DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

UNIVERSITY OF GHANA

INVESTIGATION OF DENGUE EXPOSURE AND INFECTION IN GHANAIAN CHILDREN WITH MALARIA

A dissertation submitted to the Board of Graduate Studies, University of Ghana, Legon, Ghana

In partial fulfilment of the requirements for the award of the Master of Philosophy degree in Biochemistry

BY:

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DECLARATION

The experimental work presented in this thesis was done by me at the Department of Biochemistry, Cell and Molecular Biology and the Virology Unit of Noguchi Memorial Institute for Medical Research under the supervision of Dr. Gordon A. Awandare (Department of Biochemistry, Cell and Molecular Biology, University of Ghana) and Dr. Justin Stoler (University of Miami, Coral Gables, FL, USA).

All cited references have been duly acknowledged.

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DEDICATION

To God be the Glory. Great things He has done.

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LIST OF ABBREVIATIONS

+ssRNA	Positive Sense Single Stranded Ribonucleic Acid
ACT	Artemisinin Based Combination Therapy
ADE	Antibody Dependent Enhancement
ANOVA	Analysis Of Variance
C	Capsid Protein
CDC	Centres for Disease Control
CF	Complement Fixation
CIGB	Centre for Genetic Engineering and Biotechnology
CS	Cyclisation Sequence
C_t	Threshold Cycle
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DNA	Deoxyribonucleic Acid
DSS	Dengue Shock Syndrome
E	E-Protein
EIM	Erythrocyte Invasion Mechanism
ELISA	Enzyme-Linked Immunosorbent Assay
FL	Fusion-Loop
GSK	GlaxoSmithKline
Hb	Haemoglobin
HI	Hemagglutination Inhibition
HRPO	Horseradish Peroxidase
IDRL	Infectious Diseases Research Laboratory

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPK	Pedro Kouri Tropical Medicine Institute
IRB	Institutional Review Board
KHRC	Kintampo Health Research Centre
M	Membrane Protein
MAC-ELISA	Immunoglobulin M (IgM) Capture Enzyme-Linked Immunosorbent Assay
MTPase	N-terminal S-adenosyl Methionine Methyltransferase
NHRC	Navrongo Health Research Centre
NIAID	National Institute for Allergy and Infectious Diseases
NMIMR	Noguchi Memorial Institute For Medical Research
NS	Non-Structural Protein
NT	Neutralization Test
NTPase	Nucleoside Triphosphatase
OD	Optical Density
OPD	Out-Patient Department
PAHO	Pan American Health Organisation
POC	Point Of Care
PrM	Precursor Membrane Protein
RdRp	RNA dependent RNA polymerase
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
RNase	Ribonuclease
RTP	5'-RNA-Triphosphatase

rtRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
SEM	Standard Error Of Mean
SL	Stem Loop
US NIH LID	United States National Institute of Health Laboratory of Infectious Diseases
US NMRC	United States Naval Medical Research Center
UTR	Untranslated Region
WBC	White Blood Cell
WRAIR	Walter Reeds Army Institute of Research

ABSTRACT

Background: Dengue and malaria are two important mosquito-borne diseases. Together, both diseases are indistinguishable due to similarities in clinical and laboratory characteristics. These similarities have contributed immensely to the over-diagnosis of malaria and the under-recognition of dengue in many countries in sub-Saharan Africa. No baseline study has yet been conducted in Ghana to estimate the burden of dengue. This study therefore set out to estimate the prevalence of dengue infection in a population of Ghanaian children confirmed with malaria.

Methods: Archived plasma samples, obtained from 216 study participants aged 2-14 years enrolled in a malaria study, were tested for the presence of dengue using Real-Time Reverse Transcriptase PCR, dengue Rapid Diagnostic Test (RDT) and dengue IgM/IgG capture ELISA. The study was conducted in three ecological zones of Ghana namely: Kintampo, Navrongo and Accra.

Results: Dengue RDT and dengue IgM/IgG capture ELISAs estimated a prevalence of 54% and 24%, respectively. Dengue IgG capture ELISA showed that majority of seropositive study participants were positive for IgG (20%) and this was statistically significant between Kintampo and Navrongo. Estimated IgM/IgG ratio of 0.11 indicated that dengue infection was as a consequence of a previous exposure. Further analysis showed that 22% of the study population were confirmed with exposure to dengue-malaria while exposure to malaria only, dengue only and absence of either diseases was confirmed in 70%, 2% and 6% of study population respectively. Molecular tests indicated negative for dengue in all study participants.

Conclusion: Results in the study suggest an exposure to dengue virus in a subset of Ghanaian population. However, the inability to detect and identify circulating serotype calls for more research in this regard.

CHAPTER ONE

1.0 INTRODUCTION

Malaria, though a manageable and preventable disease, is still considered one of the deadliest in the world. According to the World Health Organization estimates, there were about 207 million cases of malaria in 2012, of which 627,000 resulted in deaths. About 90% of malaria deaths occur in sub-Saharan Africa with pregnant women and children under-five years of age being the most vulnerable (World Health Organization, 2013). Ghana, located in sub-Saharan Africa is a malaria endemic country and the entire population is thus at risk of the disease, with falciparum malaria being the most dominant (World Health Organization, 2013). Malaria is the leading cause of morbidity and mortality in Ghana and it is responsible for over a third of all outpatient visits each year (Ghana Ministry of Health, 2008). Although malaria is the leading cause of mortality and morbidity in Ghana and sub-Saharan Africa, the disease is often over-diagnosed to the neglect of other febrile illnesses.

In malaria endemic countries, the case management of malaria in most health facilities is often based on clinical judgement otherwise referred to as presumptive treatment (Chandler *et al.*, 2008). Presumptive treatment provides a clinician with an approximate diagnosis of malaria based on signs and symptoms like fever, chills/rigor and hepatosplenomegaly (Chandramohan *et al.*, 2002), however, this approach has shown to have a high sensitivity but low specificity (52-61%). This low specificity is attributed to the fact that several other febrile illnesses share similar signs and symptoms with uncomplicated malaria and hence lead to a high probability of false positive diagnosis leading to the over-diagnosis and over-emphasis of malaria (Chandramohan *et al.*, 2002; Nankabirwa *et al.*, 2009). In Nigeria, a study showed that 83% of children aged

0-5 years were treated for malaria with artemisinin-based combination therapy (ACT) although microscopy results indicated negative (Oladosu and Oyibo, 2013). Similarly, in Tanzania, a recent study conducted in 870 hospital admissions showed that malaria was the clinical diagnosis given to 528 (61%) of the patients. However, only 14 (2%) implicated malaria as the cause of the fever by laboratory confirmation (Crump *et al.*, 2013). In Ghana, less than a third of diagnosed malaria cases are confirmed with laboratory tests (JSI Research and Training Institute., 2013).

The presumptive treatment approach was justified when first–line treatment for malaria (chloroquine and sulphadoxine-pyrimethamine) were cheap, effective and safe; however, this is no longer justified (Njama-Meya *et al.*, 2007). The introduction of more efficacious and expensive ACTs has necessitated the laboratory confirmation of malaria infection before commencement of treatment to avoid the rapid onset of drug resistant parasites. The approach was also justified on the basis of limited availability of microscopy which remains the practical gold standard approach for malaria confirmation in many settings (World Health Organization, 2010). The introduction of Rapid Diagnostic Tests (RDTs) were therefore welcomed as tools in aiding clinical diagnosis (Bisoffi and Van den Ende, 2008; English *et al.*, 2009). Thus, the introduction of ACTs and RDTs renders the presumptive treatment approach unnecessary. The World Health Organization (WHO) has revised malaria treatment guidelines; restricting the prescription of ACT to malaria cases only after laboratory confirmation has been established (World Health Organization, 2010). Nevertheless, a majority of patients presenting with fever to most health facilities in Africa are still diagnosed as "fever of unknown origin or malaria".

Dengue fever shares overlapping signs and symptoms with malaria and may be implicated as the cause of some fevers in patients who remain without diagnosis even when they fail to respond to anti-malarial treatment.

Dengue is a viral disease that is most commonly characterised by acute fever. It comprises of four serotypes 1, 2, 3 and 4 (DENV 1, 2, 3 and 4) and belongs to the family Flaviviridae and genus *Flavivirus* (Westaway *et al.*, 1985). It is transmitted by the *Aedes aegypti* mosquito which is also the vector for Chikungunya and yellow fever (van den Hurk *et al.*, 2012). Although the disease accounts for 3%-11% of febrile illnesses in most endemic countries, the disease still remains under-recognised and under-reported (Amarasinghe *et al.*, 2011).

In Ghana, the vector for the disease has been around for over a century (Patterson, 1979) and has persisted in most mosquito surveys throughout the 20th and 21st centuries (Appawu *et al.*, 2006; Chinery, 1995; Opoku *et al.*, 2007). The first dengue case to be reported in Ghana was the isolation of DENV 2 in Finnish travellers who had visited Ghana in the period 2000-2005 (Huhtamo *et al.*, 2008), however, no case of dengue has been diagnosed since then. Several seroprevalence studies in West Africa (Amarasinghe *et al.*, 2011; Collenberg *et al.*, 2006; Fagbami *et al.*, 1977; Jentes *et al.*, 2010; Phoutrides *et al.*, 2011; Saluzzo *et al.*, 1986) and other parts of Africa (Blaylock *et al.*, 2011; Kuniholm *et al.*, 2006; Vairo *et al.*, 2012) have all confirmed the transmission of the disease. However, in Ghana, there is no published evidence of dengue prevalence; hence this study will provide the first baseline data on dengue prevalence and will pave the way for subsequent studies.

1.1 Study Objective

1.1.1 Aim

To provide evidence of dengue virus (DENV) exposure and infection among children with malaria in Ghana.

1.1.2 Specific Objectives

- Test for dengue-specific IgM and IgG antibodies in children confirmed with malaria across three ecological zones in Ghana.
- To use differential prevalence of dengue-specific IgM versus IgG to estimate the rates of primary/recent dengue infections and secondary/previous exposure to dengue virus in study population.
- To detect and identify circulating serotypes (DENV 1-4) using Real Time Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR).
- To differentiate within study population: (i) Confirmed dengue (ii) Confirmed Malaria (iii) Absence of either diseases (iv) Confirmation of co-infection with both Pathogens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of Dengue Fever

Among mosquito-borne diseases, dengue is the fastest spreading in the world. The past 5 decades has seen a 30-fold increase in incidence expanding to new countries and from urban to rural setting in the last 10 years. The worst affected continents are South America and Asia; with parts of Africa and North America also showing prevalence of the disease (Figure 2.1). Dengue infections annually is estimated at 50 million people with approximately 2.5 billion people reported to live in dengue endemic countries (World Health Organization, 2008). Recent reports however indicate that an estimated 390 million individuals are infected each year, with 96 million developing symptoms for the different levels of disease severity (Bhatt *et al.*, 2013). Dengue is often transmitted extensively when there is a spread of the vector to new geographical locations coupled with increased international travel, unplanned urbanization and rapid population growth (Amarasinghe *et al.*, 2011).

2.1.1 Dengue in the WHO African Region

Surveillance data on the existence of dengue in the WHO African region is poor although the disease exists (Nathan and Dayal-Drager, 2007). Most of the dengue cases reported in Africa are without laboratory confirmation hence there is the probability that most of the reported cases are due to other illnesses that share overlapping clinical symptoms with dengue such as malaria, chikungunya or yellow fever (World Health Organization, 2009).

Figure 2. 1: Countries in the world at risk of dengue transmission as at 2010
Red indicates countries at risk and contour lines mark the geographical limits for year-round survival of the mosquito vector for DENV, *Aedes agypti.*
Source: World Health Organisation Map Production: Public Health Information and Geographic Information Systems (GIS), World Health Organisation

Earliest reported cases of dengue from seroprevalence studies have shown the existence of dengue since 1926-1927, when there was an epidemic in Durban, South Africa (Kokernot *et al.*, 1956). The disease has been known to have been imported from India since the 1980s (Blackburn and Rawat, 1987). Compelling evidence from Eastern Africa show that DENV 1 and 3 were implicated as the common cause of most acute fevers. There have been reported cases of outbreaks of DENV 1 and 2 in Comoros in the years 1948, 1984 and 1993 (Boisier, 1993) and DENV 3 in Mozambique from 1984-1985 (Gubler *et al.*, 1986). In West Africa, DENV 1, 2 and 3 were first isolated in human serum samples in Nigeria in the 1960s (Carey *et al.*, 1971). Burkina Faso witnessed a dengue outbreak in 1982 where DENV 2 was implicated as the cause of the outbreak (Gonzalez, 1985). Côte d'Ivoire experienced two outbreaks; one in 2006 and the other in 2008, where a sero-survey was conducted in 800 people in Abidjan. In both cases, DENV 3 was implicated as the cause of the outbreak (Veronique, 2012).

DENV 2 was isolated as the cause of an outbreak in Senegal in 1990 (Zeller *et al.*, 1992) while another outbreak was reported in 2009 (ProMED-mail, 2009).

A dengue outbreak was reported in 2011 in Guinea-Bissau and at least 2 expatriates working in the country were diagnosed with dengue back at Scandinavia (Aaby, 2011). Small pockets of dengue outbreaks were also reported in Liberia (Gubler, 2004) and Mali in 2008 where DENV 3 was suspected as the cause (World Health Organization, 2009).

A recent report by the Public Health Directorate in Angola showed 513 suspected cases of dengue as of May 31st, 2013 when tested with dengue RDT. Further confirmatory test of 43 RDT-positive and 6 RDT-negative test at Centres for Disease Control and Prevention (CDC) dengue branch also confirmed dengue infection in all RDT-positives and 50% in RDT-negatives. DENV-1 was detected as the circulating serotypes by molecular diagnostic testing. Phylogenetic analysis indicate the existence of the disease since 1968 (Centers for Disease Control, 2013). This strongly suggest the endemicity of dengue in Angola. Although surveillance of dengue is poor, it is evident that there is a drastic increase in the rate of transmission of all four serotypes of the disease since the 1980s with Eastern Africa being the worst affected (World Health Organization, 2009). West Africa has been earmarked as an emerging front for dengue surveillance and control considering the rate of population growth, presence of the vector, case report of travellers and seroprevalence studies conducted (Stoler *et al.*, 2014).

2.1.2 Dengue in Asia and the Pacific

More than 70% of the world population at risk of dengue infection live in member countries of WHO South-East Asia Region and Western Pacific Region. Due to an

increase in the spread of the disease to new geographical locations and the reported record of high mortality during early phase of outbreaks, the Asia Pacific Dengue Strategic Plan covering 2008-2015 for the two regions was prepared after consultations with development partners and member countries. This strategic plan was prepared to manage outbreaks and halt the spread of the disease to new localities (World Health Organization, 2009). The transmission of dengue in the Asia-Pacific region has been documented since 1940 where the identification of four prototypes were first made in Hawaii (Hotta, 1952; Sabin, 1952; Hammon *et al.*, 1960). Over the past 30 years, the region has witnessed an increase in transmission of all four DENV serotypes (A *et al.*, 2004; Effler *et al.*, 2005; Imrie *et al.*, 2010; Imrie *et al.*, 2006). A recent study conducted in Bali (Indonesia) in the period 2010-2012 identified an emerging new lineage of DENV 2 (Ernst *et al.*, 2015) .

2.1.3 Dengue in the WHO Eastern Mediterranean Region

Documented cases of dengue outbreak in the Eastern Mediterranean Region dates as far back as 1799 in Egypt (WHO/EMRO). Since then, there have been an increase in the frequencies of reported outbreaks. For example in Sudan an outbreak that occurred in 1985 implicated DEN-1 and -2 as the cause (Hyams *et al.*, 1986), while in Djibouti, DEN-2 was the cause of the outbreak in 1991 (Rodier *et al.*, 1996). Between 2005 and 2006, dengue outbreaks were recorded in Pakistan, Saudi Arabia, Sudan and Yemen (WHO/EMRO).

The first documented case of dengue outbreak in Pakistan occurred in 1994 (Chan *et al.*, 1995). Dengue Haemorrhagic fever (DHF) epidemic caused by DEN-3 was also reported in 2005 (Bushra, 2007). Since then, there has been an increase in frequency

and severity of the disease in urban areas of Pakistan in the North-West Frontier Province. Dengue is therefore considered a pertinent issue in Pakistan (Chan *et al.*, 1995) . In Saudi Arabia, the first case of DHF victim died in Jeddah in 1993 (World Health Organization, 2009). Jeddah is a Haj entry point and the largest commercial point in the country hence it is at high risk of a dengue transmission from travellers coming in from dengue endemic countries such as Indonesia, Malaysia and Thailand (World Health Organization, 2009).

2.1.4 Dengue in the Americas

In the 1960s and early 1970s, the WHO Region of the Americas saw a break in transmission of dengue due to campaigns to eradicate the vector, *Aedes agypti*. This was however not sustainable resulting in subsequent incursion of the mosquito. This was ultimately followed by outbreaks in Central and South America and the Caribbean (Pan American Health Organisation, 1997). Between the periods 2001 to 2007, a total of 4,332,731 cases of dengue were reported in more than 30 countries of the Americas while 106,037 cases of DHF was reported with a case fatality rate of 1.2%. All four serotypes are found in the region and were simultaneously identified in Barbados, Columbia, Dominican Republic, El Salvador, Guatemala, French Guyana, Mexico, Peru, Puerto Rico and Venezuela (Pan American Health Organisation, 2008).

