MOSQUITO DIVERSITY AND VIRUS INFECTIVITY IN KINSHASA, DEMOCRATIC REPUBLIC OF CONGO

KENNEDY MAKOLA MBANZULU

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ABSTRACT

Mosquito species distribution patterns and their ecology is gaining importance, because global climate changes are thought to lead to the emergence of mosquito-borne diseases; which are of considerable medical and veterinary importance because of their high morbidity and mortality. This study was conducted in five municipalities of Kinshasa to determine mosquito diversity, and arboviruses infection within. Mosquitoes were collected using BG-Sentinel traps, battery-powered aspirator for adult and a dipping technique for larvae. One part (adults and larvae-hatched adults) served for species identification, using morphological keys and *Ae. aegypti* were further identified by PCR using primers targeting the guanylate cyclase (GUA) and phosphoglycerate kinase (PGK) genes. Another part (adults only) was pooled into groups according to mosquitoes' genus and sampling sites. Each group was preserved in RNA later and screened for bunyaviruses, alphaviruses and flaviviruses. Positive groups were then tested for the presence of specific viruses using reverse transcriptase polymerase chain reaction (RT-PCR) assays. In total, 5714 mosquitoes were collected. Of these, 2814 adults and larvae-hatched adults were identified and belonged to 4 genera (*Culex, Aedes, Anopheles* and *Mansonia*), representing 12 mosquito species. *Culex quiquenfiasciatus* was the most predominant species, followed by *Ae. aegypti*, while *Ae. luteocephalus* seems to be reported for the first time in Kinshasa. 2900 mosquitoes were pooled in 29 groups of 100 mosquitoes and 12 pools were positive either for alphavirus or flavivirus or bunyavirus including mixed infection. Chikungunya, O'nyong'nyong and Rift valley fever viruses were mainly found in *Aedes* groups. A high frequency of arboviruses was found in agricultural areas around Ndjili River. The present study shows that mosquitoes in Kinshasa carry several arboviruses that may have serious public health implications. Such study in the human population of Kinshasa is needed.

DECLARATION

I, Kennedy Makola Mbanzulu, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for a higher degree award in any other institution.

Kennedy Makola Mbanzulu _____ Date

(Candidate: MSc. One Health Molecular Biology)

The declaration is hereby confirmed by;

Prof. Gerald Misinzo _____ Date

(Supervisor)

Prof. Roger Wumba _____ Date

(Supervisor)

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DEDICATION

I dedicate this work to God for free life I am provided with; to my grandmother, Luti Basusimina; my parents, Makola and Lusikila; my brothers and sisters and my wife Sylvie Ndilu for their advices, love and support.

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ABBREVIATIONS AND ACRONYMS

µl	microlitre
Ae	*Aedes*
AlphaV	alphavirus
AMCA	American Mosquito Control Association
An	*Anopheles*
bp	base pair
BunyaV	bunyavirus
CAR	Central African Republic
CDC	Centers for Disease Control and Prevention
ChikV	chikungunya virus
CO1	cytochrome c oxidase subunit1
Cx	*Culex*
Cytb	cytochrome b
DFV	dengue fever virus
DNA	deoxyribonucleic acid
DRC	Democratic Republic of Congo
FlaV	flavivirus
GUA	guanylate cyclase
HLC	human landing collection
ISRA	Institut Sénégalais de RecherchesAgricoles
LD	ladder
M	Mansonia
MBV	Mosquito-borne virus
NC	negative control

NSP	non-structural protein
OnyV	O'nyong'nyong virus
PC	positive control
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RVFV	rift valley fever virus
S l	sensu lato
SUA	SokoineUniversity of Agriculture
TAE	Tris Acetate EDTA buffer
USA	United States of America
UV	ultraviolet
X *g*	centrifuge rotor speed
YFV	yellow fever virus

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Global warming is modifying rainfall abundance and composition, temperature levels patterns and quantity, leading to changes on the flora and fauna and increasing contact between human and wild animals. This proximity between human and wild animals is creating new opportunities for infectious diseases to emerge in humans from wild animals, including vector-borne diseases, such as dengue fever, yellow fever, chikungunya and malaria (Poncon *et al*., 2007; Junglen *et al*., 2009).

Mosquitoes are common disease vectors because they provide an effective way to spread pathogens between humans and wildlife (Gubler, 2001). Infection risk with mosquito-borne diseases depends on several factors, including the abundance of competent vectors, the availability of vertebrate hosts and the biting rate. Mosquito prevalence, density and distribution are in turn influenced by a number of other factors including temperature, rainfall, humidity, vegetation and food supply. Because mosquitoes are dependent on ecological conditions, it is likely that habitat degradation and climate change greatly impact the abundance and richness of mosquitoes (Reiter and LaPointe, 2007). Less widely appreciated is that mosquito richness and habitat conditions can impact the abundance and richness of mosquito-vectored pathogens.

Viruses transmitted by blood-feeding arthropods are called arboviruses (arthropod-borne viruses). Arboviral diseases are among the most important emerging infectious diseases with public health threat that are facing many countries in the world including the Democratic Republic of Congo (DRC).

DRC is faced with a heavy burden of arboviral diseases because of its geographic conditions, climate, fauna and flora. Most of the previous studies conducted in DRC have focused on mosquito abundance rather than the virus prevalence in the mosquitoes. The present study investigated the diversity and abundance of mosquitoes and arboviruses in mosquitoes collected in Kinshasa, DRC.

1.2 Problem statement and justification of the study

Global demographic, societal and climate changes, have provided the mechanisms for arboviruses to break out of their natural ecology to become established in new geographic locations where susceptible arthropod vectors and hosts provide permissive conditions for arboviral disease epidemics. Little or no studies have been conducted in DRC to screen the presence of arboviruses such as yellow fever virus, dengue virus, o'nyong'nyong virus, rift valley fever virus and chikungunya in their potential mosquito vectors. The current study was conducted to provide insights on mosquito diversity and the presence of arboviruses in mosquitoes collected in selected parts of Kinshasa, DRC.

1.3 Research objectives

1.3.1 Overall objective

To determine mosquito diversity and virus infectivity in selected parts of Kinshasa, DRC.

1.3.2 Specific objectives

(i) To determine the mosquito species abundance in selected parts of Kinshasa,

(ii) To assess the mosquito breeding habitat in Kinshasa, and

(iii) To determine virus infectivity rates of mosquito in Kinshasa.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mosquitoes

2.1.1 Mosquito taxonomy, habitat and life cycle

Mosquitoes are classified into the order *Diptera* which is comprised up of two-winged flies belonging into the family *Culicidae*. The *Culicidae* family is a large and abundant group distributed throughout temperate and tropical regions of the world, and well beyond the Arctic Circle. Mosquitoes are most diverse in tropical forest environments. The *Culicidae* family includes 3546 species classified in two subfamilies (*Anophelinae* and *Culicinae*) and 112 genera. The subfamily *Anophelinae* is comprised up of three genera while the subfamily *Culicinae* has 109 genera segregated into 11 tribes (Harbach, 2008).

All mosquitoes require water to complete their life cycle (Fig. 1). This water can range in quality from melted snow water to sewage effluent and it can be in any container imaginable. The type of water in which the mosquito larva is found can be an aid in the identification of mosquito species. In addition, adult mosquitoes show a very distinct preference for the types of substrates in which to lay their eggs. They lay their eggs in places ranging from tree holes that periodically hold water, tide water pools in salt marshes, sewage effluent ponds, irrigated pastures and rain water ponds. Each mosquito species has unique environmental requirements for the maintenance of its life cycle (Rattanarithikul *et al*., 2005).

The feeding habits of mosquitoes are quite unique in that it is only the adult females that bite human and other animals. The male mosquitoes feed only on plant juices. Some female mosquito species prefer to feed on only one particular animal species while other

mosquito species feed on a variety of animal species. The female mosquitoes have to feed on an animal or human to obtain sufficient blood required for eggs development. If female mosquitoes do not obtain a blood meal, eggs do not develop and these mosquitoes have to die without laying viable eggs (McCafferty, 1983).

The flight habits of mosquitoes are species-dependent. Most species remain fairly close to their point of origin while some species can migrate far from their breeding place. The flight range for females is usually longer than that of males which can be up to 10-2000meters (Huber *et al.,* 2004; Zielke *et al*., 2015).

The length of life of the adult mosquito usually depends on several factors including temperature, humidity and sex of the mosquito and time of the year. Most males live a very short time, about a week while females live about a month depending on the above factors (McCafferty, 1983).

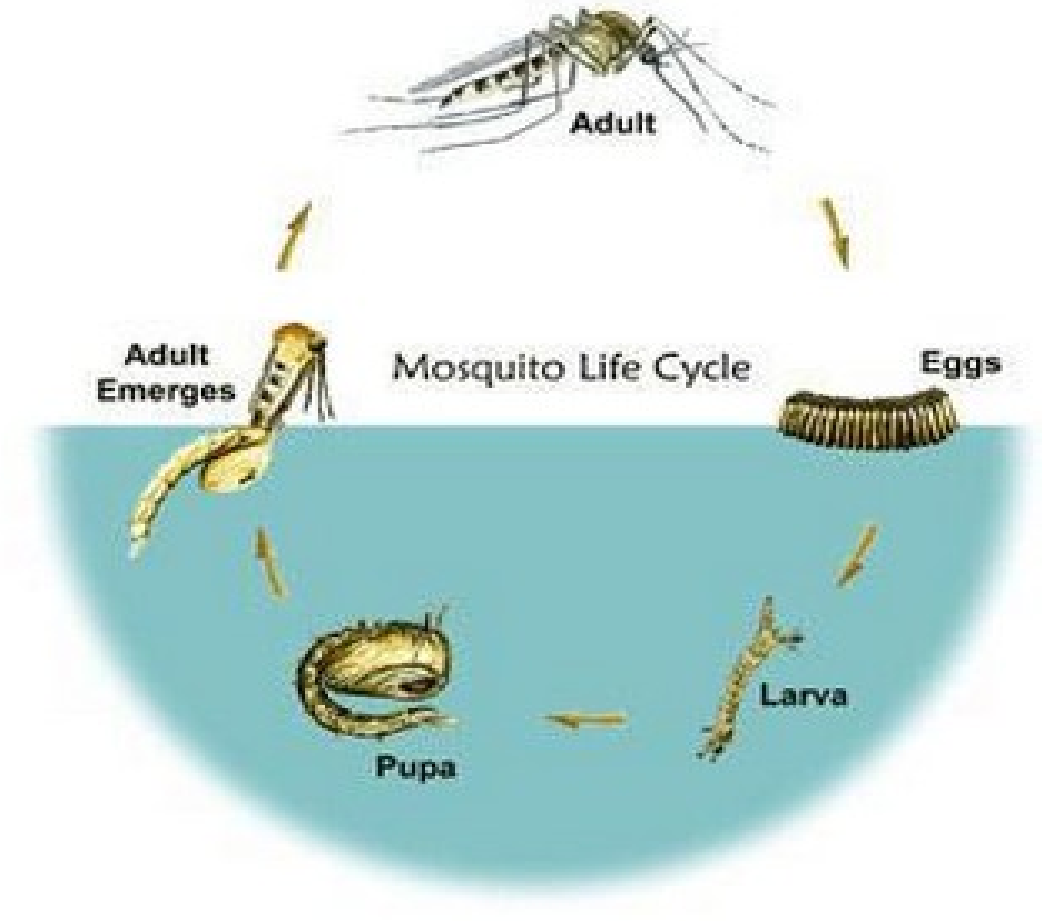

Figure 1: Mosquito life cycle. The mosquito goes through four separate and distinct stages of its life cycle including egg, larva, pupa and adult. Source: American Mosquito Control Association (AMCA). www.mosquito.org/life-cycle. Accessed on 30/07/2015.

2.1.2 The spread and emergence of mosquitoes

Mosquito species distribution patterns and ecology is affected by global climate changes that have led to emergence of invasive mosquito-borne diseases. *Aedes albopictus* (*Ae. albopictus*), currently considered as the most invasive mosquito in the world (Benedict *et al.,* 2007), originated from Southeast Asia and has nowadays been recorded in 20 European countries since its first record in Albania in 1979 (Medlock *et al.*, 2012). Similarly, *Aedes aegypti* (*Ae. aegypti*) originated from Africa and was reported for the first time in The Netherlands in 2010 although it has not established itself in Europe (Medlock, 2012; Scholte *et al.,* 2012). In the last decades, the ecology of the family *Culicidae* has been poorly neglected except few investigations on population and community ecology of invasive mosquito species. Interspecific competition between *Ae. albopictus* and *Ae. aegypti* has been investigated in artificial containers in Brazil, where both species successfully established themselves (Braks *et al.,* 2004).

