DOCUMENTATION OF THE EXPOSURE OF KENYAN RESIDENTS TO ZOONOTIC DISEASES

THIS PROJECT HAS BEEN SUBMITTED IN PART FULFILLMENT FOR THE MASTER OF SCIENCE, MEDICAL MICROBIOLOGY, UNIVERSITY OF NAIROBI

BY

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DECLARATION

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DEDICATION

Dedicated to my husband, parents and family who encouraged and supported me throughout the duration of my Master of Science course.

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To Allah who gave me strength and good health all through.

To my supervisors, Prof. Omu Anzala and Dr. Mike Drebot who were a constant help for consultation and advice.

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Thank you.

LIST OF ABBREVIATIONS

CDC: Centre for disease and control

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

FTA: Fluorescent treponemal antibody

IFA: Immunofluorescent antibody

IgM: Immunoglobulin M

IgG: Immunoglobulin G

MS: Microsoft

NML: National Microbiology Laboratory

OD: Optical density

PBS: Phosphate Buffered saline

PCR: Polymerase Chain Reaction

RNA: Ribonucleic acid

rpm: Revolutions per minute

RVF: Rift Valley Fever

SARS: Severe acute respiratory syndrome

SOP: Standard Operating Procedure

SPSS: Statistical package for the social sciences

SST: Serum Separator Tube

TMB: Tetramethylbenzidine

USA: United States of America

WNV: West Nile Virus

CHAPTER I

ABSTRACT

Introduction: Zoonoses can be transmitted from animals to humans, primarily by ingestion, bites (animal or arthropod), scratches, inhalation or direct skin/mucosal contact. They are strongly identified with emerging disease issues and are of global concern due to their link to the environment and the sudden nature in which they appear. In Kenya, it is anticipated that there may be an under-diagnosis and/or under reporting of zoonotic diseases. It is in this context that we needed to determine the prevalence of antibodies against selected zoonoses.

Methods: In a cross- sectional study conducted at Coast Provincial General Hospital and Mbagathi District Hospital between January and March 2011, we determined the seroprevalences against selected zoonoses using Enzyme linked immunosorbent assay (ELISA) from serum samples. Patients with fevers were eligible. In this study, 182 patients were recruited (91 from each hospital). A questionnaire was administered that included assessment of demographics, clinical presentations and history of recent travel. A complete blood count was also done for each patient. The data collected was entered into MS Excel and later analysed using SPSS.

Results: Sixty percent of all patients were positive for serum antibodies against a number of zoonotic disease. Dengue fever virus, IgM prevalence was 8.2% and IgG prevalence was 35.2%. Overall Flavivirus (both WNV and Dengue) IgG prevalence was 63.7%. West Nile fever virus, IgM prevalence was 1.6%. Rift Valley fever virus IgG prevalence was 7.1% and *Coxiella burnetii* IgG prevalence was 5.5%. *Rickettsia rickettsii* IgG prevalence was 2.7% and *Rickettsia typhi* IgG prevalence was 0.5%. Leptospira IgM prevalence was 0.5%. Significant factors were history of recent travel (p=0.010), age (p= 0.004), presenting symptoms (p=0.004) and region of residence (p=0.000).

Conclusion: Dengue fever had the highest serum antibody prevalence in both Mombasa and Nairobi while Leptospira and *Rickettsia typhi* had the lowest serum antibody prevalence. Some of the Dengue IgM positive patients may have been ill due to dengue fever. It is possible that a certain number of dengue acute cases might have already cleared dengue virus

from the sera at the time of testing despite negative PCRs. NS1 testing could be carried out as a supplemental when time and resources allow (33). This study showed that age, history of recent travel and abnormal haematological factors were all risk factors associated with prevalence of serum antibodies against a number of zoonotic diseases.

BACKGROUND

Zoonoses are caused by etiological agents that circulate in vertebrate animal hosts but are occasionally transmitted to humans, primarily by ingestion, bites (animal or arthropod), scratches, inhalation or direct skin/mucosal contact (1). The probable reasons for the emergence of new zoonotic diseases are many and include changes in social, dietary or cultural mores, environmental changes, and the improved recognition of neglected infections often coupled with an improved ability to diagnose infection (2). The migration of humans has provided the route of spread for infectious diseases and zoonoses (for example, plague, yellow fever, monkey pox and severe acute respiratory syndrome) (3). The diagnosis of zoonotic disease is delayed through lack of clinical suspicion or failure to obtain adequate clinical histories. There is an under diagnosis of zoonotic diseases in the Sub-Saharan Africa. This is due to poor disease surveillance coverage, poor diagnostic capacity, the geographical distribution of those most affected and lack of clear strategies to address the plight of zoonotic diseases. Awareness of medical practitioners of zoonotic diseases could be a contributing factor to their under-diagnosis and under-reporting (4). Zoonoses are a serious public health threat in both the developing and the industrialised world due to a complex multifactorial set of changing circumstances. Climatic changes have facilitated the expansion of compatible conditions for some disease vectors, remodeling dynamics for potentially new, emerging, and reemerging zoonoses (14).

CHAPTER II

LITERATURE REVIEW

Agents transmitted from animals to humans are responsible for diseases categorized as zoonotic disease or zoonoses and may include a wide spectrum of infectious (bacterial, viral, parasitic, rickettsial and fungal) and non infectious (prion) agents. Zoonoses are caused by etiological agents that circulate in vertebrate animal hosts but are occasionally transmitted to humans, primarily by ingestion, bites (animal or arthropod), scratches, inhalation or direct skin/mucosal contact (1). Animal hosts may include wild animals, food and farm animals or pets. Animals infected with a zoonotic disease agent may or may not exhibit signs of disease.

Epidemiology

Zoonoses are distributed worldwide and often spread with humans through their domestic animals. However, when humans move to new areas or come into contact with different animal species (eg moving into newly cleared natural forest areas), then new zoonoses may emerge or are recognised, and it is significant that many of the new or newly recognised emerging infections of humans are zoonotic in origin. The probable reasons for the emergence of new zoonotic diseases are many and include changes in social, dietary or cultural mores, environmental changes, and the improved recognition of neglected infections often coupled with an improved ability to diagnose infection. Zoonotic infections may be very localised in their distribution and often reflect particular associations between the natural reservoir hosts and humans – they are thus often influenced by human dietary habits, behaviour and relationships with different animal species (2).

Globalization and climate change have had an unprecedented worldwide impact on emerging and re-emerging animal diseases and zoonoses. Climate change is disrupting natural ecosystems by providing more suitable environments for infectious diseases allowing disease-causing bacteria, viruses, and fungi to move into new areas where they may harm wild life and domestic species, as well as humans. Diseases that were previously limited only to tropical areas are now spreading to other previously cooler areas e.g. malaria. Pathogens that were restricted by seasonal weather patterns can invade new areas and find new susceptible species as the climate warms and/or the winters get milder (3).

The ease of transmission of emerging pathogens is facilitated by intercontinental travel. Efficient air and land travel links make disease containment difficult as illustrated by the SARS-coronavirus outbreak (2). Tourism to more exotic locations and eco-adventure travel is gaining popularity. Volunteer work is also common in Africa and Asia. This exposes non-immune travelers to endemic diseases (7). An example of an emerging zoonotic agent that became established in North America due to the incursion of a novel strain of the virus is West Nile Virus. Unlike SARS this introduction of the NY99 genotype led a very sucessful emergence of an agent that continues to thrive in a ecological niche that is quite removed from its probable origin in Africa (5).

Transmission of zoonotic diseases

The migration of humans has provided the route of spread for infectious diseases and zoonoses (for example, plague, yellow fever, monkey pox and severe acute respiratory syndrome). Tourism constitutes a small fraction of overall movements of humans but a point worthy of note is the number of international travellers has increased by more than 300% over the last 50 years (2). In addition, over 80 million people, mostly from developing countries, are legal or illegal immigrants in the developed countries. The consequences of travel extend beyond the traveller to the population visited and the ecosystem. Tourism and immigration may constitute an interface for mixing different genetic and ecological profiles, as well as cultural and social aspects, which is of particular interest in regard to zoonoses (3).

Some zoonotic infections are unusual in their transmission (e.g., scabies infection after handling of pet guinea pigs). Other infections may have a less obvious animal link. Mowing lawns is believed to be a risk factor for acquiring tularemia (caused by *Francisella tularensis)* in disease-endemic areas where lagomorph reservoirs may be killed by mowers or hedge trimmers (3). For some infections, zoonotic transmission occurs indirectly through food. Human brucellosis is not usually acquired through animal contact but is transmitted more often by consumption of infected animal products such as unpasteurized dairy products (9). Swimming, deep sea diving, farming and fishing activities have been related to leptospirosis outbreaks as animals shed the organism in their urine. *Leptospira* spp.–infected animals excrete viable organisms in their urine, which can persist in aquatic environments for prolonged periods (2). Hunting wildlife has been associated with infections such as

brucellosis and tularemia. Exposure routes may be airborne, as demonstrated for several outbreaks of Q fever (7).

Pastoralists are predisposed to zoonoses as they have a direct contact with animals. Arthropod vectors involved in the transmission of zoonoses include mosquitoes, ticks, fleas and lice while animal vectors include cats, dogs and bats. Rift Valley fever is transmitted through a bite of a mosquito or other arthropod vectors that have fed on animals infected with the virus or contact with viremic livestock (14). Different species of mosquitoes are involved in transmitting different zoonoses. West Nile virus is transmitted by *Culex pipiens* mosquitoes and Dengue fever virus is transmitted by *Aedes aegypti*. (9).

Diagnosis of zoonotic diseases and diagnostic challenges

Most of zoonotic disease can be diagnosized using serological assays such as ELISAs and molecular techniques (PCR, sequencing). All too frequently, the diagnosis of zoonotic disease is delayed through lack of clinical suspicion or failure to obtain adequate clinical histories. There is a delayed detection and response of zoonotic diseases in the Sub-Saharan Africa. This is due to poor disease surveillance coverage, poor diagnostic capacity, the geographical distribution of those most affected and lack of clear strategies to address the plight of zoonotic diseases. Knowledge of medical practitioners of zoonotic diseases could be a contributing factor to their under-diagnosis and under-reporting (4). Studies conducted in Kenya on zoonoses focus more on Rift Valley fever and Yellow fever viruses and there is no recent information on other zoonoses. This study will give information on most of the zoonoses found in the sub Saharan Africa including Dengue Fever, Chikungunya virus, West Nile fever virus and Rift Valley fever virus. West Nile virus is transmitted by mosquitoes. Since its introduction to the Western Hemisphere in 1999, WNV had spread across North America, Central and South America and the Caribbean, although the vast majority of severe human cases have occurred in the United States of America (USA) and Canada (3).

Zoonotic disease and socio-economic burden

Zoonoses are a serious public health threat in both the developing and the industrialised world due to a complex multifactorial set of changing circumstances. To support the growing human population, we have an increasing demand for nutritional support, resulting in intensive agricultural practices, sometimes involving enormous numbers of animals, or multiple species farmed within the same region. These practices can facilitate infection to

cross species barriers. Additionally, we are witnessing increasing globalization, with persons , animals, and their products moving around the world. This movement enables unprecedented spread of infections at speeds that challenge the most stringent control mechanisms (9). Furthermore, continual encroachment of humans into natural animal habitats by population expansion or tourism brings humans into new ecologic environments and provides opportunity for novel zoonotic exposure. Climatic changes have facilitated the expansion of compatible conditions for some disease vectors, remodeling dynamics for potentially new, emerging, and reemerging zoonoses. In the next 2 decades, climate change will be the most serious issue that will dominate reemergence of pathogens into new regions (14).

Host diversity of zoonotic diseases

Many zoonotic pathogens fall into the category of generalist agents exhibiting extensive host diversity, e.g., *Coxiella burnetii*, the etiological agent of Q fever. This bacterium can successfully infect hosts ranging from domestic animals to wildlife, reptiles, fish, birds and ticks (30). Others agents have restricted specific transmission dynamics because of limited host ranges. These agents include Rift Valley virus, which is transmitted by *Aedes* spp. and *Culex* spp. mosquitoes and found in sheep and goats. For many zoonotic agents, the potential to cause infection in accidental hosts, such as humans, exists, but often this represents a dead-end host. An accidental host is a host in which the zoonotic agent cannot complete its lifecycle. Pathogens such as *Rickettsia* spp., *Bartonella* spp. and West Nile virus can be included in this group (6).

Viral haemorrhagic fevers

Viral haemorrhagic fevers include Ebola haemorrhagic fever, Marburg disease and Lassa fever. Ebola and Marburg viruses are members of the Filoviridae family and easily distinguishable from viruses of other families by the characteristic morphology of the virion which appear as long threads (34). The natural reservoir(s) of Ebola and Marburg viruses remain unknown. On the other hand, Lassa virus is a member of the Arenaviridae family and its natural reservoir is a kind of rodent of the Mastomys species, which are asymptomatically infected with the virus and continue to excrete the virus throughout their lifetime. Dengue fever is caused by four serologically related flaviviruses called dengue-1, dengue-2, dengue-3 and dengue-4 (1). The vector responsible for transmitting Dengue fever virus is the mosquito *Aedes aegypti*. The disease represents as an acute febrile illness with chills, headache, retro-

ocular pain, body aches and arthlalgia, accompanied by nausea or vomiting and a maculopapular rash. Dengue haemorrhagic fever is a less common manifestation of dengue, with about 5000 cases per year. This form of dengue is observed in patients who undergo successive infection with two different dengue viruses (9). A study to review the trend in arbovirus outbreaks and activity in Kenya done in 2001 shows an outbreak of Dengue fever in the country (11). A study on serologic evidence of arboviral infections in 2000-2004 among humans in Kenya showed a seroprevalence rate of 44% for Dengue fever virus and 29% for West Nile virus (37.) A recent outbreak of Dengue fever was observed in Coast Province. The transmission of Dengue fever virus is usually high during the dry season and many outbreaks usually occur at this time. Lassa fever represents as an acute febrile illness, headache, muscle and joint pain. Pharyngitis with a non-productive cough is a common feature. Within a few days the patient will become increasingly febrile and complain of abdominal and retrosternal pain. The patient is lethargic, with oedema of the face and neck and enlarged lymph nodes. Marburg disease represents as an acute febrile illness accompanied by malaise, myalgia and severe frontal headaches (9). The first reported case of Marburg in Kenya occurred in 1980. In 1987, an isolated case of fatal Marburg disease was recognized during routine clinical haemorrhagic fever virus surveillance in Kenya (12). The viral haemorrhagic fevers are influenced by enroachment of humans into natural populations by population expansion (8).

West Nile virus and Chikungunya virus

West Nile virus is a flavivirus and is transmitted by *Culex pipiens* mosquitoes and wild birds are the main reservoir. Humans and other mammals are incidental hosts and do not play a role in the natural preservation of WNV. The clinical picture of disease includes fever, confusion and striking muscle weakness (9). During the 2006-2007 Rift Valley fever outbreak in North Eastern Province, Kenya, a study on arboviral prevalence in Culex mosquitoes showed that 18% of the mosquito pools were positive for West Nile virus and 22% were positive for Rift Valley fever virus, and 35 were positive for both (34). Chikungunya virus is a positive stranded RNA belonging to the family Togaviridae. The vector responsible for its transmission is the mosquito *Aedes aegypti*. Chikungunya virus was first isolated from the blood of a febrile patient in Tanzania in 1953. Chikungunya in the local dialect in Tanzania means stooping or bending, describing the position assumed by patients with the illness. The illness is associated with fever, severe arthralgias, rash, headache and malaise. The vectors responsible for transmission of the virus are Aedes mosquitoes (9). An outbreak of

Chikungunya occurred on Lamu Island beginning May 2004 and peaked in July 2004. Illness cause by Chikungunya had not been recognized previously on Lamu Island. After that outbreak, other associated outbreaks occurred in Mombasa, Kenya, between November and December 2004, Comoros Islands from January to May 2005 (unpublished data), and in India in 2006 (13).

Bunyavirus group

Rift valley fever virus belongs to the Bunyavirus group and it was first isolated in 1930 from sheep during an epizootic causing abortion and death in the Rift Valley near Lake Naivasha, Kenya. The disease presents as fever, malaise and generalized bleeding. In Kenya, there was an outbreak of Rift Valley fever from November 2006 to January 2007. Humans can be infected with the virus from bites of mosquitoes or other arthropod vectors that have fed on animals infected with the virus, or contact through viremic animals, particularly livestock (14). It is unclear which, if any, animal species maintain Rift Valley fever virus during interepidemic periods, and it is possible that Rift Valley fever virus is maintained solely within arthropod vectors during these periods. (34). Rift valley fever virus outbreaks are influenced by the rainy season when mosquitoes which are the vectors breed heavily. In 1987, 1997–1998, and 2006–2007, outbreaks of infection with Rift Valley fever virus in Africa were associated with changes in river flow and flooding resulting from damming of rivers or heavy rainfall.

Crimean Congo Haemorrhagic fever virus belongs to the Nairovirus group transmitted by Hyalomma tick bites. It is a member of the Bunyaviridae family of RNA viruses and is especially common in East and West Africa. After a 1-3 day incubation period following a tick bite, flu like symptoms appear, which may resolve after one week. In up to 75% of cases, however, signs of haemorrhage appear within 3-5 days of the onset of illness (2). The main environmental reservoir for the virus is small mammals particularly European hare, hedgehogs and multimammate rats. Ticks carry the virus to domestic animal stock. Sheep, goats and cattle develop high titres of blood in body, but tend not to fall ill. The first documentation of Crimean Congo fever in Kenya was in 2000 in a previously healthy male farmer in Western Kenya (17).

