petersen *et al.,*

2001; Burt *et al.,* 2002). A series of severe cases of human meningoencephalitis due to WNV were reported in France from 1962 through 1966 (Burt *et al.,* 2002). The 1974 outbreak in South Africa which was highly epidemic reported over 10,000 cases and a mean antibody prevalence of 55% in humans was recorded in Karoo and Northern Cape provinces (Burt *et al.,* 2002).

In the 90's there was a discernable manifestation of high frequency of WNV occurrence and severe outbreaks in humans where equines and avian were identified as a major cause of WestNile fever arboviral encephalitis; the out breaks were experienced in Algeria (1994), Morocco (1996) Tunisia (1997), Italy (1998), Russia (1999-2001), Romania (1999-2000), Israel (1999 and 2000), France (2000,2003-2004) (Komar, 1999; Petersen *et al.,* 2001; Burt *et al.,* 2002; Hayes *et al.,* 2005). The most recent severe outbreak was experienced in the United States of America from 1999 to 2004 in New York with 16,706 cases reported to the Centres for Disease Control and Prevention (CDC). Of these7, 096 were diagnosed with neuroinvasive disease and 9,268 with West Nile fever (Hayes *et al.,* 2005; Rondini *et al.,* 2008).

The WNV is considerably one of the most widespread groups of arboviruses and is widely distributed in Africa, Asia America and Europe (Burt *et al.,* 2002; Balasuriya *et al.,* 2006). This arthropod-borne virus has managed to cross geographical barriers and even become established in non-endemic areas resulting to epidemics in both humans and animals (Mackenzie *et al.,* 2004; Hayes *et al.,* 2005). This is attributed to a number of factors which include; favourable ecological conditions, diversity of the host and

vectors, successful adaptations of the vectors/pathogens to new ecosystems and increased geographic range (Burt *et al.,* 2002, Mackenzie *et al.,* 2004). A good example of this is the the outbreak experienced in the United States of America (USA) where the virus became established and rapidly spread within a period of five years (Hayes *et al.,* 2005).

Natural factors as well as human activities have contributed to the spread and establishment of this virus in both endemic and non-endemic regions (Mackenzie *et al.,* 2004). Environmental factors such global warming and some human activities like deforestation enhance vector population densities (Epstein, 2001; Hubalek *et al.,* 1999). It is widely acknowledged that climate has a significant impact on the distribution of infectious diseases. Global warming and the accompanying change in weather patterns; including increased variability in climate is very favourable to the proliferation of the mosquito population and the subsequent transmission of infectious diseases, such as WNV (Epstein, 2001; Elliot *et al.,* 2003).

High temperatures have been shown to speed up the replication of WNV in mosquitoes, this rapid amplification directly affects the likelihood of the mosquito reaching maturity and subsequently infecting other hosts (Epstein, 2001). The build-up of the effects of climate on WNV vectors function to enhance the probability of vector mosquitoes and animals reservoirs living in close proximity to human populations. As such, there are variations in WNV outbreaks in animals and humans that seemingly correspond with climatic conditions. Furthermore, the recent outbreaks of WNV in Romania, Bucharest,

New York and Russia occurred following extended periods of drought (Epstein & Defilippo, 2001). A study carried out in South Africa showed that large epidemics usually occurred when high rainfall or hot weather favoured breeding of mosquito vectors (Burt *et al.,* 2002). Deforestation on the other hand creates changes in the environment which establish pressure on established ecosystem which can create conditions that favour the emergence of arboviruses (Burt *et al.,* 2002).

The virus has been restricted in range geographically by the availability of invertebrate and vertebrate hosts. However, modern transportation has introduced vectors that efficiently transmit arboviruses into new areas (Hayes *et al.,* 2005). The WNV strain circulating in America was found to be more closely related to the strain isolated from geese in Egypt with 99.7% homology in nucleotide sequence (Hayes *et al.,* 2005). This suggests that the virus was imported into America from the Middle East by an infected bird, mosquito or human (Hayes *et al.,* 2005). Similarly, a phylogenetic analysis of the complete amino acid sequence of the viral envelope glycoprotein of a Kenyan isolate done in 1998 demonstrated a sister relationship with a *Culex pipiens* mosquito isolate from Romania (Miller *et al.,* 2000). In many areas, pre-existing populations of competent vectors set the stage for successful establishment of viruses in new regions (Mackenzie *et al.,* 2004). A study done in South Africa showed that WNV was endemic in areas where the principal vector *Culex univittatus* and avian host of the virus are present (Burt *et al.,* 2002).

The origin of the WNV and other arboviruses especially of the family Flaviviridae, bunyaviridae, togaviridae and alphaviridae is Africa (Kuniholm *et al.,* 2006). The burden of inter-epidemic disease, the epidemiology of infection, and principle reservoirs of viral maintenance are however not well understood (Kuniholm *et al.,* 2006). A lot of the misconceptions stems from the fact that these arboviruses are an important cause of acute febrile illness which is similar to infections caused by *Plasmodium* and *Salmonella* (Nur *et al.,* 1999*;* Pourrut *et al.,* 2010*).* This results to a difficult in clinically discriminating between these infections.

Despite its significance and increasing public health importance in humans globally, WNV still remains poorly understood, less well controlled and less documented. Data on WNV therefore in most parts of the world let alone Kenya is not readily available. Kenya has had multiple arbovirus outbreaks including yellow fever in 1992 (Sanders *et al.,* 1998), chikungunya in 2004 (Sergon *et al.,* 2008) and Rift Valley fever in 2006 (Centre for Disease Control and Prevention 2007). Much remains unknown about the true seroprevalence of arboviruses in Kenya and the risk responsible for exposure and maintenance and transmission of these viruses (La Beaud *et al.,* 2011). In most areas in Kenya studies on arboviruses have not been carried out, however a few studies have shown that there is evidence of WNV activity in Kenya. A serosurvey conducted at the Kenyan coast in 1987 suggested that there is evidence of WNV at the Kenyan coast (Morill *et al.,* 1991). Other entomologic studies have shown that mosquitoes responsible for WNV transmission are common in Kenya (Sergon *et al.,* 2008).

This includes the isolation of WNV from the mosquitoes of the *Culex* species in Rift valley province along the Kenya-Uganda border (Miller *et al.,* 2000) and in North Eastern province (LaBeaud *et al.,* 2011).

1.2   Problem statement

The West Nile Virus has its origin in Africa (Smithburn *et al.,* 1940; Kuniholm *et al.,* 2006). It is an accurate cause of febrile illness in Kenya. Due to the fact that the febrile illness caused by arboviruses present is similar to those caused by Plasmodium and Salmonella, it is likely that WNV could be easily misdiagnosed in the country. Yet the prevalence for WNV in Kenya has never been done. This shortcoming has partially contributed to the lack of targeted preventative public health interventions. The emergence and re-emergence of certain arboviruses has become a significant public health problem, and due to the morbidity and mortality burden involved outbreaks are best identified before they occur. Regular sero-surveys are therefore an exercise long overdue. The WNV manifests clinically as a febrile illness, most of the places where this disease occur is passed off as another common disease like malaria or typhoid, the majority of the infection therefore goes undiagnosed and the frequency of the disease and the magnitude of the public health threat they pose is greatly underestimated.

1.3   Justification

West Nile Virus is a neurotropic flavivirus of public health importance (Mackenzie *et al.,* 2004). The virus has potential to cause epidemics due to the favourable environmental factors that favour the multiplication and survival of their primary

vectors. Data on incidence and prevalence of WNV is not readily available in most parts of the world. In Kenya, there is scanty data generally on arboviruses let alone WNV. Similarly serological surveillance on WNV exposure has not been regularly conducted in Kenya. However data published in some parts of the world has shown the public health risks associated with WNV outbreaks, for instance the outbreaks in Israel, South Africa and New York (Komar, 1999; Petersen *et al.,* 2001; Burt *et al.,* 2002; Hayes *et al.,* 2005). Globally WNV is one of the re-emerging infectious flavivirus causing epizootics and human outbreaks. This virus has the widest distribution of all flaviviruses (Mackenzie *et al.,* 2004). With these factors in mind, Serological surveillance for early detection of the virus will provide information beforehand for the necessary and appropriate interventions to be undertaken in case of disease outbreaks. The information obtained from this study will therefore be important in determining the seroprevalence of WNV in Trans Nzoia and equip the public health department with facts that will determine interventions. This can therefore act as tool in preventing and controlling WNV epidemics, establishing appropriate interventions to minimize the impact of the disease. It will also help to determine the importance of WNV as a cause of febrile illness in Trans Nzoia District and also act as a platform for any other future studies in this field.

1.4   Hypothesis

1.4.1   **Null hypothesis**

The WNV is not a significant cause of febrile illness in Trans Nzoia.

1.5   Objectives

1.5.1   **General objective**

To investigate West Nile Virus infection, distribution and seroprevalence in febrile patients visiting selected health facilities in the Trans Nzoia District, Kenya.

1.5.2   **Specific objectives**

1. To screen for antibodies against West Nile Virus in serum samples of febrile patients attending heath facilities in Trans Nzoia.

2. To determine the presence of West Nile virus and compare patients' clinical symptoms to laboratory findings.

3. To determine the distribution of West Nile virus in relation to age, sex and location of study participants in Trans Nzoia.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Arboviruses

Arboviruses are viruses that are transmitted to vertebrates by arthropods (Huang *et al.,* 2002; Buckley *et al.,* 2006). West Nile virus (WNV) is an arbovirus that belongs to the genus *flavivirus* within the family *Flaviviridae* which comprises over 70 viruses that are of economic importance to man (Shi *et al.,* 2001; Burt *et al.,* 2002; Hogrefe *et al.,* 2005; Tonry *et al.,* 2005). The WNV belongs to the Japanese Encephalitis serocomplex (JE that includes Japanese encephalitis (JE) Kokobera, Usutu, Koutango, Kunjin, Murray Valley encephalitis and St. Louis encephalitis (SLE) viruses (Hubalek *et al.,* 1999; Shi *et al.,* 2001; Hogrefe *et al.,* 2005). Other flaviviruses that are closely related to WNV and are significant human pathogens are Yellow Fever Virus (YFV) and four serotypes of Dengue Virus (Shi *et al.,* 2001; Hayes *et al.,* 2005; Hogrefe *et al.,* 2005).

2.2 The West Nile Virus structure and genome

The virus is an enveloped, spherical virus 40-50 nm in diameter with a single stranded positive sense 10.5 kb RNA. The genome enclosed in a 30-35 nm icosahedra core composed of multiple copies of capsid protein (**Figure 2.1**). The genomic RNA consists of the 5' and the 3' untranslated region (UTR) at the termini which are sequences that don not code for viral proteins, which has a 5' cap but it is not polyadenylated (Petersen *et al.,* 2001; Shi *et al.,* 2001). The single stranded RNA serves as m RNA

and is transcribed as a single polyprotein that is cleaved by host and viral proteases into three structural proteins; the capsid (C), pre-membrane (prM) and the envelope (E) protein and seven non-structural proteins; NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5S (**Figure 2.2**) (Petersen *et al.,* 2001; shi *et al.,* 2001; Burt *et al.,* 2006; Samuel *et al.,* 2006).

The capsid protein binds viral RNA, the prM blocks premature viral fusion ate in virus maturation, the prM protein is cleaved to membrane (M) protein by a cellular protease, and the M protein is incorporated into the mature virion) it also serves as a chaperone for the E glycoprotein during virion morphogenesis (Petersen *et al.,* 2001). The E glycoprotein mediates virus attachment to the host cell receptors, membrane fusion and viral assembly; it is the most immunologically important viral protein in regard to viral infectivity and, therefore, elicits essentially all virus-specific neutralizing antibodies (Petersen *et al.,* 2001). The viral non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5) regulate viral transcription and replication and attenuate host antiviral responses (Petersen *et al.,* 2001; shi *et al.,* 2001; Samuel *et al.,* 2006; Burt *et al.,* 2006).
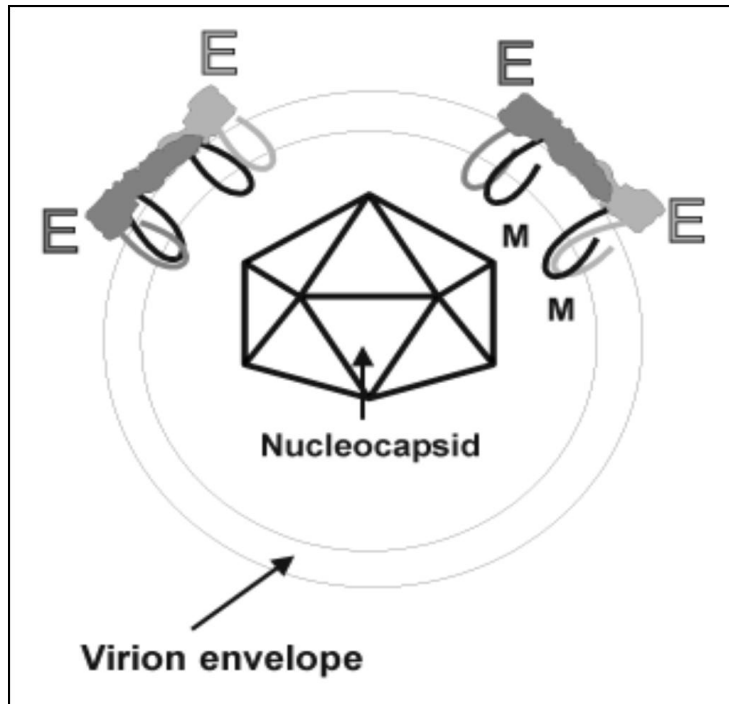
Figure 2.1: Diagram of the Flavivirus virion (Source: Petersen *et al.,* 2001)
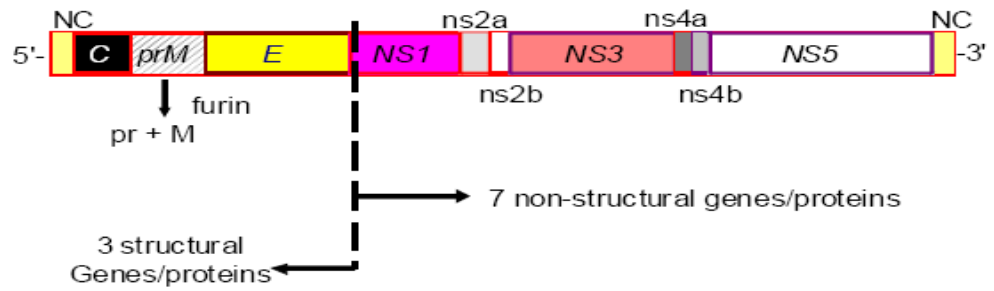
Figure 2.2: Organization of the flaviviral genome (Source: WHO/ Immunization, Vaccine and Biologicals, 2007)

2.3   The West Nile Virus transmission

The WNV is primarily transmitted by a relatively large number of mosquito vectors of the *Culex* species that acquire the virus by feeding on infected birds (Schneider *et al.,* 2007). Birds are the natural reservoir hosts (The Centre for Food Security and Public Health, 2009). The WNV circulation is confined to two basic types of cycles and ecosystems. The rural (sylvatic) cycle which encompasses wild, usually wetland birds and ornithophilic mosquitoes and the urban cycle which comprises of synanthropic or domestic birds and mosquitoes feeding on both birds and humans, mainly *Culex. pipiens/molestus* (Hubalek *et al.,* 1999; Calistri *et al.,* 2003).

