# CHIKUNGUNYA VIRUS CHARACTERIZATION AND DEVELOPMENT OF ENZYME LINKED IMMUNOSORBENT ASSAYS AS DETECTION TOOLS FOR HUMAN AND MOSQUITO SAMPLES

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**JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY**

**2016**

# Chikungunya Virus Characterization and Development of Enzyme Linked Immunosorbent Assays as Detection Tools for Human and Mosquito Samples

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**A Thesis submitted in fulfillment for the Degree of Doctor of Philosophy in Medical Virology in the Jomo Kenyatta University of Agriculture and Technology, Kenya.**

**2016**

## DECLARATION

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

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## DEDICATION

I dedicate this thesis to my dear mother, Alice Akinyi, loving husband and life time companion Dr Franklin Opijah, family and friends who touched my life.

# ACKNOWLEDGEMENT

I would like to thank the almighty God for granting me the opportunity and ability to undertake this studentship. I wish to express my appreciation to the Consortium for National Health Research (CNHR) for awarding me a fellowship to pursue my doctoral studies. I am very grateful to my mentors: Dr Lillian Musila, Dr Rosemary Sang and Dr Juliette Ongus for their patience, guidance, encouragement, enthusiasm and mentorship on all aspects of the studentship and ensuring this program is completed, God bless you. I am also grateful to Dr Lunah Kamau for her guidance. I feel indebted to Dr Shingo Inoue of Nagasaki University-Japan, for his immeasurable technical support and mentorship, ensuring that results were achieved and the support through the Science and Technology Research Partnership for Sustenable Development (SATREPs) project. I appreciate George Michuki and Cecilia Rumberia of Biosciences Eastern and Central African-International Livestock Research Iinstitute (BECA-ILRI) for assisting with the sequencing work. I wish to express my gratitude to James Kimotho and all the staff of Production Department, animal house and Viral Hemorrhagic Fever Laboratory at the Kenya Medical Research Institute (KEMRI) for their immeasurable support and friendship during this period; you were like family to me. God bless you. I would like to thank my colleagues in SATREPS project, who I worked with tirelessly, encouraging each other to ensure we finish our research work. I appreciate the University of Nairobi, especially Dr Kinyanjui, the chairman then and the entire Department of Biochemistry for their advice, encouragement and support. I am grateful to Eunice

Were for assisting with administrative issues at Jomo Kenyatta Univerity of Agriculture and Technology (JKUAT). I would like to thank my husband, Dr Franklin Opijah for supporting and encouraging me throughout this period and I appreciate my dad and mum, the late Manoah and Alice Otieno for putting me through school and believing in me and finally I appreciate my siblings and in-laws for supporting me and being interested in my studies.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **BLAST** | Basic Local Alignment Search Tool |
| **BECA** | Biosciences Eastern and Central Africa |
| **CDC ELISA** | CDC IgM Capture Enyzme Linked Immunosorbent Assay |
| **CDC** | Centers for Disease Control and Prevention |
| **CDNA** | Complementary deoxyribonucleic acid |
| **CHIKV** | Chikungunya Virus |
| **$CO_2$** | Carbon dioxide |
| **CPE** | Cytopathic effect |
| **DENV** | Dengue Virus |
| **DALYs** | Disability-adjusted life years |
| **DNA** | Deoxyribonucleic acid |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **FBS** | Fetal Bovine Serum |
| **FFU** | Focus forming units |
| **FRNT** | Focus Reduction Neutralization Assay |
| **g** | Gram |
| **hr** | Hour |
| **HRP** | Horse radish peroxidase |
| **IgG** | Immunoglobulin G |
| **IgM** | Immunoglobulin M |
| **IgM ELISA** | IgM Enzyme Linked immunosorbent Assay |
| **ILRI** | International Livestock Research Institute |
| **JKUAT** | Jomo Kenyatta University of Agriculture and Technology |
| **KEMRI** | Kenya Medical Research Institute |
| **MEM** | Minimum Essential Medium |
| **mg** | Milligram |
| **ml** | Millimetre |
| **NDUV** | Ndumu Virus |
| **ONNV** | O'nyong nyong Virus |

| | |
|---|---|
| ***p*** | Probability |
| **PBS** | Phosphate Buffered Saline |
| **PBS-T** | Phosphate Buffered Saline-Tween |
| **PFU** | Plaque forming units |
| **PRNT** | Plaque reduction neutralization assay |
| **RVFV** | Rift Valley Fever Virus |
| **RT-PCR** | Reverse Transcriptase-Polymerase Chain Reaction |
| **SATREPS** | Science and Technology Research Partnership for Sustenable Development |
| **SFV** | Semliki Forest Virus |
| **SINV** | Sindbis Virus |
| **SPSS** | Statistical Package for Social Services |
| **SSC** | Scientific Steering Committee |
| **SD** | Standard deviation |
| **USAMRIID** | United States Army Medical Research Institute of Infectious Diseases |
| **VHF** | Viral Hemorrhagic Fever |
| **µg** | Microgram |
| **µl** | Microlitre |
| **YFV** | Yellow Fever Virus |

## ABSTRACT

Chikungunya is a re-emerging disease which has become an important public health concern globally. In Kenya, there was a Chikungunya virus (CHIKV) outbreak in Lamu and Mombasa in 2004, which spread to the islands in the Indian Ocean then to South East Asia, Europe and America. Some of the challenges faced in this outbreak were due to lack of adequate surveillance and diagnostic tools to predict and facilitate early detection of the causative agent of the outbreak and the emergence of a more virulent strain of CHIKV mid-outbreak with enhanced transmission in a new mosquito species. To address some of these challenges, this study set out to characterize CHIKV from the 2005 CHIKV outbreak of Comoros Island and develop an Enzyme Linked Immunosorbent Assay (ELISA) as a diagnostic tool to detect CHIKV antigen in mosquito homogenates and anti-CHIKV antibodies in human sera. These tools would be developed in-house to ensure their availability and cost-effectiveness. The CHIKV isolate from Comoros Island, was analyzed by plaque assay to quantify the viral titre. On observing the plaques, it showed plaques of different sizes, a large (L2) and small (S8) plaque. These plaques were purified and individual plaques infected culture fluid obtained and analysed by *in vitro* growth kinetics in different cell lines and their genetic similarity assessed by whole genome sequencing, comparative sequence alignment and phylogenetic analysis. Purified CHIKV antigen was used to immunize rabbits. The rabbit serum containing CHIKV specific polyclonal antibodies were purified and conjugated to horseradish peroxidase. An antigen detection ELISA was developed and evaluated using CHIKV positive and negative mosquito homogenates and the results confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). An in-house immunoglobulin M (IgM)-capture ELISA to detect CHIKV infections serologically was also developed using these reagents and compared with an independent IgM ELISA and a neutralization test using a panel of sera from the Comoros Island 2005 CHIKV outbreak. The in-house IgM-capture ELISA was used to test human sera samples collected during the 2013 Dengue outbreak in Kenya. Phenotypic and genetic characterization of the plaque variants showed higher viral titres of S7 compared to

L2 in C6/36 mosquito cell lines and a nonsense substitution in the nsp3 of S7, which was similar to a mutation in O`nyong nyong virus that had been shown to enhance infectivity and dissemination in *Anopheles* mosquitoes. This indicated the co-circulation of two variants with potentially different characteristics during the outbreak. The developed antigen detection ELISA used to test 48 mosquito pool homogenates showed a sensitivity (100%) and specificity (93.2%) when compared to the RT-PCR, with a kappa statistic of 0.70 indicating good agreement and can therefore be used as a surveillance tool for screening of CHIKV in mosquitoes. The IgM ELISA had a sensitivity (97.6%), specificity (86.9%) and a Cohen Kappa of 77% when compared to the Centers for Disease Control and Prevention IgM ELISA and a sensitivity (91.1%), specificity (96.7%) and Cohen Kappa of 88% when compared to a neutralization test. The assay was able to detect 26 CHIKV IgM positive out of 254 Dengue suspect human samples (10.2 %) indicating the utility of this assay in the field to detect Chikungunya co-circulating with other arboviruses. In conclusion, it was demonstrated that monitoring co-circulating strains of CHIKV by plaque typing is an effective and useful tool for the detection of emergent novel strains with potentially virulent phenotypes in mosquitoes and humans. Further studies are recommended using reverse genetics to confirm the effects of the identified amino acid substitutions on virulence in humans and vector competence in various mosquito species. The successful development of in-house assays for the detection of CHIKV antigen and antibodies has provided the tools for under-resourced countries such as Kenya to conduct more robust diagnosis and surveillance for enhanced outbreak preparedness.

# CHAPTER ONE

## INTRODUCTION

### 1.1 Background

Chikungunya is an arboviral disease that is transmitted mainly by *Aedes (Ae.) aegypti* and *Ae. albopictus* mosquitoes. Chikungunya virus (CHIKV) is a member of the genus *Alphavirus*, in the family *Togaviridae.* The virus is enveloped and has a single-stranded positive-sense RNA molecule of approximately 11.8 kb and it codes for two polyproteins: the non-structural proteins (nsP1-nsP4) which are encoded at the 5'-end and the structural proteins (Capsid-E3-E2-6k-E1) at the 3'-end (Strauss & Strauss, 1994).

Globally, Chikungunya outbreaks have been reported in several countries with the first CHIKV isolated from the serum of a febrile patient reported in Tanzania in 1953 (Lumsden, 1955; Robinson, 1955; Ross, 1956). Between the 1960s and 1990, the virus was isolated repeatedly from many countries in Central and Southern Africa including Uganda, Sudan, Democratic Republic of Congo (DRC), the Central African Republic, Malawi, Zimbabwe and South Africa. CHIKV has also been isolated in Senegal, Benin, the Republic of Guinea, Cote D` Ivore and Nigeria (Powers & Logue, 2007). An outbreak of CHIKV was detected in the Democratic Republic of Congo between 1999 and 2000, (Pastorino *et al.*, 2004) with concurrent outbreaks occurring in Indonesia (Laras *et al.*, 2005).

In Kenya, an outbreak of Chikungunya was reported in May 2004, in Lamu, located in coastal Kenya (Sergon *et al.*, 2008). The outbreak peaked in July with 1,300 patients infected, accounting for ~75% of the population of the Island and no deaths related to the Chikungunya outbreak were reported. In November 2004, Chikungunya outbreak was reported in Mombasa, and genetic evidence implicated the same lineage of virus that was found in Lamu (Kariuki *et al.*, 2008).

After the Chikungunya outbreak in Lamu, Kenya in 2004 (Sergon *et al*., 2008), it spread to the Union of Comoros in 2005 (Sergon *et al*., 2007). In 2005–2006, the outbreak spread to other islands in the Indian Ocean such as Madagascar, Seychelles, Reunion Island, Mauritius, Maldives, Sri Lanka and India (Powers, 2011). The epidemic also spread to India, where it is estimated that more than 1.5 million people were infected and it was subsequently identified in South Asia, Southeast Asian countries, Europe and the United States, where it was imported by infected travelers from India and affected islands of the Indian Ocean (Warner *et al*., 2006). In New Caledonia and the Caribbean Islands, CHIKV activity has been reported from 2013 (Dupont-Rouzeyrol *et al.,* 2012).

Chikungunya outbreaks come with socio-economic and public health implications, due to prolonged chronic arthritis often suffered by patients after a chikungunya outbreak. Although chikungunya outbreak have been reported in many countries in Africa, Asia and the Americas, the disability-adjusted life years (DALYs), has only been calculated in India, Reunion Island and the Americas. Overall the estimated cost of the Reunion Island outbreak in 2005 was more than USD 80 million per case, with a direct medical cost of USD 80 to 160 per case (Soumahoro *et al.,* 2011). In addition, in the India outbreak of 2006, the foregone income for those affected was estimated to be USD 5.5 million for the year due to chronic arthritis (Krishnamoorthy, Harichandrakumar, Kumari & Das, 2009) and in Latin America, the estimated minimum burden was 151 031 DALYs in 2014, which is higher than the DALYs reported in India (25 888 DALYs). With such protracted and severe post-infection course, the economic, health and social burden on a community can be extreme and this really affects the lower socio-economic and less educated populations.

In another outbreak in the Reunion Island, the strain of CHIKV which had an amino acid substitution (A226V), in the envelope glycoprotein E1 of CHIKV, enhanced transmission of this CHIKV strain in *Ae. Albopictus (*Tsetsarkin, Vanlandingham, McGee & Higgs, 2007; Vazeille *et al*., 2007). The introduction of this mutation mid-

outbreak suggests a transition where at least two variants (226A and 226V) circulated at the same time. Other than an A226V amino acid substitution, several other amino acid substitutions have been observed, when whole genome sequences of CHIKV reference strain (S27) and CHIKV isolate from recent outbreaks were compared. However, correlation of amino acid substitutions to disease severity, increased fatality and enhanced transmissibility in a new mosquito species (*Ae. albopictus*), is still under investigation. These emerging virus strains that could have important implications for disease severity or increased transmission can be identified by screening for plaque variants in isolates from human and mosquito hosts. From as early as 1975, small- and large-plaque variants of CHIKV have been observed in different vertebrate and invertebrate cell lines (Buckley, Singh & Bhat, 1975) and plaque variants have not only been observed in CHIKV (Lim *et al*., 2009) but also in other arboviruses such as Sindbis virus (Nagata *et al*., 1967), Guaroa virus (Tauraso, 1969), Bunyamwera virus and Ngari virus (Odhiambo, Venter, Limbaso, Swanepoel & Sang, 2014). These variants can be useful in identifying genetic changes that could have phenotypic implications.Therefore, it is important to identify and analyze the virus variants using tests like cell culture, *in vitro* viral kinetics, RT-PCR and sequencing among other tests.

Chikungunya diagnosis is typically performed by testing for CHIKV specific antibodies because the viremic phase where antigen can be detected is limited and peaks around disease onset while Immunoglobulin M (IgM) and Immunoglobulin G (IgG) are usually produced during the late-acute and early-convalescent phases respectively. Detection of specific IgM using IgM-capture Enzyme–Linked Immunosorbent Assay ( IgM ELISA) in late-acute phase is commonly used for diagnosis. ELISA kits for detecting IgM are commercially available, however, these kits are expensive and therefore are not sustainable for large scale applications like routine surveillance and diagnosis in resource-limited countries.

The inaccesibility of these diagnostics means that the true incidence of Chikungunya is not known in most countries outside of major outbreaks where international resources

are often engaged for diagnostic support. Seroprevalence data from a few studies have confirmed that Chikungunya occurs in different parts of Kenya and exposure to Chikungunya by testing for IgG can be detected during the interepidemic periods. In Busia county located in Western Kenya, a prevalence of 59.9% in 2004 (Sutherland *et al*., 2011) and 11.5% in 2010 was reported (Mwongula, Mwamburi, Matilu, Siamba & Wanyama, 2013). A prevalence of 37% was observed in Coastal Kenya, 24.77% of adults in Malindi district in 2004 were affected (Mease *et al*., 2011) and in Kisumu county lowlands, 42% of the children had anti-CHIKV antibodies (Sutherland *et al*., 2011). This demonstrates that CHIKV is circulating in the population without being detected or reaching epidemic levels. Therefore it's important to have accessible and affordable CHIKV diagnostics that allow for timely diagnosis of Chikungunya during both inter epidemic periods and outbreaks, to put in place vector control measures, patient management and public health and economic concerns that come with arboviral disease outbreaks.

## 1.2 Statement of the problem

The burden of Chikungunya virus cannot be underestimated especially during an outbreak, a lot of resources from the patients own pocket and national resources are spent on hospitalization of the affected and the loss of productivity due to foregone income. In the Reunion Island outbreak in 2005, approximately USD 80 million per case was the cost of the outbreak, in the India outbreak of 2006, the foregone income for those affected was estimated to be USD 5.5 million for the year due to chronic arthritis and a disability-adjusted Life years (DALYs) of 151 031 was reported in Latin America, demonstrating the socio-economic impact of Chikungunya outbreaks.

Emerging virus strains with different growth phenotypes can be identified by the appearance of plaque variants from mosquito or human virus isolates.Therefore, its important to monitor virus evolution by screening for plaque variants from isolates from human and mosquito hosts during routine culture and isolation of viruses. These virus strains could have important implications for disease severity, increased

transmission and lead to devastating outbreaks, which may have a strain in the healthcare system and a serious negative impact on the economy of the country. Other than testing for the CHIKV, CHIKV specific antibodies can also be tested to assess circulation of this virus after the vireamic phase which is shortlived. Diagnosis of CHIKV specific antibodies is mainly done by serology. Diagnosis of Chikungunya based on the clinical presentation is challenging because the symptoms and signs are similar to other febrile diseases such as Dengue (DEN), malaria, dysentery or bacterial meningitis, therefore a diagnostic test is required to differentiate these infections. Serological cross-reactivity with other alphaviruses such as O'nyong nyong virus (ONNV) is a challenge given the close antigenic relationship in this family (Dash, Mohanty & Padhi, 2011). Neutralization tests like focus reduction neutralization test (FRNT) targetting specific neutralizing antibody produced during the infection is commonly used as a confirmatory test of virus infection, however, FRNT is tedious to perform and requires highly skilled and trained personnel.

In order to provide efficient diagnosis for Chikungunya infection there is need for diagnostic procedures that are sensitive, specific, affordable and readily available in the health facilities. Currently, the diagnosis of Chikungunya is done in central research institutions like Kenya Medical Research Institute (KEMRI) and the Centers for Disesese Control and Prevention (CDC) laboratories. The reagents used for diagnosis at KEMRI are donated by instituions such as the Diagnostic Systems Division of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and the Institute of Tropical Medicine, Nagasaki University, Japan or the CDC who produce them for their own in-house use. Therefore, these reagents are insufficient to support public health activities and routine diagnosis in Kenya. ELISA based diagnostic kits have been recently developed (in Asia, Europe and Americas) but they are not only expensive, but not locally distributed in Africa. These diagnostics are therefore not easily accessible for sustainable use in diagnosis and routine sero-surveillance, resulting in diagnostic delays that can prevent the recognition and implementation of rapid response which are critical at the onset of outbreaks. Delays in diagnosis could further be prevented by having accessible

reagents which can be stored in the regional health facilities to eliminate transportation of samples to central facilities. This study was able to characterize CHIKV and develop ELISA as diagnostic tools for the detection of CHIKV infection, which are sensitive, specific, affordable and readily available for routine surveillance in central regional centres like KEMRI and peripheral health centers.

### 1.3 Justification

Chikungunya is not typically a life threatening disease as most patients recover from it fully. Widespread Chikungunya outbreaks can have serious consequences such as reduced productivity at the individual and community level, health care disruption, socio-economic instability and exacerbation of poverty particularly in individuals from low economic backgrounds. A severe outbreak occurred in Reunion Island in 2005 as a result of an emergent strains of CHIKV which facilitated the transmission of the CHIKV in a new vector. This indicated the need to monitor virus evolution and adaptation to understand how changes in the virus could alter human disease and transmission patterns. This is particularly important in the era of global climatic changes which are expanding and altering the habitats of mosquito vectors. Identification of key genetic regions important for replication, transmission and virulence, should be monitored to facilitate the screening of isolates from different parts of the world to detect emergent strains of concern.

Arboviruses are diagnosed by virus isolation, reverse transcription-Polymerase chain reaction or by serological assays. Diagnosis of Chikungunya based on clinical presentation and the history of a patient, is difficult to monitor because, the clinical symptoms and signs resemble those of many other febrile illnesses hence easily confused with dengue, malaria, dysentery or bacterial meningitis. Where malaria is endemic and the majority of febrile illnesses are diagnosed using clinical presentation without laboratory confirmation, diseases such as CHIKV and Dengue virus (DENV) infections often are misdiagnosed as malaria. Diagnosis of Chikungunya is mainly by serology. In-house IgM-capture ELISA (IgM ELISA) is

the most widely used tool for surveillance and diagnosis because they are relatively simple to perform, do not require highly specialized equipment like RT-PCR or neutralization test, and can detect IgM antibodies which are present from a few days after infection. However, the commercial CHIKV IgM diagnostics available are very expensive, hence not sustainable for diagnosis and routine sero-surveillance. This lack of diagnostics for febrile illness of arboviral etiology has public health consequences. For example, in the 2004 Chikungunya outbreak in Kenya, diagnosis was delayed by the lack of accurate diagnostics.

Chikungunya continues to persist in Kenya during the inter-epidemic periods, with CHIKV specific antibodies being detected in the Rift Valley region, Coastal Kenya and Western Kenya among adults and children with a prevalence of between 1 – 60% (Mease *et al*., 2011, ; Morill *et al*., 1991; Mwongela *et al*., 2013 & Sutherland *et al.,* 2011 ). These data indicate that Chikungunya continues to infect individuals across the country and these people are not identified or treated adequately. The need for affordable diagnostics for this disease is apparent in order to recognize outbreaks, implement correct interventions and offer appropriate care for the infected. This study sought to characterize CHIKV antigen from the Comoros Island outbreak of 2005 and develop ELISA as diagnostic tools for Chikungunya. Once validated, these assays will be useful for routine surveillance and diagnosis of CHIKV infections in humans and mosquito vectors.

## 1.4 Research questions

1) Does Chikungunya virus isolated from Comoros Island in 2005 have phenotypic and genotypic variation?
2) How sensitive and specific would the ELISA tests be in the detection of CHIKV infections?
3) What proportion of seropositive patients would be detected using the in-house IgM ELISA?

## 1.5 Hypothesis

### 1.5.1 Null Hypothesis

1) CHIKV variants isolated from a Comoros Island outbreak of 2005 are similar phenotypically and genotypically
2) CHIKV infection is not detectable in sera of febrile patients collected during the 2013 Dengue outbreak in Kenya.

## 1.6 Objectives

### 1.6.1 General objective

To characterize CHIKV variants isolated from the Comoros Island outbreak of 2005 and develop an ELISA diagnostic assay to detect CHIKV in mosquito homogenates and CHIKV specific IgM in human serum samples

### 1.6.2 Specific objectives

1) To characterize CHIKV variants isolated from a Comoros Island outbreak of 2005 phenotypically and genotypically.
2) To develop and evaluate an antigen detection ELISA from CHIKV specific antibodies for the diagnosis of CHIKV infections in mosquitoes homogenates
3) To develop and evaluate an in-house IgM ELISA from cell culture-derived antigens and antibodies for the diagnosis of CHIKV infections
4) To validate the developed in-house IgM ELISA on febrile patient samples collected during a 2013 Dengue outbreak

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Chikungunya the disease

The name Chikungunya is derived from the Makonde word which means "that which bends up" in reference to the stooped posture developed as a result of the arthritic symptom of the disease. The joint pain which is characteristic of CHIKV infection occurs in the joints in the hands, fingers, toes, wrists, waist, elbows, knees and hips leading to difficulty in standing straight or walking and general malaise which incapacitates a patient and keeps them from their daily household and economic activities.

### 2.1.1 Pathogenesis of CHIKV

Some alphaviruses are not pathogenic to humans, while others cause clinical diseases ranging from mild to severe. Alphaviruses are subdivided into two groups; those associated with encephalitis and the other group is associated with polyarthritis and rash (Powers *et al*., 2001; Weaver and Reisen, 2010). Although CHIKV is associated with arthritis, during the Chikungunya outbreak in Reunion Island in 2006 there were documented cases of meningoencephalitis (primarily in neonates) and hemorrhagic disease (Paquet *et al*., 2006; Tandale *et al*., 2009 & Weaver & Reisen, 2009). Chikungunya virus infects infects the stromal cells of the central nervous system and, in particular, the lining of the choroid. Following transmission, CHIKV replicates in the skin and then disseminates to the liver and joints, presumably through the blood stream (Couderc *et al*., 2008 & Talarmin *et al*., 2007). In the Reunion Island CHIKV outbreak of 2005, some cases were demonstrated to be a result of maternal–fetal transmission (Gerardin *et al*., 2008). Recurrent joint pain (wrists, ankles, knees), can be experienced by 30–40% of those infected, although it is not related to chronic infection because infectious virus has not been isolated from those that are infected. Radiographic studies are typically normal or show mild swelling, as observed in joint

pain. The joint pain has been hypothesized to be due to immune-mediated antibodies ( Morrison *et al*., 2006) and auto-antibodies which have been reported in one case of CHIKV infection with severe musculoskeletal complications (Maek and Silachamroon, 2009).

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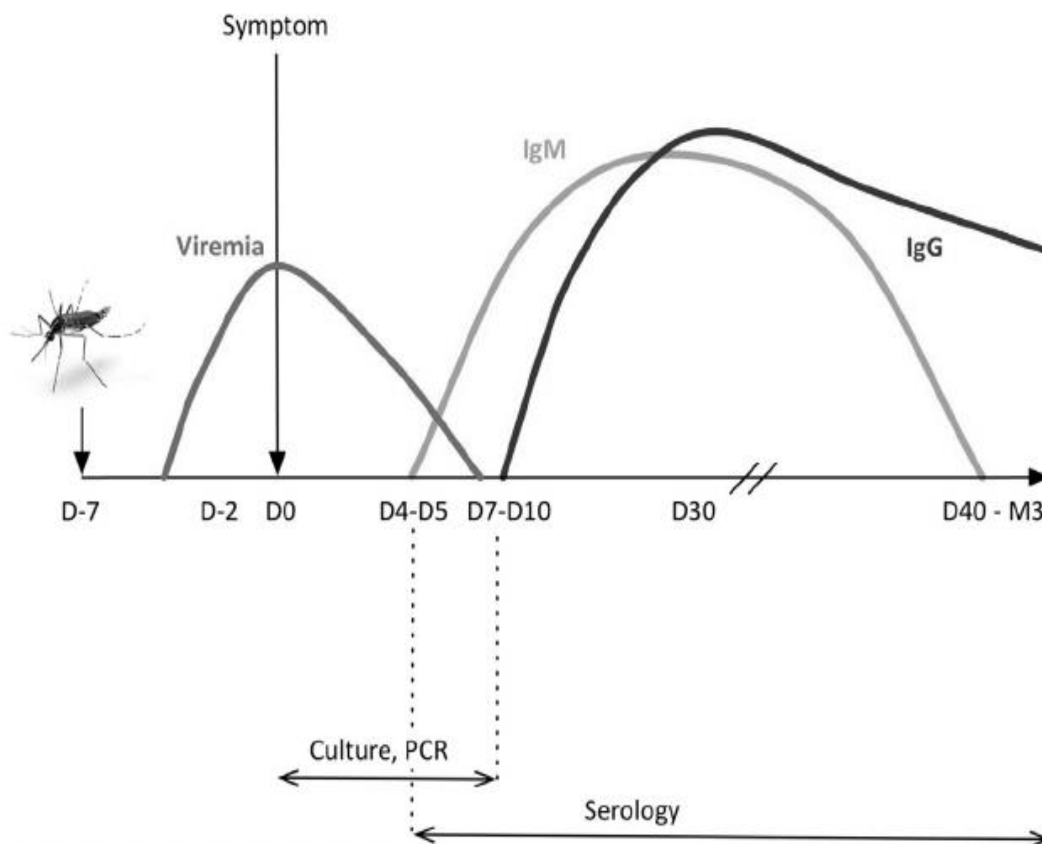


**Figure 2.1: Pathogenesis in human following Chikungunya virus infection, indicating vireamia and antibodies progression in days (D) and months (M) (Simon et al., 2015)**

Following transmission by mosquito bites, infected individuals experience an acute onset of disease 2–4 days after infection. Disease onset coincides with rising viral titre, which triggers the activation of an innate immune response, with production of type I interferons (IFNs) as shown on **Figure 2.1**. Patients successfully clear the

virus approximately 1 week after infection, and only at this time is there evidence of CHIKV-specific adaptive immunity (that is, T cell and antibody-mediated responses) (Schwartz and Albert, 2010).

## 2.2 Classification and genome organization of CHIKV

### 2.2.1 Classification of CHIKV

Chikungunya virus is a single stranded RNA, which belongs to the family *Togaviridae*, and genus *Alphavirus*. The family *Togaviridae* is comprised of two genera, *Alphavirus* and *Rubivirus*. The genus *Alphavirus* contains approximately 25 species (Weaver *et al.*, 2000) that can be classified antigenically into seven sero-complexes as shown on **Table 2.1.**

**Table 2.1: Classification of Alphaviruses into seven sero-complexes based on antigenic properties** (Weaver *et al.*, 2000).

| Complex | Virus complex | Virus |
|---|---|---|
| 1 | Semliki Forest virus complex | Chikungunya virus, Bebaru virus, virus, O'Nyong nyong virus, Ross River Mayaro virus, Semliki Forest |
| 2 | Barmah Forest complex | Barmah forest virus |
| 3 | Eastern equine encephalitis complex | Eastern equine encephalitis virus (seven antigenic types) |
| 4 | Middelburg virus complex | Middelburg virus |
| 5 | Ndumu virus complex | Ndumu virus |
| 6 | Venezuelan equine encephalitis complex | Cabassou virus, Everglades virus, Mosso das Pedras virus, Mucambo virus, Paramana virus, Pixuna virus, Rio Negro virus, Trocara virus , Venezuelan equine encephalitis virus |
| 7 | Western equine encephalitis complex | Aura virus, Babanki virus, Kyzylagach virus, Sindbis virus, Ockelbo virus and Whataroa virus |

### 2.2.2 Serological cross-reactivity of alphaviruses

Alphaviruses are classified on the basis of antigenic properties because they share antigenic sites on the capsid and at least one envelope glycoprotein. Serological cross-reactivity defines the alphaviruses and groups CHIKV within the Semliki forest virus antigenic complex consisting of; Mayaro virus, O`Nyong nyong Virus , Ross River Virus, Semliki Forest Virus, and Bebaru Virus (Weaver *et al*., 2005). Some of these viruses can be challenging to distinguish serologically, but they can be differentiated by degrees of neutralization in neutralization tests.

### 2.2.3 Genome organization of CHIKV

Like all alphaviruses, CHIKV contains a linear, positive-sense, single stranded RNA genome that is approximately 11.8 kilobases (Griffin, 2001). As such, the virus functions as a messenger RNA (mRNA) and begins replication and translation upon entry into a cell. The non-structural proteins (nsP1–nsP4) required for replication are encoded at the 5` end of the genome, and the structural proteins are encoded at the 3' end, as shown on **Figure 2.2**.

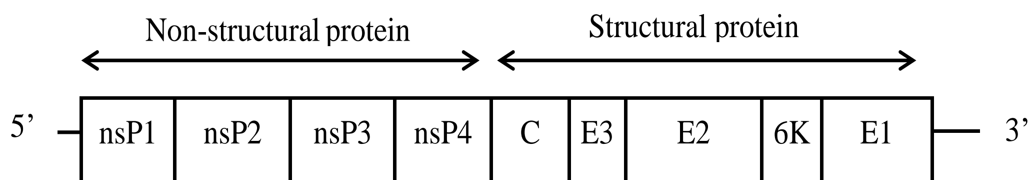


**Figure 2.2: CHIKV genome organized into non-structural proteins (nsPs) and structural proteins (Es).**

CHIKV coding sequences consist of two large open reading frames of 7422 nucleotide and 3744 nucleotide, encoding the non-structural polyprotein (2474 amino acids) and the structural polyprotein (1248 amino acids) respectively.

### 2.2.3.1 Non-structural proteins of CHIKV

Upon cell entry, genomic RNA of alphaviruses serves as mRNA for translation of the viral non-structural proteins of alphaviruses and synthesis of the complementary negative RNA strand. Non-structural proteins are synthesized and generate complexes involved in both genome replication and translation of proteins. Depending on the presence or absence of an opal stop codon between the nsp3 and nsp4, the genomic RNA is translated into one (nsP1234) or two (nsP123 and nsP1234) polyproteins (Strauss & Strauss, 1994).

The nsP123 is produced when translation is terminated at an opal stop-codon at position 524, which happens for many strains of CHIKV at the C-terminus of the nsP3 protein. The other version nsP1234, which contains the nsP4, is produced upon read-through of this opal stop codon. Not all alphaviruses have the opal stop-codon (Strauss, Levinson, Rice, Dalrymple & Strauss, 1988). However, the presence of an opal codon verses an Arginine between the nsp3 and nsp4 in the O`nyong-nyong virus (ONNV) genome has been shown to cause more efficient viral infection and facilitated earlier dissemination of the virus in its vector *Anopheles (A.) gambiae* mosquitoes (Myles, Kelly, Ledermann & Powers, 2006) indicating that amino acid change at this position may play an important role in adaptation of alphaviruses to its mosquitoes ( Ou, Strauss  & Strauss, 1983).

Non-structural protein 1(nsP1) is required for synthesis of the negative RNA strand (Hahn, Grakoui, Rice, Strauss & Strauss, 1989a) and it acts as a methytransferase and guanyltransferase in the process of 5` methy-guanosine cap formation (Durbin and Stollar, 1985). The non-structural polyprotein 1 (535 amino acids) among alphaviruses have some conserved regions, for example, seventeen amino acids (QVTPNDHANARAFSHLA), near the N terminus of nsP1 at position 31-47 (Ou *et al.*, 1983).

Non-structural protein 2 is the largest among the nsPs and possesses a number of enzymatic activities including ATPase, GTPase (Russo, White & Watowich, 2006),

helicase and RNA triphosphatase activity (Vasiljeva, Merits, Auvinen & Kaariainen, 2000; Gomez de Cedron, Ehsani, Mikkola, Garcia & Kaariainen, 1999). The C-terminal part of nsp2 has been associated with proteolytic processing of nsP and it regulates the 26S subgenomic RNA synthesis (Suopanki, Sawicki, Sawicki & Kaariainen, 1998) which down-regulates minus-strand RNA synthesis late in infection (Sawicki, Perri, Polo & Sawicki, 2006), targets nsP2 for nuclear transport and RNA triphosphatase activity (Vasiljeva *et al.,*2000).

Non-structural protein 3 (530 amino acids), plays a role in sub-genomic 26S RNA and negative strand synthesis (Hahn, Strauss & Strauss,1989b). Non-structural protein 3 acts together with nsP1 to mediate association of the replication complex with cytoplasmic membrane structures (Peranen & Kaarianinen., 1991; Peranen, Takkinen, Kalkkinen & Kaariainen, 1988) and it affects cleavage specificity of nsP2 proteinase (De Groot, Hardy, Shirako & Strauss, 1990; Strauss & Strauss 1994). Other studies have showed that nsP3 can modulate replication of sindbis virus (SINDV) *in vitro* (Lastarza, Grakoui & Rice, 1994a) and the virulence and pathogenicity of Semliki forest virus in a mouse model (Vihinen, Ahola, Tuittila, Merits & Kaariainen, 2001).

