SEROPREVALENCE AND MOLECULAR DETECTION OF CHIKUNGUNYA VIRUS AMONG FEBRILE OUTPATIENTS SEEKING HEALTH CARE IN MZUZU CITY, MALAWI

FLYWELL KAWONGA

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA

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ABSTRACT

Chikungunya is a viral disease caused by Chikungunya virus (CHIKV) that is transmitted to humans by infected *Aedes aegypti* and *Ae. albopictus* mosquitoes. The objective of this study was to determine the seroprevalence of Chikungunya infection among febrile patients seeking healthcare in Mzuzu City, Malawi. Blood samples were collected during 2019 from outpatients attending Mzuzu Central Hospital who presented with fever but who were malaria and sepsis negative. Sera from outpatients were transported to the University of Malawi for analysis of the presence or absence of CHIKV specific immunoglobulin M (IgM) antibodies and CHIKV ribonucleic acid (RNA). Enzyme- linked immunosorbent assay (ELISA) was used to detect anti-CHIKV IgM antibodies while one step reverse transcription-polymerase chain reaction (RT-PCR) was conducted in IgM positive sera in order detect the presence of CHIKV RNA. Out of 119 CHIKV-suspected samples, 73 (61.3%) tested positive for anti-CHIKV IgM antibodies by ELISA. When 14 of the IgM seropositive sera were randomly selected and screened using RT-PCR, they were all positive for CHIKV RNA. This study has therefore provided an insight of current seroprevalence of Chikungunya and circulation of CHIKV among the human population in Mzuzu city, Malawi. These results suggest an active circulation of CHIKV in the population, therefore accurate laboratory assays are highly recommended for CHIKV diagnosis and appropriate management of febrile patients.

DECLARATION

I, **Flywell Kawonga**, do hereby declare to the Senate of Sokoine University of Agriculture, that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

_____ _____

Flywell Kawonga Date

(MSc. One Health Molecular Biology Candidate)

The above declaration is confirmed

_____ _____

Prof. Gerald Misinzo Date

(Supervisor)

5th October, 2020

_____ _____

Dr. Leonard Mboera Date

(Supervisor)

24th September, 2020

_____ _____

Dr. Isaac Thom Shawa Date

(Supervisor)

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LIST OF ABBREVIATIONS AND SYMBOLS

Ae.	*Aedes*
bp	base pair
CDC	Centers for Disease Control and Prevention
CHANCO	Chancellor College
CHIKV	Chikungunya virus
DENV	Dengue virus
DNA	deoxyribonucleic acid
DMARDS	disease modifying anti-rheumatic drugs
ECSA	East/Central/South African genotype
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
E1	envelope glycoprotein 1
E2	envelope glycoprotein 2
FBC	full blood count
IgG	immunoglobulin G
IgM	immunoglobulin M
TMB	tetramethylbenzidine
LAMP	loop-mediated isothermal amplification
MRDT	malaria rapid diagnostic test
NSAID	nonsteroidal anti-inflammatory drugs
ORF	open reading frame
PCR	polymerase chain reaction
P-value	probability value

RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SACIDS	Southern African Centre for Infectious Diseases Surveillance, Foundation for One Health
SUA	Sokoine University of Agriculture
USA	United States of America
µl	microliter
µM	micromolar
WHO	World Health Organization
ZIKAV	Zika virus

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Chikungunya is a viral disease caused by the Chikungunya virus (CHIKV) and is transmitted to humans by infected mosquitoes. The name Chikungunya comes from the Makonde language meaning ‘to walk bent over’ indicating the painful arthralgia experienced by people infected with CHIKV (Robinson, 1955). Patients that are infected with CHIKV present with fever, headache, rash, malaise and muscle pains (Powers *et al.*, 2007).

The largest outbreak of CHIKV has been recorded in the south-eastern islands of Indian Ocean and later cases were recorded on the islands of Reunion, Mayotte, Mauritius, the Seychelles and Madagascar during 2005-2007 (Pongsiri *et al*., 2012). In Africa, CHIKV infection has been reported in Angola, Burundi, Cameroon, Central African Republic, Gabon, Guinea, Malawi, Mozambique, Nigeria, Republic of Congo, South Africa, Tanzania and Uganda (Diop *et al.*, 2015; Wahid *et al*., 2017; Antonio *et al.*, 2018). Although CHIKV occurrence in Malawi has been documented (Diop *et al*., 2015), its prevalence is not known with certainty. This study was therefore conducted to determine the seroprevalence and to confirm CHIKV infection by using molecular assays.

1.2 Problem statement and justification of the study

CHIKV infection presents similar signs and symptoms to other diseases such as dengue, malaria, leptospirosis and brucellosis (Waggoner *et al*., 2017). The highly specific laboratory assays are of paramount use in differentiating viral infections which greatly present with fever (Cabralcastro *et al*., 2016). Hospitals and health care providers in

Malawi do not include the differential diagnosis of arbovirus infections in patients who present with fever. Malaria is often over diagnosed in febrile patients (Waggoner *et al*., 2017). There is underestimation of the burden for mosquito-borne viral diseases such Chikungunya and thereby leading to wastage of already scarce resources in Malawi. Information of the current prevalence of CHIKV infection in Malawi is not available. The findings of this study will consolidate the knowledge of the disease and provide evidence for developing appropriate control and prevention interventions in Malawi.

1.3 Research Questions

i. What is the seroprevalence of CHIKV infection in Mzuzu city Malawi?
ii. What is the genome detection rate of CHIKV circulating in Mzuzu city, Malawi?

1.4 Research objectives

1.4.1 Main objective

The main objective was to determine the seroprevalence of CHIKV infection among febrile outpatients attending healthcare service in Mzuzu city, Malawi.

1.4.2 Specific objectives

i. To determine the seroprevalence of CHIKV infetion among febrile patients seeking health care at Mzuzu central hospital, and
ii. To examine the presence of CHIKV genome among febrile patients seeking health care at Mzuzu central hospital.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Geographical distribution

CHIKV in Africa was first identified in Tanzania in 1952 (Robinson, 1955). A second outbreak happened in South Africa in 1956 (Pialoux *et al.*, 2007) and thereafter, epidemics were reported in Angola, Burundi, Central African Republic, Democratic Republic of the Congo, Guinea, Malawi, Nigeria, South Africa and Uganda until 1960's and 1990's. In June 2004, an epidemic occurred on Lamu Island in Kenya and spread to the Comoros, La Reunion and Indian Ocean islands (Renault *et al*., 2012). Another outbreak occurred in the city of Mombasa, Kenya and CHIKV remains endemic in Kenya (Moyen *et al.*, 2014). In 2005 and 2007, Madagascar experienced persistent CHIKV circulation (Diop *et al*., 2015). In 2011, in Republic of Congo, 37 (11.7%) cases out 317 CHIKV suspects were RT-PCR positive (Irin, 2011). In northern Tanzania, 7.9% of 870 febrile patients were RT- PCR positive for CHIKV (Weller *et al.,* 2014). In Kumbo, Cameroon in 2006, 51.4% tested positive for IgM anti-CHIKV antibodies (Ansumana *et al*., 2013; WHO, 2015). In Sudan, December 2005, IgM antibodies to CHIKV were detected in patients (Gould *et al.,* 2008).

