SEROPREVALENCE OF DENGUE 2 VIRUS INFECTIONS IN PATIENTS PRESENTING WITH FEBRILE ILLNESS IN SELECTED HEALTH FACILITIES IN TRANS NZOIA REGION, KENYA , 2009

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Seroprevalence of Dengue 2 Virus Infection in Patients Presenting with Febrile Illness in Selected Health Facilities in Trans Nzoia Region, Kenya, 2009

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DECLARATION

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

Signature……………………………… Date ……………………

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This thesis has been submitted for examination with our approval as University Supervisors.

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DEDICATION

I dedicate this work to my Father, Dr. Simon Nzou Nguluu, Mother, Beatrice Nzou and Sister Grace Nzou for their love, encouragement and financial support.

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LIST OF ABBREVIATIONS AND ACRONYMS

CPE	Cytopathic Effect
DENV-1	Dengue Virus 1
DENV-2	Dengue Virus 2
DENV-3	Dengue Virus 3
DENV-4	Dengue Virus 4
DENV	Dengue Virus
D/H	District Hospital
DHF	Dengue Hemorrhagic Fever
DNA	Deoxyribonucleic Acid
DSS	Dengue Shock Syndrome
ELISA	Enzyme Linked Immunoassay
EMEM	Eagles Minimum Essential Medium
FCS	Fetal Calf Serum

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGR	Insect Growth Regulators
IV	Intravenous
ng	Nanograms
NUITM	Nagasaki University of Tropical Medicine
OD	Optical Density
OPD	*o*-Phenylene Diamine
PBS	Phosphate Buffered Saline
PBS-F	Phosphate Buffered Saline with 3% Foetal Calf Serum
PBS-T	Phosphate Buffered Saline with 0.05% Tween 20
PCR	Polymerase Chain Reaction
pfu	Plaque Forming Unit
PIII	Biosafety Level III Laboratory

RNA	Ribonucleic Acid
RPM	Revolutions per minute
RT-LAMP	Reverse Transcriptase Loop Amplification Test
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
RVF	Rift Valley Fever
ssRNA	Single Stranded Ribonucleic Acid
WHO	World Health Organization
YF	Yellow Fever

ABSTRACT

Dengue Virus and other Arbovirus infections are considered public health threats in Kenya. Although it is evident that these infections are now emerging, an accurate estimate of the magnitude of the problem has not been documented. Dengue often presents as pyrexia, hence diseases which present with febrile illness may contribute to false diagnosis of dengue or the diagnosis is never made. For the health burden to be realized, the study was aimed at investigating dengue 2 virus infection and the seroprevalence in patients presenting with febrile illness in Trans Nzoia. To describe and document the exact threat posed by this virus in Kenya, there was need to use the right laboratory tools to conduct regular serological surveys. For such surveys to be feasible in the local research environment in Kenya, a cross sectional study was done using assays which were cheap, feasible, rapid and efficient. Optimized Enzyme Linked Immunosorbent (ELISA) and Plaque Reduction Neutralization (PRNT) assays were used for this purpose. Serological surveillance of dengue 2 virus using these assays was done in Kitale and Endebess District Hospital and Andersen Medical Centre, all located in Trans Nzoia region. A total of 1121 samples were screened for dengue 2 virus infection by ELISA and PRNT and the seroprevalence found to be 0.9% with most of the positive patients coming from Kitale district hospital. The clinical information and laboratory correlated well with the laboratory diagnosis with 35 (3.1%) and 15 (1.3%) positive patients presenting with fever and headache respectively. In conclusion, the study

reveals the existence of dengue 2 virus infection that poses a danger of spreading from one area to another. The data obtained will play an important role in contributing to the management of febrile illness in Kenya generally and in particular Trans Nzoia. Dengue 2 virus will be an addition to the diagnosis of febrile illness in this area.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Dengue fever is a mosquito borne tropical infectious disease caused by the virus dengue has four serotypes, namely dengue virus (DENV) 1, 2, 3 and 4. It occurs in two forms: Dengue fever (DF) and Dengue hemorrhagic fever (DHF). Its prevalence range in urban areas is the same as in the rural areas. Sylvatic cycle also increases the spread of this infection. Human encroachment into the forests has contributed to the spread of this infection (Ryan and Ray, 2004).

In the 1960s, dengue serotypes-1, 2 and 3 were isolated for the first time from samples taken from humans in Nigeria. Subsequently, dengue has been found to occur in Senegal and Burkina Faso (predominantly being transmitted in sylvatic cycles), and possibly in other tropical rainforests in western Africa. Through virus isolation, a number of Aedes species have been associated with dengue transmission in western Africa; these include *Ae. taylori, Ae. furcifer, Ae. luteocephalus, Ae. vittatus* and *Ae. aegypti* (Gubler, 2002).

In Kenya, dengue fever infection has been reported at the coastal regions, (Morill *et al.,* 1991), and also there have been undocumented reports of dengue fever infections in Western and Northern parts of Kenya. The symptoms elicited by dengue fever are similar to majority of other diseases found in the country such as malaria and typhoid

fever. This has led to misdiagnosis of the disease. Also, lack of proper diagnosis method for the infection is also a challenge in its prevention and control. Information on dengue fever in Kenya is scanty and this has resulted to an underestimation of the disease burden in the country. This study will impact on realizing the impact of dengue 2 virus infections in Trans Nzoia, risk of an epidemic transmission and setting up an effective surveillance system

1.2 Statement of the problem

Dengue is a neglected tropical disease (NTD). The World Health Organization (WHO) receives reports of about 500,000 dengue fever cases each year, but estimates that as many as 50 million people are infected annually, in more than 100 countries, mainly across Asia, Africa and South America. Although dengue does not rival malaria as a killer, the sheer number of people infected and the fever's debilitating nature means that it has an enormous economic impact. Measured in disability-adjusted life years, which quantify disruption to quality of life and economic productivity, dengue's burden on some societies is comparable to that of HIV, tuberculosis or hepatitis. In South America, its impact rivals that of malaria and in Asia, it is a public health enemy number one (Gubler, 1999). The seroprevalence in Trans Nzoia is not known. As outbreaks and sporadic cases of dengue have continued to occur in eastern Africa, effort has been made to identify vectors and transmission cycles (sylvatic, periurban or urban) but the serological surveillance of the infection is still not well determined. Dengue often presents as pyrexia, hence diseases which present with febrile illness and are common in

Kenya such as Malaria and Typhoid fever may contribute to false diagnosis of dengue or the diagnosis is never made. Few human serology surveys have been documented in Kenya to determine the extent of the outbreaks in humans; hence the seroprevalence of dengue has very scanty information.

1.3 Justification of the Study

Although dengue appears to be spreading in Africa and in particular, Kenya, the surveillance and other research activities pertaining to dengue has been very limited. This has been mainly owing to the assumption that dengue is not a significant health problem on the continent, and this is largely attributed to the fact that severe forms of dengue illness are rarely reported. As most dengue infections are subclinical or present as dengue fever, they go undiagnosed and are commonly treated as malaria or other endemic fevers, such as typhoid and leptospirosis. This has resulted in an underestimation of the magnitude of the dengue problem in Kenya, especially in Trans Nzoia. This study will enhance understanding of dengue infection and the health burden posed by this virus. Early diagnosis, prevention and control of dengue virus infection will also be enhanced.

1.4 Null Hypothesis

There is no dengue 2 virus infection in patients presenting with fever in selected health facilities in Trans Nzoia region.

1.5 Objectives

1.5.1 General Objective

To investigate dengue 2 virus infection and prevalence in patients presenting with febrile illness in Kitale, Endebbess District hospitals and Andersen Medical Center in 2009.

1.5.2 Specific Objectives

1. To determine the seroprevalence of dengue 2 virus in patients attending selected health facilities in Trans Nzoia.
2. To correlate clinical information and laboratory diagnosis of dengue 2 virus infection in people with febrile illness visiting the selected health facilities.
3. To optimize enzyme immunosorbent assay and plaque reduction neutralization assays for dengue 2 virus.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Dengue viruses

The Dengue virus is a group IV positive-sense single stranded RNA virus whose genome is 10.5kb long. It is a member of the virus family *Flaviviridae*, genus *flavivirus* and species Dengue virus. It is an enveloped, spherical virus, measuring 40-60nm in diameter. The capsid has an indistinct symmetry, two proteins namely nucleocapsid ('C') and matrix ('M'). The envelope has one glycoprotein ('E') (Kautner *et al.,* 1997). Flaviviruses replicate in arthropod and vertebrate cells. Some of these viruses cause major diseases such as Japanese encephalitis, yellow fever and dengue hemorrhagic fever in humans. Flaviviruses are subdivided by their reactions in serological tests. Each virus contains antigenic determinants common to the group, others which specify a complex of viruses and those which determine serotype specificity (Trent, 1977).

There are four serotypes of Dengue Virus (DENV), DENV 1, 2, 3 and 4. The virus genome has about 11000 bases that codes for three structural proteins, Core (C), precursor Membrane (prM), Envelope (E); seven nonstructural proteins, Non-Structural 1(NS1), Non-Structural 2a (NS2a), Non-Structural 2b (NS2b), Non-Structural 3 (NS3), Non-Structural 4a (NS4a), Non-Structural 4b (NS4b), Non-Structural 5 (NS5); and short

non-coding regions on both the 5' and 3' ends. Infection with one of these serotypes does not provide cross-protective immunity; hence people living in a dengue-endemic area can have four dengue infections during their lifetime (Hanley and Weaver, 2010).

2.2 Geographical distribution of Dengue virus infections

The geographical spread of Dengue fever and Dengue hemorrhagic fever (Figure 2.1) is similar to that of malaria in Northern Australia, Singapore, Malaysia, Taiwan, Thailand, Vietnam, Indonesia, Philippines, Pakistan, India, Bangladesh, Puerto Rico, Brazil, Guyana, Venezuela, Trinidad and now Samoa are affected (CDC Traveler's Health, 2007). Unlike malaria, dengue is just as prevalent in the urban districts of its range as in the rural areas. Each serotype is sufficiently different to prevent cross-protection; hence epidemics caused by multiple serotypes (hyperendemicity) can occur (Ryan and Ray, 2004).

