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Prevalence and distribution of dengue and chikungunya viruses among febrile patients during inter-epidemic period in Morogoro Municipality

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PREVALENCE AND DISTRIBUTION OF DENGUE AND CHIKUNGUNYA VIRUSES AMONG FEBRILE PATIENTS DURING INTER-EPIDEMIC PERIOD IN MOROGORO MUNICIPALITY

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A dissertation submitted in partial fulfillment of the requirements for the degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

There is currently sufficient evidence available indicating that dengue and chikungunya viruses might be one of the causes of fever in Tanzania. Lately, the dengue outbreak in Dar es Salaam-Tanzania has raised concerns over the spread and distribution of these arboviruses with respect to human movements. The present study was conducted to screen for and chikungunya virus (CHIKV) dengue virus (DENV) from febrile patients in Morogoro Municipality which had no reports of outbreaks during an inter-epidemic period with high risk of transmission. Three hundred and twelve febrile individuals presenting to the outpatient department on selected health centers were tested for viral RNA to evaluate the circulation of DENV and CHIKV during March-May 2018. DENV and CHIKV detection in sera samples was performed using multiplex real-time reverse transcription polymerase chain reaction (RT-PCR). Chikungunya virus was detected in four out of 312 screened serum samples indicating acute cases while no DENV was identified. Chikungunya virus was among the causes of acute undifferentiated fever among febrile patients in Morogoro municipality with a lower prevalence of 1.3% highly prevailing amongst female aged 20-49 years. No acute cases among patients under 10 years of age were detected. The result provides insight into presence of potentially active circulation of CHIKV among febrile patients seeking medical attention, in Morogoro Municipality, Tanzania. Improvement of CHIKV case detection and reporting at the point of care routine laboratory investigations is critical in reducing misdiagnosis of mosquito-borne viral infection. Monitoring of arboviral activities in human populations, as well as mosquitoes, should be performed in order to avoid maintenance of CHIKV that may lead to future outbreaks.

DECLARATION

I, Lightness Mboya, do hereby declare to the Senate of the Nelson Mandela Institution of Science and Technology that this dissertation is my own original work and that it has not been, nor will it be presented to any other university for a similar or any other degree award, and is not previously or currently under copyright.

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CERTIFICATION

The undersigned guarantee that they have peruse and thusly suggest for assessment of an exposition entitled; “Prevalence and distribution of dengue and chikungunya viruses among febrile patients during inter-epidemic period in Morogoro Municipality”. To be acknowledged in partial fulfillment of the necessities for the Degree of Master's in Life science of the Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania.

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DEDICATION

Truly, I would like to offer my piece of work to my dearest family for their unending help, bonafide disposition and consolation; without their adoration and collaboration, I would not have finished this study. Thank you for believing in me even when I could not believe in myself, you are such an inspiration.

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LIST OF ABREVIATIONS AND SYMBOLS

%	Percentage
µl	Microlitre
Bp	Base pair
℃	Degrees centigrade
ADE	Antibody-dependent enhancement
CHIKV	Chikungunya virus
DENV	Dengue virus
DNA	Deoxyribonucleic Acid
ECSAS	East, Central and South African Strain
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	And others
MHC	Major Histocompatibility complex min
Min	Minutes
ml	Millilitre
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NM-AIST	Nelson Mandela African Institute of Science and Technology
NS	Non-structural proteins
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SUA	Sokoine University of Agriculture
WHO	World Health Organization
ZIKV	Zika Virus

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

Dengue virus (DENV) and chikungunya virus (CHIKV) infections are among the major global impact mosquito-borne viral diseases in several malaria endemic regions worldwide (WHO, 2013). According to WHO (2017), about 2.5 billion individuals (40% of the worldwide populace) are considered in danger of dengue transmission, while 50 million instances of the contamination are unsurprising to happen every year universally. In contrary, chikungunya infection has a very low death rate about (<0.1%) in hospitalized cases although individuals may experience long term effects, such as Chronic inflammatory rheumatism, that can last up to years after contradicting the infection (Bloch, 2016). Data on the event and predominance of dengue in Africa is constrained in spite of the way that flare-ups have been research center affirmed. Moreover, the total global burden of these viruses is uncertain, since symptoms and signs are very similar to other febrile illnesses and actual cases are underreported or misclassified (Bhatt *et al.,* 2012; Norrby, 2014; WHO, 2012).

There is a reported evidence of DENV and CHIKV circulating in Tanzania mainland (Ward *et al.,* 2017) and Zanzibar (Grazia *et al.,* 2012). It is suggested that Dengue is very much circulating in coastal regions, including Dar es Salaam and Zanzibar (Vairo *et al.,* 2016), while Chikungunya is highly prevalent on the northern zone (Faustine *et al.,* 2017; Hertz *et al.,* 2012; Kajeguka *et al.,* 2016). The Dengue outbreak of 2014 in Dar es Salaam was the undeviating proof of DENV transmission in Tanzania, reported to spread to seven regions of Tanzania territory, including Morogoro region (Mboera *et al.,* 2016; Mweya *et al.,* 2016). Significant levels of precipitation and temperature reasonable for DENV and CHIKV transmissions, as well as presence of primary vector *Aedes aegypti* mosquito being endemic in much of Tanzania, accounts for a shred of strong indirect indication for the existence of endemic arboviral transmission in the area (Ward *et al.,* 2017). Factors like uncontrolled and rapid urbanization which expands the urban population, poverty, ineffective public health infrastructure, increased international travel, and inadequate sewage systems which often leads to breeding sites for *Aedes* and habitually favors the spread of endemic dengue and chikungunya transmission in Tanzania (Neiderud, 2015).

It is reported that, the proportion of febrile cases due to other apart from malaria has recently increased in Sub-Saharan Africa (D'Acremont *et al.,* 2014). Studies done recently in Tanzania has shown that, victims affected with acute dengue infection are every likely misdiagnosed and regularly given plausible diagnoses of Malaria, UTI and also pneumonia (Chipwaza *et al.*, 2014; Ward *et al.,* 2017). A diminished degree of dengue and chikungunya responsiveness amongst health attendants and absence of differential pathogen diagnosis in determining febrile fever causes in dengue-endemic locales accounts for dengue underreporting and misclassification (Reyburn, 2004; WHO, 2009). These patients are often treated with anti-malaria or antibiotic drugs (Hertz *et al.*, 2012), which are largely ineffective against dengue. The implication of misdiagnosis and underreporting of diseases of such may result in decline in economy (Hume *et al.,* 2008), increase of drug-resistant malaria strains as the result of over-prescription of antimalarial drugs (Wongsrichanalai *et al.*, 2007), and increased risk of morbidity and death rates (Oladosu & Oyibo, 2013).

Early accurate arbovirus diagnosis is important in detecting viral RNA in human serum or plasma during an intense stage of infection for disease surveillance and implementation of control responses (Lai *et al.,* 2007). Reliability of arbovirus detection rely on infection stage and type of sample used, in which serology and molecular detection are the most common tests used (Korrapati *et al.,* 2015). Serology techniques were considered unsuitable for virus surveillance in the present study as the technique gives seroprevalence frequency of individuals with evidence of prior infection, on top of the reported cross-reactivity of anti-dengue antibodies between flaviviruses (Paula, 2004). The IgM assay can be detected 3-5 days and can persist for 2 weeks post onset of illness, making it unsuitable in detecting acute infection (Kim *et al.,* 2015). Virus detection by PCR offers the advantage of being able to detect virus during an severe stage of the infection since the virus persists in the infected host up-to 14 days after infection (Kong *et al.,* 2006). Additionally, PCR technique can provide insight to the circulating serotype essential for virus surveillance. Therefore, in the current study the concurrent status of DENV and CHIKV infections was comprehended on a high risk region during an inter-epidemic period.

1.2 Statement of the problem

A number of chikungunya and dengue outbreaks have been reported including the recent dengue outbreak in parts of Dar es Salaam (neighboring region) in 2018. Other studies have reported the existence of arboviruses in vector mosquitoes, highly suggesting the underlying

circulation of arboviruses in Tanzania. Most arboviral studies in Tanzania have been conducted during epidemic periods and little is known about the virus circulation during the inter-epidemic period while more attention is given to the highly endemic regions leaving the non-foci regions at high risk of transmission. Currently, no virus surveillance study has been done to screen for dengue and chikungunya viruses as one of the underlying causes of fever among febrile patients visiting health centers in Morogoro municipality. Finding from this study will assist in updating data on the present status of dengue and chikungunya infections in Morogoro municipality after an outbreak four years ago in Kilosa District and on the recent outbreak in the city Dar es Salaam that kept this study area at the risk of transmission. Screening of febrile patients across different demographic characteristics may assist in a specific focus for public health prevention and control measures in impacting febrile illnesses in Tanzania.

1.3 Rationale of the Study

Finding from this study will assist in updating data on the present status of dengue and chikungunya infections in Morogoro municipality after an outbreak four years ago in Kilosa District and on the recent outbreak in the city Dar es Salaam that kept this study area at the risk of transmission. Screening of febrile patients across different demographic characteristics may assist in a specific focus for public health prevention and control measures in impacting febrile illnesses in Tanzania.

1.4 Objectives

1.4.1 General objective

To determine prevalence and distribution of Dengue and Chikungunya infections and the associated socio-demographic characteristics in Morogoro Municipality.

1.4.2 Specific objectives

(i) To screen for Dengue and Chikungunya viruses among febrile patients in the study area.

(ii) To determine social-demographic characteristics associated with the distribution of dengue and chikungunya viruses during the inter-epidemic period in Morogoro municipality

1.5 Research questions

(i) What is the present molecular epidemiology status of DENV and CHIKV infections among the selected population in Morogoro Municipality as regards to previous DENV outbreak in Kilosa district and the recent outbreak in Dar-es-Salaam?

(ii) What are the socio-demographic characteristics associated with the distribution of DENV and CHIKV infections during the inter-epidemic period in Morogoro Municipality?

1.6 Significance of the study

Yields of this investigation will aid better understanding of the concurrent status of dengue and chikungunya viral surveillance in Morogoro region. Evidence of the concurrent status of DENV and CHIKV infections by molecular screening among the selected population in Morogoro municipality as regards to the previous outbreak in Kilosa, Morogoro and the recent outbreak in Dar-es-Salaam. This may assist clinicians in making considerable decisions when consulted with patients with symptoms that pose a diagnostic dilemma hence help in the proper prescription of medicines. Timely public health reports and investigations on disease status prior epidemics and risks of transmission may aid in attracting and avoid vanishing of significant investors and stakeholders as well as substantially improving the national economy. Scrutinizing affected individuals by both age and gender may provide stratified prevalence data of DENV and CHIKV infections that may assist in targeting specific preventive measures.

