DEVELOPMENT AND UTILIZATION OF MULTIPLEX PCR - HIGH RESOLUTION MELTING (HRM) ASSAY FOR DETECTION OF ARBOVIRAL PATHOGENS

MARTIN KOOME MBAYA

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DECLARATION

This research dissertation is my original work and has not been presented elsewhere for a degree award.

Signature ……Date…….

Martin Koome Mbaya (SC312-C003-0776/2012)

Declaration by Supervisors

This research dissertation has been submitted for examination with our approval as supervisors.

1. **Dr. Steve Nyanjom Ger**, Biochemistry Department, Jomo Kenyatta University of Agriculture and Technology

   Signature ……Date……..

2. **Dr. Jandouwe Villinger**, Martin Luscher Emerging Infectious Diseases Laboratory, African Insect Science for Food and Health- *icipe*

   Signature……Date…….

DEDICATION

This work is dedicated to my family especially my parents John Mbaya and Judith Mbaya who went out of their way to ensure that I get where I am today. To my brother and sisters who supported me morally in the course of this work. To my loving Fiancée Nancy who was patient with me when I needed to work extra hours to accomplish this task. You all played an important role in making this project a success. I am greatly indebted to you. I wish you God's blessings.

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ABBREVIATIONS AND ACRONYMS

RVF	Rift Valley Fever
HRM	High Resolution Melting
DENV	Dengue Fever virus
CHIKV	Chikungunya Virus
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	Complimentary Deoxyribonucleic acid
mRNA	Messenger Ribonucleic acid
IgM	Immunoglobulin M
IgG	Immunoglobulin G
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
dsDNA	Double Stranded DNA
NCBI	National Center for Biotechnology Information
dNTP	deoxyribonucleotide triphosphates
ICIPE	International Centre of Insect Physiology and Ecology
MBBD	Molecular Biology and Bioinformatics Unit

ABSTRACT

Arthropod borne viruses (arboviruses) are transmitted by blood sucking arthropods. Disease outbreaks caused by arboviruses are sporadic and unpredictable. Arboviral disease outbreaks in Kenya have confirmed the presence of a number of arboviral diseases within the human and animal populace. Currently, there are no approved or specific therapies for arboviral infections; treatment is limited to palliative care. Early detection and vector control are important strategies in control of arboviruses. However, there is not yet a single detection system that is capable of detecting all arboviruses simultaneously. The development of real-time thermocyclers capable of high resolution melting (HRM) analysis has enabled development of rapid, sensitive and low-cost surveillance assays. Multiplex-RT-PCR with HRM analysis offers a comprehensive means for broad range arboviral screening. I optimized a multiplex-PCR-HRM assay using sets of primers that universally amplify arboviruses within genera and utilized the assay for detection of arboviral pathogens in biological samples. The assay was able to differentiate 16 distinct arbovirus positive controls and identify Bunyamwera, Sindbis, Usutu, Wesselsbron, and Mosquito Flaviviruses from field collected mosquitoes. Moreover, the assay identified co-infections and discovered novel arboviruses. The combination of this assay with the current molecular and culture methodologies will provide powerful, rapid and cost effective diagnostic methodologies that are essential in surveillance and outbreak investigation programs in vectors, hosts and the reservoirs of arboviruses.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Arboviruses are viruses that are transmitted by infected blood sucking arthropods such as mosquitoes. More than 100 arboviruses are known to infect man and are responsible for some epidemics of emerging infectious diseases over the past decades (Dash, Bhatia, Sunyoto, & Mourya, 2013). Their global incidence has increased in the past twenty-five years, mainly due to unprecedented population growth, migration, uncontrolled urbanization and lack of effective mosquito control strategies (Gubler, 2002).

Currently, many of the diseases caused by arboviruses are considered to be emerging and re-emerging. These diseases are considered to be important as they can cause epidemics with significant economic impacts. In addition, the mortality rates caused by some of these diseases have considerable health care concerns. (Moreli & da Costa, 2013). The vast forest reserves and diverse fauna in tropics and sub tropics provide environments that are conducive to arbovirus transmission (Tadeu & Figueiredo, 2007). Arbovirus disease outbreaks have been regarded as unpredictable, often with delayed medical responses due to the difficulty in diagnosing arbiviral infections. This difficulty is partly due to clinical similarity to other diseases, presence of clinically asymptomatic disease, lack of reference laboratories that can perform differential diagnosis and cross-reactions that occur between serological tests (Moreli & da Costa, 2013)

Arboviral disease symptoms can be broadly classified into encephalitis, febrile disease and hemorrhagic fever. Currently, there are no approved or specific therapies for arboviral infections; treatment is limited to supportive care. Despite the ever-present global threat caused by arboviruses, there is not yet a single detection system that is capable of detecting all arboviruses simultaneously (Philip Samuel & Tyagi, 2006). Several outbreaks of arboviral

diseases have occurred in Kenya, confirming the presence of a number of arboviral diseases within the human and animal populace. However, much still remains unknown regarding the true prevalence of arboviral infections, in part because of spotty surveillance and clinical mis-diagnosis (Sutherland *et al*., 2011). Accurate detection of arboviral circulation and clinical cases could lead to improved prevalence and incidence estimates with more tailored patient care, rapid control intervention, and direct public health benefit for communities in disease endemic areas.

1.2 Statement of the problem

Arboviral infections are on the increase globally. Kenya, in particular has had multiple outbreaks in the last two decades. During these outbreaks there have been reported cases of morbidity and mortality leading to huge economic losses and public health distress. There are no known effective drug therapies for arboviral infections, and there are no vaccines for arboviruses other than the Yellow Fever vaccine. The control of these viruses has therefore been limited to vector control and early detection and management of cases. Despite its necessity, there is lack of an efficient detection method for both known and unknown arboviruses. The current viral detection methods are based on culture, ELISA and molecular techniques which can be costly and time consuming. Identification of these pathogens requires prior knowledge of the etiologic agents as they rely on specific oligonucleotide primers or antibodies for molecular and serological techniques, respectively. The specificity and sensitivity of these techniques also pose limitations on specific virus detection or early virus detection respectively in hosts and vectors. Arboviruses have high genetic diversity which compromises the design and use of a universal primer for some viruses belonging to the same family or genus. In cases where a number of targets have been multiplexed, targets are limited by the specificity of the primers. In cases where analysis of the multiplex PCR is

by gel electrophoresis, accurate analysis may be hindered by the tedious amplicon detection on the gels especially for target with close fragment sizes. An alternative approach has been to detect viruses within a specific genus by singleplex PCR using genus-specific primers. However, such methods require a necessary and expensive step to genotype amplicons by sequencing, which raises the assay cost beyond affordability for most resource limited laboratories. All these factors have limited surveillance programmes that could have aided in early detection, warning and control of the arboviral diseases.

1.3 Justification

Based on the rising global arboviral infections and spread, there is a need to develop a specific, sensitive, rapid, high-throughput and cost effective arbovirus detection platform for outbreak investigations as well as surveillance programmes. The platform should be able to detect both known and unknown viruses.

The advancement of molecular and bioinformatics tools as well as the huge number of virus and vector genomic data in various databases offers ample data for designing improved primer sets that universally amplify arboviral targets within specific genera. This data, coupled with the development of real-time thermocyclers capable of high resolution melting (HRM) analysis has enabled development of rapid, sensitive and high throughput surveillance assays. This is enabled further by availability of low cost HRM intercalating dyes that can allow multiplex PCRs. Utilization of multiplex-HRM technique offers a comprehensive means for arboviral screening by multiplexing different primers for different arboviruses at family or genus levels. This assay will enable detection of both known and unknown viruses. The technique will also offer a very sensitive and specific means to enable early detection of viruses. The genotyping capability of this technique will offer a fast and low cost means of identifying known viruses because sequencing will be limited to novel viruses with novel

melting profiles. When compared with the probe based methods, HRM analysis will provide savings on probe and label costs.

1.4 Hypothesis

Differences in nucleotide composition and amplicon length offer potential for arboviruses differentiation using Multiplex RT-PCR-High resolution melting (HRM)

1.5 Objectives

1.5.1 Broad objective

To develop and utilize a multiplex PCR-High resolution melting (HRM) assay for detection of arboviral pathogens in mosquitoes.

1.5.2 Specific Objectives

1. To determine the HRM thermal profiles for rapid typing of *togaviridae, flaviviridae* and *bunyaviridae* viruses.
2. To estimate the prevalence of arboviruses in mosquitoes using the multiplex RT-PCR-HRM assay.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Arboviruses

Arthropod-borne viruses (arboviruses) are responsible for many important vector-borne diseases of man and other animals (Hall *et al*., 2012). Some of the arboviruses that have been reported in East Africa include Dengue virus, Yellow fever virus, Rift Valley fever virus, West Nile fever virus, Chikungunya virus, Sindbis virus, Bunyamwera virus among others. Kenya has had multiple arboviral outbreaks in the past two decades leading to public health distress and economic losses. Some of the outbreaks include yellow fever in 1992 (Onyango *et al*., 2004), Chikungunya fever in 2004 (Sergon *et al*., 2008), Rift Valley fever (RVF) in 1997 (Woods *et al*., 2002) and 2006 (Njenga *et al*., 2009) and Dengue fever in 2011 and 2013 (Awando *et al.,* 2013)

Despite the constant arboviral activity in the region, there have been no established and standard methods for detecting and establishing the true prevalence of arboviruses in Kenya. There is also a big knowledge gap on the vectors responsible for virus maintenance and transmission especially during the inter-epidemic periods (LaBeaud *et al*., 2011b). Development and utilization of techniques that could reflect the true prevalence of arboviruses in Kenya could greatly enhance the early detection of arboviruses, thereby accelerating rapid prevention of spread through targeted control measures, public health education, and early vaccination campaigns (Sutherland *et al*., 2011). Despite the need for such techniques and programmes, there has been a major hindrance in the policies and methods used in health systems, especially in endemic areas. This has led to an underestimation of arbovirus activity in these areas (LaBea*ud et al*., 2011a). Moreover, many cases of arboviruses have been misdiagnosed for protozoan diseases such as malaria due to

overlap in clinical presentations. Reliance on specific diagnostic testing therefore becomes necessary for accurate differentiation of the viral infections and is crucial component for detection or accurate prediction of virus activity in vector populations and specific diagnosis of infection in the human or animal host. This can facilitate early warning of potential or existing outbreaks and initiation of vector management and/or vaccination programs. (Hall *et al*., 2012)

There is little that has been done in the prevention or early warning measures for arboviral infections. Many more diseases of arboviral origin have likely occurred but have gone unrecognized, especially in more remote locations (Sutherland *et al*., 2011). Arboviruses are now considered to be emerging pathogens based on their geographic spread and their increasing impact on susceptible human populations (LaBeaud *et al*., 2011b). Dengue virus infections, once rare, are now estimated to cause >50 million clinical cases per year especially in Asia and renewed spread through Central and South America.