The non-structural protein 4 (611 amino acids) is the the most highly conserved protein among the alphaviruses with an amino acid identity between CHIKV and other alphaviruses ranging from 71% (Barma Forest Virus) to 91% (ONNV). The motif Gly-Asp-Asp (GDD) is a conserved active site located at position 465-467, near the C terminus of the nsP4 sequence of CHIKV isolate. GDD is involved in catalytic activity and metal ion co- ordination of the RNA polymerase of alphaviruses thus its responsible for RNA synthesis (Kamer & Argos, 1984). Non-structural protein 4 concentration is tightly regulated in most alphaviruses by a read-through opal codon at the end of nsP3 and efficient degradation by the N-end rule pathway (Strauss & Strauss, 1994). A small proportion of nsP4 remains stable in infected cells, and it has been suggested that nsP4 associated with the replicative complex is protected from rapid degradation.

### 2.2.3.2 Structural proteins of CHIKV

The structural proteins are generated by translation of an mRNA that is generated from an internal, sub-genomic promoter immediately downstream of the non-structural open reading frame. The 5` end of the genome has a 7-methylguanosine cap, while the 3` end is polyadenylated. The structural gene products are generated by translation of a sub-genomic mRNA to produce a polyprotein that is processed to produce a capsid protein, two major envelope surface glycoproteins (E2 and E1) and 2 minor glycoproteins E3 and 6K (Simizu, Yamamot, Hashimoto & Ogata, 1984; Weaver *et al*., 2005). The capsid protein interacts with genomic RNA to form a nucleocapsid core structure beneath the viral membrane. It acts as an autoprotease, to recognize the genomic RNA and assemble it into an ordered protein shell (Warrier, Linger, Golden & Kuhn, 2008). This protein shell called 6K (58-61 amino acids), is a small hydrophobic protein associated with membranes and is incorporated in small amounts into the virion. The function of the 6K protein in virus life is not well understood. It has been shown that mutations in 6K affect virus trafficking, virus assembly, and budding (Liljestrom, Lusa, Huylebroeck & Garoff 1991; Loewy *et al.,*1995). CHIKV invasion of susceptible cells is mediated by two viral glycoproteins, E1 and E2, which carry the main antigenic determinants and form an icosahedral shell at the virion surface. Glycoprotein E2, derived from furin cleavage of the p62 precursor into E3 and E2, is responsible for receptor binding and E1 for membrane fusion (Voss *et al*., 2010).

The structural polyprotein is the precursor of protein C (261 amino acids), E3 (64 amino acids), E2 (423 amino acids), 6K (61 amino acids) and E1 (435 amino acids). CHIKV and other alphaviruses possess common features in the structural proteins, except in the E2 proteins which has two possible glycosylation sites at positions 263 and 345 assigned by the sequence Asn-X-/Thr (where X is any amino acid except proline) and contains 423 amino acids. CHIKV and ONNV E2 protein have an 82% amino acid sequence identity. The E1 protein contains 435 amino acids, and a possible glycosylation site at position 141. In the E1 protein the amino acid sequence

identity between CHIKV and ONNV is 88%. The E1 protein of CHIKV contains an uncharged tract which consist of 80-96 residues (Khan *et al*., 2002). CHIKV sequences are grouped by phylogeny into 3 distinct clades separated by geographical areas (Powers, Brault, Tesh & Weaver, 2000) designated as: the West African, East Central South Africa (ECSA) and Asian genotypes.

## 2.3 Transmission of CHIKV

Chikungunya is a viral disease transmitted to humans by the bite of infected *Aedes* species of mosquitoes. CHIKV transmission occurs in two cycles: sylvatic and urban cycle. In Africa, the virus is maintained in a sylvatic cycle comprising non-human primates (chimpanzees, monkeys and baboons) and different species of forest-dwelling mosquitoes including *Aedine* mosquitoes (*Ae. Africanus, Ae. furcifer-taylori, Ae. dalzieli, Ae. luteocephalus*) and non-*Aedine* mosquitoes (*Mansonia, Culex*, etc.) (Thiboutot *et al*., 2010) as shown on **Figure 2.3**. While CHIKV most likely evolved from this forest cycle in Central Africa, the virus developed a new urban cycle as it spread through Africa and Asia. Mosquitoes such as *Ae.aegypti* have been implicated as vectors in Africa in urban settings (Diallo, Thonnon, Traore-Lamizana & Fontenille, 1999). In Asia, transmission of CHIKV has been shown to be almost exclusively between *Ae. aegypti* mosquitoes and humans (Myers *et al*., 1965; Reuben, 1967), and in the Reunion Island, CHIKV was mainly transmitted via *Ae. albopictus,* after a mutation (E1: A226V) occurred in this CHIKV strain (Tsetsarkin *et al.,* 2007)**.** The mosquito vector implicated in the 2004 epidemics in Kenya and in Comoros in 2005 was *Ae aegypti*. In Comoros, larval surveys indicated that at least 11 different species were present on the island (Sang *et al*., 2008), three of which (*Ae. aegypti, Ae. vittatus* and *Eretmapodites chrysogaster*) had previously been shown to be competent CHIKV vectors (Gilotra and Shah, 1967; Mangiafico, 1971; Mourya and Banerjee, 1987). Outside epidemics, CHIKV is maintained in a sylvatic cycle involving monkeys, rodents, birds, and other unidentified vertebrates, and forest-dwelling *Aedes* mosquitoes, principally *Ae. furcifer* and *Ae. Africanus* (Powers *et al*., 2000).

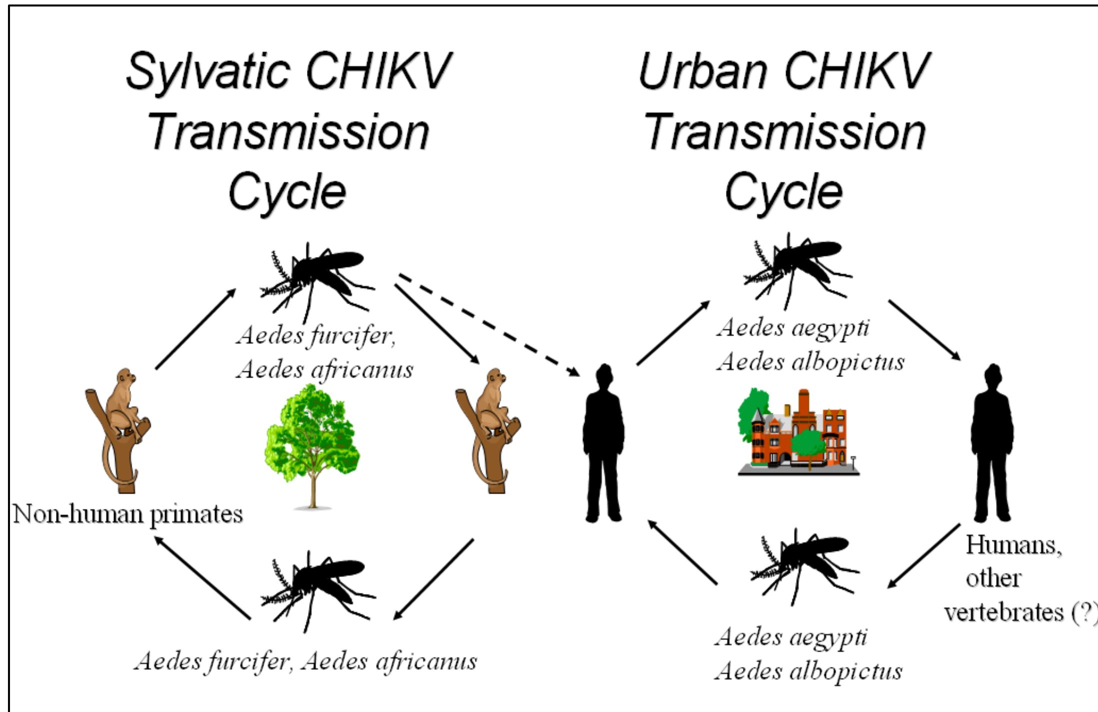


**Figure 2.3: Life cycle of CHIKV in Africa showing the interconnection between the sylvatic cycle on the left and the urban cycle on the right (Powers *et al.*, 2010)**

## 2.4 Geographical distribution of Chikungunya virus

Chikungunya has re-emerged in the 21st century in a succession of massive outbreaks, ranging from East Africa, Islands of the Indian Ocean, India, Europe, North Americas and the Caribbean Islands. Outside epidemics, circulation of Chikungunya specific antibodies have been reported in Coastal and Western regions of the country as evidenced by seroprevalence studies that have been done.

### 2.4.1 Chikungunya infection in Makonde plateau, Tanzania in 1950's

Chikungunya virus was first isolated by Ross in 1953 during an epidemic in Newala district of Tanzania (Ross, 1956), following an outbreak on the Makonde Plateau, along the border between Tanganyika and Mozambique in 1952.

### 2.4.2 Chikungunya outbreaks between 1960 and 1990

Between the 1960s and 1990, the virus was isolated repeatedly from numerous countries in Central and Southern Africa including Uganda, Sudan, Democratic Republic of Congo (DRC), The Central African Republic, Malawi, Zimbabwe, Kenya and South Africa. In western Africa, CHIKV has been isolated in Senegal, Benin, the Republic of Guinea, Cote D` Ivore and Nigeria (Powers and Logue, 2007).

### 2.4.3 Chikungunya outbreaks in Kenya in 2004

An outbreak of Chikungunya in Lamu Island, located in Coastal Kenya, occurred in May 2004 and peaked in July, where 1,300 patients were reported (~ 75% of the total population on the island). However, no deaths related to the outbreak were reported. Among the symptomatic patients, 84% were absent from work or school due to prolonged illness. In November of the same year, a Chikungunya outbreak was reported in Mombasa, and genetic evidence implicated the same lineage of virus that was found in Lamu (Kariuki *et al*., 2008).

### 2.4.4 Chikungunya outbreak in Indian Ocean Islands between 2005 to 2007

The presence of Chikungunya on the Grande Comore Island was reported in February 2005, peaking in March 2005: 5,202 cases were laboratory confirmed, representing 63% of the population with no deaths associated with the outbreak (Sergon *et al*., 2008). Dengue fever was thought to be the cause of the outbreak in Comoros Island because previous outbreaks of dengue fever had occurred in 1948,

1984 and 1993. However serological and genetic evidence from affected patients sera, confirmed CHIKV as the causative agent (Sergon *et al*., 2008).

In Reunion Island, the first documented cases were patients coming back from Comoros in March 2005 (Schuffenecker *et al*., 2006). Then, in March and July 2005 and January 2006, CHIKV caused a massive outbreak in Reunion Island , where 244,000 cases were reported representing 40% of the population. At least 213 deaths in the elderly population were associated with the Chikungunya outbreak (Renault *et al*., 2007; Schuffenecker *et al*., 2006). From Reunion Island, genetic evidence showed that the CHIKV spread to Seychelles, Mauritius, Madagascar, Mayotte, and the Maldives (Powers, 2011).

### 2.4.5 Chikungunya outbreak in South East Asia, Europe and the Americas

India had its first Chikungunya outbreak in 1963 in Kolkata (Arankalle *et al*., 2007; Pialoux, Gauzere, Jaureguiberry & Strobel, 2007). It was followed by epidemics in other parts of the country like Chennai, Pondicherry and Vellore in 1964, Visakhapatnam, Rajahmundry, Kakinada and Nagpur in 1965 and at Barsai in 1973 after 32 years (Arankalle *et al*., 2007; Pialoux *et al*., 2007). CHIKV infection emerged in outbreaks in India during 2005-2008 affecting 1.3 million people in 13 different states (Ravi, 2006). In Sri-Lanka, Chikungunya re-emerged in October 2006 and June 2008 with >40,000 suspected cases reported after a period of 40 years (Hapuarachchi, Bandara, Hapugoda, Williams & Abeyewickreme, 2008; Munasinghe, Amarasekera & Fernando, 1966). In Malaysia in 2006 (Sam, 2007), 7,000 cases were reported (Sam *et al*., 2009) and also in Indonesia in 2003 CHIKV cases were reported (Laras *et al*., 2005). In Singapore a Chikungunya outbreak occurred in January 2008( Leo *et al*., 2009 ; Ng *et al*., 2009), in the Maldives, around 12,000 suspected Chikungunya cases were reported between 2006-2007 (Yoosuf *et al*., 2009) and in Thailand between 2008 and 2009 (Theamboonlers, Rianthavorn, Praianantathavom, Wuttirattanakowit & Poovorawan, 2009).

In 2006-2007, CHIKV infections were confirmed in travelers returning to Europe, Australia, Hong Kong, Canada, Taiwan, Sri-Lanka, Corsica, Singapore, Japan, Gabon and the USA; these were directly associated with return of viremic travellers from endemic regions in the Indian Ocean Islands and Asia (Lanciotti *et al*., 2007; Warner *et al*., 2006). Of particular concern was the local transmission of CHIKV in 2007 in northern Italy, which resulted in an estimated 254 locally acquired infections (Rezza *et al*., 2007), with a report of autochthonous transmission (Bonilauri *et al*., 2008). Autochthonous transmission is where the infection is transmitted locally or in the same place between native individuals and not from migrants.

Since CHIKV is transmitted by a mosquito, local transmission can occur when the vector is introduced to a new area where it can survive or the vector is introduced into an area where it had been previously eliminated. Transmision also occurs when the environmental factors like precipitation, temperature and humidity favor vector survival in an area where the vector could not thrive previously. The re-emergence of these vectors can be pointers to an impending outbreak of CHIKV in an area that has been free from the disease. A Chikungunya outbreak was also reported in New Guinea in 2012 (Horwood *et al*., 2013), China in 2010 (Wu *et al*., 2013) and in New Caledonia in 2011 (Dupont-Rouzeyrol *et al*., 2012).These numerous outbreaks have raised a major public health concern in many countries of the world due to increasing number of Chikungunya infections globally as shown in **Figure 2.4.**

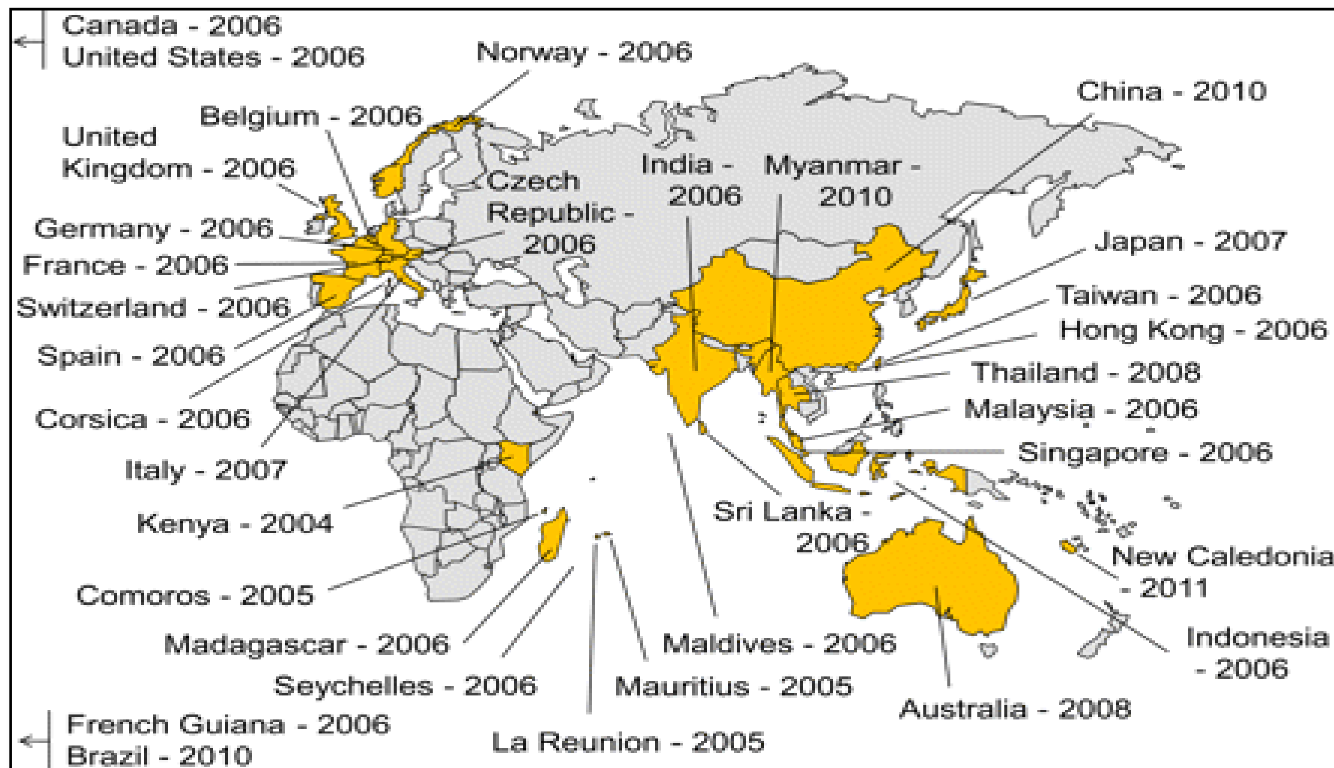


**Figure 2.4 Countries with documented CHIKV activity during the 2004 -2011 outbreak. Year listed is the year activity was first confirmed, (source : Powers , 2011) Counties reporting local transmission are shaded in grey and imported cases are shaded in yellow**

### 2.4.6 Chikungunya virus spread in the Americas

Prior to the CHIKV outbreak in the Carribean island in 2013, only cases of imported Chikungunya had been reported in the Dengue endemic areas which had *Ae. aegpyti* and *Ae. albopictus* (Lanciotti *et al.,* 2007), with no local transmission detected. The Indian Ocean lineage of CHIKV adapted to being transmitted by *Ae. albopictus* has been detected in both temperate regions like in Italy (Rezza *et al.,* 2007) and tropical regions in India (Kumar, Joseph, Kamaraj & Jambulingam, 2008 ). Therefore, the relatively naïve populations in the Americas and the presence of both *Ae. aegpti* and *Ae. albopictus*, combined with the arrival of infected travelers, raised major concerns that an epidemic in the Caribbean and /or Latin America was inevitable (Weaver & Reisen 2009). Indeed in October, 2013, CHIKV circulation was detected in Saint Martin (Leparc-Goffart, Nougairede, Cassadou, Prat & de Lamballerie, 2014) with 4,300 confirmed cases (Pan American Health Organization). Local transmission was reported in 176 cases in French Guyana on the South American mainland. The CHIKV strain that was detected in these outbreaks in the Americas was the old Asian strain which does not infect *Ae. albopictus* efficiently as compared to the Indian ocean strain, *Ae. aegpyti* (Tsetsarkin *et al.,* 2007). Since the CHIKV transmission occurred in the Americas via *Ae. aegpyti,* this limited the geographical spread of CHIKV, particularly in temperate climates where mosquitoes do not normally thrive. However re-infestation of most tropical and subtropical regions of Latin America since 1970s (Gubler, 2011) along with its persistence in the southern United States, leaves hundreds of millions of persons at risk for CHIKV infection.

### 2.4.7 Chikungunya virus burden

Chikungunya infections generally cause severe febrile illness that in the long term can cause physical disability. And because of the widespread distribution of *Aedes* species of mosquitoes, like in Kenya (Lutomiah *et al*., 2013), the populations at risk is very large. Due to chronic arthritis caused by chikungunya virus infection, its outbreak carries significant economic and public health implications. Globally, 2,548 million people, which accounts for 39% of the population in 41 endemic countries, have been

affected with Chikungunya. Estimated deaths per year of 33 to 25761 was reported and chronic cases per year is 1,193 to 46,453 (LaBeaud, Bashir & Charles 2011). In addition, the average annualized incidence of Chikungunya in 2005 was 33,000 to 93,000, a mortality of 10% to 28% (Charrel, de Lamballerie & Raoult, 2007; Tandale *et al*., 2009) and risk for disability of 5 to 50% (Borgherini *et al*., 2008;Soumahoro *et al.,* 2009) was also reported.

Although chikungunya outbreak has been reported in many countries in Africa, Asia and the Americas, The disability-adjusted life years (DALYs), has been calculated in India, Reunion Island and the Americas. The chikungunya outbreak in India in 2006 had a heavy epidemiological and economic burden to the population. The national burden of Chikungunya was approximately 25,588 disability-adjusted life years (DALYs) lost during 2006 outbreak with persistence arthralgia contributing 69% of the DALYs. The productivity loss was at a minimum of 6 million USD in terms income from missed economic activities (Krishnamoorthy *et al*., 2009). Another study done in Andhra in India reported a burden of 6600 DALYs (Cost: USD 12,400,000). Most of hospitalization was paid from patients pockets although the burden was moderate (Seyler *et al*., 2010).

Secondly, in Reunion Island in June 2006, reports indicated that those affected were 93.7% of the population, and they complained mainly of pain in the joints and bones, 6 months after the outbreak. The estimated overall cost of Reunion Island outbreak was USD 80 million, with direct medical costs of between USD 80 to 160 per case (Soumahoro *et al.,* 2011). Most working adults were disabled with loss of mobility, hand disability and depressive reaction which could last for several months and has negative consequences on the health, social and economic areas (Marimoutou, Vivier, Oliver, Boutin & Simon 2012; Queyriaux *et al*., 2008). During the Chikungunya outbreak in 2014 in Latin America, it is estimated that between 385 835 to 429 058 patients were to develop chronic inflammatory rheumatism after the outbreak (Rodriguez-Morales, Cardona-Ospina, Villamil-Gómez & Paniz-Mondolfi, 2015). The estimated minimum burden was 151 031 in 2014, which is higher than the DALYs

reported in India (25 888 DALYs). In Latin America the real Chikungunya burden and soci-econmic impact remains unknown (Cardona-Ospina, Diaz-Quijano & Rodriquez-Morales, 2015).

### 2.4.8 Chikungunya seroprevalence in Kenya

Presence of IgG in populations has been reported in coastal and lake basin regions of Kenya (Kisumu and Busia districts) as shown on **Table 2.2**, which are both located near water bodies i.e. the Indian Ocean and Lake Victoria, respectively. The presence of CHIKV specific antibodies (IgG) indicates that the population in these regions have had previous exposure to CHIKV and the antibodies generated have persisted in the population for at least one week to several months/years. In 2013, when there was a reported dengue outbreak in Kenya, Chikungunya was found to be co-circulating during this outbreak with acute cases being reported mainly along the coastal parts of Kenya (Wasonga *et al.,* 2015a). From the seroprevalence data, the populations at risk of Chikungunya infection were the elderly, children (Wasonga *et al.,* 2015a) and those that are immunocompromised like expecting mothers and individuals with complications arising from other ailments like arthritis.

The reason for the prevalence of the disease in the coastal and western regions of Kenya is unclear. This could be due to presence/absence of mosquitoes or favourable climate for mosquito breeding. Since *Ae. aegypti* is well distributed throughout Kenya (Lutomiah *et al.,* 2013) the vector distribution does not limit the disease and instead it is hypothesized that the climatic conditions could be the main factor,because change in climatic conditions favour mosquito breeding, like in the rainy season the mosquitoes are likely to thrive and possibly lead to an outbreak. This could be due to active transmission in endemic regions, movement of people across the country for trading, education and economic opportunities and great variability in the climate which could result in temperature and humidity conditions that create favourable conditions for *Aedes* mosquitoes breeding in almost any part of the country.

**Table 2.1: Seroprevalance of Chikungunya in Kenya**

| Regions | Sites | Year of study | Target population | % prevalence | References |
|---|---|---|---|---|---|
| Western Kenya | Busia district | 2010 | Pyretic children | 11.5 | Mwongula et .al., 2013 |
| | Busia district | 2004 | Adults | 59.9 | Mease et al., 2011 |
| | Kisumu District | 2004 | children | 42 | Sutherland et al., 2011 |
| | Central Nyanza | 1966-1968 | All age-groups | 54.6 | Geser et al., 1970 |
| Coastal Kenya | Msambweni district | 2000-2003 | Pregnant women | 37 | Sutherland et al., 2011 |
| | Malindi district | 2004 | Adults | 24.77 | Mease et al., 2011 |
| | Coastal Kenya | 1987 | Adults | 0.7 | Morill et al., 19191 |
| | Malindi district | 1966-1968 | All age groups | 50.8 | Geser et al., 1970 |
| Rift Valley region | Kipsamoite, Nandi district | 2004 | Children | 0 | Sutherland et al., 2011 |
| Eastern Kenya | Samburu district | 2004 | Adults | 0 | Mease et al., 2011 |
| | Kitui | 1966 - 1968 | All age groups | 1.0 | Geser et al., 1970 |

These studies tested for IgG, and some of the tests used are not clearly stated in the publications, however, serological assays show a high degree of cross-reactivity and some of these reactions over-represent CHIKV circulation (Dash, Mohanty & Padhi, 2011)

## 2.5 Role of genetic changes in host preference and vertebrate virulence

While genetic tracing of micro-evolutionary patterns can aid in tracking the epidemiology of a virus, it's equally important to investigate how individual mutations affect disease progression or the ability of the virus to be transmitted. One study identified a genetic change of a single amino acid from an Alanine to Valine substitution at E1 glycoprotein position 226 in the Reunion Island CHIKV isolate in 2006. Further studies showed that *Ae. Albopictus* facilitated the rapid transmission of the new strain of CHIKV that had the adaptive mutation (Tsetsarkin *et al*., 2007;

Vazeille *et al*., 2007). The introduction of this mutation mid-outbreak suggests a transition where at least two variants (226A and 226V) circulated at the same time.

Although laboratory studies of mosquito vectors have identified micro-evolutionary changes associated with vector susceptibility, no genetic evidence has been able to explain the unusual clinical presentations observed in Reunion Island. One study on Chikungunya pathogenesis identified `antigenic switches` (i.e. changes in expression of genes at a specific site) which were suggested to inhibit HLA class 1-restricted recognition patterns identified within the CHIKV E1 and E2 genes that had undergone substantial levels of mutation. These mutations reside in areas critical for vector infectivity and may have been involved in adaptation of the virus to *Ae. Albopictus* (Powers, 2011).

Another study demonstrated how high levels of viremia were associated with high levels of pro- inflammatory cytokines, chronic arthralgia or more severe disease (Chow *et al*., 2011). A similar study showed that patients with high levels of IFN-alpha mRNA in peripheral blood mononuclear cells, and elevated levels of circulating interleukin 12 tended to have chronic symptoms, when compared to patients with lower interleukin levels (Hoarau *et al*., 2010) . Since these studies have not matched specific genetic mutations with distinct phenotypic markers such as disease severity or persistence, these studies have only offered clues that could eventually lead to discoveries that link mutations to phenotypic changes in the mosquito or human hosts.

## 2.6 Phenotypic and genotypic identification of CHIKV.

Some of the techniques that can identify phenotypes and genotypes of CHIKV are in *vitro* kinetic assays, mice pathogenesis, virulence in mosquitoes, sequencing and reverse genetics among others.

### *2.6.1 In vitro* kinetic assays of virus infected cells

*In vitro* kinetic assays assess the rate of production of virus by infected cells. When a virus infects a cell, it hijacks the resources of the cell to manufacture and release a new

generation of progeny virus particles. The resulting cycle of virus production at the cellular level, is an important process for the spread and persistence of viral diseases (Levin, Lipsitch & Bonhoeffer, 1999) and the culture of viruses for diverse applications. One of the applications is to monitor viral growth by cell culture and virus quantification by assessing increase or decrease of viral titre over a period of time and the viability of the virus over time.

### 2.6.2 Assesing virulence in animal models

Laboratory animal models are widely used in the pre-clinical evaluation of potential vaccines, antiviral compounds and to assess virulence of virus strains. Animal models are also used to investigate the safety of vaccines, antiviral compounds and their efficacy in preventing or moderating infections or secondary transmission. In selecting an animal model, a number of factors are considered. The animal must be susceptible to the specific viral infection and facilitate its replication. The animal should also exhibit specific clinical signs of viral infection which can be observed for modification in the presence of the antiviral compound. Depending on the animal species, the clinical signs include weight loss, lethargy, pyrexia, histopathological changes in or virus recovery from tissues. These animal models must also represent humans, in terms of similarity of clinical signs, histopathological changes, virus growth kinetics or transmission. Some examples of good animal models are guinea pigs and ferrets (Bouvier & Lowen, 2010).

### 2.6.3 Assesing virulence in natural mosquitoes

The ability of a mosquito to acquire a pathogen and successfully transmit it to another susceptible host, is influenced by internal factors like mosquito survival and virus replication and by external factors including temperature, humidity, availability of vertebrate hosts, vector population density and predation. For CHIKV, horizontal transmission through saliva that is injected when a mosquito feeds on blood meal is the common mechanism of transmission, although vertical transmission via infected eggs may also occur at a low rate (Agarwal *et al*., 2014). To mimic CHIKV vector

infection, mosquitoes from endemic locations can be bred in a laboratory insectary, and fed on artificial blood meals loaded with known titres of virus. Mosquitoes that ingest the infectious blood meal are incubated at a constant temperature for at least seven days and then used to assess viral replication in the vector. The mosquitoes that become infected, develop infections that disseminate from the initial infection site in the midgut into the hemocoel. Transmission potential is measured by the presence of CHIKV in the saliva and used to extrapolate vector competence for the mosquito species.

### 2.6.4 Correlation of phenotype to genotype.

Technological advances in sequencing like automated DNA sequencing have accelerated the accumulation of whole or partial genome sequence data of many organisms in publicly available databases like GenBank. Forward genetics seeks to find the genetic basis of an observed phenotype or trait, while reverse genetics seeks to find what phenotypes arise as a result of particular genetic sequences by analyzing the phenotypic effect of a modified gene sequence. For virus strains with different phenotypes of interest, Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing is performed to generate partial or complete genomic sequences of the isolates/strains. These sequences are aligned and compared with each other and often a reference sequence and sequences from other related virus strains. Any differences in target regions or novel sites are then tested via reverse genetics by engineering specific genetic changes in a strain and observing for phenotypic changes that can then be directly attributed to the genetic change. The specific function of that protein can then be deduced by the observed change in phenotype when the genetic change is introduced.

## 2.7 Laboratory diagnosis of CHIKV infection

Diagnosis of CHIKV infection is mainly done by virus isolation, serology and molecular analysis. Neutralization assays (plaque reduction or focus reduction neutralization tests) are used as confirmatory tests for serological assays.

### 2.7.1 Animal propagation and virus culture isolation

Chikungunya virus detection is maily done by isolation of viral pathogens in cell culture and animal propagation. Chikungunya virus culture and isolation can be done by injection of the purified virus or material containing the virus into mice intracerebrally ( Sarkar, Pavri, Chatterjee, Chakravarty & Anderson, 1964; Myers *et al*., 1965) or inoculation onto mosquito cell lines (C6/36) or mammalian cell lines such as BHK-21, HeLa and Vero cells, or by mosquito inoculation by intrathoracic injection or by feeding mosquitoes on infected blood-meals or animal host.

Different viruses grow well in different cell lines. CHIKV grows well in both Vero and C6/36 cells. Culturing of CHIKV in Vero cells shows visible cytopathic effects after 1 to 3 days. In C6/36, CHIKV grows to higher viral titer than in Vero cells (Wasonga *et al.,* 2015b), although in C6/36 cells, its challenging to observe cytopathic effects. Inoculation of these cells with virus produce cytopathic effects (CPE), which are usually confirmed by a neutralization test using a CHIKV-specific anti-serum can take one to two weeks to perform.

### 2.7.2 Molecular techniques for detection of CHIKV

#### 2.7.2.1 Conventional Reverse Transcriptase Polymerase Chain Reaction

Reverse Transcriptase-Polymerase Chain Reaction allows a specific target RNA sequences to be reverse transcribed to complementary deoxyribonucleic acid (cDNA) and then amplified exponentially *in vitro*. Before initiating RT-PCR, the RNA is extracted from a sample, reverse transcribed to cDNA using reverse transcriptase, then amplified by PCR. PCR is based on an enzymatic reaction involving the use of synthetic oligonucleotides flanking the target nucleic sequence of interest in CHIKV genome. These oligonucleotides act as primers for the thermostable Taq polymerase (Freeman, Walker & Vrana, 1999)

The first step of RT-PCR which is denaturation of the template DNA (at $94^oC$) separates the double-stranded DNA into two separate strands. The high temperature

breaks the relatively weak bonds between the nucleotides that form the DNA code. Annealing of primers to their complementary sequences ($50^{o}C$) enables copying of only a very specific sequence of the genetic code targetted by the primers and primer extension which begins at regions marked by the primers, involves nucleotides in the solution being added to the annealed primers by the DNA polymerase to the new strand of DNA complementary to each of the single strands. After completing the extension, two identical copies of original DNA have been made after making 2 copies of the DNA through PCR, the cycle is repeated several times (usually 25 to 40) to yield millions of copies of the DNA sequence of interest. Repeated cycles of denaturation, annealing and extension result in the exponential production of the specific target fragment. A band is observed after gel electrophoresis to detect PCR amplification at the end-point of PCR reaction (Panning *et al*., 2009).

#### 2.7.2.2 Real time Reverse Transcriptase Polymerase Chain Reaction

Real time RT-PCR (RT-qPCR) is a technique used to monitor the progress of a PCR amplification reaction of targeted DNA molecule. The RNA is extracted and subjected to. RT-qPCR which has an additional cycle of reverse transcription that leads to formation of a cDNA molecule from an RNA molecule (Kumar *et al*., 2013; Parida, Santhosh, Dash & Lakshmana Rao, 2008). To amplify the cDNA, conventional PCR is done using DNA plate, specific primer set, deoxyribonucleotides, a suitable buffer solution and a thermostable DNA polymerase. The oligonucleotides marked with a fluorophore are added to this mixture in the thermocycler that contain sensors for measuring the florescence of the fluorophore after it has been excited, this enables measurement of specific amplified PCR products . Real time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as reaction proceeds due to accumulation of PCR product with each amplification cycle. Detection of PCR products commonly occurs in two ways : non-specific fluorescent dyes that bind to double stranded DNA (e.g. SYB Green) or sequence specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent dye which permits detection only after hybridization of the probe with its complementary sequence e.g. Taqman probes. Then, the amount of fluorescence emitted and PCR

reaction is recorded during exponential phase. If a graph is drawn between the log of starting amount of template and corresponding increases in the fluorescence of reporter dye, fluorescence real time results in a linear relationship. It needs no post PCR processing which saves resources and time. The ability of measuring kinetics in early phase of PCR provides a distinct advantage over traditional PCR which uses gel electrophoresis to detect PCR amplification at end-point of PCR reaction (Darci *et al.*, 2009).

