



MOLECULAR CHARACTERIZATION OF DENGUE VIRUS AND VECTORIAL COMPETENCE OF *Aedes* MOSQUITOES: A LOCAL MODEL OF XENOMONITORING

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ABSTRACT

Dengue is a major public health concern throughout tropical and sub-tropical regions of the world. Dengue virus is transmitted by infected female mosquitoes mainly of the species of *Aedes aegypti* and *Ae. albopictus*. Vector of *Aedes* mosquito populations may present different levels of competence for arbovirus transmission. Therefore, this study aimed at detecting DENV serotypes circulating and vectorial competence of adult field collected *Aedes* populations from selected local government areas of Kaduna State, Nigeria. A total of 233 mosquitoes were collected, identified morphologically to species level using coloured identification keys of Rueda and dissected under a dissecting microscope each species identified was tested in a pools of 20 individuals. Their bodies and head/salivary glands were separated and transferred to separate microtubes. RNA extraction was done using viral RNA extraction kit and semi-nested reverse transcription-polymerase chain reaction (RT-PCR) was used to amplified for DENV serotypes in the head/salivary glands of the *Aedes* mosquitoes. The study morphologically identified *Ae. aegypti*, *Ae. vittatus* and *Ae. Furcifer*. *Aedes aegypti* is the most abundant species 77.3% (180/233) while *Ae. furcifer* 10.3% (24/233) were the least. DENV serotypes were detected in 76.9% (10/13) of the pools of *Aedes* mosquitoes in the study areas. DENV1-3 serotypes were detected in *Ae. Aegypti*, DENV2 was detected in *Ae. vittatus* and DENV 2 and 3 were detected in *Ae. Furcifer*. Dengue virus 2 was the most detected serotypes in the study areas. PCR pools screening infectivity prevalence were found to be 7.07% (95% CI- 3.38-11.77). Thus, the finding revealed that *Aedes* species is competent enough to transmit DENV infection in the study locations.

Keywords: Molecular characterization, Dengue Virus, Serotype, Xenomonitoring, Vectorial competence

INTRODUCTION

Dengue fever is caused by Dengue Virus (DENV), which comprises of four serotypes (DENV 1- 4) that are phylogenetically and antigenically distinguishable (Gaye *et al.*, 2019; Messina *et al.*, 2014; Lundkvist, 2013). DENV is a single-stranded and positive-sense RNA virus (Hu *et al.*, 2017). The viral genome has length that is approximately 11kb composed of a single open reading frame (ORF) encoding three structural proteins (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Fahri *et al.*, 2013). Previous research findings, reported that, the transmission of the different serotypes is cyclic, with each serotypes sometimes re-emerging to dominate and the appearance of new serotypes or genotypes leads to fresh epidemics or outbreaks (Diaz *et al.*, 2019; Harapan *et al.*, 2019; Salvo *et al.*, 2019; Phadungsombat *et al.*, 2014).

Almost half of the world's population are at risk of DENV, this is due to the geographic distribution of the mosquito vectors, primarily *Aedes aegypti* (Bhatt *et al.*, 2013). Dengue symptoms varies from flu-like to life threatening forms, such as dengue haemorrhagic fever and dengue shock syndrome. Several reports also, suggested that climate change could swell up dengue transmission by 2080s to additional 2 billion people worldwide, in the absence of changes in all other determining factors (WHO, 2008).

Aedes mosquitoes are usually black, with characteristic white or silver markings on their bodies. They were formerly found in tropical and sub-tropical areas, but have now spread to all continents excluding Antarctica (Bonizzoni *et al.*, 2013). This spread was aided hugely by man-made activities, including the sale of used tyres. Their peak biting periods are early

mornings and evenings, before dusk. *Aedes* species normally breed in small water collections in containers such as tree cavities, leaf axils, bamboo stumps, rock pools, flower vases and artificial discarded containers (Chimezie *et al.*, 2018). Some studies conducted in Nigeria revealed that the major *Aedes* mosquito species found include: *Ae. aegypti*, *Ae. albopictus*, *Ae. africanus*, *Ae. luteocephalus*, *Ae. simpsoni* complex and *Ae. vittatus* (Chukwuekezie *et al.*, 2016; Adeleke, 2008; Aigbodion and Uyi, 2013).

