DETECTION OF DENGUE AND YELLOW FEVER FLAVIVARIIDS IN *AEDES* (DIPTERA: CULICIDAE) POPULATIONS IN ZARIA AND ENVIRONS

BY

DANIEL IFRAIMU

DEPARTMENT OF BIOLOGICAL SCIENCES,
FACULTY OF SCIENCE,
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

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BY

Daniel IFRAIMU, (B.Sc.) Zoology (ABU) 2006

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIAIN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN ZOOLOGY

DEPARTMENT OF BIOLOGICAL SCIENCES, FACULTY OF SCIENCE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

JUNE, 2015

DECLARATION

I declare that the work in this thesis entitled '**DETECTION OF DENGUE AND YELLOW FEVER FLAVIVARIIDS IN *AEDES* (DIPTERA: CULICIDAE) POPULATIONS IN ZARIA AND ENVIRONS**' was carried out by me in the Department of Biological Sciences under the supervision of Prof. I.S. Ndams and Dr. (Mrs.) S. A. Luka. The information containedin the literature has been duly acknowledged in text and a list of referencesprovided. No part of this thesis has previously been presented for the award of a Degree or Diplomaat this or any other institution.

DanielIFRAIMU _____ _____

Signature Date

CERTIFICATION

This thesis entitled **"DETECTION OF DENGUE AND YELLOW FEVER FLAVIVARIIDS IN *AEDES* (DIPTERA: CULICIDAE) POPULATIONS IN ZARIA AND ENVIRONS"** by Daniel IFRAIMU meets the regulations governing the award of the degree, Masters in Zoology of the Ahmadu Bello University, and is approved for its contribution to knowledge.

Prof. I.S. Ndams	_____	_____
Chairman, Supervisory Committee	Signature	Date
Department of Biological Sciences		
Ahmadu Bello University, Zaria		
Dr. (Mrs)S. A. Luka	_____	_____
Member, Supervisory Committee	Signature	Date
Department of Biological Sciences		
Ahmadu Bello University, Zaria		
Prof. A.K. Adamu	_____	_____
Head of Department	Signature	Date
Department of Biological Sciences		
Ahmadu Bello University, Zaria		
Prof. A.Z. Hassan	_____	_____
Dean, School of Postgraduate Studies	Signature	Date
Ahmadu Bello University, Zaria		

DEDICATION

This research work is dedicated to God almighty, my family and to all who have contributed to my success in life.

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All glory, praise and adoration be unto GodAlmighty for His loving kindness towards me, enabling me come this far in my pursuit for knowledge.

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ABSTRACT

Dengue virus (DENV) and Yellow fever virus (YFV) are arboviruses transmitted by *Aedes* mosquitoes. The preponderance of *Aedes* mosquitoes in Zaria has been ascertained in some studies that reported on breeding of mosquitoes. However, the incidence of these viruses in the mosquito population is not known. This study investigated the incidence of the two arboviruses in the mosquito vectors in Zaria and its surroundings. Four locations were selected: Dumbi, Kufena, Hanwa and Bomo. Mosquitoes belonging to the genus *Aedes* were collected outdoor and indoor using mouth aspirators and pyrethroid-based insecticides knock-down method, from July to October 2013. Mosquitoes collected from the locations were pooled according to species, locations and whether they were collected outdoor or indoor. Viral RNA were extracted from the pools using RNA isolation kit (Shanghai ZJ Bio Technology). Real time reverse transcriptase polymerase chain reaction was used to detect dengue and yellow fever virus from the pools of mosquitoes. The target region for the four serotypes of dengue virus was the 3′ UTR and the target region for yellow fever virus is the NS 5 gene. A total of 257 *Aedes* mosquitoes were collected. The result shows that *Aedes vittatus*(59.46%) was the most abundant species outdoor while *Aedes aegypti*(0.9%) was the least. Similarly, *Aedes furcifer*(50%) and *Aedes aegypti*(47.95%) were more preponderant indoor. Accordingly, Kufena (38.52%) had the highest collection of *Aedes* mosquitoes while Hanwa (17.12%) had the least collection. The fluorimeter channel (FAM) detected Ctvalues for 25 pool mosquito sample each for dengue and yellow fever virus. The Ct values ≤38 were positive as detected in the FAM. The Ct values show that*Aedes vittatus*

from Dumbi, Hanwa and Kufena locations were negative for Dengue virus while *Aedes furcifer*, *Aedes aegypti*, *Aedes taylori* and *Aedes metallicus* were positive for Dengue virusin all the locations except in Kufena. Similarly, *Aedes furcifer, Aedes aegypti, Aedes vittatus, Aedes taylori* and *Aedes metallicus* were all positive for Yellow fever virus in all the locations. In all, 40%of the reaction samples were positive for Dengue virus while 60% were positive for Yellow fever virus from the four locations. Bomo recorded the highest incidence (60%) for Dengue virus detected in *Aedes* mosquitoes while Hanwa had the least record (10%). Similarly, Bomo had the highest incidence (33.33%) of Yellow fever virus while Dumbi and Hanwa each had the least incidence (20%). Based on the findings of this study, it is evident that dengue and yellow fever viruses are co-circulating in the *Aedes* population around Zaria and the mosquito vectors are active vectors in the four locations. In conclusion, this study has shown that*Aedes furcifer* and *Aedes aegypti*are the most active vectors of Dengue and Yellow fever virus, whereas,*Aedes vittatus* is an active vector of Yellow fever in Kufena and Hanwa. This surveillance study has therefore brought to the fore, the urgent need for mosquito control in view of the emerging and re-emerging viral diseases.

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CHAPTERONE

1.0INTRODUCTION

1.1 Background of the study

Arboviruses are important human and veterinaryviruses that are maintained in nature through biological transmission between susceptible vertebrate hosts by blood feeding arthropods such as female mosquitoes.They are important emerging pathogens world-wide. Dengue, yellow fever, and Japanese encephalitis viruses,infect hundreds of millions of people and animals each year (Grubaugh, 2013). The feeding activities of the vectors have made them effective in viral transmission to vertebrate hosts (Grubaugh, 2013).Dengue virus (DENV) and Yellow fever virus (YFV) are enveloped arboviruses whose genome is composed of a single, positive polarity RNA molecule (Henchal and Putnak, 1990).They are single-stranded RNA viruses belonging to the Flaviviridae family, Genus Flavivirus.Dengue virus has been classified into four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, which are antigenically different viral serotypes; infection with one serotype confers a lifelong immunity only to that particular serotype (Beasley and Barrret, 2008). All the four serotypes of Denguevirus have been reported to circulate in Asia, Africa and America (Nathan and Dayal-Drager, 2006). In America, Dengue virus affected more people during the 2002 outbreak than the entire decade of 1980s (Guzman and Kouri, 2003). The epidemic of Dengue in Nicaragua in 1998, showed that of the 1,027 patients examined, 614(60%) were confirmed positive, out of which 268(44%) were classified as Dengue fever, 267(43%) as Dengue fever with haemorrhagic manifestations, 40(7%) as Dengue haemorrhagic fever and 20(3%) as Dengue shock syndrome (Harris *et al*., 2000). In

Asia, the epidemic of Dengue in 2007 in Singapore, resulted to 24 deaths giving an incidence of 192.3 cases per 100,000 and a case fatality of 0.27% (Ler *et al*., 2011). In Africa, the burden of Dengue remains poorly understood.Travellers to African countries and military personnel visiting or stationed in Africa have been identified as having laboratory-confirmed Dengue infections, indicating that the virus is circulating (Sharp, 1995; CDC, 2005). Symptoms range from a mild, febrile illness to severe Dengue haemorrhagic fever (Sanchez-Seco *etal*., 2006). Dengue fever and its more serious forms, Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS) have become important public health problems and were formally included within the disease portfolio of the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases by the Joint Coordination Board in June 1999 (WHO, 1999). Given the scope of the disease and the large numbers of persons with symptomatic infection, Dengue infection may have a tremendous impact on the health-care systems of the countries affected and on household and labour economies, especially during epidemics (Suaya *et al.,* 2006). The history of Dengue in Africa is poorly documented buta retrospective sero-survey by Kokernot and co-workers suggests that Dengue existed in Africa as far back as 1926-1927, when it caused an epidemic in Durban, South Africa (Kokernot *et al*., 1956).With poor surveillance for Dengue in Africa, it is clear that epidemic Dengue fever caused by all four Dengue serotypes has increased dramatically since 1980 with most epidemics occurring in Eastern Africa and, to a lesser extent, in Western Africa (Sang, 2006).Whilst Dengue appears to be spreading in Africa, the funding received for surveillance and other research activities pertaining to Dengue has been very limited. This has mainly been due to the assumption that Dengue is not a significant health problem on the continent, perhaps due to the rarely reported cases andsevere forms of

Dengueillness. Most Dengue infections are subclinical in nature or present as Dengue fever, resulting in wrong diagnoses and treated as malaria or other endemic fevers such as, typhoid and leptospirosis. This has resulted in an underestimation of the magnitude of the Dengue problem in Africa (Sang, 2006).

In Nigeria, DENV-1, DENV-2 and DENV-3 were firstisolated around the Jos plateau, during the 1960s from samples taken from humans (Carey, 1971).Subsequently, all the four serotypes were also reported to have been isolated in other parts of Africa (Gubler and Clark, 1995). Furthermore,Flaviviruses infection have been recorded in Nigeria among 1,816 children and adults from urban and rural areas in samples obtained mainly during the early 1970s. Virus-specific haemaglutination inhibition tests have revealed the prevalence of immunityto DENV-1 as(38%), DENV-2(45%) Yellow fever viral infection (43%) and the West Nile viral infection (49%) (Fagbami *et al.,* 1977).These four serotypes of Dengue virus (DENV) and Yellow fever virus are primarily transmitted from infected humansto susceptible humans by the female *Aedes* mosquitoes during a blood meal (Halstead, 1988).

Yellow fever virus is an important re-emerging arboviral disease and a cause of severe illness and death in South America and Africa (Deubel *et al*., 1997).In South America, all reported cases are considered the result of endemic transmission of jungle yellow fever by tree-hole breeding *Haemagogus* spp., mosquitoes that had acquired infection by feeding on an infected monkey in the forest canopy. The annual incidence of endemic jungle yellow fever in South America has varied from 46 to 524 cases, from 1990 to 2004 for an average incidence of 168 cases (Monath, 2006).

Yellow fever epidemics in Africa, caused by transmission of the virus by *Aedes aegypti* continue to occur and involve human populations both in towns and in rural villages (Nasidi *et al*., 1989). Unpredictable and explosive urban outbreaks are largely

observedin West Africa (Ellis and Barret, 2008). Itcauses severe haemorrhagic symptoms like those of Dengue although a suitable vaccine is available. The virus has however gained considerable notoriety in the last few years because of the resurgence in epidemic activity of the vector (Monath, 1997; 2001).Infections occur unnoticed during periods between epidemics, but the endemic disease burden in Africa is difficult to quantify due to insensitive surveillance. In 1986 over 5000 people died of yellow fever in eastern Nigeria; while in 1987 there were over 400 deaths from yellow fever in Western Nigeria, and over 1500 deaths were estimated to have occurred in Northern Nigeria. The most striking aspect of the incidence data was the highpeak experienced between 1987 and1994, which reflects a series of concurrent annual epidemics in West Africa, principally Nigeria (Nasidi *et al*., 1989;Monath, 2006).Official reports of yellow fever between 1965 and 2004 in South America and Africa number over 33,000 cases with overall incidence in Africa nearly 5 times that in South America (Monath, 2006). Yellow fever has two types of transmission cycles in Africa: Urban and Sylvatic. It can be transmitted from infected humans to susceptible humans by the urban mosquito vector *Aedes aegypti,*while the sylvatic cycle involves transmission from infected non-human primates to humans by mosquitoes such as *Aedes africanus, Ae.furcifer, Ae.metallicus, Ae.vittatus, Ae.simpsoni*and *Ae.taylori*. Clinical forms range from an asymptomatic form or mild febrile illness to fatal infections (Digoutte *et al*., 1995).It is difficult to differentially diagnose yellow fever with the presence ofjaundice from viral hepatitis,arboviral, haemorrhagic fevers, bacterial, parasitic diseases and diseases due to toxic substances that also cause jaundice. The virus is generally not detected in the blood during the late phase of the disease due to an increase in the neutralizing antibody titre (Deubel *et al.*, 1997).

Aedes mosquitoes have been incriminated in various yellow fever epidemics in Nigeria (Bang*et al*., 1980; 1981; Savage *et al*., 1992).Through virus isolation, a number of *Aedes* species have been associated with Dengue transmission in West Africa including:*Aedes taylori, Aedes furcifer, Aedes luteocephalus, Aedes vittatus and Aedes aegypti* (Diallo, 2003).In Zaria, studies have shownthe abundance of*Aedes*breeding in rock pools.(Adebote *et al.,* 2004 and 2008).The laboratory diagnosis of Dengue virus andYellow fever infection can reliably be made by the detection of specific virus, viral antigen, genomic sequence, and/or antibodies (Gubler, 1998; Guzman and Kouri, 2003). There are three basic methodsused by most laboratories for the diagnosis of Dengue virus and Yellow fever virus infections, these are viral isolation and characterization, detection of the genomic sequence by a nucleic acid amplification technology assay, and detection of Dengue virus specific antibodies (WHO, 1997).Dengue haemorrhagic fever has been related to historical re- infection with different serotypes, detection and typing of DENV in the species of *Aedes*has provided crucial information for prevention and control.Since both Dengue and yellow fever viruses are transmitted by the species of*Aedes,*co-circulation in a given region is possible and differential detection isvery helpful (Sanchez-seco *et al.,* 2006). The species of *Aedes*are in theFamily Culicidae, Sub family Culicinae, Order Diptera and Sub order Nematocera. They breed in transient water collections such as tree holes (Phytotelmata), domestic containersaround homes and inselbergs(Adebote *et al.,* 2008). The femalesare diurnal, mostly exophagic and exophilic, transmitting the virus during a blood meal (Adebote *et al*., 2008;Afolabi and Ndams, 2010).

1.2Statement of Research Problem

Dengue and Yellow fever are amongst the emerging and resurging diseases vectored by several *Aedes*species in Africa, Asia and America (Savage and Smith, 1995, Gratz and Knudsen, 1996). Bang *et al*. (1980; 1981) reported on *Aedes*species implicated in various yellow fever epidemics in Southern parts of Nigeria. But so far, in Zaria Northern part of Nigeria, there isno report, to our knowledge, on the detection ofYellow fever and Dengue viruses in mosquito species,even though various studies conducted in Zaria and its environs have shown the preponderance of the species of *Aedes* (Aedebote *et al*., 2008 and Afolabi and Ndams, 2010).This poses a great danger and a precursor for outbreak or epidemic should the viruses be present in the vector. Reported incidence of Dengue has increased worldwide in the past few years but little is known about its incidence in Nigeria and particularly in Zaria where the vector is abundant (Afolabi and Ndams, 2010).Yellow fever is a major threat in Nigeria, 100,000 cases with 40% case mortality were recorded in a Yellow fever outbreak in 1969. Though, this figure is regarded as an underestimation due to the fact that most cases are not reported in hospitals (WHO, 1986). Nonetheless, between the year 1985 and 2000, yellow fever outbreak have plagued various parts of Nigeria (Onyido *et al*., 2009).There is also a good reason to believe that the 2.5 billion people who live in regions at risk of Dengue infection are also at risk of yellow fever (Reiter, 2010).Despite a safe and effective vaccine, there are approximately 200,000 cases, including 30,000 deaths due to yellow fever virus each year, of which 90% are in Africa (Ellis and Barrett, 2008).An estimate of 50 to 100 million cases of Dengue fever had been reported to occur annually, resulting in 24,000 deaths corresponding to an incidence rate of 2.5–5.0% from 2.5 billion people potentially at risk (Rigau-Perez and Gubler, 1999; Gibbons and Vaughn,

2002; WHO, 2002). Hundreds of thousands of hospitalizations each year have been associated with these cases (Gubler and Meltzer, 1999).Major demographic changes resulting from factors such as rapid urbanization, failure to control mosquito vectors and environmental changes due to the global climate change particularlyin tropical regions such as here in Zaria in particular,present enormous future challenges from infectious diseases includingDengue and Yellow fever.

1.3Justification

Adult mosquito abundance is a key factor contributing to the risk of disease transmission. Monitoring the abundance of adult mosquito populations provides information on the size of the vector population and thesurveillance of arboviruses in field-collected mosquitoes. This is an important tool for detecting emerging viruses (Roiz *etal*., 2012). In Zaria and its environs, these vectors of arboviruses abound hence,molecular-based assays and the use of real time reverse transcriptase PCR which are suitable tool for effective detection will be critical in the monitoring of associated diseases in mosquito vectors. This study will help provide important information for prevention and control of arboviral diseases. Cross reaction between flaviviruses is well known and thus viral detection techniques for diagnosis are supplemented by more specific assays for precise identification (Guzman and Kouri, 2004). There could be the possibility of co-transmission of both viruses by*Aedes*mosquitoes in Zaria,hence, differential detection of Dengueand Yellow feverviruses would be very helpful in the designing of control and prevention strategies. This study will provide information that is crucial in surveillance of Dengue and Yellow fever virusesin*Aedes* mosquitoes circulating, since previous infections with one of the four Dengue serotype can be an

important risk factor for developing DHF and DSS upon infection with heterotypic serotypes (Halstead, 1998).

1.4Aim

To detectDengue and Yellow fever virusesin*Aedes*populations in Zaria and environs.

Objectives

Specific objectives are

i. To determine therelative abundance of *Aedes*mosquito species in Zaria and environs.
ii. To detect Dengue virus and Yellow fever virusin the abundant*Aedes*species in the area.
iii. To determine the prevalence of Dengue virus and Yellow fever virusin the *Aedes*species in their locations.

1.5Research Questions

i. What are the abundant species of *Aedes* in the areas?
ii. Are Dengue and Yellow fever flavivariids found in *Aedes* species in the study area?
iii. What are the prevalence of dengue and yellow fever viruses in *Aedes* species in study area?

CHAPTER TWO

2.0LITREATURE REVIEW

2.1Arbovirus

The word “arbovirus” is a contraction for the phrase arthropod-borne viruses. It was first introduced in 1942 but on the recommendation of the International Subcommittee on Viral Nomenclature in 1963, the term was officially endorsed for the arthropod-borne viruses. It has no taxonomic significance but is rather an ecologic term used to define viruses that require haematophagous (blood-sucking) arthropods for transmission between hosts (WHO, 1985). By definition, arboviruses have at leasttwo hosts; most arboviruses are maintained in complex cycles involving a non-human primary vertebrate host and a primary arthropod vector. These cycles are usually silent and undetected in nature until some environmental change allows the virus to escape the primary cycle via secondary vector or vertebrate host, or when humans’ invade or encroach in the nidus of infection. Epidemics/ epizootics in humans and domestic animals usually occur only after the virus is introduced into the periodomestic environment by a bridge vector, where it is transmitted by secondary vector to secondary reservoir host (Gubler, 2001).Infection with arboviruses represent nearly 30% of all emerging infectious diseases in the last 50 years (Jones *et al*., 2008). According to Weaver and Reisen (2010), in Grubaugh*et al*. (2013), their emergence are largely attributed to many factors, such as globalization, altering weather patterns, increased production of livestock and tropical urbanization. These factors with the failure to control vector mosquitoes have led to their re-emergence.

2.1.1Mosquito-borne RNA viruses

The diverse group of mosquito-borne RNA viruses primarily includes flaviviruses (Flaviviridae: *Flavivirus*), alphavirus (Togaviridae: *Alphavirus*), orthobunyaviruses (Bunyaviridae: Orthobunyavirus) and phlebovirus (Bunyaviridae: *Phlebovirus*)(Weaver and Reisen, 2010).

Mosquito-borne flaviviruses are phylogenetically divided into two groups: those transmitted by *Aedes* species mosquitoes, such as Dengue virus (DENV) and yellow fever virus (YFV), and those transmitted by *Culex* species mosquitoes, such as Japanese encephalitis virus (JEV) and West Nile virus (WNV). The Alphavirus are classified in two geographically isolated groups, Old World Alphaviruses, such as Chikungunya virus (CHIKV) that is found in parts of Africa, Asia, and Europe, and New World Alphaviruses, such as Eastern equine encephalitis and Venezuelan equine encephalitis (VEEV) viruses that circulate in the Americas. Orthobunyaviruses are primarily mosquito-borne and are distributed globally. The majority of Phleboviruses use Phlebotomine sand flies as their primary vectors, but many can also be transmitted by mosquitoes. One notable example is Rift Valley fever virus (RVFV), which is transmitted by a number of different mosquito species in nature (Grubaugh *et al*., 2013).

2.1.2Flavivirus

According to Pybus *et al*. (2002), in Evangelista *et al*.(2013),the genus *Flavivirus* (Family:Flaviviridae) comprises, approximately 70 virus species, although there are probably many more species yet undiscovered. Flaviviruses are small, enveloped viruses with an approximately 11 kilo base pairs (kbp) positive-sense RNA genome that encodes three structural proteins (capsid, premembrane/membrane and envelope

(E))and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), which play important roles in replication, proteolysis and maturation in infected cells (Mukhopadhyay *et al*.,2005).The E protein is currently believed to be the only protein exposed on the surface of the virion, forming an external scaffold organized in 90 E-protein dimers, which lie flat, embedded in the lipid bilayer, protecting the nucleocapsid shell that contains the genome (Kuhn *et al*., 2002). The E protein is also thought to be responsible for binding to the host cell receptor(s), involved in virus entry into the cell. The flavivirus genome encodes an uninterrupted open reading frame, flanked by 5' and 3' non-coding region (Brinton, 2002).

Flaviviruses can be categorized based on their capacity to replicate in arthropod and vertebrate hosts, described as insect-specific, mosquito-borne, tick-borne and no known vector flaviviruses (Kuno *et al*., 1998). These groupings are well supported by phylogenetic comparison of their RNA genomes and by their replication range in cell culture (Kuno*et al*., 1998; Kuno 2007).Mosquito-borne flaviviruses and tick-borne flaviviruses, including yellow fever virus (YFV), Dengue virus serotypes1–4 (DENV-1 to DENV-4), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV), are often associated with human infections and are the cause of high morbidity and mortality worldwide. In contrast, flaviviruses such as *Culex* flavivirus (CxFV), Kamiti River virus (KRV) and cell fusing agent virus (CFAV) have only been isolated from mosquitoes or mosquito cell lines but they have no recognized association with vertebrates, and have not yet been associated with human or animal disease (Evangelista *et al*., 2013).

2.1.3Dengue virus

Dengue virus is a mosquito-borne flavivirus and the most prevalent arbovirus in tropical and subtropical regions of the world (Gubler, 1997). The mature particle of the Dengue virus is spherical with a diameter of 50nm containing multiple copies of the three structural proteins, a host-derived membrane bi layer and a single copy of a positive-sense, single-stranded RNA genome. The genome is cleaved by host and viral proteases in three structural proteins (capsid, C, prM, the precursor of membrane, M, protein and envelope, E) and seven nonstructural proteins (NS); it is approximately 11kb and contains a single open reading frame, which is flanked by 5' and 3' untranslated region (UTR). Distinct genotypes or lineages (viruses highly related in nucleotide sequence) have been identified within each serotype, highlighting the extensive genetic variability of the Dengue serotypes. Purifying selection appears to be a dominant theme in Dengue viral evolution, such that only viruses that are "fit" for both human and vector are maintained. Among them, "Asian" genotypes of Dengue virus, DEN-2 and DEN-3 are frequently associated with severe disease accompanying secondary Dengue infections (Lanciotti, 1994; Leitmeyer, 1999; Messer, 2003).Over the last 2 decades, classic Dengue fever and the more severe form, DHF and DSS have emerged as the most important arthropod-borne viral disease in humans (Monath and Heinz, 1996).