In Africa, *Ae. albopictus* was first reported in Nigeria in 1991 (Savage *et al.,* 1992), where it had presumably been introduced from Asia through the used tyre trade. It was subsequently recorded in Cameroon and Equatorial Guinea, where it colonized the same larval habitats as the indigenous *Ae. aegypti*. Recent reports of *Ae. albopictus* in Gabon strongly suggested that this species now occurs in most countries of the Congo basin in Central Africa (Christophe *et al.,* 2009).

2.1.3 Mosquitoes diversity in Kinshasa

The mosquitoes fauna is less studied in Kinshasa as it is in all parts of DRC, and the only few studies carried out between the1930 and 1940's focused on malaria vectors. For instance in 1934, the anopheline diversity was determined in Kinshasa (Vincke and Henrar, 1934) and in 1948, a number of mosquito species were identified in the

surrounding areas of Congo River from Kasai, Kwamouth to Kinshasa (Fain and Henrar, 1948).

The development and the extraordinary expansion of Kinshasa City are dominated with uncontrolled urbanization and remarkable population growth. This has resulted into changes in biotope of some mosquito species due to creation of multiple collections of sewage favorable for the development of larvae of certain species such as *Culex quinquefasciatus* (*Cx. quinquefasciatus*). In all urban areas characterized bysewers, there is a heterogeneous distribution of mosquitoes compared to areas with small clear stagnant water collections, sunny and devoid of vegetation that are inhabited by *Anopheles* (*An.*) *gambiae,* a major malaria vector (Karch *et al*., 1992).

In 1993, the inventory of culicidian fauna in 24 townships including urban, suburban and rural areas of Kinshasa using morphological keys of Ethiopian region showed 20 culicidian species including (i) *Anopheles* species: *An. gambiae*, *An. funestus, An. paludis, An. brunipes, An. coustani, An. bancocki, An. nili;* (ii) *Culex* species: *Cx. quinquefasciatus, Cx. antennatus, Cx. annullioris, Cx. decens, Cx. ethiopicus, Cx. nebulosus;* (iii) *Aedes* species: *Ae. aegypti, Ae. africanus, Ae. simpsoni, Ae. argenteopnunctatus, Ae. Vittatus* and (iv) *Mansonia* species: *M. africana* and *M. uniformis*. Among the 20 species, *Cx.quinquefasciatus* was the most abundant and aggressive. *An. Gambiae* complex was predominant among *Anopheles* while *Ae. Aegypti* was established to be preponderant species among *Aedes* species in Kinshasa (Karch *et al.,*1993).

There are few recent studies conducted on mosquito diversity, which have focused on malaria vector, especially molecular identification of *An. gambaie*.

2.1.4 Medical and economic importance of mosquitoes

Mosquitoes are important because the females of many species are blood sucking, they annoy humans and other animals and they may transmit pathogens that cause human and animal diseases. The pathogens transmitted by mosquitoes include viruses (arboviruses) such as dengue, chikungunya, o'nyong'nyong, rift valley fever, west nile, japanese B-encephalitis, and yellow fever viruses; filarial worms (helminths) and protozoa (malaria). Mosquitoes are the indirect cause of morbidity and mortality among humans than any other group of organisms (Harbach, 2008).

2.1.5 Techniques for mosquito identification

Mosquito species identification can be done in several ways. The most commonly used methods are based on the study of morphological, cytogenetic analysis or DNA based methods.

2.1.5.1 Morphological identification

The identification of mosquito species is mainly done on the basis of observation of morphological characteristics. Several keys of dichotomous identification between pre-imaginal stages and adult *Culicidae* were established by many authors (Edwards, 1941). Some proposed softwares have been developed (Hervy *et al.,* 1998).

Mosquito identification based on morphological keys can be problematic because diagnostic morphological features are often damaged during collection or storage, or are not present in all developmental stages. Moreover, the morphological characteristics used to identify intact adult specimens often vary so little between species that usually only experienced mosquito taxonomists are able to distinguish mosquito species reliably (Gang *et al*., 2012).

2.1.5.2 Cytogenetic identification

The species identification can be done based on cytogenetic mapping using ovarian nurse cell polytene chromosomes. First, the polytene chromosomes are divided into regions according to banding patterns, and the identified differences in chromosome-linear and -spatial organization may serve as markers of the species identification (Coluzzi *et al.,* 1985).

2.1.5.3 Molecular DNA based methods

This technique is useful and suitable for the identification of specimens of mosquitoes collected at any stage of development, and species that are morphologically very similar or indistinguishable (Gang *et al*., 2012). Traditional morphology-based taxonomic procedures are time consuming and not always sufficient for identification to the species level, and therefore a multidisciplinary approach to taxonomy that includes morphological, molecular and distributional data is essential (Krzywinski and Bensasky, 2003). It showed that the analysis of short, standardized genomic regions (DNA barcodes) can discriminate morphologically recognized animal species. In particular, they suggest that the mitochondrial gene cytochrome c oxidase subunit1 (CO1) can serve as a uniform target gene for a bio-identification system. The ability of DNA barcodes to identify species reliably, quickly and cost-effectively has particular importance in medical entomology, where molecular approaches to species diagnose are often of great benefit in the identification of all life stages, from eggs to adults (Hebert *et al.,* 2003).

For instance several genetic approaches have been applied to the identification of mosquito species, including (i) protein electrophoresis, (ii) hybridization assays and (iii) polymerase chain reaction (PCR)-based sequence analysis (Cywinska *et al.,* 2006). The PCR has the advantage of requiring minute amounts of material for analysis. Methods

based on PCR, such as satellite DNA, restriction fragment length analysis, single-strand conformation shifts, or heteroduplex analysis, have been applied to detect diagnostic differences among PCR products in mosquito species (Garros *et al.*, 2005; Krzywinski *et al.*, 2005). Most PCR assays have examined sequence diversity in specific nuclear loci (Walton *et al.*, 1999). Other researchers have examined the taxonomic insights that can be gained by combining information from two or more genes (Linton *et al.*, 2003; Cook *et al.*, 2005). Multiplex PCR assays that included both universal (conserved) and species-specific primers are commonly performed (Phuc *et al.,* 2003). By contrast with the many studies on nuclear genes, little taxonomic work has targeted haploid mitochondrial DNA sequences in mosquitoes and less yet has examined sequence diversity in the CO1 gene (Rey *et al.*, 2001; Fairley *et al.*, 2002), despite its established potential for the diagnosis of biological diversity. The CO1 region is present in the hundreds of copies per cell, it generally lacks indels and in common with other protein-coding genes, its third position nucleotides show a high incidence of base substitutions (Hebert *et al.*, 2003). Changes in its amino acid sequence occur more slowly than those in any other mitochondrial gene, aiding resolution of deeper taxonomic affinities and primer design (Cywinska *et al.,* 2006).

Several mitochondrial genes have been used for studying *Ae. aegypti* and *Ae. albopictus* species diversity. Cytochrome b (Cytb), cytochrome oxidase 1 (COI), NADH dehydrogenase subunit 4 (ND4) and NADH dehydrogenase subunit 5 (ND5) are popular markers used for studying the genetic structure of species. In some studies, the ribosomal DNA ITS2 region was used as nuclear marker and a proportion of the nuclear housekeeping gene, phosphoglycerate kinase (PGK) and guanylate cyclase (GUA) which are not under mutation selection but with a moderate nucleotide diversity (Carol, 2007).

2.1.6 Mosquito control

There are many methods and principles involved in controlling mosquito breeding and these vary depending on the type of mosquito and the environment in which the mosquitoes breed. Control measures for mosquitoes are generally designed to act at either of two of the stages (larvae and adult) in the mosquito life cycle. Immature mosquitoes develop from eggs to larvae to pupae in standing water, and pupae develop into winged adults. Mosquito populations can be controlled in standing water using larvicides, while adulticides are used to control winged mosquitoes (Shapiro and Micucci, 2003). The immature stage of mosquito development has been found to be the easiest stage to control, since the mosquito larva is an aquatic feeding organism that does not leave the water. The most difficult stage to control is the adult winged mosquito stage because the mosquito can fly and depending on the species of mosquito, the flight range could be up 10-2000 meters (Huber *et al.,* 2004; Zielke *et al.,* 2015) Mosquito control has evolved over time to encompass a more proactive approach; moving from a past reliance on insecticide application for control to integrated pest management programs that include surveillance, source reduction, larvicides, and biological controls, as well as public relations and education (Rose, 2001).

2.1.7 Vector competence

Vector competence is the capacity of an arthropod to acquire a pathogen and transmit it to a potential host. For successful transmission of arboviruses to occur, arbovirus taken in from an infectious blood meal must overcome internal barriers in the mosquito's midgut, disseminate into other organs, and then pass through an additional barrier into the salivary glands in order to infect a new host during the next blood feeding. Vector competence can vary greatly among individuals and between mosquito populations and has been shown to be influenced by both the genetic background of a vector and environmental conditions

(Paupy *et al.,* 2001; Failloux *et al.,* 2002; Kilpatrick *et al.,* 2008). Furthermore, multiple feeding can increase the probability of concurrent infection and viral genetic mixing (Kuno and Chang, 2005).

2.2 Arbovirus

2.2.1 Classification and clinical manifestations

Vector-borne and zoonotic pathogens have comprised a significant proportion of the emerging infectious diseases in humans in recent decades. There are over 700 known arboviruses comprising *Alphavirus*, *Bunyavirus* and *Flavivirus*, at least 80 immunologically distinct types that cause diseases in humans (Smith *et al.*, 2011).

Arbovirus can cause various syndromes, ranging from benign febrile illnesses to severe systemic diseases with hemorrhagic manifestations or major organ involvement. The neurotropic alphaviruses and flaviviruses can produce severe destructive central nervous system disease with serious sequelae. Several alphaviruses (Chikungunya, Mayaro, and RossRiver) cause painful arthritis that persists for weeks or months after the initial febrile illness. Yellow fever virus has unique hepatotropic properties that cause a clinically and pathologically distinct form of hepatitis with a hemorrhagic diathesis. The dengue viruses, which cause more human illness than all other members of their family, may produce a serious, sometimes fatal, immunopathologic disease in which shock and hemorrhages occur; . *Alphavirus* is one of the two genera in the family Togaviridae; the other genus (*Rubivirus*) has rubella virus as its only member. *Flavivirus*, once classified in the *Togaviridae*, now constitutes one of three genera in the family *Flaviviridae*; the other two genera are *Pestivirus* and “Hepatitis C-like viruses”(Schmaljohn and McClain, 1996).

2.2.1.1Bunyavirus

Bunyaviruses are included in *Bunyaviridae* family that comprises over 350 isolates, grouped into five genera (i) *Orthobunyavirus*, (ii) *Phlebovirus*, (iii) *Nairovirus*, (iv) *Hantavirus* and (v) *Tospovirus*. All of these viruses are pathogens of humans and animals, except for tospoviruses, which are plant pathogens. With the exception of hantaviruses, bunyaviruses are arthropod-borne viruses and can be transmitted by a multitude of arthropod vectors such as mosquitoes, midges and thrips (in the case of plant-infecting tospoviruses) and also arachnid ticks (Saleh *et al.,* 2013).

Bunyaviruses have a particularly prominent position given the number of important pathogens in this family. Rift Valley fever virus (RVFV; *Phlebovirus*) is one of the most prominent and well-studied bunyaviruses (Ikegami, 2012). The virus has spread from Sub-Saharan Africa to Egypt, Saudi-Arabia and Yemen and there is considerable concern that it may spread into other areas for example Europe where potentially competent mosquito populations are found. The virus has a significant impact on animal health but also infects humans and can cause severe disease (Bird and Nichol, 2012). Outbreaks can have devastating effects on the local economy through restrictions on animal trade. From a European perspective, recent events have shown that outbreaks of bunyavirus infections can occur unexpectedly. The family is characterized by a three-segmented, negative sense RNA genome. The segments are named according to size L (large), M (medium) and S (small). A common feature of all bunyaviruses is that the L segment encodes an RNA-dependent RNA polymerase (RdRp), the M segment encodes the precursor to glycoproteins Gn and Gc, while the S segment encodes the nucleocapsid protein N. Genomes are encapsidated by the N protein, and complementary sequences at the 3’ and 5’ genome termini give the segments their characteristic circular “panhandle” structure (Saleh *et al.,* 2013).

