

**DETECTION AND GENETIC CHARACTERISATION OF DENGUE VIRUS
AMONG PATIENTS IN DAR ES SALAAM, TANZANIA DURING THE
2013-2014 OUTBREAK**

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

2015

ABSTRACT

The present study was carried out to confirm and genetically characterize dengue virus (DENV) present in sera collected from patients with dengue fever during the 2013 and 2014 dengue outbreaks in Tanzania. Sera were collected from patients who met the clinical case definition for dengue fever. Dengue fever was diagnosed in patients included in this study by detection of IgM and IgG DENV antibodies using a combination of a rapid test and enzyme-linked immunosorbent assay (ELISA). DENV detection in sera samples positive for anti-DENV IgM and IgG was performed using conventional and real-time reverse transcription polymerase chain reaction (RT-PCR). All sera (n = 23) tested positive for anti-DENV IgG and IgM antibodies and for the presence of DENV genomes using RT-PCR. In addition, both conventional and real-time RT-PCR showed the presence of DENV serotype 2 (DENV-2). Nucleotide sequencing of RT-PCR products after amplification of the core-pre-membrane (CprM) region of DENV using conventional RT-PCR produced a 500 bp fragment. BLASTn and phylogenetic analysis of the DENV nucleotide sequence obtained from this study clustered DENV-2 confirming the results obtained during conventional and real-time RT-PCR. The DENV-2 involved during the dengue fever outbreaks in 2013 and 2014 had 100% nucleotide and amino acid identity. This indicated that the same DENV-2 was responsible for the dengue fever outbreaks of 2013 and 2014. The phylogenetic analysis and BLASTn results of the DENV-2 CprM junction region obtained in the present study indicated that it has 98.2% nucleotide identity to SG/D2Y98P-PP1/2009 (Accession number JF327392). The SG/D2Y98P-PP1/2009 is a DENV isolate with a Phe-to-Leu alteration at position 52 in the NS4B protein of the original D2Y98P virus that was isolated in 1998 from a DENV-infected patient in Singapore. This mutation completely abolished the pathogenicity of the D2Y98P virus, as evidenced by a lack of lethality and

the absence of histological signs of disease, which correlated with reduced viral titers and intact vascular permeability. Future studies are recommended in order to fully sequence the DENV-2 isolate obtained in the present study in order to determine the presence or absence of this mutation. In addition, it is recommended that the control of *Aedes* mosquitoes in Dar es Salaam be performed in order to avoid maintenance of DENV in mosquitoes that may lead to future outbreaks.

DECLARATION

I, Gurdeep Jaswant, 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 in any other institution.

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The declaration is hereby confirmed;

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Date

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ACKNOWLEDGEMENTS

I am sincerely grateful to my supervisor Professor Gerald Misinzo and Dr. Fausta Moshia for their willingness to supervise this work. Their guidance, encouragement and advice from the development of the proposal up to the completion of this study are highly acknowledged.

I am grateful to the Ministry of Health and Social Welfare (MoHSW) for allowing me to use the collected sera samples archived at the National Health Laboratory and Quality Assurance Training Center (NHL-QATC). I also express my heartfelt gratitude and appreciation to members of staff at the NHL-QATC for their invaluable assistance during sample analysis, especially Miss Carolyn Riwa, Mrs. Mirium Joshua Matonya, Mr. Edward Samwel, Mrs. Vumilia Mwalongo and Miss Mariah Kelly for technical assistance with molecular work.

My sincere thanks go to the Southern African Centre for Infectious Disease Surveillance (SACIDS) for providing me with the scholarship and funds for undertaking this study. I am also grateful to SACIDS members of staff for their unforgettable hospitality, support and unreserved help they gave me. Finally, I wish to thank my family for their moral support, prayers and encouragement during my studies.

DEDICATION

I dedicate this work to all students of Master of Science in One Health and Molecular Biology at the Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture.

TABLE OF CONTENTS

ABSTRACT	ii
DECLARATION	iv
COPYRIGHT	v
ACKNOWLEDGEMENTS	vi
DEDICATION	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF APPENDICES	xiii
LIST OF ABBREVIATIONS AND ACRONYMS	xiv
CHAPTER ONE	1
1.0 INTRODUCTION	1
1.1 Background Information	1
1.2 Problem Statement and Justification	2
1.3 Research Objectives	2
1.3.1 Main Objective	2
1.3.2 Specific Objectives	3
CHAPTER TWO	4
2.0 LITERATURE REVIEW	4
2.1 Historical Epidemiological Background of Dengue Fever	4
2.2 Structure and Properties of Dengue Virus	4
2.2.1 Classification	4
2.2.2 Structure	5

2.2.2.2	Envelope protein	5
2.2.2.3	Membrane protein (prM/M protein)	5
2.2.2.4	NS3 protein	6
2.2.2.5	NS5 protein	6
2.3	Dengue Disease and Treatment	8
2.4	Principle of RT-PCR	8
CHAPTER THREE		10
3.0	MATERIALS AND METHODS	10
3.1	Study Area	10
3.2	Source and Storage of Samples	12
3.3	Laboratory Molecular Analyses	12
3.3.1	RNA extraction	12
3.3.2	Universal dengue virus reverse transcription polymerase chain reaction	13
3.3.3	CDC DENV-1-4 Real Time RT-PCR Assay (qRT-PCR)	17
3.3.4	Sequencing	18
3.3.5	Nucleotide sequencing, similarity search and determination of phylogenetic relationship of dengue virus	20
CHAPTER FOUR		22
4.0	RESULTS	22
4.1	Detection of DENV in Sera Samples Using by Conventional RT-PCR	22
4.2	DENV Serotyping Using CDC DENV-1-4 qRT-PCR Assay	23
4.3	Similarity of DENV RNA Sequence Against GenBank	25
4.4	Phylogenetic Analysis of DENV	25

CHAPTER FIVE	30
5.0 DISCUSSION	30
CHAPTER SIX	33
6.0 CONCLUSION AND RECOMMENDATIONS	33
6.1 Conclusion	33
6.2 Recommendations	33
REFERENCES	35
APPENDICES	40

LIST OF TABLES

Table 1: Samples selected for RNA Extraction	13
Table 2: The RT-PCR master mix for a single reaction	14
Table 3: List of oligonucleotide primers used for the detection of dengue virus:	15
Table 4: List of oligonucleotide primers and probes used for serotyping dengue virus	16
Table 5: CDC DENV-1-4 real time RT-PCR assay master mix components.....	18
Table 6: The composition of reagents for the sequencing PCR for a single reaction	20
Table 7: Sera samples used in the present study	24
Table 8: BLASTn results showing homologous gene sequences in the NCBI database to the dengue viruses with accession numbers KM892493, KM892494, KM892495 and KM892496 representing the nucleotide sequence of the DENV CprM junction region.	27
Table 9: Summary of the dengue viruses (DENV) isolates used for the construction of phylogenetic trees based on CprM junction region sequences.....	28

LIST OF FIGURES

Figure 1: The genome of organization of dengue virus (DENV). DENV genome consists of a single strand of positive RNA with an approximate length of 10.7 kb.	7
Figure 2: Map of Dar es Salaam showing the location where dengue fever samples were obtained.	11
Figure 3: Cycle sequencing polymerase chain reaction profile. Sequencing PCR was done on an ABI PCR System 9700 with a rapid thermal ramp.	20
Figure 4: Conventional PCR results of dengue virus in sera obtained from patients with dengue fever. M is 1Kb DNA ladder.....	22
Figure 5: Serotyping of dengue virus in sera using RT-PCR. The reporter dye fluorescence intensity (y-axis) is plotted against the PCR cycle number (x-axis).	23
Figure 6: Phylogenetic tree of dengue viruses based on the CprM junction region constructed using the neighbour-joining method in MEGA 6.06® software.	26

LIST OF APPENDICES

Appendix 1: Purification of Viral RNA 40

Appendix 2: Amplification profile for dengue virus real time reverse
transcription polymerase chain reaction 42

LIST OF ABBREVIATIONS AND ACRONYMS

°C	degree Celsius
x g	centrifuge rotor speed
bp	base pair
BLASTn	Basic Local Alignment Search Tool (Nucleotide)
CprM	core-pre-membrane
BLAST	Basic Local Alignment Search Tool
ELISA	enzyme-linked immunosorbent assay
DENV	dengue virus
DNA	deoxyribonucleic acid
IFM	Institute of Finance Management
IST	International School of Tanganyika
mg	milligram
min	minutes
ml	millilitre
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NS	non-structural proteins
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
TAE	Tris Acetic EDTA buffer
WHO	World Health Organization of the United Nations
µl	microliter
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Dengue is a mosquito-borne viral disease caused by the four dengue virus serotypes (DENV1-4) and is currently considered as the most important arthropod-borne viral disease in the world. Nearly half of the human population lives in risk areas, and 50-100 million infections occur yearly according to the estimates by the World Health Organization. The disease can vary from a mild febrile disease to severe haemorrhagic fever and shock. A secondary infection with heterologous serotype increases the risk for severe disease outcome. During the last three decades the impact of dengue has dramatically increased in the endemic areas including the tropics and subtropics of the world. The current situation with massive epidemics of severe disease forms in urban environments has been associated with socio-ecological changes that have increased the transmission and enabled the co-circulation of different serotypes. Consequently, an increase of dengue has also been observed in travelers visiting these areas.

Two clinical forms of dengue infection have been recognized: dengue fever, a relatively mild, self limiting febrile illness and dengue hemorrhagic fever/dengue shock-syndrome (DHF/DSS), a severe infection with vascular and haemostatic abnormalities that can lead to death (Gubler, 1998).

Virological surveillance, which involves the monitoring of dengue virus infection in humans, has been used as an early warning system to predict outbreaks in many area of the world (Gubler, 1989). Such surveillance is based on isolation of dengue virus from human serum by cell culture or mosquito inoculation and type-specific identification by immunofluorescence. Reverse transcription–polymerase chain reaction (RT-PCR), a

method with high sensitivity and specificity, has been developed for the detection of dengue virus (Seah, 1995). The RT-PCR assay has been previously shown to detect virus types in serum specimens from which dengue viruses had been isolated by the gold standard test of virus isolation in C6/36 cells followed by identification with monoclonal antibodies.