2.1.4DengueVirustransmission

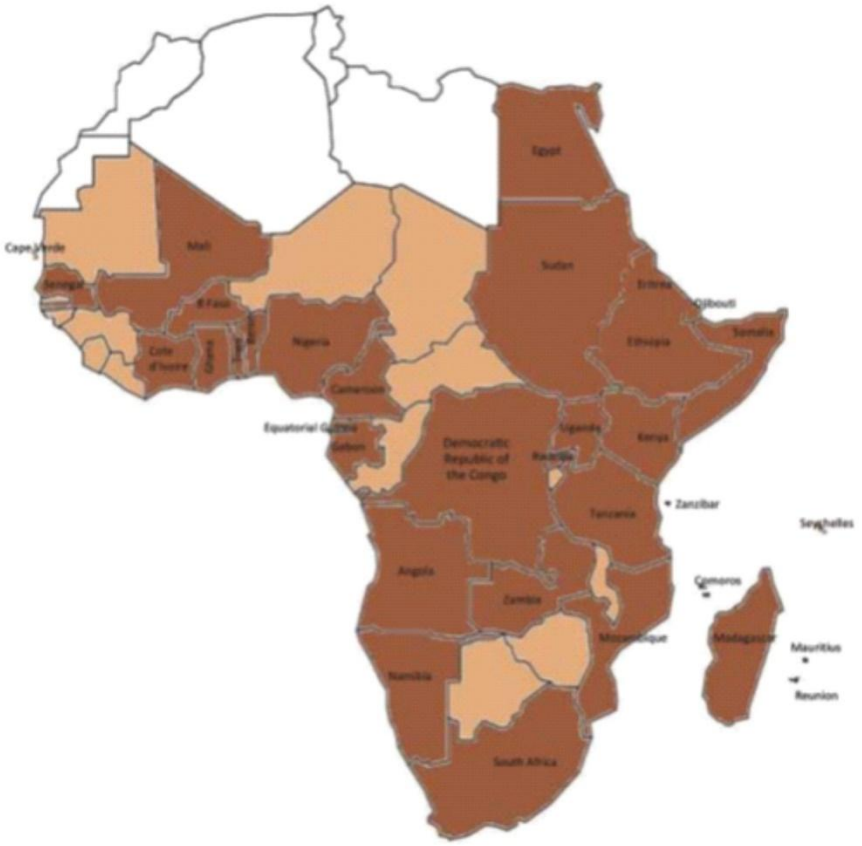
The four serotypes of Dengue virus (DENV) are transmitted from infected to susceptible humans primarily by *Aedes* mosquitoes (Halstead, 1988).Humans are the main amplifying host of the virus. Dengue virus circulating in the blood of viraemic humans is ingested by female *Aedes* mosquitoes during feeding. The virus then infects the mosquito mid-gut and subsequently spreads systemically over a period of 8-12 days.

After this intrinsic incubation period, the virus can be transmitted to other humans during subsequent probing or feeding. The intrinsic incubation period is influenced in part by environmental conditions, especially ambient temperature. Thereafter, the mosquito remains infective for the rest of its life. *Aedes aegypti* is one of the most efficient vectors for arboviruses because it is highly anthropophagic, frequently bites several times before completing oogenesis, and thrives in close proximity to humans. Vertical transmission (transovarial transmission) of Dengue virus has been demonstrated in the laboratory but rarely in the field (WHO, 2009).

2.1.5 Clinical symptoms ofdengue and factors that Influence transmission

According to Farrar (2008), in Reiter(2010), as many as 80% of all Dengue infections are asymptomatic. Among clinical cases, early stages are similar to those of yellow fever, although with excruciating arthralgia and myalgia, hence the term 'break-bone fever'. Fever and other symptoms rarely last more than seven days, but convalescence can be prolonged and debilitating. The later stages of the illness often include a widespread rash. A portion of Dengue cases, usually less than 5%, can be severe and a fraction of these may be fatal (Morens, 2009). Severe Dengue, commonly referred to as Dengue haemorrhagic fever/Dengue shock syndrome (DHF/DSS) to distinguish it from 'classic' Dengue, is associated with spontaneous haemorrhage and an increase of vascular permeability that can lead to life-threatening hypovolemic shock. The causes of this condition have been debated for decades, but remain unresolved (Rosen, 1977; Halstead *et al*., 2002; Murgue, 2010).Several factors can influence the dynamics of virus transmission including environmental and climate factors, host pathogen interactions and population immunological factors. Climate directly influences the biology of the vectors and thereby their abundance and distribution; it is consequently

an important determinant of vector-borne disease epidemics (WHO, 2009). During the acute phase of illness, the virus is present in the blood and its clearance from this compartment generally coincides with defervescence. Humoral and cellular immune responses are considered to contribute to virus clearance via the generation of neutralizing antibodies and the activation of CD_4^+ and CD_8^+ T lymphocytes. In addition, innate host defense may limit infection by the virus. After infection, serotype specific and cross-reactive antibodies and CD_4^+ and CD_8^+ T cells remain measurable for years (WHO, 2009).Although clinical diagnosis is sufficient during an epidemic, laboratory diagnosis is the definite method to confirm flavivirus infection in sporadic cases because dengue fever main symptoms may be confused with a broad range of related diseases that vary from severe malaria to leptosporosis and even Yellow fever. Rapid assays for detection of Dengue virus and Yellow fever in mosquitoes are useful for investigation of the virus and its vector in nature (Chow *et al.*, 1998). No vaccine against Dengue is available, but attenuated virus vaccines and second-generation recombinant vaccines are in active development (Webster *et al*., 2009). A large-scale trial (phase IIb) of a chimeric tetravalent vaccine (Guy *et al*., 2008) has been under way since February, 2009 (National Institute of Health, 2010). If successful, then a vaccine might be licenced by the year 2014 or 2015. The distribution of Dengue and *Aedes aegypti* mosquitoes in Africa (figure 2.1); the brown colour indicates 34 countries in which Dengue have been reported, including Dengue reported only in travelers, and *Aedesaegypti* mosquitoes. Light brown indicates 13 countries in which Dengue has not been reported but that have *Ae. aegypti*mosquitoes. White indicates 5 countries (Western Sahara, Morocco, Algeria, Tunisia, and Libya) for which data for Dengue and *Aedesaegypti*mosquitoes are not available.



Keys:

White indicates 5 countries (Western Sahara, Morocco, Algeria, Tunisia, and Libya) for which data for Dengue and *Aedes aegypti* mosquitoes are not available.

Light brown indicates 13 countries (Mauritania, The Gambia, Guinea-Bissau, Guinea, Sierra Leone, Liberia, Niger, Chad, Central African Republic, Malawi, Republic of the Congo, Zimbabwe, and Botswana) in which Dengue has not been reported but that have *Ae. Aegypti* mosquitoes.

The brown colour indicates 34 countries in which Dengue have been reported, including Dengue reported only in travelers, and *Aedes aegypti* mosquitoes.

Figure 2.1: Dengue and *Aedes aegypti* mosquitoes in Africa

Source: Amarasinghe *et al*. (2011).

2.1.6Yellow fever virus

Yellow in Latin is “flavus”, hence the name of the virus, as well as of the family (Flaviviridae) and genus (*Flavivirus*) of which Yellow fever virus is the prototype species (Barrett and Higgs, 2007). Yellow fever virus (YFV) is an enveloped virus with a positive sense, single stranded RNA genome of 10,862 bases coding for a single open reading frame(ORF) of 10,233 base pairs. This ORF encodes three structural proteins (Capsid, C; Precursor membrane, pM; and Envelop, E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (Fauquet *et al*., 2005).

2.1.7 Yellow fever virus transmission

Yellow fever is a hemorrhagic acute viral disease which is endemic in tropical areas of Africa and the Americas.Several different species of the *Aedes* and *Haemogogus* (South America only) mosquitoes transmit theyellow fever virus. These mosquitoes are either domestic (i.e., they breed around houses), wild (they breed in the jungle) or semi-domestic species (they display a mixture of habits). Any region populated with these mosquitos can potentially harbour the disease. There are three types of transmission cycles for yellow fever: sylvatic, intermediate and urban. All three cycles exist in Africa, but in South America, only sylvatic and urban yellow fever occur (Monath, 2006).In Africa, the highest incidence of yellow fever virus is in West Africa, a fact recognized as early as 1907. The virus occurs principally in the moist savannah vegetation zone where a variety of mosquito species are responsible for transmission, and in surrounding dry areas where urban type transmission occurs (Monath, 2006).

Sylvatic (or jungle) yellow fever: In tropical rainforests, yellow fever occurs in monkeys that are infected by wild mosquitoes. The infected monkeys can then pass the virus onto other mosquitos that feed on them. These infected mosquitoes bite humans entering the

forest resulting in sporadic cases of yellow fever. The majority of cases are young men working in the forest (logging, etc.). On occasion, the virus spreads beyond the affected individual.

Intermediate yellow fever: In humid or semi-humid savannahs of Africa, small-scale epidemics occur. These behave differently from urban epidemics; many separate villages in an area suffer cases simultaneously, but fewer people are infected. Semi-domestic mosquitos infect both monkey andhuman hosts. This area is often called the "zone of emergence", where increased contact between man and infected mosquitos leads to disease transmission. This is the most common type of outbreak seen in recent decades in Africa. It can shift to a more severe urban-type epidemic if the infection is carried into a suitable environment (with the presence of domestic mosquitoes and unvaccinated humans).

Urban yellow fever: Large epidemics can occur when migrants introduce the virus into areas with high human population density. Domestic mosquitos (of one species, *Aedes aegypti*) carry the virus from person to person; no monkeys are involved in transmission. These outbreaks tend to spread outwards from one source to cover a wide area.

2.1.8 Clinical symptoms of yellow fever

Yellow fever has an incubation period of 3to 6 days and severe cases classically have been noted to pass through three distinct phases: infection, remission and intoxication (Gould and Solomon, 2008). Clinical forms range from an asymptomatic form or mild febrile illness to a fatal infection (Digoutte *et al*., 1995; WHO, 2000).The fatality rate in severe cases is between 20% and 50%. Within the last decade, there have been yellow fever outbreaks in Nigeria, Ghana, Kenya, Liberia, Sierra Leone, Cameroun, Gabon and

Peru. The reemergence of yellow fever seems to be due largely to the lack of a follow-up strategy for mass vaccination and the collapse of the global effort to control *Aedes aegypti* (Robertson *et al*., 1996). Yellow fever virus has been implicated in an estimated 200,000 clinical cases and 30,000 human deaths annually in the equatorial regions of Africa and South America (Monath, 2001; Jentes *et al*., 2011). As with most viral diseases, yellow fever can present with a wide spectrum of symptoms, from mild to fatal. In clinical cases, there is generally a sudden onset of fever with severe headache, arthralgias, and myalgia. The striking yellowing of the eyes and skin, caused by hepatic dysfunction, may appear on the third day and indicates a poor prognosis. The fever often follows a 'saddleback' curve, with a brief drop in temperature and symptoms after the third day, followed by a return with increased severity that can lead to spontaneous haemorrhage ('coffee ground' vomit), delirium, renal failure, coma and death. Fatality rates of clinical cases can be as high as 80% (Monath, 2006).A safe, effective yellow fever vaccine, based on a live attenuated strain, has been available for more than half a century, and mass vaccination is a highly effective approach to prevent urban transmission, but the incidence of the disease, particularly in Africa, confirmsthat coverage is inadequate, and there is a real and present danger of a major urban epidemic (Reiter, 2010).Figure 2.2 below depicts the relevant mosquito vectors for the different transmission cycles occurring in Africa and South America.

In Africa, areas with risk of Yellow Fever virus transmission are indicated in Figure 2.3. The countries in yellow colour indicates the countries withconfirmed cases of Yellow fever or where Yellow fever case have been reported. The countries with brown colour are the countries that have not had the case of Yellow fever or where such data are not available (Jentes *et al*., 2011).

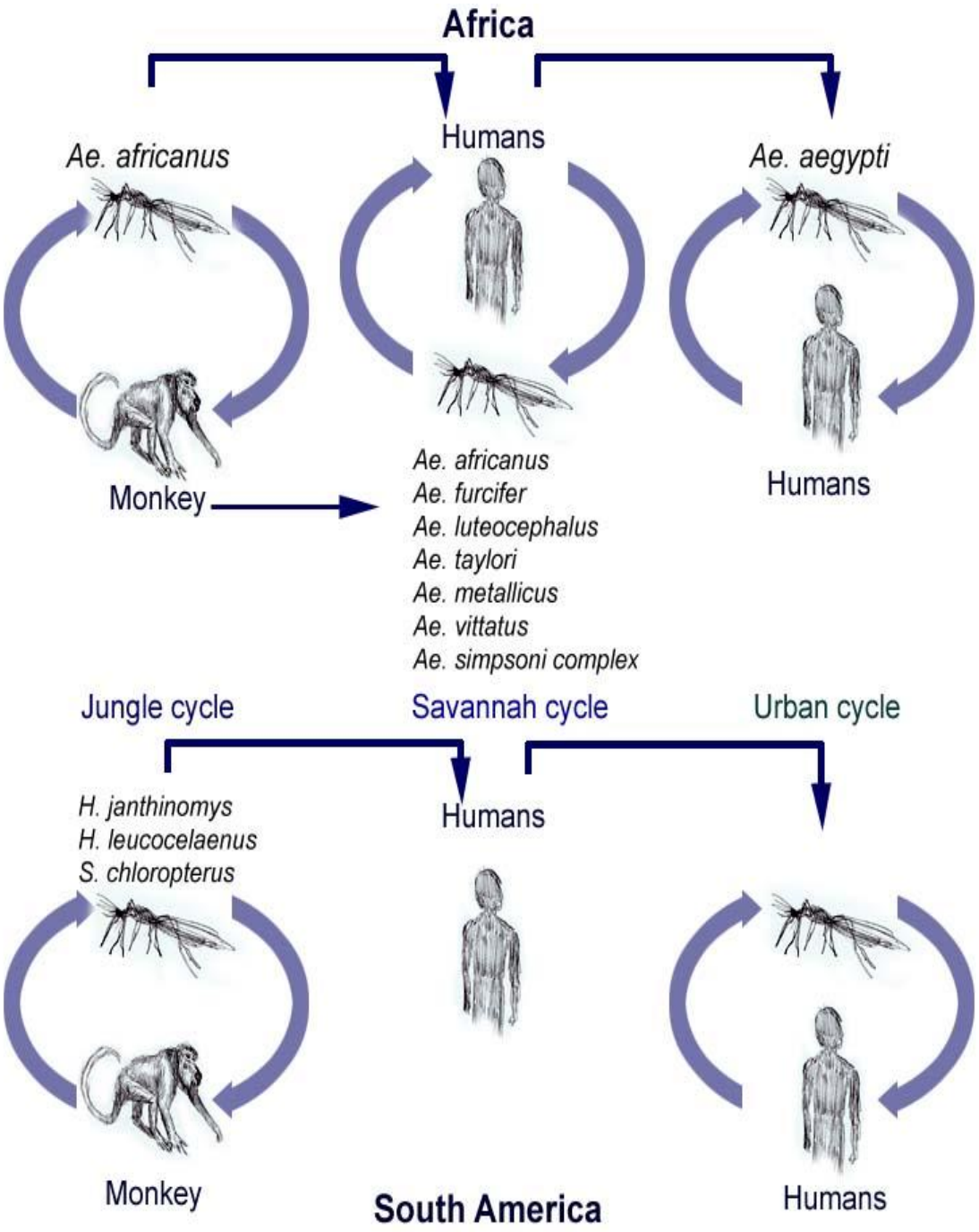


Figure 2.2: Mosquito vectors of yellow fever in Africa and South America

Source: Barret and Higgs, (2007).



Figure2.3:Areas with Risk of Yellow Fever Virus Transmission in Africa.

Source:Jentes *etal*. (2011).

2.2Mosquitoes

Mosquitoes are slender and relatively small insects, measuring about 3-6mm in length, although some species can be as small as 2-3mm while others may be as long as 10mm.The body is distinctly divided into a head, thorax and abdomen (Figure, 2.4).

The head is more or less globular in shape and has a conspicuous pair of kidney-shaped compound eyes. Between the eyes arises a pair of filamentous and segmented antennae, which in females have whorls of short hairs between the segments (that is pilose antennae) but in males the antennae have many long hairs giving them feathery or plumose appearance. Just below the antennae are a pair of palps, which may be long or short, dilated or pointed at their tips, depending on the sex of the adult and whether adults are anopheline or culicines.

The thorax is slightly humped and is covered dorsally and laterally with scales, which may be dull or shiny and metallic, white, black or almost any colour; small bristles are also present. The numbers, distribution and colour of the scales are important characters used in the identification of different mosquito species. It is this arrangement of black and white, or colourless scales on the dorsal surface of the thorax (that is the scutum or mesonotum) that gives many species especially those of the Genus *Aedes* distinctive patterns.

The abdomen is composed of ten segments but only the first eight are visible and well differentiated. They are covered dorsally and ventrally with mostly brown, blackish or whitish scales in mosquitoes of the subfamily culicinae but brightly coloured ones may be present. Their arrangement, particularly dorsally on the tergites, is used as an aid in species identification. In the Anophelinae, however, the abdomen is almost or entirely devoid of these scales. The last abdominal segment of female mosquitoes terminate in a

small pair of finger-like cerci, whereas in the males a prominent pair of claspers are present.

Mosquitoes possess only one pair of functional wings- the fore wing, the hind wings are represented by a pair of small knob-like halteres.The wings are long and relatively narrow. The number and arrangement of the wing veins is virtually the same for all mosquito species. The veins are covered with scales which are usually brown, white or creamy yellow but more brightly coloured scales may occasionally be present. The wings of resting mosquitoes are placed across each other over the abdomen in the fashion of a closed pair of scissors.

The legs of mosquitoes are long and slender, consisting of a short coxa and trochanter, along femur and tibia and a long five-segmented tarsus, which terminate in usually a pair of toothed or simple claws (Service, 2012).

2.2.1 Classification of *Aedes* mosquitoes

There are about 3530 described species of mosquitoes, which are traditionally placed in 43 genera, all contained in the family Culicidae. They are divided into three sub families: Toxorhynchitinae, Anophelinae and Culicinae. The following is the classification of *Aedes:*

PHYLUM: Arthropoda

CLASS: Insecta

ORDER: Diptera

SUB ORDER: Nematocera

FAMILY: Culicidae

SUBFAMILY: Culicinae

GENUS**:** *Aedes*

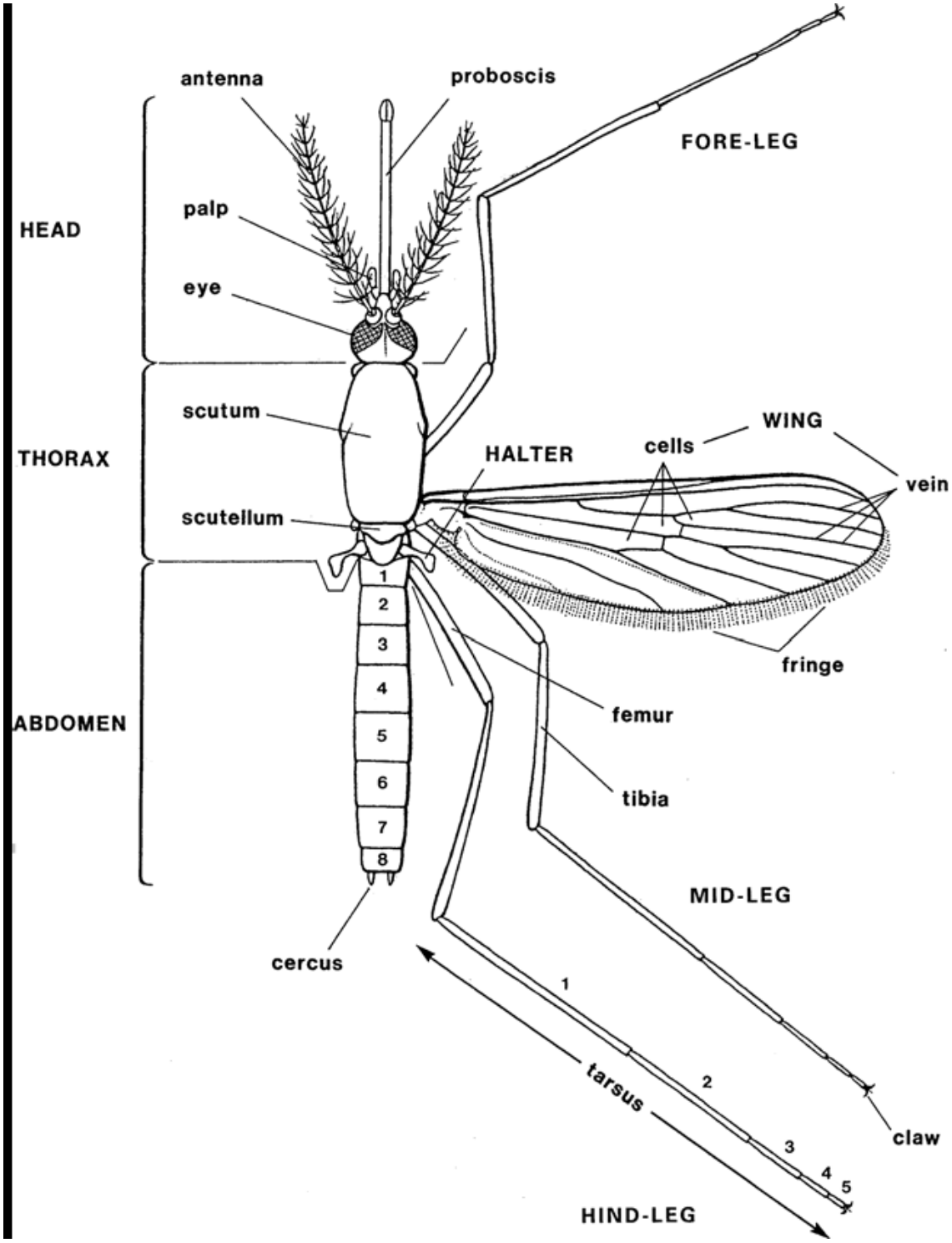


Figure2.4:Diagrammatic representation of a female adult mosquito

Source: Service, 2012

2.2.2 Biology and behaviour of adult*Aedes* mosquitoes

Mosquitoes generally have a relatively short but complex life cycle consisting of egg, four aquatic larval stages, an aquatic pupal stage and terrestrial adult stage. The immature stages are totally aquatic and without access to free-standing water of some kind, the larvae cannot complete their development to the adult phase. Larvae cannot develop out of water or in damp mud, soil or vegetation. However, adult mosquitoes do take refuge in dense vegetation. The 'host seeking' behaviour of female mosquitoes is driven by a combination of different stimuli, including carbon dioxide, body odour and body heat. Upon locating a suitable host, the female will probe the skin for a blood capillary and inject a small amount of saliva containing chemicals that prevent the blood from clotting. This is often the pathway for potential pathogens such as viruses to enter a host especially by the *Aedes* mosquitoes. The adult *Aedes* mosquitoes are diurnal being most active in the day, usually at dusk or at dawn and usually seeking refuge during the day in cool and humid sheltered habitats, such as in vegetation or inside houses (Webb and Rusell, 2007). Through virus isolation, a number of species of *Aedes* have been associated with the transmission of yellow fever and Dengue (Digoutte *et al*., 1995; Diallo, 2003). But the most efficient vector of bothDengue and yellow fever virus is the *Aedes aegyti,* a highly domesticated species that feeds almost exclusively on humans. Its geographic range has expanded with increased urbanization, resulting in increased arbovirial transmission (Kyle and Harris, 2008).

2.2.3 General morphological features used to identify *Aedes* mosquitoes

The following are the features used to distinguish *Aedes* mosquitoes from other mosquitoes:

i. Antennae of female is pilose.

ii. Proboscis are long and slinder.

iii. Maxillary palps are short.

iv. Many, but not all adults have conspicuous patterns on the thorax formed by black, white or silvery scales, in some species yellow scales are present.

v. The legs are often black with white rings.

vi. Abdomen is often covered with black and white scales forming distinctive patterns.

vii. Abdomen of female is pointed at the tip and the cerci protrude.

2.2.4 Keys used for the identification of *Aedes vittatus*

Specimens having the following features are identified as *Aedes vittatus*:

i. Scutum with three pairs of distinct, small and white spots of narrow scales present

ii. Tibiae of fore, mid and hind limb dark, each with a sub basal white spot and a white band present.

iii. Tarsomeres (1-4) of hind limb with a presence of white band.

iv. Wings with narrow dark scales on all veins with few white scales on the first vein.

2.2.5 Keys used for the identification of *Aedes aegypti*

Specimens with the following features are identified as *Aedes aegypti*:

i. A lyre-shaped white markings present on scutum of thorax

ii. White knee spot present on all femora of fore, mid and hind limb.

iii. Mid femur with a narrow white longitudinal stripe on ventral half of anterior surface.

iv. Tibiae of fore, mid and hind limbs anteriorly dark, without any white band.

v. Wings with mainly narrow dark scales on all veins.

vi. Hind tarsus with a basal white band on tarsomeres 1-4.

2.2.6 Keys used for the identification of *Aedes furcifer*

Specimens with the following features are identified as *Aedes furcifer*:

i. Proboscis with a distinct white band.

ii. Femora, tibiae and tarsomeres all sprinkled with white scales.

iii. Wings with broad dark scales mixed with broad white scales on all veins.

iv. Abdomen speckled dorsally.