Leptospira

Leptospirosis is a zoonosis and has one of the widest geographical distribution of any zoonotic disease. The highest incidence is in the tropical and sub-tropical parts of the world. *Leprospira interrogans* is the cause of leptospirosis. Leptospiras infect various animals including rats, and other rodents, domestic livestock, and household pets (2). Animals excrete leptospiras in urine, which contaminates water and soil. Swimming in contaminated water or consuming contaminated food or drink can result in human infection. Miners, farmers and people who work in sewers are at high risk. Human infection results when leptospiras are ingested or pass through mucous membranes or skin resulting in fever, headache, muscle pain, jaundice and uremia (9).

Rickettsia

Rickettsia are obligate intracellular parasites. The genus Rickettsia is divided into two antigenically distinct groups: the typhus group and the spotted fever group. Epidemic typhus is caused by *R. prowazekii* and is transmitted by louse faeces. Murine typhus is caused by *R. typhi* and is transmitted by flea faeces. Rocky Mountain spotted fever is caused by *R. ricketsii* and is transmitted by a bite of tick. African tick bite fever is caused by *R. africae* and is transmitted by bite of tick (15). The main vector of murine typhus is the rat flea *Xenopspylla cheopis* and rodents, mainly *Rattus norvegicus* and *R. rattus*, act as reservoirs. The classical cycle of infection is rat-to-rat flea with *R. typhi* being only rarely transmitted transovarially in fleas. Although fleas remain infected for life, their lifespan is not shortened by the rickettsiae, which are excreted in the faeces where they remain viable for many years. It is generally accepted that most people become infected when flea faeces containing *R. typhi* contaminate disrupted skin or are inhaled into the respiratory tract. Rarely infection may result from flea bites. Murine typhus is a worldwide disease (1).

This disease has often been associated with ports where rats and man live in close contact. Recent reports have documented murine typhus in Israel, Egypt, Thailand and Eastern African countries. The epidemiology of murine typhus is directly or indirectly related to the geographic distribution or the life cycles of their louse or flea vectors. Onset of illness is usually relatively abrupt. Murine typhus is typically a mild illness and fatalities are extremely rare. Fever, headache, chills, myalgia and diarrhea are prominent early in the illness (2).

Coxiella burnetii

Coxiella burnetii is an obligate intracellular, small gram-negative bacterium which causes Q fever. *C. burnetii* has the ability to induce persistent infections both in humans and animals. Chronically infected animals shed bacteria in faeces and urine. Such persistent infections are mostly asymptomatic but may occur in the pregnant females in the form of massive contamination of the placenta with *C.burnetii*, leading to abortion or low fetal birth weight. *C. brunetii* also causes chronic infections in humans, especially in immunocompromised patients or during pregnancy (15). The aerosol route (inhalation of infected fomites) is the primary mode of human contamination with *C. burnetii*. Contamination may also occur directly from parturient fluids of infected animals, placenta or wool. Person-to-person transmission is probably extremely rare (9). Q fever is a disease with a worldwide distribution. The reservoir is large and includes many wild and domestic mammals, birds and arthropods such as ticks. Animals are chronically infected but do not experience symptoms of *C.burnetii* infection. The uterus and mammary glands of females are sites of chronic *C. burnetii* infection. Shedding of *C.burnetii* into the environment occurs mainly during parturition (2).

Q fever has been reported in almost every country, with New Zealand remaining an exception. Onset of the disease is often abrupt, with severe fever, fatigue, chills and headaches. Acute Q fever has two primary clinical presentations: atypical pneumonia and hepatitis. Whatever mode of acquisition of *C. burnetii*, haematogenous spread of the pathogen may lead to involvement of other organs including the liver, spleen, lungs, bone marrow and female genital tract (6).

JUSTIFICATION

We are constantly exposed to a changing menu of zoonotic diseases that come to prominence from time-to-time. The recent emergence of avian flu, SARS, Dengue and West Nile viruses (3, 4, 5, 6, 7) with associated human fatalities has led to an increased focus on emerging and re-emerging zoonoses. The increasing frequencies of movements of people, their animals around the world coupled with environmental changes are conditions considered favorable for the transmission of zoonoses. Expansion of farming practices into areas where zoonotic pathogens are circulating may also lead to emergence of new agents (Eg. bats , domestic pigs (and perhaps wild boars) could replicate ebola virus and lead to increase risk for exposure and perhaps even food borne transmission (36).

In Kenya, it is anticipated that there may be an under-diagnosis and/or under reporting of zoonotic diseases. This is due to poor and/or lack of diagnostic capacity, limited disease surveillance, poor and/or limited knowledge on geographical distribution and lack of suspicion of zoonoses at the point of care.

This study was aimed at conducting a surveillance in patients presenting with fever to find out the prevalence of serum antibodies against a number of zoonotic disease agents and attempt to correlate undiagnosed febrile cases with infection by zoonotic /vector borne pathogens.

OVERALL OBJECTIVE

To investigate, the seroprevalence of selected zoonotic disease infections agents in patients presenting with fever at two referral hospitals in Kenya.

Specific Objectives

I. To determine the prevalence of infections against flaviviruses (Dengue fever virus, and West Nile virus), Rift Valley fever virus, *Coxiella burnetti*, Rickettsia species, *Leptospira interrrogans*, in patients presenting with fever at out-patient clinic at Mbagathi District Hospital in Nairobi and Coast Provincial General Hospital in Mombasa.

II. To determine the risk factors associated with the seroprevalence of the selected zoonotic disease agents.

III. To describe geographical distribution of the selected zoonotic disease in Kenya.

CHAPTER III

METHODOLOGY

STUDY SITES

Serum samples were collected from Coast Provincial General Hospital, Mombasa and Mbagathi District hospital, Nairobi. These 2 sites, distributed across Kenya and separated by distances of at least 250 miles, represent a spectrum of different habitats, environmental conditions, and population lifestyles. The study sites span a range of climates from highlands (Nairobi) to monsoonal coastal plains (Mombasa). The two tons selected for this study were urban in nature and the hospitals acted as outpatient departments.

POPULATIONS SURVEYED

A cross-sectional study to determine the prevalence of infections against arboviruses (Dengue, West Nile, Rift Valley fever), *Coxiella burnetii*, Ricketsia (spotted fever group, murine typhus) and Leptospira was done. The study was carried out between 10th January 2011 and 11th March 2011. A total of 182 samples were collected (91 samples from each site), from patients presenting with fever. Informed consent was obtained from both male and female adults presenting with fever at the outpatient clinic of both hospitals and assent was obtained from parents/guardians of children presenting with fever at the outpatient clinic of both hospitals. Questionnaires were administered that included an assessment of demographics, clinical presentations, and history of recent travel. Malaria cases were excluded by PCR methods. The samples were collected in sterile containers and serum stored at -20 degrees. One hundred and eleven samples were from female patients, and 71 samples were from male patients (Table I).

SAMPLE SIZE

Anticipated infection rate with Dengue Fever virus antibodies amongst sex workers in Majengo has been shown to be 5% (personal communication from Dr. Joshua Kimani of Majengo Clinic, Kenya and Dr. Michael Drebot, National Microbiology Laboratory, Canada). Taking this into account the assumption of other zoonotic disease infectious agents was assumed to be between 0-5%.

The study desires to attain acceptable results using Andrew Fishers method of 1994 calculated using preset standards precision of 5% and a confidence level of 95%.

$$n = \frac{z^2 pq}{d^2}$$

Where:

n is the sample size

z is the standard normal deviation (standard variate) which corresponds to 95% confidence interval

d is the degree of precision set at 0.05(5%)

p is the best prevalence estimate (5%)

q = (1-p)

Therefore substituting the values as follows;

$$n = \frac{(1.96)^2 x\ 0.05x\ 0.95}{(0.05)^2}$$

$$= \frac{0.4625}{0.0025}$$

$$= 185$$

The total sample size required is 185. Each site will recruit 91 volunteers.

Inclusion criteria

I. Male or female patient over 18 years of age who presented with fever at the out-patient clinic at any of the two selected referral hospitals.
II. Children under the age of 18 years who presented with fever at the out-patient clinic at any of the two selected referral hospitals whose parent/guardian gave assent.

Exclusion criteria

Patient who did not sign the inform consent form.

LABORATORY PROCEDURES

Sample collection and transportation

Patients presenting with fever at Coast Provincial General Hospital (Mombasa) and Mbagathi District Hospital (Nairobi) were recruited.Informed consent was obtained from both male and female adults presenting with fever at the outpatient clinic of both hospitals (Appendix I) and assent was obtained from parents/guardians of children presenting with fever at the outpatient clinic of both hospitals. Questionnaires were administered that included an assessment of demographics, clinical presentations, and history of recent travel (Appendix II).

Fifteen (15) to twenty (20) ml venous blood was collected in SST tubes and EDTA tubes. The SST tubes were centrifuged within six hours at the site of collection at 10000 rpm for 10 minutes. The serum obtained was then aliquoted into cryovials, labeled appropriately and frozen at -20^{o}C. Once 91 blood samples were collected in Mombasa and centrifuged, the frozen serum vials were put in a freezer box, packed with dry ice and shipped to Nairobi. The other 91 samples collected in Nairobi were centrifuged and frozen at -20^{o}C. The serum samples were heated at 56^{0}C for 30 min to inactivate them and shipped to the National Microbiology Laboratory, Winnipeg, Canada for testing. A complete haemogram was done on the EDTA tubes at the site of collection and results recorded for each patient.

Laboratory Diagnosis

Two laboratory diagnostic methods were used:

1. Real time PCR for the detection of nucleic acid specific for a number of zoonotic pathogens.
2. Serological tests using sandwich type Enzyme linked immunosorbent assay-ELISAs for IgM/IgG detection.

RNA extraction

RNA from selected zoonotic agents was extracted using QIAamp Viral RNA Mini Kit: Qiagen Co using manufacturers instruction.

Detection of zoonotic agents

Screening for Rift Valley Fever, Chikungunya virus and Flavivirus (Dengue and WNV) RNA was achieved through the use of Real time PCR. In addition to diagnosis of zoonotic disease, Real time PCR was also carried out to rule out confounding factors which also cause fever

including *Plasmodium falciparum.* The 480 light cycler was used and the probe mix was prepared by adding 7.15 µL of water, 9.25 µL of master mix, 1.6 µL of activator, 1.0 µL of enhancer, 0.5 µL of primer and 0.5 µL of probe. Twenty microlitre of the probe mix was added to each well and 5 µL of the RNA extract was added and plate centrifuged. The plate was then run in a Light Cycler 480 machine.

Serological screening for antibodies against the selected zoonotic diseases

a) Dengue fever and WNV diagnosis

Testing for IgM and IgG antibodies to Dengue and WNV was performed by sandwich enzyme- linked immunosorbent assays using a commercial kit (Focus diagnostic kit, USA). (Appendix III)

For detection of Dengue IgM antibodies, 96-well polysyrene plate coated antihuman antibody specific for IgM ELISA plates were filled with Wash buffer solution (Phosphate buffered saline) and allowed to soak for 5 minutes. After washing, 100µlL of the sample diluents was dispensed into the wells followed by 100 µL of each diluted specimen, calibrator and controls into the appropriate wells and incubated for 1 hour at room temperature (20 to 25^0C). After 3 washes, 100 µL of the antigen solution (inactivated dengue virus types 1-4) was added to all the wells and incubated for 2 hours at room temperature. After 3 washes, 100 µL of conjugate was added to all wells and incubated for 30 minutes at room temperature. After 3 washes, 100 µL of substrate was added to all wells and incubated for 10 minutes at room temperature and reaction stopped by adding 100 µL of Stop reagent. The absorbance was measured by a microwell spectrophotometer (Biotek) at a wavelength of 450nm. The index was calculated by dividing the sample optical density (OD) by the mean value of the calibrator. An index value of > 1.00 was presumptive for the presence of antibodies to dengue virus while an index value of < 1.00 indicated no IgM antibodies to dengue virus detected.

For detection of Dengue IgG antibodies, 96-well polysyrene coated with Dengue Virus antigen ELISA plates were filled with Wash buffer solution (PBS) and allowed to soak for 5 minutes. After washing, 100 µL of the sample diluents was dispensed into the wells followed by 100 µL of each diluted specimen, calibrator and controls into the appropriate wells and incubated for 1 hour at room temperature (20 to 25^0C). After 3 washes, 100 µL of conjugate was added to all wells and incubated for 30 minutes at room temperature. After 3 washes,

100µlL of substrate was added to all wells and incubated for 10 minutes at room temperature and reaction stopped by adding 100 µL of Stop reagent. The absorbance was measured by a microwell spectrophotometer (Biotek) at a wavelength of 450nm. The index was calculated by dividing the sample OD by the mean value of the calibrator. An index value of > 1.50 indicated IgG antibodies to Dengue virus were detected. An index value if < 1.30 indicated IgG antibodies to Dengue virus were not detected.

For detection of WNV IgM antibodies, 96-well polysyrene wells antihuman antibody specific for IgM ELISA plates were filled with Wash buffer solution (PBS) and allowed to soak for 5 minutes. After washing, 100 µL of the sample diluents was dispensed into the wells followed by 100 µL of each diluted specimen, calibrator and controls into the appropriate wells and incubated for 1 hour at room temperature (20 to 25^0C). After 3 washes, 100 µL of the antigen solution (Recombinant WNV antigen) was added to all the wells and incubated for 2 hours at room temperature. After 3 washes, 100 µL of conjugate was added to all wells and incubated for 30 minutes at room temperature. After 3 washes, 100 µL of substrate was added to all wells and incubated for 10 minutes at room temperature and reaction stopped by adding 100 µL of Stop reagent. The absorbance was measured by a microwell spectrophotometer (Biotek) at a wavelength of 450 nm. The index was calculated by dividing the sample OD by the mean value of the calibrator. An index value of > 1.10 was presumptive for the presence of IgM antibodies to WNV while an index value of < 0.90 indicated no IgM antibodies to West Nile virus detected.

For detection of WNV IgG antibodies, 96-well polysyrene coated with Recombinant West Nile Virus antigen ELISA plates were filled with Wash buffer consisting of Phosphate buffered saline (PBS) and allowed to soak for 5 minutes. After washing, 100 µL of the sample diluents was dispensed into the wells followed by 100 µL of each diluted specimen, calibrator and controls into the appropriate wells and incubated for 1 hour at room temperature (20 to 25^0C). After 3 washes, 100 µL of conjugate was added to all wells and incubated for 30 minutes at room temperature. After 3 washes, 100 µL of substrate was added to all wells and incubated for 10 minutes at room temperature and reaction stopped by adding 100 µL of Stop reagent. The absorbance was measured by a microwell spectrophotometer (Biotek) at a wavelength of 450nm. The index was calculated by dividing the sample OD by the mean value of the calibrator. An index value of > 1.50 indicated IgG antibodies to West Nile virus

were detected. An index value if < 1.30 indicated IgG antibodies to West Nile virus were not detected.

b) Leptospira Diagnosis

Testing for Leptospira IgM antibodies was done using enzyme- linked immunosorbent assay using a commercial kit (Panbio diagnostic kit, Australia).

For detection of Leptospira IgM antibodies, 96-well polysyrene coated with Leptospira antigen were used. Sample was diluted by adding 1000µL of sample diluent to 10 µL of serum sample. 100 µL of the diluted samples, controls and calibrator were added into their respective microwells and incubated for 30 minutes at 37^0C. After 6 washes with diluted was h buffer, 100 µL of horseradish peroxidase conjugated anti-human IgM into each well and incubated at 37^0C for 30 minutes. After 6 washes with diluted wash buffer, 100 µL of tetramethylbenzidine (TMB) was added into each well and incubated for 10 minutes at room temperature. 100 µL of stop solution was added into all wells and the absorbance read at a wavelength of 450 nm. The index was calculated by dividing the sample OD by the mean value of the calibrator. An index value of <0.9 indicated presence of IgM antibody. An index value of 0.9-1.1 indicated an equivocal sample while an index value of >1.1 indicated a positive sample.

c) Rift Valley Fever diagnosis

Testing for Rift Valley Fever IgG antibodies was done using antibody capture enzyme-linked immunosorbent assay (MAC ELISA). We used a positive control serum specimen obtained from a previous RVF outbreak in Kenya; a negative control serum specimen was from a person from a non-endemic region who tested negative for arbovirus infection. For RVF-specific IgG detection, 96-well polystyrene ELISA plates were coated with 100 µL RVF lysate (NML, Winnipeg) overnight at 4^0C (both positive and negative lysate). 5 µL of sample was aliquoted into each well and 125 µL of skimmed milk solution was added to wells to make a 1:25 dilution. Plates containing lysate were washed with phosphate-buffered saline (PBS) with 0.05% Tween-20. 100µL of skimmed milk was added to coated wells. Thirty three (33) µL of sample was aliquoted from wells and added to the previously coated wells to make a 1:100 dilution. The wells were serially diluted and incubated at 37^0C for I hour. Plates were

washed and 100µL of antihuman IgG was added and incubated for 1 hour at 37^0C. Plates were washed and 100 µL TMB substrate added (NML, Winnipeg) and incubated at 37^0C for 30 minutes. Absorbance was read at 450nm. Postive samples had an OD value of more than 1.00 while negative samples had an OD value of less than 1.00

d) Rickettsial testing-Immunofluorescent Antibody Test

Testing for Rickettsial IgG antibodies was bone using Immunofluorescent antibody test.