In Tanzania, infection by dengue virus has significantly increased in the last two year since 2011 and the occurrence of dengue disease has been reported by the Ministry of Health and Social Welfare and WHO in parts of Dar Es Salaam city in 2013.

1.2 Problem Statement and Justification

Several dengue outbreaks have been reported in Tanzania including the latest outbreak in parts of Dar es Salaam in 2014. During the 2013 outbreak, samples were collected from patients and screened using RT-PCR for the confirmation of the presence of the disease. So far no any molecular work has been done to study the molecular epidemiology of the dengue virus that caused the 2013 and 2014 outbreak. This study is aimed at performing the molecular characterization of dengue virus in human archived samples collected during the 2013-2014 outbreak in Dar es Salaam, Tanzania. Understanding the circulating dengue virus serotypes in Tanzania will assist in determining the molecular epidemiology of dengue in the country.

1.3 Research Objectives

1.3.1 Main Objective

To perform the molecular characterization of dengue virus in human archived samples collected during the 2013 and 2014 outbreak in Dar es Salaam, Tanzania.

1.3.2 Specific Objectives

- (i) To screen dengue virus in archived human samples collected during the 2013 and 2014 outbreak in Dar es Salaam using RT-PCR
- (ii) To determine the dengue virus serotype (DENV 1, 2, 3 and/or 4) in archived human samples collected during the 2013 and 2014 outbreak in Dar es Salaam using serotype-specific RT-PCR
- (iii) To perform sequencing and phylogenetic analysis reconstruction of dengue in archived human samples collected during the 2013 and 2014 outbreak in Dar es Salaam

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Epidemiological Background of Dengue Fever

Dengue fever and dengue hemorrhagic fever are caused by the four serotypes of dengue virus, types 1 to 4, belonging to the Flaviviridae family. In many tropical and subtropical areas of the world, these viruses are endemic and cause periodic or annual outbreaks of disease. Dengue viruses are transmitted by *Aedes aegypti* that breeds in stagnant water in all forms of receptacles in urban areas, especially following intermittent rainfall in these tropical regions. *A. aegypti* can be recognized by white markings on legs and a marking in the form of a lyre on the thorax. Dengue infection has expanded to other geographic areas such as the Americas due to changes in human ecology and behavior (Gubler and Clark, 1995; Gubler and Trent, 1994).

In previous laboratory studies, it was observed that while nearly all species of *Aedes* mosquitoes were uniformly susceptible to parenteral infection with dengue viruses, *A. aegypti* was much less susceptible to oral infection compared with several other human-biting *Aedes* such as *A. Albopictus* (Rosen *et al.*, 1985). Furthermore, different geographic strains of *A. albopictus* were found to have significant variation in susceptibility to oral infection with dengue viruses (de Lamballerie, 2002).

2.2 Structure and Properties of Dengue Virus

2.2.1 Classification

Dengue virus belongs to the Flaviviridae family, genus Flavivirus. The virus family Flaviviridae consists of small (40-60 nm in diameter) enveloped animal viruses that have a genome of a single molecule of positive sense RNA. The family is divided into four

genera: Flavivirus (including yellow fever virus, West Nile virus and dengue fever virus), Hepacivirus (including Hepatitis C virus), Pegivirus (including GB virus A, GB virus C, and GB virus D) and Pestivirus (including bovine viral diarrhea virus, classical swine fever virus).. Only the genus Flavivirus includes arthropod-borne members (arboviruses) (Knipe, 2001). Rboviruses demonstrate biological flexibility that enables them to infect and replicate in different host species, in arthropod and vertebrate cells (Karabatsos, 1995). This strategy has been advantageous by ensuring effective transmission, spreading, survival and maintenance of arboviruses in nature.

2.2.2 Structure

2.2.2.1 Envelope protein

The DENV E (envelope) protein, found on the viral surface, is important in the initial attachment of the viral particle to the host cell. Several molecules interact with the viral E protein including ICAM3-grabbing non-integrin, CD209, Rab 5, GRP 78 and the mannose receptor and have been shown to be important factors mediating attachment and DENV entry (Jindadamrongwech, 2004; Jindadamrongwech and Smith, 2004; Krishnan *et al.*, 2007; Navarro-Sanchez, *et al.*, 2003; Tassaneetrithep *et al.*, 2003).

2.2.2.2 Membrane protein (prM/M protein)

The DENV prM (membrane) protein, which is important in the formation and maturation of the viral particle, consists of seven antiparallel β -strands stabilized by three disulfide bonds. Miller JL, *et al.* (February 2008). The glycoprotein shell of the mature DENV virion consists of 180 copies each of the E protein and M protein. The immature virion starts out with the E and prM proteins forming 90 heterodimers that give a spiky exterior to the viral particle. This immature viral particle buds into the endoplasmic reticulum and eventually travels via the secretory pathway to the Golgi

apparatus. As the virion passes through the trans-Golgi Network (TGN) it is exposed to low pH. This acidic environment causes a conformational change in the E protein which disassociates it from the prM protein and causes it to form E homodimers. These homodimers lie flat against the viral surface giving the maturing virion a smooth appearance. During this maturation pr peptide is cleaved from the M peptide by the host protease, furin. The M protein then acts as a transmembrane protein under the E-protein shell of the mature virion. The pr peptide stays associated with the E protein until the viral particle is released into the extracellular environment. This pr peptide acts like a cap, covering the hydrophobic fusion loop of the E protein until the viral particle has exited the cell.

2.2.2.3 NS3 protein

The DENV NS3 is a serine protease, as well as an RNA helicase and RTPase/NTPase. The protease domain consists of six β -strands arranged into two β -barrels formed by residues 1-180 of the protein. The catalytic triad (His-51, Asp-75 and Ser-135), is found between these two β -barrels, and its activity is dependent on the presence of the NS2B cofactor. This cofactor wraps around the NS3 protease domain and becomes part of the active site. The remaining NS3 residues (180-618), form the three subdomains of the DENV helicase. A six-stranded parallel β -sheet surrounded by four α -helices make up subdomains I and II, and subdomain III is composed of 4 α -helices surrounded by three shorter α -helices and two antiparallel β -strands.

2.2.2.4 NS5 protein

The DENV NS5 protein is a 900 residue peptide with a methyltransferase domain at its N-terminal end (residues 1-296) and a RNA-dependent RNA polymerase (RdRp) at its C-terminal end (residues 320–900). The methyltransferase domain consists of an $\alpha/\beta/\beta$

sandwich flanked by N- and C-terminal subdomains. The DENV RdRp is similar to other RdRps containing palm, finger, and thumb subdomains and a GDD motif for incorporating nucleotides (Miller *et al.*, 2008).

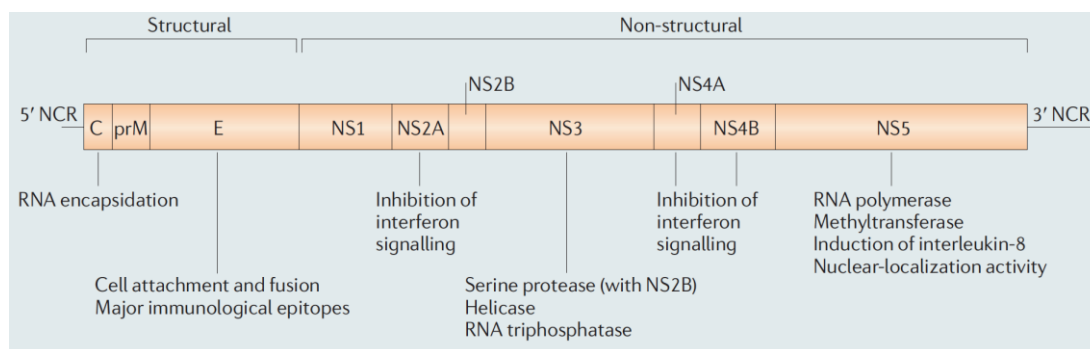


Figure 1: The genome organization of dengue virus (DENV). DENV genome consists of a single strand of positive RNA with an approximate length of 10.7 kb (Source: Vasilakis *et al.*, 2011).

This RNA encodes a single ORF, which is flanked by non-coding regions (NCRs) of 96 and ~450 nucleotides, respectively. Conserved structural elements in the 3' NCR are involved in viral replication, regulation of translation, and RNA synthesis, as well as in interactions with viral and cellular proteins. The genome serves as an mRNA for the translation of the viral proteins and encodes three structural proteins; capsid (C), membrane (prM; processed to M) and envelope (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Translation of the ORF produces a large polyprotein that is cleaved by host signal peptidases and a viral serine protease during and after translation to yield the ten viral proteins. Cleavage between NS1 and NS2A is performed by an unknown host enzyme that most probably resides in the endoplasmic reticulum. Some of the functions that are carried out by the viral proteins are indicated in the Figure 1.

2.3 Dengue Disease and Treatment

Dengue fever is characterized with sudden onset of fever; however the spectrums of the symptoms vary depending on the patient. Different types of aches and pains are common; often the headache is retro- orbital and is accompanied with rash, myalgia, loss of appetite, nausea, vomiting and abdominal pain. Additionally symptoms may include changes in taste metallic taste and flushing of the face. In addition to fever, the clinical definition of DF includes two or more of the following symptoms: headache, retro-orbital pain, muscle or joint pain, rash, haemorrhagic manifestation or leucopenia. The mild haemorrhagic symptoms of skin, such as petechiae may be observed. Additionally, other symptoms include spontaneous bleeding, such as gum bleeding, increased menorrhagic bleeding, gastrointestinal bleeding and hematuria.

Currently, no specific medication is available for treatment of dengue. However, some medicines including corticosteroids (Finsterer, 2006) have been suggested to aid dengue patients with severe symptoms. Paracetamol is preferred as antipyretic. Acetylsalicylic acid (Aspirin®) should be avoided due to its anticoagulant properties that may aggravate bleeding already caused by DENV infection. Frequent monitoring of platelet and hematocrit of DHF/DSS patients is essential for timing the treatment correctly. Several plasma volume replacement solutions have been used, including plasma expanders and electrolyte solutions. In severe cases, blood transfusions have also been used as treatment (Tomashek, 2009).