Brazil, Rio de Janeiro was the first country to report CHIKV infection in 2010 in the Americas (Albuquerque *et al*., 2012). The CHIKV infections have also been detected in Guyana, Anguilla, Antigua, French Guiana and Barbuda, Dominican Republic, British Virgin Islands, Dominica, Guadeloupe, Haiti, Martinique, Puerto Rico, Saint Barthelme, Saint Kitts Nevis, Saint Lucia, Saint Martin, Saint Vincent and the Grenadines (Fischer *et al*., 2014).

CHIKV infection in Asia was first identified in Bangkok, Thailand in 1958. In India, the outbreaks occurred in Maharashtra, Tamil Nadu Andhra Pradesh and Barsi (Fischer *et al*., 2014; Chhabra *et al.,* 2008). China's first outbreak of CHIKV occurred in September 2010 (Qiaoli *et al.,* 2012).

CHIKV cases in Europe were first identified in Italy from a traveller (Gobbi *et al.,* 2014). Other cases of CHIKV were recorded in Italy, France, Croatia, Madeira, France and Spain. The emergence of CHIKV in Europe was caused mainly by globalization including international trade (Rezza *et al.,* 2007; Sane *et al*., 2011; Parreira *et al.,* 2014).

2.2 Aetiology

2.2.1 The Virus

CHIKV belongs to genus Alphavirus and the family *Togaviridae* (Mohan *et al.,* 2010). There are 30 species of alphaviruses which are also called arboviruses, meaning arthropod-borne viruses, of which all share seven specific antigenic complexes (Caglioti *et al.,* 2013). CHIVK is closely related to other alphaviruses, such as Ross River virus, Barmah Forest virus, O'nyong-nyong virus, Sindbis viruses, and the Mayaro virus (Caglioti *et al*., 2013).CHIKV is a single stranded RNA and enveloped (Brailla *et al.*, 2017). Its genome is approximately 11.8 kb (Singh *et al.*, 2011). The genome of the CHIKV is capped at 5' and polyadenylated at 3' (Solignat *et al*., 2009). The genome of CHIKV has two open reading frames (ORF) for gene expression and protein synthesis of non-structural (nSP) and structural proteins (Bharath *et al.*, 2016). The 5' ORF is responsible for biosynthesis of nSP1, nSP2, nSP3 and nSP4 which are primarily for synthesis of RNA polymerase protein responsible for CHIKV genome replication. The 3'ORF is responsible for the production of structural proteins which include glycoprotein which protects the CHIKV genome. There are two prominent glycoproteins (E1 and E 2)

that are used for viral attachment to the host cells. The 3’ also produces E3 and 6K protein products (Bharath, 2016). The genome of CHIKV is shown in the schematic diagram in figure 1.

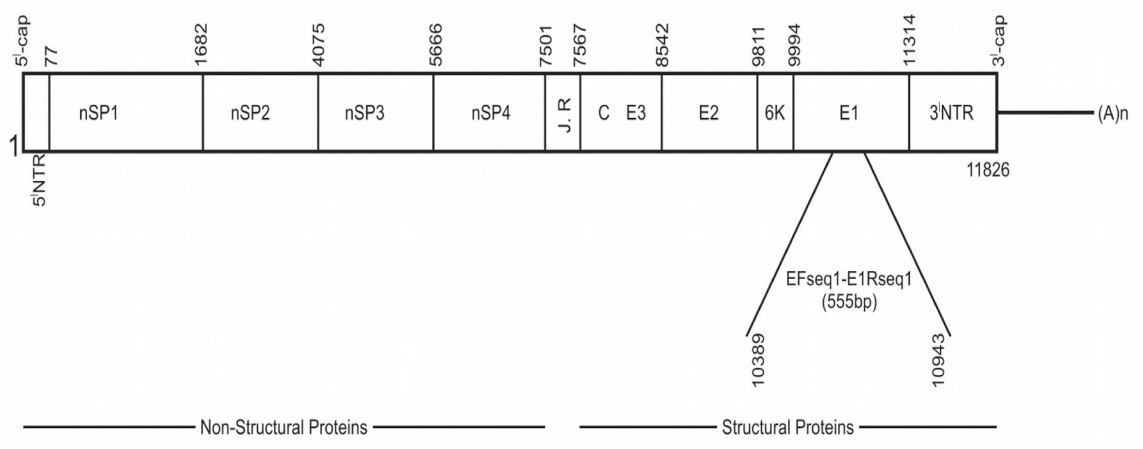

Figure 1: Schematic diagram of the structure and orientation of CHIKV genome

Source: (Bharath, 2016).

The genotypes of CHIKV are based on phylogenetic analysis of E1 gene sequence of the virus (Singh *et al.,* 2011). CHIKV genotypes are: the East/Central/South African (ECSA), West Africa, India and Asian (Pongsiri *et al*., 2012). The genotypes are named based on their primary geographical places where they were first isolated and characterised. The West African and ECSA CHIKV genotype mainly circulate in sub-Saharan Africa. The ECSA genotype has given rise to the Asian lineage which is found in urban cycles in India and Southeast Asia and also to the Indian Ocean genotype, which emerged from Kenya into the Indian Ocean Basin in 2004 (Weaver *et al*., 2015).

