

**UNIVERSITY OF GHANA**

**COLLEGE OF HEALTH SCIENCES**

**DETECTION OF DENGUE AND CHIKUNGUNYA VIRUSES IN FEBRILE  
PATIENTS AT GREATER ACCRA REGIONAL HOSPITAL**

**BY**

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**A THESIS SUBMITTED TO THE DEPARTMENT OF MEDICAL  
MICROBIOLOGY OF THE UNIVERSITY OF GHANA IN PARTIAL  
FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF A  
MASTER OF PHILOSOPHY DEGREE IN MICROBIOLOGY**

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**DECLARATION**

This study was carried out at the Virology Department, Noguchi Memorial Institute for Medical Research under the supervision of Prof. Theophilus Adiku and Dr. Joseph Humphrey Kofi Bonney. Work from other authors that were used were cited in the text and referenced. I hereby declare that, this thesis is as a result of my own research. This work neither in whole nor in part had been submitted for another degree elsewhere.

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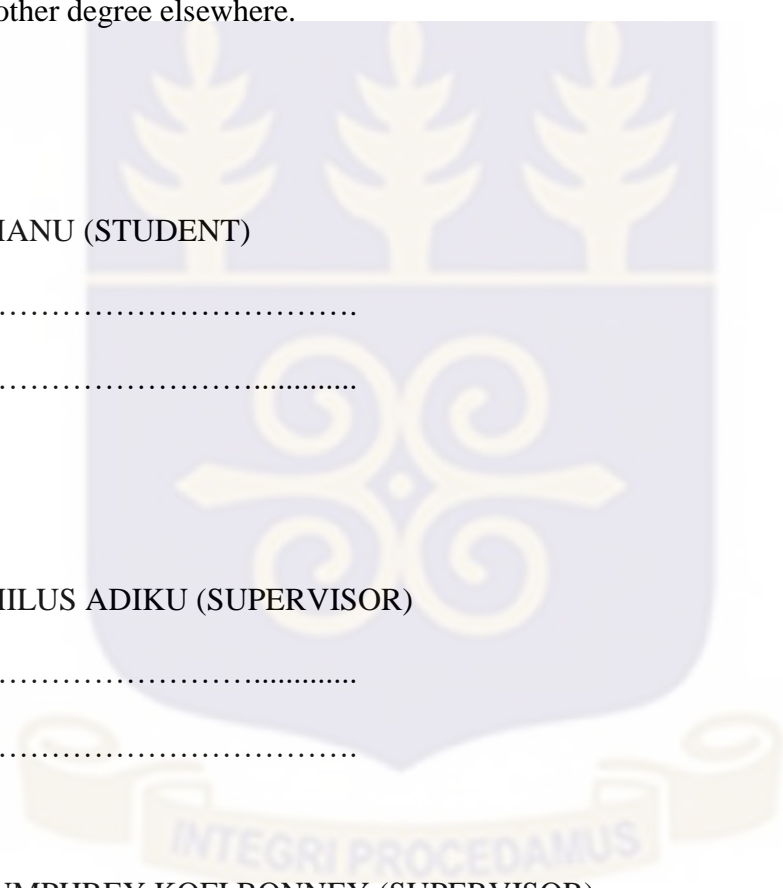
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## **DEDICATION**

This work is dedicated to my wife, Abena Anokyewaa Kyeremeh Manu.



## **ACKNOWLEDGEMENT**

I would like to thank Jehovah God for the strength to bring this work to fruition.

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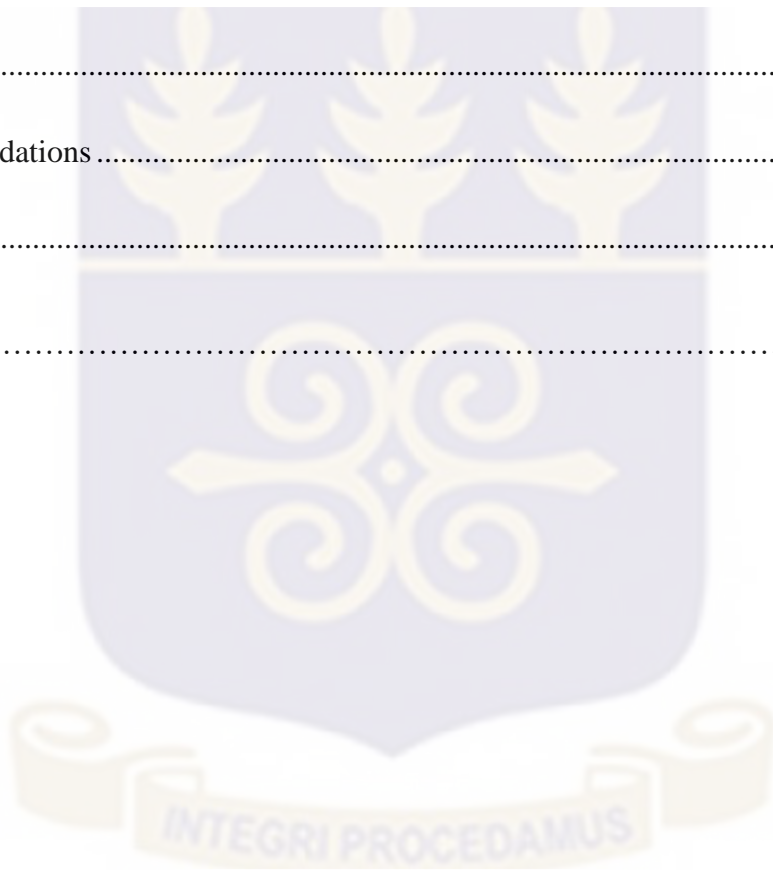
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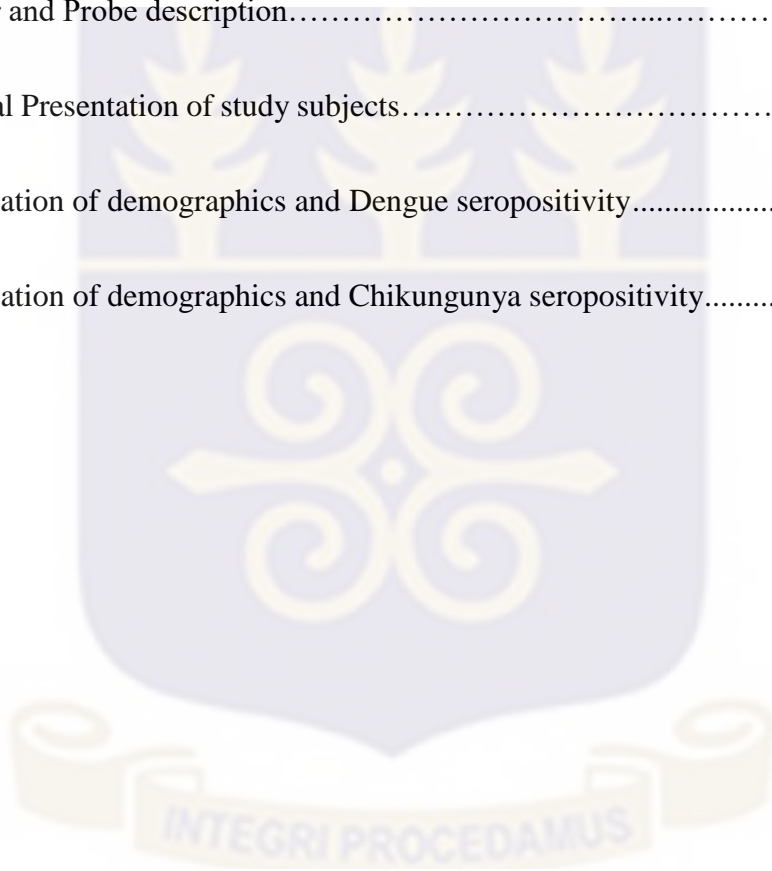
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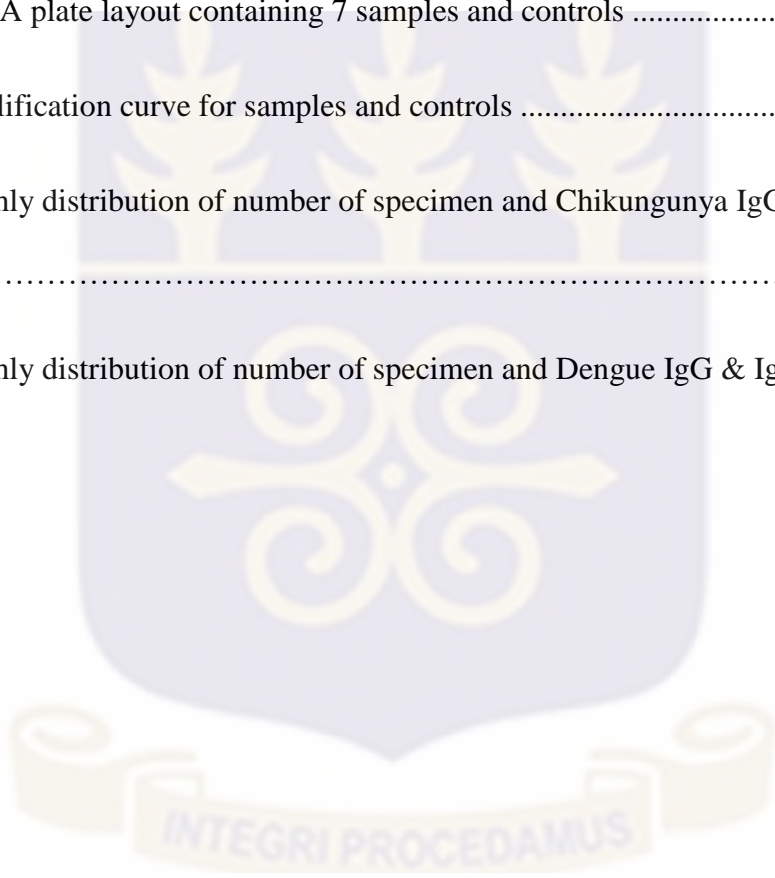
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## LIST OF ABBREVIATIONS

BHQ - Black Hole Quencher

CYD -TDV - Chimeric Dengue vaccine in a yellow fever 17D backbone

Df - Dengue fever

DHf - Dengue hemorrhagic fever

ELISA - Enzyme Linked Immunosorbent Assay

FAM - Fluorescein

HEX- Hexachloro-Fluorescein

HRP - Horseradish peroxidase

IgG- Immunoglobulin G

IgM- Immunoglobulin M

KDa - kilodalton

Ns - Non-structural protein

PCR - Polymerase Chain Reaction

PFU - Plaque-forming unit

Rpm - Revolutions per minute

rRt - Real time Reverse Transcriptase

TMB - 3, 3', 5, 5'-Tetramethylbenzidine

UTR-Untranslated region

## ABSTRACT

**Background:** Dengue and Chikungunya viruses have become major international public health concerns due to their epidemics and introduction of these viruses in new areas. Reemergence of these arboviruses exert enormous burden on populations, health systems and economies in most tropical countries. In Ghana, little is known about Dengue and Chikungunya viruses though the country has been enlisted as part of the 34 countries to which the viruses are endemic. Studies and information about these viruses are lacking though the population of the vectors are likely to become wide spread due and there is lack of investigation tools for these viruses in Hospitals and Clinics in the country.

**Aim:** To detect Dengue and Chikungunya viral markers among febrile patients, seen at Greater Accra Regional Hospital, Ghana.

**Method:** This was a hospital-based cross sectional study where whole blood was collected from febrile patients suspected of having Dengue or Chikungunya viral infection at the Greater Accra Regional Hospital. Plasma was used to test for the viral markers of Dengue and Chikungunya using Real-time Reverse Transcriptase PCR, ELISA IgG and IgM and NS1 antigen for Dengue and Chikungunya viruses. Clinical and laboratory test data on patients were also collected and analyzed.

**Results:** A total of 260 febrile patients attending Greater Accra Regional Hospital suspected of Dengue, Chikungunya viral infections or both were recruited over a period of 11 months starting from May 2016 with most of the samples coming between October and December. The study participants comprised of 84 (32%) males and 176 (68%) females. Data on the clinical presentation indicated that frequently reported symptoms other than fever were loss of appetite (92%), joint pain (86%) and muscle pain (85%).

In all, the prevalence of Chikungunya specific antibodies was 72 (27.69%) and Dengue specific antibodies was 180 (69.23%). None of the participants tested positive for Dengue by rRT-PCR and NS1 antigen testing. None also tested positive for Chikungunya by rRT-PCR.

**Conclusion:** This study provides evidence that Dengue and Chikungunya viruses are circulating in Ghana. High levels of Dengue and Chikungunya antibodies or patient exposure to these viral agents were observed, which suggests transmission at the study site. Differential diagnosis work-up in febrile patients should be made to include Dengue and Chikungunya infections.





## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 General Introduction

Dengue and Chikungunya viruses have become major international public health concern due to their epidemics and introduction of the viruses in new areas. Due to its associated increase in the incidence, distribution and clinical severity of the disease linked with these viruses, they are on the priority lists of the World Health Organization (WHO), United Nation Children Fund (UNICEF), United Nation Environment Program (UNEP), World bank and WHO special program for Research and Training in Tropical Disease (TDR) (Restrepo, Baker, & Clements, 2014).

Dengue virus is the most rapidly spreading and frequently encountered mosquito-borne viral infection in the world. The incidence of Dengue viral infection has increased 30 fold over the last decade with increasing geographical expansion to other countries with explosive outbreaks (WHO, 2009). Before 1970, severe dengue epidemics had been reported from only 9 countries but currently the virus is endemic in more than 100 countries in the WHO regions of Africa, the East Mediterranean, South East Asia and Western Pacific (WHO, 2017). Almost 2.5 billion people live in endemic areas resulting annually in an estimated 50 to 100 million cases of Dengue fever (Df), 250, 000 to 500,000 cases of Dengue haemorrhagic fever (Dhf) causing 20,000 deaths and 264 disability adjusted life years per million population per year are lost(Wichmann, Mühlberger, & Jelinek, 2003).