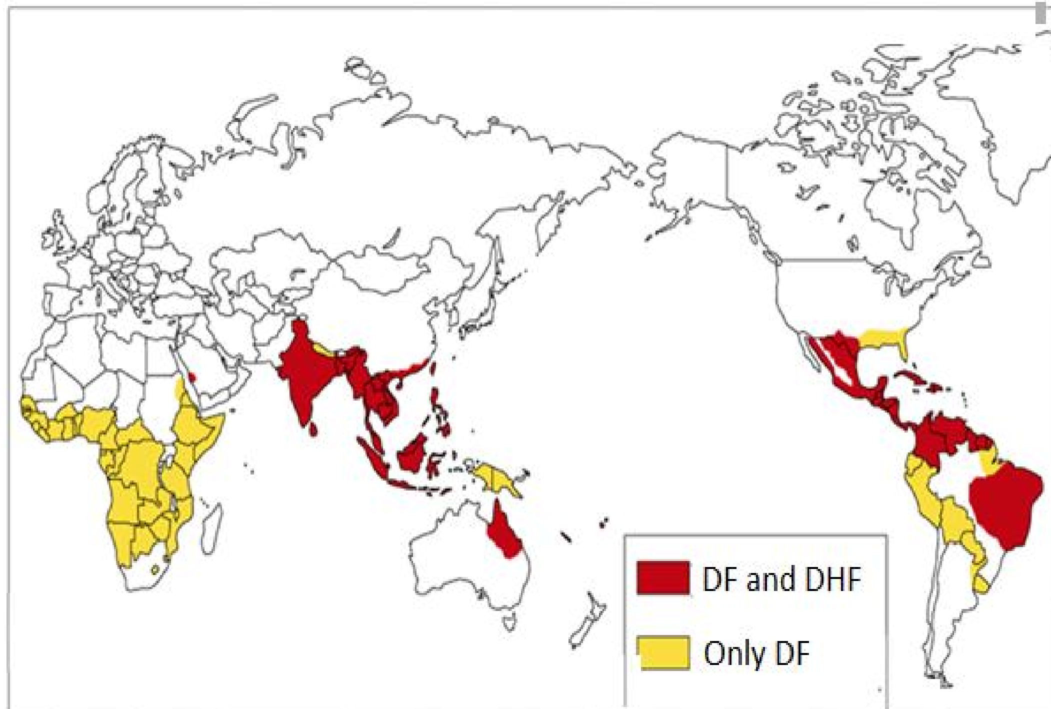


Figure 2.1: Geographical Distribution of Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF)

(Source: Nature publishing group 2007)

Each year, 100 million people become infected with dengue virus. People first reported the existence of dengue-like disease in 1779 but it was most likely present long before it first appeared in literature. The majority of deaths that result from dengue infection result from Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). People who develop DHF have a 5% chance of death but if they go on to develop DSS then the mortality rate can rise to as high as 40% (Rigau *et al.*,1998).

Massive troop deployment and refugee movements in the Second World War established South East Asia as the world's dengue hot bed (Scott, 2000). Over the past five decades, the disease incidence has grown 30-fold but its tendency to lie dormant in any given region before exploding into a severe outbreak has contributed to dengue's neglect by public health experts (Gubler, 2002).

There was a serious outbreak in Rio de Janeiro in February 2002 affecting around one million people and killing sixteen. On March 20, 2008, 23,555 cases of dengue, including 30 deaths were recorded in Rio de Janeiro in less than three months (Gubler, 2002). In Singapore, there are 4,000–5,000 reported cases of dengue fever or dengue hemorrhagic fever every year. In the year 2004, there were seven deaths from dengue shock syndrome. An epidemic broke out in Bolivia in early 2009, in which 18 people died and 31,000 infected (Halstead, 2007).

Since the first outbreak of dengue in South Africa in 1926–1927, cases of the disease imported from India were detected in the 1980s (Metselaar, 1980). The threat of dengue in South Africa has been evaluated in vector studies and competent vectors have been identified. This, together with the occurrence of imported cases, led to recommendations for continuous surveillance to obvert outbreaks in the country (McMeniman *et al*., 2009).

2.3 Epidemiology of Dengue Viruses

All types of dengue virus are re-emerging worldwide, causing larger and more frequent epidemics, especially in cities in the tropics. The emergence of dengue as a major public health problem has been most dramatic in the western hemisphere. Dengue fever has reached epidemic levels in Central America and is threatening the United States (Pandey *et al.,* 2003).

Dengue virus remains the most significant and wide spread flavivirus disease to have emerged globally. A 2001-2002 epidemic in Hawaii without fatalities is a reminder that dengue has also re-emerged in locations once considered to be dengue free. Usually transmitted by *A. aegypti* mosquitoes, dengue has recently been transmitted by *A. albopictus* a vector switch of potential significance with respect to dengue re-emergence (Gubler*,* 2002).

In America including many US southern states, *A. albopictus* has been spreading into areas where *A. aegypti* mosquitoes are not found and persisting for longer seasonal periods, putting tens of millions more people at risk of dengue infection. Dengue re-emergence is further complicated by disturbing increases in a serious and formerly rare form of the disease DHF (DSS being its highly fatal form). These severe complications are thought to result from the evolution of dengue viruses to escape high population immunity seen in increased viral virulence and human immunopathothogenesis due to antibody dependent enhancement of viral infection (Moreus*,* 1994).

The Dengue virus in East Africa was first isolated in 1982 after an epidemic of febrile illness in the Kenyan coastal towns of Malindi and Kilifi (Johnson *et al.,* 1982). The outbreak was believed to have spread from the Seychelles outbreak that occurred between 1977 and 1979 (Metselaar, 1980).

2.4 Transmission of Dengue Virus

Dengue virus is transmitted through the bite of the mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Upon biting an infected person, the viruses in the blood infect the mosquito and travels from the mosquito's stomach to its salivary glands where it multiplies. The virus is then injected into another person when the mosquito injects anticoagulants that prevent blood clotting while feeding. The mosquito remains able to transmit dengue for its entire life (Rigau *et al.,* 1998). The virus is now believed to be the most common arthropod-borne disease in the world, and is mainly found in the tropics because the mosquitoes require a warm climate. A major fear of epidemiologists is that the mosquitoes will adapt to cooler climates and then be able to infect people in the United States and other temperate climates (Rigau *et al.,* 1998).

Dengue virus may also be transmitted via infected blood products such as blood transfusions, plasma, and platelets (Wilder *et al.,* 2009), and in countries such as Singapore, where dengue virus is endemic, the risk was estimated to be between 1.6 and 6 per 10,000 blood transfusions (Teo *et al.,* 2009).

A female *A. aegypti* may need five blood meals to reproduce successfully, and compared with the single blood meal required by most mosquito vectors. This finding confirmed by individual marking female mosquitoes and recapturing them around people's homes, helps to explain why dengue can persist even when very few mosquitoes are present. It also shows that just a few infected mosquitoes or people are needed to kick start an outbreak (Scott, 2000).

Several factors which contribute to the resurgence of dengue fever include: No effective mosquito control efforts are underway in most countries with dengue, public health systems to detect and control epidemics are deteriorating around the world. Rapid growth of cities in tropical countries has led to overcrowding, urban decay and substandard sanitation, allowing more mosquitoes to live closer to more people. The increase in non-biodegradable plastic packaging and discarded tires is creating new breeding sites for mosquitoes and increased jet air travel is helping people infected with dengue viruses to move easily from city to city (Scott, 2000).

2.5 Pathogenesis, clinical signs and pathology of Dengue virus infections

The origin of the word *dengue* is not clear, but one theory is that it is derived from the Swahili phrase "Ka-dinga pepo", which describes the disease as being caused by an evil spirit. The Swahili word "dinga" may possibly have its origin in the Spanish word "dengue" meaning fastidious or careful, which would describe the gait of a person suffering the bone pain of dengue fever (Harper, 2001). Alternatively, the use of the

Spanish word may derive from the similar-sounding Swahili also known as "Dandy Fever", slaves in the West Indies who contracted dengue were said to have the posture and gait of a dandy (Gubler and Meltzer, 1998).

The first reported epidemics of DF occurred in 1779-1780 in Asia, Africa, and North America. The near simultaneous occurrence of outbreaks on three continents indicates that these viruses and their mosquito vector have had a worldwide distribution in the tropics for more than 200 years. During most of this time, DF was considered a mild, nonfatal disease of visitors to the tropics. Generally, there were long intervals (10-40 years) between major epidemics, mainly because the introduction of a new serotype in a susceptible population occurred only if viruses and their mosquito vector could survive the slow transport between population centers by sailing vessels (Putnak, *et al.,* 1996).

The incubation period of dengue fever is approximately four days. The person will come down with fever and present a discrete macular or maculopapular rash. It is difficult to distinguish dengue fever from other viral diseases and the person usually recovers in 5 days. In more severe cases, fever and rash are accompanied by headache, retro orbital pain, myalgia, backache, sore throat, and abdominal pain. The patients become lethargic and experience anorexia and nausea (Kautner *et al.,* 1997).

Infection confines a patient to bed for more than a week with a raging temperature and the agonizing limb pains (Gubler, 1999). Some suffer loss of hair and develop a measles like rash, bleeding gums and depression that can last for weeks. In a small percentage of

cases, mostly infants, the infection causes fatal DHF. The victims vomit and pass blood in the faeces and urine as their capillaries leak (Gubler, 1999).

The disease manifests as a sudden onset of severe headache, muscle and joint pains (myalgias and arthralgia; severe pain that gives it the nickname break-bone fever or bone crusher disease), fever, and rash. The dengue rash is characteristically bright red petechiae and usually appears first on the lower limbs and the chest; in some patients, it spreads to cover most of the body. There may also be gastritis with some combination of associated abdominal pain, nausea, vomiting, or diarrhea (Ryan and Ray, 2004).

Some cases develop much milder symptoms which can be misdiagnosed as influenza or other viral infection when no rash is present. Thus travelers from tropical areas may pass on dengue inadvertently, having not been properly diagnosed at the height of their illness. Patients with dengue can pass on the infection only through mosquitoes or blood products and only while they are still febrile. The classic dengue fever lasts about two to seven days, with a smaller peak of fever at the trailing end of the disease (the so-called "biphasic pattern"). Clinically, the platelet count will drop until the patient's temperature is normal. Cases of DHF also show higher fever, variable hemorrhagic phenomena, thrombocytopenia, and hemoconcentration. A small portion of cases lead to DSS which has a high mortality rate (Parida *et al.,* 2005).

After inoculation of the virus into the bloodstream by the infected mosquito, DENV attacks the dendritic cells and keratinocytes as the first targets to infection. This is

followed by infection of monocytes and macrophages in the lymph nodes that serve as the major targets of the infection dissemination for the virus through the lymphatic system to cells of the mononuclear lineage. As the viral load increases due to inability of the immunological response to control DENV replication, it results to apoptosis and necrosis in various tissues and this triggers the development of imbalance in profile of soluble mediators and cytokines resulting in endothelial cell dysfunction and blood coagulation abnormalities observed in DHF and DSS (Halstead, 1988).

2.5.1 Dengue hemorrhagic fever (DHF)

Dengue hemorrhagic fever is an acute febrile diseases, found in the tropics and Africa, and caused by four closely related virus serotypes of the genus family *flaviviridae*. It is also known as break bone fever (Parida *et al.,* 2005). It has a similar incubation period as dengue fever and many of the same symptoms. However, the fever is more severe and the drowsiness and lethargy is more extreme. The patient has increased vascular permeability and abnormal homeostasis. This can cause the individual to lose blood volume, resulting in hypotension, followed by shock, DSS and finally death results (Rigau *et al.,* 1998). If spotted early, DHF responds to intensive hospital treatment but mortality can reach 15% (Gubler, 1999).