2.4 Principle of RT-PCR

In principle, viral RNA is extracted from serum sample, transcribed to cDNA in a reverse transcription (RT) reaction either in a separate reaction or in a one step format and amplified by polymerase chain reaction (PCR). Depending on the purpose, the

amplification targets include highly conserved regions of the DENV genome, such as the NS5 and 3' UTR, or areas with more variability, such as the C-preM and E-gene regions. The products of the PCR will be sequenced to know the evolution of the virus.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was the laboratory based study where by it used the archived sample from the National Health Laboratory and Quality Assurance Training Centre in Dar es Salaam. The original sera samples were collected from patients by Muhimbili National Hospital and International School of Tanzania (including samples from patients residents of Masaki and IFM) as well as NHLQATC (for samples from the IFM students).It was conducted in Dar es Salaam due to its geographical position, which favor the presence of coursing agent of the disease, The city is close to the equator and the warm Indian Ocean, the city experiences generally tropical climatic conditions, typified by hot and humid weather throughout much of the year. It has a tropical wet and dry climate. Annual rainfall is approximately 1,100 mm (43 inches), and in a normal year there are two rainy seasons: “the long rains” in April and May and “the short rains” in October and November. In the present study, samples were obtained from patients residing in Masaki, The International School of Tanganyika (IST), The Institute of Finance Management (IFM) and Muhimbili (Fig. 2). Masaki is one of the posh suburbs located along the central beach where many diplomats and expatriates reside. The IST is an international school located within Masaki enrolling approximately 1040 students from around the world. The IFM is the oldest higher learning financial institution in Tanzania located within the city centre enrolling about 8,400 students while Muhimbili National Hospital is the main referral hospital in Tanzania located in Upanga, Dar es Salaam.

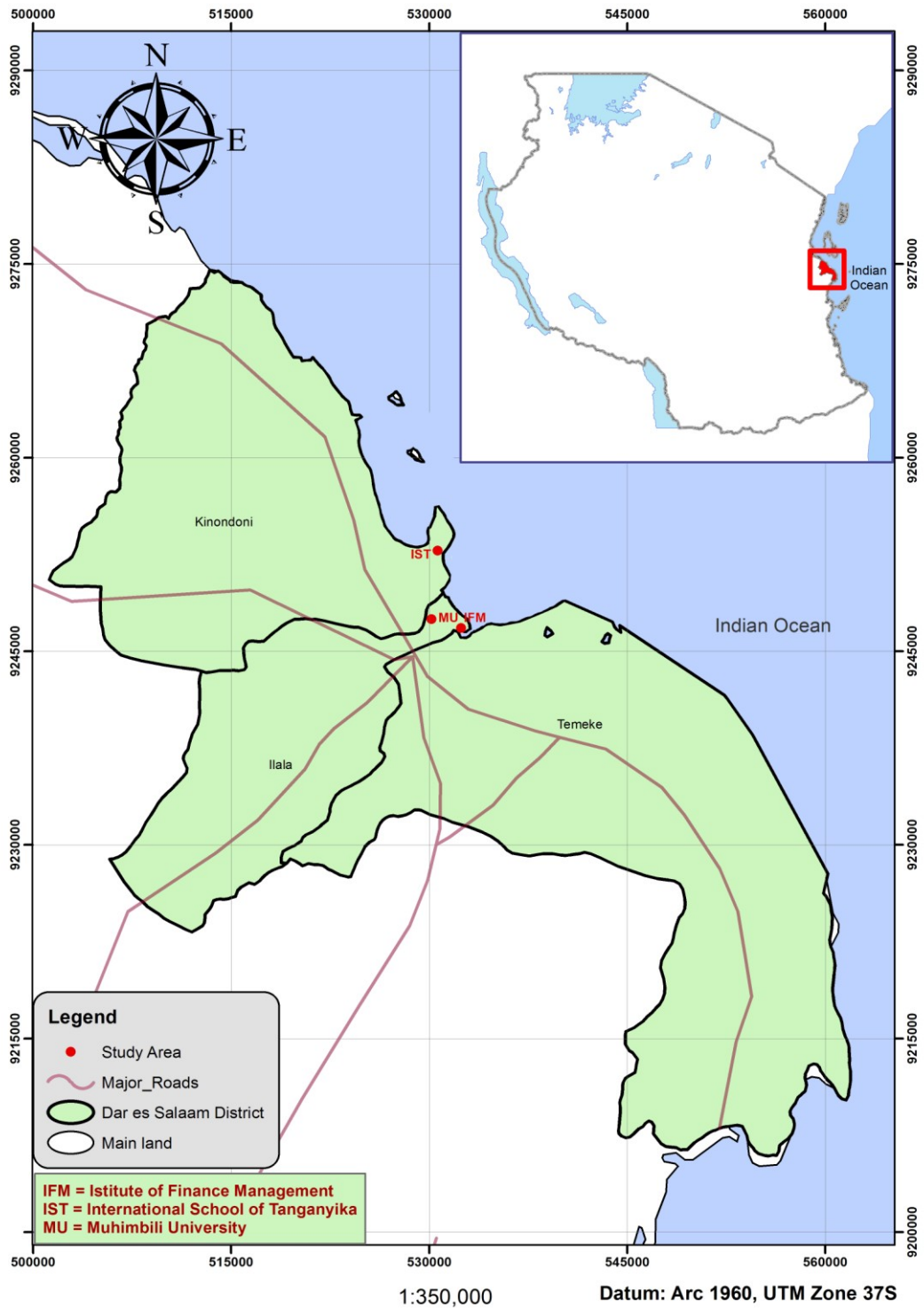


Figure 2: Map of Dar es Salaam showing the location where dengue fever samples were obtained.

3.2 Source and Storage of Samples

Sera samples obtained from patients diagnosed with dengue fever by a combination of a rapid and an antigen enzyme-linked immunosorbent assay (ELISA) IgG and IgM antibody tests were used in the present study. This included four (4) sera samples from dengue fever patients at the Institute of Finance Management (IFM) in June 2013 and nineteen (19) samples from dengue fever patients from the International School of Tanganyika (IST) hospital, Masaki and Muhimbili National Hospital (MNH) between January and April 2013 (Table 1). Sera were stored at -80 °C before performing RNA extraction.

3.3 Laboratory Molecular Analyses

3.3.1 RNA extraction

Twenty three samples were selected from a pool of 165 samples, and only those positive sample for rapid dengue diagnostic and ELISA antibody tests were selected for RNA extraction as shown on Table 1.

Viral RNA was recovered from sera using QIAamp Viral RNA Mini extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions (Appendix 1). Briefly, samples were lysed using a lysis buffer to lyse cells and viral envelope followed with chemical protein precipitation using ethanol. Protein precipitates were pelleted by centrifugation and the supernatant was passed through a silica column to trap the RNA based on charge differences. Column-bound RNA was washed with two different buffers and the column dried by high speed centrifugation. Afterwards, column-bound RNA was eluted with RNase free water. Extracted viral RNA was stored at -80 °C until reverse transcription polymerase chain reaction (RT-PCR) was performed.

Table 1: Samples selected for RNA Extraction

<i>No</i>	<i>Sample ID</i>	<i>Sex</i>	<i>Age</i>	<i>Location</i>	<i>Rapid test</i>	<i>ELISA test</i>
1	TDS0018267	F	39	Masaki	+	+
2	TDS0018268	F	43	IST	+	+
3	TDS0018269	M	52	IST	+	+
4	TSD0018280	M	34	IST	+	ND
5	TDS0018281	F	13	Masaki	+	ND
6	TDS0018299	F	36	Masaki	+	+
7	TDS0018303	F	46	Masaki	+	+
8	TDS0018905	M	25	Masaki	+	+
9	TDS0018907	F	53	Masaki	+	+
10	TDS0018914	M	56	Masaki	+	+
11	TDS0018915	M	53	Masaki	+	+
12	TDS0018909	M	53	IST	+	ND
13	TDS0019210	M	46	Masaki	+	+
14	TDS0020701	F	16	Masaki	+	+
15	TDS0020702	F	40	Masaki	+	ND
16	TDS0020703	F	34	Masaki	+	+
17	TDS0012226	F	32	Masaki	+	+
18	TDS0019053	F	50	Masaki	+	+
19	TDS0021567	F	4	Muhimbili	+	ND
20	NHL3603	M	20	IFM	+	+
21	NHL3602	M	NA	IFM	+	+
22	NHL3601	M	24	IFM	+	+
23	NHL3626	M	23	IFM	+	+

Key: F, Female; M, Male; IST, International School of Tanganyika; IFM, Institute of Finance Management; ND, not determined; and NA, not available.

3.3.2 Universal dengue virus reverse transcription polymerase chain reaction

A universal RT-PCR for the detection of all DENV serotypes (DENV-1 to DENV-4) was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, USA) using AgPath-ID one-step RT-PCR kit (Applied Biosystems, Courtaboeuf, France). The RT-PCR master mix for a single reaction is shown in Table 2. Briefly, reverse transcription was performed using a reverse transcriptase for 30 minutes at 45 °C followed by initial denaturation of DNA for 10 minutes at 94 °C. Afterwards, 40 PCR cycles consisting of denaturation of DNA at 94 °C, annealing of primers at 55 °C and DNA extension by a DNA polymerase, each for 30 seconds. PCR was followed by a final extension at 72 °C for 7 minutes. Detection of all DENV-1 to DENV-4 serotypes was carried out by amplification of the core-pre-membrane (CprM) junction region

using universal DENV primers D1 and D2 (Applied Biosystems, Carlsbad, USA; Table 2), previously described by Lanciotti *et al.* (1992). All reactions were run with DENV-1 to DENV-4 vaccine strain as a positive control, and nuclease-free water during RNA extraction and RT-PCR as the negative control. The primer sequences used in this study were previously reported by Lanciotti *et al.*, (1992) as described in Table 3.