There are various factors that can be associated with the global dynamics of arboviral infections. International travel has been implicated for dissemination of the viruses in new regions. This has been evidenced by the recent outbreaks of Chikungunya virus. It was noted that before 2006, all three CHIKV genogroups, namely Asian, Central/East African and West African genotypes (Schuffenecker *et al*., 2006) were restricted to the geographical areas denoted by their names (Yusof *et al*., 2011). Since these genogroups were defined, many new strains of CHKV have been found, some in regions in which CHKV has been absent (Townson & Nathan, 2008). The geographical distribution of CHIKV has changed worldwide. Endemicity of arboviruses is no longer the only factor involved in epidemics.

Other factors that can explain the viral dynamics is the evolutionary processes in viruses include mutations, reassortments and recombinations. A point mutation of A226V in CHKV (Rianthavorn *at al*., 2010) enabled the virus to infect a new vector, ***A. albopictus*** in addition

to ***A. aegypti***. This mutation enables the CHKV to be more adaptive to the new mosquito vector that can facilitate its spread.

The global spread of arboviral infections could also be due to the presence of a large naive population. These populations lack prior exposure to and immunity against the virus and in the presence of competent vectors among such populations, there could be introduction of the virus (Parola *at al.*, 2006).

2.2 Transmission

The three major modes for animal virus transmission between hosts are direct, mechanical and/or vertical (Kuno & Chang, 2005).

Direct transmission is the most widespread among all virus groups and is considered the fundamental mechanism in all animal virus groups (Kuno, G, 2001)**.** The common routes for direct transmission are intranasal, oral, venereal and exposure of skin with abrasion, cornea, reproductive tissue or any mucous tissue. Oral transmission includes ingestion of infected vectors as found in insectivorous animals such as bats. These bats become infected with viruses such as RVF, YFV and JEV upon ingestion of infected vectors. The direct transmission has been found to be very important in maintaining the no-vector group of flaviviruses in nature (Kuno & Chang, 2005). These viruses infect small mammals that have high turnover which provides a large number of immunologically naive hosts. Direct transmission has been associated with transmission of viruses to mosquito larvae through feeding activity in virus-contaminated aquatic environments such as dambos in Africa (Turell MJ *et al.*, 1990). Viruses associated with direct transmission have however been found to be less zoonotic (Woolhouse, Taylor, & Haydon, 2001).

Mechanical transmission on the other hand occurs through contaminated mouth parts of the blood sucking arthropods. Most of these arthropods have multiple vertebrate blood-meal

hosts and may switch hosts within short periods (Kuno & Chang, 2005). Vectors with high frequency of interrupted feeding patterns (insect vectors) are more efficient mechanical transmitters compared to those with low frequency of interrupted feeding (acarines). There are various mosquito vectors for arboviruses. ***Aedes, Anopheles, Mansonia*** **and** ***Culex*** species are principal mosquito vectors for a number of arboviruses. ***A. aegypti****,* for example are the principal arboviral vectors for Dengue Fever, CHKV and YF viruses. They originated in Africa and spread to other countries in the tropics in the 17th and 18th centuries (Gubler, 2004). Several other species of ***Aedes****,* including ***Ae. albopictus, Ae. africanus, and Ae. luteocephalus****,* are found in Africa and are potential arbovirus vectors. Among other factors, high virus titer in skin or blood is required for efficient mechanical transmission because normally less than 20 nanolitres constitutes the blood that contaminates the mouthparts of the vector.

2.3 Classification of Arboviruses

Arboviruses are classified into five families: *Togaviridae* (genus *Alphavirus*), *Flaviviridae* (genus *Flavivirus*), *Bunyaviridae* (genus *Orthobunyavirus, Nairovirus* and *Phlebovirus*), and *Reoviridae* (genus *Orbivirus*, *Seadornavirus* and *Coltivirus*) families.

The majority of the arboviruses with high economic and health impact are found in the *Togaviridae, Flaviridae and Bunyaviridae* families.

Togaviridae

The *Togaviridae* family belongs to group IV of the Baltimore classification of viruses. The genome is linear, single-stranded, positive sense RNA that is 10,000-12,000 nucleotides long. The 5'-terminus carries a methylated nucleotide cap and the 3'-terminus has a polyadenylated tail, therefore resembling cellular mRNA. The virus is enveloped and forms spherical

particles (65-70nm diameter), the capsid within is icosahedral. The vector for *Togaviridæ* is primarily the mosquito.

Flaviviridae

The *Flaviviridae* are a family of viruses that are primarily spread through arthropod vectors (mainly ticks and mosquitoes). *Flaviviridae* have monopartite, linear, single-stranded RNA genomes of positive polarity, 9.6 to 12.3 kilobase in length. The 5'-termini of flaviviruses carry a methylated nucleotide cap, while other members of this family are uncapped and encode an internal ribosome entry site. Virus particles are enveloped and spherical, about 40-60 nm in diameter.

Bunyaviridae

Bunyaviridae is a family of negative-stranded, enveloped RNA viruses. Though generally found in arthropods or rodents, certain viruses in this family occasionally infect humans. Some of them also infect plants. *Bunyaviridae* are vector-borne viruses. With the exception of Hantaviruses, transmission occurs via an arthropod vector (mosquitos, tick, or sandfly). Incidence of infection is closely linked to vector activity. Human infections with certain *Bunyaviridae*, such as *Crimean-Congo hemorrhagic fever virus*, are associated with high levels of morbidity and mortality.

2.4 Surveillance techniques

Based on the current arbovirus epidemiology, it is clear that they will continue to be of major public and animal health concern. There is a need to use diagnostic tools that are both specific and sensitive for surveillance, outbreak investigation, or clinical applications. These diagnosis tools are categorized as either live virus, protein or nucleic acid based assays.

Cell culture (Live virus)

Arboviruses may be grown in a cell culture and the cells affected with virus will evolve morphologic changes, often specific for the type of virus involved. The disadvantages of conventional viral culture are that it is labor intensive, moderately expensive, and slow (McAdam & Riley, 2009). Cell culture is considered to be the gold standard for virus detection.

Antibody detection (Protein based assay)

Antibody detection is based on specific antibodies produced by the vertebrate host upon being infected by a virus. It is an adaptive immune system which works by binding the antibodies to the virus thus rendering it non-infectious. Two types of antibodies are important, IgM and IgG. The presence of IgM in the blood of the host is used to test for acute infection, whereas IgG indicates an infection sometime in the past.

Antigen detection (Protein based assay)

Enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and colour change to identify a substance. Antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate. Performing an ELISA involves at least one antibody with specificity for a particular antigen. Antigen detection is insufficiently sensitive and/or specific (Choudhar*y at al*., 2013).

Nucleic acid detection

Polymerase Chain Reaction (PCR) assays may be used to detect a target DNA or RNA sequence in a sample. The advent of molecular technologies, such as reverse transcription-PCR (RT-PCR), and the application of these technologies in diagnostic and reference laboratories provide a time-efficient alternative to traditional serological methods for the identification of virus isolates (Lambert & Lanciotti, 2009). Such technologies also provide an inherent flexibility of the design which allows a researcher to develop an assay with various degrees of specificity (Lambert & Lanciotti, 2009).

2.5 General Challenges in arboviral infection diagnosis

The diagnosis of arboviral diseases can be difficult due to factors such as clinical similarity to other diseases and difficult access to reference laboratories that can perform differential diagnosis. The clinical presentation for Dengue fever for example can easily be confused with yellow fever or malaria (Moreli & da Costa, 2013). In Kenya this poses a big challenge because of the endemicity of malaria and lack of proper facilities for further diagnosis.

Traditional viral detection and surveillance methods including PCR can be costly and time consuming and generally require prior knowledge of the etiologic agent, as they rely on virus-specific primers or antibodies. Therefore, these techniques are unsuitable for situations when the causative agent of an outbreak is entirely novel or is an unknown sequence variant (Bishop-Lilly *at al*., 2010). The pitfalls of using specific PCR targets were vividly demonstrated when it was found that a new variant of a genital *Chlamydia trachomatis* strain had escaped detection for several years because it had acquired a deletion in the region of a virulence plasmid that was a target for a commonly used real-time PCR assay (Seth-Smith *at al*., 2009).