2.2.2 Pathophysiology of CHIKV infection

Humans are the primary host of CHIKV which is spread from one person to another via infected mosquito bites. CHIKV infection in humans targets specific cell types that are

susceptible for their attachment and viral replication. The cells that are vulnerable to CHIKV infection and viral replications are; human epithelial and endothelial cells, primary fibroblasts and macrophages derived from monocytes. Lymphoid and Monocytoid cells, early lymphocytes, monocytes and dendritic cells were unable to demonstrate CHIKV replication during a study conducted by Sourisseau *et al.* (2007). However, CHIKV-positive Monocytes have been detected in vivo from CHIKV-infected patients (Kam *et al.*, 2009). The virus attaches to the host cell receptors by using mainly E1 and E2. The virus genome information then directs the cell to make the proteins for the use of the virus for replication and transcription of its own proteins (Gorchakov *et al.*, 2005). After the primary infection, there is a number of immune reactions but the virus goes to the lymph nodes and then to other tissues via the cardiovascular system (Kam *et al.*, 2009).

2.3 Transmission

2.3.1 Vectors

Ae. *aegypti* and *Ae*. *albopictus* are the main vectors of CHIKV (Chhabra *et al.*, 2011; Bharath *et al.*, 2016). CHIKV is transmitted in two different pathways which include urban and sylvatic cycles. The urban cycle is the transmission of CHIKV from human to mosquito to human while sylvatic transmission refers to the transmission of CHIKV from animal to mosquito to human (Singh *et al.*, 2011). The sylvatic cycle is the primary form of maintenance in Africa (Chhabra *et al*., 2008).

2.4 The disease

2.4.1 Risk factors

A study conducted in Senegal found that all species of *Aedes*. responsible for transmitting CHIKV were higher at the periphery of villages than at the center, suggesting a high risk of CHIKV infection at the edges of villages (Diawo *et al.*, 2012). A study done in North-eastern Tanzania found that environmental factors such as living in a house with uncovered containers, keeping hoofed animals and vegetation (<100 m) were associated with high CHIKV IgM seropositivity (Kajeguka *et al.*, 2017).

A study conducted by Diawo *et al.* (2012) in Southeastern Senegal reported that absolute vector abundance has a considerable seasonal variation with *Ae. vittatus*, *Ae. luteocephalus* and *Ae*. *aegypti* reaching their peak abundance in June at the beginning of rainy season and decline during the following months . Total vector abundance peaks at the start of wet season and declines after rainfall increases and temperature decreases. Vector abundance increases in November as rainfall drops and temperatures start to increase. Vector abundance increases the risk of CHIKV transmission to humans.

2.4.2 Signs, symptoms and complications of Chikungunya

Signs and symptoms of CHIKV infection generally appear after an incubation period of 3–7 days after exposure to CHIKV (Lum *et al*., 2015). Acute fever and polyarthralgia are highly indicative of CHIKV infection although they are non-specific (Lum *et al.*, 2015). Joint pain is mostly bilateral, symmetric and debilitating. Ophthalmic, neurological and cardiac symptoms also occur in CHIKV infections (Martínez *et al*., 2016). Complications of Chikungunya include myocarditis, hepatitis, ocular and neurological disorders (Farnon *et al.*, 2008).

CHIKV infection course is divided into an acute stage which lasts approximately one week. The acute stage of CHIKV infection is noted to have a high viremic load of the virus with an average of 107 pfu/mL (Thiberville *et al*., 2013). A chronic stage lasts from months to years in CHIKV infected patients. During this stage, small viral loads persist for a long time causing continued signs and symptoms of CHIKV (Lum *et al*., 2015). Chronic CHIKV infections cause socio economic and health complication for those infected and public health problems for the societies (Gérardin *et al.*, 2011). In a study by Manimunda and others (2010) it was found that symptoms of one-month post-infection were rheumatism (75%) and fatigue (30%), with joint pain, fatigue, and neuritis being present after ten months. A study by De Brito *et al*. (2016) reported that 50% of people infected with CHIKV will experience chronic symptoms. The disease has more severe effects on neonates and the elderly (Gérardin *et al.*, 2008).

2.4.3 Diagnosis of CHIKV

Diagnosis of arboviruses including CHIKV is difficult in endemic areas where they co-exist with malaria because they are all characterised by similar signs and symptoms (Cabralcastro *et al.*, 2016). The duration of viremia and the response of the host immune response should be taken into consideration when selecting the detection tests and sampling (Chusri *et al.*, 2014). CHIKV is diagnosed by detecting the identification of the specific immune responses of the host to CHIKV and viral RNA by RT-PCR. ELISA is used for diagnosis of CHIKV infection by detecting either IgM or IgG antibodies specific antibodies are detectable in serum 5–7 days after onset of clinical symptoms (Lanciotti *et al.*, 2007). There are also commercial immunochromatographic antigen detection kits that detect E 1 antigen of CHIKV with high sensitivity in the early phase (up to 4–5) days after the onset of symptoms. The tests have high specificity and no

cross-reactions with other Arboviruses such as dengue virus (DENV) have been established to exist (Jain *et al.*, 2018).

Viral RNA is detected by real-time RT-PCR in the first week after onset of clinical symptoms, (Lanciotti *et al.*, 2007). Molecular diagnostic tests used for the diagnosis of CHIKV detect and amplify fragments such as nSP1, nSP2, nSP3, nSP4 and E1 genes of the CHIKV genome (Lanciotti *et al.*, 2007).

Loop-mediated isothermal amplification (LAMP) amplifies CHIKV target genome under isothermal conditions without the use of a thermal cycler and is a fast, specific, and cheap technique for CHIKV diagnosis (Reddy *et al.*, 2014).

2.4.4 Treatment

The current treatment targets on alleviating severity of symptoms and not curing the disease. Treatments that are mainly used are antipyretics and nonsteroidal anti-inflammatory drugs (NSAIDs) (Rougeron *et al.*, 2015). The corticosteroids are used as a treatment for acute CHIKV cases (Goupil *et al*., 2016). Maintaining proper fluid levels is important in CHIKV infections. Anti-rheumatic drugs such disease modifying anti-rheumatic drugs (DMARDs) including methotrexate, hydroxychloroquine or sulphasalazine, have been proposed for treating severe chronic arthralgia (Bouquillard *et al*., 2009; Foissac *et al.*, 2016).

2.4.5 Control and prevention of CHIKV

Public health education on environmental management practices is needed to eliminate the risks by removing uncovered water containers that serve as breeding sites for *Aedes*.

mosquitoes. Avoiding animal husbandry in domestic environment and clearing of vegetation surrounding houses prevents the breeding of mosquitoes responsible for CHIKV transmission (Kajeguka *et al.*, 2017)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design, sample collection and storage

The present study used archived samples that were stored at -80 °C collected in 2019 from outpatients at Mzuzu Central Hospital, Mzuzu city, Malawi. The outpatients were examined by a qualified clinician at the health facility and enrolled as participants according to the inclusion criteria. The whole blood sample was collected from consenting participants using venipuncture technique by qualified laboratory technologists/technicians. Serum was separated by centrifugation and aliquoted into cryovials and temporarily stored in -80 °C ultralow freezers at The University of Malawi, Chancellor College (CHANCO), Department of Biological Sciences, Vector-Borne Disease Laboratory in Zomba, Malawi.