RT-PCR products were separated by electrophoresis on a 1.5% agarose gel in 0.5% TBE buffer (SERVA Electrophoresis, Heidelberg, Germany) stained with GelRed nucleic acid stain (Phenix Research Products, Candler, USA). Each well was loaded with five μ l of the PCR product and three μ l of blue/orange 6X DNA loading dye (Promega, Madison, USA). Samples were separated along with a 1,000bp DNA ladder (Promega, Madison, USA) at 150 volts for 30 minutes. The agarose gel was visualized by ultraviolet fluorescence light using a transilluminator (Sigma-Aldrich, St. Louis, USA) and images were acquired using a digital camera.

Table 2: The RT-PCR master mix for a single reaction

<i>No.</i>	<i>Component</i>	<i>Volume (μl)</i>
1.	2x RT-PCR buffer	12.5
2.	10 μ M Universal D1 primer	1.0
3.	10 μ M Universal D2 primer	1.0
4.	Nuclease-free water	9.0
5.	25x RT-PCR enzyme Mix	0.5
6.	Extracted RNA template	1.0
	Total volume per reaction	25.0

Table 3: List of oligonucleotide primers used for the detection of dengue virus:

DENV serotype	Primer name	5' → 3' oligonucleotide sequence	Genome position	Gene	Amplicon size (bp)	RT-PCR primer pair
DENV1-4	D1	TCAATATGCTGAAACGCGCGAGAAACCG	134-161		511	D1 and D2
DENV1-4	D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	616-644		511	D1 and D2
DENV-1	TS1	CGTCTCAGTGATCCGGGGG	568-586		482	D1 and TS1
DENV-2	TS2	CGCCACAAGGGCCATGAACAG	232-252		119	D1 and TS2
DENV-3	TS3	TAACATCATCATGAGACAGAGC	400-421		290	D1 and TS3
DENV-4	TS4	CTCTGTTGTCTTAAACAAGAGA	506-527		392	D1 and TS4

Table 4: List of oligonucleotide primers and probes used for serotyping dengue virus

DENV serotype	Oligonucleotide name	5' → 3' oligonucleotide sequence^a	Fluorophore^b	Genome position	Gene^c	Amplicon size (bp)
DENV-1	D1-F	CAAAAGGAAGTCGYGCAATA		8936-8955	NS5	112
	D1-R	CTGAGTGAATTCTCTCTGCTRAAC		9023-9047	NS5	112
	D1-probe	CATGTGGYTGGGAGCRGC	FAM/BHQ1	8961-8979	NS5	112
DENV-2	D2-F	CAGGCTATGGCACYGTCACGAT		1426-1447	E	78
	D2-R	CCATYTG CAGC ARCACCATCTC		1482-1504	E	78
	D2-probe	CTCYCCRAGAACGGGCCTCGACTTCAA	HEX/BHQ1	1454-1480	E	78
DENV-3	D3-F	GGACTRGACACACGCACCCA		701-720	prM	74
	D3-R	CATGTCTCTACCTTCTCGACTTGYCT		749-775	prM	74
	D3-probe	ACCTGGATGTCGGCTGAAGGAGCTTG	TR/BHQ2	722-747	prM	74
DENV-4	D4-F	TTGTCCTAATGATGCTRGTCG		884-904	prM	89
	D4-R	TCCACCYGAGACTCCTTCCA		953-973	prM	89
	D4-probe	TYCCTACYCCTACGCATCGCATTCCG	Cy5/BHQ3	939-965	prM	89

^aNucleotide Y is C or T and R is A or G; ^bTaqman probes were labeled at the 5' end with reporter dyes including FAM (6-carboxyfluorescein), HEX, TR (Texas Red) and Cy5 and at the 3' end with black hole quenchers BHQ1, 2 and 3; ^cDengue virus non-structural gene 5 (NS5), envelope (E) and membrane precursor (prM).

3.3.3 CDC DENV-1-4 Real Time RT-PCR Assay (qRT-PCR)

Sera samples that were positive for dengue virus after testing using real time RT-PCR (qRT-PCR) were serotyped using the CDC DENV-1-4 RT-PCR assay (Santiago *et al.*, 2013). The CDC DENV-1-4 qRT-PCR Assay is an in vitro diagnostic platform approved by the US Food and Drug Administration (FDA) for detection and serotyping of DENV serotypes DENV-1, -2, -3 and -4 in patients with signs or symptoms of mild or severe dengue (Santiago *et al.*, 2013). The primers and probes of the CDC DENV-1-4 qRT-PCR assay that were also used in this study are shown in Table 3.

Serotype specific one step qRT-PCR was carried out in an Applied Biosystems 7500 Fast real-time PCR System (Applied Biosystems, Carlsbad, USA) using AgPath-ID one-step RT-PCR kit without Rox (Applied Biosystems, Courtaboeuf, France). The qRT-PCR master mix for a single reaction used in the present study is shown in Table 4. Fourplex serotype-specific dengue virus primers and four fluorogenic probes including the DEN-1 probe labeled with 6-carboxyfluorescein (FAM) reporter dye at the 5' end and with the black hole quencher 1 (BHQ-1) at the 3'end, the DEN-2 probe labeled with HEX and BHQ-1, the DEN-3 probe labeled with Texas Red (TR) and BHQ2 and DEN-4 probe labeled with Cy5 and BHQ-3 were used (Table 3). The standard cycling method was selected and fluorescence capture set to detect emissions through the FAM, HEX, TR and CY5 channels in each well. Thermocycling parameters included reverse transcription at 50 °C for 30 minutes, reverse transcriptase inactivation at 95 °C for 2 minutes and fluorescence detection for 45 cycles of 95 °C for 15 seconds and a combined annealing and extension at 60 °C for 1 minute (Appendix II). All reactions were run with (i) 1:10 dilutions of quantified (GCE/mL) laboratory-adapted, heat inactivated DENV1-4 strains (DENV-1 Hawaii 44, DENV-2 New Guinea C 44, DENV-3 H87 (Phillipines 56), DENV-4 H241 (Phillipines 241)) as a positive control for

DENV1-4 detection, (ii) a human specimen control for use as an RNA extraction procedural control to demonstrate successful recovery of RNA from human serum after detection of ribonuclease P (RNase P) using RP-F primer, RP-R primer and RP-probe, and (iii) nuclease-free water during RNA extraction and RT-PCR was used as the negative control as shown in table 5.

Table 5: CDC DENV-1-4 real time RT-PCR assay master mix components.

<i>No.</i>	<i>Component</i>	<i>Volume (μl)</i>
1.	2X PCR master mix*	12.5
2.	Nuclease-free water	5.55
3.	10 μM Forward primer (D1-F, D2-F, D3-F or D4-F)	0.5
4.	10 μM Reverse primer (D1-R, D2-R, D3-R or D4-R)	0.5
5.	Reverse transcriptase and Taq DNA polymerase	0.5
6.	Probe (D1-probe, D2-probe, D3-probe or D4-probe)	0.45
7.	Extracted RNA template	5.0
	Total volume per reaction	25.0

3.3.4 Sequencing

PCR products (5 μl) obtained using universal dengue virus primers D1 and D2 (Table 2) were treated with exonuclease I (1 μl) and alkaline phosphatase (1μl) (USB ExoSAP-IT) in order to remove unused primers and dNTPs, respectively. Enzyme treatment was performed at 37 °C for 15 minutes followed by inactivation of the enzymes at 80 °C for 15 minutes. Afterwards, PCR products were sequenced directly with universal dengue virus primers D1 or D2 using the Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA). The sequencing PCR master mix is shown in Table 5 while the sequencing PCR thermocycling conditions are shown in Fig. 3. After completion of the sequencing PCR reaction, DNA fragments were precipitated using isopropanol (75%) followed by incubation in the dark for 15 minutes. Then the DNA fragments in the plate were pelleted by centrifugation at 1,200 rpm for 45 minutes and the isopropanol was discarded. Thereafter, the DNA fragments were re-suspended in

15 μ l of Hi-Di® Formamide (Applied Biosystems, Foster City, CA) before being separated on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

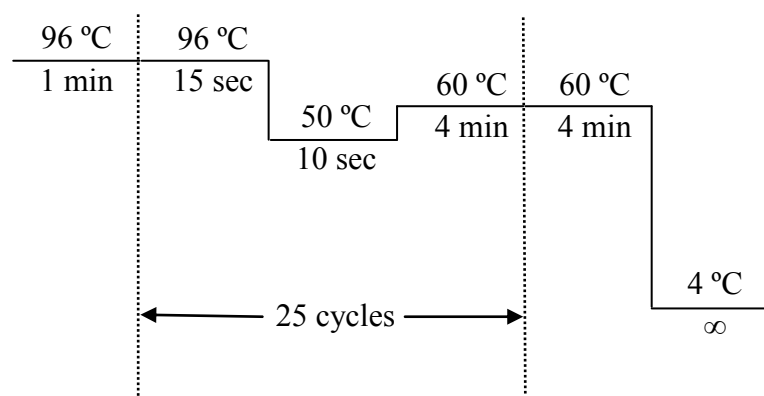


Figure 3: Cycle sequencing polymerase chain reaction profile. Sequencing PCR was done on an ABI PCR System 9700 with a rapid thermal ramp.

Table 6: The composition of reagents for the sequencing PCR for a single reaction

No.	Component	Volume (μ l)
1	Nuclease free water	11
2	Primers (D1 or D2)	2
3	Big Dye Terminator buffer	4
4	Big Dye Terminator enzyme	1
5	PCR product DNA template	2
	Total volume per reaction	20

3.3.5 Nucleotide sequencing, similarity search and determination of phylogenetic relationship of dengue virus

The forward and the reverse complement nucleotide sequences delimited by D1 forward and D2 reverse primers of CprM junction region PCR products of DENV were aligned to obtain a consensus nucleotide sequence. The nucleotide sequences of DENV of CprM junction region RNA were submitted to GenBank, and compared with other sequences using BLASTn (Altschul *et al.*1990). BLASTn compares nucleotide sequences to sequence databases and calculates the statistical significance of matches and can be used to infer functional, and evolutionary relationships between sequences. A set of sequences representing DENV1-4 serotypes, together with those obtained from the present study, were used for phylogenetic analysis. Sequences were aligned using

ClustalW algorithm in BioEdit (Ibis Biosciences, Carlsbad, CA) and clustering pattern was determined by neighbor-joining method using the Kimura-2-parameter option implemented within MEGA 6.06 (Tamura *et al.*, 2011).