The virus multiplies in the mosquito vector and after an incubation period of two days the vector becomes infective for active transmission to a susceptible host (Indian Council of Medical Research, 2002). Transmission occurs when the infected mosquito bites and infects birds; the birds then amplify the virus to efficient viral levels to transmit to other birds, humans and animals (Komar, 1999; Wong *et al.,* 2004). Humans, horses and other mammals are usually "dead-end" hosts, because they do not produce sufficient viraemia to contribute to the transmission cycle (Brown *et al.,* 2008; The Centre for Food Security and Public Health, 2009). Humans however, may transmit or acquire virus in utero, through breast milk, via blood transfusion or organ transplantation, or through occupational exposure (Gould and Fikring, 2004).
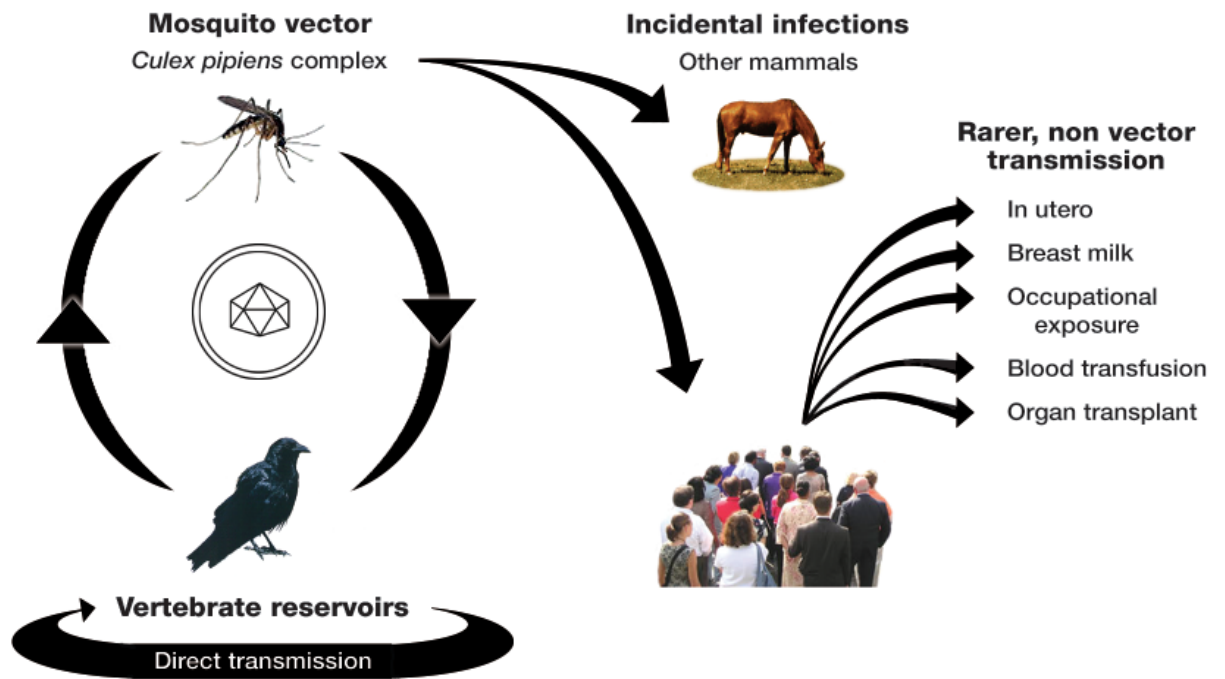
Figure 2.3: West Nile Virus transmission cycle (Source: Gould and Fikring, 2004)

Transmission in humans is highly influenced by human behaviour like migration that influence exposure to mosquito vectors and abundance and feeding patterns of competent mosquitoes (Hayes *et al.,* 2005). Migrating birds also carry the virus into new geographic areas. Human infections are high in areas where ecological factors like high rainfall and hot weather favour the breeding of the mosquito vectors and high viral amplification of the virus, significant number mosquitoes that feed on both mammals and birds ("bridge mosquitoes") become infected and spread the virus to other hosts like humans and horses (Burt *et al.,* 2002; Brown *et al.,* 2008).

Humans often become infected via mosquito bites as an infectious mosquito probes the skin for blood, depositing saliva and virus in extravascular tissue and circulatory system (Hubalek *et al.,* 1999; Hayes *et al.,* 2005). The virus replicates at the site of inoculation in the skin Langerhans and dendritic cells; primary viraemia then occurs as the virus disseminates into the lymph nodes, this then leads to infection of peripheral tissues (Hayes *et al.,* 2005; Samuel *et al.,* 2006). Stimulation of toll like receptors and increased level of TNF-α increases the permeability of the blood brain barrier leading to the viral penetration of the CNS (Hayes *et al.,* 2005). Studies carried out shows persistent infection of the CNS in patients with viraemia for more than 60 days (Hayes *et al.,* 2005). The virus traverses the blood-brain barrier and infects the brain parenchyma, clinically manifesting as viral encephalitis, the virus may also affect the leptomeninges, resulting in a clinical presentation of aseptic meningitis (viral meningitis) (Hayes *et al.,* 2005). Patients with West Nile encephalitis may present with features of both encephalitis and aseptic meningitis (meningoencephalitis) (Hubalek *et al.,* 1999; Hayes *et al.,* 2005; Samuel *et al.,* 2006).

2.4   The epidemiology of the West Nile Virus

The WNV is widely distributed in Europe, the Middle East, Africa, parts of Asia, Australia in the form of Kunjin virus, a subtype of WNV, North America, and parts of Central America and the Caribbean (shi *et al.,* 2001; Burt *et al.,* 2002; Rondini *et al.,* 2008). This makes WNV one of the most wide spread member of the Japanese Encephalitis Virus Complex **(Figure 2.4)**.
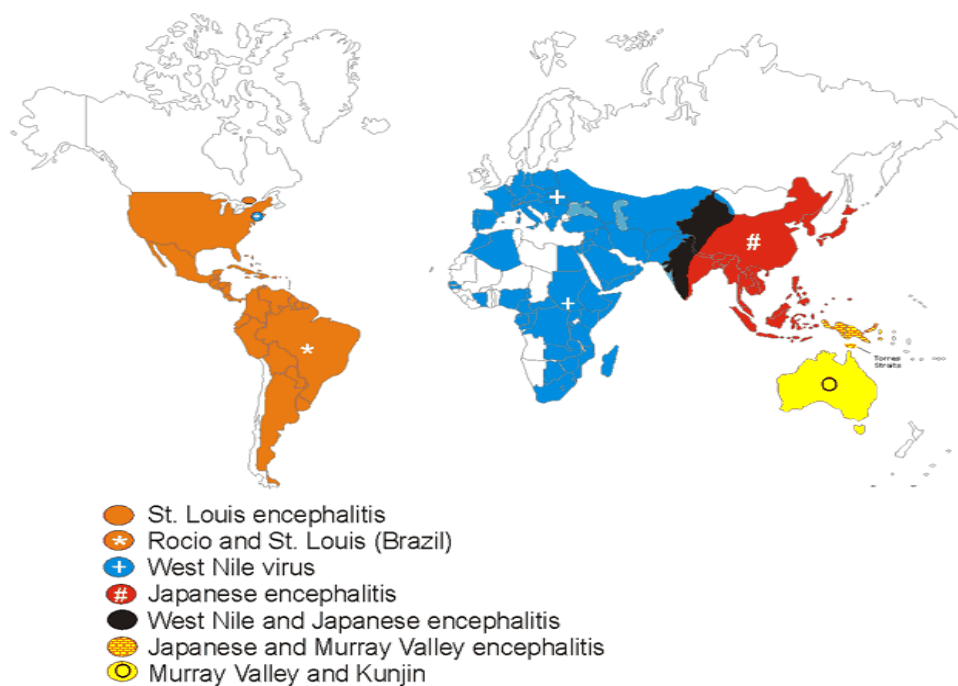
Figure 2.4: Geographical distribution of the Japanese encephalitis virus serocomplex (Source: CDC 2000)

Phylogenetic studies have identified that WNV consists of at least two separate genetic lineages or strains, lineage 1 which is divided into three clades 1a, 1b and 1c and lineage 2 (Komar, 1999; Shi *et al.,* 2001; Hayes *et al.,* 2005). Lineage 1 consists of strains from Asia, North America, Australia, Middle East, Europe and some African strains **(Figure 2.5)**. Clade 1a is widespread and most of the virulent viruses in recent outbreaks belong to this clade, its predominant in the U.S and Canada **(Figure 2.5)**. Clade 1b consists of Kunjin virus predominant in Australia while clade 1c consists of viruses from India. Lineage 2 remains as an African strain **(Figure 2.5)**.

Only members of lineage 1 viruses have been associated with clinical human encephalitis, lineage 2 viruses consist of attenuated and virulent viruses (Komar, 1999; Burt *et al.,* 2002; Rondini *et al.,* 2008). Among lineage 1 WN viruses, the viruses causing the recent human and equine outbreaks throughout Europe and Asia have been most closely related to a WNV first isolated in Romania in 1996 (ROM96) and subsequently in Kenya in 1998 (Hayes *et al.,* 2005). In addition to this diversity, new strains of WNV are being recognized other than the two existing lineage 1 and 2 strains, a new lineage 2 strain was recently reported in Central Europe, Czech Republic, Russia and Malaysia (Rondini *et al.,* 2008).
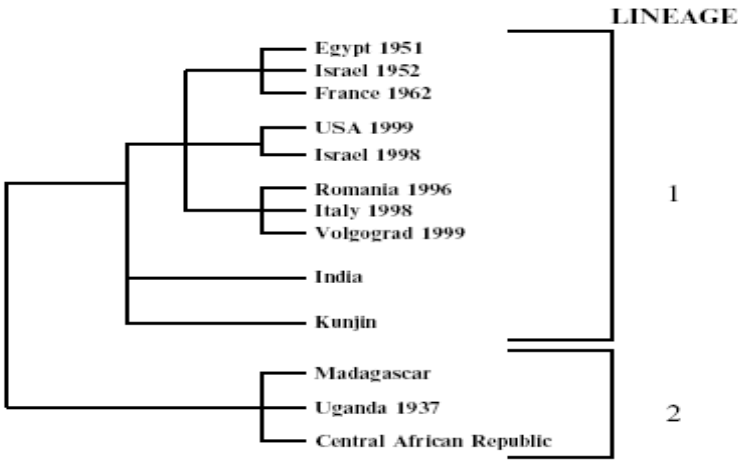
Figure 2.5: Simplified representation of a Phylogenetic tree showing the genetic relatedness of different strains of WNV, including separation into two distinct lineages (Source: Komar, 1999)

2.5 Clinical signs associated with West Nile virus Infection

The incubation period for WNV is 2 to 14 days after which clinical signs start to manifest (Hayes *et al.,* 2005). However about 80% of the human infections are asymptomatic (Hayes *et al.,* 2005). The WNV infection is classified into; West Nile Fever (WNF) and West Nile Neuroinvasive Disease (WNND)/West Nile Encephalitis (WNE) (Hubalek *et al.,* 1999; Hayes *et al.,* 2005; Samuel *et al.,* 2006).

West Nile Fever is characterized by fever, headache, general malaise, lymphadenopathy, body aches and muscle pains, anorexia, nausea, diarrhoea, vomiting, a transient macular rash and gastrointestinal symptoms. Most uncomplicated forms are resolved within six days (Hubalek *et al.,* 1999; Samuel *et al.,* 2006). A recent follow-up study of WNF patients who sought medical attention found that difficulty concentrating and neck pain or stiffness were also prominent symptoms, and that fatigue and muscle weakness frequently lasted for approximately one month after onset (Hayes *et al.,* 2005). Of the 98 patients interviewed, 31% were hospitalized, 79% missed school or work because of their illness, and the median time before patients felt fully recovered was 60 days (Hayes *et al.,* 2005). This shows that even without neurologic manifestations, WNV infection clearly can cause a notable public health problem.

Only 2% of Patients with WNF proceed to the severe form of WNV infection, West Nile Neuroinvasive Disease or West Nile Encephalitis which in some cases is life threatening (Hubalek *et al.,* 1999; Hayes *et al.,* 2005). This form is characterized by three

syndromes; encephalitis, meningitis and acute flaccid paralysis. Headache, fever, stiff neck and photophobia characterize meningitis (Hayes *et al.,* 2005). Encephalitis on the other hand is characterized by in coordination tremors, ataxia, symptoms that resemble Parkinson's disease tremors and involuntary movement. Seizures and comma may occur. Patients who recover have persistent neurological dysfunction (Hayes *et al.,* 2005). Patients with acute flaccid paralysis present with paralysis which is similar to polio which appears suddenly and progresses rapidly (Hayes *et al.,* 2005). It may affect more than one limb which darkens at the peak of paralysis. This syndrome is also accompanied by muscle aches in the lower back, bowel and bladder dysfunction (Hayes *et al.,* 2005).

The death rates among patients with neuroinvasive disease in recent epidemics have averaged to 10% (Tonry *et al.,* 2005). A study carried out in the United States of America by Hayes *et al.,* 2005 showed that persons of all ages are susceptible to WNV infection, but the incidence of neuroinvasive disease and death increases by age and is slightly higher in males (Hayes *et al.,* 2005). The effects of WNV infection can be very devastating in case of an outbreak; this is evident from the 1999 trough 2004 outbreak in the United States of America (Hayes *et al.,* 2005). **Table 2.1** shows Neuroinvasive cases and West Nile fever cases reported to the CDC between 1999 and 2004.

Table 2.1: Human WNV disease cases by clinical syndrome in the United States, 1999-2004. (Hayes et al., 2005)

Year	Total cases	Neuroinvasive cases	West Nile fever	Other clinical/ unspecified cases	Deaths
1999	62	59	3	0	7
2000	21	19	2	0	2
2001	66	64	2	0	9
2002	4,156	2,946	1,162	48	284
2003	9,862	2,866	6,830	166	264
2004	2,539	1,142	1,269	128	100
Total	**16,706**	**7,096**	**9,268**	**342**	**666**

2.6   Diagnosis of West Nile Virus

Serological tests are often used for WNV diagnosis (Wong *et al.,* 2004; Balasuriya *et al.,* 2006). Presence of WNV specific immunoglobulin, IgM in serum or CSF indicates recent infection. Anti-WNV IgM may however persist in the serum for over six months (Hogrefe *et al.,* 2004; WHO, 2007). This is therefore used as the basis for serological testing.

ELISA is used to detect the presence of IgM, IgG antibodies, but this is not a definitive method of diagnosis due to cross reactivity with other related flavivirus (Hayes *et al.,*

2005; Balasuriya *et al.,* 2006; WHO, 2007*)*. All IgM/IgG reactive serum is usually further tested with the plaque reduction neutralization test (PRNT) which is the gold standard (Balasuriya *et al.,* 2006). This test detects the presence of antibodies against the NS1 protein of the virus (Buckley *et al.,* 2006). This test can therefore be used to detect presence of WNV, to confirm positive ELISA results and it may also help distinguish serologic cross-reactions among the flaviviruses; this is because the test measures the biological parameter of *in vitro* virus neutralization (Wong *et al.,* 2004; Hayes *et al.,* 2005; WHO, 2007). Virus detection by nucleic acid amplification tests can also be used for WNV diagnosis and they include reverse transcriptase-polymerase chain reaction (RT-PCR) and loop mediated isothermal amplification (LAMP) (Hayes *et al.,* 2005).

2.7   Treatment of WNV infections

Currently there is no specific and effective therapy available therefore management of WNV illness remains supportive (Hayes *et al.,* 2005; The Centre for Food Security and Public Health, 2009). However, various therapies are undergoing clinical trials and they include; Ribavirin, interferon-α, WNV-specific immunoglobulin, and antisense gene–targeted compounds have all been considered as specific treatments for WNV disease, but no rigorously conducted clinical trials have been completed (Hayes *et al.,* 2005; The Centre for Food Security and Public Health, 2009). Only two vaccines are available for vaccinating equines: an inactivated WNV vaccine and a recombinant vaccine that uses canary pox virus to express WNV antigens (Hayes *et al.,* 2005). An inactivated vaccine is also being studied for use in humans (Hayes *et al.,* 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

Trans-Nzoia District is one of the administrative districts of Rift Valley Province in Kenya. Parts of the district are heavily forested, with significant human activity in and around the forests; the main agricultural activity carried out in this region is large scale farming. Serum samples were collected from three sites; Kitale District Hospital, Andersen Medical Clinic and Endebess Sub-District Hospital. Kitale District Hospital is located in Kitale town which is an agricultural town situated between Mount Elgon and the Cherengani Hills. It is the administrative centre of the Trans-Nzoia District of Rift Valley Province. Kitale District Hospital serves the whole of Trans Nzoia. Andersen Medical Clinic is a private health facility run by Mt Elgon Orchards; it is located at the border between Kenya and Uganda and serves communities from both countries. Endebess Sub-District Hospital is located in Endebess which is both an administrative division and a small town that houses the headquarters for the division in Trans-Nzoia District. Endebess is one of the towns nearest to Mount Elgon

3.2 Study population

The population in these areas is mainly rural. Many people are peasant farmers and herdsmen going into forests and woodland from time to time to graze cattle or women

who collect firewood. Patients resided in all areas of the districts served by these health facilities.