Consequently, due to the importance of *Ae. aegypti* and *Ae. albopictus* in the transmission of dengue and other diseases, such as urban yellow fever, chikungunya fever and zika, these mosquitoes species has been the subject of numerous studies with special focus on population genetics and vector competence, in order to seek new alternatives for its control (Pimenta *et al.*, 2014). In addition, recent studies have reported *Ae. aegypti* and *Ae. Albopictus* in Ghana and Senegal as a competent vectors of dengue. Senegal, have suffered outbreaks in the past (Amoa-Bosompem *et al.*, 2021).

The life cycle of DENV in a mosquito begins when it takes a blood meal from an infected person. However, the virus must have the capacity of infecting the *Aedes* mosquito midgut before it can disseminate accordingly in the *Aedes* mosquito salivary glands. Once the salivary glands are infected, the *Aedes* mosquito can then successfully transmit the DENV to other humans during subsequent blood meals (Choy *et al.*, 2020; Pompon *et al.*, 2017).

However, despite the impacts of dengue fever on the global health systems, the vector competence (VC) of adult field-derived *Ae.* species populations to transmit DENV needs to be better understood, especially regarding mosquitoes in Kaduna State. There is paucity of information on the evaluation of the

ability of local field populations of *Ae.* mosquitoes to be infected and transmit DENV. Moreover, different vector mosquito populations may present different levels of vector competence for arbovirus, both at large and small geographical scales. There is however, paucity of data on the *Aedes* mosquito vector competence in the dengue transmission, despite reports of outbreaks in West Africa countries such Senegal.

Therefore, due to the increasing number of cases of dengue fever in Nigeria from several published and unpublished reports, basic biology studies become essential to be prepared in case of an epidemic outbreak. Understanding DENV transmission among mosquito populations may contribute to future vector-borne disease control programs by adding information about mosquito susceptibility and capability of transmitting the virus. Hence, it become necessary to determined the vectorial competency of bloodfed field-captured *Ae* species populations in order to assessed and

established the risk of DENV transmission or outbreak in Kaduna State, Nigeria.

MATERIALS AND METHOD

Study Area

Kaduna State is in the center of Northern Nigeria and more than 1000 km from the Atlantic Ocean (Ukegbu, 2005). It is located on a Latitude of 10° 36' 33.5484" N and Longitude of 7° 25' 46.2144" E. The natural vegetation of Zaria and its environs is typical 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.

Purposive sampling method was used for the selection of the study locations. This was based on certain characteristic such as proximity to the River, security, vegetation, availability of large public health facilities, environmental activities and population.