CHAPTER FOUR

4.0 RESULTS

4.1 Detection of DENV in Sera Samples Using by Conventional RT-PCR

These were the gel obtained after running the 23 positive sera samples from after performing a rapid test (IgM and IgG) and ELISA and rapid test of dengue virus (IgM and IgG) were tested for the presence of DENV genomes using conventional RT-PCR. A total of 20 out of the 23 sera samples (45 %) tested positive in RT-PCR. Positive samples produced a PCR fragment of approximately 500 bp, an expected band size for all DENV serotypes. Primers amplified DENV-1, DENV-2, DENV-3 and DENV-4 producing PCR fragments of approximately 480, 110, 290 and 390 bp, respectively, were included in the multiplex reaction. DENV was detected in patients 1 to 7 and 9 while no DENV was detected in patient 8. In addition, DENV-2 was strongly detected in patients 6, 7 and 9 and weakly detected in patients 4 and 5 as shown in Figer.4. Positive samples originated from all four sampling locations including IST, IFM, Masaki and Muhimbili sampled both in 2013 and 2014. Furthermore, males and females of all ages tested positive for DENV.

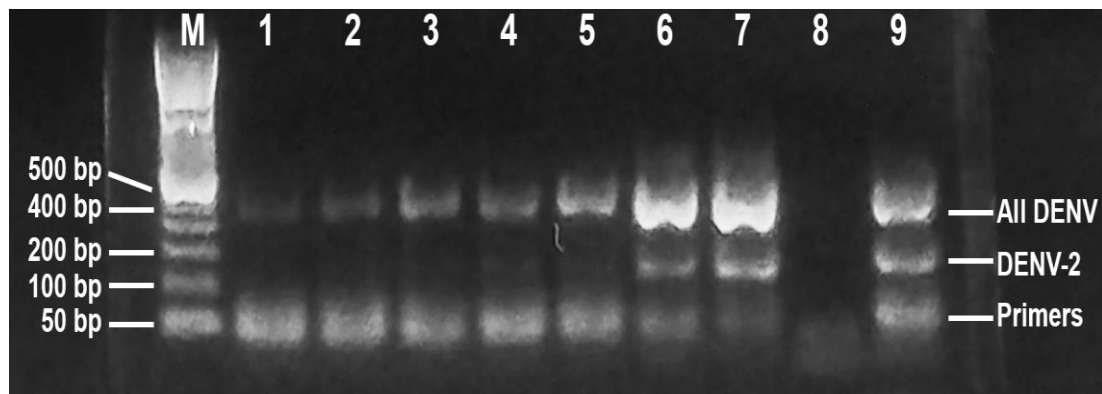


Figure 4: Conventional PCR results of dengue virus in sera obtained from patients with dengue fever. M is 1Kb DNA ladder.

4.2 DENV Serotyping Using CDC DENV-1-4 qRT-PCR Assay

Sera samples that were positive for DENV on conventional RT-PCR were serotyped using the CDC DENV-1-4 qRT-PCR assay. DENV-2 serotype was detected in each of the sera sample tested. Seven sera samples tested strongly positive for DENV-2 (Ct values ranging between 15 and 32) while four samples were weakly positive for DENV-2 (Ct values ranging between 33 and 40) (Fig. 5; Table 2). DENV-1 Hawaii 44, DENV-2 New Guinea C 44, DENV-3 H87 (Phillipines 56) and DENV-4 H241 (Phillipines 241) were detected in each of the DENV-1, DENV-2, DENV-3 and DENV-4-specific reporter dyes, indicating specificity in detection. (Table 2).

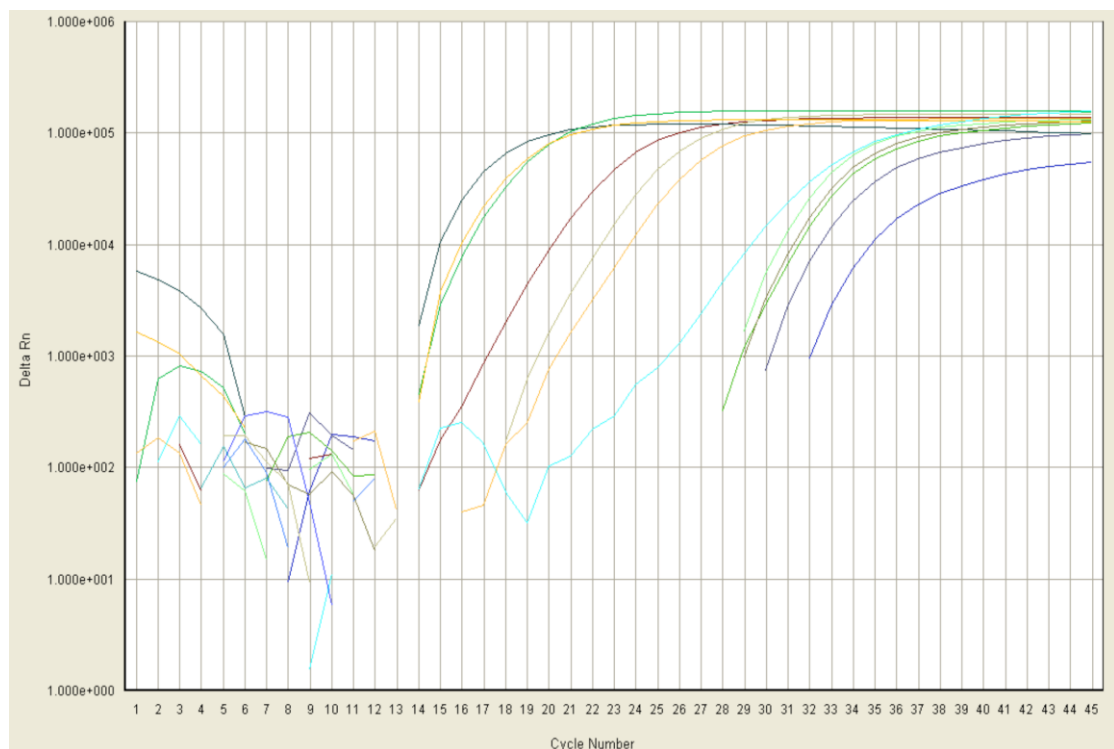


Figure 5: Serotyping of dengue virus in sera using RT-PCR. The reporter dye fluorescence intensity (y-axis) is plotted against the PCR cycle number (x-axis).

Table 7: Sera samples used in the present study

No	Sample ID	Sex	Age	Location	Rapid test	ELISA test	DENV1-4 RT-PCR ^a	DENV1-4 Real Time RT-PCR Assay ^b (CT value)			
								DENV-1 FAM	DENV-2 HEX	DENV-3 TR	DENV-4 Cy5
17	TDS0012226	F	32	Masaki	+	+	+	- ^c	-	-	-
1	TDS0018267	F	39	Masaki	+	+	+	-	-	-	-
2	TDS0018268	F	43	IST	+	+	+	-	-	-	-
3	TDS0018269	M	52	IST	+	+	+	-	-	-	-
4	TSD0018280	M	34	IST	+	ND	+	-	-	-	-
5	TDS0018281	F	13	Masaki	+	ND	+	-	-	-	-
6	TDS0018299	F	36	Masaki	+	+	+	-	-	-	-
7	TDS0018303	F	46	Masaki	+	+	+	-	-	-	-
8	TDS0018905	M	25	Masaki	+	+	+	-	-	-	-
9	TDS0018907	F	53	Masaki	+	+	+	-	-	-	-
10	TDS0018914	M	56	Masaki	+	+	+	-	-	-	-
11	TDS0018915	M	53	Masaki	+	+	+	-	-	-	-
12	TDS0018909	M	53	IST	+	ND	+	-	(+) 17.81	-	-
18	TDS0019053	F	50	Masaki	+	+	+	-	-	-	-
13	TDS0019210	M	46	Masaki	+	+	+	-	(+) 17.41	-	-
14	TDS0020701	F	16	Masaki	+	+	+	-	(+) 22.28	-	-
15	TDS0020702	F	40	Masaki	+	ND	+	-	-	-	-
16	TDS0020703	F	34	Masaki	+	+	+	-	(+) 25.77	-	-
17	TDS0020976						+		(+) 15.78		
18	TDS0021567	F	4	Muhimbili	+	ND	+	-	(+) 24.19	-	-
19	NHL/13/3601	M	24	IFM	+	+	+	-	(+) 33.63	-	-
20	NHL/13/3602	M	-	IFM	+	+	+	-	(+) 34.95	-	-
21	NHL/13/3603	M	20	IFM	+	+	+	-	(+) 39.40	-	-
22	NHL/13/3605						+		(+) 32.20		
23	NHL/13/3626	M	23	IFM	+	+	+	-	(+) 33.35	-	-
	DENV-1 Haw 44	ND	ND	ND	ND	ND	+	28.56	-	-	-
	DENV-2 NGC44	ND	ND	ND	ND	ND	+	-	(+) 19.13	-	-
	DENV-3 H87	ND	ND	ND	ND	ND	+	-	-	30.20	-
	DENV-4 H241	ND	ND	ND	ND	ND	+	-	-	-	28.19
	RP	ND	ND	ND	ND	ND	+	-	-	-	-

^aConventional RT-PCR using primers from *Lanciotti et al.*, 1992 (Table 3). ^bCDC DENV-1-4 Real Time RT-PCR Assay using primers and probes from Santiago *et al.*, 2013 (Table 4). ^cA (-) sign indicates negative results and the Ct value was undetermined by the machine.

4.3 Similarity of DENV RNA Sequence Against GenBank

All the eleven RNA nucleotide sequence of DENV from dengue fever patients collected in 2013 and 2014 were identical. The obtained nucleotide sequence were submitted to GenBank and provided with the accession number *KM892493*, *KM892494*, *KM892495* and *KM892496* for the dengue virus strains *D2/TZ/IFM/2013*, *D2/TZ/Masaki/2014*, *D2/TZ/IST/2014* and *D2/TZ/Muhimbili/2014*. The *KM892493*, *KM892494*, *KM892495* and *KM892496* nucleotide sequences were 100% identical. BLASTn of the DENV obtained from the present study showed high identity with a number of published DENV-2 sequences, although none of these DENV sequences at Gen Bank had 100% identity (Table 4). However, Tanzanian DENV showed highest identity with other DENV from Singapore, China, Guam, Brunei, Indonesia, Vietnam, Australia and East Timor (Table 1).