2.2 Dengue Case Classification

Dengue fever is a self-limiting viral disease with patients recovering after a non-severe clinical course (Tripathi *et al.*, 2011). However if not managed, a proportion of these cases progress into the severe form of the disease which is characterised by plasma leakage with or without haemorrhage. In severe cases, intravenous rehydration is the

appropriate therapy reducing case fatality rate to less than 1% (World Health Organization, 2009). Two schemes have been proposed for the classification of dengue: the 1997 and 2009 World Health Organisation (WHO) dengue case classification. The later has however demonstrated clear advantages for clinical use (Horstick *et al.*, 2014). The WHO 1997 dengue case classification classifies dengue as dengue fever (DF) and dengue haemorrhagic fever (DHF). DHF is further classified into four severity grades (grades 1, 2, 3, 4). DHF 1 and 2 are referred to as DHF while DHF 3 and 4 are collectively referred as dengue shock syndrome (DSS) (World Health Organization, 1997). The 1997 WHO dengue case classification has some limitations, namely: (i) misdirect clinicians in identifying severe disease (ii) difficult to use, as tests required are not available (iii) differences in reporting globally due to the difficulty of use of this classification scheme (iv) does not encourage triage in times of outbreak (World Health Organization, 1997). Based on these shortcomings, the 2009 WHO dengue case classification was introduced for the timely identification and management of severe dengue by clinicians. This saves resources and leads to a reduction in dengue mortality (Horstick *et al.*, 2014). The 2009 WHO dengue case classification scheme classifies dengue as dengue with/without warning sign and severe dengue (Horstick *et al.*, 2014). Figure 2.2 summarises criteria for dengue and severe dengue diagnosis based on WHO 2009 dengue classification scheme. Patients presenting to health facilities with fever, rash, aches and pain, nausea and vomiting are diagnosed as probable dengue since other diseases such as malaria and yellow fever also show these clinical signs and symptoms. However, patients presenting with warning signs such as mucosal bleed, abdominal pain and tenderness should be observed strictly since they are at risk of developing severe dengue which is characterised by plasma leakage, severe bleeding and severe organ failure.

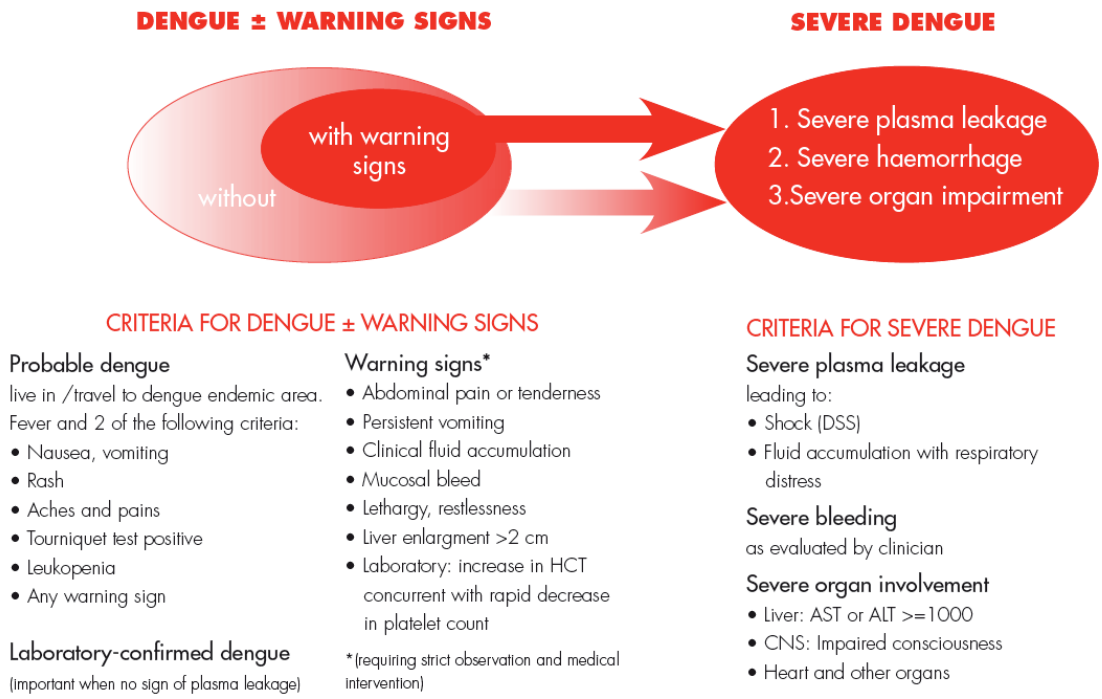

Figure 2. 2: Dengue case classification based on levels of severity
Source: (World Health Organisation, 2009)

2.3 Dengue Virus Transmission

2.3.1 Structural Morphology and Replication Machinery

DENV is a positive-sense single stranded RNA virus enveloped in a capsid protein. The RNA genome consists of approximately 10,700 nucleotides and measures ~11kb in length. It encodes 3,411 amino acids and can be translated directly as a long precursor polyprotein which contains three structural proteins (capsid C, precursor membrane prM processed into membrane protein M, and envelop E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Perera and Kuhn, 2008) (Figure 2.3). Viral RNA is encapsulated in C-protein (11KDa) to form viral nucleocapsid (Ma *et al.*, 2004; Jones *et al.*, 2003; Chang *et al.*, 2001). Surrounding the nucleocapsid is the lipid bilayer with M and E attached (180 copies). The M protein (approximately 8kDa) is the proteolytic fragment of its precursor form, the prM. The size of the E-protein is approximately 53KDa which facilitate attachment and entry of

virus by membrane fusion (Rey *et al.*, 1995; Nybakken *et al.*, 2006; Modis *et al.*, 2003; Modis *et al.*, 2005).

The structural proteins play no role during replication of the virus and are only synthesized to package the matured virus. The NS proteins are essential for replication of the virus and are often expressed in cytoplasm of infected cells (Pang *et al.*, 2001; Alvarez *et al.*, 2006).

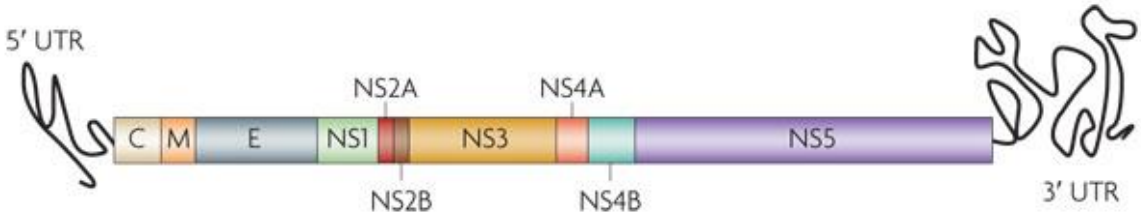

Figure 2. 3: Genome structure of dengue virus (DENV)
The DENV encodes three (3) Structural protein: (Capsid, C, Membrane, M and Envelope, E) and seven (7) non-structural proteins: (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). It contains two untranslated regions (5' and 3'). 5' UTR is capped but the 3' UTR lacks Poly (A) tail and folds into a conserved stem loop (SL). Source: (Guzman et al., 2010a)

NS1 is a glycoprotein and possesses two glycosylation sites which are conserved among flaviviruses. During viral replication, the role of NS1 is unknown however it has been suggested to facilitate viral infection. NS1 has also been shown to be capable of eliciting an immune response (Avirutnan *et al.*, 2010; Schlesinger *et al.*, 1987). NS3 proteins have three enzymatic properties namely: 5' RNA-triphosphatase (RTP), Nucleoside triphosphatase (NTPase) and a helicase. It interacts with NS5 protein and aids in viral replication. It also interacts with its co-factor NS2B for processing of polyproteins into structural and non-structural components (Bazan and Fletterick, 1989; Miller *et al.*, 2010). NS2A, NS4A and NS4B are less characterised and their role in viral replication remains unknown.

NS5 is the largest protein measuring approximately 103KDa. It has three major functional roles:

(i) N-terminal S-adenosyl methionine methyltransferase (MTPase): Responsible for guanine 7- N- and ribose 2'-O-methylation, required for DENV capping which is recognised by host cell translational machinery.

(ii) Nuclear Localisation Sequence: Interact with NS3 viral helicase for transport of protein to nucleus.

(iii) RNA-dependent RNA polymerase in C-terminal domain: This acts as an NS5 polymerase responsible for the synthesis of new vRNA genome (Miller *et al.*, 2010).

The vRNA genome contains a single open reading frame which is flanked by 5' and 3' untranslated regions (5'and 3' UTR). The 5'UTR contains approximately 95-135 nucleotides and a type I cap which is similar to cellular mRNA (Yu *et al.*, 2008). The cap structure is involved in a cap dependent translation initiation of viral RNA by scanning 5'UTR for initiation codons. The 3' UTR contains 114-650 nucleotides and folds into a conserved stem loop (SL) but lacks a Poly (A) tail. An essential RNA element known as conserved sequence is found upstream of the SL (Men *et al.*, 1996). This element contains cyclization sequence (CS) which has a complementary sequence at the 5' end of the genome (Hahn *et al.*, 1987). Both untranslated regions are essential for efficient replication and translation of the viral RNA (Gamarnik, 2010; Padmanabhan and Strongin, 2010). Among the different DENV serotypes, the sequences of the UTR's are highly conserved and their characteristic secondary structure gives them distinct functions. Aside the cap structure at the 5' UTR, there is also a large stem loop (SLA) which has been proposed to act as a promoter for RNA polymerase-methyl transferase NS5 (Yu *et al.*, 2008). The 5' and 3' UTR have

complementary upstream AUG regions (UAR) and cyclization sequence regions which hybridize during genome cyclization and RNA synthesis (Gamarnik, 2010).

2.3.2 The Replication Cycle

Dengue virus primarily infects immune cells such as monocytes, macrophages and dendritic cells (Jessie *et al.*, 2004) . The virus enters the host cell through a receptor mediated endocytosis. A cell surface receptor responsible for viral entry has not yet been identified, however, candidate cellular receptors required for the entry are various glycoproteins (i.e. heparin sulfates), dentritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) or a mannose receptor (Chen *et al.*, 1997; Tassaneetrithep *et al.*, 2003; Miller *et al.*, 2008). Also, the human C-type lectin-like molecule, CLEC5A, which contributes to lethal diseases in mice by acting as a proinflammatory receptor for DENV has been suggested to act as a possible critical microphage receptor (Chen *et al.*, 2008; Watson *et al.*, 2011). The viral E glycoproteins which are major targets of humoral immunity affect host cell receptor binding and viral entry. The E-glycoproteins are composed of three domains, domain I and II which possess fusion peptides at the distal tips, and domain III responsible for receptor-binding activity. In its matured state, the E-protein exists as a homodimer which renders its fusion peptides inaccessible. During RNA replication, the fusion peptides are exposed by low pH (Modis *et al.*, 2004). Two asparagine (N) linked glycosylation sites occur at position Asn-67, which is conserved among DENV, and Asn-153, which is conserved among all flaviviruses (Heinz and Allison, 2003). These ensure proper folding of the protein after translation (Modis *et al.*, 2003; Bryant *et al.*, 2007). The replication cycle of dengue virus is depicted in Figure 2.4.

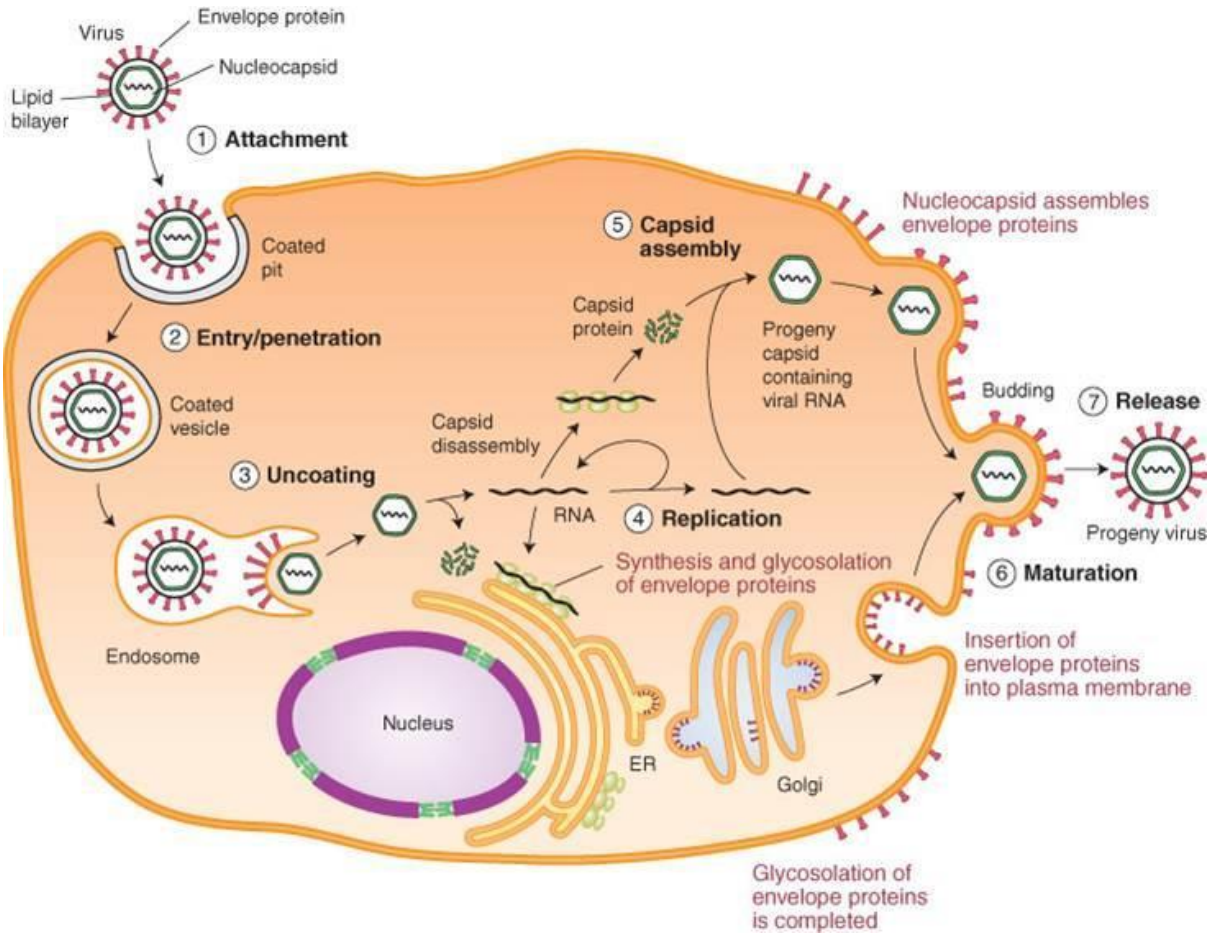

Figure 2. 4: Replication cycle of dengue virus
(1) DENV binds and enters cell through receptor mediated endocytosis. (2) Virus is internalised into a bubble-like structure known as endosome. (3) Proton pumps lower the pH within the endosome and virus responds by changing conformation of E-proteins to form spike-like hydrophobic structures releasing the capsid into the cytoplasm. (4i) Uncoating of Nucleocapsid releases viral RNA which travels to endoplasmic reticulum where translation occurs. (4ii) Synthesis of viral replication complex initiate the start of RNA replication. (5) After synthesis of many copies of RNA, assembly of virus occurs at the ER followed by (6) maturation in the Golgi apparatus and (7) finally release of matured virus from the cell.
Source: *http://www.studyblue.com/notes/note/n/virus-replication-cycles/deck/6581282.* Retrieved on: 29th July, 2014.

Entry of the virus into the cell is followed immediately by internalisation of virus in an endosome. Acidification in the endosome causes conformational change on the E-protein exposing the fusion peptide to facilitate membrane fusion (Heinz and Allison, 2003). Viral nucleocapsid is released into the cytoplasm followed by uncoating and release of viral RNA (Heinz and Allison, 2003). The positive sense single stranded viral RNA (+ssRNA) travels to the endoplasmic reticulum and is translated to a single

polyprotein (Clyde *et al.*, 2006). The C-terminal region of the structural protein contains hydrophobic amino acids that serve as signal sequence and directs the insertion of the viral genome into the ER (Mackenzie *et al.*, 1996). An ER signal protease together with NS2B-NS3 protease cleaves polyproteins into structural and non-structural proteins (Lobigs, 1993; Yamshchikov and Compans, 1993).

At this stage, translation is switched off and synthesis of viral RNA (vRNA) commences. Using the +ssRNA as a template, the negative sense single stranded RNA is synthesized. The pair of RNA forms a double helix and subsequent rounds of synthesis produces many copies of +ssRNA which become translated to proteins. vRNA and viral proteins are assembled to produce virion progeny which are released to infect other immune cells (Clyde *et al.*, 2006). Prior to the release of the viral particle, prM is processed into matured M proteins by furin host protease. The prM covers the distal end of the E-protein, thereby protecting it from premature fusion back into the cell (Guirakhoo *et al.*, 1991; Guirakhoo *et al.*, 1992; Zhang *et al.*, 2003).