1.7 Delineation of the study

The study was a cross-sectional hospital based study which was conducted to screen for dengue DENV and CHIKV in sera from (312) patients with fever and malaria-like symptoms on selected health centres in Morogoro municipality (n = 5) during March-May 2018. Prevalence of these viruses was determined by using Multiplex real-time reverse transcription-polymerase chain reaction. And the distribution of CHIKV and DENV during the inter-epidemic period in Morogoro municipality was analyzed from the social-demographic distributions of the study participants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Dengue virus infection

2.1.1 Dengue fever

It has been anticipated that 500 000 people influenced with serious dengue call for hospitalization every one year and nearly 2.5% of those affected with dengue fever die (WHO, 2012). Dengue is the furthermost quickly spreading mosquito-borne viral disease in the world, and it has expanded 30-fold in the past 50 years and in the current decade because of geographical extension to different nations from urban to rural settings (WHO, 2014). Every one of the four DENV serotypes have been separated in Africa, with DENV2 reported to be associated with most outbreaks (Amarasinghe *et al.*, 2011; Were, 2012).

Dengue fever may be categorized clinically as (a) Probable dengue, (b) Dengue which has cautioning signs and (c) Severe dengue (WHO, 2012). The disease manifestation has three stages: febrile, critical, and convalescence or recovery. The period of incubation only lasts for about 4-10, followed by the febrile stage, which lasts for about 2-7 days causing unspecified signs and symptoms. These includes high grade fever (38.8-40.5℃), pain on the front head, retro-ocular pain (pain behind the eyes), malaise, maculopapular rash, arthralgia, myalgia, nausea, vomiting. It is at this phase that the diseases is clinically tough to differentiate it from other fever-like diseases and the assessment whether the disease is in extreme or non-serious form is difficult. In the febrile phase, the total blood count in the earliest abnormality is a gradual decrease in the all-out number of leucocytes in the body. This should raise concern that the victim has signs and symptoms of dengue. On the other hand, critical phase lasts for about 24-48 hours and it occurs when the fever diminishes and followed by bleeding and a raise in capillary permeability (Epelboin *et al.*, 2012; WHO, 2016).

The final results in plasma leakage clinically demonstrated as pleural effusion and ascites together with increased hematocrit levels, leukopenia with neutropenia, decreased platelet count, and lymphocytosis with 15-20% atypical shapes. These resemble to dengue shock syndrome or severe dengue as when plasma levels decreases, there is high risk of an individual getting shock. If shock prolongs, progressive organ deterioration may occur resulting into death. Promisingly, if patient survives the critical phase there is steady restoration of body fluids, general well-being improves and hemodynamic status stabilizes

within 48-72 hours. There is an increase in total white blood cell count, neutrophils and decreased lymphocytes. The increased platelet count subsequently increases leucocytes. This parallels to the recovery stage (Beltrán-Silva *et al.,* 2016; Rückert *et al.,* 2017). Generally, indications and signs of dengue infection may be very similar to Chikungunya infection and other febrile illnesses (WHO, 2009).

2.1.2 Virus structure and survival

The (+)ssRNA enveloped virus that causes Dengue comprises of four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), which belong to the family *Flaviviridae* and genus *Flavivirus* (Taslim *et al.,* 2018). All four serotypes circulate in similar ecological niche and they affect closely identical syndromes in humans although they are antigenically dissimilar (Guzman *et al.,* 2010). Infection with only one serotype gives only limited immunity against other serotypes but a permanent immunity to that strain (Lupi, 2011). Dengue virus structure is almost spherical in structure, with a width of approximately 50 nm. The virus core is the nucleocapsid, a structure that is made of the viral genome along with C proteins. This nucleocapsid is surrounded by a viral envelope, which is a lipid bilayer that is derived from the host. Embedded in the viral envelope are 180 copies of the E and M proteins that span throughout the lipid bilayer. These proteins form a protective outer layer that controls virus entry into human cells (Lopez & Arias, 2010).

The viral genome is a single strand of RNA. It is referred to as positive-strand RNA because it can be directly translated into proteins. This viral genome encodes ten genes (Coffey *et al.,* 2013). The viral genome is translated as a single, long polypeptide and then cut into ten encoded proteins. The genome encodes three structural (capsid [C], membrane [M], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins that are involved in viral replication and assembly (Jhan *et al.,* 2017). The NS1 protein amongst these structural proteins interacts with the host immune system and thereby evoking T cell responses. This attained response has been used as a diagnostic marker in detecting dengue fever infection (Erickson *et al*., 2010; Mukhopadhyay *et al.,* 2005).

The infection replication process starts when the infection joins to a human skin cell. The skin cell's membrane then encloses around the virus forming a pouch (endosome) which covers around the virus particle. The dengue virus is able to enter a host cell using the normal cell process of nourishment by engulfing large particles from outside (Lopez & Arias, 2010).

The virus then joins with the endosomal membrane once it is inside the cell and it is released into the cytoplasm in which the virus particles releases the viral genome. Viral RNA is then translated into a single polypeptide that is cut into ten proteins, followed by replication of the viral genome. The latter is the virus assembly process which occurs on endoplasmic reticulum (ER) surface in which the structural proteins and recently synthesized RNA are released out from the ER (Navarro-Sanchez *et al*., 2003). The immature viral particles are then conveyed via the trans-Golgi network where they develop and transform to their infectious state. On the final stage, matured viruses are then released out from the cell and can ready to go to infect other cells (Mukhopadhyay *et al.*, 2005).

2.1.3 Vector organism

Dengue and chikungunya viruses are spread via a human-to-mosquito-to-human cycle of transmission (Fig. 1). Infections spread by a vector necessitates that a vector organism should be able to get a pathogen, maintain it and effectively transmit it to a susceptible host. Temperature, accessibility of vertebrate hosts and vector population density accounts for external factors necessary for vector-borne infection, whereas mosquito survival and virus replication abilities count for internal factors (Coffey *et al.*, 2013). The internal physiology and innate behavioral traits of the vector, its ability to transmit an agent along with feeding duration and host preferences are the important intrinsic factors that the viral pathogen use to overcome obstacles before diffusion to another host (Agarwal *et al.*, 2014).

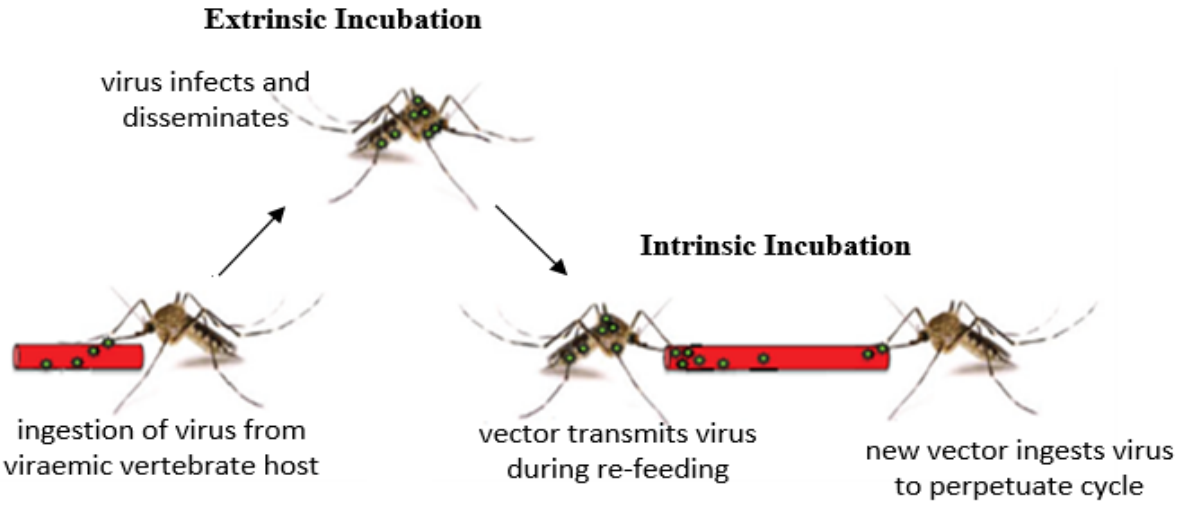

Figure 1: Mosquito-borne virus infection and transmission process (Coffey *et al.*, 2014)

The mosquito species *Aedes aegypti* and, to a lesser magnitude, *Aedes albopictus* are the prime vectors in dengue and chikungunya viruses (Fig. 2) in sub-Saharan Africa, although these viruses are also retained in in sylvatic cycles including primates and forest dwelling *Aedes* species in some parts of Africa (Hertz *et al.*, 2012).

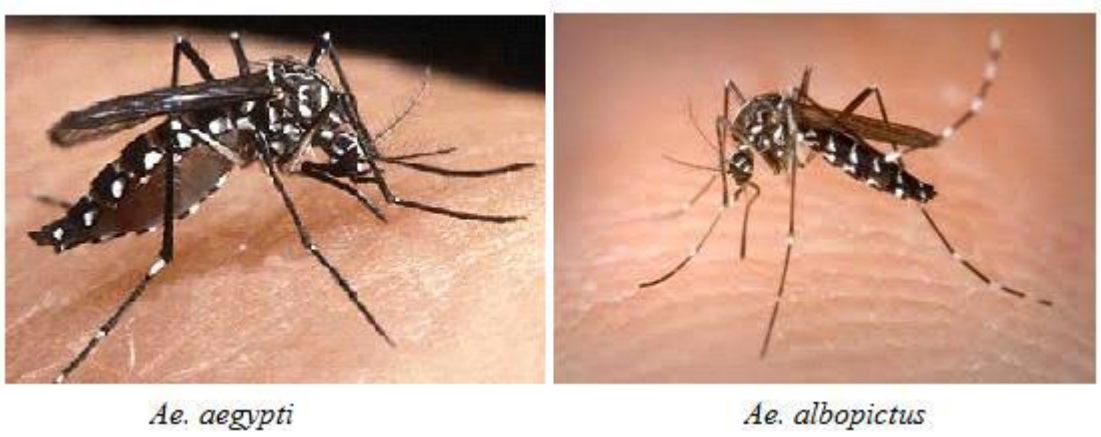

Figure 2: Mosquito vectors for DENV transmission (Rodhain and Rosen, 1997)

Aedes aegypti is a small, dark mosquito identified by the shinning white bands on its legs and a silver-white pattern of scales on the body. It dwells in tropical and subtropical regions across the world, primarily in latitudes ranging from 35°N to 35°S. Temperatures within 5°C-40°C are essential for mosquito survival although they are incapable of surviving cold winters since *Aedes aegypti* needs a warm climate, these mosquitoes cannot live at elevatons above 1000m where the temperature is cooler. *Aedes aegypti* feeds mainly on human blood during daytime and it thrives in close contiguity to humans where they spend their whole lives in and around the houses and incubate eggs (Brady *et al*., 2014; Erickson *et al*., 2010). It is an impatient mosquito as the minor movement interrupts its feeding process, and therefore in a short interval of time several people may be bitten for one blood meal. *Aedes aegypti* takes more than one blood meal before laying eggs that is during a gonotrophic cycle, unlike most mosquitoes (Gibbons & Vaughn, 2002). Adult mosquitoes feeding frequency increases as the temperature increases about 18°C with a reduced humidity. High mosquito densities are usually expected during and after a rainy times of a year, this is highly attributed by high precipitation, rainfall accumulation, intensity and frequency. Moreover, the presence of variable manmade breeding habitats to maintain water can affect mosquito numbers (Eastin *et al.*, 2014).

