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RESEARCH ARTICLE

A Simple PCR Assay for Discrimination of Dengue Vectors from Nsukka LGA, Nigeria

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ABSTRACT

Mosquitoes of *Aedes* species are vectors of several arboviral diseases which continue to be a major public health problem in Nigeria. This study among other things, morphologically identified *Aedes* mosquitoes collected from Nsukka LGA and used an allele specific PCR amplification for discrimination of dengue vectors. Larval sampling, BG-sentinel traps and modified human landing catches were used for mosquito sampling in two selected autonomous communities of Nsukka LGA (Nsukka and Obimo). A total of 124 *Aedes* mosquitoes consisting of five (5) different species were collected from April to June, 2019 in a cross-sectional study that covered 126 households, under 76 distinct geographical coordinates. Larvae was mainly collected from plastic containers 73% (n=224), metallic containers 14% (n=43), earthen pots 9% (n=29) and used car tyres 3% (n=9), reared to adult stage 69.35% (n=86), and all mosquitoes were identified using standard morphological keys. Five (5) *Aedes* mosquito species were captured; *Aedes aegypti* 83(66.94%), *Aedes albopictus* 33(26.61%), *Aedes simpsoni* (4.48%), *Aedes luteocephalus* (≤1%) and *Aedes vittatus* (≤1%). Nsukka autonomous community had higher species diversity than Obimo. Allele specific amplification confirmed dengue vectors, *Aedes aegypti* and *Aedes albopictus* species on a 2% agarose gel. Since the most recent re-emergence of arboviral diseases is closely associated with *Aedes* species, findings of this study therefore, give further evidence about the presence of potential arboviral vectors in Nigeria and describe the role of a simple PCR in discriminating some. Further entomological studies should integrate PCR assays in mosquito vector surveillance.

Keywords: *Aedes aegypti*, *Aedes albopictus*, Dengue

1 Introduction

Mosquito borne diseases have long plagued humanity. Globally, they infect 96 million people [1] and the disease landscape is continually changing owing to several factors [2], [3]. A number of mosquito species serve as vectors of several arboviruses, with majority belonging to *Aedes* species, which cause clinical disease symptoms in humans and animals [4]. The most prevalent arboviruses of latest times include: Dengue virus (DENV), Chikungunya virus, Yellow fever virus, Zika virus, West Nile virus and Rift Valley fever virus [4], [5]. Dengue fever (also called Dengue or break bone fever) is caused by four distinct but closely related dengue viruses called serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) [6]. More serious manifestations of this disease are called dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), while mild manifestations are called dengue fever (DF) [7]. DF and DHF are the most rapidly spreading vector-borne diseases with approximately 50 million cases of infection worldwide [8], [9]. The disease has witnessed the most dramatic spread from nine to 128 countries,

with the number of cases exploding from just 15,000 per year in the 1960s, to an estimated 390 million in 2012 [1], [10], [11]. The recent geographic distribution of dengue now includes countries which had not reported dengue for 20 or more years and several have no known history of the disease and this situation has worsened during the coronavirus pandemic [3], [12], [13]. Dengue mainly occurs in tropical and subtropical areas around the globe therefore, America, Southeast Asia and the Western Pacific regions are the most affected regions [8], [14]. Dengue is likely under recognized and underreported in Africa because of low awareness by health care providers, other prevalent febrile illnesses, and lack of diagnostic testing and systematic surveillance [15]. Dengue viruses are transmitted to humans through the bites of infective female *Aedes* mosquitoes after acquiring the virus through feeding on the blood of an infected person [16]. *Aedes aegypti* has exceptionally been shown to be the principal vector of DENVs in tropical areas of Africa, Central and South America [4], [17]. The other most important *Stegomyia* mosquito vector is *Aedes albopictus*, which often times is a secondary vector of these arboviruses [18]. It is generally believed to be a less competent vector of arboviruses than *Aedes aegypti* because it is not well adapted to urban domestic environments and is less anthropophilic however, because of its rapidly changing global distribution, it is becoming an increasing important vector associated with dengue outbreaks [19], [20]. Once infected, these mosquitoes capable of transmitting DENV to susceptible individuals for the rest of their life, during probing and blood feeding and to the next generation of mosquitoes by trans-ovarial transmission [7], [16]. Dengue is driven by climate change, trade and travel; annual mean temperatures of 15°C for *Aedes aegypti* and 11°C for *Aedes albopictus* seem to be indicative thresholds for the persistence of these vector populations in European regions, for example [13], [21]. The development and survival of all these mosquito vectors are closely linked with rainfall events [22] and the worldwide trades in second hand tyres has strongly allowed them to spread beyond their ranges [20], [23]. Several studies have shown that these vectors are abundant in the South Eastern parts of Nigeria [24]–[26]. Despite the reports of the presence of *Aedes* species in the region, molecular methods have not been employed to identify *Aedes* mosquitoes. Yet owing to the limitations of morphological techniques like, being specific to only a few developmental stages and impossible to identify damaged specimens, DNA-based technologies that use cyto-taxonomic features are increasingly being accepted for identification worldwide and sometimes the only option for distinguishing sibling species [27], [28]. The PCR method uses genetic markers to distinguish between sibling species in defined populations [29]. This study in particular investigated the mosquito vector species present in Nsukka LGA, their breeding habitats and used a simple PCR method to discriminate the distinct dengue vector species using the target primers.

