PREVALENCE OF MALARIA AND SELECTEDARBOVIRAL INFECTIONSIN PATIENTS PRESENTING WITH UNDIAGNOSED FEBRILE ILLNESS IN RUSINGA ISLAND, KENYA

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and has not been submitted for award in any institution of learning to the best of my knowledge.

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RECOMMENDATION

We confirm that this thesis has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

To my loving parents Mr. and Mrs. Kipanga, my siblings, Augustine Muthiani and Phillip Mutukuwhose prayers and support have propelled me this far.

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ABSTRACT

Onsetof uncomplicated malaria is characterized by fever, headache, joint pains, myalgia and lack of appetite. These non-specific signs and symptoms also presentin patients with arthropod borne viral (arboviral) infections complicate differential diagnoses. The lack of diagnosticsthat can detectarboviral infections in Kenyan public hospitals coupled with malaria diagnostic tools incapable of detecting low *Plasmodium*parasitemia,has led to diagnosis based on clinical symptoms only,favouring malaria diagnosis at the expense of arboviral infections detection.Investigations were conducted to detect*Plasmodium*parasites undetected by microscopy and rapid diagnostic tests (RDTs) and determine Sindbis and Bunyamwera viruses neutralizing antibodies among undiagnosed febrile ill patients in Rusinga Island. Human blood and serum samples (n=92) were collected from patients without malaria (as confirmed by microscopy and RDTs) fromTom MboyaHospital in the island. The blood samples were screened for *Plasmodium* parasites by nested PCR coupled to high resolution melting analysis (nPCR-HRM), and serum samples screened for neutralizing antibodies by plaque reduction neutralization test (PRNT). Association between risk factors and exposure to infections was determined by Chi square and Logistic multivariate analyses. *Plasmodium* parasites were detected in 36 (39.1%) of the 92 patients. Out of these 36 patients with *Plasmodium* infections, only 16 (44.4%) were correctly treated with antimalarial medication with the rest being treated with antibiotics, antihelminthes and amoebicides. Conversely, a majority of non-malaria febrile patients (n=32) were treated with antimalarial medication. *Plasmodium falciparum* was the major malaria-causing parasite detected in Rusinga Island (29 out of 36).Individuals involved in outdoor activities (farmers and fishermen) were 2.24-2.43 folds more likely to get malaria infections than those involved in indoor-based (teachers/students)occupations.Neutralizing antibodies against Sindbis virus were detected in five (5.4%) patients, three of whom had malaria co-infection. No antibodies against Bunyamwera virus were detected. Theseresults demonstrate limitations of differential diagnostics of febrile illness in rural malaria endemic settings that undermineproper acute febrile illness managementand patient care. The under-appreciation of arboviral infections is of great concern, in a country where active arbovirus circulation has been demonstrated, resulting in poor health outcomes for non-malaria febrile patients. This study highlights the need for improved diagnostics deployable in rural malaria endemic settings to counter the increasing challenges of low parasitemia malaria and non-malaria undiagnosed acute febrile illnesses.

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LIST OF ABBREVIATIONS

Arbovirus	Arthropod borne virus
CHIKV	Chikungunya virus
CPE	Cytopathic effects
DDSR	Division of disease surveillance and response
EIP	Extrinsic incubation period
HRM	High resolution melting
ICIPE	International Center for Insect Physiology and Ecology
MM	Maintenance media
NMAUF	Non malarial acute undifferentiated fevers
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PRNT	Plaque reduction neutralization test
RDTs	Rapid diagnostic tests
SSA	Sub-Saharan Africa
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background information

Febrile illnesses are characterized by a sudden onset of fever, which in addition to joint pains, vomiting, myalgia and headache havebeen readily taken to infer uncomplicated malaria in sub-Saharan Africa (SSA) where malaria is endemic(WHO, 2010a; Lubell *et al*., 2008; Perkins and Bell, 2008; Amexo *et al*., 2004).Other multipleand potentially deadly diseases characterized by similar symptoms include typhoid, arthropod borne viral (arboviral) infections, leptospirosis, meningococcal meningitis and lower respiratory infections(mainly in children) like pneumonia(Gwer *et al*., 2007; Chandramohan *et al*., 2002; Bojang *et al*., 2000), complicating differential diagnoses. Clinicians often follow clinical algorithms to diagnose malaria, which though sensitive, have low specificity, especially in malaria endemic areas (WHO, 2010a;Chandramohan *et al*., 2002; Barat *et al*., 1999).Tendencies of over-diagnosis and over-treatment of malaria even by qualified doctors are common in Africa, (Olaleye *et al*., 1998), leading to wastage of antimalarial drugs, deaths from unknown illnesses, increased perception of anti-malarial drug resistance, presentation of undesirable side effects and economic burden to the poor (Amexo *et al.,* 2004).

Although many governments in SSA have embraced laboratory diagnosis of malaria using microscopy or rapid diagnostic test kits (RDTs) as recommended by the World Health Organization (WHO), misdiagnosis of malaria still continues, hampering the true prevalence of malaria from being established (Barat *et al.,* 1999). The misdiagnosis has further been attributed to untrained personnel without the needed expertise in microscopy usage, poor servicing and quality control for microscopes (Hanscheid, 2003). Additionally, RDTs may be of poor quality due to lack of appropriate storage conditions or low quality manufacturing standards (WHO, 2010b). The unreliability of standard malaria screening protocols permits many clinicians to prescribe anti-malarial medication even in the presence of laboratory reports indicative of absence of malaria (Chinkhumba *et al.,* 2010; Petti *et al*., 2006; Hanscheid, 2003). An unambiguous, sensitive and reliable technique is necessary to sufficiently decompose sources of illnesses, especially malaria and arboviral infections with similar and often overlapping presentations. This can potentially be achieved by evaluating existing PCR based contemporary techniques (Nicastri *et al.,* 2009).

The lack of follow-ups on the real causes of fever in SSA is indeed wanting (Joshi *et al*., 2008; Reyburn *et al*., 2004). Though numerous studies on bacterial caused infections that range from typhoid, pneumonia, meningitis, have been carried out both in Kenya and other countries in SSA to explain the cause of non-malaria febrile illness in patients and especially in children (Nadjm *et al*., 2010; Berkley *et al*., 2005a; Berkley *et al*., 2005b; Parent du Châtelet *et al*., 2004; O'Dempsey *et al*., 1993), little has been done to ascertain the role of arboviruses in causing fever in non-malaria febrile patients in Kenya. Not much is known about the etiology of non-malaria fevers in SSA (Hawkes *et al.,* 2009; Perkins and Bell, 2008), especially after bacterial infections have been ruled out. Determining the causes of these fevers is equally challenging due to resource limitations in hospitals in developing countries. In research centres, there is little surveillance on arboviruses during inter-epidemic periods with much publicity during epidemics and epizootics.

The Malaria Atlas Project (Hay and Snow, 2006) and the Ministry of Health in Kenya (2010) have categorized the western part of the country around the lake Victoria region as a malaria endemic region. The altitude and presence of water throughout the year are key factors that favor the expansive growth of the mosquito population. The lake offers a permanent proliferation site for different species of mosquitoes while the warm temperatures are important for facilitating the mosquito breeding cycle. These different species of mosquitoes are responsible for transmitting not only malaria, but also arboviruses. Previous studies in other areas of the western part of Kenya have shown prevalence of Chikungunya virus (42%-59%), Rift Valley fever (1-19%) and (4%-9%) West Nile virus (Mease *et al.,* 2011; Sutherland *et al.,* 2011; LaBeaud *et al*., 2007). It is thus important to determine whether there are other viruses that play any significant role in causing febrile illnesses in Rusinga Island.

1.2 Statement of the problem

There isgrowing concernover the increase in numbers of undiagnosed febrile patients in Rusinga Island,western Kenya. Malaria and arboviral infections are common among patients in the island. Unfortunately, both diseases have similar and often overlapping clinical symptoms. Existing diagnostic tools such as microscopy and RDTs are predominantly biased towards detection of malaria, often resulting in over-diagnosis of malaria at the expense of arboviral infections. Additionally,the sensitivity and specificity of microscopy and RDT techniques to detect minimal *Plasmodium*parasitemia that seeds subsequent explosion of parasite populations is inadequate.

There is need to develop new tools that can efficiently differentiate malaria and arboviral infections, and improvemalarial detection efficiency.

1.3 Objectives

1.3.1 General objective

To determine malariaand arboviral infection rates among patients presenting with undiagnosed febrile illness in Rusinga Island, Kenya.

1.3.2 Specific objectives

1. To detect *Plasmodium* infections undetected by microscopy or RDTs using PCR, in patients presenting with undiagnosed febrile illnesses in Rusinga Island, western Kenya.
2. To determine seroprevalence of Sindbis and Bunyamweraviruses neutralizing antibodies in patients presentingwith undiagnosed febrile illnesses in Rusinga Island, western Kenya.

1.4 Hypotheses

1. *Plasmodium* infections undetected by microscopy or RDTs cannot be detected by PCR in patients presenting with undiagnosed febrile illnesses in Rusinga Island.
2. Sindbis and Bunyamweraviruses neutralizing antibodies are not prevalent in serum samplesof patients presenting with undiagnosed febrile illnesses in Rusinga Island.

1.5 Justification

Malaria has been over-diagnosed in clinics in Rusinga Island, Mbita constituency, leading to neglect of arboviral and other febrile related infections. Primary causative reason for the over-diagnosis is overlapping clinical presentations of the two diseases, and relative over-investment by the government and community in diagnosis and treatment of malaria while neglecting arboviral diagnostics. In this respect, most cases presenting febrile related manifestations have by default been subjected to anti-malaria medication, even when microscopy and RDT examination of blood sampled from the patients have not detected malaria causative agents. The febrile related manifestations may be due to infection by *Plasmodium* undetectable by the classical microcopy and RDT techniques, or arboviral infection. The actual state of infections can be established by interrogating the samples for *Plasmodium* infection using more specific and sensitive molecular tools. Tools such as nestedPCR(nPCR) followed by high resolution melting analysis (HRM) and assessment of seroprevalence of Sindbis and Bunyamwera viruses neutralizing antibodies in the samples using established techniques were used. These approaches were interrogated in this study

to provide insight on the prevalence of the two ailments among patients visiting Tom Mboya hospital in Rusinga Island, western Kenya.

CHAPTER TWO
LITERATURE REVIEW

2.1 Malaria diagnosis

The WHO recommends that apart from clinically diagnosing malaria using the symptomatic approach, parasitological tests should be performed to confirm the presence of parasitemia. Microscopy and RDTs are the two parasitological tests recommended for use (WHO, 2010a). Before this, the use of clinical algorithms was widespread as the way to diagnose malaria in many malaria struck regions of Africa (Chandramohan *et al.,* 2002). Kenya is among many other countries in SSA that adopted this policy to better manage malaria within its borders (Kenyan Ministry of Health, 2010).