Chikungunya has been reported from over 60 countries in Africa, Asia, Europe and the Americas. After its detection in 1953 from Africa , the virus has been circulating at a relatively low levels in Africa till 2000 where large outbreaks occurred in Democratic Republic of Congo, Gabon and Kenya (WHO, 2016).

Re-emergence of these arboviruses exert enormous burden on populations, health systems and economies in most tropical countries. The spread of these viruses to the Americas, Africa, East Mediterranean regions and Asia represent a global pandemic threat (WHO, 2011). Reemergence of these viruses in areas where the incidence had been controlled or eradicated is largely due to vector management associated with reduced allocation of funds towards vector and increased resistance in mosquitoes (Charette, Berrang-Ford, Llanos-Cuentas, Cárcamo, & Kulkarni, 2017).

Dengue and Chikungunya viruses share vectors and have similar transmission patterns. Both viruses are transmitted by *Aedes aegypti* and *Aedes albopictus* which have adapted to peri-domestic setting making transmission more pronounced in the tropical urban centres with high population. More than 50 % of the world's population lives in areas infested with these mosquitoes. The presence of these vectors together in high population density represent a high probability of an outbreak and spread of these viruses (Musso, Van Mai, & Gubler, 2015).

## **1.2 Problem Statement**

Transmission of Dengue and Chikungunya viruses have been reported to be endemic in 34 countries in all African regions (Amarasinghe, Kuritsky, Letson, & Margolis, 2011). These viral infections have also been diagnosed in travelers from Europe and North America returning from several West Africa countries including Ghana (Baronti, Touret, Lamballerie, Nougairède, & Piorkowski, 2016)(Huhtamo *et al.*, 2008).

Re-emergence of these viruses now poses greater risk due to the increase in number of cases and its associated severity and complications. Though the disease burden has increased, Dengue and

Chikungunya epidemiology in African region have not well been documented. There is paucity of information, public health interventions and health system preparedness.

Many febrile cases are presumptively diagnosed and treated for malaria due to lack of investigation tools for these viruses in hospital facilities. Enormous attention given to malaria and malaria burden in Africa contributes to a minor concern for Dengue and Chikungunya cases (D'Acremont, Lengeler, & Genton, 2010).

Severe form of the illnesses caused by Dengue virus (DENV) and Chikungunya virus (CHIKV) especially the latter is often characterized by haemorrhagic manifestations. However in Ghana, haemorrhagic manifestations are routinely tested for Yellow fever without testing for other probable infections like Dengue and Chikungunya though they share a common vector.

Work done by Appawu *et al.*, 2006, identify Ghana as a potential risk for transmission of arbovirus including DENV and CHIKV due to high larval indices and biting rates of *Aedes aegypti* recorded in the study areas yet DENV and CHIKV are not part of the routine workout in the hospital laboratory investigations.

Although Dengue and Chikungunya virus detection have not been documented in Ghana, it has been reported in neighboring countries, Burkina Faso and Cote d'Ivoire. On 22nd November 2016, Ghana received a WHO notification and alert of Dengue fever outbreak in Burkina Faso ( Ministry of Health, Ghana, 2016 ) With increasing movement of people across borders between Ghana and neighboring countries and the presence of the transmitting vector, it is likely there has been exposures with undetected apparent and/or subclinical infections in the country.

### **1.3 Justification**

Dengue and Chikungunya cases share symptoms as malaria and can easily be misdiagnosed as malaria. Misdiagnosis hinders epidemiological importance of Dengue and Chikungunya diseases and greatly affect the clinical picture of, and outcome for infected patients. Again misdiagnosis risk delaying of specific supportive treatment and in the case of Dengue can lead to ten-fold impact on the likelihood to the progression of Dengue fever to Dengue haemorrhagic fever which can lead to death. This therefore place much importance on the investigation of non-malaria cause of fever such as Dengue and Chikungunya which are not part of the routine hospital laboratory investigation in Ghana which this study seeks to address.

This study may inform authorities and policy makers to include Dengue and Chikungunya in the differential diagnoses of unexplained febrile cases in Ghana. This will improve awareness of these agents, case detection and management, promote surveillance and prevent inappropriate prescription of non-steroidal anti-inflammatory drugs which could lead to severe bleeding in patients with severe thrombocytopenia.

Data from this study to some extent supports the role played by Dengue and Chikungunya virus in the occurrence of febrile illnesses in Ghana and highlight the circulation of these arthropod borne infections other than malaria.

#### **1.4 Aim**

To determine the prevalence of Dengue and Chikungunya viral infections among febrile non-malaria cases at a secondary health facility.

#### **1.5 Objectives**

- To determine the prevalence of Dengue and Chikungunya viral IgM and IgG.
- To determine active infections of Dengue and Chikungunya viruses.



## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Dengue virus

Dengue virus is a member of the family *Flaviviridae* and of the genus *Flavivirus*. It is a spherical virus of about 50 nanometers in diameter consisting of 3 structural proteins; the capsid (C), the pre membrane/membrane (prM/M) and an envelope (E). The 10.7 kilo base positive sense single stranded ribonucleic acid (RNA) genome also encodes for 7 non-structural proteins ( NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The order of gene is in 5-CprM(M)-E-NS1-NS2A-NS2b-NS3-NS4A-NS4B-NS5-3 (Diamond & Pierson, 2015). Upon infection with the virus, it activates the interferon signaling pathway but the virus develops resistance by NS2A, NS4A and NS4B that blocks the interferon cascade to escape the immune response (Idrees & Ashfaq, 2012).

There are 4 known serotypes of the Dengue virus circulating in the tropical and subtropical region in the world. These serotypes (Dengue virus 1 -4) differ from one another by 25-40% amino acid level (Bhatt *et al.*, 2013). The co-circulation of diverse serotypes is common in hyperendemic regions and mostly an outbreak arise from a dominant serotype in a 2-4 year cycle (Moi, Takasaki & Kurane, 2016).

The serotypes are further separated into subtypes that differ by 3%. There are 3 subtypes of Dengue virus 1, 6 for Dengue virus 2 (one of which is found in non-human primates), 4 for Dengue virus 3 and 4 for the serotype 4. Phylogenetic studies have shown that subtypes normally circulate within a defined area (Lambrechts *et al.*, 2012).

### **2.1.1 Epidemiology and burden of Dengue virus**

Dengue viral infection is an important public health concern in both developing and developed countries due to the morbidity and mortality associated with the virus. The virus is spreading at an alarming rate and the disease incidence has increased 30 fold over the last decade (WHO, 2017). Before 1970, 9 countries had reported cases of severe dengue, the disease has spread to more than 100 countries in Asia, Africa, Europe and the Pacific after 2014 (WHO, 2017). In 2010, it was estimated that about 2.5 billion people live in endemic countries resulting annually in an estimated 50 to 100 million Dengue fever infections (Wichmann *et al.*, 2003). There were approximately 390 million Dengue infections worldwide in 2010, of which 96 million manifest clinically. Of the apparent infections, 70% occurred in Asia and 14% in Africa and America each (Brady *et al.*, 2012). Dengue infections are underreported in Africa due to inadequate surveillance and difficulty in the differential diagnoses as against other endemic infections in the continent (Bhatt *et al.*, 2013).

The average number of severe cases of Dengue reported to WHO continue to increase exponentially from an average of 1,000 cases in 1950 to more than 3 million cases per year globally in 2013. From 2000 to 2008, 1,656,870 Dengue fever/Dengue Haemorrhagic fever (DF/DHF) cases were reported to WHO compared to 479,848 cases in 1990 to 1999. A record of 69 countries from the WHO regions of South-East Asia (SEA), Western Pacific (WP) and the Americas in 2008 reported cases of Dengue fever (WHO, 2011). It is estimated that 9,221 people died of Dengue infections per year between 1990 and 2013. Mortality rate is higher in children less than 1 year and in patients more than 45 years. A total of 576,900 years of life lost to premature mortality (YLL) and 56,600 years lived with disability (YLD) is credited to Dengue in 2013 (Stanaway *et al.*, 2016). In 2015, WHO regions of SEA, WP and Americas reported 3.2 million dengue activity

with 2.5 million of these cases from the Americas alone of which 10,200 were diagnosed as severe resulting in 1181 deaths (WHO, 2017).

Dengue is hyperendemic in South East Asia. In 2013, 8 countries in the region had reported cases of Dengue by 2009 the infection has spread to all member countries except Democratic people of Korea. Timor-Leste, Bhutan, Nepal, Indonesia, all in SEA reported outbreaks in 2004 (WHO, 2011). Indonesia in 2004 reported 58,301 cases of Df/DHf resulting in 658 deaths thus case fatality rate of 1.1% (WHO, 2004). In Asia, trends indicate surge in Dengue cases in China, the Cook Island, Fiji, Malaysia and Vanatu. In 2015, Malaysia reported 111,000 suspected Dengue cases, 16% higher than the number of cases reported in 2014. India in 2015 also recorded its worst outbreak of Dengue since 2006 (WHO, 2017).

Dengue also continues to affect several American countries despite concerted Dengue control efforts. Epidemics have occurred in Mexico, Honduras, Costa Rica, Brazil, Ecuador and Venezuela. Of the 14.2 million cases of Dengue with 7000 deaths reported in this region between 2000 and 2014, 70% were from Brazil, Columbia and Mexico (PAHO, 2014).

In Europe reports of Dengue have been cases imported by travelers or expatriates returning from endemic countries. According to European Network on Imported Infectious Diseases Surveillance (TropNetEurop), the number of cases increased from 64 in 1999 to 224 in 2002. For 2008, there was a slight decline to 116 cases (Jelinek, 2009). Among travel-acquired Dengue virus cases reported from Europe, 45% was acquired by patients who travelled to countries in South East Asia, 19% from South and Central America, 16% from India, 12% from the Caribbean and 8% from Africa (Wichmann *et al.*, 2003). Ghana and other 26 African countries have been reported as locations where expatriates and travelers from countries which Dengue was not endemic has

acquired the infection (Amarasinghe *et al.*, 2011). In Europe , Dengue has been ranked second after Malaria for frequent admission into hospital after returning from abroad (WHO, 2014).

Imported viral cases coupled to competent vectors and immunological naïve individuals have led to the report of autochthonous cases in Europe. France and Croatia reported 2 and 17 autochthonous cases in 2010 respectively. The outbreak of Dengue in Portugal in 2012 led to the report of 2,200 confirmed laboratory cases (Schaffner & Mathis, 2014).

In Africa, after the virus was first detected in 1960 in Nigeria, it caused outbreak in more than 20 countries (Amarasinghe *et al.*, 2011). Thousand and sixty six (1066) probable cases and case fatality rate of 1.2% has been reported from the recent outbreak in Burkina Faso (WHO, 2016). Literature suggests that the virus is endemic in 34 countries in all regions of Africa. Twenty two (22) out of the 34 have reported confirmed laboratory cases (Amarasinghe *et al.*, 2011). In Ghana a seroprevalence of 3.2 % and 21.6% to IgM and IgG respectively to the virus were detected in 2015 in malaria parasite positive children in urban centres in Ghana which suggest exposure and transmission of the virus (Stoler *et al.*, 2015).

### **2.1.2 Clinical manifestations**

After an incubation period of 4 to 10 days, infection by any of the 4 serotypes can cause a wide range of illnesses. The infection can be unapparent or may cause undifferentiated febrile illness, Dengue fever, Dengue haemorrhagic fever and Dengue Shock syndrome. The severity of the disease depends on the age of the patient, race and secondary infection by a different serotype (Simmons *et al.*, 2015).

### **2.1.2.1 Dengue Fever**

Following the incubation of the virus, the infection goes through 3 phases; the febrile, the critical and the recovery phase. The early phase of the infection characterized by fever lasts for 4 to 10 days and it is followed by non-specific signs and symptoms such as headache, retro-ocular pain, malaise, myalgia, arthralgia, anorexia and vomiting. The early symptom of the infection is variable and makes it difficult to differentiate it from other febrile illnesses. At the critical phase of the infection the body is flushed with a visible rash that may be maculopapular. Towards the end of the febrile phase, the rash is replaced by petechiae which may appear on the feet, and on the hands of the patient. Occasionally there is an unusual haemorrhagic manifestation such as gastrointestinal bleeding, hypermenorrhea, and massive epistaxis. Diagnosis of the infection at the acute stage is made by tourniquet testing, testing for the non-structural protein antigen (NS1) and complete blood count. A positive tourniquet test, increase in hematocrit, thrombocytopenia and leucopenia with neutropenia with lymphocytosis is suggestive of Dengue infection. Positive NS1 antigen confirms Dengue virus as the cause of the infection. Dengue Fever is common in adults, adolescent and children. The disease is self-limiting, only small proportion progress to the severe form (Hadinegoro, 2012).

### **2.1.2.2 Severe Dengue (Dengue haemorrhagic fever & Dengue shock syndrome)**

Dengue haemorrhagic fever is characterized by high fever, haemorrhage, defects in homeostasis, hepatomegaly and evidence of vascular leakage such as ascites, pleural effusion and hypoalbuminaemia which can lead to shock. WHO has graded the severity of the Dengue Hemorrhagic Fever. Grade I and II are differentiated by presence of thrombocytopenia and concurrent haemoconcentration. Circulatory failure and profound shock with undetectable blood pressure and shock differentiate grade III from grade IV. Grading of the severity of the disease has

been found clinically and epidemiological important in epidemics in SEA, WA and America regions of WHO (WHO, 2011).

Dengue haemorrhagic fever is common in infants that acquired maternal antibodies and have been subsequently been affected by the virus. In adults, it is caused by secondary infection by a different serotype of the virus. Primary infection by Dengue virus 1 and virus 3 have also been documented to cause DHf (Sanyal, Sinha, & Halder, 1991). Besides secondary infection, congenital heart disease, haemolytic disease, chronic disease such as bronchial asthma and diabetes has been implicated as risk for developing DHf. The risk is also higher in whites than in blacks. The Dengue virus 2 is known to replicate in a greater concentration in peripheral blood of whites than in blacks (Kouri, Guzmán, & Bravo, 1987). In DHf, there is plasma leakage which can lead to hypovolemic shock if not quickly managed. Patients in shock are at greater risk of dying if no prompt treatment and management is given (Sanyal *et al.*, 1991).