2.2.7 Keys used for the identification of *Aedes metallicus*

Specimens with the following features are identified as *Aedes metallicus*:

i. Scutum with a distinct patch of white markings on scutal fossa.

ii. Wing with mainly narrow dark scales on all veins.

iii. Prescutellar area with all broad, flat metallic silvery white scales.

iv. White knee spot absent on fore femur, present on mid and hind femora.

v. Hind tibia without a white stripe base.

vi. Fourth hind tarsomeres distinctly dark.

vii. Scutellum with broad white scales on all lobes.

2.2.8 Keys used for the identification of *Aedes taylori*

Specimens with the following features are identified as *Aedes taylori*:

i. Proboscis with a distinct white band.

ii. Femora, tibiae and tarsomeres all sprinkled with white scales

iii. Wings with broad dark scales mixed with broad white scales on all veins.

iv. Erect, forked scales numerous, not restricted to occiput.

v. Abdomen not speckled dorsally.

(Huang and Ward 1981; Highton, 1983; Rueda, 2004;Huang, 2004; Service, 2012)

2.3 Molecular Methods

2.3.1 Polymerase chain reaction (PCR)

The detection of viruses (flaviviruses) by rapid and reliable techniques is still one of the most demanding tasks in virus diagnosis. Cell culture-based methods which determine the number of infectious virus particles, are time consuming and complex. The need for alternative fast and sensitive diagnostic tools has provided thePolymeraseChain Reaction (PCR) (Niesters, 2001). Recently, due to its considerable sensitivity, PCR has become a promising method for virus detection hence, for diagnostic purposes, a rapid, specific and sensitive virus PCR is preferable. However, whenever the identification of infectious virus particle is required, for example during vaccine production and control, the plaque assay, although time consuming and laborious, it is most appropriate. This is essentially due to the fact that the real time PCR can only detect a biological molecule, i.e., the genome, but not a biological feature i.e. the infectious potential of a cell (Bae *et al*., 2003). Bessoff *et al.* (2008)reported that the development and modification of nestedreverse transcription-PCR (RT-PCR) and a real timeRT-PCR techniques has significantly reduced processing time.This rapid detection of the flavivirus genomic sequence by these methods has now become a trend. This is because the real-time PCR or RT-PCR assay has many advantages include: rapidity, the ability to provide quantitative measurements, a lower contamination rate, a higher sensitivity, a higher specificity, and easy standardization. This, therefore, has made real-time PCR to be the new gold standard for a rapid diagnosis (Shu and Huang, 2004).

In Real time PCR, there are five main chemical formats (the DNA binding fluorophores, the 5' nuclease, adjacent linear and hairpin oligonucleotide probes, and self-fluorescing amplicons) that are used to detect the PCR product during real-time PCR. Among these, the most widely used format is the 5'→3' nuclease oligonucleotide probe (TaqMan

assay). The TaqMan real-time PCR is highly specific due to the sequence-specific hybridization of the probe.The most commonly used fluorogenic oligoprobes rely upon fluoresecence resonance energy transfer (FRET) between fluorogenic labels or between one fluorophore and a dark or black-'hole' non-fluoresecent quencher, which disperses energy as heat rather than fluorescence (Mackay *et al*., 2002).Along with the development of fluorophores, nucleotide labeling chemistries, and instrumentation, has the potential to develop multiplex PCR protocol with up to four fluorophores in a single tube. In contrast to the TaqMan assay, the SYBR Green real-time RT-PCR assay has the advantage of simplicity in primer design and uses universal RT-PCR protocols suitable for the detection of multiple target sequences, although it is theoretically less specific (Kuno, 1998).

2.3.2Molecular diagnosis

Currently, molecular diagnosis of viruses is based on reverse transcription PCR, such as one-step or nested RT-PCR, nucleic acid sequence based amplification (NASBA), or real-time RT-PCR. This has gradually replaced the virus isolation method as the new standard for the detection of flavivirus. The field of molecular diagnosis has changed significantly over the past decade, leading to assays that are much more reliable for the detection and characterization of various pathogens. Previously, rapid laboratory diagnosis did not contribute significantly to clinical treatment, aetiological investigation, or control of virus infections due to the lack of a reliable and sensitive assay system for the detection of virus in acute-phase serum. However, several laboratories have published various RT-PCR protocols for Dengue virus identification (Lanciotti *et al*., 1992;Guzman and Kouri, 1996,). Among these, the two-step nested RT-PCR protocols originally reported by Lanciotti *et al.* (Lanciotti *et al.*, 1992) and

later modified to a single step multiplex RT-PCR for the detection and typing of Dengue virus by Harris *et al*. (Harris *et al*., 1998) are well known. These assays used the virus core to pre membrane gene regions as the target sequence for virus detection. For example, in Dengue virus, they have the advantage of detecting and differentiating the four Dengue virus serotypes by analysing the unique sizes of the amplicons in the agarose gel. Alternatively, the NASBA assay, an isothermal RNA-specific amplification assay, has been developed for the detection of viral and bacterial RNA in clinical samples. Wu and co-workers, (Wu *et al.*, 2001) reported on the detection of Dengue viruses by the NASBA assay, which had high degrees of sensitivity and specificity. Since the amplification procedure used with the NASBA assay is entirely isothermal and is conducted at 41°C, it would be suitable for epidemiological studies in the field. Similarly, the laboratory diagnosis of Dengue virus infection can be made by the detection of specific virus, viral antigen, genomic sequence, and/or antibodies (Gubler, 1998; Guzman and Kouri, 2003). At present, the three basic methods used by most laboratories for the diagnosis of Dengue virus infection are viral isolation and characterization, detection of the genomic sequence by a nucleic acid amplification technology assay, and detection of Dengue virus specific antibodies (WHO, 1997).

2.3.3 Virus isolation and characterization

The rapid progress in molecular biology techniques have helped in resolving many virologic puzzles and yielded highly valuable data hitherto unavailable, such as characterization of virus (Kuno and Chang, 2005). The reverse transcription polymerase chain reaction is a rapid, easy and reliable technique based on nucleic acid detection because of the high variability of the RNA viruses; it is retro transcription and the first amplification are generic for all viruses and the nested amplification is a multiplex

reaction that specifically identifies each virus (Sanchez-Seco *et al*., 2006). Diagnosis of viruses is made by virus isolation followed by specific identifications. Several methods were available which included the inoculation of suckling mice, the intrathoracic inoculation of mosquitoes or inoculation of cell cultures (Vero cell) (WHO,1985). These methods have gradually being replaced by RNA isolation kits which uses guanidinium thiocyanate denaturation and isopropanol precipitations(Kuno and Chang, 2005). Virologic techniques to identify Yellow fever have relied on PCR techniques specific to Yellow fever virus. Multiplex assays have been developed for the diagnosis and differentiation of multiple flaviviruses from each other (including YFV, Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis, and the four Dengue virus serotypes) (Chao*et al.*, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in four selected settlements namely:Dumbi (Dumbin Dutse), Kufena, Hanwa and Bomo, located between Latitude 11˚ 04□ N and Longitude 7˚ 43□ E, inZaria and Sabon Gari Local Government Areas of Kaduna State (Figure 3.1). The natural vegetation of Zaria and its environs istypical of Northern Guinea Savanna, characterized mainly by grasses and shrubs; with scattered trees;it lies about 686m above sea level (Musa *et al*., 2009). Weather reports from the Nigerian Meteorological Agency, Zaria, shows an average temperature of 19.7°C to 32.4°C, average humidity of 53.7 and total rainfall of 1100mm. Dumbi and environs is associated with shrubs, grasses and inselbergs that serve as breeding sites for mosquitoes.These were observed during a 300 level local field trip course of the Department of Biological Sciences, Ahmadu Bello University, Zaria in 2006 (Anonymous).It alsohas permanent settlements of nomadic cattle herdsmen at the foot of the hill in addition to providing shelter for monkeys, birds and goats. Kufena environs has a poorly-flooded paddy fields suitable for farming.The famous Kufena hill is located thereand serves as a veritable shelter for wild animals, especially monkeys that were sighted during the field collection of mosquitoes. Thehuman settlements engage in farming and quarry activities.Hanwa is a cosmopolitan urban settlement with more residential buildings than the other study areas. It also has a hills which are suitable breeding sites for mosquitoes.Bomo is situated at the edge of a large permanent lake (Bomo Lake). The activity of people there is mainly farming with maize, sorghum, millet and cowpea mostly cultivated in the area. The selection criteria for the four locations were based on the abundance of *Aedes* mosquitoas reported by Adebote *et al.* (2006, 2008).

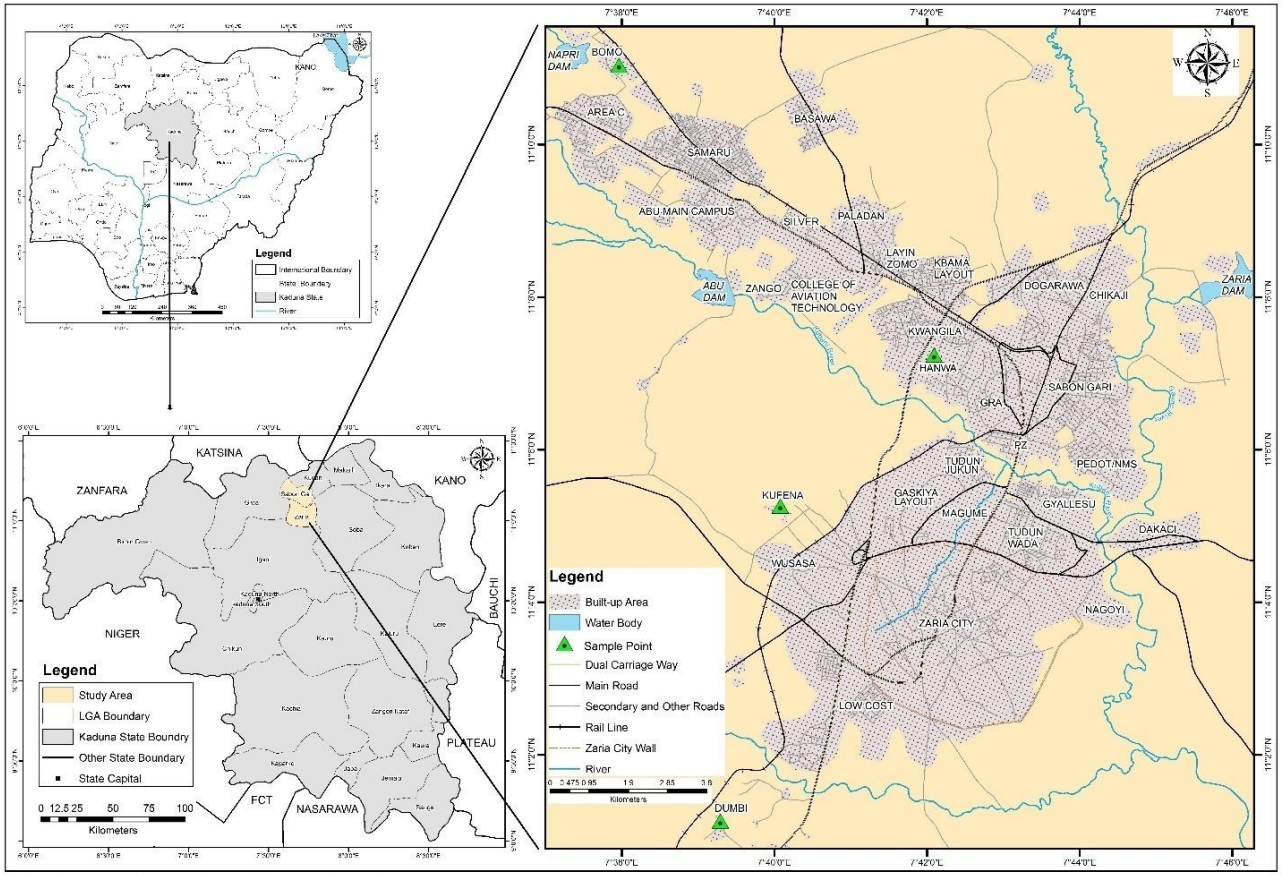


Figure 1: Map of the Study Area showing Sample Points
Source: Adapted and modified from the administrative map of Kaduna state

Figure 3.1: Map of Study area.

3.2*Aedes*Mosquito Collection

Aedes specieswere sampled both outdoors and indoorsfrom the four locations (Dumbi, Kufena, Hanwa and Bomo) from July to October 2013. According to Focks (2003),adult mosquito surveillanceand population estimates of mosquitoes primarily relied on ovitraps, visual attractants, human landing count, sticky traps and mouth aspirator collections due to diurnal feeding behaviour of *Aedes* species. In this research, the use of mouth aspirators and pyrethroid (WHO, 1985)were employed to collect those *Aedes*mosquitoes landing on humans with humans used as bait while those that rested or feedindoorswere collected by pyrethroid-based insecticide knock down method.

3.2.1 Collection of *Aedes* species outdoors

The outdoor collection of *Aedes*mosquitoes was carried out using a mouth aspirator which was designed using two hosepipes;one measuring 30 cm and the other measuring 60cm in length. A net was attached to one end of the smaller hosepipe and inserted to the bigger hosepipe, the bigger hosepipe end is put in the mouth while the other end of the smaller hosepipe is held to close up mosquitoes landing on humans or at their resting sites and suck them up to the hosepipe. A container was constructed with a net tightly covering the open end and a retractable opening by the side big enough for the smaller hosepipe to pass through when inserted and to quickly cover when removed.Outdoor collections were carried out using the mouth aspiratorin accordance with World Health Organization standard procedures which adopts the stationed human bait collector method (WHO, 1985). Mosquitoes landing on parts of the body such as arm and the legs, a flash light andthe mouth aspirator were used to gently locate and aspirate the mosquitoeswhich were then blown into the container with a closed net. The collection was done twice a week in each sampling locationwith three collectors (three volunteers from each of the village) and collection was between 5:00pm to 7:00pm.This

is because *Aedes* mosquitoes are mostly diurnal, feeding late in the evening and resting outdoors in tree and shrub shades, around rock pool breeding sites and by standing water with clogged vegetation (Service, 2012). These were the sites visited during the collections. The *Aedes*mosquitoes were caught and kept in the containers with closed net and transported to the Parasitology and Entomology Laboratory of the Department of Biological Sciences Ahmadu Bello University, Zaria for Identification.

3.2.2 Collection of *Aedes*mosquito indoor

The indoor mosquitocollection was carried out using a pyrethroid-based insecticide knock down method. The insecticides were purchased from the local market and distributed to the residents in the fourlocations,having obtained the consent of the people with the help of the village leaders.Volunteers, were informed andeducated on the essence of the research and how to carry out the collection of mosquitoes. Each of the consentinghouseholds were given packs of pyrethroid insecticides but were told to collect and keep the mosquitoes in a container since entering their houses was not welcomed by the residents.

*Aedes*mosquitoes were collected from the room where at least one person slept.Before the pyrethroid was used; the windows and doors were properly closed and spread sheets were laid covering immovable items in the room. The pyrethroid insecticide were burned on a stainless plate on the floor of the room to allow the smoke to fill the room and allowed to stay for 30 minutes. Thereafter, a flash light was used to locate themosquitoes knocked down and they were gently picked and placed in containers. Collections were done twice a week in all the four locations.Mosquitoes collected were transported to the Parasitology and Entomology Laboratory of the Department of Biological Sciences, Ahmadu BelloUniversity, Zaria for identification.

3.3 MosquitoIdentification

Morphological identifications were carried out using dissecting microscope and pictorial identification keys for culicines to identify the mosquitoes to species level (Huang and Ward 1981; Highton, 1983; Rueda, 2004;Huang, 2004; Service, 2012). Gross morphology features of the species such as antennae, proboscis, maxillary palp, occiput, scutum, scutellum, targite, wings, foreleg, midleg, hindleg, fermur, tibia, tarsomeres, tarsus and cercus were used to identify Female *Aedes* species.

3.3.1Pools of *Aedes*mosquitoes

Correctly identified mosquitoes were pooled according to the species, month andlocation and then preserved dry in plastic containers with silica gel at room temperature in theParasitology and Entomology Laboratory of the Department of Biological Sciences, Ahmadu BelloUniversity, Zaria until viral extraction.A totalof 48 pools of *Aedes* mosquitoeswere used for the sample analysis.

3.4 SamplesAnalyses

Yellow Fever Virus (YFV) Real Time RT-PCR kit,Dengue Virus General-type Real Time RT-PCR kitand RNA isolation kit were purchased from Liferiver®, Shanghai, China and upon receiving the kits, Yellow Fever Virus (YFV) Real Time RT-PCR kit andDengue Virus General-type Real Time RT-PCR kit were keptat -38ºC to maintain the quality of the reagents while the RNA isolation was kept at room temperature (25°C)in the Parasitology and Entomology Laboratory of the Department of Biological Sciences, Ahmadu BelloUniversity, Zaria. These, together with the48 poolsof *Aedes* mosquitoes, were transportedto the National Influenza Reference Laboratory/ Nigeria Centre for Disease Control (NIRL/NCDC), Federal Ministry of Health, Abujafor

analysis. The Yellow fever virus and Dengue virus kits were swiftly placed in a -38ºC refrigerator.

3.4.1 RNA extraction

Analysis of the 48 pooled*Aedes* samples wascarried out in a class B biosafety hood of the NationalInfluenza Reference Laboratory/ Nigeria Centre for Disease Control (NIRL/NCDC), Federal Ministry of Health, Abuja.The48 pooled samples were prepared for reverse transcriptase Real Time-Polymerase Chain Reaction (RT-PCR) amplification, by subjecting them to viral RNA extraction with care being taken not todegradethe RNA or inhibit the PCR amplification process. This was carried out using RNA isolation kit.

The procedure below were followed according to Gonzalez-Reiche *et al*. (2010) and WHO(2014):

3.4.1.1*Grindingmosquito samples*.

Each of the 48 pooled samples of*Aedes*wasmacerated in clean mortar with a pestleinto which100µl of normal saline wasadded to maintain a constant pH. The homogenatewascollected in anRNase free tube andlabelled 1-48accordingly, noting the location, species and month collected.Buffer AVL® was used as the Lysis buffer to lysis the cell (Buffer AVL is a trade name, manufactured by QIAGEN), 100µl of Buffer AVL, which containsguanidinium thiocynate, a chaotropic agent that lysis the cell and inactivate RNases; thiswas added to each labelled RNase free tube and vortex for 3seconds and then centrifuged at 8,000revolutions per minute (rpm) at 4ºC for 1 minute as described in a method by Harris *et al.* (1998)to clarify thesample. The supernatant was thenused for the RNA extraction.

3.4.1.2 . *Binding solution*

In preparing for RNA extractions, a binding solution was prepared for the samples. These were first prepared as follows; 350µl of 100% elution buffer was added to the dried carrier RNA and briefly shaken. RNA binding beads A was vortexed for 30 seconds to obtain a uniform suspension.42ml of 100% ethanol was added to the 14ml washing buffer W and was well mixed. This is to ensure the precipitation of nucleic acids since RNA is not soluble in ethanol.The Binding solution was prepared by the addition of 350µl of carrier RNA, to1.1ml of RNA binding beads A to 26ml of binding buffer making up a total stock solution of 27.45ml (table 3.1).

From this, 526µl of the binding solution was pipetted into 1.5ml Eppendorf tubes and numbered 1- 48, for the 48 pooled samples. These were briefly shaken to allow for a uniform mixture.

3.4.1.3 *Extraction procedure*

140µl of the supernatant from each ofthe labelled RNase free tube was pipetted into1.5ml Eppendorf tubes containing the 526µl binding solution labelled 1-48 to a total volume of 666µl.Each of the samples in the 1.5ml Eppendorf tubes were vortexed for 10seconds and allowed to settle for 3 minutes at room temperature. Binding columns were then numbered 1-48 for all the samples and

Table 3.1 Constituents of the Binding solution

Reagents	Reaction volume
Binding Buffer	26ml
RNA binding beads A	1.1ml
Carrier RNA	350µl
Total volume	27.45ml

666µl was pipetted from each of the 1.5ml Eppendorf tubes to the binding columns accordingly and centrifuged at13000rpm at 4ºC for 1minute.

Thisprocedure captures the RNA on the binding beads in the column whilethe flow-through in each of the collection tube was discarded and the column placed in another collection tube. 500µl of washing buffer A was added to each of the column and centrifuged at 13000rpm for 40 seconds at 4ºC and the flow-through were discarded. The column was placed into another collection tube and additional washing was done with 500µl of washing buffer A, centrifuged at 13000rpm for 40 seconds at 4ºC and the flow-through discarded. This procedure was done to wash away protein and other contaminants whereas nucleic acid were captured on the binding beads (magnetic beads). The columns were then placed in another collection tube for washing with the second washing buffer W.

500µl of washing buffer W was added to each sample and centrifuged at 13000rpm for 15seconds at 4ºC and the flow-through discarded, the columns were placed in another collection tube and additional 500µl of washing buffer W was again dispensed into each of the columns. Centrifugation was done at 13000rpm for 15 seconds at 4ºC and the flow-through discarded. This was done to remove residual binding solution and to precipitate RNA from the solution given that nucleic acids are insoluble in ethanol.

3.4.2 Total RNA isolation

The columns were then placed in a collection tube and air dried for 2 minutes by centrifuging at 13000rpm. Elution buffer was warmed to 65ºC in a bath and 50µl was pipetted to each column and mixed through tips to make beads completely dissolved in elution buffer and them settled at room temperature for 2 minutes. Elution buffer has low ionic strength to elute the nucleic acids. The columns were placed in the rotor and

centrifuged for 2 minutes at 13000rpm, to ensure complete elution of total RNA from the binding columns. The flow-through was immediately stored at -20ºC to ensure the integrity of the total RNA extracted from the mosquito samples. All the 48 samples of 50 µl each were stored awaiting amplification. Thus, a highly purified total RNA was isolated from this method of extraction.

Nucleic acids were eluted in a small volume of elution solution. This procedure ensured total recovery of nucleic acids.

3.4.3 Real time reverse transcription polymerase chain reaction

The real time kits of Dengue and yellow feverare designed for the detection of viruses in serum, plasma or mosquito sample by quantitative or qualitative real time RT- PCR. In this research, the qualitative approach was used for the detection of viruses in the*Aedes* mosquitoes.

3.4.3.1 *Principle of real time PCR*

The principle of the real time detection is based on the fluorogenic 5´ nuclease assay. This consist of a probe- a short oligonucleotide that has a fluorescence reporter at the 5´ end and a quencher at the 3´ end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. This is based on a phenomenon known as frequency resonance energy transfer (FRET). During the PCR reaction, the DNA polymerase cleaves the probe at the 5´ end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by cleaved reporter dye, which is monitored real time by the PCR detection systems. The PCR cycle at which an increase in the fluorescence signal is detected initially is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities in real time allows the detection of the

accumulating product without having to re-open the reaction tube after the amplification.

3.4.3.2 *One- step real time RT-PCR*

The one-step real time RT-PCR was used in the reaction setup for this research. The step combines the first-strand complimentary DNA (cDNA) synthesis reaction and the real time PCR reaction in the same tube, simplifying the reaction set up and reducing the possibility of contamination.

3.4.4 Detection of viruses

For the purpose of maximizing the available reaction in each of the Dengue and yellow fever real time kit, 48 isolatedRNA extracted from the*Aedes* mosquito samples stored in the refrigerator at -20°C were pooled based on location, species and place of collection. These were numbered 1-25 for Dengue virus detection and 1-25 for yellow fever virus detection, making a total real time sample reaction of 50 with each reaction being a duplicate i.e., reaction numbered "1" is the same for yellow fever and for Dengue virus detection.Applied Biosystem 7300 real time PCR machine was used with a 96 well-standard plate.

The layout of the Real time sample reaction (Table 3.2) indicates the locations (Dumbi, Kufena, Bomo and Hanwa), species and place of collection (Outdoor or Indoor collection) withreaction number 1-25. This layout was used for the Dengue virus and Yellow fever virus detection.