2.1.4 Dengue virus transmission

After a mosquito has fed on the dengue virus infected blood, it becomes a dengue vector. During the viremic period when the sullied individual has raised degrees of the dengue disease in the blood it is at that point that the mosquito takes the infection during its blood supper. The virus then spreads all the way through the mosquito's body for about 8-12 days. Subsequently at this interval of time, the affected mosquito can then re-infect dengue virus to a new individual upon a mosquito bite during feeding. After getting dengue infection, the mosquito remains infected with DENV for the rest of its life about 3-4 week period that is why the infected mosquitoes continues spreading the virus to healthy individuals in their entire life spans period (Hume *et al.,* 2008; Lupi, 2011; Rückert *et al.,* 2017).

Female mosquitoes feeds on humans as they require blood for eggs production. During their lifetime, each and every female mosquito lay various lots of eggs, and regularly *Aedes aegypti* take a considerable amount of blood dinners before laying a group of eggs. An affected female mosquito carries the dengue virus in its salivary glands (Cao-Lormeau, 2009; Sim *et al.,* 2012). During feeding, a diseased female mosquito infects the host with the dengue virus by inoculating its saliva into the human host so as to avoid blood clotting to the host and to facilitate the feeding process. The greater part of dengue diseases are transmitted by mosquito bite in spite of uncommon occasions in which a contaminated expecting mother can transmit the infection to the embryo and during organ transplantations or blood transfusions from tainted contributors (Friedman *et al.,* 2014).

The eggs of *Aedes aegypti* mosquitoes can survive dry environments for quite a lot of months. Eggs laid in a dry container can allow development of new mosquitoes only when the container is filled with water (Hanley *et al.,* 2008; Kuno *et al.,* 1997). Because of this adaptation elimination of mosquito populations has become difficult. Globally, dengue outbreaks occur annually during the rainy season, the time at which mosquito breeding conditions are conducible. In highly populated regions dengue fever poses a particular health threat as epidemics are more likely to occur on zones with an enormous number of people interacting with a high numbers of mosquito vectors than in more secluded and remote areas such as in the mountains (WHO, 2009).

2.1.5 Virulence mechanisms

Dengue infection contaminate juvenile dendritic cells in the skin by means of the vague receptor dendritic cell-explicit ICAM3-getting non-integrin (DC-SIGN). The tainted dendritic cells at that point develop and move to local or local lymph nodes to proceed to introduce viral antigens to T cells and along these lines starting cell and humoral immune reactions. The virus multiplies and then enters the bloodstream. The infection replication likewise happens in macrophages in lymph nodes, liver parenchymal cells, spleen alongside in fringe blood monocytes. Antibody-dependent enhancement (ADE) is performed by both vitro and in vivo, macrophages. This happens when safe edifices that structure between dengue infections and non-killing antibodies contaminates mononuclear phagocytes through their Fc receptors. Virus uptake by macrophage cells proliferates and may result into a disease severity forms including dengue shock syndrome (Brady *et al*., 2014; Erickson *et al*., 2010; Navarro-Sanchez *et al*., 2003).

Upon obtaining dengue antibodies either by maternal or natural forms against one serotype prevention against infection with another dengue serotype is not guaranteed. Past exposure of the disease to one serotype could expand the disease triggered by exposure to a second strain as the four distinct serotypes do not cross-neutralize but cross-react. On re-infection with the disease, the body then reacts to the first strain with which it was infected while putting up a solid immune response (Stephan *et al.,* 2002). An elevated level of DENV in the blood (viremia) is then observed four days after an affected *Aedes aegypti* mosquito bite, this complaint persists for about five to twelve days. An individual displays no indications of dengue fever on day one of viremic phase then five days later an individual develops dengue fever symptoms that lasts about a week or more.

2.2 Chikungunya virus infection

Chikungunya fever is caused by the *Alphavirus* of the family *Togaviridae* (WHO, 2017) and comprises of three different strains: the East, Central and South African strain (ECSAS), West African strain, and Asian strain (Pongsiri *et al.,* 2012). *Alphaviruses* are enveloped single-stranded positive-sense RNA genome with a spherical virion 60 to 70 nm in diameter (Schmaljohn & McClain, 1996). The genome is about 11.7 kb long and comprises of two regions, a non-structural domain (Fig. 3) which encodes four viral non-structural proteins (nsP1- nsP4), essential for replication, polyprotein processing and the structural domain. The

non-structural proteins also synthesize the 26S sub-genomic mRNA which ultimately produces five individual structural proteins which consist of the viral capsid (C), two envelope glycoproteins (E1 and E2), and two peptides (E3 and 6K) (Hernandez *et al.*, 2014).

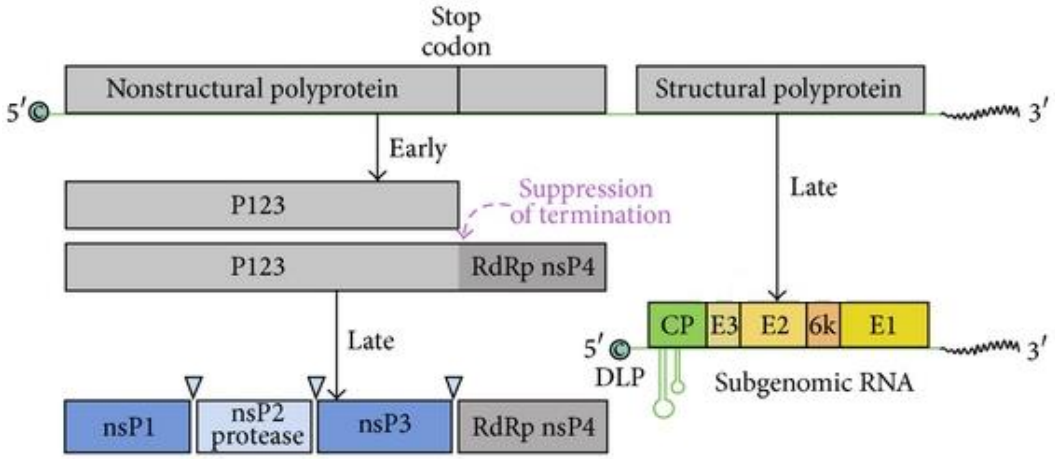

Figure 3: Alphavirus genome organization (Hernandez *et al.*, 2014)

Chikungunya virus may cause acute, subacute, and chronic infections. Upon biting by an infected mosquito an individual usually develops signs and symptoms of the disease 3–7days after the bite. Acute chikungunya fever typically lasts for 3–10 days and is regarded as a sudden onset of high fever that takes up to a week and severe joint pain which is commonly observed in the hands and feet but can manifest to other joints. This fever (39–40°C) is mostly accompanied by irregular shaking chills (Fig. 4). Other signs and symptoms include diffuse back pain, headache, rash, myalgia with back and shoulder pain, nausea, vomiting, conjunctivitis and slight photophobia may occur.

Most patients develop maculopapular rash 2–5 days after fever onset. On the other hand, joint pain and swelling can be severe enough to affect the individual's ability to walk (Johnson *et al.*, 2008; Tilak *et al.*, 2016). Individuals with chronic Chikungunya infection usually improves after the ten first days of symptoms onset, whereas these symptoms can reoccur 2–3 months after the initial infection. This usually involves exacerbation of pain in previous injured joints and bones, distal polyarthritis and tenosynovitis in wrists and ankles (ADHS, 2016). In most cases the viremia is present during the first 48 hours of illness, although in some patients this may carry on for up to 4 days. Most patients recover completely within a few weeks although up to 10% of individuals experience chronic joint pain, swelling and stiffness which may last about a year or more (Kraemer *et al.*, 2015; Rückert *et al.*, 2017).