### **2.1.3 Treatment and Control of Dengue virus**

There is no specific antiviral agent for the treatment of Dengue illnesses. Clinical management is by supportive treatment where intravascular volume is replaced (WHO, 2015). Supportive treatment has reduced the case fatality rate of Dengue from 20% to less than 1% prevention of Dengue illnesses approach targeted against the vector (Simmons *et al.*, 2015).

#### **2.1.3.1 Vaccine**

Currently the CYD-TDV is the only approved vaccine for use against the prevention of Dengue infection by the 4 different serotypes of the Dengue virus. This vaccine has been evaluated in phase

III randomized trials conducted in Asian countries and Latin America. The results for vaccine efficacy against symptomatic confirmed Dengue in Asia and Latin America was 56.5% and 60.8% respectively (WHO, 2015).

The vaccine under the trade name DENGVAXIA is a prophylactic, tetravalent, live attenuated chimeric Dengue vaccine in a Yellow fever 17D backbone developed by Sanofi Pasteur, the vaccine division of the pharmaceutical company Sanofi. The live attenuation of the CYD-TDV is obtained by replacing the genes that encode the prM and E proteins of the attenuated Yellow fever 17D virus genome with the corresponding genes of the 4 different serotype of the Dengue virus. The vaccine contains no adjuvant or preservative and can last for 36 months when stored between 2 °C and 8°C and secured from light (WHO, 2016b).

The vaccine is approved for use in endemic areas in individuals between the age of 9 and 60 years. The vaccine has been reported not advisable for use in individuals who are allergic to any of the component of the Dengue virus, immunocompromised, pregnant and lactating mothers (WHO, 2016c).

#### **2.1.3.2 Vector, vector control and disease transmission**

*Aedes aegypti* and *Aedes albopictus* are the main vectors of Dengue. Spatial and ecological existence of these vectors have been reported from several countries in the world. *Aedes aegypti* is a dark mosquito with white and black striped legs and a silvery lyre shaped pattern of scales on the dorsal side of the thorax. It is common and found to infect almost all countries in Sub Sahara Africa. It is present in a wide range of environment preferably poor sanitation and densely crowded areas. The adults are mostly within or near human habitat and bite during day time (Kamgang *et al.*, 2010).

*Aedes albopictus* is common in South East Asia and Americas preferably in the suburban and rural settings. Like the *Aedes. aegypti*, they have white dorsal stripes but not lyre shaped pattern. Though it has a high affinity for biting humans, it also feed on dogs, cats, squirrels and deer. Its peak biting time is early morning and late afternoon (CDC, 2012).

Literature has report of positive correlation between outbreak of Dengue and the population density of *Aedes* species (Kim Lien *et al.*, 2015). Failure of control programs and certain socio-environmental condition has led to population rise of the vectors and invasion into places which they were originally not present (Medronho *et al.*, 2009).

WHO recommends an integrated vector control program which encompass biological, chemical, source reduction and larviciding, environmental management and manipulation to reduce or interrupt the virus transmission by the *Aedes* species (WHO, 2011).

Transmission of the virus occurs when an *Aedes aegypti* mosquito feeds on infected human during the viraemic phase of the illness that manifest 2 days before the onset of the fever and lasts for 4 to 5 days after the onset of the fever. The minimum virus concentration needed to establish infection in the mosquito has not been formally established. Based on the dose  $4 \times 10^4$  PFU/ml and  $5 \times 10^4$  PFU/ml that cause infection in chick-skin membrane and monkeys respectively, the lower limit of  $10^4$  PFU/ml has been deduced to cause infection in mosquitoes (Runtuwene *et al.*, 2014). After the ingestion of the virus contaminated blood, the virus binds to receptors on the cellular surface of the midgut epithelium. The virus replicates within the midgut and are shed through the hemocoel to affect other secondary tissues such as the salivary glands. When the salivary glands becomes sufficiently infected, upon the next blood meal, the virus may be transmitted to a new host through the saliva of the infected host (Carrington & Simmons, 2014).

Human Dengue viral infection in the absence of the vector has been reported by literature. Cases of the transmission through needle stick injury (Chen & Wilson, 2004), blood transfusion (Matos *et al.*, 2016), bone marrow and solid organ transplant (Sabino *et al.*, 2016) and perinatal during pregnancy (Sinhabahu, Sathananthan, & Malavige, 2014) have been documented. In perinatal transmission, the virus has been detected in placenta and cord blood of infected infants. The virus can also be shed through breast milk of infected mothers (Ribeiro *et al.*, 2013).

#### **2.1.4 Diagnostic method for detection of Dengue infection**

Rapid and accurate diagnosis of Dengue infection is key to clinical management for appropriate clinical care, epidemiological survey for determining the virus during an outbreak for prompt public health intervention and for research and vaccine trials. Laboratory diagnosis is also important for differential diagnosis of Dengue from other febrile illnesses such as leptospirosis, meningococemia, influenza and sepsis. Laboratory diagnosis is central to clinicians to identify patients who should be closely monitored for signs of severe Dengue which can lead to shock and death. Laboratory diagnosis method for detection and confirmation of the virus include virus isolation by culture, viral nucleic acid detection and serological methods. The choice of the method is dependent upon the reason for testing (clinical diagnosis, survey or vaccine development), the phase or stage of the infection (acute or convalescent) and the technical expertise available (Plennevaux *et al.*, 2016).

#### **2.1.4.1 Isolation of Dengue virus**

Virus isolation is the most reliable method for detecting and confirming infection. It provides direct and definitive evidence of virus circulation and critical for viral characterization and determination of epidemiological characterization (Phanthanawiboon *et al.*, 2014).

Cell culture is the commonly used method for Dengue virus isolation. C6/36 and Vero Cells are the most widely used cell lines for isolating Dengue virus though the virus can replicate in other vertebrate and invertebrate cell lines. C6/36 cell line is derived from the *Aedes albopictus* and the Vero cells from the African green monkey kidney epithelia cells. Cell culture is most appropriate when samples are taken at the early phase of the disease and processed without delay (Chonticha *et al.*, 2007) Though it has the advantage of detecting virus which may occur in low concentrations in viraemic sera, it is time consuming, require substantial skill and competency and infrastructure with Biosafety level 2 or 3. Specimen appropriate for virus isolation include acute phase serum, plasma or washed buffy coat from the patient, autopsy tissue from fatal cases ( liver, spleen, thymus and lymph nodes) and mosquitoes collected from affected areas. A marked cytopathic effect is seen in the cell lines when samples are positive for any of the serotype for the Dengue virus (WHO, 2011).

#### **2.1.4.2 Viral nucleic acid detection**

Molecular diagnosis has contributed significantly to Dengue fever investigation and clinical treatment due to its sensitivity, specificity and reliable system for the detection and characterization of the virus with a much more rapid turnaround time. Compared to the virus isolation, the sensitivity of the molecular methods varies from 80% to 100% depending on the

region of the genome targeted by primers, the procedure used to amplify and detect the products, and the methods for subtyping (Shu & Huang, 2014).

A number of reverse transcriptase – polymerase chain reaction (RT- PCR) assays have been reported for detecting the Dengue virus (Kuno, 1998), , among these the two step nested RT-PCR reported by Lanciotti. et. al, 1992 and later improved to a single step multiplex RT-PCR for detection and typing of the virus by Harris *et al.*, 1998, is well known. This assay use the Dengue virus core to pre-membrane gene regions as the target sequence for detecting the 4 serotypes of the Dengue virus by analyzing the unique sizes of the amplicons in an agarose gel. Currently the automated real time reverse transcriptase polymerase chain reaction (rRT-PCR) has replaced the conventional (RT-PCR) methods due to its ability to provide quantitative measurements, a lower contamination rate, high sensitivity and specificity and easy standardization. The rRT has steadily replaced the conventional RT-PCR as the new gold standard for the rapid diagnosis of Dengue virus infection with acute phase serum samples (Mackay *et al.*, 2002). The rRT –PCR uses primer pairs, fluorescent probes and 5' nuclease to detect a single nucleotide a time (singleplex) or a multiple or all the 4 at time in a specialized PCR machine without electrophoresis (Shu & Huang, 2004).

#### **2.1.4.3 Viral antigen detection**

The NS1 protein, a product of the NS1 gene is a glycoprotein of about 50 kDa. The protein is secreted by only mammalian cells upon infection with the virus. The NS1 protein when produced do not form part of the virus assembly but is released from the Dengue virus infected cells. The antigen appears as early as first day after the onset of the febrile illness and decline to undetectable levels by 5- 6 days (Wang & Sekaran, 2010). It has been demonstrated that the antigen can be

detected in the acute phase serum of patients with either primary or secondary Dengue infections (Oyero & Ayukekbong, 2014).

Lateral flow immune-chromatographic assay is available for the detection of NS1 antigen however it is disadvantaged such that it cannot differentiate between the different serotypes of the virus (WHO, 2009). However, the sensitivity for the lateral flow immune-chromatographic test range from 81% to 94% and makes it a feasible method for early diagnosis of Dengue fever (Ferraz *et al.*, 2013).

#### **2.1.4.4 Immunological response and serological testing**

A few days after the onset of the fever, specific IgM antibody to the virus appears as the initial response to the primary infection. It is detected as early as 3-5 days after the onset of the fever, mostly suppressing the viraemia and may last for 30 -90 days. Since IgM may persist in the serum for more than 30 days, a positive result on a single serum sample is only provisional and does not necessarily mean the infection is current and ongoing. The most reliable way to confirm an active infection is by a significant four fold or greater rise in the antibody in the paired sera. IgG appears 2 weeks after the infection, reaching its peak at the 3<sup>rd</sup> week. The IgG antibody is type specific and does not give protection against reinfection by a different serotype. The IgG titre gradually decreases without disappearing from the serum maintaining an immunological memory. The IgG and IgM antibodies give immune protection by blocking cellular attachment, viral fusion or by antibody dependent cellular cytotoxicity (WHO, 2006).

In the secondary infection by heterogenic serotype, the antibodies directed against the previous infection do not confer immunity against the current infection. These antibodies from the previous

infection enhances severe form of the infection by forming immune complex with the virus that binds to the Dengue target cells in a phenomenon called antibody dependent enhancement. Antibody dependent enhancement is mostly experienced by infants born to dengue-immune mothers and adults with waning homotypic antibodies (Moi *et al.*, 2016).

Primary and Secondary infections are differentiated by IgM/IgG ratio. Where the ratio is greater than 1.2, the infection is defined as primary and the vice versa for the secondary infection (WHO, 2011).

Different serological methods are used to detect and describe the Dengue virus specific antibodies. These methods include the Hemagglutination test, indirect immunofluorescent antibody test, complement fixation, Western blotting and the enzyme linked immunosorbent assay (ELISA). Among the methods, the most widely used for routine diagnosis of Dengue infections is the capture IgM and the indirect IgG ELISA. These methods are now common due to their high sensitivity, simplicity, specificity and feasibility for automation. The capture IgM ELISA and the indirect IgG ELISA are key methods used for surveillance. In areas where the virus is not endemic, these methods are used for clinical surveillance for viral illness where positive results are treated as recent infection (Muianga *et al.*, 2016).

## **2.2 Chikungunya virus**

The Chikungunya virus, first isolated from the serum of febrile patients in 1953 (Mason & Haddow, 1957) is a member of the genus *Alphavirus* and of the family *Togaviridae*. It is an enveloped virus of about 60-70 nm in diameter. It has a genome size of 11.8kb which is linear and positive sense. The genome has 2 open reading frames. The first open reading frame encodes for

the 4 nonstructural proteins (nsp1, nsp2, nsp3, nsp4) that are involved in the replication of the virus. While the nsp1 functions as the cytoplasmic RNA capping enzyme, the nsp2 has a helicase and protease activity. The nsp3 is involved in the negative strand synthesis and nsp4 as the RNA dependent RNA polymerase (Solignat *et al.*, 2009). The other open reading frame encodes for the structural proteins which include the surface glycoproteins (E1 & E2), the capsid protein (E3) and the small peptides (6K) (Santhosh *et al.*, 2008). Between the 2 open reading frames is a junction (J) of an untranslated region of 65 nucleotide. The order of the gene is 5'-nsp1-nsp2-nsp3-nsp4-J-E3-E2-6K-E1-3'. There are 3 genotypes of the virus based on the whole genome and the E1 sequence. The 3 genotypes are the East Central South African (ESCA), the Western African and the Asian (Deeba *et al.*, 2016).

### **2.2.1 Epidemiology of Chikungunya virus**

The virus originated from East Africa around Tanzania during an epidemic of fever in 1952-1953 and then spread to the temperate and the rest of the tropics (Mason & Haddow, 1957). It is more common in Africa, Asia and the Indian subcontinent. The virus has since 1953 circulated at relatively very low levels in Africa until 1999 to 2000 that an outbreak was reported in Democratic Republic of Congo involving 50,000 people. In 2007, there was an outbreak in Gabon involving 17,618 people of which 808 were hospitalized (Deeba *et al.*, 2016). In 2015, the Ministry of Health and Social action of Senegal notified World Health Organization of 810 confirmed Chikungunya cases (WHO, 2015b). In 2016, the Ministry of Health of Kenya also alerted WHO of an outbreak of the virus with 7 laboratory confirmed cases from Mandera East subcountry (WHO, 2016d).

There have been reported cases and outbreaks of Chikungunya in several countries in Africa including Cameroon, Nigeria, Angola, Kenya, Zambia and Zimbabwe (CDC, 2014). The virus emerged in the Indian Ocean region in 2015 causing one of the largest outbreak in La Reunion Island involving approximately 300,000 people almost 40 % of the inhabitants and 254 deaths (WHO, 2015c).

In Asia, after first detected in Bangkok in Thailand in 1958, the virus has spread to India, Singapore, Malaysia, China and Japan. India recorded one of the largest epidemics in 2006 affecting more than 1.3 million people. In Malaysia, the 2 outbreaks in 1998 and 2006, were driven by the circulation of ECSA (Rougeron *et al.*, 2015).