Antigens (CDC Atlanta) were pipetted into 12 well teflon coated slides. Three (3) µL of sample antigen was pipetted onto each well. The slides were air-dried for an hour and fixed in cold acetone on a rocking platform for 15 minutes.

Sample serum was screened at a 1:32 dilution. A positive and a negative control was added on the first slide. R. rickettsii positive control was used at a 1:512 dilution and the R.typhi positive control was used at a 1:32 dilution. An initial dilution of 1:32 was made by diluting 5 µL of serum in 155 µL of Fluorescent treponemal antibody (FTA) buffer (Becton Dickinson Co. 211248) using Nunc plates (cat# 62409-114 from VWR). For positive sera at 1:32, serum was titrated in 50 µL FTA buffer.

Fifteen µL of diluted serum was added to each antigen well and incubated at 37^0C for 1 hour in a humid chamber. Slides were washed in FTA buffer for 15 minutes and air dried. IgG conjugated secondary antibody (Evans Blue dye E2129-10G and FTA buffer). A 1:32 IgG secondary antibody was prepared by adding 50 µL of goat anti-human IgG (Sigma product #F3512) to the BlueDye/FTA solution. Fifteen µL of the 1:32 secondary antibody solution was added into each well and incubated at 37^0C for 1 hour in a humid chamber. Slides were washed in FTA buffer for 15 minutes and agitated. The slides were rinsed in distilled water and air dried. A drop of mounting media (IFA mounting media-Focus) was added to each well and viewed under fluorescent microscope at 40X magnification.

e) Q-fever Testing

Serology IgG testing of Q-fever was carried out by using a commercial kit (Focus Diagnostic kit, USA).

Sera were screened at a 1:32 dilution for IgG. A positive and a negative control (supplied in the kit) were put on the first slide. The positive control was diluted with PBS at a 1:2 dilution and the negative control was used full strength. For IgG test, 155 µL of the 5 X IgG sample diluent working solution was pipetted into the microtitre plate (Nunc plate cat#62409-114) at

full strength and 5 μL of serum was added. The slides were incubated in a humid chamber at 37^0C for 30 minutes. The slides were washed with PBS on rocking platform for 10 minutes and air dried. IgG conjugate was added to the slides and incubated for 30 minutes. Slides were then washed in PBS and air dried. A drop of mounting medium was added and slides viewed at 40X under fluorescent microscope.

BIOHAZARD CONTAINMENT

As the transmission of these pathogens can occur through contact with contaminated needles, blood and blood products, appropriate biosafety precautions were employed by myself in drawing of blood, testing and handling of all specimens for this study using standard universal precautions. Safety measures include use of personal protective equipment (gloves, laboratory coats, wrap-around gowns and protective eyewear), use of handwashing sinks and use of masks in the collection and processing of samples. Laboratory precautions included the use of a biosafety cabinet.

ETHICAL CONSIDERATIONS

This study was conducted in compliance with the protocol. The study was approved by Kenyatta National Hospital Ethics committee (Approval P313/09/2010) dated 10th December 2010. The patients with fever attending the two hospitals were approached by myself. The patients were informed of the study. The purpose of the study was explained to each patient individually. She/he was informed that the information given and materials such as questionnaires would be confidential. The prosedure which included blood withdrawal was explained. The languages used were English and Kiswahili. Children below 18 years were explained the procedure together with their parents or guardians and all their questions answered. The patients were informed of risks involved in blood withdrawal such as mild pain and site discomfort. They were informed that there was no financial reimbursement and the benefit that may be accrued by the paticipant will contribute in the understanding of zoonoses. Once the consent process was explained and reading of the written informed consent done, the patient was requested to sign the consent in duplicateand one copy given back (Appendix I). The patient was informed that the study involvement was fully voluntary and could withdraw at any time without any consequences. The patient was informed that she/he would be

identified only by a unique number known by him and myself. The patients identity would not be disclosed in any publication or presentation of this study.

DATA ANALYSIS PLAN

All study data was collected by myself at each site and entered in a code-secured MS Excel spreadsheet. It was cleaned continually as the study progressed. A questionnaire was administerd to each patient to capture information on demographics of the patient such as age, sex, socio-economic status, marital status and history of recent travel.

For analysis, the MS Excel spreadsheet of the data collected was imported into SPSS statistical software. Demographic characteristics of volunteers such as age, sex and socio-economic status were summarized by site. For each site and overall, prevalence of infectious agents and its two sided 95% confidence interval was calculated. The prevalence was also calculated by relevant characteristics such as age and sex. Chi-square test was used to evaluate the association of volunteer characteristics with prevalence of the infectious agents.

STUDY LIMITATIONS

1. The study was expensive to conduct as all the reagents involved in conducting the serological and molecular work were expensive.
2. The prevalence obtained only reflects a small sample size.
3. The study failed to capture data on animal contacts of the study participants which is vital for a study on zoonotic diseases.

CHAPTER IV

RESULTS

The study was done on January-March 2011 and the number of patients was 182 (71 males and 111 females). The population demographics was as follows:

Table I: Patients Demographics Of All Patients

Demographic Characteristics	No.	(%)
Gender		
Male	71	39
Female	111	61
Education level		
None	18	9.9
Primary	80	44.0
Secondary	75	41.2
University/College	9	4.9
Marital Status		
Single never married	62	34.1
Married monogamous	100	54.9
Married polygamous	2	1.1
Divorced/separated	9	4.9
Widowed	9	4.9

Table II: Patients Demographics Per Region

Demographic Characteristics	Mombasa		Nairobi	
	No.	(%)	No.	(%)
Gender				
Male	30	33.0	41	45.1
Female	61	67.0	50	54.9
Education level				
None	14	15.4	4	4.4
Primary	36	39.6	44	48.4
Secondary	34	37.4	41	45.1
University/College	7	7.7	2	2.2
Marital Status				
Single never married	23	25.3	39	42.9
Married monogamous	58	63.7	42	46.2
Married polygamous	2	2.2	0	0
Divorced/separated	4	4.4	5	5.5
Widowed	4	4.4	5	5.5
Age Distribution				
Mean	31.30			
Median	31.00			
Mode	31			
Minimum	71			
Maximum	3			
Range	68			

The outcome of the selected zoonotic diseases was as follows:

Table III. Prevalence of various Zoonotic Pathogens (N/182)

Disease	**No. Positive**	**Prevalence (N/182)**
Dengue IgM	15	8.2%
Dengue IgG	64	35.2%
Flavivirus (Dengue and WNV) IgG	116	63.7%
WNV IgM	3	1.6%
Chikingunya RNA	0	0
Flavivirus RNA	0	0
RVF RNA	0	0
RVF IgG	13	7.1%
Coxiella burnetii IgG	10	5.5%
R.ricketsii IgG	5	2.7%
R. typhi IgG	1	0.5%
Leptospira IgM	1	0.5%

The highest prevalences in viral zoonoses were Flavivirus IgG.

The highest prevalence in bacterial zoonoses is *C. burnetii* IgG.

Table IV. Regional Prevalence of various Zoonotic Pathogens (N=91)

Disease	**Mombasa**	**N/91**	**Nairobi**	**N/91**
	No. Pos	**Prevalence**	**No. Pos**	**Prevalence**
Dengue IgM	10	11%	5	5.5%
Dengue IgG	51	56%	13	14.3%
Flavivirus (dengue and WNV) IgG	92	50.5%	24	13.1%
WNV IgM	1	1.1%	2	2.2%
Chikingunya RNA	0	0	0	0
RVF RNA	0	0	0	0
Flavivirus RNA	0	0	0	0
RVF IgG	5	5.5%	8	8.8%
C. burnetii IgG	6	6.6%	4	4.4%
R. ricketsii IgG	2	2.2%	3	3.3%
R.typhi IgG	1	1.1%	0	0
Leptospira IgM	0	0	1	1.1%

Table V : Co morbidity in patients

Infection	Number	Percent
Dengue IgM and Dengue IgG	2	3.3
Dengue IgM, Dengue IgG,WNV IgM and WNV IgG	1	1.7
Degue IgM, Dengue IgG and WNV IgG	2	3.3
Dengue IgM, Dengue IgG, WNV IgG and RVF IgG	1	1.7
Dengue IgM, Dengue IgG, WNV IgG and Q-fever IgG	1	1.7
Dengue IgM and WNV IgM	1	1.7
Dengue IgM and RVF IgG	1	1.7
Dengue IgM and Q fever IgG	1	1.7
Degue IgM and R-ricketsii IgG	1	1.7
Dengue IgG and WNV IgG	**41**	**68.3**
Dengue IgG, WNV IgG and RVF IgG	1	1.7
Dengue IgG, WNV IgG and Q fever IgG	3	5.0
Dengue IgG, WNV IgG, R-rickettsii IgG and R typhi IgG	1	1.7
Dengue IgG and RVF IgG	1	1.7
WNV IgM and RVF IgG	1	1.7
WNV IgG and Q fever IgG	1	1.7
Total	**60**	**100.0**

The highest cross infection in patients was Dengue IgG and WNV IgG. Co-dengue WNV exposures are probably not additative due to flavivirus cross reactivity in IgG ELISA. Flavivirus IgG ELISAs are notorious for being cross reactive and so it is quite possible that the majority of positives generated by the WNV IgG ELISA are in fact dengue antibody reactors despite the use of WNV antigen. The presence of IgG means that the infection has persisted for more than 2 weeks. Additional tests including neutralisation assay should be performed to confirm co-infections as these infections may have occured at different times and it is difficult to prove co-infection unless one dtects both antigens in a sample.

Table VI: Hematology and Dengue IgM

Variable	Dengue_IgM Neg.		Dengue_IgM Pos.		P-value
	No.	(%)	No.	(%)	
WBC ranges					
Normal (6.3-8.3)	137	(91.3)	13	(8.7)	0.652
Abnormal					
High (15.9-19.9)	20	(62.5)	2	(6.3)	
Low (2-2.8)	10	(31.2)	0	(0)	
RBC ranges					
Normal (4.5-4.9)	138	(90.2)	15	(9.80	0.078
Abnormal					
High (5.1-6.1)	16	(55.1)	(0)	(0)	
Low(2.1-2.5)	13	(44.9)	(0)	(0)	
HGB ranges					
Normal (13.4-14.4)	152	(91.6)	14	(8.4)	0.762
Abnormal					
High (16-17.20	3	(18.7)	1	(6.3)	
Low (5.9-6.7)	12	(75)	0	(0)	
PLT ranges					
Normal (235-295)	155	(91.7)	14	(8.3)	0.940
Abnormal					
High (450-550)	5	(44.7)	1	(7.7)	
Low (62-102)	7	(48)	0	(0)	
NEU ranges					
Normal (52.5-72.5)	142	(91.0)	14	(9.0)	
Abnormal					0.379
High (69.4-89.4)	20	(77)	1	(3.8)	
Low (42-51)	5	(19.2)			
LYM ranges					
Normal (24-40)	133	(91.1)	13	(8.9)	
Abnormal					0.513
High (55-70)	20	(60)	0	(0)	
Low(17-24)	14	(34.7)	2	(5.6)	
MO ranges					
Normal (0-3)	137	(91.3)	13	(8.7)	
Abnormal					0.652
High (0-3.6)	25	(75)	2	(6.3)	
Low (0-3)	5	(18.7)	0	(0)	
EO ranges					
Normal (1-7.5)	148	(93.1)	11	(6.9)	
Abnormal	19	(82.6)	4	(17.4)	0.088

High (0.3-9.3)	15	(65.2)	4	(17.4)	
Low(0-4)	4	(17.4)	0	(0)	
BA ranges					
Normal (58-74)	107	(92.2)	9	(7.8)	
Abnormal					0.753
High (80.3-95.3)	30	(45.4)	6	(9.1)	
Low (40-58)	30	(45.4)	0	(0)	

There was no significant association between any Haematology parameter and Dengue IgM.

Table VII: Hematology and Flavivirus IgG

Variable	Flavivirus_IgG Neg. No.	(%)	Flavivirus_IgG Pos. No.	(%)	P-value
WBC ranges					
Normal (6.3-8.3)	101	(67.3)	49	(32.7)	0.126
Abnormal	17	(53.1))			
High (15.9-19.9)	7	(20.1)	15	(46.9)	
Low (2-2.8)	10	(33)	0	(0)	
RBC ranges					
Normal (4.5-4.9)	101	(66.0)	52	(34)	0.445
Abnormal					
High (5.1-6.1)	10	(38)	12	(41.4)	
Low(2.1-2.5)	7	(20.6)	0	(0)	
HGB ranges					
Normal (13.4-14.4)	108	(65.1)	58	(34.9)	
Abnormal					0.838
High (16-17.20	5	(31.2)	6	(37.5)	
Low (5.9-6.7)	5	(31.2)	0	(0)	
PLT ranges					
Normal (235-295)	109	(64.5)	60	(35.5)	0.731
Abnormal					
High (450-550)	4	(30.8)	4	(30.8)	
Low (62-102)	5	(38.4)	0	(0)	
NEU ranges					
Normal (52.5-72.5)	103	(66.0)	53	(34.0)	
Abnormal					0.410
High (69.4-89.4)	4	(17.7)	11	(42.3)	
Low (42-51)	9	(40)	0	(0)	
LYM ranges					
Normal (24-40)	99	(67.8)	47	(32.3)	
Abnormal	19	(52.8)			0.091
High (55-70)	10	(30)	17	(47.2)	
Low(17-24)	9	(18.8)	0	(0)	
MO ranges					
Normal (0-3)	105	(70.0)	45	(30)	
Abnormal					**0.002**

High (0-3.6)	3	(10)	15	(30)	
Low (0-3)	3	(10)	4	(12)	
EO ranges					
Normal (1-7.5)	102	(64.2)	57	(35.8)	
Abnormal					0.611
High (0.3-9.3)	10	(41.6)	7	(30.4)	
Low(0-4)	6	(28)	0	(0)	
BA ranges					
Normal (58-74)	80	(69.0)	36	(31.0)	
Abnormal					0.122
High (80.3-95.3)	20	(30)	28	(42.4)	
Low (40-58)	18	(27.6)	0	(0)	

Monocytes had a significant association with Flavivirus IgG. Flavivirus IgG positive patients had a high monocyte count (30%) compared to the Flavivirus IgG negative patients who had a lower monocyte count (10%).

Table VIII: Hematology and WNV IgM

Variable	WNV IgM Neg.		WNV IgM Pos.		P-value
	No.	(%)	No.	(%)	
WBC ranges					
Normal (6.3-8.3)	147	(98.0)	3	(2.0)	0.420
Abnormal					
High (15.9-19.9)	12	(37.5)	(0)	(0)	
Low (2-2.8)	20	(62.5)			
RBC ranges					
Normal (235-295)	150	(98.0)	3	(2.0)	
Abnormal					
High (450-550)	10	(34)	0	(0)	0.447
Low (62-102)	19	(66)	0	(0)	
HGB ranges					
Normal (13.4-14.4)	163	(98.2)	3	(1.8)	
Abnormal					
High (16-17.20	15	(95)	0	(0)	0.588
Low (5.9-6.7)	1	(5)	0	(0)	
PLT ranges					
Normal (235-295)	166	(98.2)	3	(1.8)	
Abnormal					
High (450-550)	3	(34)	0	(0)	0.628
Low (62-102)	10	(76)	0	(0)	
NEU ranges					
Normal (52.5-72.5)	153	(98.1)	3	(1.9)	
Abnormal					0.476
High (69.4-89.4)	20	(78)	0	(0)	
Low (42-51)	6	(22)	0	(0)	
LYM ranges					
Normal (24-40)	144	(98.6)	2	(1.4)	0.552
Abnormal	35	(97.2)	1	(2.8)	
High (55-70)					
Low(17-24)					
MO ranges					
Normal (0-3)	148	(98.7)	2	(1.3)	0.470
Abnormal					

High (0-3.6)	21	(63)	1	(3)	
Low (0-3)	10	(30)	0	(0)	
EO ranges					
Normal (1-7.5)	157	(98.7)	2	(1.3)	
Abnormal					0.277
High (0.3-9.3)	15	(64.5)	1	(4.3)	
Low(0-4)	7	(31.2)	(0)	(0)	
BA ranges					
Normal (58-74)	114	(98.3)	2	(1.7)	
Abnormal					0.915
High (80.3-95.3)	20	(30)	1	(1.5)	
Low (40-58)	45	(67.5)	(0)	(0)	

There was no significant association between any Haematology parameter and WNV IgM.

Table IX: History of recent travel and Zoonotic Diseases

Variable	Travelled		Not travelled		P-value
	No.	(%)	No.	(%)	
Dengue_IgM					
Negative	18	10.8	149	89.2	0.284
Positive	3	20.0	12	80.0	
WNV_IgM					
Negative	21	11.7	158	88.3	0.528
Positive	0	0	3	100	
Flavivirus_IgG					
Negative	10	7.7	41	78.8	**0.010**
Positive	11	21.2	120	92.3	
RVF_IgG					
Negative	19	11.2	150	88.8	0.652
Positive	2	15.4	11	84.6	
Q Fever IgG					
Negative	20	11.6	152	88.4	0.876
Positive	1	10.0	9	90.0	
R ricketsii IgG					
Negative	20	11.3	157	88.7	0.548
Positive	1	20.0	4	80.0	
R typhi IgG					
Negative	21	11.6	160	88.4	0.717
Positive	0	0	1	100	
Leptospira IgM					
Negative	21	11.6	160	88.4	0.717
Positive	0	0	1	100	

There is a significant association between Flavivirus IgG and history of recent travel. Flavivirus IgG positive patients who had travelled were more than the Flavivirus IgG negative patients who had not travelled. IgG positivity is evidence of past exposure to flavivirus. The time of travel was 2 months before the testing was done. This can be correlated well with IgG positive patients.