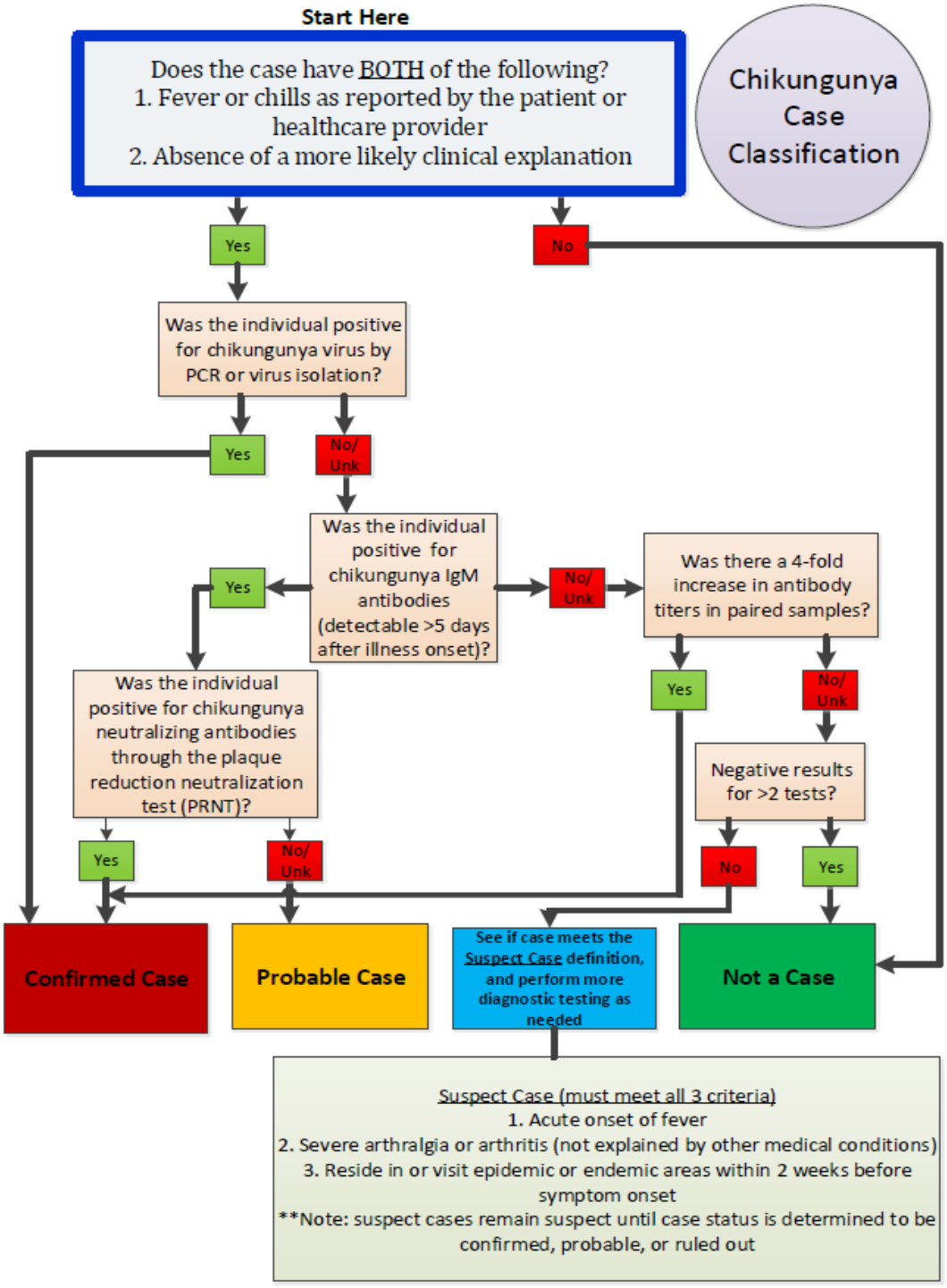

Figure 4: Chikungunya case classification algorithm (ADHS, 2016)

2.3 Dengue and chikungunya virus screening

Monitoring arboviral incidence and prevalence with time is crucial so as to avoid unexpected rise in the proportion of severe cases as the procedure measures the disease background rate which will alert and promote investigation, intervention and prevention measures. Prompt

warning of a disease epidemic alerts clinicians on the necessity of diagnosis and properly treat DENV and CHIKV cases while enabling health services in allocating human and material resources more efficiently and to healthier engaging the community on prevention and control measures, and consecutively reducing disease transmission and refining clinical practices (WHO, 2012; ADHS, 2016). Dengue plagues diminishes the beneficial limit and financial development of numerous segments of society because of their quick extension and delayed spans (WHO, 2009). For instance, certain groups of people require special attention as they depend on others who take care of them and together with the immediate surroundings (e.g. older people who stays alone, travelers, children in daycare programmes, migrant workers and soldiers). These individuals if not accurately screened may also pose high risk of dengue virus transmission to health care locations.

Early anticipation is the best measure in dengue and chikungunya avoidance and control since currently no vaccine is available. The current dengue and chikungunya prevention strategies are reactive rather than anticipatory as they are every so often implemented late thereby reducing the chances for preventing transmission and subsequent controlling the epidemic (Viennet *et al.,* 2016; WHO, 2009). These reactive strategies results into short-term behavioral change as individuals may fail to uphold government efforts in partnership with personal and community responsibility for dengue and chikungunya preventions and control (Reich *et al.,* 2016).

Most outbreaks brings about the uncertainty, misperception and a sense of urgency. Public risk communication during outbreaks has a core task of announcing the epidemic timely while giving precise and transparent information on what measures people can do to prevent themselves from getting the disease. This helps in building public trust in which subsequently permits them to respond to the dangers of transmission with even added rational and logical reactions. Additionally, media involvement prior a rise in number of cases enhances the chance in raising public awareness of the disease while fostering community and individual mitigations against spread of the disease (Parks *et al.,* 2004; WHO, 2005).

2.3.1 Status of dengue infection

The study on epidemiology of dengue: past, present and future prospects by Murray *et al.* (2013) revealed that globally, dengue fever is the furthermost critical mosquito-borne viral illness because of its wide distribution of the virus and its vectors throughout tropical and

subtropical regions. This geographic spreading out has been favored with prompt increases in epidemics and incident cases resulting in the more severe forms of dengue.

The current data available to ascertain the global true impact of dengue most likely underestimates the economic and social burden of the disease since there is insufficient disease surveillance data, underreporting and misdiagnosis cases (Murray *et al.,* 2013). Dengue is anticipated to increase due to aspects like the current changing aspects of environmental change, travel, exchange, globalization, settlement and viral movement.

The presence of other prevalent febrile diseases such as Malaria, poor surveillance system, low level of attentiveness among health care practitioners and lack of differential diagnosis capacity to rule out dengue accounts for the misdiagnosis and underreporting of dengue in Africa (Amarasinghe *et al.,* 2011). The clinical presentation of DENV infections is challenging as it is similar to other various causes of febrile illnesses such as malaria (Gubler, 1998). It is important to report the raising threat of DENV fever as no specific therapy or vaccine is available upto date therefore early detection of epidemic cases and accurate clinical management may assist in reducing mortality cases of dengue (Sim *et al.,* 2012). Another preventive measure is the effective control of the vector organism. Amended reporting of dengue cases and disease surveillance are crucial to comprehend the true global impact (WHO, 2012). Increasingly exact information will illuminate the prioritization of wellbeing approach, inquire about, and monetary resources toward controlling the disease (Murray *et al.,* 2013).

2.3.2 Prevalence of dengue in Morogoro Region

Causes and dynamics of dengue may be addressed by the environmental and climatic situations on the respective area throughout the relevant period. The role of indigenous temperature variation, rainfall timing and magnitude of the rain episodes observed in April 2018 was insufficient to engage in a precarious role on the spread of Dar-es-Salaam outbreak to Morogoro Region.

The decreased convenience of breeding spots in flower pots, car tires and plastic containers as it was elaborated in an entomology study of Mboera *et al.* (2016), following community education for malaria prevention strategies may have reduced the impact of vector breeding, thereby limiting virus survival. In the rural areas of Kilosa and Kilombero districts, it was previously observed that majority of water containers around the houses were sheltering *A.*

aegypti larvae whereas in the same surroundings, buckets, clay vessels, tins, and loading water drums were the most collective breeding sites (Mathias *et al.,* 2017). Transmission of dengue infection depend on the productivity rate of female *Aedes* mosquitoes from breed dwellings which eventually defines the quantity of adult mosquitoes.

The extension of topographical dispersion of *A. aegypti* and potential risks to spreading dengue virus is highly facilitated with the changing climatic condition. Temperature influences on the extrinsic incubation duration, adult transience and water developmental rates of the vector (Butterworth *et al.,* 2017; Kraemer *et al.,* 2015). Temperatures on cold autumn weather during the rainy season early on 2018 may have caused a decrease in the quantity of adult mosquitoes dropping below the critical threshold in which the incubation period is diminutive than the typical mosquito lifespan. This may have effectively reduced vector capacity and reduced viral transmission, resulting in a significant reduction in dengue cases in Dar-es-Salaam and subsequently banned further spread of the disease.

To avoid the unpredicted rise in cases of DENV and CHIKV upon an outbreak, it is important for the government to monitor arboviral incidence and prevalence with time (Fritzell *et al.,* 2018) as most arboviral preventive approaches are highly implemented late instead of defensive (Tambo *et al.,* 2017). Such approaches result into a flood use of resources during outbreaks, temporary behavioral changes in which at worse individuals may fail to cooperate with the government efforts in prevention schemes and tackling outbreaks (WHO, 2009). Screening and surveillance records are of public health and economic importance at the National level (Nsubuga *et al.,* 2006) so as to protect investors' interests. Production of surveillance data which are highly underreported and the capability to convert these records into usable information is vital to applicable public health action (Groseclose & Buckeridge, 2017).

2.4 Molecular detection of DENV and CHIKV

2.4.1 Multiplex real-time RT-PCR

For viruses with an RNA genome such as DENV and CHIKV, reverse transcription (RT) is combined with Polymerase Chain Reaction (PCR) in making RNA into a complementary DNA (cDNA) since RNA cannot directly serve as a PCR template. Reverse transcriptase enzyme is an RNA dependent DNA polymerase that can synthesize a DNA strand from RNA template (Viljoen *et al.,* 2005). According to Korrapati *et al.* (2015), several real time RT-

PCR methods exploiting different combinations of primers, probes and fluorescents in detecting, serotyping, and quantification of DENV and CHIKV have been described. Hemi-nested RT-PCR which was initially developed in 1992 and improved to date is one of the PCR currently in use. The assay involves two cycles of amplification followed by gel electrophoresis process in detecting amplicons. Challenge involved is that these tests require about a day or more to accomplish significantly resulting in a limitation of the clinical utility of the assay and a high risk of contamination (Waggoner *et al*., 2013).

2.4.2 AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR

The Bioneer's Dual Hot start technology has established a high specific and sensitive AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit for detecting viral RNA in clinical samples. Dual- Hot Start™ excludes non-specific complementary DNA and non-specific DNA amplifications so as to allow the currently available most sensitive one-step RT-PCR assays. This kit is intended to enhance reproducibility and fasten usage by vacuum-drying the entire PCR reagents together with primers, probes, dNTPs, DNA polymerases, and salts. The primer-probe set demonstrates efficiency at maximum value in amplification by the use of bioinformatics algorithms which matched to the AccuPower® diagnostic kits.

The AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit is a diagnostic kit intended for the qualitative detection of Zika, Dengue and, Chikungunya Virus RNA in human samples including plasma and serum through real-time (RT-PCR) (WHO, 2016). The system comprises of an ExiPrep device used for an automatic nucleic acid preparation and an Exicycler for detection and amplification of nucleic acid. Real-time PCR selectively amplifies the target sequence while monitoring the amplification progress in real-time through a fluorescent dye. The amount of ZIKV, DENV and CHIKV in target sequence available to each amplification cycle is relatively direct to the generated signal from the fluorescent labeled oligonucleotide probes which is bound to the amplified target. Specific primers together with hydrolysis probe brings about the specificity of the assay. Labelling of the hydrolysis probe (5'-Fluorescent reporter; 3'- Quencher) with a corresponding pair of fluorescent dye was essential in monitoring amplified products (WHO, 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Material, reagents or tools used in this study

The equipment, reagents and materials used in this study are indicated in Appendix 1.