2.4 Control of Dengue Fever

Currently, the only method of dengue prevention is through the control of its vector. However, this method has been deemed both expensive and ineffective (Gubler, 1998; Halstead and Deen, 2002). The increasing spread of dengue virus has therefore renewed global interest in the development of vaccines and therapeutic drugs.

2.4.1 Vaccine Development

Several factors must be taken into consideration when designing an ideal dengue vaccine. First, the vaccine must be able to elicit antibodies against each of the four dengue serotypes (DENV 1-4) to prevent the risk of antibody-dependent enhancement

of infection (ADE). ADE is the phenomenon observed during a secondary dengue infection where pre-existing sub neutralising antibodies from a primary infection cross-react with heterologous serotypes facilitating infection of immune cells (Guzman *et al.*, 2010b). Secondly, the vaccine should be safe for use in immunization thus causing little or manageable side effects. Thirdly, the vaccine must be affordable (Whitehead *et al.*, 2007; Thomas and Endy, 2011). The development of dengue vaccine is still hindered by several obstacles such as: inadequate knowledge on the pathogenesis of the disease, difficulty in developing vaccines capable of eliciting neutralising antibodies to all DENV 1-4 and insufficient investment from vaccine developers (Guzman *et al.*, 2010a).

There are no licensed dengue vaccines yet available. However, current vaccine development approaches include: live attenuated virus, live chimeric virus, inactivated virus and live recombinant (DNA and subunit) (Murrell *et al.*, 2011). Several preclinical/clinical trials are currently underway in many endemic countries to evaluate the safety and efficacy of the most advance dengue vaccine candidates (Table 2.1).

2.4.2 Treatment and Therapeutic Approaches

There are no antiviral drugs against dengue viral infection and treatment consist primarily of supportive care including bed rest, analgesics and antipyretics. In a case of DSS, urgent resuscitation with intravenous fluid to replace lost intravascular fluids is necessary. Starch and dextran are administered in severe cases while Ringer's lactate is used in moderately severe cases (Wills *et al.*, 2005). Due to plasma leakage, aspirin and salicylates should be avoided (World Health Organization, 2009).

Table 2. 1: Current dengue vaccine candidates at various stages of preclinical/clinical trials.
Source: (Guzman *et al.*, 2010a)

Vaccine Approach	Developer	Status
Live attenuated tetravalent chimeric yellow fever-dengue vaccine	Sanofi Pasteur	Phase II
Live attenuated tetravalent viral isolated vaccine	WRAIR and GSK	Phase II
Live attenuated chimeric DENV 2-DEN vaccine	CDC and Inviragen	Phase I
Recombinant E subunit vaccine	Merck	Phase I
Live attenuated tetravalent vaccine comprising 3' deletion mutations and DEN-DEN chimeras	US NIH LID and NIAID	Phase I
Subunit recombinant antigen (domain III) vaccine	IPK/CIGB	Preclinical
Live attenuated chimeric yellow fever-dengue vaccine	Oswaldo Cruz Foundation	Preclinical
Tetravalent DNA vaccine	US NMRC and GenPhar	Preclinical
Purified inactivated tetravalent vaccine	WRAIR and GSK	Preclinical

2.5 Laboratory Diagnosis

Diagnosis of dengue viral infection by laboratory techniques is often not available at the time of care. Hence most clinicians make diagnosis based on presenting clinical symptoms and the travel history of a patient (Rigau-Perez *et al.*, 1998). The detection

of dengue infection is usually from the onset of fever to 10 days post-infection; however not all patients are diagnosed within this period. Regardless of the stage of infection, it is imperative that an ideal diagnostic test should be sensitive (Vordam and Kuno, 1997). A laboratory confirmation of dengue virus infection can be made by one of the following methods;

- Isolation of the virus in cell culture
- Identification of viral nucleic acid (using PCR)
- Identification of antigen (NS1 based Enzyme-Linked Immunosorbent Assay (ELISA)
- Serologic assay for the detection of virus specific antibody (Vordam and Kuno, 1997).

A summary of the differences in relative advantages and optimal time frame of use of the different diagnostic tools (grouped under direct and indirect methods) used in dengue confirmation is elaborated in Figures 2.5 and 2.6.

Direct laboratory methods of viral detection (virus isolation, genome detection, antigen detection) could be employed to effectively diagnose early and serotype-specific dengue viral infections during the acute phase of the disease. Components of viral particles can be detected in samples such as plasma, serum, whole blood and infected tissues during 0-5 days following the onset of symptoms. However, the use of direct virus detection procedures are not often routinely employed in laboratories and very few commercial kits are available to aid in this regard (Peeling *et al.*, 2010).

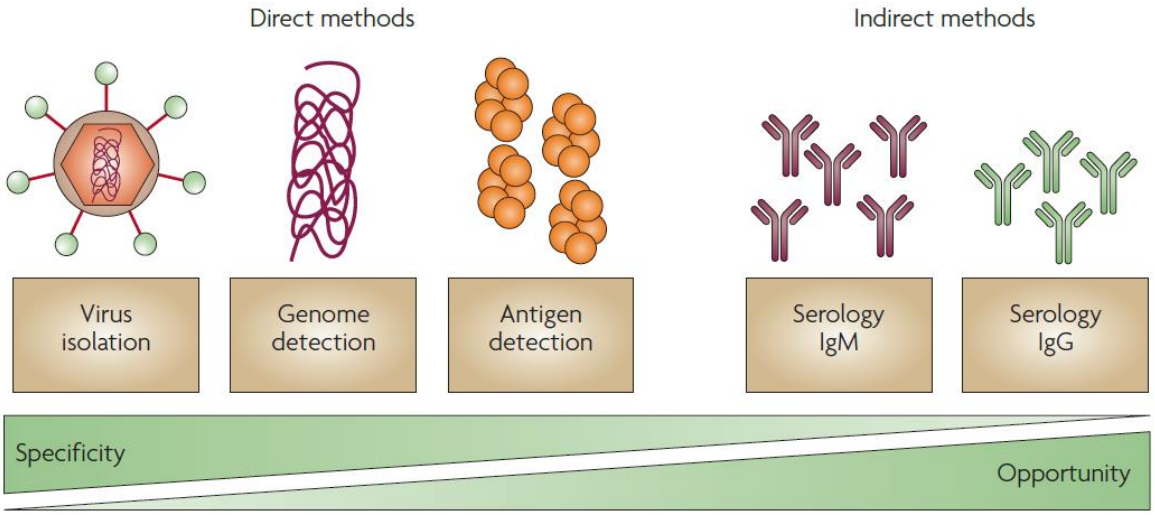

Figure 2. 5: Comparison of the advantages between direct and indirect laboratory methods for dengue diagnosis
(Opportunity refers to the fact that antibody testing is usually the most practical diagnostic option)
Source: Adapted from (Peeling *et al.*, 2010)

The common diagnostic methods routinely used to diagnose dengue infections are serological tests (Indirect laboratory methods) because of their ease of use. During a first (primary) or subsequent (secondary) dengue infection, different antibody responses are observed. Primary dengue infections are characterized by a detection of immunoglobulin M (IgM), 5 or more days following the onset of illness and immunoglobulin G (IgG) is detected 10-15 days following the onset of symptoms (Figure 2.6) (Vaughn *et al.*, 2000). Similarly during a secondary infection, there is the early detection of IgM but at lower titres or levels as compared to primary infections (in some cases IgM are absent). There is however a rapid increase in IgG due to its presence in previous infections (Vaughn *et al.*, 2000). Hence detection of IgM suggests that the infection is a recent one although it is still present 2-3 months post-infection (World Health Organization, 1997; Kuno *et al.*, 1991).

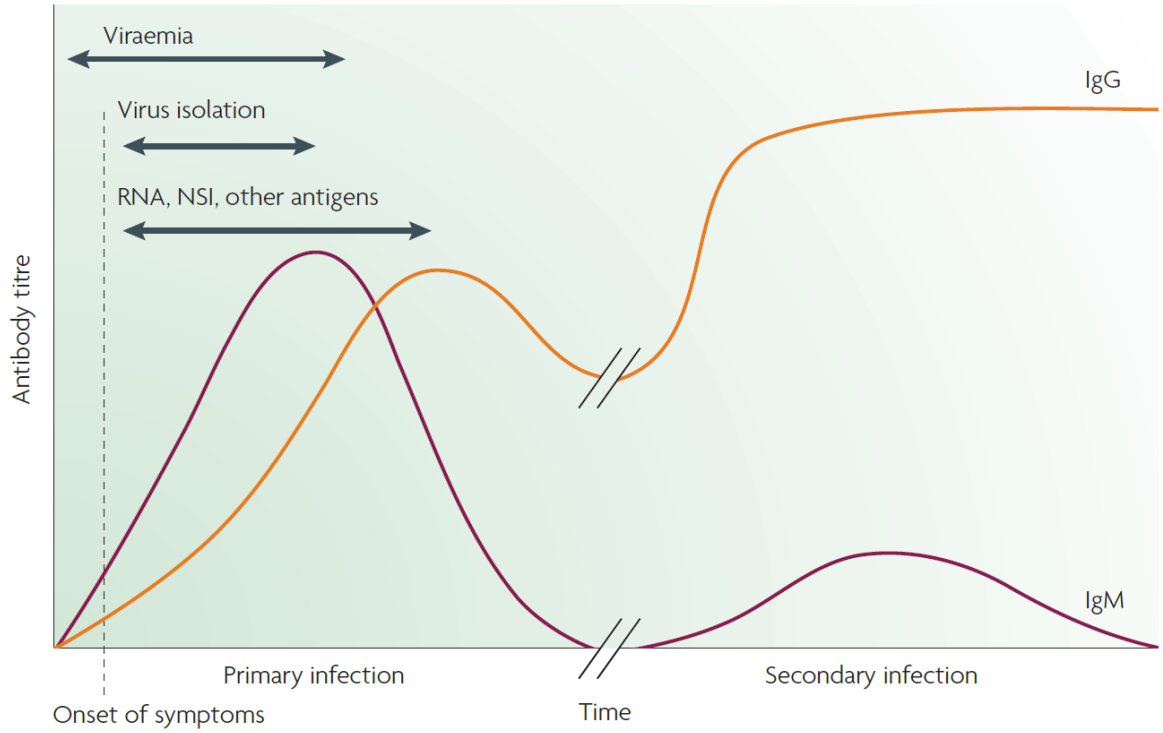

Figure 2. 6: Major diagnostic markers for dengue infection
The titres of the IgM and IgG response vary, depending on whether the infection is primary or secondary. Source: Adapted from Peeling *et al.*, 2010.

2.5.1 Virus Detection

The most common method of virus isolation is the inoculation of serum into mosquito cell lines such as *Aedes albopictus* (C6-36) and *Aedes pseudoscutellaris* (AP61) (Guzman and Kouri, 1996; Race *et al.*, 1979; Kuno *et al.*, 1985) or mammalian cell lines such as LLC-MK2 and Vero cells, or inoculation into intra-cerebra of suckling mice (Peeling *et al.*, 2010). In terms of sensitivity, the best isolation system is direct inoculation of samples into larval or adults of *Toxorchunchitis spp* mosquitoes (*T. ambionesis*, *T. splendens*) (Gubler and Rosen, 1976; Rosen and Shroyer, 1985). The direct inoculation into *Toxorchunchitis spp* mosquitoes is however not available in most endemic countries. The C6-36 cell line is the widely used method for most routine diagnosis. Immunofluorescent assay (with serotype-specific monoclonal antibodies) is performed to identify the virus, either 7-10 days after inoculation in cell lines or 14 days

after inoculation in mosquitoes (Guzman and Kouri, 1996; Henchal *et al.*, 1983; Vordam and Kuno, 1997). The use of C6-36 cell lines will give a viral isolation rate of up to 36% while there is an 80% viral isolation rate with the use of direct mosquito inoculation (Kuno *et al.*, 1985; Vordam and Kuno, 1997).

Culturing of DENV comes with a lot of limitations. It requires obtaining specimen from patients experiencing the acute phase of the disease (with sufficient viral loads) and isolating the virus immediately as the period for successful isolation of the virus is short. There is a peak in viremia before the onset of the disease however this drops considerably after medical care is sought. The drop in viremia coupled with rising levels of antibodies results in fever clearance. The culturing of DENV is both time consuming and labour intensive which requires that the infectious samples must be kept at cold temperatures. Also, culturing must be performed only in a Bio-safety Level 3 laboratory and this requires professional training for personnel (World Health Organization, 1997).

2.5.2 Viral RNA Detection

The detection of viral RNA can be achieved through the use of nucleic acid amplification test (NAAT). Specimen used for these tests include: tissues, whole blood or sera obtained from patients during the acute phase of the disease. Majority of protocols developed for the detection of the DENV employ the use of primers that target regions of the genome that are specific to each serotype (Lanciotti *et al.*, 1992; Johnson *et al.*, 2005; Wu *et al.*, 2001). However, most protocols differ in RNA extraction methods, specificity, sensitivity, methods to determine serotypes and PCR products (Lanciotti *et al.*, 1992; Harris *et al.*, 1998; Morita *et al.*, 1994; Rosario *et al.*, 1998).

Lanciotti *et al*, (1997) developed a rapid assay which could detect virus in sera by using consensus primers that target the viral C and prM genes for the first round amplification. This is followed by a second round amplification which uses specific primers to determine the serotype (different sizes of PCR product indicate specific dengue serotype). The combination of PCR with nucleotide sequencing and restriction enzyme analysis has proved effective for characterisation of dengue strains (Rico-Hesse, 1990; Deubel *et al.*, 1993; Chungue *et al.*, 1995; Lanciotti *et al.*, 1997; Balmaseda *et al.*, 1999; Tolou *et al.*, 2000). Classification of DENV (according to their nucleotide sequence) into different genotypes has been made possible through nucleotide sequence studies. Rico-Hesse (1990) showed 5 genotypes of dengue 1 and 2 while studying the E/NS1 gene junction. Similar results obtained by others while studying fragments encoding amino acids 29-94 in E proteins of 28 isolates of dengue 2 and amino acids 28-87 in 35 isolates of dengue 1 (Deubel *et al.*, 1993; Talisuna *et al.*, 2004). Sequencing the M and E genes of 23 distinct strains, dengue 3 has been classified into four subtypes while dengue 4 has been classified into 2 genotypes by studying the complete E gene of 19 strains (Talisuna *et al.*, 2004; Chungue *et al.*, 1995; Lanciotti *et al.*, 1997)

Detection of dengue RNA can be rapidly performed with primers specific to all four dengue serotypes in a one-step TaqMan real-time reverse transcriptase-PCR (Kong *et al.*, 2006). Johnson *et al*., 2005 developed a fourplex real-time reverse transcriptase PCR which could detect all four dengue serotypes in one reaction run. The real-time RT-PCR assay was effective in detecting dengue in patients with both low and high viremia, and thus used in this study for the detection of viral RNA in plasma samples.

2.5.3 NS1-based Assay

The NS1-based assay is a simple method which targets the viral antigen NS1 in the bloodstream during the acute phase of a primary dengue infection. However, in secondary infection, antigen detection is hampered by pre-existing virus IgG antibodies. All flaviviruses synthesize NS1 antigen during infection of mammalian cells (Peeling *et al.*, 2010). A study to evaluate performance of enzyme-linked immunosorbent assay (ELISA) and rapid immunochromographic assay targeting NS1 antigen have shown high concentrations of this antigen 0-9 days after onset of clinical manifestations (Dussart *et al.*, 2006). Sensitivities of NS1 assay varies between primary and secondary infection. A study conducted in Indonesia on the performance of a commercial dengue NS1-based ELISA kit showed a sensitivity of 68% and 48% in primary and secondary infections, respectively. Specificity in dengue-naive samples was 100% (Aryati *et al.*, 2013).

2.5.4 Serological Diagnosis

Five serological tests are known for use in dengue diagnosis namely: hemagglutination-inhibition (HI) test, complement fixation (CF) test, neutralisation test (NT), immunoglobulin M (IgM) capture ELISA (MAC-ELISA) and indirect immunoglobulin G ELISA. Although these test are sensitive for detection of dengue, they have some limitations. They are known to cross-react with other flaviviruses, specifically: yellow fever, Japanese and St. Louis encephalitis virus. They also cross-react with other viruses that cause similar clinical manifestations such as Chikungunya, Oropouche or Mayaro infections (Guzman and Kouri, 1996). Furthermore, these assays can only detect antibodies five days after the onset of disease, making them unsuitable for rapid diagnosis (Guzman and Kouri, 1996).

Hemagglutination-inhibition test (HI)

The HI test has been the standard method for dengue diagnosis due to its high sensitivity and ease of use. It is often used in seroepidemiological studies and for differentiation of primary from secondary dengue infections. Paired samples (acute phase and convalescent phase samples) are used for the detection of dengue-specific antibodies. In primary infections, acute phase antibodies are detectable 5 or 6 day after onset of disease and antibody titres are usually above 1:10 dilutions. In convalescent phase samples however, the antibody titres are below 1: 640 dilutions. In secondary infection, there is an increase in antibody levels. A titre of 1:1280 dilutions or higher in samples collected during an acute or convalescent phase is indicative of a secondary dengue infection. Limitations of HI include lack of specificity, need for paired samples, and inability to detect virus serotypes (Vordam and Kuno, 1997).