2.2.1.2 Alphavirus

Alphavirus is one of two genera in the family *Togaviridae*; *Rubivirus* (rubella virus), the other *togavirus* genus. The genus *alphaviruses* comprises 29 species which are classified on the basis of antigenic properties. All *alphaviruses* share antigenic sites on the capsid and at least one envelope glycoprotein, but viruses can be differentiated by several serological tests, particularly neutralization assays. *Alphaviruses* species are nearly globally distributed and include three major categories: aquatic viruses, arthralgic viruses and encephalitic viruses (Powers *et al.,* 2011). All *alphaviruses* are mosquito-borne, except the aquatic viruses (i) salmon pancreatic disease virus and (ii) southern elephant seal virus, which are either water-borne or vectored by ectoparasitic lice. The other *alphaviruses* are transmitted between mosquitoes and avian or mammalian hosts. The arthralgic alphaviruses are primarily found in the Old World, with the exception of Mayaro virus, which occurs in South America. Of the arthralgic *alphaviruses*, the most important human pathogen is chikungunya virus. Of the encephalitic *alphaviruses*, the most important human pathogens are *Venezuelan equine encephalitis virus* (VEEV) and *eastern equine encephalitis virus* (EEEV) (Nasar *et al.,* 2012). *Alphaviruses* are 70 nm in diameter, with nucleocapsids and enveloped outer shells that assemble into icosahedral structures with T-4 symmetry. The single-stranded, messenger sense, approximately 12-kb RNA genome is 5'-capped and 3'-polyadenylated. It encodes two open reading frames (ORFs) separated by an intergenic region and flanked by 5'- and 3'-untranslated regions (UTRs). Four non-structural proteins (nsP1–4) are expressed as a polyprotein during cap-dependent translation of the 5′-ORF, and form the replicative complex that is responsible for viral genomic and subgenomic RNA replication. A subgenomic RNA encodes three main structural proteins: capsid, E2 and E1 (Weaver *et al*., 2012).

2.2.1.3 Flavivirus

The genus *Flavivirus* consist of nearly 80 viruses, many of which are arthropod-borne human pathogens. *Flaviviruses* cause a variety of diseases including fevers, dengue fever, Japanese encephalitis, and yellow fever. Other *flaviviruses* of regional or endemic concern include Kyasanur forest disease, Murray Valley encephalitis, St. Louis encephalitis, Tick-borne encephalitis, and West Nile viruses (Schmaljohn and McClain, 1996; Kuno *et al.,* 1998).

The flavivirus genome is encapsidated within an electron-dense core surrounded by a lipid bilayer, forming small spherical particles approximately 50 nm in diameter. The single-stranded, positive sense 11-kb RNA genome contains a single ORF that is flanked by UTRs ranging from approximately 100 nucleotides at the 5'-UTR to approximately 400–700 nucleotides at the 3′-UTR. Genomic RNA is translated to generate all viral proteins, including three structural proteins capsid C, pre-membrane/membrane (prM/M) and envelope (E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Weaver*et al*., 2012).

2.2.2 Transmission and life cycle of arbovirus

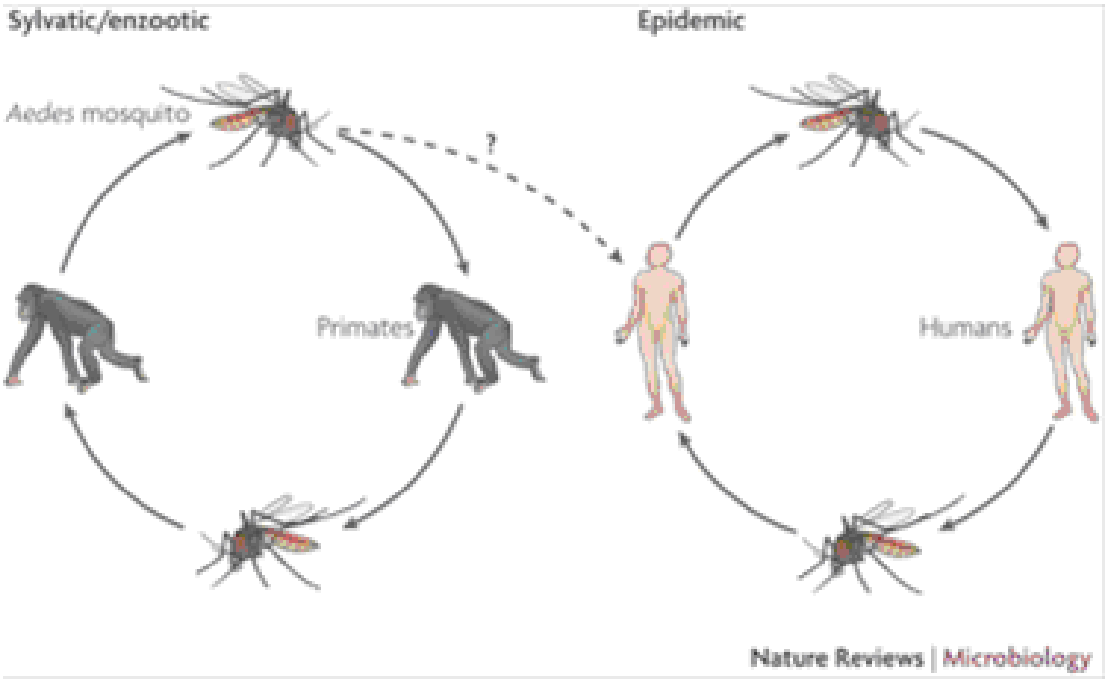

Figure 2: Sylvatic/enzootic and epidemic transmission cycles of dengue virus.

Source: Whitehead *et al.,* 2007.

Most *alphaviruses* and *flaviviruses* survive in nature by replicating alternately in a vertebrate host and a hematophagous arthropod (mosquitoes or, for some *flaviviruses*, ticks). Arthropod vectors acquire the viral infection by biting a viremic host, and after an extrinsic incubation period during which the virus replicates in the vector's tissues, they transmit virus through salivary secretions to another vertebrate host. Virus replicates in the vertebrate host, causing viremia and sometimes illness. The ability to infect and replicate in both vertebrate and arthropod cells is an essential quality of *alphaviruses* and *flaviviruses*. The principal vertebrate hosts for most are various species of wild mammals or birds. The natural zoonotic cycles that maintain the virus do not usually involve humans (Fig. 2). However, a few viruses (yellow fever virus, dengue virus types 1, 2, 3 and 4 and chikungunya virus) can be transmitted in a human-mosquito-human cycle. As a result of being pathogenic for humans and capable of transmission in heavily populated areas, these viruses can cause widespread and serious epidemics. Because of their high transmission

potential, these viruses are major public health problems in many tropical and subtropical regions of the world where appropriate mosquito vectors are present (Schmaljohn and McClain, 1996).

2.2.3Molecular detection

Molecular detection of arboviruses can be done by:

(i) Nucleic acid hybridization

Nucleic acid hybridization, using RNA extracted from either arbovirus-infected cell culture supernatants or pools of infected *Ae. albopictus*, hybridized either with biotinylated probes or ^{32}P-labelled probes, is used primarily in epidemiological studies. It can also be used for viral diagnosis in tissues obtained in autopsies. The detection method using biotinylated probes is less sensitive than using radiolabelled probes, and it has not been used for direct viral identification in clinical samples. RNA-RNA hybridization is a sensitive technique that can be applied either directly on fresh samples or on retrospective analyses of fixed samples. Due to the difficulties in working with RNA, as experienced technicians are required to obtain reproducible results, this method has been more often used as a research tool than a routine diagnostic method (Sérgio and Benedito, 2004).

(ii) Reverse transcription - polymerase chain reaction (RT-PCR)

RT-PCR has been developed for the diagnosis of several diseases, and during the last years it has been revolutionizing the laboratorial diagnosis of infectious diseases. This method is rapid, sensitive, simple, and if correctly standardized, it can be used for genome detection in human clinical samples, biopsies, autopsy tissues or mosquitoes (Deubel *et al.,* 1990). These PCR methods vary somewhat in terms of the amplified gene regions of the genome, in the ways they detect RT-PCR products, and the virus typing methods.

The results of several studies suggest that RT-PCR depends on the region of the genome that is chosen to be amplified and on the primers used to achieve this goal. Then it was developed an RT-PCR test using consensual primers, designed to amplify the C and prM genes of dengue viruses, with a 511 bp fragment through a protocol consisting of a conventional RT-PCR, followed by a nested-PCR, using specific primers for each dengue virus serotype (Lanciotti *et al.,* 1992). The sensitive and specific real-time RT-PCR assays for use on the rapid instrument for detection of Chikungunya virus and O'nyong-onyong in mosquitoes that can be used in a field,targeting different genes E1, NSP1, NSP2, 5'NTR was developed (Darci *et al.,* 2009). The same for other arboviruses, RT-PCR amplifying different genes were developed.

RT-PCR and restriction enzyme digestion of amplified DNA have been used in combination, aiming at the development of a fast and simple virus identification method. In some studies, a nested-PCR followed by restriction enzyme digestion of the amplicons indicated that nested-PCR provides a high yield of arbovirus genome amplification, even in the presence of IgM antibodies, and that restriction enzyme digestion rapidly defines the circulating serotype (Sérgio and Benito, 2004). In addition to specific amplification and restriction enzyme analyses, other studies have demonstrated that nucleotide sequencing of gene fragments amplified by RT-PCR can be used as a fast method of genetic classification of arboviruses such as dengue virus serotypes (Deubel *et al.,* 1993; Chow *et al.,* 1994).

2.2.3. Arboviruses in DRC

Chikungunya virus was isolated in the DRC for the first time in 1958 ina village called Doruma, Eastern Congo province (Osterrieth and Blanes-Ridaura, 1960). Co-infection chikungunya with yellow fever viruses and chikungunya with dengue viruses have been

observed in DRC (Osterrieth *et al.,* 1961). During the last 39 years, there has been no further report on chikungunya virus isolation in the DRC. However in February 1998, IgM antibodies against chikungunya virus were found in 12 patients during an outbreak of west nile fever among migrants in Kisangani, city town located in East part of DRC (Nur *et al.,* 1999).

A recent study in Congo basin assessed the role of wildlife species as reservoirs for arthropod-borne viral pathogens (*flaviviruses* and *alphaviruses*) by testing archived serum samples from various animals such as buffalo , elephants, duikers mandrills , gorillas, monkeys, and chimpanzees collected between 1991 and 2009 were tested for antibodies against chikungunya virus, o'nyong'nyong virus, west nile virus, dengue 2 virus, and yellow fever virus by plaque reduction neutralization test. Overall, results demonstrate a high prevalence of neutralizing antibodies against multiple arboviruses in wildlife in equatorial Africa (Kading *et al.,* 2013).