3.2 Study area

The investigation was conducted in Morogoro Municipality in Morogoro region, Tanzania. The district occupies a total of 260 square kilometers of land covered with a population of about 315 866 (URT, 2013). Morogoro Region occupies about 8.2% of the entire area of mainland Tanzania. The climate of the region can be described as a bimodal rainfall dispersal characterized by two rainfall peaks annually, associated with a dry season to separate the short rains (October–December) from the long rains (March-May). The cool-dry season records a temperature as low as 12℃ averagely, while a temperature of 38℃ is observed during the hot-dry seasons.

Sampling was conducted in Mazimbu, Mafiga, Sabasaba, Nunge and SUA health centers (Fig. 5) in Morogoro municipality, Morogoro region. The municipality has a moderately dense human population with tertiary institutions and industries which attracts many people from all walks of life thus several characteristics of the area favors *Aedes* mosquito breeding and transmission of arboviral diseases. Nunge is a business area characterized by a high number of traders. Mazimb u has vegetation cover with high-populated neighborhoods and inadequate sewage systems which often leads to breeding sites for *Aedes*. Located in this ward is the busiest bus terminal in which affected vectors are likely to be transported along with human movement from the epidemic region of Dar es Salaam. Mafiga is a highly populated area with middle-class individuals. The area is characterized by vegetation. Sokoine University of Agriculture (SUA) hospital is where located and are surrounded by rural at the at the fringe of the Uluguru Mountain, described by low human populace however with high vegetation

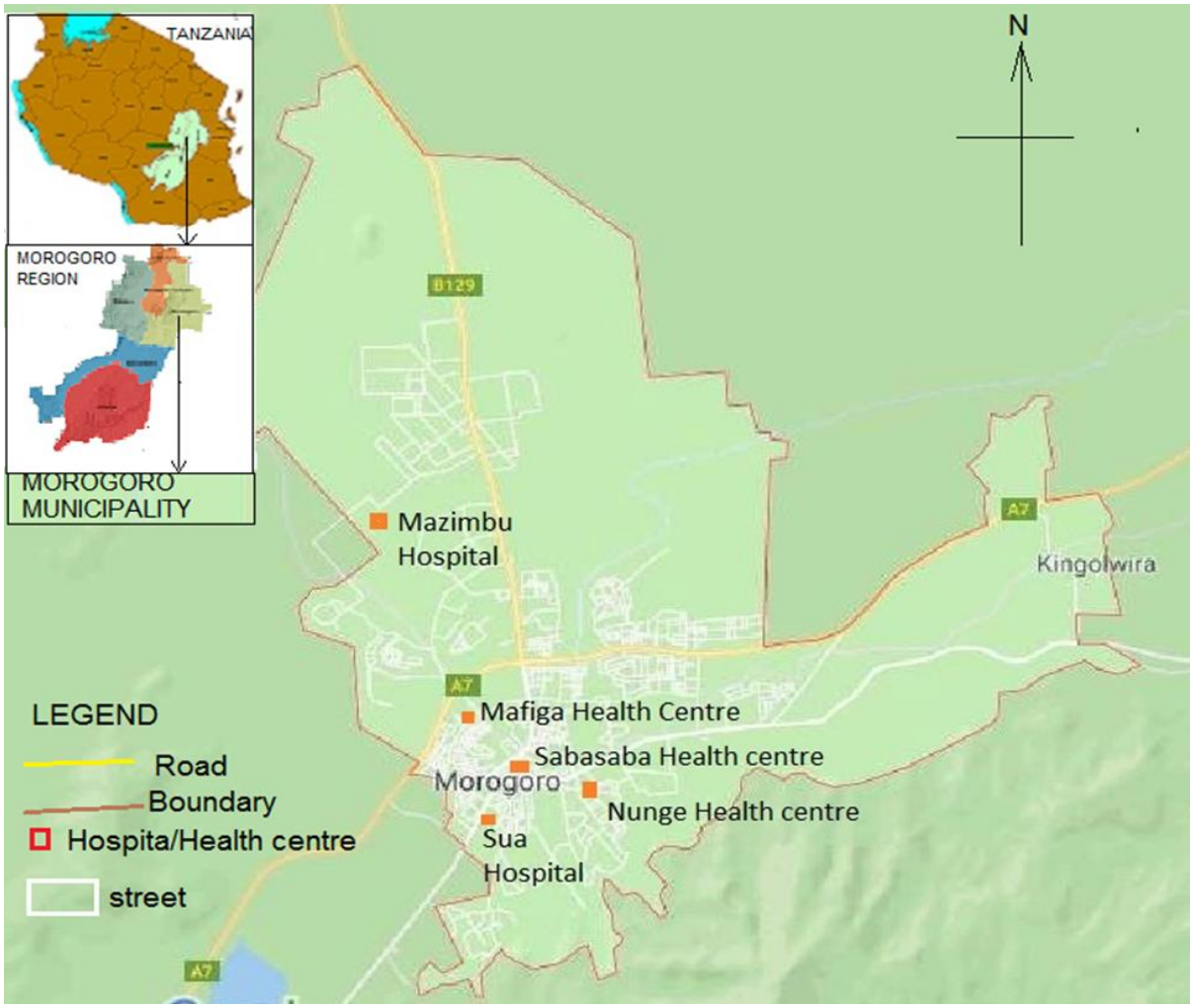

Figure 5: Map of Morogoro municipality indicating the five Health centers of this study (indicated by orange color)

3.3 Study design

This was a cross-sectional hospital-based study that was conducted from March-May 2018 among individuals presenting to the outpatient department with febrile condition (fever ≥ 37.5 °C) in five Health centers in Morogoro municipality.

3.4 Study population

The study participants were febrile individuals presented at the selected health facilities in Morogoro municipality, Morogoro region.

3.4.1 Sample size determination

The desired sample size was derived by the formula $n=Z^2PQ/d^2$. Where n is the sample size; P is the expected prevalence in the target population, Q is 1-P; Z is 1.96; standard error, d is

the level of statistical significance (0.05). Prevalence value of 20.9% was used representing maximum uncertainty (Vairo *et al*., 2016). Hence, the estimated minimum sample size was 255 with an added 10% samples for data inconsistencies, giving a total sample size of 293.

3.4.2 Inclusion criteria

The inclusion criteria for enrolling individuals into this study were, Presentation with fever ≥ 37.5 °C, malaria-like symptoms and patient agreeing to consent.

3.4.3 Exclusion criteria

The exclusion criteria used for this study were individuals with chronic and debilitating illnesses.

3.5 Sampling technique

Individuals who met the above criteria were successively enrolled based on the attendance ratio at each of the health centers until the desired sample size was attained. Samples were obtained from 5 health centers in Morogoro Municipality: SUA Hospital, Nunge Health center, Mafiga Health center, Sabasaba Health center and Mazimbu Hospital. The health facilities were selected on the basis of providing primary and advanced healthcare services and possessing sufficient laboratory facilities for routine diagnostics.

3.5.1 Blood sample collection

Samples from peripheral venous blood (up to 4 ml) were drawn into plain or EDTA anticoagulant tubes at the time of enrollment as per attached SOP. Each plain and EDTA tube was labeled with patient name, age, sex and the date the sample was taken. All samples were collected and centrifuged on site to obtain serum/plasma and furthermore aliquoted into cryo-vials (2 ml each) and later transported in cold chain to NIMR-Morogoro laboratory analysis. At the laboratory, these samples were barcoded and stored in freezers at -20 °C in anticipation of laboratory analysis (Fig. 6).

Figure 6: Blood serum samples all set for RNA extraction

3.6 Laboratory work

3.6.1 RNA extraction and RT-PCR

Stored serum/plasma was collected and subjected to an in vitro automated diagnostic system a one-step multiplex RT-PCR by using AccuPower® RT-PCR kit (Bioneer, Seoul, Republic of South Korea) following manufacturer's instructions and with some few modifications. The premix contained specific primers and probe for chikungunya and dengue RNA viruses. It comprised of a strip of eight tubes with all components required for cDNA synthesis and PCR, including M-MLV RT, thermostable DNA polymerase, RNAse inhibitor, and deoxy-ribonucleotide triphosphates (Fig. 7). Enzyme viability is preserved for a long period even further than the usual storage conditions by the freeze-dried format.

Figure 7: ZIK-1111 PCR premix (96 reactions) consisting of PC, IPC, NTC and the Optical sealing film

The RT-PCR was carried out in accordance to the kit company's instructions as per the following preparations. The premix enclosed optimum concentrations of the entire essential components needed for complementary DNA synthesis and amplification in one tube of 0.2 ml. Every single premix tube contained a stabilizer and was well-preserved in a lyophilized system. The primer mixture was arranged through mixing conserved forward and reverse primer 400 nM and 200 nM respectively with DEPC-treated distilled water.

The lyophilized content was then dissolved, vortexed and then briefly spinned. Briefly, 400 µL of NTC (Non-template control) was added into both PC (positive control) and NC (negative control) sample loading tubes, 7 µL of PC were added into the PC sample loading tubes and 400 µL of blood serum were then added to the remaining sample loading tubes, the quantity used ensured that a maximum amount of serum was available for virus detection and subsequently reducing contamination risks from duplicating runs. Furthermore, 14 blood serum sample loading tubes, 1NTC loading tube and 1PC loading tubes (A total of 16 loading tubes) were placed in one ExiPrep for RNA extraction. A 30 minutes RT-PCR step was performed at 50 °C followed by a 15 min Taq polymerase activation at 95 °C, 40 cycles of PCR denaturation at 95°C for 30 s, 60 °C of annealing for 30 s and 72 °C extension for one minute. The RT-PCR was conducted in a Master cycler gradient machine (Eppendorf, Hamburg, Germany) for 2½ hours.

Figure 8: Automated Multiplex real-time RT-PCR workflow Bioneer, 2017 (WHO, 2016)

The use of AccuPower RT-PCR premix highly facilitated the diagnosis work as each solo tube contained all the necessary parts for the RT-PCR premix and only fewer steps are needed in the process. This helped to reduce the time required to prepare the assays and consequently minimizing risks of cross-contamination and potential errors (Fig. 8).