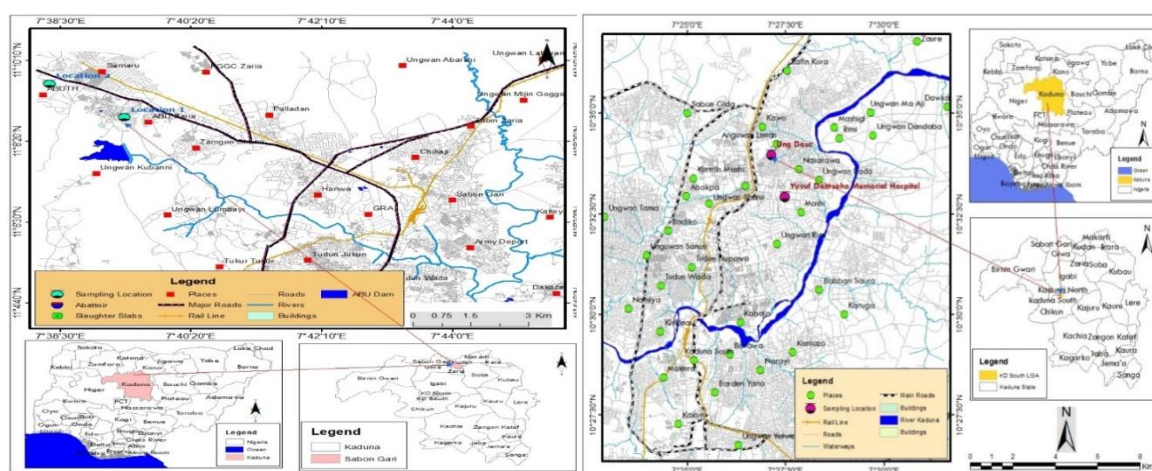


Figure 1: Zaria and Kaduna with Sampling Location

Source: GIS Lab Department of Geography ABU Zaria, Using ArcGIS 10.3 Software

Mosquito Collection, Identification and Preparation for detection of DENV Serotypes

Trained volunteers were used to collect adult *Aedes* mosquitoes using human baits between 6:00 - 10:00 am, while the evening collection was from 3:00 - 7:00 pm during the study periods. To collect mosquitoes, the lower legs of the collector was exposed such that when they perch to take a blood meal the mosquitoes were collected using test tubes. The opening of the tube was plugged with cotton wool to prevent escape of the mosquito. Also some indoor resting mosquitoes were collected using aspirators and sweep nets.

Aedes Mosquitoes Collection and Preparation for Detection of DENV Serotypes

The collection was carried out by human-landing catch techniques. Adult *Aedes* mosquitoes were collected both indoors and outdoors using human landing collector, in the morning hours between 6:00 –10:00 am and in the evening between 3:00 -7:00 pm. To collect *Aedes* mosquitoes, the lower legs of the collector was exposed, such that when they perch to take a blood meal, a test tube was used to collect the *Aedes* species. However, some indoor resting mosquitoes was collected using sweep nets and aspirator. The opening of the test tube was plugged with cotton wool to prevent escape of the mosquito. The mosquitoes then were transported to Entomology and Parasitology Laboratory of the Department of Zoology, ABU, Zaria for further processing.

Identification, Dissection and DENV RNA Extraction

A total of 233 female *Aedes* mosquitoes were collected, morphologically identified to species level with the help of coloured identification/taxonomic keys of Rueda (2004). The identified mosquitoes were carefully dissected under a dissecting microscope and with bodies and heads placed in separate vials. The *Aedes* species were pooled and labeled according to species and place of collection. Each pool contained at least 20 females *Aedes* mosquitoes. The heads recovered from the dissection were then used in the pool screen PCR assay. Poolscreen provides estimates of the prevalence of infection in the vector population together with user selectable confidence intervals for these estimates, based upon the pool size, the number of pools examined and the number of negative pools. A total of 13 pools were used for the study, consisting of 8 and 5 pools from Zaria and Kaduna town respectively. *Aedes* species pooled samples were stored in - 35°C for RNA extraction.

RNA Extraction from Pools of *Aedes* Mosquito Species

The viral RNA was extracted using the QIAamp Viral RNA extraction kit (QIAGEN, Hilden, Germany) based on the manufacturer's protocol. Each of the 13 pools were homogenized thoroughly with a mini handheld homogenizer in 350 µL buffer RLT (lysis buffer). The homogenate was then centrifuged for 3 mins at 13000 rpm. Which was then transferred to an QIAamp viral RNA extraction kit, in a 2 mL

collection tube. One volume (350 μ L) of 70% ethanol was added to the flow-through and mixed consistently by pipetting. Then lysate was carefully transferred into an RNAeasy spin column placed in a 2 mL collection tube and centrifuged for 15 s at 13000 rpm. This was followed by double washing steps using RW1 and RPE buffers in spin columns. The flow-through was then discarded. The column was dried by centrifuging for 1 min at 13000 rpm. Thereafter, 30 μ L of elution buffer was used to elute the RNA. The RNA was quantified using Nanodrop 1000 apparatus (Thermo Scientific, Dreieich, Germany) at a wavelength of 260 nm. The extracted RNA was stored at -45°C until used.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