2.5.2 Dengue shock syndrome (DSS)

A syndrome due to the dengue virus that tends to affect children under 10, causing abdominal pain, hemorrhage (bleeding) and circulatory collapse (shock). It starts abruptly with high continuous fever and headache plus respiratory and intestinal

symptoms with sore throat, cough, nausea, vomiting, and abdominal pain. Shock occurs after 2 to 6 days with sudden collapse, cool clammy extremities, weak thready pulse, and blueness around the mouth (circumoral cyanosis). There is bleeding with easy bruising, blood spots in the skin (petechiae), spitting up blood (hematemesis), blood in the stool (melena), bleeding gums and nosebleeds (epistaxis). Pneumonia and heart inflammation (myocarditis) may be present. The mortality is appreciable ranging from 6 to 30%. Most deaths occur in children. Infants under a year of age are especially at risk of death. It is also called Philippine or Southeast Asian hemorrhagic fever (Gubler, 2000).

2.6 Diagnosis of dengue infection

2.6.1 Clinical diagnosis

The diagnosis of dengue mainly depends on clinical diagnosis. The classic picture is high fever with no localizing source of infection, a petechial rash with thrombocytopenia and relative leucopenia - low platelet and white blood cell count. Care has to be taken as diagnosis of DHF can mask end stage liver disease and vice versa (Ryan and Ray, 2004).

Clinical manifestations that lead to clinical diagnosis include: fever, bladder problem, constant headaches, eye pain, severe dizziness and loss of appetite. These may be accompanied by hemorrhagic tendency (positive tourniquet test, spontaneous bruising, bleeding from mucosa, gingiva, injection sites; vomiting blood, or bloody diarrhea). Thrombocytopenia (<100,000 platelets per mm^3 or estimated as less than 3 platelets per high power field) and evidence of plasma leakage (hematocrit more than 20% higher

than expected, or drop in hematocrit of 20% or more from the baseline following intravenous (IV) fluid, pleural effusion, ascites, hypoproteinemia) and encephalitis occurrences may also be observed (Gubler and Meltzer, 1998).

2.6.2 Laboratory diagnosis of Dengue virus

Laboratory diagnosis of dengue infection is by indirect IgG, IgM and IgA ELISA which is able to detect chronic and acute dengue infections. Immediate diagnosis of dengue can be performed in rural areas by the use of Rapid Diagnostic Test kits, which differentiate between primary and secondary dengue infections. Serology and polymerase chain reaction (PCR) studies are available to confirm the diagnosis of dengue if clinically indicated (Kautner *et al.,* 1997).

2.7 Prevention and control of Dengue virus infections

2.7.1 Vector control

Primary prevention of dengue mainly resides in mosquito control. There are two primary methods: larval control and adult mosquito control (Vu *et al.,* 1998). In urban areas, *Aedes* mosquitos breed on water collections in artificial containers such as plastic cups, used tyres, broken bottles and flower pots. Periodic draining or removal of containers is the most effective way of reducing the breeding grounds for mosquitos. Larvicide treatment is another effective way to control the vector larvae, but the larvicide chosen should be long-lasting and preferably have World Health Organization clearance for use

in drinking water (Harper, 2001). There are some very effective insect growth regulators (IGRs) available which are both safe and long-lasting such as pyriproxyfen. For reducing the adult mosquito load, fogging with insecticide is somewhat effective. Prevention of mosquito bites is another way of preventing disease. This can be achieved by using insect repellent, mosquito traps or mosquito nets (Vu *et al.,* 1998).

2.7.1.1 Biological control

Infecting *Aedes* mosquitos with the bacterium Wolbachia, the adult lifespan is reduced by half (McMeniman *et al.,* 2009). Once an insect is infected, the bacterium will spread via its eggs to the next generation. A pilot release of infected mosquitoes could begin in Vietnam within three years. If no problems are discovered, a full-scale biological attack against the insects could be launched within five years (Teo *et al.,* 2009).

2.7.2 Mosquito Mapping

The technology, named Intelligent Monitoring of Dengue, uses traps with kairomones that capture *Aedes* gravid females, and upload insect counts with a combination of cell phone, GPS and internet technology. The result is a complete map of the mosquitoes in urban areas, updated in real time and accessible remotely, that can inform control methodologies (Hanley and weaver, 2010).

2.7.3 Potential antiviral approaches

Possible laboratory modification of the yellow fever vaccine YF-17D to target the dengue virus via chimeric replacement has been discussed extensively in scientific literature (Lai and Monath, 2003) but as of 2009 no full scale studies had been conducted (Querec *et al.,* 2006). Molecular replication mechanism of the virus can be specifically attacked by disrupting the viral RNA polymerase (Filomatori *et al.,* 2006). In cell culture (Kinney *et al.,* 2005) and murine experiments (Burrer *et al.,* 2007) morpholino antisense oligomers have shown specific activity against Dengue virus (Stein *et al.,* 2008). In 2007 virus replication was attenuated in the laboratory by interfering with activity of the dengue viral protease, and a project to identify drug leads with broad spectrum activity against the related dengue, hepatitis C, West Nile, and yellow fever viruses was launched (Webster *et al.,* 2009).

2.7.4 Management of Dengue Infection

The mainstay of treatment is timely supportive therapy to tackle shock due to hemoconcentration and bleeding. Close monitoring of vital signs in critical period (between day 2 to day 7 of fever) is critical. Increased oral fluid intake is recommended to prevent dehydration (Kautner *et al.,* 1997). Supplementation with intravenous fluids may be necessary to prevent dehydration and significant concentration of the blood if the patient is unable to maintain oral intake. A platelet transfusion is indicated in rare case if the platelet level drops significantly (below 20,000) or if there is significant bleeding.

The presence of melena may indicate internal gastrointestinal bleeding requiring platelet and/or red blood cell transfusion. Aspirin and non-steroidal anti-inflammatory drugs should be avoided as these drugs may worsen the bleeding tendency associated with some of these infections. Patients may receive paracetamol preparations to deal with these symptoms if dengue is suspected (CDC 2007). In Brazilian traditional medicine (Alternative Medicine), *Uncaria tomentosa* (cat's claw herb) is used to treat patients with dengue. In Philippines, the *Euphorbia hirta Linn* (tawa-tawa herb) is used to treat patients with dengue (Rothman, 2004).

2.7.5 Vaccine for Dengue Viruses

Work on dengue vaccine begun in the 1970's, but progress has been slow. One of the major challenges is that unlike yellow fever, dengue is not caused by a single virus but by 4 distinct viral serotypes. Each is equally infectious and pathogenic and the catch is that once a person's immune system has fought off and memorized an infection by one dengue serotype, a secondary infection with a different serotype is thought to be the biggest single risk factor for developing DHF (Halstead, 2007).

Lack of a good animal model with which to study dengue and DHF is another problem. Mice get infected but they do not develop the disease. Although monkeys produce antibodies against the virus and sometimes develop fever, they never get the hemorrhagic form. Hence, research into dengue vaccines involves a largely blind leap from the test tube to clinical trials (Vaughn, 2000).

Despite the obstacles, dengue vaccines are now edging towards clinical use. Two of them, one developed at Mahidol University in Bangok, Thailand (Bhamarapravati and Sutee, 2000) and another at Walter Reed Army Institute of Research in Silver Sporing, Maryland (Eckels *et al.,* 1980) are based on living weakened dengue viruses, selected by growing all four serotypes through multiple generations in cultured cells. The Bangok vaccine is licensed to Aventis Pasteur of Lyon, France. Safety and efficacy trials show that the vaccine is safe and protects up to 90% of recipients from all four serotypes. The Walter Reed vaccine is licensed to GlaxoSmithKline Biologicals in Rixensart, Belgium (Chang *et al.,* 2001).

2.8 Dengue virus infections in Kenya

Although dengue epidemics are infrequent in Eastern Africa when compared with South-East Asia, the Americas and the Caribbean, all four serotypes of dengue have caused outbreaks in this region. The dengue virus in East Africa was first isolated in 1982 after an epidemic of febrile illness in the Kenyan coastal towns of Malindi and Kilifi and was found to be dengue 2 virus serotype (Johnson *et al.,* 1982).The clinical presentation was consistent with classical dengue fever, with no severe dengue reported (Metselaar, 1980). Since then there have been sporadic cases of dengue reported in Kenya (unpublished observations) and a serology survey carried out in 2005 (unpublished) revealed the occurrence of dengue transmission in coastal and inland parts of Kenya. The 1982 outbreak in Kenya is believed to have spread from the Seychelles

outbreak that occurred between 1977 and 1979 (Metselaar, 1980). However, dengue has been reported in Sudan, Djibouti, Seychelles, Somali and Comoros which are neighboring countries to Kenya.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site description

The study was carried out in Trans Nzoia region, with Trans Nzoia District as an administrative District of Rift Valley Province, Kenya. Located between the Nzoia River and Mount Elgon, Kitale is the town center. The district has three constituencies: Cherangani Constituency, Kwanza Constituency and Saboti Constituency. Samples from both outpatients and inpatients were collected from Kitale District Hospital (D/H), Endebess District hospital (DH) and Anderson Medical Clinic (AMC).

3.2 Study design

This was a hospital based cross-sectional study done between November 2009 and November 2010. Sections of the population who attended the hospitals and gave consent were tested and the data obtained was used to determine the seroprevalence.

3.3 Sample size

There is some evidence that in many parts of Kenya the seroprevalence of certain arboviruses ranges between 0 and 3% (Morill *et al.,* 1991). Using an expected prevalence of three percent, the sample size was estimated using Fisher's Exact Test

(1983) to determine the minimum number of samples required to obtain the true seroprevalence of dengue 2 virus in the region.

- $N = Z^2 \ X \ pq / d^2$

N = Desired sample size (if target population is greater than 10,000)

Z = Standard normal deviation at the required confidence interval (1.96)

p = proportion in the target population estimated to have measured character (q = 1 – p)

d = level of statistical significance at 95% confidence level = 0.05

$$n = \frac{1.96 \times 1.96 \times 0.03 \times 0.97}{0.0025}$$

= 44.72 = 45

Samples were collected from 3 facilities hence the total number of patients recruited for the study were:

45x3 = 135

A total of 1205 samples were collected in the study but 84 were missing or hemolyzed hence 1121 were tested. This was because the samples were collected as part of a larger study. A sample size of 1121 was used to determine the true

seroprevalence of dengue 2 virus infections in Trans Nzoia. This was supported by the fact that when one has a higher sample size, the likelihood of encountering Type-I and Type-II errors occurring reduces, at least if other parts of the study are carefully constructed and problems avoided. Higher sample size allows the researcher to increase the significance level of the findings, since the confidence of the result are likely to increase with a higher sample size. This is to be expected because
the larger the sample size, the more accurately it is expected to mirror the behavior o f the whole group (Kalla and Siddharth, 2009).

Data on calculation of seroprevalence of the positive cases of Dengue 2 virus was based on the following model:

Prevalence = (a/a+b) x 100%

Where: a= Number of individuals in the population with the disease at a given time.

b= Number of individuals in the population at risk of developing the disease at a given time, not including those with the disease.