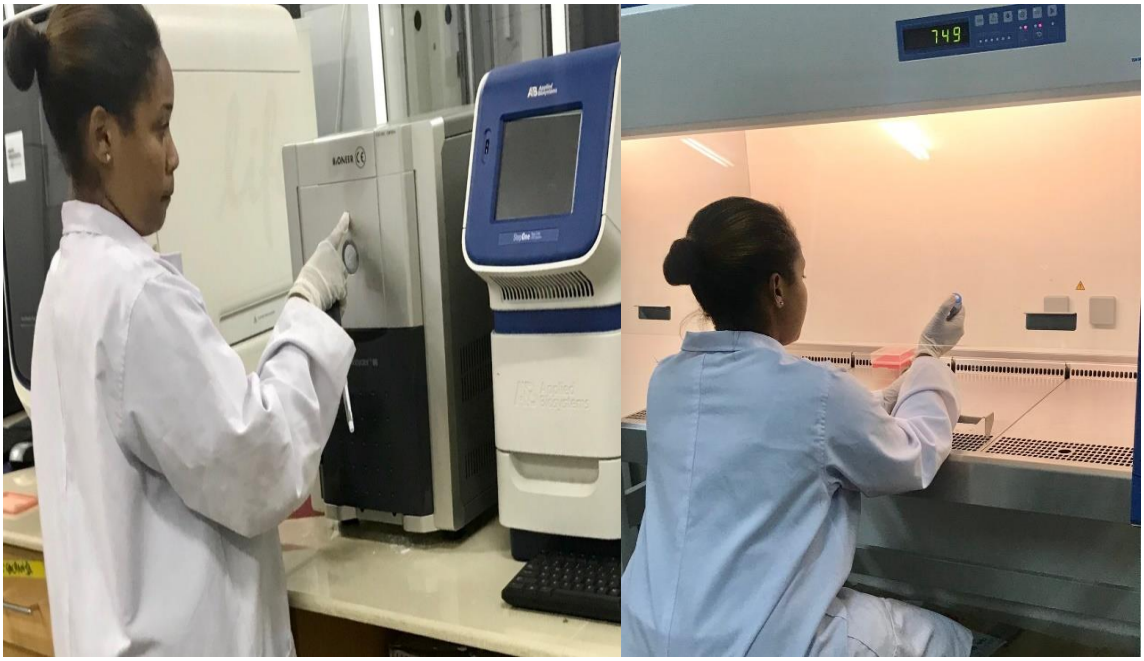

Figure 9: Laboratory activities involving preparing serum sample to be loaded in the ExiPrep

3.7 Data analysis

Data collected in this study were recorded and analyzed using Microsoft Office-Excel 2016 (Microsoft, California, USA) for cleaning and descriptive statistics. The demographic backgrounds including, age, sex and locality were analyzed. Multiplex real-time RT-PCR data analysis was done by calculating the threshold cycle (CT) value represented the positive amplification of the gene in RT cycle numeral.

3.8 Ethical considerations

Ethical approval to perform the study was obtained from the Medical Research Coordinating Committee (MRCC) of the National Institute of Medical Research (NIMR/HQ/R.8a/Vol.IX/1896) and go-ahead to perform the study in the health facilities was pursued from the Morogoro Region Health Authority. Participants were requested to affirm in participating in the study through the informed consent form and participants who did not contribute in the study received normal routine care according to their condition and presentations. All data collected was further coded for confidentiality purposes as well as blinding before analysis and later securely archived for future reference.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Study participants characteristics

During the sample collection period, from March to May 2018, the study enrolled 312 febrile individuals from the five selected health centers in Morogoro municipality.

The majority of enrollments were from Mazimbu Hospital (42%) followed by Nunge (21%), Mafiga (14%), Sabasaba (12%) and SUA (12%) health centers. The majority of patients enrolled (40%) were adults aged (20-29) years of the study participants, (74%) were females and (26%) were males. The exact number of participants and percentages is as summarized in Table 6 below:

Table 1: Social demographic characteristics of study participants (N = 312)

Variables	Frequency	Percentage
Hospital		
Mazimbu Hospital	130	42
Mafiga Health Centre	43	14
Nunge Health Centre	64	21
Sabasaba Health Centre	38	12
SUA hospital	37	12
Age in years		
≤ 9	17	6
10-19	48	15
20-29	125	40
30-39	60	19
40-49	29	9
>50	33	11
Sex		
Male	82	26
Female	230	74

4.1.2 Screening for DENV and CHIKV by RT-PCR

Three hundred and twelve (312) serum/plasma samples were detected for DENV and CHIKV using AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time –PCR (Fig. 10 & 11), results summarized in Table 7. Chikungunya and Dengue cases were categorized in accordance to the World Health Organization (2009) criteria and confirmed acute chikungunya was well-defined as a positive PCR result. Among all febrile participants, only 4 cases with a prevalence of (1.28%) met the WHO criteria for CHIKV confirmed cases (Fig. 12). None of the cases were positive for dengue by Multiplex Real-Time RT-PCR.

Table 2: Results of samples screened for DENV and CHIKV by multiplex real-time RT-PCR

Variables	Confirmed cases	
	DENV	CHIKV
Health Centre		
Mazimbu Hospital	0	2
Mafiga Health Centre	0	0
Nunge Health Centre	0	1
Sabasaba Health Centre	0	0
SUA hospital	0	1
Age in years		
0-9	0	0
10-19	0	0
20-29	0	2
30-39	0	1
40-49	0	1
>50	0	0
Sex		
Male	0	0
Female	0	4

The results from the amplification plot of tested samples representing (1) CHIKV positive and negative results as compared with CHIKV positive samples and positive control (Fig. 10).

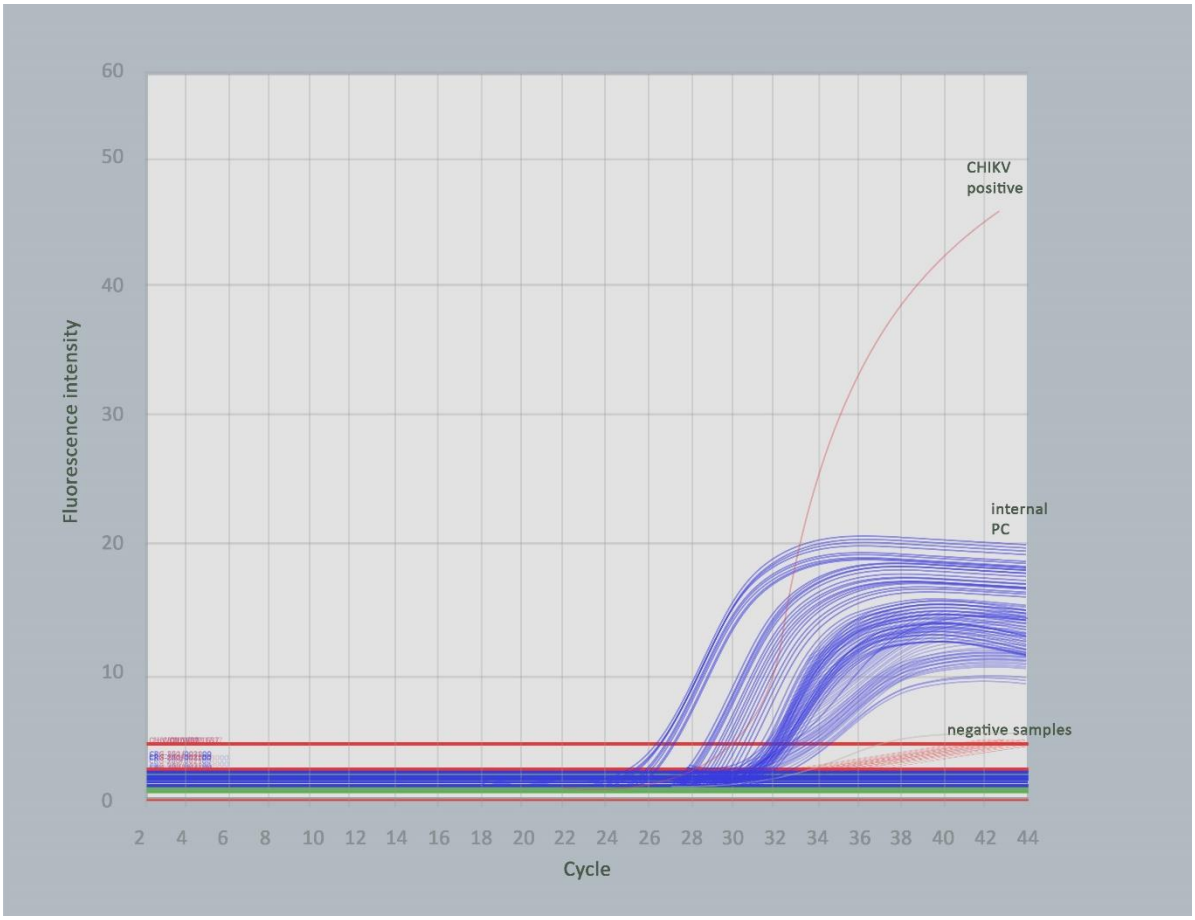

Figure 10: The amplification plot of Multiplex Real-time RT-PCR showing the CHIKV positive sample, the IPC and negative CHIKV and DENV samples

4.2 Discussion

Monitoring arbovirus infections as part of virological surveillance in humans including virus isolation from human serum and mosquito inoculations has greatly influenced outbreak predictions globally (Gubler, 1998). It has been previously reported that DENV and CHIKV are amongst the viral causes of fever in patients on the lookout for health care in Morogoro region (Chipwaza *et al.*, 2014).

The study has demonstrated a prevalence of CHIKV infection (1.28%) in 312 febrile victims seeking medical care in health facilities during the inter-epidemic period. Because of poor accessibility to diagnostic capabilities in areas with malaria epidemics, most febrile cases have been previously and recently treated as cases of malaria (D'Acremont *et al.*, 2014; Kinimi *et al.*, 2018a; Mishra *et al.*, 2011; WHO, 2009).This is attributed by the similar

clinical manifestations of indications and signs in the early stages of dengue, chikungunya and other febrile illnesses. The diagnostic capacity to rule-out chikungunya, dengue and other infections has remained an obstacle to their control and prevention in resource-limited settings (Mardekian & Roberts, 2015).

This study has revealed about 1.3% of CHIKV prevalence, suggesting very minimal active CHIKV transmission in Morogoro Municipal. This prevalence is less than the previously reported chikungunya prevalence in different regions of the country (Chipwaza *et al.,* 2014; Faustine *et al.,* 2017; Kajeguka *et al.,* 2016; Kinimi *et al.,* 2018b). The variation in many studies may be endorsed by the seasonal variations, climatic changes, timing of blood sampling in an acute infection, and type of test used for virus detection (Hertz *et al.,* 2012). Furthermore, CHIKV infection symptoms last for about 2-10 days, the time in which virus is circulating in blood and it can be identified by PCR only within this limit. The sensitivity of acute infection diagnosis after the viremic phase becomes minimal as virus levels are significantly reduced (Kajeguka *et al.,* 2016).