Complement fixation (CF) test

CF is not routinely used for dengue diagnosis and often requires highly qualified and trained personnel. The principle of this test is that, complement proteins will react with antigen-antibody complexes and hence be depleted to confirm a positive test. Test is very specific for confirming primary infections and hence detection of infecting serotype (Vordam and Kuno, 1997).

Neutralisation Test (NT)

The NT is a very sensitive and specific serological test for diagnosis of dengue virus. It can be used for the detection of infecting serotypes in primary infections. However, the test is not reliable for detecting serotypes in secondary infections. Some limitations of this test are that, it is time consuming and very expensive.

Enzyme-linked immunosorbent assay (ELISA)

ELISA has been the test of choice for dengue diagnosis due to its high degree of sensitivity and ease of use. It can be used to detect both IgM and IgG antibodies specific to dengue. It is sensitive for the detection of acute phase antibodies hence no need for convalescent samples since dengue-specific IgM antibodies are produced five days after the onset of disease (Guzman and Kouri, 1996; Vordam and Kuno, 1997; Burke *et al.*, 1982; Innis *et al.*, 1989). ELISAs are designed to detect E proteins from all four serotypes to enable detection of any dengue serotype. Dengue ELISAs can be grouped into IgM based assay, IgG based assay or rapid immunochromatographic test.

IgM based assay

Detection of dengue-specific IgM is a useful diagnostic tool. It has a sensitivity and specificity of ~90% and 98%, respectively; IgM is often detectable 3-5 days after the onset of clinical manifestation. A study conducted in Puerto Rico showed that 80% of patients showed detectable levels of IgM after day 5 of onset of illness (Peeling *et al.*, 2010). This was further confirmed by hemagglutination-inhibition test or viral isolation in acute phase samples (Kuno *et al.*, 1991). In non-endemic areas, a positive result for IgM can be suggestive of a current infection (within the past 2-3 months). However, in an endemic area, a demonstration of seroconversion (four fold or greater changes in antibody titres) of paired samples is required to diagnose a current infection (World Health Organization, 2009). The assay uses a 96 IgM capture polystyrene microwell each coated with anti-human antibodies specific for IgM (μ-chain). Upon incubation of diluted control and plasma samples in the microwells, the IgM present in the samples bind to the anti- human antibody (IgM specific) in the wells. The plates are washed to remove non-specific reactants and DENV antigens are added to the wells and incubated.

If anti-DENV IgM is present, the DENV antigen binds to the anti-DENV. The wells are washed again to remove unbound DENV antigens, and mouse anti-DENV conjugated with horseradish peroxidase (anti-DENV: HRPO) is added to the wells and incubated. If there are bound anti-DENV-DENV antigens in the wells, the mouse anti-DENV: HRPO will bind to the DENV antigen. Excess conjugate are removed by washing. Enzyme substrate and chromogen are added, and the colour is allowed to develop. The resultant colour change after adding stop reagent is quantified by a spectrophotometric reading of optical density (OD) which is directly proportional to the amount of antigen-specific IgM present in the sample. The sample OD reading are compared to a reference cut-off value to determine results.

IgG based assay

IgG based assays are very sensitive for the detection of dengue IgG antibodies in humans. They are employed to establish a previous exposure to dengue virus (Peeling *et al.*, 2010). During a primary infection, IgG levels are often low but increase over a period, measuring as long as 60 years post infection (Halstead and Papaevangelou, 1980). One limitation to the use of the IgG-based assay is the exhibition of cross-reactivity with other flaviviruses, making them unfavourable for identifying infecting dengue virus serotype (Peeling *et al.*, 2010). The test principle for IgG assay is similar to that for IgM assays. The assay uses 96 polystyrene microwells coated with equal portions of inactivated and purified dengue virus types 1-4. The diluted plasma and control samples are incubated in the wells to allow specific binding of IgG antibodies present in the sample to the antigens in the wells. Antibodies that did not specifically bind are removed by washing and peroxidase-conjugated anti-human IgG is added to react with the specific IgG. Excess conjugate are removed by washing, and enzyme

substrate and chromogen are added, and the colour allowed to develop. Addition of the stop reagent will stop the reaction and the resultant colour change is quantified by a spectrophotometric reading of optical density which is directly proportional to the amount of antigen-specific IgG present in the sample. The sample optical density readings are then compared with the reference cut-off OD reading to determine results.

Rapid Immunochromatographic test

The rapid immunochromatographic test is a rapid membrane based test designed to detect antibodies to dengue. It is very simple and easiest to use point of care assay. Test results are usually ready within 15 minutes (Vaughn *et al.*, 1998). The test can be done using either serum or whole blood. Plasma may be used depending on the anticoagulant. It employs the use of an antibody binding protein conjugated to colloidal gold particle and a unique combination of dengue antigens immobilized on the membrane. Upon addition of the sample and the diluent into the sample wells of the test cassettes, the mixture passes through the antibody binding/ gold complex. Immunoglobulin, if present in the sample, will bind to this complex. When this complex passes over the immobilized antigen, it will capture any dengue specific antibodies (IgG or IgM) present in the complex which will produce a pink/purple band in the T (test) zone of the test cassette. The remaining complex continues to migrate to the control area on the test cassette which again produces a pink/purple band in the C area. The control band is a confirmation that the test has been performed properly.

CHAPTER THREE

3.0 MATERIALS AND METHODS

This study leveraged 220 archived plasma samples from two on-going Erythrocyte Invasion Mechanisms (EIM) projects namely; 'Alternative molecular mechanisms for erythrocyte invasion by *Plasmodium falciparum* in Ghana' (NMIMR-IRB CPN 004/11-12) and 'Role of complement receptor 1 in erythrocyte invasion by *Plasmodium falciparum* in semi-immune Ghanaians'(NMIMR-IRB CPN 003/11-12). The principal investigator for these projects is Dr. Awandare who also doubles as the principal investigator for this study. These samples were obtained from three study sites namely: Navrongo, Kintampo and Accra, processed and stored at -80°C at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

3.1 Ethical Consideration

Since this study used archived plasma samples from two on-going project, proposals were re-submitted to site-specific Independent Ethics Committees or Institutional Review Boards (IRB) of the Ghana Health Service, the Kintampo Health Research Centre, the Navrongo Health Research Centre and the Noguchi Memorial Institute for Medical Research. The conduct of the study commenced only after ethical approval was granted. The study was also explained to parents/guardians attending the health facility with children aged 2-9 years old and signed/thumb printed informed consent was obtained before enrolment into the study. Children aged 10-14 years were asked to sign Assent forms before enrolment.

3.2 Study Sites

The study was conducted in three ecological zones of Ghana namely: Kintampo North Municipality (Brong Ahafo Region), Kassena Nankana District (Upper East Region) and Ledzokuku-Krowo Municipality (Greater Accra Region). These sites were selected based on the malaria transmission intensities in these zones and the presence of well-established clinical and laboratory research facilities: Kintampo Health Research Centre (KHRC, Kintampo), Navrongo Health Research Centre (NHRC, Navrongo) and Infectious Diseases Research laboratory/Noguchi Memorial Institute for Medical Research (IDRL/NMIMR, Greater Accra).

Kintampo North municipality of Ghana lies within the forest-savannah transition belt and has malaria transmission all year round (holoendemic region). Parasite prevalence in Kintampo is >50% in children with an annual entomological inoculation rate estimated at 261-269 bites per person per year (Dery *et al.*, 2010). The incidence of malaria for children under 5 was 7 attacks per child per year and peaked in children aged 12-35 months (8.6 per child per year) (Dery *et al.*, 2010; Owusu-Agyei *et al.*, 2009). Malaria transmission in Kassena Nankana district is highly seasonal with the heaviest transmission occurring from June to October (Appawu *et al.*, 2004). Greater Accra experiences a relatively low malaria transmission. The mosquito vector for DENV (*Aedes agypti)* has been reported to be present in all the three study sites (Appawu *et al.*, 2006; Chinery, 1995; Opoku *et al.*, 2007).

3.3 Study Procedure

Children presenting with suspected acute malaria at the out-patient departments (OPD) of Kintampo Municipal Hospital in Kintampo, War Memorial Hospital in Navrongo

and Ledzokuku-Krowo Municipal Assembly Hospital in Accra were recruited only after satisfying the following inclusion criteria:

- Aged between 2-14 years old
- Being a resident in the area for at least the preceding six months
- Parent or guardian willing to give consent for child's participation by signing informed consent form.
- Willingness of children aged 10-14 years to receive and sign assent form to participate in the study.

Eligible study participants who met the inclusion criteria were enrolled and individual informed consents were obtained. Enrolment forms were administered to participants' parents/legal guardians and 0.5mL of peripheral blood was taken to estimate full blood count, perform haemoglobin electrophoresis, perform rapid test for malaria using First Response® Malaria Ag HRP2 (Premier Medical Corporation Ltd) and determine parasitaemia using Giemsa-stained thin and thick blood smears. Parasitaemia (number of parasites/µL of blood) was estimated by counting the number of parasites per 200 WBC on a thick smear of blood. Parasitaemia calculation was done using the following formula and this is based on WBC count of patients obtained from a haematology analyser.

Parasitaemia (parasite/ µL) calculation: $\frac{\text{Number of parasites counted}}{200} \times \text{WBC}/\mu\text{L}$

After screening, an additional 5ml of venous blood was obtained from malaria positive participants and processed for cryopreservation of malaria parasites and harvesting/storage of plasma and WBC depletion to obtain red cell pellets at the various clinical research laboratories.

3.4 Sample Processing

The blood samples collected were processed at laboratories in Kintampo Health Research Centre-Kintampo, Navrongo Health Research Centre-Navrongo and Department of Biochemistry, Cell and Molecular Biology, University of Ghana-Accra. Upon receipt of 5ml venous blood, it was centrifuged immediately to separate the erythrocytes, buffy coats (leukocytes) and plasma. The plasma was harvested and stored at -80°C to be used subsequently for antibodies assays by RDT and ELISA.

3.5 Diagnostic Tools

Serological tests are most commonly used to diagnose dengue infection because of their ease of use, high degree of sensitivities and cost-effective benefits.

In this study two diagnostic test methods were employed; serological tests (dengue rapid diagnostic test and dengue IgM/IgG capture ELISAs) and Molecular Test (RT-PCR). These diagnostic test methods were employed for the confirmation of the presence or absence of dengue in a total of 220 archived plasma samples.

3.5.1 Serological Test

Dengue Rapid Diagnostic Test (dengue RDT)

The Rapid Test One Step® Dengue IgG/IgM test kit (Panbio, Australia) which was employed for the rapid detection of antibodies to dengue virus is a membrane based screening test kit. It is a newer generation lateral flow immunochromatographic type assay, and among the simplest and easiest to use POC (Point of Care) assays.

Test Procedure

All plasma samples were allowed to thaw completely and mixed well before commencing the test. Plasma (5µL) was added to sample wells using a micropipette. Followed by 3 drops of sample buffer/diluent also to the sample wells. The results were then read within 5 minutes for strong positives, and up to 30 minutes for weaker positives as well as to confirm negative results. A single pink/purple band appearing at the C (control) line area of the test cassette is indicative of a negative result whereas an appearance of two (2) lines, one in the C (control) line and the other in the T (Test) line of the cassette is an indication of a positive result. A test result is invalid if a single line appears at the T area or no band appears at the C area. In such circumstances, the test was repeated using a fresh test cassette. The manufacturer reports 98.0% sensitivity and 98.0% specificity as compared with gold standard ELISA test for dengue.

Dengue IgM/IgG capture ELISAs

The dengue virus IgM capture DxSelect™ ELISA and dengue virus IgG capture DxSelect™ ELISA (Focus Diagnostics, Inc., Cypress, CA, USA) were used for the detection of dengue IgM and IgG antibodies respectively to DENV types 1-4 in human plasma. These test kits were used in strict adherence with the manufacturer's instructions with only minor modifications.

Dengue virus IgM capture ELISA

Dengue virus IgM capture ELISA is a qualitative assay for the detection of human plasma IgM antibodies to all four subtypes of the dengue virus. This assay is specific for IgM and is often used to establish the presence of a current infection. It uses a flavivirus group monoclonal horseradish peroxidase conjugated with

tetramethylbenzidine (TMB) as substrate. Each capture well is coated with anti-human IgM. The antigen solution contains equal proportions of inactivated DENV types 1-4 and the following virus were used; DENV 1: TH-Sman, DENV 2: TH-36, DENV 3: H87, DENV 4: H241.

Materials supplied (See appendix 1.1)

Test Procedure

All reagents were allowed to adjust to room temperature before use. The antigen solution was prepared by adding 6 mL of reconstitution solution provided to each vial containing inactivated lyophilized dengue virus antigen as per the instructions in the protocol. Plasma samples, controls (detectable and non-detectable) and cut-off calibrators were diluted with sample diluent provided using a 1:101 dilution factor in labelled test tubes. All the dilutions were done in duplicates to a final volume of 1.10 mL.

Wells in the microwell plates were filled with wash buffer and allowed to soak for 5 minutes. The wells were then decanted and sample diluent (100 µL) was dispensed into "blank" wells while 100 µL of diluted plasma samples, controls and cut-off calibrators were dispensed into appropriate wells of a blank microtiter plate in the location corresponding to that in the ELISA wells. The samples were then efficiently transferred into the ELISA wells with a 100 µL 8-channel pipettor. The plates were covered with sealing tapes and incubated for 60 minutes at room temperature (20-25°C). The contents of the wells were discarded and washed 4 times with wash buffer. The prepared antigen solution was added to all wells using a 100 µL 8-channel pipettor. The plates again were sealed and incubated for another 60 minutes at room temperature. The content of the wells were discarded and washed 4 times. The IgM conjugate was

added to all wells, sealed and incubated for 30 minutes at room temperature. The plates were washed 6 times after discarding the content. Substrate reagent was added and incubated for 10 minutes at room temperature. The reaction was stopped by adding stop reagent to all the wells in the same sequence and pace as the substrate reagent was added. An antibody-positive well indicated a change in colour from blue to yellow. The absorbance of each well was measured within an hour after stopping the reaction using the multiskan FC microplate photometer (Thermo Scientific, USA) at 450 nm. The optical density (OD) reading of the blank wells were subtracted from each plasma sample, detectable/non-detectable controls and cut-off calibrator OD reading. An average of the OD readings of each duplicate was obtained. Each average OD reading was divided by the absorbance value of the cut-off calibrator to obtain an index value. The index values of the test and control samples were compared to the index value of the corrected cut-off calibrator (corrected cut-off calibrator is always 1.00). An index value of >1 suggested the presence of IgM antibodies to dengue virus whereas an index value of < 1 was indicative of the absence of IgM antibodies to dengue virus. To ascertain the validity of the assay, the detectable control index value must be between 1.5 and 3.5 while the non-detectable control index value should be less than 0.8.

Dengue virus IgG capture ELISA

Dengue virus IgG capture ELISA is a qualitative assay for the detection of dengue IgG antibodies in humans. It can be used to establish previous exposure to dengue virus or can be employed as an epidemiological tool for surveys to determine dengue virus sero-prevalence. The manufacturer's (Focus Diagnostics, Inc., Cypress, CA, USA) instructions on the use of the kit was strictly adhered to, with minor modifications.

Materials supplied (See appendix 1.2)

Test Procedure

The test procedure for the dengue virus IgG capture ELISA was very similar to the dengue virus IgM capture ELISA except, the dengue virus IgM capture ELISA provided antigen solution for coating of the microwell plates, whereas the dengue virus IgG captured ELISA used microwell plates pre-coated with inactivated and purified dengue virus types 1-4 and therefore did not require coating prior to addition of conjugates. All other steps were the same as that for the dengue IgM capture ELISA.

3.5.2 Molecular Test (Dengue viral RNA Detection Test)

As a further confirmatory test to detect and identify specific serotypes (DENV 1-4) of dengue virus, viral RNA was extracted from all 220 plasma samples, purified and used for real-time reverse transcriptase Polymerase Chain Reaction (RT-PCR) assay. This was conducted at the biosafety level 3 facility at the Department of Virology, Noguchi Memorial Institute for Medical Research.

Viral RNA Extraction

The QIAamp Viral RNA Mini Kit supplied by QIAGEN Group was used for the extraction and purification of viral RNA from plasma.

Principles and Procedure

The plasma membranes were first lysed by adding 140 µl of the sample to 560 µl of prepared Buffer AVL (AVL + RNA carrier), mixed by pulse-vortexing for 15 seconds and incubated at room temperature for 10 minutes. Highly denaturing conditions provided by Buffer AVL enhances the lysing process. Addition of RNA carrier enhances binding of viral nucleic acids to QIAamp mini membrane and ensures the

inactivation of RNases (reducing the chance of viral RNA degradation) and isolation of intact viral RNA.