2.6 Advances in Molecular detection of Arboviruses.

PCR based techniques provides an inherent flexibility of the design which allows a researcher to develop an assay with various degrees of specificity (Lambert & Lanciotti, 2009). Sensitivity and specificity of virus detection have improved considerably with the advent of nucleic acid amplification tests (Choudhary *at al*., 2013). One of the versions of PCR based techniques is multiplex PCR to detect a group of viruses of interest. It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes (targets) at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. This technique has gained popularity because of its experimental simplicity and greater cost effectiveness (Giridharan *at al*., 2005). Recently, several groundbreaking studies have been published that use *de novo* high-throughput bead-based pyrosequencing of DNA to provide putative identification of viral disease agents (Towner *at al*., 2008). The technique is however limited by the need to generate tens of millions of high quality reads, ideally greater than 200 bases in length. This approach is not cost effective. However, with the advancement in sequencing technology in the last five years, the day may come when it can be fully integrated in surveillance or genotyping studies (O'Brien & Stelling, 2011).

2.7 High Resolution Melting

High Resolution Melting Analysis (HRM or HRMA) is a recently developed technique for fast, high-throughput post-PCR analysis of genetic mutations or variance in nucleic acid sequences. It enables researchers to rapidly detect and categorize genetic mutations (e.g. single nucleotide polymorphisms (SNPs)), identify new genetic variants without sequencing (gene scanning) or determine the genetic variation in a population (e.g. viral diversity) prior

to sequencing. The first step of the HRM protocol is the amplification of the region of interest, using standard PCR techniques, in the presence of a specialized double-stranded DNA (dsDNA) binding dye. These specialized dyes are highly fluorescent when bound to dsDNA and poorly fluorescent in the unbound state. This change allows the user to monitor the DNA amplification during PCR (as in quantitative PCR). HRM uses same concept as the classical melting curve analysis but its superior in its ability to give a more detailed analysis of the denaturation. It characterizes nucleic acid samples based on their disassociation (melting) behavior. Sample discrimination is based on the sequence, length, GC content or strand complementarity. According to QIAGEN, even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified. By combining multiplex PCR with HRM, multiple amplicons targeting different loci may be differentiated (Seipp *at al*., 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1 Mosquitoes samples and virus isolates

Archived mosquito homogenate samples collected from North Eastern province, Kenya were used. The mosquitoes were collected during rainy seasons between January, 2011 and December, 2012. Mosquito genera were identified on basis of microscopic morphologic appearance and stored in pools of 5-25 mosquitoes. The mosquitoes were of known arbovirus vectorial capacities mostly from *Aedes* and *Culex* species. Homogenization was done using established protocols **(Appendix 3).** The homogenates were stored in minus 80°C until processing to ensure the integrity of the viruses. Frozen viral isolates from each of the three families (*Bunyaviridae, Togaviridae* and *Flaviviridae*) were used for standardization.

3.2 RNA extraction and cDNA synthesis

Viral RNA from the virus isolates and the mosquito homogenates was extracted using MagNa Pure 96 (Roche) extractor. MagNA 96 Pure DNA and Viral NA Small Volume Kits were used according to manufacturer's instructions with an elution volume of 50 µL. The starting volume of the sample was 200 µL but less volume (up to 50 µL) of the starting volume was used for the virus isolates by topping up with H_20 because of their higher viral copies compared with the field collected mosquito homogenate samples. 5 µL of the extracted RNA was reverse transcribed immediately in 10 µL Transcriptor cDNA synthesis kit (Roche) reaction volumes. We used 0.6 mM reaction concentrations of non-ribosomal random hexamers as primers instead of the RT-kit supplied random hexamers (Endoh *et al*., 2005). The final concentrations in the reactions were 1X Transcriptor RT Reaction Buffer, 1mM of each dNTP, 20U of RNase inhibitor and 10Units of Transcriptor Reverse

Transcriptase. The RNA was reverse transcribed on a block thermocycler (Eppendorf Master cycler) set at 95^0C for 10 minutes for enzyme activation, 57^0C for 40 minutes for cDNA synthesis and 85^0C for 5 minutes for inactivation of the enzyme. The synthesized cDNA was stored in a minus 80^0C freezer awaiting PCR assays. All the biosafety and good laboratory practices and requirements were observed strictly.

3.3 Primer design and assay optimization.

Based on multiple alignments of arboviruses within genera (**Appendix 2**), we modified existing primers for universal *Alphavirus*, *Phlebovirus*, and *Orthobunyavirus* amplifications, and designed new primers for universal *Nairovirus*, *Flavivirus*, and *Thogotovirus* amplifications. Primers were designed manually, targeting 100-400 base pair (bp) polymorphic regions flanked by relatively conserved regions in which universal primers could be designed. We allowed for up to 36-fold degeneracy and for A-C and G-T mismatches (except within the last five 3' bases), and maintained annealing temperature differences, calculated according to Rychlik *et al.,* 1990 within 1°C between primer pairs and within 4°C across the multiplex panel of primers. To minimize overall degeneracy further for the *Nairovirus* and *Flavivirus* primers, we mixed cocktails of primers with different degeneracies for each of the forward and reverse primers (Appendix 2). We analyzed potential primer sequences using the OligoCalc online oligonucleotide properties calculator, (Kibbe, 2007) and avoided primers with 3' self-dimerization and hairpin formations. We further evaluated and minimized primer-primer interactions based on *in silico* reactions performed using Amplify 3X software for Macintosh.

3.4 Assay optimizations

Assay optimization involved singleplex and multiplex PCRs as well as the HRM temperature shifts selections. The performance efficiency of each generic primer was first evaluated by amplifying its target using standard singleplex PCR cycling conditions in 10 µL reactions volumes in a thermocycler (Bio-Rad DNA Engine). The reactions were performed using QIAGEN Multiplex PCR kit with 5 µL of the mastermix, 1 µL of the Q-solution, 0.5 µL of the 10 µM forward and the reverse primers, 1 µL of the cDNA and topped up with 2 µL molecular grade H_2O so as to provide a final concentration of 1x of the mastermix, 0.5 µM of the Q-solution and 0.5 µM of the primers. The kit's mastermix contained HotStarTaq DNA Polymerase, Multiplex PCR Buffer and dNTP Mix providing a final concentration of 3 mM $MgCl_2$. The cycling programme entailed an initial activation step at 95°C for 5 minutes and 40 cycles consisting of 3 steps cycling programme of denaturation at 95°C for 30 seconds, annealing step at 55°C to 65°C dependent on the primer under use, and an extension step at 72°C for 90 seconds before a final extension at 72°C for 7minutes.

3.5 Agarose Gel Electrophoresis

1.5% Agarose gels were cast by dissolving 1.5 grams of agarose in 100ml of 1X TAE buffer in a plastic beaker and boiling in a microwave until the agarose particles were well dissolved. The solution was then let to cool to about 60°C before adding ethidium bromide to a final concentration of 0.5 µg/ml so as to enable visualization of the DNA migration and separation. The solution was then poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel had solidified, the comb was removed and the gel, still in its casting tray placed horizontally into the electrophoresis chamber and covered with the 1X TAE running buffer.

5 µL of the PCR products from each reaction was mixed with 1 µL of 5% carol load dye

(QIAGEN) and loaded in the gel wells. The electrophoresis chamber was then connected to the gel electrophoresis power pack set at 80 volts and ran for 1 hour. Visualization was done on Gel Doc imaging system and a photo taken using the equipped camera.

Primers that were found not to work efficiently were optimized by changing their concentration or their cycling parameters. Those that did not work were re-designed or newly designed using new targets. Once all the primers were confirmed to amplify efficiently, two-plexes were performed for primers that had close cycling conditions and gradually the rest of the primers were introduced. During these optimizations a Touch-down PCR cycling program that targeted all the primers optimal cycling conditions was developed and used. For optimal performance, the various optimization parameters e.g. primer concentration and cycling conditions were changed until the panel was working optimally. The verification for specificity and efficiency was by agarose gel electrophoresis as stipulated above.

3.6 Singleplex and Multiplex High Resolution Melting (HRM) Assays

Singleplex PCRs with HRM were performed in duplicates for each of the primers using QIAGEN multiplex PCR kit using the protocol stipulated above. SYTO9 saturating intercalating dye (Life technologies) was added to a final concentration of 1µm/µL in 10 µL reaction volumes. The reactions were performed in real-time HRM capable thermocycler (Rotor-Gene Q, QIAGEN) using the touchdown cycling program developed above (Appendix 3) and detection performed on the green channel for which the SYTO-9 dye is detected.

The data collection at the HRM component was set from 75°C to 95°C. Temperature increments readings at the HRM step were evaluated from 0.02°C to 0.5°C. The smallest increment temperature that gave similar thermo profiles for replicate samples was chosen and

used thereafter. The PCR and HRM setups were repeated until all the virus isolates could be amplified and HRM genotyping results could show characteristic profiles at the species level. Multiplex PCR-HRM assays were then performed in duplicates for all the 16 virus isolates available. The following methods of analysis were performed for the data obtained in either singleplex or multiplex assays.

3.7 Analysis

3.7.1 Analysis of HRM data by HRM thermal profiles

The potential of the HRM to type the viruses was evaluated using the Rotor–Gene Q operating software. The software was used to first normalize the raw florescence values relative to the positive and negative control values, which provided signal levels between 0 and 100%. The software generated a graph of normalized fluorescence against melting temperatures. Temperature shifts were performed until each of the isolate produced a characteristic thermal profiles while maintaining a very close or similar profiles for the individual replicate.

3.7.2 Analysis by melt curve peaks

The analysis was also based on the melt curve peaks. They were generated by the Rotor–Gene Q operating software by plotting dF/dT against the melting temperatures. The peaks represented the highest change in florescence. The peaks were grouped into bins and a threshold set to which those below the set threshold were discarded from analysis. The bins were mapped to the genotypes for auto-calling based on the positive controls included in the run.