Age and sex are proxy features termed as the explicit behavior that cause higher exposure to mosquito bites (Bisimwa *et al.,* 2016; Furuya-Kanamori *et al.,* 2016). In this study, molecular detection of CHIKV was significantly lower in children than in adults, which may indicate current virus exposure. Most adults aged between 18 and 55 years practices a lifestyle of staying outdoors during dusk and dawn hours which favors *Aedes* mosquito bites. Consequently, such exposure to mosquito propensity makes them vulnerable to mosquito-borne diseases as well as dengue and chikungunya (Dhimal *et al.,* 2015; Kinimi *et al.,* 2018). Moreover, employed individuals and livestock keepers who are associated with daily movements from home to work have an increased chance of contracting *Aedes* mosquito bites at early morning and at dusk from different locations (Camara *et al.,* 2018).

In this study, chikungunya cases were exclusively present in adult's female ranging from 20-49 years of age. Similarly, other studies reported adulthoods as a potential risk factor to DENV and CHIKV infections (Mardekian & Roberts, 2015; Ndosi *et al.,* 2016). Cultural dynamics in Tanzania where females are mostly responsible for farming, housekeeping, cooking, fetching water and fire woods, together with other risk factors such as reduced body immunity in lactating and pregnant mothers may influence female individuals to mosquito-borne exposure. In contrary, these findings differ from a study conducted in Magugu district in the Northern zone (Faustine *et al* ., 2017), which reported that male sex had five times

higher odds of being CHIKV positive. This may be attributed by a different lifestyle practiced between people of two different locations as most of individuals in the North-east zone are pastoralists in which men are highly involved than women.

Our findings may suggest the possibility of affected *Aedes* mosquitoes that minimally circulate CHIKV active cases in Morogoro Municipality. It has been ascertained by the unpublished study conducted by Ahedor (2016) that there is a relatively high abundance of *Aedes aegypti* mosquitoes in Morogoro region. Suitable weather and climate conditions are essential for the persistence, distribution, and transmission of pathogens, hosts and vectors for the disease. Fluctuations in weather conditions may pose an influence on communicable diseases by affecting the pathogens, hosts, vectors, and the earth (Wu *et al.,* 2014). Many arboviral infectious diseases are extremely subtle to climatic and weather fluctuations (Tian *et al.,* 2015). The low prevalence of CHIKV may also be attributed by the possible fact that, may be the CHIKV was minimally present in blood when the samples were collected for this study, hence a sero-prevalence study should have indicated possible presence of CHIKV antibodies during the sample collection period, just as it was for serological evidence of presence of DENV infection in Morogoro (Chipwaza *et al.,* 2014).

This study has shown that there were no DENV cases at the selected health centers despite the recent outbreak in Dar es Salaam region and its serological evidence in Morogoro (Chipwaza *et al.,* 2014). Most of chikungunya and dengue studies which were reported in Tanzania were conducted during outbreaks, and they used serological tests and solitary, but only a few reported virological prevalence of these arboviruses (Bisimwa *et al.,* 2016; Faustine *et al.,* 2017; Kajeguka *et al.,* 2016). Sampling procedure was conducted within a short time in March up to May 2018 during the inter-epidemic period. This was the period in which a dengue outbreak had occurred in Dar es Salaam city, chances of human movements between the two regions could have facilitated spread of the virus, but this did not happen to large extent.

Similarly, another findings conducted in Tanga and Hai in North Tanzania reported no prevalence of acute DENV (Hertz *et al.,* 2012). Other study conducted in Morogoro municipality to assess the viral infectivity in the vector population by molecular detection method reported no DENV was detected (unpublished data) (Ahedor, 2016). Nevertheless the results in this study are likely similar with a study performed by Faustine *et al.* (2017) which reported a less dengue virus prevalence of 1 (0.8%) in Magugu district in Northern Tanzania.

In contrary, the results from the inter-epidemic study differed from other epidemic findings in Tanzania. In 2014, Dengue fever outbreak occurred in Dar es Salaam with more than 400 confirmed cases and at least three died (Mboera *et al.*, 2016). Additionally, the recent outbreak in Dar es Salaam in March 2018 reported 11 confirmed cases (as reported from the Ministry of Health). Thus, the samples tested were not detected with DENV but there was a probability of affected individuals who did not seek medical attention during this study frame. Additionally, dengue patients undergo dehydration upon severe diarrhea and as dengue is mostly misdiagnosed in most dengue-endemic areas, these patients tend to recover easily after receiving oral rehydration solutions and sufficient resting.

Limitations in this study were the fact that only a single diagnostic technique was used. This kit targeted virus RNA on current infections thereby omitting the chances of finding out past exposure to the viruses through serology tests. Moreover, virus detection on vector organisms was not involved. The study focused more on screening for these arboviruses which is highly underreported and misdiagnosed among patients with fever.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This investigation was performed on selected health centers in Morogoro municipality to screen for DENV and CHIKV from febrile patients during the inter-epidemic period using multiplex real-time RT-PCR. Result from this study provides insight into presence of minimally active circulation of chikungunya virus (1.3% prevalence) among febrile patients seeking medical attention, which may be transmitted in Morogoro Municipality, Tanzania. The findings show that CHIKV may also be important cause of fever among febrile patients in Morogoro municipality during dengue inter-epidemic period, with a low prevalence. Only 4 cases of acute chikungunya among 312 studied samples were exclusively prevailing amongst female patients aged between 20 and 49 years. The present study findings may show the significance of reporting age and gender stratified data as factors (variables) to consider in chikungunya surveillance, to help in targeting specific preventive measures. There was no detection of DENV at this sampling period, the time in which high risk of transmission of dengue epidemics in the neighboring Dar es Salaam was likely to occur.

5.2 Recommendations

The present study recommends the following:

(i) The ministry of health should integrate reliable and affordable arboviral screening and diagnostic tests at the point of care routine laboratory investigations to facilitate early and accurate diagnosis and management of chikungunya patients and reduce misdiagnosis of mosquito-borne viral infections.

(ii) Clinicians ought to think about chikungunya case discovery when given patients having febrile ailment and fever of obscure beginning in order to advance patient supervision and add to a progressively vivacious appraisal of infection trouble in Tanzania.

(iii) PCR based diagnosis of both CHIKV and DENV should be carried out together with serological tests to avail their possible past infection (sero prevalence), which may be overlooked by absence of active CHIKV and DENV in collected blood. This may

possibly be the reason for observed low prevalence of CHIKV (1.3%) and zero prevalence of DENV herein.

(iv) The government should initiate regular surveillance programmes in monitoring arboviral activities and febrile illness viruses in human populations, as well as mosquito vectors and embark an integrated vector control programme at the municipal and National level in order to avoid maintenance of the viruses in mosquitoes that may breach future outbreaks.

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APPENDICES

Appendix 1: Materials and Reagents used in the study

-	Vacutainer tubes and needle	- Permanent marker pen/ pencil
-	Vacutainer holders	- Staining jar/ holder
-	Cotton wool	- Tube rack
-	Pipettes	- Appropriate PPE
-	70% Ethanol	-Xylene cleaning solution
-	Bioneer kits	-Two-ply adsorbent tissue

Bioneer kits and instruments used in this study

Cat no.	Kit name	Instrument	Tests
Bioneer kits			
ZIK-1111	AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit	Exicycler™ 96	96
K-4473	ExiPrep™ Dx Viral RNA Kit	ExiPrep™ 16 Dx Fully Automated Nucleic Acid Extraction System	16
Bioneer instruments			
A-2060	Exicycler™ 96 Real-Time Quantitative Thermal Block	Exicycler™ 96 Real-Time Quantitative Thermal Block	96
A-5050	ExiPrep™ 16 Dx Fully Automated Nucleic Acid Extraction System	ExiPrep™ 16 Dx Fully Automated Nucleic Acid Extraction System	16
A-5150	ExiPrep™ 48 Dx	ExiPrep™ 48 Dx	48
A-7040	ExiSpin™	ExiSpin™	32

Contents of the Viral RNA kit (K-4473) for use with BIONEER's ExiStation™ System

Reagents	**Units**
Buffer Cartridge 1 and 2	1 pack each
Disposable filter tips	3 packs
Elution tube	1 pack
Contamination shield filter paper	1 pack
Protection cover	4 packs
Waste tray	1 pack
Sample loading tube	6 packs

Contents of the Multiplex Real-Time RT-PCR kit (ZIK-1111) for use with BIONEER's ExiStation™ System

Reagents	Unit	Quantity (96 test kit)
ZIKV Premix	1 Aluminum Foil Bag	8 tubes x 12 strips (96 tubes)
Positive Control RNA	15 µl / tube (Natural 8-tube strip)	8 tubes x 2 strips (16 tubes)
IPC RNA (Internal Positive Control)	15 µl / tube (Yellow 8-tube strip)	8 tubes x 2 strips (16 tubes)
DEPC-DW (No Template Control)	15 µl / tube (Purple 8-tube strip)	8 tubes x 2 strips (16 tubes)
DEPC-DW	1800 µl / tube	4 tubes
SL Buffer	1800 µl / tube	8 tubes
Optical Sealing Film	-	1sheet
Quick Manual	-	1sheet

Materials required but not provided by ZIK-1111 Kit.

Material	Product name/description
Real-Time PCR instrumentation required for kit ZIK-1111	BIONEER's ExiStation™ Universal Molecular Diagnostic System (Cat. No. A-2200): (Integrated platform of Real-Time PCR instrument (Exicycler™ 96) and nucleic acid extraction Instrument (ExiPrep™ 16 Dx)) Exicycler™ 96 Real-Time Quantitative Thermal Block
Reagent requirements for kit ZIK-1111	ExiPrep™ 16 Dx (Cat. No. A-5050) ExiPrep™ Dx Viral RNA Kit (Cat. No. K-4473) ExiSpin™ (Cat. No. A-7040)
Materials requirements for kit ZIK-1111	Disposable powder-free gloves Appropriate volume pipette set Sterilized pipette tips with filters 1.5 ml micro tubes or 15 ml conical tubes

The test kits were stored between -25°C and -15°C.

Appendix 2: Informed consent form

Respondent's ID and Year …….

Consent to participate in this study

Greetings! My name is

……

(Name of a person administering the questionnaires)

The purpose of this research is to investigate the etiologies of acute febrile infections in Morogoro Region. You are being asked to participate in this study because you have particular knowledge and experiences that may be important to the study.