Absolute ethanol was added and mixed by pulse-vortexing. The sample was transferred into a spin column and centrifuged at 6000 g (8000 rpm) for 1 minute. The filtrate tube was discarded and the column placed in a new collection tube. The filtrate was washed twice with AW1 buffer and AW2 buffer and centrifuged at 6000 g (8000 rpm) for 1 minute and 20000 g (14000 rpm) for 4 minutes, respectively. The two washes were to remove all residual contaminants and improve the purity of eluted RNA. After wash, collection tubes were discarded and 60 µl of AVE buffer added, incubated for 1 minute and centrifuged at 6000xg (8000rpm) for 1 minute to elute pure viral RNA. The purified viral RNA was stored at -20°C to be used for RT-PCR assay.

Confirmation of successful extraction of viral RNA via Nano-drop spectrophotometer

The Nanodrop 2000/2000c spectrophotometer (Thermo Scientific, USA) was used to quantify the amount and purity of viral RNA isolated from each specimen. RNA absorbs at 260 nm and the ratio of absorbance at 260 nm and 280 nm is often used to access the purity of RNA; a ratio of ~ 2.0 is generally accepted as "pure" for RNA. The test was done in strict adherence with the manufacturer's instructions.

Real-Time reverse transcriptase-PCR (RT-PCR) Assay

For the detection of dengue 1-4 specific serotypes, the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Foster, CA) was used. The kit includes RT-PCR buffer, RT-PCR enzyme mix and nuclease free water. PCR primers and TaqMan® probes used in the real-time assay were previously designed by Johnson *et al*., (2005). DENV 1 and 3 probes used FAM/TAMRA while DENV 2 and 4 probes used FAM/ None as reporter

and quencher dyes, respectively. Table 3.1 shows the sequences of the PCR primers and TaqMan® probes used in the real-time RT-PCR assay.

Procedure

Primers and probes were diluted using 1:10 dilution factor; to 90 µl of nuclease free water, 10 µl of primers (forward and reverse) and probes were added. RT-PCR master mix was prepared as shown in Table 3.2.

Table 3. 1: Oligonucleotide primers and fluorogenic probes used in the serotype-specific DENV real-time RT-PCR

Oligonucleotides (primers and probes)	Nucleotide sequence	Genome position
DEN-1 F	CAAAAGGAAGTCGTGCAATA	8973
DEN-1 R	CTGAGTGAATTCTCTCTACTGAACC	9084
DEN-1 Probe	CATGTGGTTGGGAGCACGC	8998
DEN-2 F	CAGGTTATGGCACTGTCACGAT	1605
DEN-2 R	CCATCTGCAGCAACACCATCTC	1583
DEN-2 Probe	CTCTCCGAGAACAGGCCTCGACTTCAA	1008
DEN-3 F	GGACTGGACACACGCACTCA	740
DEN-3 R	CATGTCTCTACCTTCTCGACTTGTCT	813
DEN-3 Probe	ACCTGGATGTCGGCTGAAGGAGCTTG	762
DEN-4 F	TTGTCCTAATGATGCTGGTCG	904
DEN-4 R	TCCACCTGAGACTCCTTCCA	992
DEN-4 Probe	TTCCTACTCCTACGCATCGCATTCCG	960

DEN-1-4 F means a forward primer for detecting dengue virus serotype 1-4
DEN-1-4 R means a reverse primer for detecting dengue virus serotype 1-4

Table 3. 2: Dengue 1-4 Real Time RT-PCR Master Mix pipetting protocol

Reagents	Unit volume [1x (µl)]
Nuclease free water	8.82
2x RT-PCR Buffer	12.5
Dengue 1-4 forward Primer	0.25
Dengue 1-4 Reverse Primer	0.25
Dengue 1-4 Probe	0.18
Enzyme mix	0.5
Total	22.5

RT-PCR master mix was prepared on ice (total volume of 22.5 µl per reaction) and distributed to 96-well plates. Extracted viral RNA samples were then added (2.5 µl) to the master mix in a total reaction volume of 25 mL. Positive control virus mix was added and these constituted: heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87 and DENV-4 H241.

RT-PCR assay was run in singleplex, i.e. each DENV serotype assayed in a separate reaction. The thermal cycle was run and data analysed on an ABI 7300 Fast Dx Real-Time PCR instrument (Applied Biosystems, Foster, CA). Amplification conditions for the reaction are shown in Table 3.3.

Table 3. 3: Thermal cycling conditions for DENV 1-4 Real-Time RT-PCR assay

Steps	Stage	Cycles	Temp	Time
Reverse transcription	1	1	50 ºC	10min
RT inactivation/initial denaturation	2	1	95 ºC	5min
Amplification	3	45	95 ºC	15 sec
			60 ºC	35sec

Interpretation of RT-PCR results

In RT-PCR assay, a positive reaction is detected by accumulation of a fluorescent signal. The C_t (Cycle threshold) is define as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background levels). C_t values are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the C_t, the greater the amount of target nucleic acid in the sample). A specimen is considered positive for DENV 1, 2, 3, 4 if the fluorescent amplification curve crosses the threshold line within 37 cycles ($C_t \leq 37$ cycles). To determine amplification, the RT-PCR underwent 45 cycles (Figure 3.1).

3.6 Statistical Analysis

Data were analysed using SPSS version 20 software. Descriptive and simple statistical analyses were carried out using student's unpaired t test for comparisons between any two subject groups with continuous variables and Chi-square test for categorical variables. Analysis of variance was also performed for comparisons of continuous variables among multiple groups. Consequently, a multiple comparison test (Bonferroni test) was performed to determine which combination of two groups are

different. All tests were two-tailed and a P <0.05 was considered statistically significant.

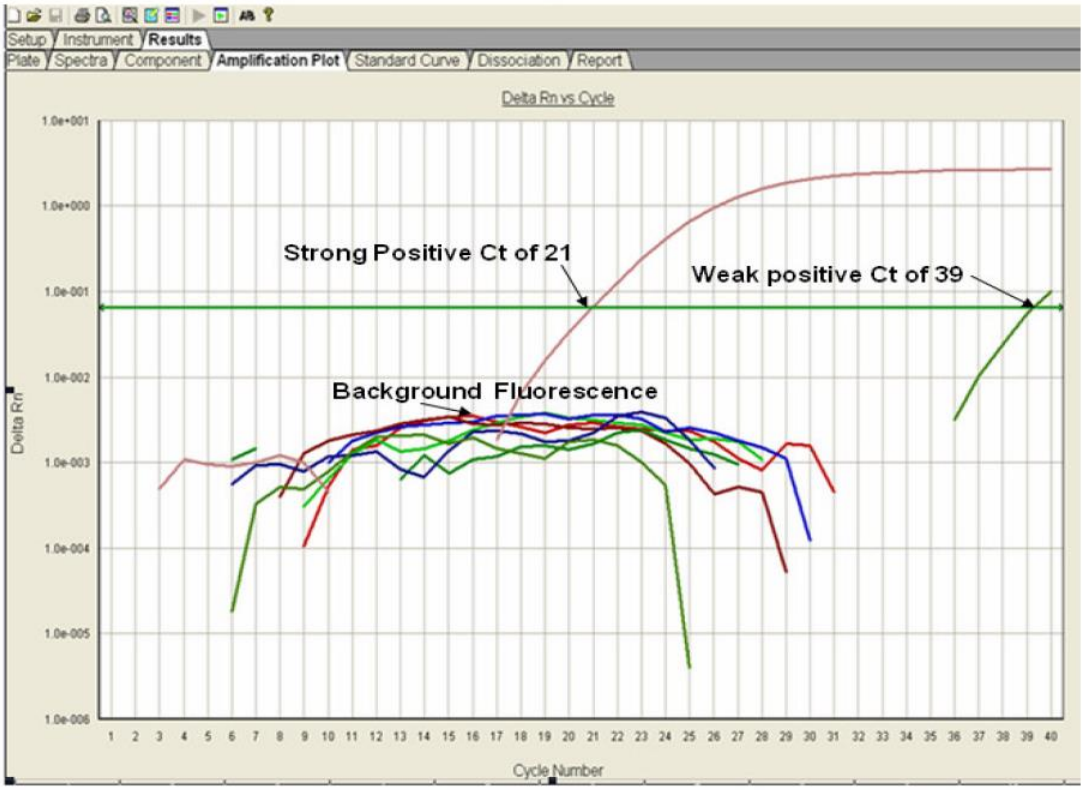

Figure 3. 1: An example of an amplification plot displaying Ct values and their interpretations

C_t values ≤ 21 are strong positive reactions indicative of abundant target nucleic acid in sample.

C_t values of 39 and above are weak reactions indicative of minimal amount of target nucleic acid in sample.

CHAPTER FOUR

4.0 RESULTS

This study obtained 220 archived plasma samples from children aged 2-14 years, diagnosed with malaria, to detect the presence of dengue IgG and IgM antibodies which are molecular markers for the confirmation of a previous or a current dengue viral infection, respectively. Missing data for four study participants were excluded from the total number of enrolled participants, hence, data analysis was performed on 216 study participants who had complete datasets. Real-time RT-PCR was performed for the identification of serotype-specific dengue viral RNA. The samples were collected from three ecological zones of Ghana: Kintampo, Navrongo and Accra during the peak malaria seasons.

4.1 Characteristics of Study Participants

The clinical and laboratory characteristics of the children involved in the study on the day of enrolment are summarized in Table 4.1. Of the 216 study participants, 51.4% were males while 48.6% were females. Although the percentage of males was higher in Kintampo and Accra relative to Navrongo, these differences were not statistically significant.

In sub-Saharan Africa, children 5 years or below are more susceptible to malaria and since majority of the enrolled study participants were diagnosed with malaria, the ages (2-14 yrs.) of the enrolled study participants were further categorized into two age groups: 2-5 years and 6-14 years. This was done to determine the age distribution of study participants to dengue exposure. Out of the 216 study participants, 60.2% were within the 2-5 years age group while 39.8% were within the 6-14years age group.

Kintampo and Navrongo had more study participants in the 2-5 years age group than the 6-14 years age group (62.8% vs. 37.23%, 62.50% vs. 37.23%, respectively), while Accra recorded an equal percentage of study participants in both age group (50% vs. 50%). The mean age in all 3 study sites was 5.2 ± 3.0 (Mean ±S.D). Statistical test showed a significant difference in the proportions of participants between the two age groups.

Mean parasite density recorded was highest in Kintampo followed by Navrongo and Accra. An ANOVA test of the mean parasite density followed by multiple comparison test showed a strong statistically significant difference in mean parasite density between Kintampo and Navrongo (P=0.00); Kintampo and Accra (P=0.00). However, no statistically significant association was observed in mean parasite density between Navrongo and Accra (P=0.72). There was no statistically significant difference in the mean haemoglobin levels across all three study sites and differences between sites were minimal. The mean axillary temperature recorded also showed minimal difference between study sites, however, statistical analysis indicated a significant association between Kintampo and Accra (P=0.007), and Navrongo and Accra (P=0.012). There was however no observed statistically significant association between Kintampo and Navrongo (P=1.00).

Table 4. 1: Baseline characteristics of study participants according to study sites

Study Sites / Variables		Kintampo (n=94)	Navrongo (n=80)	Accra (n=42)	Total (n=216)	P-value
Gender	Males: n (%)	51 (54.3)	38 (47.5)	22 (52.4)	111 (51.4)	0.87
	Females: n (%)	43 (45.7)	42 (52.5)	20 (47.6)	105 (48.6)	
Age	2-5 years: n (%)	59 (62.8)	50 (62.5)	21 (50.0)	130 (60.2)*	0.0034
	6-14 years: n (%)	35 (37.2)	30 (37.5)	21 (50.0)	86 (39.8)*	
Parasite Density (parasite/µL) Mean (SEM)		118236.16$^{\infty}$ (16213.70)	44005.29$^{\infty}$ (3852.74)	20738.10$^{\infty}$ (3326.67)	60993.18 (7797.70)	0.000
Haemoglobin Levels (g/dL) Mean (SEM)		10.06 (0.18)	10.03 (0.18)	10.73 (0.29)	10.27 (0.21)	0.074
Axillary Temperature (ºC) Mean (SEM)		37.68 (0.13)§	37.72 (0.29)§	38.81 (0.16)§	38.07 (0.58)	0.006

Data are presented as the mean and standard error of mean (SEM) for each variable except for age and gender which are presented as the number of enrolled study participants in each category. "n" represents the number of participants enrolled.* denotes statistically significant difference between proportion of participants in the two age groups (2-5yrs and 6-14yrs) (P=0.0034, Chi-squared test). $^{\infty}$ denotes statistically significant difference in mean parasites density between Kintampo and Navrongo and between Kintampo and Accra (P=0.00 for both, ANOVA and Bonferroni's multiple comparison tests). § denotes statistically significant difference in mean axillary temperature between participants in Kintampo/Accra and Navrongo/Accra (P=0007 and 0.012, respectively).

4.2 Results of Dengue RDT

For the rapid detection of dengue antibodies, the plasma samples were tested for presence of IgG and IgM antibodies to dengue types 1-4 using the Rapid Test One Step® Dengue IgG/IgM test kit.

The dengue RDT showed that out of the total number of 216 study participants tested, majority of the study participants were positive (53.7%) for dengue antibodies. Within the study sites, the majority of participants in Kintampo (57.4%) and Accra (59.5%) tested positive for dengue antibodies whereas in Navrongo, the majority of participants tested negative (53.8%) (Figure 4.1). However, these differences were not statistically significant across the study sites.

4.3 Dengue IgM/IgG Capture ELISA Results

To confirm the results of the RDTs, plasma samples were further tested for dengue-specific IgG and IgM antibodies using the dengue antibody capture ELISAs.

Of the 216 study participants analysed, 23.6% tested positive for dengue specific antibodies and 76.4% tested negative. Within each study site, majority of the study participants tested negative. Chi-square test showed a statistically significant difference between seropositive participants in Kintampo and Navrongo (33.8% vs. 13.8%) with P=0.006 (Figure 4.2).

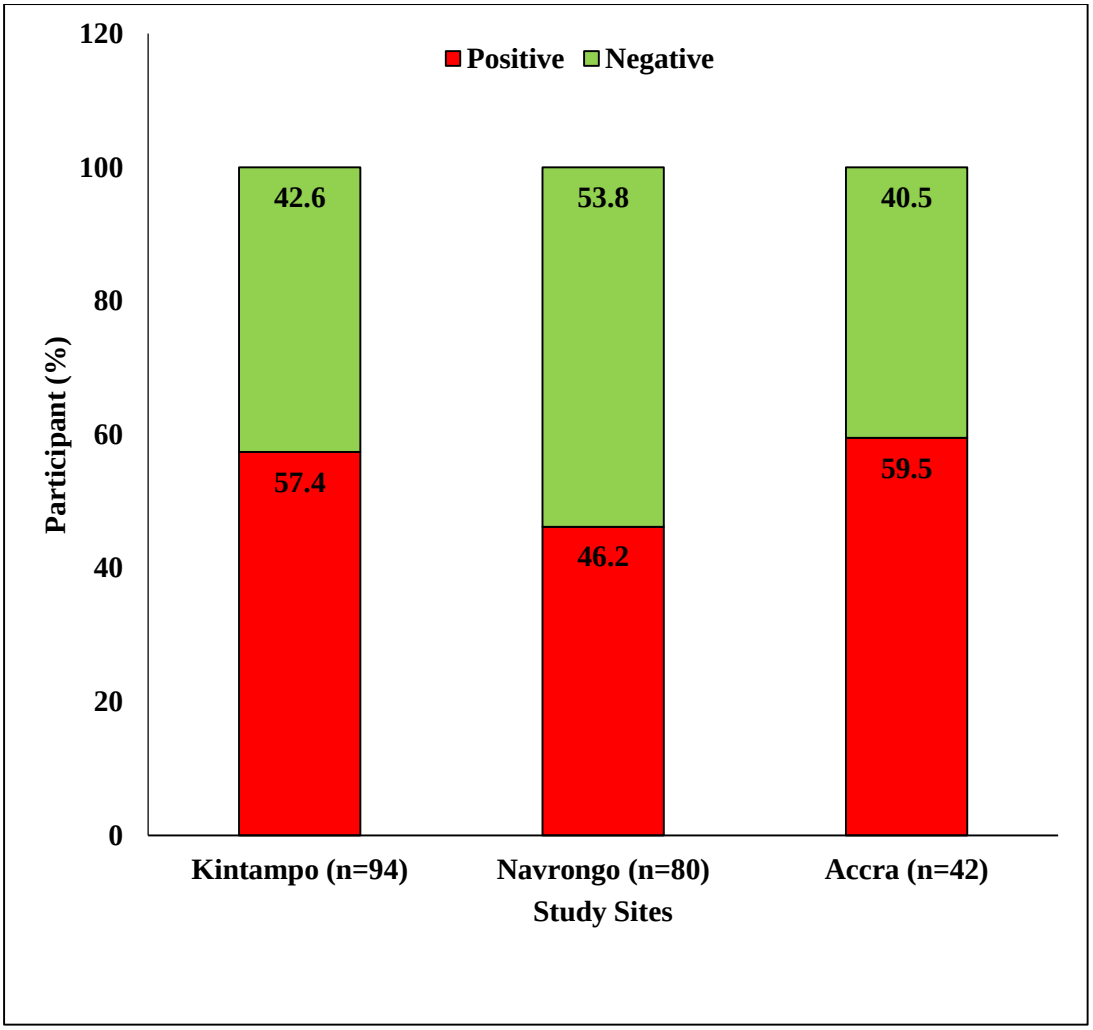

Figure 4. 1: Percentages of dengue seropositive participants by rapid test as per study site

Plasma samples were tested for IgG and IgM antibodies to DENV 1-4 using the Rapid Test One Step® Dengue IgG/IgM test kit. Data are presented as percentage of seropositive and seronegative participants.