4.4 Phylogenetic Analysis of DENV

A phylogenetic tree for the CprM junction region constructed using the neighbour-joining method using DENV sequences retrieved from GenBank representing all the four DENV serotypes (Fig. 6). The tree revealed that the DENV sequence obtained from this study clustered into serotype II and closely related to the *SG/D2Y98P-PP1/2009* (Accession number JF327392) (Grant *et al.*, 2011). The *SG/D2Y98P-PP1/2009* is a DENV isolate with a Phe-to-Leu alteration at position 52 in the NS4B protein of the original *D2Y98P* virus that was isolated in 1998 from a DENV-infected patient in Singapore (Grant *et al.*, 2011). Dengue viruses strains *D2/TZ/IFM/2013*, *D2/TZ/Masaki/2014*, *D2/TZ/IST/2014* and *D2/TZ/Muhimbili/2014* characterized in this study (indicated in bold) clustered into dengue virus serotype 2 (DENV-2) along with many other DENV-2 isolates including the *GZ40* strain isolated from Guangdong,

China. Phylogeny was inferred following 1000 bootstrap replications as shown in figure 6.

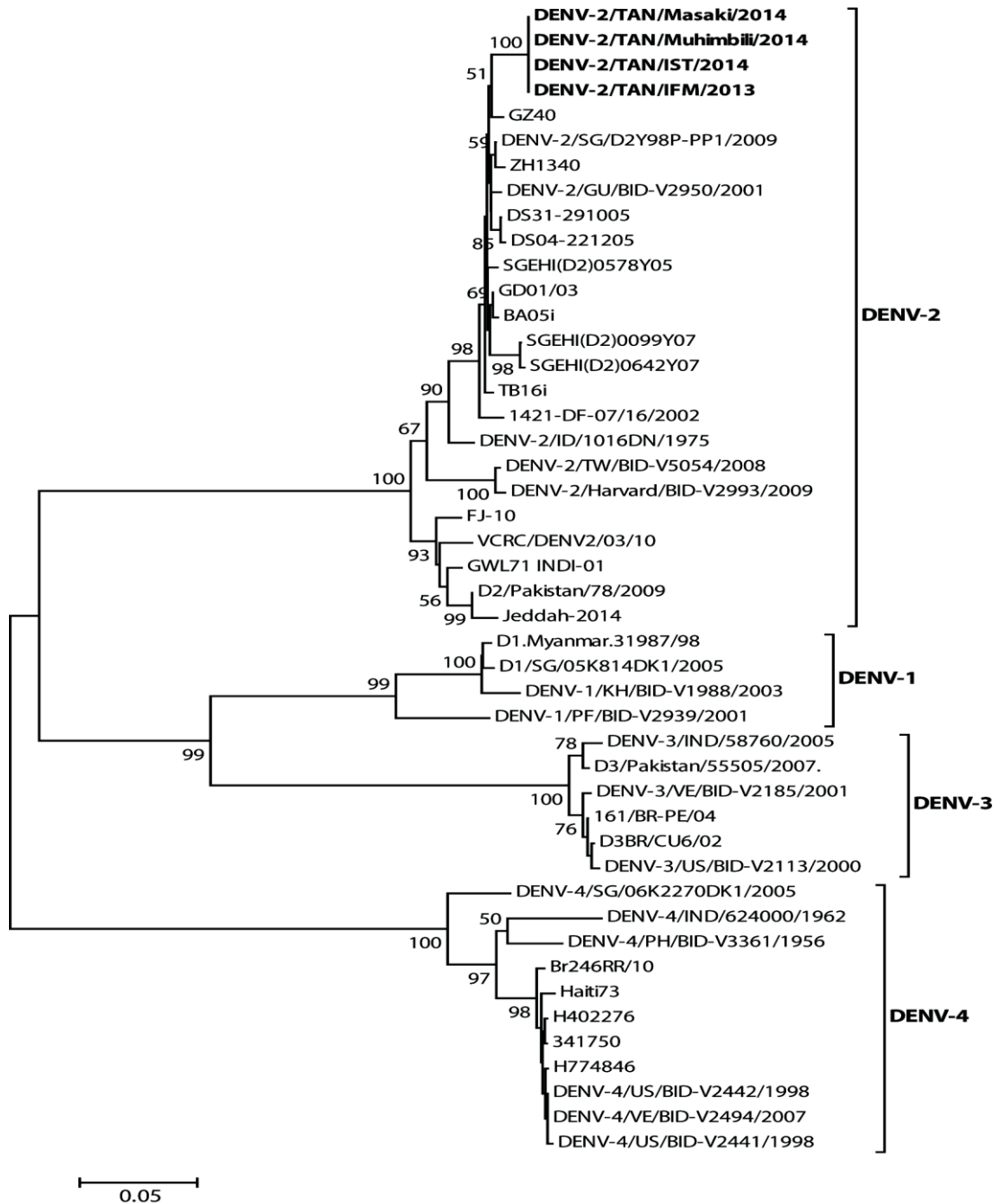


Figure 6: Phylogenetic tree of dengue viruses based on the CprM junction region constructed using the neighbour-joining method in MEGA 6.06® software.

Table 8: BLASTn results showing homologous gene sequences in the NCBI database to the dengue viruses with accession numbers KM892493, KM892494, KM892495 and KM892496 representing the nucleotide sequence of the DENV CprM junction region.

DENV isolate/strain	Country of origin	Year of isolation	Serotype	Nucleotide identity (%)	Mismatches (Alignment length)	Accession No.	Reference
SG/D2Y98P-PP1/2009	Singapore	2009	DENV-2	98.20	9 (500)	JF327392	Grant <i>et al.</i> , 2011
GZ40	China	2010	DENV-2	98.00	10 (500)	JX470186	Zhao <i>et al.</i> , 2012
GD01/03	China	2003	DENV-2	98.00	10 (500)	FJ196853	Wu <i>et al.</i> , 2011
GU/BID-V2950/2001	Guam	2001	DENV-2	98.00	10 (500)	HM488257	Unpublished
DS31-291005	Brunei	2005	DENV-2	98.00	10 (500)	EU179857	Osman <i>et al.</i> , 2008
SGEHI(D2)0578Y05	Singapore	2005	DENV-2	97.80	11 (500)	JN851126	Unpublished
ZH1340	China	NA	DENV-2	97.80	11 (500)	EU359009	Unpublished
DS04-221205	Brunei	2005	DENV-2	97.80	11 (500)	EU179858	Osman <i>et al.</i> , 2008
TB16i	Indonesia	NA	DENV-2	97.80	11 (500)	AY858036	Unpublished
BA05i	Indonesia	NA	DENV-2	97.80	11 (500)	AY858035	Unpublished
SGEHI(D2)0685Y04	Singapore	2004	DENV-2	97.60	12 (500)	JN851127	Unpublished
VN/BID-V703/2006	Vietnam	2006	DENV-2	97.60	12 (500)	EU482640	Unpublished
DS09-280106	Brunei	2006	DENV-2	97.60	12 (500)	EU179859	Osman <i>et al.</i> , 2008
TSV01	Australia	1993	DENV-2	97.59	12 (498)	AY037116	Johansson <i>et al.</i> , 2001
SGEHI(D2)0270Y05	Singapore	2005	DENV-2	97.40	13 (500)	JN851130	Unpublished
SGEHI(D2)0377Y04	Singapore	2004	DENV-2	97.40	13 (500)	JN851123	Unpublished
VN/BID-V735/2006	Vietnam	2006	DENV-2	97.40	13 (500)	EU482672	Unpublished
ET300	East Timor	2000	DENV-2	97.40	13 (500)	EF440433	Unpublished
ZH413-2	China	2012	DENV-2	97.20	14 (500)	KC131142	Unpublished
SGEHI(D2)0232Y06	Singapore	2006	DENV-2	97.20	14 (500)	JN851128	Unpublished
SGEHI(D2)0345Y05	Singapore	2005	DENV-2	97.20	14 (500)	JN851125	Unpublished
SGEHI(D2)0462Y05	Singapore	2005	DENV-2	97.20	14 (500)	JN851124	Unpublished
SG/05K4155DK1/2005	Singapore	2005	DENV-2	97.20	14 (500)	EU081180	Schreiber <i>et al.</i> , 2009
SG/05K4137DK1/2005	Singapore	2005	DENV-2	97.20	14 (500)	EU081179	Schreiber <i>et al.</i> , 2009
SG/05K3330DK1/2005	Singapore	2005	DENV-2	97.20	14 (500)	EU081178	Schreiber <i>et al.</i> , 2009

Table 9: Summary of the dengue viruses (DENV) isolates used for the construction of phylogenetic trees based on CprM junction region sequences.