3.8 Screening and Genotyping of the field samples

The cDNA synthesized from the mosquito homogenates extracts were first amplified using the multiplex panel developed above. The samples that showed amplification were analyzed by comparing at least two of the analysis methods discussed above to identify them. Those identified were confirmed by amplifying them using singleplex panels to which the positive controls were included. Those that could not be clearly identified were amplified in all singleplex panels. These analysis methods were however confirmed by manual analysis which entailed close observation of the profiles and associating each with the known viruses to ascertain the genotype of the virus. Re-PCR was utilized for samples with low amplification. Samples suspected to have co-infections were amplified further in singleplex PCR panels. Those that could be identified were reported while those that did not follow any of the known virus profiles were purified for sequencing

3.9 Product Purification

5 µL of the PCR product was mixed with 2 µL of ExoSAP-IT enzyme (Affymetrix) in a 0.2 mL PCR tube. ExoSAP-IT utilizes two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase to remove unutilized dNTPs and primers. The Exonuclease I degrades residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase hydrolyzes the remaining dNTPs from the PCR mixture which would interfere with the sequencing reaction.

The tubes were incubated at 37°C for 15 minutes for the treatment and then at 85°C for 15 minutes for the enzyme inactivation. This was achieved in a thermocycler (Bio-Rad DNA Engine). The tubes were then sealed using parafilm and sent for sequencing at Macrogen (Seoul, Korea) according to Vázquez *et al*., 2012

CHAPTER FOUR

4.0 RESULTS

4.1 Conventional PCR results

Conventional PCRs were performed for empirically optimizing the concentrations of each of the primers used in the multiplex panel. cDNA from the available virus isolates were used. Once optimized, the multiplex assay was performed using the procedure described earlier. The gel photo below shows the results of the multiplex PCR. In total, the assay amplified all the 16 isolates available as our positive controls.

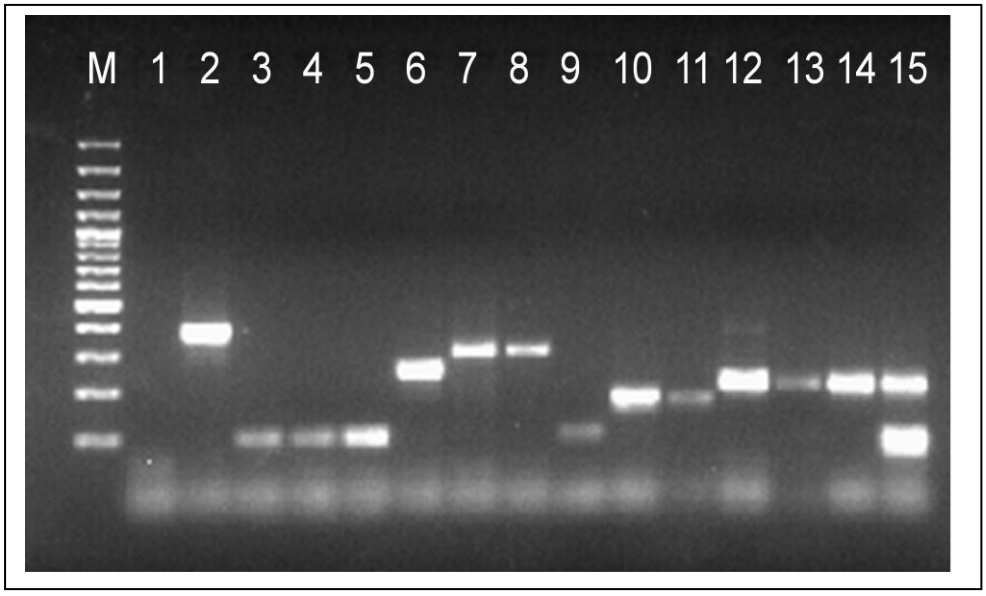

Figure 4.1.1 Gel image of the Multiplex RT-PCR

M: 100bp molecular marker, **1**-NTC. **2**-RVF, **3**-CHKV, **4**-SFV, **5**-Middleburg virus, **6**-Bunyamwera virus, **7**-Sindbis Virus, **8**-Thogoto Virus, **9**-Dhori virus, **10**-Dugbe Virus, **11**-Hazara Virus, **12**-YFV, **13**-WNV, **14**-Usutu Virus, **15**-CCHF (PCR Product re-amplified)

4.2 Multiplex RT-PCR-HRM analysis.

The results of the multiplex RT-PCR-HRM have been reported below using the melting profiles. The profiles indicate clear and distinct HRM thermal profiles that can clearly differentiate the 16 viral isolates used in this study. Some of the isolates were performed in duplicates so as to show the reproducibility of the assay.

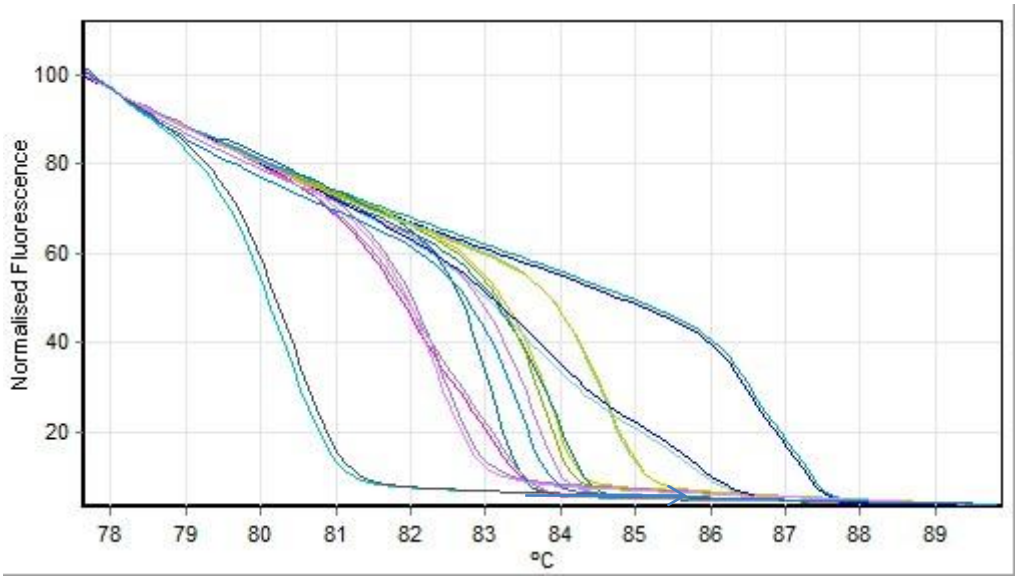

Figure 4.2.1: Normalized HRM profiles of diverse arboviruses represented as percent fluorescence

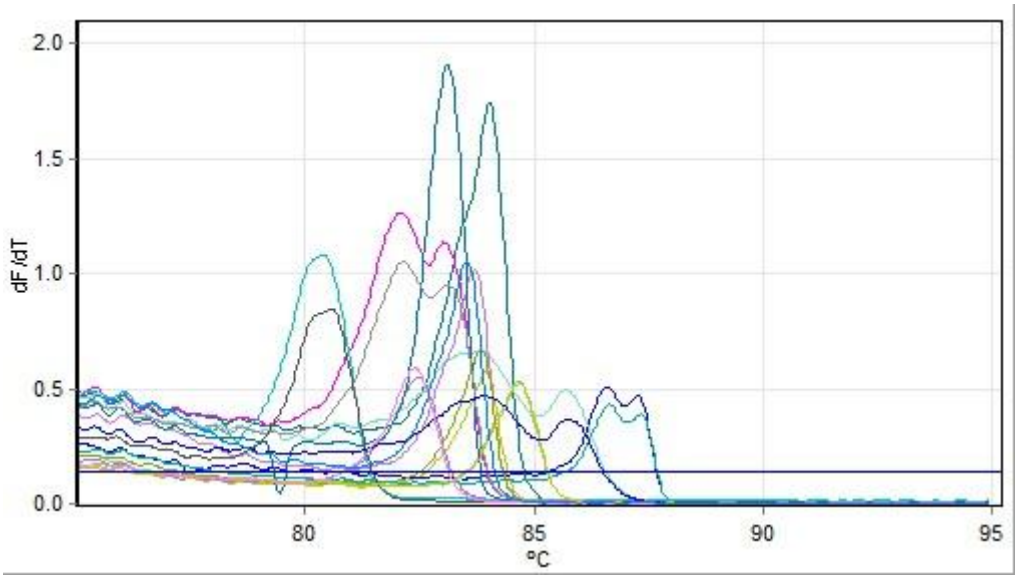

Figure 4.2.2 Melt rates of diverse arboviruses represented as change in fluorescence units

with increasing temperature (df/dt)

No.	Colour	Name
1		Sindbis
2		RVF
3		Babanki
4		WN
5		Dugbe
6		Usutu
7		Chik
8		Dhori
9		Middleburg

No.	Colour	Name
10		Sindbis-2
11		RVF-2
12		Bunyamwera
13		WN-2
14		Dugbe-2
15		Usutu-2
16		Chik-2
17		Dhori-2
18		Middleburg-2

Table 4.2.1: Colour coded keys representing the multiplex RT-PCR-HRM thermo profiles

4.3 Screening and Genotyping of the field samples

A total of 651 mosquito homogenates were screened for arboviruses using the optimized conditions and the procedure detailed in this report. Positive samples were confirmed by running singleplex PCR of the positive samples alongside the positive controls of the genus under suspicion. Samples showing identical profiles to the positive controls were identified with the said control. Those showing profiles outside the expected profiles of the positive controls were sequenced at Macrogen (South Korea).

From the analysis of the HRM, melt peak and sequencing results, one sample was identified as Bunyamwera by HRM and confirmed by sequencing. Forty-five Culex flaviviruses were

identified by HRM. One Mosquito Flavivirus and one Wesselsbron virus were confirmed by sequencing. Four samples produced HRM profiles that were identical to Usutu virus while two samples were reported as Sindbis virus due to their profile similarity to the Sindbis positive control and a further confirmation by sequencing.