In 1999 and 2000, two important outbreaks of febrile illness were reported following heavy rain falls in the Matete and Kingabwa townships of the capital city Kinshasa. An estimated 50 000 persons were infected and chikungunya virus identified as the main causative agent (Muyembe-Tamfum *et al.,* 2003) while only a total of 76 blood samples from dengue-like fever patients were examined at the laboratory in Marseille and 44 out of the 76 blood samples analysed were found positive for chikungunya infection by the presence of IgM specific for chikungunya virus and negative for other tested viruses (dengue, west nile, and bunyamwera). And latter viruses were isolated in 9 out of 21 sera from patients from Kingabwa during the February 2000 outbreak. Partial genomic sequences were determined for each isolated virus, in order to evaluate the origin and diversity of this re-emerging virus after 39 years. The nine isolates from the DRC

clustered together and constituted a homogenous group regarding the 10% of the chikungunya virus genome sequenced. They may represent a DRC genotype, closer to Central African Republic (CAR) and Ugandan isolates than those from Tanzania and South Africa (Pastorino *et al.*, 2004). And they suggested further characterization of potential vectors present in the DRC and their competence for transmitting chikungunya virus could serve to anticipate the resurgence of chikungunya virus in humans.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

Mosquito samples were collected in Kinshasa (Fig. 3) the capital city of DRC, located at 4°19'30''S and 15°19'20'E. The climate of Kinshasa is characterized by two seasons including a dry season from the second half of May to September and a rainy season from October to the first half of May with short break in February. Kinshasa is divided into24 municipalities. Five sampling sites were selected: (i) Kimwenza in western Kinshasa, a semi-rural area of Mont-Ngafula municipality, where the last epidemic of chikungunya occurred, (ii) Kingabwa, an urban area of Limete municipality in northeastern part of Kinshasa where rice agriculture is practiced, located along Congo River, where previous urban chikungunya outbreaks have been reported, (iii) Ndjili municipality including Ndjili Brasserie in Southeastern Kinshasa, along Ndjili river where some farms and agriculture are practiced, (iv) a suburban area located in Kimbanseke municipality, in eastern part of Kinshasa and (v) Ngaba municipality around the market in central part of Kinshasa.

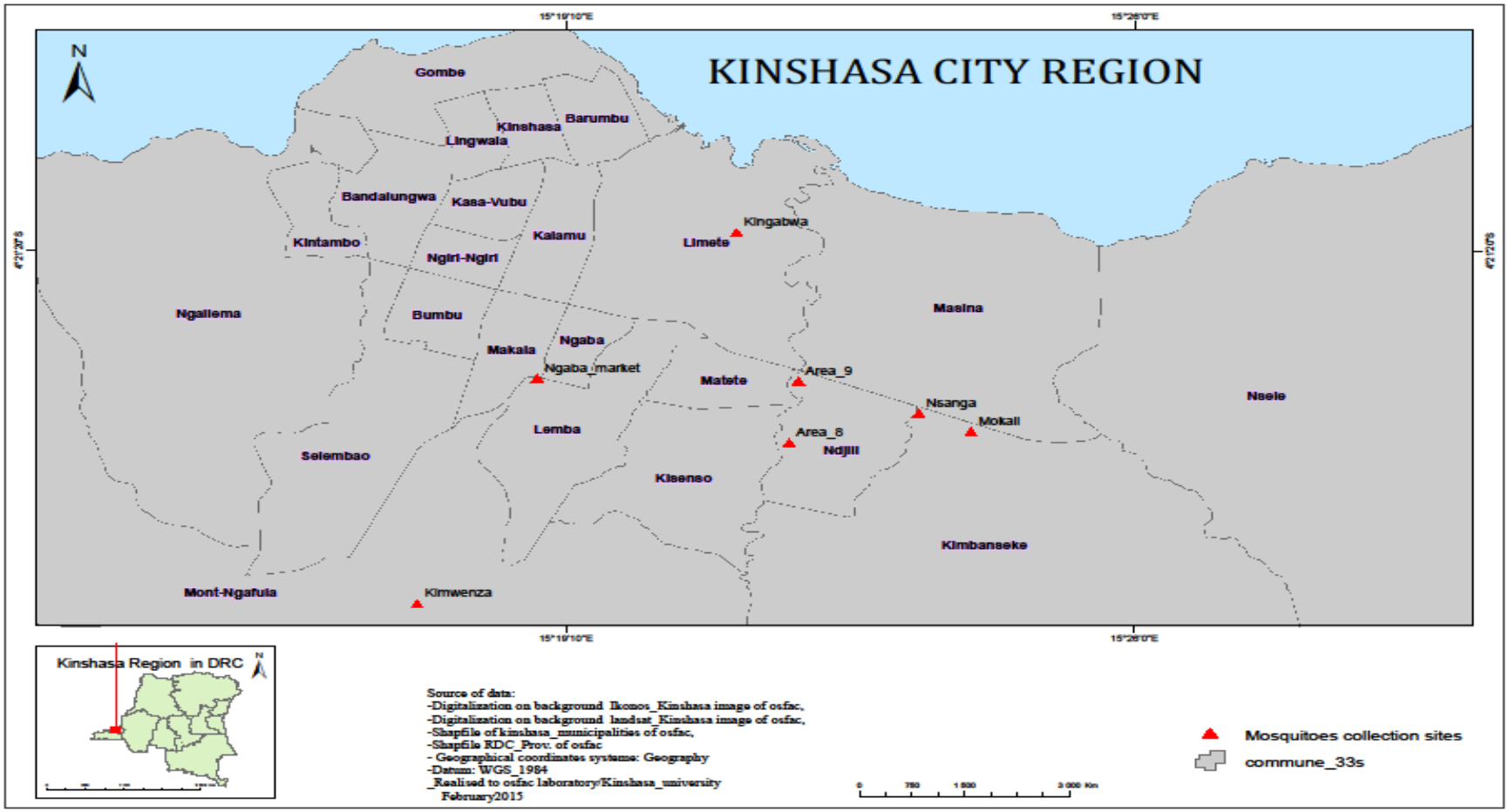

Figure 3: Map of Kinshasa city region showing mosquitoes collection sites

3.2 Mosquitoes sampling

3.2.1 Adult mosquitoes collection

The present study was carried out between March and May 2014. Firstly to study mosquito diversity, adult mosquitoes were collected using the BG-Sentinel trap, a battery-powered aspirator and human landing collection (HLC).The BG-Sentinel trap was set from 18:00pm to 06:00amindoor and 06:00am to 18:00pm outdoor, the aspirator was used for both HLC and catches of resting mosquito from 5:00am to 18:00pm and from 18:00 pm to 6:00 am outdoor and indoor respectively, with additional hand catches every day for an average of four days per site. The trapped adult mosquitoes were killed using 100 % alcohol, labeled and kept with silica gel until identification.

Secondly, for the screening of arboviruses in mosquitoes, the BG-Sentinel trap was used daily, outdoor from sunrise to sunset, to collect mosquitoes of the *Aedes* genus; and set indoor overnight to collect other genera. The battery-powered aspirator was used for both HLC and catches of resting mosquito from sunrise to sunset, with additional hand catches. Those targeted mosquitoes were killed using alcohol vapor, and only females were pooled according to genus and location and preserved in RNA later.

3.2.2Collections and rearing of mosquito larvae

From the same sites where adults were collected, larvae were collected from different habitats using the standard dipping technique or pipette. Ecological data related to each habitat were recorded (Appendix1). Those larvae within water from larvae habitat were transferred into plastic bottles in insectariums of Entomology Unit of Tropical Medicine Department of University of Kinshasa for rearing. At the insectariums, larvae collected within same water from the field were transferred to plastic tub. Once larva emerged in pupa, it was taken from the plastic tub and placed individually in a small plastic container

with a little water from field using a pipette. The small container was labeled with the location of larvae collection, date and a unique mosquito number; and covered to prevent the mosquito escaping while allowing respiration. The emerged adults were killed using ethyl-acetate, pinned and labeled in the same way with its corresponding pupae, and kept for identification.

3.3 Laboratory analyses

Laboratory analyses were done at SUA, Morogoro in Tanzania.

3.3.1 Mosquito morphological identification

All mosquitoes pinned and those preserved with silica gel were identified to genus and species level using morphological keys for *Anophelinae* and *Culicinae* (Edwards, 1941; Gilles and Coetzee 1987; Rueda, 2004) under a stereomicroscope.

3.3.2 Molecular identification of mosquitoes

3.3.2.1 Mosquitoes DNA extraction

DNA was extracted after grinding mosquito legs in PBS medium and using QIAamp DNA Mini extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, samples were lysed using a lysis buffer followed by protein precipitation using ethanol. The lysate was then passed through a Qiagen column followed by washing and cleaning of DNA. Afterwards, the DNA was eluted with RNase free water. The extracted DNA was stored at- 80°C until amplification.

3.3.2.2 *Aedes aegypti* identification using PCR

Polymerase chain reaction (PCR) targeting the coding exon region (conserved region) of the nuclear gene guanylate cyclase (GUA) and phosphoglycerate kinase (PGK) of *Ae.*

Aegypti was carried out using two different primer sets in two different reactions (Table 1).

Table 1: *Aedes aegypti* PCR primers

Primer	Sequence
PGK-F	5'-CAAGGCTTTCCGCGAAAGTCTTGC-3'
PGK-R	5'-TAAGCGCTGCTCAAGGCGTCGACA-3'
GUA-F	5'-GCCTTCACATCTCGGACATCCCGC-3'
GUA-R	5'-CCGACCCCATTTTGACGTTCCGCG-3'
PGK nest-F	5'-GCATAGTTCCATGATGGGTGAGGG-3'
PGK nest-R	5'-TGCTCAAGGCGTCGACACCAGGTA-3'
GUA nest-F	5'-TGCTGTCCGAGCAGTTCGAAGCCG-3'
GUA nest-R	5'-CCATGTCCATCATGTCCAGCGCCA-3'

Table 2: Mosquito PCR master mix components and DNA template

No.	**Component**	**Volume(µl)**
1.	2X PCR Buffer	10.0
2.	10µM Forward primer	1.0
3.	10 µM Reverse primer	1.0
4.	RNA free water	7.0
5.	Extracted DNA template	1.0
	Total volume per reaction	**20.0**

PCR conditions were set for an initial denaturation at 94 ºC for 10 min. The following 36 subsequent cycles were as follows: denaturation at 92 ºC for 1 min, primer annealing at 60 ºC for 1 min, and primer extension at 72 ºC for 2 min. The final extension was set at 72 ºC for 10 min and the reactions were cooled at 4 ºC.

PCR reactions for the nested PCR used nested primers shown in table 1 and same conditions as above. In both rounds, the master mix components were as shown on the table 2 above.

3.3.2.3 Agarose gel electrophoresis and visualization of PCR products

PCR products were separated by electrophoresis on a 1.5% agarose gel in 0.5% TAE buffer (Serva Electrophoresis, Heidelberg, Germany) stained with GelRed nucleic acid stain (Phenix Research Products, Candler, USA). Each well was loaded with 5µl of the PCR product and 1µl of blue/orange 6X DNA loading dye (Promega, Madison, USA) at 100 volts for 30 minutes. The agarose gel was visualized using a gel documentation system (EZ Gel Doc, Bio Rad, USA).

3.3.3 Arboviruses screening

3.3.3.1 Viral RNA extraction

After grinding the mosquitoes preserved with RNA later using a tip, RNA was extracted using Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions (Appendix 2). Briefly, samples were lysed using a lysis buffer followed with protein precipitation using ethanol. The lysate was then passed through a column followed by washing and cleaning of RNA. Afterwards, the RNA was eluted with RNase free water. The extracted RNA was stored at -80°C until amplification.

3.3.3.2 Arbovirus detection by RT- PCR

The viral RNA was detected using one step RT-PCR (Invitrogen One step RT-PCR kit). The final volume for this reaction was 15 µl, which was used for various PCR amplifications using primers targeting virus genera or specific arboviruses and the master mix for each PCR tube was prepared as shown in table 3. First, the presence of arboviruses was tested using primers targeting *Flavivirus, Alphavirus*, and *Orthobunyavirus* of arbovirus genera (Table 4). Samples which tested strongly positive for arbovirus genus were further tested with primers that target conserved genes in the specific virus species belonging to the concerned genus, except for dengue primers which were used for all samples (Table 5). The PCR cycling conditions were performed after optimization for each primer pair as shown on figure 4.

3.3.3.3 Gel electrophoresis and visualization of RT-PCR products

RT-PCR products were separated by electrophoresis on a 1.5% agarose gel in 0.5% TAE buffer (Serva Electrophoresis, Heidelberg, Germany) stained with GelRed nucleic acid stain (Phenix Research Products, Candler, USA). Each well was loaded with 5µl of the PCR product and 1µl of blue/orange 6X DNA loading dye (Promega, Madison, USA) at 100 volts for 30 minutes. The agarose gel was visualized by ultraviolet fluorescence light using a gel documentation system (EZ Gel Doc, Bio Rad, USA).