2.1.1 Microscopy in diagnosis of malaria

Microscopic screening of Giemsa-stained thick and thin blood smears is the standard tool for parasitological diagnosis of malaria in majority of hospitals worldwide (Njama-Meya *et al*., 2007;Mangold *et al*., 2005; Milne *et al*., 1994). Microscopy has its use in malaria diagnosis, speciation of malaria parasites, parasite quantitation, ability to assess response to antimalarial treatment and identification of other causes of fever (WHO, 2010a). Even with the ability to perform such functions, microscopy has been faulted in several occasions (Salwa *et al*., 2009; Johnston *et al*., 2006; Mangold *et al*., 2005; Milne *et al*., 1994). Incorrect diagnosis and even incorrect species identification undermine the realization of the primary goal in malaria control and case management which is to reduce morbidity,progression to severe disease and mortality (Kenyan Ministry of Health, 2010;Njama-Meya *et al*., 2007). Poorly trained laboratory staff, poorly maintained microscopes that give poor results, unavailability of good quality reagents, poor supervision, quality control and inability to detect low parasitemia belowabout 100 parasites per microlitreare some of the downfalls of microscopy(Salwa *et al*., 2009; Amexo *et al*., 2004; Hanscheid, 2003; Trampuz *et al*., 2003; Moody, 2002). According to Zurovac*et al* (2006), they found out that the sensitivity and specificity of these microscopes was 68.6% and 61.5% respectively.

2.1.2 Rapid diagnostic test (RDT) approaches in detection of malaria

These methods utilize an immune-chromatographic technique to detect parasite specific antigens such as the specific histidine-rich protein 2 on *Plasmodium falciparum* and *Plasmodium*aldolase or lactate dehydrogenase to detect the other *Plasmodium* species (Moody, 2002). As reported by Fançony *et al* (2013), RDTs can be used in community based malaria cross-sectional studies as

they favour malaria detection in the absence of expert microscopists. A similar study by Salwa*et al*(2009), observed that RDTs had slightly better sensitivity and specificity compared to microscopy as they can be used for validation/confirmation of microscopic diagnosis. Additionally, a study by Nicastri*et al* (2009) further showed that RDTs were able to detect an additional three samples as being malaria positive which had been missed by microscopy. There is however conflicting information observed by Chinkhumba*et al* (2010) that microscopy was able to pick 44 samples that were negative by RDT, 59% (26) of which had > 5,000 parasites per microlitre. Moody (2002), also states that a negative result by RDT cannot be taken as such until confirmed by microscopy. He further states that the sensitivity of these RDTs below 100parasites/µl is usually low. In areas where laboratory microscopic services are not available or of poor quality, such as outside of formal health systems, RDTs usually come in handy as a cost effective and alternative means of malaria diagnosis that is easy to use with no intensive training required. However, they too have up to a certain level of accuracy, especially compromised by low parasitemia that normally ends up giving false negative results (Baiden *et al*., 2011; Ishengoma *et al*., 2011). Furthermore, they cannot be used to give information on malaria parasite density and follow up on treatments to check if the disease is clearing as they pick up all antigens of living or dead parasites (Kenyan Ministry of Health, 2010). Also, according to WHO (2010b), quality assurance is a challenging issue especially due to humidity and temperature inconsistencies during transportation and storage.

2.1.3 Molecular based techniques for malaria detection

Polymerase Chain Reaction (PCR),a molecular based technique, is capable of amplifying nucleic acid molecules millions of times; producing multiple copies and is a more reliable technique compared to microscopy and RDTs. Though not used in Kenyan hospitals for clinical management of diseases (Kenyan Ministry of Health, 2010), it is for sure the best diagnostic tool to use in malaria detection, giving true positive or negative results with minimal inconsistencies. This technique has several variations in terms of primers, nucleic acid extraction procedures, resulting in different experimental assays (Bass *et al*., 2008, Boonma *et al*., 2007, Oyedeji *et al*., 2007). Numerous studies have compared results from microscopyand RDT diagnosis of malaria with those from PCR on the same and the results are greatly refined and more accurate from the latter (Harris *et al*., 2010, Johnston *et al*., 2006). A study carried out in Tanzania by Nicastri and colleagues (2009), evaluated blood samples for the presence of malaria using microscopy, RDTs and nested PCR, the latter being used as the reference gold standard. The results showed that the

PCR was able to detect false positives by microscopy (12 out of 32) and an additional 5 malaria cases that had been missed by both microscopy and RDT. The nested PCR also gave a higher parasite density than that reported by microscopy. *Plasmodium* detection of mixed infections and even very low parasitemia is possible by PCR, not overlooking the fact that species differentiation and parasitic quantitation is accurate and precise. Conventional PCRs that are often labour-intensive, requiring post- PCRprocesses such as gel electrophoresis and having to work with the carcinogenic ethidium bromide, have long turnaround times and are greatly susceptible to contamination. The nested PCR with melting curve analysis addresses these limitations and those of RDTs and microscopy (Mangold *et al.,* 2005) though relatively expensive.

2.2 High resolution melting analysis

High resolution melting (HRM) analysis characterizes double-stranded DNA samples based on their dissociation (melting)behavior. It is a novel, homogeneous, close-tube, post-PCR method that enables individuals to analyze genetic variations (Single Nucleotide Polymorphisms (SNPs), mutations, species identification, epigenetic studies for analysis of DNA methylation status) in PCR amplicons. It is similar to classical melting curve analysis, but due to small increments in temperature (0.008-0.2˚C), far more information on the melting behavior of a particular sample can be studied. Samples can be discriminated according tosequence, length, GC content, or strand complementarity,down to single base-pair changes.HRM analysis can only be performed on instruments thathave HRM hardware and software installed. Data is acquiredusing specialized HRM sources and detectors (Rotor-gene Q ® user manual, 2012).

Fluorescence decreases as DNA intercalating dye is released during thermal induced double stranded (ds) DNA dissociation. The sharp decrease in fluorescence marks the greatest rate of change in fluorescence and at its midpoint; the melting temperature (Tm) of a particular DNA sample is established.In terms of cost-benefit analysis, HRManalysis provides accurate results and savings on probe andlabel costs compared to other methods.

As the temperature increases, the double stranded DNA molecule containing intercalated dye such as Evagreen dye™ dissociatesuniquely into single stranded DNA.

Different species will produce distinct melting profiles enabling their identification. **Figure 1** below illustrates this principle.

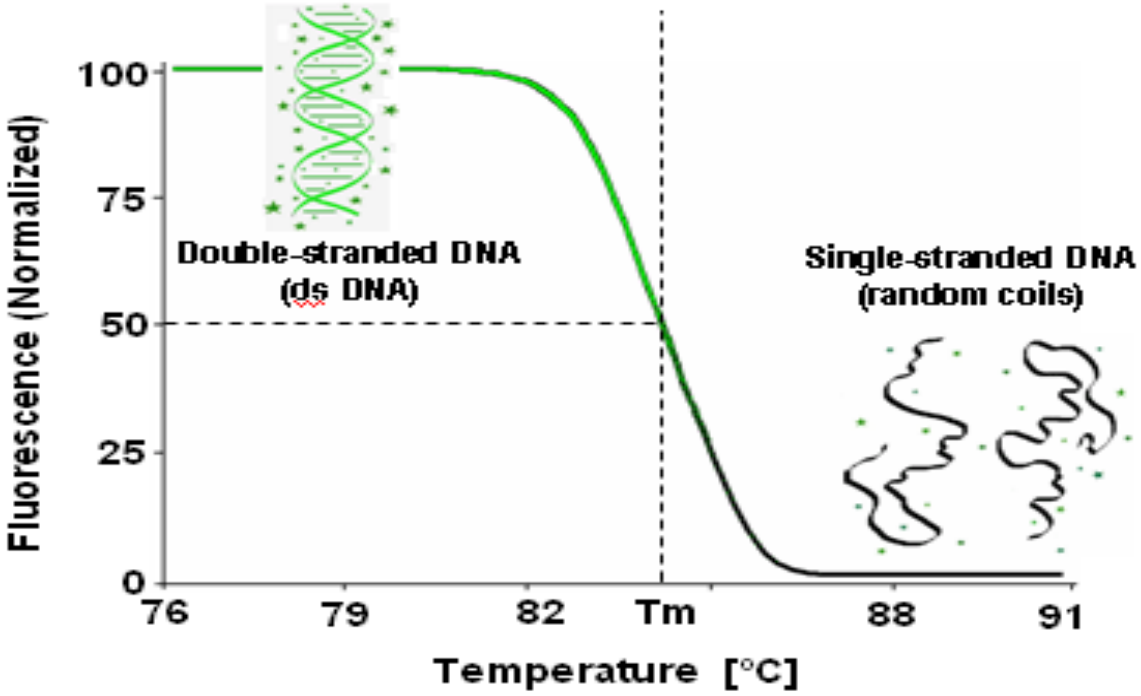


Figure 1: Normalized HRM curve (Source:http://hrm.gene-quantification.info/).

2.3 Malariaover-diagnosis

The SSA region is a malaria endemic region and most of the fevers are diagnosed as malaria (Perkins and Bell, 2008). Even in the event of a negative malaria result from microscopy or RDT, the clinicians still go ahead to prescribe the artemether based combined treatment (ACTs) (Joshi *et al*., 2008, Reyburn *et al*., 2004). The WHO report on malaria (2009) noted more usage of ACTs compared to RDTs that had been procured that year in many malaria endemic African countries, showing just how much malaria has been overrated.

Mbita constituency in Homabay county has been classified by the Malaria Atlas Project (Hay and Snow, 2006) as a malaria endemic region with *Plasmodium falciparum* the main parasite. This fact, compounded with the fact that the Kenyan ministry of health (2010) has continued to classify this Lake Victoria region as a high malaria endemic area, is seen by extension to likely give clinicians in the region more confidence in their diagnosis of malaria and prescription of antimalarial drugs even if laboratory results come negative for malaria. This practice of giving antimalarial drugs and even antibiotics to febrile patients, without consideration of laboratory

results or further examination is wide spread not only in Kenya (Zurovac *et al.,* 2008), but also in the rest of SSA region. A study carried out in Tanzania showed that although a total of 201 slides tested negative for malaria, 22% (44) of these patients were treated with antimalarial drugs alone, 34% (68) with antibiotics alone, 26% (52) on a combination of the two and 18% (37) left the centers with no medication (Reyburn *et al.,* 2006).