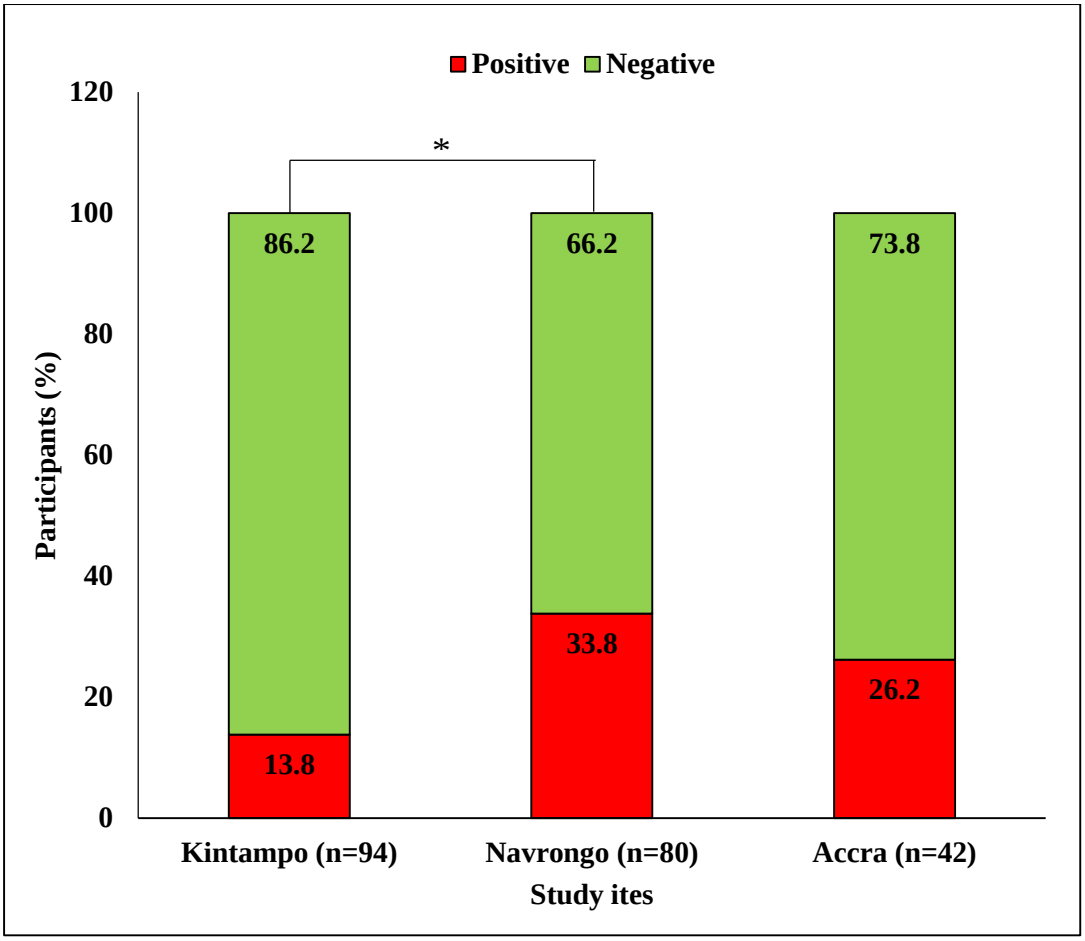

Figure 4. 2: Dengue IgM/IgG capture ELISA results of study participants shown per study sites

Plasma samples were tested for the presence of IgG antibodies to confirm a previous infection and IgM antibodies to confirm an active dengue infection using dengue virus IgG capture ELISA and dengue virus IgM capture ELISA, respectively**.** Data is presented as percentage of study participants testing positive or negative. * indicates a statistically significant association between test results in Kintampo and Navrongo (p=0.006).

4.3.1 Prevalence of Dengue IgM and IgG Antibodies

Out of the 23.6 % seropositive study participants, 20.4% were positive for IgG antibodies while 2.3% tested positive for IgM antibodies and 0.90% tested positive for both IgG and IgM antibodies. All the subjects tested positive for both antibodies in Navrongo whereas Kintampo and Accra only showed prevalence of either IgG or IgM antibodies but not both. All three study sites recorded a high prevalence of IgG antibodies compared to IgM antibodies (Figure 4.3). Chi-square test showed a

statistically significant association between Kintampo IgG seropositive study participants and those in Navrongo (P=0.034).

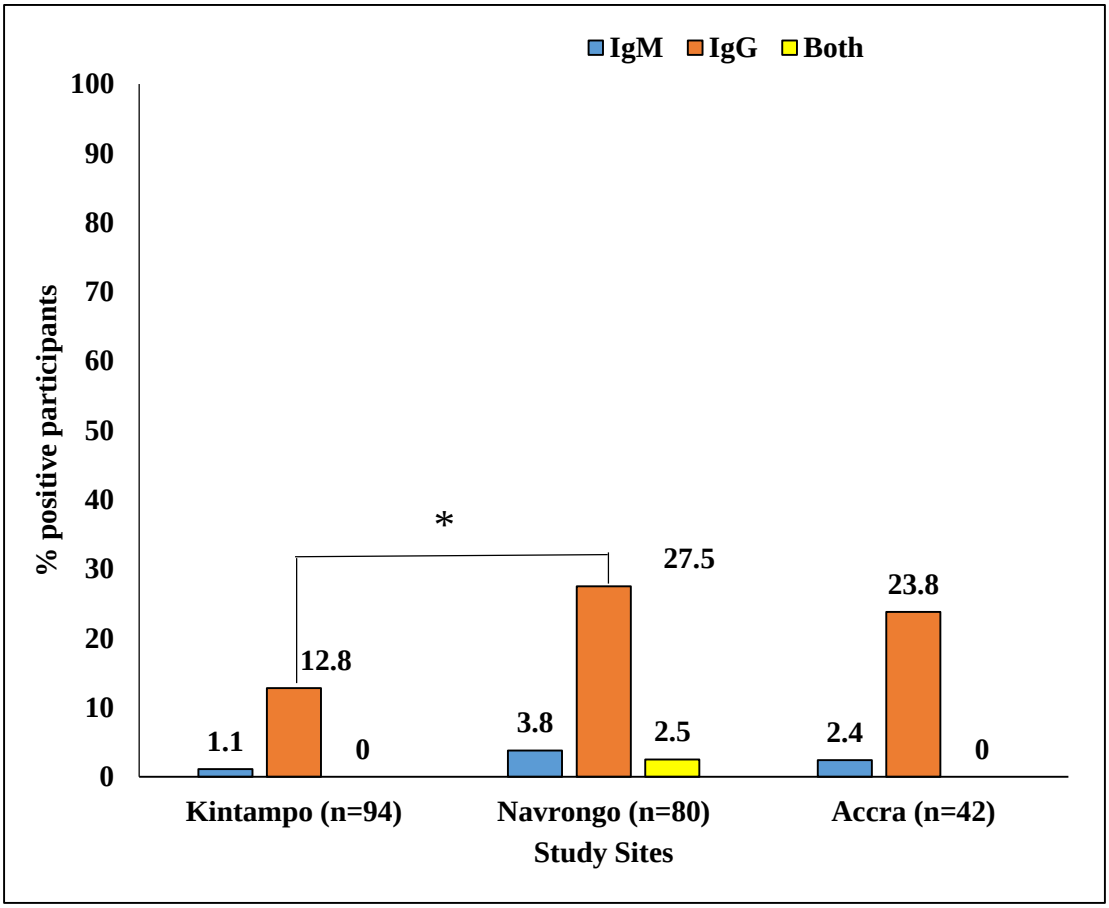

Figure 4. 3: Prevalence of IgM and IgG antibodies among seropositive study participants

Dengue seropositive study participants were categorized into IgG, IgM or both (IgG and IgM) antibodies**.** This confirms whether dengue infection is as a result of current or previous infection. Data are presented as percentages of study participants who tested positive for IgG, IgM or Both. *denotes statistically significant difference between IgG positive participants in Kintampo and Navrongo (p=0.034)

4.3.2 Classification of Dengue According to Gender

Previous studies have shown that males were more predisposed to dengue than females (Teixeira *et al.*, 2002; Gupta *et al.*, 2006; Goh, 1998) hence the study investigated the association between dengue and gender. The number of males who tested positive for dengue was 26.1% and that for females was 21.0%. The trend was the same within

study sites except for Accra were 5.7% of females tested positive and 4.5% of males tested positive (Table 4.2). There was no statistically significant association between dengue and gender.

Table 4. 2: Classification of dengue seropositive study participants by gender

Study Sites	Gender	
	Males (N=111)	Females (N=105)
Kintampo n (%)	9 (8.1)	4 (3.8)
Navrongo n (%)	15 (13.5)	12 (11.4)
Accra n (%)	5 (4.5)	6 (5.7)
Total n (%)	29 (26.1)	22 (21.0)

Data are presented as the number and percentage of seropositive study participants categorized according to gender in each study site. "n" represents the number of seropositive study participants. "N" denotes total number of enrolled male or female participants.

4.3.3 Classification of Dengue According to Age

Classification of seropositive study participants according to age categories showed that the percentage of seropositive study participants in the 6-14 years age group (27.9%) was slightly more than the 2-5 years age group (20.8%). Navrongo showed very minimal difference between the two age groups (12.3% vs. 12.8%). Accra observed more seropositive participants in 6-14 years (9.3%) compared to 2-5years (2.3%) (Table 4.3). There was no statistically significant association between dengue infection and age.

Table 4. 3: Classification of dengue seropositive study participant by age categories

Study Sites	Age categories	
	2-5 years (N=130)	6-14 years (N=86)
Kintampo n (%)	8 (6.2)	5 (5.8)
Navrongo n (%)	16 (12.3)	11 (12.8)
Accra n (%)	3 (2.3)	8 (9.3)
Total n (%)	27 (20.8)	24 (27.9)

Data are presented as number of seropositive study participants categorized under two age categories in each study site. "n" denotes number of seropositive study participants. "N" denotes total number of enrolled study participants aged 2-5year or 6-14 years.

4.4 Comparison of Dengue RDT and Dengue IgM/IgG Capture ELISA

Results obtained by dengue RDT were compared with those obtained by dengue IgG/IgM capture ELISA (Figure 4.4). There was a higher percentage of seropositive study participants obtained with dengue RDT (53.7%) when compared to those tested with dengue IgM/IgG capture ELISA (23.6%). This observation was statistically significant only in Kintampo (57.4% vs. 13.8%) (P=0.036). Navrongo and Accra showed similar trends but the differences were not significantly different statistically (46.2% vs. 33.8% and 59.5% vs. 26.2% respectively) (Figure 4.4). Since dengue IgM/IgG ELISA was a more standardized test than the dengue RDT, the sensitivity and specificity of RDT was low when compared to dengue IgM/IgG ELISA (42.31% and 43.45%, respectively).

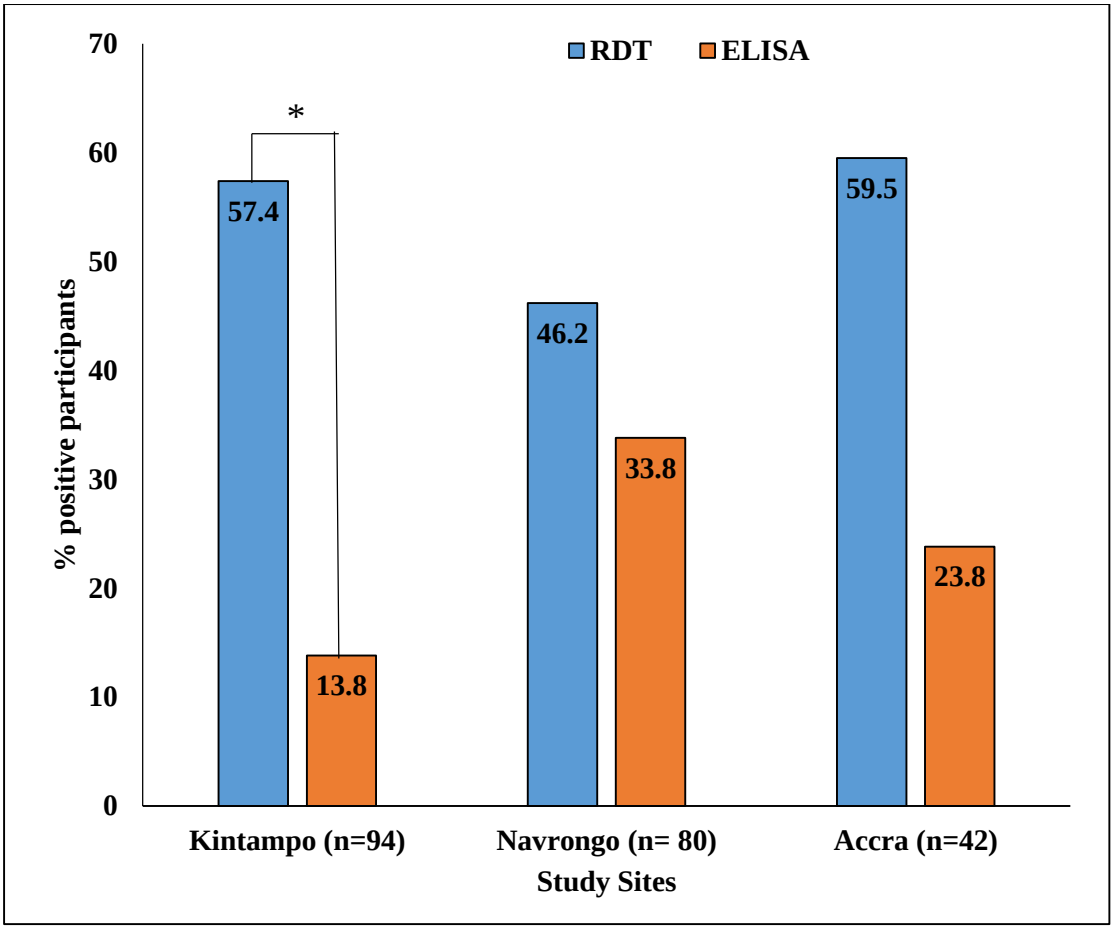

Figure 4. 4: Comparison of seropositive results of dengue rapid test and dengue IgG/IgM capture ELISA

Dengue RDT results were compared with results from dengue IgM/IgG capture ELISAs. Data are presented as percentage of study participants who tested positive when either test was conducted. * Indicates statistically significant difference in the percentage of seropositive participants between the two tests in Kintampo (P=0.036).

4.5 Malaria Distribution by Study Site

Children enrolled into the study were first screened for malaria positivity by RDT and later confirmed by microscopy. A few of these children who tested positive via RDT, however, tested negative via microscopy. Hence, the study investigated the distribution of malaria among the study participants.

Out of 216, 92.1% tested positive for malaria and 7.9% tested negative for malaria. Similar trends were observed in Kintampo (83.0%) and Accra (97.6%) with majority of study participants testing positive for malaria and a few testing negative. All the

study participants in Navrongo tested positive for malaria (100%) (Figure 4.5). Malaria status of study participants in Kintampo was significantly different statistically from those in Navrongo (P=0.00).