Table 3: RT-PCR master mix components for arbovirus detection and RNA template

No.	Component	Volume(µl)
1.	5X RT- PCR Buffer	3.0
2.	dNTP mix	0.6
3.	Forward primer	0.5
4.	Reverse primer	0.5
5.	RNase free water	6.8
6.	RT- PCR enzyme	0.6
7.	Extracted RNA template	3.0
	Total volume per reaction	**15.0**

Table 4: DNA sequences of the primers for screening of different arbovirus genera

Virus	Target gene or protein	Primer	Sequence (5'→3')	Position	PCR Product size (bp)
Bunyavirus	Nucleocapsid protein	BCS82C	ATG ACT GAG TTG GAG TTT CAT GAT GTC	86-114	251
		BCS332V	GCTGT TCC TGT TGC CAG GAA AAT	309-329	
Alphavirus	NSP4	VIR 2052 F	TGG CGC TAT GAT GAA ATC TGG AAT GTT	6971–6997	150
		VIR 2052R	TAC GAT GTT GTC GTC GCC GAT GAA	7086–7109	
Flavivirus	NS5	FU 1	TAC AAC ATG ATG GGA AAG AGA GAG AA	9007-9032	220
		CFD2	GTG TCC CAG CCG GCG GTG TCA TCA GC	9308-9283	

Table 5: DNA sequences of the primers used for detection of specific viruses

Virus	Target gene or protein	Primer	Sequence (5'→3')	Position	PCR product size (bp)
RVF	Glycoprotein M gene	RVF1 RVF2	GAC TAC CAG TCA GCT CAT TAC C TGT GAA CAA TAG GCA TTG G	777-7981 309-1327	551
Yellow fever	Polyprotein	CAG YF7	CGA GTT GCT AGCAA TAA ACA CAT TTG GA AAT GCT CCC TTT CCC AAA TA	43-71 1293-1312	1250
Chikungunya	5′NTR	CHIK3F CHIK3R	CACACGTAGCCTACCAGTTTC GCTGTCAGCGTCTATGTCCAC	14-112	98
O'nyong'nyong	5′NTR	ONN3F ONN3R	GATACACACACGCAGCTTACG-3 TACATACACTGAATCCATGATGGG	11-97	80
Dengue	Structural polyprotein	D1 D2	TCA ATA TGC TGA AAC GCG CGA GAA ACC G- TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	38-65 455-483	511

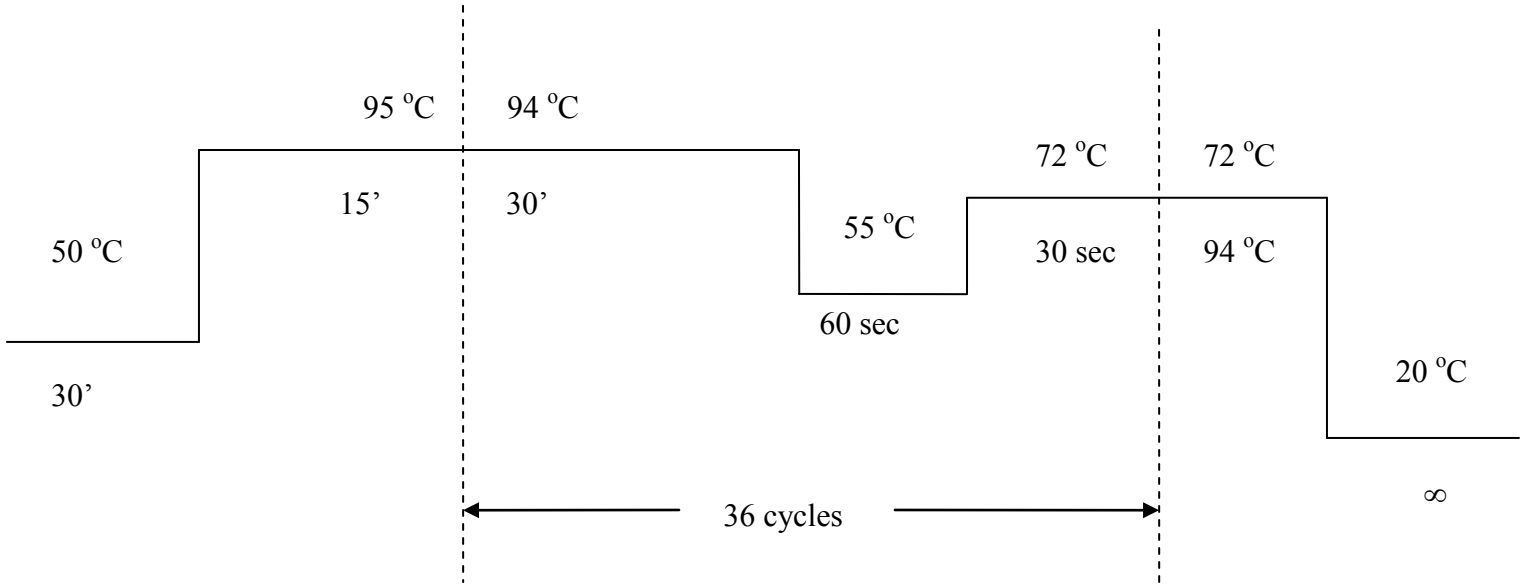

Figure 4. : RT-PCR cycling conditions for arboviruses detection

3.4 Data Analysis

The collected data were entered and stored in MS Excel 2007 Spreadsheet. Later exported to EpiInfo software version 7 for analysis, the proportions of mosquitoes by genus and species, and proportions of positive pools of arboviruses were calculated.

CHAPTER FOUR

4.0 RESULTS

4.1 Number of collected mosquitoes

In the present study, mosquitoes were collected from Ndjili, Kimwenza, Ngaba, Kingabwa and Kimbanseke. Larvae were collected from different habitats in order to study mosquito diversity and larvae breeding habitats. A total of 1031mosquitoes werehatched from collected larvae. To assess the diversity, a total of 1783 adult mosquitoes were collected from abovementioned different areas.

A total of 2900 mosquitoes were collected from Ndjili, Kimwenza, Ngaba, Kingabwa and Kimbanseke in order to screen for the presence of arboviruses. These mosquitoes were pooled into 29 pools based on their genus and location. These pools included nine, seven, five, four and four pools from, Kimwenza, Ndjili, Kingabwa, Ngaba and Kimbanseke respectively.

4.2 Mosquitoes diversity

Based on morphological identification, a total of 12 species of culicidae belonging to four genera including *Culex, Aedes, Anopheles,* and *Mansonia* were identified. Five species of *Culex*, three of *Aedes*, two of *Anopheles* and two from *Mansonia* genus were identified (Table 6). Out of 2814 mosquitoes identified, the *Culex* represented the most abundant genus (70.5%; 54.3%), followed by genera of *Aedes* (21.6%; 23.5%), *Anopheles* (6.7%; 22.2%) and *Mansonia* being the minority (1.2%; 0.0%) respectively from adults mosquitoes collected and larvae-hatched adults (Tables 6 and 7). In general, *Cx. quinquefasciatus* was widely predominant species among all culicidian species while *An.gambaie* ssp. was the mostly found species within the *Anopheles* genus. The

abundance of *Aedes* fauna by species included *Ae. aegypti* (95.9%), *Ae. africanus* (3.5%) and *Ae. luteocephalus* (0.6%) (Table 8). *Culex. quinquefasciatus* was widely found at all study sites, while other species were rare and not homogenously distributed. The occurrence of *Ae. Aegypti* (21.4%) was common and higher than other culicidian species except *Cx. Quinquefasciatus* (51.1%) in Kinshasa (Figure 5). *Ae. luteocephalus* was only found in semi-rural area of Kimwenza (Table 8).

4.3 Characterization of breeding sites surveyed and mosquitoes distribution

The larvae habitats were defined by their nature, water movement, turbidity, presence or absence of shade and aquatic vegetation. We recorded 12 types of larvae habitats including sunny pond emptied with clear water, gutter with polluted and stagnant water, small puddles with sunny clear water, small puddles with sunny turbid water, stream with polluted water, clear sunny paddy water, outdoors container with clear water, outdoors container with turbid water, tires with clear water, tires with turbid water, axil of banana plant's branch containing clear water, and bamboo (Table 9).

An. Gambiae was collected almost in water more or less clear and sunny, except at Kimbanseke, where it was fortuitously found in a collection of polluted water that is normally favorable for the *Culex* spp. larvae. *Culex* was found almost in all types of larvae habitats while *Aedes* was found more in artificial habitat (plastic, metal or tyres thrown in nature), but was less found in the bamboo and axial branches of banana. However, it was not possible to identify the type of larvae habitat for *Mansonia* spp.

Table 6: Mosquito adults distribution by genus from each sampling site in Kinshasa, DRC

Site	Geographical Coordinates	*Culex*		*Aedes*		*Anopheles*		*Mansonia*		**Total**
		n	%	n	%	n	%	n	%	
Kimwenza	S04°29'15"E15°15'47.1"	192	69.11	69	24.8	13	4.7	4	1.	**278**
Kingabwa	S04°24'38.6" E15°33'59.1"	204	68.9	58	19.6	28	9.5	6	2	**296**
Kimbanseke	S04°24'34"E15°32'50"	316	64.5	117	23.9	47	9.6	10	2.0	**490**
Ndjili	S04°24'26"5E015°23'35.3	302	67.6	124	27.7	19	4.3	2	0.4	**447**
Ngaba	S04°26'31"E15°23'43"	243	89.3	17	6.3	12	4.4	0	0	**272**
Total		**1257**		**385**		**119**		**22**		**1783**
Proportion		**70.5%**		**21.6%**		**6.7%**		**1.2%**		

Table 7: Mosquito larvae reared distribution by genus from each sampling site in Kinshasa, DRC

Site	Geographical coordinates	*Culex*		*Aedes*		*Anopheles*		*Mansonia*		**Total**
		n	%	n	%	n	%	n	%	**n**
Kimwenza	S04°29'15"E15°15'47.1"	84	50.9	35	21.2	46	27.9	0	0.0	**165**
Kingabwa	S04°24'38.6"E15°33'59.1"	98	49.0	42	21.0	60	30.0	0	0.0	**200**
Kimbanseke	S04°24'34"E15°32'50"	130	53.5	68	28.0	45	18.5	0	0.0	**243**
Ndjili	S04°24'26.5"E15°23'35.3"	112	43.6	77	29.9	68	26.5	0	0.0	**257**
Ngaba	S04°26'31"E15°23'43"	136	81.9	20	12.1	10	6.0	0	0.0	**166**
Total		**560**		**242**		**229**		**0**		**1031**
Proportion		**54.3%**		**23.5%**		**22.2%**		**0.0%**		

Table 8: Different species identified and their abundance at each sampling site in Kinshasa, DRC

Species	**Kimwenza**	**Kingabwa**	**Kimbanseke**	**Ndjili**	**Ngaba**	**Total**
Cx.quinquefasciatus	224	243	332	287	351	**1437**
Cx. antennatus	34	38	79	88	19	**258**
Cx. decens	11	16	25	31	5	**88**
Cx. nebulosus	3	2	8	3	4	**20**
Cx. annulliosis	4	3	2	5	0	**14**
Ae. aegypti	98	97	176	193	37	**601**
Ae. africanus	2	3	9	8	0	**22**
Ae. luteocephalus	4	0	0	0	0	**4**
An. gambaie ssp	59	88	84	81	22	**334**
An. funestus	0	0	8	6	0	**14**
M. africana	4	2	10	2	0	**18**
M. uniforminus	0	4	0	0	0	**4**

Table 9: Localization and description of different larvae habitats related to genus of mosquito larvae collected

Types of larvae habitat	**frequency of types of larval habitats**	**Mosquito identified**	**Site of sampling**
Sunny pond emptied with clear water	4	*Anopheles*	Kimwenza, Ndji Kingabwa
Gutter with polluted and stagnant water	7	*Culex*	Kimbanseke, Ng Ndjili, Kingabw
Small puddles with sunny clear water	6	*Anopheles* and *Aedes*	Kimbanseke, Ndjili, Kingabw
Small puddles with sunny polluted water	5	*Culex* and *Anopheles*	Kimbanseke
Stream with polluted water	2	*Culex*	Kimbanseke
Clear sunny paddy water	1	*Anopheles*	Kingabwa
Outdoors plastic and metal container with clear water	5	*Aedes, Culex* and *Anopheles*	Ndjili, Kimbans kingabwa
Outdoors plastic and metal container with turbid water	6	*Culex and Aedes*	Ndjili, Kimbans
Tyres with clear water	2	*Aedes, Anopheles* and *Culex*	Ndjili
Tyres with turbid water	3	*Culex* and *Aedes*	Ndjili, Kimbans
Axil of branch banana plant containing clear water	3	*Aedes,*	Kimwenza, ndjil
Bamboo	2	*Aedes* and *Culex*	Kimwenza, Ndji