Table 3.2: Layout of Real time sample reaction with locations, species and place of collection

Reaction No.	Location	Species	Place of collection
1.	Dumbi	*Aedes vittatus*	Outdoor
2.	Dumbi	*Aedes aegypti*	Indoor
3.	Dumbi	*Aedes furcifer*	Outdoor
4.	Dumbi	*Aedes furcifer*	Indoor
5.	Dumbi	*Aedes aegypti*	Indoor
6.	Kufena	*Aedes vittatus*	Outdoor
7.	Kufena	*Aedes furcifer*	Indoor
8.	Kufena	*Aedes furcifer*	Outdoor
9.	Kufena	*Aedes aegypti*	Indoor
10.	Kufena	*Aedes spp*	Indoor
11.	Kufena	*Aedes vittatus*	Outdoor
12.	Kufena	*Aedes furcifer*	Indoor
13.	Bomo	*Aedes furcifer*	Outdoor
14.	Bomo	*Aedes furcifer*	Indoor
15.	Bomo	*Aedes aegypti*	Indoor
16.	Bomo	*Aedes taylori*	Outdoor
17.	Bomo	*Aedes metallicus*	Outdoor
18.	Bomo	*Aedes furcifer*	Outdoor
19.	Bomo	*Aedes furcifer*	Indoor
20.	Hanwa	*Aedes aegypti*	Indoor
21.	Hanwa	*Aedes vittatus*	Outdoor
22.	Hanwa	*Aedes furcifer*	Outdoor
23.	Hanwa	*Aedes furcifer*	Indoor
24.	Hanwa	*Aedes aegypti*	Outdoor
25.	Hanwa	*Aedes aegypti*	Indoor

3.4.4.1 *Master mix for dengue virus detection*

The Dengue virus general-type real time RT-PCR kit contains a specific ready-to-use system for the detection of the Dengue virus (for serotypes1-4).The Master Mix for Dengue virus detection reaction was prepared (Table 3.3). The reagents were vortex and kept on ice for the duration of the master mix preparation. Super mix contains $MgCl_2$,forward and reverse primers, a labelled probe, DNA polymerase, polymerase buffer,deoxynucleotide triphosphates (dNTP's) and RNase free water. This was pipetted into an Eppendorf tube followed by internal control (a house keeping gene) andanenzyme mix (reverse transcriptase). After the preparation, 20µl Master Mix were vortex, spanned down briefly and aliquot into each 27 wells of the 96 well real time PCR reaction plate. Separately, 5µl of molecular grade water (No Template Control, NTC) serving as the negative control was aliquot to the1stwell (A1) of the 96 well real time PCR reaction plate,5µl ofthe isolated RNA sample werealiquot to 25 wells of the 96 well real timeand5µl of Dengue fever virus positive control (Positive Template Control, PTC) was aliquot to the 12th well (A12) of the reaction plates making a total reaction volume of 25µl for each well.Detection for Dengue virus were from the25 wells in which 5µl each of isolated RNA were aliquot. Whereas wells A1 and A12 serve as Negative and Positive controls, in which5µl of molecular grade water and 5µl of Dengue fever virus positive controlwere aliquot, respectively.

3.4.4.2 *Master mix for yellow fever virus detection*

The Yellow fever virus real time RT-PCR kit contains a specific ready to use system for the detection of Yellow fever virus. Internal controls in the reaction mix allows the determination and control of the possibility of PCR inhibition.Master Mix for Yellow fever virus detection reaction was prepared (Table 3.4.). The reagents were vortex and kept on ice for the duration of the master mix preparation.

Table 3.3 Master mix preparation for each single reaction for dengue

No.	Single reaction	X 27 Reaction
1	18µl of Super Mix	490µl of Super Mix
2	1µl of Internal control	30µl of Internal control
3	1µl of RT-PCR Enzyme Mix	28µl of RT-PCR Enzyme Mix
Total Volume	20µl	548µl

Table3.4Master mix preparation for each single reaction for yellow fever

No.	Single reaction	X 27 Reaction
1	18µl of Super Mix	490µl of Super Mix
2	1µl of Internal control	30µl of Internal control
3	1µl of RT-PCR Enzyme Mix	28µl of RT-PCR Enzyme Mix
Total Volume	20µl	548µl

The Super mix in the master mix preparation contains $MgCl_2$, forward and reverse primers, a labelled probe, DNA polymerase, polymerase buffer, dNTP's and RNase free water. These, together with the internal control and enzyme mix, were pipetted into an Eppendorf tube and vortex and spanned down briefly. The20µl master mix preparedwere eachaliquot to 27 wells of 96 well real time PCR reaction plate.Separately, 5µl of molecular grade water was pipetted into well B1 as the negative control(NTC) making a total reaction volume of 25µl, 5µl of the isolated RNA sample were each aliquot to 25 wells of the 96 well-standard plate, making a total reaction volume of 25µl for the detection of Yellow fever virus and 5µl of positive control was added to the reaction tube in well B12 as the positive control (PTC), making a total reaction volume of 25µl. Dilution was not performed with the positive controls to generate a standard curve, since it was not needed in qualitative detection. All the tubes were immediately micro sealed to avoid contamination and spin down for 20 seconds to collect all the reaction volume at the bottom of the reaction tube. The reaction plate was then placed in an ABI 7300 cycler using Sequence Detection systems (SDS) v1.4 software and programmed according to protocol. Figure 3.4 shows the layout of the 96 well real time PCR reaction plate. Wells of sample reactions are labelled 1-25 for Dengue and Yellow fever detection induplicates. The 96 standard well plate for the real time reverse transcriptase polymerase chain reaction is numbered vertically from 1 to 12 while horizontally, the plate were labelled alphabetically from well A to H.

3.4.4.3 *Protocol performed*

The following were performed based on the protocol from Liferiver®, Shanghai, China.

On theSequence Detection Systems (SDS) software, the detector name was set as

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC Undet.	1 Undet.	2 Undet.	3 Undet.	4 Undet.	5 Undet.	6 Undet.	7 Undet.	8 Undet.	9 Undet.	10 Undet.	PTC Undet.
B	NTC Undet.	1 Undet.	2 Undet.	3 Undet.	4 Undet.	5 Undet.	6 Undet.	7 Undet.	8 Undet.	9 Undet.	10 Undet.	PTC Undet.
C												
D	11 Undet.	12 Undet.	13 Undet.	14 Undet.	15 Undet.	16 Undet.	17 Undet.	18 Undet.	19 Undet.	20 Undet.	21 Undet.	22 Undet.
E	11 Undet.	12 Undet.	13 Undet.	14 Undet.	15 Undet.	16 Undet.	17 Undet.	18 Undet.	19 Undet.	20 Undet.	21 Undet.	22 Undet.
F												
G	23 Undet.	24 Undet.	25 Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.
H	23 Undet.	24 Undet.	25 Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.

KEY: Undet. =Undetermined, NTC= Negative control, PTC= Positive control.

Figure 3.2. Layout of96 well real time PCR reaction plate.

Dengue and Yellow fever for the wells accordingly, fluorimeter channel(FAM) was selected for the reporter dye. The TaqMan® MGB (minor –groove binding molecule) probe uses fluorescent quenchers hence, "none" was selected for the quencher dye. The threshold was set at 0.2 just above the maximum level of molecular grade water. The baseline start was setat 3 and the baseline end was set at 15.The reaction set up was a one-step reaction, hence it was performed as follows:

Stage 1. Reverse transcription of RNA to complementary DNA (cDNA):

The temperature was set at 45°C for1 cycle for 10 minutes. This activates the reverse transcriptase enzyme to transcribe RNA to cDNA. Where the reaction containedDengue or yellow fever virus it was transcribed into a complementaryDNA.

Stage 2. Activation of DNA polymerase:

The temperature was set at 95°C for 1 cycle for 15 minutes. This activates the thermo stableDNA polymerase.

Stage 3. Step 1.Denaturation:

The temperature was set at 95°C for 15 seconds. This temperature separates the strands of the cDNA.

Step 2. Annealing/ extension: The temperature was set at 60°C for 1 minute for 40 cycles.The primer and probes binds to their complimentary sequence. The DNA polymerase commences the extension at the same time DNA polymerase has an exo nuclease activity as it encounters a strand in its path it dismantles it hence, it cleaves the probe at the 5´ end and dismantle it and continues in the addition of nucleotides, hence in the process the reporter dye is separated from

the quencher dye and it fluorescence. The fluorescence emitted is measured at 60°C by the real time systems optical unit during the PCR. Monitoring the fluorescence intensities in real time allows the detection of the accumulated product without having to re-open the reaction tube after the amplification as the temperature reduces to 60°C for a minute and temperature heated up again to 95°C for 40 cycles. The detection of amplifiedDengue virus cDNA fragment and yellow fever virus cDNA fragments was performed in fluorimeter channel FAM. Data collections was at stage 3 step 2.

The interpretations of the threshold cycle values(Ct) values, indicating the positive samples from the reaction and the negative or undetermined samples (Table 3.5.) The FAM is the reporter dye that shows the fluorescent of the samples and their corresponding threshold cycle values. The HEX/VIC/JOE is reporter dyes for the internal control with its corresponding values.

The Applied Biosystem 7300 real time PCR system uses fluorescent based PCR chemistries to provide quantitative or qualitative detection of nucleic acid in real time. In this work, the system was used for the qualitative detection of Dengue and yellow fever virus. The target region for the Dengue virus general type (DENV-1, DENV-2, DENV-3 andDENV-4) was 3' untranslated region (3' UTR) genome region of all the four serotypes, while the target gene for the yellow fever virus was the non-structural protein 5 (NS 5).

Table 3.5: Interpretation of the data (Ct values)

	FAM	HEX/VIC/JOE	RESULT ANALYSIS
1	UNDET	25≈35	Below the detection limit or negative
2	≤38	—	Positive; and the software displays the quantitative value
3	39≈40	25≈35	Re-test; if it is still 38≈40. Report as in 1
4	UNDET	UNDET	PCR inhibition; no diagnosis can be concluded

The sequence detection system (SDS) software mathematically transforms the raw fluorescence data to establish a comparative relationship between the spectral changes in the passive reference dye and those of the reporter dyes. Based on that comparison, the software generates four results: Plate, Spectra, Amplification plot and component. The plate showed the arrangement of each pooled sample inmicro-titre wells labelled. The first wells, A1 and B1contained no template control (NTC) and wells A12 and B12 contained the positive template control (PTC).The spectra showed the raw data that displays thefluorescence spectra of each sample in the well (A1-H12). The fluorescence was plotted on the y-axis whereas the filters are on the x-axis. The amplification plot showed the plot of normalized reporter dye fluorescence$R_n$as a function of cycle number for selected detector and wells. It also showed the magnitude of the signal generated by the specified set of PCR condition in delta Rn vs cycle. Amplification plots were created when the fluorescent signal from each sample was plotted against cycle number; therefore, representing the accumulation of product over the duration of the real time PCR experiment.

The component showed the fluorescence and cycle run displaying the complete spectra contribution of each dye in a selected well over the duration of the PCR run. Here, only the first selected well A1 was shown and since the probe was a TaqMan MGB product; only two components (ROX and FAM reporter dye) were shown**.**

3.5Data Analysis

Data obtained from the collections of mosquito were analysed using descriptive statistics.The abundance of species of *Aedes* collected indoorsandoutdoors in the four locations: Dumbi, Kufena, Hanwa and Bomo were expressed aspercentages and presented in graphs. The differences in mosquito abundance among species and

locations were subjected to analysis of variance (ANOVA) to determine significant differences. The results of the amplification of the isolated viral RNA transcribed to complimentary DNA on real time instrument are shown by the values of the threshold cycle (Ct) values which represents the precise number of PCR cycles at which the fluorescence signal begins to increase exponentially and crosses the detection threshold, i.e., the background; in relation to the positive template control in the fluorimeter channel FAM.

Analysis of variance (ANOVA) was used to determine significant difference in the prevalence of the virus in the mosquitoes collected and from the different locations.

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito Collections

4.1.1 Monthly collections

The four months collection of*Aedes* mosquitoes, from July to October, 2013 in Dumbi, Kufena, Bomo and Hanwa showed the behaviour of the species in the different locations over the period of study.A total of 257 female *Aedes*mosquitoes were collected outdoor and indoor.Collections were carried out twice in a week or bi-weekly in each location, for both outdoor and indoor during the period of study. The total outdoor collections for the period of study were 111 *Aedes* mosquitoes, while those for the indoor collections were 146 *Aedes*mosquitoes in the four locations.In July,a total of 41 *Aedes* mosquitoes were collected outdoor and indoor in the four locations (Appendix I). The *Aedes* mosquitoes collected outdoor were 25 while 16 *Aedes* mosquitoes were collected indoor. *Aedes vittatus* were only found outdoors in two locations: Dumbi and Kufena whereas *Aedes furcifer* were found both outdoor and indoor in Bomo,*Aedes aegypti* were found indoor in Dumbi and Hanwa for this month.In the month of August, 73*Aedes*mosquitoes werecollected with 30 *Aedes* mosquitoes collected outdoor and 43 collected indoor; in this,*Aedes taylori* and *Aedes metallicus*were collected outdoor in Bomobut were not found in the three other locations (Appendix II).The month of September, recorded the highest collections for the period of study asa total of 92 *Aedes* mosquitoes were collected. In this collections, 32 *Aedes* species were collected outdoor while 60 were collected indoor.*Aedes furcifer*was found either indoor or outdoor in all the four locations (Appendix III). In October, a total collection of 51 *Aedes*mosquitoes were collected from the four locations with 24 *Aedes* mosquitoes collected outdoor and

27 collected indoor.*Aedes aegypti* and *Aedes furcifer* were found to be predominant indoor whereas*Aedes vittatus* were only found outdoor and was not found in Bomo (Appendix IV).

The occurrence and relative abundanceofthe monthly collections,in all the four locations (Figures 4.1 and 4.2) for outdoor and indoor collections describes the apparent density for each *Aedes* mosquitoes in each location. These apparent densities were calculated as the mean of the collections of each of the *Aedes* mosquito.In figure 4.1, the highest apparent density for outdoor collections for the four months was recorded in*Aedes vittatus* (1.625) in Kufena in August,while the least apparent densitieswere*Aedes furcifer* in Dumbi and Kufena in August; *Aedes metallicus* and*Aedes taylori* in Bomo in August and *Aedes aegypti* in Hanwa in September (0.125).*Aedes taylori* and *Aedes metallicus* were exclusively encountered in outdoor collections in Bomo.

In Figure 4.2, the highest apparent densityfor indoor collections for the four months was *Aedes furcifer*(1.625) and *Aedes aegypti* (1.625) in September in Kufena. Similarly, *Aedes aegypti* were found in all the locations entirely indoors. While the lowest mean for indoor collections was *Aedes furcifer* (0.125) in Dumbi in October.

4.1.2 Collections according to species

The apparent densities of the *Aedes* mosquito species collected in the four locations for the period of study indicates that, *Aedes furcifer* (3.1875) was the most predominant species encountered(Figure 4.3). *Aedes metallicus* (0.03125) was the least abundant species collected. *Aedes vittatus* were found in Dumbi, Kufena and Hanwa but none in Bomo. Similarly, *Aedes metallicus* and *Aedes taylori* were only encountered in Bomo. Whereas, *Aedes furcifer* and *Aedes aegypti* were found in all the four locations.

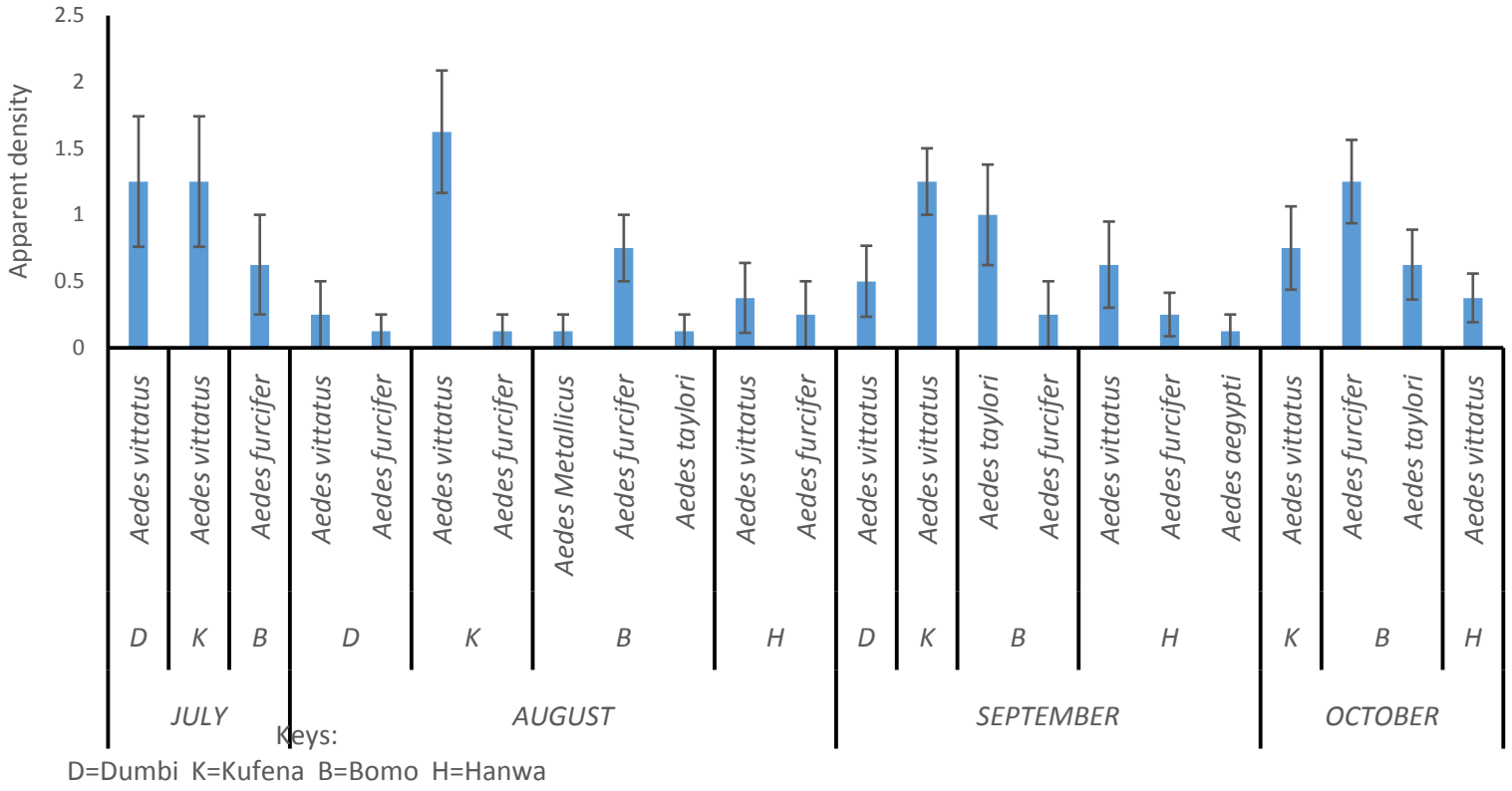


Figure 4.1.Occurrence and relative abundance of *Aedes* mosquito species in selected outdoor locations in Zaria

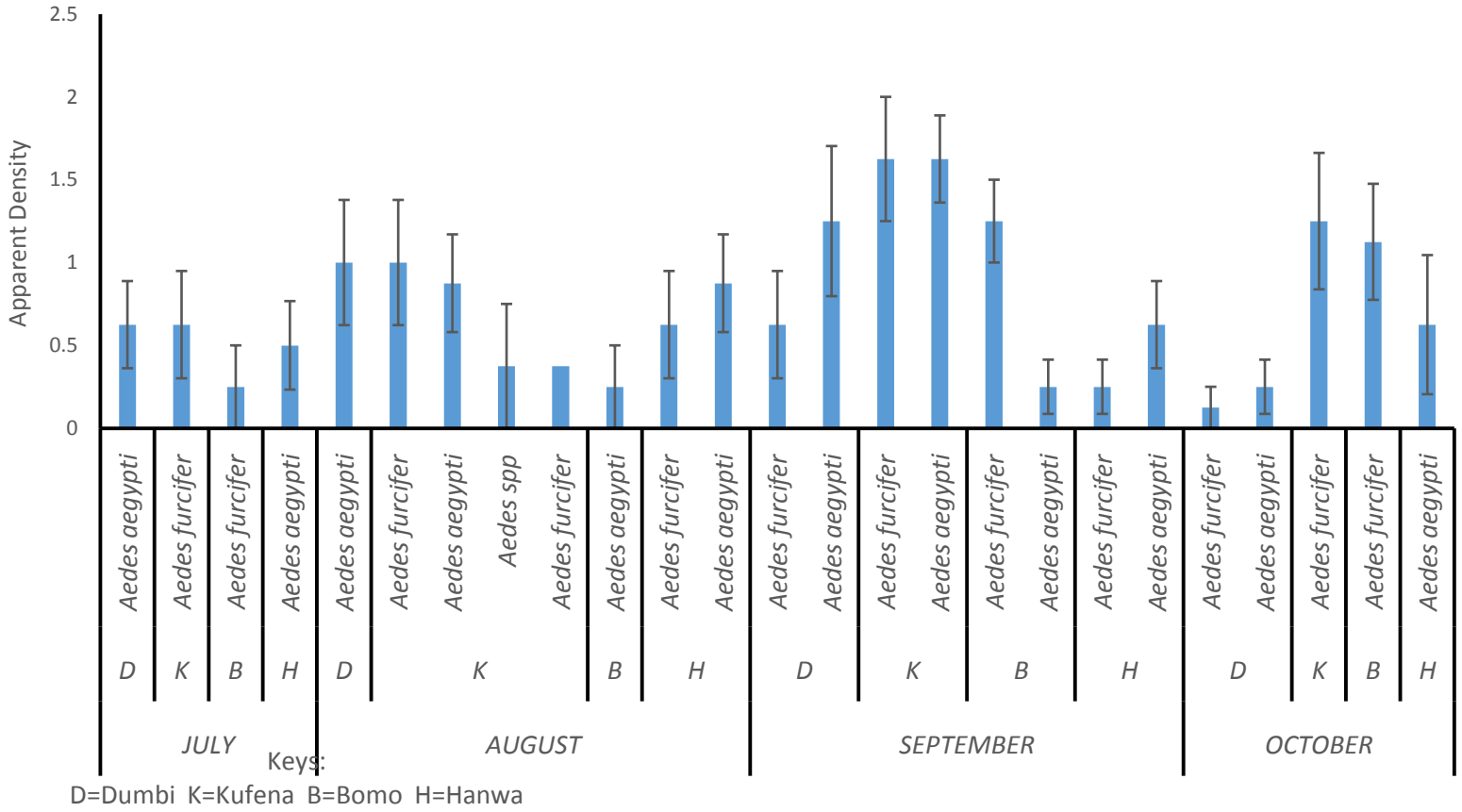


Figure 4.2.:Occurrence and relative abundance of *Aedes* mosquito species in selected indoor locations in Zaria.

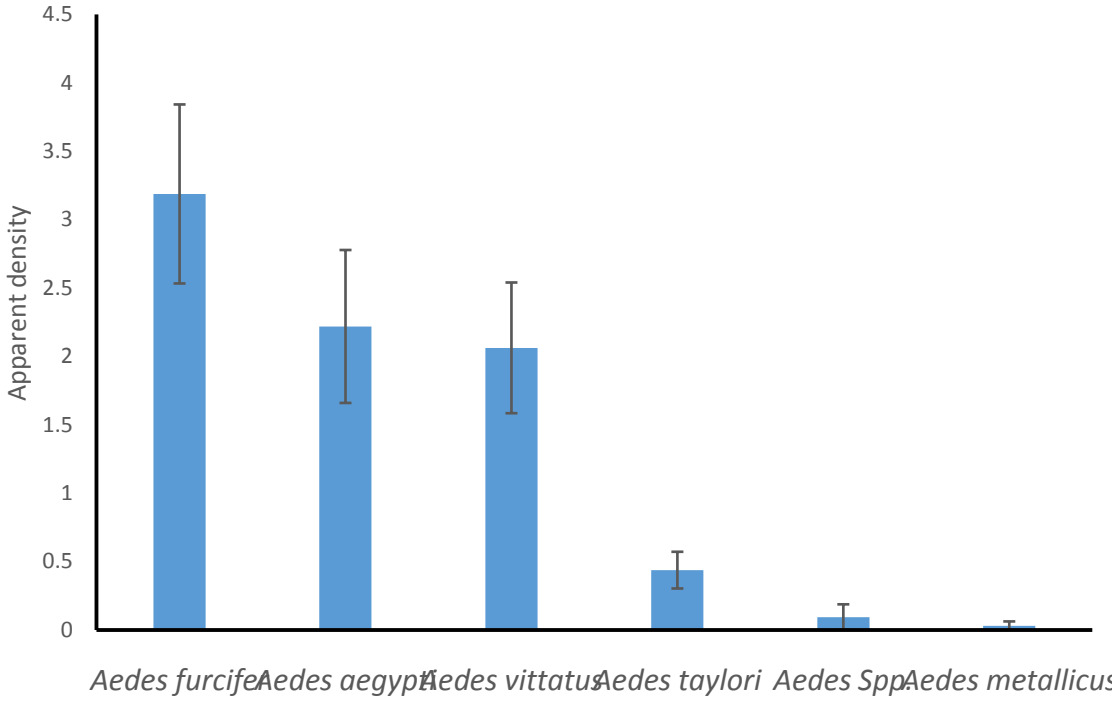


Figure 4.3.Relative abundance of *Aedes* mosquito species in the four location in Zaria

4.1.3 Collections according to locations

The collections from the four locations, showed that more *Aedes*mosquitoes were collected from Kufena over the period of study. Hanwa had the least collection of *Aedes* in all the four locations shown in the apparent density of the species in the four locations (Figure 4.4).