There is evidence to show that malaria transmissions and fevers due to malaria are generally on the decrease in Kenya and even in other highly endemic areas of the SSA region due to the numerous mosquito eradication programmes(Griffin *et al*., 2010; WHO, 2010b; WHO, 2009; Ceesay *et al*., 2008; O'Meara *et al*., 2008;WHO, 2008; Okiro *et al*., 2007).However, the practice of administering antimalarial drugs indiscriminatelycauses neglect ofother febrile illnesses which are on the increase and may be fatal (D'Acremont *et al.,* 2009). A study carried out in Tanzania in 2004 (Reyburn*et al.,* 2004), confirmed that there were more mortalities in non-malaria febrile patients than in malaria confirmed patients, indicating the fact that the other non-malarial acute undifferentiated fevers (NMAUF), especially the viral ones have been greatly ignored (Joshi *et al*., 2008). Moreover, the team found that about half (43%) of the deceased patients (non-malaria) were not treated with antibiotics while at the hospital and though they did not know the cause of the deaths, they suggested that the intervention of antibiotics might have helped avoid or reduce the mortalities (Reyburn *et al.,* 2004).

Non-malarial acute undifferentiated fevers (NMAUF) refer to febrile illnesses with no indication of an organ-specific disease after diagnosis of malaria has been excluded. In developing countries, such acute undifferentiated fevers include those caused by arboviruses that depend on arthropod vectors for their transmission (Joshi *et al.,* 2008). In Ecuador, a study that was carried out to determine the causes of fever found out that arboviral infections such as dengue and yellow fever contributed to fever though not as much as leptospirosis and malaria (Manock *et al.,* 2009).

2.4Arbovirustransmission

Arboviruses are Arthropod borne virusesthat are biologically transmitted by hematophagous (blood feeding) arthropods. Arboviral replication is characterized by a biological cycle in the arthropod vector and vertebrate host. The arthropods become infected after a blood meal from a viremic vertebrate and remain infectious for the rest of their lives. The virus is amplified in these arthropods during an incubation period that results in viral replication in the arthropods salivary glands. Later, the virus is transmitted to non-immune vertebrate hosts during feeding by the

arthropod (Weaver and Reisen, 2010, Sang and Dunster, 2001). Arboviruses circulate in wild animals and cause disease to humans and/ or domestic animals which in some cases are incidental/dead-end hosts that produce viremias inadequate to cause arthropod infections after spillover transmissions occur (Weaver and Reisen, 2010).

The transmission of the arboviruses by these vectors can either be vertical or horizontal. In vertical transmission, the arboviruses are transmitted to the arthropod progeny transovarially whereas in horizontal transmission, there may be either oral transmission by a competent vector to a vertebrate host or sexually, whereby the female transmits the arbovirus to the male during mating (Weaver and Reisen, 2010). While most of the documented arboviruses cause zoonoses, about 50% of these viruses (about 100 out of the 535 that infect humans) are transmitted by mosquitoes (Sang and Dunster, 2001). Three families of arboviruses that cause great concern to public health are Togaviridae, Bunyaviridae and Flaviviridae. The infections by these arboviruses range from mild febrile illnesses that are self-limiting and last for a short duration, to more severe encephalitis and hemorrhagic fevers that are fatal. Sang and Dunster (2001) noted in their study that especially in malaria endemic regions, majority of arboviral caused infections remain undiagnosed and that their effect to public health has been greatly underestimated. Factors such as non-specific symptoms seen in arboviral infections, lack of specialized diagnostic services and active surveillance systems were largely sited as the main reasons for misdiagnosis of these infections. Similar observations were made by LaBaeud and colleagues (2011).

In Kenya, there has been evidence of arboviral activity detected in human serum and even in mosquitoes from different parts of the country such as the coastal region, the north-eastern region, the Rift valley and even the western regions(LaBeaud *et al*., 2011, Mease *et al*., 2011, Sutherland *et al*., 2011, LaBeaud *et al*., 2008, Woods *et al*., 2002, Reiter *et al*., 1998, Morrill *et al*., 1991, Johnson *et al*., 1983, Bowen *et al*., 1973) . In 1992/1993, Yellow fever outbreak was reported in Kerio Valley in Rift valley Province, Chikungunya virus outbreak was reported in Lamu Island in the coastal region in 2004, and two Rift valley fever outbreaks were reported in the North-Eastern parts of the country in 1997 and 2006/2007. Similarly,entomologicstudies on arboviral circulation in mosquitoes have also been done (LaBeaud*et al.,* 2011, Miller *et al.,* 2000). These studies have been able to link specific species of mosquitoes as being responsible for transmission of specific arboviruses in different geographical regions of the country.*Culexquinquefasciatus* was identified as one of the mosquito vectors responsible of transmitting both Rift valley fever and West Nile viruses (LaBeaud*et al.,* 2011).

Sang and Dunster (2001) attribute the emergence and re-emergence of arboviruses in Kenya to five variables namely: The vector, the virus, humans, the wild vertebrate host and finally to environmental factors. The presence of a water body either due to heavy rainfall that causes flooding, a natural feature such as a lake, still water in boats, a water container that is not closed, tyres that collect water or even gutters around the homestead that remain with water, provide a breeding ground for the arthropods that rely on water for their larval stages to thrive. Warm temperatures have equally been established to favour the activity of arthropods. With the increasing reality of global warming, there has been increased distribution of these arthropods especially the flying ones as they venture into new warm territories that were cold previously. Global warming has also been documented to cause a reduction in the extrinsic incubation period (EIP) of these arboviruses. Extrinsic incubation period refers to the period of time between when a vector ingests the arbovirus in the blood meal and when it transmits the arbovirus to a vertebrate host. Thus, with this period being shortened, there is increase probability of the vector transmitting the virus multiple times in its lifetime. Commerce and migration of humans, import of livestock from other regions has also been implicated in the arboviral threat witnessed in the country. Virus mutation can result in the changing of a previously preferred vector for another vector that is competent enough to transmit the virus. An example of a change in vector preference was seen in the Reunion Islands during the 2005/2006 Chikungunya virus (CHIKV) outbreak where the known CHIKV vector *Aedesaegypti*was absent or in scarce numbers while *Aedesalbopictus* was noted to be the principal CHIKV vector (Tsetsarkin *et al.,* 2007).

2.4.1 Sindbisvirus

Sindbis virus is an enveloped single stranded virus of positive polarity that is transmitted to humans by the bite of an infected mosquito (*Culex* species). Their genomic RNA of about11.7kb nucleotides encodes four non-structural proteins, a capsid and two envelope proteins (Strauss *et al*., 1984). A member of the Western equine encephalitis complex, the Sindbis virus belongs to the *Togaviridae* family and shares the *alphavirus* genus position with other viruses such as Chikungunya, Semliki Forest virus, Onyong'nyong' virus, Ross River virus, Venezuelan equine encephalitis and Eastern equine encephalitis viruses. The virus was first isolated in 1952 in Sindbis health district near Cairo, Egypt from pools of ornithophilic*Culexunivittatus* and *Culexpipiens* mosquitoes (Taylor *et al*., 1955). Infection with the virus is characterized by rash, arthralgia and fever. Although the symptoms last for a short duration (less than a week) and

recovery is complete, some patients still suffer recurrent joint swelling and tenderness for months (Tesh, 1982).

In nature, the virus is maintained by vertebrate hosts (birds) and invertebrate vectors (mosquitoes). Sindbis virus is prevalent in South and East Africa, Egypt, Israel, Phillipines and parts of Australia. Different regions have different names for the disease caused by Sindbis virus. In Sweden it is called Ockelbo, Pogosta in Finland, Karelian fever in Russia and Babanki virus in much of SSA. It was only in 2004 that the causative agent of Pogosta disease was isolated in Finland directly from human isolates and confirmed to be Sindbis virus(Kurkela *et al.*, 2004). Passerines and tetraonid birds have been greatly implicated in transmitting the virus to different geographical regions over long distances (Jöst *et al*., 2010, Kurkela *et al.*, 2008).

A systematic mosquito surveillance study carried out in Kenya between 2007-2012, established the circulation of Sindbis virus in *Culex* mosquitoes found around large water bodies in Kisumu associated with the Lake Victoria water basin and Naivasha associated with Lake Naivasha(Ochieng *et al*., 2013). Important to note is that this virus was not found in the arid and semi-arid areas of Kenya, where most of the arbovirus diversity and abundance was recorded. This was attributed to the fact that migratory birds which are the vertebrate hosts of the virus, usually swam around these water points during their stop over breeding seasons (Ochieng *et al*., 2013). As already established, the virus is of public health importance and it would be further interesting to see whether there is a correlation between seroprevalence in human subjects and the indication of it being present in mosquitoes from this region.

2.4.2 Bunyamwera virus

Bunyamwera virus is an enveloped, segmented, single stranded virus of negative polarity. It is a member of the family *Bunyaviridae* and genus *Orthobunyavirus*. Its genome and that of the other members of this family consist of three linear genomic RNA segments: Small (S),Medium (M) and Large (L) segments. They encode for six proteins in total: The nucleocapsid protein (N), a non-structural protein (NS), three envelope gycoproteins and a viral RNA dependent RNA polymerase.

Due to the presence of the three segments, reassortments frequently occur and lead to an increase in the number of members in this family. Other viruses in this family that are of public health and agricultural importance include Ngari virus; a reassortment of Bunyamwera virus with the M segment of Batai virus (Briese*et al*., 2006), Rift Valley Fever virus and Crimean-

Congo Hemorrhagic Fever (Gerrard *et al*., 2004). Bunyamwera virus was first isolated from *Aedes*mosquitoes that were caught in Semliki forest in Uganda (Smithburn *et al*., 1946). It is transmitted to humans by infected mosquitoes and very likely ticks (Lwande *et al*., 2013) that have fed on infected vertebrate blood. Infection by the virus results to a mild febrile illness characterized by headache, fever, joint and back pain, rash and mild involvement of the central nervous system. Serological evidence of the infection has also been largely reported in SSA, but most infections go unrecognized (LeDuc and Porterfield, 2005). Outbreaks have also occurred in North America, South America, Africa(Gerrard *et al*., 2004), and Europe. More recently in Kenya, a five year surveillance study on mosquitoes also indicated circulation of the virus in Garissa and Magadi(Ochieng *et al*., 2013) .