3.4 Inclusion criteria

The study recruited patients presenting with *febrile illness* (fever of 37.5^{o}C and above), patients who gave consent for sample collection and signed the informed consent form (Appendix II) as proof of consent and those 5 years of age and above. To be infected by

dengue virus, one needs exposure to mosquitoes, which implies that all persons are eligible. However, it is expected that the immune system is well developed in older children, for which reasons an arbitrary age cutoff of 5 years was used.

3.5 Exclusion criteria

The study did not include patients without febrile illness and children below 5 years of age.

3.6 Social – demographic and clinical characteristics

Data on social – demographic and clinical characteristics was obtained through the use of a questionnaire which was administered by the clinician in the hospitals (Appendix I).

3.7 Sample collection, transportation and processing

A venous blood sample (5ml) was collected in a vacutainer tube containing no anticoagulant, centrifuged at 3,000 rpm for 5 minutes, and the serum stored in a refrigerator at -80°C. The samples were collected once a month and transported to the Biosafety level (III) laboratory on dry ice. In the PIII laboratory at the Centre for Microbiology Research (CMR) the serum was aliquoted into four cryovials. Two cryovials were used for virus isolation, plaque reduction assays and two tubes were used for ELISA.

3.8 Laboratory methods

3.8.1 Indirect IgG, IgM and IgA capture ELISA

An Immunoglobulin G, M and A (IgG, IgM and IgA) capture ELISA (MAC-ELISA) was conducted, with modifications (Bundo and Igarashi, 1985). Briefly, test, positive and negative control sera were diluted 1:100 and 1:1000 in phosphate buffered saline (PBS-T) buffer and dispensed into microtitre wells already coated with goat anti-human IgG, IgM and IgA (Cappel, USA). The control wells contained anti-flavivirus antibody negative serum; blockace; no anti-human IgG, IgM and IgA, or PBS-T buffer only (Figure 3.2). The final reaction product was measured in an ELISA reader at a wavelength of 492 nm. Positive/Negative (P/N) values were determined, and a factor of one or more was considered positive.

3.8.1.1 IgG, IgM and IgA indirect ELISA Procedure

Phosphate buffered saline (PBS) 0.1M, (Sigma) solution pH 7.45 was prepared by adding 10 tablets (10mg/tablet) of PBS into one litre of distilled water (1 tablet/100 ml water) and autoclaved. This solution was used as a diluent for preparation of virus dilution (antigen solution) which was from the strain 00st-22A isolated from dengue 2 virus from an acute patient in Philippines in the year 2000.

Additional buffers prepared were:

1) PBS, 0.05% Tween 20 (PBS-T) for ELISA plate washing. PBS-T consists of 0.5ml of tween 20 in 1000ml of PBS

2) PBS, 3% FCS (PBS-F) for blocking, serum dilution and antibody dilution. This was purchased from sigma and was heat inactivated at 56□C for 30 minutes.

Virus dilution with PBS 1:500 was made and used to coat ELISA plate. Virus solution (in PBS), 100 µl was added into each well of 96-well ELISA plate in rows A, B, E and F except in the last two wells of E and F (figure 3.1) in which 100µl of positive control was added. This was followed by addition of 100 µl of PBS, 3% FCS (PBS-F) into each well of 96-well ELISA plate in rows C, D, G and H (for background reaction to be subtracted later), the plates were wrapped in Para film and stored at 4 °C overnight.

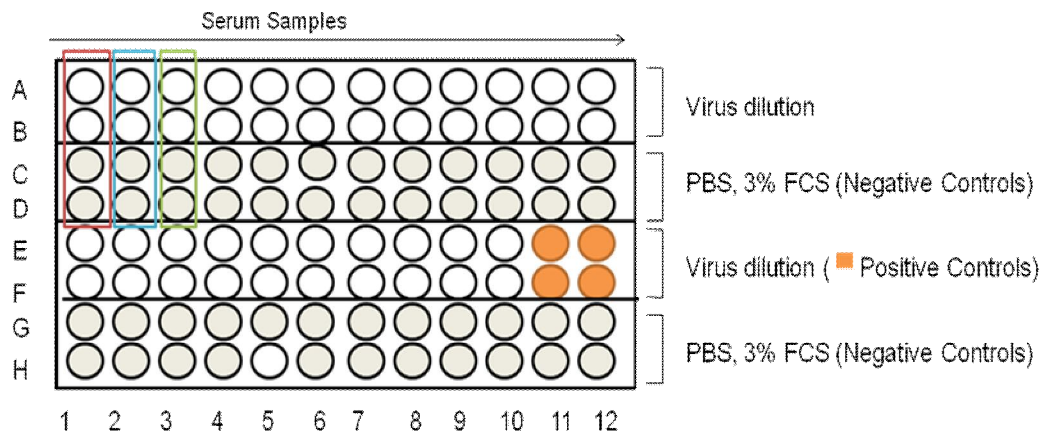


Figure 3.1: Layout of a 96 well plate ELISA procedure

The following day, the virus solution was discarded from the plate by tapping and washing three times with PBS-T using an ELISA washer. This was followed by addition of 100 µl of PBS, 3% FCS (PBS-F) (Blocking solution) into every well of the plate and

incubated for 30 minutes at room temperature. Blocking solution was discarded by tapping the plate and 100 µl/well of diluted patient serum added in duplicate into virus antigen coated wells and also duplicate in PBS, 3% FCS coated well. The plate was then incubated for one hour at 37°C. Serum dilution was discarded from the plate by tapping and plate washed three times with PBS-T. After adsorption of the plate on an adsorbent paper, 100 µl/well of diluted antibody solution (1:5000 with PBS, 3% FCS) was added in every well and then the plate incubated for one hour at 37°C.

Antibody solution from the plate was discarded by tapping and the plate washed three times with PBS-T. After blotting the plate on the paper, 100 µl/well of 2M *o*-phenylene diamine (OPD), (sigma) solution was added in every well. The plate was incubated for 15 minutes at room temperature in the dark. Stop solution 100 µl/well of one normal (N) Sulfuric acid was added to stop the reaction. The optical density (OD) was measured within 20 minutes after adding the stop solution. The OD was then measured with an ELISA plate reader at 492 nm.

OD specific for DEN-2 was calculated as:

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

If the specific OD reading was more than 1.0, that serum was regarded as anti-DEN – 2 virus antibody positive.

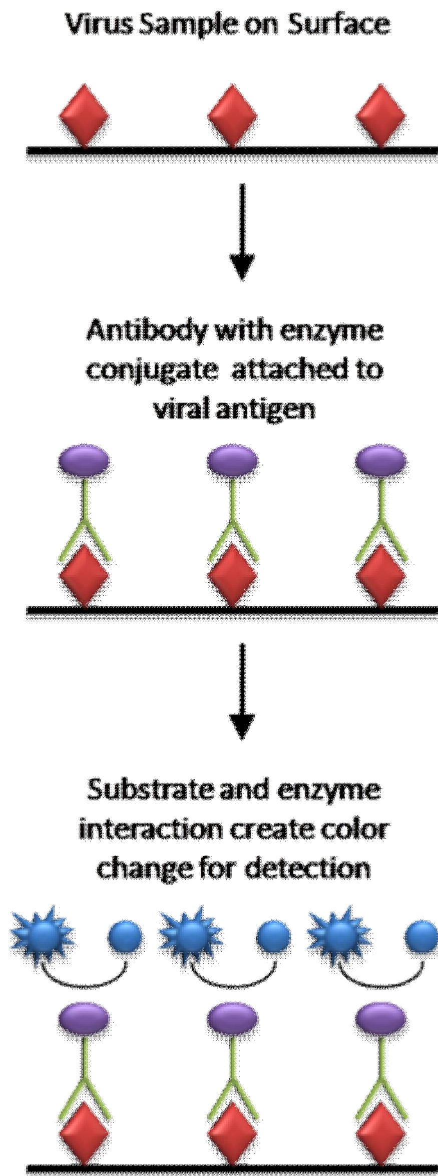


Figure 3.2: Indirect ELISA process

(Source: Kaufmann and Kabelitz, 2002)

3.8.2 Plaque reduction neutralization test (PRNT)

A plaque reduction neutralization test (PRNT) was used to confirm the presence of dengue 2 virus specific neutralizing antibodies in test sera already regarded as positive by ELISA.

3.8.2.1 PRNT Procedure

PRNT plates were made by seeding Vero cell line at a concentration of 1 – 2 x 10^5 cells/ ml into 6-well plates (Nunc) at a volume of 2 ml/ well. Cells were cultured in growth medium for 5 – 6 days at 37ºC, 5% CO_2. The cells were observed under the microscope and if Vero cells had made an even monolayer on the bottom of the 6-well plate (Cells should cover at least 80% of the surface), that was the timing to perform PRNT.

Test serum was diluted with maintenance medium (MM) (EMEM, 2% FCS, P/S supplemented) into 1:20 dilutions. At the same time, standard virus dilution of 2000 PFU/ml made with MM. Then equal volume of serum dilution and standard virus dilution was mixed. For positive control wells, equal volume of MM and standard virus dilution were mixed together.

The virus-serum mixture was incubated for one hour at 37ºC. Thereafter, 1 ml of culture medium was aspirated from each well of the 6-well plates (leaving 1 ml), following which 100 µl/well of serum/virus mixture was added to Vero cells in duplicate wells. Plates were incubated for 1.5 - 2 hours in the 37ºC, 5% CO_2 incubator. Overlay medium

(EMEM, 2% FCS, 1.4% Methylcellulose, P/S supplemented) added into each well, and incubated the plates at 37ºC, 5% CO_2 for 2-7 days.

On harvesting of the plate, overlay medium was removed as much as possible with a plastic pipette (discarded overlay medium into 2% SDS solution and autoclaved later). Plates were fixed by adding 1 ml of 10% formaldehyde in PBS (1/10 diluted Formalin with PBS) over the cell and incubated for one hour at room temperature in the safety cabinet with UV light on to fix the plate completely.

Formaldehyde was removed with a plastic pipette and discarded into a specific waste bottle. The surfaces of the plates were wiped with 70% Ethanol. The plates were washed twice with tap water. The plates were blotted on paper and staining solution (0.5 ml of 1% Crystal Violet solution in water) added and waited for 5 minutes at room temperature. The dye was discarded and the plates washed gently with tap water. The plates were adsorbed on paper and air-dried at room temperature. The plates were examined for the formation of plaques which were counted for each set of duplicate wells and the percentage reduction calculated by comparing with the positive control virus well (100% plaque formation). More than 90% plaque reduction was regarded as positive.