3.3   Inclusion and exclusion criteria

Only consenting, febrile patients above five years of age were included in the study. Guardians of children below 18 years provided consent on behalf of the children. Non-consenting and non-febrile patients below five years of age were excluded from the study.

3.4   Sample size determination

The sample size was determined using Fisher's exact test (Fisher *et al.,* 1985).

It has been documented that the seroprevalence of some arboviruses ranges from 0-3% (Morill *et al.,* 1991). Therefore assuming that the seroprevalence of WNV antibodies will be up to 3 %; by substituting the formula (N= $(z^2 \text{xpq})/d^2$

Where;

N=Desired sample size, Z =Standard normal deviation at the required confidence interval (95%), p= proportion in the target population which is 3% (Morill *et al.,* 1991), q=1-p, d= level of statistical significance at 95% confidence level.

$$\frac{1.96^2 \times 0.03(1-0.03)}{0.05^2}$$

=44.7

The study was conducted in three sites therefore (44.7x3) hence the total number of samples was 134.

This was the minimum number of samples required for the study however with the assumption that the seroprevalence would not be more than 3% it was determined that a sample size of 1000 would be enough to achieve the true seroprevalence. When adjusted by 15 % for haemolysed samples difficult to process, the sample size becomes 1150.

3.5   Sample collection, storage and transportation

Two to five millilitres of venous blood was collected in plastic vacutainer serum tubes (BD Vacutainer$^®$ Plastic and SST^{TM} Tubes). The whole blood was centrifuged for five minutes at 3000 rpm. The gel in the tubes formed a physical barrier between the serum and the red blood cells during centrifugation. The sera were stored at -20°C in Kitale District Hospital, Andersen Medical Clinic and Endebess Sub-District Hospital. Once every month, the vacutainer tubes containing the sera were transported in dry ice to KEMRI laboratories in the Centre for Microbiology Research- Biosafety Level III (CMR-P3) laboratory. The sera in the vacutainer tubes was then carefully aliquoted into cryovials and stored at -80°C for further processing.

3.6   Experimental methods

3.6.1   Cell cultures

For this work, Vero cells from the African green monkey kidney cell line and c6/36 cells from mosquito cell lines were used (Igarashi, 2000). Vero cells were cultured for use in

plaque assay and Plaque Reduction Neutralization Test (PRNT). The c6/36 cells on the other hand were cultured for use in virus propagation and virus isolation. Both cells lines were maintained in Growth Medium (GM) containing 1x Minimum Essential Medium (MEM), 5% Foetal Calf Serum (FCS), 7.5% sodium bicarbonate and 1 % L-glutamine /Penicillin/streptomycin. Vero cells were cultured in tightly capped rectangular canted neck cell T75 culture flasks with a vented cap (Corning[®] CellBIND[®] 75 cm²) at 37°C/ 5% CO_2. C6/36 cells were cultured in tightly capped rectangular canted neck cell T75 culture flasks with a vented cap (Corning[®] CellBIND[®] 75 cm²) at 28°C. The cells were split every 48-72 hours with trypsin-EDTA for to prevent overgrowth. This was when the appearance of the cells when (80-90% confluent) coincided with the normal morphology of the cell line. The cells were considered clean/good when no bacterial, fungal or even viral contamination was found in the monolayer.

3.6.2   **Large scale cell culture and virus propagation of West Nile virus**

This assay was adapted from the Igarashi manual for arboviruses (Igarashi, 2000). Cells from the mosquito cell line C6/36 were propagated in four T150 cm^2 culture flasks with a vented cap (Corning[®] CellBIND[®] 150 cm²) at 28°C with growth medium (Minimum Essential Medium (MEM), 5% Foetal Calf Serum). All the cell suspensions were transferred from the culture flasks to a one litre spinner bottle and 500 ml of GM added to the cell suspension. The cells were cultured on a low speed magnetic stirrer (high humidity model) at a speed of 20 rpm at 28°C for 3days, this is important for the proper circulation of air to the cells. The number of cells in the spinner bottle were counted in

order to determine the cell concentration (a cell concentration of 1.0×10^6 cells/ml is recommended) for the addition of cytodex. Cytodex are small glass beads that aid to increases the number of cells by increasing the surface area of the cells cultured. An aliquot of 100 ml of cytodex-normal saline was added into the C6/36 cell suspension. An aliquot (100 µl) of the cell suspension was observed under inverted microscope to determine that 50 to 100 % of the cytodex was covered with cells. The magnetic stirrer was stopped for a while to let the cytodex with attached cells settle down. Spent medium was gently discarded from the spinner bottle.

An aliquot of 100 ml of WNV (NY99 vaccine strain) infected C6/36 cells was added into the spinner bottle. The cells were then incubated at 28°C for 2 hours gently mixing every 20 minutes. An aliquot of 800ml of maintenance medium (Minimum Essential Medium (MEM), 5% Foetal Calf Serum) was then added. The cells were then cultured at a speed of 20 rpm at 28°C for 5 days while monitoring the virus titre regularly before harvest.

On the day of virus harvesting, the infected culture fluid (ICF) was gently filtered in a biosafety cabinet using a ten diameter funnel and Watman filter paper to eliminate the dead cells and cytodex from the ICF. The filtered ICF was then centrifuged at 8,000 rpm for 30minutes at 4°C. All supernatant was pooled in one glass jar. A total volume of 22.2g of NaCl and 60g of PEG6000 (Polyethylene Glycol) per I litre of ICF was added into the jar containing the ICF and the suspension stirred using a low speed magnetic

stirrer (high humidity model) at 20 rpm overnight at 4^oC. The ICF was then centrifuged at 8,000 rpm for 30 minutes at 4^oC. The supernatant was discarded and the faint precipitate re-suspended on the surface centrifuge tubes with Sodium Chloride – Tris – EDTA (STE) buffer. The re-suspended virus precipitate was centrifuged at 10,000 rpm for 20 minutes at 4^oC and the supernatant harvested. The precipitate was further re-suspended with another 18 ml of STE buffer. Both the supernatant and the precipitate were layered in separate tubes onto 15–60% (w/w) sucrose gradients prepared in Tris-EDTA buffer pH7.4. The gradients were centrifuged at 20,000 rpm for 14 hours at 4^oC. The tubes were then fractionated by upward displacement using a fractionating column. The flow rate of the samples was kept at 100 ml/minutes. The protein concentration of the virus was then measured using a spectrophotometer. The entire protocol is summarised in **figure 3.1** below.
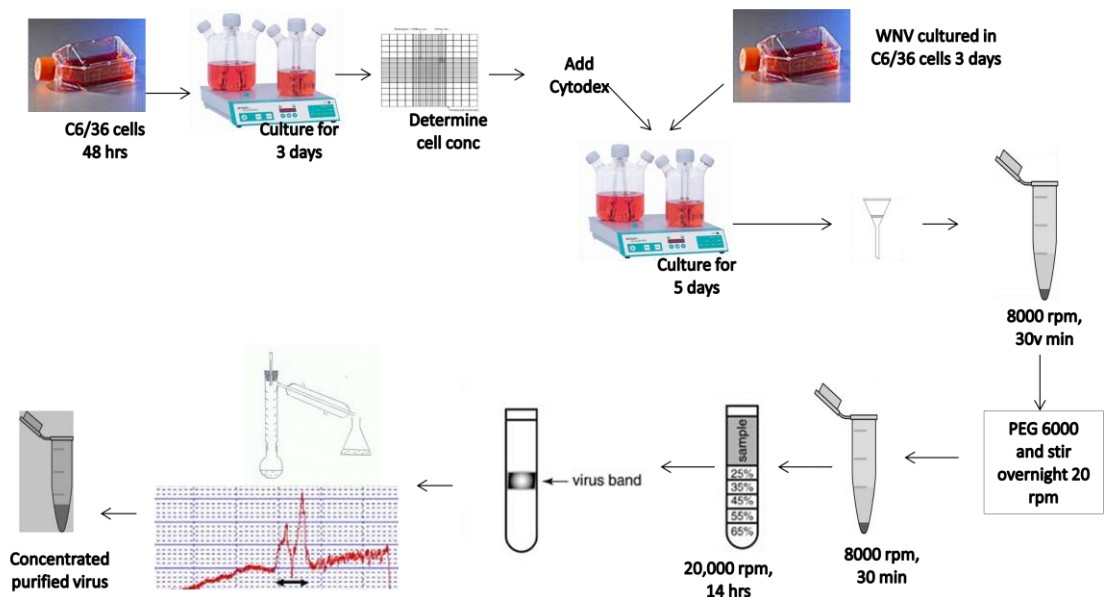
3.1: Large scale cell culture and purification of WNV

3.6.3 Indirect IgG ELISA

The assay developed for this survey uses purified WNV propagated locally in a standard indirect ELISA (Igarashi, 2000). Sera collected from the field were tested according to well-established and optimized protocols as outlined in **3.6.4**.

3.6.4 Optimization of ELISA

The virus used for this protocol was cultured using Vero cells and purified by sucrose density gradient ultra-centrifugation (Igarashi, 2000). Protein concentration of the purified WNV antigen was determined as 0.55 mg/ml. Vials containing purified whole WNV were stored at -80°C and thawed when needed.

Suitable concentration of antigen in a coating solution and corresponding serum and secondary antibody dilution for this study were established using check board titrations. The Virus solution (0.55 mg/ml) was re-suspended in 1xPBS. Serial dilutions of 1mg/well (1 mg/100ml) to 250 ng/well were made. An aliquot of 100 µl of the antigen was dispensed in each well in rows A, B, C, D, E and F of a 96-well poly-styrene flat bottomed clear ELISA plates (Nunc-Immuno 96 Microwell ^{TM} Solid Plates) as illustrated in **Figure 3.2**. An aliquot of 1x PBS was dispensed in all wells in rows G and H as illustrated in **Figure 3.2**. The plates were then incubated at 4°C overnight. Three plates were coated. The virus solution was then discarded from the plates by tapping on absorbent paper towels and the plates washed 3 times with 200µl /well PBS, 0.05% tween-20 (PBS-T) by an ELISA washer. All the wells were blocked by adding 100 µl of

the blocking solutions PBS- 3% FCS diluted in 0.05% Tween-20 into every well of the first plate, the second plate was blocked using blockace and the third plate was blocked using skimmed milk. All three plates were incubated for 30 minutes at room temperature. The blocking solution was then discarded from the plates by tapping.

The optimal sera concentration to be used for this protocol was also determined. Serial dilutions of the sera of 1:100 to a final dilution of 1:1000 using PBS- 3% FCS diluted in 0.05% Tween-20 was made. An aliquot of 100 µl of the sera was dispensed in the ELISA plate as shown in (**Figure 3.2**). The plate was then incubated at 37°C for one hour. The serum dilution was discarded from the plates by tapping and washed 3 times with 200µl/well of PBS-T with an ELISA washer.

The optimum working dilution for the secondary antibody, goat anti-human IgG+M+A antibody horse radish peroxide labelled (American Qualex, A139PN), was determined by re-suspending the secondary antibody with PBS- 3% FCS diluted in 0.05% Tween-20 starting with a dilution of 1:1000 to a final dilution of 1:16,000. An aliquot of 100 µl/well of the dilute antibody was then distributed as shown in (**Figure 3.2**). The plates were then incubated at 37°C for one hour. The antibody solution was discarded from the plates by tapping and washed 3 times with 200µl/well of PBS-T with an ELISA washer.
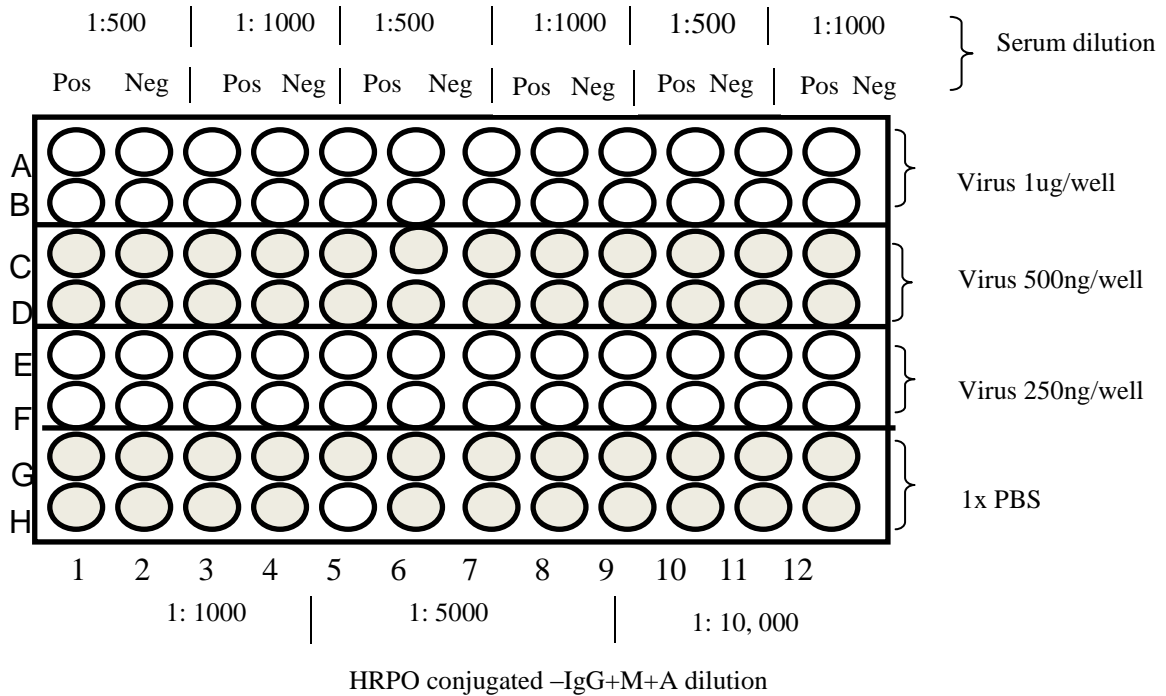
Figure 3.2: Check board ELISA for the determination of optimal dilutions of antigen, serum and HRPO-conjugate.

The substrate, sigma fast o-phenylenediamine (OPD) was prepared by dissolving 1 buffer tablet and 1 OPD tablet of the Sigma fast OPD into 20 ml of distilled and allowed to dissolve completely. To every well 100 μl/well of the substrate was then added and incubated at room temperature in the dark for 15 minutes. To stop the reaction 100 μl/well of 1 N Sulphuric acid (Stop solution) was added to each well. The ELISA reaction coloured product was then measured as absorbance with ELISA plate reader (Thermolabsystems Multiscan JX, China) at 492 nm using Ascent Software Version 2.6 (Thermo Scientific, Tokyo, Japan).

Optical density specifics for WNV were calculated as:

(Mean OD of virus coated wells) – (Mean OD of PBS-F coated wells).

The best dilution with the highest Positive/Negative (P/N) ratio and difference was selected for use.

3.6.5 Test procedure

Into each well of flat-bottomed 96-well ELISA plate 100µl of WNV antigen (Ag) solution diluted in PBS was added in rows A, B, E and F and incubated overnight in a - 4^0 C. 100µl of 1xPBS was then added into each well of flat-bottomed 96-well ELISA plate in rows C, D, G and H for background reaction to be subtracted later **(Figure 3.3)**. The plates were then wrapped in aluminium foil and incubated at 4°C overnight.