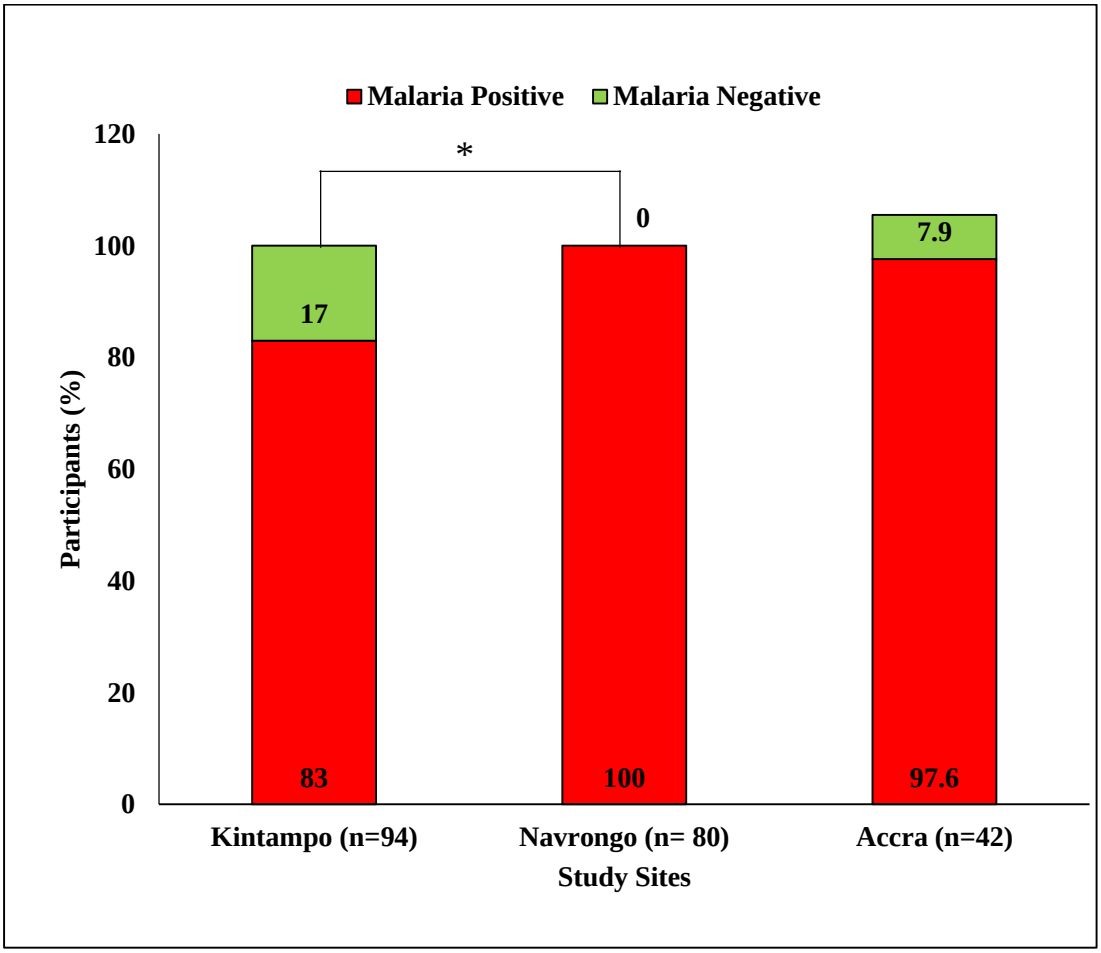

Figure 4. 5: Distribution of positive/negative malaria in study participants according to study sites

Study participants were diagnosed for malaria using both malaria rapid test and microscopy. Results presented are solely based on parasitaemias of study participants. Pararsitaemias (number of parasites/µL of blood) were calculated by counting number of parasites per 200 WBC on a thick blood smear. Data are presented as percentage of study participants testing positive or negative for malaria. * denotes statistically significant difference in malaria status between study participants in Kintampo and Navrongo (P=0.00).

4.6 Exposure to Dengue and Malaria

Out of the 216 study participants, 21.76% were confirmed with an exposure to both dengue and malaria while 70.37% were confirmed as having had an exposure to malaria

only. The number of study participants who had had an exposure to dengue only was 1.85% while 6.02% were confirmed without exposure to neither diseases. The trend was the same in Kintampo. Navrongo and Accra recorded no (0%) exposure and 2.4% exposure to dengue only, respectively (Figure 4.6). There was no statistically significant difference in proportions of malaria only, dengue only, absence of either disease or exposure to dengue-malaria across study sites.

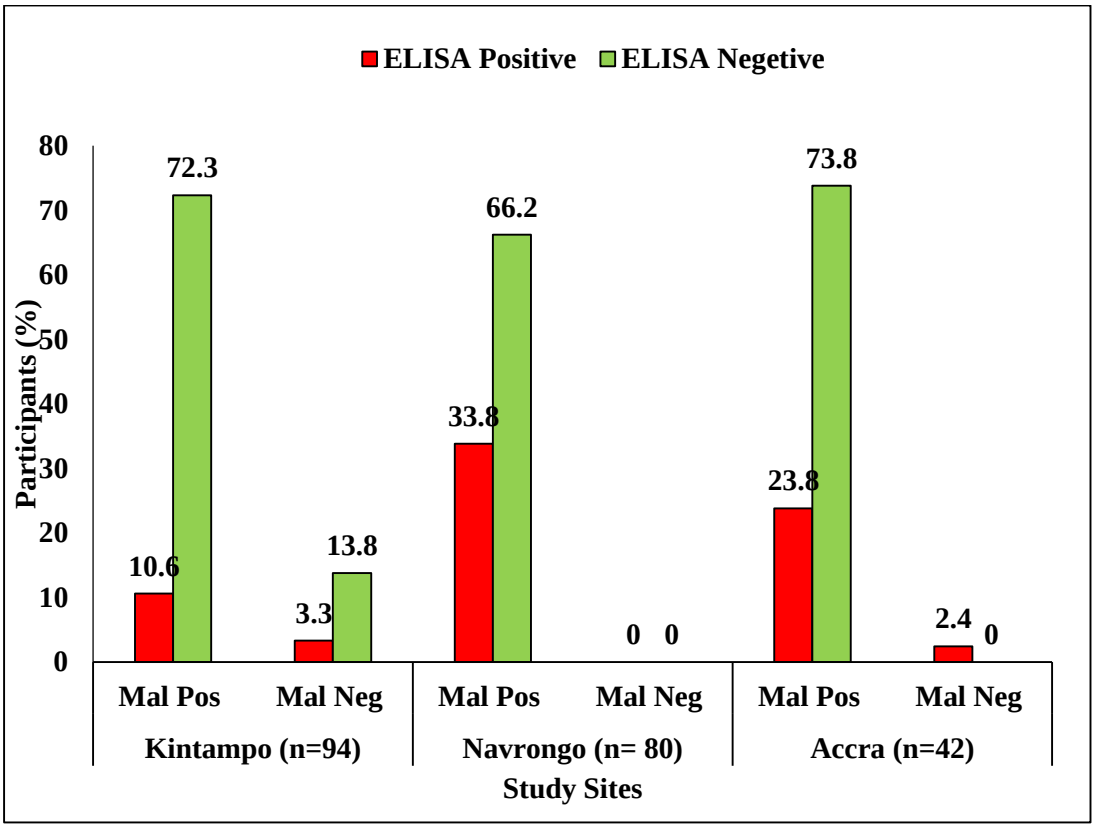

Figure 4. 6: Dengue and malaria co-exposure according to study sites
Malaria and dengue co-exposure analysis was based on the proportion of study participants who tested positive for malaria that also tested positive or negative for dengue. Co-exposure was defined as proportion of study participants who tested positive for both malaria and dengue. Data are presented as the percentage of study participants co-exposed to dengue and malaria or exposed to either disease alone. Missing parasitaemia data for 4 study participants were not included in the calculations.

4.7 Confirmation of Nucleic Acid Extraction via Nanodrop

To confirm successful extraction of nucleic acid from plasma samples, 17 randomly selected specimen were tested by Nanodrop for purity and quantification of concentrations (ng/µl) of the extracted viral RNA. All 17 showed high purity (2.9- 4.09) however, concentrations varied between 4.6- 80.5 ng/µl (Table 4.4).

4.8 Real-Time Reverse Transcriptase-Polymerase Chain Reaction

To detect viral RNA using serotype-specific primers and fluorogenic probes targeting specific regions of the viral genome, all 216 plasma samples tested negative. A specimen is considered positive for DENV 1, 2, 3, 4 if the fluorescent amplification curve crosses the threshold line within 37 cycles ($C_t \leq 37$ cycles). Figure 4.7 shows results for some selected samples after performing the assay. It can be observed that fluorescent amplification curves for samples were below threshold line while serotype-specific positive controls (PC 1, 2, 3 and 4) were above the threshold. Hence, all the positive controls exhibited the expected performance (i.e. fluorescent signal crossed the cycle threshold ($C_t \leq 37$). All non-template control did not cross the fluorescent amplification curve within 37 cycles The C_t values are inversely proportional to the amount of target nucleic acid in the sample (i.e. a low C_t value means greater amount of target nucleic acid in the sample).

Table 4. 4 Concentrations and Purity of extracted viral RNA

Sample ID	Nucleic Acid Concentration (ng/µl)	260/280 (nm)
8	35	3.44
28	14	3.5
38	67	3.4
52	4.6	2.9
57	11.3	3.52
79	63.9	3.46
84	72.5	3.39
97	74.9	3.46
110	8.2	4.09
128	68.7	3.46
148	80.5	3.47
151	69.7	3.73
163	74.5	3.54
188	72.1	3.19
200	73.1	3.5
201	65.6	3.37
202	13	3.25

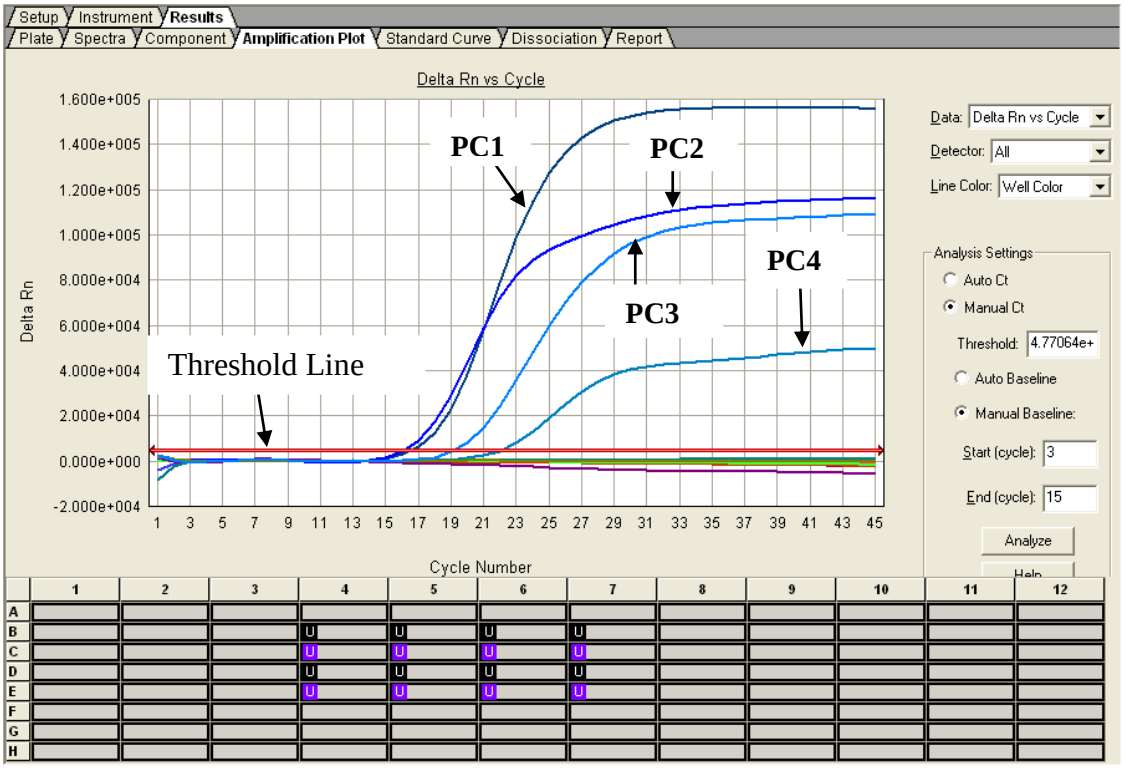

Figure 4. 7: Amplification plot for some selected samples
Non-template control for DENV 1, 2, 3 and 4 did not show amplification curve. "PC 1, 2, 3 and 4" denotes positive controls for DENV 1, 2, 3 and 4, respectively. A positive result is one with an amplification curve crossing the threshold line within 37 cycles. None of the tested samples had an amplification curve above the threshold line.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

5.1 Discussion

This is the first seroprevalence study to be conducted in Ghana covering the northern, middle and coastal belts of the country. The study sought to investigate the prevalence of dengue viral infection through laboratory diagnostic methods namely; dengue RDT, dengue IgG/IgM capture ELISAs and RT-PCR assays.

A total of 216 study participants between ages 2-14 were recruited. Recruitment was solely randomized and did not discriminate between males and females. Further categorization into 2-5 and 6-14 years, showed majority of the participants were within the 2-5 year group. Study participants were recruited only after testing positive for malaria by RDT, however, a few who tested positive by RDT but were later found to be negative by microscopy were included in the study. All the study participants presented with fever and the mean axillary temperature recorded confirms the presence of fever at the time of enrolment. The mean axillary temperature was statistically significant between participants in Accra and Kintampo/Navrongo.

The three study sites have different malaria transmission intensities and this is evident in the mean parasite densities recorded for the various study sites. Kintampo which is holoendemic has malaria transmission all year round (Dery *et al.*, 2010) and recorded the highest mean parasite density as compared to Navrongo where malaria transmission is highly seasonal and occurs mostly during rainy season (Appawu *et al.*, 2004). Though malaria transmission in Accra is seasonal, it is relatively lower than Navrongo. This may be due to patronage on the usage of insecticide treated mosquito nets by people

living in Accra as oppose to those in Navrongo. The mean parasite densities recorded in the various study sites were reflective of their respective malaria transmission intensities. The mean haemoglobin levels of the study participants at all study sites were within normal range (haemoglobin > 8.0g/dL).

Plasma samples were first screened for the presence of IgG or IgM antibodies to dengue serotypes 1-4 using the dengue RDT. The results showed that majority of study participants (54%) were positive for dengue specific antibodies. Interestingly, of the 54% seropositive study participants; 49% were positive for dengue IgM antibodies, 3% positive for dengue IgG antibodies and 2 % were positive for both dengue IgM and IgG antibodies. These results may have suggested an active dengue infection and an onset of a wave of dengue epidemic considering the high seropositivity of dengue IgM antibodies. However, the results of the dengue RDT could not be reliable because RDT was only used to screen for dengue specific antibodies prior to the use of the more expensive dengue ELISA.

To verify the results of the RDTs, the same plasma samples were tested with dengue IgG and IgM capture ELISAs. The results of the dengue ELISAs showed that 20.4% were positive for IgG antibodies while 2.3% were positive for IgM antibodies and 0.90% tested positive for both and this gives a prevalence of 24%. These results were considered more reliable than the RDT results since ELISAs are more specific than rapid tests, and remain the diagnostic tools of choice in seroprevalence and epidemiologic studies (Blaylock *et al.*, 2011).

A study conducted to evaluate the performance of different test kits against reference standard results showed that Focus ELISA demonstrated a strong agreement with

reference ELISA results and a high mean sensitivity (98.6%, 95% CI 98.0%-99.2%) (Hunsperger *et al.*, 2009). Reference ELISAs were designed by CDC and Armed Forces Research Institute of Medical Science (Hunsperger *et al.*, 2009). These results lend credence to the accuracy of the dengue ELISAs used in this study. This study showed a prevalence rate of 24% with dengue ELISAs. These results however are contrary to two similar studies conducted: one in Tanzania and Zanzibar in 2007 which showed a lower seroprevalence rate of 5% (Vairo *et al.*, 2012) and the other in Western Kenya which also showed a low seroprevalence of 1% (Blaylock *et al.*, 2011). Another study conducted in Sierra Leone between October 2006 and October 2008 using serum samples from suspected Lassa fever patients showed a 4.3% prevalence of DENV and 4.0% prevalence of CHIKV (Schoepp *et al.*, 2014). These differences in prevalence between the various studies could be due to variability in the study participants' inclusion criteria or diagnostic test used. Whereas this study recruited children as study participants, the above mentioned studies were conducted in adults.

The high seroprevalence rate recorded in this study as compared to other studies could also be attributed to the probability of a cross-reactivity of dengue ELISAs with antibodies specific for other flaviviruses or malaria. The phenomenon of cross-reactivity can be explained by similarities in antigenic epitopes on fusion loop (FL) of the domain II of E proteins capable of inducing cross-reactive host antibodies. The FL of E proteins are highly conserved in different flaviviruses and all four DENV serotypes (Calisher *et al.*, 1989; Lin *et al.*, 2012; Crill and Chang, 2004; Lai *et al.*, 2013).

Given the fact that Ghana is a malaria and yellow fever endemic country (World Health Organization, 2013), there is a potential for false-positive results when testing with

dengue IgM/IgG capture ELISAs. However, the odds of a cross-reaction with antibodies to yellow fever as a consequence of prior vaccination can be ruled out because a free national vaccination exercise conducted in November 2011 targeted people aged above 10 years excluding pregnant women, persons with HIV and AIDS, persons on anti-cancer treatment and steroid treatment (VibeGhana, 2011). Majority of the seropositive study participants were below 10 years (n=45, 87%) and hence were excluded in this exercise. However, there could still be the possibility of cross-reaction with antibodies to yellow fever as a consequence of natural infections. Houghton-Triviño *et al.*, (2008) in their study revealed that there was a high cross-reactivity between DENV and yellow fever. The study showed a 46% and 42% cross-reactivity when dengue IgM ELISA was tested on yellow fever patients and prior vaccinated participants, respectively (Houghton-Trivino *et al.*, 2008). Previous studies have also established cross-reactivity between DENV and yellow fever. Examples include a study evaluating PanBio DENV IgG ELISA which observed cross-reactivity with antibodies to yellow fever after a vaccination exercise (Schwartz *et al.*, 2000), while another study established a high cross-reactivity rate of 84% among flaviviruses: yellow fever, West Nile virus and Japanese encephalitis (Koraka *et al.*, 2002).