Participation involvement

If you agree to participate in this study, the following will occur:

1. You will be interview about previous illness history or your baby and a blood sample from your vein or blood from your baby may be collected.
2. In addition, some personal information will be gathered like your age, height and weight level of education, occupation and ethnicity.
3. Your identity (name) will be stored at a confidential place in the hospital together with your medical files, and researchers have only access to a code in which nobody will be able to recognize.
4. In case you are willing to participate in future with further research within this or future projects you can specify.
5. You will be interviewed only once for approximately 10 minutes in a private setting.

Benefits

There will be no direct benefits to you; however, the information you provide will help to increase the understanding on febrile illness. This will help to formulate prevention and control strategies aimed at reducing the acute febrile infections.

Confidentiality

I assure you that all the information collected from you will be confidential. Only the main research in the project and your medical doctor will have access to the information. The final report will contain responses and laboratory results from several participants without any

references to individuals. We will not put your name or others identifying information on the records of the information you provide.

Risks

You will be interview as described above, in case some questions make you feel uncomfortable, you are free not to respond to them. There are no risks associated with the collection of blood samples taken from you or your child.

Rights to withdraws and alternatives

Your participation in the study is on a voluntary basis. If you choose not to participate in the study or decide to stop participating in the study there will not be any problem refusal to participate or withdraws from the study will not affect the quality of health service you get from the hospital.

In case of injury

We do not anticipate that any harm will occur to you or your baby as a result of participation in this study.

Whom to contact

If you have questions about this study, you may contact the following:

Principal Investigator of the research project Dr. Lucas E Matemba NIMR laboratory, P.O Box 476, Morogoro. Tel 0757 313 626.

National Health Ethics review Committee (NATHREC), 2448-Baraka Obama Avenue, and P.O Box 9653, Dar es Salaam.

Certification of consent

I have been invited to take part in the study of "investigation of Acute Febrile Infections in Morogoro region, Tanzania" I have read the foregoing information or it has been read to me and I have understood I agree to participate in this study.

Signature

Name of the participating mother (in capital letter) …….

Signature (or thumbprint of participant)……..

Signature of witness (if a participant cannot read)…….

Signature of research assistant ……

Date consent

Signed …….....Are you willing to be in contact for future research?

YES ☐

☐ NO

Address P.O Box …….

Hamlet …… Village…… Ward ……..……

District …… Region ……

Appendix 3: Laboratory protocol for AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit

ExiStation™ Procedure

The device is able to perform Nucleic acid extraction from 48 samples within an hour with a combination of 3 ExiPrep™ 16 Dx and 1 Exicycler™ 96. It has enhanced usability with automated processes such as decapping and capping of sample tubes and a built-in automatic cartridge puncher.

Preparation;

1) Turn on the computer pre-installed with ExiStation™ Manager Software.
2) Execute the ExiStation™ Manager Software by clicking the icon located on the desktop.
3) Turn on the ExiPrep™16 Dx (A-5050) by pressing the main power button located at the front of the instrument. Press the 'STARTING' image displayed on the LCD to initiate instrument startup.
4) Press the 'MISC SET' button on the LCD screen (or the 'Load' button on the software). Attach the filter paper onto the Contamination Shield. Attach the prepared Contamination Shield then the Tip Protector in the instrument. Press the 'Misc Set' button again.
5) The ExiStation™ Manager Software has six distinct parts. Prep - control nucleic acid extraction (ExiPrep™16 Dx instrument). Assign PCR - transfer sample information from 'Prep' to 'PCR' (Exicycler™ 96) and assign for PCR run. The result after the PCR run present experiment information and sample result information
6) Click the 'Prep' tab on the upper left of the main screen to initiate the nucleic acid extraction process.
7) Click the pull-down arrow for 'Diagnosis Kit 1'. A popup will appear.
8) Inspect the Buffer Cartridge and mark the used well by clicking on the corresponding location to exclude the used well from sample assignment. Select 'OK' to finish.
9) The program will automatically allocate the NTC and PC wells. The default setting is one (1) for each of NTC and PC.
10) Input each lot number of the diagnostic kit and the prep kit. Click the 'Sample ID' column and enter the sample information by using a barcode reader (optional) or a

keyboard.

11) Check that all necessary materials and accessories are present before proceeding.

12) Clean the surface (preferably a positive pressure BSC) where work will be performed.

13) Remove the shrink-wrap enclosing both Buffer Cartridges and then remove the acryl lids in a positive pressure BSC.

14) Punch the film with the Hole Punch according to the layout mapped on the software.

15) Cover Buffer Cartridges and with the acryl lids after film punching is complete. Take the necessary number of strips of the Diagnostic Kit Tubes from the freezer. Remove the foil covering the tubes. Insert appropriate numbers of Diagnostic Kit Tubes into the Elution Tube Rack.

Sample Loading

1) Clean the negative pressure Biosafety Cabinet (BSC) on which the nucleic acid extraction preparation will be performed. Clean the surface with 70% Ethanol or 5% nitric acid solution before and after use in order to prevent contamination. After each use, turn on the UV lamp to eliminate contaminants.

2) Add 400 $\mu\ell$ of Sample Loading (SL) Buffer, 1x PBS or normal saline into pretreated samples and completely dissociate the cell pellet using a pipette.

3) Take out the necessary number of Sample Loading Tubes from the magazine and insert them into rack holes.

4) Take the original clinical sample containers or Sample Loading (SL) buffer (for NTC and PC) and pipette into the Sample Loading Tubes by following steps 5) through 8).

5) For the tube that is assigned as control (NTC and PC), add 400 $\mu\ell$ Sample Loading (SL) buffer. (supplied with the AccuPower® Diagnostic Kit)

6) Additionally, add 5 $\mu\ell$ of PC only into the appropriate PC wells. (supplied with the AccuPower® Diagnostic Kit)

7) Move the filled Sample Tube into the Sample Tube Rack.

8) Uncap the original clinical sample container and pipette 400 $\mu\ell$ of sample into the next available Sample Loading Tube. Move the Sample Loading Tube into Sample Tube Rack after pipetting each sample.

9) Repeat step 8) until all samples are loaded.

10) Take the Sample Tube Rack and load onto the Setup Tray.

11) Place the Waste Tray onto the Setup Tray. Slide the base plate in and close the door of the ExiPrep™16 Dx.

12) Click the 'RUN (▶)' button of the ExiStation™ Manager Software. Double check whether all accessories for the extraction are loaded properly according to the 'Check ExiPrep Setting' list and check the boxes. Click the 'OK' button to initiate the prep process. Nucleic acid extraction process takes between 80 minutes and 100 minutes depending on the type of nucleic acid.

PCR Preparation

1) Click 'Assign PCR' tab and check the box of each 'Prep Work List' to assign PCR position. Checked 'Prep Work List' is assigned to the PCR position corresponding to the prep instrument 1 to 3 in order.

2) After the nucleic acid extraction process is finished, the cooling block is automatically turned off. Select the 'Prep' tab again and click "Store ON" on the control panel to turn the cooling block on for long-term storage.

3) Open the door of the ExiPrep™16 Dx (A-5050) after the nucleic acid extraction process is complete, and then remove the Elution Tube Rack.

4) Remove Protection Cover according to Protection Cover Separation Tool utility method.

5) Seal PCR Tube using Optical sealing film and proceed to the next step.

6) Taking care not to flip the orientation of the tubes, Place the Elution Tube Rack on the PCR Preparation Plate with the corresponding ExiPrep™16 Dx number.

7) Seal the Diagnostic Tubes with the adhesive Optical Sealing Film.

8) Right before the PCR reaction, completely mix the tube contents using ExiSpin™ (A-7040) for 2500rpm for 5 sec. Hard vortex for 20 sec. / 20 cycles

9) While the ExiSpin™ is operating, you can start the Exicycler™ 96.

10) Turn the Standby Power Switch, located at the rear of the instrument ON. The front ring-LED status light should turn on RED.

11) Press the front Operation Power Switch for 3 seconds. A brief self-test sequence will initiate. If the self-test passes, the front ring-LED will blink GREEN with a short beep.

12) Push the Door Switch for 2 seconds to slide the 96-well thermal block out. Insert the reaction tubes in their pre-determined locations. After sample loading is complete, push the Door Switch for 2 seconds to close the door.
13) Place the mixed premix tubes into the assigned well position of Exicycler™ 96 right after the ExiSpin™ cycling is complete.
14) Remove all consumables and components, starting with the Buffer Cartridges and various racks from the instrument and discard all liquids and consumables in their appropriate containers.
15) Press the 'Misc Set' button, remove the Tip Protector and Contamination Shield, and then press the 'Misc Set' button again.
16) Push the Base Plate in, shut the instrument door and initiate UV sterilization by clicking "UV ON" on the control panel.
17) Select 'Assign PCR' tab and confirm the assigned 'Prep Work List'. After the 'Prep' process, 'Current Step' will be presented as 'Prep End' and the upper-status bar will be changed to 'Ready to PCR'. Initiate PCR run by clicking the activated 'PCR Start' button at the bottom right of the window.
18) A popup will appear prompting the user to enter a Work List Name. Click 'OK' after entering a name to generate a Work List for Real-Time PCR.
19) After entering the Work List Name, 'PCR' tab will be activated and the Exicycler™ 96 will automatically initiate PCR run.

Data Analysis

After the PCR run is finished, select 'Result' tab to check the results of each sample.

Interpretation of Results

The test uses 2 wells of each NTC and PC to determine the validity of the experiment, and each reaction includes IPC in wells of samples as well as NTC and PC to check whether PCR works. NTC: to determine whether the sample is contaminated in the process of sample pretreatment, nucleic acid extraction, and PCR preparation (prevent false-positive error) PC: to determine whether target RNA is properly amplified (prevent false-negative error) IPC: to check whether PCR is inhibited by the sample and to determine the amplification of nucleic acids in each well. High concentrations of target DNA can lead to a reduced or absent fluorescence signal of IPC due to PCR competition.

The validity of IPC is determined by Ct value of IPC signal. If its Ct value is within the specified range, it is valid. If the Ct value is out of the specified range, it is invalid. The validity of PC and NTC is determined by the Ct value of the target signal. If the assay is valid, target Ct will be 'undetermined' in NTC well and PC Ct value will be within its specified range. If the control results are invalid, take measures according to User's Guide 9 troubleshooting. The result of IPC determines the validity of the test, and Ct value of the target signal determines whether the target is 'Detected' or 'Non-detected'. Cut-off value: to classify results as positive or negative. The cut-off value is determined to utilize statistical technique, probit analysis. The low value of confidence interval (CI) of LoD is converted into Ct value derived from the LoD test. The cut-off Ct value determines the target RNA detection results as positive with 97.5% probability.