#### 2.7.2.3 Multiplex polymerase chain reaction of CHIKV with other arboviruses

A duplex/multiplex real time RT-PCR (rRT-PCR) uses the principle of RT-qPCR as described above and it allows for detection, quantification and identification of more than one arbovirus. For example, a multiplex for CHIKV and four serotypes of Dengue has been developed (Cecilia *et al.*, 2015). Both reverse transcription and cDNA amplification can be done in one-step and in one tube containing: sample, all primers and probes that are specific to both viruses, then it can simultaneously detect and quantify RNA of both viruses. The primers and probes are designed to target specific regions of the genome which are conserved for each virus to ensure detection of both in a single reaction.

#### 2.7.2.4 Reverse transcription Loop Mediated isothermal amplification

Reverse transcription (RT) Loop Mediated isothermal amplification (RT-LAMP) developed for CHIKV targeted the E1 gene for the rapid and real-time detection. (Parida *et al.*, 2006). RT-LAMP is a technique that amplifies nucleic acid with high specificity, efficiency, and rapidity under isothermal conditions with a set of six specially designed primers that recognize eight distinct sequences of the target genome. The whole procedure is simple, rapid and can be done within one hour by incubating mixture of samples, primers, DNA polymerase with strand displacement activity and substrate in a single tube, in a single step at a constant temperature of 63°C (Notomi *et al.*, 2000). LAMP yields large amounts of double stranded DNA, and leads to the formation of a white precipitate of magnesium pyrophosphate in the reaction mixture that can be observed with the naked eye without cyclic denaturation of the template. Detection of gene amplification can be accomplished by gel

electrophoresis or by real-time monitoring of turbidity in a turbidimeter. Since the amplification of DNA is directly correlated with the production of magnesium pyrophosphate, real time monitoring of RT-LAMP reaction is possible by measurement of turbididty. RT-LAMP is highly sensitive and comparable to real time PCR, with a detection of limit of ~10 RNA copies per assay. CHIKV RT-LAMP is also fast, efficient, inexpensive and it's suitable for field diagnosis of tropical diseases (Parida *et al*., 2006)

### 2.7.3 Serological assays

Enzyme Linked-immunosorbent Assays utilizes a specific antibody linked to an enzyme to detect the presence of an unknown amount of antigen (virus) or antibody in a sample. The antibody-antigen binding is detected and/or quantified through the enzyme's ability to convert a reagent to a detectable signal that can be used to calculate the concentration of the antibody or antigen in the sample. Horseradish peroxidase (HRP) is a common enzyme utilized in ELISA schemes due to its ability to amplify signal and increase assay sensitivity. There are many types of ELISA assays which can generally be classified as either indirect, or sandwich ELISA systems (Kuby, 2007).

#### 2.7.3.1 Indirect ELISA

Indirect ELISA is a two-step ELISA system which involves binding of primary and labelled secondary antibodies. It involves attachment of the CHIKV antigen to polystyrene plate, then the sample is applied and CHIKV specific primary antibody added. An enzyme-labelled secondary antibody, directed at the first antibody is added and detection is done by a substrate. This technique is used mostly to detect CHIKV specific IgG antibodies in sera. This technique is flexible since different primary detection antibodies can be used with a single labelled secondary antibody (Hundekar, Thakare, Gokhale & Barde, 2002; Shukla *et al*., 2009)

#### 2.7.3.2 Sandwich ELISA/IgM ELISA

The sandwich ELISA quantifies antigens detected between 2 layers of antibodies (capture and detector antibodies). It involves attachment of the capture antibody to a polystyrene plate. The sample is applied, then CHIKV antigen added (the antigen should have more than one antigenic epitopes capable of binding to atleast two antibodies). The CHIKV specific primary antibody and the enzyme labelled CHIKV specific antibody (detector antibody) are added and finally the substrate is used to quantify the analyte. CHIKV specific Polyclonal or monoclonal antibodies can be used as the capture and detection antibodies in this ELISA system. Monoclonal antibodies recognize a single epitope, thus are less sensitive but more specific, while polyclonal antibodies which recognize multiple epitopes are more sensitive. The advantage of this technique is that test samples do not have to be purified before analysis and the assay is more sensitive than indirect ELISAs. It's highly efficient in CHIKV antigen detection and many commercial ELISA kits are based on the sandwich ELISA system.

In Sandwich ELISA, two antibodies are used; therefore the antigen is specifically captured and detected, making it suitable for complex samples, since antigen does not require prior purification. The sandwich ELISA is flexible, because it can be adapted as a direct sandwich ELISA (antigen detection ELISA, which is used to detect antigens in serum or mosquito supernatants) or an indirect sandwich ELISA (IgM capture ELISA used to detect anti-CHIKV antibodies in sera) detection method (Wasonga *et al*., 2015a) as shown on **Figure 2.5.**

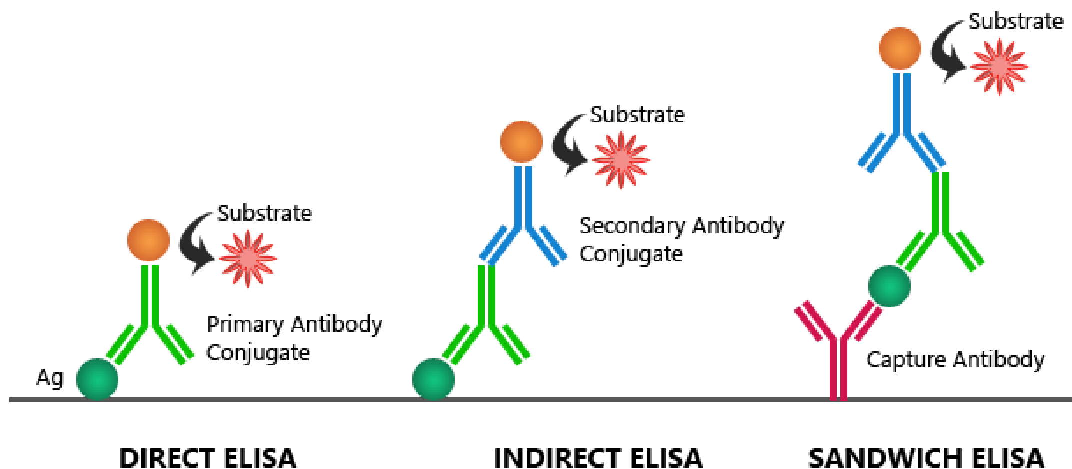


**Figure 2.5: A schematic representation of direct, indirect and sandwich ELISA (https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle)**

### 2.7.4 Confirmatory tests for sero-positive serum samples

Neutralization tests are currently considered to be the gold standard for detecting and measuring antibodies that can neutralize the viruses that cause many diseases (Ratnam *et al*., 1995; Thomas *et al*., 2009-11) and are used to confirm positive samples that have been analyzed by serological tests. Neutralizing antibodies are secreted by B-cells primarily to respond to infection involving a particular antigen and proliferate to produce a colony of cells, which can clear away the infection by neutralization of the antigen by the antibody. The rest of the memory B-cells persist and can survive for years or even a lifetime (Parham, 2005). Complete neutralization of virus after incubation with test serum proves the presence of neutralizing antibodies in that sample, which are formed from activated B cells that are specific to the virus encountered during the primary immune response. These B-cells are able to live for months or even years and can respond quickly following subsequent exposure to the same virus (Bona *et al.,*1996).

Neutralizing antibodies can be produced in the acute phase (IgM) or during the chronic phase (IgG) after infection with a virus. Neutralization tests can be done to measure neutralizing antibodies. Because of the specificity of antigen-antibody

reactions, measurement of neutralization antibodies using neutralization tests is able to differentiate viruses including the cross-reactive viruses particularly alphaviruses (CHIKV and ONN) which are antigenically very similar (Powers *et al.,* 2000). Examples of neutralization tests are Plaque reduction neutralization test (PRNT) and Focus reduction neutralization assay(FRNT). PRNT and FRNT apply a similar principle but differ in methodology. PRNT uses a 6 to 24-well cell culture plate measures dead cells that are detached from the cell-culture plate, takes slightly longer time to perform and only 1 to 4 sample can be tested per plate. FRNT on the other hand, is performed in a 96-cell culture plate, measures infected cells that are not detached from the cell culture plate, takes a shorter time to incubate after innoculation, and can test more samples per plate compared to PRNT.

#### 2.7.4.1 Plaque reduction neutralization test

The plaque reduction neutralization test (PRNT) is used to quantify the titer of neutralizing antibody for a virus. PRNT has been widely used in assessing the protective neutralizing antibody response for arboviruses. The serum sample to be tested is diluted and mixed with virus-infected culture fluid. This is incubated to allow the antibody to react with the virus. The incubated sample is poured over a confluent monolayer of host cells in a petri dish, 6-well, 12-well or 24-well cell culture plate. The surface of the cell layer is covered in a layer of overlay e.g. methylcellulose, carboxymethyl cellulose or agar to prevent the virus from spreading indiscriminately. The plaques are visualized by staining using crystal violet or neutral red. The concentration of plaque forming units can be estimated by the number of plaques (regions of infected cells) formed after a few days depending on the virus e.g. 4 days for CHIKV. The plaque forming units are measured by microscopic observation of the plaques.

#### 2.7.4.2 Focus reduction neutralization test

The focus reduction neutralization test is used to quantify the titer of neutralizing antibody for a virus using a 96-well cell culture plate. The serum sample to be tested is diluted and mixed with a viral infected culture fluid and then incubated to allow the antibody to react with the virus. The serum-virus solution is inoculated onto confluent

monolayer of host cells. The surface of the cells is then covered in a layer of agar or methylcellulose to prevent the virus from spreading indiscriminately. The concentration of focus forming units can be estimated by the number of foci (region of infected cells) formed after a few days (1 day for CHIKV, RFV). Unlike PRNT where plaques are visualized by staining using crystal violet or neutral red, FRNTs' foci are visualized by immnostaining of the plate by fixing, permeabilization of the cells, incubation with virus specific antibodies and detection with a substrate.

### 2.7.5 Commercial IgM ELISA for detection of Chikungunya

For serological diagnosis of acute infection, serum has to be obtained in the period between the 10-14 days that separates the acute phase (immediately after the onset of illness) from the convalescent phase, when CHIKV specific IgM are at their peak. The antibodies can be detected by IgM ELISA and ICT (immunochromatographic tests). Several in-house tests for IgM specific ELISA have been developed, for example, IgM ELISA developed by the Diagnostic Systems Division of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), USA, National institute of Virology, Pune, India,Department of Virology, Institute of Tropical Medicine, Nagasaki University Sakamoto, Japan and many others. However, in the last five years, commercial IgM Capture ELIS kits have also been developed as shown in the **Table 2.3**. Despite kits for detecting IgM antibodies against CHIKV being available commercially, they are very expensive. Assuming samples are run in duplicate, only 45 samples including the controls can be run with one ELISA kit costing for example: $585 (Ksh. 52,650) which works out to ~13$ (Ksh 1,300) per sample.

**Table 2.2: Commercial CHIKV IgM Capture ELISA kits**

| | Product name | No. of samples | Price USD |
|---|---|---|---|
| **1.** | IgM Capture ELISA | 1 x 96 assay | $ 585 |
| **2.** | IgMμ-capture ELISA | 1 x 96 assay | $ 585 |
| **3.** | Human IgM ELISA kit | 1 x 96 assay | $ 555.4 |
| **4.** | IgM Human ELISA kit | 1 x 96 assay | $ 561 |
| **5.** | IgMμ-capture ELISA | 1 x 96 assay | [a] |
| **6.** | IgM-ELISA | 1 x 96 tests | [a] |

[a]Represents commercial ELISA kits for which information were not readily available.

This is not affordable in a resource constrained country and is definitely not sustainable for outbreak response, routine diagnosis or for routine surveillance which should be done throughout the inter-epidemic period. Since, these anti-CHIKV ELISA kits have been developed for commercial purposes in Asia, Europe and Americas, they are not easily available for purchase in Africa and many have short shelf lives making it difficult to purchase or store in bulk.

### 2.7.6 Management of Chikungunya

There is no vaccine or specific antiviral treatment for Chikungunya, so only palliative care can be provided to manage the symptoms associated with this infection. During the outbreak in Reunion Island, some patients were treated with chloroquine because it has been effective in treatment of chronic arthralgia (Staikowsky *et al.*, 2008) and it also inhibits viral replication. The study on chloroquine in Reunion Island concluded that treatment of acute infection with chloroquine was not effective (De Lamballerie, Ninove & Charrel, 2009). In Reunion Island, analgesics especially paracetamol, combined with non-steroid anti-inflammatory drugs were commonly used to manage pain (Staikowsky *et al.*, 2008). Corticosteroids were used occasionally to manage

incapacitating CHIKV infections (Staikowsky *et al*., 2008). Interferon gamma and ribavirin worked well when used as a combination therapy and showed antiviral activity in vitro (Briolant, Garin, Scaramozzino, Jouan & Crance, 2004). Investigations are ongoing to discover an effective antiviral drug against CHIKV (de Lamballerie *et al*., 2009). In addition, prophylaxis for exposed immunocompromised patients using anti-CHIKV immunoglobulin has been proposed in a study (Couderc *et al*., 2008 ) where anti-CHIKV immunoglobulin was obtained from convalescing patients. This study is a promising first step towards applying immunoprophylaxis for the treatment and prevention of CHIKV illness.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study site

The bulk of the study was done at KEMRI, Nairobi. Chikungunya virus propagation, all molecular analysis by RT-PCR to confirm the identity of CHIKV seed stock and as a confirmatory test for CHIKV positive mosquito homogenates was done in the Arbovirology/Viral Hemorrhagic Fever (VHF) laboratory. Development of the ELISA tests, *in vitro* growth kinetics and neutralization tests were done in the Production Department in KEMRI. The rabbits were housed in the animal house and whole genome sequencing of the CHIKV plaque variants was done on the Biosciences Eastern and Central Africa - International Livestock Research Institute's (BecA-ILRI-Hub) genomics platform

## 3.2 Study design and experimental animals

This is a laboratory-based study and cross-sectional study ,where a panel of serum collected from the dengue outbreak were analyzed from one time point. The study involved the use of rabbits purchased from the National rabbit breeding centre located at the Ngong Veterinary farm. The rabbits 3 month old females weighing 3 to 6kg, were used to raise CHIKV specific antibodies for development of two ELISA tests, an antigen detection ELISA which was evaluated using mosquito homogenates and in-house IgM ELISA which was evaluated using human serum samples from the Comoros Island CHIKV outbreak of 2005. Serum collected during the dengue outbreak of 2013 was used to field test the IgM ELISA assay developed in this study.

## 3.3 Study Samples

### 3.3.1 Chikungunya virus stock

To select a CHIKV stock to use in this study, 5 independent archived viral samples (CHIKV 72, CHIKV 73, CHIKV 74, CHIKV 75 and CHIKV 76) that were identified from the KEMRI VHF laboratory, were cultured in Vero cells (African green monkey kidney derived cells ATCC, CCL81), to assess their viability. These CHIKV samples were isolated from the human samples received in the VHF laboratory as part of outbreak response. These samples had been stored in liquid nitrogen at -80°C. The cultured viruses were analyzed by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to confirm their identity as CHIKV using specific primers on **Table 3.1**. One of the isolates (CHIKV 75) which represented the latest documented circulating strain in the region was selected for further research in this study. This sample, CHIKV 75 was later renamed Com5 to obtain a unique ID to be used during submission of its sequences to the GenBank. The Com5 was isolated from a positive serum sample obtained from the Grande Comore Island, one of the islands in the Comoros Island archipelago during the 2005-2006 Chikungunya outbreaks. Com5 was propagated in Vero cells in the VHF laboratory, Centre for Virus Research, KEMRI.

### 3.3.2 Passage of Vero and C6/36 cells

Vero cells and C6/36 (*Ae. aegypti* derived) cells were passaged for use in further research like inoculation of virus, plaque assay or focus assay**.** The Vero cells and C6/36 cells were grown in growth media (Eagle's Minimum Essential Media (EMEM) (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 3.5% sodium bicarbonate, 292 µg/ml L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin) in 75 $cm^2$ tissue culture flasks. When they attained 80-90% confluence, the growth media was discarded, the cells were washed with 5 ml of fresh growth media, and then 5 ml of 1 % EDTA-trypsin added to the tissue culture 75 $cm^3$ flasks and incubated at 37 °C for approximately 4 minutes. The trypsin causes the cells which are adherent to dislodge from the flask. Five millilitres of growth media was

then added to stop the action of trypsin which if prolonged can be harmful to the cells and the tissue culture flask was tapped from the side to ensure that the cells are completely dislodged. A sterile serological pipette was used to pipette the cells in and out of the flask to break up the clumps of cells. The cells, EDTA trypsin and growth media was transferred to a centrifuge tube, centrifuged at 410 g for 4 minutes, the supernatant was aspirated off and the cells re-suspended in 25 ml of fresh growth media, then seeded in a culture flasks for propagation of virus or on 6-12 cell-culture plates for plaque assay and incubated at 37°C. The Vero cell monolayers were used to prepare virus stock (Com 5) and for large scale culture of CHIKV, to determine the in *vitro* viral kinetics of purified plaques and for plaque purification. C6/36 cells were cultured in EMEM containing 2% FBS and incubated at 28°C with 5% $CO_2$ and used for *in vitro* growth kinetics of purified plaques.

### 3.3.3 Serum samples

A total of 148 sera from patients with febrile illness that were received in the VHF laboratory as part of the 2004-2005 CHIKV outbreak response in Lamu, Kenya and Comoros Island were used to validate the in-house IgM capture ELISA (in-house IgM ELISA). In-house IgM ELISA was also used to test 254 dengue negative febrile patient samples collected during the 2013 dengue outbreak in Kenya as part of outbreak response. The sera were aliquoted and stored at -80 °C.

## 3.4 Chikungunya virus propagation

The five independent viral samples [CHIKV 72, CHIKV 73, CHIKV 74, CHIKV 75 (Com5) and CHIKV 76] were propagated in Vero cells as follows: The viral samples were prepared by inoculating 100µl of CHIKV infected culture fluid (ICF) in confluent monolayers of Vero cells in a 6-well plate , which were then incubated at 37°C with 5% $CO_2$ for 1.5 hours for 1 to 2 days while cultured in maintenance media EMEM containing 2% FBS, 3.5% sodium bicarbonate, 292 µg/ml L-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin in 25 $cm^2$) tissue culture flasks. The flasks were monitored daily by observing under a microscope for the development of

cytopathic effect (CPE). Infected culture fluid was harvested when 70-80% CPE was observed. The ICF was harvested at 80% CPE and not beyond, so as to harvest the ICF when the virus titer is at peak and before the cells start dying. Beyond 80% CPE, most viral particles do not have cells in which to replicate and begin to die causing the viable viral titre to plateau and then decline. The flasks were frozen at -80°C for 1 hour to release viruses from the cells. The ICF was thawed and centrifuged at 410 g for 10 minutes to separate ICF containing virus from cellular debris. Com5 ICF was used as antigen in the development of IgM capture ELISA, in neutralization tests, *in vitro* growth kinetic studies and for immunization of the animals.

## 3.5 Confirmation of identity of CHIKV seed stock by Reverse Transcriptase-Polymerase chain reaction.

### 3.5.1 RNA extraction

To confirm the identity of the viral isolates as CHIKV and not any other arbovirus, RT-PCR was done. RNA was extracted as follows: 250µl of the CHIKV infected culture fluid was transferred into a micro-centrifuge tube containing 750 µl of TRIZOL$^{R}$ LS reagent (Life Technologies, Grand Island, NY, USA), then vortexed to mix. The sample was allowed to stand at room temperature for 5-10 minutes to allow for complete lysis of the virus. Two hundred microlitres of chloroform was added (Reagent grade) to the sample supernatant and vortexed. The sample was incubated at room temperature for 5-10 minutes, centrifuged at 18,500g for 10 minutes at 4 º C, then 500-550µl of the clear aqueous solution removed and put in clean RNase free 1.5ml snap cap micro-centrifuge tube.

One microlitre of glycogen and 500µl of reagent grade isopropanol was added to the sample, vortexed for 60 seconds and incubated at room temperature for 10 minutes. The sample was centrifuged at 18,500g for 10 minutes at 4º C. The liquid was emptied into a discard container leaving a white RNA pellet at the bottom of the tube. Five hundred microlitre of 75% ethanol was added to the pellet, the tube capped and inverted to gently wash the inside of the tube. The tube containing the pellet was

centrifuged for 2 minutes at 18,500g at 4ºC and the liquid emptied into a discard container. Centrifugation was done for another 30 seconds, and a pipette used to remove excess liquid (approx. 20 µl). Eleven microlitres of nuclease free water was added to the pellet to resuspend the RNA. The tube was incubated at room temperature for 1 minute, vortexed to mix, spun down at 185g for 30 seconds, then the resuspended RNA used as a template for first strand cDNA synthesis.

### 3.5.2 DNA Template preparation

To prepare the cDNA, the first strand cDNA Synthesis from viral RNA (20µl reaction) was done as follows: 10 µl of the RNA of interest and 2 µl of 50ng/ µl random hexamers were pippetted into domed topped 0.2 ml thin walled PCR reaction tube, the mixture spun down in a mini-centrifuge (18,500g for I minute), then put in a thermocyler to heat to 70ºC for 10 minutes to denature the sample, then cooled to 4 ºC for 5 minutes. The following was then added to the sample: 4 µl of 5X First strand buffer, 2 µl of 0.1M dithiothreitol, 1 µl of Superscript III reverse transcriptase (Invitrogen, Grand Island, NY, USA) and 1 µl of 10mM dNTP. The sample was spun down in a mini-centrifuge, heated on a thermocycler to 25 ºC for 15 minutes, 42 ºC for 50 minutes, followed by 70 ºC for 15 minutes, cooled to room temperature. The generated cDNA was then used as a template for PCR amplification.

### 3.5.3 RT-PCR Amplification

PCR was conducted using the following procedure for a 25 µl reaction volume. A master mix was prepared consisting of nuclease free water (9.5µl/reaction), forward primer (0.5 µl/reaction), reverse primer (0.5 µl/reaction) and amplitaq mastermix (12.5 µl/reaction) (Life Technologies, CA, USA). The primer pair targeted the envelope gene E1 based on CHIKV strain S27 (Accession number AF369024.1) to generate a 787 bp product. The sequence and position of the forward and reverse primers are indicated in **Table 3.1**

**Table 3.1 Primers used for PCR Amplification of CHIKV RNA**

| | Primer | 5` -3` sequence | Fragment size |
|---|---|---|---|
| **1** | CHIKV 10133 F | ACCGGCGTCTACCCATTCATGT | 787bp |
| | CHIKV 10920 R | GGGCGGGTAGTCCATGTTGTAAGA | |
| **2** | CHIKV 7028 F | TGC GCG GCC TTC ATC GGC GAC TAC | 1260bp |
| | CHIKV 8288R | CCA GGT CAC CAC CGA GAG GG | |

*Positions are numbered with respect to S27-African prototype sequence (GenBank Accession AF369024).

Twenty three microliters of the cocktail/ master mix was added to each PCR tube, then 2.0 µl of cDNA was added to each tube, loaded to the thermocycler and denaturated at 95°C for 10 minutes. The reaction then ran for 43 cycles which included 94°C for 30 seconds, annealing at 55°C for I minute followed by an extension at 72°C for 7 minutes. The RT-PCR products were then separated and visualized by electrophoresis on a 2% agarose gel.

#### 3.5.3.1 Agarose gel preparation

A two percent agarose gel was prepared as follows: two grams of agarose powder was mixed with 100ml of 0.1M Tris Borate EDTA (TBE) buffer. This solution was heated in a microwave for 2 minutes or until the powder was dissolved. Five microliters of ethidium bromide was added into the agarose gel solution and mixed by swirling. A comb was placed in the gel tank and the agarose was poured into the gel tank avoiding inhalation of the fumes and formation of bubbles. The gel was left to set. Once the gel had set, the combs were removed carefully without disrupting the gel. The gel was transferred to the electrophoresis tank and TBE buffer added until it was three quarter full. Ten microliter of a 100 bp DNA ladder was added to the first well, 3 µl of loading dye was mixed with 10 µl of each DNA sample and loaded on subsequent wells on the agarose gel. The power source was connected at 150V and the gel run for one hour. The DNA fragments were visualized on a UV transilluminator and photographed with

a cannon digital camera. When a band of 787bp was observed on the 2% agarose gel , it meant that the sample was a CHIKV positive sample.

### 3.5.4 Purification of the PCR amplicons

To sequence the PCR amplicons for confirmation of the identity of the virus, the PCR products were purified. The QIAquick PCR Purification Kit (Qiagen, Germany) was used following the manufacturers protocol. Five volumes of Buffer PB was added to 1 volume of the PCR sample and mixed. A QIAquick spin column was placed in a 2 ml collection tube and the sample was applied to the QIAquick column to bind the DNA and centrifuged for 30–60 seconds at 21,700 g. The flow-through was discarded; the QIAquick column was placed back into the same collection tube. Buffer PE was then added to the QIAquick column and centrifuged for 30–60 seconds to wash the DNA. The flow-through was discarded again and placed back into the QIAquick column in the same tube. The column was centrifuged for an additional 1 minute at maximum speed to remove residual ethanol from Buffer PE completely. The QIAquick column was placed in a clean 1.5 ml micro-centrifuge tube. To elute DNA, 50 μl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the column for complete elution of DNA bound to the QIAquick membrane and the column centrifuged for 1 minute. The PCR sample was then run on 2% agarose gel as described in section 3.5.3.2 to confirm that a single PCR product was present.

### 3.5.5 Sanger Sequencing of PCR amplicons to confirm viral identity

Purified RT- PCR amplicons of the five independent viral samples were submitted for sequencing in an automated ABI 3500 series Genetic Analyzer (Applied Biosystems) using the ABI Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturers' instructions. The first step in a chain termination sequencing experiment was to anneal a short oligonucleotide labelled primer on to the DNA template. The chain synthesis is started by adding DNA polymerase plus each of the four deoxynucleotides (dATP, dCTP, dGTP, dTTP), a single labelled dideoxynucleotide (e.g. dideoxy dCTP) and buffers. The dideoxynucleotides blocks

further strand synthesis causing the chain termination. The strand synthesis reactions is carried out four times in parallel (ddATP, ddCTP, ddGTP and ddTTP). Four separate reactions are performed when using dye primer-based sequencing. Each reaction contains a primer labelled at its 5' with one of the different flourescent labelled dyes and dideoxynucleotides (ddNTPS), and randomly terminate protein synthesis, creating DNA fragments of varying lengths. Since fluorescently-labeled primer are used for extension, all terminated fragments are fluorescently labelled. Following a sufficient number of cycles to allow for optimal generation of extended products, the 4 reaction are combined and separated on one capillary electrophoresis-based genetic analyzer. During electrophoresis, the products of the cycle sequencing are injected electronically into capillaries filled with polymer. The fluorescent labelled DNA fragments, separate by size, move through the laser beam. An optical detection device on the Applied Biosystems genetic analyzer detects the florescence signal. Sequencing primers were the same forward and reverse primers used for RT- PCR amplification **(Table 3.1)**

## 3.6 Phenotypic and genotypic characterization of CHIKV isolated from Comoros Island CHIKV outbreak of 2005

### 3.6.1 Plaque assay to quantify CHIKV viable titres

The viral titre was assessed using plaque assay. Confluent Vero cell monolayers in a 6-well plate were inoculated using 100µl of diluted virus ($10^{-3}$ – $10^{-8}$), to infect the cells and the plate was gently rocked back and forth, then side to side to evenly distribute the inoculated virus and incubated at 37°C in an incubator with gentle rocking every 20 minutes for 1.5 hours, innoculated cells were overlayed with 5ml of 1.25 % methyl cellulose 4000 (Wako, Osaka, Japan) solution in 2X EMEM supplemented with 2% FBS, 3.5% sodium bicarbonate and 1 % antibiotics/antifungal agent. The plates were incubated at 37°C for 4 days. The 6-well plates were fixed with 5ml of 10% formalin overnight, the formalin was pippeted out and staining done using 3ml of crystal violet overnight. The plate was rinsed with tap water, air-dried and the plaques visualized by inverting the plates on a light box. The viral titre measures biological activity of virus and is expressed as plaque forming units (PFU) per

millilitre. To calculate the viral titre of the seed stock, the total number of isolated plaques were counted, then divided by the the dilution factor with the highest number of plaques and volume of diluted virus that was added to the wells.

### 3.6.2 Purification of CHIKV variants by plaque assay

To purify the plaques observed in Com5 for further research, a standard plaque assay was performed using serially diluted Com5 ICF. A hundred microliters of appropriately diluted ($10^{-3}$ – $10^{-8}$) Com5 ICF was inoculated to confluent Vero cell monolayer in each well of a 6-well plate to infect the cells and the plate was gently rocked back and forth, then side to side to evenly distribute the inoculated virus. The plates were incubated at 37°C with gentle rocking every 20 minutes for 1.5 hours. The plated cells were overlayed with 3 ml of 1.5% SeaKem LE agarose solution (Lonza, Rockland, ME, USA) in 2 X EMEM supplemented with 2% FBS, bicarbonate and 1 % antibiotics/antifungal agent, incubated at 37% for 1.5 hours, to allow the overlay to solidify and then the plates were incubated overnight at 37°C. The following day, to facilitate visualization of plaques, the second overlay; 1.5 ml of 1.5% agarose in 2X EMEM supplemented with 2%FBS, 3.5% sodium bicarbonate, 1% antibiotics and L-Glutamine mixed with 6% neutral red dye (Sigma–Aldrich, St. Louis MO, USA) was added to the wells. On day 2 after inoculation, the plaques were visualized by inverting the plates on a light box. The plaques of different sizes were marked using a black pen.

### 3.6.3 Amplification of virus from the plaque plug

On the 6-well plate with plaques, the plaque positions were marked from the bottom of the plate. Using a sterile glass pipette tip, an agarose plug was removed directly over the marked plaque and placed in a micro centrifuge tube containing 1 ml growth medium. Approximately 7- 10 small and large plaques were picked in this manner. The virus particles were eluted out of the agarose by vortexing the tube for 30 seconds at a speed of 410 g. The supernatant was inoculated on confluent Vero cell and after 2-3 passages harvested as described in section 3.4. The ICFs from the harvested plaque

variants were stored at -80°C for downstream assays. Out of 10 clones of large plaques and 8 clones of small plaques, L2 and S7 variants were selected from each group for further analysis because they exhibited stable plaque sizes.

### 3.6.4 In vitro viral growth kinetic analysis

To determine *in vitro* viral growth patterns and survival of plaque variants in different cell lines over time, ICF containing equal titres of Com5 and its the Large (L2) and Small (S7) plaque variants were inoculated to C6/36 cells (28°C) and Vero cell (37°C) monolayers in 75 $cm^3$ tissue culture flasks that were confluent as described in section 3.4., at a multiplicity of infection of 1. Eight samples were cultured: 4 samples for each cell-line (a negative control, Com5, L2 and S7). Each sample was run in three independent growth experiments therefore a total of 24 cell-culture flasks were used for this experiment. One ml of ICF was removed from the culture flask every 24 hours for 3 days including day 0 during the incubation period at 28-37°C. The ICF was centrifuged at 410 g and stored at - 80°C.

### 3.6.5 Quantification of viral growth using focus assay

To determine the titre of viable virus particles in the parent sample (Com5) and plaque variants (L2 and S7) after they were purified, the virus titres of the ICF collected daily from each flask was determined using focus assay as described below: One hundred microlitres of CHIKV ICF (Com5, L2 and S7) was used to inoculate a confluent Vero cell monolayer in a 96-well plate. After incubation at 37˚ C for 1.5 hours, the infected cells were overlayed with 150 µl of 1.25% methylcellulose 4000 in 2% FBS in Minimum Essential Media. The plates were then incubated at 37˚C for 1 day. Then methycellulose was pippeted out and the plates washed three times with 0.1M Phosphate buffered saline (PBS) to remove methylcellulose, then fixed with 10% formalin in phosphate buffer solution (Wako, Osaka, Japan) overnight at room temperature. The plates were washed 3 times with PBS, and permeabilized with 200 µl of 1% Nonidet P-40 solution (Sigma Aldrich, St Louis, MO, USA) in PBS per well for 30 minutes at room temperature. Three washes of the plates with PBS were followed

by blocking with original concentration of Blockace (UK-B 80, Yukijirushi, Sapporo, Japan) for 30 minutes at room temperature. Focus immuno-staining was performed after the blocking. One hundred microlitres of rabbit sera with a high titre for anti-CHIKV IgG (diluted 1:500) was added and incubated for 1 hour at 37˚C, followed by a wash with PBS. Subsequently, 1:500 diluted HRP conjugated goat anti-rabbit IgG (American Qualex, San Clemente, CA) was added to the plates at 100 µl/well followed by incubation at 37˚C for 1 hour. The staining was visualized by the addition at 100 µl/well of a 0.5 mg/ml solution of substrate 3,3, diaminobenzidine tetrahydrochloride (DAB), (Wako, Osaka, Japan) in PBS with 0.03% of $H_2O_2$ added at room temperature for 30 minutes, and the staining reaction was allowed to proceed. After washing the stained cells with distilled water and air drying the plates, the number of foci per well were counted using an inverted microscope.

### 3.6.6 Whole genome sequencing of CHIKV isolates (Com5, L2 and S7) using Roche 454 pyrosequencing technology.

The parent sample (Com5) and its variants (L2 and S7) were sequenced to identify differences in the RNA sequences. CHIKV genomic RNA was extracted from the ICF of each isolate using TRIZOL LS Reagent (Life Technologies, Grand Island, NY, USA) as described in section 3.5.1. The isolated RNA was reverse transcribed into double stranded cDNA (ds-cDNA) using the modified random priming mediated sequence independent single primer amplification (RP-SISPA) methodology (Chen, Miller, DeRisi & Chiu, 2011).