The first case of local transmission of Chikungunya in Europe was reported in 2007 from Italy. France in December, 2013, reported 2 laboratory confirmed autochthonous cases in the French part of the Caribbean island of St. Martin. Since then autochthonous cases have spread to more than 43 countries and territories in the WHO regions of the America. In 2015, 693,489 suspected and 3,7840 laboratory confirmed cases were reported to Pan American Health Organization; Colombia recorded the highest of these cases. Of the confirmed cases in the Americas, 21 were from Argentina. This was the first outbreak and first autochthonous transmission in the area (WHO, 20117b).

### **2.2.2 Co-infections of Dengue and Chikungunya**

Dengue and Chikungunya viruses are transmitted by the same vector and exhibit similar clinical manifestations and characteristics. This makes it very difficult to recognize patients infected with single or both infections by clinical manifestations only. Nevertheless with advanced diagnostic

tools and robust surveillance system, both viruses have reported to be co-circulation /co-infections in humans and the vector (Furuya-Kanamori *et al.*, 2016).

Co-infections of Dengue and Chikungunya are prevalent in SEA, Central and West Africa, the Pacific Islands and the Americas. Co-infections of Dengue and Chikungunya viruses have been reported from 13 of the 98 countries to which the viruses are endemic (Edwards *et al.*, 2016).

In Africa co-infections range from 1.03% in 2007 (Leroy *et al.*, 2009), 0.9% (Gabor, Schwarz, Esen, Kremsner, & Grobush, 2016) in 2010 in Gabon to 17.8% in Nigeria (Baba *et al.*, 2013). The co-circulation of the co-infections in 2007 in Gabon was the Central African lineage and the Dengue virus 2. The Central African lineage with mutation at A226V site and the Dengue virus 2 was the cosmopolitan genotype (Leroy *et al.*, 2009). The E1-A226V is a new variant of the ECSA reported to have caused outbreaks in China (Wu *et al.*, 2013)

In India, during the 1964 outbreak at Vellore, co-infection of Dengue and Chikungunya were reported among 14 patients (Myers & Carey, 1967). At Delhi, co-infection was reported among 6 people. Of the 6, one died and two showed severe Dengue infection. Dengue virus 3 and Dengue virus 4 were the circulating serotypes against the ECSA genotype of the Chikungunya virus (Jain, Dubey, Shrinet, & Sunil, 2017). During the 2013, outbreak of Chikungunya at Laos, 3 people were found to be positive for ECSA and Dengue virus 2 genotype Asian 1 and 2 co-infected with the ECSA Dengue virus 3 genotype II (Phommanivong *et al.*, 2016).

The Americas are facing number of Dengue infections that coincided with the emergence of Chikungunya infections though few co-infections have been reported. In 2015, 46 (32%) of the 144 samples referred to the Guatemala national reference lab tested positive by PCR for co-infection of Dengue and Chikungunya (Edwards *et al.*, 2016).

Though in Europe no autochthonous case of co-infection has been reported yet, a case in Portugal from an expatriate returning from Angola tested positive for concurrent Dengue serotype 4 and Chikungunya virus (Parreira *et al.*, 2014).

### **2.2.3 Clinical manifestation of Chikungunya**

Following the bite and injection from the vector *Aedes* species infected mosquito, the incubation period may last from 1 to 12 days. After the incubation, the disease coincides with high viraemia. The disease normally presents with a sudden high fever ( $>40^{\circ}\text{C}$ ), rash, arthralgia, arthritis and myalgia. The fever lasts 3-4 days and is accompanied with chills and rigors. The fever wanes after 2 to 3 days and then comes again with bradycardia. The fever is connected to symptoms such as headache, photophobia, conjunctivitis, anorexia, nausea and abdominal pains. The most common symptoms of Chikungunya virus infection are the fever associated myalgia, arthralgia and arthritis. Arthralgia and arthritis can be very severe, painful, crippling and may involve more than 10 joints including small and large joints. The pain is intense and prevent sleeping and walking properly. The virus derived its name from the intense joint and muscle pains experienced by patients during the acute phase of the disease. Chikungunya literally in Swahili means “he who walks with stooped posture”. The arthralgia and arthritis persists for several months and years after the viraemic phase of the disease has subsided. Dermatological manifestation mostly observed is a morbilliform rash that appears at the upper extremities. The rash may change into vesiculobullous and occasionally pupuric exanthema in children. Atypical manifestations of Chikungunya include oral candidiasis, encephalitis, meningitis, acute flaccid paralysis and haemorrhagic manifestations (Cavrini, Gaibani, Pierro, Rossini, & Landini, 2009).

Severe form of the disease occurs in the aged and neonates. Adults who manifest the severe form of the disease mostly have underlying conditions such as diabetes, liver dysfunction, epilepsy, hypertension and renal dysfunction (Economopolou *et al.*, 2009). Neonates and children who got infected through intrapartum at the time of delivery exhibit severe form of the disease (Gerardin *et al.*, 2008). In La Reunion island where cases of Mother-to-child transmission were first reported, the disease has been associated with neurological manifestations such as encephalitis, seizures, acute encephalopathy and Guillain-Barre syndrome. The mother-to fetal transmission rate at La Reunion island as at 2008 was 48.7% (GAZerardin *et al.*, 2008).



## CHAPTER THREE

### 3.0 MATERIALS AND METHOD

#### 3.1 Study design

This was a hospital-based cross sectional study where clinical specimen of whole blood were collected from febrile patients suspected of having Dengue or Chikungunya viral infection. The study was a collaborative study between Noguchi Memorial Institute for Medical Research and Ghana Health Service and part of a broader study where surveillance for same viral agents are extended to other health facilities across the country.

#### 3.2 Study site and subjects

The study was conducted at the Greater Accra Regional Hospital, otherwise known as the Ridge Hospital. The hospital has 192 bed capacity and offer services such as Internal medicine, Obstetrics and Gynecology, Pediatrics, Ophthalmology and Outpatients.

#### 3.3 Sample size

In all a total of 260 eligible out- patients were recruited from Greater Accra regional hospital from May 2016 to April 2017.

### 3.4 Case definition

#### 3.4.1 Inclusion criteria

Probable Dengue case was defined as an acute febrile illness ( $>38.5^{\circ}\text{C}$ ) ongoing within 5 days of clinical symptoms with two or more of the following clinical manifestations; headache, retro-orbital pain, leucopenia, myalgia, arthralgia, rash, heamorrhagic manifestations (WHO, 2009).

Probable Chikungunya was defined as an acute febrile illness ( $>38.5^{\circ}\text{C}$ ) and severe arthralgia not explained by malaria or any other medical conditions and having reported transmission within 15 days.

Confirmed acute Chikungunya or Dengue case was defined as acute febrile episode with positive rRt PCR results and/or ELISA results .

#### 3.4.2 Exclusion criteria

- i. Patients who declined consent or refused to part of the study.
- ii. Patients who refuse to submit samples after consenting.

### 3.5 Sample size

Based on the formula  $n = \frac{Z^2 * P (1-P)}{M^2}$ , a minimum sample size was for the study was determined.

$n =$  sample size

$Z = 1.96$  which is the standard score for the 95% confidence interval.

$P = 21.6\%$ , estimated seroprevalence of Dengue Fever in malaria positive children in three urban centres in Ghana (Stoler *et al.*, 2015).

### **3.6 Sample Collection**

After obtaining informed consent from patients, whole blood was collected from each of the 260 study subjects by venipuncture into a clean anticoagulant-free Vacutainer tubes. Each tube was labeled with a patient identification number and date of sample collection.

A total of 5ml of whole blood was collected from each patient into an anticoagulant-free test tube. The samples were spun at 8000rpm for 5minutes and the serum was aliquoted into 2 separate vials of 1ml each. The serum specimens were kept in a standard freezer at  $-20^{\circ}\text{C}$  until they were transported to the Noguchi Memorial Institute for Medical Research for processing. Information on demographics and clinical symptoms were captured with case investigation forms.

### **3.7 Ribonucleic acid (RNA) extraction and purification**

The extraction and purification of the ribonucleic acid was done for all plasma samples using QIAamp Viral Mini Extraction kit (Qiagen, Hilden, Germany). The manufacturer's protocol was followed as outlined below.

A volume of 560 $\mu\text{l}$  of buffer AVL containing RNA carrier was added to 140 $\mu\text{l}$  of each serum and control. The buffer AVL was prepared by reconstituting 310 $\mu\text{l}$  of lyophilized carrier RNA with 310 $\mu\text{l}$  of AVE buffer. The buffer AVL and sample mixture was pulse-vortexed for 15 seconds and incubated at room temperature for 10 minutes. A volume of 560 $\mu\text{l}$  of absolute ethanol was then added and pulse vortexed for 15 seconds. A volume of 630 $\mu\text{L}$  of the solution was dispensed from the microcentrifuge tube into the 2ml QIAamp Mini column without wetting the rim. This was followed with centrifuging at 8000rpm for 1 minute. After the centrifugation the tube containing

the filtrate was discarded and the QIAamp mini column was transferred in to a 2ml collection tube. The rest of the 630µl of the solution was transferred into the same QIAamp mini column and spun at 8000rpm for 1 minute. The QIAamp mini column was transferred into another 2ml collection and the old collection tube containing the filtrate discarded. The QIAamp mini column was opened and 500µL of buffer AWL was added. It was then centrifuged at 8000rpm for 1 minute. The column was placed into another 2ml collection tube and it was opened to add 500µl of buffer AW2. It was then centrifuged at 14,000rpm for 3 minutes. The QIAamp mini column was placed into a new 2ml collecting tube and centrifuge at 14,000rpm for 1 minute. The QIAamp mini column was then placed into a clean sterile labelled 1.5 microcentrifuge tube. The column was open and 60µl of buffer AVE was added and incubated at room temperature for 1 minute and spun for 8000rpm for 1 minute. The purified ribonucleic acid solution contained in the labelled 1.5ml microcentrifuge tube was stored at -70°C prior laboratory analysis.

### **3.8 Trioplex Real-time RT-PCR Assay**

#### **3.8.1 Mastermix and plate set up**

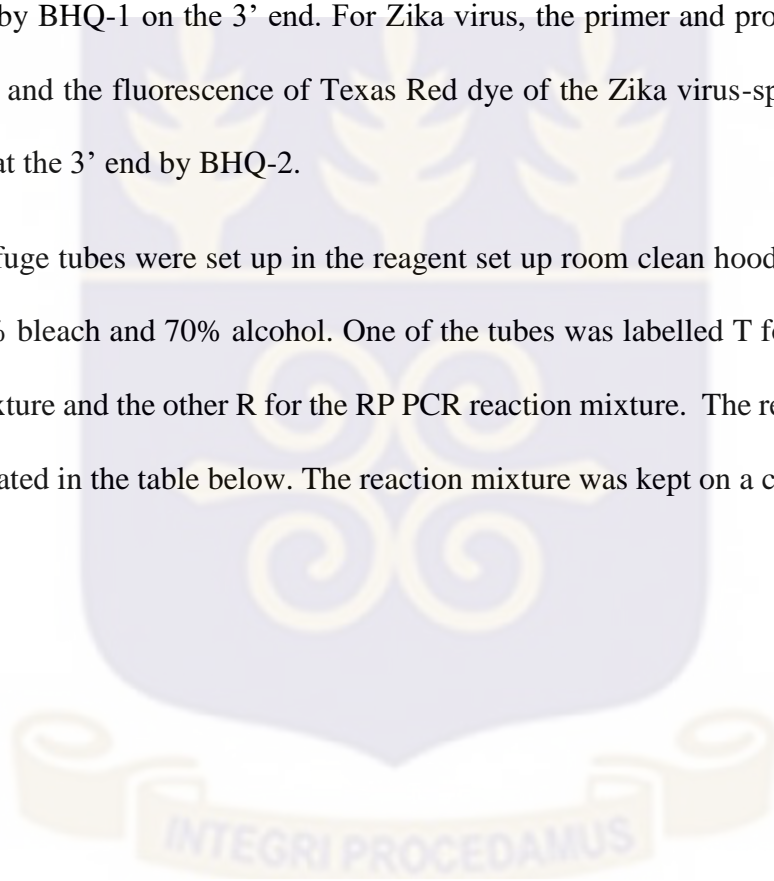
The Trioplex Real-time RT-PCR assay tests for Dengue, Chikungunya and Zika viruses in the same plate (multiplex) in the same run.

The reaction mixture was prepared using the AgPath-ID™ One Step RT-PCR kit (Applied Biosystems, California USA). The lyophilized primer and probe set of each viral agent in a vial was reconstituted with 250µl of nuclease free water and allowed to rehydrate for 15 minutes at room temperature in the dark. It was then vortexed for 15 seconds to obtain a uniform mixture. The mixture was then placed in a cold block in the reagent set up room clean hood to keep the

mixture cold during the mastermix preparation. The nuclease free water and the 2x Reaction buffer were thawed at room temperature and mix thoroughly prior the mastermix preparation.

The primers and probe of the DENV were directed to the 5'UTR of the genome as indicated in **Table 3.3** For the Dengue virus-specific probe, the reporter dye (FAM) attached to the 5' end of the probe is quenched by BHQ-1 on its 3' end. The primers and probe of the CHKV were directed to the nsP1 gene. For Chikungunya virus-specific probe, the fluorescence from the HEX on the 5' end is quenched by BHQ-1 on the 3' end. For Zika virus, the primer and probe were directed to the envelop gene and the fluorescence of Texas Red dye of the Zika virus-specific dye at the 5' end is quenched at the 3' end by BHQ-2.

Two microcentrifuge tubes were set up in the reagent set up room clean hood after the hood was cleaned with 10% bleach and 70% alcohol. One of the tubes was labelled T for the Trioplex RT-PCR reaction mixture and the other R for the RP PCR reaction mixture. The reaction mixture was prepared as indicated in the table below. The reaction mixture was kept on a cold block.