Table X: Age in years and Zoonotic Diseases

Variable	1 – 30 years		31 years and above		P-value
	No.	(%)	No.	(%)	
Dengue_IgM					
Negative	86	96.6	81	87.1	
Positive	3	3.4	12	12.9	**0.019**
Flavivirus_IgG					
Negative	67	75.3	51	54.8	
Positive	22	24.7	42	45.2	**0.004**
WNV_IgM					
Negative	87	97.8	92	98.9	
Positive	2	2.2	1	1.1	0.535
RVF IgG					
Negative	77	86.5	92	98.9	**0.001**
Positive	12	13.5	1	1.1	
Q Fever IgG					
Negative	82	92.1	90	96.8	0.170
Positive	7	7.9	3	3.2	
R ricketsii IgG					
Negative	87	97.8	90	96.8	0.686
Positive	2	2.2	3	3.2	
R typhi IgG					
Negative	89	100	92	98.9	0.327
Positive	0	0	1	1.1	
Leptospira IgM					
Negative	89	100	92	98.9	0.327
Positive	0	0	1	1.1	

Three Zoonotic diseases (Dengue IgM, Flavivirus IgG and RVF IgG) had a significant association with age. Dengue IgM, Flavivirus IgG and RVF IgG positives were more in people aged 31 years and above.

Table XI: Gender and Zoonotic Diseases

Variable	Males		Female		P-value
	No.	(%)	No.	(%)	
Dengue_IgM					
Negative	62	87.3	105	94.6	
Positive	9	12.7	6	5.4	0.082
Flavivirus_IgG					
Negative	51	71.8	67	60.4	
Positive	20	28.2	44	39.6	0.114
WNV_IgM					
Negative	69	97.2	110	99.1	
Positive	2	2.8	1	0.9	0.322
RVF IgG					
Negative	66	93.0	103	92.8	0.966
Positive	5	7.0	8	7.2	
Q Fever IgG					
Negative	67	94.4	105	94.6	0.947
Positive	4	5.6	6	5.4	
R ricketsii IgG					
Negative	70	98.6	107	96.4	0.377
Positive	1	1.4	4	3.6	
R typhi IgG					
Negative	70	98.6	111	100	0.210
Positive	1	1.4	0	0	
Leptospira IgM					
Negative	70	98.6	111	100	0.210
Positive	1	1.1	0	0	

There was no significance association between Gender and any Zoonotic disease.

Table XII: Presenting Symptoms (Fever, Headache , joint pains) and Zoonotic diseases.

Variable	Symptoms presented		No Symptoms presented		P-value
	No.	(%)	No.	(%)	
Dengue_IgM					
Negative	96	91.4	71	92.2	
Positive	9	8.6	6	7.8	0.850
Flavivirus_IgG					
Negative	59	56.2	59	76.6	
Positive	46	43.8	18	23.4	**0.004**
WNV_IgM					
Negative	103	98.1	76	98.7	
Positive	2	1.9	1	1.3	0.751
RVF IgG					
Negative	99	94.3	70	90.9	
Positive	6	5.7	7	9.1	0.382
Q Fever IgG					
Negative	100	95.2	72	93.5	
Positive	5	4.8	5	6.5	0.613
R ricketsii IgG					
Negative	104	99.0	73	94.8	
Positive	1	1.0	4	5.2	0.084
R typhi IgG					
Negative	104	99.0	77	100	
Positive	1	1.0	0	0	0.390
Leptospira IgM					
Negative	105	100	76	98.7	
Positive	0	0	1	1.3	0.242

Flavivirus IgG had a significant association with presenting symptoms (Fever, headaches, joint pains, vomiting). The presence of IgG indicates that the patient was infected atleast 2 weeks previously since IgG antibody usually appears apprximately 14 days after exposure.

Table XIII: Region and Zoonotic Diseases

Variable	Nairobi		Mombasa		P-value
	No.	(%)	No.	(%)	
Dengue_IgM					
Negative	86	94.5	81	89.0	
Positive	5	5.5	10	11.0	0.178
Flavivirus_IgG					
Negative	78	85.7	40	44.0	
Positive	13	14.3	51	56.0	**0.000**
WNV_IgM					
Negative	89	97.8	90	98.9	
Positive	2	2.2	1	1.1	0.560
RVF IgG					
Negative	83	91.2	86	94.5	
Positive	8	8.8	5	5.5	0.388
Q Fever IgG					
Negative	87	95.6	85	93.4	
Positive	4	4.4	6	6.6	0.515
R ricketsii IgG					
Negative	88	96.7	89	97.8	
Positive	3	3.3	2	2.2	0.650
R typhi IgG					
Negative	91	100	90	98.9	
Positive	0	0	1	1.1	0.316
Leptospira IgM					
Negative	90	98.9	91	100	
Positive	1	1.1	0	0	0.316

Flavivirus IgG had a significant association with region. It was higher in Mombasa and could be due to a higher number of mosquitoes present at the Coast. The presence of IgG indicates that the patient was infected atleast 2 weeks previously since IgG antibody usually appears apprximately 14 days after exposure.

DISCUSSION

The zoonotic diseases have been shown to have high prevalences worldwide. In developing nations, the prevalence rates are not well known due to lack of diagnostic capacity. In this study, the total seroprevalence rate of the selected zoonotic diseases was 60%. This rate was higher than the estimated seroprevalence rate of RVF virus in Garissa which was 14% as per the CDC report (18). This could be explained by the fact that knowledge on most of the zoonotic diseases is not known and has limited disease surveillance.

Flaviviruses (Dengue and WNV) IgM ELISAs used during this study are reasonably specific, however, Flavivirus IgG ELISAs are notorious for being cross reactive and so its quite possible that the majority of positives generated by the WNV IgG ELISA are in fact dengue antibody reactors despite the use of WNV antigen

In this study, Dengue prevalence with IgM was 8.2% and Dengue IgG prevalence was 35.2%. This seroprevalence may compare with urban prevalence found elsewhere e.g. 29.5% in Brazil during epidemics (19). This urbanisation of Dengue fever is due to open container waste such as plastic cans, tyres, flat rooftops which harbour rain water during the rainy season and become good breeding grounds for Aedes mosquitoes which are vectors of Dengue virus. This was the major reason of Dengue outbreak in Brazil in early 2008. With these demographic changes and subsequent increases in Aedes spp. populations, increased Dengue fever virus transmission is likely to occur. For example, in Ghana, Aedes spp. mosquito densities and biting rates seem high enough to support outbreaks of yellow fever and dengue (38). *Aedes aegypti* mosquitoes are more present at the coast and the warmer temperatures are favourable for the breeding of these mosquitoes. The tests done were ELISA using IgM and IgG commercial kits. The presence of IgM means that the infection is acute while the presence of IgG means that the infection is chronic. The IgM prevalence was lower compared to IgG. This can be explained by 2 reasons; In normal prevalence of IgG and IgM, IgM appears first in acute infections and clears between day 60 and 90 (34). IgG shows chronicity as IgM has disappeard making IgG persistent in populations living in endemic areas. The other explanation is there is extensive cross reactivity amongst flaviviruses in secondary and chronic infections and IgG outcome may be higher due to false positives (20).

It has been found that there is worlwide increased incidence and emergence of epidemic Dengue haemorrhagic fever since 1950s due to ecological changes, massive human movement during World War II and uncontrolled urbanisation (21). Therefore, the number of

countries and human population affected has increased and dengue is considered a global public health problem due to epidemics involving large human populations (22, 23).

Monocytes had a significant association with Flavivirus IgG ($p=0.002$). This significance was that the Flavivirus IgG positive patients had high monocyte rate (30%) compared to the Flavivirus IgG negative patients who had a lower moncyte rate (10%). This could be explained through immunopathogenesis of Flavivirus infections. After a mosquito bite, the initial sites of viral replication is the immature skin dendritic cells. This stimulates dendritic cell maturation and activation along with production of TNF-α and IFN- α, but little IL-12. The activated mature dendritic cells migrate to lymphoid organs activating T cells releasing cytokines and chemokines. IFN-γ becomes the major cytokine enhancing the activation of Flavivirus virus infected dendritic cells. IFN- γ will also activate macrophages and monocytes release and this may explain the high monocyte counts in Flavivirus infected patients (24, 25). The high monocyte count could also be due to another infectious agents besides Flavivirus as infection with Flavivirus could have occurred years ago despite the statistical correlation.

In this study, WNV seroprevalence based upon IgM was 1.6%. Flavivirus (Dengue and WNV) IgG seroprevalence was 63.7%. WNV IgG ELISA number was not used as this may be misleading due to cross reaction of dengue antibody with WNV antigen. Most of the dengue IgG positives were also WNV IgG positive therefore it is probable that Dengue was the actual exposure in most cases. This could only have been confirmed if a neutralisation assay was performed in the study. Urbanisation of WNV is due to open water waste cans that harbour water during rainy season becoming good breeding grounds for *Culex* mosquitoes. Testing was done using ELISA by commercial kits. WNV IgM prevalence was much lower than Dengue IgM prevalence. The presence of IgM may be indicative in certain instances, an acute infection linked to the febrile illness, despite negative PCRs.

WNV is maintained in nature between mosquitoes and birds. The isolation of WNV from male *Culex* spp. mosquitoes in Rift Valley Province suggests a natural transovarian transmission cycle among some mosquito vectors but is unlikely to contribute greatly to virus maintenance between enzootic periods (35). The presence of WNV in local mosquitoes in a study done in North Eastern Kenya suggested that the virus is maintained in a natural cycle yet to be elucidated and that the actual incidence of WNV in human populations in the region could be underestimated (34).

Until 1999, the geographic distribution of WNV included the Mediterranean countries, Africa, West Asia, the Middle East and parts of Europe. In 1999, WNV was introduced into the

Western hemisphere, where it spread rapidly throughout the region. Associated with this rapid geographic spread has been an increased incidence of severe and fatal neurological disease in humans, horses and birds. WNV is transmitted in enzootic cycles involving a variety of *Culex* species mosquitoes and birds (26).

Information on history of recent travel had a significant association with Flavivirus IgG (p=0.010). This significance was that 52% of Flavivirus IgG positive patients had travelled compared to 26% of Flavivirus IgG negative patients who had not travelled. This could be explained through the diagnosis of Flavivirus infection in which a detailed medical history, patient's recent travel history and blood tests for specific antibodies are important factors that assist in diagnosis (1). Patients with an extensive travel history are more likely to be exposed to arboviral diseases such as Dengue fever and WNV.

In this study, RVF IgG prevalence was 7.1%. Testing was done using ELISA. RVF IgG ELISA antibodies are believed to last decades after infection and so provide a reliable index of prior RVF exposure (20). In contrast, though less well studied, it appears that IgM is lost in 50% of patients by day 45 and is absent in 100% by 4 months after infection (27). Addition of IgM data in this study would have yielded important information about acute RVF infection in our populations, but such a study could not be performed. This prevalence rate was lower compared to a reported rate of 14% done in Garissa (18). This could be explained by the fact that Garissa has a hot climate and is considered an endemic locality while the study areas had no reported outbreaks and were both non endemic areas. The prevalence rate in Nairobi was 8.8% which was higher than the rate in Mombasa (5.5%). This can be explained by the close proximity of Nairobi to Rift Valley province, where it is cultural to rear animals. Nairobi is also more cosmopolitan compared to Mombasa hosting populations from epidemic and endemic areas like Garissa, Baringo. Cattle rearing is also not quite common in Mombasa as compared to Nairobi.

RVF virus is native to sub-Saharan Africa and natural epizootic/epidemic outbreaks of RVF have been detected 8 times during the second half of the 20th century in Kenya. After sheep and cattle were introduced into Africa, the disease pattern became one of apparently low-level enzootic and endemic activity punctuated by massive transmission during periods of very high rainfall. Introduction of RVF to new areas has been linked to livestock movement, environmental modification and weather changes (28).

Humans can be infected with the RVF virus from bites of mosquitoes or other arthropod vectors that have fed on animals infected with the virus, or contact with viremic animals,

particularly livestock (14). Alternative forms of human exposure, such as aerosol and direct contact, may be more critical for transmission during epidemics. (27).
In a study to determine arbovirus prevalence in mosquitoes in Northern Kenya, 22% of the mosquito pools were positive for RVF virus. (34)The study confirms that RVF virus disseminates to the legs of wild *Culex quinquefasciatus* mosquitoes and suggests that these mosquitoes, promiscuous feeders, could play a role in the transmission of RVF virus in disease endemic regions (9).

This study is a new reporting in Kenya on Rickettsial disease. Testing was done using the IFA test for IgG which was the reference test. Tests on *Coxiella burnetii* which causes Q fever, *R. ricketsii* and *R. typhi* were done. The prevalence rate of Q fever IgG was 5.5%, *R. ricketsii* IgG was 2.7% and *R. typhi* IgG was 0.5%. The prevalence rate of Q fever in this study was lower as compared to the prevalence rate documented in Spain was 15.3% (29). This could be because Q fever is endemic in Spain. There prevalence rate of Q fever in Mombasa was more (6.6%) than in Nairobi (4.4%). This could be due to different epidemic and climatic environments as Mombasa has a much warmer climate than Nairobi. .
Q fever has a worldwide distribution and is considered a public health problem in many countries. *C. burnetii* has an extensive reservoir including many wild and domestic mammals, birds, and arthropods such as ticks. The most frequent sources of human infection are farm animals, especially cattle, goats and sheep. Animals are often chronically infected, shedding bacteria in faeces, milk, urine and especially birth products of mammals (30).
The aerosol route (inhalation of infected fomites) is the primary mode of human contamination with *C. burnetii*. Contamination by c. burnetii aerosols may occur directly from parturient fluids of infected animals, which may contaminate newborn animals, placenta or wool. Ingestion of raw milk is also a factor in transmission of *C. burnetii* (29).

Transmission of *R. rickettsii* occurs through the bite of an infected tick or through contamination of abraded skin or mucous menbranes. *R. typhi* is transmitted when flea faeces (*Xenopsypylla cheopis*) containing *R. typhi* contaminate disrupted skin or are inhaled into the respiratory tract (9).
The prevalence rate of *R. ricketsii* in this study was lower as compared to the prevalence rate documented in Colombia which was 40.3% (31). The prvalence rate of *R. typhi* in this study was lower as compared to the prevalence rate documented in Greece which was 20.6% (32). This low prevalence rate of *C. burnetii*, *R. rickettsii* and *R. typhi* in this study could be

explained by the small sample size conducted and that patients were from urban areas. If the study was done in a rural setting, the outcome would have been different. The prevalence rate of *R. typhi* in Mombasa (1.1%) was higher than in Nairobi (0%). This could be because *Rattus norvegicus* which is the natural host of vector Xenopsylla flea is more prevalent in the coastal areas than in inland areas (32).

In this study, Leptospira IgM prevalence rate was 0.5%. Testing was done by ELISA using a commercial kit. The rate was lower than expected and could be explained by the fact that the patients were from urban areas.

Age had a significant association with Dengue IgM (p=0.019). This significance is such that Dengue IgM positives was more in people aged 31 years and above. This could be explained by the sociocultural activities of the adults who tend to stay more outdoors and less mode of clothing subjecting them to mosquito bites more than the younger people.
Age also had a significant association with Flavivirus IgG (p= 0.004) and RVF IgG (p=0.001). This significance is such that Flavivirus IgG and RVF IgG positives were more in people aged 31 years and above. Significant prevalences of past infections in older ages could be explained because of longer exposure time experienced by older people. It could also be explained by sociocultural practices such as cattle rearing, slaughtering of animals and ownership of cattle are associated with mature persons in a society. This could be compared to a study on serologic evidence of Dengue fever virus and WNV infections in Kenya in which older persons were more likely to be seropsitive than children (37).

Presenting symptoms (fever, headaches, joint pains, vomiting, diarrhoea) had significant association with Flavivirus IgG (p=0.004)). This significance was such that more Flavivirus IgG positives had presenting symptoms. This could be explained by the fact that manifestations of most tropical infections are protean. They are varied due to various organs involved and have similar clinical manifestations with diseases such as malaria and typhoid.

Flavivirus IgG (p=0.000) had significant association with region. This significance was such that more Dengue IgG and WNV IgG postives were found in Mombasa than in Nairobi. This could be explained by the fact that the 2 cities have different climatic conditions. Mombasa has a hot and humid climate favouring mosquito breeding while Nairobi has a cooler climate.

CONCLUSIONS

Individuals from both Mombasa and Nairobi had the highest seroprevalence rates to flaviviruses (most likely dengue) while Leptospira and *Rickettsia typhi* had the lowest serum antibody prevalence. This study showed that age, history of recent travel and abnormal haematological factors were all risk factors associated with prevalence of serum antibodies against a number of zoonotic diseases. It is possible that some of the individuals with Dengue IgM were infected recently with the virus and these particular infections may have been associated with the undiagnosed febrile illness documented in the enrolled patients.