The virus solution was discarded from the plates by tapping and the plate washed three times with 200µl /well PBS-T by an ELISA washer. The plates were blocked by adding 100 µl of PBS- 3% FCS diluted in 0.05% Tween-20 (Blocking solution) into every well of the plate and incubated for 30 minutes at room temperature. The blocking solution was then discarded by tapping. Test and control sera were diluted to 1/1000 with PBS-3% FCS diluted in 0.05% Tween-20 and 100µl/well added in duplicate into antigen coated wells and also in 1xPBS coated wells. The plate was then incubated at 37°C for one hour. The serum dilution was discarded from the plates by tapping and washed three times with 200µl/well of PBS-T by an ELISA washer.
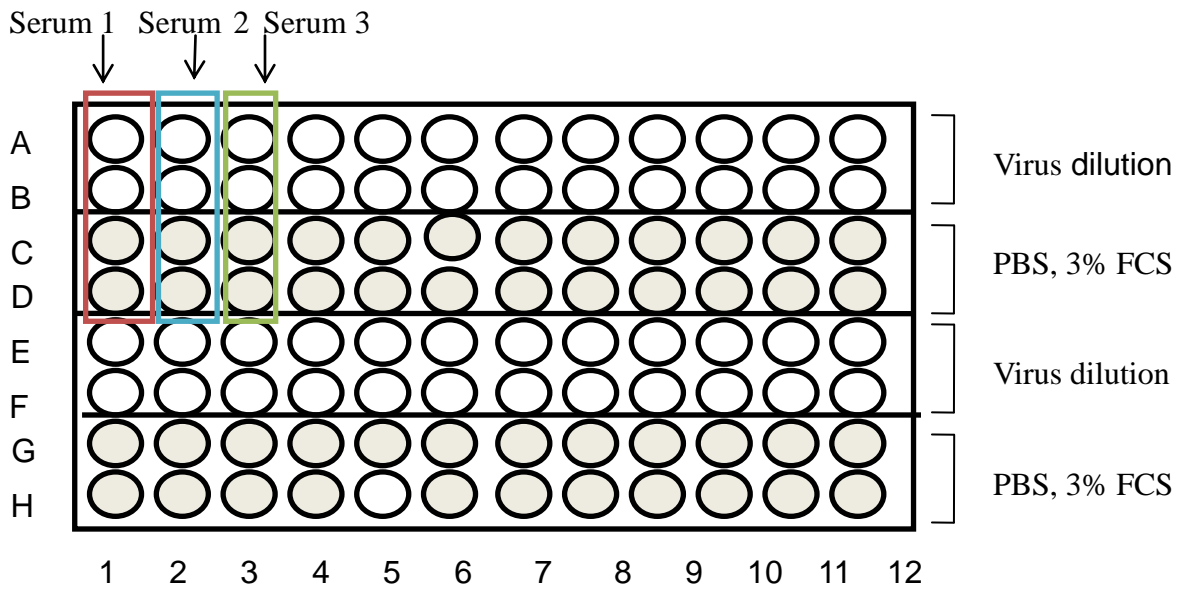
Figure 3.3: ELISA protocol for detecting antibodies against WNV.

The secondary antibody, goat anti-human IgG+M+A antibody horse radish peroxidase labelled, was diluted to 1/5000 with PBS- 3% FCS diluted in 0.05% Tween-20 and 100μl/well of the dilute antibody added into every well. The plates were then incubated at 37°C for one hour. The antibody solution was discarded from the plates by tapping and washed three times with 200μl/well of PBS-T by an ELISA washer.

The substrate, sigma fast OPD was prepared by dissolving one buffer tablet and one OPD tablet of the Sigma fast OPD into 20 ml of distilled and allowed to dissolve completely. To every well 100μl/well of the substrate was then added and incubated at room temperature in the dark for 15 minutes. To stop the reaction 100 μl/well of 1 N Sulphuric acid (Stop solution) was added to each well. The ELISA reaction coloured product was then measured as absorbance with ELISA plate reader (Thermolabsystems

Multiscan JX, China) at 492 nm using Ascent Software Version 2.6 (Thermo Scientific, Tokyo, Japan).

Optical density specifics for WNV were calculated as:

(Mean OD of virus coated wells) – (Mean OD of PBS-F coated wells)

If the specific OD reading exceeded 1.0, that serum was regarded as anti-WNV antibody positive as long as the positive control was above 1.0 and the negative control less than 1.0

3.6.6 Plaque assay

The plaque assay can be used to purify a clonal population of virus or to determine viral titre as plaque-forming units per ml (pfu/ml) so that known amounts of virus can be used to infect cells during subsequent work like the plaque reduction neutralization test (PRNT) (Igarashi, 2000).

Vero cells at a concentration 2×10^5 cells/ml were seeded into 6-well plates (Nunc) at a volume of 2 ml/well. Cells were cultured in Growth Medium (GM) for two days at 37°C, 5% CO_2. Only Vero cells that made an even monolayer (over 80% convergence) at the bottom of the 6-well plate were used to carry out the plaque assay. To perform the plaque assay, 10-fold dilutions of the virus stock (NY99 strain) were prepared in maintenance media (MM) - MEM, 2% FCS, P/S supplemented. Approximately 1 ml of culture medium from each well of 6-well plates was aspirated and discarded. Aliquots of

100 μl/well of the virus mixture was then inoculated onto susceptible cell monolayers in duplicate wells, and allowed to adsorb at 37^oC, 5% CO_2 by spreading inoculum every 30 minutes for 1.5 hours.

In order to localize the spread of the new viruses and restrict entry into neighbouring cells, 4 ml of overlay medium (EMEM, 1.5% FCS, 1.2% Methylcellulose, P/S supplemented) was added into each well, and the plates incubated at 37^oC, 5% CO_2 for 4-6 days. When the plates are incubated, the original infected cells release viral progeny. Consequently, the infection results to cytopathic effect in cells characterized by cell death. This leads to formation of a circular patch of dead cells called a plaque. Eventually the plaque became large enough to be visible to the naked eye. On the fourth day, the cells were fixed with 10% formaldehyde in PBS. Aliquots of 2ml of formaldehyde was poured over the overlay medium and incubated for 1 hour at room temperature. After one hour incubation the formaldehyde was dispensed and the wells rinsed with water. Staining solution (0.5 ml of 1% Crystal Violet solution in water) was used to stain each well for 10 minutes at room temperature, after which the dye was discarded, the plates were then rinsed with water and air-dried at room temperature. Dyes that stain living cells are often used to enhance the contrast between the living cells and the plaques.

The titre of the virus stock was then calculated in plaque-forming units (PFU) per millilitre. To determine the virus titre, the average number of plaques for each dilution was counted, only plates containing between 10 and 100 plaques were counted to

33

minimize error. The average was multiplied by the dilution factor and divided by the inoculum volume. The resulting value was taken to be infectivity by plaque forming unit/ml (PFU/Ml). The virus dilution with a virus titre of 2000 PFU/Ml was used for subsequent work in plaque reduction neutralization test (PRNT). The working dilution for PRNT was optimized to 1:400.

3.6.7 **Plaque Reduction Virus Neutralization Tests (PRNT)**

Plaque reduction Neutralization Test (PRNT) was used to determine the presence of WNV specific neutralizing antibodies in test sera already regarded as positive by ELISA (Igarashi 2000). Briefly, Vero cells at a concentration 2 x 10^5 cells/ml were seeded into 6-well plates (Nunc^{TM} Δ) at a volume of 2 ml/well. Cells were cultured in Growth Medium for two days at 37°C, 5% CO_2. Only Vero cells that made an even monolayer (over 80% convergence) at the bottom of the 6-well plate were used to carry out PRNT.

Sera was diluted with Maintenance Medium (MEM, 2% FCS, P/S supplemented) in 1:20 dilutions and then mixed with equal volume of standard virus solution of 2000 PFU/ml. For the positive control wells, equal volumes of MM and standard virus dilution were mixed. The virus-serum mixture was incubated for 1 hour at 37°C. To each well 100 μl of the mixture was then added to Vero cells in duplicate wells, and allowed to adsorb by spreading inocula every 30 minutes for 1.5 hours in the incubator. In order to minimize the spread of the new viruses and restrict entry into neighbouring cells, 4 ml of overlay

medium (EMEM, 1.5% FCS, 1.2% Methylcellulose, P/S supplemented) was added into each well, and the plates incubated at 37°C, 5% CO_2 for 4-6 days.

When the plates were incubated, the original infected cells released viral progeny. Consequently, each infectious particle produced a circular zone of infected cells called a plaque. Eventually the plaque became large enough to be visible to the naked eye. On the fourth day, 2 ml of 10% formaldehyde in PBS was poured over the overlay medium and incubated for 1 hour at room temperature, and the wells rinsed with water. Staining solution (0.5 ml of 1% Crystal Violet solution in water) was used to stain each well for 10 minutes at room temperature, after which the dye was discarded, the plates were then rinsed with water and air-dried at room temperature. The average number of plaques was counted for each serum; only wells with less than 200 plaques were counted to minimize error. The percentage plaque reduction was calculated for each serum sample was by using the number of plaques in the control as 100%. Those that reduced the number of plaques by $\geq$70% ($PRNT_{70}$) were further titrated.

3.6.8   Antibody titration

All the serum samples that reduced the number of plaques by $\geq$70% ($PRNT_{70}$) were titrated (Igarashi 2000). A twofold dilution of the serum samples was prepared starting with a dilution of 1:80 to a final dilution of 1:640 (as shown in **Figure 3.4)** and the procedure in **section 3.6.7** was repeated.
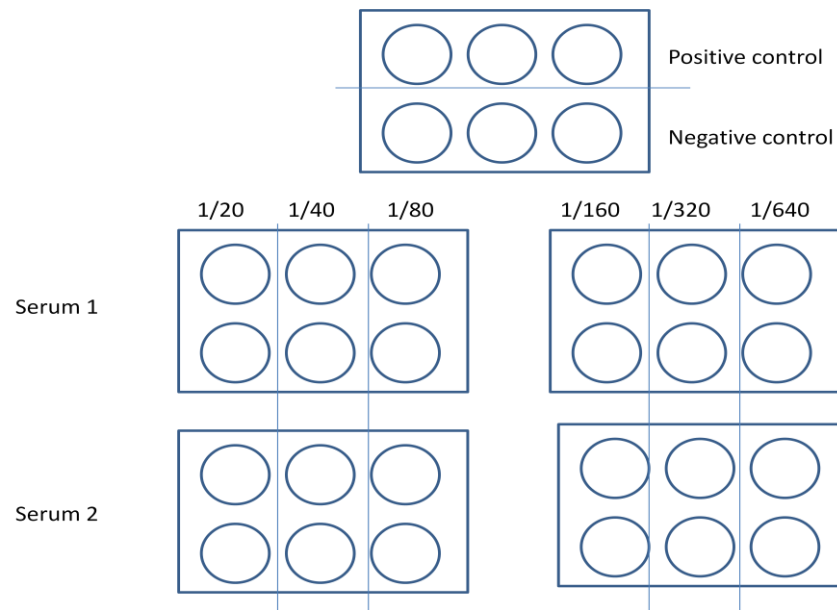
Figure 3.4: Antibody titration for the determination of end point titres for neutralised samples.

The number of plaques for each dilution was counted, 70% reduction of the number of plaques was the cut off of positivity in this assay. Those that reduced the number of plaques by $\geq$70% ($PRNT_{70}$) were confirmed positive. Antibody titters were expressed as the reciprocal of serum dilutions yielding $\geq$70% reduction in the number of plaques ($PRNT_{70}$). For etiologic diagnosis, the $PRNT_{70}$ antibody titre to the respective virus was required to be at least fourfold greater than that to the other flaviviruses tested.

3.6.9 Virus isolation

Mosquito cell lines C6/36 cells (1.0×10^5 cells/ ml) were cultured in T25 flasks with growth media (GM) (EMEM, 5% FCS, P/S supplemented) (Igarashi, 2000). The cells were incubated at 37°C for 2-3 days until they were 80 % confluent. Growth media was

then discarded from the flask and 2ml of maintenance media (MM) added into the flask to avoid drying up. An aliquot of 20 µl of serum sample was then inoculated and gently shaken. After 1hour incubation at 28°C the fluid (MM and serum) was discarded this is because the existence of human serum may damage the cells. An aliquot of 4ml of MM was then added into the flask and incubated at 28°C. The cells were observed for cytopathic effect (CPE) on the third day after which CPE and cell condition was observed for on daily basis under an inverted microscope until the seventh day. In the event that CPE appears the infected culture fluid (ICF) is to be collected and centrifuged at 3000 rpm and stored at -80°C after which virus in ICF can be detected by hemagglutination test, antigen detection ELISA, RT-PCR , neutralization test. The entire protocol is summarised in **figure 3.5.**

C6/36 cells
48 hrs

Inoculate with
patient's sera,
Culture for 5days

Observe for
CPE daily

Centrifuge
and
maintain
supernatant

Confirmatory test
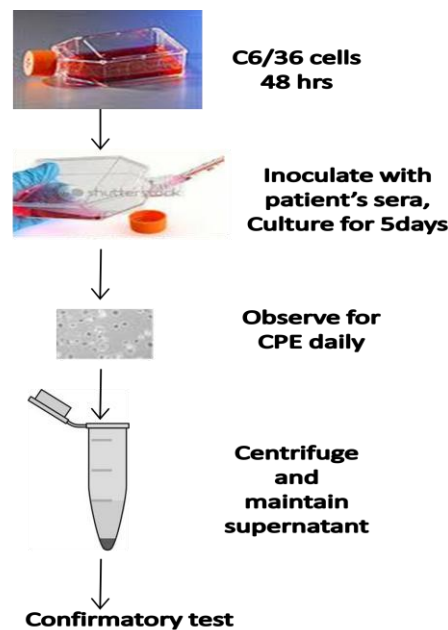
Figure 3.5: Virus isolation

3.6.10 **Interpretation of results**

Serum samples that were reactive or that showed evidence of antibodies against WNV on testing with Indirect IgG ELISA only, were considered to be either probably WNV seropositive or had a past Flavivirus infection/vaccination. A ruling on WNV seroconversion was only made if the serum sample was reactive on testing with both Indirect IgG ELISA and PRNT. The PRNT is done to confirm seropositive patients determined by ELISA, a confirmatory test is significant to rule out cross reactivity with other Flaviviruses.

Table 3.1: Interpretation of WNV serology results

Indirect IgG ELISA	PRNT	Interpretation of WNV results
Non-reactive	Not tested	**No serological evidence of past WNV infection**
Reactive	Non-reactive	**Possible past Flavivirus infection or vaccination** The IgG ELISA cannot differentiate between members of the Flavivirus Family.
Reactive	Reactive	**Evidence of past West Nile Virus infection** The West Nile Virus PRNT is highly specific for West Nile Virus, indicating definitive evidence of West Nile Virus infection.

CHAPTER FOUR

4.0 RESULTS

4.1 Sample collection

To determine whether WNV is a significant cause of febrile illness in Trans Nzoia, 1150 serum samples were collected from febrile patients from three different study sites in Trans Nzoia. Of these, 96 % were clean sera, haemolysis was observed in 4% of the samples which were difficult to process therefore giving a total of 1114 serum samples which were processed **(Table 4.1)**. The three sites were; Andersen Medical Clinic (457 samples), Endebess Sub-District Hospital (144 samples) and Kitale District Hospital (513 samples) **(Table 4.1)**.

Table 4.1 : Distribution of study participants from the three study sites

Study site	Number of study participants (N = 1114)
Kitale District Hospital	513
Andersen Medical Clinic	457
Endebess Sub-District hospital	144

The sample population mainly consisted of female patients with a total of 570 female patients and 407 male patients for the remaining 137 patients the field entry questionnaire was missing response for "sex" **(Table 4.2)**. The age distribution of the patients was children above five years to adults over 61 years and the mean age was 28 years **(Table 4.2)**.

Table 4.2: socio-demographic characteristics of study participants

Characteristic	Number of study patients (N= 1114)
Sex	
Male	407
Female	570
[1]Missing	137
Age in years	
Mean	28
Median	33
Range	5 - 80

---

[1] The field questionnaire for the 137 participants was missing response for sex.
[2] The field questionnaire for the 365 participants was missing response for the specific

The entry point for this study was febrile illness. All participants received a physical examination by a clinician and mainly patients who were found to be having pyrexia of unknown origin (≥38°c) were considered for the study. Additional symptoms relevant for this study which included rash, headache, jaundice, eye infection, meningitis and bleeding were also considered upon examination.