HRM results also identified that some samples were co-infected. One sample was found to have both Sindbis virus and Culex Flavivirus while another sample was found to have Semiliki Forest virus and Culex Flavivirus. Three samples that had amplified with *Nairovirus* primers and *Alphavirus* primers did not produce quality sequences to enable sequence and BLAST analysis. Nine samples amplified with *Alphavirus* primers but the profiles did not match the profiles of the positive controls available. Six samples on the other hand had low amplifications with *Alphavirus* primers and could thus not be analyzed further.

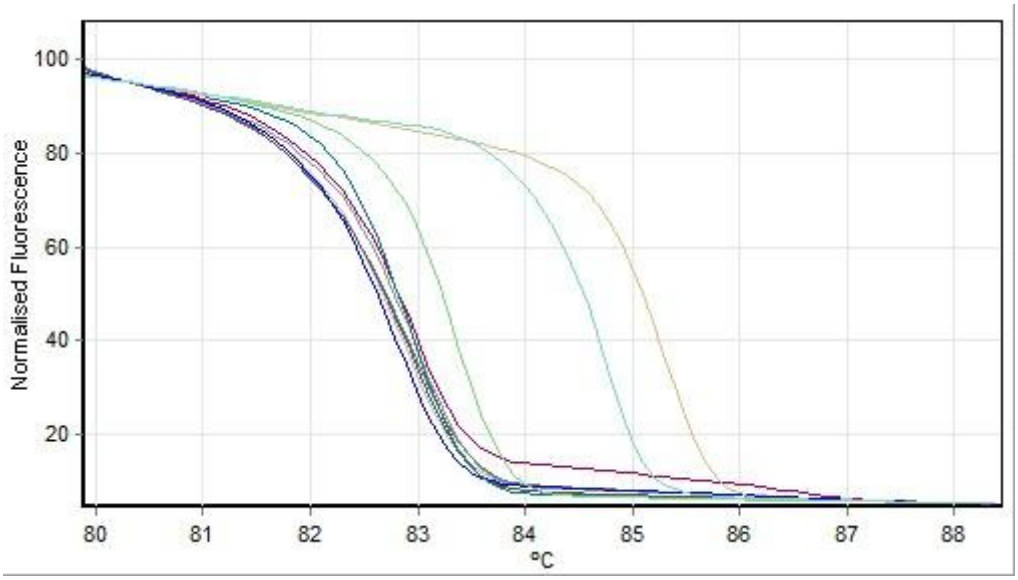

Figure 4.3.1: Normalized HRM profiles of flaviviruses represented as percent fluorescence

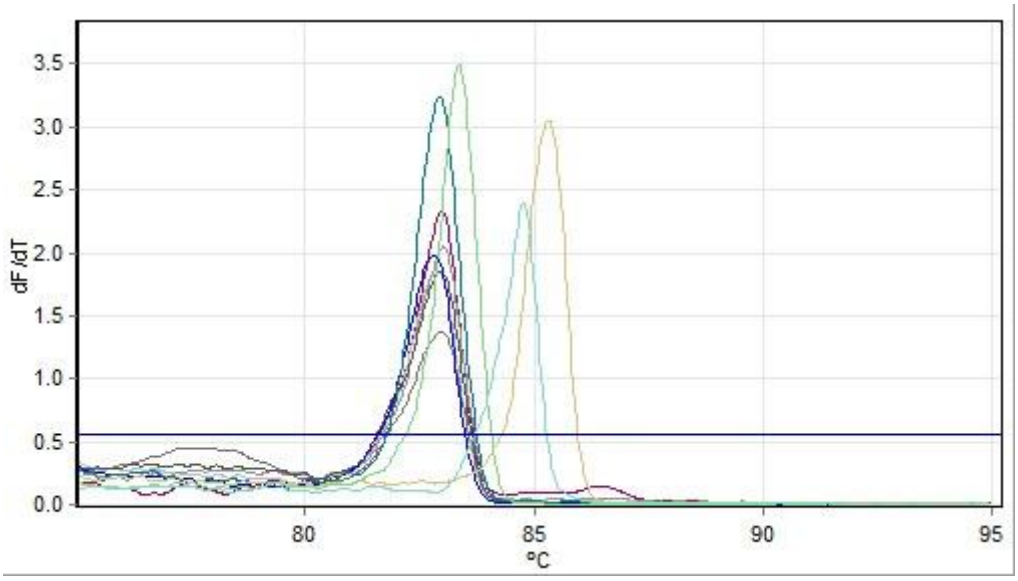

Figure 4.3.2 Melt rates of Flaviviruses represented as change in fluorescence units with increasing temperature (df/dt)

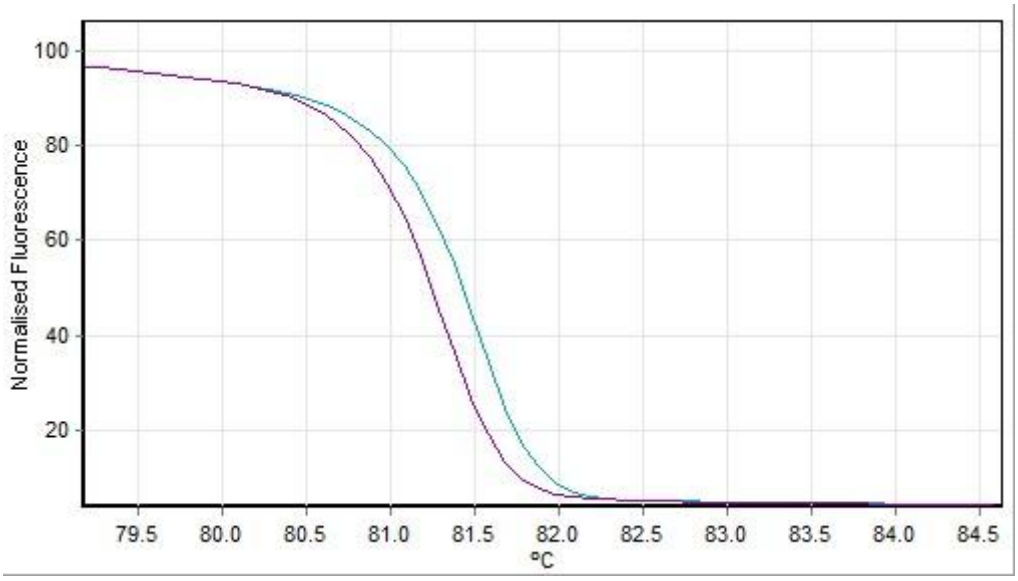

Figure 4.3.3: Normalized HRM profiles of Orthobunyaviruses represented as percent fluorescence

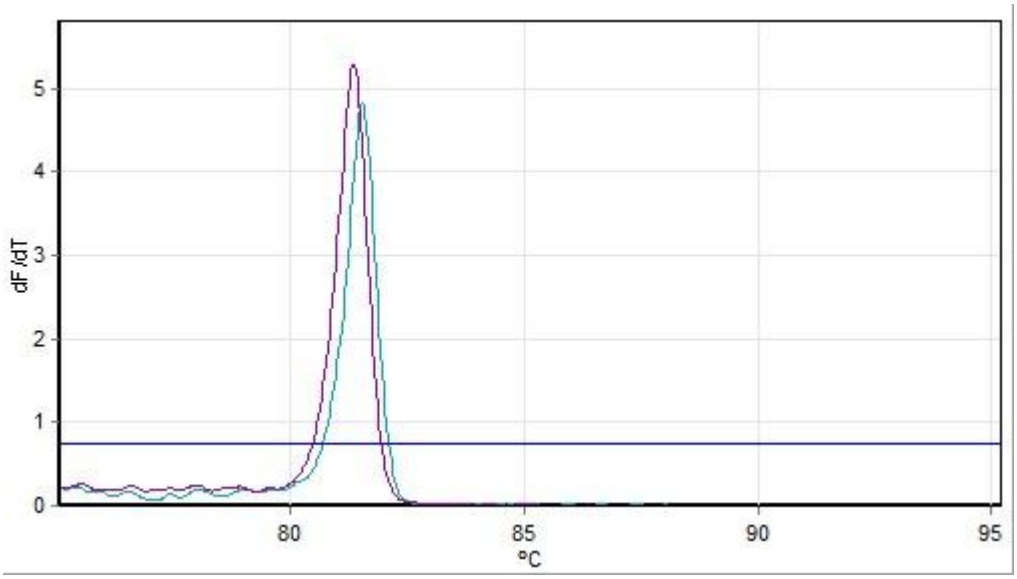

Figure 4.3.4: Melt rates of Orthobunyaviruses represented as change in fluorescence units with increasing temperature (df/dt)

No.	Colour	Name	Genotype	Peak 1
1		AMH003692		81.52
2		Bunyamwera		81.35

Table 4.3.1 Colour coded keys representing the Orthobunyaviruses amplified RT-PCR-HRM thermo profiles