The ds-cDNA was purified using a mini-elute kit (Qiagen, Hilden, Germany) and libraries constructed according to the Roche rapid library preparation manual. The libraries were sequenced using the GS FLX Titanium Sequencing Kit XLR70 (Roche, Branford, CT, USA) in combination with the matching GS FLX Titanium PicoTitrePlate Kit 70 × 75 (Roche) in a 454 Genome sequencer FLX (Roche, Branford, CT, USA) according to the manufacturer's instructions. The sequences were de-multiplexed to individual samples identity. Sequencing reads were trimmed using inbuilt sff tools (Roche) to remove barcodes and RP-SISPA primers. Reads below 100

bases and of low quality were discarded using custom scripts and Next generation sequencing quality control (NGS QC) Toolkit v.2.3.2. Assembly of contigs was performed using gs Assembler v 2.5.3 and the contig identities determined by searching the non-redundant (nr) database (NCBI) using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov). The contigs were further mapped against a reference sequence using CLC genomics workbench v 5.1 (http://www.clcbio.com/;CLCBio, Amino acidrhus, Denmark).The resulting partial genomes are available in GenBank under accession numbers for Com5, L2 and S7 are KP702297, KF283986 and KF283987 respectively. http://blast.ncbi.nlm.nih.gov/Blast.cgi]. These are 97.7 - 99.2 % complete genome sequences except for small regions in the 5' and 3' UTR regions

## 3.7 Development and evaluation of an antigen detection ELISA from CHIKV specific antibodies for the detection of CHIKV infections in mosquitoes homogenates .

### 3.7.1 Production and purification of CHIKV antigen for use in rabbit immunization

To prepare purified antigen for use in immunization of the rabbits to produce CHIKV specific antibodies, the CHIKV ICF was produced in large quantities requiring large scale Vero cell culture and then purified by sucrose gradient precipitation as described below.

#### 3.7.1.1 Preparation of Cytodex with normal saline

To increase the surface area on the culture flasks for vero cell attachment and growth, a micro-carrier (cytodex) was used. Cytodex is formed by biochemically coupling a thin layer of denatured collagen to the cross-linked dextran matrix and it's used to increase surface area for anchorage of adherent cells which increases the yield of the culture cells. In this study, ten grams of Cytodex (GE Healthcare Bio Science, Uppsala, Sweden) was mixed with 500 ml of normal saline, shaken and cytodex left to settle. The supernatant was removed and 300ml to 400ml of normal saline added.

Cytodex solution was shaken and left to settle. Washing of cytodex was repeated three times and finally 500ml of cytodex-Normal saline (10g in 500ml) solution was made, the solution was autoclaved at $121^o$ for 20 minutes, then stored at room temperature.

#### 3.7.1.2 Vero cell preparation

Large scale Vero cell propagation was done in culture flasks containing 150 $cm^3$ of the media. Vero cells were propagated in 2 culture flasks with a capacity of $150cm^3$. The cells were then passaged as described in section 3.3.2, from 2 to 4 culture flasks and incubated at 37° C for 2-3 days. The cells from the 4 culture flasks were passaged and then transferred to a 1 litre spinner bottle containing 500ml of growth media and 100ml of cytodex-normal saline. The cells were cultured in the spinner bottle with a magnetic stirrer bar at 37° C for 2 to 3 days, to achieve cells confluency. After three days, vero cell counts in the spinner bottle were performed to determine if the optimal cell concentration on the cytodex of 1 x $10^6$cells/ml was achieved by removing 1ml of cell suspension observing under the microscope to see what percentage of the surface of cytodex was covered by the cells (70% to 100% coverage is preferable).

#### 3.7.1.3 Inoculation of CHIKV to spinner bottle

To generate ICF for inoculation to spinner bottle, the virus was inoculated in two $75cm^3$ cell culture flasks and harvested as described in section 3.4. To inoculate the ICF to the spinner bottle, in the biosafety cabinet, the magnetic stirrer was stopped for a while to enable the un-attached and attached cells to cytodex to settle down. The spent medium was gently discarded from the spinner bottle (carefully to avoid discarding of the cytodex with cells) and 50 ml of the virus infected Vero cells added into one spinner bottle as shown on **Figure 3.1**, and incubated at $37^oC$ for 2 hours while gently mixing every 20 minutes. Eight hundred millimetres of EMEM with 2% FBS was then added into the spinner bottle, the cells were cultured with the magnetic stirrer running at a minimum speed and incubated at $37^oC$ for 2 to 5 days and the virus titre was monitored daily before harvest.

**Figure 3.1 Cell Culture in a spinner bottle for use in purification of CHIK**

#### 3.7.1.4 Harvesting of the CHIKV from the large scale culture.

To harvest the CHIKV from the ICF, the magnetic stirrer was stopped and the spinner bottles carried gently to the biosafety cabinet. Infected culture fluid was filtered using a large funnel (10 cm diameter) and Whatman filter paper to remove the dead cells and cytodex from the ICF. The filtered ICF was centrifuged at 8, 230 g for 30 minutes at 4°C. All the supernatants were pooled into one 2L or 3L jar, containing a big magnetic stirrer bar, 22.2 g of sodium chloride and 60 g of Polyethylene glycol- 6000 per 1L of 1CF. The solution was stirred very slowly at 4°C overnight, then transferred to 2 centrifuge tubes, centrifuged at 8,230 g for 30 minutes at 4°C and then the supernatants discarded. The faint precipitate on the surface of 2 centrifuge tubes was re-suspended with 0.1M saline Tris- EDTA (STE) buffer and then pooled together in one centrifuge tube. The pooled re-suspended virus (from 2 centrifuge tubes) was centrifuged at 12,860 g for 20 minutes at 4°C. After this centrifugation step, both the supernatant and precipitate were used in the preceding purification steps of the ICF. The second harvested supernatant was approximately 18 ml. The precipitate was re-suspended again with another 18 ml of STE buffer. Previously, a sucrose gradient of different concentrations (15 – 50% of sucrose) and different volumes as shown on Table 3.2, were prepared in two Beckman centrifuge two tubes. The total volume of the different concentrations of sucrose was 20 ml in each tube. To each tube containing the sucrose gradient, 18 ml of the supernatant was added to one centrifuge tube and the precipitate added to the second tube gently, giving a total of 40 ml per tube.

**Table 3.2  Preparation of a sucrose gradient for purification of viral antigen**

| Sucrose gradient (%) | 50 | 45 | 40 | 35 | 30 | 15 | sample | Total |
|---|---|---|---|---|---|---|---|---|
| Volume (ml) | 3 | 4 | 4 | 4 | 3 | 2 | 20 | 40 |

The two tubes containing sucrose gradient, supernatant/precicipate of CHIKV ICF, was ultracentrifuged at 33,500g for 14 to 16 hours at $4^{o}$C, and fraction collection done as shown on **Figure 3.2**.

**Figure 3.2  CHIKV purification using sucrose gradient and fraction collection**

The concentration of the purified CHIKV ICF that was collected as fractions, were measured using a spectrophotometer (OD280 and OD 260), the fractions with the highest titres were pooled together, aliquoted into 1 ml cryovials and the stored at -80° C. The protein concentration was determined using spectrophotometer (OD280 and OD 260), Protein concentration was determined using the following formular [Protein (mg/ml) = [(1.45 x A280) - (0.74 x A260) x Dilution Factor]. The purified CHIKV ICF was used for immunization of animals and as antigen for the indirect IgG ELISA.

### 3.7.2 Generation of rabbit serum containing CHIKV specific Pabs

#### 3.7.2.1 Inactivation of the purified CHIKV for immunization of rabbits:

One millilitre of purified CHIKV was mixed with 100µl of 10% formalin, and left overnight to inactivate. Equal volumes of inactivated antigen (0.25mg/ml) and Freunds complete adjuvant were mixed using two syringes and a three way stopcock.

#### 3.7.2.2 Housing and Immunization of rabbits

Two New Zealand white rabbits were obtained and housed in stainless steel cages in the KEMRI animal house where they were fed on pelleted rabbit diet and water. An initial blood collection was performed on each rabbit to test pre-immune serum. The rabbit was placed in a rabbit restraining box and the ear gently shaved and swabbed with 70% alcohol to disinfect it, then sample drawn using 22 to 26 gauge needle about 2-3 ml of blood. The needle was removed and pressure was applied to the blood vessel to stop the bleeding. To harvest post-immune sera, the same procedure was used. The rabbits were injected repeatedly subcutaneously with antigen/adjuvant mix to generate anti-CHIKV polyclonal antibodies (anti-CHIKV Pabs). In the first injection, the mixture was administered subcutaneously in 4 sites over the back and rump. A 22 to 26 gauge needle was used to inoculate purified virus at a concentration of 0.25 mg/mL (0.5mL /shot). After injections were completed, the immunization sites were checked for inflammation or any injury, the rabbit was monitored for several minutes and then returned to its cage. The rabbit was observed daily to check the immunization sites for inflammation. The subsequent immunizations were performed in 2-week interval.Ten additional immunizations were given, test bleeds were drawn later to check antibody titre. After a sufficient antibody response measuring an 0D490 of 3.00 after running the samples using IgG Indirect ELISA, a final blood sample was drawn by intra-cardiac (IC) method under anesthesia using ketamine at a concentration of 50mg/kg, intramuscularly, at a level suitable for surgery. Following the IC method, the rabbit was euthanized with ketamine without recovery from anesthesia. The remains of the rabbits were disposed of by incineration. The blood was dispensed from the syringes to a centrifuge tube containing heparin, then centrifuged at 410g, the serum aspirated off and then stored at -80 °C

### 3.7.3 Purification of rabbit serum containing CHIKV specific IgG

The rabbit serum containing CHIKV polyclonal antibodies (anti-CHIKV Pabs) was purified using saturated ammonium sulphate precipitation and protein G column chromatography using the following procedure. An equal volume of saturated ammonium sulphate was mixed with the anti-CHIKV Pabs (50%: final concentration of ammonium sulphate) and centrifuged at 9,800 g for 15 minutes at 4°C to remove the albumin. The precipitate was re-suspended in phosphate buffered saline (PBS) (pH 7.2), mixed with half the volume of saturated ammonium sulphate (33%: final concentration of ammonium sulphate) and then centrifuged at 9,800 g for 15 minutes at 4°C to remove the pseudoglobulin. The re-suspended precipitate was filtered through a 0.45 µm pores nitrocellulose membrane.

Protein G was used to purify anti-CHIKV Pabs further. Protein G purifies antibodies through its binding to the fragment antibody binding (Fab) and fragment crystallizable (Fc) region of antibody. The anti-CHIKV Pabs were bound and eluted using protein G column chromatography (HiTrap; GE Healthcare, Uppsala, Sweden) using the following protocol. A syringe was filled with distilled water, the top cap of the column was removed and connected to the syringe through the luer connector (or to a pump). The snap off end of the column outlet was removed and the ethanol preservative washed out using 5 ml of distilled water at approximately 1 drop/sec (approximately 2 ml/min). The column was equilibrated with at least 3 ml of binding buffer and the sample applied using a syringe or by pumping onto the column. The column was then washed with 5 to 10 ml binding buffer or until no material appeared in the effluent and eluted with 5 ml of elution buffer. The column was reconditioned with 5 ml binding buffer. The purified anti-CHIKV Pabs 1 ml fractions were collected and then stored at -80°C. The amount of IgG in each fraction was determined using the absorbance readings at $OD_{280nm}$ and $OD_{260nm}$ [IgG concentration (mg/ml) = (1.45 x $OD_{280nm}$ – 0.74 x $OD_{260nm}$) x dilution factor]. Peak fractions were selected and pooled and the purified anti-CHIKV Pabs was stored at -80 °C. The purified anti-CHIKV Pabs was later conjugated with HRP.

### 3.7.4 Conjugation of anti-CHIKV Pabs to Horse Radish peroxidase

To link the anti-CHIKV Pabs with HRP, conjugation was done (Nakane and Kawaoi, 1974) as follows, 4 mg of HRP (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 1ml of molecular grade water. 0.2 ml of freshly prepared 0.1M sodium periodate (Wako, Osaka, Japan) was added and mixed using a rotator for 2 hours at room temperature during which the brown colour of the HRP changed to green. This solution was dialysed using slide-A-Lyzer dialysis cassette G2 (Thermo Scientific - USA) in I mM sodium acetate buffer pH 4.4 at 4°C overnight. Buffer was changed before the end of the day, and this was routinely done on all overnight incubations. Dialysis of 8 mg anti-CHIKV IgG (5 ml) in 0.2M sodium carbonate buffer pH- 9.5 was done at 4°C and incubated overnight. The buffer was changed before the end of the day.

To conjugate HRP to anti-CHIKV Pabs , 20µl of 0.2M sodium carbonate buffer pH- 9.5 was added to the dialyzed HRP, then the mixture immediately added to the dialyzed anti-CHIKV Pabs and mixed using a rotator for 2 hours at room temperature. The tube with the solution (dialyzed HRP and dialyzed anti-CHIKV Pabs) was wrapped with aluminum foil, 0.1 ml of freshly prepared sodium tetrahydroborate (Wako, Osaka, Japan) added to the solution and mixed using a rotator for 2 hours at 4° C. The mixture was then dialyzed in phosphate buffered saline at 4° C overnight

To remove unconjugated anti-CHIKV Pabs, the conjugated anti-CHIKV Pabs was precipitated using saturated ammonium sulphate. The volume of dialyzed conjugated anti-CHIKV Pabs recovered was 5 ml to which 2.5 ml of saturated ammonium sulphate was added. This mix was kept at room temperature for 15 minutes and then centrifuged at same temperature at 4,600 g for 15 minutes. The precipitate was suspended in 5 ml PBS. Incubation and centrifugation of conjugated anti-CHIKV Pabs done above, at room temperature for 15 minutes was repeated twice. The solution was dialyzed in PBS at 4°C overnight, 40 mg of bovine serum albumin and an equal volume of biostab stabilizer (Sigma-Aldrich, St Louis, MO) were added to the

conjugated anti-CHIKV Pabs. The purified conjugated anti-CHIKV Pabs was stored in aliquots of 1 ml cryovials at -80 ºC. The optimal dilution of the newly prepared conjugate was determined by antigen detection ELISA.

## 3.8 Development of the antigen Detection ELISA using conjugated anti-CHIKV Pabs

### 3.8.1 Determination of best dilution of the HRP conjugated anti-CHIKV Pabs by antigen detection ELISA

To propagate CHIKV as one of the components of the antigen detection ELISA, the CHIKV seed stock (Com5) was cultured in Vero cells as described in section 3.4 above. The CHIKV seed stock was propagated and used to determine the best dilution of anti-CHIKV Pabs in antigen detection ELISA. The optimal dilution of the conjugated anti-CHIKV Pabs was determined as follows: Ninety six-well plates were coated with 20 µg/ml of unconjugated anti-CHIKV Pabs. After incubation at 4°C overnight, undiluted blockace was added to all the wells except the blank, incubated at room temperature for one hour, followed by four washing steps with PBST (PBS containing 0.05% [vol/vol] Tween 20). The CHIKV ICF was added to the wells except the blank. The plates were incubated for 1 hour at 37°C and washed four times. Conjugated anti-CHIKV Pabs at different dilutions (10x, 20x, 40x, ~ 1280x) were added to the wells, except the blank. The plate was incubated for 1 hour at 37°C and then washed. A peroxidase substrate buffer containing *o*-phenylenediamine (0.4 mg/ml in 0.1 M citrate-phosphate buffer [pH 5.0]) was added. The optimal concentration of anti-CHIKV Pabs was determined based on a desired ratio of positive to negative sera (P/N) of >2.0, in the positive control group (confirmed CHIKV ICF) and negative control of vero cells supernatant. The resulting color change was quantified by reading the optical density at 492 nm ($OD_{490}$) using an ELISA Reader (Multiskan Ex-Thermo Scientific-China). The $OD_{492}$ values were calculated by subtracting the absorbance in the blank with that of the wells with viral antigen. The optimum dilution of conjugated anti-CHIKV Pabs to use was 160 x dilution.

### 3.8.2 Evaluation of the developed antigen detection ELISA

Sensitivity of the developed antigen detection ELISA was obtained by doing a ten-fold dilution of CHIKV ICF (Com5), the various dilutions ( $10^0$ to $10^6$) were analyzed by antigen detection ELISA test and confirmed by RT-PCR to determine the detection limit. Specificity of the antigen detection ELISA was obtained by analyzing different viruses: alphaviruses (Sindbis virus, Ndumu virus, O`nyong nyong virus, Semliki forest virus) and bunyaviruses (yellow fever virus, dengue virus, rift valley fever virus) by the ELISA test and confirmed by RT-PCR.

### 3.8.3 Application of the antigen detection ELISA to CHIKV innoculated mosquito homogenates

#### 3.8.3.1 Mosquito inoculation and homogenization

The ability of the CHIKV specific antigen detection ELISA to detect pools of *Ae. Aeygpti* mosquitoes which were inoculated by artificial feeding with a CHIKV spiked blood meal is described below.These mosquitoes were collected as part of routine vector surveillance done by the VHF Laboratory. The mosquitoes were starved of sucrose and deprived of water for 24 hours prior to being fed for 1hour on infectious blood in membrane feeders covered with mouse skin and maintained at 37°C. The blood meal contained 1:1 ratio of CHIKV stock suspension and defibrinated rabbit blood containing 2.5 % sugar. Fully engorged mosquitoes were aspirated into 1 gallon plastic cages and reared for 21 days before sacrificing them. The mosquitoes were immobilized by exposure to triethylamine (TEA) and then the head, abdomen and legs separated on an ice block. The mosquito parts were put into pools of 25 heads, 25 abdomen and 25 legs, and then stored at - 80° C in the VHF laboratory. The mosquitoes pools were retrieved from storage and homogenized as described below.
Uninfected *Ae. aegypti* mosquitoes were used for negative controls. The mosquitoes were homogenized by adding one 4.5-mm-diameter, copper-clad steel beads and 1 ml of homogenization media (EMEM supplemented with 15% FBS and 2% Penicillin/streptomycin and 2% glutamine) into a 1.5-ml cryogenic, polypropylene,

round-bottom vials containing a pool of 25 mosquitoes. The tube was vortexed until all the mosquitoes was ground. The mosquito homogenate and steel beads were centrifuged in a microcentrifuge at 4$^{o}$C, at a speed of 18,500g for 5 minutes to create a pellet. Two hundred and fifty microliters of the supernatant were aliquoted into sterile cryovials, labeled and stored at -80$^{o}$. Aliquots of the supernatant were tested using antigen detection ELISA as described in section 3.8.1 and confirmed by RT-PCR as described in section 3.5.

#### 3.8.3.2 RT-PCR as a confirmatory test for samples analyzed by antigen detection ELISA

The confirmatory test for the CHIKV positive mosquito homogenates identified by antigen detection ELISA was RT-PCR. The pools of mosquito homogenates that were positive for CHIKV, were also analyzed by RT-PCR by amplifying a 1260 bp fragment located in the envelope region to confirm the results as described in section 3.5 using primers shown on **Table 3.1**.

## 3.9 Development and evaluation an in-house IgM ELISA from cell culture-derived antigens and antibodies for the diagnosis of CHIKV infections

Reagents required for development of the IgM Capture ELISA are: anti-human IgM capture antibody, CHIKV ICF as an antigen, conjugated anti-CHIKV Pabs, and substrate for colour development. CHIKV antigen, CHIKV HRP conjugated antibodies were developed in this study and anti-human IgM capture antibody and substrate for colour development were commercially sourced.

### 3.9.1 Evaluation of the CHIKV antigen as a component of the in-house IgM ELISA.

The CHIKV (Com5) was used as antigen for the the IgM capture ELISA. The antigen was titrated by a slight modification of the protocol described in section 3.8.1 above. The CHIKV antigen was used at different dilutions (10x, 20x, 40x, ~ 1280x), to determine its titre and ascertain that it would be sufficient for use as an antigen in IgM ELISA. The CHIKV antigen was found to be 160 ELISA units. The minimum

recommended concentration of an ELISA antigen is ≥ 100 ELISA units (Inoue *et al.*, 2010).

### 3.9.2 Optimization of the HRP conjugated anti-CHIKV Pabs for IgM Capture ELISA.

Using the CHIKV antigen titrated in the section 3.9.1 above and various dilutions of the conjugated anti-CHIKV Pabs (500x, 1000x, 1500, 2000x) made with (0.1M Phosphate buffered saline containing 0.05%Tween 20) PBS-T & 10% blockace, the working concentration of conjugated anti-CHIKV Pabs to be used in the IgM capture ELISA, was determined as described below.

A 96-well flat-bottomed microtitre ELISA plate (Maxi-sorp, Nalgene International, Roskilde, Denmark) was coated with 5.5 µg/100µl of anti-human IgM (µ-chain specific) goat IgG (MP Biomedicals LLC, France) diluted with coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) and incubated at 4°C overnight. The wells were blocked with Blockace (Yukijirushi Sapporo, Japan) at room temperature for 1 hour, and then washed four times with PBS-T. The test sera were diluted (1:100) in PBS-T and 100 µl aliquots of serum were distributed into duplicate wells. Human serum previously tested and known to contain anti-CHIKV antibody and negative sera were run on each plate as positive and negative controls. The plate was incubated at 37°C for 1 hour and then washed as described above. One hundred microlitres of CHIKV antigen (160 ELISA units) was added and incubated at 37°C for 1 hour. After washing, conjugated anti-CHIKV Pabs (500x, 1000x, 1500, 2000x diluted in PBS-T with 10% Blockace) was added to the wells and incubated for 1 hour at 37°C. After washing, 100µl of the substrate solution; *o*-phenylenediamine hydrochloride substrate (Sigma Aldrich) (final concentration 0.5mg/mL) and 0.03% hydrogen peroxide reconstituted in 0.05M citrate phosphate buffer (pH 5.0), was added to each well and incubated for 1 hour at room temperature in the dark. The reaction was stopped using 100 µl of 1N sulphuric acid and colour change detected at 492 nm ($OD_{492}$) on an ELISA Reader (Multiskan Ex, Thermo Scientific, China). A positive (or sample)

$OD_{492}$ /negative control $OD_{492}$ (P/N) ratio $\geq$ 2.0 was considered positive. The optimal dilution of HRP-conjugated anti-CHIKV rabbit pAbs was determined as 1,500x.

### 3.9.3 Evaluation of the in-house IgM ELISA using a panel of sera for detection of CHIKV.

To validate the in-house IgM ELISA, 148 archived human serum samples from the 2005 Comoros Island CHIKV outbreak (a mix of antibody positive and negative samples) which were previously tested by CDC IgM-capture ELISA (CDC ELISA), were also tested using the new in-house IgM ELISA and FRNT used as the gold standard.

#### 3.9.3.1 CDC IgM Capture ELISA

CDC ELISA is a monoclonal based ELISA system used in a previous study (Sergon *et al.,* 2008) and as described below.

For CHIKV-specific IgM detection, 96-well polystyrene ELISA plates were coated with a 1:1,000 dilution of anti-human IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD) overnight at 4°C. After five washes with phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBS-T), non-specific binding was blocked by adding 5% non-fat dry milk in PBS with 0.5% Tween-20 and incubated for 30 minutes at room temperature. Test serum was added at 1:400 dilution and incubated for 1 hour at 37°C. Each diluted test serum sample was added in quadruplicate, with two wells serving as positive control (with CHIKV antigen) and two wells serving as negative control. After adding a 1:40 dilution of CHIKV antigen (S-27; Centers for Disease Control and Prevention, Fort Collins, CO), plates were incubated overnight at 4°C before adding a HRP–conjugated alphavirus-specific monoclonal antibody 2A2C-3 (Centers for Disease Control and Prevention) at 1:6,000 dilution and further incubated for 1 hour at 37°C. The presence of CHIKV-specific antibodies was detected by adding ABTS [2,2′ amino-bis(3-ethylbenthiazoline-6-sulfonic acid)] substrate (Kirkegaard and Perry Laboratories), and the absorbance was read at 405 nm. Positive samples required a mean optical density (OD) value ≥ 0.2 above that of the negative control for each sample.

### 3.9.3.2 Focus Reduction Neutralization Test

Vero cells were passaged and 5 x $10^5$ cells were seeded per well in a 96-well cell culture plate. The plates were incubated at 37°C in 5% $CO_2$-atmosphere for 1 to 2 days until the cell monolayer was confluent. Using CHIKV titre determined previously by plaque assay, CHIKV was diluted up to 50-100 FFU/100µl with EMEM 2% FBS. Twenty to forty microlitres of human sample sera was diluted 10X in EMEM 2% FBS and incubated in a 56°C water bath for 30 minutes to inactivate the serum. The 10 x diluted and heat treated sera was 2-fold serially diluted from 20 x to 1,280 x with maintenance medium (EMEM 2% FBS). Equal volumes (200 µl each) of 50-100 FFU/100µl virus solution and the serially diluted sera (10 upto 1,280 x) were mixed in 48 wells plate, then incubated at 37°C for 1 hour in 5% $CO_2$ incubator.

All growth medium was discarded from 96 well -cell culture plate seeded with Vero cells, taking care not to scratch the monolayer with the pipette. Virus/serum solution (100 µl) was inoculated in each well, including control wells (one which had 50 FFU/100 µl virus only which would be 100% foci and one which contained no virus at all that would give 0 % foci) and the plates incubated at 37°C in 5% $CO_2$-atmosphere for 30 minutes. The virus/serum solution would generate % foci that is reduced, if the serum neutralized the virus. During incubation, the plates were repeatedly tilted every 20 minutes to disperse the inoculum. After 1.5 hours incubation, 250 µl of the 1.25% methylcellulose 4000 in 2% FBS in EMEM, was added to the wells, the plates incubated at 37° C in 5% $CO_2$-atmosphere for 1 day. After inoculation of the virus-antibody mixed solution, CPE would be observed after 36 hours.

Immunostaining was done as follows: After 1 day incubation period, the overlay medium was removed using a transfer pipette. Two hundred and fifty microlitres of 10% formalin solution was added to each well to fix the cells overnight, then the monolayer was rinsed once with PBS, 200 µl of 1% NP-40 solution was added, left at room temperature for 30 minutes, then rinsed thrice with PBS. The wells were blocked with undiluted Blockace for 30 minutes at room temperature and rinsed thrice with PBS. One hundred microliters of high titre unconjugated anti-CHIKV

Pabs (diluted 1:500) was added and incubated for 1 hour at 37˚ C, followed by a wash in PBS. Subsequently, 1:500 diluted HRP conjugated goat anti-rabbit IgG (American Qualex, San Clemente, CA) was added to the plates at 100 µl/well followed by incubation at 37˚C for 1 hour. The staining was visualized by the addition of 100 µl/well of a 0.5 mg/ml solution of substrate 3, 3, diaminobenzidine tetrahydrochloride (Wako) in PBS with 0.03% of $H_2O_2$ at room temperature for 30 minutes, and the staining reaction was allowed to proceed. After washing the stained cells with distilled water and air drying the plates, the number of foci per well were counted using an inverted microscope. The $FRNT_{50}$ was calculated by counting the number of foci in each well. The number of foci in the negative control well was considered as 100%. The dilution of serum which showed 50% reduction of focus number compared to the negative control well was considered the $FRNT_{50}$ and the titre was indicated using the reciprocal of dilution factor as the neutralization antibody titre.

## 3.10 Evaluation of the developed in-house IgM ELISA on febrile patient samples collected during a 2013 Dengue outbreak

After development and evaluation of the in–house IgM ELISA, the ELISA test was tested using 254 dengue negative febrile patient samples that were received in the VHF laboratory during the 2013 dengue outbreak in Kenya as part of outbreak response. These 254 outbreak samples had been tested and found to be DEN negative using a DENV IgM-capture ELISA developed by the Diagnostic Systems Division of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), USA and DENV RT-PCR. The sera were aliquoted and stored at -80 °C. These human samples were analyzed by the in-house IgM ELISA as described in section 3.9.2 and the samples that were positive for CHIKV IgM were confirmed using a neutralization test (FRNT) as described in section 3.9.3.2

## 3.11 Data Analysis

### 3.11.1 Sanger sequence analysis

To confirm the identity of the 5 independent viral isolates used in this study, the partial nucleotide and deduced amino acid sequence of E1 gene of CHIKV from Sanger sequencing were compared with the sequences of other CHIKV isolates/strains reported from different geographical regions of Asia and Africa. Nucleotide sequences generated were assembled into contiqs using DNA baser v3.5 (SRL 2011) and identified by Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast) search against Gen bank databases. Comparison of nucleotide and deduced amino acid sequences with reference strains of previously characterized CHIKVes was conducted using Fasta algorithm (Pearson and Lipman, 1988) applying default parameters. Multiple sequence alignments and phylogenetic analysis were performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 (Tamura *et al*., 2011).

### 3.11.2 454 Roche sequence analysis

The whole genome sequences of the parent strain and the plaque variants were obtained using a 454 Roche sequencer. A mapping assembly of the nucleotide sequence data from the 454 sequencer was done using GS Runmapper (Roche 454). Database homology search of the sequence was done using Blast version 2.2.4. Multiple sequence alignment was done using CLUSTAL X software version 2.0. Molecular Evolutionary Genetics Analysis (MEGA) software version 6.05 (Tamura *et al*., 2011) was used for phylogenetic analysis of the aligned genomes. Bootstrap resampling to determine confidence values on the groupings within trees was performed with one thousand replicates (Felsenstein, 1985) by use of neighbour joining tree method based on the number of nucleotide sequences. The sequences were aligned to available sequences of the 3 main lineages of CHIKV from East Central and South Africa, West Africa and Asia genotypes and other reference sequences available in the GenBank database using clustalW (Thompson, Higgins & Gibson, 1994)

### 3.11.3 Evaluation of serological diagnostics

To evaluate the antigen detection ELISA and in-house IgM ELISA, data was entered into IBM® SPSS® Statistics 20 software. A 2x2 contigency tables was used to calculate diagnostic parameters such as sensitivity and specificity. Sensitivity and specificity of antigen detection ELISA was defined by comparison with RT-PCR and in-house IgM ELISA was defined by comparison with CDC-ELISA and $FRNT_{50}$. Sensitivity is the proportion of true positive samples as identified by the reference assay, that are correctly identified as reactive by the test under evaluation (ELISA assays) and specificity is the proportion of true-negative samples that are identified by the reference assay, that are correctly identified as non-reactive by the test under evaluation (ELISA assays). The samples (serum and mosquito homogenates) with positive or negative diagnostic results were classified as true positives or true negatives if the same results were obtained by both laboratory tests: the serological test (IgM ELISA and antigen detection ELISA) and the reference tests ($FRNT_{50}$ and RT-PCR), respectively. The cut-off values for the 2 ELISA assays are a positive negative ratio (P/N ratio) of 2.00. Positive samples should have a P/N ratio of $\geq 2$ and Negative samples should have a P/N ratio of $\leq 2.00$. Agreement was assessed using Cohen`s Kappa statistic. Significance was determined at a $p<0.05$ at 95% confidence interval. The Cohan Kappa statistic value for each test was determined to measure the agreement of each test's results against the reference panel (Vidal, Yokomi, Moreno, Bertolini & Cambra, 2012). Cohan Kappa statistic value , $< 0.3$ is a weak agreement , 0.3 to 0.7 is a moderate agreement and .$> 0.7$ is a strong agreement. The correlation between the IgM titres and FRNT was obtained by drawing a correlation curve. The pearson correlation coefficient (r), $< 0.3$ is a weak correlation, 0.3 to 0.7 is a moderate correlation and $> 0.7$ is a strong correlation.

## 3.12 Ethical statement

The study was approved by the Kenya Medical Research Institute's Ethics Review Committee and animal care and use committee (SSC No. 1940). All human sera from CHIKV Comoros Island outbreak of 2005 and suspected dengue outbreak samples

collected in 2013 in Kenya, were archived samples, which were received in the VHF laboratory as part of response to arbovirus outbreak and no consent forms therefore was used. These samples were anonymized by the removal of all identifiers and they were assigned new codes without reference to patient information.

# CHAPTER 4

# RESULTS

## 4.1 Confirmation of the identity of 5 archived independent viral isolates and quantification of Com5 viral titre

In this study, 5 independent viral isolates were amplified by RT-PCR to confirm their identity and they were all positive for CHIKV as shown on **Plate 4.1.**

### 4.1.1 Molecular analysis of CHIKV isolates

The envelope 1 region of the 5 independent viral isolates were amplified by RT-PCR, then run on a 2% agarose gel to confirm their identity.

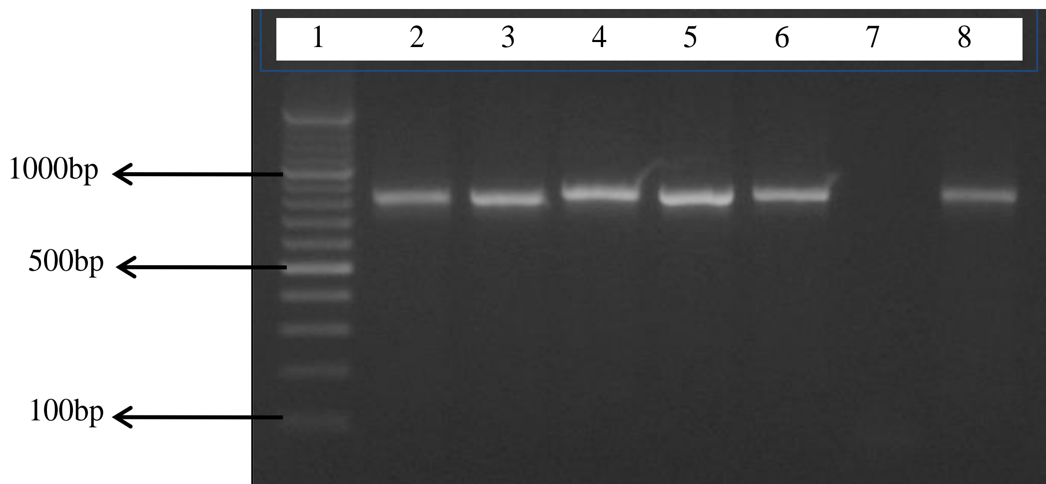


**Plate 4.1:Reverse transcriptase-polymerase chain reaction (RT-PCR) based detection of CHIKV RNA of 5 independent viral isolates. (1-100bp DNA Ladder, five viral isolates: 2 (72), 3 (73), 4 (74), 5 (75), 6 (76) were positive for CHIKV, 7 - Negative control (cell culture supernatant) and 8 -Positive control (CHIKV isolate). The expected product size of ~787bp of CHIKV envelope gene was detected.**

### 4.1.2 Phylogenetic analysis of the 5 independent viral isolates

Phylogenetic analysis was done using E1 sequences of the 5 independent viral isolates that were screened for this study, together with other selected CHIKV isolates from the GenBank. Phylogenetic analysis confirmed two distinct CHIKV lineages, one belonged to the West African genotype and the rest clustered with ECSA genotype. Three samples from Comoros Island (72, 74 and 75), clustered together with other Comoros Island strains and were closely related to Reunion Island and Lamu strains of CHIKV. The other 2 isolates (73 and 76) clustered together with the CHIKV S27, an African strain as shown in the **Figure 4.1**.