The viral RNA was converted to a complementary DNA copy (cDNA) prior to enzymatic DNA amplification in a single tube, by the use of reverse transcriptase (RT). Dengue virus downstream consensus primers D1-5'-TCAATATGCTGAAACGCGAGAAACCG-3' and D2-5'-TTGCACCAACAGTCAATGTCTTCAGGTTTC-3' homologous to the genomic RNA of the four serotypes of DENV-1 - DENV-4 were used. The envelope and non-structural protein 1 (E/NS1) junction of the DENV genome was the gene of interest. The RT-PCR and Multiplex semi nested PCR was performed according to a previous protocol (Hakami et al., 2018) with some modifications. The Target gene was amplified in 25 μ L volumes containing the following components: 800mM deoxynucleotide triphosphates (dNTPs), 8mM dithiothreitol, 0.24 μ M each of primers D1 and D2, 0.5U of AMV RT (Promega, Madison, WI, USA), and 0.625U of Dreamtaq DNA polymerase (Fermentas Inc., USA). The reactions were allowed to proceed for 1 h at 42°C and then to proceed with 95°C for 3 minutes for initial denaturation followed by 35 cycles of denaturation (95°C for 30 sec), primer annealing (55°C for 1min) and primer extension (72°C for 2min) along with final extension (72°C for 5 min).

For the amplification of DENV 1-4 serotypes, the PCR product obtained from the amplification using consensus primers D1 and D2 was further used as a template. DENV 1-4 serotypes was amplified in a 20 μ L of reaction mixture consisting of 2 μ L of diluted (1:10 in ddH₂O) first PCR products, 0.8mM dNTPs, 0.5U of Dreamtaq DNA Polymerase and 0.3 μ M of primer D1 and 0.3 μ M of dengue

virus type specific primers: TS1 5'-CGTCTCAGTGATCCGGGGG-3', TS2 5'-CGCCACAAGGGCCATGAACAG-3', TS3 5'-TAACATCATCATGAGACAGAGC-3' and TS4 5'-CTCTGTTGTCTTAAACAAGAGA-3'. The samples were subjected to initial denaturation (95°C for 3min) followed by 20 cycles denaturation (95°C for 30s), primer annealing (55°C for 1min), and primer extension (72°C for 1min) along with final extension (72°C for 5min). The PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide. The expected sizes of the amplified products were 482 for DENV1, 119 for DENV2, 290 for DENV3 and 392 for DENV4 (Hakami et al., 2018).

Evaluation of Vector Competence

In addition, the Vector Competence (VC) was evaluated as the proportion of all experimentally infected mosquitoes (n = 233) for which DENV was detected in the head/salivary glands. The analysis of vector competence was performed according to Bennett et al. (2002).

Data Analysis

Descriptive statistics was used to calculate the composition and distribution of DENV serotypes in *Aedes* species using SPSS version 24. The prevalence of infection in the population was also determined using the PoolScreen computer program.

RESULTS NAD DISCUSSION

Composition and Relative Abundance of *Aedes* Species in Kaduna State

An overall of 233 *Aedes* mosquitoes species were used for the study, with 62.2% sampled from Zaria (Table 1). The species collected in this study were *Ae. aegypti*, *Ae. vittatus* and *Ae. furcifer*. *Aedes aegypti* accounted for 77.3% of the total mosquitoes collected in the study area while *Ae. furcifer* (10.3%) had the least representation (Table 1). In terms of relative abundance, *Aedes aegypti* was the most abundant species sampled in Zaria (82.8%) and Kaduna (68.2%). The relative abundance of *Ae. vittatus* and *Ae. furcifer* were 10.3% and 6.9%, respectively in Zaria, and 15.9% in Kaduna city. As shown in figure 1, comparatively, *Ae. aegypti* recorded higher abundance at Zaria, whereas *Ae. vittatus* and *Ae. furcifer* was higher in Kaduna.