4.1.4 Outdoor collections

The *Aedes* mosquitoes collected outdoor were *Aedes vittatus*, *Aedes furcifer*, *Aedestaylori*, *Aedes metallicus* and *Aedes aegypti*. This outdoor collections shows that *Aedes vittatus* was the species mostly encountered (Figure 4.5). *Aedes aegypti* was the least encountered(Figure 4.5). This is indicated in the apparent density of the *Aedes* mosquitoes over the period of the study.

4.1.5 Indoor collections

The indoor collections show three species were abundant: *Aedesfurcifer*, *Aedes aegypti* and *Aedes* spp, encountered in the locations. From these, *Aedes furcifer* was the most abundant species, followed by*Aedes aegypti*which were encountered in all four locations. Figure 4.5 shows the apparent density of the Aedes mosquitoes collected outdoor and indoor from all the locations

4.2 Abundance of *Aedes* Mosquitoes

The abundance of the species in each of the locations, indoor or outdoor over the period of study; July to October, 2013 are presented in percentages.

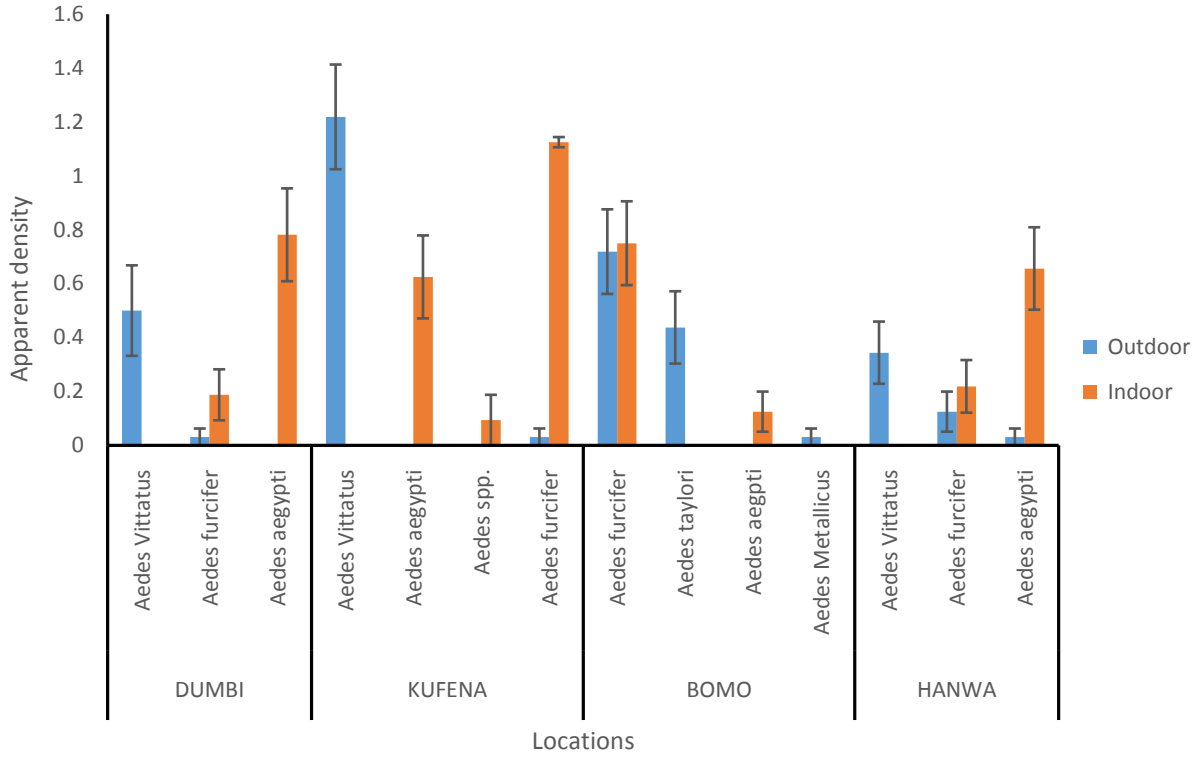


Figure 4.4. Spatial distribution of *Aedes* mosquitoes in selected locations in Zaria

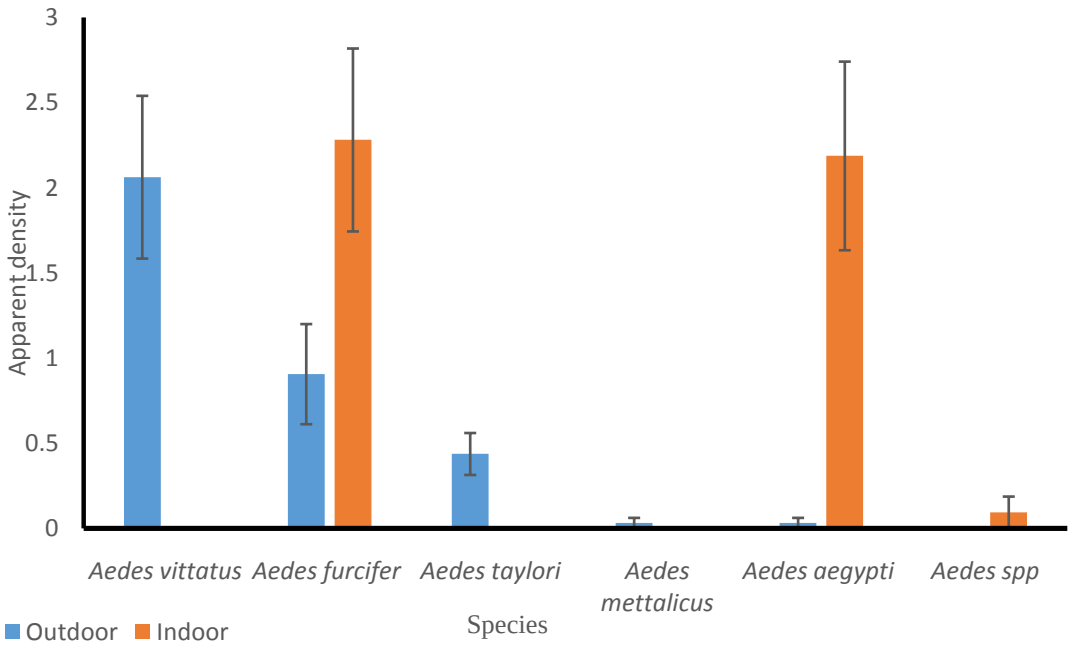


Figure 4.5. Relative abundance of *Aedes* mosquito species from outdoor and indoor collections made in four locations in Zaria

4.2.1.1*Abundance of Aedes in july*

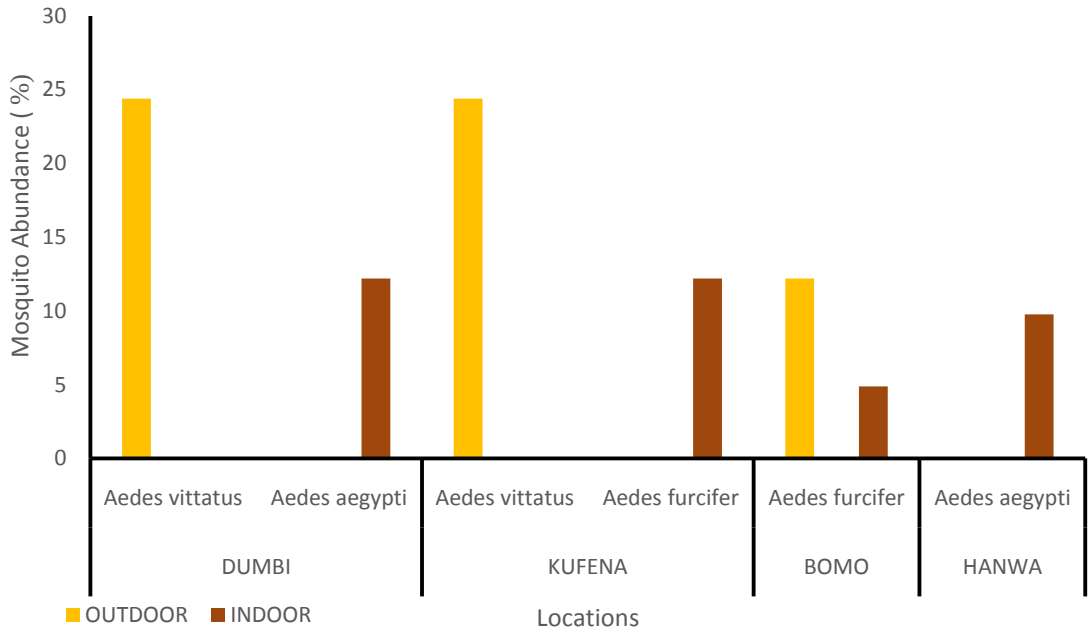
The abundance of *Aedes*mosquitoes inJuly,2013 (figure 4.6),*Aedes vittatus*outdoor in Dumbi and Kufena were the most abundant (24.39 %). In Bomo*Aedes furcifer*indoor was the least abundant (4.88%). Nonetheless, in the four locations there was no significance difference in the abundance both outdoor and indoor. Amongst the *Aedes* mosquitoes, there was no significant difference outdoor and indoor (P-value ≥0.05).

4.2.1.2 *Abundance of Aedes in august*

The abundance of *Aedes* mosquitoes in August, 2013 (figure 4.7),*Aedes vittatus*outdoor in Kufena was the most abundant (17.81%).*Aedesfurcifer*outdoor in Dumbi, Kufena; *Aedes metallicus*and *Aedes taylori* outdoor in Bomo were the least abundant (1.37%)from the locations.However, in the four locations there was no significance difference in the abundance both outdoor and indoor(P-value ≥0.05).Amongst the *Aedes* mosquitoes indoor, there was a significant difference (P-value ≤0.05)whereas, there was no significant difference outdoor.

4.2.1.3 *Abundance of Aedes in september*

The abundance of *Aedes* mosquitoes in September, 2013 (figure 4.8)*Aedes furcifer*and *Aedes aegypti* (14.13%) indoor in Kufena were the most abundant. *Aedes aegypti*outdoor in Hanwa was the least abundant (1.09%). Nonetheless, in the four locations there was no significance difference in the abundance both outdoor and indoor (P-value ≥0.05).Amongst the *Aedes* mosquitoes indoor, there was a significant difference (P-value ≤0.05) whereas there was no significant differenceoutdoor.

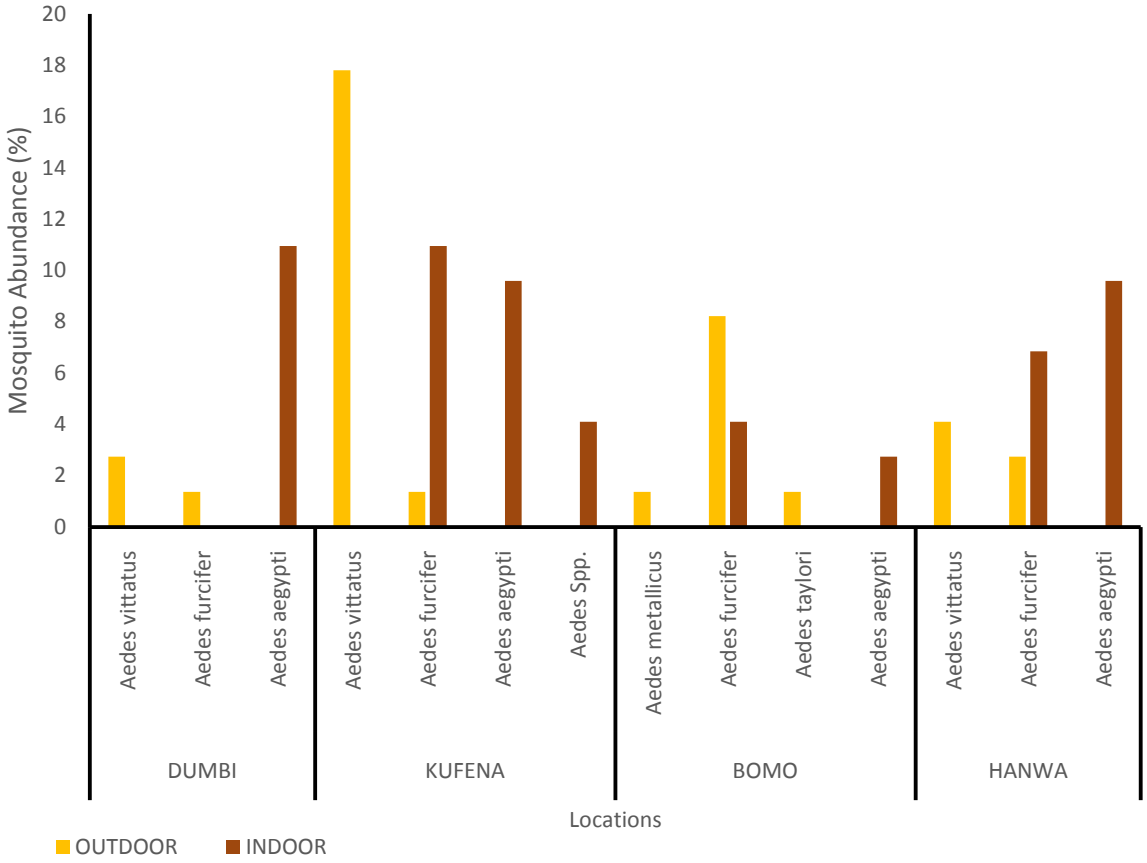


ANOVA **P-value**

Locations: outdoor: 0.7133 indoor: 0.6660

Species: outdoor: 0.3759 indoor: 0.1035

Figure 4.6Relative abundance of *Aedes* mosquito species during the month of July, 2013 in Zaria

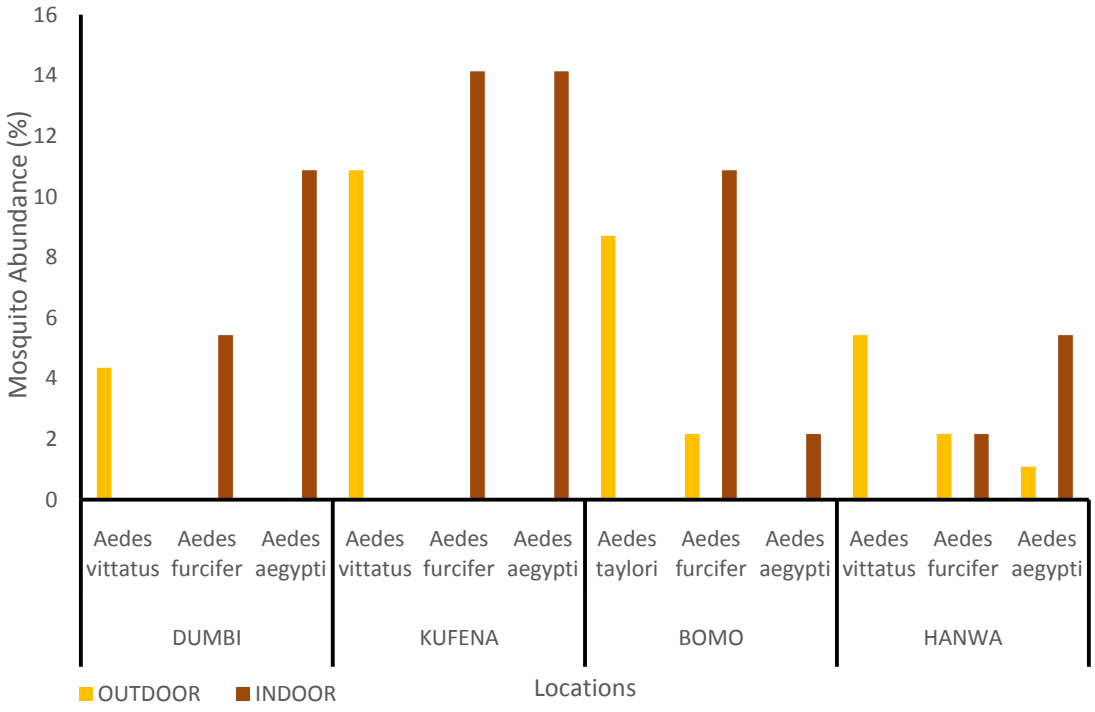


ANOVA **P-value**

Locations: outdoor: 0.667 indoor:0.2135

Species: outdoor: 0.1510 indoor: 0.0005

Figure 4.7Relative abundance of *Aedes* mosquito species during the month of August, 2013 in Zaria



ANOVA **P-value**

Locations: outdoor: 0.8941 indoor:0.2754

Species: outdoor: 0.1302indoor: 0.0008

Figure 4.8Relative abundance of *Aedes* mosquito species during the month of September, 2013 in Zaria

4.2.1.4 *Abundance of Aedes in october*

The abundance of *Aedes*mosquitoes in October, 2013 (figure 4.9)*Aedes furcifer* indoor in Kufena and outdoor in Bomo were the most abundant (19.61%).*Aedes furcifer*indoor was the least abudant (1.96%) in Dumbi. In the four locations there was no significance difference in the abundance both outdoor and indoor. Also, Amongst the *Aedes* mosquitoes, there was no significant difference outdoor and indoor (P-value ≥0.05).

4.2.2 Abundance of *Aedes*mosquitoes in the locations

From the four locations as shown in figure 4.10 Kufena (38.52%) had the most abundant species of*Aedes* over the period of study. Hanwa (17.12%) is the location where fewer species of *Aedes*were collected during the study period.

4.2.3Abundance of *Aedes* mosquitoes outdoor

The abundance of *Aedes* outdoor as shown in figure 4.11 shows that *Aedes vittatus* (59.46%) was the most abundant species collected while, *Aedes metallicus* (0.90%) and *Aedes aegypti* (0.90%) were the least abundant species collected outdoor.

4.2.4 Abundance of *Aedes vittatus* outdoor in each of the four locations

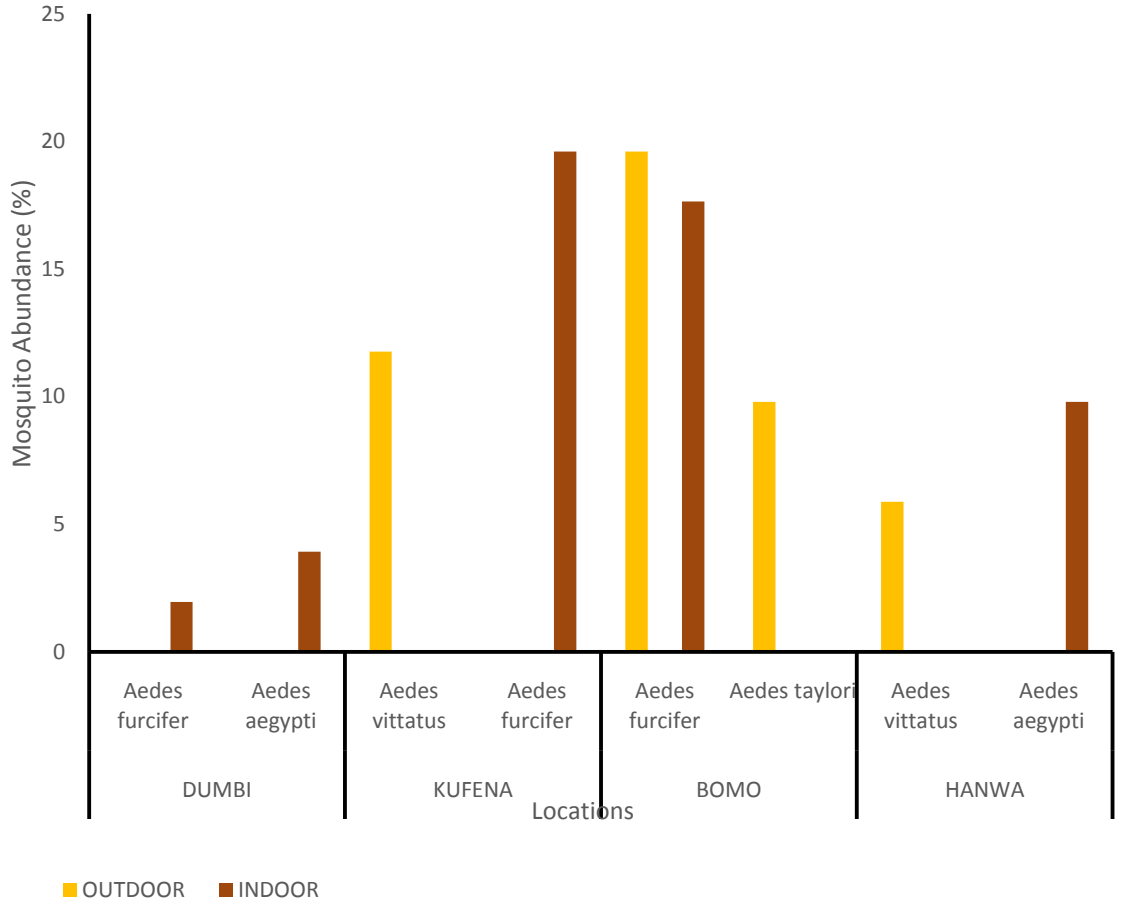
From the four locations as shown in figure 4.12, *Aedes vittatus* was most abundant in Kufena (59.09%) but none was found in Bomo (0%).

4.2.5 Abundance of *Aedes furcifer*outdoor from the four locations

In the four locations, as shown in figure 4.13, *Aedes furcifer* was most abundant in Bomo (79.31%) whereas Dumbi (3.45%) and Kufena (3.45%) had the least abundance.

4.2.6 Abundanceof *Aedes taylori*outdoor from the four locations

In the four locations, as shown in figure 4.14, *Aedes taylori* was only recovered in Bomo (100%) and absent in the other locations.