3.8.3 Optimization of assays

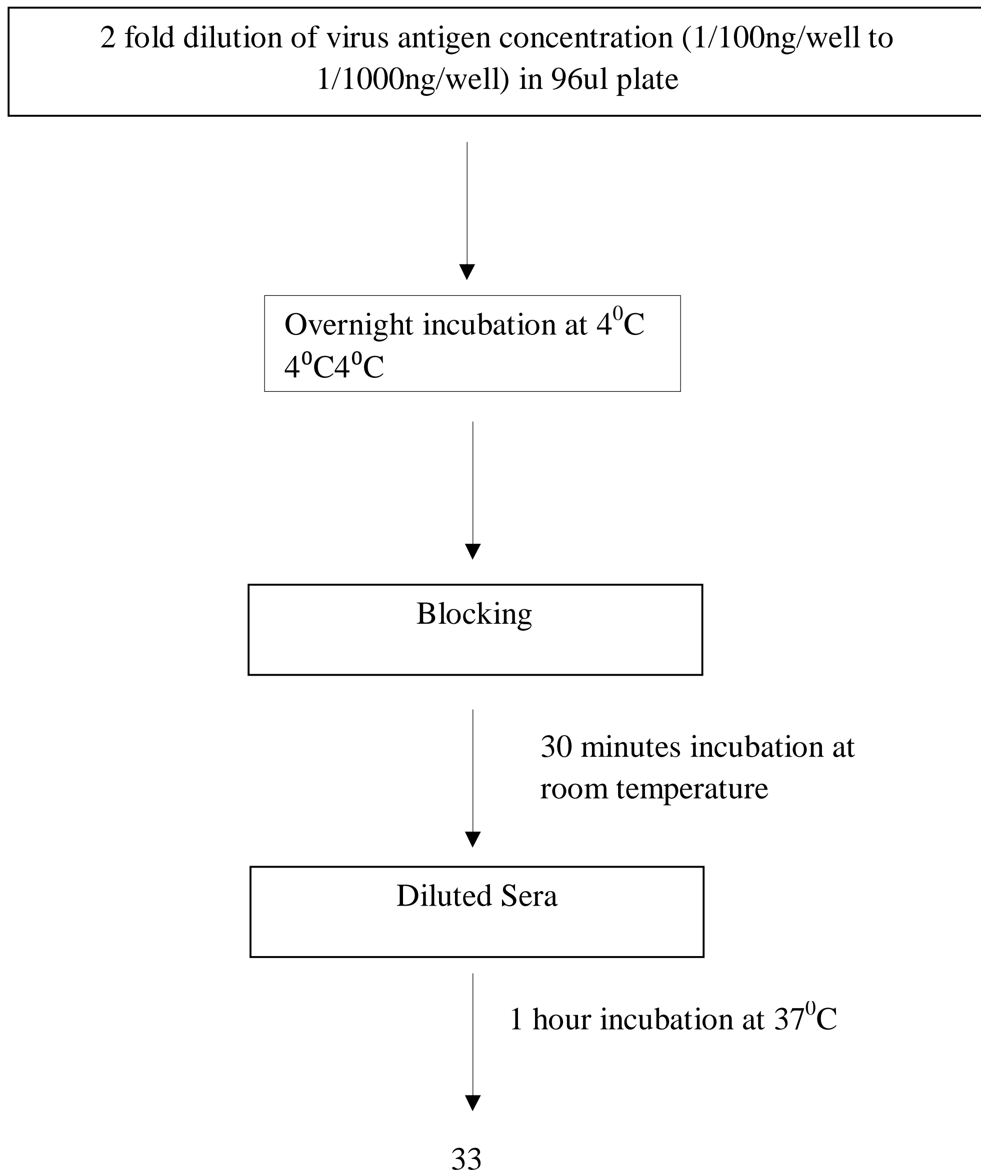
3.8.3.1 Indirect IgG, IgM and IgA capture ELISA

ELISA was optimized through duplicate two fold varying dilution of virus antigen concentration into microtitre well plates with the order 1/100ng/well (1/200ng/well, 1/400ng/well up to 1/1000ng/well) and incubated at 4° C overnight. Virus solution was discarded from the plate by tapping and washing the plate three times with PBS-T using an ELISA washer. Blocking solution 100 µl of 3% FCS was dispensed into every well of the plate and incubated for 30 minutes at room temperature. Blocking solution was discarded by tapping and 100 µl/well of diluted standard sera added into the virus antigen coated wells. The plate was then incubated for one hour at 37°C. Serum dilution was discarded from the plate by tapping and the plate washed three times with PBS-T. After adsorption of the plate on adsorbent paper, 100 µl/well of diluted antibody solution (1:5000 with PBS, 3% FCS) was added in every well and then the plate incubated for one hour at 37°C.

Antibody solution from the plate was discarded by tapping and the plate washed three times with PBS-T. After blotting the plate on the paper, 100 µl/well of substrate solution was added in every well. The plate was incubated for 15 minutes at RT in the dark. Stop solution 100 µl/well of one normal (N) Sulfuric acid was added to stop the reaction (Figure 3.3). The optical density (OD) was measured within 20 minutes after adding the stop solution. The optical densities (OD) were measured using an ELISA reader at a wavelength of 492nm and the cut off point for positive and negative determined by

calculating positive and negative (P/N) ratio [OD of positive serum/OD of negative serum] and difference [OD of positive serum – OD of negative serum] for each combination.

The dilution with the highest P/N ratio and difference was selected for determination of the cut off points.



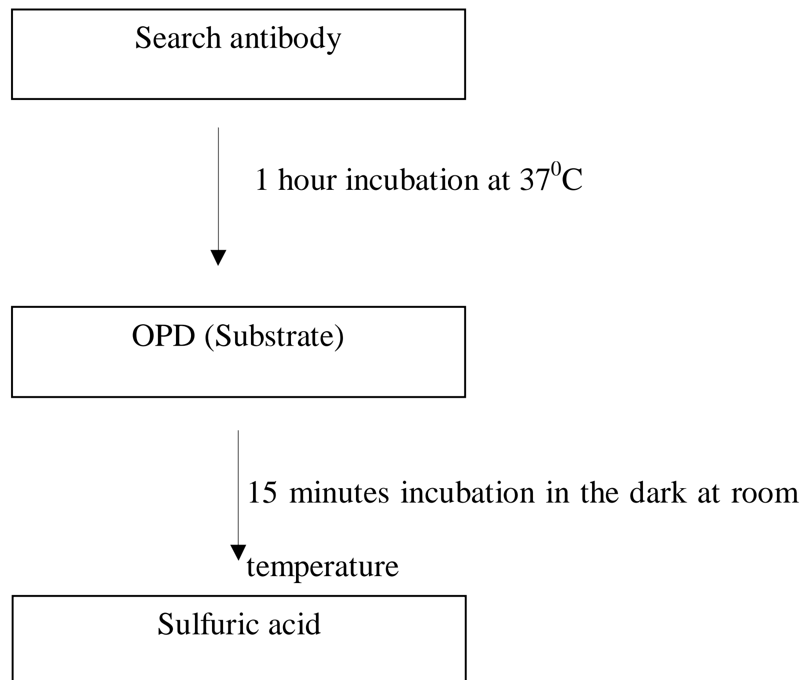


Figure 3.3: Elisa optimization process

3.8.3.2 Plaque reduction neutralization test (PRNT)

Two fold dilutions of the test serum samples and the standards (negative and positive control sera) were prepared. Vero cells (Seeded volume of cells) was prepared on a 24 well plate and reacted with the samples (test and controls) through inactivation at $56^{o}C$ for 30 minutes. Stock virus was diluted to a predetermined titer to 200 plaque forming units (pfu)/0.1ml in virus diluents, then mixed one volume of each diluents sera (100 µl) with equal volume of the diluted virus (100 µl) (virus control: diluted virus + equal volume virus diluents). The mixture was incubated at 37° C for one and a half hours.

Six well plates were seeded with Vero cells, 200,000 cells/ml, and grown to over 80% confluence. The incubated virus and sera were then inoculated into the six well as shown in figure 3.3. The plate was incubated in a 5% CO_2 incubator at 37° C for one hour. 3

mls of 1.25% Methylcellulose were used as an overlay and the plates incubated in a 5% CO_2 incubator at 37° C (Figure 3.4). The plates were harvested after three days. Fixed with 10% formalin and stained with 0.5% crystal violet in 99% ethanol.

Estimation of serum PRNT titer was done through counting the number of plaques and calculating the percentage plaque reduction for each serum dilution using the number of plaques in the control as 100%. Reciprocal of this dilution was 50% PRNT titer ($PRNT_{50}$) and at this dilution, the sera were considered to contain one $PRNT_{50}$ unit of neutralizing antibody.

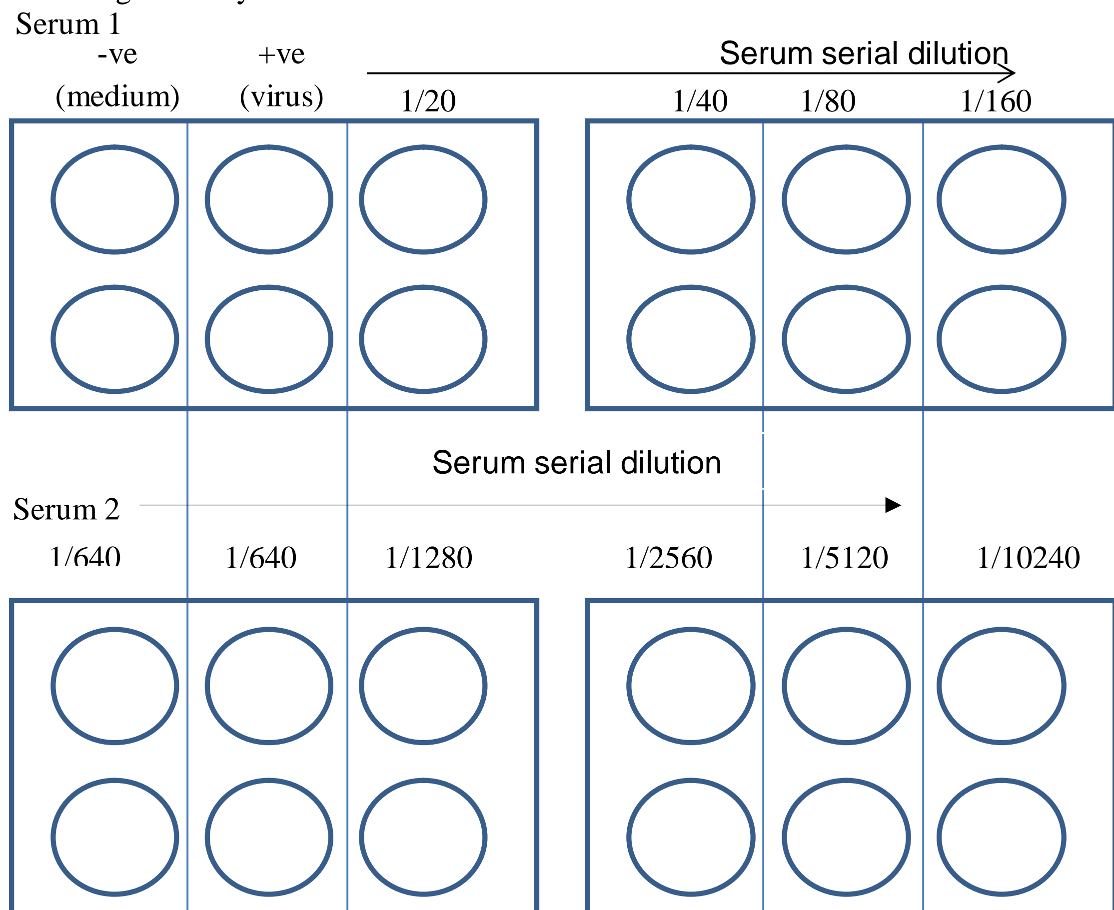


Figure 3.4: Layout of PRNT optimization process

3.9 Ethical Considerations

The study was undertaken only after obtaining approval from both the Scientific Steering Committee (SSC) of the Kenya Medical research Institute (KEMRI) where the work was done, and from the National Ethical Review Committee (ERC) which grants approval for research studies involving human subjects under the SSC No.1626.