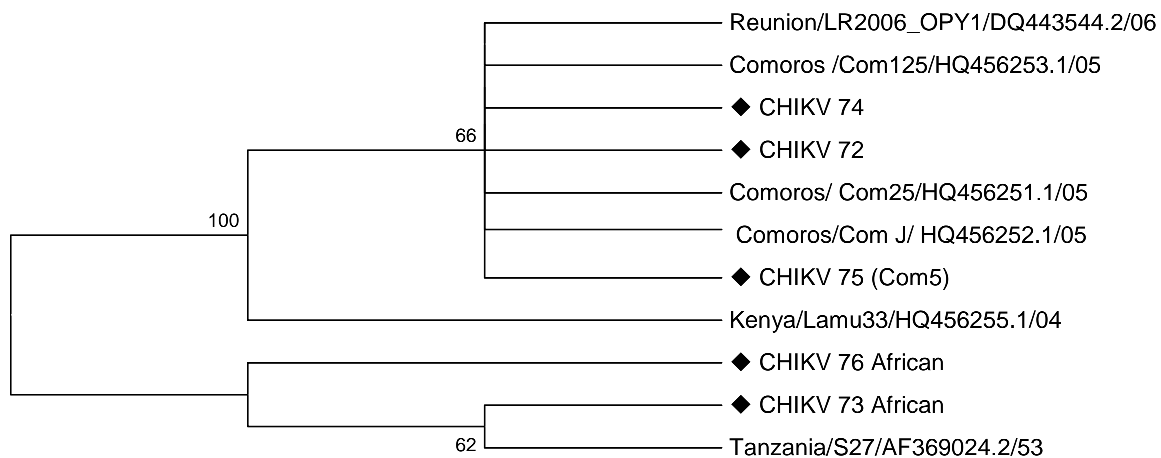


**Figure 4.1: Neighbor joining tree of CHIKV strains showing the genetic relationship of the envelope 1 region sequences of the 5 independent viral isolates from the repository that were screened for this study and sequences from other CHIKV strains obtained from the GenBank. The samples used in this study are indicated as◆. Isolates are labeled in this order: Country/strain name/accession number/year of sample collection.**

### 4.1.3 Quantification of Com5 using plaque

Out of the five independent viral isolates, one isolate (Com5) was selected for further analysis because it reflected the latest documented circulating strain among the 5 independent isolates. Quantification of the viral titre of the selected study isolate (Com5), was done by plaque assay. The viral titre of Com5 was 1.7 x $10^8$ PFU/ml. The plaques were of different sizes suggesting a co-circulating strain/virus in the Comoros Island isolate (Com5) as shown in **Plates 4.2 and 4.3.**

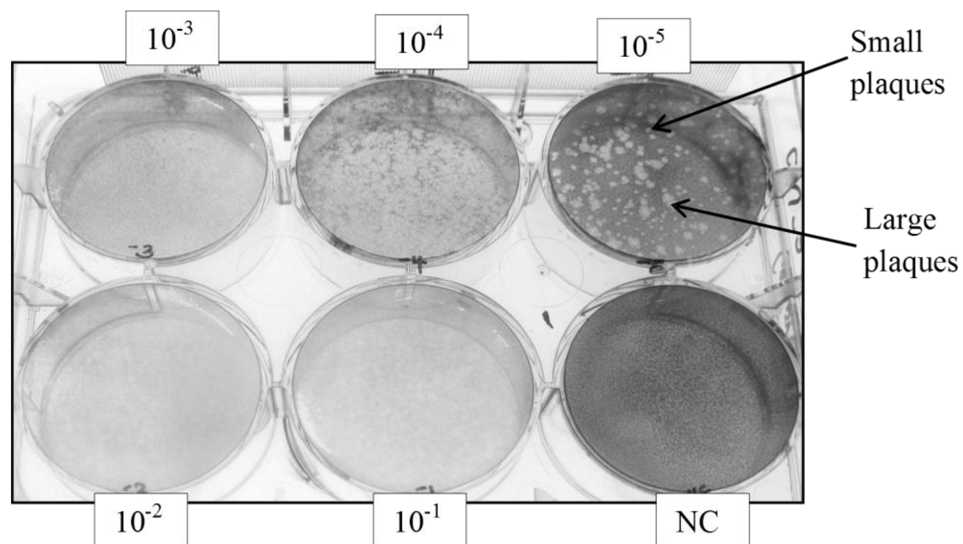


**Plate 4.2:Pictorial presentation of titrated Chikungunya virus, Com5 strain by plaque assay. The negative control was un-innoculated vero cells.**

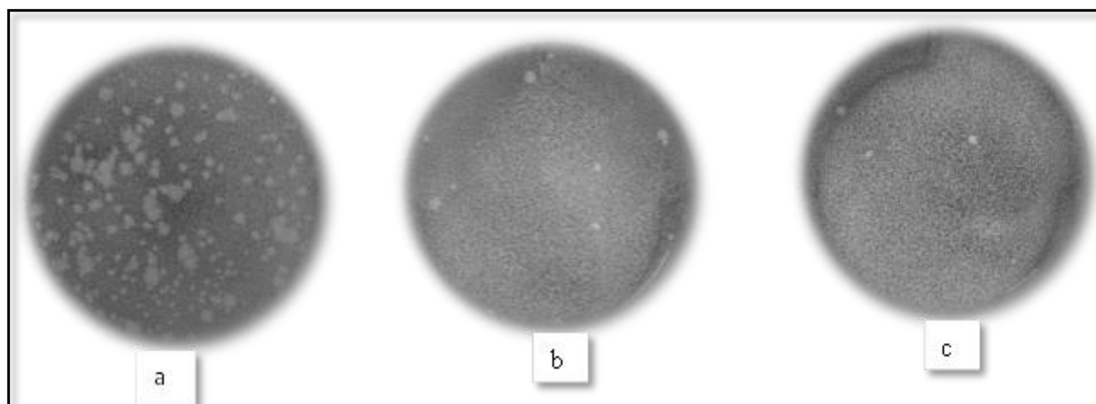


**Plate 4.3:Quantification of CHIKV titre, Com5 strain in PFU/ml.**

The wells that had quantifiable plaques were dilutions $10^{-5}$ (a), $10^{-6}$ (b) and $10^{-7}$ (c) as shown on **Plate 4.3**. The viral titres are as shown below.

**Table 4.1:Calculation of plaque forming units per millilitre for CHIKV, Com5 strain.**

| Dilutions of virus | - 5 | - 6 | - 7 | Total | Viral titre (pfu/ml) |
|---|---|---|---|---|---|
| Small plaques | 122 | 8 | 2 | 132 | $1.189 \times 10^{8}$ |
| Large plaques | 51 | 4 | 3 | 58 | $5.225 \times 10^{7}$ |
| Large and Small Plaques | 173 | 12 | 5 | 190 | **$1.7 \times 10^{8}$** |

**Plate 4.3** and **Table 4.1** shows the number of plaques that were quantifiable in different dilutions. This data was used to calculate the viral titre of the virus isolate used in the study.

## 4.2 Characterization of the CHIKV variants isolated from Comoros Island in the 2005 outbreak phenotypically and genotypically

### 4.2.1 Purified plaque variants

While performing plaque assays to titrate CHIKV in the parent strain, plaques of different sizes were observed. To separate the different plaque types, a single plaque of each size was selected using a glass capillary pipette and re-grown by cell culture. Two purified plaque variants were obtained and they retained their sizes after 2 to 3 passages confirming a stable phenotype (**Plate 4.4).**

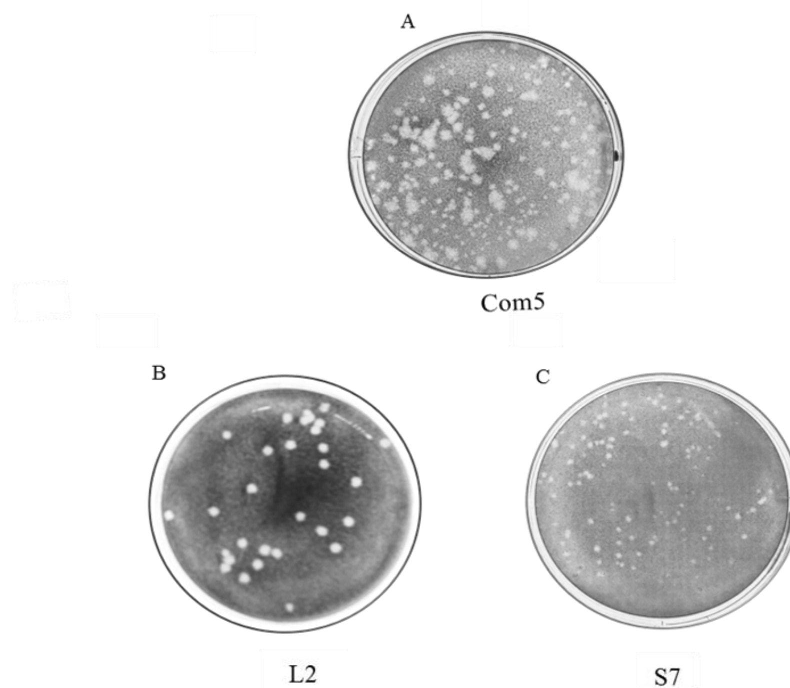


**Plate 4.4: Plaque phenotypes of the parent CHIKV isolate (Com5), the large (L2) and small (S7) plaque variants, 4 days post infection in Vero cells. Plaques of different sizes were observed in Com5, were plaque purified and re-analyzed by plaque assay to obtain the two purified plaque isolates. The parent strain is represented by A. The large plaque variant (L2) measured 1.5 ± 0.3 mm (B) and small plaque variant (S7) measured 0.5 ± 0.3 mm (C).**

### 4.2.2 In vitro growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7)

CHIKV replicates steadily up to day 2 of *in vitro* culture following inoculation in most cell lines until CPE reaches 100%, after which viral replication plateaus and virus viability begins to decline. Plaques of the same size demonstrates that the viral isolate is a pure strain, however, when the plaque assay shows plaques of different sizes, it implies there could be more than one strain in a viral isolate. Different viruses may present with different plaque sizes. A large plaque does not necessarily mean virulence. A virus can become virulent if a mutation occurs in its genome, affecting protein function. CHIKV S27 which is the reference strain has plaques of equal size. To test if the plaque sizes represented differences in growth kinetics, the virus titres at different time points of the 3 day growth cycle were calculated. The *in vitro* growth kinetics of the three virus isolates in Vero cells, showed S7 having the highest viral titre throughout the experimental period (**Figure 4.2**). At 1 to 2 DPI, S7 had the highest viral titre followed by L2 and then Com5, the difference in viral titre between the parent strain and its variants was however insignificant ($p>0.05$) . The viable viral titre for all the isolates declined at 3 DPI, with L2 and S7 having a higher titre than Com5 ($p>0.05$).

In C6/36 cells, S7 had the highest viral titre throughout the experimental period (**Figure 4.3**). At 1 DPI, S7 had the highest viral titre followed by Com5 then L2. The difference in viral titres between the parent strain and its variants was significant ($p=0.02$) at 1 DPI. The $p$ value was determined using student T-test. The viral titre for all the isolates declined at 2 DPI, with L2 and S7 having higher titres than Com5. These results confirmed that all the three virus isolates had higher viral titres in C6/36 than in Vero cells ($p=0.03$).

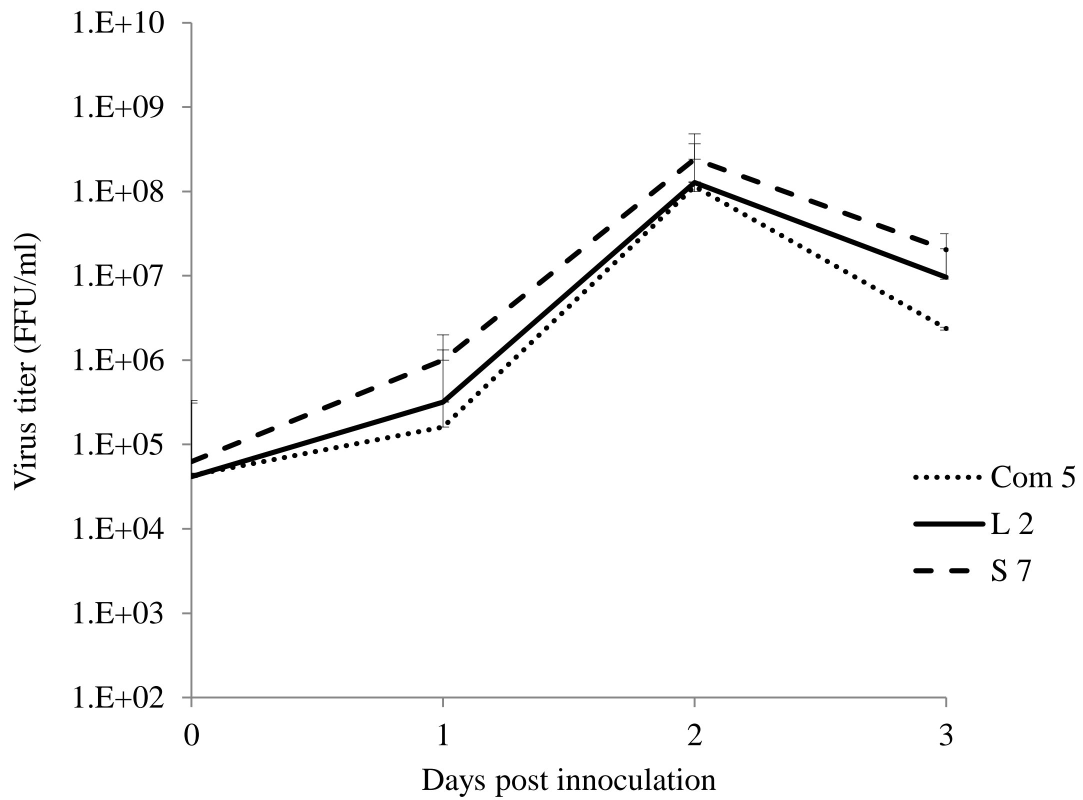


**Figure 4.2**:***In vitro*** **growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7) cultured on Vero, African green monkey kidney derived cells. Equal viral titre of each CHIKV variants were inoculated and cultured for 3 days. Viral titres at different time points post-inoculation were quantified by focus assay. Com5 (dotted line) = original isolate, L2 (solid line) = large plaque variant and S7 (dashed line) = small plaque variant. The data indicate an average of three independent experiments ± standard deviation. The differences in viral titre between the Com5, L2 and S7 at all the time points were not significant ($p>0.05$).**

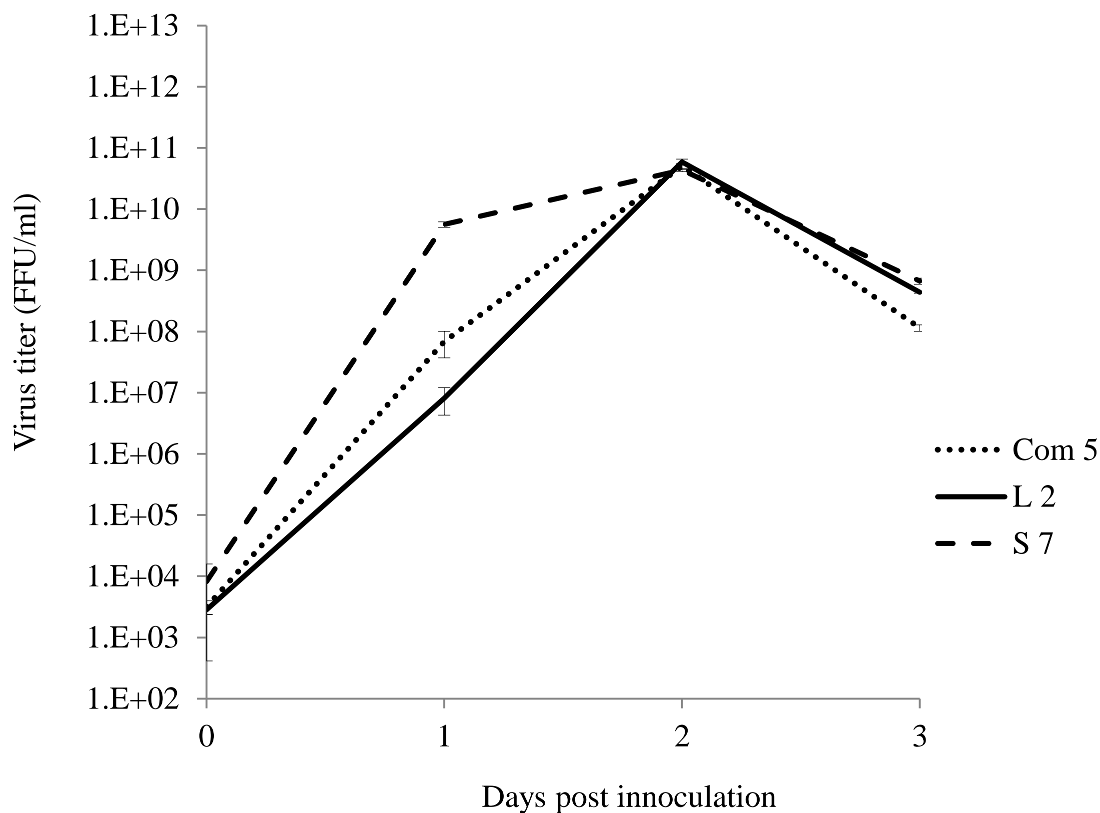


**Figure 4.3**: ***In vitro* growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7) cultured on C6/36, *Aedes albopictus* derived cells. Equal viral titre of each of the CHIKV strains was inoculated, cultured for 3 days and viral titres quantified by focus assay. Com5 (dotted line) = original isolate, L2 (solid line) = large plaque variant and S7 (dashed line) = small plaque variant. The data indicate an average of three independent experiments ± standard deviation. The difference in viral titre between the Com5 and the plaque variants (L2 and S7) at 1 DP1 was significant ($p<0.05$). S7 had the highest viral titre throughout the experimental period.**

### 4.2.3 Phylogenetic analysis of Com5, L2, S7 and selected isolates from CHIKV outbreaks in the Indian Ocean Islands and surrounding countries

Plaque assay and *in vitro* growth kinetics established the fact that, there were differences in infection pattern between the parent strain and plaque variants. This prompted the analysis of the nucleotide sequences of these plaque variants along with sequences of other CHIKV isolates obtained from the GenBank, to determine their genetic relationship by performing phylogenetic analysis . The plaque variants (L2 and S7) clustered closely with ComJ and with the Indian Ocean Island clade of CHIKV strains which consist of isolates from Comoros Island, Reunion Island, India and Sri Lanka **(Figure 4.4).** The other Comoros Island isolates (Com25 and Com125), Reunion Island and Kenyan isolates (Lamu33 and KPA15), clustered together reflecting both a close genetic and geographic relationship between these strains **(Figure 4.5).** Large plaque variant, Small plaque variants and ComJ clustered in a sub-clade within the ECSA genotype. The bootstrap values less than 70% have been shown on **Figure 4.5** to demonstrate the minor difference between the study isolates.

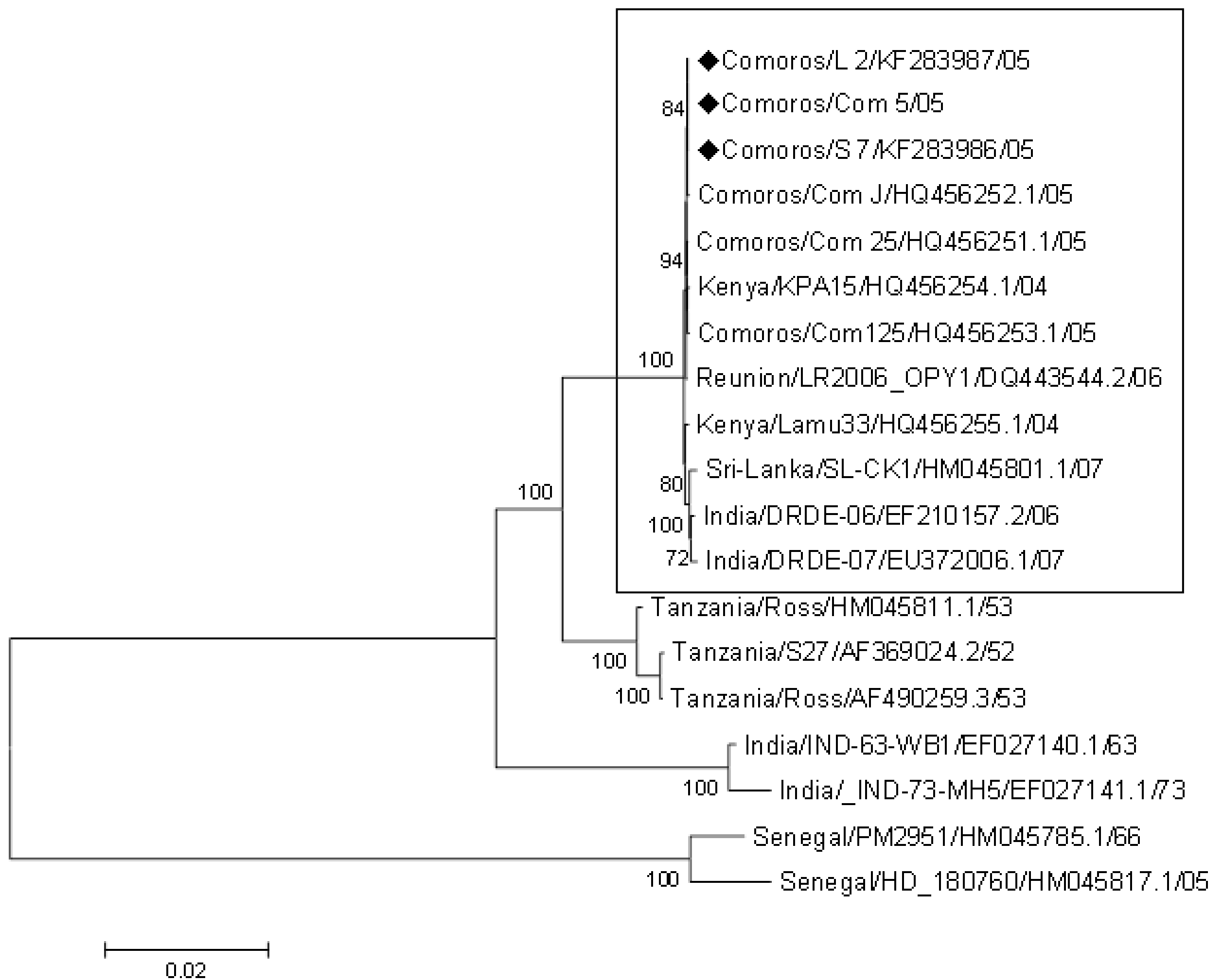


**Figure 4.4**:**Neighbour joining phylogenetic tree of nucleotide sequences of whole genome of Com5, L2, S7 and S27 prototype sequences. Sequences of plaque variants under study indicated by (♦) were compared with selected CHIKV strains from the GenBank. Whole genome sequences of plaque variants and reference sequences were aligned using clustal W and a phylogenetic tree constructed using MEGA v5.05. Numbers on the internal branches indicate bootstrap values of 1000 replicates. CHIKV genotypes represented in the phylogenetic tree are the ECSA, West African and Asian genotypes.**

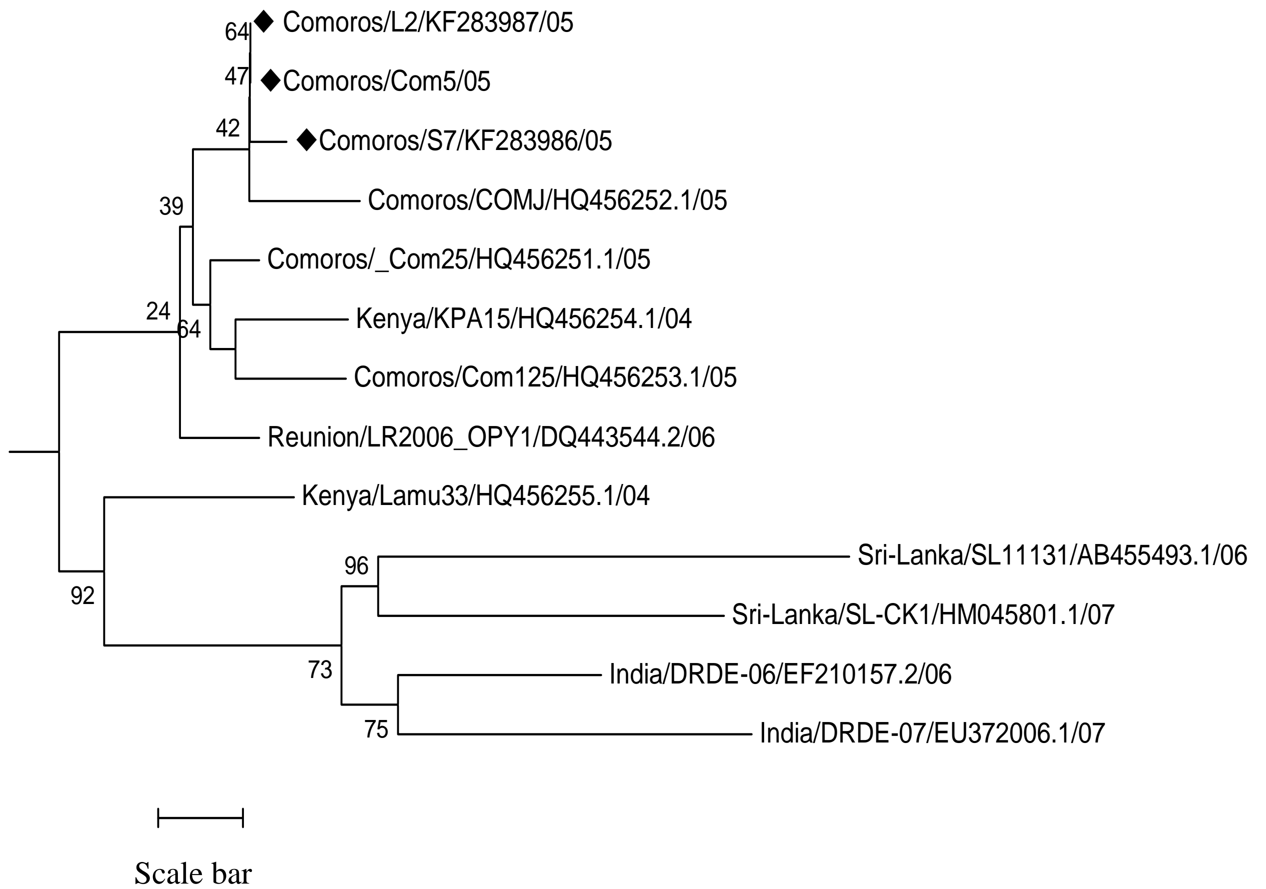


**Figure 4.5:A magnified phylogenetic sub-tree of nucleotide sequences of whole genome of CHIKV isolates in the ECSA lineage. This sub-tree is highlighted in Figure 4.5. Sequences of plaque variants under study indicated by (◆) were compared with selected CHIKV strains from the GenBank. Bootstrap values are indicated on branches. Scale bar indicates nucleotide substitutions per site. The plaque variants obtained in this study clustered closely with CHIKV Comoros isolates and Re-union Island.**

### 4.2.4 Genotypic variations in the plaque variants

Comparative analysis of the parent and plaque variants nucleotide and protein sequences with other CHIKV sequences from the GenBank was done, to determine if the amino acids changes observed among the plaque variants are new or have been observed previously from isolates in the same outbreak. Near complete whole genome sequences were obtained, deposited in GenBank and assigned the following accession numbers: Com5 (KP702297), L2 (KF283986) and S7 (KF283987) [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. The length of the genomic sequence obtained for Com5 is 11,171 nucleotides, for L2 is 11,537 nucleotides and 11,712 nucleotides for S7 which excludes small regions of the 5' and 3' UTR regions for which sequences were not obtained. When the RNA sequences were aligned, the overlapping regions of the two plaque variants were 99.93% identical. The polyproteins were identical except for two amino acid substitutions. One was a missense amino acid substitution at amino acid position 642 located in the nsP2 region, where L2 had a Tyrosine (Y642) and S7 (C642) had a Cysteine. The second one was a nonsense amino acid substitution at amino acid position 524 in the nsP3 region, where L2 had an arginine (R524) and S7 had an opal stop codon (X524) with reference to the S27 prototype strain **(Table 4.2)**. On examining these substitutions and the E1: A226V mutation in other Kenyan and Indian Ocean CHIKV isolates, Cysteine was observed in S7 and S27 prototype strain only and not in any other isolate whereas Arginine (R524) was unique to L2 and the S27 prototype strain, the stop codon (CGA)/(X524) was present in all other isolates. Envelope 1: A226V was only present in the Reunion Island isolate and one Indian (DRDE-07) isolate (**Table 4.2**). Genome wide alignments of amino acids of the plaque variants (L2 and S7) using the S27 prototype strain as the reference sequence was carried out to monitor evolution of the CHIKV overtime. Thirty amino acid changes in the non-structural protein (nsP) sequences (**Table 4.2**) and 22 amino acid changes in the structural proteins (sP) sequences. (**Table 4.3**) were observed.

**Table 4.2: Comparisons of amino acid sequences of the non-structural proteins of Com5, L2 and S7 and selected CHIKV Indian Ocean isolates using S27 prototype strain as the reference sequence**

| Non-structural protein | nsP1 | | | | | | | | nsP2 | | | | | nsP3 | | | | | | | | | | | | nsP4 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polypeptide position | 171 | 172 | 234 | 383 | 384 | 481 | 488 | 507 | 589 | 909 | **1177** | 1178 | 1328 | 1508 | 1550 | 1659 | 1664 | 1670 | 1685 | 1709 | 1715 | 1794 | 1795 | 1804 | **1857** | 1938 | 2117 | 2363 | 2377 | 2418 | 2467 |
| Amino acid position | 171 | 172 | 234 | 383 | 384 | 481 | 488 | 507 | 54 | 374 | **642** | 643 | 793 | 175 | 217 | 326 | 331 | 337 | 352 | 376 | 382 | 461 | 462 | 471 | **524** | 75 | 254 | 500 | 514 | 555 | 604 |
| Tanzania/S27/53 | R | L | E | M | I | T | Q | L | S | H | **C** | S | A | V | Y | P | V | T | K | I | A | L | S | P | **R** | T | T | Q | I | V | V |
| Kenya/Lamu33/04<br>Kenya/KPA15/04<br>Comoros/Com 25/05<br>Comoros/Com 125/05<br>Reunion/LR-2006/06<br>India/DRDE-06 /06<br>India/DRDE-07/07 | R | V | K | L | L | I | R | R | N | Y | **Y** | N | V | I | H | S | A | I | E | T | T | P | N | S | **X** | A | A | L | T | I | I |
| Comoros/Com J/05<br>**Comoros /L2/05** | Q | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | V | – | – | – | – | – | – | – | **R** | – | – | – | – | – | – |
| **Comoros /S7/05** | Q | – | – | – | – | – | – | – | – | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Sri-Lanka/SL-CKI/07 | Q | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

Isolate and the two amino acid substitutions of the present study are indicated in bold. (–) denotes similarity to amino acids of the Lamu33 strain at corresponding positions. Selected isolates are Indian Ocean outbreak isolates that occurred before and after the Comoros Island isolates that are under study.

**Table 4.3: Comparisons of amino acid sequences of the structural proteins of Com5, L2 and S7 and selected CHIKV Indian Ocean isolates using S27 prototype strain as the reference sequence**

| Structural protein | C | E3 | E2 | | | | | | | | | | | | | | 6K | | E1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polypeptide position | 63 | 284 | 382 | 399 | 404 | 485 | 489 | 506 | 519 | 536 | 592 | 624 | 637 | 669 | 700 | 711 | 756 | 802 | 1035 | 1078 | 1093 |
| Amino acid position | 63 | 23 | 57 | 74 | 79 | 160 | 164 | 181 | 194 | 211 | 267 | 299 | 312 | 344 | 375 | 386 | 8 | 54 | 226 | 269 | 284 |
| Tanzania/S27/53 | K | I | G | I | G | N | A | L | S | I | M | S | T | A | S | V | V | I | A | M | D |
| Kenya/Lamu33/04<br>Kenya/KPA15/04<br>**Comoros/L2/05**<br>**Comoros /S7/05**<br>Comoros /Com J/05<br>Comoros /Com 25/05<br>Comoros/Com 125/05<br>India/DRDE-06 /06<br>Sri-Lanka/SL-CKI/07 | R | T | K | M | E | T | T | M | G | T | R | N | M | T | T | A | I | V | A | V | E |
| Reunion/LR-2006/06<br>India/DRDE-07/07 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | V | – | – |

Isolate and plaque variants of the present study are indicated in bold. (–) denotes similarity to amino acids of the Lamu33 strain at corresponding positions. Selected isolates are Indian Ocean outbreak isolates that occurred before and after the Comoros Island isolates that are under study. Accession numbers of the selected isolates showed in Table 4.2 and Table 4.3 are: S27 (AF369024.2), Lamu33 (HQ456255.1), KPA15 (HQ456254.1), Com J (HQ456252.1), COM25 (HQ456251.1), COM125 (HQ456253.1), DRDE-06 (EF210157.2), SL-CKI( HM045801), LR2006-OPYI (DQ443544.2), DRDE-07 (EU372006.1). The isolates are named in this order: country of origin/strain name/ year of sample collection.