ANOVA **P-value**

Locations: outdoor- 0.3889 indoor- 0.8299

Species: outdoor-0.5382 indoor-0.0668

Figure 4.9Relative abundance of *Aedes* mosquito species during the month of October, 2013 in Zaria

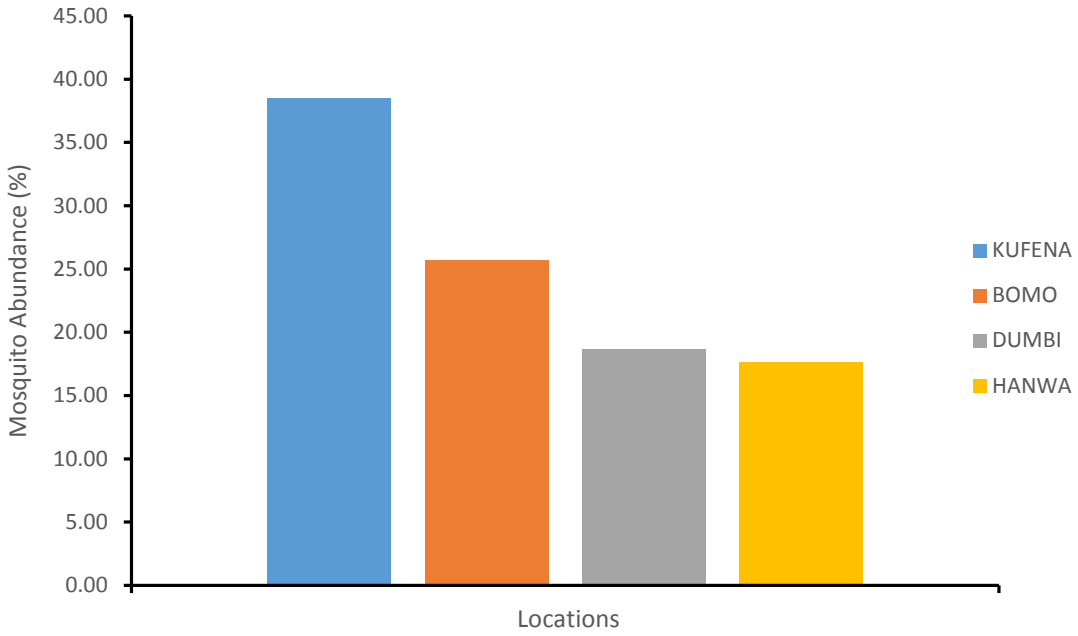


Figure 4.10Abundance of *Aedes*mosquitoes from four locations in Zaria and Environs

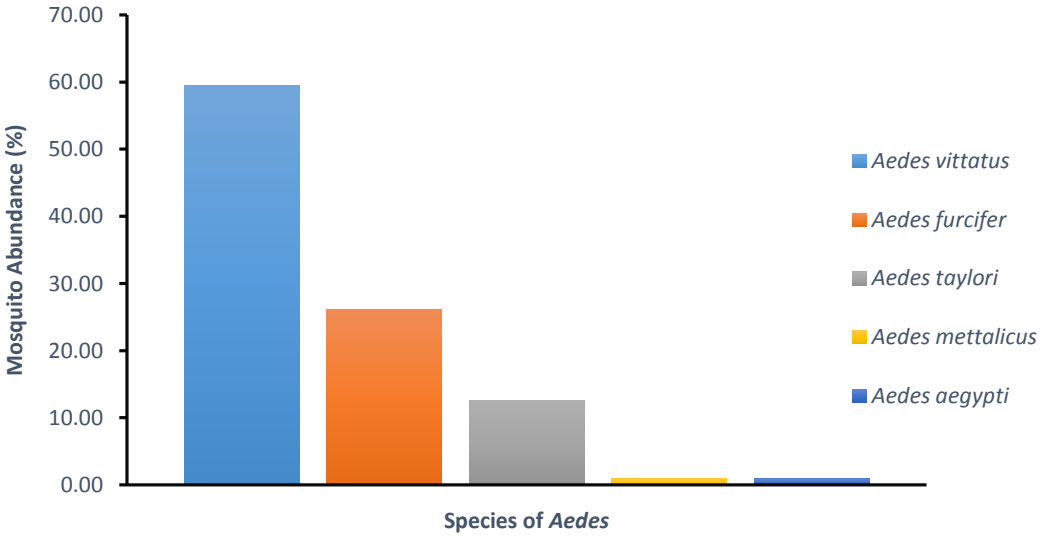


Figure 4.11Outdoor Abundance of *Aedes*mosquitoes in Zaria and environs from July-October, 2013

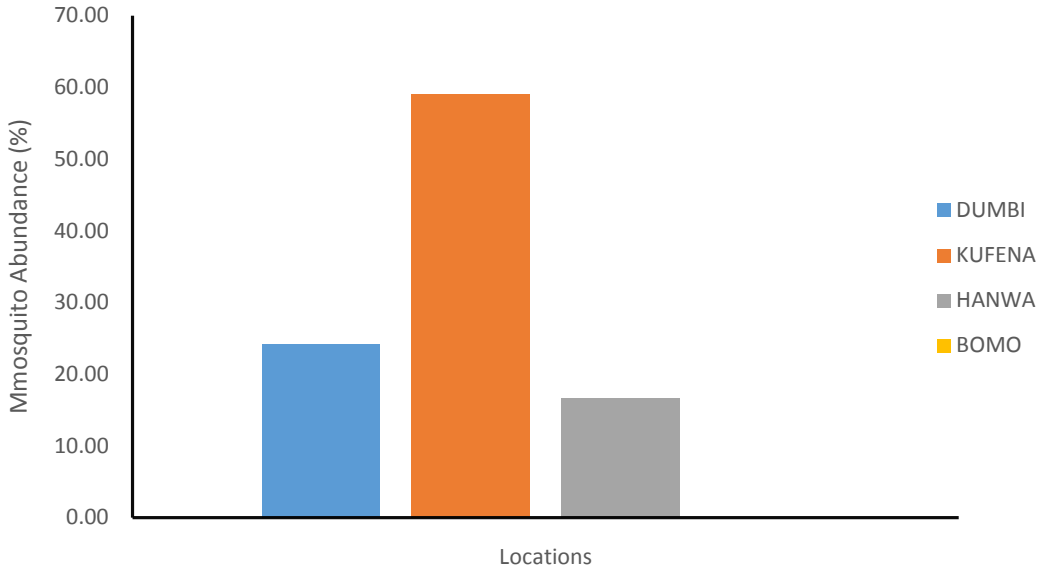


Figure 4.12Abundance of *Aedes vittatus* outdoor from four locations in Zaria and environs

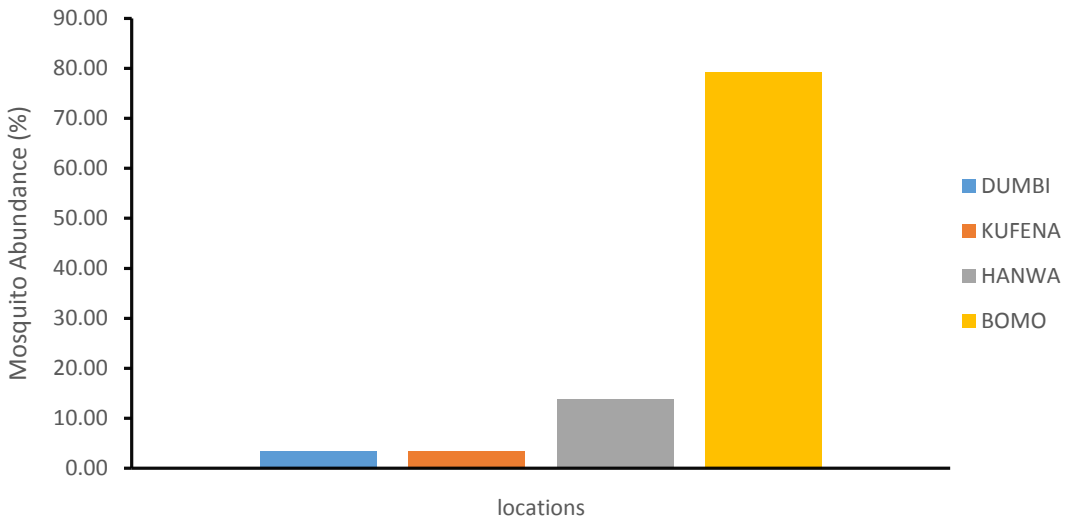


Figure 4.13Abundance of *Aedes furcifer*in outdoor mosquito collections from different locations in Zaria and environs

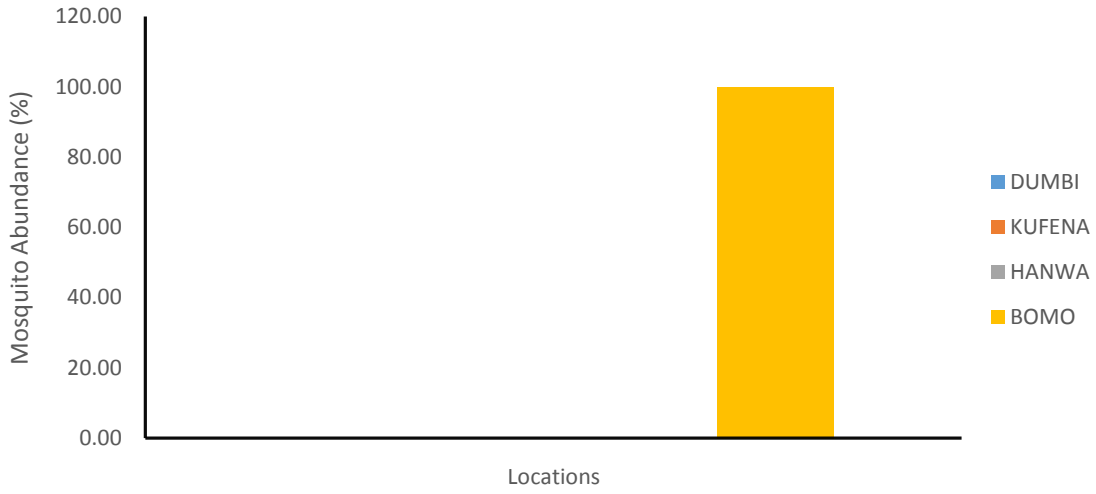


Figure 4.14Abundance of *Aedes taylori*in outdoor mosquito collections from different locations in Zaria and environs

4.2.7 Abundance of *Aedes metallicus*outdoor from the four locations

From the four locations, as shown in figure 4.15, *Aedes metallicus* was only encountered in Bomo (100%) and absent in other locations.

4.2.8 Abundance of *Aedes aegypti* outdoor from the four locations

From the four locations as shown in figure 4.16, *Aedes aegypti* was only encountered in Hanwa (100%).

4.2.9Abundance of *Aedes* mosquitoes indoor

As shown in figure 4.17, *Aedes furcifer* (50%) was the most abundant species in indoor collection from the four locations.

4.2.10Abundance of *Aedes furcifer* indoor from the four locations

From the four locations as shown in figure 4.18, *Aedes furcifer* was most abundant in Kufena (49.32%).

4.2.11Abundance of *Aedes aegypti* indoor from the four locations

From the four locations, as shown in figure 4.19, *Aedes aegypti* was most abundant in Dumbi (35.71%) but it was least abundant in Bomo (5.71%). Whereas it was 30.00% and 28.57% abundant in Hanwa and Kufena respectively.

4.2.12Abundance of *Aedes*spp indoor from the four locations

From the four locations as shown in figure 4.20, *Aedes* spp was only encountered in Kufena (100%).

4.3 Viral RNA Isolation

With the extraction method using a guanidium thiocynate based technique following manufactures protocol, viral RNA were isolated from 50 pools (25 each for Dengue virus and yellow fever virus) of *Aedes* mosquitoes. These viral isolates were identified using the real timereverse transcriptase PCR for Dengue virus and yellow fever virus.

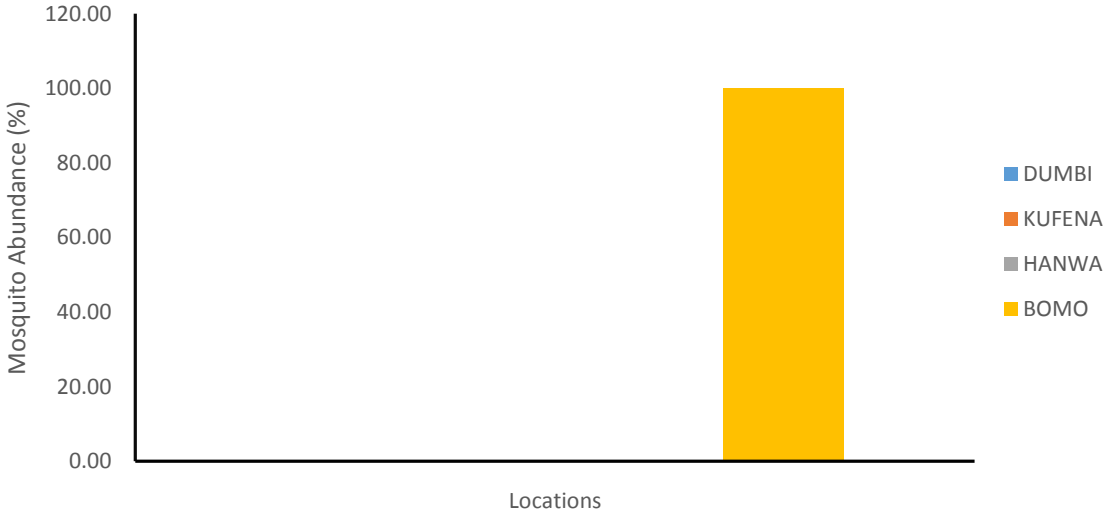


Figure 4.15Abundance of *Aedes metallicus*in outdoor mosquito collections from different locations in Zaria and environs

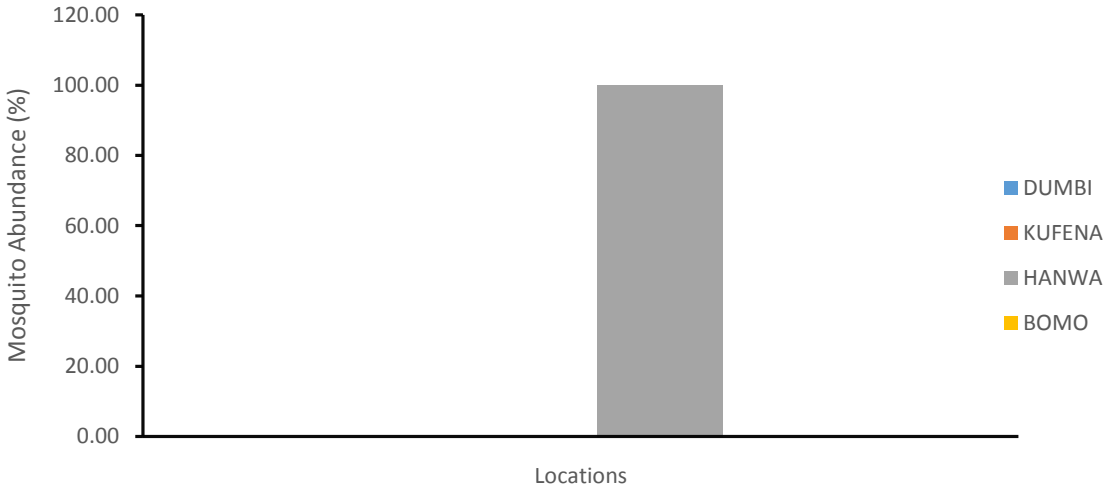


Figure 4.16Abundance of *Aedes aegypti*in outdoor mosquito collections from different locations in Zaria and environs

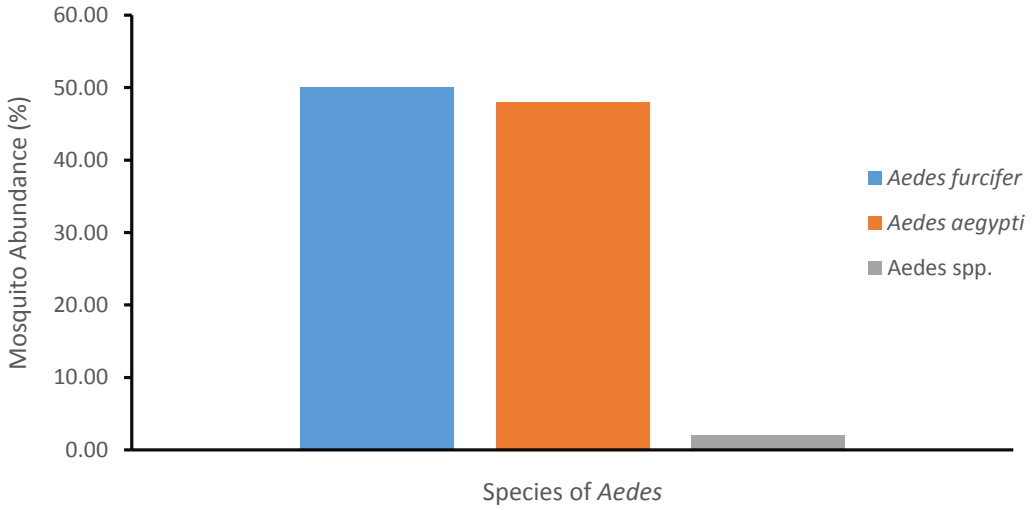


Figure 4.17Indoor Abundance of *Aedes* species in Zaria and environs, from July to October, 2013

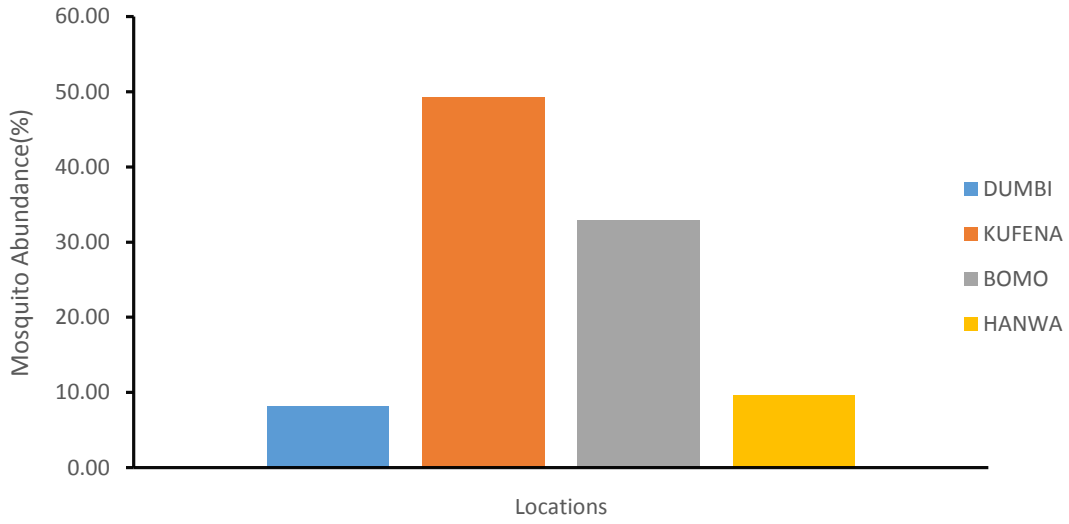


Figure 4.18Abundance of *Aedes furcifer*in indoor mosquito collections from different locations in Zaria and environs

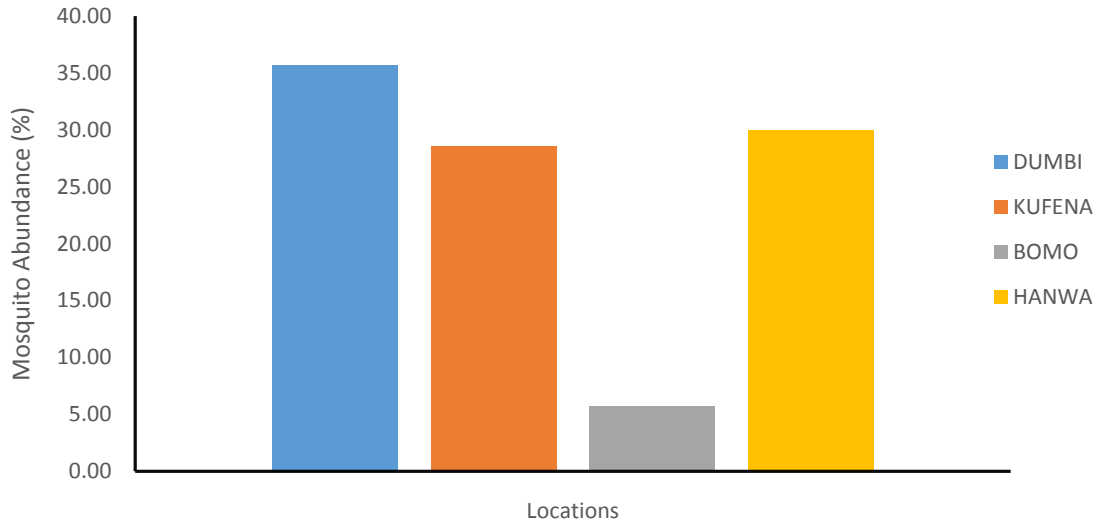


Figure 4.19Abundance of *Aedes aegypti*in indoor mosquito collections from different locations in Zaria and environs

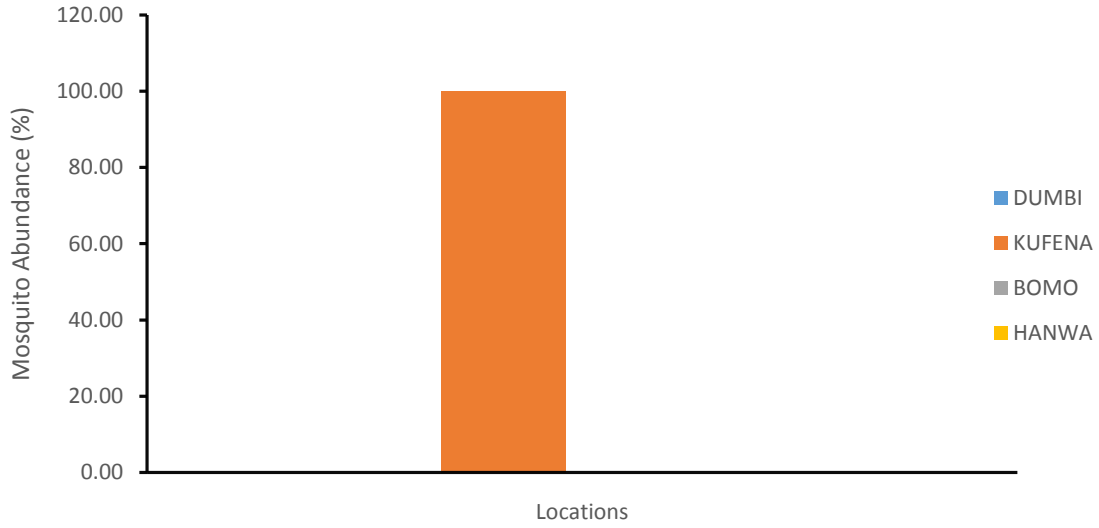


Figure 4.20Abundance of *Aedes* spp. In indoor mosquito collections from different locations in Zaria and environs

4.4 Dengueand Yellow fever Viruses Detection Using Real Time RT-PCR

4.4.1 Dengue virus detection

From table 4.1, positive template control (PTC) of Dengue virus has a Ct value of 17.16. The negative template control (NTC) which was molecular grade water and the pool mosquito samples that were negative had Ct values above 38 hence the software displays ‘’undetermined’’ in the fluorimeter channel FAM. 10 samples from the reaction were positive for Dengue virus; their Ct values were below 38 as shown in FAM for the target region. This implies that from the 25 pool mosquito samples, 40% of the pool mosquito samples were positive for Dengue virus. The number of positive pool mosquito samples for Dengue virus in the locations and whether they were collected outdoor or indoor(Table 4.2). From the four locations, three locations-Dumbi, Bomo and Hanwa - were found to have positive samples for Dengue virus. Samples from Kufena were not positive for Dengue virus.

The prevalence of Dengue virus in *Aedes* species in the four locations(Tables 4.3), indicates that the *Aedes* mosquitoes with the most prevalence in the study population for Dengue virus were *Aedes aegyti*(40%)and*Aedes furcifer*(40%).In the positive samples of Dengue virus, two pools of *Aedes aegyti*collected indoor were positive in Dumbi, one pool in Bomo and another pool in Hanwa. One pool of *Aedes furcifer*collected outdoor was positive for Dengue in Dumbi and two pools in Bomo. One pool of *Aedesfurcifer* collected indoor in Bomo was positive for Dengue virus. Two pools of species collected outdoor that were also positive for Dengue virus are *Aedes taylori* and *Aedesmetallicus* found only in Bomo

The prevalence of Dengue virus in the four locations(Table 4.4)point out the highest prevalence of Dengue virus in Bomo, as 6 of the samples tested were positive for

Table 4.1: Exporting mode based on selected wells for Dengue virus General-type

Well	Sample Name	Detector	Ct values	Species	Location	Place of collection	Interpretation
A1	NTC	DENGUE	UNDET.				NEGATIVE
A2	1	DENGUE	UNDET.	*Aedes vittatus*	DUMBI	OUTDOOR	NEGATIVE
A3	2	DENGUE	21.9657	*Aedes aegypti*	DUMBI	INDOOR	POSITIVE
A4	3	DENGUE	2.60933	*Aedes furcifer*	DUMBI	OUTDOOR	POSITIVE
A5	4	DENGUE	UNDET.	*Aedes furcifer*	DUMBI	INDOOR	NEGATIVE
A6	5	DENGUE	26.9527	*Aedes aegypti*	DUMBI	INDOOR	POSITIVE
A7	6	DENGUE	UNDET.	*Aedes vittatus*	KUFENA	OUTDOOR	NEGATIVE
A8	7	DENGUE	UNDET.	*Aedes furcifer*	KUFENA	INDOOR	NEGATIVE
A9	8	DENGUE	UNDET.	*Aedes furcifer*	KUFENA	OUTDOOR	NEGATIVE
A10	9	DENGUE	UNDET.	*Aedes aegypti*	KUFENA	INDOOR	NEGATIVE
A11	10	DENGUE	UNDET.	*Aedes* spp.	KUFENA	INDOOR	NEGATIVE
A12	PTC	DENGUE	17.16				POSITIVE
D1	11	DENGUE	UNDET.	*Aedes vittatus*	KUFENA	OUTDOOR	NEGATIVE
D2	12	DENGUE	UNDET.	*Aedes furcifer*	KUFENA	INDOOR	NEGATIVE
D3	13	DENGUE	16.9456	*Aedes furcifer*	BOMO	OUTDOOR	POSITIVE
D4	14	DENGUE	UNDET.	*Aedes furcifer*	BOMO	INDOOR	NEGATIVE
D5	15	DENGUE	23.468	*Aedes aegypti*	BOMO	INDOOR	POSITIVE
D6	16	DENGUE	13.863	*Aedes taylori*	BOMO	OUTDOOR	POSITIVE
D7	17	DENGUE	15.6238	*Aedes metallicus*	BOMO	OUTDOOR	POSITIVE
D8	18	DENGUE	23.0773	*Aedes furcifer*	BOMO	OUTDOOR	POSITIVE
D9	19	DENGUE	29.4189	*Aedes furcifer*	BOMO	INDOOR	POSITIVE
D10	20	DENGUE	15.5305	*Aedes aegypti*	HANWA	INDOOR	POSITIVE
D11	21	DENGUE	UNDET.	*Aedes vittatus*	HANWA	OUTDOOR	NEGATIVE
D12	22	DENGUE	UNDET.	*Aedes furcifer*	HANWA	OUTDOOR	NEGATIVE
G1	23	DENGUE	UNDET.	*Aedes furcifer*	HANWA	INDOOR	NEGATIVE
G2	24	DENGUE	UNDET.	*Aedes aegypti*	HANWA	OUTDOOR	NEGATIVE
G3	25	DENGUE	UNDET.	*Aedes aegypti*	HANWA	INDOOR	NEGATIVE
G4		DENGUE	UNDET.				
G5		DENGUE	UNDET.				
G6		DENGUE	UNDET.				
G7		DENGUE	UNDET.				
G8		DENGUE	UNDET.				
G9		DENGUE	UNDET.				
G10		DENGUE	UNDET.				
G11		DENGUE	UNDET.				
G12		DENGUE	UNDET.				