4.2 Large scale cell culture and virus propagation

The profiles of the resulting sucrose gradient are shown in **Figure 4.1**. The protein content of the virus banded (**A** and **B**) distinctively in the region centring around 15 to 25 % sucrose gradient concentration. About 10 fractions (area labelled **C** in the **Figure 4.1**) were collected by the fractionating column via upward displacement. Only two fractions with high optical density absorbance were used as the purified concentrated virus. The virus concentration of these fractions was then determined using a spectrophotometer to be 0.55 mg/ml.
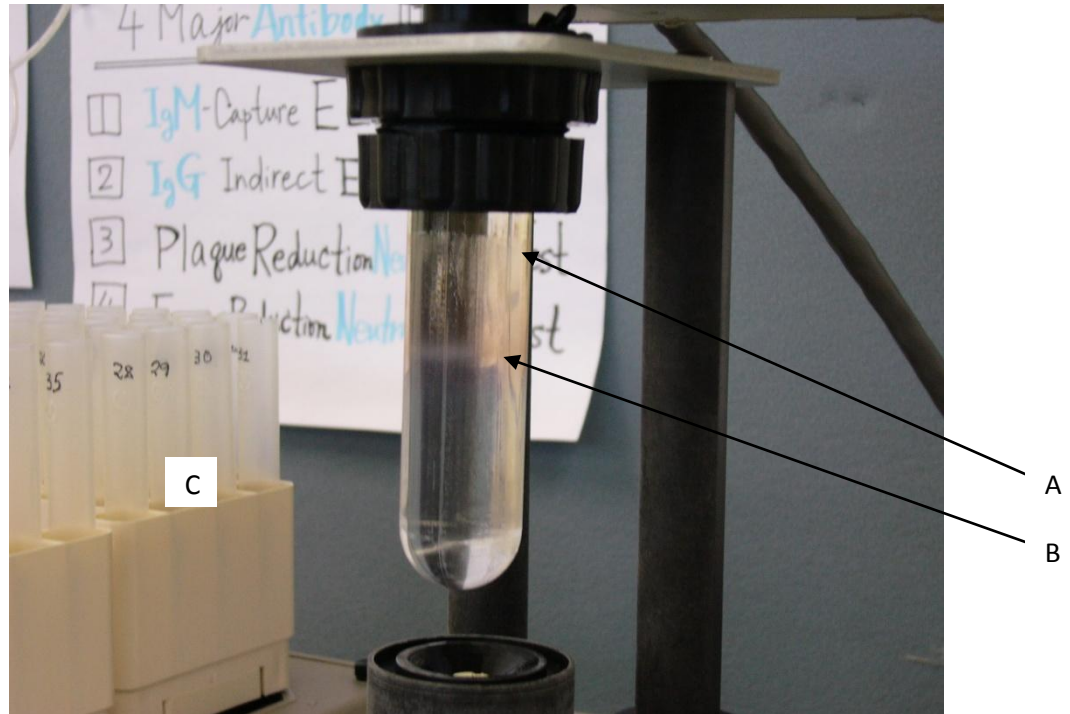
Figure 4.1: Profiles of the resulting sucrose gradient showing recovery of the virus after ultra centrifugation (A and B shows the viral protein bands at different sucrose gradients while C shows the fractions collected).

4.3 Optimization of ELISA results

The antigen proteins from **section 4.2** were attached to the plate by passive adsorption which is mediated primarily by hydrophobic interactions. Suitable concentration of antigen in a coating solution and corresponding serum and secondary antibody dilution were established using check board titrations **(Table 4.3)**. Realistic and economically viable combination of values was chosen from these experiments. The combination chosen for this test was: antigen concentration of 500 ng/well, sera dilution of 1: 1000

and conjugate dilution of 1: 5000 **(Table 4.4)**. The standardised ELISA developed here

was used for this study to screen for seroprevalence of WNV in Trans Nzoia district.

Table 4.3: Optical density of the individual wells measured at an absorbance of 492 nm.

| | 1/ 500 | | 1/1000 | | 1/ 500 | | 1/1000 | | 1/ 500 | | 1/1000 | | Serum dilution |
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	
	1	2	3	4	5	6	7	8	9	10	11	12	
A	3.25	1.41	2.33	0.59	3.04	1.11	2.17	0.49	2.97	0.93	1.99	0.52	virus 1µ g/ 100 µl
B	3.27	1.37	2.34	0.57	2.91	0.99	1.96	0.43	2.79	0.85	1.89	0.45	
C	2.90	0.91	1.84	0.38	2.54	0.64	1.66	0.29	2.40	0.57	1.53	0.31	virus 500 ng / 100 µl
D	2.81	0.94	1.82	0.38	2.50	0.64	1.52	0.28	2.40	0.58	1.45	0.30	
E	2.55	0.72	1.43	0.32	2.16	0.51	1.20	0.21	1.82	0.38	1.16	0.21	virus 250 ng / 100 µl
F	2.59	0.66	1.61	0.36	2.15	0.50	1.24	0.21	1.99	0.38	1.18	0.21	
G	0.82	0.40	0.41	0.17	0.52	0.25	0.32	0.13	0.40	0.19	0.28	0.30	
H	0.63	0.33	0.42	0.14	0.45	0.25	0.29	0.14	0.57	0.28	0.39	0.22	3 % PBS- F

| 1:1000 | 1:5000 | 1:10,000 |

HRPO conjugate IgG+M+A dilution

Table 4.4: Average optical density of the serum samples (controls) at different concentrations of antigen, serum and HRPO conjugated IgG+M+A dilutions.

| 1/ 500 | | 1/1000 | | 1/ 500 | | 1/1000 | | 1/ 500 | | 1/1000 | | serum dilution |
Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	
2.53	1.02	1.92	0.43	2.49	0.80	1.76	0.33	2.40	0.66	1.60	0.22	virus 1µg/100 µl
2.13	0.56	1.41	0.23	2.04	0.39	1.29	0.15	1.92	0.34	1.15	0.05	virus 500ng/100 µl
1.85	0.32	1.10	0.19	1.67	0.26	0.91	0.07	1.42	0.15	0.83	0.05	virus 250ng/100 µl

| 1:1000 | 1:5000 | 1:10,000 |

HRPO conjugate IgG+M+A dilution

An array of blocking buffers was used ranging from block ace, skimmed milk (non-fat dry milk) in PBS with 0.05% Tween 20, to PBS- 3% FCS diluted in 0.05% Tween 20. There was no significant difference among the blocking buffers used in terms of background noise and signal amplification. Due to economic reasons, the blocking buffer used for this work was PBS- 3% FCS diluted in 0.05% Tween 20.

4.4   Sample screening for WNV exposure by ELISA

All the study participants ($n = 1114$) were screened by indirect ELISA for the presence of antibodies against WNV. Of these 105 (9.4%) patients were reactive and 1009 (90.6%) were negative (**Figure 4.2**). For patients with detectable IgG or IgM or IgA antibodies, the levels of antibodies were high as indicated by high OD values of >1.0.
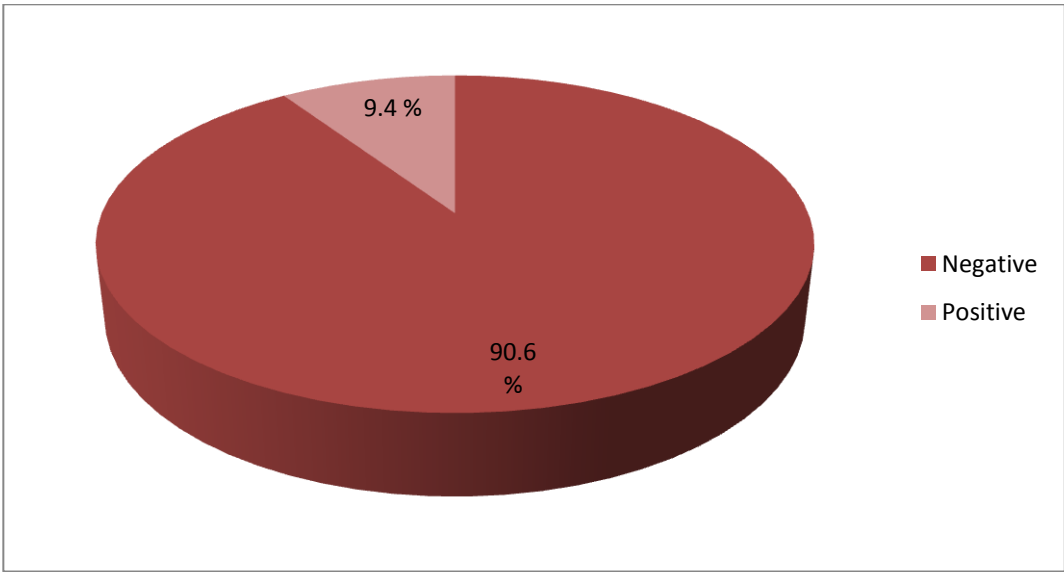
Figure 4.2: WNV seropositive patients by ELISA in Trans Nzoia

4.5   Screening for neutralizing antibodies to WNV

All sera positive by ELISA were further tested for the presence of virus-specific neutralising antibodies by plaque reduction neutralisation tests (PRNT$_{50}$) against the NY99 strain of WNV. A subset of sera ($n = 105$) that were reactive by ELISA **(Figure 4.2)** were further screened by PRNT using WNV for neutralising antibodies. Of those tested to be positive by ELISA 12 (1.1%) patients were found to have neutralizing antibodies against WNV and confirmed to be seropositive for WNV **(Figure 4.3)**.
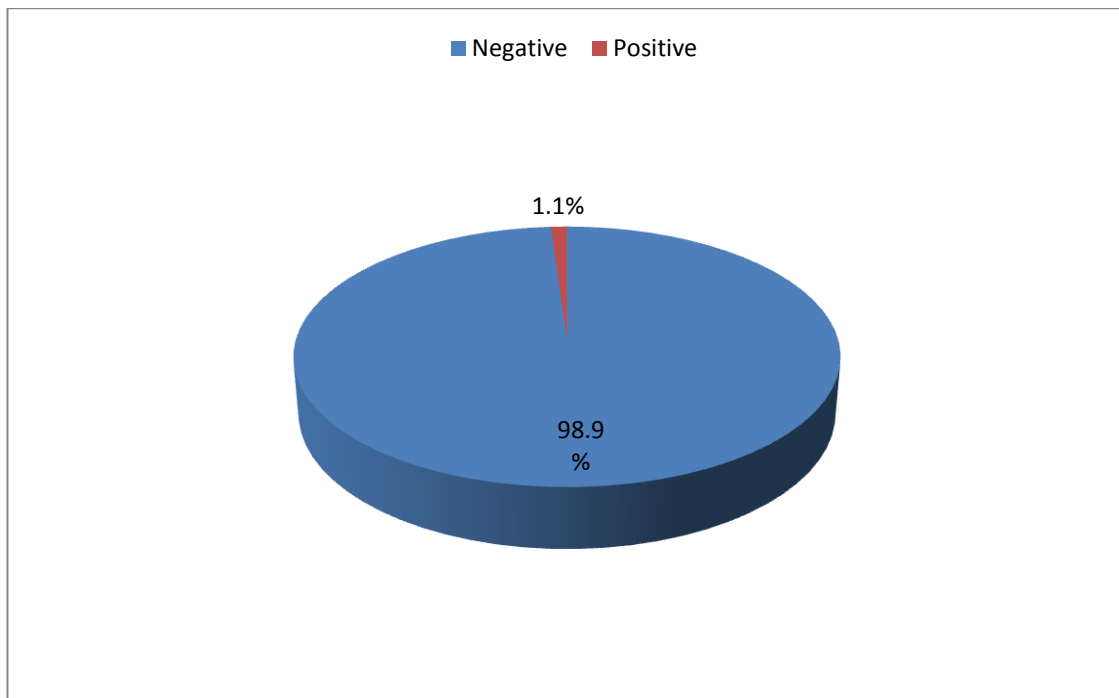
Figure 4.3: WNV seroprevalence by gold standard PRNT in Trans Nzoia

The World Health Organization (WHO) standard method based on 50% plaque reduction was employed to detect positive virus-neutralizing sera (Weingartl *et al.,* 2003; Buckley *et al.,* 2006; WHO/IVB, 2007). The outline of the resulting neutralization test for one serum sample is shown in **Figure 4.4**. The clear spots in the positive control wells **(A** and **B)** are the plaques, indicating cell destruction by the virus and the absence of neutralising antibodies to neutralise the virus and prevent cell death. The serum sample **(**in wells **C**and**D)** showed complete neutralisation since no plaques were observed therefore $\geq$50% (PRNT$_{50}$) reduction in the number of plaques, indicating that the patient's serum had neutralizing antibodies against WNV.
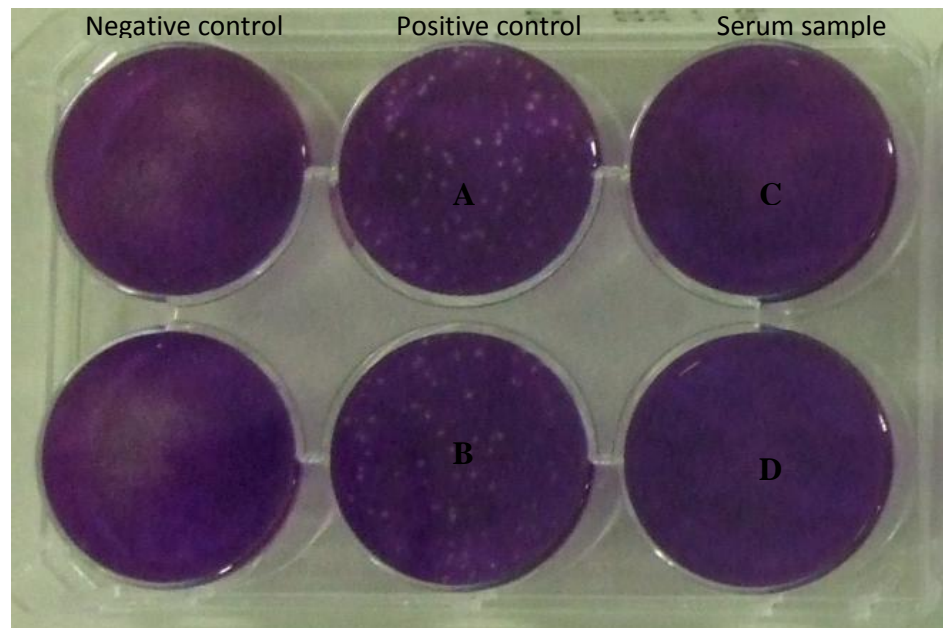
Figure 4.4: A diagram showing the PRNT results of one serum sample.

A subset (*n=12*) of samples that neutralised and reduced the number of plaques by $\geq 50\%$ ($PRNT_{50}$) were further titrated in order to determine the antibody titre in theses samples. The serum samples listed in **Table 4.5** were tested with WNV and endpoint titers at 70% reduction in plaques were determined. The endpoint titer represents the reciprocal of the serum dilution at which the test sera neutralizes the challenge inoculum by 70%.

Table 4.5: End point titters at 70% reduction in plaques.