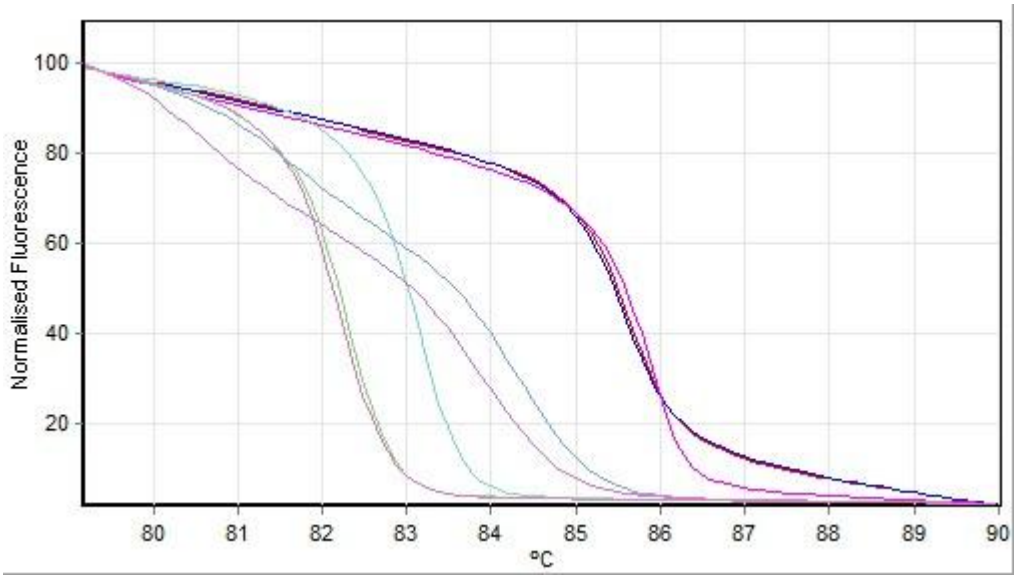

Figure 4.3.5: Normalized HRM profiles of Alphaviruses represented as percent fluorescence

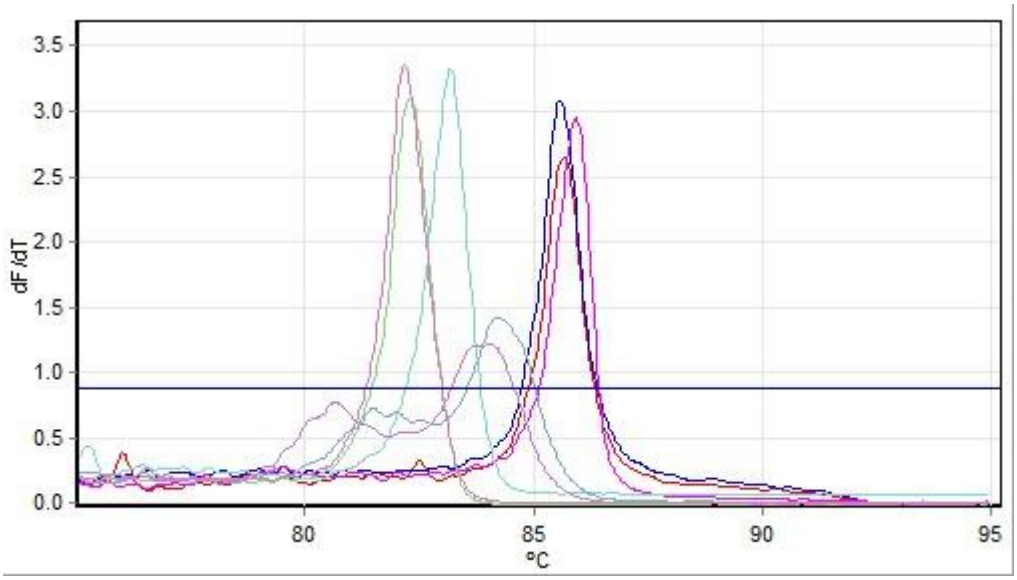

Figure 4.3.6 Melt rates of Alphaviruses represented as change in fluorescence units with increasing temperature (df/dt)

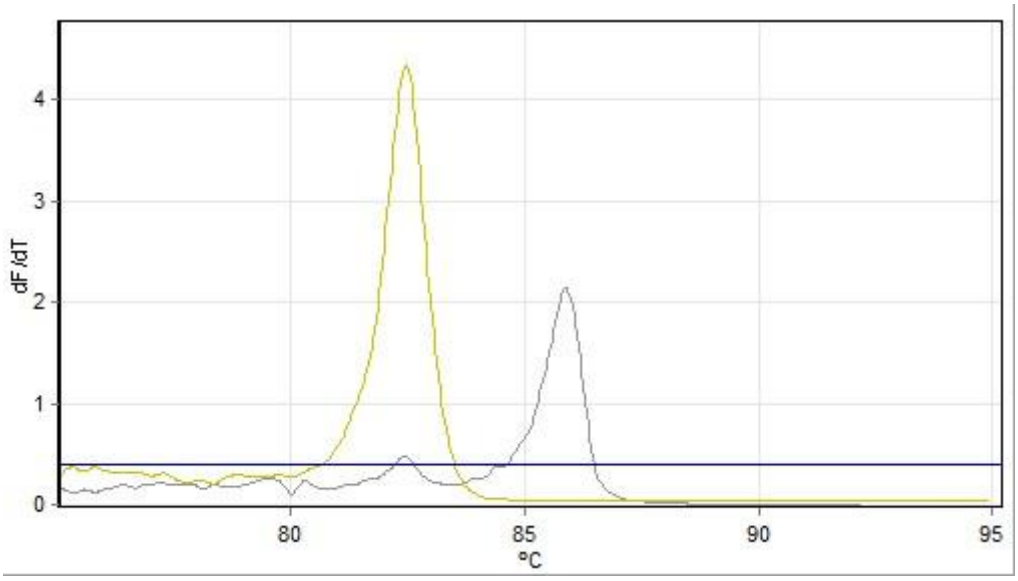

Figure 4.3.7: Melt rates of a co-infected sample represented as change in fluorescence units with increasing temperature (df/dt).

It shows a co-infection of Sindbis (first peak) with unknown Alphavirus (second peak).

No.	Colour	Name	Genotype	Peak 1	Peak 2
1		AMH007404		82.43	85.87
2		Vir2052 Sindbis		82.45	

Table 4.3.2: Colour coded keys representing the Alphaviruses amplified RT-PCR-HRM thermo profiles.

CHAPTER FIVE

5.0. DISCUSSION, CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

5.1 DISCUSSION AND CONCLUSION

The results of this study have confirmed that Multiplex RT-PCR-HRM can be used as a tool for detecting and genotyping arboviruses in mosquitoes. The assay developed in this study was able to differentiate all of the 16 virus isolates that were available. These isolates fall under the genera *Alphavirus, Flavivirus, Orthobunyavirus, Nairovirus, Phlebovirus* and *Thogotovirus.*

The results indicate that there is active circulation of arboviruses in Kenya. The study has shown circulation of *Alphaviruses, Flaviviruses, Orthobunyaviruses* and other viruses that could not be established. Further work shall be carried out to establish their identity.

The Multiplex RT-PCR-HRM assay developed in this study allows for the detection of multiple viruses using multiple sets of primers and can therefore be adopted for screening of arboviruses during outbreaks and surveillance programmes prior to sequencing.

Outbreak investigation or surveillance programs require screening of thousands of samples within a short period. Automated extraction of nucleic acids has gained popularity in the recent past because of its high throughput capabilities coupled with better yield and efficiency compared with manual methods. In this study MagNa Pure 96 (Roche) extractor was used for extraction of the nucleic acid from the viral isolates and the mosquito homogenates.

The optimization of the multiplex assay was however complex because of the many parameters that required optimization. This was influenced greatly by the use of multiple

primers for the various arbovirus genera. Sequence analysis during primer designs revealed high level of genetic variability which complicated the use of a consensus region for viruses falling under the same genus. Therefore, degenerate primers were used to constitute primer mixes for the forward and reverse primers of *Flaviviruses* and *Nairoviruses*. The assay therefore required optimization of these degenerate primer mixes before they were added to the multiplex panel. Due to the genetic variability of the target regions coupled with GC content variability of the primers in the multiplex panel, it was not possible to get a single annealing and extension PCR cycling temperature. We therefore utilized a touch down cycling program as represented in Appendix 3. This program ensured the amplification of primer targets with annealing temperatures between 51°C and 60°C. Other parameters that were investigated were the concentration of the templates being used. While we did not quantify our cDNA, 1 µL of the 70 µL eluate was found to be optimal in a 10 µL reaction volume.

Unlike probe based detection methods in real time PCR, fluorescence increments with the non-specific dyes does not necessarily indicate the target amplification. The increment could be due to non-specific amplification or formation of the primer dimers. It was therefore important that the optimization of this assay be validated by agarose electrophoresis so as to evaluate the amplification efficiency of the primers. This efficiency was confirmed by the ability to produce amplicons of expected sizes as well as bands with good intensity which was an indication of the quantity of the amplicons as seen in the gel image (Figure 4.1.1).

Upon establishment and confirmation of optimal PCR setup and cycling conditions, a HRM capable thermocycler (Rotor-Gene Q, QIAGEN) was used to evaluate the ability of HRM to differentiate the virus isolates. The results of this assay were found to be able to differentiate the 16 known positive arbovirus isolates under this study as seen in Figures 4.2.1 and 4.2.2. However, the multiplex HRM profiles of the isolates were clustered together making it hard

to analyze and differentiate the profiles efficiently. In this regard, singleplex panels were utilized. The combined use of the HRM thermal profiles and the melt peaks provided a powerful genotyping tool. The *Flavivirus* isolates (i.e. West Nile, Yellow Fever and Usutu viruses) had distinct profiles and could be easily differentiated by both their HRM profiles and melt peaks. On the other hand Chikungunya and Semiliki Forest Viruses had close HRM profiles and required closer visual assessment of the profiles coupled with the melt peak analysis for accurate differentiation. Middleburg, Sindbis, Babanki and Ndumu viruses had close resemblance in both the HRM profiles and the melt peaks. We used replicates in the controls so as to check on the reproducibility thus increasing our reporting confidences. While we lacked variety of positive controls for *Orthobunyaviruses* and *Phleboviruses*, Bunyamwera, RVF and CCHF viruses had clear, distinct and reproducible profiles. Dubge and Hazara viruses (*Nairoviruses*) and Thogoto and Dhori viruses *(Thogotoviruses)* also had distinct and reproducible profiles therefore providing confidence in their identification. However, Dhori virus was not consistent in amplification despite changing the primers and raising the primer concentrations. This could be due to poor quality of the cDNA. Further tests should be done using high quality cDNA.