RECOMMENDATIONS

To Hospitals:

1. Both hospitals need to install biological safety cabinets, especially Class II.
2. The tests done in this study should be included in routine medical diagnostic testing for patients presenting with a fever.

For Research:

1. There is need for further studies capturing a bigger population samples in rural settings to determine prevalence of antibodies against zoonoses.
2. There is need for further studies on monocytes and the probable magnitude they contribute to the symptoms and signs in patients with Dengue fever virus and WNV infections.

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APPENDICES

Appendix I: Informed Consent form

Title: Documentation of the exposure of Kenyan Residents to zoonotic disease agents

Introduction:

Agents transmitted from animals to human may be responsible for disease categorised as zoonotic disease or zoonoses and may include a wide spectrum of infectious (bacterial, viral, parasitic, rickettsial and fungal) and non infectious (prion) agents. Zoonoses are caused by etiological agents that circulate in vertebrate animals hosts but are occasionally transmitted to humans, primarily by ingestion, bites (animals or arthropod), scratches, inhalations or direct skins/mucosal contacts. Animals hosts may include wild animals, food and farm animals or pests. Animals infected with a zoonotic disease agent may or may nor exhibit signs of disease.

Zoonoses are strongly identified with emerging disease issues given the fact that they have repeatedly been identified as ranking among the most important emerging infections and are of global concern due to their link to the environment and the sudden nature in which they appear often in new areas of the world where they are unexpected.

We are constantly exposed to a changing menu of zoonotic disease that come to prominence from time to time. The recent emergence of avian flu, SARS, Ebola , Dengue and West Nile viruses with associated human fatalities has led to an increased focus on emerging and re-emerging zoonoses. The increasing frequencies of movements of people, their animals around the world coupled with environmental changes are conditions considered favourable for the transmission of zoonoses.

The purpose of the study:

This study aims to investigate the prevalence of specific antibodies against a number of zoonotic diseases, determine the risk factors associated with the prevalence of selected zoonotic diseases and determine its distribution, in selected referral hospitals in Kenya.

How do you join?

You will be recruited in the study if you present with a fever when you visit a hospital. A total of 182 volunteers will be recruited into this study over a period of 2 months from 2 public hospitals across the country: Each hospital will recruit 91 volunteers each. These include Mbagathi district hospital (Nairobi) and Coast General Provincial Hospital (Mombasa).

You should be over 18 years and be able to sign an informed consent form. Volunteers who are below 18 years, their parent/guardian will have to give assent

Procedure:

- You will be at the study clinic for about one hour.
- The study will be explained to you in a language that you understand. Time will be allowed for any questions. Once you understand and you agree to join, you will sign or mark 2 copies of the Informed Consent Form confirming that you have been informed about the study and voluntarily agree to take part. One copy is yours to keep and the other will be kept in our confidential study file. If you do not wish to keep your copy, you will sign or mark a form that states you do not want to take it, and we will keep it for you.
- For children, informed consent will be provided by the parent/ guardian
- You will be asked questions about your general health and a medical examination will be performed by a doctor
- 15 to 20 ml (about 2 tablespoons or about 4 tubes) of your blood will be drawn for laboratory to test circulating antibodies.

What are the Risks and/or Discomforts?

There may be some risk involved in drawing blood for the laboratory tests and from being a volunteer in this study such as:

- You may have pain and bruising where the needle goes into your arm.
- You may feel dizzy or faint.

There are no anticipated physical risks in participating in this study. However if there are any injuries that may arise due to your participation, you will be offered treatment by the study doctor free of charge.

What are the benefits of study participation?

There are no direct benefits as a result of participation in this study. However information obtained in this study will contribute in the understanding of Zoonotic disease agents.

When can you leave the study?

Your participation in this study is completely up to you. You can leave this study at any time without giving a reason. Withdrawal will NOT compromise any rights you had before entry into the study or influence any current or future medical care you may need.

You may be removed from the study without your consent for the following reasons:

- If the study is stopped or cancelled
- Other reasons as given by the Study Sponsor
- You are not doing what is needed for the study.

If you leave or are asked to leave the study after lab tests have been done, you may still get your test results from the study site.

How much will it cost?

You do not have to pay to be in the study.

Confidentiality

Your participation in the study, all information collected about you, and all laboratory test results will be available to no one except the study team. You will be identified only by your own unique identity number, which is known only by you and the clinic staff. Apart from the study team members that you meet, other staff from National or International government regulatory agencies, members of the Ethics Committee, study monitors, auditors, inspectors, and representatives of the Sponsor may check the records to make sure that the study was conducted properly. They are equally bound to respect your confidentiality. Your identity will not be disclosed in any publication or presentation of this study.

Contact Numbers

If you have any questions regarding the study or your participation in the study, you can Call **Munira Antar** the Investigator, at mobile: 0722410018.

If you have a question about your rights as a research volunteer you should contact **Prof Guantai, the Chairman of the Ethics Committee at Kenyatta National Hospital, Tel: 726300-9.**

INFORMED CONSENT DOCUMENT FOR ADULTS OVER 18 YEARS

I, (name of volunteer)

……..……...…

Of (address)

……..……

Agree to take part in the research project entitled: **Documentation of the exposure of Kenyan Residents to zoonotic disease agents**

I have been told in detail about the study and know what is required of me. I understand and accept the requirements. I understand that my consent is entirely voluntary and that I may withdraw from the research study for any reason, and this will not affect the legal rights I may otherwise have. My questions have been answered to my satisfaction.

Participant: Print Name: ……..….

Signature/Mark or Thumbprint: …….…….

Date: |__|__|/|__|__|__|/|__|__|__|__|

Person Obtaining Consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer and answered his/her questions:

Print Name: ……..

Signature: …….…….

Date: |__|__|/|__|__|__|/|__|__|__|__|

Impartial Witness: *(only necessary if volunteer was not able to read and understand the Consent Information Sheet and Informed Consent Document):*

I affirm that the Informed Consent Document has been read to the volunteer and he/she understands the study, had his/her questions answered, and I have witnessed the volunteer's consent to study participation.

Print Name: ……..…….

Signature/Mark or Thumbprint: …….…….

Date: |__|__|/|__|__|__|/|__|__|__|__|

INFORMED CONSENT DOCUMENT FOR CHILDREN UNDER 18 YEARS

I, (name of parent/guardian)
……..……...…

Of (address)
……..……

Agree for my chid to take part in the research project entitled: **Documentation of the exposure of Kenyan Residents to zoonotic disease agents**

I have been told in detail about the study and know what is required of my child. I understand and accept the requirements. I understand that my child's consent is entirely voluntary and that he/she may withdraw from the research study for any reason, and this will not affect the legal rights my child may otherwise have. My questions have been answered to my satisfaction.

Participant: Print Name: ……..….

Signature/Mark or Thumbprint: …….…….

Date: |__|__|/|__|__|__|/|__|__|__|__|

Person Obtaining Consent:

I have explained the nature, demands and foreseeable risks of the above study to the parent/guardian and answered his/her questions:

Print Name: …….

Signature: …….…….

Date: |__|__|/|__|__|__|/|__|__|__|__|

Impartial Witness: *(only necessary if volunteer was not able to read and understand the Consent Information Sheet and Informed Consent Document):*

I affirm that the Informed Consent Document has been read to the parent/guardian and he/she understands the study, had his/her questions answered, and I have witnessed the volunteer's consent to study participation.

Print Name: ……..…….

Signature/Mark or Thumbprint: ……..…….

Date: |__|__|/|__|__|__|/|__|__|__|__|

MWISHONI *(APPENDICES)*
Mwisho wa I: FOMU YA WARAKA WA IDHINI

Kichwa:
"Nyaraka wa mfiduo ya wakazi wa Kenya kwa viini vya ugonjwa wa zonootic"
Documentation of the exposure of Kenyan Residents to zoonotic disease agents.
Utangulizi:

Viumbe vinavyo ambukizwa kutoka kwa wanyama hadi binadamu ndivyo vinavyo sababisha ugonjwa kwa ujumla unaoitwa ugonjwa wa *zoonotic* au *zoonoses* na ni pamoja na jumla ya viumbe vya magonjwa ya kuambukizana (bakteria, virusi, vidoea, *rickttsial* na uyoga) na yale yasiyo ya viumbe vya kuambukizana (*prion*). *Zoonoses* zina sababishwa na viumbe vya *etiological* ambavyo husambaa kwa wanyama wenyeji *vertebrates* (wanyama wenye mjongojongo) na mara kwa mara kuambukizwa kwa binadamu, sana sana kupitia kumeza, kuumwa (na wanyama au *arthropod*), kukwaruzwa, kuvuta pumzi au moja kwa moja kupitia kukutana kwa ngozi/tundu za mwili. Wanyama walioambukizwa viumbe vya ugonjwa wa zoonotic wanaweza kuonyesha au kuto onyesha dalili ya ugonjwa huo. Zoonoses hutambulika sana na ugonjwa unaojitokeza na ukweli ni kwamba zimetambulika kama maambukizi muhimu yanayopatikana kwa sasa na yanaleta wasiwasi wa kimataifa kutokana na uhusiano wao na mazingara na tabia yao ya kujitokeza kwa ghafla mara kwa mara katika maeneo ya ulimwengu ambapo hayatarajiwi.

Mara kwa mara sisi hujiweka wazi daima kwa menyu inayobadilika ya magonjwa ya zoonotic ambayo huja kuwa maarufu mara kwa mara. Kutokea kwa hivi karibuni kwa mafua ya avian, SARS, Ebola, Dengue na virusi vya West Nile vinavyo husishwa na vifo vya binadamu vimesababisha ongezeko la kuzingatia zoonoses zinazojitokeza kwa mara ya kwanza au zile zinazo jitokeza tena. Ongezeko la mwenendo wa mara kwa mara wa watu, wanyama wao duniani kote pamoja na mabadiliko ya mazingara ni hali zinazofanya kuwa nzuri kwa ajili ya kuambukizwa zoonoses.

Madhumuni ya utafiti:

Lengo la utafiti huu ni kuchunguza maambukizi ya antibodi maalumu dhidi ya magojwa kadhaa ya zooonotic, kujua hatari zinazohusiana na magonjwa fulani ya zoonotic na usambazaji wake, katika hospitali za rufaa zitakazo chaguliwa nchini Kenya.

Jinsi ya kujiunga na utafiti

Utaingizwa kwenye utafiti huu ukiwa na homa wakati unapotembelea hospitali. Jumla ya 182 watakao jitolea watajiunga na utafiti huu kwa muda wa miezi 2 kutoka kwa hospitali 2 za

umma nchini kote: Kila hospitali itaandikisha watu mia tano watakao jitolea. Hii ni pamoja na hospitali ya Kiwilaya ya Mbagathi (Nairobi) na Hospitali Kuu ya mkoa wa Coast General (Mombasa).

Lazima uwe na umri wa zaidi ya miaka 18 na uwe unaweza tia sahihi fomu ya waraka wa idhini. Watakao jitolea na walio na miaka chini ya 18, wazazi/walezi wao ndio watakao peana kibali.

Utaratibu:

- Utakuwa kwenye kiliniki takribani saa moja.
- Utaelezwa kuhusu utafiti huu kwa lugha unayoielewa. Utapewa wakati wa kuuliza maswali yoyote. Wakati utakapokuwa umeelewa na kukubali kujiunga na utafiti, utatia sahihi au kuweka alama kwa nakala 2 za fomu ya waraka wa idhini ili kuthibitisha yakwamba umepewa habari za kuhusu utafiti huu na umejitolea kushiriki kwa hiyari. Nakala moja ni yako ujiwekee na nakala nyengine itawekwa kwenye faili zetu za utafiti kwa njia ya usiri. Ikiwa hautaki kuweka nakala yako, utaweka sahihi au kuweka alama kwenye fomu ambayo inaeleza kuwa hautaki kuweka nakala yako ya fomu, ma nakala hiyo utawekewa.
- Kwa watoto, idhini ya kushiriki itatolewa na wazazi/mlezi.
- Utaulizwa maswali kuhusiana na haliyako ya kiafya kwa ujumla na ufanyiwe uchunguzi wa kimatibabu na daktari.
- Kiasi cha mililita 15 hadi 20 (kiasi cha vijiko 2 vikubwa au mirija 4) ya damu yako kitachukuliwa kwa uchunguzi wa maabara ili kufanyia uchunguzi wa kingamwili zilizoko.

<u>Nini hatari na/au usumbufu?</u>

Kunaweza kuwa na hatari inayohusiana na utoaji wa damu kwa uchunguzi wa maabara na kwa kuwa mtu aliyejitolea kwenye utafiti huu kama vile:

- Unaweza hisi maumivu na chubuko mahali ambako sindano huingia mukononi mwako.
- Unaweza kuhisi kusulika au kuzirai.

Hakuna hatari za kimwili zinazo tarajiwa kwenye utafiti huu. Hata hivyo ikiwa kutatokea majeraha kutokana na kushiriki kwako kwenye utafiti huu, utapewa matibabu bila malipo na daktari wa utafiti huu.

Nini manufaa ya kushiriki kwenye utafiti huu?

Hakuna manufaa ya moja kwa moja kwa ajili ya kushiriki kwenye utafiti huu. Hata hivyo habari tutakazo pata kwenye utafiti huu zitachangia kuelewa kwa viumbe vya kusababisha magonjwa ya Zoonotic.

Wakati gani unaweza kutoka kwenye utafiti huu?

Wewe mwenyewe ndio utaamua kushiriki kwenye utafiti huu. Unaweza kujitoa kwenye utafiti huu wakati wowote bila kupeana sababu. Kujitoa HAKUTA badilisha haki zako ulizokuwa nazo kabla ya kujiunga na utafiti huu au kuathiri huduma za kimatibabu unazohitaji sasa au siku zijazo.

Unaweza kutolewa kwenye utafiti huu bila idhini yako kwa sababu zifuatazo:

- Ikiwa utafiti umesimamishwa au futiliwa mbali
- Sababu nyenginezo zitakazo peanwa na mfadhili wa utafiti
- Kama hufanyi vile unavyopaswa kufanya ukiwa kwenye utafiti huu

Ukijitoa au ukitolewa kwenye utafiti huu baada ya uchunguzi wa maabara kufanywa, unaweza kupewa majibu yako ya utafiti kwenye kituo cha utafiti.

Itagarimu nini?

Hautahitajika kulipa ili kujiunga na utafiti huu.

Uaminifu

Ukiwa kwenye utafiti huu, habari zozote zitakazochukuliwa kukuhusu wewe, na majibu yako ya uchunguzi zitawekwa kwa usiri na hazitaonekana na watu wengine isipokuwa wafanyikazi wa utafiti huu. Utatambulika kwa kutumia nambari yako ya utafiti, ambayo itajulikana na wewe na wafanyikazi wa kiliniki. Mbali na wafanyikazi wa utafiti huu utakao kutana nao, wafanyikazi wengine wa Kitaifa na Kimataifa wa udhibiti na uendeshaji wa utafiti, wanachama wa kamati ya maadili, wachunguzi wa utafiti, wakaguzi wa utafiti, inspectors wa utafiti na wawakilishi wa wafadhili pia wanaweza kuangalia rekodi ili kuhakikisha yakuwa

utafiti unafanywa vizuri. Hao pia watahitajika kuheshimu siri zako. Hutatambulika kwenye chapisho au habari zitakazo peanwa kuhusu utafiti huu.

Nambari za mawasiliano

Ukiwa na swali lolote kuhusu utafiti huu au kujiunga na utafiti huu, unaweza piga simu **Munira Antar** mtafiti mkuu, nambari ya simu 0722-410018

Ukiwa na swali kuhusu haki zako kama mshiriki wa utafiti huu unaweza mjulisha **Prof. A Guantai**, katibu mkuu wa Kamati ya Maadili na Utafiti ya Hospitali kuu ya Kenyatta/Chuo kikuu cha Nairobi kwenye afisi zao katika Hospitali kuu ya Kenyatta Nambari 726300-9

UKURASA WA KUWEKA SAHIHI WARAKA WA IDHINI KWA WENYE MIAKA 18 NA ZAIDI

Anayejitolea:
Mimi, (jina la anayejitolea) .....

Wa (Anwani) .....

nakubali kushiriki kwenye utafiti huu uitwao:

Nyaraka wa mfiduo ya wakazi wa Kenya kwa viini vya ugonjwa wa zonootic"
Documentation of the exposure of Kenyan Residents to zoonotic disease agents.

Nimeambiwa kwa kirefu kuhusu taratibu za utafiti na ninajua nini kinachohitajika kwangu. Naelewa na nakubali yanayohitajika. Naelewa najiunga na utafiti huu kwa hiyari na kuwa naweza kujitoa wakati wowote na sababu yeyote. Nikijitoa kwa utafiti huu, haki zangu za kisheria nilizonazo hazitaathiriwa.

Anayejitolea:

Jina la anayejitolea: .....

Sahihi/Alama ya kidole gumba: .....

Tarehe:___ ___ /___ ___ ___/ ___ ___ ___ ___

Mtu anaye chukua idhini:
Nimeeleza asili, madai na hatari zisizoeleweka za utafiti huu zilizoandikwa hapo juu kwa anayejitolea na nimeyajibu maswali yake:

Jina:..... .....

Sahihi: .....

Tarehe:___ ___ /___ ___ ___/ ___ ___ ___ ___

Shahidi: (*Ikiwa anayejitolea hawezi kusoma na kuelewa ukurasa wa habari za waraka, na waraka wa idhini ya kujitolea)*
Nathibitisha kwamba waraka wa kujitolea umesomewa anayejitolea, na anaelewa utafiti huu na nimeshuhudia akitoa idhini ya kushiriki kwa utafiti.