**Table 3.1: Trioplex rRT-PCR Reaction Mixture**

Trioplex Reaction mix	
Component	Quantity/ Reaction ( $\mu\text{L}$ )
Nuclease free water	$N * 0.5\mu\text{L}$
2 X reaction Buffer	$N * 12.5\mu\text{L}$
DENV (Primers/Probe)mix	$N * 0.5\mu\text{L}$
CHIKV (Primers/Probe)mix	$N * 0.5\mu\text{L}$
ZIKV (Primers/Probe)mix	$N * 0.5\mu\text{L}$
Enzyme mix	$N * 0.5\mu\text{L}$
<b>Subtotal</b>	<b><math>N * 15\mu\text{L}</math></b>
Sample RNA	$N * 5\mu\text{L}$
<b>Total</b>	<b><math>N * 20\mu\text{L}</math></b>

N = the total number of reactions (samples and controls) set up

**Table 3.2: RP –PCR Reaction Mixture**

RP-PCR Reaction mix	
Component	Quantity/Reaction ( $\mu\text{L}$ )
Nuclease free water	N*1.5 $\mu\text{L}$
2X reaction Buffer	N*12.5 $\mu\text{L}$
RP (Primers/Probe) mix	N*0.5 $\mu\text{L}$
Enzyme	N*0.5 $\mu\text{L}$
<b>Subtotal</b>	<b>N*15<math>\mu\text{L}</math></b>
Sample RNA	N*5 $\mu\text{L}$
<b>Total</b>	<b>N*20<math>\mu\text{L}</math></b>

N = the total number of reactions (samples and controls) set up

A 96 PCR well mastermix layout as in Figure 3.1 served as a guide in dispensing the reaction mixture into the Micro Amp® Fast Optical 96-well Reaction plate with barcode (0.1ml) (Applied Biosystems, California, USA). A total volume of 15 $\mu\text{L}$  of the Trioplex rRT-PCR reaction mixture was dispensed into wells.

A volume of 5 $\mu\text{L}$  of nuclease free water was added to the negative control wells. The plate was loosely covered with an optical strip caps and the plate was moved to the nucleic acid handling area on a cold block. The optical strip caps were removed and 5 $\mu\text{L}$  of the extracted sample RNA (template) was added to each corresponding well. The pipette tips were changed after each sample

addition. A volume of 5µL of DENV 1-4 positive control, CHIKV positive control, ZIKV positive control and HSC were added to wells for controls as shown in Figure 3.2.

**Table 3.3: Primer and Probe Descriptions**

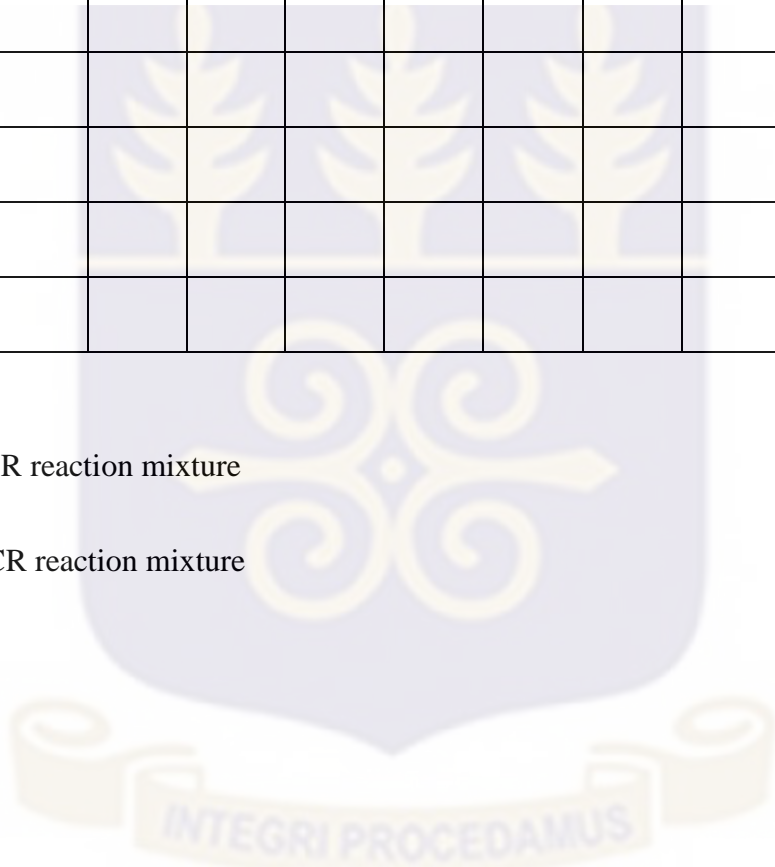
Sequence Designator	Part Number	Gene Location
DENV-F	SO3684	5'-UTR
DENV-R1		
DENV-R2		
DENV-P		
CHIKV-F	SO3685	NSP1
CHIKV-R		
CHIKV-P		
ZIKV-F	SO3686	Envelope gene
ZIKV-R		
ZIKV-P		
RP-F	SO3687	Human Ribonuclease P
RP-R		
RP-P		

**Figure 3.1: Triplex rRT-PCR Mastermix layout.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	Tx	Tx	Tx	Tx					Tx	Tx	Tx	Tx
B	RP	RP	RP	RP					RP	RP	RP	RP
C												
D												
E												
F												
G												
H												

Tx – Triplex PCR reaction mixture

RP – RNase P PCR reaction mixture



**Figure 3.2: Triplex rRT-PCR Template layout.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC								HSC	DENV-PC	CHKV-PC	ZKV-PC
B	NC								HSC	DENV-PC	CHKV-PC	ZKV-PC
C												
D												
E												
F												
G												
H												

NC – Negative control

HSC – Human serum control

DENV-PC – Dengue virus 1-4 positive control

CHKV-PC – Chikungunya positive control

ZIKV-PC – Zika virus positive control

 - Samples

### 3.8.2 rRT-PCR RUN

Amplification was performed on the Applied Biosystems 7500 Fast Dx Real-time instrument (Life Technologies, California, USA). Cycling conditions for the primer and probe sets for this multiplex rRT-PCR run consisted of a reverse transcriptase step at 50<sup>0</sup> C for 30 minutes to produce cDNA from the RNA present in the sample. This was followed by a Taq polymerase activation step at 95<sup>0</sup> C for 2 minutes and then 45 cycles at 95<sup>0</sup> C for 15 seconds for denaturation. The extension step were performed at 60<sup>0</sup> C for 1 minute.

After 2 hours the run was over .The data was saved and analyzed based on the instrument manufacturer's instructions. Analysis was performed separately for each target using the manual threshold setting. The thresholds were adjusted to fall within the beginning of the exponential phase of the fluorescence curves and above any background signal.

### 3.9 Anti Dengue virus IgM Enzyme Linked Immunosorbent Assay

The anti-Dengue virus IgM was tested using the Abcam® Anti-Dengue Virus IgM (Abcam, Cambridge, UK). The assay has a sensitivity of 97.6% and 90% specificity. Manufacturer's instructions were followed as outlined below.

All samples, reagents and controls were brought to room temperature and were allowed to thaw. The 1X washing solution was prepared by diluting 50 ml of 20x washing solution with 950mL of deionized water. A volume of 10µL of each sample was diluted in 1000µl of IgM Sample Diluent. A 96 well plate map as in the **Figure 3.3** aided in the dispensing of samples, controls and reagent into the wells. A volume of 100µl of controls and the diluted samples were dispensed into the appropriate wells. The plate was covered with foil and incubated for 60 minutes at 37<sup>0</sup>C. After the

incubation the foil was removed and the contents of the well were aspirated and washed 3 times with 300µl of 1X washing solution using the BioTek instrument ELx50 plate washer. After the wash, a volume of 100µl of the Dengue virus anti-IgM HRP conjugate were dispensed into the wells except for the blank well. The plate was incubated at room temperature in the dark for 30 minutes. The plate was washed again after the incubation. After the last wash, the plate was inverted and blotted on clean paper towel, 100µl of TMB substrate solution was dispensed in the well and incubated for 15 minutes at room temperature in the dark. A volume of 100µl of Stop solution was then dispensed into the wells and any blue colour developed during the incubation turned into yellow. Absorbances of the specimens were measured immediately after the addition of the stop solution using the Biotek® instrument ELx808 plate reader at wavelength of 450 nm and 650 nm.

### **3.10 Anti Dengue virus IgG Enzyme Linked Immunosorbent Assay**

The anti-Dengue virus IgM was tested using the Abcam® Anti-Dengue Virus IgM (Abcam, Cambridge, UK). The assay has a sensitivity of 93% and specificity >90%. Manufacturer's instructions were followed as outlined below.

All samples, reagents and controls were brought to room temperature and were allowed to thaw. The 1X washing solution was prepared by diluting 50ml of 20x washing solution with 950mL of deionized water. A volume of 10µl of each sample was diluted in 100µl of IgG Sample Diluent. A 96 well plate map as in the Figure 3.3 aided in the dispensing of samples, controls and reagent into the wells. A volume of 100µl of controls and the diluted samples were dispensed into the appropriate wells. The plate was covered with foil and incubated for 60minutes at 37°C. After the incubation the foil was removed and the contents of the well were aspirated and washed 3 times

with 300µl of 1X washing solution using the BioTek instrument ELx50 plate washer. After the wash, a volume of 100µl of the Dengue virus anti-IgG HRP Conjugate were dispensed into the wells except for the blank well. The plate was incubated at room temperature in the dark for 30minutes. The plate was washed again after the incubation. After the last wash and the excess removed by inverting and blotting the plate on clean paper towel, 100µl of TMB substrate solution was dispensed in the well and incubated for 15minutes at room temperature in the dark. A volume of 100µl of Stop solution was then dispensed into the wells and any blue colour developed during the incubation turned into yellow. The absorbance of the specimens were measured immediately after the addition of the stop solution using the Biotek® instrument ELx808 plate reader at wavelength of 450 nm and 630 nm.

The following criteria were met for the anti-Dengue virus IgG and IgM human ELISA absorbance values and were considered valid as per the manufacturer protocol.

Substrate blank: Absorbance value < 0.100

Negative control: Absorbance value < 0.200 and < cut-off

Cut-off control: Absorbance value 0.150 – 1.300

Positive control: Absorbance value > cut off

### **3.11 Anti-Chikungunya virus IgM Enzyme Linked Immunosorbent Assay**

The anti-Chikungunya virus IgM was tested using the Abcam Anti Chikungunya Virus IgM ELISA kit (Abcam, Cambridge, UK). The assay has specificity and sensitivity >90%.

All samples, reagents and controls were brought to room temperature and were allowed to thaw. The 1X washing solution was prepared by diluting 50mL of 20x washing solution with 950mL of deionized water. A volume of 1mL of 1X Chikungunya Virus Solution 1 was prepared by adding 1mL to the concentrate. The 1X Chikungunya virus Solution 1 was incubated at 15minutes at room temperature whiles it was mixed gently intermittently.

A volume of 10µl of each sample was diluted in 1000µl of sample Diluent. A 96 well plate map as in the figure 3.3 aided in the dispensing of samples, controls and reagent into the wells. A volume of 50µl of controls or diluted samples were dispensed into the appropriate wells except the blank well. The plate was covered with a foil and incubated at 37<sup>0</sup>C for 60minutes. After the incubation, with BioTek instrument ELx50 plate washer, the contents of the well were aspirated and each well washed three times with 300µl of 1X washing solution. The plate was inverted and blotted on a clean paper towel to remove the excess fluid after the wash. A volume of 50µL of 1X Chikungunya Virus Solution 1 was added to all wells except for the blank and a 30 minutes incubation at 37<sup>0</sup>C was then performed. Washing was performed after the incubation and 50µL of the Chikungunya Virus Solution 2 was added to all wells except the blank well and incubated for 30 minutes at room temperature. A volume of 50µl of SP Conjugate was added to all wells except the blank well and incubated at 30 minutes at room temperature. The plate was washed again and 100µL of TMB Substrate Solution was added to all wells including the blank well and incubated for 15 minutes at room temperature. A volume of 100µl of Stop Solution was then added to all wells and any blue colour developed during the incubation turned yellow. The absorbance of the specimens were measured immediately after the addition of the stop solution using the `Biotek® instrument ELx808 plate reader at 450 nm and 630 nm wavelengths.

### **3.11 Anti-Chikungunya Virus IgG Enzyme Linked Immunosorbent Assay**

The anti-Chikungunya virus IgM was tested using the Abcam Anti Chikungunya Virus IgM ELISA kit (Abcam, Cambridge, UK).. The assay has specificity and sensitivity >90%.

All samples, reagents and controls were brought to room temperature and were allowed to thaw. The 1X washing solution was prepared by diluting 50ml of 20x washing solution with 950mL of deionized water. A volume of 1ml of 1X Chikungunya Virus Solution 1 was prepared by adding 1mL to the concentrate. The 1X Chikungunya virus Solution 1 was incubated at 15minutes at room temperature while it was mixed gently intermittently.

A volume of 10µl of each sample was diluted in 1000µL of sample Diluent. A 96 well plate map as in the Figure 3.3 aided in the dispensing of samples, controls and reagent into the wells. A volume of 50µl of controls or diluted samples were dispensed into the appropriate wells except the blank well. The plate was covered with a foil and incubated at 37<sup>0</sup>C for 60 minutes. After the incubation, with BioTek instrument ELx50 plate washer, the contents of the well were aspirated and each well washed three times with 300µl of 1X washing solution. The plate was inverted and blotted on a clean paper towel to remove the excess fluid after the wash. A volume of 50µl of 1X Chikungunya Virus Solution 1 was added to all wells except for the blank and a 30 minutes incubation at 37<sup>0</sup>C was then performed. Washing was performed after the incubation and 50µL of the Chikungunya Virus Solution 2 was added to all wells except the blank well and incubated for 30 minutes at room temperature. A volume of 50µl of SP Conjugate was added to all wells except the blank well and incubated at 30 minutes at room temperature. The plate was washed again and 100µl of TMB Substrate Solution was added to all wells including the blank well and incubated for 15 minutes at room temperature. A volume of 100µl of Stop Solution was then added to all wells and any blue colour developed during the incubation turned yellow. The absorbance of the

specimens were measured immediately after the addition of the stop solution using the Biotek® instrument ELx808 plate reader at 450 nm and 630 nm wavelengths

The following criteria were met for the anti-Chikungunya virus IgG and IgM human ELISA absorbance values and were considered valid as per the manufacturer protocol.