The inclusion criteria were outpatients with fever ≥38 °C, for not more than 5 days and with symptoms of chills, headache, severe joint pains, dizziness, nausea/vomiting, arthralgia and rash were recruited into the study. Malaria negative patients with a non-raised neutrophil count (1.5-8 x 10^9/L) were screened for CHIKV infections.

3.2 Onsite laboratory tests

Malaria screening was done using malaria rapid test, SD Bioline Malaria Ag- Pf/Pan kit (Standard Diagnostics, Suwon city, Republic of Korea). Blood samples were drawn by finger prick from the patient. Malaria tests were performed as per the standard operating procedure for the kit. Sample of blood (5ml) were drawn from all consenting malaria negative patients and put in ethylene diamine tetra-acetic acid (EDTA) and red top plain tubes. Full blood Count (FBC) tests were done to rule out sepsis. Testing followed the

approved standard operating procedures at the facility. Patients with normal FBC parameters were recruited into the study.

The SD Bioline Chikungunya IgM rapid test (Standard Diagnostic, Kyonggi-do, Korea) was used to screen for anti-CHIKV IgM antibodies onsite. This is a rapid test that is one step *in vitro* immunochromatographic test for detection of anti-CHIKV antibodies in patient's blood. When positive results were established using the rapid test, the patient's sample was included in the study for further testing by ELISA and RT-PCR. The sample was span at 400*g* for 5 minutes to separate serum from cells. The separated serum was stored at -20 °C and cells stored at 2-8 °C. Both were transferred to the Vector-Borne Disease Laboratory at The University of Malawi, (CHANCO) in Zomba for ELISA and RT -PCR testing.

3.3 Laboratory analysis

The stored samples were analysed for the presence or absence of CHIKV specific antibodies and CHIKV specific viral genes. The ELISA test was used to detect anti-CHIKV IgM antibodies to determine seroprevalence. Then IgM seropositive samples were subjected to RT-PCR using specific primers for confirmation of CHIKV infections.

3.3.1 Serological analysis of anti-CHIKV IgM antibodies

The ELISA test was used to detect anti-CHIKV IgM antibodies to infer recent and active infections. Stored samples were tested for anti-CHIKV antibodies using Abcam's ELISA Kit (Abcam, Cambridge, UK) by strictly following manufacturer's instructions. Briefly, microtiter strip wells were precoated with anti-human IgM to bind corresponding antibodies of the specimen. Afterwards, 50µl of samples or control were added to the relevant wells and incubated at 37^{o}C for 1 hour. The wells were washed to remove all

unbound sample and control material. Chikungunya antigen solution 1 was added and incubated for 30 minutes at room temperature. The wells were washed again and Chikungunya antigen solution 2 was then added and incubated for 30 minutes at room temperature. The wells were washed again. Streptavidin conjugate was added and incubated for 30 minutes at room temperature. The wells were then washed again. After washing, the captured Chikungunya-specific immuncomplex was visualized by adding tetramethylbenzidine (TMB) substrate and incubated at room temperature for 15 minutes. The intensity of this product was proportional to the amount of CHIKV-specific IgM antibodies in the patient specimen. Sulphuric acid was added to stop the reaction. This produced a yellow endpoint colour. Absorbance at 450 nm was read using an ELISA microwell plate reader (Bio-Rad, California, USA).

3.3.2 Molecular detection of CHIKV

3.3.2.1 RNA extraction

Viral RNA was extracted from fourteen randomly selected Chikungunya seropositive samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 560 µL prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 mL micro centrifuge tube. A total of 140 µL serum was added to the buffer AVL–carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 seconds and incubated at room temperature for 10 minutes. Briefly the tube was centrifuged to remove drops from the inside of the lid. Afterwards, 560 µL ethanol (96–100%) was added and mixed by pulse-vortexing for 15 seconds. After mixing, briefly the tube was centrifuged to remove drops from inside the lid. Then 630 µL of the solution from previous mixture was applied to the QIAamp Mini column (in a 2 mL collection tube) without wetting the rim. The cap was closed and centrifuged at 6000 x *g* /8000 revolutions per minute (rpm) for 1 minute. Then the QIAamp Mini

column was placed into a clean 2 mL collection tube, and the tube containing the filtrate was discarded. The QIAamp Mini column was opened and the previous step was repeated. Then the QIAamp Mini column was carefully opened and 500 µL buffer AW1 was added. The cap was closed and centrifuged at 6000 x *g* / 8000 rpm for 1 min. Then the QIAamp Mini column was placed in a clean 2 mL collection tube and the tube containing the filtrate was discarded. The QIAamp Mini column was carefully opened and 500 µL Buffer AW2 was added. The cap was closed and centrifuged at full speed (20 000 x *g*/ 14 000 rpm) for three minutes. Then QIAamp Mini column was placed in a new 2 mL collection tube and the old collection tube with the filtrate was discarded and centrifuged at full speed for one minute. The QIAamp Mini column was placed in a clean 1.5 mL microcentrifuge tube. Then old collection tube containing the filtrate was discarded. The QIAamp Mini column was opened, 60 µL buffer AVE was added and equilibrated at room temperature. The cap was closed and incubated at room temperature for 1 minute and centrifuged at 6000 x g (8000 rpm) for 1 min to elute extracted DNA. The elution with 60 µL buffer AVE contained at least 90% of the viral RNA from the QIAamp Mini column. Viral RNA was then stored at -20 °C until used for one step RT-PCR.

3.3.2.2 RT-PCR

Extracted viral RNA was used in one step RT-PCR using titan one tube RT-PCR Kit (Roche, California, USA) in GeneAmp PCR System 9700 (Applied Biosynthesis, Foster city, California, USA). Then nSP1 gene was amplified using primers 5’-TAG AGC AGG AAA TTG ATC CC-3’ as sense primer and 5’-CTT TAA TCG CCT GGT GGT AT-3’ as anti-sense primers respectively, adapted from Futoshi and others (2002). Preparation of a 25 µL reaction mixture containing 12.5 µL of 2x reaction mix, 1 µL of enzyme mix (reverse transcriptase/*Taq* DNA polymerase), 0.5 µL of 10 µM forward primer, 0.5 µL of

10 µM reverse primer, 0.5 µL magnesium salt, µM 8 µL of nuclease-free water and 2 µL of RNA template. Amplification conditions were as follows ; reverse-transcription reaction at 42 ºC for 60 minutes , inactivation of reverse transcription at 94 ºC for 3 minutes; denaturing at 94 ºC for 1 minute, annealing at 54 ºC and extension at 68 ºC for 2 minutes for 35 cycles, and final extension at 68 ºC for 7 minutes.