Sample ID	Neutralisation titres against WNV at $PRNT_{70}$
END 007	> 1280
END 071	640
AMC 059	320
AMC 344	160
KDH 128	160
KDH 189	320

The outline of the resulting antibody titration of the serum sample in **Figure 4.4** is shown in **Figure 4.5.** In this illustration the corresponding dilution with 70% reduction in the number of plaques was 1/320, the titre of neutralizing antibodies was therefore regarded as 320.
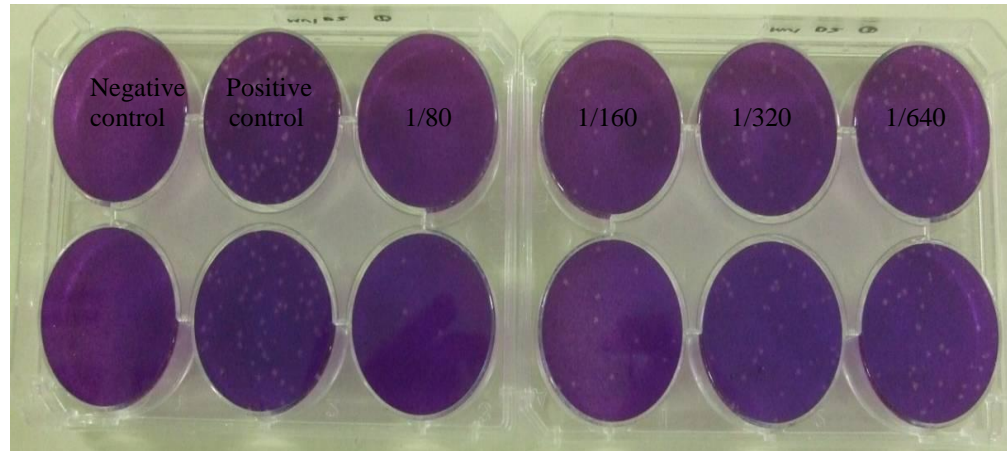
Figure 4.5: Diagram showing antibody titration of a serum sample.

4.6   Clinical findings

Overall, 267 patients presenting with fever were tested, 269 patients presenting with headache, 113 patients presenting with rash, 79 patients presenting with eye infection, 61 jaundiced patients, 4 encephalitic and 4 patients with signs of haemorrhage were tested **(Table 4.6)**. The field questionnaire for the remaining 325 participants had a missing response for the specific clinical symptoms. Out of the 105 samples positive by ELISA, a total of 15 (5.6%) patients had fever, 15 (5.7%) had headache, 4 (3.5%) had rash, 4 (5.2%) had eye infection and 2 (3.3%) had jaundice; the remaining 65 patients had missing response for the specific clinical symptoms **(Table 4.6)**. None of the patients presenting with signs of meningitis or bleeding were reactive **(Table 4.6)**. All the 12 samples that neutralised were of patients presenting with febrile illness. For most of the clinical signs, the symptoms lasted for a period of about one month after the onset of the clinical signs.

Table 4.6: Summary of the subset of symptomatic patients tested by ELISA

	Patient's Status	
Presenting symptoms	**Seropositive** IgG or IgM or IgA	**Seronegative** IgG or IgM or IgA
Fever	15	252
Rash	4	109
Eye Infection	4	73
Jaundice	2	59
Headache	15	249
Meningitis	0	4
Bleeding	0	2
[2]Missing	65	287

4.7 Evaluation of WNV distribution in Trans Nzoia using socio-demographic characteristics of the study participants

4.7.1 Geographical distribution of WNV in Trans Nzoia

To estimate for location specific risk for WNV exposure, samples were collected from three different sites **(Table 4.1)**. Of the 105 cases which were positive by ELISA **(Figure 4.2)**, 41 (0.08%) were from Kitale District Hospital (KDH), 58 (0.13%) from Andersen Medical Clinic (AMC) and 10 (0.07%) from Endebess Sub-District Hospital (END) **(Figure 4.6).**

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[2] The field questionnaire for the 365 participants was missing response for the specific clinical information.
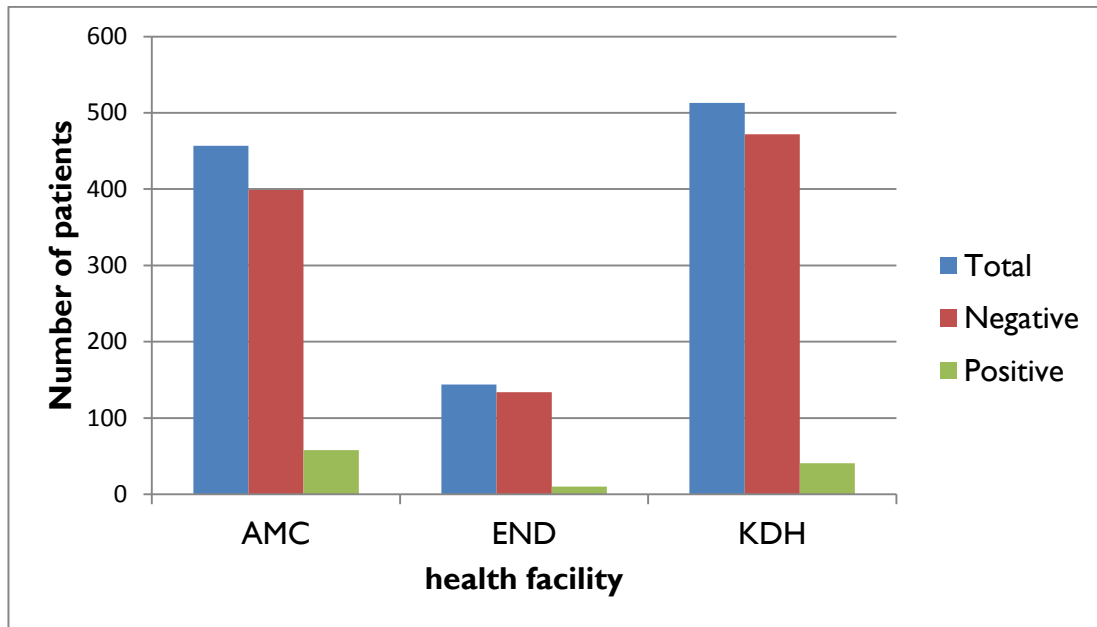
Figure 4.6: A summary of WNV seropositivity by ELISA per study site.

4.7.2   **Distribution of WNV in relation to the age of study participant**

To estimate for risk of exposure across different ages, samples were collected from patients of ages 5 to >61 years. None of the patients of 5 years was positive for the presence of anti-WNV antibodies **(Figure 4.7)**. Antibodies against WNV were detected in all patients from the age six years to 60 years **(Figure 4.7)**. The highest seropositivity ratio was in the 21-25 and 26-30 years age group, with 20.7 % and 24.1 % of the exposed patients respectively in these age groups **(Figure 4.7)**. None of the patients in the age group of 61 years and above were positive of anti-WNV antibodies **(Figure 4.7)**.
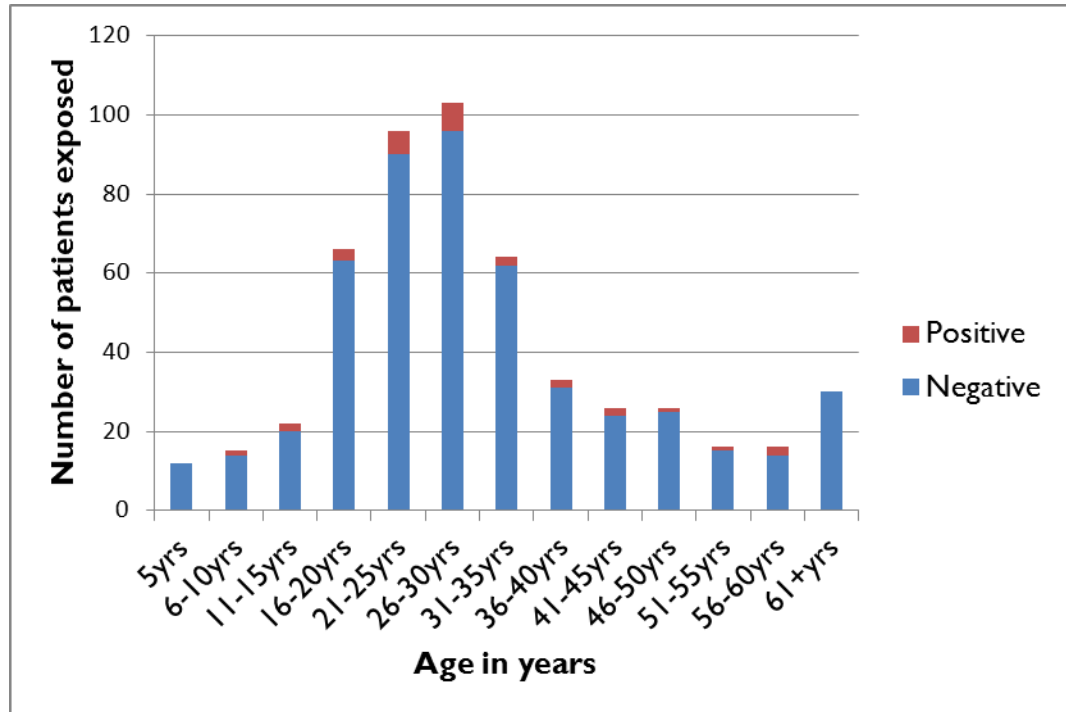
Figure 4.7: Exposure pattern by age.

4.7.3   **Distribution of WNV in relation to the gender of study participant**

Study participants were drawn from both sexes. Both female and male patients were affected, however female patients were disproportionately affected with 69% of the total WNV sero-positive patients female and 31% of them male **(Figure 4.8)**. Female patients were at a significantly increased risk of exposure than the male patients (odds ratio = 1.2, 95% CI= 0.75-1.41)
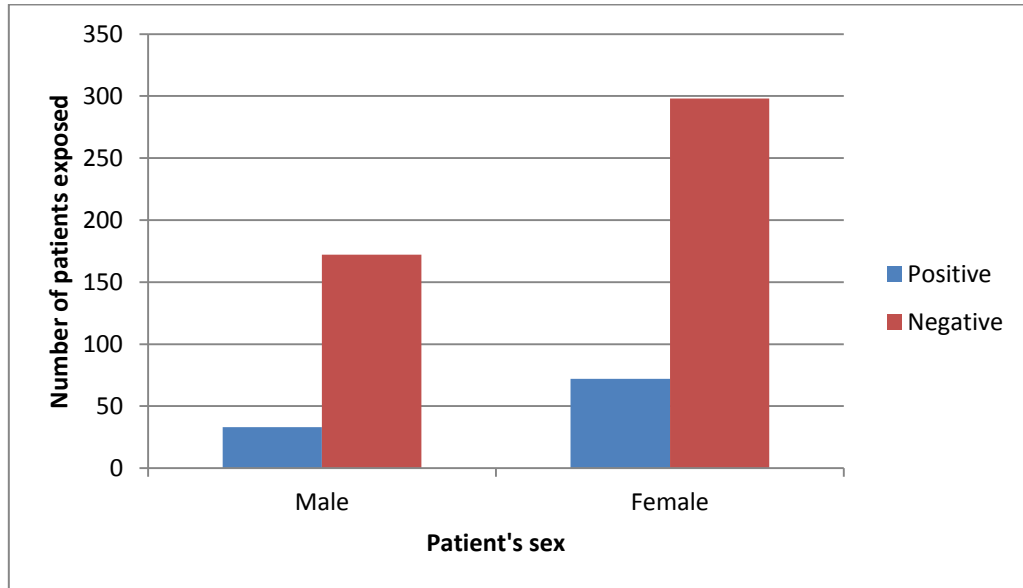
Figure 4.8: Exposure pattern by gender of study participants.

4.8 Virus isolation

Attempts to isolate WNV from these samples using C_{636} mosquito cell lines were not successful. No cytopathic effect was observed in the cells cultures which were inoculated with the serum samples.

CHAPTER FIVE

5.0 DISCUSSION

Many cases in Africa that present with fever are documented as fever of unknown origin, especially if they fail to treatment against malaria, typhoid and antimicrobial drugs; the majority of these conditions remain undiagnosed (Nur *et al.,* 1999). However, several arboviruses such as West Nile Virus, Chikungunya Virus, Dengue Virus, Rift Valley Fever Virus and Yellow Fever Virus are frequently considered in the aetiology of acute febrile illness (Nur *et al.,* 1999; Kuniholm *et al.,* 2006). A number of factors that favour the emergence of vector-borne diseases were present in this study site, which led to consideration of a possible role of WNV infection in the aetiology of febrile illness in the area. Some of these factors include; the area receives high rainfall and is heavily forested which makes it ideal for the breeding of the *Culex* mosquito. People in this area have encroached forested land for agriculture and settlement which increases their risk of exposure to sylvatic mosquitoes that are vectors to these viruses. The virus has previously been isolated from the *Culex univittatus* complex mosquito in this region, which was found to be the most abundant species in the area (Miller *et al.,* 2000).

Precise diagnosis can be made when serological tests are used in combination with clinical observations and epidemiological history, and/or when seroconversion is demonstrated. Serodiagnostic techniques are also widely used to demonstrate freedom from a disease, and in epidemiological investigations (Martin *et al.,* 2000). The Indirect

ELISA is one of the simplest immunoassay techniques for the detection of antibodies, but its routine application is often impeded by non-specific signals arising from the use of semi or un-purified antigens (Gravell *et al.,* 1977; Forghani and Schmidt, 1979; Martin *et al.,* 2000). The WNV replicates to high titres in cell cultures and therefore production of purified and concentrated stocks of the virus for ELISA antigen is possible however it is expensive and laborious.

Large scale purification of WNV

The findings of this study have demonstrated that large volumes of WNV propagated in C_{636} cells can be easily purified by use of sucrose gradient, ultracentrifugation and polyethylene glycol 6,000 combined with the use of a fractionating column (**Figure 4.1**). Cell debris was almost all separated from the viral suspension, and consequently the total amount of non-viral protein in the viral suspension decreased. This is consistent with other studies where viruses of the Japanese encephalitis complex have been purified using this method (Okuda *et al.,* 1975; Hasegawa *et al.,* 1980). The viral suspension purified locally by the method mentioned above was sufficient for use as antigen for ELISA assay, PRNT standard, monoclonal antibodies and also as a vaccine source with respect to both the antibody response and purity from the host protein.

Optimisation of ELISA

Since the mid-1970's, there has been an increasing demand for sensitive, relatively simple assays for use in basic research and clinical diagnosis. Over the same period,

many practical considerations have led to the need to adapt existing assays (Karaszkiewicz, 2010).

 For this ELISA flat bottomed plates with a clear base made of polystyrene were used. The absorbance of colorimetric substrates is measured by shining a laser up through the base of each well, so it was essential to use a flat bottomed plate with a clear base.

The use of virus-specific antigens of optimal concentration in combination with optimal serum dilutions and concentrations of affinity purified secondary antibody creates a system in which antibody to WNV can be efficiently screened for **(Table 4.2)**. Use of purified antigens at optimal concentration reduces cross-reactivity **(Table 4.2)**.

An optimal blocking buffer maximizes the signal-to-noise ratio and does not react with the antibodies or target protein (Martin *et al.,* 2000). The blocking buffer used for this work was PBS- 3% FCS diluted in 0.05% Tween20. For an antibody to work successfully in ELISA, it should react specifically with the antigen but not cross-react with a component of the blocking buffer in ELISA applications (Martin *et al.,* 2000). Some systems may benefit from the addition of a surfactant such as Tween®-20 (a gentle non-ionic detergent) to the blocking solution. Surfactants can help to minimize hydrophobic interactions between the blocking protein(s) and the antigen or the antibodies (Martin *et al.,* 2000).

The concentration of the test sera is one of the most crucial parameters in the optimization process. Too little sera produces a weak signal and this could result to a false negative result as illustrated in the results for the positive sample used **(Table 4.4)**. Too much of the sera produces a high back ground noise even in the internal control wells **(Table 4.3)** and this result to a false positive results as illustrated in **table 4.4** for the negative sample used.