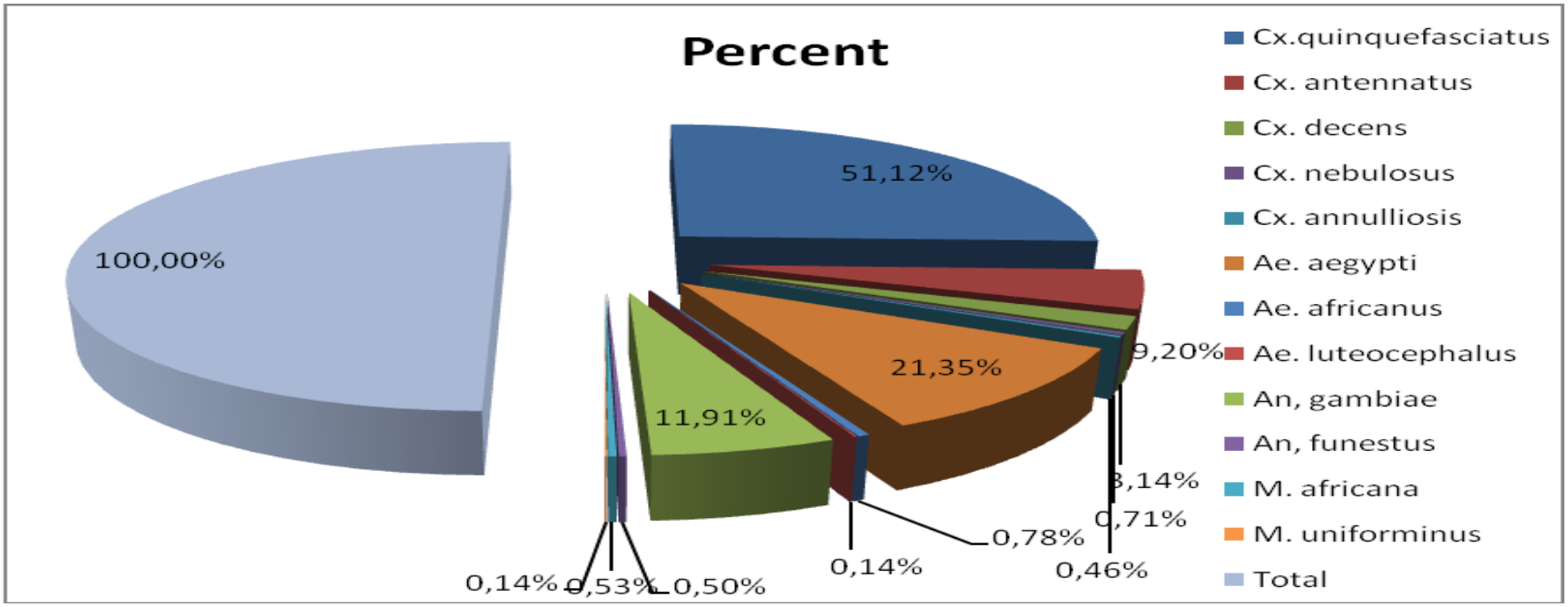

Figure 6: Different species identified and their abundance in Kinshasa, DRC

4.4 Molecular identification of *Aedes aegypti*

Aedes aegypti were identified based on morphology and using PCR targeting the GUA and PGK genes. The primers used in the present study specifically amplify GUA and PGK genes of *Ae. aegypti*. The morphological identification of *Ae. aegypti* was mostly confirmed by PCR (Fig.6).

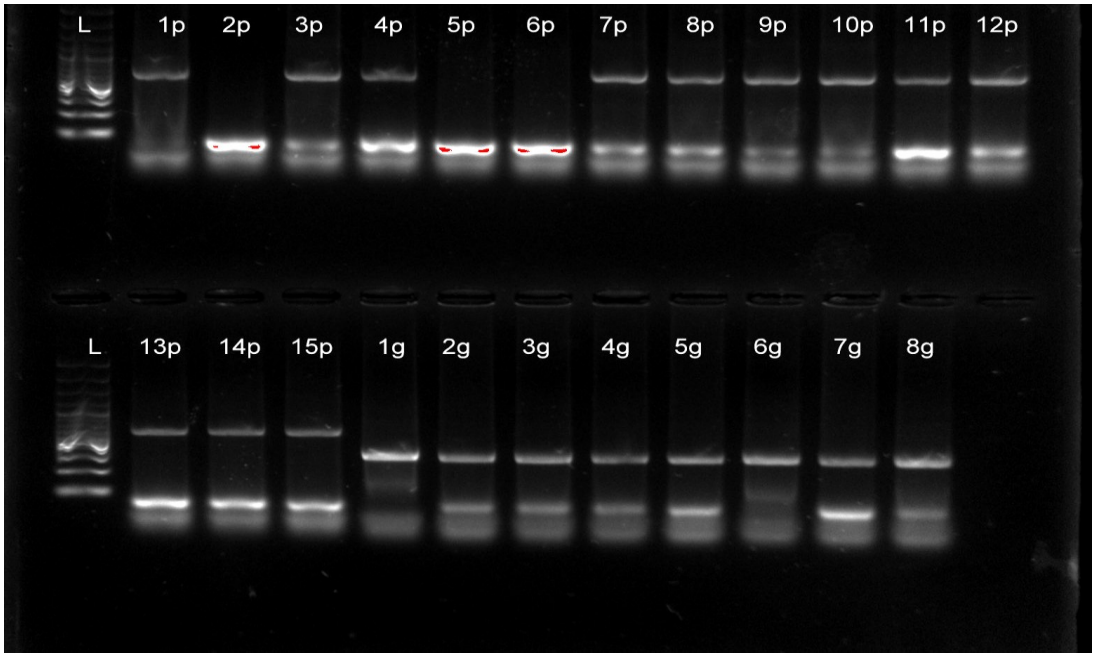

Figure 7: *Aedes* mosquito identification using primers targeting the GUA and PGK genes. LD: DNA marker;1p-15p: samples amplified with PGK primers; 1g-8g: samples amplified with GUA primers. PCR products expected size were 683bp and 411bp for PGK and GUA respectively. Samples 2, 5 and 6 were not amplified by PGK while they were amplified by GUA suggesting that different strains of *Ae. aegypti* are circulating in Kinshasa.

4.5 Arbovirus detection in mosquitoes by RT-PCR

A total of 29 pools of mosquitoes including twenty, six and three pools of *Aedes, Culex* and *Anopheles*, respectively were stored in RNA later and screened for *Flavivirus, Alphavirus* and *Orthobunyavirus* arbovirus genera (Fig.7 and 8). Samples number 4,10,12,16 and 22 tested strongly positive with genus primers, and were then further tested with primers targeting conserved genes in the specific virus belonging to the concerned genus (Fig. 9-11), except for dengue primers which were used to screen all samples as shown in table 10.

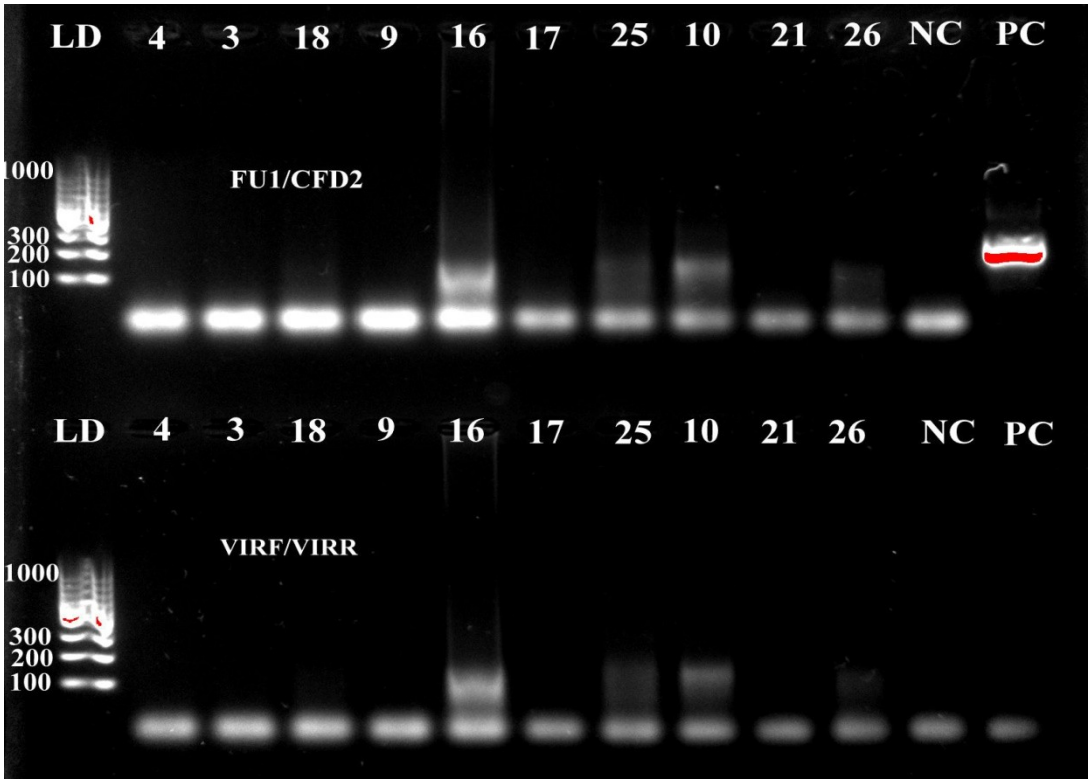

Figure 8: Visualization of RT-PCR products of the flavivirus NS5 gene and alphavirus NSP4 gene. A positive sample from a dengue patient was used as a positive control for flavivirus. The expected PCR product sizes are 220 bp and150 bp for flavivirus (top) and alphavirus (bottom), respectively. LD: DNA marker; NC: negative control; PC: positive control; DEN-2 virus was used as NC for VIRF/VIRR primer (alphavirus).

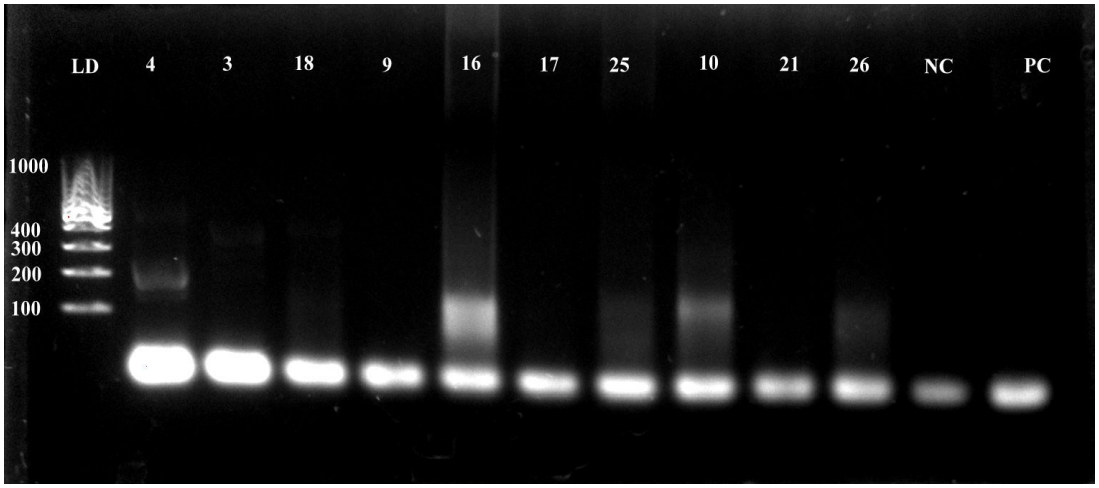

Figure 9: Amplification of the nucleocapsid protein of bunyavirus. The expected PCR product size was 251bp. LD: DNA marker; NC: negative control; PC: positive control to test the specificity of the BCS82C/BCS332V primers, DEN-2 virus was used as the positive control.