Upon screening of field-collected mosquitoes sampled from Kenya, the assay identified Bunyamwera, Sindbis, Usutu, Wesselsbron, Mosquito Flaviviruses and Culex Flaviviruses. The assay also identified co-infections with Sindbis virus and Culex Flaviviruses and Semiliki Forest virus and Culex Flaviviruses. Other samples had novel profiles with *Nairovirus* and *Alphavirus* primers. These unidentified samples shall form the basis of further identification assays in the subsequent tests. These results from field collected mosquitoes confirms active circulation of arboviruses in Kenya as previously reported (LaBeaud *at al*., 2011a, Ochieng *at al*., 2013)

This assay has further demonstrated its utility in pathogen discovery. It is through this aspect

that a virus was identified with 97% identity to Wesselsbron virus (GenBank Accessions: JX423783, JX423791), an arbovirus that has not previously been isolated in Kenya and has livestock disease symptoms that overlap with those for Rift Valley Fever (Coetzer JA & Barnard BJ, 1977). The utility of the assay was further demonstrated by identification of Mosquito Flaviviruses and Culex Flaviviruses that had homologies of only 67-77% to the nearest *Flavivirus* sequences available on GenBank. These results confirms the postulation by Lambert & Lanciotti (2009) that multiplex PCR using genus primers would detect additional species outside the evaluated ones or uncharacterized viruses. The results support the study by Vázquez *et al*. (2012) on the prevalence of insect Flaviviruses in mosquitoes. Crochu *et al.* (2004) also noted that cell fusing agent virus (CFAV) and other genetically related viruses are widespread in nature and occur in a variety of mosquito species.

However, incidences of integrated sequences for mosquito borne viruses have been reported (Crochu *at al*., 2004).Integration of sequences is a theoretical suggestion in which intracellular reverse transcriptase copy the viral RNA into a DNA form before incorporation into the host genome (Zhdanov, 1975). Further tests should be performed to ascertain whether the mosquito borne viruses identified in this study were as a result of infection or integrated sequences. Such tests would entail performing DNAse treatment of the extracted nucleic acid and thus eliminating the genomic DNA so as to be left with the RNA only. This way the positive amplification of the Flaviviruses would be considered to be due to viral RNA and not integrated DNA. Alternatively, longer fragments could be amplified or amplification targeting different segments of these viral genomes. Growth of the virus through cell culture could also be considered.

5.2 LIMITATIONS

During this study, we encountered a number of challenges. One challenge was the effect of the concentration of the viral RNA on the HRM profiles of the positive samples. It was found that samples with low amplification had the melting temperatures shifted to the right. In such cases, it required a close examination of the HRM profiles and the melt peaks in order to establish the identity. Further work should focus on automatic genotype analysis on the software to provide objective results not requiring expert evaluation as suggested by (Seipp *at al*., 2009).

The cDNA templates also used in this study were crude and it was therefore not possible to establish their initial concentration and evaluate its effect on the HRM profiles. In the future, it will be advisable to estimate the RNA concentration by a Nanodrop or spectrophotometer so as to guide in standardizing the templates first.

HRM requires distinct thermal profiles. We had to evaluate and re-design some primers so as to have profiles with distinct profiles. Further, we had to perform visual inspection coupled with the melt peak analyses to increase on the reporting confidences. This required experience and may not be applicable for investigators with little experience. Such work requires primers that focus on genome areas that will give distinct profiles for a given group of viruses. This will avoid instances where two or more viruses have close profiles creating chances of misdiagnosis.

The Rotor-Gene Q has the potential to record data at increments of up to 0.02°C. In this study, we used 0.1°C because lower increments were found to give variability even in replicates. In future, it will be necessary to establish the correlation between temperature increments and the genetic variability of the cDNA.

5.3 RECOMMENDATIONS

Despite these shortcomings however, this study has provided and utilized a multiplex RT-PCR-High resolution melting (HRM) assay for detection of arboviral pathogens in mosquitoes. It is among the few that can provide for a cost effective and fast method of diagnosis in addition to high throughput that is essential in surveillance and outbreak investigation programs.

Further, the assay provides a platform for arbovirus discovery.

This novel assay shall be utilized in detection of arboviruses in *togaviridae, flaviviridae* and *bunyaviridae* genera which have the highest economic and health impacts. The assay shall not be limited to mosquitoes as other arbovirus vectors as well as the vertebrate hosts and reservoirs can be investigated using the assay. In order to be used for clinical diagnostics there shall be need to evaluate its sensitivity and specificity against ELISA, singleplex PCR, sequencing and other methods currently under use.

I recommend the incorporation of this multiplex HRM assay in an algorithm that includes other molecular assays as well as cell culture and antibody detection methodologies for comprehensive identification of arboviruses in vectors, hosts and reservoirs.

REFERENCES

Awando, J. A., Ongus, J. R., Ouma, C., & Mwau, M. (2013). Seroprevalence of Anti-Dengue Virus 2 Serocomplex Antibodies in out-patients with fever visiting selected hospitals in rural parts of Western Kenya in 2010-2011: a cross sectional study. *Pan African Medical Journal*, ***16,*** 1–10**.**

Bishop-Lilly, K. a, Turell, M. J., Willner, K. M., Butani, A., Nolan, N. M. E., Lentz, S. M., … Read, T. D. (2010). Arbovirus detection in insect vectors by rapid, high-throughput pyrosequencing. *PLoS Neglected Tropical Diseases*, ***4***(11).

Choudhary, M. L., Anand, S. P., Heydari, M., Rane, G., Potdar, V. a, Chadha, M. S., & Mishra, A. C. (2013). Development of a multiplex one step RT-PCR that detects eighteen respiratory viruses in clinical specimens and comparison with real time RT-PCR. *Journal of Virological Methods*, ***189***(1), 15–9.

Crochu, S., Cook, S., Attoui, H., Charrel, R. N., De Chesse, R., Belhouchet, M., … de Lamballerie, X. (2004). Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of Aedes spp. mosquitoes. *The Journal of General Virology*, ***85***(Pt 7), 1971–80.

Dash, a P., Bhatia, R., Sunyoto, T., & Mourya, D. T. (2013). Emerging and re-emerging arboviral diseases in Southeast Asia. *Journal of Vector Borne Diseases*, ***50***(2), 77–84.

Endoh, D., Mizutani, T., Kirisawa, R., Maki, Y., Saito, H., Kon, Y., … Hayashi, M. (2005). Species-independent detection of RNA virus by representational difference analysis using non-ribosomal hexanucleotides for reverse transcription. *Nucleic Acids Research*, ***33***(6)**.**

Eshoo, M. W., Whitehouse, C. A., Zoll, S. T., Massire, C., Pennella, T. D., Blyn, L. B., … Ecker, D. J. (2007). Direct broad-range detection of alphaviruses in mosquito extracts, ***368,*** 286–295.

Giridharan, P., Hemadri, D., Tosh, C., Sanyal, A., & Bandyopadhyay, S. K. (2005). Development and evaluation of a multiplex PCR for differentiation of foot-and-mouth disease virus strains native to India. *Journal of Virological Methods*, ***126***(1-2), 1–11.

Gubler, D. J. (2002). The global emergence/resurgence of arboviral diseases as public health problems. *Archives of Medical Research*, ***33***(4), 330–42.

Gubler, D. J. (2004). The changing epidemiology of yellow fever and dengue, 1900 to 2003: full circle? *Comparative Immunology, Microbiology and Infectious Diseases*, ***27***(5), 319–30.

Hall, R. a, Blitvich, B. J., Johansen, C. a, & Blacksell, S. D. (2012). Advances in arbovirus surveillance, detection and diagnosis. *Journal of Biomedicine & Biotechnology*, **32**(2)-123-127.

Kibbe, W. a. (2007). OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research*, ***35***(Web Server issue), W43–6.

Kuno, G., & Chang, G. J. (2005). Biological Transmission of Arboviruses : Reexamination of and New Insights into Components , Mechanisms , and Unique Traits as Well as Their Evolutionary Trends, ***18***(4), 608–637**.**

LaBeaud, a D., Sutherland, L. J., Muiruri, S., Muchiri, E. M., Gray, L. R., Zimmerman, P. a, … King, C. H. (2011a). Arbovirus prevalence in mosquitoes, Kenya. *Emerging Infectious Diseases*, ***17***(2), 233–241.

LaBeaud, Sutherland, L. J., Muiruri, S., Muchiri, E. M., Gray, L. R., Zimmerman, P. a, … King, C. H. (2011b). Arbovirus prevalence in mosquitoes, Kenya. *Emerging Infectious Diseases*, ***17***(2), 233–241.

Lambert, A. J., & Lanciotti, R. S. (2009). Consensus Amplification and Novel Multiplex Sequencing Method for S Segment Species Identification of 47 Viruses of the Orthobunyavirus, Phlebovirus, and Nairovirus Genera of the Family Bunyaviridae. *Journal of Clinical Microbiology*, ***47***(8), 2398–2404.

McAdam, A. J., & Riley, A. M. (2009). Developments in tissue culture detection of respiratory viruses. *Clinics in Laboratory Medicine*, ***29***(4), 623–34.

Moreli, M. L., & da Costa, V. G. (2013). A systematic review of molecular diagnostic methods for the detection of arboviruses in clinical specimens in Brazil and the importance of a differential diagnosis. *Virology Discovery*, ***1***(1).

Njenga, M. K., Paweska, J., Wanjala, R., Rao, C. Y., Weiner, M., Omballa, V., … Breiman, R. F. (2009). Using a field quantitative real-time PCR test to rapidly identify highly viremic rift valley fever cases. *Journal of Clinical Microbiology*, ***47***(4), 1166–71.