Isolate	Country of origin	Town	Host species	Year of outbreak	Accession number	Serotype	References
D1.Myanmar.31987/98	Myanmar	Unknown	Unknown	1998	AY726554	1	Unpublished
D1/SG/05K814DK1/2005	Singapore	Unknown	Human	2005	EU081226	1	Schreiber <i>et al.</i> , 2009
DENV-1/KH/BID-V1988/2003	Cambodia	Phnum Pénh	Human	2003	FJ639676	1	Unpublished
DENV-1/PF/BID-V2939/2001	French Polynesia	Tahiti	Human	2001	FJ898448	1	Unpublished
DENV-2/TAN/IFM/2013	Tanzania	Dar es Salaam	Human	2013	KM892493	2	This study
DENV-2/TAN/Masaki/2014	Tanzania	Dar es Salaam	Human	2014	KM892494	2	This study
DENV-2/TAN/IST/2014	Tanzania	Dar es Salaam	Human	2014	KM892495	2	This study
DENV-2/TAN/Muhimbili/2014	Tanzania	Dar es Salaam	Human	2014	KM892496	2	This study
GZ40	China	Guangdong	Human	2010	JX470186	2	Zhao <i>et al.</i> , 2012
DENV-2/SG/D2Y98P-PP1/2009	Singapore	-	C6/36 cells	2009	JF327392	2	Grant <i>et al.</i> , 2011
ZH1340	China	-	Human	Unknown	EU359009	2	Unpublished
DENV-2/GU/BID-V2950/2001	Guam	-	Human	2001	HM488257	2	Unpublished
DS31-291005	Brunei	-	Albopictus	2005	EU179857	2	Osman <i>et al.</i> , 2008
DS04-221205	Brunei	-	C6/36 cells Albopictus	2005	EU179858	2	Osman <i>et al.</i> , 2008
SGEHI(D2)0578Y05	Singapore	-	Human	2005	JN851126	2	Unpublished
GD01/03	China	Guangzhou	<i>Aedes albopictus</i> cell line (C6/36)	2003	FJ196853	2	Wu <i>et al.</i> , 2011
BA05i	Indonesia	Jakarta	Human	2004	AY858035	2	Ong <i>et al.</i> , 2008
SGEHI(D2)0099Y07	Singapore	-	Human	2007	GU370050	2	Unpublished
SGEHI(D2)0642Y07	Singapore	-	Human	2007	JN851115	2	Unpublished
TB16i	Indonesia	Jakarta	Human	2004	AY858036	2	Ong <i>et al.</i> , 2008
1421-DF-07/16/2002	Taiwan	-	Human	2002	DQ645549	2	Unpublished
DENV-2/ID/1016DN/1975	Indonesia	-	Human	1975	GQ398258	2	Christenbury <i>et al.</i> , 2010
DENV-2/TW/BID-V5054/2008	Taiwan	-	Human	2008	HQ891023	2	Unpublished
DENV-2/Harvard/BID-V2993/2009	Unknown	-	Human	2009	FJ906969	2	Unpublished
FJ-10	China:	Fujian Province	Unknown	Unknown	AF276619	2	Unpublished
VCRC/DENV2/03/10	India	Kanjirappalli, Kottayam, Kerala	Human	2010	JN935384	2	Kumar <i>et al.</i> , 2013

GWL71 INDI-01	India	Unknown	Human	2001	DQ448233	2	Unpublished
D2/Pakistan/78/2009	Pakistan	Karachi	Human	2009	KF041237	2	Koo <i>et al.</i> , 2013
Jeddah-2014	Saudi Arabia	Jeddah	Human	2014	KJ830750	2	Unpublished
DENV-3/IND/58760/2005	India	Pune, Maharashtra	Human	2005	JQ922556	3	Unpublished
D3/Pakistan/55505/2007	Pakistan	Hyderaad	Human	2007	KF041255	3	Koo <i>et al.</i> , 2013
DENV-3/VE/BID-V2185/2001	Venezuela	Aragua	Human	2001	FJ639756	3	Unpublished
161/BR-PE/04	Brazil	Pernambuco	Human	2004	JX669499	3	Unpublished
D3BR/CU6/02	Brazil	Cuiaba (Mato Grosso)	Human	2002	JF808127	3	Unpublished
DENV-3/US/BID-V2113/2000	USA	Puerto Rico	Human	2000	FJ547078	3	Unpublished
DENV-4/SG/06K2270DK1/2005	Singapore	-	Human	2005	GQ398256	4	Christenbury <i>et al.</i> , 2010
DENV-4/IND/624000/1962	India	Vellore, Tamil Nadu	Human	1962	JQ922558	4	Unpublished
DENV-4/PH/BID-V3361/1956	Philippines	-	Human	1956	GQ868594	4	Unpublished
Br246RR/10	Brazil	Boa Vista, Roraima State	Human	2010	JN983813	4	Naveca <i>et al.</i> , 2012
Haiti73	Haiti	-	Human	1994	JF262782	4	Rossi <i>et al.</i> , 2012
H402276	Brazil	Boa Vista-RR	Human	1982	JN559740	4	Unpublished
341750	Colombia	-	Human	1982	GU289913	4	Kelly <i>et al.</i> , 2010
H774846	Brazil	Boa Vista-RR	Human	2010	JQ513333	4	Nunes <i>et al.</i> , 2012
DENV-4/US/BID-V2442/1998	USA	Puerto Rico	Human	1998	FJ882596	4	Unpublished
DENV-4/VE/BID-V2494/2007	Venezuela	Aragua	Human	2007	FJ882585	4	Unpublished
DENV-4/US/BID-V2441/1998	USA	Puerto Rico	Human	1998	FJ882595	4	Unpublished

CHAPTER FIVE

5.0 DISCUSSION

The present study was carried out to confirm and genetically characterize DENV present in sera collected from patients with dengue fever during the 2013 and 2014 dengue outbreaks in Tanzania. Sera were collected from patients who met the clinical case definition for dengue fever. Clinical signs and symptoms of patients included in this study included sudden onset of fever, headache, retro-orbital pain, muscle or joint pain, rash, haemorrhagic manifestation and/or leucopenia. Mild haemorrhagic symptoms including cutaneous petechiae, spontaneous bleeding of gums, increased menorrhagia, gastrointestinal bleeding and hematuria were observed in some of the patients. Dengue fever was diagnosed in patients included in this study by detection of IgM and IgG antibodies using a combination of a rapid test and enzyme-linked immunosorbent assay (ELISA). The ELISA and rapid test were used to screen serum for the presence of anti-DENV IgG and IgM antibodies. In the present study, DENV detection and serotyping in sera samples was performed using conventional and real-time RT-PCR previously described by Lanciotti *et al.* 1992 and Santiago *et al.*, 2013, respectively. All sera that tested positive for anti-DENV IgG and IgM antibodies also tested positive for the presence of DENV genomes in RT-PCR. In addition, both conventional and real-time RT-PCR showed the presence of DENV serotype 2. Nucleotide sequencing of RT-PCR products after amplification of the CprM region of DENV using conventional RT-PCR produced a 500 bp fragment. BLASTn and phylogenetic analysis of the DENV nucleotide sequence obtained from this study clustered DENV into serotype II confirming the results obtained during conventional and real-time RT-PCR.

The DENV-2 involved during the dengue fever outbreaks in 2013 and 2014 had 100% identity at nucleotide and protein level. This indicated that the same DENV-2 was

responsible for the dengue fever outbreaks of 2013 and 2014. Each of the four DENV serotypes is maintained in two ecologically and evolutionary distinct transmission cycles: a sylvatic cycle and a human cycle. The human cycle involves the domestic *Aedes aegypti subsp. aegypti* and peridomestic *Aedes albopictus* mosquitoes and can be found in a diverse range of environments throughout the tropics and subtropics (Vasilakis *et al.*, 2011). In some mosquito species, DENV has been shown to persist in mosquito populations by transovarial transmission, in which virus-infected mosquitoes transfer the virus to their eggs (Vasilakis *et al.*, 2011). It can be speculated from the results obtained from the present study that transovarial transmission of DENV occurred between the 2013 and 2014 outbreaks in Dar es Salaam. However, it is also possible that DENV infection continued undetected between 2013 and 2014 owing to misdiagnosis of dengue as malaria (Gubler *et al.*, 1986) and other clinically similar viral diseases like chikungunya disease, o'nyong-nyong fever and Zika fever (Moore, 1975; Jentes, 2010).

Most DENV infections are subclinical or result in classical dengue fever, which is characterized by fever, muscle and joint pain, and rash. However, approximately 0.5% of infections result in the most severe manifestation of the disease, dengue haemorrhagic fever, which can be fatal in as many as 5% of cases. Infection with a given serotype results in lifelong homologous immunity to that serotype but increases the risk of haemorrhagic fever upon infection by a heterologous serotype (Halstead, 2007). The principal risk factors for developing DHF include the strain of infecting DENV (Watts *et al.*, 1999; Vaughn *et al.*, 2000; Wang *et al.*, 2003; de Araujo, 2009).

The phylogenetic analysis and BLASTn results of the DENV-2 CprM junction region obtained in the present study indicated that it has 98.2% nucleotide identity to SG/D2Y98P-PP1/2009 (Accession number JF327392) (Grant *et al.*, 2011). The

SG/D2Y98P-PP1/2009 is a DENV isolate with a Phe-to-Leu alteration at position 52 in the NS4B protein of the original D2Y98P virus that was isolated in 1998 from a DENV-infected patient in Singapore (Grant *et al.*, 2011). This mutation completely abolished the pathogenicity of the D2Y98P virus, as evidenced by a lack of lethality and the absence of histological signs of disease, which correlated with reduced viral titers and intact vascular permeability. This observation may be related to the reduced severity of clinical signs and lack of dengue haemorrhagic fever in dengue patients in Dar es Salaam. Future studies are recommended in order to fully sequence the DENV-2 isolate obtained in the present study in order to analyse all virulence determinants.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Dengue fever outbreaks of 2013 and 2014 in Dar es Salaam were caused by DENV serotype 2, closely related to other DENV-2 isolated in Singapore and China in 2009 and 2010, respectively. The same DENV-2 was responsible for the 2013 and 2014 outbreaks indicating that DENV was maintained either in mosquitoes and/or humans between 2013 and 2014 outbreaks in Dar es Salaam presumably through transovarial route in *Aedes* mosquitoes or misdiagnosed dengue infections that went undetected between the years. The DENV-2 responsible for the outbreak in Dar es Salaam has high similarity to isolates known to cause less severe disease, and might be related to the less mortality observed in dengue fever patients during the outbreaks.

6.2 Recommendations

The following recommendations can be put forward based on the results obtained from the present study:

- The control of *Aedes* mosquitoes in Dar es Salaam has to be performed in order to avoid maintenance of DENV in mosquitoes that may lead to future outbreaks.
- The genetic identity of DENV in mosquitoes collected during the outbreak has to be performed in order to ascertain the role of *Aedes* mosquitoes in the transmission of DENV.
- Full genome sequencing of the DENV-2 partially characterized in the present study has to be performed in order to determine its relatedness to other DENV full genomes and establish its virulence.

- More studies on the type of DENV in mosquitoes in Tanzania need to be performed in order to understand the DENV serotypes circulating in Tanzania.
- Dengue fever diagnosis should be included at point of care in order to reduce its misdiagnosis to malaria.