## 4.3 Development and evaluation of antigen detection ELISA from CHIKV specific antibodies using CHIKV innoculated mosquito homogenates

### 4.3.1 Purification of anti-CHIKV Pabs ( rabbit serum containing CHIKV specific IgG)

CHIKV was cultured, propagated in large scale in Vero cells and the ICF was harvested, filtered, ultra-centrifuged and ran on a sucrose gradient to obtain purified CHIKV. The total amount of purified virus obtained was 2.444 mg/9ml and 3.48 mg/6ml in the two trials. The purified virus was used for immunization of rabbits. After repeated injections of the rabbits, anti-CHIKV antibodies were collected and purified using ammonium sulphate precipitation and protein G chromatography. The fractions collected during purification of these antibodies are shown in **Figure 4.6.** The fifth fraction gave the highest peak and had the highest anti-CHIKV antibody concentration; therefore it was used for conjugation to HRP.

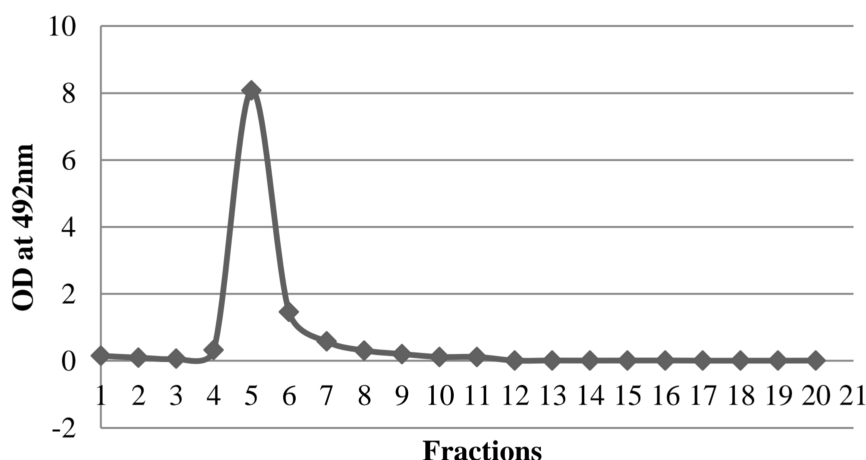


**Figure 4.6:Absorbance at 492nm of fractions collected after ammonium sulphate precipitation and protein G purification of CHIKV Polyclonal antibody .**

### 4.3.2 Development of the antigen detection ELISA to detect CHIKV in mosquito homogenates

A sandwich antigen detection ELISA protocol was developed using purified anti-CHIKV Pabs as the coating antibody, various dilutions of CHIKV ICF were added (negative was
un-innoculated Vero cell supernatant) and conjugated purified anti-CHIKV Pabs were used as detector antibody.The results are shown on **Table 4.4**. The best dilution of the detector antibody was 160x. This was arrived at by considering the negative controls OD which should be below 0.1 and positive controls OD that should be between 1.5 to 2.0.

**Table 4.4:Determination of the optimum dilution of anti-CHIKV Pabs for use as a detector antibody in antigen detection ELISA. Positive control was CHIKV ICF and negative control was un-inoculated Vero cell supernatant**

| Dilution of conjugate | Positive | Negatives | P/N Ratio |
|---|---|---|---|
| 10x | 2.96 | 1.60 | 1.85 |
| 20x | 2.86 | 1.28 | 2.24 |
| 40x | 2.35 | 0.87 | 2.70 |
| 80x | 1.90 | 0.53 | 3.59 |
| 160x | 1.40 | 0.30 | 4.69 |
| 320x | 0.88 | 0.17 | 5.11 |
| 640x | 0.57 | 0.11 | 5.10 |
| 1280x | 0.45 | 0.07 | 6.06 |
| 2560x | 0.31 | 0.04 | 7.90 |
| 5120X | 0.18 | 0.03 | 7.16 |
| *10, 240X* | 0.1025 | 0.02 | 5.13 |

### 4.3.3 Testing specificity of antigen detection ELISA using different arboviruses

To evaluate the antigen detection ELISA, the assay was used to test different arboviruses to assess its specificity. Different alphaviruses were used to check for cross-reactivity that is common among viruses in this family/genus. Flaviviruses and bunyaviruses were included in the test to exclude their reactivity with the assay. This assay was developed to detect CHIKV in serum or mosquito homogenates.The specificity of the assay was tested using ICF from different arboviruses, with positive results (P/N Ratio $\geq$ 2.00) being observed in the positive control (CHIKV ICF). Negative results (P/N Ratio $\leq$ 2.00) were shown by the flaviviruses, Sindbis virus and Ndumu virus. While, O`nyong nyong virus showed a weak (**Figure 4.7**).

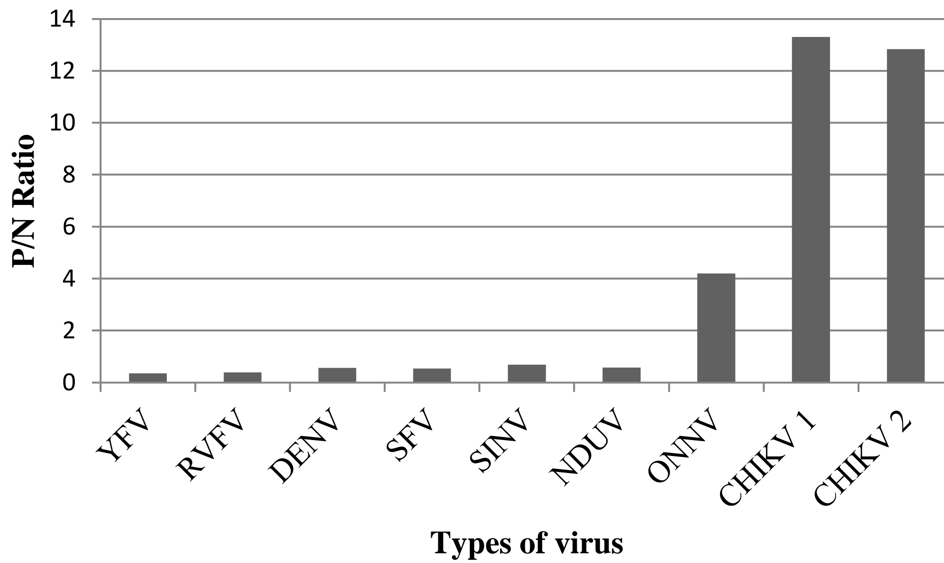


**Figure 4.7:Specificity of antigen detection ELISA was done using different alphaviruses and flaviviruses and bunyaviruses**

### 4.3.4 Testing sensitivity of antigen detection ELISA at different dilutions of CHIKV antigen.

The sensitivity of the assay was assessed using a serially diluted CHIKV isolate (initial concentration of 1.7 x$10^8$ PFU/ml), tested using the antigen detection ELISA protocol. A standard curve for the 10-fold diluted CHIKV stock was constructed. Supernatant from un-inoculated Vero cells was used to set the baseline. The result showed that the minimum concentration of virus detectable by the assay is 1.7 x $10^{3}$ PFU/ml (**Figure 4.8).** The serially diluted CHIKV ICF, was also analyzed by RT-PCR to determine the detection limit. The detection limit of RT-PCR was 1.7 x $10^{5}$PFU/ml (**Plate 4.5**).

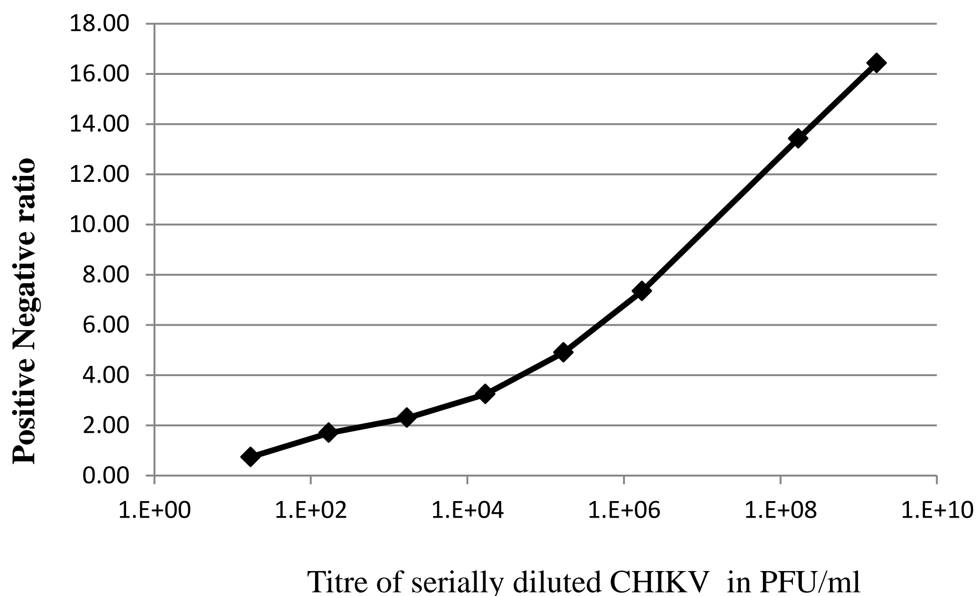


**Figure 4.8:Graph of 10-fold serially diluted CHIKV (1.7 x108 PFU) tested by antigen capture ELISA to assess sensitivity of the virus isolate. The cell culture supernatant cultured in Vero cells was used as the negative control. Cut-off is P/N ratio ≥ 2.00.**

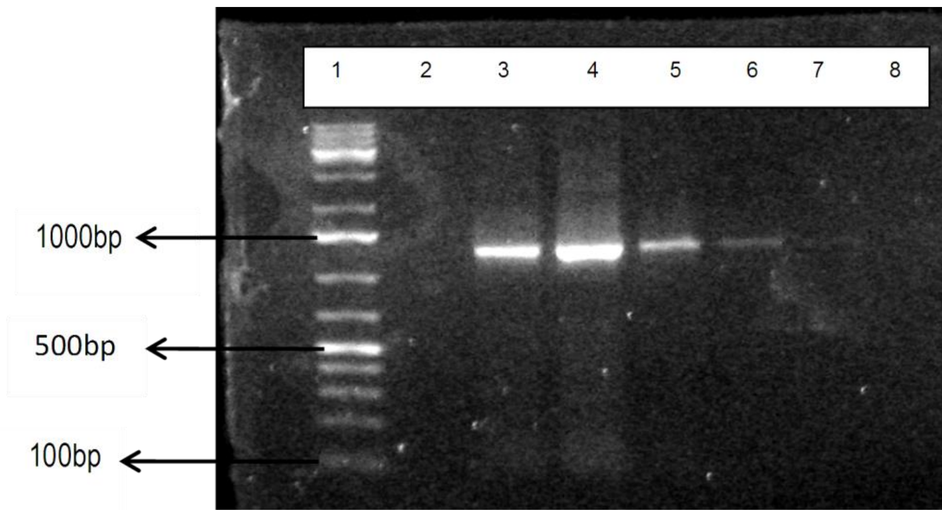


**Plate 4.5:Bands of serially diluted CHIKV, amplified by RT-PCR to observe the expected product size of ~787bp in the envelope 1 region of the CHIKV genome to obtain the detection limit of Com5. 1 (DNA Ladder), 2 (negative control), 3 (1.7 x 108 PFU/ml), 4 (1.7 x 107 fu/ml), 5 (1.7 x 106 PFU/ml),6 (1.7 x 105 PFU/ml), 7 (1.7 x 104 PFU/ml), 8 (1.7 x 103 PFU/ml).**

### 4.3.5 Mosquito homogenates tested by the antigen detection ELISA

Having assesed the detection limit of the antigen detection ELISA and confirmed by CHIKV RT-PCR using CHIKV ICF, the assay was used to detect CHIKV *in vivo* in mosquitoes. Laboratory fed-mosquitoes's, that were pooled and homogenized, both the un-innoculted mosquitoes (negative controls) and those that were fed/inoculated with CHIKV were tested using the developed antigen detection ELISA and 7 out of 48 samples were positive by ELISA as shown on **Table 4.5.**

**Table 4.5:Positive/Negative OD (P/N) Ratio of antigen detection ELISA results of CHIKV positive mosquito homogenate pools and their status by both ELISA and RT-PCR.**

| Sample ID | P/N ratio (ELISA) | ELISA status | RT-PCR Status |
|---|---|---|---|
| 5 | 5.32 | pos | pos |
| 6 | 3.7 | Pos | Neg |
| 10 | 2.47 | pos | pos |
| 13 | 3.56 | pos | pos |
| 14 | 3.15 | Pos | Neg |
| 15 | 3.9 | Pos | Neg |
| 16 | 3.7 | pos | pos |

The same panel of laboratoty fed mosquito homogenates were analysed by RT-PCR (**Plate 4.6** and **Plate 4.7**). Four out of 48 samples (5, 10, 13 and 16) were positive as shown in Plate 4.5. All the remaining mosquito supernatants were all negative.

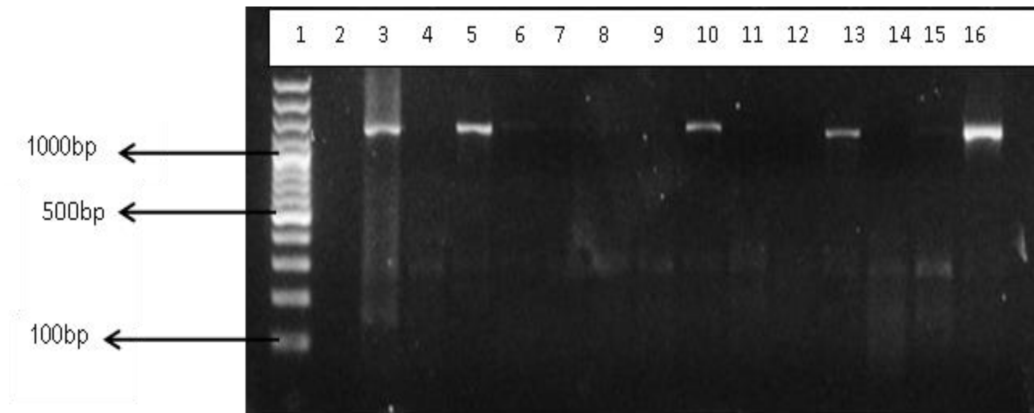


**Plate 4.6: RT-PCR based detection of the E1 segment of the envelope gene of CHIKV RNA from mosquito homogenates. The positive samples showed the expected band size of ~1260 bp. 1 - DNA Ladder, 2 -Negative control (un-innoculted homogenized mosquitoes pool), 3- positive controls (fed/innoculted homogenized mosquitoes pool) and 4-13 mosquito homogenates under investigation.**

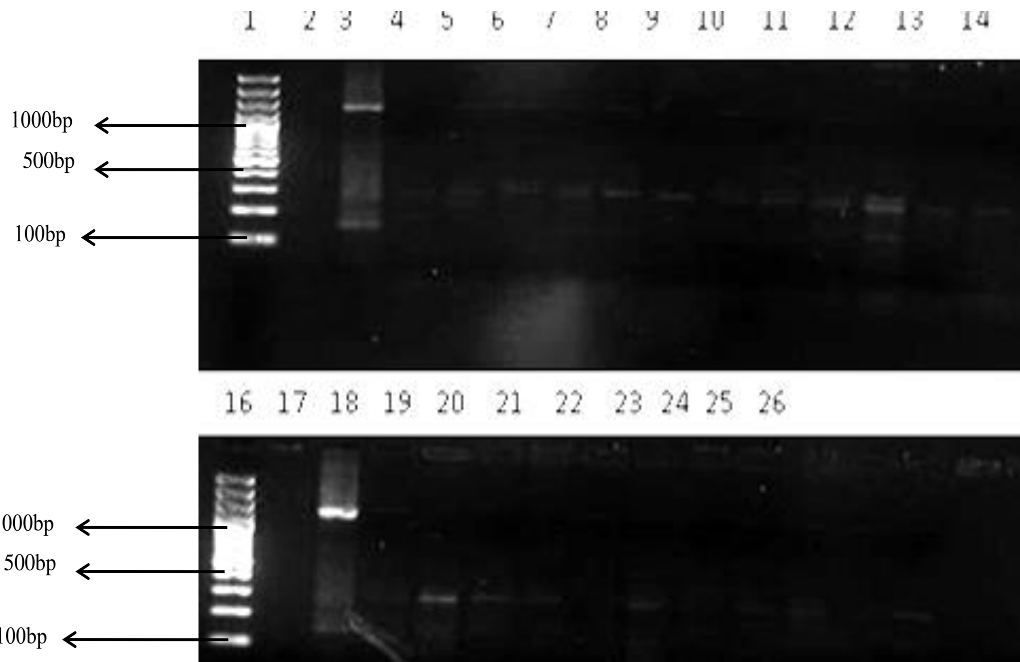


**Plate 4.7:RT-PCR based detection of CHIKV RNA from mosquito homogenates. The positive control showed expected band size of ~1260bp. 1-DNA ladder , 2- Negative control (un-innoculted homogenized mosquitoes pool), 3-positive controls (fed/innoculted homogenized mosquitoes pool) and 4-23 laboratory infected mosquito homogenates under investigation. All the samples run on this gel were negative. All the remaining 18 samples were also negative.**

### 4.3.6 Assessing sensitivity and specificity of the antigen detection ELISA using RT-PCR as a comparative assay.

Comparative analysis of antigen detection ELISA and RT-PCR was done using mosquito homogenates. Four (4) samples were positive and 43 negative by both tests. Sensitivity of 100% and specificity of 93.2 % was achieved. Cohan kappa agreement of 0.7 was calculated fro the data shown in **Table 4.6**.

**Table 4.6:Test of mosquito homogenates using antigen detection ELISA and confirmed by RT-PCR**

| | | RT-PCR (n = 48) | | |
|---|---|---|---|---|
| | | Positive | Negative | **Total** |
| **Antigen -detection ELISA** | Positive | 4 | 3 | 7 |
| | Negative | 0 | 41 | 41 |
| | **Total** | 4 | 44 | 48 |

## 4.4 Development and evaluation of in-house IgM-Capture ELISA

### 4.4.1 Assessing the best dilution of CHIKV antigen as one of the reagents for use in-house IgM ELISA

The optimum dilution of CHIKV antigen for in-house IgM ELISA protocol was obtained by titrating the antigen using antigen detection ELISA (**Table 4.7**). The antigen was equivalent to 160 ELISA units which is sufficient concentration for an in–house IgM ELISA antigen (recommended titre is above 100 ELISA units).

**Table 4.7:Titration of CHIKV antigen using antigen detection ELISA as an antigen for in-house IgM ELISA.**

| Dilution of antigen | | ELISA units |
|---|---|---|
| Original CHIKV | 2.1155 | 160 |
| 10X | 0.891 | 16 |
| 20X | 0.61 | 8 |
| 40X | 0.427 | 4 |
| 80X | 0.2485 | 2 |
| 160X | 0.154 | 1 |
| 320X | 0.1 | Baseline |
| 640X | 0.07 | |
| 1280X | 0.079 | |
| 2560X | 0.0375 | |
| 5120x | 0.035 | |
| 10240x | 0.039 | |

### 4.4.2 Determining the optimal dilutions of the newly developed conjugated anti-CHIKV Pabs using the inhouse IgM ELISA

To determine the optimal concentration of the conjugated CHIKV Pabs for use in the in-house IgM ELISA, the conjugated antibody was used at different dilutions (**Figure 4.9).** The best dilution for the serially diluted HRP conjugated anti-CHIKV Pabs for in-house IgM ELISA Protocol was 1:1500. Its recommended that the best dilution should be selected based on negative controls OD which should be below

0.1 and positive controls OD that should be between 1.5 to 2.0.

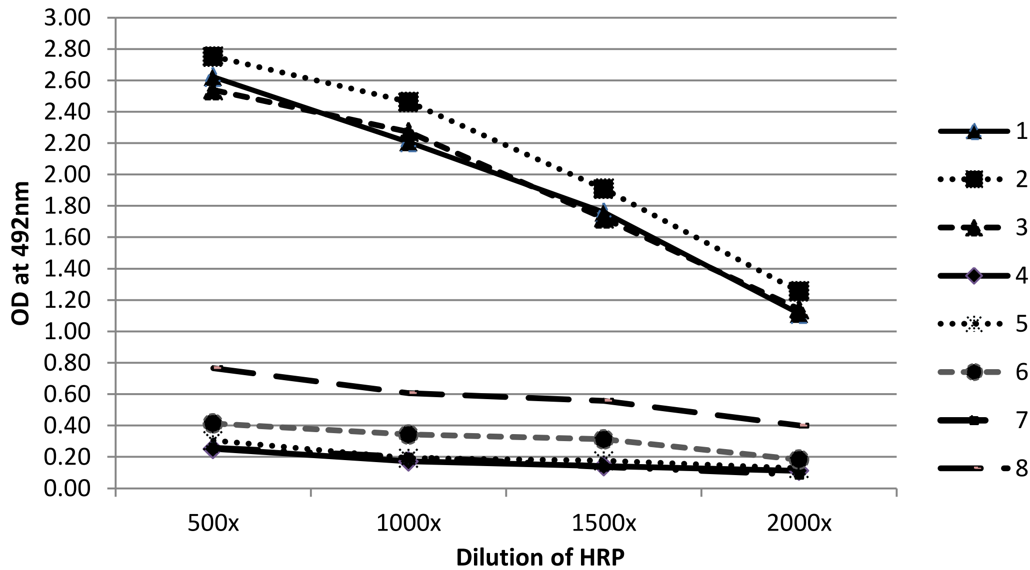


**Figure 4.9:Serial dilutions of conjugated anti-CHIKV Pabs used as detector antibody in the in-house IgM ELISA assay to determine the assay`s optimal concentration,where 1 to 3 represent positive sera and 4 to 8 represent negative sera.**

### 4.4.3 Assessing the sensitivity and specificity of the in-house IgM ELISA.

The in-house IgM ELISA was evaluated by analyzing a panel of febrile patient sera from the Chikungunya outbreak of 2004-2005 in Lamu and Comoros Island. The CDC ELISA and $FRNT_{50}$ were used as comparative assay and gold standard, respectively as shown on **Appendix V, VI and VII**. Comparative analysis of in-house IgM ELISA and CDC ELISA, showed 40 febrile patient sera were positive and 93 febrile patient sera were negative by both tests, with a sensitivity of 97.6% and specificity of 86.9 %, with a Cohan kappa agreement of 0.77, the data in **Table 4.8** were used to make this calculation. The same panels of serum were also tested by $FRNT_{50}$. Comparative analysis of in-house IgM ELISA and $FRNT_{50}$ showed, 51 febrile patient sera as

positive and 89 febrile patient sera were negative by both tests, with a sensitivity of 91.1% and a specificity of 96.7 % and a Cohan kappa agreement of 0.88, the data in **Table 4.9** were used to make this calculation.

**Table 4.8: Comparative analysis of a panel of serum samples analyzed by the in-house IgM ELISA and the CDC ELISA**

| | | **CDC ELISA (n = 148)** | | |
|---|---|---|---|---|
| | | Positive | Negative | **Total** |
| **In-house IgM ELISA** | Positive | 40 | 14 | 54 |
| | Negative | 1 | 93 | 94 |
| **Total** | | 41 | 107 | 148 |

**Table 4.9: Comparative analysis of a panel of serum samples analyzed by in-house IgM ELISA and FRNT**

| | | $FRNT_{50}$ (n =148) | | |
|---|---|---|---|---|
| | | Positive | Negative | **Total** |
| **In-house IgM ELISA** | Positive | 51 | 3 | 54 |
| | Negative | 5 | 89 | 94 |
| **Total** | | 56 | 92 | 148 |

### 4.4.4 Assessing the correlation of the Positive Negative Ratio of the in-house IgM ELISA and FRNT50

To ascertain the relationship of the positive OD / negative OD (P/N) ratio optical density and neutralization titre at 50% reduction (FRNT50), the **c**orrelation of P/N ratio of IgM capture ELISA and $FRNT_{50}$ was determined by constructing a solid line, which indicates the correlation curve of all 148 Chikungunya positive cases ($Y= 0.8468\ln(x) + 0.2103$, $R^2 = 0.6156$) (**Figure 4.10**). The correlation co-efficient was 0.78, which show a strong agreement between the two test.

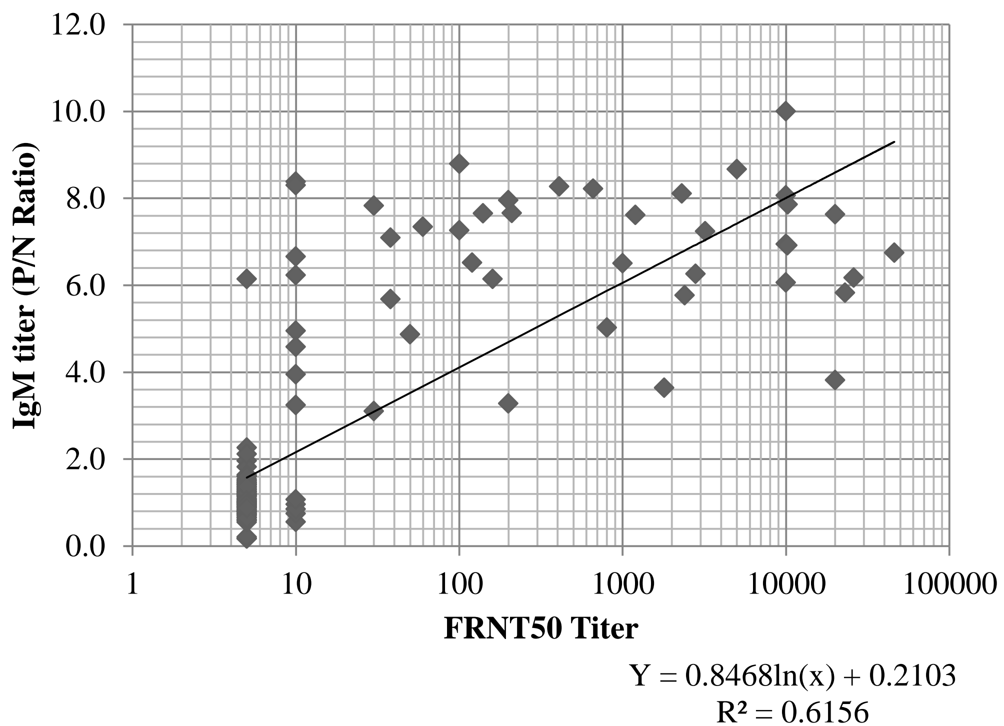


**Figure 4.10:Correlation of results of a panel of 148 serum samples analyzed by both CHIKV specific in-house IgM ELISA Positive/Negative ratio and Focus Reduction Neutralization Test 50% reduction reduction (FRNT50)** .

## 4.5 Application of the IgM ELISA to the dengue outbreak of 2013 in Kenya

A total of 254 human samples collected during the dengue outbreak in Mombasa and Northern Kenya in 2013, were tested using the IgM ELISA were analyzed by the in-house IgM ELISA and confirmed by $FRNT_{50}$ (**Table 4.10**). The cut-off for the in-house IgM ELISA is a P/N ratio of 2.00 and for the $FRNT_{50}$ was a dilution of 1:10. Out of 254 human samples, 26 (10.2%) were IgM positive. Out of these 26 samples, 17 samples were further analyzed by $FRNT_{50}$, 14 (82.4%) became positive (**Appendix VIII**). Age distribution and infection rate among confirmed Chikungunya and non- Chikungunya cases in 2013 in Eastern Kenya was determined. Chikungunya cases were detected in all age-groups, with a higher

positive ratio being observed in children 14 years and below and in adults 55 years and above. A significant difference ($p<0.05$) was observed between the 8 yr and below [5/26 (19.2 %)] and above 8 yr [19/213 (8.9%)] age groups. The age-group of above 8 years old (and especially those between 14 to 54 years old), reported a lower positive ratio. Most of the Chikungunya cases were males (80%) with most of cases being between the ages of 14 to 35 years (**Figure 4.11**).

**Table 4.10:Geographical distribution and laboratory test results of dengue suspected cases from Kenya tested for CHIKV–specific IgM**.

| Districts | Total no. of samples | In-house IgM ELISA) | FRNT | Age group (years) | % Seropositives In house IgM ELISA |
|---|---|---|---|---|---|
| Mombasa | 174 | 21 | 11/13 | 3 to 75 | 12.1 |
| Wajir West | 14 | 2 | 2/2 | 5 to 14 | 14.3 |
| Nairobi | 4 | 2 | 1/1 | 5 to 6 | 50.0 |
| Mandera East | 14 | 1 | 0/1 | 35 | 7.1 |
| others areas | 48 | 0 | 0 | N/A | 0.0 |
| **Total** | **254** | **26** | **14/17** | | **10.2** |

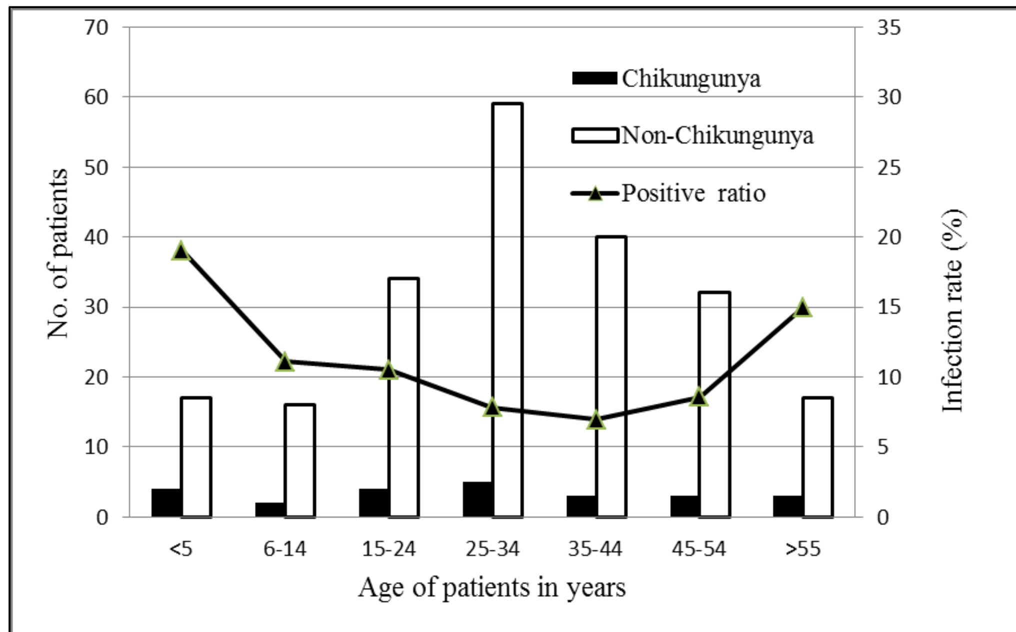


**Figure 4.11:Age distribution of laboratory confirmed Chikungunya assay positive and Chikungunya negative patients from Eastern Kenya in 2013. Black bars indicate confirmed Chikungunya cases, white bars indicate Chikungunya assay negative cases and the smooth curve indicates the ratio of positive OD divided by the negative OD of the in-house IgM ELISA across the age-groups.**

# CHAPTER FIVE

# DISCUSSION, CONCLUSION AND RECOMMENDATION

## 5.1 Discussion

For decades, Chikungunya was known to have mild symptoms which did not cause any fatalities, and it was localized in Africa and Asia only (Enserink, 2007). However in the last 10 years, CHIKV has become an arbovirus of greater public health importance, because it has been recognized to cause severe symptoms in infected humans like hemorrhagic manifestations, chronic arthritis and rash (Paquet *et al*., 2005 & Tandale *et al*., 2009). The virus has spread beyond Africa and Asia, to Europe and the Americas, making it one of the infections of global public health concern (Leparc-Goffart *et al* ., 2014). The aim of this study, was to characterize CHIKV isolated from a patient serum in Comoros Island during the 2005 CHIKV outbreak and develop an two ELISA systems. One of the ELISA systems was to detect CHIKV antigen in mosquito homogenates and the other was to detect antibodies in serum. The development of this assay coincided with a dengue outbreak in Kenya in 2013, hence, the developed in-house IgM ELISA was used to test for CHIKV IgM in the dengue suspect samples and this assay was able to detect CHIK IgM in these outbreak samples.

### 5.1.1 To characterize CHIKV variants isolated from a Comoros Island outbreak of 2005 phenotypically and genotypically

After the CHIKV outbreak in Kenya in 2004, there were other CHIKV outbreaks between 2004-2007 in East Africa (Powers &Logue, 2007; Schuffenecker *et al*., 2006), where a strain of CHIKV with an A226V substitution in the envelope glycoprotein E1 (Tsetsarkin *et al*., 2007) was associated with more efficient dissemination, infectivity and transmission of the virus by *Ae. albopictus* (Tsetsarkin *et al*., 2007). This demonstrated how changes arising from genetic drifts can contribute to real changes in disease presentation, transmission levels and host

adaptation. Plaque variants which represent mixed virus populations have been reported not only in CHIKV (Lim *et al*., 2009) but also in several other viruses such as Sindbis virus (Nagata *et al*., 1967), Guaroa virus (Tauraso, 1969), Bunyamwera virus and Ngari virus (Odhiambo *et al*., 2014). In this study, plaque variants were identified that reproduced their phenotype when passaged in culture. This allowed for examination of the phenotype and genetic difference associated with plaque variants to determine if potentially significant emerging virus variants could be detected in the course of an outbreak.

To examine the phenotype further, *in vitro* growth kinetics was done to establish whether the difference in plaque size was a function of different viral infection and/or replication rates. There was a significant difference in viral growth rate among the parent strain and plaque variants in the mosquito cell line, with higher viral titre observed in the parent strain and its variants in C6/36 cells than in Vero cells which is a mammalian cell line. This observation agrees with previous findings which found that CHIKV titres were higher in C6/36 cells compared to Vero cells (Li *et al*., 2012; Lim *et al*., 2009; Wikan *et al*., 2012). The small plaque variant had a slightly higher viral titre in both cell lines, contrary to other studies where the large plaque variants typically replicates faster and to a higher viral titre than small plaques (Sundstrom *et al*., 2011). This indicates that the S7 variant could have a growth advantage in mosquitoes, that would enable it to grow to a higher viral titre than the L2 virus variant.