Informed consent was sought from all participating children and adults by the clinician. In the case of children, consent was sought from their parents or guardians. Samples from volunteers were identified only by the Clinic Code Number and their names did not appear in the final data files. All those who consented were given the benefit of additional testing, whose findings might help explain their illness. It was clearly explained to participants that the blood drawn was not a treatment. It was also explained that the diagnostic procedures that were carried out are still research in nature, although they had a high predictive value. Patients were at liberty to refuse consent with or without explanation, and without penalty or prejudicial action towards them.

3.10 Limitations of the study

Lack of a native antigen or isolate, limited information about dengue 2 virus in Kenya and lack of a gold diagnosis method in the country limited the study.

3.11 Dissemination of findings

The results were returned to the attending clinician, but due to the nature of the assays, these results could not be returned in real-time. If it turned out that dengue 2 virus is a

common cause of febrile illness in a particular area, then clinicians will be able to justify this as one of their differential diagnosis in febrile illness.

3.12 Data management and analysis

The labels placed on the samples collected had Out Patient/In Patient numbers, but no patient names. Identification of the patients through the sample identification was not possible, but the clinician filtering the patients was able to link the result. Patient details included exact domicile for purposes of mapping probable clusters of high dengue 2 virus prevalence. This data was linked to the results of the laboratory tests performed and stored in an access 2007 database and analyzed using Pearson's correlation coefficient and SPSS for Windows.

CHAPTER FOUR

4.0 RESULTS

4.1 Patient social-demographic characteristics

The study was undertaken in Trans Nzoia region on patients who visited the selected health facilities out of which 1086 (97%) patients were natives of Trans Nzoia region and 35 (3%) of the patients were from other different parts of the country. Patients came from 29 districts in the area out of which dengue 2 virus infection was found to be in 12 (41.4%) of those districts. These are the districts which had positive patients with Trans Nzoia West and East recording the highest number of positive patients of 22 (44%) and 9 (18%) respectively.

The highest percentage of positive patients was women (Fig.4.1). Age interval of 5 years was chosen to enhance analysis of the data (Fig.4.2). Most of the patients were in the 26-30 years age bracket, and had majority of those positive.

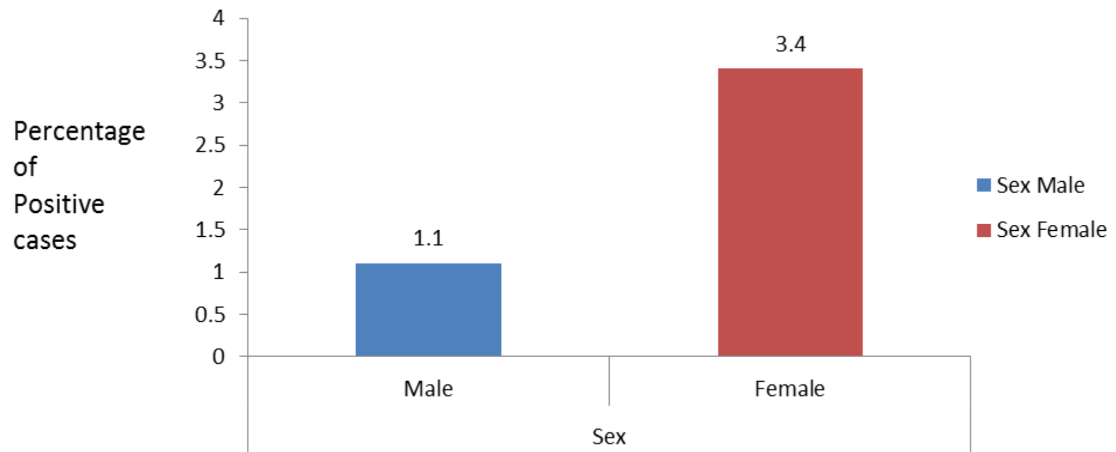


Figure 4.1: Percentage of dengue 2 virus positive cases by sex

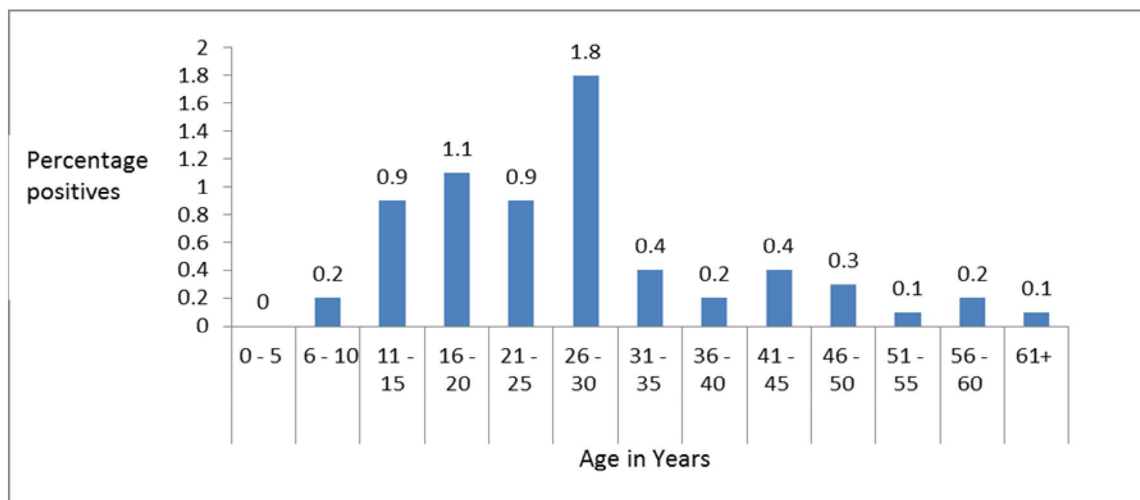


Figure 4.2: Percentage of positive dengue 2 patients by age bracket

4.2 Sample characteristics

A total of 1205 samples were collected from the sites with 570 (47.3%) from Kitale District Hospital, 470 (39%) Andersen Medical Centre and 165 (13.7%) Endebess District Hospital. There were 84 missing or hemolysed samples; hence 1121 samples were analyzed in the study. A total of 5ml venous whole blood was collected from each participant. Serum samples were obtained from whole blood, of which most were normal with a few jaundiced and lipidemic.

4.3 Clinical presentation

There were seven symptoms presented by the patients, with some of the symptoms more common than others (Table 4.1). Fever was the most common symptom in all the patients with 35 (3.1%) positive and 555 (49.5%) negative patients reporting fever which was between an average range of 37.8 -38.8 ºC.

Table 4.1: Frequency of Symptoms in Patients

Presenting Symptoms	Negative	Positive	Total
Fever	555 (49.5%)	35 (3.1%)	590 (52.6%)
Rash	107 (9.7%)	6 (0.4%)	113 (10.1%)
Eye Infection	73 (6.5%)	4 (0.4%)	77 (6.9%)
Jaundice	59 (5.2%)	2 (0.2%)	61 (5.4%)
Headache	259 (23.1%)	15 (1.3%)	274 (24.4%)
Meningitis	3 (0.3%)	1 (0.1%)	4 (0.4%)
Bleeding from mucosal sites	2 (0.2%)	0 (0%)	2 (0.2%)
Total	1058 (94.5%)	63 (5.5%)	1121 (100%)

4.4 Screening for exposure

A total of 1121 patients were screened for Dengue 2 virus infection from Trans Nzoia by Enzyme Linked Immunosorbent Assay (ELISA) which is a specific test. The patients were from the 3 selected health facilities in Trans Nzoia region. The blood samples

extracted from the patients were analyzed, out of which 50 (4.5%) were found to be positive (Fig. 4.3). Kitale district hospital had the highest positive cases followed by Andersen medical clinic. Endebess district hospital had only two cases.

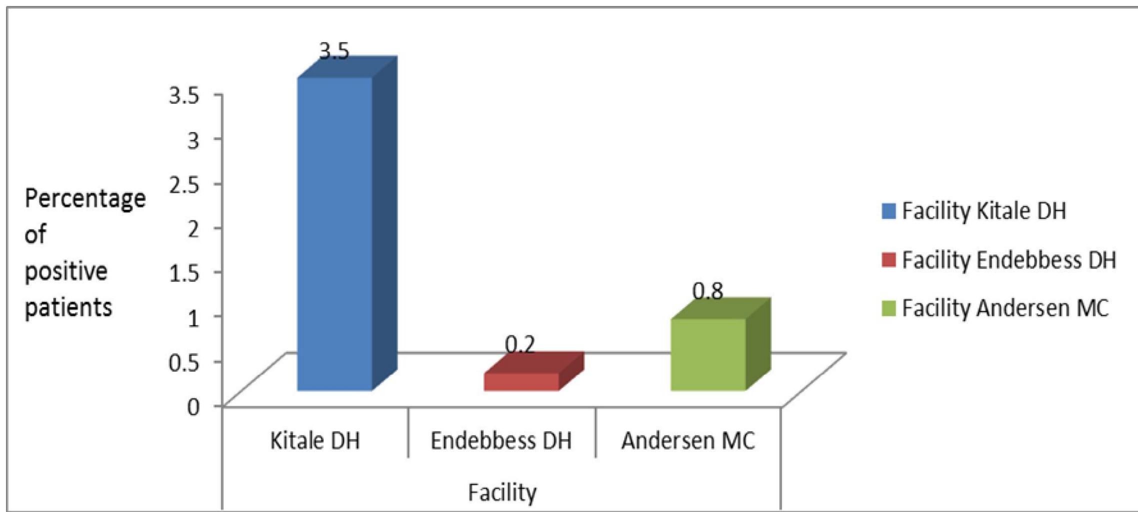


Figure 4.3: Percentage of positive patient's in each of the selected health facility

4.5 Seroprevalence calculation

The seroprevalence of Dengue 2 virus by mathematical calculation was found to be 0.9 % in Trans Nzoia.