Table 1: Species Composition and Relative Abundance of *Aedes* Mosquitoes Collected from Zaria and Kaduna

Species	Zaria		Kaduna		Overall	
	N	%	N	%	N	%
<i>Aedes aegypti</i>	120	82.8	60	68.2	180	77.3
<i>Aedes vittatus</i>	15	10.3	14	15.9	29	12.4
<i>Aedes furcifer</i>	10	6.9	14	15.9	24	10.3
Total	145	62.2	88	37.8	233	100

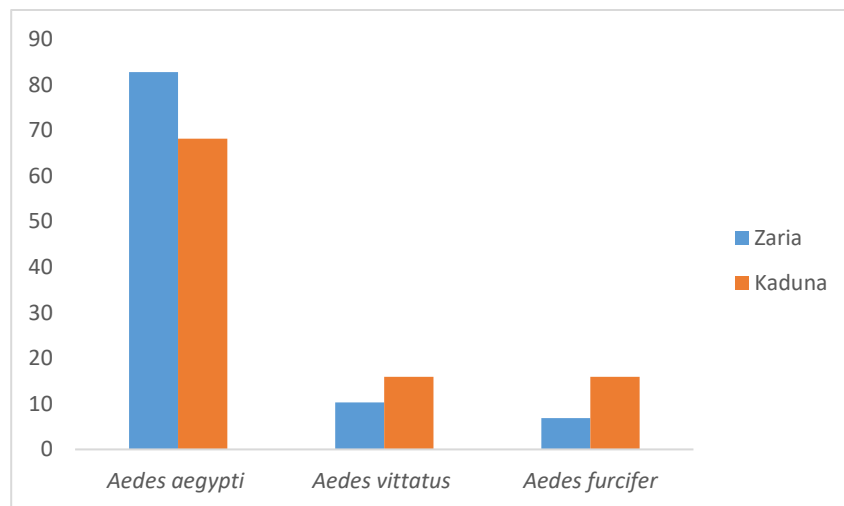


Figure 1: Relative Abundance of *Aedes* Species in the Study Locations

DENV Serotypes in *Aedes* Species

A total of 13 pools were obtained from the 233 *Aedes* mosquitoes samples collected from the study locations. The pooling was done according to species and locations of collection. The pools were analyzed for possible infection with dengue virus serotypes. The electrophoregrams of the PCR products is shown in plate 1. Lanes M; standard size marker of 50bp, NC= Negative control, lanes 1 and 9 are amplicons for DENV 2 and 3 infections obtained in the pools of *Aedes*

furcifer while lanes 2,3,4,6,7,8 and 10 were amplified DENV serotypes 1,2 and 3 detected in *Aedes aegypti*. Lane 5 shows the amplification of DENV-2 in *Aedes vittatus* pools.

Lanes 1,4 are test samples of DENV-2 obtained from Zaria and while lanes 2,3,5 were DENV-2 test samples gotten in Kaduna. Lanes 6,7 and 10 were DENV-1 test pools from Zaria, Lanes 8 and 10 were test samples for DENV-3 obtained in Zaria and Kaduna town respectively.