2 Research Methodology

The study was carried out in two selected autonomous communities of Nsukka Local Government Area (LGA), Enugu State, South Eastern Nigeria as shown in Figure 1. This area is located between 6°51'24"N and 7°23'45"E, at an elevation of 1,810 feet, with an approximate population of 309,633 people, 17.52 square miles total land area and a home to members of the Igbo ethnic group [30].

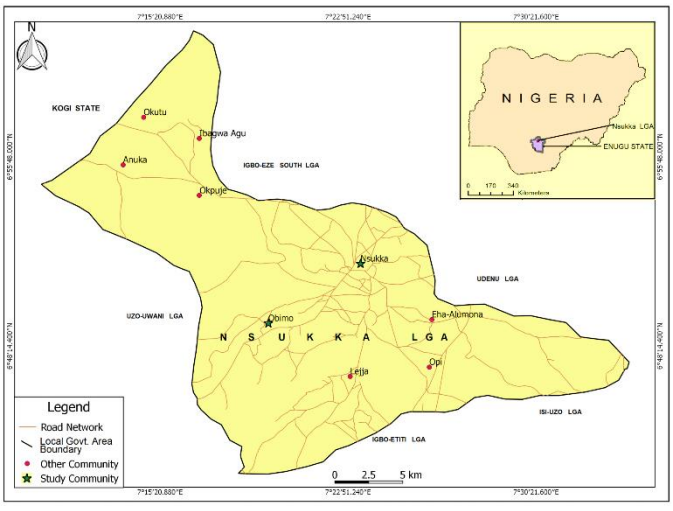


Figure 1: *Map of Nsukka LGA showing the study locations (Nsukka and Obimo).*

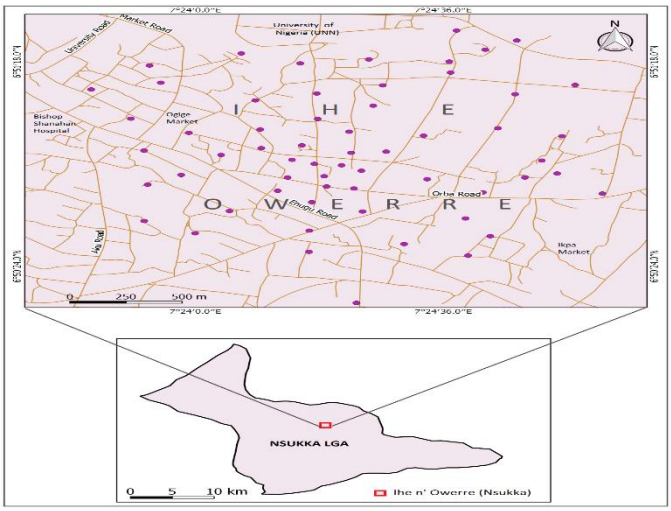


Figure 2A: *Map of Nsukka LGA showing the sampling points in Nsukka autonomous community.*

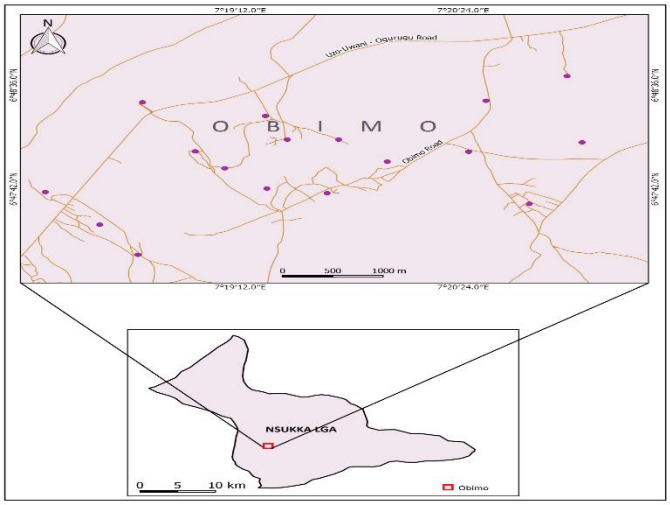


Figure 2B: *Map of Nsukka LGA showing the sampling points in Obimo autonomous community.*

Nsukka LGA has a tropical climate, with an average annual rainfall of about 1579 mm, the driest month is January, with 10 mm precipitation and in September, precipitation reaches its peak with an average of 299 mm [31]. Nsukka LGA has a warm climate with mean annual temperature of 24.9 °C, March being the warmest month of the year at 27.0 °C average temperatures and August is the coldest month of the year, at 22.9 °C on average [31]. These sites were selected based on ecological and demographical characteristics such as breeding habitats, the topography, vegetation and areas of high human habitation. Nsukka autonomous community (Figure 2A) is a peri-urban business area having a number of shops, garages, university, schools and gas stations while Obimo (Figure 2B) is situated in the sparsely-populated areas of Nsukka LGA, with a high vegetation cover and comprising rural settings.