Table 4.2: Mosquito sample pools positive for Dengue virus, in Zaria and environs from July to October, 2013

SPECIES	LOCATION	OUTDOOR COLLECTION	INDOOR COLLECTION
Aedes aegypti	Dumbi	0	2
	Kufena	0	0
	Bomo	0	1
	Hanwa	0	1
	TOTAL	0	4
Aedes furcifer	Dumbi	1	0
	Kufena	0	0
	Bomo	2	1
	Hanwa	0	0
	TOTAL	3	1
Aedes metallicus	Dumbi	0	0
	Kufena	0	0
	Bomo	1	0
	Hanwa	0	0
	TOTAL	1	0
Aedes taylori	Dumbi	0	0
	Kufena	0	0
	Bomo	1	0
	Hanwa	0	0
	TOTAL	1	0

Table 4.3 Prevalence of dengue virus in *Aedes* species in Zaria and environs, from July to October, 2013

SPECIES	NUMBER OF POSITVE POOLS (%)	DUMBI (%)	KUFENA (%)	BOMO (%)	HANWA (%)
Aedes aegypti	4 (40.00)	2(66.67)	0 (0.00)	1 (16.67)	1 (100.00)
Aedes furcifer	4 (40.00)	1 (33.33)	0 (0.00)	3 (50.00)	0 (0.00)
Aedes metallicus	1 (10.00)	0 (0.00)	0 (0.00)	1 (16.67)	0 (0.00)
Aedes taylori	1(10.00)	0 (0.00)	0 (0.00)	1 (16.67)	0 (0.00)
TOTAL	10 (100.00)	3 (100.00)	0 (0.00)	6 (100.00)	1 (100.00)

Table 4.4 **Prevalence of dengue virus in the four locations in Zaria and environs, from July to October, 2013**

LOCATIONS	NUMBER OF POSITIVE POOLS (%)	*Aedes aegypti (%)*	*Aedes furcifer (%)*	*Aedes metallicus (%)*	*Aedes taylori (%)*
Dumbi	3 (30.00)	2 (50.00)	1 (25.00)	0 (0.00)	0 (0.00)
Kufena	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Bomo	6(60.00)	1 (25.00)	3 (75.00)	1 (100.00)	1 (100.00)
Hanwa	1(10.00)	1 (25.00)	0 (0.00)	0 (0.000	0 (0.00)
TOTAL	10 (100.00)	4 (100.00)	4 (100.00)	1 (100.00)	1 (100.00)

Dengue virus (60%) in this locations. Dumbi had 3 samples positive for Dengue virus (30.00%) while Hanwa had only 1 sample positive for Dengue virus (10.00%). There were no positive samples for Dengue virus in Kufena in all the samples tested.

Two-way analysis of variance (ANOVA) of the presence of Dengue virus in the four locations and based on the *Aedes* species found in those locations(Table 4.5). From the four locations, it shows that there was no significant difference (P>0.05) in the means of positive pools samples of Dengue virus in those four locations. Similarly, there was no significant difference amongst the *Aedes* species found positive for Dengue virus in the pools from the four locations. Kufena had no positive sample for Dengue virus in all the samples tested for Dengue virus.

4.4.2 Yellow fever virus detection

Positive template control (PTC) for yellow fever virus had a Ct value of 14.6694.The negative template control(NTC) had Ct values above 38 hence the software displays ‘‘undetermined’’ in the fluorescence channel(Table 4.6). The pool mosquito samples that were found positive for yellow fever virus were 15 from the 25 reaction samples, having Ct values below 38 as shown from the fluorescence channel FAM. This shows that the positive pool mosquito samples were 60% of the total pool mosquito pool. The number of positive pool of *Aedes* mosquitoes sample for Yellow fever virus in the four locations and where the sample were found positive(Table 4.7).In Tables 4.8, the prevalence of Yellow fever virus in the*Aedes* mosquitoes showed the most prevalent being *Aedes furcifer*(46.67%)followed by *Aedes aegypti* (26.67%). Two pooled species of *Aedes furcifer* were positive for Yellow fever virus collected indoor and outdoor in Dumbi.

Table 4.5 Two-way ANOVA of the presence of dengue virus across locations and based on *Aedes*species, in Zaria and environs from July to October, 2013

Hypotheses	F-statistics	Critical Values	P-value	Decision
$\boldsymbol{H_0}^{(1)}$	3.71	3.86	0.06	Accept
$\boldsymbol{H_0}^{(2)}$	1.59	3.86	0.26	Accept

Source: *Laboratory Data (2014) p-value ≤ 0.05*

Note: $H_0{}^{(1)}$ *= There is no significant difference in the means of positive pools across locations.* $H_0{}^{(2)}$ *= There is no significant difference in the means of positive pools across the species*

Table4.6Exporting modebased on selected wells for yellow fever virus

Well	Sample Name	Detector	Ct values	Species	Location	Place of collection	Interpretation
B1	NTC	YELLOW FEVER	UNDET				NEGATIVE
B2	1	YELLOW FEVER	UNDET	*Aedes vittatus*	DUMBI	OUTDOOR	NEGATIVE
B3	2	YELLOW FEVER	UNDET	*Aedes aegypti*	DUMBI	INDOOR	NEGATIVE
B4	3	YELLOW FEVER	1.62942	*Aedes furcifer*	DUMBI	OUTDOOR	POSITIVE
B5	4	YELLOW FEVER	25.0694	*Aedes furcifer*	DUMBI	INDOOR	POSITIVE
B6	5	YELLOW FEVER	16.5625	*Aedes aegypti*	DUMBI	INDOOR	POSITIVE
B7	6	YELLOW FEVER	20.5568	*Aedes vittatus*	KUFENA	OUTDOOR	POSITIVE
B8	7	YELLOW FEVER	22.8115	*Aedes furcifer*	KUFENA	INDOOR	POSITIVE
B9	8	YELLOW FEVER	15.4014	*Aedes furcifer*	KUFENA	OUTDOOR	POSITIVE
B10	9	YELLOW FEVER	23.6713	*Aedes aegypti*	KUFENA	INDOOR	POSITIVE
B11	10	YELLOW FEVER	UNDET	*Aedes spp.*	KUFENA	INDOOR	NEGATIVE
B12	PTC	YELLOW FEVER	14.6694				POSITIVE
E1	11	YELLOW FEVER	UNDET	*Aedes vittatus*	KUFENA	OUTDOOR	NEGATIVE
E2	12	YELLOW FEVER	UNDET	*Aedes furcifer*	KUFENA	INDOOR	NEGATIVE
E3	13	YELLOW FEVER	UNDET	*Aedes furcifer*	BOMO	OUTDOOR	NEGATIVE
E4	14	YELLOW FEVER	20.7743	*Aedes furcifer*	BOMO	INDOOR	POSITIVE
E5	15	YELLOW FEVER	16.914	*Aedes aegypti*	BOMO	INDOOR	POSITIVE
E6	16	YELLOW FEVER	10.5021	*Aedes taylori*	BOMO	OUTDOOR	POSITIVE
E7	17	YELLOW FEVER	17.6994	*Aedes metallicus*	BOMO	OUTDOOR	POSITIVE
E8	18	YELLOW FEVER	UNDET	*Aedes furcifer*	BOMO	OUTDOOR	NEGATIVE
E9	19	YELLOW FEVER	15.5825	*Aedes furcifer*	BOMO	INDOOR	POSITIVE
E10	20	YELLOW FEVER	UNDET	*Aedes aegypti*	HANWA	INDOOR	NEGATIVE
E11	21	YELLOW FEVER	20.6439	*Aedes vittatus*	HANWA	OUTDOOR	POSITIVE
E12	22	YELLOW FEVER	15.6923	*Aedes furcifer*	HANWA	OUTDOOR	POSITIVE
H1	23	YELLOW FEVER	UNDET	*Aedes furcifer*	HANWA	INDOOR	NEGATIVE
H2	24	YELLOW FEVER	20.47	*Aedes aegypti*	HANWA	OUTDOOR	POSITIVE
H3	25	YELLOW FEVER	UNDET	*Aedes aegypti*	HANWA	INDOOR	NEGATIVE
H4		YELLOW FEVER	UNDET				
H5		YELLOW FEVER	UNDET				
H6		YELLOW FEVER	UNDET				
H7		YELLOW FEVER	UNDET				
H8		YELLOW FEVER	UNDET				
H9		YELLOW FEVER	UNDET				
H10		YELLOW FEVER	UNDET				
H11		YELLOW FEVER	UNDET				
H12		YELLOW FEVER	UNDET				

Table 4.7Mosquito sample pools positive for Yellow virus, in Zaria and environs from July to October, 2013

SPECIES	LOCATION	OUTDOOR COLLECTION	INDOOR COLLECTION
Aedes aegypti	Dumbi	0	1
	Kufena	0	1
	Bomo	0	1
	Hanwa	1	0
	TOTAL	**1**	**3**
Aedes furcifer	Dumbi	1	1
	Kufena	1	1
	Bomo	0	2
	Hanwa	1	0
	TOTAL	**3**	**4**
Aedes vittatus	Dumbi	0	0
	Kufena	1	0
	Bomo	0	0
	Hanwa	1	0
	TOTAL	**2**	**0**
Aedes taylori	Dumbi	0	0
	Kufena	0	0
	Bomo	1	0
	Hanwa	0	0
	TOTAL	**1**	**0**
Aedes metallicus	Dumbi	0	0
	Kufena	0	0
	Bomo	1	0
	Hanwa	0	0
	TOTAL	**1**	**0**

Tables 4.8 **Prevalence of yellow fever virus in *Aedes* species in Zaria and environs, from July to October, 2013**

SPECIES	NUMBER OF POSITVE POOLS (%)	DUMBI (%)	KUFENA (%)	BOMO (%)	HANWA (%)
Aedes aegypti	4 (26.67)	1 (33.33)	1 (25.00)	1 (20.00)	1 (33.33)
Aedes furcifer	7 (46.67)	2 (66.67)	2 (50.00)	2 (40.00)	1 (33.33)
Aedes vittatus	2 (13.33)	0 (0.00)	1 (25.00)	0 (0.00)	1 (33.33)
Aedes metallicus	1 (6.67)	0 (0.00)	0 (0.00)	1 (20.00)	0 (0.00)
Aedes taylori	1(6.67)	0 (0.00)	0 (0.00)	1 (20.00)	0 (0.00)
TOTAL	15 (100.00)	3 (100.00)	4 (100%)	5 (100.00)	3 (100.00)

Two pools of *Aedes furcifer* collected indoor and outdoor were positive for Yellow fever virus in Kufena. Two pooled species of *Aedes furcifer* collected outdoor were positive for Yellow fever virus in Bomo and one pool in Hanwa. One pool each of *Aedesaegypti*collected indoor in Dumbi, KefenaandBomo were positive for Yellow fever virus and a pool collected outdoor in Hanwa was positive for Yellow fever virus. One pool each of *Aedes vittatus*collected outdoor was positive for Yellow fever virus in Kufena and Hanwa. The pools of *Aedes taylori* and *Aedes metallicus* found only in Bomo outdoor were positive for Yellow fever viruses.*Aedes vittatus* were negative for Dengue virus in all the three locations where they were collected but in Hanwa and Kufena they were positive for yellow fever virus.

The number of positive pooled samples for each species of *Aedes* mosquito found either outdoor or indoor from the four locations (Table 4.9).From the four locations were positive for Yellow fever virus. Bomo had the highest prevalence of Yellow fever virus with 5 (33.33%) samples positive. Kufena showed 4 (26.67%) samples positive for Yellow fever virus while Dumbi had 3 (20%) samples positive for Yellow fever virus and Hanwa showed 3 (20%) samples positive for Yellow fever virus.

In Table 4.10 the two-way analysis of variance of the presence of Yellow fever virus in the four locations and based on the *Aedes* species found in those locations showed that there was no significant difference in the means of the positive pools sample in the four locations but there was significant difference in the means of the positive pools amongst the *Aedes* species from the locations. In the four location irrespective of the prevalence the Yellow fever virus, the virus is in circulations in those locations within the species of *Aedes* populations but amongst the *Aedes* species there were difference in terms of their prevalence.

Table 4.9 **Prevalence of yellow fever virus in the four locations in Zaria and environs, from July to October, 2013**

LOCATIONS	NUMBER OF POSITIVE POOLS (%)	*Aedes aegypti*(%)	*Aedes furcifer*(%)	*Aedes vittatus*(%)	*Aedes metallicus*(%)	*Aedes taylori*(%)
Dumbi	3 (20.00)	1 (25.00)	2 (28.57)	0 (0.00)	0 (0.00)	0 (0.00)
Kufena	4 (26.67)	1 (25.00)	2 (28.57)	1 (50.00)	0 (0.00)	0 (0.00)
Bomo	5(33.33)	1 (25.00)	2 (28.57)	0 (0.00)	1 (100.00)	1 (100.00)
Hanwa	3(20.00)	1 (25.00)	2 (28.57)	1 (50.00)	0 (0.00)	0 (0.00)
TOTAL	15 (100.00)	4 (100.00)	7 (100.00)	2 (100.00)	1 (100.00)	1 (100.00)

Table 4.10 Two-way ANOVA of the presence of Yellow fever virus across locations and based on*Aedes* species in Zaria and environs from July to October, 2013

Hypotheses	F-statistics	Critical Values	P-value	Decision
$H_0^{(1)}$	0.815	3.490	0.510	Accept
$H_0^{(2)}$	7.222	3.259	0.003	Reject

Source: *Laboratory Data (2014) p-value ≤ 0.05*

Note: $H_0^{(1)}$ *= There is no significant difference in the means of positive pools across locations.* $H_0^{(2)}$ *= There is no significant difference in the means of positive pools across the species*

4.4.3 Co infection of dengue and yellow fever virus

In the pools of *Aedes* mosquitoes, using the real time reverse transcriptase polymerase chain reactions, 6 samples (12%) were positive for both Dengue and yellow fever virus. This was for both pools of 25 each of Dengue and Yellow fever viruses. This was observed in two locations; Dumbi and Bomo. In Dumbi, a pool of *Aedes furcifer* outdoor was positive for both viruses and a pool of *Aedes aegypti* was positive for both viruses.In Bomo, a pool of *Aedes metallicus* and *Aedes taylori* were positive for Dengue and yellow fever viruses. A pool of *Aedes aegypti* and a pool of *Aedes furcifer* were also positive for both viruses outdoors.

The prevalence of the co-infections of the viruses in the four locations and in the *Aedes* species in those locations (Table 4.11). *Aedes aegypti* (33.33%) and *Aedes furcifer* (33.33%) had the most prevalenceof the viruses as they were found in Dumbi and Bomo locations, respectively. In the 6 samples positive, Bomo had 4 samples positive for both viruses.

The two way analysis of variance (ANOVA) of the presence of Dengue and Yellow fever viruses in the four locations and based on the *Aedes* species positive for both viruses in those locations (Table 4.12). In the locations it was found that, there was significant difference in the means of the positive pools sample in the four locations.Amongst the *Aedes* species there was no significant difference in the means of the positive pools of Dengue and Yellow fever viruses.

Table 4.11 **Co- infection ofdengue virus and yellow fever virus in *Aedes*mosquitoes in Zaria and environs, from July to October, 2013**

SPECIES	NUMBER OF POSITVE POOLS (%)	DUMBI (%)	KUFENA (%)	BOMO (%)	HANWA (%)
Aedes aegypti	2 (33.33)	1 (50.00)	0 (0.00)	1 (25.00)	0 (0.00)
Aedes furcifer	2 (33.33)	1 (50.00)	0 (0.00)	1 (25.00)	0 (0.00)
Aedes vittatus	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Aedes metallicus	1 (16.67)	0 (0.00)	0 (0.00)	1 (25.00)	0 (0.00)
Aedes taylori	1 (16.67)	0 (0.00)	0 (0.00)	1 (25.00)	0 (0.00)
TOTAL	6 (100.00)	2 (100.00)	0 (0.00)	4 (100.00)	0 (0.00)

Table 4.12 Two-way ANOVA of the presence of dengue and yellow fever viruses across locationsand based on *Aedes*species in Zaria and environs from July to October, 2013

Hypotheses	F-statistics	Critical Values	P-value	Decision
$\boldsymbol{H_0}^{(1)}$	6.769	3.490	0.006	Reject
$\boldsymbol{H_0}^{(2)}$	1.615	3.259	0.234	Accept

Source: *Laboratory Data (2014) p-value ≤ 0.05*

Note: $H_0{}^{(1)}$ *= There is no significant difference in the means of positive pools across locations.* $H_0{}^{(2)}$ *= There is no significant difference in the means of positive pools across the species*

5.0 DISCUSSION

Mosquitocollection carried out during the period of study has revealed the species population of *Aedes* and its behaviour in the four locations (Dumbi, Kufena, Hanwa and Bomo) of the study. *Aedes furcifer*was found to be the most abundant species in the locations due to its preponderance both indoor and outdoor. Although, there was no significant difference in its abundance. This speciesmay well have expanded itshabitat to human dwellings like the *Aedes aegypti* which is known to breed in containers around human dwellings. Although, according to Adebote *et al*. (2006) *Aedes aegypti* was the most abundant species breeding in villages around Zaria, *Aedes furcifer* may likely be breeding just around human dwellings hence their abundance indoor.*Aedes furcifer* is a known sylvatic vector, but in the locations studied, it may have expanded its flight range and is in sympatry with *Aedes aegypti*, a species known to thrive around human dwellings. This is similar to the findings of Diallo *et al*. (2013) who reported that *Aedes aegypti* and *Aedes furcifer* were the abundant species in Bele Doki and Daron Minam, Senegal.

This study also, found*Aedes vittatus*to be the dominant species collected outdoor. Although, it was only found in three locations (Dumbi, Kufena and Hanwa) these are locations associated with Inselbergs and rocksclearly showing that *Aedes vittatus* is limited to rocky areas and its flight range could be restricted to such areas. This results agrees with the findings of Adebote*et al*. (2008) on the abundance of *Aedes vittatus* in Inselbergs surrounding Zaria. *Aedesaegypti* was found in all the locations almost entirely indoors which suggest that the mosquito is endophagic. This outcome agrees with the findings of Afolabi and Ndams (2010) on the abundance of *Aedes aegypti* in

the study locations. *Aedes taylori* and *Aedes metallicus* were only found in Bomo; this could be due to the vegetation of the location and the Bomo lake that serves for irrigation farming and has lots of conserved orchards and sugar cane plantations that could well serve as good breeding sites for these mosquitoes since *Aedes taylori* and *Aedes metallicus* are known to breed in leaf axil of plants (Service, 2012).

From all the four locations,*Aedes furcifer* was found to be both exophagic and endophagic.*Aedes vitattus, Aedes taylori* and *Aedes metallicus* were the only species found entirely outdoor during the period of study, showing that these three species are exophilic and exophagic. Kufena and Bomo had the most abundant of the species of *Aedes* sampled from all the locations. This could be due to the vegetation of the locations.While, Bomo is a grassland vegetation with several plantations, Kufena has a heavy forest with inselberg, with wild animals sheltered in the rocks.

This study assessed the presence of Dengue and Yellow fever flaviviruses in the vector population of *Aedes* mosquito in four locations. Total RNA extraction carried out on the pools of *Aedes*mosquitoes using a guanidinium thiocynate-based technique with twowashing steps, isolated viral RNA in aqueous phase detected by real time PCR technique was similar to RNA extraction on mosquitoes carried out by Liotta *et al.* (2005) who carried out alcohol purification steps retaining RNA in aqueous phase eliminating non desirable genomic DNA. Dengue virus was detected in pools of *Aedes furcifer, Aedes aegypti*, *Aedes taylori* and *Aedes metallicus* in three of the four locations in this study.Dengue virus was not detected in samples from Kufena, implying that; though Dengue is circulating in the three other locations Kufena is potentially at risk of Dengue virus.Therefore, since *Aedes furcifer* and *Aedes aegypti*are abundant in this locations, any introduction of Dengue virus from the outside population would certainly lead to the circulation of Dengue virus in the location. This findings agree with theresult

of a study carried out by Diallo (2003) who reported the isolation of Dengue virus in *Aedes taylori, Aedes furcifer, Aedes leuteocephalus, Aedes aegypti* and*Aedesvittatus* in Senegal. Although in this study, three locations where *Aedes vittatus* were found, Dengue virus was not detected in any of the pools; indicating that *Aedes vittatus* in this locations may not be a vector of Dengue virus. The detection of Dengue virus in the outdoor collections of*Aedes* mosquito agrees with the findings of Hull *et al*. (1984) on the isolation of Dengue virus from *Aedes* mosquitoes in the field or nature. In Nigeria, Baba and Talle (2011) and Idris *et al*. (2013) conducted separate studies on patients who visited the University of Maiduguri Teaching Hospital (UMTH) Maiduguri, which serves as a reference Centre for six states in North eastern Nigeria and other neighbouring West Africa countries (Chad, Niger and Cameroun) and found the circulation of Dengue virus with Dengue virus serotype 3 isolated.

Yellow fever virus was detected in pools of*Aedes furcifer*, *Aedes aegypti*, *Aedes vittatus*, *Aedes taylori* and *Aedes metallicus*. In all four locations, Yellow fever virus was detected in at least one of the pools of mosquitoes. This showed that *Aedes* mosquitoes in the locations are active vectors of Yellow fever virus supporting the findings of Bang *et al.* (1980 and 1981), on *Aedes* mosquitoes being responsible for transmission of Yellow fever virus in parts of Nigeria. This activity of the vectors could lead to unpredictable and explosive outbreak since all the locations have shown the circulation of the Yellow fever virus in the vector population. From this study, besides*Aedes vittatus*being prevalent for Yellow fever virus, all the *Aedes* species are vectors of both Dengue and Yellow fever virus with *Aedesfurcifer* and *Aedes aegypti* being the most active. This study confirms the assertion made by Adedote *etal*.(2006) that villages around Zaria such as Bomo may be Yellow fever prone. This is also in line with the findings of Sanchez-Seco *et al*. (2006) on the detection of Dengue and Yellow

fever viruses using degenerate primers, confirming the circulation of both viruses in *Aedes* mosquitoes. It is imperative to note therefore,that these two viruses: Dengue and Yellow fever virus are co-circulating within the *Aedes* population in Zaria and its surrounding settlements affirming a study conducted in Maiduguri by Baba *et al*. (2013) statingthat four important arbovirus,Dengue virus, Chikungunya virus, West Nile virus and Yellow fever virus were causing disease in humans and are co-circulating in malaria and typhoid endemic areas of the North eastern parts of Nigeria. Similarly, Dawurung *et al,* (2010) reported the circulation of Dengue viruses among febrile patients attending Plateau State Specialist Hospital, Jos North Central Nigeria.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The abundance of *Aedes* mosquitoes, from the four locations (Dumbi, Kufena, Hanwa and Bomo) studied,indicates that*Aedes furcifer*was the most abundant species in the population. While, *Aedes metallicus* was the least abundant of the species in the populations from the locations, although there was no significant difference within the four months of study. Detection of viruses in vector population is an important element in an early alert system of surveillance and epidemiological investigation. This study employed the use of qualitative real time RT-PCR to detect Dengue and Yellow fever virus in field caught population of *Aedes* mosquitoes in four locations (Dumbi, Kufena, Hanwa and Bomo). The detection of Dengue and Yellow fever viruses in thesefour locations, reveals the circulation of the viruses in the vector population of *Aedes* mosquitoes within Zaria and environs.In these mosquito species, *Aedes aegypti* and *Aedes furcifer* were the most prevalent forDengue and Yellow fever viruses. Other species that were also found prevalent for both viruses are*Aedes taylori* and *Aedes metallicus*.Whereas,*Aedes vittatus* was only associated withYellow fever virus.These circulating viruses establish a potential health risk to the human populations residing in these settlements.Accordingly, Dengue and Yellow fever viruses are in active circulation within the population of *Aedes* species in these locations.Our findings in these locations, hassubstantiatedthe argument put forth by Stoler *et al*. (2014) for greater attention for Dengue fever virus surveillance and other arboviruses in West Africa.