2.5 Neutralizing antibodies

According to biology online, neutralizing antibodies refer to antibodies that are capable of keeping an infectious agent, usually a virus, from infecting a cell by blocking the cell's receptors or neutralizing the virus's biological effect by interfering with its receptors. This results in the inactivation of the virus such that it is no longer able to infect and replicate in cell cultures or animals(WHO, 2007). Several studies have shown that detecting the presence of a particular arbovirus is possible by serologically identifying neutralizing antibodies in the sera of study subjects (LaBeaud *et al*., 2008, Buckley *et al*., 2003). When a virus infects a cell within a fixed cell monolayer, it produces a viral plaque which is formed due to cell lysis. The lysed cell then spreads the infection to adjacent cells where the infection-to-lysis cycle is repeated. The infected cell area thus creates a plaque; an area of infection surrounded by uninfected cells (Kaufmann and Kabelitz, 2002). It is assumed that one plaque is representative of a single virus particle. Therefore, when serum containing neutralizing antibodies against a specific virus is mixed and incubated with a predetermined virus dilution, it is thus expected that the number of viral plaques that were formed initially in the absence of the serum will now be reduced due to the presence of these neutralizing antibodies. This principle is the basis of plaque reduction neutralization test (PRNT) also referred to as serum dilution neutralization test.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

The study was conducted in Tom Mboya Hospital, a public hospital in RusingaIsland (0 ° 24′S 34 ° 12′E) on Lake Victoria basin,Mbita constituency, Kenya. The constituency is a malaria endemic region in western Kenya consisting of mainland and about sixteen islands, with Mfangano (50 Km^2) and RusingaIslands (46Km^2) **Figure 2** as most prominent**.**

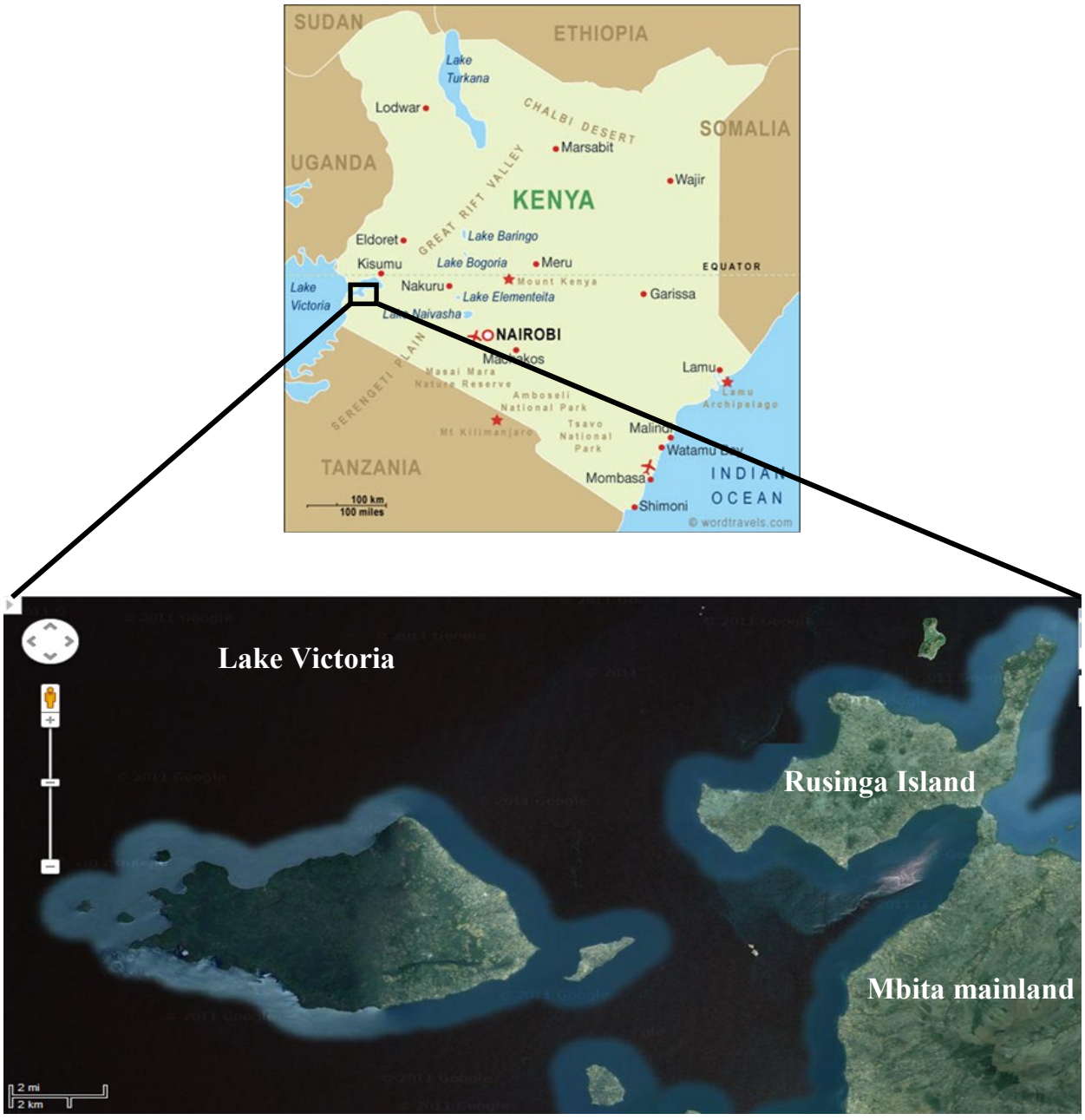


Figure 2: Map showing the location of Rusinga Island on Lake Victoria. Source: Google Maps.

Malaria prevalence in Rusinga Island was about 50% in 2007 with malaria transmission rates fluctuating with seasons though sustained throughout the year (Opiyo*et al*., 2007). *Anopheles gambiae*, *A. arabiensis* and *A*. *funestus* are primary vectors of malaria in the area (Minakawa*et al*., 1999). Rusinga Island is connected to the mainland (Mbita point) via 250 meters causeway constructed in the early 1980s through rock and earth filling. Vegetation in the island consists of mainly short shrubs and scattered grass due to deforestation and overgrazing. The natives here speak mainly Dholuo language and are involved in fishing which is their major economic activity. Subsistence farming (animals and crop) is also practiced. The area has typically two rainy seasons; the long rains that extendfrom March to May and theshort rains in August to December. The temperatures range from 17°C to 34°C with annual rainfall ranging between 700 mm to 1,200mm(Gouagna *et al.,* 2003). The lake offers a great point of interaction between the mosquito vector, numerous bird species (migratory and native), human-beings and domestic animals, thereby increasing the chances of arboviral transmission. The climatic conditions in this region coupled with the permanent water source, provide a favorable breeding site for a large and diverse population of mosquitoes, which are responsible for about 50% of arboviral infections in man (Sang and Dunster, 2001).

3.2Studydesign

3.2.1 Human blood sampling

Blood was sampled from individuals visiting Tom MboyaHospital presenting with febrile symptomsand screened for presence of *Plasmodium* parasitesusing microscopy (thick blood smear)or RDTs (CareStart™ malaria HRP2 *Plasmodium falciparum*). Patients with malaria were treated, allowed to go home and were not included in this study. Blood(4 -6 ml) was drawn from consenting patients (12years old and above), in whom *Plasmodium*parasites were not detected. Adults accompanying patients 12-17 years old consented on their behalf. None of these patients had taken any antimalarial treatment two weeks prior to seeking medical attention. Risk factor data was collected by trained laboratory technicians using questionnaires onpatients whom *Plasmodium* parasites were not detected. Heparinized (lavender capped) and non-heparinized (red capped) collection tubes were used to collect patients' blood and serum respectively. The blood and sera were aliquoted into labeled cryovials in three replicates and immediately stored in liquid nitrogen shippers. Filled shippers and questionnaires were transported back to the Emerging Infectious laboratoryat ICIPE's duduville campus in Nairobi for analyses. To protect patient anonymity, all blood samples and questionnaires were labeled

with barcode identifiers. The samples were collected between May 28, 2012 and Feb 28, 2013and the process facilitated by staff in the Kenyan Ministry of Public Health and Sanitation and Division of Disease Surveillance and Response (DDSR) of the Government of Kenya. Ethicalclearance to work on human samples was provided byKEMRI's National Ethical Review Board, see**appendix 1**.

3.2.2 Extraction of total DNA from blood

Total DNA in blood samples was extracted as described by Kawasaki (1990) with few modifications. Briefly, 50 µl of human blood was aliquoted from a labeled 2 ml cryovial tube and placed into its respective labeled 1.5 ml eppendorf tube. 0.5 ml of Tris-EDTA (TE) buffer pH 7.5 was then added and spun for five minutes at 13,000 relative centrifugal force (rcf) at 4°C. The resultant supernatant was discarded, pellet re-suspended in TE buffer and vortexed. The procedure was repeated three times. The final pellet was re-suspended in 100 µl of K buffer (see **appendix 2**),vortexed and incubated at 55°C for an hour. The extracted DNA was incubated at 95°C for 10 minutes to inactivate proteinase K, and then stored at -20°C until when required.

3.2.3 Detection of *Plasmodium* parasitesby nested PCR-HRM (nPCR-HRM)

Plasmodium DNA in the total extracted DNA was amplified using nested PCR. For the primary amplification step, a forward primer (PL 1459 out F) CTG GTT AAT TCC GAT AAC and a reverse primer (PL 1706 out R)TAA ACT TCC TTG TGT TAG AC were used. Similarly, a second pair of primers described elsewhere (Mangold*et al*., 2005) was used for the secondary amplification reaction. These primers targeted the 18S rRNA gene marker. Hot Firepol® HRM mix kit (Solis BioDyne, Estonia) was used for the two amplification processes. Optimal DNA amplification for each of the two reaction steps was carried out in a 10µl final reaction volume that consisted of 1µl DNA template, 2µl HRM mix, 0.5µl of 0.5µM of both primers and 6µl nuclease free PCR water. The PCR thermal conditions consisted of an initial denaturation at 95°C for 5 minutes, 45 cycles of denaturation at 94°C for 20 seconds, decreasing annealing temperatures from 65°C-50°C for 25 seconds (cycles 1-5), 50°C for 40 seconds (cycles 6-10), 50°C for 50 seconds (cycles 11-45), and extension at 72°C for 30 seconds. A final extension of 72°C for 3 minutes was included before HRM analysis.Upon completion, the amplification process then transitioned into the melting phase (HRM) in the same closed tube system yielding distinct melting profiles. These profiles were indicative of various *Plasmodium* species present in the samples. The set of conditions for HRM included a stepwise temperature increase of 0.2°C/sec from 75°C to 90°C, with fluorescence acquisition at each temperature transition. The

Rotorgene Q® machine(QIAGEN, Germany)facilitated both the amplification process and HRM analysis. *Plasmodium falciparum* infected blood and PCR water were used as positive and negative controls respectively in the PCR process. The other *Plasmodium* species that could not be detected using the HRM platform due to lack of positive controls, were deduced by sequencing at Macrogen, Korea.Using Geneious software (6.1.5 version, Biomatters; Kearse*et al.* 2012), the resultant chromatograms were trimmed and curated sequences were aligned with known *Plasmodia* sequences obtained from GenBank (Accession numbers: AF145336 and AB489195 for *P. malariae*, AB182489 and AB182493 for *P. ovale* and JQ627152 and JF681166 for *P. falciparum*), see **appendix 3**.

3.2.4Virusisolation

Clean Vero cell lines (from the kidney of green African monkey:*Chlorocebussabaeus*) were propagated and maintained in T25 and T75 culture flasks. To establish viral stocks of Sindbis and Bunyamwera viruses, confluent cells were trypsinized and plated onto a twenty-four well plate. After two days, the cells were confluent and 50 µl of suspected mosquito field samples of Bunyamwera and Sindbisviruses that had been homogenized were plated onto these wells. The plates were incubated for one hour at 37°C in 5% CO_2then 1 ml of maintenance media was added. The plate was returned to the incubator and presence of cytopathic effects (CPE) observed as from the next day until when the CPE were spotted. The infected cells with the media were then harvested, placed into cryovials and frozen at -80 °C.