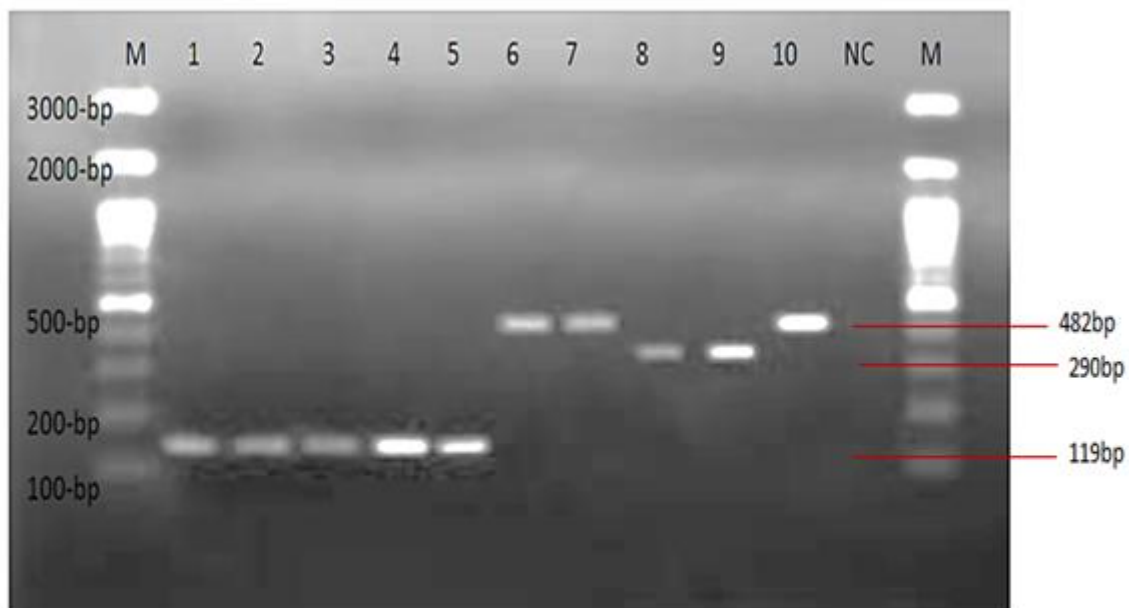


Plate 1: Electrophoregram of amplified cDNA by RT-PCR. DENV 1, 2, 3 and 4 serotypes specific primer sets were used with specific amplicons of 482 bp, 119 bp, 290 bp and 392 bp respectively. Lane M: Standard size marker of 100 bp, NC

Vector Competence (proportion of DENV in the head/salivary glands of total haematophagous *Aedes* species)

Eight pools were screened from Zaria and five from the Kaduna town (Table 2). Of the 13 pools screened, 10 were positive for DENV 1-3 while 3 pools were negative for DENV serotypes (Fig. 2). The overall Vector competency (VC) was calculated to be 76.9% (95% CI: 46.2, 94.9). Highest VC was

obtained from Kaduna town (80.0%) while the lowest was found in Zaria (75.0%). There was no statistical significant difference ($\chi^2 = 0.043$, $p = 0.83$) in the study locations (Table 2). *Aedes aegypti* had seven positive pools, *Aedes vittatus* had one and *Aedes furcifer* had two positive pools. In addition, the PCR pools screening infectivity prevalence was found to be 7.07% - 95%CI- 3.38-11.77.

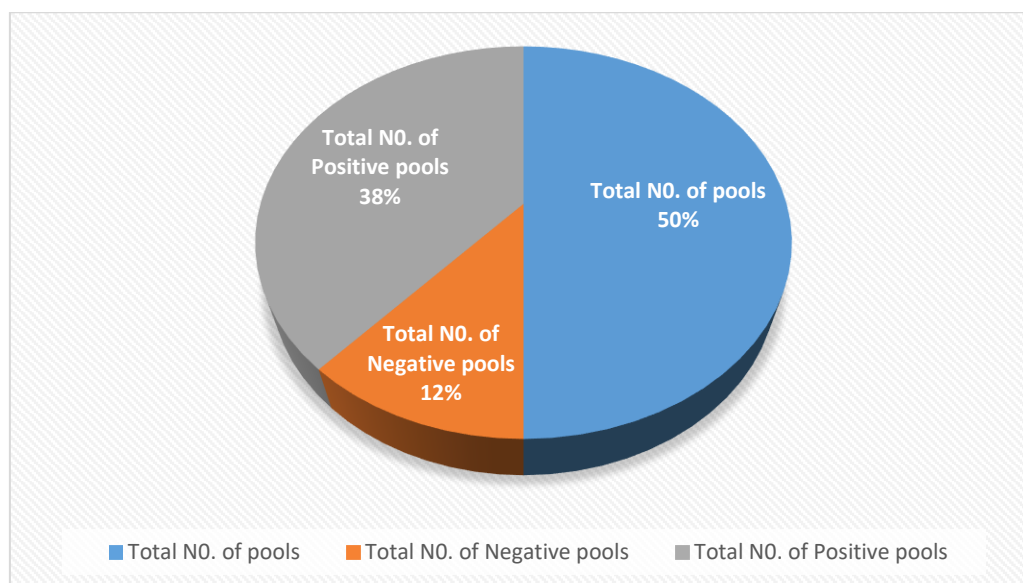


Figure 2: Pools of *Aedes* species used and their infectivity on the basis of PCR pool screen analysis