Substrate blank: Absorbance value < 0.100

Negative control: Absorbance value < cut-off

Cut-off control: Absorbance value 0.150 – 1.300

Positive control: Absorbance value > cut off

**Figure 3.3: ELISA plate layout containing samples and controls**

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC											
B	NC											
C	CC											
D	CC											
E	PC											
F	PC											
G	BB											
H	BB											

NC – Negative control

CC – Cut-off control

PC – Positive control

BB – Blank

 - Samples

### **3.13 Qualitative detection of Dengue virus NS 1 antigen**

The qualitative detection of the Dengue virus NS 1 antigen was performed using the Dengue NS 1 Ag STRIP (Bio-Rad, Marnes-la-Coquette, France) rapid test. The Dengue NS 1 Ag STRIP is a disposable test that uses the lateral immunochromatography. It has a sample pad, conjugate pad and a membrane where the immunological reaction with two lines corresponding respectively to the Test line (anti-NS 1 monoclonal antibodies) and the Control line (biotin). The procedure was followed as instructed by the manufacturer.

The samples and the Dengue NS 1 Ag STRIPS were brought from the refrigerator to room temperature and allowed to thaw. A clean and non-coated microtitre plate was labelled with respective sample identification numbers. A volume of 50µl of each sample was transferred from the sample tubes into the appropriate wells. One strip of the Dengue NS 1 Ag STRIP was vertically positioned in each well the arrows directed to the bottom of the well. Using the dropper supplied with the kit, one drop of the migration buffer was added to each well. The microtitre plate was gently agitated to ensure adequate mixing. With the strips maintained in the mixture for 15 minutes at room temperature the results for each strip was recorded.

The appearance of blue or purple line at the Test line and the Control line (C) indicated positive results. The appearance of a blue or purple line only at the control line indicated negative results.

The test was repeated if the control line was absent.

### **3.14 Ethical Considerations**

Ethical approval was sought from College of Health Sciences Ethical and Protocol Review for this study under the protocol identification number: CHS-Et/M.5-P3.3/2016-2017. After study participants were provided with detailed explanation of the study in a plain and understandable language, their informed consent were sought before they were enrolled. Parents' consent was sought for participants less than 18 years old. Participation in the study was voluntary.

### **3.15 Data Handling**

Demographics and clinical data were captured on case investigation forms. All case investigation forms were kept in a locked file and the electronic data were stored in a password-secured Microsoft Excel database to which only the authors had access.

### **3.16 Statistical Analysis**

Data analysis were performed using Statistical Package for Social Sciences (SPSS) V 22. Univariate analysis including frequency and measure of tendency were calculated to show the fraction of infection and exposure among the participants. Categorical variables were expressed in the form of percentages and frequencies. Chi-square test were used to determine significant differences in categorical variables. A *p* value less than 0.05 were considered significant.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Demographic characteristics of study participants

A total of 260 febrile patients attending Greater Accra regional hospital suspected of Dengue, Chikungunya viral infections or both were recruited over a period of 11 months starting from May 2016 with most of the samples coming between October and December. The study participants comprised of 84 (32%) males and 176 (68%) females. One hundred and fifty-five which represents (60%) of the patients were between the ages of 21 and 40, and data on age were not available for 7 of the participants.

#### 4.2 Clinical presentation

Frequently reported symptoms other than fever were loss of appetite (92%), joint pain (86%) and muscle pain (85%). Other presentations recorded at different frequencies and percentages included diarrhea, extreme weakness after rehydration, nausea, vomiting, conjunctivitis, chest pains, rapid respiration and recent loss of hearing as shown in Table 4.1. Four of the participants (2%) reported haemorrhagic manifestations.

**Table 4.1: Clinical presentation of study subjects**

VARIABLES	FREQUENCY	PERCENT
Nausea	115	44%
Muscle pain	222	85%
Joint pain	223	86%
Diarrhea	148	57%
Extreme weakness after rehydration	44	17%
Vomiting	105	40%
Loss of appetite	239	92%
Conjunctivitis	10	4%
Chest pain	121	47%
Rapid respiration	132	51%
Recent loss of hearing	4	2%
Bleeding	4	2%

#### 4.3 Distribution of Anti Dengue antibodies by age and gender

The prevalence of Dengue specific antibodies (IgM and/or IgG) was 69.23%. Overall, of the 69.23%, 172 were positives for Dengue IgG antibodies representing (66.15%) of the patients. Dengue IgG prevalence was 66.28% in females and 33.72% in males. There was no statistical difference between Dengue IgG prevalence and gender ( $p = 0.427$ ). Participants in the age group 21-30 years presented the highest Dengue IgG prevalence of 65.84% (54/82) compared to children

and the geriatric group. There was significant association between Dengue IgG seropositivity and age groups ( $p=0.01$ ). Dengue IgG prevalence decreased with increase in age after 21-30 years age group. Participants below 5 years and in the age group 5-14 years age group reported no positive case of IgG antibodies.

Anti-Dengue virus IgM results revealed that 15% (39/260) of the samples were positive. Of these 8 samples tested positive for only Dengue-specific IgM and 31 tested positive for both IgM and IgG. No significant difference was noted in the proportion of suspected cases that were positive for IgM when analyzed for various age groups ( $p = 0.289$ ). Of the samples that tested positive for both IgM and IgG, the highest prevalence 25% (8/31) was observed in the 21-30 age group compared to children  $\leq 5$  years and the aged  $\geq 51$  years. The highest prevalence of Dengue IgM antibodies was observed in males 15.48% (13/84) than in females 14.77 % ( 26/176) though no significant association was observed for IgM antibodies and gender. Analysis of Dengue seroprevalence by gender and age group is presented in Table 4.2.



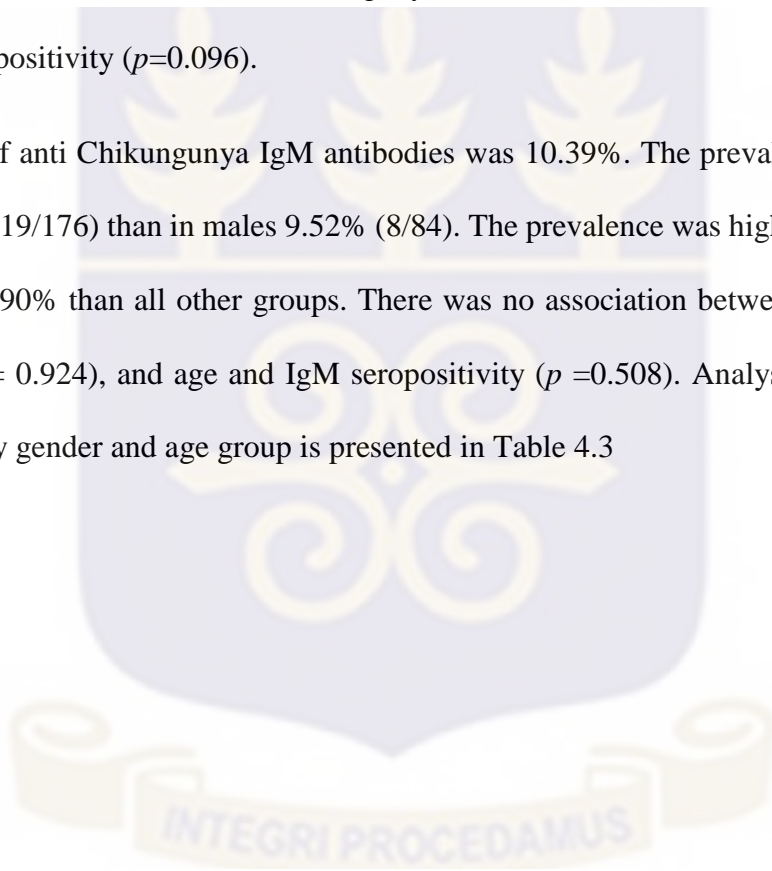
**Table 4.2: Association of demographics and Dengue seropositivity**

Characteristics	Total Number of patients	Dengue virus IgM	P-value	Dengue virus IgG	P value	IgM and IgG
Overall Total	260	39		172		31
Sex						
Male	84	13	0.236	58	0.427	10
Female	176	26		114		21
Age						
< 5 years	3	0	0.289	0	<0.001	0
5-14 years	11	1		0		0
15-20 years	14	5		6		4
21-30 years	82	12		54		8
31-40 years	73	8		45		7
41-50 years	27	8		23		7
51-60 years	22	1		18		1
61-70 years	12	0		11		0
>70years	9	2		8		2
Unknown	7	2		7		2

#### 4.4 Distribution of Anti-Chikungunya antibodies by age and gender

The prevalence of Chikungunya specific antibodies was 27.69%. The Anti-Chikungunya IgG antibodies was 20.76% of the total prevalence. The IgG prevalence was higher in males 21.4% (18/84) than in females 20.69% (36/176) though there was no association between gender and IgG seropositivity. While the prevalence was higher in the 21-40 age group (31/54) than in the geriatrics above 50 years, age groups within the bracket of preschool, adolescent and young adults (15-20) years presented no case of anti Chikungunya antibodies. There was no association between age and IgG seropositivity ( $p=0.096$ ).

The prevalence of anti Chikungunya IgM antibodies was 10.39%. The prevalence was higher in females 10.80% (19/176) than in males 9.52% (8/84). The prevalence was higher in the age group (21-40) years 12.90% than all other groups. There was no association between gender and IgM seropositivity ( $p=0.924$ ), and age and IgM seropositivity ( $p=0.508$ ). Analysis of Chikungunya seroprevalence by gender and age group is presented in Table 4.3



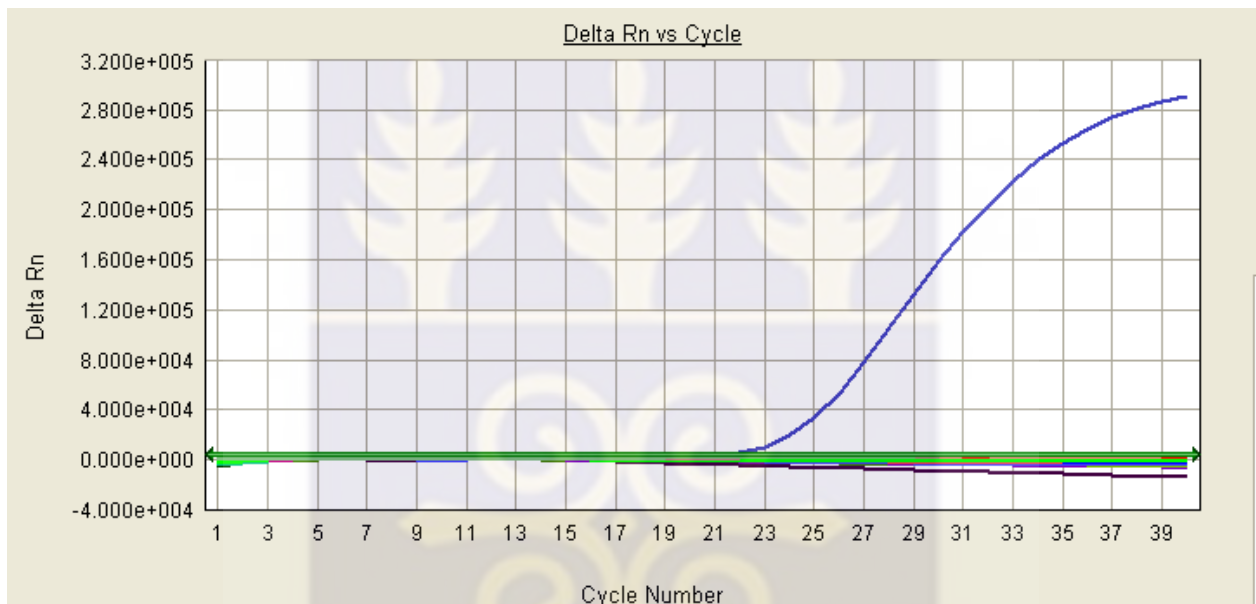
**Table 4.3: Association of demographics and Chikungunya seropositivity**

Characteristics	Number of patients	No. positive for Chikungunya IgM	P-value	No. positive for Chikungunya IgG	P value	No. positive for both IgM and IgG
Overall Total	260	27		54		9
Sex						
Male	84	8	0.924	18	0.32	4
Female	176	19		36		5
Age						
< 5 years	3	0	0.508	0	0.096	0
5-14 years	11	0		0		0
15-20 years	14	0		0		0
21-30 years	82	11		10		3
31-40 years	73	9		21		3
41-50 years	27	1		9		1
51-60 years	22	4		7		2
61-70 years	12	0		4		0
>70years	9	2		1		0
Unknown	7	0		2		0

#### 4.5 Confirmed Dengue and Chikungunya cases

None of the participants tested positive for Dengue by PCR and /or NS1 antigen testing. None also tested positive for Chikungunya by PCR.