O'Brien, T. F., & Stelling, J. (2011). Integrated Multilevel Surveillance of the World's Infecting Microbes and Their Resistance to Antimicrobial Agents**.** *Clinical Microbiology Reviews*, ***24***(2), 281–95.

Ochieng, C., Lutomiah, J., Makio, A., Koka, H., Chepkorir, E., Yalwala, S., … Sang, R. (2013). Mosquito-borne arbovirus surveillance at selected sites in diverse ecological zones of Kenya; 2007 -- 2012. *Virology Journal*, ***10***(1), 140.

Onyango, C. O., Grobbelaar, A. a, Gibson, G. V. F., Sang, R. C., Sow, A., Swaneopel, R., & Burt, F. J. (2004). Yellow fever outbreak, southern Sudan, 2003. *Emerging Infectious Diseases*, ***10***(9), 1668–70.

Philip Samuel, P., & Tyagi, B. K. (2006). Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes. *The Indian Journal of Medical Research*, ***123***(5), 615–28.

Rianthavorn, P., Prianantathavorn, K., Wuttirattanakowit, N., Theamboonlers, A., & Poovorawan, Y. (2010). An outbreak of chikungunya in southern Thailand from

2008 to 2009 caused by African strains with A226V mutation. *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases*, ***14*** *Suppl 3*, e161–5.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M.-C., … Brisse, S. (2006). Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Medicine*, ***3***(7), e263.

Seipp, M. T., Durtschi, J. D., Voelkerding, K. V, & Wittwer, C. T. (2009). Multiplex amplicon genotyping by high-resolution melting. *Journal of Biomolecular Techniques : JBT*, ***20***(3), 160–4.

Sergon, K., Njuguna, C., Kalani, R., Ofula, V., Onyango, C., Konongoi, L. S., … Breiman, R. F. (2008). Seroprevalence of Chikungunya virus (CHIKV) infection on Lamu Island, Kenya, October 2004. *The American Journal of Tropical Medicine and Hygiene*, ***78***(2), 333–7.

Seth-Smith, H. M. B., Harris, S. R., Persson, K., Marsh, P., Barron, A., Bignell, A., … Clarke, I. N. (2009). Co-evolution of genomes and plasmids within Chlamydia trachomatis and the emergence in Sweden of a new variant strain. *BMC Genomics*, *10*, 239.

Sutherland, L. J., Cash, A. a, Huang, Y.-J. S., Sang, R. C., Malhotra, I., Moormann, A. M., … LaBeaud, a D. (2011). Serologic evidence of arboviral infections among humans in Kenya. *The American Journal of Tropical Medicine and Hygiene*, ***85***(1), 158–161.

Tadeu, L., & Figueiredo, M. (2007). Emergent arboviruses in Brazil Arboviroses emergentes no Brasil, ***40***(2), 224–229.

Towner, J. S., Sealy, T. K., Khristova, M. L., Albariño, C. G., Conlan, S., Reeder, S. a, … Nichol, S. T. (2008). Newly discovered ebola virus associated with hemorrhagic fever outbreak in Uganda. *PLoS Pathogens*, ***4***(11).

Townson, H., & Nathan, M. B. (2008). Resurgence of chikungunya. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, ***102***(4), 308–9.

Vázquez, A., Sánchez-Seco, M.-P., Palacios, G., Molero, F., Reyes, N., Ruiz, S., … Tenorio, A. (2012). Novel flaviviruses detected in different species of mosquitoes in Spain. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)*, ***12***(3), 223–9.

Woods, C. W., Karpati, A. M., Grein, T., McCarthy, N., Gaturuku, P., Muchiri, E., … Peters, C. J. (2002). An outbreak of Rift Valley fever in Northeastern Kenya, 1997-98. *Emerging Infectious Diseases*, ***8***(2), 138–44.

Woolhouse, M. E., Taylor, L. H., & Haydon, D. T. (2001). Population biology of multihost pathogens. *Science (New York, N.Y.)*, ***292***(5519), 1109–12.

Yusof, M. A., Kuen, L. S., Adnan, N., Izmawati, N., Razak, A., Zamri, A., … Saat, Z. (2011). Epidemiology and molecular characterization of chikungunya virus involved in the 2008 to 2009 outbreak in Malaysia, ***3***(April), 35–42.

APPENDICES

APPENDIX 1: Protocol for mosquito pool homogenization

A one, 4.5-mm diameter copper clad steel bead (BB-caliber airgun shot) is placed in the tube containing the pooled mosquitoes and a diluent and shaken vigorously. The diluent contains Eagle's Minimum Essential Media (MEM) (Sigma-Aldrich, St. Louis, MO) with Earle's salts and reduced $NaHCO_3$, supplemented with 10% heat-inactivated fetal bovine serum (FBS), (Sigma-Aldrich), 2% L-Glutamine (Sigma-Aldrich), and 2% antibiotic/ antimycotic solution with 10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B per ml (Sigma-Aldrich). The supernatant is harvested by spinning the homogenate in 1.5ML tubes at 12,000 rpm for 15 min (bench top centrifuge eppendorf 5417R) to remove the suspended solids, without removing the beads. The homogenate is then pippeted out and put in clean 2ml cryovials for PCR or inoculation in cell culture. When the homogenate is not for immediate use, it is stored frozen in a minus 80^0C freezer.

APPENDIX 2: Multiplex primer table with target sequence alignment accession numbers

	Alignments Gene Bank Accessions	**Primer Names**	**Primer Sequence**	**Opti-mal Ta(^{o}C)**	**Reaction Conc (nM)**
Phlebovirus	DQ380143-82, EF201833-35, EU312103-47, EU574070-87, FJ153285-6, NC_014395	Phlebo JV3aF (Lambert & Lanciotti, 2009)*	AGTTTGCTTATCAAGGGTTTGATGC	59.86	500
		Phlebo JV3bF (Lambert & Lanciotti, 2009)*	GAGTTTGCTTATCAAGGGTTTGACC		500
		Phlebo JV3 R (Lambert & Lanciotti, 2009)*	CCGGCAAAGCTGGGGTGCAT		500
Nairovirus	AY389508, AY675240, AY720893, AY947890, AY995166, DQ076419, EU257628 EU697949-51, GQ,337055, GU477492, JF785543, NC_004159	Nairo L 1a F	TCTCAAAGATATCAATCCCCCCITTACCC	56.2	375
		Nairo L 1b F	TCTCAAAGACATCAATCCCCCTTWTCCC		375
		Nairo L 1a R	CTATRCTGTGRTAGAAGCAGTTCCCATC		150
		Nairo L 1b R	GCAATACTATGATAAAAACAATTMCCATC		150
		Nairo L 1c R	CAATGCTGTGRTARAARCAGTTGCCATC		150
		Nairo L 1d R	GCAATGCTATGGTAGAAACAGTTTCCATC		150
		Nairo L 1e R	CRAKGCTGTGGTAAAAGCAGTTRCCATC		150
Bunyamwera Group *Orthobunya virus*	AF325122, AM711130, AM709778-81,AY593729, EU564831,	Bunya group F (Lambert & Lanciotti, 2009)	CTGCTAACACCAGCAGTACTTTTGAC	58.92	167
		Bunya group R (Lambert & Lanciotti, 2009)	TGGAGGGTAAGACCATCGTCAGGAACTG		167

	JX846604, KC608151, M19420, NC_001927				
Alphavirus	AB455493-4, AF339477, AF079456-7, AY112987, DQ189079-86, EU703759-62, FJ000063-9, FJ445426-504, GQ428210-5, GU013528-30, HM045784-823, HM147989, J02363, JF972635, JQ771797-9, EF536323, HM147989, M20303, M69205, NC_001547, NC_004162, X04129, Y14761	Vir 2052 F(Eshoo *et al.*, 2007) Vir 2052 R(Eshoo *et al*., 2007)	TGGCGCTATGATGAAATCTGGAATGTT TACGATGTTGTCGTCGCCGATGAA	58.39	400 400
Thogotovirus	AF527529-30,	Thogoto S6 F	GATGACAGYCCTTCTGCAGTGGTGT	60.28	300

	NC_0065404 GU969311, M17435	Thogoto S6 R Dhori S5 F Dhori S5 R	RACTTTRTTGCTGACGTTCTTGAGGAC CGAGGAAGAGCAAAGGAAAG GTGCGCCCCTCTGGGGTTT	56.51	300 800 800

***primers modified from the cited primers**

APPENDIX 3: Multiplex cycling conditions

Cycle	**Denaturation**	**Annealing**	**Extension**
Initial Denaturation	**95°C (5 min)**		
1	94°C (20s)	63.5° (25s)	72° (5s)
2	94°C (20s)	62.5° (25s)	72° (5s)
3	94°C (20s)	61.5° (25s)	72° (10s)
4	94°C (20s)	60.5° (25s)	72° (11s)
5	94°C (20s)	59.5° (25s)	72° (12s)
6	94°C (20s)	58.5° (40s)	72°(15s)
7	94°C (20s)	57.5° (40s)	72°(15s)
8	94°C (20s)	56.5° (40s)	72°(20s)
9	94°C (20s)	55.5° (40s)	72°(25s)
10	94°C (20s)	54.5° (50s)	72°(30s)
11-15	94°C (20s)	53.5° (50s)	72°(30s)
16-20	94°C (20s)	52.5° (50s)	72°(30s)
21-25	94°C (20s)	51.5° (50s)	72°(30s)
26-30	94°C (20s)	50.5° (50s)	72°(30s)
31-40	94°C (20s)	49.5° (50s)	72°(30s)
41-50	94°C (20s)	47.5° (50s)	72°(30s)
Final Extension			72° (3 min)