Sequencing to determine if there was a genetic basis for the observed phenotypes, showed two amino acid differences. The first difference was a a change from a Cysteine to Tyrosine (C642Y) in nsP2 which was observed in the L2 variant while the S7 variant had a Cysteine in the same position. Asparagine has also been observed in the same position, in a 06.27 strain (accession number AM258993.1) from the CHIKV outbreak in Reunion Island in 2005 (Schuffenecker *et al*., 2006). The nsP2 region is essential for viral RNA replication and transcription and contains three highly conserved proteins: a helicase, a protease and RNA triphosphatase

(Schuffenecker *et al.*, 2006). The observed amino acid substitution is located in a region that codes for the nsP2 protease, but located outside the active site (1013-1015) of the enzyme. The three amino acids (cysteine, tyrosine and asparagine) that have been observed at this position are all uncharged polar residues representing conservative mutations unlikely to affect enzyme function.

The second substitution was detected at amino acid position 524, where L2 had an Arginine and S7 had a stop codon (CGA). The presence of the stop codon between the nsp3 and nsp4 results in translation into either one (nsP1234) or two (nsP123 and nsP1234) polyproteins (Strauss & Strauss, 1994). The nsP123 is produced when translation is terminated at the stop-codon at position 524 upstream of nsP4. The polyprotein P1234 is produced when translational read-through occurs or when the opal termination codon has been replaced by a sense codon in the alphavirus genome (Myles *et al.,* 2006). Non-structural protein 3 plays a role in sub-genomic 26S and negative strand synthesis, while nsP4 codes for RNA dependent RNA polymerase of alphaviruses and it's expression is tightly regulated in most alphaviruses by a read-through stop codon at the end of nsP3 (Strauss & Strauss, 1994). The presence of a stop codon upstream of nsP4 in ONNV genome has been shown to cause more efficient infection and earlier dissemination of the virus in its vector *Anopheles (A.) gambiae* mosquitoes, relative to viruses encoding an arginine at the same position by regulating alphavirus replication. This amino acid substitution (R524X) plays an important role in adaptation of ONNV to its mosquito vector (Myles *et al*., 2006). Chikungunya and O'Nyong-nyong viruses are both alphaviruses in the Semliki Forest antigenic complex (Powers *et al*., 2000) and are antigenically very closely related and difficult to differentiate serologically. Despite being antigenically closely related, ONNV and CHIKV have different mosquito vectors, *A. gambiae* and *Ae. aegpyti* respectively.

Most of the CHIKV isolates examined in the study (both study isolates and CHIKV sequences from the GenBank) had the stop codon indicating the predominance of a highly adapted virus in the 2004-2006 CHIKV outbreak. The combination of amino

acids at the sites of the substitution (C642, R524) in the two variants were not the amino acids that were present in the majority of the outbreak samples and earlier isolates from Kenya, suggesting emergence of these variants in the course of the Comoros outbreak. The close phylogenetic relationship of L2 and S7 virus isolates to other outbreak strains also suggests that they were existing or emergent variants rather than independent introductions of an exogenous strain into the CHIKV population circulating during the Comoros outbreak.

When the amino acid sequences of all virus isolates examined in this study were compared with the S27 African prototype strain, the majority of the changes observed were similar indicating no major evolutionary drifts. One of these amino acid substitutions (R171Q) however was of particular interest as it was only identified within a few 2005 Comoros Island isolates and in a traveller diagnosed with Chikungunya returning to Japan from Sri Lanka in 2006 (Lim *et al*., 2009). Interestingly, Comoros Island isolates (L2, S7, Com5 and ComJ) and Sri Lankan isolates (strains: SL11131 and SL-CKI) share two amino acid substitutions (A226 and 171Q) and belong to the ECSA genotype. These amino acids patterns suggest that different CHIKV variants could have spread to Sri Lanka, some with R171 and 226V and others with 171Q and A226 amino acid substitutions through different pathways. CHIKV with the 171Q and A226 amino acid substitutions could have been transmitted directly from Comoros Island in 2005 to Sri Lanka in 2006, in addition to the previously described evolutionary pathway which has the virus with the R171 and 226V amino acids substitutions spreading from Comoros Island in 2005, Mayotte Island in 2006, Reunion/Mauritius in 2005-2006, Seychelles 2005 - 2006, India and Sri Lanka in 2006 (Enserink, 2007).

Since the complete genome sequences of plaque variants were not obtained in this study, due to limitations of the sequencing platform that could have been brought about by short read lengths, or high background, not so pure sample RNA or sequencing reagents, the potential role that the 3` and 5` UTR region could play in gene regulation cannot be eliminated. Given that the E1:A226V mutation allowed better adaptation of CHIKV to

*Ae. albopictus* in a previous study (Tsetsarkin *et al.,* 2007). Further research is recommended on the functional significance of the amino acids substitutions(C642Y, R524X) observed in this study. This can be done by reverse genetics, so as to correlate the amino acids changes and the biological characteristics on virulence in mammalian hosts and mosquito species, to allow for monitoring of potentially virulent emergent CHIKV strains during outbreaks.

### 5.1.2 To develop and evaluate an antigen detection ELISA from CHIKV specific antibodies for the diagnosis of CHIKV infections in mosquitoes homogenates

The development of effective assays to detect arbovirus infections has had many challenges and include antigen cross-reactivity between arboviruses in the same family contributing to low specificity and unavailability of positive controls in areas where diseases are episodic not endemic. To circumvent cross-reactivity, highly specific monoclonal antibodies developed against specific epitopes, requiring more advanced technological expertise, have been used to enhance specificity but often with a loss in sensitivity due to emerging virus variants with different antigenic epitopes. In this study we used polyclonal antibodies which are sensitive, therefore able to detect most or all the positive samples that are assayed, require less technological expertise and less investment to develop, when compared to monoclonal antibodies, therefore Pabs can be widely used to develop diagnostics for other emerging viruses even with the limited resources available for research in Africa. On the other hand, polyclonal antibody have a limitation of not differentiating closely related viruses. This limitation can be solved by confirming the positive samples by conducting a neutralization test which is able to differentiate cross-reactive viruses or for acute cases using highly specific PCR techniques to detect viral nucleic acid are usually used.

Currently, there is need for a rapid diagnostic method for detection of CHIKV infection in both mosquito and human samples for disease detection and surveillance. The developed Antigen detection ELISA in this study was evaluated using a panel of

alphaviruses, selected flaviviruses and bunyaviruses, which showed no reactivity, except for ONNV which showed a weak signal due to serological cross-reactivity of alphaviruses, which is a challenge given the close antigenic relationship in this family (Dash *et al*., 2011). The differentiate cross-reactive samples, a neutralization test has been used to identify the causative agent. The high sensitivity with this antigen capture ELISA for detecting CHIKV antigen could be due to introduction of a horseradish peroxidase labeled anti-CHIKV Pabs as an identifying detector antibody, which amplified the reaction signals in the whole system and improved the detection capabilities of the assay.

Although the antigen detection ELISA provides a convenient method for detecting CHIKV in a large number of mosquito homogenates, it has a limitation of having a high rate of false positives because of its high detection level. The false positives could be due to non-specific reactions, giving rise to unexpected results. The assay would therefore be useful for screening large numbers of mosquitoes during vector surveillance for CHIKV. The antigen detection ELISA had a reasonably high sensitivity and specificity with a kappa of 0.70, which indicates good agreement (Sim &Wright, 2005), when compared to the RT-PCR. This protocol was able to detect CHIKV infection from a mosquito pool that had only one infected abdomen among many negative abdomens. Therefore, the antigen detection ELISA was sensitive and can be used for detecting CHIKV infections in mosquito homogenates.

### 5.1.3 To develop and evaluate an in-house IgM ELISA from cell culture-derived antigens and antibodies for the diagnosis of CHIKV infections

In addition to the antigen detection ELISA, an in-house IgM ELISA was also developed for detection of CHIKV IgM in human sera. In comparison to the conventional IgM-capture ELISA protocol which has a secondary detector antibody as the final step, this assay was shortened by one hour due to the direct conjugation of the primary antibody to the detector. Even though conjugation of CHIKV specific primary antibody shortened the ELISA protocol and reduced background reactions during the assay, this conjugation also has limitations. It can be expensive when

several viruses are under investigation, because all the viruses under study will have there antibodies generated separately and conjugated, therefore all these factors should be considered before designing an ELISA assay. Shortened protocols or rapid tests are critical during outbreaks, since time between outbreak onset and release of diagnostic results defines the outbreak response that will be taken such as vector control and patient management. This prompt outbreak response has economic and public health implications/effects. In this study, an in-house IgM ELISA protocol for the detection of CHIKV IgM in serum developed using cell-culture derived CHIKV antigen and HRP conjugated anti-CHIKV Pabs was developed and evaluated using two currently used assays as confirmatory tests. One was developed by CDC and the other a neutralization test ( FRNT) to compare the diagnostic utility of the conjugated polyclonal antibody based ELISA protocol.

The sensitivity (97.6%) and specificity (86.95%) between the in-house IgM ELISA and the CDC ELISA was good. However, the relatively low specificity between the in-house IgM ELISA and the CDC ELISA was due to a number of factors that differed between the two assays. First, the CDC ELISA protocol called for a serum dilution of 1:400 compared to 1:100 in the in-house IgM ELISA. Secondly, there was a difference in the assay antigens used: The CHIKV Comoros 5 strain in the in-house IgM ELISA but CHIKV S-27 the prototype strain in CDC ELISA. Thirdly, there was a difference in the positive/negative criteria. The assay antigen was used in the entire 96-well ELISA plate in the in-house IgM ELISA and the P/N Ratio calculated using a single negative control serum but the CDC ELISA employed an OD with assay antigen minus OD with control antigen of each serum sample. Besides our main objective was to detect all positive samples, which would then be confirmed by a neutralization test

In spite of these differences, the in-house IgM ELISAs' sensitivity (91.1%) and specificity (96.7%) when compared to the neutralization test gave a good agreement between the two tests,. The agreement between In-house IgM ELISA and the comparative assays achieved a kappa of 0.69 and 0.88, which indicates good to

excellent agreement (Sim & Wright, 2005), when compared to the CDC ELISA and FRNT. The in-house IgM ELISA was determined to be as good as these two reference tests for the diagnosis of Chikungunya infections in human samples.

### 5.1.4 To validate the developed in-house IgM ELISA on febrile patient samples collected during a 2013 Dengue outbreak

The development of the in-house IgM assay coincided with a Dengue outbreak in Eastern Kenya in 2013. After evaluating the in-house IgM ELISA and achieving good agreement between these assays and the confirmatory tests, in-house IgM ELISA was used to test samples from the 2013 Dengue outbreak and determine if there was co-circulation of CHIKV with DENV infection, given that both viruses have been reported in the coastal area in the past and the two viruses are transmitted by the same mosquito vector, Ae. albopictus. Co-circulation of CHIKV and DENV has been widely observed in many countries like India (Chahar *et al.*, 2009), Sri Lanka (Hapuarachchi *et al.*, 2008) , Malaysia (Nayar *et al.*, 2007) and Gabon (Leroy *et al.*, 2009).

Among the 254 outbreak samples collected from Mombasa, Nairobi and Northern parts of Kenya, that had been tested and found to be DEN negative were also tested using the in-house IgM ELISA assay and any positive samples were confirmed using FRNT. The assay was able to detect 10% of the samples as positive and this was confirmed by FRNT, demonstrating its utility in detecting active CHIKV infections. This assay also demonstrates how multiple arboviruses can be circulating in the same outbreak leading to misdiagnosis and wrong treatment. In the case of Dengue and Chikungunya where the same vector are involved, vector control would be similar but for other co-circulation of arboviruses which are transmitted by different vectors (*Ae. aegypti and A. gambiae*) like CHIKV and ONNV respectively, this may lead to incorrect vector control measures, thus failing to address the root cause of the outbreak. The three samples that were IgM positive by the in-house IgM ELISA and

negative by FRNT could have been due to cross-reaction with other alphaviruses such as ONNV, as serological cross-reactivity of alphaviruses is a challenge given the close antigenic relationship in this family (Dash *et al*., 2011). This limitation can be reduced by use of virus specific recombinant antigen and monoclonal antibodies to develop the ELISA protocol to improve cross-reaction of closely related virus, and ultimately to confirm the individual positive serum samples, a neutralization can be used.

Demographic data was analyzed to determine if the 10% of CHIKV cases were localized geographically and associated with any risk factors. Mombasa County located along the Eastern coastline had the highest number of Chikungunya positive cases with a few cases reported from Wajir West and Mandera East in Northern Kenya and Nairobi, the capital city located in Central Kenya. This distribution could be because CHIKV has been reported along the eastern coast before and is likely hypoendemic in that region. High human traffic between Mombasa and Nairobi could have introduced the virus to both Mandera and Nairobi accounting for the few cases in those regions. This is in contrast to DENV which in this recent outbreak was first detected in Wajir and Mandera where it is currently considered endemic and then followed by Mombasa and Nairobi indicating that the two arboviruses have distinct geographical foci.

Chikungunya cases were detected in all age-groups, with a higher positive ratio being observed in children and among the elderly (55 years and above). The higher number of positive cases observed among the children could be due to the naïve population who had not been born during the Chikungunya outbreak in 2004 in Kenya. The high positive ratio observed in the elderly , could be due to lowered immunity with advanced age. The age-group of above 8 years old (and especially those between 14 to 54 years old), reported a lower positive ratio, which could be attributed to immunity developed during the previous Chikungunya outbreak in the same region in 2004. By contrast, during the Chikungunya outbreak in Lamu Island, Kenya in 2004 which was the first documented outbreak in the coastal region, all age-groups

were equally infected (Sergon *et al.*, 2008) indicating that the population at that time were immunologically naïve and all equally susceptible to CHIKV infection. In summary, the demographic data among this small sample of cases tested from the 2013 Dengue outbreak indicated that the children and elderly and those residing in Mombasa were the most vulnerable to CHIKV infection. This finding implies that the vulnerable population which includes the children and elderly should be given preference during interventions such as management of the Chikungunya symptoms, control of vectors among others in case of an outbreak.

## 5.2 Conclusion

1. The plaque phenotypes that were observed and explored in this study, were associated with specific amino acid changes. This amino acid differences have justified the need to examine their *in vivo* effects in terms of disease severity and increased fatality, virulence in mammalian and vector competence in various mosquito species.
2. The developed CHIKV antigen detection ELISA was able to detect low viral tire in the homogenized mosquito pools. Therefore this test can used during routine suirvellance to detect CHIKV in mosquitoes before an outbreak actually occurs.
3. The developed CHIKV IgM capture ELISA has ensured a sustainable supply of a locally produced CHIKV ELISA system to assist with differential diagnosis of Dengue and Chikungunya in Kenya or any other co-infection with Chikungunya.
4. The CHIKV IgM capture ELISA was able to detect co-circulation of Chikungunya and Dengue during the 2013 Dengue outbreak.

## 5.3 Recommendations

1) It is important for investigators to enhance surveillance activities to predict outbreaks and monitor genetic evolution during routine culture, virus isolation and other viral tests, so that variants that enhance virulence through enhanced transmission, vector or host infectivity can be identified.

2) There should be investments in diagnostic development by governments in endemic regions for arboviruses, so that specific diagnostics can be developed to detect and distinguish arboviruses including Chikungunya, Dengue, West Nile and O'nyong nyong which appear in similar endemic zones and particularly in sub Saharan Africa.
3) Incorporation of CHIKV and arbovirus testing into the healthcare system, among patients who present with febrile illness like malaria, typhoid, brucellosis etc. This would enhance accurate diagnosis of febrile illnesses and improve patient care.
4) During outbreaks, it is important to test for a range of arboviruses, in case there is a co-circulation or co-infection of 2 viruses, especially where the viruses are closely related antigenically and/or do not share vectors so that vector control and response to outbreak is properly implemented.

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## APPENDICES

### Appendix I :Approval letter from the KEMRI Animal Care and use Committee

**KENYA MEDICAL RESEARCH INSTITUTE**

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**KEMRI/ACUC/ 01.01.11** **11TH January 2011**

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Wasonga Carolyne,

**RE: Development and Evaluation of stable ELISA Reagents for the detection of Chikungunya Virus Infection**

Following the receipt of the above mentioned proposal to the KEMRI ACUC, it has been established that all aspects of animal care and use have been addressed appropriately.

The committee grants you the approval to proceed with your study after obtaining all the other necessary approvals and expects you to adhere to all the animal handling procedures in KEMRI as described in your proposal.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso,
Chairperson, KEMRI ACUC

In Search of Better Health

## Appendix II:Approval letter from the Steering Scientific committee

**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

**ESACIPAC/SSC/9059** **14th February, 2011**

**Carolyne Wasonga**

**Thro'**
**Director, CVR**
**NAIROBI**

Forwarded
DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

**REF: SSC No.1940 (Revised) –Development of replenishable, cost-effective and stable ELISA reagents for the detection of Chikungunya Virus Infection.PI: Carolyne O. Wasonga (CVR)**

I am pleased to inform you that the above-mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC) during its **175th meeting** held on **1st February, 2011** and amendments therein approved for implementation.

The SSC however, advises that amendments can only be effected when ERC approval is received.

**Sammy Njenga, PhD**
**SECRETARY, SSC**

## Appendix III:Approval letter from the KEMRI Ethics Review Committee

# 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** **April 21, 2011**

**TO:** **Ms. CAROLYNE WASONGA (PRINCIPAL INVESTIGATOR)**

**THROUGH :** **DR. FRED OKOTH,**
**THE DIRECTOR, CVR**
**NAIROBI.**

forwarded April 28th 2011
FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

Dear Madam,

**RE: -SSC PROTOCOL No. 1940-REVISED (*RE-SUBMISSION*): DEVELOPMENT AND EVALUATION OF REPLENISHABLE AND STABLE ELISA REAGENTS FOR THE DETECTION OF CHIKUNGUNYA VIRUS INFECTION**

Reference is made to your letter dated April 19, 2011.

We acknowledge receipt of the revised proposal clearly describing the level of containment (Biosafety Level 2) designed to maximize safety when working with agents of moderate risk to humans and the environment. The Committee is satisfied that the issue raised at the 187th meeting of 15 March 2011 has been adequately addressed.

The study is granted approval for implementation effective this **21st day of April 2011**. Please note that authorization to conduct this study will automatically expire on **19th April 2012**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat on **8th March 2012**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

Sincerely,

**Christine Wasunna,**
**FOR: SECRETARY,**
**KEMRI/NATIONAL ETHICS REVIEW COMMITTEE**

In Search of Better Health

**Appendix IV: An in-house IgG indirect ELISA used to test rabbit serum for CHIKV specific IgG**

An in-house IgG indirect ELISA modified by Inoue *et al.,* 2010, was used in detecting CHIKV specific IgG to determine CHIKV infections in rabbit serum. In this protocol, the 96-well micro-plate was coated with purified antigen, CHIKV Com5 strain diluted with ELISA coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) in all wells except the blank. The plates were incubated at 4˚C overnight. The wells were blocked with 100 µl of original concentration of blockace except for the blank and were incubated at room temperature for 1 hour. The plates were washed 3 times with PBS-T. The test sera were then diluted at 1:1,000 in PBS-T in 10% blockace and added in duplicate to the plates. Control serum known to contain CHIKV specific antibody was run on each plate as a positive control. After incubation at 37˚ C for 1 hour, followed by washing and addition of 100 µl of substrate solution in each well. The substrate solution consisted of 5 mg of OPD and 0.03% hydrogen peroxide in 10 ml of 0.05 M citrate phosphate buffer at pH 5.0. The plate was incubated at room temperature for 15–30 minutes in the dark, and then the reaction was stopped by the addition of 100 µl of 1 N sulfuric acid per well. A standard curve was prepared using the OD492 values of the CHIKV positive control serum starting with a 1,000 dilution, followed by serial twofold dilutions down to 1:1280 in PBS-T in 10% Blockace. Then the IgG titers of patient sera were determined from a positive standard curve. A sample titer 1:3,000 was considered to be a CHIKV positive IgG infection.

**Appendix V: Laboratory results of Focus Reduction Neutralization Test ($FRNT_{50}$) and in-house IgM ELISA positive negative (P/N) ratio of sera from the CHIKV outbreak of Comoros Island of 2005**

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| **1** | 38 | 7.09 | 0.515 |
| **2** | 410 | 8.27 | 0.52 |
| **3** | 5,000 | 8.67 | 0.81 |
| **4** | 10,000 | 6.06 | 0.42 |
| **5** | 38 | 5.68 | 0.235 |
| **6** | 210 | 7.66 | **0.475** |
| **7** | 350 | 6.04 | **Neg** |
| **8** | 10 | 0.56 | Neg |
| **9** | 5 | 0.95 | Neg |
| **10** | 5 | 1.45 | Neg |
| **11** | 450 | 7.52 | 1.16 |
| **12** | 5 | 1.41 | Neg |
| **13** | 20,000 | 7.63 | 0.64 |
| **14** | 30 | 3.11 | Neg |
| **15** | 10,000 | 6.95 | 1.225 |
| **16** | 3,200 | 7.24 | 0.295 |
| **17** | 10,200 | 6.92 | 1.225 |
| **18** | 5 | 0.84 | Neg |
| **19** | 2,300 | 8.11 | 0.8 |
| **20** | 1,800 | 3.64 | 0.58 |

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| **21** | 140 | 7.65 | 1.21 |
| **22** | 160 | 6.14 | 1.145 |
| **23** | 60 | 7.35 | 1.305 |
| **24** | 23,000 | 5.82 | 0.325 |
| **25** | 10 | 2.46 | 0.45 |
| **26** | 9,500 | 7.43 | 1.51 |
| **27** | 120 | 6.52 | 0.78 |
| **28** | 800 | 5.02 | 0.56 |
| **29** | 200 | 3.28 | Neg |
| **30** | 5 | 0.79 | Neg |
| **31** | 5 | 0.75 | Neg |
| **32** | 2,400 | 5.77 | 0.515 |
| **33** | 100 | 8.79 | 0.85 |
| **34** | 5 | 1.96 | Neg |
| **35** | 20 | 4.29 | 0.275 |
| **36** | 2,800 | 6.26 | 0.23 |
| **37** | 10 | 3.41 | 0.985 |
| **38** | 10 | 4.11 | 0.82 |
| **39** | 46,000 | 6.75 | 0.7 |
| **40** | 10,000 | 10 | Pos |

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| **41** | 5 | 0.17 | Neg |
| **42** | 5 | 0.17 | Neg |
| **43** | 5 | 0.8 | Neg |
| **44** | 5 | 1.17 | Neg |
| **45** | 5 | 0.71 | Neg |
| **46** | 5 | 0.74 | Neg |
| **47** | 250 | 0.17 | 0.245 |
| **48** | 30 | 7.83 | Pos |
| **49** | 5 | 1 | Neg |
| **50** | 5 | 1.41 | Neg |
| **51** | 5 | 1.46 | Neg |
| **52** | 5 | 1.36 | Neg |
| **53** | 5 | 0.17 | Neg |
| **54** | 5 | 1.47 | Neg |
| **55** | 5 | 1.35 | Neg |
| **56** | 5 | 1.26 | Neg |
| **57** | 5 | 1.3 | Neg |
| **58** | 100 | 7.27 | 0.21 |
| **59** | 5 | 1.09 | Neg |
| **60** | 5 | 0.9 | Neg |

**Appendix VI:Laboratory results of Focus Reduction Neutralization Test ($FRNT_{50}$) and in-house IgM ELISA positive negative (P/N) ratio of sera from the CHIKV outbreak of Comoros Island of 2005.. ...Continue**

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| 61 | 10 | 0.75 | Neg |
| 62 | 50 | 4.87 | **0.345** |
| 63 | 20 | 6.03 | **0.455** |
| 64 | 5 | 0.78 | Neg |
| 65 | 10 | 4.95 | **0.585** |
| 66 | 200 | 1.3 | Neg |
| 67 | 10 | 6.66 | Pos |
| 68 | 1,200 | 7.62 | Neg |
| 69 | 5 | 1.82 | Neg |
| 70 | 20,000 | 3.82 | Neg |
| 71 | 10,000 | 8.06 | Pos |
| 72 | 200 | 7.96 | Pos |
| 73 | 5 | 2.01 | Neg |
| 74 | 10 | 4.59 | Neg |
| 75 | 10 | 6.23 | Neg |
| 76 | 5 | 0.6 | Neg |
| 77 | 10 | 8.38 | Neg |
| 78 | 150,000 | 0.8 | Neg |
| 79 | 5 | 0.9 | Neg |
| 80 | 10,240 | 7.86 | Pos |

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| 81 | 26,000 | 6.17 | Neg |
| 82 | 660 | 8.22 | Neg |
| 83 | 10 | 8.3 | Pos |
| 84 | 10 | 0.96 | Neg |
| 85 | 1,000 | 6.5 | Pos |
| 86 | 5 | 0.66 | Neg |
| 87 | 5 | 1.42 | Neg |
| 88 | 5 | 1.24 | Neg |
| 89 | 5 | 0.98 | Neg |
| 90 | 5 | 1.51 | Neg |
| 91 | 5 | 1.5 | Neg |
| 92 | 5 | 0.92 | Neg |
| 93 | 5 | 0.98 | Neg |
| 94 | 5 | 0.95 | Neg |
| 95 | 5 | 0.75 | Neg |
| 96 | 5 | 0.78 | Neg |
| 97 | 5 | 0.79 | Neg |
| 98 | 5 | 2.26 | Neg |
| 99 | 5 | 1.19 | Neg |
| 100 | 5 | 1.01 | Neg |

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| 101 | 5 | 1.02 | Neg |
| 102 | 5 | 1.03 | Neg |
| 103 | 5 | 0.96 | Neg |
| 104 | 5 | 0.85 | Neg |
| 105 | 5 | 6.14 | Neg |
| 106 | 5 | 1.64 | Neg |
| 107 | 5 | 0.8 | Neg |
| 108 | 5 | 0.9 | Neg |
| 109 | 5 | 0.8 | Neg |
| 110 | 5 | 1.4 | Neg |
| 111 | 5 | 1 | Neg |
| 112 | 5 | 0.9 | Neg |
| 113 | 5 | 0.7 | Neg |
| 114 | 5 | 1.17 | Neg |
| 115 | 5 | 0.21 | Neg |
| 116 | 5 | 1.17 | Neg |
| 117 | 5 | 1.2 | Neg |
| 118 | 5 | 1 | Neg |
| 119 | 5 | 1.54 | Neg |
| 120 | 5 | 1.01 | Neg |

**Appendix VII:Laboratory results of Focus Reduction Neutralization Test (FRNT50) and in-house IgM ELISA positive negative (P/N) ratio of sera from the CHIKV outbreak of Comoros Island of 2005**

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| **121** | 5 | 1.02 | Neg |
| **122** | 5 | 1 | Neg |
| **123** | 5 | 1.6 | Neg |
| **124** | 5 | 0.83 | Neg |
| **125** | 5 | 2.12 | Neg |
| **126** | 5 | 0.97 | Neg |
| **127** | 5 | 0.8 | Neg |
| **128** | 5 | 0.88 | Neg |
| **129** | 10 | 1.07 | Neg |
| **130** | 10 | 3.24 | Neg |
| **131** | 5 | 0.59 | Neg |
| **132** | 5 | 0.66 | Neg |
| **133** | 5 | 1.03 | Neg |
| **134** | 5 | 1.3 | Neg |
| **135** | 5 | 0.55 | Neg |
| **136** | 5 | 1.03 | Neg |
| **137** | 5 | 1.06 | Neg |
| **138** | 5 | 1.32 | Neg |
| **139** | 5 | 0.96 | Neg |
| **140** | 5 | 0.6 | Neg |

| Sample ID | FRNT | in-house ELISA | CDC ELISA |
|---|---|---|---|
| **141** | 5 | 0.87 | Neg |
| **142** | 5 | 1.24 | Neg |
| **143** | 5 | 1.16 | Neg |
| **144** | 5 | 1.22 | Neg |
| **145** | 5 | 0.72 | Neg |
| **146** | 5 | 1.37 | Neg |
| **147** | 5 | 0.6 | Neg |
| **148** | 5 | 0.91 | Neg |

…

Appendix VIII:Geographical distribution and laboratory test results of Dengue suspect cases from Kenya tested for Chikungunya virus IgM using the IgM ELISA**.**

| sample ID | New Sample ID | DISTRICT | AGE | Gender | P/N Ratio | FRNT (NT50) | Status |
|---|---|---|---|---|---|---|---|
| 1 | 1 | Nairobi | 5 | .... | 2.79 | 26 | positive |
| 2 | 2 | | **64** | **female** | 2.6 | 14 | positive |
| 3 | 3 | Mombasa | **....** | **Male** | 2.81 | >160 | positive |
| 4 | 4 | | **45** | **Male** | 2.56 | >1280 | positive |
| 5 | 5 | | **49** | **female** | 2.48 | >80 | positive |
| 6 | 6 | | **75** | **Male** | 2.72 | 16 | positive |
| 7 | 7 | | | **Male** | 2.74 | >1280 | positive |
| 8 | 8 | | **...** | **Male** | 3.21 | 350 | positive |
| 9 | 9 | | **3** | **Male** | 2.93 | 34 | positive |
| 10 | 10 | | **6** | **Male** | 2.88 | 12 | positive |
| 11 | 11 | | **40** | **Male** | 3.49 | >1280 | positive |
| 12 | 12 | | **3** | **Male** | 3.36 | 40 | positive |
| 13 | 13 | | **35** | **Male** | 2.28 | 40 | positive |
| 14 | 14 | | **...** | **Male** | 2.23 | 160 | positive |
| 15 | 15 | | **24** | **female** | 2.42 | 28 | positive |
| 16 | 16 | | **23** | **female** | 2.51 | * | positive |
| 17 | 17 | | **29** | **Male** | 2.43 | * | positive |
| 18 | 18 | | **50** | **Male** | 2.84 | * | positive |
| 19 | 19 | | **.....** | **Male** | 2.45 | * | positive |
| 20 | 20 | | **60** | **Male** | 2.51 | * | positive |
| 21 | 21 | | **17** | **Male** | 2.38 | * | positive |
| 22 | 22 | | **26** | **Male** | 2.49 | * | positive |
| 23 | 24 | | **29** | **Male** | 2.23 | * | positive |
| 24 | 28 | Wajir west | **14** | **Male** | 3.78 | 36 | positive |
| 25 | 29 | | **5** | **Male** | 2.5 | >80 | positive |
| 26 | 29 | Mandera East | **35** | **Male** | 2.17 | 40 | positive |

*Represents serum that were not tested by FRNT due to insufficient sample.

# Appendix IX:Reprints of published papers

Virus Genes (2015) 51:323–328
DOI 10.1007/s11262-015-1243-4

## Genetic divergence of Chikungunya virus plaque variants from the Comoros Island (2005)

Caroline Wasonga[1,2] · Shingo Inoue[4] · Cecilia Rumberia[5] · George Michuki[5] · James Kimotho[3] · Juliette R. Ongus[6,7] · Rosemary Sang[2] · Lillian Musila[8]

Received: 17 June 2015 / Accepted: 23 August 2015 / Published online: 7 September 2015
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**Abstract** Chikungunya virus (CHIKV) from a human sample collected during the 2005 Chikungunya outbreak in the Comoros Island, showed distinct and reproducible large (L2) and small (S7) plaques which were characterized in this study. The parent strain and plaque variants were analysed by in vitro growth kinetics in different cell lines and their genetic similarity assessed by whole genome sequencing, comparative sequence alignment and phylogenetic analysis. *In vitro* growth kinetic assays showed similar growth patterns of both plaque variants in Vero cells but higher viral titres of S7 compared to L2 in C6/36 cells. Amino acids (AA) alignments of the CHIKV plaque variants and S27 African prototype strain, showed 30 AA changes in the non-structural proteins (nsP) and 22 AA changes in the structural proteins. Between L2 and S7, only two AAs differences were observed. A missense substitution (C642Y) of L2 in the nsP2, involving a conservative AA substitution and a nonsense substitution (R524X) of S7 in the nsP3, which has been shown to enhance O'nyong-nyong virus infectivity and dissemination in *Anopheles* mosquitoes. The phenotypic difference observed in plaque size could be attributed to one of these AA substitutions. Phylogenetic analysis showed that the parent strain and its variants clustered closely together with each other and with Indian Ocean CHIKV strains indicating circulation of isolates with close evolutionary relatedness in the same outbreak. These observations pave way for important functional studies to understand the significance of the identified genetic changes in virulence and viral transmission in mosquito and mammalian hosts.

**Keywords** Chikungunya virus · Variants · Genetic variation · Comoros Island

Edited by Keizo Tomonaga.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11262-015-1243-4) contains supplementary material, which is available to authorized users.

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## Introduction

Chikungunya virus (CHIKV) is a single-stranded, positive sense RNA which belongs to the genus *Alphavirus*, in the family *Togaviridae*. Chikungunya (CHIK) is an arboviral disease that is transmitted mainly by *Aedes* mosquitoes. Due to the ubiquitous distribution of the CHIKV mosquito vector, emergence of new strains with new virulence features has become a global concern. A CHIK outbreak emerged in Lamu, Kenya in 2004 [1], in Comoros Island in 2005 [2] and in 2005–2006, the outbreak spread to other islands in the Indian Ocean including Madagascar, Seychelles, Reunion, Mauritius, and Maldives [3]. The epidemic also spread to India and subsequently to Europe, the United States, South Asia and Southeast Asian countries, where it was imported by infected travellers from India and affected Indian Ocean Islands [4–6]. In Reunion Island, the CHIKV was more virulent than in previous CHIK outbreaks [7]. A novel mutation in the Reunion Island resulted in a strain of CHIKV (E1:A226V) that could be easily transmitted by *Aedes albopictus* [7, 8]. Emergent virus strains with different growth phenotypes can be identified by the appearance of plaque variants from mosquito or human virus isolates. Plaque variants which represent mixed virus populations have been reported in CHIKV [4], Sindbis virus [9], Guaroa virus [10], Bunyamwera, and Ngari viruses [11]. During routine culture and titration of CHIKV (Strain: Com5) from 2005 outbreak in the Comoros Island, two distinct plaque sizes were observed from one isolate. The CHIKV isolate was from the serum of a febrile patient from the Grande Comore Island, within the Comoros Island archipelago during 2005 CHIK outbreak. This led to further investigation to determine if the phenotype in these CHIKV variants could be correlated with genetic changes.