The enzyme conjugate as well requires optimization, the optimal range being partially determined by the form and origin and also by the substrate used for signal generation (Martin *et al.,* 2000). The amount of enzyme that binds directly influences the amount of signal that is generated. Too little enzyme and the signal may be very weak with a poor signal-to-noise ratio **(Table 4.4)**. Too much and the background may be too high, again resulting in a poor signal-to-noise ratio and little distinction between standards of different concentrations **(Table 4.4)**

Ensuring exact incubation times, especially in the critical substrate step, yields reliable results. The ELISA assay was optimized according to well established protocols to perform efficiently in the local environment for the serological diagnosis of WNV infection. The results shows that it was more reliable to screen for WNV using an antigen concentration of 500 ng/ ml and a serum dilution of 1:1000 coupled with enzyme conjugate of 1:5000 **(Table 4.4)**

Exposure to WNV

An array of antibody types and subclasses is produced by a normal host humoral response to viral infection (Pier *et al.,* 2004). Protection of the host from systemic viral infection usually involves antiviral antibodies of the IgG, IgM and IgA classes (Pier *et al.,* 2004). The IgM antibody is produced early in the immune response, the appearance of IgM antiviral antibodies is generally indicative of a new or recent viral infection (Martin *et al.,* 2000; Pier *et al.,* 2004). The IgG antibody on the other hand is the most important effecter of immunity and is an indicator of past viral infection (Martin *et al.,* 2000; Pier *et al.,* 2004). The IgA antibodies also play a role in antiviral immunity, since they are typically found in secretions of the mucosal surfaces which represent a common point of entry into the host of many viral pathogens (Pier *et al.,* 2004).

This indirect ELISA detected IgM, IgG and IgA allowing for timely diagnosis of disease. The detection of antibodies against WNV in 9.4 % of the selected patient population suggests possible exposure to a past WNV infection or active transmission **(Figure 4.2)**. Studies have shown that sera collected from individuals exposed to WNV 6 - 8 months previously will have IgG antibodies that bind strongly to viral antigen (Pourrut *et al.,* 2010). The WNV IgM antibody may persist for more than a year and the demonstration of IgM antibodies in a patient's serum, particularly in residents of endemic areas is diagnostic of an acute WN viral infection. The test screened for IgA, IgG and IgM antibodies; it was therefore not possible to distinguish between the three

different Ig's. A clear distinction of which cases were due to active transmission or previous exposure was not possible.

The serological data obtained from this study suggests that there is WNV activity in Trans Nzoia District with a seroprevalence of 1.1% as determined by PRNT (**Figure 4.3**). The presence of antibodies to NS1 protein infers that the virus had replicated in the patients since non-structural proteins are only produced in infected cells after virus replication, meaning that they would not be present in an introduced virus (Buckley *et al.,* 2006). This data leads to conclusion that there is transmission of WNV in this area though at low levels. This is consistent with a previous study in the Rift Valley province where WNV was isolated from mosquito vectors demonstrating WNV activity and transmission in the area (Miller *et al.,* 2000). The occasional disparity that was noted between results of the two tests (ELISA and PRNT) is explained by the fact that the antibody types detected can be different; for instance IgM produced early in infection does not always possess neutralizing activity (Calisher *et al.,* 1986). Therefore, taken together these findings suggest that the patients had a prior WNV infection, but this virus was not responsible for all the clinical signs observed at the time of presentation except for the 1.1%. The identification of neutralizing antibodies in these patients therefore suggests a recent incidence of WNV.

Clinical findings

The entry point for this study was febrile illness, anti-WNV antibodies were detected in some of the febrile patients. Of the subset of febrile patients screened by indirect ELISA 1.1% neutralized WNV **(Figure 4.3)**. This therefore infers that WNV was responsible for only 1.1% of the febrile cases in this study. This suggests that the febrile illness in the other patients could have been due to exposure to either other pathogens. In most of the cases clinical signs lasted for a period of about one month. Most cases of febrile illness associated with WNV are self-limiting; however uncomplicated, the illness can be a public health problem (Hayes *et al.,* 2005). A recent follow-up study of West Nile Fever patients who sought medical attention found that difficulty concentrating and neck pain or stiffness was also prominent symptoms, and that fatigue and muscle weakness frequently lasted for approximately one month after onset (Hayes *et al.,* 2005). In that study, of the 98 patients interviewed, 31% were hospitalized, 79% missed school or work because of their illness, and the median time before patients felt fully recovered was 60 days (Hayes *et al.,* 2005). This shows that even without neurologic manifestations, WNV infection clearly can cause a notable public health problem.

Only 2% of Patients with febrile illness proceed to the severe form of WNV infection, West Nile Neuroinvasive Disease or West Nile Encephalitis which is life threatening (Hubalek *et al.,* 1999; Hayes *et al.,* 2005). This form is characterized by three syndromes; encephalitis, meningitis and acute flaccid paralysis (Hayes *et al.,* 2005). For this study, none of the cases that presented with signs of meningitis or bleeding were

exposed to WNV **(Table 4.6)**. This concludes that no patient had severe infection, complications or neuroinvasive disease as a result of exposure to WNV. The detection of low levels of WNV and the low intensity of the severity of the clinical symptoms could suggest that; a less virulent strain is in circulation. Less virulent strains of WNV mostly lineage two strains have not been associated with severe disease (Porrut *et al.,* 2010). It could also suggest that the patients have been previously exposed to other flaviviruses. In this regard, laboratory studies have shown that prior immunization of rodents, monkeys, and pigs with heterologous flaviviruses reduces the severity of subsequent WNV infection (Price *et al.,* 1971, Goverdhan *et al.,* 1992, Ilkal *et al.,* 1994, Tesh *et al.,* 2002; Rodriguez *et al.,* 2010.)

Evaluation of WNV distribution using socio-demographic characteristics of the study participants.

The distribution of WNV is dependent on the occurrence of susceptible avian reservoir hosts and competent mosquito vectors, mosquito host preference, and availability of hosts (Burt *et al.,* 2006; Gibbs *et al.,* 2006; Brown *et al.,* 2008). These factors can be influenced by geographic variables such as land use/land cover, elevation, human population density, physiographic region, and temperature (Gibbs *et al.,* 2006). The potential influence of environmental and social factors on WNV transmission has been of great interest since the discovery of the virus.

A study in the Chicago area found that risk factors associated with clusters of human cases and dead birds included vegetation, age, income, race and distance to reported

WNV positive dead birds, age of housing, mosquito control activities, and geological factors (Ruiz *et al.,* 2004).

Environmental conditions affecting both avian reservoir hosts and the mosquito vector populations may regulate WNV amplification. Identifying such factors will not only aid in understanding WNV epidemiology, but also will serve in predicting and possibly reducing the risk of WNV infection (Gibbs *et al.,* 2006).

For the few cases that were found to be exposed to WNV in this study, there were unique risks.

Geographical distribution of WNV in Trans Nzoia.

The association between urban land use and human cases indicates that urban/suburban land use enhances environmental conditions for both enzootic and bridge transmission (Gibbs *et al.,* 2006). Urbanization and deforestation have been linked to emergence of arboviruses (Wilson 1994).

It was observed that the antibody seroprevalence rates were higher in patients attending AMC as compared to the other health facilities **(Figure 4.6).** This facility is located at the Kenya-Uganda border and serves people around this community. The area receives high rainfall and is heavily forested with high vegetation cover which is favourable for mosquito breeding. The people in this area have encroached forested land for agriculture and settlement increasing their exposure to the sylvatic mosquito that transmit this virus. This indicates increasing risk for WNV disease with decreasing forested lands as

humans continue to expand and modify their environment. This result is consistent with other studies carried out in South Africa (Burt *et al.,* 2002) where WNV cases where found to be clustered in areas with high rainfall and high vegetation cover. In a more recent study, spatial analysis of WNV case distribution in the New York City area in 1999 revealed that vegetation abundance was significantly and positively associated with human WNV cases (Brownstein *et al.,* 2002). This association was used to predict areas of greatest human risk for WNV infection.

Distribution of WNV in relation to the age of study participant

It was observed that all patients from the age of six years to 60 years were exposed and none of the patients below six years or 61 years and above were exposed to WNV **(Figure 4.7)**. The antibody seroprevalence rates increased exponentially from six years to 30 years after which the rates decreased until 60 years **(Figure 4.7)**. The linear increase in WNV IgG and IgM seroprevalence with age suggests continuous exposure of these populations to this virus. It was expected that children below five years would not be exposed since children at this age are usually kept indoors therefore reducing their risk of exposure. The findings of the study confirms this since none of the patients below six years were exposed **(Figure 4.7)**. Patients at the age group 21 - 25 years and 26 - 30 years were more affected than the other age groups. This age category (21 - 30 years) is the most active age group in the society and therefore practices like herding cattle, fetching water and firewood raises their likelihood of exposure to infected mosquito. The older people could be showing immunity due to a previous exposure to WNV or any

other related flavivirus. This is study is consistent with a study in Gabon where study subjects at the age of 13-35 years were more exposed than the other age groups (Pourrut *et al.,* 2010).

Distribution of WNV in relation to the gender of study participant

Human activities not only support mosquito populations, but also provide food, nesting, and roosting habitat for both native and introduced birds (Burt *et al.,* 2002). The most important risk factor for acquiring WNV is exposure to infected mosquitoes.

It was observed that female patients seemed to be more susceptible than the male patients; this was evident from the findings in that 69 % of the seropositive patients were female patients **(Figure 4.8)**. This could be attributed to the kind of activities they are involved in. It is a common practice in most cultures in Kenya that some duties like collecting firewood from the forest and fetching water from rivers running through the forest are left to women. This therefore exposes the women to a higher risk of acquiring WNV since there are high population densities of the mosquito vectors in the forest vegetation. As demonstrated by a number of arboviruses, disease emergence is most often related to human activities that increase disease vector habitats or change the density of non- human vertebrates involved in virus amplification (birds in the case of WNV) (Mackenzie *et al.,* 2004).

Virus isolation

Attempts to isolate WNV from these samples using C_{636} mosquito cell lines were not successful. No cytopathic effect was observed in the cells cultures which were

inoculated with the serum samples. This could be attributed to the fact that samples collected at the site were stored at -20° C due to lack of -80°C freezers at these sites. Samples for virus isolation should be stored at -80°C immediately after collection due to the labile nature of RNA.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

1. This study has established that WNV replicates in high titres in C_{636} cells and can be purified by using sucrose gradient, ultracentrifugation and polyethylene glycol 6,000 combined with the use of a fractionating column .

2. This study has established that the use of virus-specific antigens of optimal concentration in combination with optimal serum dilutions and concentrations of affinity purified secondary antibody creates a system in which antibody to WNV can be efficiently screened for.

3. The findings of this study have determined the seroprevalence of WNV in Trans Nzoia to be 1.1% and that the virus was responsible for only a small percentage (1.1%) of the febrile illness in this area.

4. This study has established that that there is WNV activity in the area though at low levels and this suggest that the virus will continue to circulate in the environment. There is therefore a great risk of an outbreak occurring in this area.

5. This study has also established that risks associated with exposure appear to be increased in areas where humans have encroached forested land where the environment becomes increasingly impacted by human population expansion.

6.2   Recommendations

1. This study was not able to discriminate between IgG and IgM antibodies. Further screening of the serum samples by IgM capture ELISA would be recommended to discriminate between previous exposure and active transmission.

2. The study identified presence of WNV in study samples from study subjects originating from other areas away from Trans Nzoia. Therefore screening for the virus in other parts of the country would be recommended.

3. Isolation of the virus from serum samples for further characterisation of the strain in order to determine the strain/lineage in circulation would be important in determining the virulence of the strain.

4. Comparison of isolated to strains isolated in other parts of the country and also in other parts of the world would be important in determining if the same strain is in circulation and if so the mode of introduction into these areas.

5. Since 90% of the PRNT assay did not have neutralising antibodies, this study provides basis to screen for other viruses closely related to WNV in the area.

6. Effective surveillance and proper reporting systems should be put in place by the public health in order to control the virus in selected areas in case of an outbreak.

7. Differential diagnostic tools would help both in clinical diagnosis and disease surveillance.

8. Prevention and control measures should also be put in place and based on surveillance reports.

9. This information may be used in addressing regional public health needs and mosquito control programs; priority should be placed on campaigns aimed at decreasing manmade mosquito habitats in urban/suburban areas.

6.3   Study limitations

1. The Lack of -80°C freezers at the collection sites was an impediment therefore attempts to isolate the virus from the serum sample were not successful. Due to the labile nature of RNA, no further characterization, PCR or RT-LAMP would be attempted. Samples for virus isolation should be stored at -80°C immediately after collection.

2. Reliance on field workers (clinician) to fill in patient information, who at times left out some of the necessary patient information needed for the study. Failure to have complete patient information is however not a rationale for rejecting already collected patient's sample since the patient willingly consented to have their sample taken and expects to get their results back.

3. Another study limitation was the necessary reliance on study participant recall who at times gave disputing information.

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APPENDICES

APPENDIX I: ROLE OF INVESTIGATORS

Joyce Mwongeli Ngoi is the principal investigator for this work; and a MSc. Student at ITROMID KEMRI. She was involved in conducting the entire assays for this project. She is also conducting this as part of her degree requirement.

Dr. Matilu Mwau is the Principal Investigator of the KEMRI/Nagasaki Arbovirology Collaborative Project. He is the main contact person, and will provide the overall leadership of this project. He will supervise the postgraduate student, the data collection, laboratory experimentation, data analysis, and dissemination of the findings.

Prof. Ann Muigai is the co-investigator from Jomo Kenyatta University of Agriculture and Technology. She will provide the research team with a key link between JKUAT and the KEMRI administration. She will also supervise the postgraduate student and provide assistance and advice on academic issues.

APPENDIX II: INFORMED CONSENT DOCUMENT

General information of the study to the participant

TITLE OF STUDY: SEROLOGICAL SURVEY FOR HUMAN WEST NILE VIRUS EXPOSURE IN FEBRILE PATIENTS ATTENDING SELECTED HEALTH FACILLITIES IN TRANS NZOIA DISTRICT, KENYA.

INTRODUCTION

The proposed study seeks to validate the seroprevalence of West Nile Virus clinical diagnosis through screening of 1114 samples collected from patients from the Kitale District Hospital, Andersen Medical Clinic and Endebess District Hospital, by ELISA and PRNT.

PARTICIPATION

Participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you (your child) are otherwise entitled. You (your child) may discontinue your (your child's) participation at any time without penalty or loss of benefits. The principal investigator may decide to withdraw you (your child) from the study if we are unable to obtain a blood sample from you (your child).

PROCEDURE TO BE FOLLOWED

You are here to have a little blood drawn from you because your clinician has recommended some tests. If you agree to be a participant in this study, we will take two

and a half extra milliliters (which is equivalent to one teaspoonful) of blood during the procedure. We will use sterile and disposable instruments that are clean and safe. The extra blood taken from you will be transported to the KEMRI laboratories in Nairobi for analysis of West Nile Virus Status. In order to ensure complete confidentiality of the test results, no names will be attached to the blood samples, but an identification number assigned to you will be used to label the sample.

INCONVENIENCES AND BENEFITS

The patients targeted are those already determined by the clinician as needing laboratory tests. Only a minimal additional amount of blood (2.5 mls) will be collected which is equivalent to one teaspoonful, the phlebotomist will also illustrate the amount on the syringe to show how much of the patient's blood will be drawn. Phlebotomy is a well-established procedure that causes only minimal discomfort. For this reason no additional risks and inconveniences are anticipated. Although there will be no immediate benefits for the patient, this study will help determine the importance of West Nile fever as a cause of febrile illness in selected health facilities in Trans Nzoia District. Such information is important for the clinicians in their differential diagnosis of febrile illnesses. The study will also provide data on WNV epidemiology, this will help to determine what necessary intervention and resources should be provided, distributed and also the appropriate measures of prevention to be undertaken.

PARTICIPANT'S RIGHTS

Your participation in this study is voluntary and if you decline to participate, you will not be denied any services that are normally available to you.

COMPENSATION

No direct compensation in the form of salary will be paid for participating in the study and no special incentive will be offered to persuade persons to participate. Patients will be at liberty to refuse consent with or without explanation, and without penalty or prejudicial action towards them.

DURATION OF PARTICIPTION

This study only requires one to blood draw .There is no follow-up or further information needed.

WHO CAN PARTICIPATE IN THIS STUDY

Inclusion Criteria:

1. Blood samples will be collected from children above 5 years of age and adults. To be infected by West Nile Virus one needs exposure to mosquitoes, this implies that all persons are eligible. However, we expect that the immune system is well developed in older children, for which reasons we will use an arbitrary age cutoff of 5 years.