3.3.2.3 Separation of RT-PCR products by agarose gel electrophoresis

After conducting one step RT-PCR, PCR products were visualized after separation using agarose gel electrophoresis. Briefly, 2% agarose gel was prepared and run in Tris-acetate EDTA buffer (Sigma-Aldrich, St. Louis, Missouri, USA). The DNA ladder (Bio-Rad Laboratories, California, USA) of 100bp was loaded into first well followed with14 samples (DNA PCR products) on position 1-14. The negative and positive controls were loaded at the end of the gel but on separate wells as well. Positive control was the last to be loaded into the gel to prevent contamination. Agarose gel electrophoresis was performed by connecting the electrophoretic tank to electric current for 60 minutes at 100 Volts. After agarose gel ectrophoresis, the agarose gel was stained with Gel Red (Bio-Rad, California, USA) and visualized on an ultraviolet transilluminator (Bio-Rad, California, USA) and photos taken by digital camera.

3.4 Ethical clearance

Ethical approval to conduct the study was obtained from the College of Medicine Research and Ethics Committee ((P.02/20/2956). All study participants were consented to participate in the study.

3.5 Data analysis

Data collected from the clinical history were entered and analyzed using Microsoft Office-Excel 2007 (Microsoft, California, USA) and Epi Info version 7.0.8.0 (CDC, Atlanta, USA). Sex, present fever (fever of ≥38 °C measured by the clinician at the hospital), previous of fever (fever presented in patients before the onset of other symptoms at home), abdominal pains, joint pains, bleeding (from the nose and gums), nausea, backache, body weakness, chest pain chest pain/backache, dizziness, fast breathing/cough, heart palpitations, itchy legs, leg swelling, redness of eyes, sweating, throat pain and yellow eyes were used to draw frequency tables in relationship to seropositivity of ELISA test.

CHAPTER FOUR

4.0 RESULTS

4.1 Socio-demographic characteristics of the study participants

A total 119 participants who met the inclusion criteria were recruited into the study. Of these, 79 (66.39 %) were females and 40 (33.61%) were males. The participant's mean age was 31 years (2-83 years). The proportion of patients who tested positive for anti-CHIKV IgM antibodies by Elisa test among different age groups is presented in table 1 below.

4.2 Seroprevalence of Chikungunya virus infection among age groups

Of the total 119 sera analysed for anti-CHIKV IgM antibodies using ELISA, 73 (61.3%) tested positive. The highest proportion (19.2%; n=14) of seropositivity among anti-CHIKV IgM positive individuals were observed among individual aged 30-39 years. Age groups of 1-9, 40-49 and ≥50 years were the second to be detected to have high proportions of anti-CHIKV anti-IgM antibodies. Age of 20-29 years had 11 (15.1%) cases that were anti-CHIKV IgM antibodies positive. Individuals aged group of 10-19 years had the lowest prevalence (12.3%, n=9).After running Chi-square independence test using SPSS software, the results showed no any significance difference among age groups in association with the observed seropositive results (p-value <0.05) (Table 1).

Table 1: The proportion of anti-CHIKV IgM antibodies among seropositive individuals by age group

Age group (years)	Number of participants (n=119)	Number of anti-CHIKV IgM seropositive No. (n=73)	Chi square value (X2)	P-value
1 to 9	23	13	1.59	0.9
10 to 19	13	9		
20 to 29	20	11		
30 to 39	20	14		
40 to 49	21	13		
50 and above	22	13		

4.3 Seroprevalence and clinical presentation of study participants

Studies have shown symptoms and signs such as joint pains, fever, polyarthralgia, abdominal pains, bleeding from the nose and gums etc. are associated with Chikungunya infection (Lum *et al.*, 2015; Martínez *et al*., 2016). In this present study, of the total 119 sera analysed for anti-CHIKV IgM antibodies using ELISA, 73 (61.3%) tested positive. A total of 33 (45.21%) patients who presented with joint pains were positive for anti-CHIKV IgM antibodies .The proportions (30.14%) of those with present fever were not seropositive for CHIKV. Patients without abdominal pains (58.33%, n=42) had higher seropositivity of anti-CHIKV IgM antibodies than those with abdominal pains (41.67%, n=30). Thirty-eight (52.05%) patients with history of previous fever tested positive for anti-CHIKV IgM antibodies. Sixty-four (87.67%) patients with no bleeding history were positive for anti-CHIKV IgM antibodies. Majority (83.56 %; n=61) of patients without history of vomiting tested positive for anti-CHIKV IgM antibodies. All the results showed no any significance after running Chi-square test (p-value <0.05) (Table 2).

Table 2: Clinical presentations among anti-chikungunya seropositive in patients Clinical presentation of patients and positive anti-CHIKV IgM antibodies detection

Clinical presentation	Participant 's response	Number of participants n=119	Number of anti-CHIKV IgM positive n=73	Chi square X2	P-value
Acute fever	Yes	34	22	0.227	0.634
	No	85	51		
Previous fever	Yes	63	38	1.892	0.169
	No	56	35		
Bleeding	Yes	12	9	1.049	0.306
	No	107	64		
Vomiting	Yes	20	12	0.018	0.892
	No	99	61		
Joint pain	Yes	55	33	0.078	0.78
	No	64	40		
Abdominal pain	Yes	47	30	0.202	0.653
	No	72	43		

Backache, body weakness, chest pain, fast breathing, itchy legs, leg swelling, nausea, redness at eyes, sweating, throat pain and yellow eyes had a frequency of 1(5%) each as clinical presentation among anti-CHIKV IgM seropositivity. Chest pain and heart palpitation had a frequency of 2(10%) each, and dizziness had a frequency of 3(15%) among anti-CHIKV IgM seropositivity.

4.4 Presence of CHIKV in sera

A total of 14 sera that were IgM positive for anti-CHIKV on ELISA were randomly selected for one step RT-PCR (this was done to detect the presence of CHIKV RNA in patients, only 14 samples were randomly selected). All 14 samples tested positive for RT-PCR upon separation on gel electrophoresis. The results of agarose gel electrophoresis were captured by a digital camera (Figure 2).