Chikungunya virus (CHIKV), transmitted by *Aedes agypti* and *Aedes albipoctus*, the mosquito vectors responsible for DENV transmission, share similar symptoms with DENV (Vairo *et al.*, 2012). Hence there is the probability that the high seroprevalence of DENV could also be as a result of cross-reaction with antibodies to CHIKV. A test to detect CHIKV or the other flaviviruses would have been helpful.

Primary (current) or secondary (previous) dengue infections can be distinguished by the dengue virus E/M protein-specific IgM/IgG ratio. A primary (current) infection is established if the IgM/IgG ratio is greater than 1.2 (using sera at 1:100 dilution factor) while a secondary (previous) infection is defined as IgM/IgG ratio less than 1.2 (Falconar *et al.*, 2006). The IgM/IgG (2.3%/20.4%) ratio calculated for this study was 0.11 indicating a secondary or previous exposure to dengue infection. This further suggests that majority of the study participants sought medical attention 10-15 days after the onset of fever while a few visited 5 days or within 2-3 months after onset of fever since dengue IgG and IgM antibodies are detectable within these time frames, respectively (Vaughn *et al.*, 2000).

Classification of seropositive study participants by gender showed that a slight majority were males as compared to females. However, these differences were not statistically significant. This is congruent with other studies conducted in tropical/subtropical countries which showed that males were more predisposed to dengue than females (Gupta *et al.*, 2006; Teixeira *et al.*, 2002; Goh, 1998). The Aedes mosquito bites in the day time (World Health Organization, 2009). A possible explanation to the observation that males are more predisposed to dengue infection that females could be that males are more active and tend to engage in outdoor games which exposes them to the bites of Aedes mosquitoes more frequently than females.

Majority of the seropositive study participants were within the 2-5 years old age group. However, the observation that more of the seropositive study participants were within the 2-5 year age group was not consistent with that of a study conducted in Pakistan which showed a higher prevalence of dengue (IgM=39.4%, IgG=22.4%) in the age

group of 21-30 as compared to ≤ 10 years and older age groups (≥ 51 years) (Ali *et al.*, 2013). The Pakistan study used ELISA kits to test for dengue specific IgM and IgG antibodies in 612 study participants. The differences observed between the two studies highlights the limitations on the choice of age brackets and the sample size used in this study.

Dengue and malaria are two very important diseases transmitted by mosquitoes. Dengue diagnosis has remained off the diagnostic lens of many clinicians largely due to similar clinical and laboratory characteristics it shares with malaria thereby contributing to the over-diagnosis of the later (Assir *et al.*, 2014). Out of 216 study participants, the percentages of an exposure to malaria only, dengue only, dengue-malaria and absence of either diseases were 70.37%, 1.85%, 21.76% and 6.02%, respectively. These results were not consistent with those of a study conducted in Pakistan by Assir *et al*., 2014 which showed that out of 52 probable cases of dengue, 35% were isolated malaria, 10% were isolated dengue and 33% were mixed dengue and malaria infection.

Viral RNA detection with RT-PCR tested negative for all 216 plasma samples. A possible explanation to this could be that the samples were not obtained during the acute phase of the disease. RT-PCR is very sensitive and produces the highest yield during 0-5 days after onset of disease. However, sensitivity of RT-PCR decreases over the course of the illness (Peeling *et al.*, 2010; Vaughn *et al.*, 2000). The detection of higher percentages of IgG antibodies in serum indicate that study participants were previously exposed to dengue and the viral RNA might have been cleared from their blood hence the inability of RT-PCR to detect the viral RNA. Since there was successful extraction

of nucleic acid from plasma specimen (Table 4.4), it stands to suggest that another possible explanation to the unsuccessful detection of viral RNA could also be due to contamination by ribonuclease (RNase). This could be from the field when samples were collected, during extraction of viral RNA or during addition of vRNA to master mix for RT-PCR assay.

5.2 Conclusion

The results from the study suggest an exposure to dengue virus in febrile Ghanaian children with a prevalence of 24%. Majority of the seropositive participant were positive for IgG and recorded an IgM/IgG ratio of 0.11 further confirming a previous dengue exposure. Difference in IgG proportions were statistically significant only between participants in Kintampo and Navrongo. The rate of dengue-malaria co-exposure was moderately high (22% ~ 1 in every 4) in malaria confirmed study participants although detection of viral RNA was negative for all study participants suggesting that none of the study participants had an acute dengue infection.

5.3 Limitations

- The sample size used in the study was too small and thus cannot reflect the entire population. Hence inferred prevalence may either be under- or over-estimated.
- Despite the high IgG recorded by the dengue IgG ELISA, plaque reduction neutralisation test (PRNT) which would eliminate the potential for cross-reactivity with antibodies for other flaviviruses in the plasma samples was not considered at the time.

5.4 Recommendations

- In order to determine the real magnitude of the problem, there is the need to conduct further seroprevalence studies on a large scale and increase the age limit to include older aged participants.
- In future studies, plaque reduction neutralisation test (PRNT) should be considered as an option to eliminate the possibility of a cross-reactivity between dengue antibodies and antibodies for other flaviviruses as was highlighted as a limitation of the dengue ELISAs.
- There is also the need to embark on a strong campaign to sensitize the Ghanaian populace about the threat of dengue virus and also to draw-up and implement rigorous preventive measures to avert the spread of the disease.

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APPENDIX

1.1 Focus Diagnostic Dengue Virus IgM Capture DxSelect™

Materials supplied

The Focus Diagnostics Dengue Virus IgM DxSelect™ Test kit contains material to perform 96 determinants. Before they were used, supplied reagents which were stored at 20 C were allowed to adjust to room temperature.

- **Antigen, lyophilized**: 2 vials containing inactivated lyophilized dengue virus antigen (equal portions of DENV 1-4). Each 6ml antigen vial could perform approximately 60 tests. To reconstitute the antigen, exactly 6ml of reconstitution solution provided was added and allowed to rehydrate at room temperature.
- **Antigen reconstitution solution, 13ml**: 1 vial containing cell culture water and 0.1% sodium azide.
- **IgM Capture Wells, 96 wells**: 12 Eight-well polystyrene break-apart microwell strips on a frame. Each well is coated with anti-human IgM. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets.
- **IgM Conjugate, 16 mL**: 1 vial of affinity-purified and peroxidase-conjugated mouse anti-flavivirus. Contains protein, buffer, and preservatives.
- **IgM Detectable Control, 0.30 mL**: 1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

- **Non-Detectable Control, 0.30 mL**: 1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).
- **IgM Cut-Off Calibrator, 0.30 mL**: 1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).
- **Sample Diluent, 100 mL**: 1 vial of protein, surfactant, and preservatives in PBS.
- **10X Wash Buffer, 100 mL**: 1 vial of surfactant in PBS with preservatives. Prepare a 1X wash buffer solution before use. To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled (or deionized) water and rinse out any crystals. Use only the highest grade purified water for reconstitution of the wash buffer. It has been observed that some sources of deionized water contain materials which can interfere in the assay. Swirl until well mixed and all crystals are dissolved.
- **Substrate Reagent, 16 mL**: 1 vial of tetramethylbenzidine (TMB) and horseradish peroxide in buffer. A dark blue colour indicates contamination with peroxidase; and, if this occurs, use a fresh bottle. Ready to use.
- **Stop Reagent, 16 mL**: 1 vial 1 M sulfuric acid.
- **Sealing Tape**: 3 sheets of sealing tape.

MATERIALS REQUIRED, BUT NOT SUPPLIED

- Distilled water
- 250 or 500 mL wash bottle

- 1 L graduated cylinder
- Test tubes for serum dilutions
- 10 µL pipettors with disposable tips
- 100 µL pipettors with disposable tips (100 µL 8- or 12-channel pipettor recommended for runs over 48 wells)
- 1 mL pipet or dispenser
- 5 mL pipet
- Timer
- Paper towels or absorbent paper
- Sink
- Vortex mixer or equivalent
- ELISA plate spectrophotometer, wavelength = 450 nm

1.2 Focus Diagnostic Dengue Virus IgM Capture DxSelect™

Materials supplied

The Focus Diagnostics Dengue Virus IgG DxSelect™ Test kit contains sufficient materials to perform 96 determinations. Allow the supplied reagents to warm to room temperature before use. All un-opened materials are stable at 2 to 8°C until the expiration date stated on the reagent label.

- **IgG Antigen Wells, 96 wells:** 12 eight-well polystyrene microwell strips on a frame. Each well is coated with equal proportions of inactivated and purified dengue virus types 1-4. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets.

- **IgG Conjugate, 16 mL:** 1 vial of affinity-purified and peroxidase-conjugated goat anti-human IgG (Fc fragment specific). Contains protein, buffer, and preservatives.

- **IgG Detectable Control, 0.30 mL:** 1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

- **Non-Detectable Control, 0.30 mL:** 1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

- **IgG Cut-off Calibrator, 0.30 mL:** 1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

- **Sample Diluent, 100 mL:** 1 vial of protein, surfactant, and preservatives in PBS.
- **10X Wash Buffer, 100 mL:** 1 vial of surfactant in PBS with preservatives. Prepare a 1X wash buffer solution before use. To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled (or deionized) water and rinse out any crystals. Use only the highest grade purified water for reconstitution of the wash buffer. It has been observed that some sources of deionized water contain materials which can interfere in the assay. Swirl until well mixed and all crystals are dissolved.

- **Substrate Reagent, 16 mL:** 1 vial of tetramethylbenzidine (TMB) and hydrogen peroxide in buffer. The Substrate Reagent may turn slightly blue while stored cold, however, the colour should return to slightly amber after warming to room temperature. A dark blue colour indicates contamination with peroxidase; and, if this occurs, use a fresh bottle.

- **Stop Reagent, 16 mL:** 1 vial 1 M sulfuric acid.

- **Sealing Tape:** 2 sheets of sealing tape.

MATERIALS REQUIRED, BUT NOT SUPPLIED

1. Distilled water

2. 250 or 500 mL wash bottle

3. 1 L graduated cylinder

4. Test tubes for serum dilutions

5. 10 µL pipettors with disposable tips

6. 100 µL pipettors with disposable tips (100 µL 8- or 12-channel pipettor recommended for runs over 48 wells)

7. 1 mL pipet or dispenser

8. 5 mL pipet

9. Timer

10. Paper towels or absorbent paper

11. Sink

12. Vortex mixer or equivalent

13. ELISA plate spectrophotometer, wavelength = 450 nm

Table A 1: Summary Statistics of Axillary temperature, Parasite densities and Haemoglobin (Hb) levels

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Hb level	Kintampo	94	10.06	1.658	0.175	9.71	10.40	5	14
	Navrongo	80	10.03	1.632	0.180	9.67	10.39	5	13
	Accra	42	10.71	1.849	0.285	10.13	11.29	6	14
	Total	216	10.17	1.700	0.116	9.94	10.40	5	14
Parasite density	Kintampo	94	118236.16	155516.273	16213.692	86029.66	150442.67	0	752100
	Navrongo	80	44005.29	34888.082	3852.744	36339.54	51671.04	1617	206000
	Accra	42	20738.10	21559.304	3326.673	14019.74	27456.45	761	78840
	Total	216	71097.97	111847.956	7610.289	56097.64	86098.30	0	752100
Axillary Temperature	Kintampo	94	37.68	1.240	.129	37.43	37.94	35	40
	Navrongo	80	37.72	2.594	.290	37.14	38.29	17	40
	Accra	42	38.81	.931	.157	38.49	39.13	37	40
	Total	216	37.89	1.889	.131	37.63	38.14	17	40

“N” denotes number of enrolled study participants

Table A 2: Analysis of variance (ANOVA) for Axillary temperature, Parasite density and Haemoglobin levels

		Sum of Squares	Df	Mean Square	F-Statistcs	p-value
Axillary Temperature	Between Groups	36.073	2	18.037	5.264	.006
	Within Groups	702.476	205	3.427		
	Total	738.550	207			
Parasite density	Between Groups	371130843271.624	2	185565421635.812	17.048	.000
	Within Groups	2318511707957.148	213	10885031492.757		
	Total	2689642551228.773	215			
Hb Level	Between Groups	15.007	2	7.504	2.637	0.074
	Within Groups	600.398	211	2.845		
	Total	615.405	213			

Table A 3: Bonferroni multiple comparison of Haemoglobin level, Parasite density and Axillary Temperature

Dependent Variable	(I) Site	(J) Site	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
						Lower Bound	Upper Bound
Hb level	Kintampo	Navarongo	0.025	0.258	1.000	-0.60	0.65
		Accra	-0.654	0.315	0.118	-1.41	0.11
	Navarongo	Kintampo	-0.025	0.258	1.000	-0.65	0.60
		Accra	-0.679	0.320	0.105	-1.45	0.09
	Accra	Kintampo	0.654	0.315	0.118	-0.11	1.41
		Navarongo	0.679	0.320	0.105	-0.09	1.45
Parasite density	Kintampo	Navarongo	74230.870*	15844.868	.000	35996.63	112465.11
		Accra	97498.068*	19428.921	.000	50615.38	144380.76
	Navarongo	Kintampo	-74230.870*	15844.868	.000	-112465.11	-35996.63
		Accra	23267.197	19796.760	.724	-24503.10	71037.50
	Accra	Kintampo	-97498.068*	19428.921	.000	-144380.76	-50615.38
		Navarongo	-23267.197	19796.760	.724	-71037.50	24503.10
Axillary Temperature	Kintampo	Navrongo	-.030	.282	1.000	-.71	.65
		Accra	-1.126*	.367	.007	-2.01	-.24
	Navrongo	Kintampo	.030	.282	1.000	-.65	.71
		Accra	-1.096*	.375	.012	-2.00	-.19
	Accra	Kintampo	1.126*	.367	.007	.24	2.01
		Navrongo	1.096*	.375	.012	.19	2.00
*. The mean difference is significant at the 0.05 level.							

Table A 4: Association between RDT, ELISA, prevalence of IgG/IgM and Study sites

	Study site N (%)			
RDT	Kintampo (n=94)	Navrongo (n=80)	Accra (n=42)	p-value
Positive	54 (57.4)	37 (46.2)	25 (59.5)	
Negative	40 (42.6)	43 (53.8)	17 (40.5)	0.213
ELISA				
Positive	13 (13.8)$_a$	27 (33.8)$_b$	11 (26.2)$_{ab}$	
Negative	81 (86.2)$_a$	53 (66.2)$_b$	31 (73.8)$_{ab}$	0.006
IgG/IgM ELISA				
Negative	81 (86.2)	53 (65.3)	31 (73.8)	
IgM	1 (1.1)$_a$	3 (3.8)$_a$	1 (2.4)$_a$	
IgG	12 (12.8)$_a$	22(27.5)$_b$	10 (23.8)$_{ab}$	
Both IgG and IgM	0 (0.0)	2 (2.5)	0 (0.0)	0.031

Chi-squared test with Yates' correction for continuity used to test the relationship between two classification factors (e.g. RDT result and study site). The null hypothesis is that the two factors are independent. If the calculated P-value is small (<0.05), then the null hypothesis is rejected and the alternative hypothesis that there is a relation (association) between the two factors must be accepted. For significant associations, a z-test was used to compare column proportions (e.g. positive ELISA in Kintampo vs positive ELISA in Accra). Alphabetic subscripts were used to denote subsets of study site categories whose column proportions differ significantly from each other at the 0.05 level. Two columns with the same subscript show that the respective column proportions are not significantly different whereas different subscript prove significantly different proportions. A significant association exist between ELISA seropositive participants in Kintampo and Navrongo (p=0.006) but not either with Accra. Also there is a significant association between IgG positive participants in Kintampo and Navrongo (p=0.031).

Table A 5: Association between RDT and ELISA

		ELISA, N (%)			
		positive	Negative	Total	p-value
RDT	Positive	22 (43.1)	94 (57.0)	116 (53.7)	
	Negative	29 (56.9)	71 (43.0)	100 (46.3)	
	Total	51 (100)	165 (100)	216 (100)	0.081

Table A 6: Sensitivity and specificity of RDT

Sensitivity (%)	42.31	28.73 -56.80
Specificity (%)	43.45	35.84 - 51.30
Positive Likelihood Ratio	0.75	0.53 - 1.06
Negative Likelihood Ratio	1.33	0.99 - 1.77
Disease prevalence (%)	23.64	18.19 - 29.81
Positive Predictive Value (%)	18.80	12.18 - 27.07
Negative Predictive Value (%)	70.87	61.10 - 79.41

Table A 7: Dengue-malaria co-exposure according to study sites

	Site		
	Kintampo, N (%)	Navrongo, N (%)	Accra, N (%)
Dengue and Malaria	10 (10.6) $_{a}$	27 (33.8) $_{a}$	10 (23.8)$_{a}$
Malaria only	68 (72.3)$_{a}$	53 (66.2)$_{a}$	31 (73.8)$_{a}$
Dengue only	3 (3.2)a	0 (0)a	1(2.4)a
Absence of either disease	13 (13.8)a	0 (0)a	0 (0)a
TOTAL	94 (100)	80 (100)	42 (100)

"N" denotes number of study participants. "a" denotes subsets of study site categories whose column proportions differ significantly from each other at the 0.05 level. Two columns with the same subscript show that the respective column proportions are not significantly different whereas different subscript prove significantly different proportions.