2. Patients referred by the clinician for laboratory blood tests, either due to an illness they have, or for routine examinations e.g. blood group.

3. Patients able and willing to give informed consent.

Exclusion criteria:

1. Unable or unwilling to give consent

2. Aged below 5 years

ASSURANCE OF CONFIDENTIALITY OF VOLUNTEER'S IDENTITY

Records relating to your (your child's) participation in the study will remain confidential. Your (your child's) name will not be used in any report resulting from this study. All computerized records and laboratory specimens will contain only a unique study number, not your name. You will receive a signed copy of this consent form.

USE OF BLOOD SAMPLES

The blood samples obtained in this study will not be used for any other purpose other than the ones stated in the protocol and consent form. The results of all testing performed will be shared with the medical or clinical officer caring for you.

REVIEW OF RESEARCH RECORDS

It should be noted that consent forms will be kept in a locked file at KEMRI or a designated storage facility for not less than 10 years following completion of the study. These data sheets will be made available only to the Principal Investigator and the Co-investigators, clinical personnel who require this information to treat the patient, or to members of the Ministry of Health who require this information for legal reasons or to investigate an outbreak.

DATA MANAGEMENT:

Data Storage

Most data entry will be performed at KEMRI on a computer provided by this study. The only identifier used in this computerized database will be the subject's study number. Clinical data sheets will be kept on file at KEMRI or a designated storage facility for not less than 5 years following completion of the study. These data sheets will be made available only to the Principal Investigator and the Co-investigators, clinical personnel who require this information to treat the patient or to members of the Ministry of Health who require this information for legal reasons or to investigate an outbreak.

Data Management

As this is primarily a surveillance project, the only data analysis planned is the determination of circulating West Nile Virus in selected health facilities in Kenya that is; Kitale District Hospital, Andersen Medical Clinic and Endebess DH.

Dissemination of Data

Publication or presentation of any data resulting from this study will be a joint collaboration between Japan and Kenyan researchers. Nothing will be published or presented without a review from KEMRI. Manuscripts submitted for review to either organization will be completed and approval or recommendations for changes given.

PERSONS AND PLACES FOR ANSWERS REGARDING THE RESEARCH STUDY ITSELF

If you have questions now or in the future regarding the research study Contact Joyce Mwongeli Ngoi: 02027222541 ext. 2256/2290

PERSONS AND PLACES FOR ANSWERS IN THE EVENT OF RESEARCH RELATED INJURY

If you think you (your child) has a medical problem related to this study, please report to Joyce Mwongeli Ngoi: 02027222541 ext. 2256/2290

PERSONS AND PLACES FOR ANSWERS REGARDING YOUR RIGHTS AS A RESEARCH SUBJECT

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 Secretary, KEMRI/National Ethics Committee, P.O BOX 54840-00200, Nairobi; Telephone number : 020-272 2541; 0722 205901, 0733 400003.

APPENDIX III: CONSENT FORMS FOR ADULTS

Informed Consent for Adults aged 18 and above, in English.

My name is…………and I work in this health facility. We think that West Nile Virus is an important cause of febrile illness in this area. The information we gather is useful to the government and other policy makers who may consider preventative programs in this community or other communities in the future. We will summarize our findings from this study and disseminate it to various stakeholders including Ministry of Health, KEMRI, and others. The KEMRI's ethical review committee, who are responsible for conducting such reviews at national level, has approved this study.

Research Procedures: You are here to have a little blood drawn from you because your clinician has recommended some tests**.** If you agree to be a participant in this study, we will take 2 extra milliliters (which is equivalent to one teaspoonful) of blood during the procedure. We will use sterile and disposable instruments that are clean and safe. The extra blood taken from you will be transported to the KEMRI laboratories in Nairobi for analysis of WEST NILE VIRUS Status. In order to ensure complete confidentiality of the test results, no names will be attached to the blood samples, but an identification number assigned to you will be used to label the sample.

Risk/benefits: During this procedure there will be no long-lasting effect. However, you may feel a brief moment of pain or fear. You will not be given any monetary benefits; neither will you incur any costs. The study will benefit your community since by helping

us and the government to understand the problems your community is facing as a result of West Nile Virus, we will be able to recommend and design appropriate interventions to minimize the impact of this disease

Participant's Rights: Your participation in this study is voluntary and if you decline to participate, you will not be denied any services that are normally available to you.

Confidentiality: We will make every effort to protect your identity. You will not be identified in any report or publication of this study or its results.

Contact Information: If you have questions now or in the future regarding your rights or this study, you may ask any of the field officers involved in this study or contact Joyce Mwongeli Ngoi, of KEMRI at 02027222541 ext. 2256/2290

Consent for the individual for blood sample:

May I now ask if you would like to participate in the study?

The above details about the study and the basis of participation have been explained to me and **I agree** to take part in the study. I understand that I am free to choose to be part of the study. I also understand that if I do not want to go on with the study, I can withdraw at any time. **I give my consent** for my blood to be tested for West Nile Virus.

Please sign here or put your right hand thumb mark if you agree:

Signature/ Thumb mark--

Date -------------------------

Ridhaa ya watu waliozidi umri wa miaka 18, kwa Kiswahili

Jina langu ni…………... Mimi ni mfanyakazi wa kituo hiki cha afya. Tunafanya utafiti juu ya ugonjwa uitwao West Nile Virus ambao tunafikiri ni mojawapo ya magonjwa yanayoambukiza jamii hii. Ili tujue umuhimu wa West Nile Virus huku, tumeamua kufanya utafiti. Matokeo ya utafiti huu yatasambazwa kwa serikali na wadau kama Wizara ya Afya na KEMRI na wengineo, ambao watatengeneza miradi mbalimbali itakayolenga kupunguza maambukizi na kuboresha huduma za walioathirika katika jamii hii, na nyingine hapo baadaye. Utafiti huu umepitishwa na kuruhusiwa na kamati inayohusika na utoaji wa vibali vya utafiti ya KEMRI, yaani ERC.

Utaratibu:

Daktari wako ameshakueleza kwamba anahitaji upimwe damu ili ajue kama una magonjwa fulani. Ukikubali kukuendelea na utafiti huu, tutatoa kiasi cha 2 mls (hii ni sawa na kijiko cha chai) zaidi cha damu, zoezi ambalo litachukua muda mfupi tu. Tutatumia vifaa visafi na salama ambavyo vitafunguliwa mbele ya macho yako, na vitatumika kwako tu na kutupwa mara tu baadaye. Baadaye, damu tutakayotoa kwa huu utafiti itapelekwa maabara kwa upimaji wa West Nile Virus. Ili kuhakikisha usiri wa jina lako katika utafiti huu, jina na maelezo yako hayataandikwa kwenye sampuli ya damu, bali sampuli itatambulishwa na nambari tu. Ni utaratibu wa utafiti huu kuwa watafiti hawatakujulisha majibu yako.

Faida/Mapungufu:

Utasikia maumivu kidogo ama woga wakati unachomwa sindano, lakini hutapata maumivu ya muda mrefu. Hutapata malipo yoyote ya kifedha, na pia hutatumia pesa

zako mwenyewe katika utafiti huu. Utapewa neti ya kuzuia kuumwa na mbu. Utafiti huu utasaidia jamii yako kwa sababu tukifahamu matatizo ya jamii hii, tutaweza kushauri na kutengeneza miradi mbalimbali ya kupunguza athari za West Nile Virus.

Haki za mshirika:

Ushiriki wako katika utafiti huu ni wa hiari kabisa. Ukikataa kushiriki, hutanyimwa huduma zinazotolewa kwa kawaida.

Usiri/Utunzaji wa taarifa:

Katika utafiti huu, tutahakikisha kuwa maelezo yako, jina lako ni siri kabisa. Jina lako halitaandikwa au kuhusishwa kwa hii fomu, sampuli, na popote ndani ya ripoti nzima tutakayotoa baadaye.

Mawasiliano

Iwapo utakuwa na swali kuhusiana na haki zako ama utafiti huu, unaruhusiwa kuwasiliana na afisa yeyote wa utahini, au Mkuu wa utafiti huu, Joyce Mwongeli Ngoi wa KEMRI, Nambari ya simu 0202722541 ext 2256/2290

Ridhaa ya kutolewa damu:

Napenda kukuuliza ridhaa yako ya ushiriki wako katika zoezi la utoaji damu

Nimeelewa maelezo ya hapo juu yanayohusu utafiti huu, na ninakubali kushiriki katika zoezi hili. Naelewa kuwa ushiriki wangu ni wa hiari, na pia kama sitakubaliana muda wowote naruhusiwa kujitoa katika zoezi hili. Natoa ridhaa damu yangu itumike katika upimaji wa West Nile Virus.

Sahihi/dole gumba…………………………

Tarehe………………..

APPENDIX IV: CONSENT FORMS FOR MINORS

Informed Consent for minors

My name is…………and I work in this health facility. We think that West Nile Virus is an important cause of febrile illness in this area. In order to be sure about the exact magnitude of the problem, we are conducting a study to determine the prevalence of West Nile Virus. The information we gather is useful to the government and other policy makers who may consider preventative programs in this community or other communities in the future. We will summarize our findings from this study and disseminate it to various stakeholders including Ministry of Health, KEPI, KEMRI, and others. The KEMRI's ethical review committee, who are responsible for conducting such reviews at national level, has approved this study.

Research Procedures: Your child is here to have a little blood drawn because your child's clinician has recommended some tests**.** If you allow your child to participate in this study, we will ask you a few questions regarding where your child resides and his/her vaccination status. Then we will take 2 and half extra milliliters (which is equivalent to one teaspoonful) of blood during the procedure. We will use sterile and disposable instruments that are clean and safe. The extra blood taken from your child will be transported to the KEMRI laboratories in Nairobi for analysis of your child's West Nile Virus Status. In order to ensure complete confidentiality of the test results, no names will be attached to the blood samples, but a number assigned to your child will be used to label the sample.

Risk/benefits: During this procedure there will be no long-lasting effect. However, your child may feel a brief moment of pain or fear. Your child will not be given any monetary benefits; neither will he/she incur any costs. The study will benefit your community since by helping us and the government to understand the problems your community is facing as a result of West Nile Virus, we will be able to recommend and design appropriate interventions to minimize the impact of this infectious disease.

Your child's Rights: Your child's participation in this study is voluntary and if you disallow participation, you will not be denied any services that are normally available to you.

Confidentiality: We will make every effort to protect your child's identity. Your child will not be identified in any report or publication of this study or its results.

Contact Information: If you have questions now or in the future regarding your rights or this study, you may ask any of the field officers involved in this study or contact Joyce Mwongeli Ngoi, of KEMRI at 02027222541 ext 2256/2290.

Consent for the individual for blood sample:

May I now ask if you will allow your child to participate in this study?

The above details about the study and the basis of participation have been explained to me and **I allow my child** to take part in the study. I understand that I am free to allow my child to be part of this study. I also understand that if I do not want him/her to go on with the study, I can withdraw at any time. **I give my consent** for my child's blood to be tested for West Nile Virus.

Please sign here or put your right hand thumb mark if you agree:

Signature/ Thumb mark---

Date ------------------------

Ridhaa ya watoto walio chini ya miaka 18, kwa Kiswahili

Jina langu ni…………... Mimi ni mfanyakazi wa kituo hiki cha afya . Tunafanya utafiti juu ya ugonjwa uitwao West Nile Virus ambao tunafikiri ni mojawapo ya magonjwa yanayoambukiza jamii hii. Ili tujua umuhimu wa West Nile Virus huku, tumeamua kufanya utafiti. Matokeo ya utafiti huu yatasambazwa kwa serikali na wadau kama Wizara ya Afya, KEPI, na KEMRI na wengineo, ambao watatengeneza miradi mbalimbali itakayolenga kupunguza maambukizi na kuboresha huduma za walioathirika katika jamii hii, na nyingine hapo baadaye. Utafiti huu umepitishwa na kuruhusiwa na kamati inayohusika na utoaji wa vibali vya utafiti ya KEMRI, yaani ERC.

Utaratibu:

Daktari wako ameshakueleza kwamba anahitaji mtoto wako apimwe damu ili ajue kama mtoto huyu ana magonjwa fulani. Ikiwa utakubali mtoto wako ahusike na utafiti huu, kwanza tutakuuliza maswali machache juu ya umri wake, anapoishi, na kama ameshapewa chanjo. Baadaye, tutatoa kiasi cha 2.5 mls (Hii ni sawa na kijiko cha chai) zaidi cha damu, zoezi ambalo litachukua muda mfupi tu. Tutatumia vifaa visafi na salama ambavyo vitafunguliwa mbele ya macho yako, na vitatumika kwako tu na kutupwa mara tu baadaye. Ili kuhakikisha usiri wa jina la mtoto wako katika utafiti huu, jina na maelezo yake hayataandikwa kwenye sampuli ya damu, bali sampuli itatambulishwa na namba tu.

Faida/Mapungufu:

Mtoto wako atasikia maumivu kidogo ama woga wakati anachomwa sindano, lakini hatapata maumivu ya muda mrefu. Hatapata malipo yoyote ya kifedha, na pia hatatumia pesa zako katika utafiti huu. Utafiti huu utasaidia jamii yako kwa sababu tukifahamu matatizo ya jamii hii, tutaweza kushauri na kutengeneza miradi mbalimbali ya kupunguza athari za West Nile Virus.

Haki za mtoto wako:

Ushiriki wa mtoto wako katika utafiti huu ni wa hiari kabisa. Ukikataa ashiriki, motto wako hatanyimwa huduma zinazotolewa kwa kawaida.

Usiri/Utunzaji wa taarifa:

Katika utafiti huu, tutahakikisha kuwa maelezo ya mtoto wako na jina lake ni siri kabisa. Jina lake halitaandikwa au kuhusishwa kwa hii fomu, sampuli, na popote ndani ya ripoti nzima tutakayotoa baadaye.

Mawasiliano

Iwapo utakuwa na swali kuhusiana na haki zako ama utafiti huu, unaruhusiwa kuwasiliana na afisa yeyote wa utahini, au Mkuu wa utafiti huu, Joyce Mwongeli Ngoi wa KEMRI, Nambari ya simu 0735 561 002 ama 0202722541 ext 2256/2290

Ridhaa ya kutolewa damu:

Napenda kukuuliza ridhaa yako ya ushiriki wa mtoto wako katika zoezi la utoaji damuya ushiriki wako katika zoezi la utoaji damu

Nimeelewa maelezo ya hapo juu yanayohusu utafiti huu, na ninakubali motto wangu ashiriki katika zoezi hili. Naelewa kuwa ushiriki wa motto wangu ni wa hiari, na pia kama sitakubaliana muda wowote naruhusiwa kumtoa katika zoezi hili. Natoa ridhaa damu ya motto wamnu itumike katika upimaji wa West Nile Virus.

Sahihi/dole gumba…………………………

Tarehe………………..

APPENDIX V: QUESTIONNAIRE

SEROLOGICAL SURVEILLANCE FOR HUMAN WEST NILE VIRUS EXPOSURE IN FEBRILE PATIENTS ATTENDING SELECTED HEALTH FACILLITIES IN KENYA.

Date of hospital visit ………………………….

Study Number ……………….….

Village …………………………….…

Sub-location …………………………….

Location …………………………….

Division …………………………………

District ………………………………….

Province …………………………………

Gender:

Male ( ) Female ( )

Age (years) ( ) Date of birth ( )

Have you ever been vaccinated for Yellow Fever?

Yes ( ) No ( )

If Yes, when?

Year ( ) Date ( ) Age ( )

Presenting Complaints:

Fever:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Rash:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Eye infection:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Jaundice:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Headache:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Symptoms suggestive of meningitis-meningoencephalitis

Yes ( ) No ( )

Duration of symptoms…………………………………..

Bleeding diathesis:

Yes ( ) No ( )

Duration of symptoms…………………………………..

List any other symptoms

1. ……………………………….

2. ……………………………….

3. ……………………………….

Any other remarks:

……………………………………………………………………………………………………

……………………………………………………………………………………………………

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………………………………………………………………………….............................

Name of person completing form: ………………………...…………………………..

Signature ………………………….. Date ……………….. Time ………………………