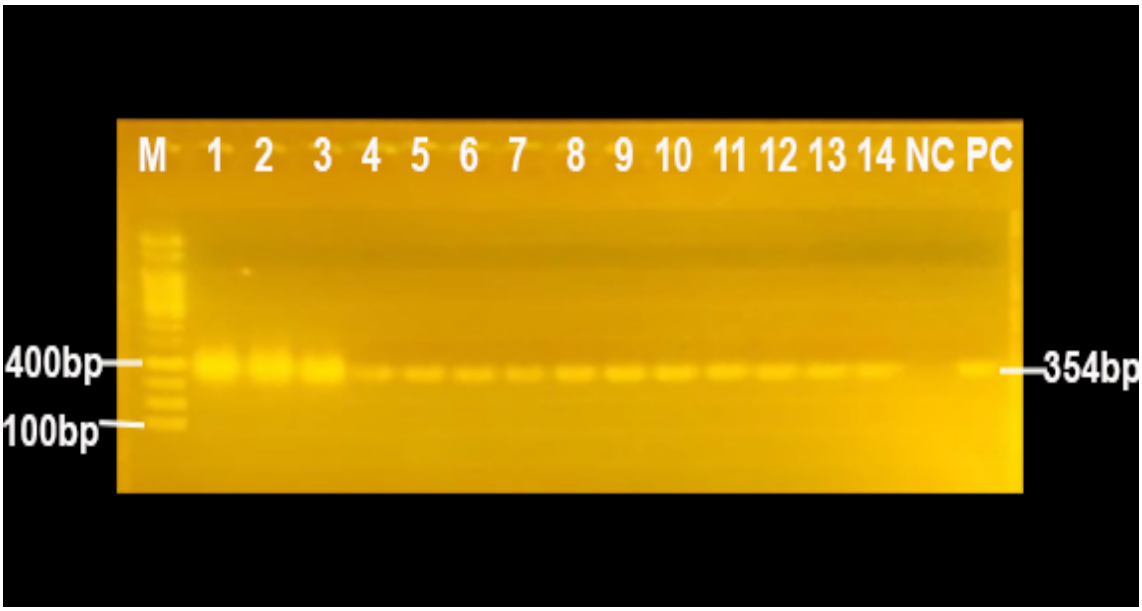

Figure 2: Detection of CHIKV RNA in chikungunya seropositive cases. **The expected size after separation by agarose gel electrophoresis is 354 bp. M is a 100 bp DNA ladder, lanes 1 to14 were loaded with samples, NC is a negative control and PC is a positive control.**

CHAPTER FIVE

5.0 DISCUSSION

The study has established the presence of antibodies against Chikungunya virus amongst febrile patients seeking health care at Mzuzu Central Hospital. In addition, the present study confirmed the existence of circulation of CHIKV in Mzuzu city, Malawi by detection of CHIKV RNA using RT-PCR, a very sensitive and specific technique in diagnosing infectious disease (Shailendra *et al.,* 2017). The presence of anti-chikungunya IgM antibodies confirmed the presence of recent CHIKV infection in patients.

The results indicate a high seroprevalence of Chikungunya (61.3%) as detected by presence of anti-CHIKV IgM antibodies patient's sera. The seroprevalence of Chikungunya in this study is slightly lower but similar to a study in Eastern Sudan, where a seroprevalence of Chikungunya of 73.1% was reported (Nahla *et al.,* 2019). The seroprevalence reported in this study is slightly higher but similar with seroprevalence reported among febrile patients in Cameroon and Zambia with a seroprevalence of 51.4% (anti-CHIKV IgM antibodies) and 36.9% (anti-CHIKV IgG antibodies) respectively (Pistone *et al.,* 2009; Chisenga *et al*., 2020). The difference in seropositivity in this study and other studies may be due to differences in sample sizes and methodologies used. The high seroprevalence in this study is likely to be attributed to the strict inclusion criteria of suspected patients of CHIKV infection whereby patients with malaria and sepsis were excluded for enrolment into the study. Therefore, only samples that were very suspicious of CHIKV infection and other febrile illnesses apart from malaria and sepsis were enrolled into the study to be confirmed by more specific and sensitive assays such as ELISA and RT-PCR and thereby decreasing the unnecessary number of suspects, leading to higher seropositivity. Furthermore, by not including

malaria positive samples, differential diagnosis was narrowed down to CHIKV infection and leading to high seroprevalence determined by ELISA assay. This argument is supported by a study done by Kinimi *et al.,,* (2018) in Tanzania where they found a higher seropositivity of anti-CHIKV antibodies of 91.75% among malaria negative patients. However in the same proceeding study, in Tanzania by Kinimi *et al*., 2018 found a co-infection of Chikungunya and malaria to be 7.4 %.

This study has established that relying on clinical presentation of patients with probable suspicion of CHIKV infection is unreliable. Slightly below half (45.21%) of the patients who presented with joint pains were positive for anti-CHIKV IgM antibodies and more than half of those without joint pains (54.7%) were positive for anti-CHIKV IgM antibodies. Although joint pain is the critical symptom for CHIKV infection clinically as its name suggest for Chikungunya (Schilte, *et al*., 2013) , this study has established that even without joint pain, CHIKV infection is likely to be prevalent in infected patients. This has further shown that some patients do not present joint pains but have CHIKV infections and therefore it is only laboratory analyses that can elucidate the differential diagnosis of CHIKV infection. This therefore calls for the highly specific laboratory techniques such as serology and molecular nucleic acid amplification for their reliability to differentiate viral and other infections which greatly present with fever (Cabralcastro *et al*., 2016). This study has therefore proved the hypothesis that CHIKV is prevalent in Mzuzu city and that people have been exposed to the virus. The study has provided an insight of the burden of Chikungunya among non-malaria and non-septic cases in Mzuzu city, Malawi.

The present study indicates the cases of CHIKV were recent ones through detection of IgM anti-CHIKV antibodies. By detecting anti-CHIKV IgM antibodies, the results

suggest a possibility of acute infection. IgM antibodies appear first and can be detected during the first week of the disease particularly from day 5 (Panning *et al.,* 2006).