6.2 Recommendations

It is therefore, imperative in view of the rapidly changing ecological dynamics and human activities due largely to urbanization, that preventive and control measures be sustained in mosquito control. The use of insecticides and other control measures is encouraged alongside proper sanitation that would reduce the breeding site of mosquitoes especially in household surroundings. Efforts should be sustained by relevant authorities in active surveillance as outbreaks do occur when no precautions are taken regarding vector surveillance.

Furthermore, in view of the current trend of emerging and resurging viruses, this study recommends that routine vaccination againstYellow fever virus be carried out or be intensified in view of the potential risk of the active vectors to protectthe human population without immunity that would be vulnerableto infection. This will ultimately guide against potential outbreak or even diseases caused by these viruses. Even though no vaccine has yet been made available for Dengue, it is recommended that proper sanitation be maintained and breeding habitats of mosquitoes be eliminated to reduce the danger posed to human health by these vectors.

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APPENDICES

APPENDIX I:

Mosquito collection for the month of July

LOCATION	SPECIES	PLACE OF COLLECTION	
		OUTDOOR	INDOOR
DUMBI	*Aedes vittatus*	10	0`
	Aedes aegypti	0	5
KUFENA	*Aedes vittatus*	10	0
	Aedes furcifer	0	5
BOMO	*Aedes furcifer*	5	2
HANWA	*Aedes aegypti*	0	4
TOTAL		25	16

APPENDIX II:

Mosquito collection for the month August

LOCATION	SPECIES	PLACE OF COLLECTION	
		OUTDOOR	INDOOR
DUMBI	*Aedes vittatus*	2	0
	Aedes furcifer	1	0
	Aedes aegypti	0	8
KUFENA	*Aedes vittatus*	13	0
	Aedes furcifer	1	8
	Aedes aegypti	0	7
	Aedes spp	0	3
BOMO	*Aedes Metallicus*	1	0
	Aedes furcifer	6	3
	Aedes taylori	1	0
	Aedes aegypti	0	2
HANWA	*Aedes vittatus*	3	0
	Aedes furcifer	2	5
	Aedes aegypti	0	7
TOTAL		30	43

APPENDIX III:

Mosquito collection for the month of September

LOCATION	SPECIES	PLACE OF COLLECTION	
		OUTDOOR	INDOOR
DUMBI	*Aedes vittatus*	4	0
	Aedes furcifer	0	5
	Aedes aegypti	0	10
KUFENA	*Aedes vittatus*	10	0
	Aedes furcifer	0	13
	Aedes aegypti	0	13
BOMO	*Aedes taylori*	8	0
	Aedes furcifer	2	10
	Aedes aegypti	0	2
HANWA	*Aedes vittatus*	5	0
	Aedes furcifer	2	2
	Aedes aegypti	1	5
TOTAL		32	60

APPENDIX IV:

Mosquito collection for the month of October

LOCATION	SPECIES	PLACE OF COLLECTION	
		OUTDOOR	INDOOR
DUMBI	*Aedes furcifer*	0	1
	Aedes aegypti	0	2
KUFENA	*Aedes vittatus*	6	0
	Aedes furcifer	0	10
BOMO	*Aedes furcifer*	10	9
	Aedes taylori	5	0
HANWA	*Aedes vittatus*	3	0
	Aedes aegypti	0	5
TOTAL		24	27

APPENDIX V: Mouth Aspirator and CollectionContainer



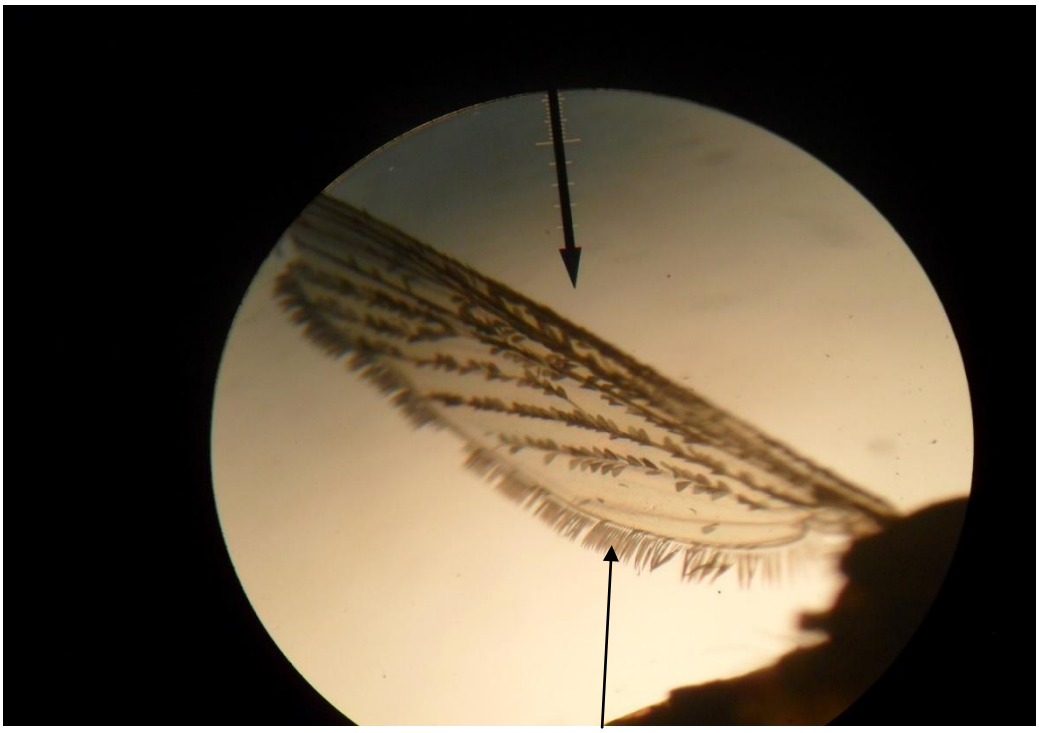
APPENDIX VI: Pyrethriod Based Insecticides



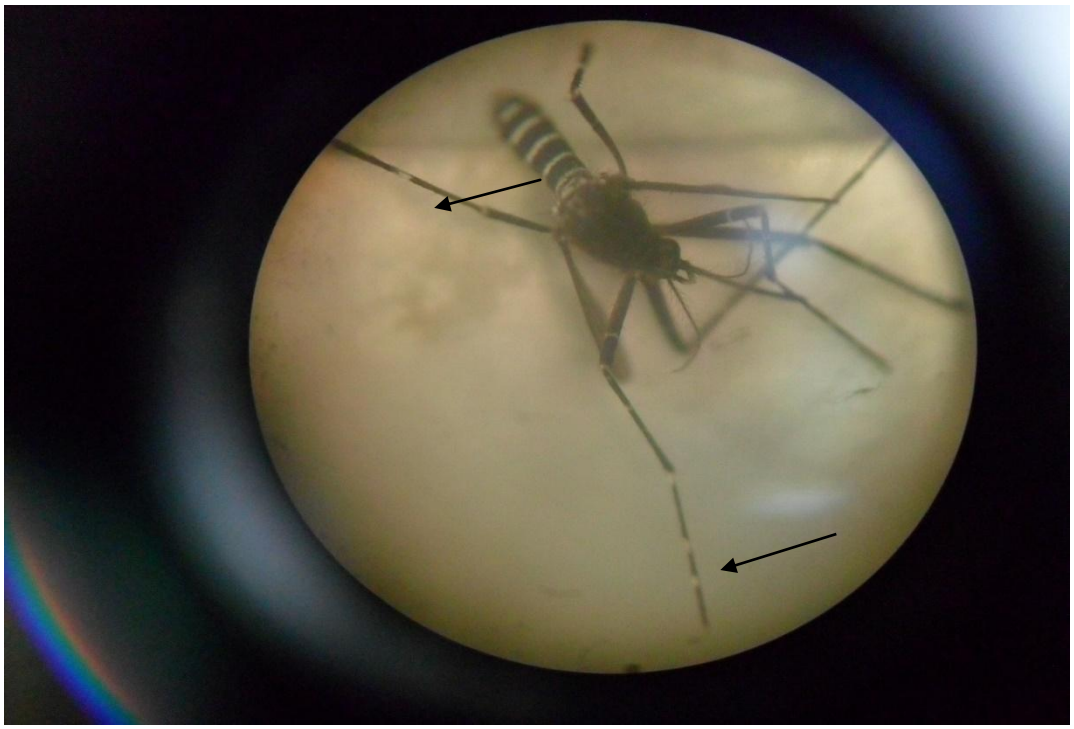
APPENDIX VII: Mosquito Identification



APPENDIX VIII: Wing of *Aedes furcifer*with broad dark and white (pale) scales on all veins



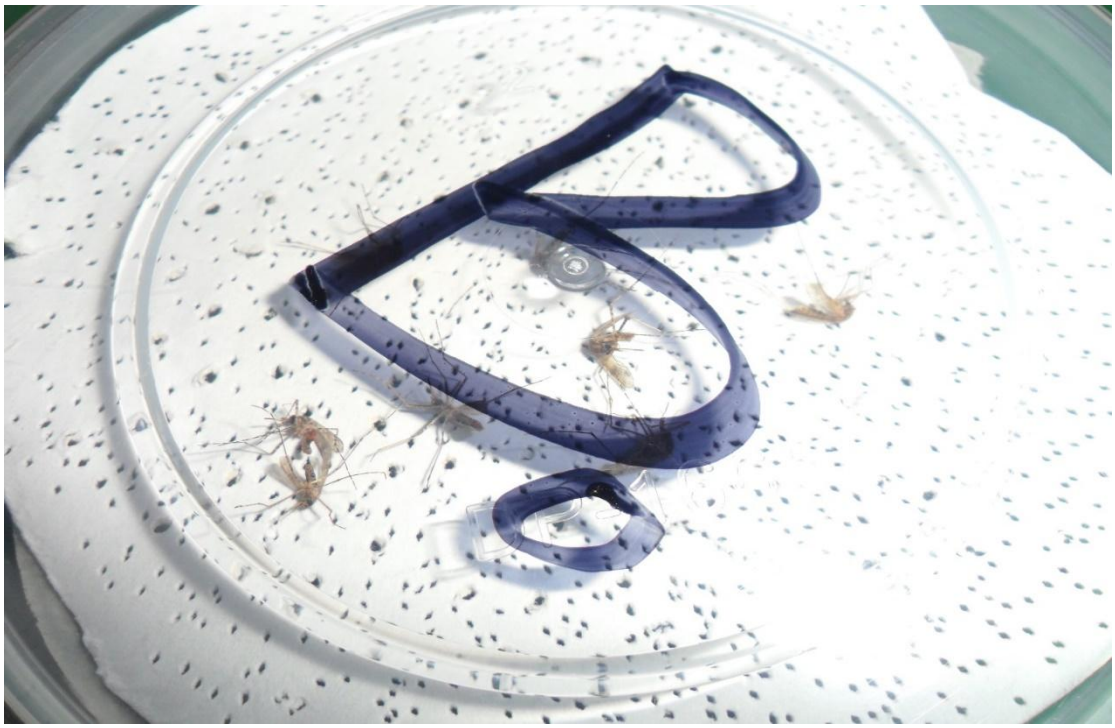
APPENDIX IX: *Aedes vittatus*showing the Tibiae of fore, mid and hind limb dark with a sub basal white band



APPENDIX X: Proboscis of *Aedes furcifer*showing a distinct white band



APPENDIX XI: Mosquitoes Stored in Silica Gel



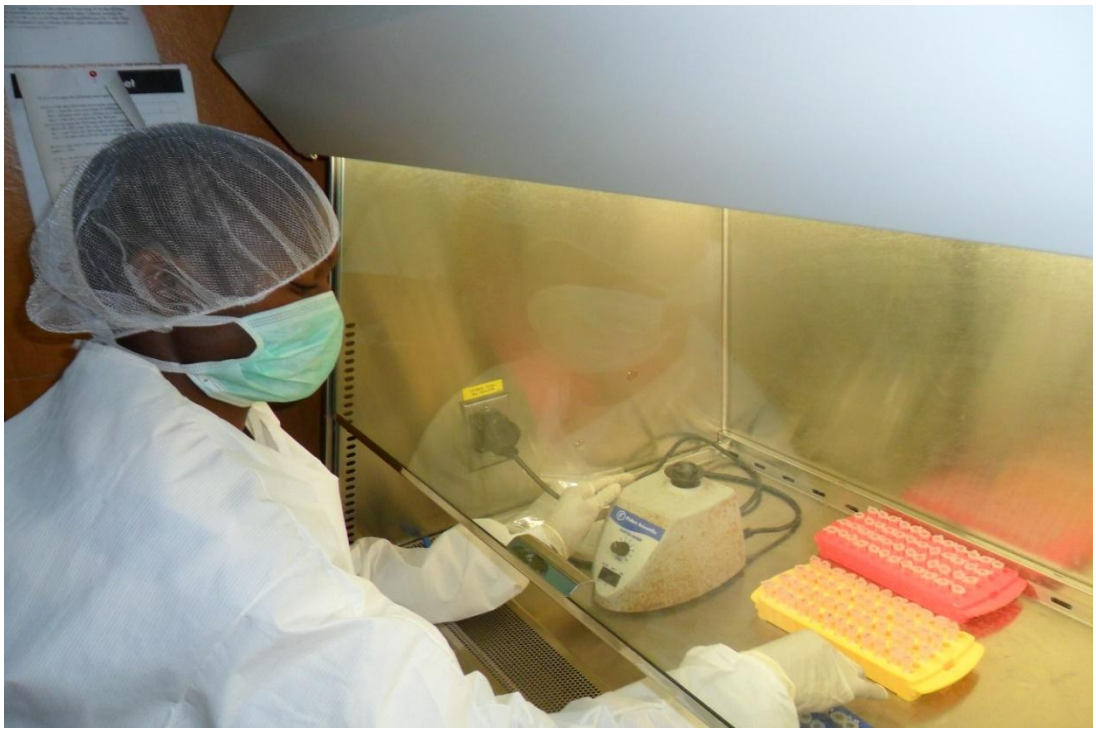
APPENDIX XII: Silica Gel Beads



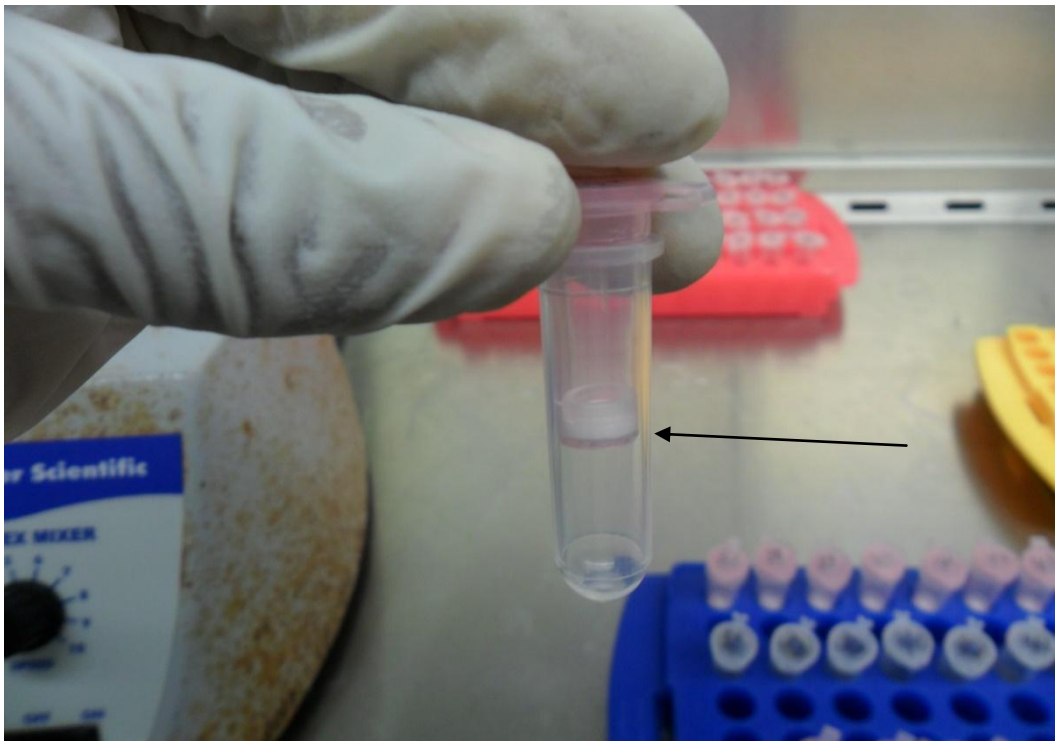
APPENDIX XIII: Stored Mosquitoes



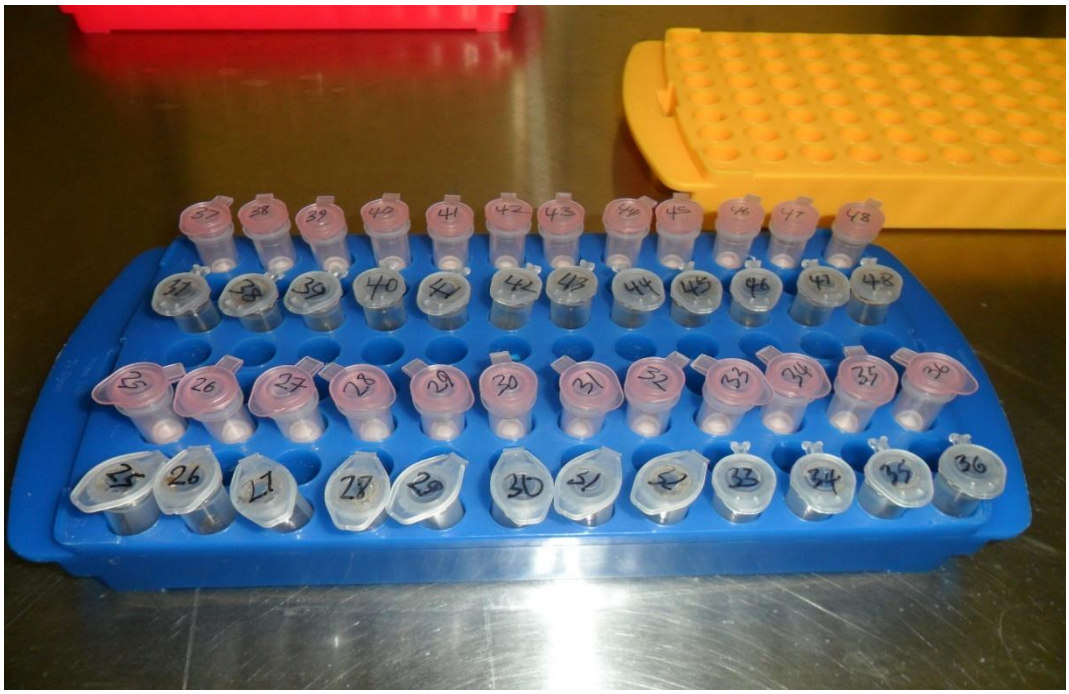
APPENDIX XIV: In a Biosafety Laboratory



APPENDIX XV: Binding Columns



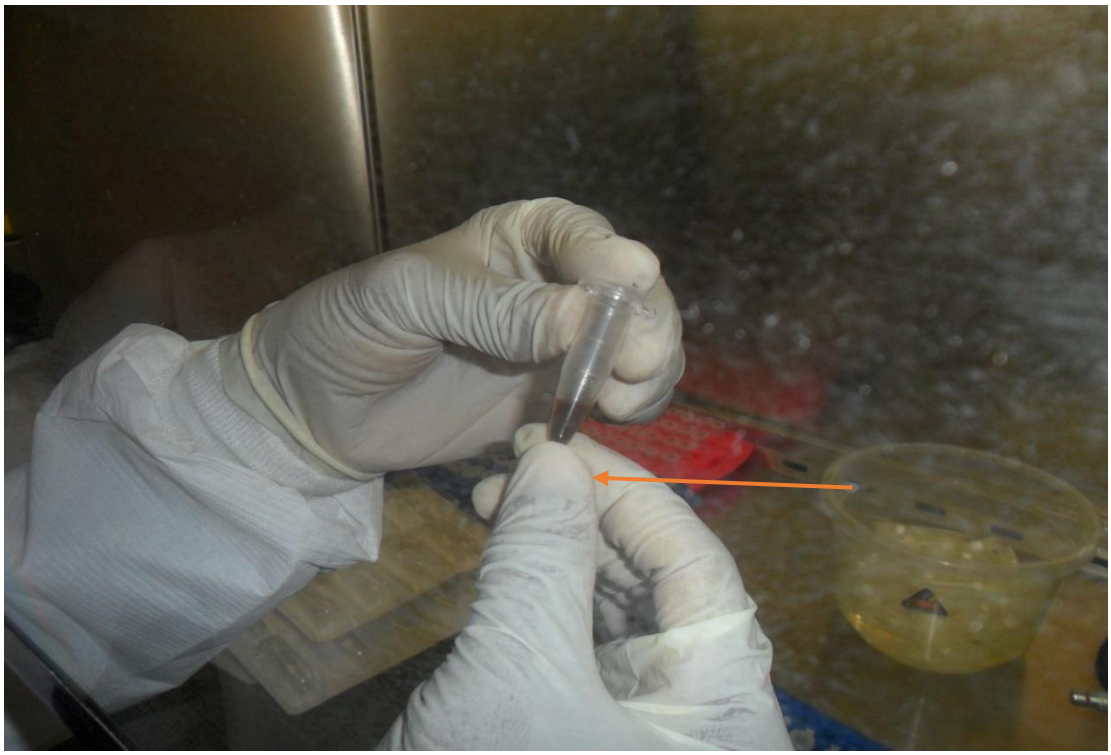
APPENDIX XVI: Numbered Columns and Eppendorf Tubes



APPENDIX XVII: Yellow Fever Virus and Dengue Fever Virus Kits



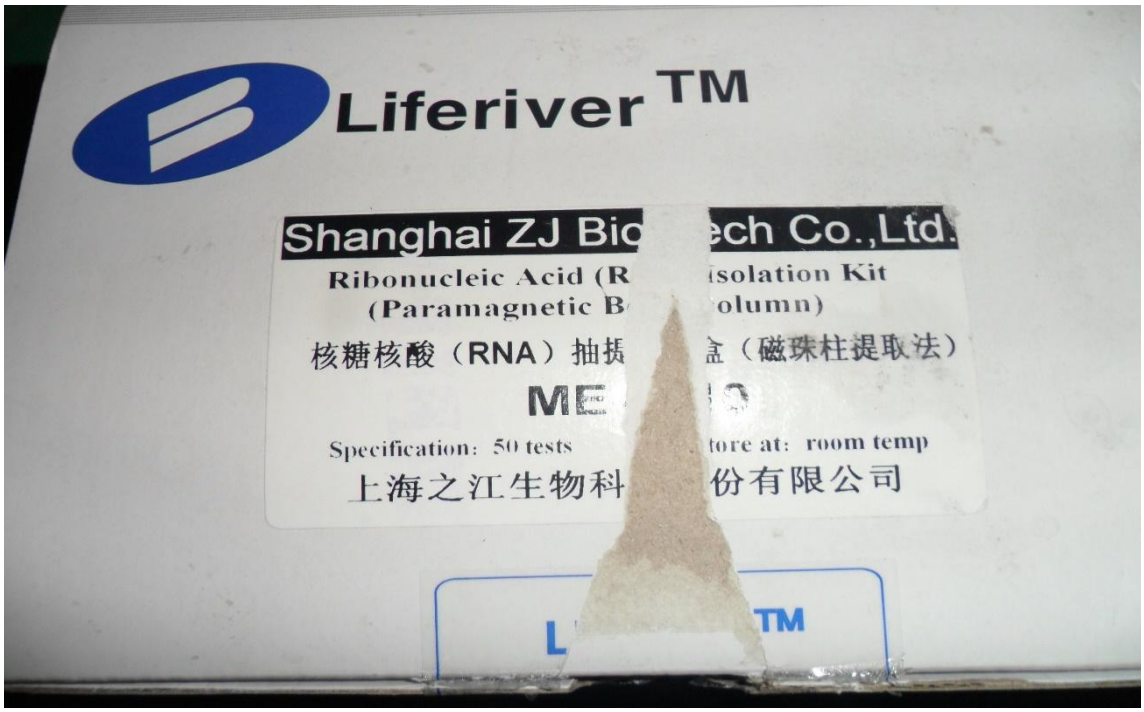
APPENDIX XVIII: Homogenized Mosquitoes



APPENDIX XIX: Applied Biosystem 7300 Real Time Machine with 96 well standard plate



APPENDIX XX: RNA Isolation Kit


Liferiver TM
Shanghai ZJ Bio
ech Co.,Ltd.
Ribonucleic Acid (R
(Paramagnetic B
isolation Kit
olumn)
核糖核酸（RNA）抽提
盒（磁珠柱提取法）
ME
Specification: 50 tests
tore at: room temp
上海之江生物科
份有限公司
TM