**Figure 4.1: Amplification curve for sample and controls**



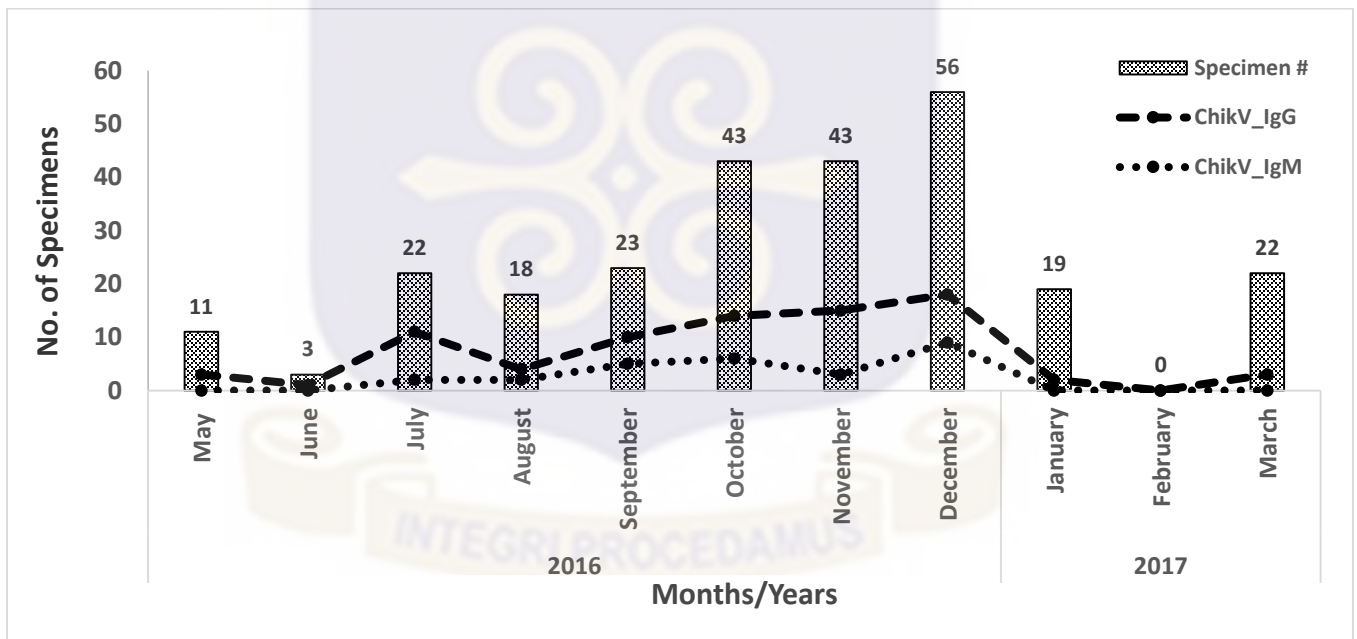
The horizontal line with arrows depicts the threshold. The blue sigmoid curve that crossed the threshold shows the positive control. The negative control and samples are depicted by the irregular lines below the threshold. The amplification of the positive control shows a positive run.

#### 4.6 Seasonality of Dengue and Chikungunya antibodies

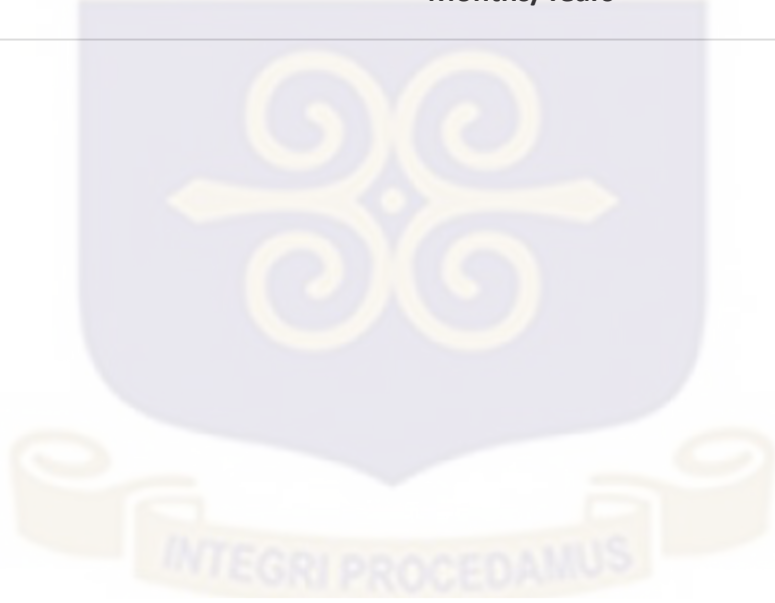
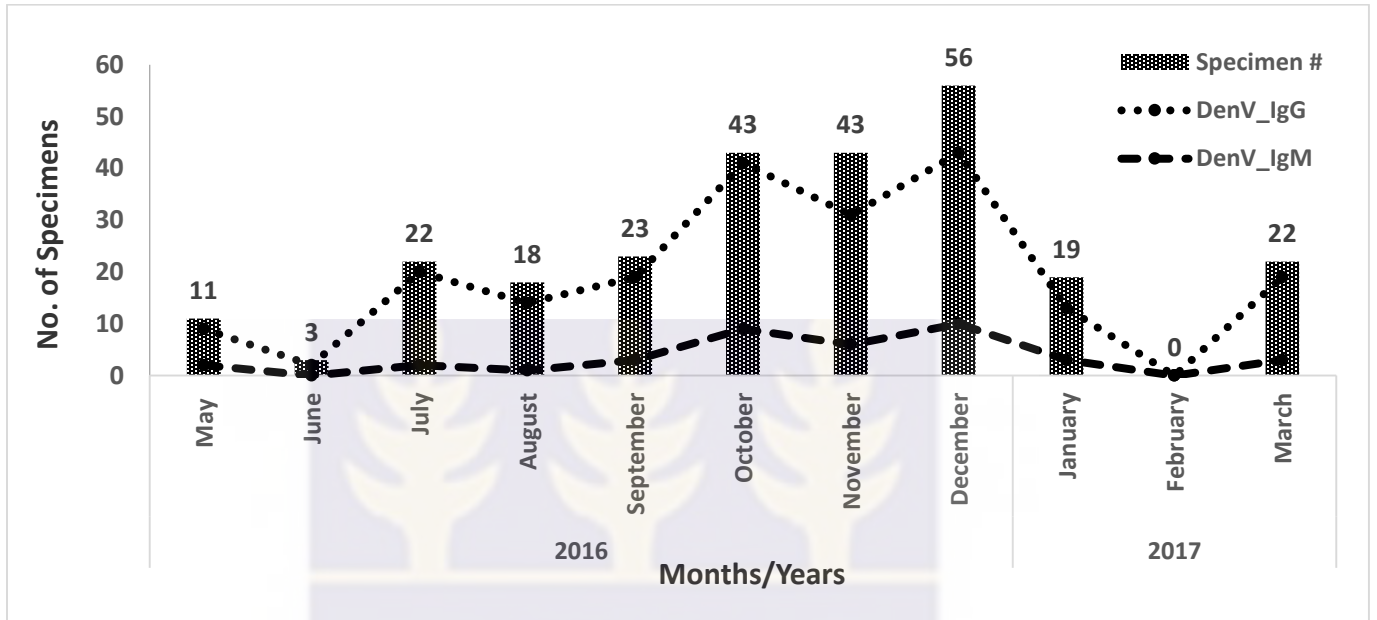
Figure 4 and 5 show month by month proportion of number of cases, IgG and IgM antibodies of Dengue and Chikungunya reported during the study period. Though the period for sample

collection and analysis is limited to give conclusive indication of seasonality, observations were made. From Figure 4.4 there was a rise in both Chikungunya IgG and IgM cases from August till it peaked at December. There was a narrow peak at the month of July. Figure 4.5 also shows a rise in IgM and IgG antibodies of Dengue with major peaks at October and December. According to the seasonal report forecast of 2016 by Ghana Meteorological agency, the rainy season was experienced April to October and dry season in November to March. In all most of the IgG and IgM antibodies to both viruses were reported in the late rainy season and early part of the dry season.

**Figure 4.2: Monthly distribution of number of specimen and Chikungunya IgG and IgM antibodies**



**Figure 4.3: Monthly distribution of number of specimen and Dengue IgG and IgM antibodies**



## CHAPTER FIVE

### 5.0 DISCUSSIONS AND CONCLUSION

#### 5.1 Study Background

Dengue and Chikungunya viruses are major public health issue in sub-Saharan Africa and increasingly becoming the most common agents of febrile illnesses yet there is a challenge in case detection and reporting due to paucity of information to understand the disease burden and economic impact, lack of awareness among health practitioners and limited diagnostic systems and tools (Mazaba-Liwewe *et al.*, 2014).

The study sought to investigate with molecular and serological tools, Dengue and Chikungunya viruses in febrile patients at the outpatient department at the Greater Accra Regional Hospital. The study recorded no positive signal by Real-time PCR for Dengue and Chikungunya viruses by the Triplex Real-time RT-PCR assay though literature suggest that the viral nucleic acid to these viruses can be detected from day 0 to day 7 following infection when the patient exhibit clinical manifestation which correspond to the duration of the fever. The negative PCR results of this study to Dengue and Chikungunya viruses might be due to sample collected after the acute phase of the disease beyond the viral RNA detection (Peeling *et al.*, 2010). Although Dengue and Chikungunya viruses detection have not been documented in Ghana, it has been reported in neighboring countries, Burkina Faso and Cote d'Ivoire. The negative PCR results in this study which is indicative of absence of active transmission could be due to the protective role of herd immunity

and environmental factors. This negative results is consistent with work done by (Kajeguka, Kaaya, Mwakalinga, & Al, 2016) and (Vairo *et al.*, 2012) in Tanzania.

## **5.2 Dengue seroprevalence**

The entire prevalence of Dengue specific antibodies (IgM and IgG) was 69.23%. IgM was found positive in 15% (n=39) and IgG in 66.15 % ( n = 172). These findings demonstrated high intensity of Dengue transmission in urban area (Accra) of Ghana. This observed level of Dengue exposure was comparable to endemic countries of Central America and South-East Asia : 56.2% in Yogyakarta (Rodríguez-Barraquer *et al.*, 2014) Indonesia and 52.2% in Mexico (Amaya-Larios *et al.*, 2014). In Africa the seroprevalence of Dengue differ significantly based on the patient population evaluated. In a study conducted in Nigeria in children between 1 month and 5 years reported 50% of the population exposure to the Dengue virus 2 (Vasilakis *et al.*, 1966) while the prospective study of Operation Restore Hope done in Somalia reported 43% in Military troops between the ages of 19-25 years (Sharp *et al.*, 1993).

The IgG prevalence of 66.15% in this study was higher than studies in Kenya with IgG of 12.5% (Ochieng *et al.*, 2015), 9.4% in Sudan (Himatt *et al.*, 2015) and in Ghana with 21.6% prevalence of IgG in malaria positive children (Stoler *et al.*, 2015). The difference in the seroprevalence in Ghana in this study and that by (Stoler *et al.*, 2015) could be explained by the difference in the study population and the fact that this study was based in tertiary hospital receiving cases from a lot of catchment area while the other was conducted in health facilities serving local communities in each site. The higher prevalence of 66.15% in this study may be due to the possibility of cross reactivity of the Anti-Dengue IgG ELISA test with other flaviviruses, autoimmune diseases, malaria and leptospirosis (Blaylock *et al.*, 2011).

The highest prevalence of Anti-Dengue IgG antibody was seen in 21-30 years age group compared to other age groups in the preschool, the adolescent and the geriatrics. This high prevalence of Dengue infection in the young age group of 20-30 years was consistent with previous studies in Zambia and Burkina Faso (Mazaba-Liwewe *et al.*, 2014) (Ridde *et al.*, 2016) but different from the pattern in Sudan where children between 5-14 years old were found to be the most affected (Himatt *et al.*, 2015). The decline in prevalence in the Dengue IgG prevalence in the age group above 50 years old may be indicative of the less likelihood of the persons in this category to present for health care and examination. This observation was also consistent with a study done in Barbados (Kumar & Nielsen, 2015). Absence of Dengue IgG antibodies in the < 5 years old group might be explained that there might have been no transplacental transfer of Dengue antibodies from mother to baby in this study. The absence of Dengue IgM in this group could be that the patients had had fever for less than 5 days. Literature suggests that patients sampled before day 5 may have negative IgM results.

### **5.3 Chikungunya seroprevalence**

The total seroprevalence of Chikungunya in this study was 27.69%. IgM was positive in 10.39% (n= 27/260) and IgG in 20.76% (n=54/260). This observation was lower than that observed in the Comore Island with a total prevalence of 63% (Sergon *et al.*, 2007). In a similar study, 13% and 36.1% of Chikungunya IgG antibodies were reported in Nigeria and Benin respectively compared to 20.76% in Ghana (Kolawole, Bello, Seriki, & Irekeola, 2017) (Bacci *et al.*, 2015). This established exposure of the virus and prevalence of the immune response against the virus within the West African states (Kolawole *et al.*, 2017). The IgM prevalence of 10.39% support the concept that there are recent infections yet the virus is neglected in the hospital and laboratory

work out in Ghana. In this study there was no significant association between gender and Anti-Chikungunya antibodies distribution. This outcome contrast study pattern by Azami *et al.*, 2013 and Sissoko *et al.*, 2008, where they found significant association between Anti-Chikungunya antibodies distribution and gender. Chikungunya infection seemed lower in prevalence (27.69%) compared to Dengue (69.23%). This was contrast to study by (Jain *et al.*, 2017) where Chikungunya infection was found to be higher than Dengue.

Literature had reports of joint pains and muscle pains to be prominent clinical features associated with Chikungunya infection. In a study by Sissoko *et al.*, 2010, 96.3% and 92.5% of the study population reported polyarthralgia and myalgia respectively. This was consistent with 86% arthralgia and 85% myalgia in this study.

#### **5.4 Monthly distribution of Dengue and Chikungunya antibodies**

Chikungunya and Dengue diseases are climate sensitive. Climate factors provide an important drive that influences directly or indirectly virus replication, propagation and transmission to man. Results of this study though eleven months and no additional statistical method applied to examine the time-series relationship, it provides valuable information about season when the seroprevalence of the viruses peaked. The gradual rise in the seroprevalence of Chikungunya from the month of August till it peaked at December and major peaks in the October and December with respect to Dengue seroprevalence coincided with the latter part of the rainy season and early part of the dry season in Ghana. This was contrast to a study by (Schwartz *et al.*, 2008) where peak transmission was between February to March. This finding might be due to appropriate rainfall produced around that time coupled to the temperatures in the early dry season that might have enhanced transmission. This information might be important to travelers and expatriates who

would have had exposure to prevent the peak transmission times so as to minimize the risk of developing the severe form of the infection which specially affects those with previous exposure.