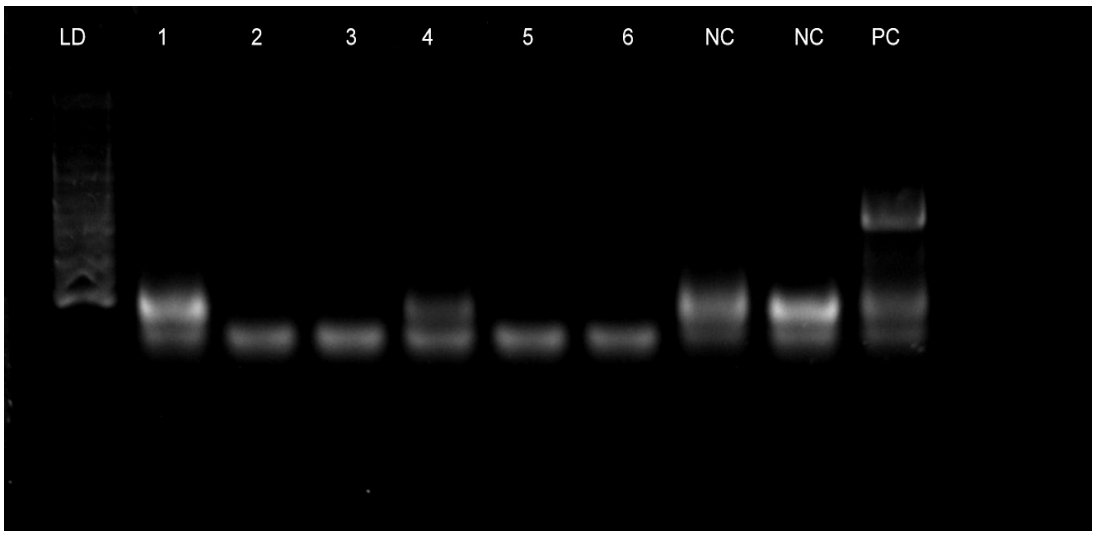

Figure 10: Visualization of RT-PCR products of universal conserved region of Structural polyprotein of dengue virus. DEN-2 virus was used as positive control (PC), producing an expected band size of 511 bp. LD: DNA marker. All other samples including the negative control (NC) tested negative.

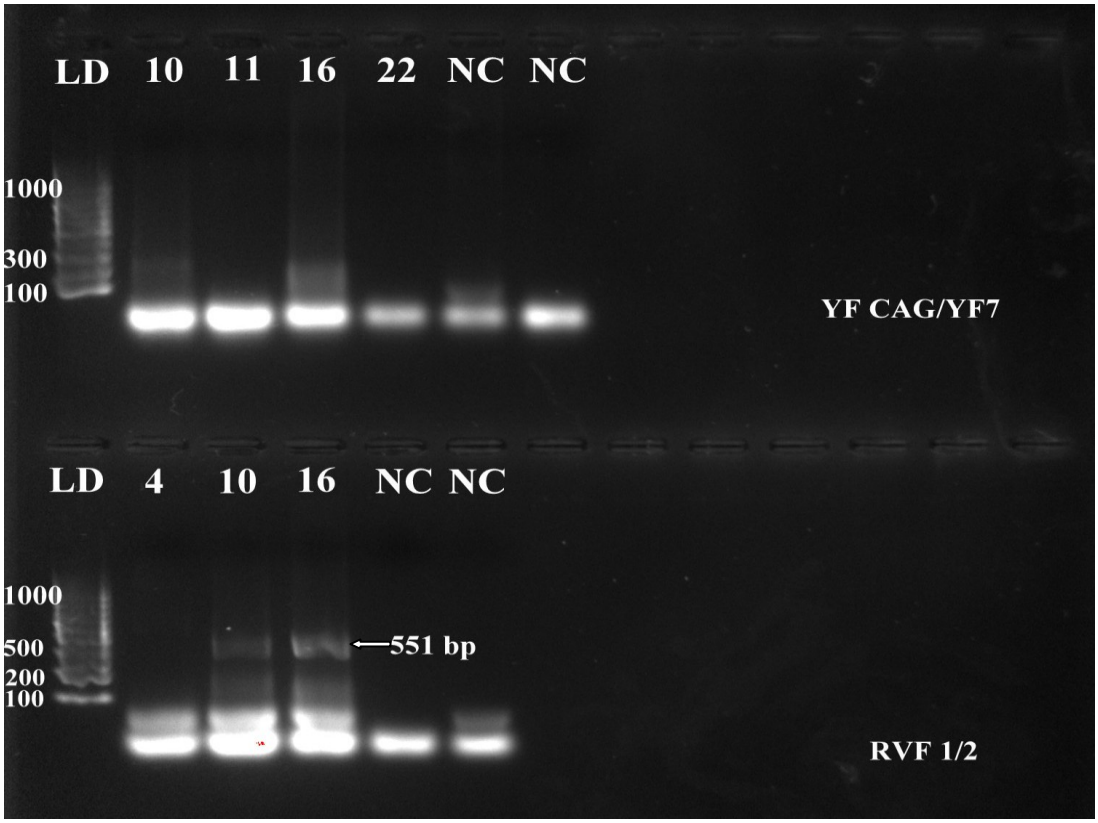

Figure 11: Visualization of RT-PCR product for polyprotein of yellow fever virus and Glycoprotein M gene for RVF. LD is the DNA marker, 4-22 pools, NC represent a negative control. The expected sizes of amplicons were 1250 bp for Yellow fever and 551 bp for Rift valley fever, respectively. All samples were negative for Yellow fever, while samples 10 and 16 were positive for Rift valley fever.

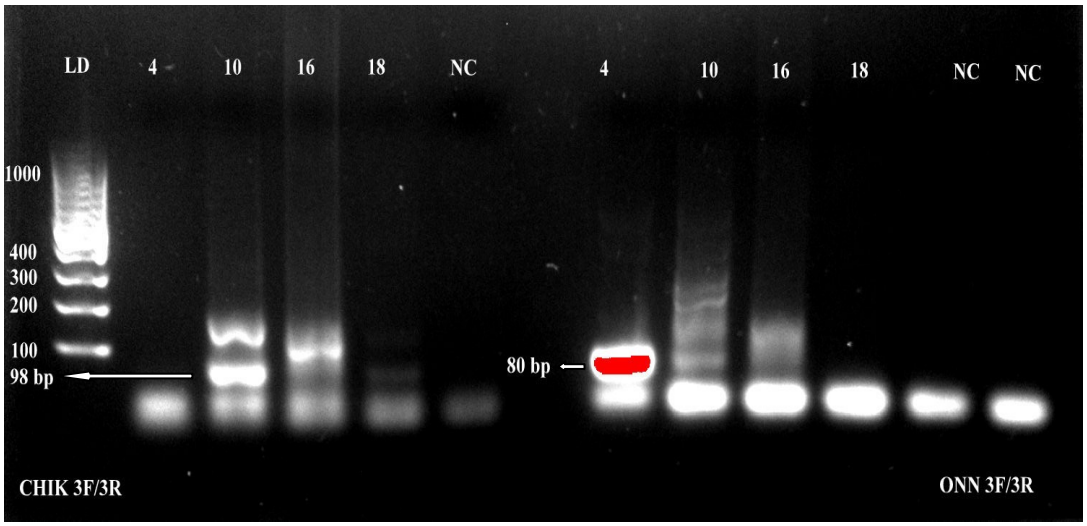

Figure 12: Visualization of RT-PCR product for 5′NTR for Chikungunya and O'nyong'nyong viruses. The expected PCR product size for Chikungunya was 98 bp while it was 80 bp for O'nyong'nyong virus. Pools 10, 16, 18 tested positive for Chikungunya virus while pools 4, 10 and 16 tested positive for O'nyong'nyong virus. NC: negative control; LD: ladder.

4.6 Arbovirus infection rates and distribution

From 29 pools screened, arboviruses were detected in 12 (41.4%), including alphavirus (8 pools), flavivirus (9 pools) and orthobunyavirus (5 pools). Some pools showed mix infection with two or three arboviruses of different genus or species. Amplification with specific primers of different members of each genus showed the presence of chikungunya virus, o'nyong'onyong virus, rift valley fever virus in *Aedes* spp. pools. The surrounding agriculture area of Ndjili river showed predominated positive pool (5) and high diversity of arboviruses, followed by Kimbanseke, Kimwenza, and Kingabwa (Table 10).

Table 10: List of arboviruses screened in mosquitoes using RT-PCR

Location	Mosquito genus	Pool #	FlaV	AlphaV	BunyaV	ChikV	OnyV	YFV	RFV	DFV
Kimb	*Cx.*	1	-	-	-					-
	Ae.	2	±	±	±					-
	An.	3	-	-	-					-
	Ae.	4	-	-	+	-	+		-	-
	Ae.	5	±	±	-					-
Kingw	*Ae.*	6	-	-	-					-
	Ae.	7	-	-	-					-
	Cx.	8	±	-	-					-
	Ae.	9	-	-	-					-
Ndjili	*Ae.*	10	+	+	+	+	+	-	+	-
	Ae.	11	-	-	-			-		-
	Ae.	12	+	-	-					-
	An.	13	-	-	-					-
	Cx.	14	-	-	-					-
	Ae.	15	-	-	-					-
	Ae.	16	+	+	+	+	±	--	+	-
Kimw.	*An.*	17	-	-	-					-
	Ae.	18	-	-	-					-
	Ae.	19	-	-	-					-
	Cx.	20	-	-	-					-
	Ae.	21	-	-	-					-
	Ae.	22	-	+	-			-		-
	Ae.	23	-	-	-					-
	Ae.	24	±	±	±					-
	Ae.	25	±	±	-					-
Ngaba	*Ae.*	26	±	±	-					-
	Ae.	27	-	-	-					-
	Cx.	28	-	-	-					-
	Cx.	29	-	-	-					-
	Dengue	30	+	-	-	-	-	-	-	+
	RNase free water	31	-	-	-	-	-	-	-	-

(+): strongly positive reaction, (-): negative and (±): weakly positive. Sample number 30: dengue positive sample used as positive control; sample number 31: RNase free water used as negative control. Kimb. (Kimbanseke), Kingw. (Kingabwa), Kimw (Kimwenza).

CHAPTER FIVE

5.0 DISCUSSION

In the present study, a total of 5714 mosquitoes were collected using BG- Sentinel traps, battery-powered aspirator for both catches of resting mosquitoes and HLC at five sampling sites in Kinshasa. Collected mosquitoes were identified based on morphology. The results obtained from the present study indicate that mosquitoes in Kinshasa belong to *Culex, Aedes, Anopheles* and *Mansonia* genera representing 12 species of mosquitoes. To our knowledge, *Ae. luteocephalus* is reported for the first time in Kinshasa. *Cx. quiquenfiasciatus* was the most predominated species, followed by *Ae. aegypti*. The presence of 12 species of *culicidae* identified in this study have already been reported by Karch *et al*. in 1993, except *Ae. luteocephalus*. While in their study, Karch *et al*. (1993) found high number of species of *culicidae* in the same city province of Kinshasa, the less number of species in our study could bedue to the difference of sample size, their study area being almost representative of whole city province of Kinshasa. As it has been reported, we found that the distribution of species in this city province was not homogeneous (Karch *et al.,* 1993).

In addition to the lack of drainage system of sewage, uncontrolled population growth in the suburban areas of Kinshasa as well as in its urban areas is affecting the level of environmental pollution in both areas. This could actually explain the abundance of *Cx. quinquefasciatus* compared to *Cx. antennatus* that Karch *et al*. (1993) found to be highly predominant in suburban areas of Kinshasa such as Mont-Ngafula and Kimbanseke than *Cx. quinquefasciatus.* However, our findings corroborate with findings by Wat'senga and Manzambi in the same suburban areas (Wat'senga and Manzambi, 2002).

The current study showed the predominance of *Cx. quinquefasciatus* in both suburban and urban areas (around Ngaba market) where the level of pollution is highly increasing. These observations are corroborated by studies from Kisangani city in DRC (Mbongu-Sodni, 1983), Cameroon (Hougard *et al.,* 1993), Ivory Coast (Konan *et al.,* 2003) and Senegal (Darriet *et al*., 1986) that showed that the strong presence of *Cx. Quinquefasciatus* can be considered as a biological marker of urbanization.