REFERENCES

- Carey, D.E. (1971) Chikungunya and dengue: a case of mistaken identity? *Journal of Histological Medicine and Allied Sciences* 26, 243–262.
- Christenbury, J.G.; Aw, P.P.; Ong, S.H.; Schreiber, M.J.; Chow, A.; Gubler, D.J.; Vasudevan, S.G.; Ooi, E.E. and Hibberd, M.L. (2010) A method for full genome sequencing of all four serotypes of the dengue virus. *Journal of Virology Methods* 169:202-206.
- de Araujo, J. M. (2009). Quantification of dengue virus type 3 RNA in fatal and non-fatal cases in Brazil, 2002. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 103, 952–954.
- Grant, D.; Tan, G.K.; Qing, M.; Ng, J.K.; Yip, A.; Zou, G.; Xie, X.; Yuan, Z.; Schreiber, M.J.; Schul, W.; Shi, P.Y. and Alonso, S.A (2011); single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. *Journal of Virology* 85:7775-7787.
- Grant, D.; Tan, G.K.; Qing, M.; Ng, J.K.; Yip, A.; Zou, G.; Xie, X.; Yuan, Z.; Schreiber, M.J.; Schul, W.; Shi, P.Y. and Alonso, S. A, (1986). single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. *Journal of Virology* 85:7775-7787.

- Gubler, D. J.; Sather, G. E.; Kuno, G. and Cabral, J.R (2002). Dengue 3 virus transmission in Africa. *Am. Journal Tropical Medicine and Hygiene* 35, 1280–1284.
- Jentes, E. S. (2010) Acute arboviral infections in Guinea, West Africa, 2006. *American Journal Tropical Medicine and Hygiene* 83, 388–394.
- Johansson, M.; Brooks, A.J.; Jans, D.A. and Vasudevan, S.G, (2001) A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-beta and the viral helicase, NS3. *Journal General Virology* 82:735-745.
- Kelly, E.P. ; Puri , B.; Sun, W. and Falgout, B.(2009) Identification of mutations in a candidate dengue 4 vaccine strain 341750 PDK20 and construction of a full-length cDNA clone of the PDK20 vaccine candidate. *Journal of Vaccine* 28:3030-3037.
- Koo, C.; Nasir, A.; Hapuarachchi, H.C.; Lee, K.S.; Hasan, Z.;, Ng,L.C. and Khan, E.(2013) Evolution and heterogeneity of multiple serotypes of Dengue virus in Pakistan,. *Virology Journal* 4: 275 - 280.
- Kumar, N.P.; Jayakumar, P.R.; George,K.; Kamaraj, T.; Krishnamoorthy, K.; Sabesan, S. and Jambulingam, P.(2013) Genetic characterization of dengue viruses prevalent in Kerala State, India. *Journal Medicine Microbiology* 62:545-552.

- Moore, D. L. (1975) Arthropod-borne viral infections of man in Nigeria, 1964 –1970. *Annals Tropical Medicine Parasitology* 69: 49–64.
- Naveca ,F.G.1.; Souza,V.C.; Silva, G.A.; Maito, R.M.; Granja, F.; Siqueira, T.and Acosta,P.O. (2012), Complete genome sequence of a Dengue virus serotype 4 strain isolated in Roraima, Brazil. *Journal Virology* 86:1897-1898.
- Ong, S.H.1.; Yip, J.T.; Chen, Y.L.; Liu, W.; Harun, S.; Lystiyaningsih, E.; Heriyanto, B.; Beckett, C.G.; Mitchell, W.P.; Hibberd, M.L.; Suwandono, A.; Vasudevan, S.G. and Schreiber, M.J.(2008) Periodic re-emergence of endemic strains with strong epidemic potential-a proposed explanation for the 2004 Indonesian dengue epidemic. *Infection Genetics Evolution* 8:191-204.
- Osman, O.; Fong, M.Y. and Devi, S. (2008) Complete genome sequence analysis of dengue virus type 2 isolated in Brunei. *Virus Resistance* 135:48-52.
- Rossi, S.L.; Nasar, F.; Cardoso, J.; Mayer, S.V.; Tesh, R.B.; Hanley, K.A.; Weaver, S.C. and Vasilakis, N. (2012) Genetic and phenotypic characterization of sylvatic dengue virus type 4 strains. *Virology* 423:58-67.
- Santiago, G.A.; Vergne, E.; Quiles, Y.; Cosme, J.; Vazquez, J.; Medina, J.F.; Colon, C.; Margolis, H.; Munoz-Jordan, J.L. (2013). Analytical and Clinical Performance of the CDC Real Time RT-PCR Assay for Detection and Typing of Dengue Virus. *PLoS Neglected Tropical Diseases* 7:2311-2316.

Schreiber, M.J.; Holmes, E.C.; Ong, S.H.; Soh, H.S.; Liu, W.; Tanner, L.; Aw, P.P.; Tan, H.C.; Ng, L.C.; Leo, Y.S.; Low, J.G.; Ong, A.; Ooi, E.E.; Vasudevan, S.G. and Hibberd, M.L.(2009) Genomic epidemiology of a dengue virus epidemic in urban Singapore. *Journal Virology* 83:4163-4173.

Schreiber, M.J.1.; Holmes, E.C.; Ong, S.H.; Soh, H.S.; Liu, W.; Tanner, L.; Aw, P.P.; Tan, H.C.; Ng, L.C.; Leo, Y.S.; Low, J.G.; Ong, A.; Ooi, E.E.; Vasudevan, S.G. and Hibberd, M.L.(2009) Genomic epidemiology of a dengue virus epidemic in urban Singapore. *Journal Virology* 83: 4163-4173.

United Republic of Tanzania (URT), 2013. Population Distribution by Age and Sex. National Bureau of Statistics Ministry of Finance Dar es Salaam and Office of Chief Government Statistician President's Office, Finance, Economy and Development Planning Zanzibar. Pp 471.

Vasilakis, N.; Cardoso, J.; Hanley, K.A.; Holmes, E.C. and Weaver, S.C. (2011) Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. *Nature Reviews Microbiology* 13: 9532-9541.

Vaughn, D. W. (2000) Dengue viremia titer, antibody response pattern and virus serotype correlate with disease severity. *Journal Infectious Diseases* 181, 2-9.

- Wang, W. K. (2003). High levels of plasma dengue viral load during defervescence in patients with dengue hemorrhagic fever: implications for pathogenesis. *Virology* 305, 330–338.
- Watts, D. M.(1999). Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. *Lancet* 354: 1431–1434.
- Wu, W.1.; Bai. Z.; Zhou, H.; Tu, Z.;; Fang, M.; Tang, B.; Liu, J.; Liu, L. and Chen, W. (2011) Molecular epidemiology of dengue viruses in southern China from 1978 to 2006. *Virology Journal* 26:318-322.
- Zhao, H.1.; Deng, Y.Q.; Hong, W.X.; Yu, X.D.; Jiang, T.; Yu, M.; Hu, F.Y.; Zhu, S.Y.; Li, X.F.; Song, K.Y.; Qin, E.D.; Zhang, F.C. and Qin, C.F.(2012). Complete genome sequence of dengue virus serotype 2 Cosmopolitan genotype strain in Guangdong, China. *Journal Virology* 86:13808-13809.

APPENDICES

Appendix 1: Purification of Viral RNA

In a 1.5 ml microcentrifuge tube, 560 μ l of prepared buffer AVL containing carrier RNA and 140 μ l of either homogenized tissue, buffy coat, supernatant of nasal or ocular swabs was added. The contents were pulse vortexed for 15 seconds to ensure efficient lysis and incubated at room temperature for 10 minutes for complete viral particle lysis. After incubation, the tube was centrifuged for 10 seconds at 6000 x g to remove drops from the inside of the lid. Five hundred and sixty microlitres of absolute ethanol (96–100%) was added to the sample mixture, then mixed by pulse-vortexing for 15 seconds and centrifuged for 10 seconds at 6000 x g to remove drops from inside the lid.

After centrifugation, 630 μ l of the sample mixture were transferred to the QIAamp mini column placed in a 2 ml collection tube. The column was centrifuged at 6000 x g for one minute. The collection tube with the filtrate was discarded and replaced with a clean 2 ml collection tube. If the solution has not completely passed through the membrane, centrifugation was repeated at a higher speed until all of the solution had passed through. The final volume of sample mixture was transferred to the column, centrifuged at 6000 x g for one minute after which the collection tube with filtrate is discarded and replaced with a clean 2 ml collection tube.

The bound nucleic acid was washed by adding 500 μ l of buffer AW1 to the column and centrifuging at 6000 x g for one minute. The collection tube with the filtrate was discarded and replaced with a clean collection tube. The second wash, added 500 μ l of buffer AW2 and centrifuged at 17 000 x g for three minutes. The collection tube with the filtrate was discarded and replaced with a clean 1.5 ml microcentrifuge tube.

RNA was eluted by adding 60 μ l of buffer AVE equilibrated to room temperature to the column. Column contents were incubated at room temperature for one minute, then centrifuged at 6000 x g for one minute. The filtrate, viral RNA, was immediately stored at -80°C until amplification.

Appendix 2: Amplification profile for dengue virus real time reverse transcription polymerase chain reaction

7500 Fast System SDS Software - [DENV IST RESEARCH(20032014).sds (Standard Curve)]

File View Tools Instrument Analysis Window Help

Setup Instrument Results

Instrument Control

Start Stop Disconnect Extend...

Estimated Time Remaining (hh:mm):

Status:

Temperature

Sample: Cover: Heat Sink: Block:

Cycle

Stage: Rep: Time (mm:ss): Step: State:

Thermal Cycler Protocol

Thermal Profile	Auto Increment	Ramp Rate
Stage 1	Stage 2	Stage 3
Reps: 1	Reps: 1	Reps: 45
50.0	95.0	95.0
30:00	2:00	0:15
		60.0
		1:00

Add Cycle Add Hold Add Step Add Dissociation Stage Delete Help

Settings

Sample Volume (µL): 25

Run Mode: Fast 7500 Expert Mode Select/View Filters...

Data Collection: Stage 3, Step 2 (60.0 @ 1:00)