## **5.5 CONCLUSION**

This study provided evidence that Dengue and Chikungunya viruses are circulating in Ghana, outside of an epidemic. High levels of Dengue and Chikungunya antibodies or exposure were observed, which indicates transmission intensity at the study site. Increased awareness of these viruses should be built up and differential diagnosis work-up in febrile patients in Ghana should include Dengue and Chikungunya infections. This study may inform the burden of these viruses and design models and policies to control and prevent these viruses.

## **5.6 Limitation**

A comparison of the acute serum with convalescent serum from the same patients would have been useful. The used anti-Dengue ELISA method was not serotype specific making the circulating serotype unidentified and the number of people at risk of infections to the other serotypes unknown. The study did not include entomological investigation which would have been possible to determine the presence of the viruses within the vectors in the study site.

## **5.7 Recommendations**

Plaque reduction neutralization test should be performed in subsequent studies to assist in the identification and confirmation of circulating Dengue virus serotypes which is useful in the early prediction of epidemics. Hospitals and clinics should be prepared to deal with severe form of the clinical presentation. Disease surveillance and proper vector control programs along with

community awareness and participation should be adopted and strengthened to interrupt disease transmission cycle and facilitate appropriate public health responses.



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## APPENDICES

### APPENDIX A

## INFORMATION AND CONSENT FORM

**Title: Detection of Dengue and Chikungunya viruses from febrile patients at Greater Accra**

**Regional Hospital**

**Investigator:** Simon Kofi Manu

**Institution:** University of Ghana

College of Health Sciences

School of Biomedical and Allied Health Sciences

Department of Medical Microbiology

**Introduction:** Dengue and Chikungunya virus are arboviruses transmitted by mosquitoes of the *Aedes* species. Infections caused by these viruses cause lethal diseases characterized by acute febrile illness with headache, muscle and joint pains. Cases of Dengue can develop into life threatening haemorrhagic fever resulting in bleeding, low levels of platelet and blood plasma leakage into Dengue shock syndrome.

In Ghana, Dengue and Chikungunya, which are febrile diseases caused by viruses, are not part of the routine workout in hospital laboratory investigations.

In this study, I want to screen for Dengue and Chikungunya virus in clinical samples of febrile patients at Greater Accra Regional Hospital.

In this study, I will require 5 millimetres of venous blood from consented participants which will be collected by venipuncture. The blood will be collected once on the day of visit.

### **Risk or Discomfort**

No harm is expected to occur in the course of the study. However you may experience or feel pain at the site where the blood sample will be taken. All procedure pertaining to the sample taken will be performed under aseptic conditions to prevent infection.

### **Benefit**

This study may not benefit you o by improving or making your medical conditions better. It may benefit patients in future who contract Dengue and/ or Chikungunya viral infections by helping improve on diagnosis of these infections.

### **Confidentiality**

Information that will be collected from you will be used solely for the study. Your name and given information that makes it possible for you to be identified will not be used in the writing or reporting of the study. All case investigation form will be kept in locked file. Blood samples taken from you will be coded.

### **Future Use of Biological Specimen**

You will be asked if we could store the leftover of your specimen for future studies and testing. Your name will not be recorded on the specimen. You can still participate if you do not want your specimen to be stored.

### **Voluntary Participation and Right to Leave the Study**

Participation in the study is exclusively voluntary. You are at liberty to leave at any time or decide not to answer any question. If you decide not to be part of the study, it will not affect you or negatively influence your course of medical care to be rendered to you or your ward.

### **Your Right as a Participant**

The proposal has been reviewed and approved by the Ethical and Protocol review Committee of the College of Health Sciences of University of Ghana. You can forward your concerns about your right as participant to the study to the chairman of the committee.

### **VOLUNTEER AGREEMENT**

The above document describing the benefits, possible risks and procedures for this study entitled **“Detection of Dengue and Chikungunya viruses in febrile patients at Greater Accra Regional Hospital”** has been read and explained to me. I have been given an opportunity to ask any question about the research and have been answered to my satisfaction. I willingly agree to participate in this study

\_\_\_\_\_  
Date

\_\_\_\_\_  
Name and signature or mark of volunteer

**If volunteers cannot read the form themselves, a witness must sign here:**

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to be part in the study.

---

Date

---

Name and signature of witness

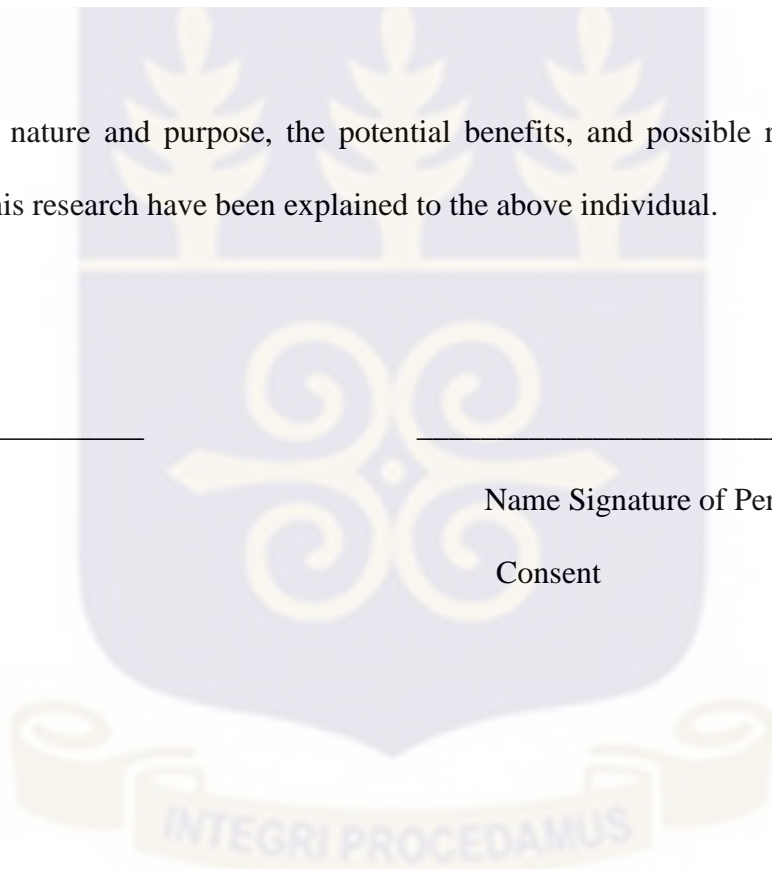
I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

---

Date

---

Name Signature of Person Who Obtained  
Consent



## **GUARDIAN OR PARENTAL CONSENT FORM**

**Title: Detection of Dengue and Chikungunya viruses from febrile patients at Greater Accra**

**Regional Hospital**

**Investigator:** Simon Kofi Manu

**Institution:** University of Ghana

College of Health Sciences

School of Biomedical and Allied Health Sciences

Department of Medical Microbiology

**Introduction:** Dengue and Chikungunya virus are arboviruses transmitted by mosquitoes of the *Aedes* species. Infections caused by these viruses cause lethal diseases characterized by acute febrile illness with headache, muscle and joint pains. Cases of Dengue can develop into life threatening haemorrhagic fever resulting in bleeding, low levels of platelet and blood plasma leakage into Dengue shock syndrome.

In Ghana, Dengue and Chikungunya, which are febrile diseases caused by viruses, are not part of the routine workout in hospital laboratory investigations. After testing for malaria, all cases which tested negative are treated for bacterial and parasitic infections, without testing for these agents.

In this study, I want to screen for Dengue and Chikungunya virus in clinical samples of febrile patients at Ridge Hospital in Greater Accra region of Ghana.

In this study, I will require 5 millimetres of venous blood from consented participants which will be collected by venipuncture. The blood will be collected once on day of visit.

### **Risk or Discomfort**

No harm is expected to occur to your child in the course of the study. However he/she may experience or feel pain at the site where the blood sample will be taken. All procedure pertaining to the sample taken will be performed under aseptic conditions to prevent infection.

### **Benefit**

This study may not benefit your child by improving or making his/her medical conditions better. It may benefit patients in future who contract Dengue and/or Chikungunya viral infections by helping improve on diagnosis of these infections.

### **Confidentiality**

Information that will be collected from your child will be used solely for the study. His/her name and any given information that makes it possible for him/her to be identified will not be used in the writing or reporting of the study. All case investigation form will be kept in locked file. Blood samples taken from your child will be given an identification number.

### **Future Use of Biological Specimen**

You will be asked if we could store the leftover of your child's specimen for future studies and testing. His/her name will not be recorded on the specimen. Your child can still participate in the study if you do not want his/her specimen to be stored.

### **Voluntary Participation and Right to Leave the Study**

Participation in the study is exclusively voluntary. Your child is at liberty to leave at any time when you decide he/she can no longer be part of the study. This will not affect your child or negatively influence his/her course of medical care to be rendered him/her.

### **Your Child's Right as Participant**

The proposal has been reviewed and approved by the Ethical and Protocol review Committee of the College of Health Sciences of University of Ghana. You can forward your concerns about your child's right as participant to the study to the chairman of the committee.



## **VOLUNTEER AGREEMENT**

The above document describing the benefits, possible risks and procedures for this study entitled **“Detection of Dengue and Chikungunya viruses in febrile patients at Greater Accra Regional Hospital”** has been read and explained to me. I have been given an opportunity to ask any question about the research and have been answered to my satisfaction. I willingly agree that my child should participate in this study

---

Date

---

Name and signature or mark of volunteer

**If volunteers cannot read the form themselves, a witness must sign here:**

I was present while the benefits, risks and procedures were read to the child’s parent or guardian. All questions were answered and the parent/guardian has agreed to be part of the study.

---

Date

---

Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

---

Date

---

Name Signature of Person Who Obtained  
Consent



**Appendix B**

**CASE INVESTIGATION FORM**

**Detection of Dengue and Chikungunya viruses in febrile patients at Greater Accra Regional Hospital**

Case identification number: .....

**Identification**

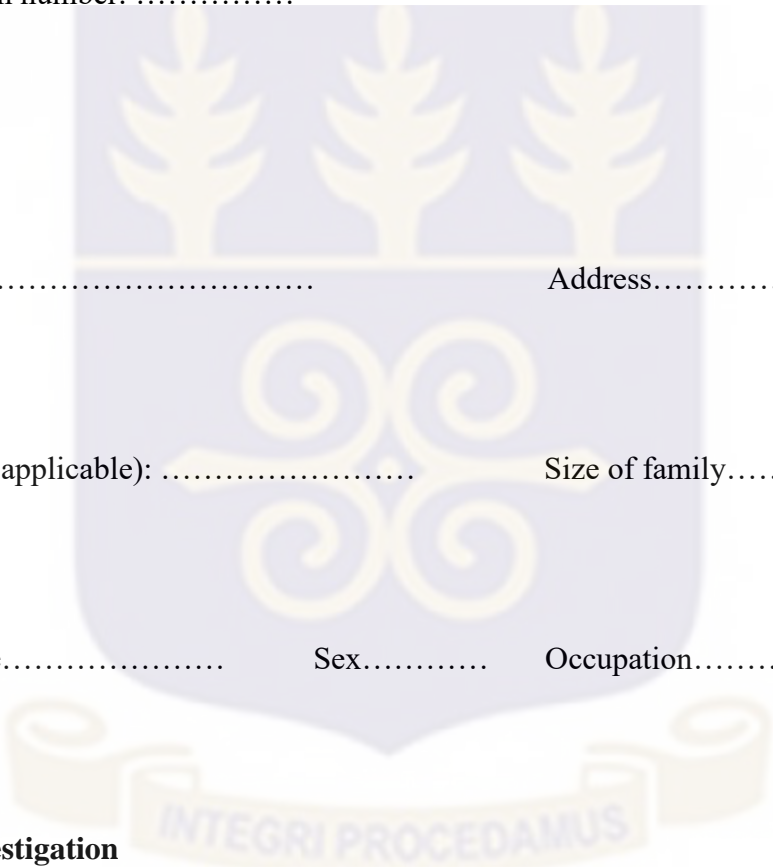
Name of Patient: ..... Address.....

Father/Mother (if applicable): ..... Size of family.....

Date of Birth/Age..... Sex..... Occupation.....

**Notification/Investigation**

Date of notification: ...../...../..... Notified by: .....Date investigated:  
.....




Symptoms(Check each if present)	Yes	No	Date of appearance
Fever $\geq 38^{\circ}\text{C}$			
Diarrhoea			
Extreme weakness after rehydration			
Nausea			
Vomiting			
Loss of appetite			
Muscle pain			
Joint pain			
Conjunctivitis			
Chest pain			
Rapid respiration			
Recent loss of hearing			
<b>Bleeding, specify below:</b>			<b>Date of first appearance</b>
Black or bloody vomits			
Black or bloody stool			
Mouth			

Symptoms(Check each if present)	Yes	No	Date of appearance
Bloody urine			
Skin or puncture site			
Other bleeding, specify			
Other observations, specify			



APPENDIX C



**UNIVERSITY OF GHANA**  
**COLLEGE OF HEALTH SCIENCES**  
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:..... 13<sup>th</sup> March, 2017.

**Mr. Simon Kofi Manu**  
Department of Medical Microbiology  
School of Biomedical and Allied Health Sciences  
University of Ghana  
Korle-Bu, Accra

**ETHICAL CLEARANCE**

Protocol Identification Number: **CHS-Et/M.5 – P 3.3/2016-2017**

The Ethical and Protocol Review Committee of the College of Health Sciences on the 9<sup>th</sup> of March, 2017 unanimously approved your research proposal.

TITLE OF PROTOCOL: **“Serological and molecular detection of Dengue and Chikungunya Viruses from Febrile patients attending Ridge Regional Hospital”**

PRINCIPAL INVESTIGATOR: **Mr. Simon Kofi Manu**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.


Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

**This ethical clearance is valid till 28<sup>th</sup> February, 2018.**

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: ..... 

**PROFESSOR ANDREW A. ADJEI**  
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS  
Dean, SBAHS  
Head of Department

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