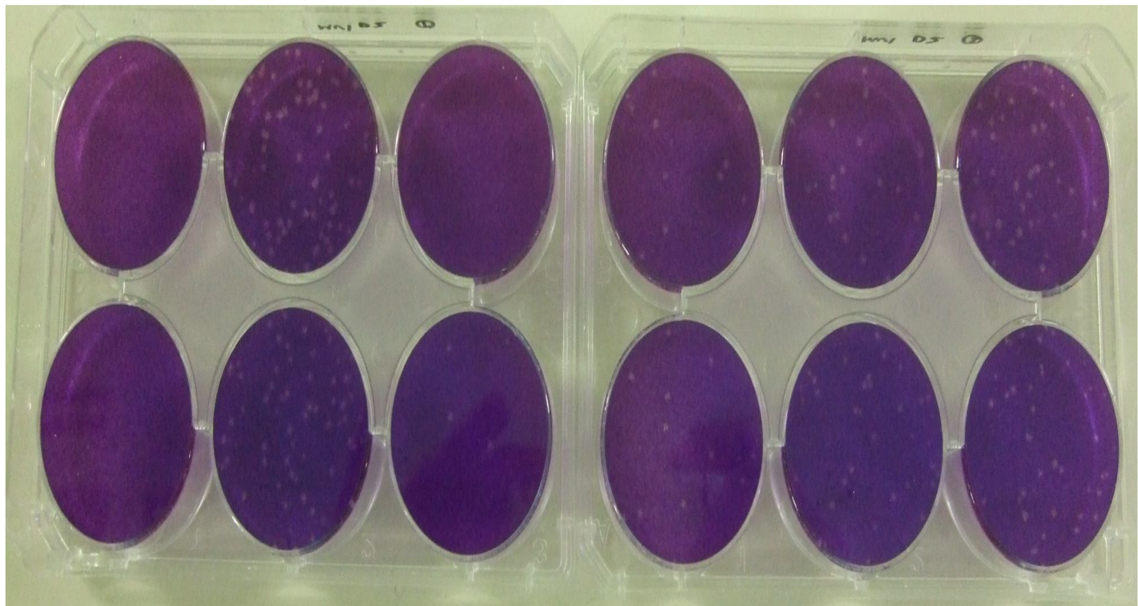
4.6 Correlation of clinical and laboratory diagnosis

Mathematically the correlation was calculated using the Pearson correlation coefficient (Appendix IV) as: $1121(1058x63) – (1058x63)/ (\sqrt{}1121x395378 – (1058)^2 \sqrt{}1121x1507 – (63)^2) = 2.7$ ($p< 1$)

The correlation was not statistically significant as it gave a correlation of 2.7.

4.7 Optimization of assays

Plaque reduction neutralization test (PRNT) optimized best when there was no plaques formed in the 6-well plates. 50% neutralization was considered to contain $PRNT_{50}$ and 75% neutralization considered to be partial neutralization.



C V 1/80 1/160 1/320 1/640

C: negative control (cell culture medium only), V: positive control (virus standard only without serum) 70% reduction of the number of plaques was the cut off of positivity in this assay. So the titre of neutralizing antibody was regarded as 320.

Plate 4.1: Layout of a 6-well plate PRNT optimization

4.8 Confirmation of positive samples by plaque reduction neutralization assay (PRNT)

Out of 1121 samples, 5 (0.4%) completely neutralized indicating they were positive. The others did not completely neutralize meaning they were negative (Table 4.2).

Table 4.2: Patient's status - PRNT status tabulation

	DENGUE – PRNT			Total
DENGUE-STATUS	No Neutralization	Partial Neutralization	Neutralization	
Negative	1068	1	2	1071
Positive	43	2	5	50
Total	1111	3	7	1121

4.9 Borderline testing

Out of the 3 borderline cases by ELISA, one completely neutralized on PRNT. This was the reason for one partial neutralized and two neutralized negative samples (Table 4.3) and were included in the calculation to determine the seroprevalence.

Table 4.3: Borderline Neutralized Samples

	Borderline Neutralized Samples	
Sample	**Optical Density**	**Neutralization**
1	0.81	Complete
2	0.94	Partial
3	0.77	Partial

Table 4.4: Sensitivity and specificity of assay

		PRNT		
		Positive	Negative	**Total**
ELISA	Positive	7	43	50
	Negative	3	1068	1071
	Total	10	1111	1121

Positive predictive value = tp/(tp+fp)x100=14

Negative predictive value =tn/(tn+fn)x100=99.7

sensitivity=7/10(100)=70

Specificity=43/1111(100)=3.9

Sensitivity of the PRNT assay was 70% while the specificity was 4%.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The study was carried out in Trans Nzoia region which is in the Rift Valley Province. Presence of patients from other provinces in this region is an indication of interaction of people from different regions of the country. Patients from the other provinces captured in the study could have been visiting the area or are in the region on the basis of work.

Majority of patients visiting the selected health facilities were from Kwanza (16.7%) and Trans Nzoia West (12.8%) districts, which is the reason for the majority of positive cases in these districts. Trans Nzoia East had the second highest number of positive patients with nine out of 48 patients (18.8%) testing positive for dengue 2 virus infection in that area. Dengue 2 virus positive patients have febrile illness forcing them to visit the hospitals for treatment. This is similar to a study carried out by Gubler, (2002) which showed that febrile illness was the cause for patients with dengue virus infection to visit the hospital.

Kitale town, where most of the positive cases (3.5%) were reported is the biggest and most developed in the Trans Nzoia region. The low incidence of positive cases in some of the districts may be due to low virus circulation in these areas. These findings are

similar to a study carried out by Gubler, (1999) which found that areas with low virus circulation had low transmission of the virus.

Kitale District hospital is the largest hospital in the region. It is equipped with good medical facilities, which means that most of the advanced and complicated illnesses are referred there, hence, the reason for the hospital having the highest number of patients with the infection. Andersen Medical Clinic (AMC) is in a remote area at the Kenya - Uganda border and is located in a rural setting. It has vastly populated environs with dense vegetation and large tracks of land covered by forest. The hospital is also privately owned and is equipped to handle majority of the diseases found in the area. People in the area visit the hospital when sick because the rest of the health facilities are far away. The hospital also serves patients from the neighboring Uganda. This explains why the hospital had the second highest rate of infection. Endebess District Hospital (END) is between KDH and AMC and had only two positive patients (1.2%). This is explained by the fact that the patients who are ill attended the other selected health facilities. In addition, the hospital is not well equipped to handle the diseases around the area, a probable reason why the hospital had only 165 (14.7%) patients participating in the study. The location of the health facility is also not convenient to the locals. Although it is located at the heart of Endebess, people living far from the market had an option of visiting either AMC or KDH depending on whichever health facility was nearer to their residence. A study by Rigau *et al.,* (1998) showed that dengue is as prevalent in the

urban settings as in the rural setting. Trans Nzoia region where the study was done covered both rural and urban setting and dengue 2 virus was found in both setting.

Females were more infected (56.8%) than males due to their indoor and outdoor activities that expose them to the virus, such as fetching water, fire wood and cleaning the homesteads, which are areas that enhance the spread of the virus (Halstead, 2007). The ages of 26 – 45 years are regarded as the most highly active, productive and people mostly on transit. These patients might have been infected during transit where they had visited and carried the virus with them and the symptoms developed when they had returned home. This scenario was similar to the report by Gubler (1999) who categorized the disease as being an occupation hazard. Patients in the age bracket of 46 – 50 years are at the edge of productivity and retirement; hence the reasons for higher infection rates in the years immediately before and after apply.

The seroprevalence of dengue 2 virus in Trans Nzoia was found to be 0.9%. A similar survey conducted during September 1987 for evidence of human arboviral infections in the Coast Province in Kenya revealed that the seroprevalence of dengue 2 virus was 1.0% (Morill *et al.,* 1991). This shows that the infection is still active and there is a possibility of spreading from one area to another. Various reasons have been advanced to explain the emergence of the virus and other arboviruses. These include increasing migration to the area by people from disease endemic regions, a high level of vector infestation, lack of eradication measures and changing climatic patterns (Morill *et al.,*

1991). Most importantly, it may well be that the encroachment of human populations into bush and forest areas leads to increased exposure to the virus.

Presence of dengue 2 virus in Trans Nzoia region gives a strong indication that dengue has emerged in locations once considered to be dengue free (Gubler, 2002). The seroprevalence shows that dengue 2 virus is an emerging infection considering that reported cases of dengue are an under-representation of all cases when accounting for subclinical cases and where the patient did not present for medical treatment. Although DHF has never been reported in Kenya, it could eventually occur if dengue infections are not controlled. This is supported by the fact that, at first it was thought that dengue virus was transmitted only by mosquitoes of the *A. aegypti* species which bite at night but *A. albopictus* species which bite during the day have also been shown to transmit the virus (Gubler, 2002).

The correlation between the clinical and laboratory diagnosis was not statistically significant. This is because the Pearson's correlation coefficient only detects linear dependencies but the clinical presentations are similar to the one from Kautner *et al.,* 1997 study of dengue virus infection. This strengthens the reliability of the laboratory findings of this study.

Frequency of fever was high in most of the patients (52.6%). Majority of the symptoms such as fever, rash, eye infection, jaundice and headache were present in all positive patients with little occurrence of meningitis and bleeding. This was due to the fact that

the positive patients had not developed the severe form of dengue hemorrhagic fever. The fact that patients had not been given any medication for dengue infection indicated a strong immune system that was able to fight and clear the infection early enough hence preventing the disease from progressing to the severe form. Presence of these symptoms emphasized the importance in clinical diagnosis of dengue 2 virus infection which was confirmed by laboratory diagnosis. The symptoms were the same as described by Gubler, 1999 study.

Neutralizing antibodies are secreted by B cells produced in the bone marrow, which primarily respond to infection involving a particular antigen and proliferate to produce a colony of cells and clear away the infection, and the rest persist as the memory cells that can survive for years or even a life time (Parham, 2005). Plaque Reduction Neutralization Test (PRNT) was done to check for the presence of neutralizing antibodies in the patient's serum. Lack of neutralization in most patients was due to the fact that the patients had been acutely infected for the first time; hence no memory cells had been formed. The type of B cells present at this stage of infection is usually the B – 1 cells, which express IgM in greater quantities than IgG, and its receptors show polyspecificity, meaning that they have low affinities for many different antigens, but have a preference for other immunoglobulin's, self-antigens and common bacterial polysaccharides (Montecino *et al.,* 2009). The IgM antibodies produced during the early phase of infection were produced in low numbers, such that they could not neutralize the virus by making them easy targets for phagocytes and activation of the complement

system. Cross contamination cannot be completely ruled out, since ELISA is an enzyme based assay and there could have been cross reaction of antigens leading to false positives.

Complete neutralization proved the presence of neutralizing antibodies which are formed from activated B cells that are specific to the virus encountered during the primary immune response. These cells are able to live for a long time and can respond quickly following subsequent exposure to the same virus (Bona *et al.,* 1996). Duration of the neutralizing antibodies in this study is not likely to be life long, (although in literature, infection with dengue virus provides lifetime immunity) due to the partial neutralization of some of the positive samples because the partiality could be caused by the fact that these patients had been infected a long time ago and the immune system is deteriorating, hence the reduction in the number of neutralizing antibodies. Some of the patients had been infected once and after the memory cells were formed, no subsequent infections with the same virus occurred; hence the neutralizing antibodies were not significant in the immune system and are now slowly deteriorating.

Complete neutralization and partial neutralization of borderline samples confirmed the presence of neutralizing antibodies in small quantities which could not be detected by ELISA. This means that neutralizing antibodies are present in more people than captured in the study; hence plaque reduction neutralization assays are important in the confirmation of neutralizing antibodies against dengue 2 virus infection.