To confidently confirm CHIKV circulation in the study population, the study also employed molecular techniques to detect viral RNA. CHIKV viral RNA (nSP 1 gene) was amplified and detected in sample patients using one step RT-PCR. All of the 14 randomly selected sera were RT- PCR positive. This test was carried to confirm the serology results by ruling out false positive results as a result of possible cross reactivity of host antibodies against arboviruses. Some of the positivity of ELISA results needs to be carefully interpreted as we may not rule out possibility cross-reactivity of other antibodies as only a few seropositive samples were subjected for RT-PCR test as the test was only done to confirm and detect CHIKV in patients sera. There is a possibility of other arboviruses cross reactivity when IgM antibodies are used for detection (Lanciotti *et al.,* 2008). Furthermore, co-infection of DENV, CHIKV and Zika virus among study patients cannot be ruled out from positive results detected in serology. The occurrence of cross-reactivity and co-infection of DENV and CHIKV have been reported in Columbia (Villamil *et al.,* 2015). There is need to develop multiplex assays for detection of all arboviruses of medical importance so that correct and reliable diagnosis is achieved where these viruses are endemic. By employing molecular assays such as RT-PCR in this study, we confidently report the occurrence and prevalence of CHIKV in Mzuzu city, Malawi with certainty.

This study therefore indicates that CHIKV is prevalent and contributes to the burden of febrile illnesses in Mzuzu city, Malawi. Accurate laboratory assays with high specificity and sensitivity as molecular assays are of paramount importance for differential diagnosis of febrile illnesses in endemic areas. The study has established that there an

underestimation of arbovirus infections and misdiagnoses which lead to improper treatments and wastage of already scarce resources for a low income country as Malawi. Therefore, clinicians should consider CHIKV infection as a differential diagnosis in febrile cases.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The findings of this study have determined the current seroprevalence status of CHIKV infection in Mzuzu city, Malawi to be high through detection of anti-CHIKV IgM antibodies. The results presented in this study have also confirmed CHIKV circulation as evidenced by presence of CHIKV RNA in patient's sera. Overall, this study therefore concludes that Chikungunya is prevalent in Mzuzu city, Malawi and that serological and molecular assays are of paramount use in detection and confirmation of Chikungunya infections.

6.2 Recommendations

i. The governments need to mobilise facilities and laboratory tests for detection of CHIKV among febrile patients.
ii. Physicians should consider including Chikungunya in their differential diagnosis among febrile patients. This will ensure correct diagnosis and correct management of patients and therefore avoid unnecessary use of already scarce resources.
iii. Vector control strategies need to be established to control the mosquitoes responsible for CHIKV transmission.
iv. Education need to be provided to the clinicians on how to recognize suspects of Chikungunya.
v. There is need to promote community awareness to reduce risk exposure to mosquito bites during the day and other potential risk factors.

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APPENDICES

Appendix 1: Demographic, clinical characteristics and CHIKV infection

Site	ID	Sex	Fever Now	Temperature	Fever Previous	Rash	Bleeding	Vomiting	Joint pain	Abdominal pain	Other	ELISA Results	RT-PCR Results
MZZ	001	Female	0		1		0	0	0	0		0	N/A
MZZ	002	Female	0	37.5	1		0	0	0	0		0	N/A
MZZ	003	Female	0		0		0	0	0	0		1	1
MZZ	004	Female	0	36.8	1		0	0	1	1		1	ND
MZZ	005	Female	1	37.1	0		1	0	1	0	Backache	0	N/A
MZZ	006	Female	1	36.2	1		0	0	0	1	Dizziness	1	1
MZZ	007	Female	0	36.6	0		0	0	0	1		0	N/A
MZZ	008	Male	1		1		0	1	0	0	Cough	0	N/A
MZZ	009	Female	0	37.9	1		0	0	0	0	Dizziness	0	N/A
MZZ	010	Female	1	38.1	1		0	1	0	0	Backache	1	ND
MZZ	011	Female	0	36.2	1		0	0	0	1		1	ND
MZZ	012	Male	0	36.4	0		0	0	0	1	Nausea	1	ND
MZZ	013	Female	1	38.6	1		0	0	0	1		1	1
MZZ	014	Female	0	36.4	0		0	1	1	1		1	ND
MZZ	015	Female	0	38.5	1		0	0	0	0		1	
MZZ	016	Male	1	38.5	1		0	0	0	0	Chest pain	1	1
MZZ	017	Male	0		0		0	0	0	0		0	N/A
MZZ	018	Female	0	37	0		0	0	0	0	Heart papitations	1	ND
MZZ	019	Male	0	36.4	0		0	0	1	1		1	ND
MZZ	020	Male	0		0		0	0	0	0		1	ND
MZZ	021	Female	1	38.6	**1**		0	1	1	1		1	ND

MZZ	102	Male	0	37.7	1		0	0	0	0		1	ND
MZZ	103	Female	0	37.9	0		0	0	0	0		1	ND
MZZ	104	Male	0		1		0	0	0	0			N/A
Site	**ID**	**Sex**	**Fever Now**	**Temperature**	**Fever Previous**	**Rash**	**Bleeding**	**Vomiting**	**Joint pain**	**Abdominal pain**	**Other**	**ELISA Results**	**RT-PCR Results**
MZZ	105	Female	0		0		0	0	0	0	Heart papitations	1	ND
MZZ	106	Male	0		1		0	1	1	1		1	ND
MZZ	107	Female	1		0		0	0	0	0	Facial Edema	0	N/A
MZZ	108	Female	0		0		0	0	0	0		0	N/A
MZZ	109	Male	1		1		0	0	1	0			N/A
MZZ	110	Female	0		1		0	1	0	1		1	ND
MZZ	111	Female	1		1		0	0	1	0		0	N/A
MZZ	112	Male	1		1		0	0	1	0		1	ND
MZZ	113	Female	0	37.8	0		0	0	1	0		0	N/A
MZZ	114	Male	0	0	0		0	0	1	1	Diarrhea, weakness	0	N/A
MZZ	115	Male	1		0		0	0	0	1		0	N/A
MZZ	116	Female	0	37.8	0		0	0	0	0		1	ND
MZZ	117	Female	1		1		0	0	1	1		0	N/A
MZZ	118	Female	0	37.9	1		0	0	1	0		1	ND
MZZ	119	Male	1		1		1	0	1	0		1	ND
MZZ	022	Male	0		0		0	0	1	0		1	ND
MZZ	023	Female	0		0		0	0	1	1		0	N/A
MZZ	024	Female	0		0		0	0	0	0		1	ND
MZZ	025	Female	0		1		0	0	1	0		1	ND
MZZ	026	Female	0	37.5	1		0	0	1	1	Sweating	1	ND
MZZ	027	Male	1	38	**1**		0	0	0	0	Convulsions	**0**	N/A
MZZ	028	Male	1	39.2	1		0	0	0	1	Redness at eyes	1	ND
MZZ	029	Female	0		0		0	0	1	0	Backache	0	N/A
MZZ	030	Male	1		1		0	0	1	1		1	1