Jina: .....

Sahihi: .....

Tarehe:___ ___ /___ ___ ___/ ___ ___ ___ ___

UKURASA WA KUWEKA SAHIHI WARAKA WA IDHINI KWA WATOTO CHINI YA MIAKA 18

Anayejitolea:
Mimi, (jina la Mzazi/Mlezi wa mtoto): .....

Wa (Anwani) .....

nakubali kushirikisha mtoto wangu kwenye utafiti huu uitwao:

Nyaraka wa mfiduo ya wakazi wa Kenya kwa viini vya ugonjwa wa zonootic"
Documentation of the exposure of Kenyan Residents to zoonotic disease agents.

Nimeambiwa kwa kirefu kuhusu taratibu za utafiti na ninajua nini kinachohitajika kwa mtoto wangu. Naelewa na nakubali yanayohitajika. Naelewa mtoto wangu anajiunga na utafiti huu kwa hiyari na kuwa anaweza kujitoa wakati wowote na sababu yeyote. Akijitoa kwa utafiti huu, haki zake za kisheria alizonazo hazitaathiriwa.

Mzazi/Mlezi wa anayejitolea:

Jina la Mzazi/Mlezi wa anayejitolea: .....

Sahihi/Alama ya kidole gumba: .....

Tarehe:___ ___ /___ ___ ___/ ___ ___ ___ ___

Mtu anaye chukua idhini:
Nimeeleza asili, madai na hatari zisizoeleweka za utafiti huu zilizoandikwa hapo juu kwa mzazi/mlezi wa anayejitolea na nimeyajibu maswali ya mzazi/mlezi:

Jina:..... .....

Sahihi: .....

Tarehe:___ ___ /___ ___ ___/ ___ ___ ___ ___

Shahidi: (*Ikiwa Mzazi/Mlezi wa anayejitolea hawezi kusoma na kuelewa ukurasa wa habari za waraka, na waraka wa idhini ya kujitolea)*
Nathibitisha kwamba waraka wa kujitolea umesomewa Mzazi/Mlezi wa anayejitolea, na anaelewa utafiti huu na nimeshuhudia akitoa idhini ya kushiriki kwa mtoto wake kwa utafiti.

Jina: .....

Sahihi: .....

Tarehe:___ ___ /___ ___ ___/ ___ ___ ___ ___

Appendix II: Questionnaires

zoonotic disease study – VOLUNTEER DEMOGRAPHICS **Study Site:**

Volunteer Study ID Number: |__|__|__|__|

Zoonotic disease Study	**Demographics** *Complete for volunteers at Study Entry visit.*

Date of Visit: ¦___¦___¦ / ¦___¦___¦___¦ / ¦___¦___¦___¦ ___¦
(DD/MMM/YYYY)

1. Gender: |__| Male |__| Female
2. Date of birth dd/mon/yyyy: |__|__|-|__|__|__|-|__|__|__|__|
3. Race: Black ❒ Asian ❒ White ❒ Other ❒ If Other, specify: _____

4. Region/district currently living in: _____
5. How many years of school completed in each category? *(Enter 00 if no education in a given category)*

   |__|__| Completed years of primary school

   |__|__| Completed years of secondary school
   |__|__| Completed years of tertiary school (university or college)
   |__|__| Completed years of other school or apprenticeship
   Other (specify): _____
6. What is current marital status?
   |__| Single (never married) |__| Married, monogamous
   |__| Married, polygamous |__| Divorced/separated
   |__| Widowed
7. History of recent travel?

Comments:_____
_____
_____
_____
_____

Interviewer Signature:……

Date : ¦___¦___¦ / ¦___¦___¦___¦ / ¦___¦___¦___¦___¦ dd/MMM/yyyy

zoonotic disease study - BASELINE MEDICAL HISTORY **Study Site:**

Volunteer Study ID number |__|__|__|__|

Baseline Medical History & Complete Physical Exam

To be completed by Study Physician at study entry.

Date of Visit: ¦____¦____¦ / ¦____¦____¦____¦ / ¦____¦____¦____¦ ____¦ (DD/MMM/YYYY)

Sex: Male ☐ Female ☐ **DOB**: ¦____¦____¦ / ¦____¦____¦____¦ / ¦____¦____¦____¦ ____¦

(DD/MMM/YYYY)

Presence of fever No Yes *(If yes, describe history of presence fever, onset, other related symptoms)*

……

……

……

Past Medical History (in last X Months prior to onset of fever) *By Diagnosis (presumptive or confirmed) including start & stop dates, and highest severity for significant acute and all chronic medical conditions, surgery and major hospitalizations-*

Diagnosis	Presumed / Confirmed	Severity	Start Date (dd/mon/year)	Stop Date (dd/mon/year)
……	❒		……	……
……	❒		……	……
……	❒		……	……
……	❒		……	……
……	❒		……	……
……	❒		……	……
……	❒		……	……

Family History *(history of fever, bloody diarrhea vomiting etc in family members):* None Yes *(If yes, specify)*

……

……

Additional Comments:

……

……

……

……

Study Physician's Initials/date:……..¦____¦____¦/¦____¦____¦____¦/¦____¦____¦dd/MMM/yy

Appendix III: ELISA kit methodology

Dengue Virus IgM Capture DxSelect™ (OUS)

Product Code EL1500M
Rev. P

Indirect Enzyme-linked immunosorbent assay (ELISA) for the qualitative detection of human IgM class antibodies to dengue virus

This package insert is for export only and not for distribution in the United States.

Outside of the United States:
For *in vitro* Diagnostic Use.

FOCUS Diagnostics

INTENDED USE

The Focus Diagnostics Dengue Virus IgM Capture DxSelect™ is a qualitative assay for the detection of human serum IgM antibodies to dengue virus (DV) infections to be used in support of the diagnosis of acute dengue virus infections in humans.

SUMMARY AND EXPLANATION OF TEST

Dengue fever (DF) is an acute, self limiting, viral disease that is characterized by fever, headache, body pains, rash, lymphadenopathy, and prostration. In its most severe form, dengue hemorrhagic fever (DHF), infected patients will experience severe fever and renal failure leading to the often fatal dengue shock syndrome (DSS).[1] It is estimated that approximately 2 billion people are at risk for DF world wide, and that over 1 million people per year are infected.[2] This, combined with the hundreds of thousands of cases of DSS, make dengue the most important arbovirus disease in the world.[2]

Dengue virus (DV) is a flavivirus and is closely related to the yellow fever virus, Japanese encephalitis virus and other group B Arboviruses. Members of this group possess single stranded RNA which is surrounded by an icosahedral nucleocapsid covered with a 10 nm deep lipid envelope.[3] There are 4 strains of dengue virus, each serologically distinct. Infection with 1 strain does not protect the host from infection by the others. In fact, one report suggests DHF and DSS occurs most commonly in individuals that have been infected previously by another strain.[4] The presence of circulating, non-neutralizing, cross-reactive DV antibody may act as an immune infection enhancement factor.[4] However, non-neutralizing, cross-reactive antibodies against other non-DV flaviviruses are not associated with immune infection enhancement.[4]

Dengue fever epidemics have been reported regularly throughout the world. The largest epidemics have occurred in the southern United States (1922 affecting over 1 million people), Australia (1925 and 1942), Greece (1927) and Japan (1942-1945).[2] Peruvian and CDC officials have reported a major DF outbreak that occurred between March to July 1990.[5] This epidemic centered around Iquitos, Peru, involving DV types 1 and 4, is the first laboratory confirmation of indigenous transmission of dengue in Peru.[5]

Dengue fever can be transmitted wherever the mosquito vectors, *Aedes aegypti* and *Aedes albopictus,* are found. *A. aegypti* is primarily localized to tropical and subtropical Americas and is indigenous to the southern part of the United States.[2] The primary vector for DF in Asia is *A. albopictus.* This mosquito has recently established itself in the United States as far north as central Illinois; however, DV transmission has not been associated with it to date.[2]

In most patients, suspected cases of DF are most rapidly diagnosed using serological methods. Traditionally, hemagglutination inhibition and plaque reduction neutralization have been used.[3] However, IgM Capture enzyme-linked immunosorbant assays (ELISA) have become the method of choice. In most individuals the IgM response to DF is strain specific and persists as long as 90 days.[6]

IgG antibody to dengue virus has been detected in patients as long as 60 years post infection.[7] Therefore, a single antibody determination should not be considered conclusive. However, the IgG ELISA is useful as an epidemiological tool when used to establish sero-prevalence. This information can also be valuable in studies designed to determine the role immune enhancement plays in DSS. As with most flaviviruses, IgG is highly cross-reactive among most members of the flavivirus group.

The Focus Diagnostics Dengue Virus IgM Capture DxSelect™ is intended for the detection of human serum IgM antibodies to DV types 1-4. This assay is specific for IgM and uses a flavivirus group monoclonal horseradish peroxidase conjugate with TMB as substrate. Each Capture Well is coated with antihuman IgM. The antigen solution contains equal proportions of inactivated DV types 1-4. The following virus strains are used: Type 1: TH-Sman; Type 2: TH-36, Type 3:H87; and Type 4: H241.

TEST PRINCIPLE

In the Focus Diagnostics Dengue Virus IgM Capture DxSelect™, the polystyrene microwells are coated with anti-human antibody specific for IgM (pt-chain). Diluted serum samples and controls are incubated in the wells, and IgM present in the sample binds to the anti-human antibody (IgM specific) in the wells. Focus has shown that the 2-hour incubation period performs substantially equivalent to the overnight incubation procedure.[8] Nonspecific reactants are removed by washing. Dengue virus (DV) antigen is then added to the wells and incubated; and, if anti-DV IgM is present in the sample, the DV antigen binds to the anti-DV in the well. Unbound DV antigen is then removed by washing the well. Mouse anti-DV conjugated with horseradish peroxidase is then added to the wells and incubated; and, if DV antigen has been retained in the well by the anti-DV in the sample, the mouse anti DV: HRPO binds to the DV antigen in the wells. Excess conjugate is removed by washing. Enzyme substrate and chromogen are added, and the color is allowed to develop. After adding the Stop Reagent, the resultant color change is quantified by a spectrophotometric reading of optical density (OD) which is directly proportional to the amount of antigen-specific IgM present in the sample. Sample optical density readings are compared with reference cut-off OD readings to determine results.

Background Subtract Procedure

The Focus Diagnostics Dengue Virus IgM Capture DxSelect™ kit employs capture wells coated with anti-human IgM. Captured IgM with heterophilic antibody activity may directly bind the reporter reagent. One way to help mitigate these false reactions is through use of a background subtraction procedure. Published data have shown that low level reactive samples may benefit from implementation of background subtraction; however, each laboratory must define its own reflex testing algorithms for analytes measured by μ-capture ELISA.[12]

MATERIALS SUPPLIED

The Focus Diagnostics Dengue Virus IgM Capture DxSelect™ kit contains sufficient materials to perform 96 determinations. Allow the supplied reagents to warm to room temperature before use. All un-opened materials are stable at 2 to 8°C until the expiration date stated on the reagent label.

Antigen, Lyophilized | REF | EL1522 | Ag |

2 vials containing inactivated lyophilized dengue virus antigen (equal portions of DV types 1-4). Each 6 mL antigen vial will perform approximately 60 tests.

To reconstitute the antigen, add exactly 6 mL of the reconstitution solution provided. DO NOT USE DISTILLED WATER OR ANOTHER REAGENT OTHER THAN THE REAGENT PROVIDED FOR RECONSTITUTION. ASSAY RESULTS ARE INVALID IF ANY OTHER MATERIAL IS USED FOR RECONSTITUTION. Allow the antigen to rehydrate at room temperature for 1 hour prior to use: the antigen must be completely dissolved before use. Store the remaining antigen at 2 to 8°C for up to 30 days following reconstitution. If the remaining antigen will not be used within 30 days, aliquot and freeze at –70°C or colder. Thaw only once.

Antigen Reconstitution Solution, 13 mL | REF | EL1523 | SOLV | Ag |

1 vial containing cell culture water, surfactant and 0.1% sodium azide.

IgM Capture Wells, 96 wells | REF | EL1521 | Ab | IgM |

12 Eight-well polystyrene break-apart microwell strips on a frame. Each well is coated with anti-human IgM. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets.

IgM Conjugate, 16 mL | REF | EL1502 | CONJ | IgM |

One vial of affinity-purified and peroxidase-conjugated mouse anti-flavivirus. Contains protein, buffer, and non-azide preservatives.

IgM Detectable Control, 0.30 mL | REF | EL1515 | CONTROL | > |

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

Non-Detectable Control, 0.30 mL | REF | EL1512 | CONTROL | < |

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

IgM Cut-Off Calibrator, 0.30 mL | REF | EL1503 | CONTROL | CAL |

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

Sample Diluent, 100 mL | REF | EL1608 | DIL | SPE |

1 vial of protein, surfactant, and non-azide preservatives in PBS.

10X Wash Buffer, 100 mL | REF | EL0405 | BUF | WASH |

1 vial of surfactant in PBS with non-azide preservatives. Prepare a 1X wash buffer solution before use.

To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled (or deionized) water and rinse out any crystals. Use only the highest grade purified water for reconstitution of the wash buffer. It has been observed that some sources of deionized water contain materials which can interfere in the assay. Swirl until well mixed and all crystals are dissolved.

Substrate Reagent, 16 mL | REF | EL0009 | SUBS | TMB |

1 vial of tetramethylbenzidine (TMB) and hydrogen peroxide in buffer. A dark blue color indicates contamination with peroxidase; and, if this occurs, use a fresh bottle.

Stop Reagent, 16 mL | REF | EL0105 | SOLN | STOP |

1 vial 1 M sulfuric acid.

Sealing Tape

3 sheets of sealing tape.

MATERIALS REQUIRED, BUT NOT SUPPLIED

1. Distilled water
2. 250 or 500 mL wash bottle
3. 1 L graduated cylinder
4. Test tubes for serum dilutions
5. 10 µL pipettors with disposable tips
6. 100 µL pipettors with disposable tips (100 µL 8- or 12-channel pipettor recommended for runs over 48 wells)
7. 1 mL pipet or dispenser
8. 5 mL pipet
9. Timer
10. Paper towels or absorbent paper
11. Sink
12. Vortex mixer or equivalent
13. ELISA plate spectrophotometer, wavelength = 450 nm

SHELF LIFE AND HANDLING

1. Kits and kit reagents are stable through the end of the month indicated by the label expiration date when stored at 2 to 8°C.
2. Do not use test kit or reagents beyond their expiration dates.
3. Do not expose reagents to strong light during storage or incubation.
4. Allow reagents to warm to room temperature before use.

WARNINGS AND PRECAUTIONS

1. This package insert is for export only and not for distribution in the United States. Outside of the United States this kit is for *in vitro* diagnostic use.
2. All blood products should be treated as potentially infectious. Source materials from which this product (including controls) was derived have been screened for Hepatitis B surface antigen, Hepatitis C antibody and HIV-1/2 (AIDS) antibody by FDA-approved methods and found to be negative. However, as no known test methods can offer 100% assurance that products derived from human blood will not transmit these or other infectious agents, all controls, serum specimens and equipment coming into contact with these specimens should be considered potentially infectious and decontaminated or disposed of with proper biohazard precautions.

3. The lyophilized antigen contains 4 strains of inactivated DV; however, the reagent should be considered potentially infectious and handled accordingly.
4. The stop reagent contains sulfuric acid. Do not allow to contact skin or eyes. If exposed, flush with copious amounts of water.
5. Sodium azide at a concentration of 0.1% has been added to some reagents as an antibacterial agent. To prevent buildup of explosive metal azides in lead and copper plumbing, controls should be discarded into sewerage only if diluted and flushed with large volumes of water. Use copper-free and lead-free drain systems where possible. Occasionally decontaminate the drains with 10% sodium hydroxide (CAUTION: caustic), allow to stand for 10 minutes, then flush with large volumes of water.
6. Do not substitute or mix reagents from different kit lots or from other manufacturers.
7. Use only protocols described in this insert. Incubation times or temperatures other than those specified may give erroneous results.
8. Cross-contamination of patient specimens can cause erroneous results. Add patient specimens and handle IgM Capture Well strips carefully to avoid mixing of sera from adjoining wells. Avoid contamination of the substrate reagent with traces of the enzyme conjugate.
9. Bacterial contamination of serum specimens or reagents can produce erroneous results. Use aseptic techniques to avoid microbial contamination.
10. Perform the assay at room temperature (approximate range 20 to 25°C).
11. Use proper pipetting techniques, maintaining the pipetting pattern throughout the procedure to ensure optimal and reproducible values.

SPECIMEN COLLECTION AND PREPARATION

Serum is the preferred specimen source. No attempt has been made to assess the assay's compatibility with other specimens. Hyperlipemic, heat-inactivated, hemolyzed, or contaminated sera may cause erroneous results; therefore, their use should be avoided.

Specimen Collection and Handling

Collect blood samples aseptically using approved venipuncture techniques by qualified personnel. Allow blood samples to clot at room temperature prior to centrifugation. Aseptically transfer serum to a tightly closing sterile container for storage at 2 to 8°C. If testing is to be delayed longer than 5 days, the sample should be frozen at –20°C or colder. Thaw and mix samples well prior to use.