3.2.5Virus isolates confirmation by passage

200µl of the infected cells that had been harvested from the original inoculation were re-inoculated into confluent T-25 flasks and monitored until the CPE was reproduced. The flasks were then frozen at -80°C for a day then thawed and the contents transferred into 15ml centrifuge tubes. The tubes were centrifuged at 2500-3000rpm for 10min, the supernatant collected and aliquoted into cryovials of 1 ml each then stored at -80°C.

3.2.6 Virus Confirmation by PCR

To confirm that the CPE observed was actually due to the presence of the intended viruses, 250 µl of each of the harvested viruses was put into a sterile cryovial and RNA extracted using the Trizol (Invitrogen, Carlsbad, CA, USA) extraction method following manufacturer's instructions. About 5 µlof the extracted RNA was converted into complementary DNA (cDNA)through reverse transcriptase PCR. Here, the final reaction volume of 10µl consisted of

5 µl RNA template, 0.5 µl water, 2 µl transcriptase®buffer, 1µl dNTPs and random hexamer primer, 0.25 µl RNase inhibitor and reverse transcriptase from the Roche kit (Mannheim, Germany).The forty-five minute reaction conditions were 25 °C for 10 min, 55°C for 30 min and 85°C for 5 min. This was followed by real time PCR and HRM (section 3.2.3) using CTG CTA ACA CCA GCA GTA CTT TTG AC (OrthoBun F1) and TGG AGG GTA AGA CCA TCG TCA GGA ACT G (OrthoBun R1) forward and reverse primers for Bunyavirus and TGG CGC TAT GAT GAA ATC TGG AAT GTT (Vir 2052 F) and TAC GAT GTT GTC GTC GCC GAT GAA (Vir 2052 R) forward and reverse primers for Sindbis virus. The resultant melting profiles of the samples were compared with the melting profiles of Sindbis and Bunyamwera virus controls, a positive sample being one with similar profile to the controls. Absence of similar profiles meant that the entire process from cell culture to molecular work had to be repeated.

3.2.7 Determination of viral titer (Plaque Assay)

Plaque assay was used to determine known amounts of virus (viral titer) that produced recommended plaque forming units per well. To achieve about 800pfu/ml (80plaques in a well when 100µl of virus is added per well), tenfold dilutions of each virus were prepared as described at (www.bdbiosciences.com › Resources › Baculovirus Protein Expression). Briefly, fifty microlitres of a viral stock (e.g. Sindbis) was added onto 450 µl 2% MEM (Sigma Aldrich, St. Louis, USA) over a tenfold dilution series upto 10^{-10}. A hundred microlitre of these dilutions was then plated onto their respective confluent Vero cells in 6 well plates. After one hour incubation at 37°C, 3 ml of methyl cellulose overlay medium was added to each well and incubated at 37°C, 5% CO_2 for threedays for Sindbis virus and four days for Bunyamwera virus. After the overlay was removed, 10 % formaldehyde was added to the wells and placed under UV for 30 min for fixing and to inactivate the virus.The plates were placed under slow running tap water to remove the formaldehyde and stained immediately with 0.5 % methyl violet dye then washed off and left overnight to dry.

The plaques were then counted and plaque forming units per ml (pfu/ml) calculated using the formula:

$$\text{Plaque forming units per ml} = \text{number ofplaques/volume of diluted virus per well}$$

As shown in **Figure 3**, the numbers of plaques reducewith increase in virus dilution. This facilitates the selection of the well (and hence the dilution) to be used for the next assay which is plaque reduction neutralization test (PRNT).

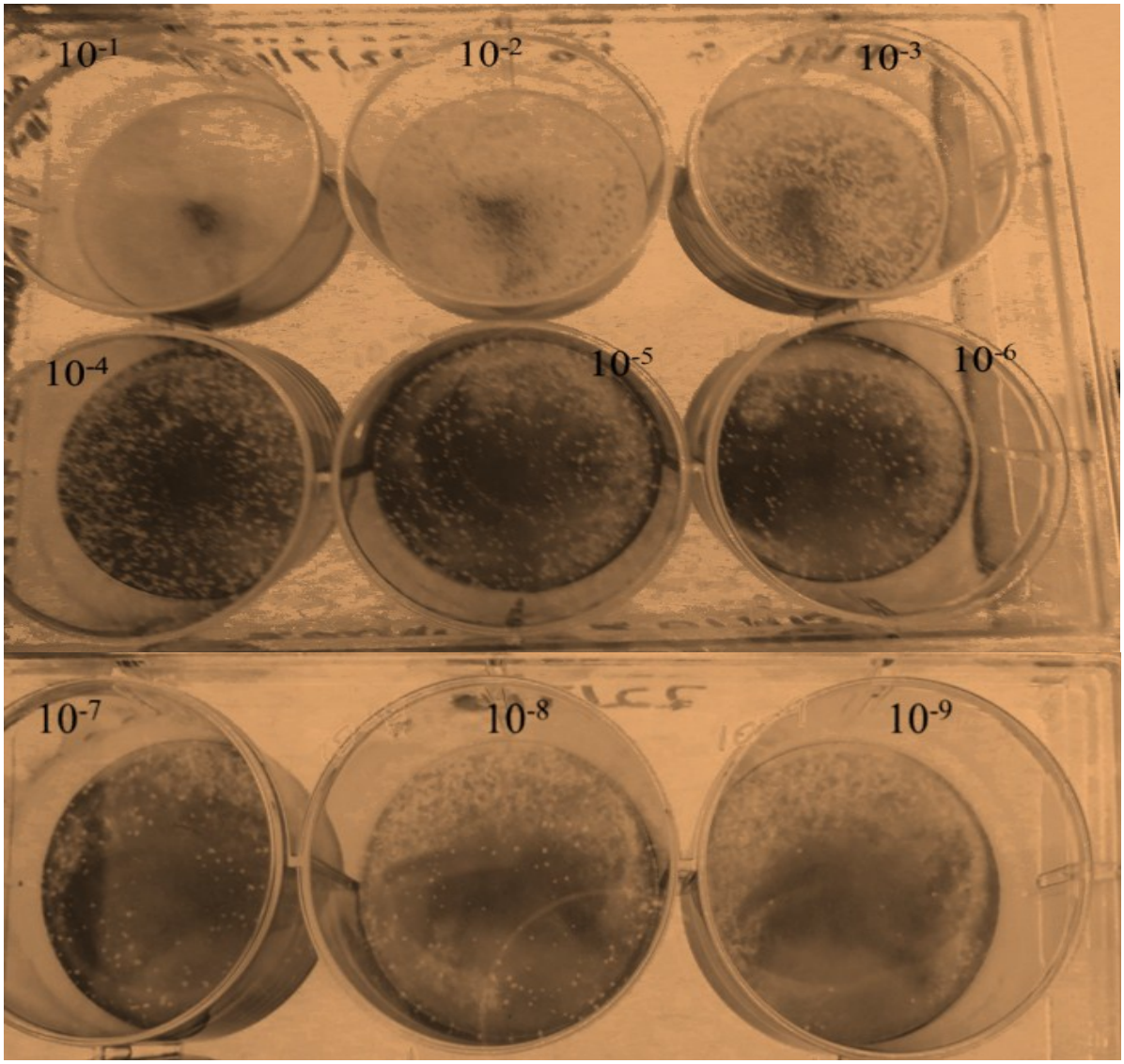


Figure 3: Plaque assay for Bunyamwera virus showing virus dilution and corresponding number of plaques formed; number of plaques reduce with increase in virus dilution.

For Bunyamwera and Sindbis viruses, 10^{-8} dilution and 10^{-7} dilutions respectively produced the desired number of plaques and thus were used for the plaque reduction neutralization procedure.

3.2.7 Determination of Neutralization Activity (Plaque Reduction Neutralization Test)

All the sera samples were heat inactivated at 56°C for 30min. Twelve microlitres of a serum sample was diluted into 108 µl of 2 % MEM (1:10 dilution)virus. A previously unthawed vial of virus was thawed on ice. The virus stock was then diluted as previously determined from the plaque assay. Sixty microlitres of the diluted virus was then mixed with an equal volume of the serum in a 24 well plate and incubated for one hour. Other controls incubated included a negative control (MEM alone), backtitrated controls (10^{-9} and 10^{-8} for Bunyamwera and Sindbis viruses respectively) and a working dilution of the virus. A 100 µl of the incubated mixture and the controls were inoculated onto confluent cells in 6 well plates and incubated for one hour again. Three milliliters of methyl cellulose overlay medium was finally added to the wells and the plates incubated for three days for Sindbis and four days for Bunyamwera virus and later stained as described above (3.2.6).

3.3 Data Analysis

Descriptive statistics including frequencies and proportions for both numerical (age) and categorical variables (gender, occupation, interaction with domestic animals and location) constituted the data collected from questionnaires supplied. Proportion values of malaria prevalence,arboviralprevalence, drug prescription patterns and fever proportions were calculated. Chi-square test was used to analyze significance of variables (age, gender andoccupation) in relation to malaria and arboviral infection. A multivariable logistic regression model was used to investigate risk factors associated with testing positive for malaria, Sindbis and Bunyamwera virus antibodies. The significance level was set at $p<0.05$. The analyses were performed using STATA v10.1 (StataCorp, College Station, TX, USA).

CHAPTER FOUR
RESULTS

4.1 Characteristics of study subjects

The study comprised 92 subjects aged between 12 and 70 years, with a mean age of 34 years. There were 45 females and 45 males, with the gender of two individuals not reported.Mean body parameters of these patients such as body temperature and body weight were 37.7 ºC and 59.23 Kg respectively, with 66 of them having fever. The subjects were recruited from Tom Mboya clinic between May 28, 2012 and Feb 28, 2013.

4.2 Malaria prevalence amongthe undiagnosed febrile patients

Prevalence of malaria in Rusinga Island and a summary of the *Plasmodium* species present in the island are illustrated in **figure 4**.