MZZ	031	Female	0		0		0	0	0	0	Dizziness	0	N/A
MZZ	032	Male	0		1		0	0	0	1		1	ND
Site	**ID**	**Sex**	**Fever Now**	**Temperature**	**Fever Previous**	**Rash**	**Bleeding**	**Vomiting**	**Joint pain**	**Abdominal pain**	**Other**	**ELISA Results**	**RT-PCR Results**
MZZ	033	Female	0		1		0	0	1	1		1	ND
MZZ	034	Female	0		1		0	0	1	1		0	N/A
MZZ	035	Male	0		0		1	0	1	0	Chest pain	1	ND
MZZ	036	Female	0		0		0	0	0	0	Chest pain/Backache	1	1
MZZ	037	Female	0		0		0	1	0	0	Dizziness	0	N/A
MZZ	038	Female	0		0		0	0	0	0		0	N/A
MZZ	039	Female	0	36	0		0	0	1	0		0	N/A
MZZ	040	Female	0		0		0	0	1	1		0	N/A
MZZ	041	Female	1		1		0	0	0	0		1	ND
MZZ	042	Female	0		0		0	0	0	0		0	N/A
MZZ	043	Female	1	39.7	1		0	0	1	1		0	
MZZ	044	Female	1	38.1	0		0	1	0	0		1	ND
MZZ	045	Male	0	37.9	0		1	0	0	0	Sores on buttocks	0	N/A
MZZ	046	Male	0		0		0	0	0	1		1	ND
MZZ	047	Female	1	38.1	0		0	0	0	0		1	1
MZZ	048	Female	1	38.2	1		0	0	1	0		1	ND
MZZ	049	Female	1	38	1		0	0	1	0		1	ND
MZZ	050	Male	0	37.1	1		0	0	0	0	Itchy legs	1	ND
MZZ	051	Male	0		**1**		0	0	0	0		**1**	ND
MZZ	052	Female	0	36	0		0	0	0	0		0	N/A
MZZ	053	Female	0	36	0		0	0	1	0		0	N/A
MZZ	054	Female	0	36	1		0	0	1	0		0	N/A
MZZ	055	Female	1	38.9	1		0	0	1	0		0	N/A
MZZ	056	Male	1	38.4	0		0	0	0	0		1	ND
MZZ	057	Male	0		1		0	1	1	1		0	N/A

MZZ	058	Female	0		0		0	0	0	0		0	N/A
Site	**ID**	**Sex**	**Fever Now**	**Temperature**	**Fever Previous**	**Rash**	**Bleeding**	**Vomiting**	**Joint pain**	**Abdominal pain**	**Other**	**ELISA Results**	**RT-PCR Results**
MZZ	059	Male	0		0		0	1	1	1	Diarrhea	0	N/A
MZZ	060	Female	0		0		0	1	0	0	Dizziness	1	1
MZZ	061	Female	0		0		0	1	0	1		1	ND
MZZ	062	Male	0		0		0	0	0	1	Abnominal pause	1	ND
MZZ	063	Female	0		0		0	0	0	0	Leg swelling	1	ND
MZZ	064	Female	0		0		0	0	0	1		1	1
MZZ	065	Male	0	36.1	0		1	0	1	1		1	ND
MZZ	066	Female	0	36.5	1		0	0	1	0	Throat pain	1	ND
MZZ	067	Female	1	40	1		1	1	0	1		1	ND
MZZ	068	Female	0		0		1	0	0		Menstruation bleeding	1	ND
MZZ	069	Female	0		0		0	0	1	0		1	ND
MZZ	070	Male	1		1		0	0	0	1	Abd. Pain/shivering	0	
MZZ	071	Male	1		1		0	0	0	0	Fast breatihng/cough	1	1
MZZ	072	Male	1		1		0	0	1	1		1	ND
MZZ	073	Female	1		1		0	1	0	0		1	ND
MZZ	074	Female	0		0		0	0	0	0	Dizziness	1	ND
MZZ	075	Female	0		0		0	0	1	1		1	1
MZZ	076	Female	0		0		0	0	1	1		1	ND
MZZ	077	Male	0	36	0		0	0	1	1		1	ND
MZZ	078	Female	0		0		1	1	1	1		1	ND
MZZ	079	Female	0		0		0	0	0	1	Nausea/sweating	0	
MZZ	080	Female	0		0		0	0	0	0		1	ND
MZZ	081	Female	0	36	1		0	0	1	0		0	

MZZ	082	Female	0		1		0	0	1	1		1	ND
MZZ	083	Female	0		0		0	0	1	0		0	
Site	**ID**	**Sex**	**Fever Now**	**Temperature**	**Fever Previous**	**Rash**	**Bleeding**	**Vomiting**	**Joint pain**	**Abdominal pain**	**Other**	**ELISA Results**	**RT-PCR Results**
MZZ	084	Female	1		1		0	0	0	0		1	ND
MZZ	085	Male	1		1		0	0	0	0		1	1
MZZ	086	Male	0		1		1	1	0	0		1	ND
MZZ	087	Male	0		1		1	0	1	0	Yellow eyes	1	ND
MZZ	088	Male	0		0		0	0	1	1		1	ND
MZZ	089	Male	0		0		0	0	1	0		1	ND
MZZ	090	Male	0		1		0	0	1	1		1	ND
MZZ	091	Female	1		1		1	1	1	0	Vomiting bloody stuff	0	
MZZ	092	Female	0		1		0	0	1	1		1	1
MZZ	093	Female	0		1		0	0	0	1		0	
MZZ	094	Female	0		0		0	0	0	0		0	
MZZ	095	Male	0		0		0	1	0	1			
MZZ	096	Female	0		0		0	0	0	1		0	
MZZ	097	Female	1	38.1	0		0	0	1	0		1	ND
MZZ	098	Female	0	36.3	1		0	0	1	0		1	1
MZZ	099	Female	0	38	**0**		1	0	1	0	Body weakness	1	ND
MZZ	100	Female	0	38	0		0	1	1	1	Dizziness/chills	0	
MZZ	101	Female	0	37.8	0		0	1	1	1	Dizziness	0	

(1=positive; 0=Negative; ND= Not determined; N/A=Not applicable) of participants