## Results and discussion

To examine the phenotype and genetic differences associated with the plaque variants, the different plaques observed were isolated by plaque purification using Vero cells (African green monkey kidney derived cell line, ATCC, CCL81). Out of 10 clones of large plaques and 8 clones of small plaques, L2 and S7 variants were selected from each group for further analysis because they exhibited stable plaque sizes. Plaque assay of Com5 on Vero cells produced distinct and reproducible plaques of two sizes,

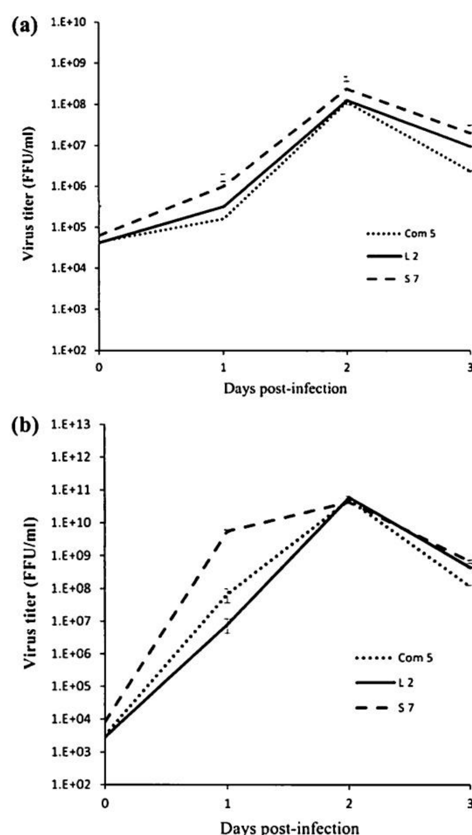


**Fig. 1** **a** In vitro growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7) cultured in Vero, African green monkey kidney derived cells. Equal viral titre of each CHIKV strains was inoculated and cultured for 3 days and viral titres quantified by focus assay. Com5 (*dot*) = original isolate, L2 (*solid*) = large plaque variant and S7 (*long dash*) = small plaque variant. The data indicate an average of three independent experiments ± standard deviation. The difference in viral titre between the Com5, L2 and S7 was not significant at all time points. ($p > 0.05$). **b** In vitro growth kinetics of the original CHIKV isolate (Com5) and the two plaque variants (L2 and S7) cultured on C6/36, *Aedes albopictus* derived cells. Equal viral titre of each CHIKV strains was inoculated and cultured for 3 days and viral titres quantified by focus assay. Com5 (*dot*) = original isolate, L2 (*solid*) = large plaque variant and S7 (*long dash*) = small plaque variant. The data indicate an average of three independent experiments ± standard deviation. The difference in viral titre between the Com5 and the plaque variants (L2 and S7) at 1 DPI was significant ($p < 0.05$). S7 had the highest viral titre throughout the experimental period

1.5 ± 0.3 mm (L2) and 0.5 ± 0.3 mm (S7) in diameter (Fig. S1). The small plaques predominated in the Com5 virus population, representing 68 % of all plaques.

To examine differences in the growth kinetics of the parent strain and plaque variants, confluent monolayers of C6/36 (mosquito, *Aedes albopictus* derived) cells (28 °C) and Vero cells (37 °C) were inoculated with virus infected culture fluid (ICF) containing equal titres of Com5, L2 and S7 at a multiplicity of infection of 1. One millilitre of ICF was collected from the culture flask at 0 day post infection (DPI) and every 24 h for 3 days and CHIKV titre determined by focus assay [12]. Three independent growth experiments were performed for each isolate. When in vitro growth kinetics of Com5, L2 and S7 were examined, S7 had the highest viral titre in Vero cells (Fig. 1a) and in C6/36 cells at 1-2 DPI (Fig. 1b). The difference in viral titre between Com5, L2 and S7 was insignificant ($p > 0.05$) in Vero cells but significant ($p < 0.05$) at 1 DPI in C6/36 cells. All the three virus isolates had higher viral titres in C6/36 than in Vero cells ($p = 0.03$). The significant difference in viral growth rate among the parent strain and plaque variants in C6/36 cells agrees with previous findings [13–15]. S7 had a slightly higher viral titre in both cell lines, contrary to other studies where the large plaque variants typically replicates faster and to a higher viral titre than small plaques [15]. This suggests that S7 has a growth advantage than L2 in both cell lines.

The genomes of Com5, L2 and S7 were sequenced to assess if any genetic changes could be responsible for the observed plaque size difference between the CHIKV variants, this was done using the 454 Genome sequencer (Roche Branford, CT, USA) according to manufacturer's instructions. The partial genomic sequences excluding the 3′ and 5′ UTR region were obtained and deposited in Genbank with the following accession numbers: Com5 (KP702297), L2(KF283986) and S7 (KF283987) [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. When the RNA sequences were aligned, the overlapping regions of the two plaque variants were 99.93 % similar. Phylogenetic analysis was performed using nucleotide sequences of L2 and S7 and other complete genome sequences of CHIKV strains available in the GenBank. L2 and S7 clustered closely with ComJ, Indian Ocean Islands, India and Sri Lanka CHIKV isolates and they all belong to the East Central South African (ECSA) genotype (Fig. S2), reflecting a close genetic and geographic relationship of strains in the same outbreak.

Alignment of amino acid (AA) sequences of L2 and S7 using S27 prototype strain as reference showed that these variants were identical except for two AA substitutions. The first was a missense AA substitution at position 642 located in the nsP2 region where L2 had a Tyrosine (Y642) and S7 a Cysteine (C642) (Table 1). When we examined this substitution in Kenyan and other Indian Ocean CHIKV

**Table 1** Comparisons of amino acid sequences of the non-structural proteins of Com5, L2 and S7 and selected CHIKV Indian Ocean isolates using S27 prototype strain as a reference sequence

| Non-structural protein | nsP1 | | | | | | | | nsP2 | | | | | nsP3 | | | | | | | | | | | | nsP4 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polypeptide position | 171 | 172 | 234 | 383 | 384 | 481 | 488 | 507 | 589 | 909 | 1177 | 1178 | 1328 | 1508 | 1550 | 1659 | 1664 | 1670 | 1685 | 1709 | 1715 | 1794 | 1795 | 1804 | 1857 | 1938 | 2117 | 2363 | 2377 | 2418 | 2467 |
| Amino acid position | 171 | 172 | 234 | 383 | 384 | 481 | 488 | 507 | 54 | 374 | 642 | 643 | 793 | 175 | 217 | 326 | 331 | 337 | 352 | 376 | 382 | 461 | 462 | 471 | 524 | 75 | 254 | 500 | 514 | 555 | 604 |
| Tanzania S27/53 | R | L | E | M | I | T | Q | L | S | H | C | S | A | V | Y | P | V | T | K | I | A | L | S | P | R | T | T | Q | I | V | V |
| Kenya/Lamu33/04<br>Kenya/KPA15/04<br>Comoros/Com 25/05<br>Comoros/Com 125/05<br>Reunion/LR-2006/06<br>India/DRDE-06/06<br>India/DRDE-07/07 | R | V | K | L | L | I | R | R | N | Y | Y | N | V | I | H | S | A | I | E | T | T | P | N | S | X | A | A | L | T | I | I |
| Comoros/Com J/05<br>**Comoros /L2/05** | Q | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | V | – | – | – | – | – | – | – | R | – | – | – | – | – | – |
| **Comoros /S7/05** | Q | – | – | – | – | – | – | – | – | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Sri-Lanka/SL-CK1/07 | Q | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

Isolates and the two amino acid substitutions of the present study are indicated in bold. (–) denotes similarity to amino acids of the Lamu33 strain at corresponding positions

**Table 2** Comparisons of amino acid sequences of structural proteins of Com5, L2 and S7 and selected CHIKV Indian Ocean isolates using S27 prototype strain as a reference sequence

| Structural protein | C | E3 | E2 | | | | | | | | | | | | | | 6K | | E1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polypeptide position | 63 | 284 | 382 | 399 | 404 | 485 | 489 | 506 | 519 | 536 | 592 | 624 | 637 | 669 | 700 | 711 | 756 | 802 | 1035 | 1078 | 1093 |
| Amino acid position | 63 | 23 | 57 | 74 | 79 | 160 | 164 | 181 | 194 | 211 | 267 | 299 | 312 | 344 | 375 | 386 | 8 | 54 | 226 | 269 | 284 |
| Tanzania/S27/53 | K | I | G | I | G | N | A | L | S | I | M | S | T | A | S | V | V | I | A | M | D |
| Kenya/Lamu33/04<br>Kenya/KPA15/04<br>**Comoros/L2/05**<br>**Comoros /S7/05**<br>Comoros /Com J/05<br>Comoros /Com 25/05<br>Comoros/Com 125/05<br>India/DRDE-06 /06<br>Sri-Lanka/SL-CKI/07 | R | T | K | M | E | T | T | M | G | T | R | N | M | T | T | A | I | V | A | V | E |
| Reunion/LR-2006/06<br>India/DRDE-07/07 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | V | – | – |

Plaque variants of the present study and other CHIKV isolates are indicated in bold and are named in this order: country of origin/strain name/year of sample collection. (–) denotes similarity to amino acids of the Lamu33 strain at corresponding positions. Selected isolates are Indian Ocean outbreak isolates that occurred before and after the Comoros Island isolates that are under study. Accession numbers of the selected isolates shown in Table 2 are S27 (AF369024.2), Lamu33 (HQ456255.1), KPA15 (HQ456254.1), Com J (HQ456252.1), COM25 (HQ456251.1), COM125 (HQ456253.1), DRDE-06 (EF210157.2), SL-CKI(HM045801), LR2006-OPYI (DQ443544.2), DRDE-07 (EU372006.1)

isolates from the same outbreak, the Cysteine (C642) was observed in S7 and S27 prototype strain only and not in any other isolate. Asparagine has also been observed in the same position, in 06.27 strain (accession number AM258993.1) from the CHIKV outbreak in Reunion Island in 2005 [16]. This AA substitution is located in a region that codes for the nsP2 protease, but outside the active site (478–480) of the enzyme. The three AAs observed at this position represent conservative mutations unlikely to affect enzyme function.

In the second AA substitution at position 524 in the nsP3 region, L2 had an Arginine (R524) and S7 a stop codon (X524). Arginine (R524) was unique to L2 and the S27 prototype strain and the stop codon (CGA)/(X524)) was present in all other isolates (Table 1). The opal stop codon is located between the nsP3 and nsP4. The nsP3 is essential for minus strand and sub-genomic 26S mRNA synthesis while nsP4 codes for RNA-dependent RNA polymerase of alphaviruses and its expression is tightly regulated by a read-through stop codon at the end of nsP3 [17]. The presence of a stop codon upstream of nsP4 in O'nyong-nyong virus (ONNV) genome causes more efficient infection and earlier dissemination of the virus in its vector *Anopheles gambiae* mosquitoes, relative to viruses encoding an Arginine (R524) at the same position [18], therefore, we hypothesize that the opal stop codon could improve Chikungunya virus infectivity in its vector *Aedes* mosquito. CHIKV and ONNV are both alphaviruses in the Semliki Forest antigenic complex [19], are antigenically very closely related and have different mosquito vectors. The E1: A226V mutation was present in the Reunion Island isolate and one Indian (DRDE-07) isolate (Table 2). However, the variants in this study did not have this mutation.

To monitor CHIKV evolution overtime, whole genome alignment of AA sequences of the plaque variants using the S27 strain as the reference was done. In total, 30 AA changes in the nsP sequences (Table 1) and 22 AA changes in the structural protein (Table 2) were observed. Notably, within the Comoros Island, 2 variants were circulating (nsP1:R171 and Q171) around the same period. Arginine (R) was present in other Comoros Island isolates (Com 25 and COM125 strains), S27 strain and the Indian Ocean CHIK outbreaks between 2004 and 2006, while, Glutamine (Q) was present in the Com5, L2, S7 and Com J. This substitution (R171Q) was also observed in a traveller diagnosed with CHIK returning to Japan from Sri Lanka in 2006 [15]. Since the Comoros Island isolates (L2, S7, Com5 and ComJ) and Sri Lankan isolates (strains: SL11131 and SL-CKI) share two AA substitutions (171Q and A226) and belong to the ECSA, it is likely that these isolates originated from Kenya (Lamu33), then spread to Comoros Island, India and then Sri Lanka in 2005–2006 (Fig. S2), unlike the majority of other strains which could have spread from Kenya (KPA15-Mombasa), then to Comoros Island, Reunion, Mauritius, Seychelles in 2005–2006, India and Sri Lanka in 2006 [20]. It is possible that different CHIKV variants could have

spread to Sri Lanka, some with R171 and 226V and others with 171Q and A226 AA substitutions through different pathways.

In conclusion, since the complete genome sequences of plaque variants were not obtained in this study, we cannot eliminate the potential role that 3′ and 5′ UTR region could also play in gene regulation. Given that the E1:A226V mutation allowed better adaptation of CHIKV to *Aedes albopictus* in a previous study (10), we recommend further research on the functional significance of the AA substitutions observed in this study by reverse genetics, so as to correlate the AA changes and the biological characteristics on virulence in mammalian hosts and mosquito species, to allow for monitoring of potentially virulent emergent CHIKV strains during outbreaks.

**Acknowledgments** The project was funded by the Consortium for National Health Research (CNHR), Kenya (Project Number RLG09-001/031). This study was also supported with reagents and equipment by JICA-JSPS Project and JICA-JST-SATREPS Project. We acknowledge the Biosciences Eastern and Central Africa - International Livestock Research Institute's (BecA-ILRI-Hub) genomics platform for supporting the sequencing work. We also appreciate the support given by the Government of the Comoros Island. This work was submitted with the permission of the Director, KEMRI, Nairobi, Kenya.

**Author contributions** CW, SI contributed in the laboratory work, data analysis and drafted the manuscript. CR, GM participated in sequencing the variants and data analysis; JK and JO contributed in study design and data analysis. RS and LM contributed to study design, data analysis, overall supervision, implementation and management of the project. All authors reviewed and approved the final manuscript.

**Disclaimers** The views expressed are not to be considered as official, or as reflecting the views of USAMRU-K, the Walter Reed Army Institute of Research or the United States Departments of the Army or Defence.

**Compliance with ethical standards**

**Conflict of interests** The authors hereby declare that they have no competing interests.

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Jpn. J. Infect. Dis., 68, 410-414, 2015

**Short Communication**

# Development and Evaluation of an in-House IgM-Capture ELISA for the Detection of Chikungunya and Its Application to a Dengue Outbreak Situation in Kenya in 2013

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**SUMMARY:** Chikungunya (CHIK) is a mosquito-borne viral disease. In the 2004 CHIK outbreak in Kenya, diagnosis was delayed because of the lack of accurate diagnostics. Therefore, this study aimed to develop and evaluate an in-house IgM-capture enzyme linked immunosorbent assay (ELISA) (in-house ELISA) for the detection of chikungunya virus (CHIKV) infections. Anti-CHIKV antibodies were raised in rabbits, purified and conjugated to horseradish peroxidase. These anti-CHIKV antibodies and cell-culture derived antigen were used to develop the ELISA. To validate the in-house ELISA, 148 patient sera from the 2005 Comoros CHIK outbreak were tested with centers for disease control and prevention (CDC) IgM-capture ELISA (CDC ELISA) and focus reduction neutralization test (FRNT) as reference assays. The in-house ELISA had a sensitivity of 97.6% and specificity of 81.3% compared to the CDC ELISA and a sensitivity of 91.1% and specificity of 96.7% compared to FRNT. Furthermore, 254 clinically suspected dengue patient samples from Eastern Kenya, collected in 2013, were tested for CHIKV IgM using the in-house ELISA. Out of the 254 samples, 26 (10.2%) were IgM positive, and of these 26 samples, 17 were further analyzed by FRNT and 14 (82.4%) were positive. The in-house ELISA was able to diagnose CHIKV infection among suspected dengue cases in the 2013 outbreak.

Chikungunya (CHIK) is a re-emerging disease that has become an important cause of acute febrile illness in Africa, Southeast Asia, the Western Pacific, and India (1). Diagnosis of CHIK based on its clinical presentation is challenging, because the clinical symptoms resemble those of other febrile illnesses such as dengue (DEN), malaria, and typhoid (2). Chikungunya virus (CHIKV) belongs to the family *Togaviridae* and the genus *Alphavirus*. CHIK and DEN have caused epidemics in diverse geographical regions (3,4). Both diseases are transmitted to humans by the *Aedes* species of mosquitoes. Although CHIK can be associated with hemorrhagic manifestations and arthritis in severe cases, both diseases have similar clinical symptoms, including fever, rash, joint pain, headache, and fatigue. Co-circulation of CHIKV and dengue virus (DENV) has been widely observed in many countries such as India (5), Sri Lanka (6), Malaysia (7), and Gabon (8). Therefore, a laboratory test is required to distinguish these 2 infections. Serological testing is the primary method of diagnosing CHIK because the viremic phase is limited during the course of infection. Detection of CHIKV specific Immunoglobulin M (IgM) using commercial enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test kits are used for diagnosis in Europe and in limited areas in South East Asia. However, these kits are expensive and are not readily available in African countries, thus CHIKV infections can go undetected until they reach outbreak proportions while cases can also be misdiagnosed and mismanaged.

This study aimed to develop an in-house CHIKV IgM-capture ELISA (referred to from here as "in-house ELISA") and evaluate it using 2 reference tests: an IgM-capture ELISA (CDC ELISA) developed by the Centers for Disease Control and Prevention (Fort Collins, CO, USA) and a focus reduction neutralization test (FRNT). Once validated, the in-house ELISA was then used to diagnose febrile patients from Eastern Kenya during the DEN outbreak in 2013. Two hundred and fifty four of these outbreak samples were tested and found to be DEN negative using a DENV IgM-capture ELISA, developed by the Diagnostic Systems Division of the United States Army Medical Research Institute of Infectious Diseases, USA, and DENV RT-PCR. These febrile patient samples were selected for testing using the in-house ELISA to determine if there was co-circulation of CHIKV with DENV, given that the coastal area has previously been affected by both viruses (9,10). Ethical approval for the use of animals and human samples was sought and granted by the Ethics Review Committee of

Received October 30, 2014. Accepted January 5, 2015.
J-STAGE Advance Publication April 10, 2015.
DOI: 10.7883/yoken.JJID.2014.482

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the Kenya Medical Research Institute (KEMRI) (SSC 1940).

The CHIKV Comoros 5 strain used was isolated during the 2005 CHIK epidemic in the Union of Comoros from a febrile patient from Grand Comore Island. The CHIKV was propagated on a large scale using Vero cells (African green monkey kidney derived cells) (American Type Culture Collection—ATCC, CCL81). CHIKV infected culture fluid (ICF) was concentrated with polyethylene glycol 6000 and sodium chloride. The concentrated virus was purified by sucrose-gradient ultracentrifugation at 50,000 × g for 14 h at 4°C (11).

The polyclonal antibody (pAb) against CHIKV was then generated in 2 New Zealand white rabbits by repeated subcutaneous inoculation of 0.25 mg/mL (0.5 mL/shot) of the purified CHIKV antigen 9 times. The pAb was purified by saturated ammonium sulfate precipitation and protein G column chromatography using the following procedure: an equal volume of saturated ammonium sulfate was mixed with the rabbit pAb (50% final concentration of ammonium sulfate), and centrifuged at 9,800 × g for 15 min at 4°C to remove the albumin. The precipitate was re-suspended in phosphate buffered saline (PBS) (pH 7.2), mixed with half the volume of saturated ammonium sulfate (33% final concentration of ammonium sulfate) and then centrifuged at 9,800 × g for 15 min at 4°C to remove the pseudoglobulin. The re-suspended precipitate was filtered through a 0.45 $\mu$m nitrocellulose membrane. To further purify the pAb, it was bound and eluted using protein G column chromatography (HiTrap; GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The amount of IgG in each fraction was determined using the absorbance readings at $OD_{280\,nm}$ and $OD_{260\,nm}$ (IgG concentration [mg/mL] = [1.45 × $OD_{280\,nm}$ − 0.74 × $OD_{260\,nm}$] × dilution factor). Peak fractions were selected and pooled, and the purified pAb was then conjugated with horse radish peroxidase (HRP) (Sigma-Aldrich, St Louis, MO, USA), using a published protocol (12). The HRP conjugated rabbit pAb was used as the detector antibody and CHIKV ICF at 160 ELISA units was used as the antigen component of the in-house ELISA. All other components were commercially sourced.

The in-house ELISA was performed as follows: a 96-well flat-bottom microtiter ELISA plate (Maxi-sorp, Nalgene International, Roskilde, Denmark) was coated with 5.5 $\mu$g/100 $\mu$L of anti-human IgM ($\mu$-chain specific) goat IgG (MP Biomedicals LLC, Kaysersberg, France) diluted with coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6), and incubated at 4°C overnight. The wells were blocked with Block Ace (Yukijirushi, Sapporo, Japan) at room temperature (r.t.) for 1 h, and then washed 4 times with PBS containing 0.05% Tween 20 (pH 7.2) (PBS-T). The test sera were diluted 1 : 100 in PBS-T, and 100 $\mu$L aliquots were distributed into duplicate wells. Sera known to contain antibodies against the antigen and negative sera were run on each plate as positive and negative controls, respectively. The plate was incubated at 37°C for 1 h and then washed as described above. CHIKV antigen (100 $\mu$L: 160 ELISA units) was then added and incubated at 37°C for 1 h. After washing as described above, HRP-conjugated anti-CHIKV rabbit pAb (1,500 × dilution in PBS-T with 10% Block Ace) was added to the wells and incubated for 1 h at 37°C. After washing, 100 $\mu$L of the substrate solution (*o*-phenylenediamine hydrochloride substrate; final concentration 0.5 mg/mL; Sigma Aldrich) and 0.03% hydrogen peroxide reconstituted in 0.05 M citrate phosphate buffer (pH 5.0) was added to each well and incubated for 1 h at r.t. in the dark. The reaction was stopped using 100 $\mu$L of 1N sulfuric acid and color change was detected at 492 nm ($OD_{492}$) on an ELISA Reader (Multiskan Ex, Thermo Scientific, Beijing, China). A P/N (positive [or sample] $OD_{492}$/negative control $OD_{492}$) ratio of $\geq 2.0$ was considered positive.

A total of 148 serum samples collected during the 2005 CHIK outbreak in the Union of Comoros, which had been previously tested using the CDC ELISA (13), were used to evaluate the in-house ELISA. This test panel had a combination of CHIK IgM positive and negative samples of which all were CHIK IgG negative. The 148 samples were then tested using the in-house ELISA and $FRNT_{50}$. $FRNT_{50}$ was performed to confirm the neutralizing activity of any CHIKV-specific IgM using a published protocol (14). The serum samples were first heat inactivated at 56°C for 30 min and serially diluted 4-fold (10× to 163,840×).

The sensitivity and specificity of the in-house ELISA results compared with the CDC ELISA and FRNT was calculated using IBM® SPSS® Statistics 20 software. Significance was determined at a $P$ value of $<0.05$ at a 95% confidence limit and a correlation curve between in-house ELISA titers and $FRNT_{50}$ titers was generated. Agreement was assessed using the Cohen's Kappa statistic.

The sensitivity and specificity between the in-house ELISA and the CDC ELISA were 97.6% and 81.3%, respectively (Table 1). This relatively low specificity was due to a number of factors that differed between the 2 assays. First, the CDC ELISA protocol called for a serum dilution of 1 : 400 compared to 1 : 100 in the in-house ELISA. Second, there was a difference in the assay antigens used. In the in-house ELISA, the CHIKV Comoros 5 strain was used, whereas the CHIKV S-27 prototype strain was used in CDC ELISA. Third, there was a difference in the positive/negative criteria. The assay antigen was used in the entire 96-well ELISA plate in the in-house ELISA and the P/N ratio was calculated using a single negative control serum, but the CDC ELISA calculations involved subtracting the OD with control antigen of each serum sample from the OD with assay antigen. Despite these differences, there was good agreement between the 2 tests with a Cohan's kappa

Table 1. Comparative analysis of a panel of serum samples analyzed by the in-house CHIKV IgM-capture ELISA and the CDC IgM-capture ELISA

| | | CDC IgM-capture ELISA ($n$ = 148) | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| In-house IgM-capture ELISA | Positive | 40 | 20 | 60 |
| | Negative | 1 | 87 | 88 |
| | Total | 41 | 107 | 148 |

Sensitivity of 97.6% and specificity of 81.3% was achieved. Cohan kappa agreement was 0.69.

agreement statistic ($\kappa$) of 0.69 (95% CI, 0.56 to 0.82, $P < 0.05$).

The sensitivity and specificity between the in-house ELISA and FRNT were 91.1% and 96.7%, respectively (Table 2). There was good agreement between these 2 tests with a $\kappa$ of 0.88 (95% CI, 0.80 to 0.97, $P < 0.05$). The in-house ELISA titers (P/N ratio) and $FRNT_{50}$ titers (Fig. 1) were positively and significantly correlated ($Y = 0.84681 \ln(x) + 0.2103$, $R^2 = 0.6156$, $P = 0.007$). The in-house ELISA was determined to be as good as the CDC ELISA and FRNT reference tests for the diagnosis of CHIKV infections in human samples, given that it had a reasonably high sensitivity and specificity with a $\kappa$ of 0.69 and 0.88, which indicates good to excellent agreement (15), compared to the CDC ELISA and FRNT.

After validation of the in-house ELISA, field samples from the DEN outbreak reported in Eastern Kenya in 2013 were analyzed. These 254 samples were tested using the in-house ELISA and any positive samples were confirmed using FRNT.

Table 2. Comparative analysis of a panel of serum samples analyzed by the in-house CHIKV IgM-capture ELISA and FRNT

| | | FRNT ($n$ = 148) | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| In-house IgM-capture ELISA | Positive | 51 | 3 | 54 |
| | Negative | 5 | 89 | 94 |
| | Total | 56 | 92 | 148 |

Sensitivity of 91.1% and specificity of 96.7% was achieved. Cohan kappa agreement was 0.88.

Twenty-six (10.2%) of the 254 samples were positive for CHIKV IgM (Table 3). Out of the 26 IgM positives, 17 samples (9 samples had insufficient amounts remaining) were further analyzed by FRNT and 14 (82.4%) were positive, with a geometric mean titer of neutralizing antibody of 1 : 39. The 3 samples that were determined to be IgM positive by the in-house ELISA and negative by FRNT could have been due to cross-reaction with other alphaviruses such as the O'nyong nyong virus since serological cross-reactivity of alphaviruses is a challenge, given the close antigenic relationship in this family (16). In summary, 26 (10.2%) of the DEN IgM negative febrile patients were positive for CHIKV IgM and the remaining 89.8% of the cases remained unidentified. These febrile cases could be other arboviral infections, malaria, or typhoid fever. These results confirmed the co-circulation of DENV and CHIKV in the 2013 DEN outbreak in Kenya. This study also addresses the need for more accurate diagnosis of febrile illness in Kenya.

Since demographic data were available for the outbreak samples, the data were analyzed to determine if the 10% of CHIK cases were localized geographically and associated with any risk factors. Mombasa County located along the Eastern coastline had the highest number of CHIK positive cases with a few cases reported from Wajir West and Mandera East in Northern Kenya and Nairobi, the capital city located in Central Kenya (Table 3).This distribution could be because CHIKV has previously been reported along the Eastern Coast and is likely hypoendemic in that region. High human traffic

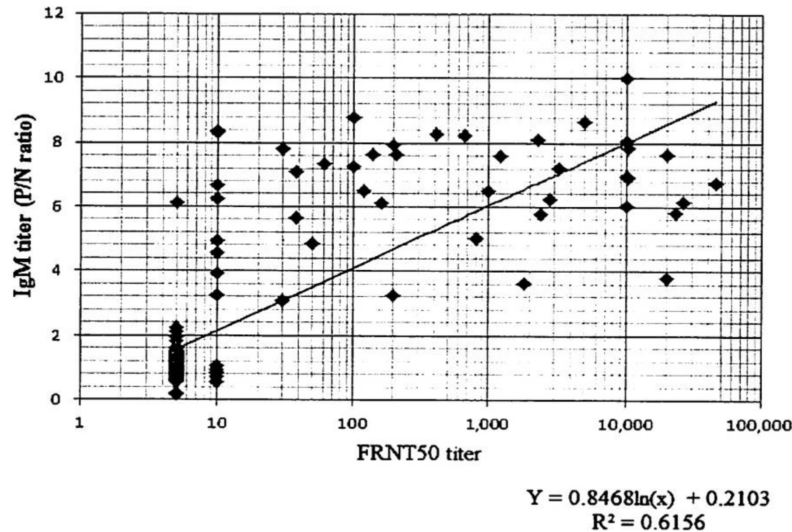


Fig. 1. Correlation of anti-chikungunya virus (CHIKV), the in-house IgM ELISA positive control optical density/negative control optical density (P/N) ratio and 50% focus reduction neutralization assay titers ($FRNT_{50}$). The solid line indicates the correlation curve of all 148 serum samples between CHIKV IgM-capture ELISA and FRNT ($Y = 0.8468 \ln(x) + 0.2103$, $R^2 = 0.6156$, $P = 0.007$).

between Mombasa and Nairobi could have introduced the virus to both Mandera and Nairobi, accounting for the few cases in those regions. This is in contrast to DENV, which in this recent outbreak was first detected in Wajir and Mandera where it is currently considered endemic, followed by Mombasa and Nairobi, indicating that the 2 arboviruses have distinct geographical foci.

CHIK cases were detected in all age groups, with a higher positive ratio being observed in children 14 yr and below, and in adults 55 yr and above (Fig. 2). A significant difference ($P < 0.05$) was observed between the 8 yr and below (5/26 [19.2%]) and above 8 yr (19/213 [8.9%]) age groups. The higher number of positive cases among the young could be due to the naïve population who had not been born during the CHIK outbreak in 2004 in Kenya. The high positive ratio observed in the ≥55 yr age-group, could be due to lowered immunity with advancing age. The age group of above 8 yr old (and especially those between 14 to 54 yr) reported a lower positive ratio, which could be attributed to immunity developed during the previous CHIK outbreak in the same region. By contrast, during the CHIK outbreak in Lamu Island, Kenya in 2004, which was the first documented outbreak in the coastal region, all age groups were equally infected (9), indicating that the population at that time were immunologically naïve and all individuals were equally susceptible to CHIKV infection. In summary, the demographic data among this small sample of cases tested from the 2013 DEN outbreak indicate that children and elderly, and those residing in Mombasa were the most vulnerable to CHIKV infection.

Table 3. Distribution of CHIK cases analyzed from clinically suspected dengue sera in Kenya and the results of Laboratory tests

| District | Total no. of sample | In-house IgM-capture ELISA | FRNT positive /total tested | Age group of CHIK positive case (yr) | % Seropositive in-house IgM-capture ELISA |
|---|---|---|---|---|---|
| Mombasa | 174 | 21 | 11/13 | 3 to 75 | 12.1 |
| Wajir West | 14 | 2 | 2/2 | 5 to 14 | 14.3 |
| Nairobi | 4 | 2 | 1/1 | 5 to 6 | 50.0 |
| Mandera East | 14 | 1 | 0/1 | 35 | 7.1 |
| Other areas | 48 | 0 | 0 | N/A | 0.0 |
| Total | 254 | 26 | 14/17 | 3 to 75 | 10.2 |

FRNT, focus reduction neutralization test; N/A, not applicable because there were no positive cases.

In this study, we generated a CHIKV IgM ELISA by producing the key components of the assay, namely, the CHIKV antigen and the HRP-conjugated anti-CHIKV polyclonal antibody. This ensures a sustainable supply of a locally produced CHIKV ELISA system to assist with differential diagnosis of DEN and CHIK in Kenya. The test was validated against 2 reference assays and was able to detect CHIK in febrile patient sera collected during the 2013 DEN outbreak. The CHIK cases were masked by the larger number of DEN cases. The ability to differentiate CHIK and DEN is critical for long-term care and prognosis of patients, since CHIK can cause prolonged arthralgia/arthritis, whereas DEN can cause hemorrhage and plasma leakage. This assay has made it possible to sustain active surveillance, support the diagnosis of febrile cases, and to monitor the incidence of CHIK. Similar assays can be easily developed in under-resourced countries to detect endemic diseases of public health importance for which only limited or costly commercial assays are available.

**Disclaimers** The opinions and views in this manuscript are the private views of the authors. The views expressed are not to be considered as official, or as reflecting the views of USAMRU-K or the United States Departments of the Army and Defense.

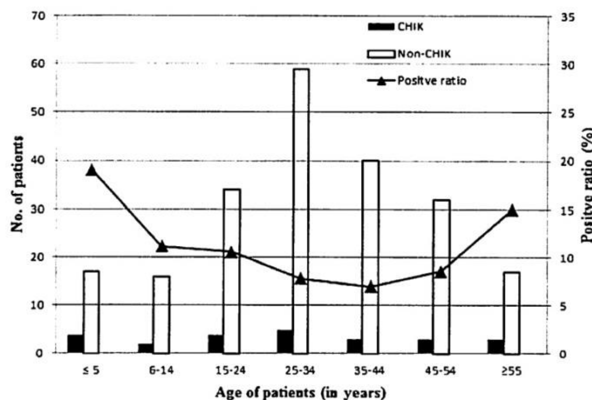


Fig. 2. Age distribution of laboratory confirmed CHIK and non-CHIK patients in 2013 in Eastern Kenya. Black bars indicate confirmed CHIK cases, white bars indicate non-CHIK cases and smooth curve indicates the positive ratio across the age-groups.

**Acknowledgments** The project was funded by the Consortium for National Health Research (CNHR), Kenya (Project number RLG09-001/031) and supported with reagents and equipment by the JICA-JSPS and JICA-JST-SATREPS projects. We appreciate technical support provided by the Production Department and Arbovirus/Viral Hemorrhagic Fever Laboratory, KEMRI. This work has been submitted by permission of the Director, KEMRI, Nairobi, Kenya.

**Conflict of interest** None to declare.

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