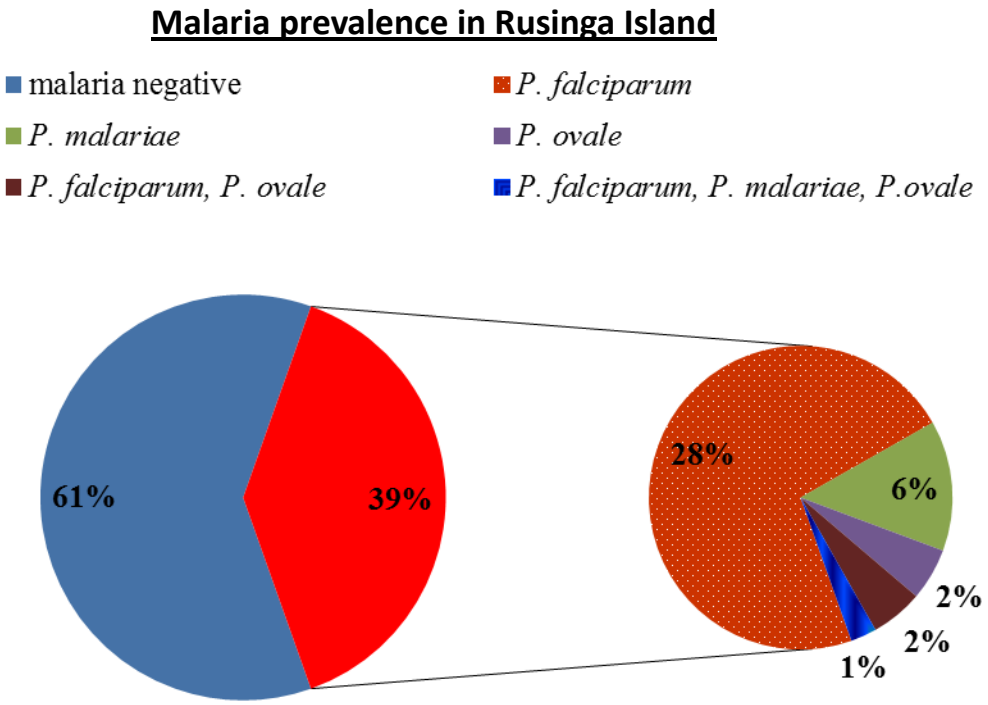
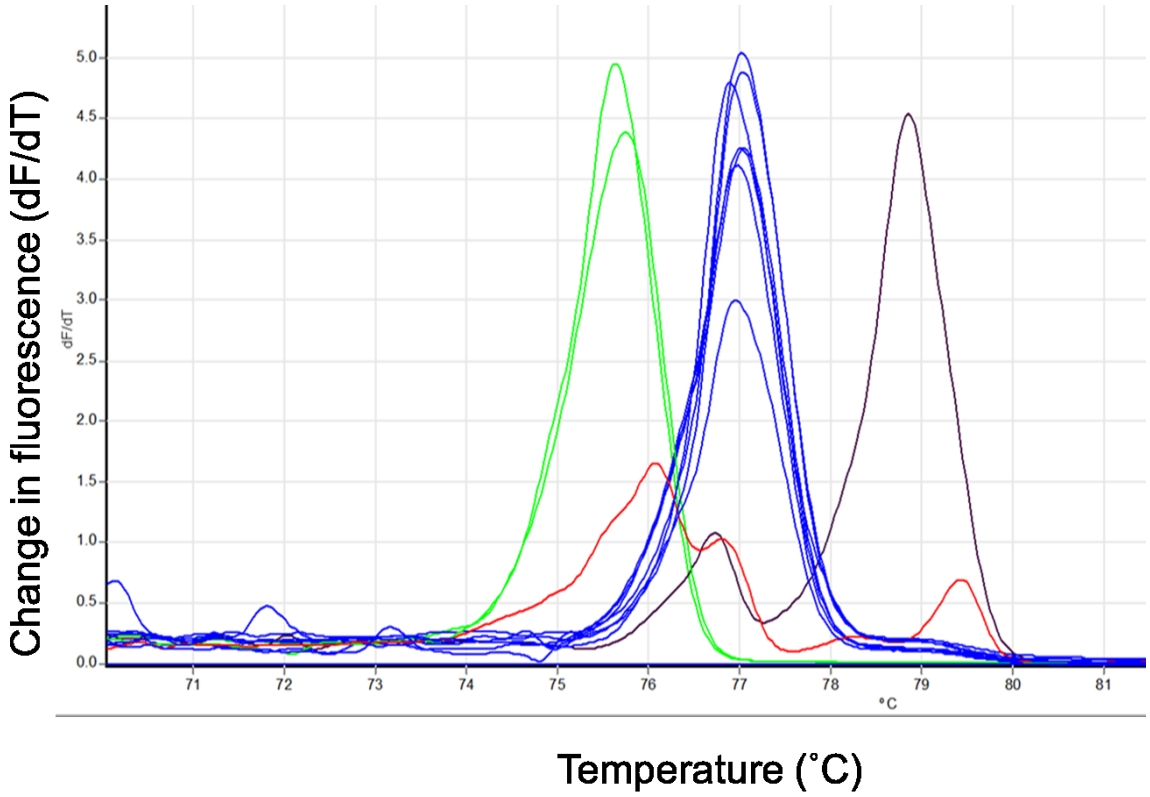


Figure4: Malaria prevalence rates and abundance of *Plasmodium* species in Rusinga Island.

Based on the nPCR-HRM analyses, 36(39.1%, 95% CI 29.1-49.1) of the 92 subjects enrolled in the study had *Plasmodium* parasites, of which 26 (28.2%, 95% CI 19.1-37.5) were exclusively infected with *P. falciparum* parasites. Among mixed infections, two (2%) were double infections (*P. falciparum* and *P. ovale*) and one (1%) had triple infection (*P. falciparum*, *P. malariae*, and *P. ovale)*. Two (2%) *Plasmodium ovale* and five (6%) *P. malariae* pure infections were also

detected in the samples.The unique HRM profiles of the three *Plasmodium* parasites are represented in **figure 5**.

HRM melting profiles of *Plasmodium* species



Plasmodium malariae

Plasmodium falciparum

Plasmodium falciparum and *Plasmodium ovale*

Plasmodium malariae, Plasmodium falciparum and *Plasmodium ovale*

Figure 5: Distinct melting profiles of *P.malariae*, *P. falciparum* and *P. ovale* found in human blood samples collected from Tom Mboya hospital, Rusinga Island.

Among 92 patients presenting with febrile illness enrolled in this study, 48 (52.2%) were treated with antimalarial drugs. However, of the 36 patients with detectable *Plasmodium* parasites, only 16 (44.4%) patients were correctly treated with antimalarial drugs. Conversely, of the 56 patients without detectable *Plasmodium* parasites, 32 (57.1%) were incorrectly treated with antimalarial drugs.

Additionally, 20 (55.6%) patients who had detectable *Plasmodium* parasites were incorrectly treated with other drugs like antibiotics, antihelminthes and amoebicides(**Figure 6**).

Drug prescription patterns in Tom Mboya Hospital

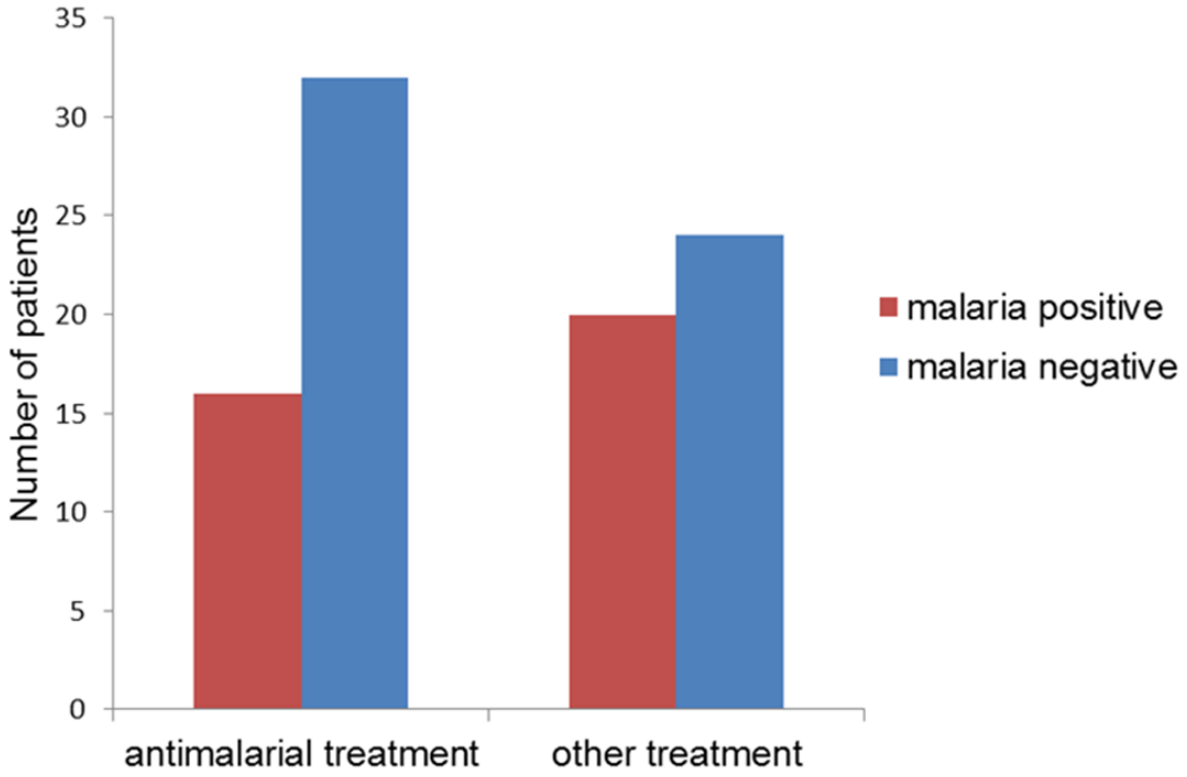


Figure 6: Prescription patterns of antimalarial medication and other treatments such as antibiotics, antihelminthes and amoebicides administered to patients with malaria and without malaria.

Out of the 66 patients who presented with fever at Tom Mboya Hospital,24patients (36%)had malaria directly implicated as the cause of their fever, with the remaining 42 (64%) patients having fever as a result of other non-malaria infections(**Figure 7**). Additionally, 11 patients among the remaining 26 patients who did not complain of fever initially also had malaria.Based on gender, similar numbers of males (n=18) and females (n=17) had *Plasmodium*parasitemia (Pearson chi-square = 0.0173, p = 0.895), though the odds of men having malaria relative to women was 1.61. Individuals involved in farming and fishing (outdoor activities) had the highest numbers of malaria infection and an increased chance of approximately 2.24-2.43 of getting infected with malaria, compared to those involved with indoor based occupations.

These variables were however not significant in contributing to malaria infection (**Table 1)**.