Table 2: Proportion of Vector Competence in Kaduna State

Study Location	No. of Pools Screened	Positive Pools		χ^2	P-value
		N	% (95% CI)		
Zaria	8	6	75.0 (34.9 - 96.8)	0.043	0.830
Kaduna	5	4	80.0 (28.3 - 99.5)		
Total	13	10	76.9 (46.2 - 94.9)		

Discussion

Mosquito collection carried out within the period of the study has revealed the species population of *Aedes* in the two locations (Zaria and Kaduna town). Three species were distinctively identified which are *Aedes aegypti*, *Aedes furcifer* and *Aedes vittatus*. *Aedes aegypti* was found to be the most abundant species in the Locations while *Aedes furcifer* were the least species found. The findings showed that *Aedes aegypti* was present in all the locations. This agrees with the findings of (Surtees, 1967), who reported that *Aedes aegypti* occurs in all parts of Nigeria. Also Chukwuekezie *et al.* (2018) reported that *Aedes aegypti* is found in all the Ecozones. The breeding of these species as observed in the study area were predominantly found in man-made containers such as cans, tyres, earthen pots among others. Nwoke and Nwoke (2006) observed that the habit of littering the environment with discarded containers in the community provides good breeding sites for these mosquitoes. A near uniform peak biting period between 7 – 9am and 5 – 7pm) which was observed during the day in the study locations, this further buttresses their proclivity for diurnal biting activities. This study detected high prevalence of DENV 1-3 serotypes in the female adult collected *Aedes* mosquitoes species in the study areas. The high prevalence obtained might be as a result of changes in behaviours of the *Aedes* mosquitoes species interaction with infected individuals. This is because the *Aedes* mosquitoes were collected at the height of the raining season (August - October). This is in line with the reports of Isa *et al.* (2021) and Halstead *et al.* (2008) that, DENV peak transmission is usually known to be connected with the times of high rainfall in endemic countries.

Also, it might have been worsened due to the rate of industrialization and development that is obvious in the past couple of years thereby making the city more populated with

humans, and so creating an avenue for the *Aedes* mosquitoes and human interaction in the study locations. It is possible that the protracted humanitarian crisis and people behaviour in the Kaduna State might have account for more human and *Aedes* mosquito interaction. Furthermore, the high prevalence obtained could also be due to transovarial transmission, as *Aedes* does that as a way of maintenance of DENV in nature (Isa *et al.*, 2021).

However, the prevalence obtained in this study is higher than 58.54% reported in Adamawa State in *Aedes* species (Isa *et al.*, 2021). The variation observed could be due to differences in the study areas and humans behaviour which encourages mosquitoes breeding in the study locations.

The study also, demonstrated that most of the *Ae.* populations were highly infected with DENV and have also developed DENV infection of the heads/salivary glands. Knowing the association and interaction between DENV and *Ae.* mosquitoes species, is very crucial for epidemiological purpose and this is also important in the control of dengue disease. Understanding the vector competence is very important since the *Aedes* mosquito populations differs in their tolerant ability for DENV development (Pimenta *et al.*, 2014). This is similar to the obstacles faced by the DENV from the points of infection of the *Aedes* mosquito species to the periods when it is liberated in saliva during blood feeding (Mercado-Curiel, *et al.*, 2008). Thus, this knowledge, should assist in focusing control efforts on *Aedes* species populations with higher capacity for transmission of DENV and will also give idea of a specific locations with higher risk. However, *Aedes* species with DENV in the thorax and abdomen, but not found in the heads/salivary glands, does not have the capacity enough to transmit the viruses by bite to a new hosts such as humans.

CONCLUSION

The study identified *Aedes aegypti*, *Aedes furcifer* and *Aedes vittatus* in the study location with *Aedes aegypti* as the most abundant species (77.3%). The finding also detected DENV1-3 serotypes in *Aedes mosquitoes* species in Kaduna State and couldn't report any occurrence of DENV4 serotype. It was also noted that the species were competent enough to transmit the virus during blood meal.

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