Specimen, Controls and Calibrator Preparation

Dilute each specimen, control and calibrator 1:101 as follows: label tubes and dispense 1 mL of **Sample Diluent** into each labeled tube. Add **10 µL of specimen, control or calibrator** to each appropriate tube containing the 1mL Sample Diluent and mix well by vortex mixing.

TEST PROCEDURE

The Focus Diagnostics Dengue Virus IgM Capture DxSelect™ may be used in either of 2 ways. The classical CDC protocol uses an overnight capture antigen incubation step; or, as an alternative, the capture antigen incubation step can be shortened to 2 hours at room temperature.

1. Allow all reagents to warm to room temperature before use. Remove the IgM Capture Well packet from cold storage. To avoid condensation, allow micro-well strips to reach room temperature before opening the foil packet. If less than a full plate is to be used, return unused strips to the foil packet with desiccant and reseal completely. Store unused IgM Capture Wells at 2 to 8°C. (Note: At the end of the assay, retain the frame for use with the remaining strips.)
2. Prepare Antigen Solution (make sure reagent has reached room temperature). If the kit is being used for the first time, reconstitute sufficient antigen (see MATERIALS SUPPLIED, above).
3. Fill wells with 1X Wash Buffer solution (see MATERIALS SUPPLIED, above) and allow to soak for 5 minutes. Decant (or aspirate) the antigen wells and tap vigorously to remove Wash Buffer. Blot the emptied Capture Wells face down on clean paper towels or absorbent paper to remove residual Wash Buffer.
4. Dispense 100 µL of the Sample Diluent into the "blank" wells and 100 µL of each diluted specimen, control or calibrator (see Specimen, Controls, and Calibrator Preparation, above) into the appropriate wells. (Note: For runs with more than 48 wells it is recommended that 250 µL of each diluted sample first be added to a blank microtiter plate in the location corresponding to that in the ELISA wells. The samples can then be efficiently transferred into the Antigen Wells with a 100 µL 8- or 12-channel pipettor.)
5. Cover plates with sealing tape, and incubate for 60 ± 1 minute at room temperature (20 to 25°C).
6. Remove sealing tape, and empty the contents of the wells into a sink or a discard basin.
7. Fill each well with a gentle stream of 1X Wash Buffer solution then empty contents into a sink or a discard container.
8. Repeat wash (step 7) an additional 2 times, allow the last wash to soak for 5 minutes before decanting or aspirating.
9. Tap the Capture Wells vigorously to remove 1X Wash Buffer. Blot the emptied Capture Wells face down on clean paper towels or absorbent paper to remove residual 1X Wash Buffer.
10. Add 100 µL of the prepared (see step 2, above) Antigen Solution to all wells, using a 100 µL 8- or 12-channel pipettor.
11. Cover plates with sealing tape and incubate for 2 hours at room temperature (20 to 25°C).
12. Repeat wash steps 6 through 9.
13. Add 100 µL of IgM Conjugate to all wells, using a 100 µL 8- or 12-channel pipettor.
14. Cover plates with sealing tape, and incubate for 30 ± 1 minute at room temperature (20 to 25°C).
15. Repeat wash steps 6 through 9.
16. Add 100 µL of Substrate Reagent to all wells, using a 100 µL 8- or 12-channel pipettor. Begin incubation timing with the addition of Substrate Reagent to the first well. (Note: Never pour the substrate reagent into the same trough as was used for the conjugate.)
17. Incubate for 10 ± 1 minutes at room temperature (20 to 25°C).
18. Stop the reaction by adding 100 µL of Stop Reagent to all wells using a 100 µL 8- or 12-channel pipettor. Add the Stop Reagent in the same sequence and at the same pace as the Substrate was added. In antibody-positive wells, color should change from blue to yellow.
19. Gently blot the outside bottom of wells with a paper towel to remove droplets that may interfere with reading by the spectrophotometer. Do not rub with the paper towel as it may scratch the optical surface of the well. (Note: Large bubbles on the surface of the liquid may affect the OD readings.)
20. Measure the absorbance of each well within 1 hour of stopping the assay. Set the microwell spectrophotometer at a wavelength of 450 nm. Zero the instrument on the blank wells.

West Nile Virus IgM Capture DxSelect™ (English)

Enzyme-linked Immunosorbent Assay (ELISA)

Product Code EL0300M

Rev. K

IgM Capture Enzyme-linked immunosorbent assay for qualitatively detecting human serum IgM antibodies to West Nile virus

For *in vitro* Diagnostic Use

FOCUS Diagnostics

INTENDED USE

The Focus Diagnostics West Nile Virus IgM Capture DxSelect™ is intended for qualitatively detecting IgM antibodies to West Nile virus in human serum. In conjunction with the Focus Diagnostics West Nile Virus IgG DxSelect™, the test is indicated for testing persons having symptoms of meningioencephalitis, as an aid in the presumptive laboratory diagnosis of West Nile virus infection. Positive results must be tested using the background subtraction method (either on the initial test or on a repeat test). Positive results must be confirmed by neutralization test, or by using the current CDC guidelines for diagnosing West Nile encephalitis.[1] This test is not intended for self-testing, and this test is not FDA cleared nor approved for testing blood or plasma donors. Assay performance characteristics have not been established for automated instruments.

Caution: IgM assay cross-reactivity has been noted with some West Nile IgM assays testing specimens containing antibody to enteroviruses. Reactive results reported from children must contain a caution statement regarding possible cross-reactivity with enteroviruses.

SUMMARY AND EXPLANATION OF TEST

Most people who are infected with West Nile virus (WNV) will not have any type of illness. Experts estimate that 20% of the people who become infected will develop West Nile fever: mild symptoms, including fever, headache, and body aches, occasionally with a skin rash on the trunk of the body and swollen lymph glands.[2] Symptoms of mild disease will generally last a few days. About 1 in 150 of West Nile virus infections (<1%) result in meningitis or encephalitis.[2] Case fatality rates among patients hospitalized during recent outbreaks have ranged from 4% to 14%.[3] Advanced age is the most important risk factor for death, and patients older than 70 years of age are at particularly high risk.[3] The case fatality rates for WNV are similar to St. Louis encephalitis (SLE) virus and Western equine encephalitis (WEE) virus (5-15%), but much lower than Eastern equine encephalitis (EEE) virus (30-70%), and higher than La Crosse (LAC) virus (<1%).[4,5]

WNV is an arbovirus. Arboviruses are zoonotic, and are transmitted through complex life cycles involving a vertebrate (e.g., birds) and an arthropod (e.g., mosquitoes).[3] Humans and domestic animals can develop clinical illness but usually are "dead-end" hosts because they do not produce significant viremia. Infection is usually not transmitted from person to person. Arbovirus infections can be prevented in two major ways: personal protective measures to reduce contact with mosquitoes and public health measures to reduce the population of infected mosquitoes in the environment.[2]

WNV can be detected by culturing the organism, by detecting viral antigen or by detecting viral nucleic acid in cerebrospinal fluid, tissue, blood, or other body fluid. Although a positive culture or a positive nucleic acid detection test are highly specific, experts do not recommend their use for screening because of their limited sensitivity.[2] Viral culture of cerebrospinal fluid or brain tissue has had very low yield among U.S. patients. Nucleic acid amplification testing has been positive in up to 55% of samples of cerebrospinal fluid and 10% of serum samples.[2] Centers for Disease Control and Prevention (CDC) recommends serology for detecting WNV and other arboviruses.[1]

CDC reports that although the antibody response to human infection with WNV has not been thoroughly or systematically studied, the following are reasonable assumptions, based on extensive experience with other flaviviruses, or preliminary conclusions based on empirical observations made during the 1999 and 2000 epidemics:

- IgM antibody in serum: By the eighth day of infection, a large majority of infected persons will have detectable serum IgM antibody to WNV; in most cases it will be detectable for at least 1-2 months after illness onset; and in some cases it will be detectable for 500 days or longer.[6]
- IgG antibody in serum: By three weeks post-infection (and often earlier), virtually all infected persons should demonstrate serum IgG antibody to WNV by enzymatic immunoassay (EIA) for 500 days or longer.[6]

Positive results are known to occur with persons vaccinated for flaviviruses (e.g., yellow fever, Japanese encephalitis, dengue), with persons infected with other flaviviruses, and with persons previously infected with WNV. Because closely related arboviruses exhibit serologic cross-reactivity, sometimes it may be epidemiologically important to attempt to pinpoint the infecting virus by conducting plaque reduction neutralization tests using an appropriate battery of closely related flaviviruses.

TEST PRINCIPLE

In the Focus Diagnostics West Nile Virus IgM Capture DxSelect™, the polystyrene microwells are coated with anti-human antibody specific for IgM (μ-chain). Diluted specimen samples and controls are incubated in the wells, and IgM present in the sample binds to the anti-human antibody (IgM specific) in the wells. Non-specific reactants are removed by washing. Recombinant WNV antigen is then added to the wells and incubated; and, if anti-WNV IgM is present in the sample, the WNV antigen binds to the anti-WNV in the well.

Unbound WNV antigen is then removed by washing the well, Mouse anti-flavivirus conjugated with horseradish peroxidase (HRPO) is then added to the wells and incubated; and, if WNV antigen has been retained in the well by the anti-flavivirus in the sample, the mouse anti-flavivirus: HRPO. binds to the WNV antigen in the wells. Excess conjugate is removed by washing. Enzyme substrate and chromogen are added, and the color is allowed to develop. After adding the Stop Reagent, the resultant color change is read by a spectrophotometer. The color intensity is compared to the Cut-off's to determine if antigen-specific IgM is present in the sample.

Background Subtract Procedure

All IgM reactive samples must be tested with the background subtract procedure to check for false positives caused by cross-reacting antibodies (e.g., RF and heterophilic antibodies) and other substances. Heterophile antibodies are antibodies that can be present in the patient specimen and can bind to animal antibodies (for example the Capture Wells contain rabbit antibody and the Anti-flavivirus Conjugate contains mouse antibody). The background subtract procedure detects false positives by testing initially positive samples with and without West Nile Antigen and comparing the reactivity. If heterophile antibodies are present in the sample, they will cross-link the Capture Well antibodies to the Anti-flavivirus Conjugate, and both wells will be reactive. If heterophile antibodies are absent, then only the well with Antigen will be reactive. The background subtraction method will not eliminate false positive results due to cross-reactive antibodies to other flaviviruses (e.g. St. Louis encephalitis, dengue etc).

MATERIALS SUPPLIED

The Focus Diagnostics West Nile IgM Capture DxSelect™ Test kit contains sufficient materials to perform 96 determinations. Allow the supplied reagents to be used to warm to room temperature before use. All un-opened materials are stable at 2 to 8°C until the expiration date stated on the reagent label.

Antigen (lyophilized) | REF | EL0322 | Ag | + |

2 vials containing lyophilized recombinant West Nile virus antigen. Each 8mL antigen vial will perform approximately 80 tests.

To reconstitute one vial of the antigen, add exactly 8mL of the Sample Diluent provided.

DO NOT USE DISTILLED WATER OR ANOTHER REAGENT OTHER THAN THE REAGENT PROVIDED FOR RECONSTITUTION. ASSAY RESULTS ARE INVALID IF ANY OTHER MATERIAL IS USED FOR RECONSTITUTION.

The lyophilized antigen appears as a "pearl" and it must be "tapped" to the bottom prior to opening and reconstituting. Allow the antigen to re-hydrate at room temperature for one hour prior to use: the antigen must be completely dissolved before use. Store the remaining antigen at 2 to 8°C for up to 60 days following reconstitution. **Avoid storing the reconstituted antigen at room temperature: remove from 2 to 8°C, withdraw the amount needed immediately, and return the unneeded antigen immediately to 2 to 8°C. Do not freeze.**

IgM Capture Wells, 96 wells | REF | EL1521 | Ab | IgM |

12 eight-well polystyrene break-apart microwell strips on a frame. Each well is coated with rabbit anti-human IgM. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets.

Anti-flavivirus Conjugate, 16 mL | REF | EL0302 | CONJ | IgM |

One vial of affinity-purified and peroxidase-conjugated mouse monoclonal anti-flavivirus. Contains protein, buffer, and non-azide preservatives. Ready to use.

Positive Control, 0.3 mL | REF | EL0315 | CONTROL | + |

One vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see **Specimen, Controls and Calibrator Preparation**, below).

Negative Control, 0.3 mL | REF | EL0312 | CONTROL | – |

One vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see **Specimen, Controls and Calibrator Preparation**, below).

Cut-Off Calibrator, 0.3 mL REF EL0303 CONTROL CAL

One vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see **Specimen, Controls and Calibrator Preparation**, below).

Sample Diluent, 112 mL REF EL1608-112 DIL SPE

One vial of PBS containing protein, surfactant, and non-azide preservatives. Ready to use.

10X Wash Buffer, 100 mL REF EL0405 BUF WASH

One vial of surfactant in PBS with non-azide preservatives. Prepare a 1X wash buffer solution before use.

To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled (or deionized) water and rinse out any crystals. Swirl until well mixed and all crystals are dissolved.

Substrate Reagent, 16 mL REF EL0009 SUBS TMB

One vial of tetramethylbenzidine (TMB) and hydrogen peroxide in buffer. A dark blue color indicates contamination with peroxidase; and if this occurs, then use a fresh bottle. Ready to use.

Stop Reagent, 16 mL REF EL0105 SOLN STOP

One vial 1 M sulfuric acid. Ready to use.

Sealing Tape

Three sheets of sealing tape.

MATERIALS REQUIRED, BUT NOT SUPPLIED

1. Distilled or deionized water
2. 250 or 500 mL wash bottle *or* automated EIA plate washer
3. 1 L graduated cylinder
4. 12 x 75 mm borosilicate glass test tubes or equivalent
5. 10 µL pipettor with disposable tips
6. 100 µL pipettor with disposable tips (100 µL eight-channel pipettor recommended for runs over 48 wells).
7. 1 mL pipet or dispenser
8. 5 mL pipet
9. Timer
10. Paper towels or absorbant paper
11. Sink
12. Vortex mixer or equivalent
13. ELISA plate spectrophotometer, wavelength = 450 nm

SHELF LIFE AND HANDLING

1. Kits and kit reagents are stable through the end of the month indicated by the label expiration date when stored at 2 to 8°C.
2. Do not use test kit or reagents beyond their expiration dates.
3. Do not expose reagents to strong light during storage or incubation.
4. Allow reagents to warm to room temperature before use.

WARNINGS AND PRECAUTIONS

1. This kit is for *in vitro* diagnostic use only.
2. All blood products should be treated as potentially infectious. Source materials from which this product (including controls) was derived have been screened for Hepatitis B surface antigen, Hepatitis C antigen and HIV-1/2 (AIDS) antibody by FDA-approved methods and found to be negative. However, as no known test methods can offer 100% assurance that products derived from human blood will not transmit these or other infectious agents, all controls, serum specimens and equipment coming into contact with these specimens should be considered potentially infectious and decontaminated or disposed of with proper biohazard precautions. Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.[7]
3. The Capture Wells are produced with anti-human IgM antibodies. After adding patient or control specimens, the strips should be considered potentially infectious and handled accordingly.
4. Sodium azide at a concentration of 0.1% has been added to some reagents as an antibacterial agent. To prevent buildup of explosive metal azides in lead and copper plumbing, those reagents (see Materials Supplied, above) should be discarded into sewerage only if diluted and flushed with large volumes of water. Use copper-free and lead-free drain systems where possible. Occasionally decontaminate the drains with 10% sodium hydroxide (CAUTION: caustic), allow to stand for 10 minutes, then flush with large volumes of water.
5. Do not substitute or mix reagents from different kit lots or from other manufacturers.

6. Use only protocols described in this insert. Incubation times or temperatures other than those specified may give erroneous results.
7. Cross-contamination of patient specimens can cause erroneous results. Add patient specimens and handle strips carefully to avoid mixing of sera from adjoining incubation tray wells. Decant carefully.
8. Bacterial contamination of serum specimens or reagents can produce erroneous results. Use aseptic techniques to avoid microbial contamination.
9. The Stop Reagent contains sulfuric acid. Do not allow to contact skin or eyes. If exposed, flush with copious amounts of water.
10. Sodium azide inhibits conjugate activity. Clean pipette tips must be used for the conjugate addition so that sodium azide is not carried over from other reagents.

SPECIMEN COLLECTION AND PREPARATION

This test must be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established. Performance characteristics have not been established with hyper-lipemic, heat inactivated, hemolyzed, icteric, or contaminated sera. It is unknown if such specimens will cause erroneous results. Hyper-lipemic, heat inactivated, hemolyzed, icteric, and contaminated sera must not be tested.

Specimen Collection and Handling

Collect blood samples aseptically using approved venipuncture techniques by qualified personnel. Allow blood samples to clot at room temperature prior to centrifugation. Aseptically transfer serum to a tightly closing sterile container for storage. Separated serum should remain at 22°C for no longer than 8 hours. If the assay will not be completed within 8 hours, refrigerate the sample at 2 to 8°C. If the assay will not be completed within 48 hours, or for shipment of samples, freeze at –20°C or colder. [8] Thaw and mix samples well prior to use.

Serum Specimen, Controls and Calibrator Preparation

Dilute each serum specimen, serum control and calibrator 1:101. For example, label tubes and dispense 1000 µL of Sample Diluent into each labeled tube. Add 10 µL of specimen, control or calibrator to each appropriate tube containing the 1000 µL Sample Diluent and mix well by vortex mixing.