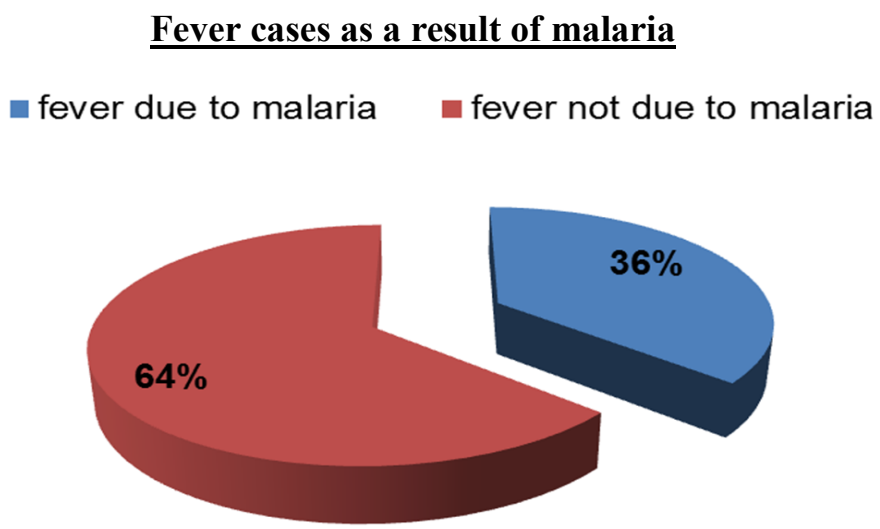


Figure 7: Causes of fever include malaria and other undetermined non-malaria infections.

Table 1: Relative contribution of various variables implicated in malaria infection

Variable		Odds Ratio	95% CI	P-value
Gender				
	Female	1	_	_
	Male	1.61	0.6-4.31	0.343
Age		0.99	0.95-1.03	0.555
Occupation				
	Businessperson	1	_	_
	Farming	2.43	0.47-12.51	0.288
	Fishing	2.24	0.51-9.78	0.284
	Teaching/School	1.19	0.24-6.04	0.83
	Unemployed	2.21	0.33-14.62	0.41

4.3 Prevalence of arboviral infections in Rusinga Island

Out of the 92 patients enrolled in the study, Sindbis virus neutralizing antibodies weredetected in five (5.4%; 95% CI 1.1%-9.5%) patients. No Bunyamwera virus neutralizing antibodies were

detected. Relatively similar number offemales, n=2 (2.2%) andmales, n=3 (3.3%) had Sindbis virus neutralizing antibodies as shown in **Table 2**. Among the individuals with Sindbis virus neutralizing antibodies, three out of the five had co-infection with *Plasmodium* parasites as illustrated in **Table 2**and **figure 8**.

Table 2: Data on individuals with Sindbis virus neutralizing antibodies and co-infection with malaria in Rusinga Island.

Serial Number	Gender	Occupation	Treatment	Malaria status
SCHU12028	F	Housewife	antimalarial	*P. falciparum*
SCHU12029	F	Housewife	antimalarial	0
SCHU12030	M	Farming	antibiotic, amoebicide	*P. falciparum*
SCHU12083	M	Fishing	antibiotic, amoebicide	*P. falciparum, P. malariae, P. ovale*
SCHU12092	M	Businessman	antimalarial,antibiotic	0

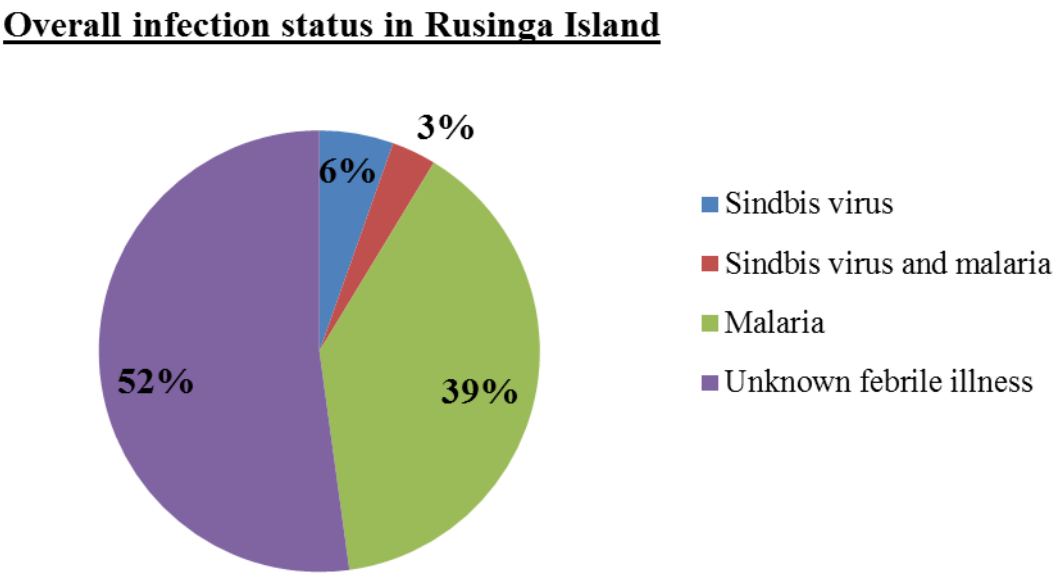
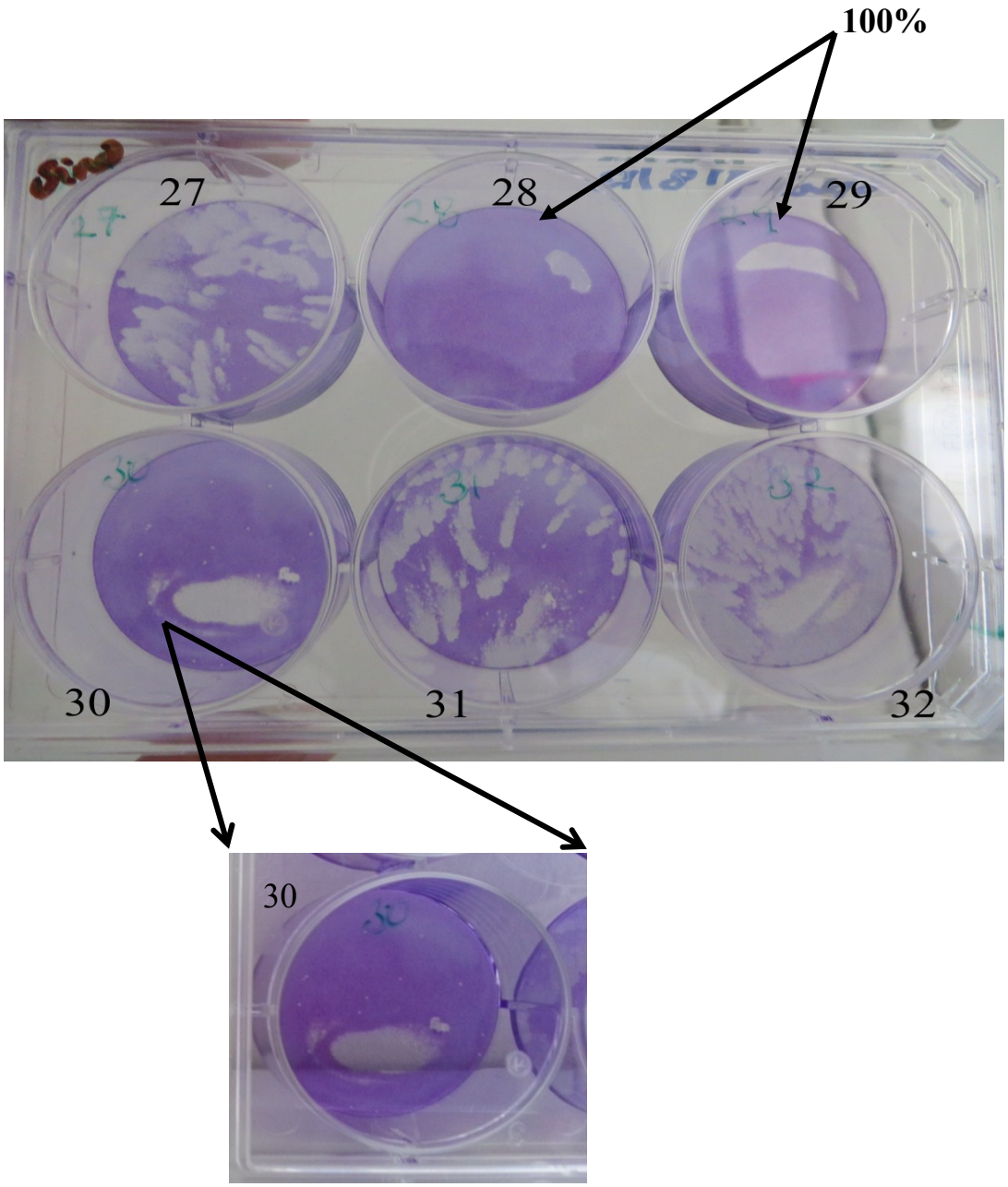


Figure 8: A pie chart illustrating the acute febrile illnessstatus in Rusinga Island.

Plaque reduction is illustrated in **Plate 1**, where in wells 28 and 29 there was complete (100%) neutralization activity by these serum samples. Serum sample, number 30 shows 90% neutralization activity.

Plate 1: Plaque reduction in wells 28, 29 and 30 due to action of neutralizing antibodies.



CHAPTER FIVE
DISCUSSION

A sensitive and specific diagnostic tool that is capable of precise detection of low *Plasmodium*parasitemia is a desirable asset in any health facility. From the results, 39.1% malaria prevalence was recorded from 36 patients in Tom MboyaHospital,Rusinga Island. Ironically, this value constituted individualswho had been previously diagnosed as not having malaria by microscopy and RDTs,butin whom *Plasmodium* parasites were later detected by nPCR-HRM technique. The inability of microscopy and RDTs to detect low *Plasmodium*parasitemia of below 100parasites/µl (Trampuz et al., 2003; Moody, 2002) was noted as the most probable reason. This is because even the nPCR-HRM technique that is highly sensitive yielded only small amounts of fluorescence from the amplified samples, indicating the low amounts of parasitemia in the samples. By combining the high sensitivity of nested PCR with real-time species differentiating HRM analysis, the reporting of true malaria prevalence for epidemiological studies can be synergistically enhanced. nPCR-HRM enhances low-parasitemia malaria diagnosis and can potentially surmount the deficiencies of microscopy and RDT based results in determining malaria parasitemia, and evaluating epidemiology of the disease.Several studies carried out in the Kenyan highlands of Nandi and Kisii and also in Iran noted increased detection of *Plasmodium*parasites by nested PCR that had been initially missed by microscopy (Zoghi*et al*., 2012; Wangai*et al*., 2011). Though microscopy is regarded as the standard diagnostic tool for malaria detection in SSA, complementary use of molecular based methods is important especially in rural endemic areas. This will enable clinicians rule out malaria in differential diagnoses and prevent the over-use of antimalarial medication and the effects that come with it.