The larvae ecology was ranging from small puddle water, clean or unclean water, sunny to stream or pond with various turbidity including artificial container suggesting that unplanned urbanization and human activities are playing a major role in mosquito emergence. *An. gambiae* which is uncommon in turbid water was fortuitously collected from polluted water in Kimbanseke. This supports the results from Nigeria and Cameroon (Kamgang, 2006).This could reflect a good adaptation of this species to the dynamism of urbanization.

In this study, we could not find the larval stage of *Mansonia* spp. while in the study conducted in Kimbanseke in 2002, Wat'senga managed to collect larval stage of *Mansiona* spp. This could be explained by the fact that our almost breeding sites did not contain aquatic vegetation, also in most of case the larval stages live attached to submerged parts of aquatic plants and the collection method could fail to reach them.

The significant presence of *Ae. aegypti* reported in this study is most linked to human activities, mismanagement of container after use, presence of old tyres that can be attributed to lack of environmental hygiene. This was demonstrated in the work realized in Pakistan where tyre trade had contributed to the reinvading of *Ae. aegypti* in areas where it was already eradicated and it is spreading to new areas where it was unknown. Therefore, the emergence of murderous diseases such as dengue (Rasheed, 2012).

Although they were very poorly represented in this study both *Ae. africanus* and *Ae.luteocephalus* are also known as arboviruses vectors for dengue virus, chikungunya virus, yellow fever virus, especially in their sylvatic cycle as reported by many studies done in Africa (Robert *et al*.,1993; Digoutte, 1999; Ngoagouni *et al.,* 2012). If *Ae. africanus* was already described in previous studies conducted in Kinshasa (Karch *et al.*, 1993;Wat'senga and Manzambi, 2002), for *Ae .luteocephalus,* it might be the first time to be reported in Kinshasa precisely at Kimwenza which is a semi-rural location with several surrounding bushes.

Mosquitoes collected and analyzed for assessing arboviruses carried in the selected areas of Kinshasa have provided an opportunity to report for the first time certain arboviruses in Kinshasa. From 29 pools in which *Aedes* pools predominated, arboviruses were detected in 12 including *Alphavirus* (8), *Flavivirus* (9) and *Orthobunyavirus* (5) genera. Amplification with specific primers of different viruses showed the presence of *Chikungunya* virus, *O'nyong'nyong* virus, Rift valley fever in *Aedes* spp. pools. All samples were negative for Dengue and Yellow fever viruses. The surrounding agriculture area of Ndjili River where rearing of pigs is common, showed predominated positive cases and high diversity of arboviruses, followed by Kimbanseke, Kimwenza and Kingabwa.

The study done in 2000 during outbreaks of febrile illness in Matete township and Kingabwa neighbourhood of Kinshasa province city allowed to detect only antibodies of *Chikungunya* virus in sera from 44 of 76 dengue-like fever patients in Kingabwa. Later, nine samples out of 21 seropositives samples were confirmed by RT-PCR. But no antibody of other arboviruses belonging to *Flavivirus* genus or *Bunyavirus* genus (Dengue virus, Bunyamwera virus, West Nile Virus) was detected (Pastorino *et al.*, 2004). In this study we found the virus members of three genera of aborviruses (*Flavivirus, Alphavirus,*

and Bunyavirus) with species such as Chikungunya, O'nyong'nyong and Rift valley viruses. These findings suggest that diverse arboviruses are circulating in Kinshasa, and the number of positive pools (12 from a total of 29 pools) in this study led to believe that the prevalence of arboviruses is high in Kinshasa, compared to the results of studies conducted in other countries such as Kenya (Caroline *et al.,* 2013), Thailand (Worawit, 2004) and from independent european arbovirus surveys undertaken in the Czech Republic, Italy, Portugal, Spain and the United Kingdom (Mattia *et al*., 2012).

In addition, the presence of most of these arboviruses seems to be unnoticed in malaria endemic regions where most of time all febrile illnesses including arboviral ones are considered as malaria because of the similarity in their clinical signs and by lack of adequate sanitary structures and appropriate laboratory for diagnosis. These febrile illnesses are making difficult the diagnosis and treatment of arboviral diseases. This ignorance and disadvantage are contributing to high mortality in Africa, and huge economic losses resulting in worsening poverty. Arboviral infection can be most effectively controlled by use of vaccines. However, this is limited by lack of registered vaccines for the majority of circulating arboviruses in the region except the yellow fever vaccine and Rift valley fever vaccine for livestock use only. Early detection of virus activity or detection of increased virus activity in the vector populations can be a key indicator or early warning for appropriate action to prevent occurrence of outbreaks. Surveillance programs designed to monitor virus activity in vectors also provides a system for mapping disease distribution and information needed not only to assess risk but also to identify vector species for targeted control (Hall *et al.,* 2012).

Our results suggest also that the environment is playing an important role in the occurrence of these arboviruses, such revealed by the high frequency of positive pools in

the surrounding areas of the Ndjili River where agriculture and pigs husbandary, and poultry are practiced. This finding is corroborating the findings from Kenya, where the pastoral regions of Garissa and Marigat showed highest frequency and diversity of arboviruses compared to other regions (Caroline *et al.,* 2013).

In this study, we could not detect this *Chikungunya* in Kingabwa where occurred the previous outbreak. This could be supported by the fact that mosquitoes collection was done when there was no active outbreak. In addition, it has been demonstrated in some works that the mosquito infection rate by arboviruses is low. But the presence of chikungunya at Ndjili can arouse some questions, is it a new emerging strain of this virus in a new area? Does dispersal distance of *Aedes* spp. play a role? Because the isolated strain of chikungunya from Kingabwa could end up in Ndjili zone through contiguity. In addition, the population movement should also play a role. Maybe it is a primary focus of chikungunya virus of less virulence as no outbreak has been reported in this area. Thus the need of making arboviruses characterization from these sites, as last outbreak showed the emergence of a new strain (Pastorino *et al*., 2004).

The abundance of mosquito vectors in an environment can favour the spreading of the virus in several vectors. Furthermore, multiple feeding can increase the probability of concurrent infection and viral genetic mixing (Kuno and Chang, 2005). The isolation of other arboviruses such as O'nyong'nyong and Rift valley fever viruses in mosquitoes circulating in Kinshasa signs that arbovirus emergence is gaining importance in Kinshasa, capital city of DRC; so more attention is needed to address this challenge. No pool was positive for Yellow fever virus, maybe because of most people are vaccinated and as they are serving as reservoir for yellow fever virus, the mosquitoes are feeding on uninfected blood.

There have not been reported any case of dengue fever in humans in Kinshasa and no dengue virus was detected in mosquitoes collected during the present study. This suggests that mosquito collection was done in an environment that seems to be free from dengue virus unless otherwise specified. This negative result could be also due to our small sample size. In Cameroon and Gabon, all *Ae. aegypti* field samples were negative for dengue virus, but a few was positive for chikungunya virus; however that study showed experimentally in laboratory a possibility of those *Aedes* to be infected with dengue virus (Christophe *et al.,* 2009).

In addition, if dengue virus circulates in Kinshasa, it is worth considering the vector competence. The study conducted with the aim of assessing geographical variability of genetics population of *Ae. aegypti* had shown that *Ae. aegypti* originating from Central Africa is less competent compared to those circulating in East Africa and other locations of the world (Failloux *et al.*, 2002). This was also supported by experimental and field studies from Central Africa conducted in Gabon and Cameroon showing a less susceptibility of *Ae. aegypti* to be infected with dengue virus and chikungunya virus than *Ae. albopictus* (Christophe *et al.*, 2009). This variability in vector competence between populations of *Ae. aegypti* from different geographical origins is intricately linked to the genetic heterogeneity of the species. The genetic differentiation of *Ae. aegypti* populations is highly dependent on environmental factors, human activities, insecticide treatment and modalities of storage of water supplies (Paupy *et al.,* 2005).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In the present study, a total of 5714 mosquitoes from five selected areas of Kinshasa were collected and analyzed with overarching objective of determining their diversity and screening for arboviruses presence in these mosquitoes. Culicidian fauna represented by 12 species of mosquitoes including *Ae. luteocephalus* which we believe is reported for the first time in Kinshasa, was characterized by the predominance of *Cx. quiquenfiasciatus* species followed by *Ae. aegypti*. The larvae ecology ranged from small larvae habitat with water, clean or unclean water, sunny to gutter, stream or pond with various turbidity, including artificial containers suggesting that uncontrolled urbanization and human activities are playing a major role in mosquito emergence. Twelve pools out of 29 were positive for *Alphavirus, Flavivirus* and *Orthobunyavirus*. Specific primers used to detect arboviruses species showed the presence of *Chikungunya, O'nyong'nyong* and *Rift valley fever* viruses, mainly in *Aedes* spp. pools. The surrounding agriculture area of Ndjili River showed predominated positive cases and high diversity of arboviruses. We conclude that various and prevalent arboviruses of public health importance are carried by mosquitoes circulating in Kinshasa, and the environment is playing a role for their emergence**.**

6.2 Recommendations

This preliminary study showed that the attention of health authorities in Kinshasa should be also focused on testing for emerging arboviruses, to establish effective prevention measures, adequate monitoring and appropriate treatment. Healthcare providers must take account of arboviral diseases in their differential diagnosis of febrile illnesses. In addition, vector control means should be applied in all parts of Kinshasa. For the future, it would be

interesting to make a large and representative sampling of the whole Kinshasa province and other parts of DRC to detect areas at risk for better control and prevention. Further in-depth molecular characterization and evolutionary studies of these viruses including vector competence will give more light on the dynamics of transmission, molecular epidemiology and the potential for future emergence of serious pathogens.

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APPENDICES

Appendix 1: Mosquito collection form

COUNTRY: **Collectors:**

Collection No.	**Longitude/Latitude**	**Date**
State/Province	**Locality**	

TERRAIN
0 Mountain
0 Hill
0 Valley
0 Plateau
0 Plain

ENVIRONMENT
0 Rain Forest
0 Evergreen Forest
0 Deciduous Forest
0 Coniferous Forest
0 Swamp-Forest
0 Swamp-open
0 Beach
0 Mangrove
0 Orchard-Plantation
0 Rice Paddy
0 Bamboo grove
0 Village
0 Urban
0 _____

ENVIRON. MODIFIERS
0 Primary
0 Secondary
0 Palm
0 Orchard
0 Plantation
0 _____

COLLECTION METHOD
0 Biting/Landing
0 Larval
0 Light trap
0 _____

ELEVATION
_____

LARVAL HABITAT
0 Pond-Lake
0 ground pool
0 flood pool
0 marsh
0 stream margin
0 stream pool
0 rock pool
0 seepage-spring
0 swamp
0 ditch
0 pit
0 well/cistern
0 tree hole
0 bamboo_____
0 Heliconia axil
0 Bromeliad axil
0 fallen leaf
0 crab hole
0 _____

DIMENSIONS OF SITE
_____
_____

WATER
0 Permanent
0 semi-permanent
0 temporary
Ph _____
Conductivity_____
Temperature_____

WATER MOVEMENT
0 Standing
0 Slow
0 Moderate flow
0 Fast

TURBIDITY
0 Clear
0 Turbid
0 Polluted
0 Colored_____

SHADE
0 None
0 Partial
0 Heavy

AQUATIC VEGETATION
0 None
0 Emergent_____
0 Floating_____
0 Submerged_____
0 Algae_____

Appendix 2: Purification of viral RNA

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

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

The bound nucleic acid was washed by adding 500 µl of buffer AW1 to the column and centrifuging at 6000 X *g* for one minute. The collection tube with the filtrate was discarded and replaced with a clean collection tube. The second wash, added 500 µl of buffer AW2 and centrifuged at 17 000 X *g* for three minutes. The collection tube with the filtrate was discarded and replaced with a clean 1.5 ml microcentrifuge tube. RNA was eluted by adding 60 µl of buffer AVE equilibrated to room temperature to the column. Column contents were incubated at room temperature for one minute, then centrifuged at

6000 X *g* for one minute. The filtrate, viral RNA, was immediately stored at -80 °C until amplification.