Optimization of the assays was done for the purpose of developing a functional and effective method for dengue 2 virus diagnosis. Treatment of serum removes complement hence the 56□C for one hour. Setting of virus pfu was done by plaque assay titration and 200 pfu settled on for ease of calculation of percentage neutralization.

Optimal inoculation time was perceived as one hour after poor plaques formed at times 20, 30 and 40 minutes. PRNT assays have a limitation in differentiating IgG and IgM antibodies (Bundo and Igarashi, 1985). The strength of PRNT for dengue work is that the serotypes can be differentiated through the use of virus lysate/antigen specific to each serotype. Optimum serum dilution was determined through comparison with plaques formed by the positive control. Partial neutralization was determined by 75% neutralization of plaques in a 6 well plate. This was done by counting plaques on the well plate with few plaques and dividing the number of plaques by the number of plaques present in the positive control well plate, then multiplying the result by 100. Completely neutralized samples had no plaques in the well plate. The cutoff points for ELISA and PRNT positive and negative controls were similar to the ones got by a study done by Dr. Igarashi (Bundo and Igarashi, 1985).

PRNT assay target neutralizing antibodies while the indirect ELISA captures both neutralizing and non-neutralizing antibodies. Hence in the study there was observed difference between the two assays. Non-neutralizing antibodies do not persist for long in the body hence PRNT remains an indispensable assay and is considered a gold standard for many arboviral diagnostic platforms (Putnak, 2005).

Optimization of the assays was of great importance in the study since there was development of a standard operating procedure (SOP) for both ELISA and PRNT for dengue 2 virus detections in Kenya. This would greatly reduce time and enhance consistency when it comes to diagnosis of this infection in the country. It also creates a foundation for further development and research of these methods for the diagnosis of the virus which is regarded as one of the neglected tropical disease.

Presence of dengue 2 virus is evidenced by the finding of positive patients in the region and neutralization of patient's serum. PRNT detects the presence of neutralizing antibodies which are majorly IgG and not IgM (Moreus, 1994).

5.2 Conclusion

- The seroprevalence of dengue 2 virus infection was found to be 0.9%.
- Clinical information and laboratory diagnosis did not give a significant statistical correlation.
- Optimization of both ELISA and PRNT was successful.

5.3 Recommendations

The following ways if put in place can help prevent, control and reduce or eliminate the rate of spread of the virus:

1. Nationwide surveillance systems should be put in place and seroprevalence of dengue virus determined so that it might help to know the necessary measures needed to control the spread of the virus.
2. Patients who present with febrile illness and are negative for malaria or other common related diseases should be tested for dengue infection and other arboviruses.
3. The optimized methods should be improved to enhance reproducibility.

The study findings reject the null hypothesis which states that there is no dengue 2 virus infection in patients presenting with fever in selected health facilities in Trans Nzoia region.

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APPENDICES

APPENDIX I:

Questionnaire for clients participating in the study

A SEROPREVALENCE SURVEY FOR DENGUE - 2 VIRUS EXPOSURE IN HUMANS AT SELECTED HEALTH FACILITIES IN KENYA

Date of hospital visit ………………………….

Study Number ………………….…

Village ………………………………

Sub-location …………………………….

Location …………………………….

Division …………………………………

District ……………………………….

Province …………………………………

Gender:

Male ( ) Female ( )

Age (years) ( ) Date of birth ( )

Presenting Complaints:

Fever:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Temperature in degrees centigrade: (   )

Muscle pains:

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…………………………………..

Neck pains

Yes (   )   No (   )

Duration of symptoms…………………………………..

Bleeding diathesis:

Yes (   )   No (   )

Duration of symptoms…………………………………..

List any other symptoms

1. ………………………………..

2. ………………………………..

3. ………………………………..

Have you ever been vaccinated for Yellow Fever?

Yes ( ) No ( )

If Yes, when?

Year ( ) Date ( ) Age ( )

Any other remarks:

……………………………………………………………………………………………

……………………………………………………………………………………………

……………………………………………………………………………………………

……………………………………………………………………………………………

…………………………………………………………………………………

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

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

Role of Investigators

Samson Muuo Nzou is an MSc degree student at ITROMID KEMRI and Principal Investigator for this work. He will conduct the assays in this protocol as part of his degree requirements.

Dr. Matilu Mwau (KEMRI) 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 oversee the development of the protocols necessary for this collaboration. He will supervise the postgraduate student, the data collection, laboratory experimentation, data analysis, and dissemination of the findings.

Dr. Toru Kubo (Nagasaki University) is the co-investigator from Nagasaki University. He will provide technical oversight of this project, including the development of the assays and the analysis of samples from the field. He will play a key role in the interpretation of findings and future publications

Dr. Rebecca Waihenya (JKUAT) is also co-investigator from Jomo Kenyatta University of Agriculture and Technology and university supervisor of the Msc student. She will provide the research team with a key link between JKUAT and the KEMRI administration. She will provide technical assistance and advice.

APPENDIX II:

Informed consent document

TITLE OF STUDY: Seroprevalence of dengue virus infections in patients presenting with febrile illness in selected health facilities in Trans Nzoia district, Kenya.

NAME AND INSTITUTIONAL AFFILIATION OF ALL INVESTIGATORS:

Kenya Medical Research Institute (KEMRI) Investigators

Samson Muuo Nzou Principal Investigator, Matilu Mwau Co-investigator.

Nagasaki University Investigators

Toru Kubo Co-investigator

Jomo Kenyatta University of Agriculture and Technology Investigator

Rebecca Waihenya Co-investigator

PRINCIPAL INVESTIGATOR:

Samson Muuo Nzou

Contact: 0733 454 059, 0202722541 ext 2256/2290

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).

INTRODUCTION

The proposed study seeks to validate the seroprevalence of dengue virus infections in clinical diagnosis through screening of 246 samples collected from patients from the Kitale District Hospital, Andersen Medical Clinic and Endebess District Hospital by ELISA and PRNT.

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 2 and a half extra milliliters 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 DEN 2 virus Status. It is also possible that during this analysis, other viruses may be found in your blood. It may also be necessary to conduct some of the procedures in a country other than Kenya.

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 ml) will be collected. Phlebotomy is a well-established procedure that causes only minimal discomfort. For this reason no additional risks and inconveniences are anticipated. There will be no immediate benefits for the patient.

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 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 DEN 2 Virus one needs exposure to mosquitoes implying that all persons are eligible. However, it is expected that the immune system is well developed in older children, for which reasons an arbitrary age cutoff of 5 years will be used.

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

3. Clients who are 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 will also be stored in compact disks and flash disks.

Data Management

As this is primarily a surveillance project, the only data analysis planned is the determination of circulating DEN 2 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 IN THE EVENT OF RESEARCH RELATED INJURY

If you think you (your child) has a medical problem related to this study, please report to Samson Muuo Nzou, Contact: 0733 454 059, 0202722541 ext 2256/2290 or Secretary, KEMRI/National Ethics Committee, P.O. BOX 54840-00200, Nairobi; Telephone number : 020-272 2541; 0722 205901, 0733 400003.

PERSONS AND PLACES FOR ANSWERS REGARDING THE RESEARCH STUDY ITSELF

Contact Samson Muuo Nzou,0733 454 059, 0202722541 ext 2256/2290 or 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 DEN 2 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 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 DEN 2 Virus Status. It is also possible that during this analysis, other viruses may be found in your blood. It may also be necessary to conduct some of the procedures in a country other than Kenya. 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 DEN 2 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. You can discuss with other members as you wish and you will be given a copy of the consent form to go with it.

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 Samson Muuo Nzou 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 DEN 2 Virus.

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

Signature/ Thumb mark---

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

Witness 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 Dengue 2 ambao tunafikiri ni mojawapo ya magonjwa yanayoambukiza jamii hii. Ili tujue umuhimu wa Dengue 2 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 ml 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 Dengue 2 na virusi vingine jinsia. Inawezekana kufanya utafiti huu katika nchi zingine mbali na Kenya. 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 Dengue 2.

Haki za mshirika:

Ushiriki wako katika utafiti huu ni wa hiari kabisa. Ukikataa kushiriki, hutanyimwa huduma zinazotolewa kwa kawaida. Ukohuru kuzungumza na washiriki wengine na utapewa nakala sawia uende nayo.

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, Samson Muuo Nzou 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 Dengue 2.

Sahihi/dole gumba…………………………

Tarehe………………..

Sahihi/dole gumba ya shahidi………………….

Tarehe……………….

APPENDIX IV

Consent forms for Minors

Informed Consent for minors

My name is………..and I work in this health facility. We think that Dengue 2 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 seroprevalence of Dengue 2 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 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 Dengue 2 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 Dengue 2 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. A copy of the consent form will be given to you to go with it.

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 child's rights or this study, you may ask any of the field officers involved in this study or contact Samson Muuo Nzou 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 Dengue 2 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 Dengue 2 Virus ambao tunafikiri ni mojawapo ya magonjwa yanayoambukiza jamii hii. Ili kujua umuhimu wa Dengue 2 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. Baadaye, tutatoa kiasi cha 2.5 ml 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. Damu tutakayotoa kwa huu utafiti itapelekwa maabara kwa upimaji wa Dengue 2 Virus. 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 Dengue 2 Virus.

Haki za mtoto wako:

Ushiriki wa mtoto wako katika utafiti huu ni wa hiari kabisa. Ukikataa ashiriki, mtoto wako hatanyimwa huduma zinazotolewa kwa kawaida. Utapewa nakala sawia uende nayo

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 za mtoto wako ama utafiti huu, unaruhusiwa kuwasiliana na afisa yeyote wa utahini, au Mkuu wa utafiti huu, Samson Muuo Nzou wa KEMRI at 02027222541 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 mtoto wangu ashiriki katika zoezi hili. Naelewa kuwa ushiriki wa mtoto wangu ni wa hiari, na pia kama sitakubaliana muda wowote naruhusiwa kumtoa katika zoezi hili. Natoa ridhaa damu ya mtoto wangu itumike katika upimaji wa Dengue 2 Virus.

Sahihi/dole gumba…………………………

Tarehe………………..

Table 7.1: Clinical and laboratory diagnosis correlation and Mathematical calculation

Presenting Symptoms	Negative (x)	X^2	Positive (y)	Y^2	XY	Mean X	Mean Y
Fever	555	308025	35	1225	19425	151.1429	9
Rash	107	11449	6	36	642		
Eye Infection	73	5329	4	16	292		
Jaundice	59	3481	2	4	118		
Headache	259	67081	15	225	3885		
Meningitis	3	9	1	1	3		
Bleeding	2	4	0	0	0		
	1058	**395378**	**63**	**1507**	**24365**		

$$r_{xy} = \frac{\sum x_i y_i - n\bar{x}\bar{y}}{(n-1)s_x s_y} = \frac{n\sum x_i y_i - \sum x_i \sum y_i}{\sqrt{n\sum x_i^2 - (\sum x_i)^2}\ \sqrt{n\sum y_i^2 - (\sum y_i)^2}}.$$

Where:

χ= number of negative patients

y= number of positive patients

n= sample size

r= result