A major medical implication evident from the results is that febrile illness misdiagnosis and misadministration of antimalarial drugs can be significant in malaria endemic settings, which result in a myriad of downstream issues. They include improper treatment and patient care, chronic suffering of patients, drug wastageand development of parasite resistance to these drugs (Amexo*et al*., 2004). Due to the inability of microscopy and RDTs to detect low *Plasmodium*parasitemia (Okell*et al*., 2009),92 febrile patients were diagnosed as not having malaria but were still given different medications on the basis of clinical symptoms alonedespite negative microscopy and/or RDTs results for malaria(Roucher*et al*., 2012; Chinkhumba*et al*., 2010; Chandler *et al*., 2008).Due to limited diagnostic platforms for diagnosis of other non-

malaria febrile illnesses and the low sensitivity and specificity of the present malaria screening protocols, clinicians are limited to heuristic methods of clinical diagnoses(Chandramohan*et al*., 2002), mostly having a bias towards malaria diagnosis and treatment. This is despite the fact that there are other potential differential diagnoses for febrile illness, including arthropod borne viral (arboviral) infections (Crump *et al*., 2013; Hertz *et al*., 2012; Joshi *et al*., 2008).The drug prescriptions have inadvertently contributed to parasite resistance to anti-malarials (Na-Bangchangand Karbwang,2013) and mortalities from other undiagnosed illnesses (Reyburn*et al*., 2006).Only 16 (44.4%)patients with low-parasitemiamalaria were correctly treated with antimalarial drugsat Tom MboyaHospital,with a majority of the antimalarial drugs being prescribed to 32 (57.1%) patients in whom *Plasmodium*parasitemiawas not detected. The scenario of over-prescription of antimalarial drugs in non-malaria febrile patients and under-prescription of antimalarial drugs in low-parasitemia malaria patients was noted.It is therefore necessary to establish diagnostic techniques that detect low-parasitemia to clearly discriminate malaria related febrile clinical symptoms from those related to other differential illnesses, and improve case management of febrile illness.

As earlier stated, fever used to be synonymous to malaria (Perkins and Lubell, 2008; Chandramohan*et al*., 2002), however,this perception has changed over time due to implementation of proper policies that encourage parasitological testing before drug prescription (WHO, 2010a).In this study, malaria was cited as the cause of fever in only 24 (36%) of the 66 febrile patients who initially presented to the hospital with fever. Moreover, we also found out that of the remaining 26 patients that did not present with fever initially, 11 patients did in fact have *Plasmodium* infections. Fever is thus a poor indicator of malaria.These results are similar to a systematic review carried out by D'Acremont and associates (2010) that reviewed 39 journal papers carried out in the past 20 years in Africa on malaria related fevers. This large-scale review showed that there had been a reduction in the number of fevers related to malaria over time; in that, only about a fifth of all fevers were found to be directly as a result of malaria. This was attributed to a change in diagnosis from clinical to laboratory based.

Nearly all malaria cases (29 out of 36, 80.6%) were caused by *P. falciparum,* the most dangerous malaria causing parasite. These results show that *P. falciparum* is still the main species responsible for malaria in this malaria endemic region as earlier established (Kenyan Ministry of Health, 2010; Hay and Snow, 2006). Additionally, the melting curve profiles for the

three *Plasmodium* species were similar to those by Mangold and colleagues (2005). We however improved the sensitivity of the assay by adopting the advantages of nested PCR.

Table 1 shows that individuals involved in fishing and farming (outdoor) activities were more susceptible to being infected with malaria compared to those who work indoors such as shopkeepers, housewives, carpenters, teachers and students. This can be attributed to the fact that while outdoors and especially in areas that have water (artificially made or naturally present) which are breeding sites for mosquitoes, chances of getting bitten are increased especially during dawn and dusk (Imbahale*et al*., 2011). Fishermen usually spend nights out fishing and hence are even more prone to being bitten by mosquitoes. This may also explain further the 1.61 increased chance of men getting malaria infection as opposed to women because fishing is mainly carried out by men (Ukoroije and Abowei, 2012).

The estimated seroprevalence of Sindbis virus neutralizing antibodies was 5.4%(n=5) of whom 3 had co-infection with malaria. The value (5.4%) is indicative of exposure to Sindbis virus, resulting in the production of antibodies specific to the virus.The primary reservoir hosts implicated in maintaining the virus are the birds. The abundant supply of fish provided by the lake ensures a constant presence of both migratory and native birds which use Rusinga Island as their nesting place, bringing the infection nearer to the humans. A study carried out in Finland noted a relatively similar prevalence rate in humans of 5.2% between the years 1999-2003 (Kurkella*et al*., 2008). Increased interaction between reservoirs, humans and mosquito vectors (Hall *et al*., 2012) could be responsible for the relatively higher numbers of Sindbis infection in men than women in this study. During 2007-2012 period, Ochieng and colleagues (2013) reported the presence of Sindbis and Sindbis-like viruses in *Culex* and *Culiseta* mosquitoes from Kisumu; a town located 73Km from Rusinga Island in the Kenyan Lake Victoria basin. In their study, they did not find Bunyamwera virus in any of the sampled mosquitoes from this lake side region (Ochieng*et al*., 2013), similar to this study. However, this study clearly indicates that Sindbisvirus is likely to contribute to febrile illness in the region.

The data collected at Tom Mboya Hospital (data not shown as further work is ongoing) showed that no further tests were carried out to determine the cause of febrile illness once malaria was ruled out. Febrile illnesses can have many etiologies ranging from bacterial, fungal, mycobacterial, arboviral, protozoan, bacterial zoonoses and even viral infections. These unknowns complicate appropriate febrile illness diagnosis and treatment.In resource limited malaria endemic areas such as Rusinga Island, diagnostic services for detecting non-malarial

acute febrile illnesses are limited (Crump *et al*., 2013, Joshi *et al*., 2008). This situation leads tonon-malaria febrile illness cases being often heuristically treated, based on clinical symptoms, with anti-malarial drugs (Crump *et al*., 2013; Roucher*et al*., 2012, Icke*et al*., 2005) despite the stated range of unrecognized differential diagnoses (Crump *et al*., 2013; Hertz *et al*., 2012). Prescription of antibiotics normally follows as the clinicians try to clinically diagnose the non-specific symptoms exhibited in febrile illnesses (Reyburn*et al*., 2006). Arboviral infections have been underappreciated in Kenya despite the fact that they are prevalent and of great public health importance. This is mainly due to absence of diagnostic tools for detecting arboviruses coupled with limited guidelines on how to manage acute febrile illnesses.Plate 1 shows evidence that individuals in Rusinga Islandhave been exposed to Sindbis virus and that's why they have antibodies against the virus. The chance that there may be unnoticed but active transmission of Sindbis virus is also greatly possible, exposing the population to harmful effects of the infection such as arthralgia. The etiology of a huge percentage (52%) of febrile illnesses still remains unknown in Rusinga Island. Several studies have highlighted how malaria is over-diagnosed and treated at the expense of arboviral infections and other febrile illnesses, resulting in poor health outcomes for patients without malaria (Crump *et al*., 2013; Manock*et al*., 2009). Therefore, there's need for active surveillance of a wide range of arboviruses in this population to assist in better understanding of arbovirus epidemiology in Rusinga Island and the Lake Victoria basin.

Finally, these findings demonstrate the limitations of differential diagnostics of febrile illness in rural malaria endemic settings that preventproper acute febrile illness management and patient care. This causes inadvertent over-prescription of antimalarial drugs in non-malaria febrile patients and under-prescription of antimalarial drugs in low-parasitemia malaria patients, neglecting arboviral infections in the process. Clear and proper guidelines on the management of non-malaria acute febrile illnesses in Kenya should be developed and implemented to guide clinicians on differential diagnoses (Nyandigisi*et al*., 2011).

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The findings of this study can be concluded as follows:

1) Out of a total of 92 patients identified as not having *Plasmodium* parasites by microscopy and RDTs, *Plasmodium* parasites were detected in 36 (39%) of them by nPCR-HRM technique. This demonstrates the high specificity and sensitivity of our tool over microscopy and RDTs in the detection of low *Plasmodium*parasitemiain the rural malaria endemic area of Rusinga Island. As a result of misdiagnosis, misadministration of drugs occurred. Only 16 (44.4%) patients out of the 36 patients with malaria were correctly treated with antimalarial medication. A majority of febrile patients (n= 32, 57.1%) without malaria were incorrectly treated with antimalarials,demonstrating the limitations of differential diagnostics of febrile illness in the rural malaria endemic setting of Rusinga Island.
2) Additionally, a seroprevalence of 5.4% (n=5) of Sindbis virus neutralizing antibodies was detected in Rusinga Island. The presence of these specific Sindbis antibodies is indicative of exposure to Sindbis virus. The likelihood of Sindbis virus being in active circulation though unnoticed is high. No Bunyamwera virus neutralizing antibodies were detected in this cohort (n=92). There is increased interaction between the vector for Sindbis virus (*Culex* mosquitoes), the primary host (birds) and man resulting in the transmission of the virus and hence the detection of the antibodies. The situation is converse for Bunyamwera virus.

6.2 Recommendations

1. Sensitive diagnostic platforms for malaria diagnosis should be adopted or developed and implemented to curb the increased cases of undetected low *Plasmodium*parasitemia and ensure rational use of antimalarial drugs.For example, adoption of the nPCR-HRM technique to Kenyan hospitals.
2. Active human and entomological arboviralsurveillance of a wide range of arbovirusesshould be stepped up in Rusinga Island and the Lake Victoria basin,to better inform policy makers on arboviralepidemiology and management.
3. Development of affordable arboviral diagnostics and their use in hospitals should be considered in Kenya. This will improve acute febrile illness management in the country and rule out arboviral infections in differential diagnoses.

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APPENDICES

Appendix 1: Ethical clearance form from Kenya Medical Research Institute (KEMRI):

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 February 16, 2012

TO: PROF. RICHARD MUKABANA
ICIPE, INTEGRATED VECTOR & DISEASE MANAGEMENT
P. O. BOX 30772 – 00100,
NAIROBI, KENYA

Dear Sir,

RE: NON-SSC PROTOCOL No. 310 (*RE-SUBMISSION*): AN INVESTIGATION OF ARBOVIRUS PREVALENCE AND ECOLOGY IN SUBA DISTRICT (*VERSION 0.1; FEBRUARY 2012*)

Reference is made to your letter dated February 15, 2012. We acknowledge receipt of the revised proposal.

This is to inform you that the Committee determines that the issues raised at the initial review are adequately addressed. Consequently, the study is granted approval for implementation effective this **16th day of February 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **February 14, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **January 7, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

Please note that any unanticipated problems resulting from the implementation of this study must be reported to the ERC. You are required to submit any proposed changes to this study to the SSC and ERC for review and approval prior to initiation and advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix2: Components of K Buffer

- 10mM Tris-HCl, pH 8.3
- 50mM KCl
- 1.5mM $MgCl_2$
- 0.5% Tween 20
- 100µg/ml proteinase K (freshly dissolved enzyme)

  -Proteinase K: at 20mg/ml in 10mM Tris-HCl, pH 7.5

Appendix 3:*Plasmodia* species sequence alignment