TEST PROCEDURE

Perform the assay at room temperature (approximate range 20 to 25°C). Use proper pipetting techniques, maintaining the pipetting pattern throughout the procedure to ensure optimal and reproducible values. Performance characteristics have not been established for procedures that are different from the procedure described below. Different procedures, e.g., different times, volumes, temperatures, or others, may produce invalid results.

1. Allow all reagents to warm to room temperature before use. Remove the **IgM Capture Well** packet from cold storage. To avoid condensation, allow micro-well strips to reach room temperature before opening the foil packet. If less than a full plate is to be used, return unused strips to the foil packet with desiccant and reseal completely. Store unused **IgM Capture Wells** at 2 to 8°C. (**Note:** At the end of the assay, retain the frame for use with the remaining strips.)
2. Prepare Antigen Solution (make sure reagent has reached room temperature). If the kit is being used for the first time, reconstitute sufficient antigen (see **Materials Supplied**, above).
3. Fill wells with **1X Wash Buffer solution** (see **Materials Supplied**, above) and allow to soak for 5 minutes. Decant (or aspirate) the **IgM Capture Wells** and tap vigorously to remove Wash Buffer. Blot the emptied Capture Wells face down on clean paper towels or absorbant paper to remove residual Wash Buffer.
4. Dispense 100 µL of the **Sample Diluent** into the "blank" well(s) and 100 µL of each **diluted specimen, control or calibrator** (see **SPECIMEN COLLECTION AND PREPARATION**, above) into the appropriate wells. (**Note:** For runs with more than 48 wells it is recommended that 250 µL of each diluted sample first be added to a blank microtiter plate in the location corresponding to that in the ELISA wells. The samples can then be efficiently transferred into the Antigen Wells with a 100 µL 8 or 12-channel pipettor.)
5. Cover plates with sealing tape, and incubate for 60 ± 1 minute at room temperature (20 to 25°C).
6. Remove sealing tape, and empty the contents of the wells into a sink or a discard basin.
7. Fill each well (250 µL) with a gentle stream of **1X Wash Buffer** solution then empty contents into a sink or a discard container.
8. Repeat wash (step 7) an additional 2 times, allow the last wash to soak for five minutes before decanting or aspirating.
9. Tap the Capture Wells vigorously to remove 1X Wash Buffer. Blot the emptied Capture Wells face down on clean paper towels or absorbant paper to remove residual 1X Wash Buffer.
10. Add the prepared (see Step 2, above) **100 µL Antigen Solution** to all wells, using a 100 µL 8 or 12-channel pipettor.
11. Cover plates with sealing tape and incubate for 2 hours at room temperature (20 to 25°C).
12. Repeat wash steps 6 through 9.
13. Add 100 µL of **IgM Conjugate** to all wells, using a 100 µL 8 or 12-channel pipettor.
14. Cover plates with sealing tape, and incubate for 30 ± 1 minute at room temperature (20 to 25°C).
15. Repeat wash steps 6 through 9.

16. Add 100 µL of **Substrate Reagent** to all wells, using a 100 µL 8 or 12-channel pipettor. Begin incubation timing with the addition of Substrate Reagent to the first well. (**Note**: Never pour the substrate reagent into the same trough as was used for the conjugate.)
17. Incubate for 10 ± 1 minutes at room temperature (20 to 25°C).
18. Stop the reaction by adding 100 µL of **Stop Reagent** to all wells using a 100 µL 8 or 12-channel pipettor. Add the Stop Reagent in the same sequence and at the same pace as the Substrate was added. In antibody-positive wells, color should change from blue to yellow.
19. Gently blot the outside bottom of wells with a paper towel to remove droplets that may interfere with reading by the spectrophotometer. Do not rub with the paper towel as it may scratch the optical surface of the well. (**Note**: Large bubbles on the surface of the liquid may affect the OD readings.)
20. Measure the absorbance of each well within 1 hour of stopping the assay. Set the microwell spectrophotometer at a wavelength of 450 nm. Zero the instrument on the blank wells.

IgM Procedure (condensed version)

1. Dilute samples
   Serum samples and Controls: 1:101 in Sample Diluent. (e.g., 10 µL + 1000 µL)
2. Soak Wells for **5 minutes** with **1X Wash**, decant.
3. **100 µL** of **sample** for **60 minutes**, decant.
   Background subtract only: 100 µL of diluted sample is added to each of two wells. One well (the "Ag" well, "Ag" for antigen) gets WNV antigen in Step 5, and the other well (the "SD" well, "SD" for diluent) gets Sample Diluent in Step 5. Important note: Background subtract is one way to check for heterophile antibodies present in positive samples. Therefore, background subtract should not be performed unless the patient sample was initially positive.
4. Wash 3 times.
5. **100 µL** of **Antigen** for **120 minutes**, decant.
   Background subtract only: Add 100 µL of Antigen to the "Ag" well, and add 100 µL Sample Diluent to the "SD" well, incubate for 120 minutes.
6. Wash 3 times.
7. **100 µL** of **Conjugate** for **30 minutes**, decant.
8. Wash 3 times.
9. **100 µL** of **Substrate Reagent** for **10 minutes**.
10. **100 µL** of **Stop Reagent**, read at $\lambda = 450$ nm.

Please see the **PROCEDURE** section for important details.

QUALITY CONTROL

Each plate run (or strips or wells from a single plate) must include the Cut-off Calibrator and the two Controls. If multiple plates are run, include the Cut-off Calibrator and both controls on each plate. It is recommended that until the user becomes familiar with the kit performance, all specimens, controls and the Cut-off Calibrator should be run in duplicate with the Cut-off Calibrator run twice for a total of four wells. If single wells are used, the Cut-off Calibrator should be run in triplicate. Include a minimum of 1 blank well (containing sample diluent only) for instrument calibration purposes.

The Cut-off Calibrator has been formulated to give the optimum differentiation between negative and positive sera. Although the absorbance value may vary between runs and between laboratories, the mean value for the Cut-off Calibrator wells must be within 0.100 to 0.700 OD units. All replicate Cut-off Calibrator ODs should be within 0.10 absorbance units from the mean value. Report results as index values relative to the Cut-off Calibrator. To calculate index values, divide specimen and control optical density (OD) values by the mean of the Cut-off Calibrator OD values.

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Not for Sale or Distribution in the United States of America

LEPTOSPIRA IgM ELISA

Cat No. E-LEP01M / E-LEP01M05

INTENDED USE

The Panbio *Leptospira* IgM ELISA is for the qualitative detection of IgM antibodies to *leptospira* in serum as an aid in the clinical laboratory diagnosis of patients with clinical symptoms consistent with leptospirosis.

INTRODUCTION

Leptospirosis is an acute infectious illness affecting both man and animals. It is caused by spirochaetes of the genus *leptospira*. At present, there are more than 250 pathogenic serovars of the species, *leptospira interrogans*[1].

Infection may range in presentation from subclinical to severe[2]. Onset is usually sudden, with symptoms including headache, fever, muscle pains, conjunctival infection, meningitis and abdominal pains. Severe complications such as hepato-renal failure and central nervous system involvement may arise, particularly if diagnosis and treatment is delayed. Other documented symptoms include depression and irritability[3].

Man is the accidental host in leptospirosis and becomes infected through contact with urine or tissues from infected animals (eg. livestock), or water and soils contaminated with infected animal urine. The organism enters the body through cuts or abrasions of the skin, or through mucosal membranes. Infections may therefore be related to occupational or recreational activities. Person to person transmission is rare.

IgM antibodies appear as early as 3 days after infection and may persist for up to 5 months, although there have been reports of IgM antibodies persisting for years or life[4].

Detection of specific antibody of the IgM class to *leptospira* genus specific antigen by ELISA is a valuable screening procedure for the diagnosis of acute infection[5,6]. It is an acceptable alternative to genus specific complement fixation and agglutination tests. The presence of a significant or rising level of IgM is considered presumptive evidence of active *leptospira* infection.

The Panbio *Leptospira* IgM ELISA has been demonstrated to detect infections caused by a number of *L. interrogans* serovars including: *hardjo, pomona, copenhageni, australis, madanesis, kremastos, nokolaevo, celledoni, canicola, grippotyphosa, szwajizak, djasiman* and *tarassovi*[4].

PRINCIPLE

Serum containing antibodies to *leptospira* antigen, when present, combine with *leptospira* antigen attached to the polystyrene surface of the microwells. Residual serum is removed by washing and peroxidase conjugated anti-human IgM is added. The microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of IgM antibodies to *leptospira* in the test sample.

MATERIALS PROVIDED

Note: E-LEP01M05 = E-LEP01M x 5

1. *Leptospira Antigen-Coated Microwells* - (12x8 wells). Ready for use. Unused microwells should be resealed immediately and stored in the presence of the desiccant. Stable at 2-8°C until expiry.
2. *Wash Buffer (20x)* - One bottle, 60 mL of 20x concentrate of phosphate buffered saline (pH 7.2-7.6) with Tween 20 and preservative (0.1% Proclin™). Crystallisation may occur at low temperatures. To correct, incubate at 37°C until clear. Mix well. Dilute one part Wash Buffer with 19 parts of distilled water. Diluted buffer may be stored for one week at 2-25°C.
3. *Sample Diluent* - Two bottles, 50 mL **(Pink)**. Ready for use. Tris buffered saline (pH 7.2-7.6) with preservatives (0.1% Proclin™) and additives. Stable at 2-8°C until expiry.
4. *HRP Conjugated Anti-human IgM* - One bottle, 15 mL **(Yellow)**. Ready for use. Horseradish peroxidase conjugated goat anti-human IgM with preservative (0.1% Proclin™) and protein stabilisers. Stable at 2-8°C until expiry.
5. *TMB Chromogen (TMB)* - One bottle, 15 mL. Ready for use. A mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric-acid citrate buffer (pH 3.5-3.8). Stable at 2-8°C until expiry.
6. *Reactive Control* - One **Black**-capped vial, 200 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8°C until expiry.
7. *Calibrator* - One **Orange**-capped vial, 400 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8°C until expiry.
8. *Negative Control* - One **White**-capped vial, 200 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8°C until expiry.
9. *Stop Solution* - One **Red**-capped bottle, 15 mL. Ready for use. 1M Phosphoric acid. Stable at 2-25°C until expiry.

Proclin™ 300 is a registered trademark of Rohm and Haas Company.

Xn - Harmful (Xn - Harmful) Control and Calibrator Safety Precaution:

Concentration of sodium azide in these components is classified as harmful and subject to the following risk phrases (R22, R32) "Harmful if swallowed" and "Contact with acids liberates very toxic gas."

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

(1) Accurate adjustable micropipettors with disposable pipette tips (5-1000 µL capacity)
(2) Deionised water
(3) Microplate washing system
(4) Microplate reader with 450 nm filter
(5) Timer
(6) Graduated cylinder
(7) Flask
(8) Test tubes or microtitre plate for serum dilutions

PRECAUTIONS FOR *IN VITRO* DIAGNOSTIC USE

(i) All human source material used in the preparation of controls has been tested for antibody to human immunodeficiency virus 1 & 2 (HIV 1&2), hepatitis C (HCV) as well as hepatitis B surface antigen and found to be negative. However no test method can offer complete assurance and all human controls and antigen-coated wells should be handled as potentially infectious material. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2[7].

(ii) This test should be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.

(iii) Icteric or lipaemic sera, or sera exhibiting haemolysis or microbial growth should not be used.

(iv) Do not heat-inactivate sera.

(v) All reagents must be equilibrated to room temperature (20-25°C) before commencing the assay. The assay will be affected by temperature changes. Do not remove microwells from closed bag until they have reached room temperature (20-25°C).

(vi) Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.

(vii) Unused microwells should be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.

(viii) Substrate System:
  (a) As TMB is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces.
  (b) Avoid prolonged exposure to direct light.

(c) Some detergents may interfere with the performance of the TMB.
(d) The TMB may have a faint blue colour. This will not affect the activity of the substrate or the results of the assay.

(ix) Some kit components contain sodium azide, which may react with lead or copper plumbing to form highly explosive metal azide compounds. When disposing of these reagents through plumbing fixtures, flush with a large volume of water to prevent azide build-up in drains.

(x) Sodium azide inhibits conjugate activity. Clean pipette tips must be used for the conjugate addition so that sodium azide is not carried over from other reagents.

FOR FURTHER SAFETY INFORMATION PLEASE REFER TO THE MATERIAL SAFETY DATA SHEET (MSDS) AVAILABLE FROM PANBIO.

SPECIMEN COLLECTION AND PREPARATION

Blood obtained by venipuncture should be allowed to clot at room temperature (20-25°C) and then centrifuged according to the Clinical and Laboratory Standards Institute (CLSI) (Approved Standard - Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, H3-A5, 2003).

The serum should be separated as soon as possible and refrigerated (2-8°C) or stored frozen (≤ -20°C) if not tested within two days. Self-defrosting freezers are not recommended for storage. The use of icteric sera or sera exhibiting haemolysis, lipaemia or microbial growth is not recommended. The CLSI provides recommendations for storing blood specimens, (Approved Standard - Procedures for the Handling and Processing of Blood Specimens, H18-A3, 2004).

TEST PROCEDURE

Note: Ensure all reagents are equilibrated to room temperature (20-25°C) before commencing assay. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.

ELISA PROCEDURE

(i) Remove the required number of microwells from the foil sachet and insert into strip holder. Five microwells are required for Negative Control (N), Reactive Control (R) and Calibrator (CAL) in triplicate. Ensure the remaining unused microwells are sealed tightly in the foil sachet.

(ii) Using suitable test tubes or a microtitre plate, dilute the Negative Control, Reactive Control, Calibrator in triplicate and patient samples.
   (a) To 10 µL serum add 1000 µL of Sample Diluent. Mix well. **Alternatively,**
   (b) To 10 µL serum add 90 µL of Sample Diluent. Take 20 µL of the diluted serum and add 180 µL Sample Diluent. Mix well.

(iii) Pipette 100 µL of diluted patient samples, Controls and Calibrator into their respective microwells.

(iv) Cover plate and incubate for 30 minutes at 37°C ± 1°C.

(v) Wash six (6) times with diluted Wash Buffer (refer to washing procedure).

(vi) Pipette 100 µL HRP Conjugated Anti-human IgM into each well.

(vii) Cover plate and incubate for 30 minutes at 37°C ± 1°C.

(viii) Wash six (6) times with diluted Wash Buffer (refer to washing procedure).

(ix) Pipette 100 µL TMB into each well.

(x) Incubate for 10 minutes at room temperature (20-25°C), timing from the first addition. A blue colour will develop.

(xi) Pipette 100 µL of Stop Solution into all wells in the same sequence and timing as the TMB addition. Mix well. The blue colour will change to yellow.

(xii) Within 30 minutes read the absorbance of each well at a wavelength of 450 nm with a reference filter of 600-650 nm.

Note: If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. Reading the microwells at 450 nm without a reference filter may result in higher absorbance values due to background.

WASHING PROCEDURE

Efficient washing to remove uncomplexed sample or components is a critical requirement of the ELISA procedure.

A. Automated Plate Washer

(1) Completely aspirate all wells.
(2) Fill all wells to rim (350 µL) during wash cycle.
(3) On completion of six (6) washes, invert plate and tap firmly on absorbent paper towel to ensure all Wash Buffer is removed.
(4) Automated plate washers must be well maintained to ensure efficient washing. Manufacturer's cleaning instructions should be followed at all times.

B. Manual Washing

(1) Discard contents of plate in appropriate waste container.
(2) Fill wells with Wash Buffer using a suitable squeeze bottle. Avoid bubbling of Wash Buffer as this may reduce wash efficiency. Discard Wash Buffer from wells immediately.
(3) Refill wells with Wash Buffer and discard immediately.
(4) Repeat step (3) another four times. This will make a total of six (6) washes with Wash Buffer.
(5) After the final wash, discard contents of wells and tap the plate on absorbent paper towel to ensure all Wash Buffer is removed.

CALCULATIONS

IMPORTANT NOTE: The calibration factor is batch specific and is detailed in the specification sheet. Obtain the calibration factor value before commencing calculations.

(1) Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off Value.

(2) An index value can be calculated by dividing the sample absorbance by the Cut-off Value (calculated in step (1) above).

Alternatively,

(3) Panbio Units can be calculated by multiplying the index value (calculated in step (2) above) by 10.

Index Value = $\frac{\text{Sample Absorbance}}{\text{Cut-off Value}}$

Example: Sample A Absorbance = 0.949
Sample B Absorbance = 0.070

Mean absorbance of Calibrator = 0.802
Calibration Factor = 0.62
Cut-off Value = 0.802 x 0.62 = 0.497

Sample A (0.949/0.497) = 1.91 Index value
Sample B (0.070/0.497) = 0.14 Index value

Panbio Units = Index Value X 10

Sample A 1.91 X 10 = 19.1 Panbio Units
Sample B 0.14 X 10 = 1.4 Panbio Units

QUALITY CONTROL

Each kit contains Calibrator, Reactive and Negative Controls. Acceptable values for these are found on the accompanying specification sheet. The Negative and Reactive Controls are intended to monitor for substantial reagent failure. The Reactive Control will not ensure precision at the assay cut-off. The test is invalid and should be repeated if the absorbance readings of either the Controls or the Calibrator do not meet the specifications. If the test is invalid, patient results cannot be reported.

Quality Control (QC) requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard QC procedures.

It is recommended that the user refer to CLSI C24-A and 42 CFR 493.1256 for guidance on appropriate QC practices.