2.1 Sample collection

Mosquitoes of *Aedes* species were successfully collected as adults and larvae in Nsukka LGA, from April to June 2019. Mosquito sampling forms obtained from NAVRC were used to record data and each sampling site was geo-referenced using a global positioning system gadget.

2.2 Mosquito larvae collection

Mosquito larva was collected using a larval sampler/scooper with the aid of a pair of hand lenses and a torchlight for clear vision. Stagnant water collected in household outdoor containers were searched for the presence of larva. Spotted larvae were identified to genus using morphological characters by a Research Entomologist. All the instars of mosquito larvae were scooped into a small collection bowl, sorted out and transferred into a larval collection bottle using a dropper. An average of 4 mosquito larvae were collected from each household container type. Larval sampling was carried out between 800hrs and 1600hrs and dispatched to the NAVRC laboratory for mosquito rearing.

2.3 Collection of adult mosquitoes

Adult mosquitoes were collected using battery-operated BG-sentinel traps and modified human landing catches. The BG-sentinel traps were set up outdoors at 600hrs in appropriate and strategic locations, preferably cool and shady and collected at 1800hrs, from the premises of randomly selected households in each community. The live adult mosquitoes collected were euthanized with ethyl acetate in test tubes, sorted and identified by a qualified Entomologist using a stereomicroscope. The samples were immediately preserved in RNA-later in properly labelled sterile screw top cryovial tubes. Adult mosquitoes were also collected using human baits. The mosquitoes were immobilized using ethyl acetate in cotton wool-closed test tubes and identified under the stereomicroscope. All the samples were dispatched to the laboratory for storage at -20 ^{o}C and further processing.

2.4 Mosquito rearing and identification

The mosquito larvae samples from Nsukka LGA were reared to adult stage under appropriate conditions. The larvae were fed on appropriate animal feed locally prepared. Upon emergence, the mosquitoes were transferred from larval bowls to well labelled test-tubes using an aspirator. The mosquitoes were later morphologically identified using the gross standard morphology of the species, external morphology of the head, mouthparts, antennae, proboscis, stripes on the thorax, patches of pale and black scales on the wings and legs and the terminal abdominal segments as described by Gillett (1972) under a stereomicroscope by a qualified Entomologist [32]. Mosquitoes collected using HLC, BG-sentinel traps and those from larval emergence, were systematically pooled together into vials according to their sex, species, collection method and location, before being homogenized.

2.5 Genomic DNA extraction

Total DNA from mosquito tissues was extracted using the Quick-DNA™ Tissue/Insect Miniprep Kit (Zymo Research, USA), according to manufacturer's instructions. Briefly, BashingBead™ Buffer (Zymo Research, USA) was first added to the Eppendorf tube containing the mosquito tissues and homogenized completely. The samples were then lysed by BashingBead™ Buffer (Zymo Research, USA), mixed thoroughly using DAIGGER Vortex-Genie (Scientific Industries, USA), centrifuged in a thermal micro-centrifuge (TGL-16, Scientific Industries), and the proteins were precipitated using Genomic Lysis Buffer in Zymo-Spin™ III Filter tubes (Zymo Research, USA), under an airflow chamber (PV-CH60BF). The lysate was then passed through Zymo-Spin™ IICR Columns and washed with DNA washing buffers. The eluted solution of the Eppendorf tube containing the extracted mosquito DNA was stored at -20 °C for subsequent PCR analysis.

2.6 PCR Amplification

Primers targeting the second internal transcribed spacer region (ITS2) were used for amplification. The multifunctional forward primer 18SFHIN (GTAAGCTTCCTTTGTACACACCGCCCGT) and reverse primers TAACGGACACCGTTCTAGGCCCT and GTACTAGGCTCACTGCC were used for amplification for *Aedes aegypti* and *Aedes albopictus* respectively. The primers were supplied by New England BioLabs, Inc., USA and Inqaba Biotech Africa Ltd. The master mix was made according to manufacturer's instructions by adding 12.5 µl One Taq Master Mix (2X), 2 µl 20 µM Forward Primer, 2 µl 20 µM Reverse Primer, 5 µl of the DNA template and nuclease free water to a total volume of 25µl, in PCR tubes. They were inserted in a MyGene™ Series Peltier thermal cycler (LongGene Scientific Instruments Co. Ltd) and ran using the following amplification conditions; 97°C, 4 min, 1 cycle, 96°C, 30 s, 48°C, 30 s, 72°C, 2 min, 30 cycles; 72°C and 72°C, 4 min for 1 cycle. The positive and negative controls were used in all PCR reactions.

2.7 Molecular Analysis

The PCR products were analyzed by agarose gel electrophoresis. Gel electrophoresis of the amplicons was performed using a 2% agarose gel in TAE buffer, stained with 5 µl Ethidium Bromide. Each gel-well was loaded with 7 µl of the PCR product and 3 µl purple DNA loading dye (6X) (New England BioLabs, Inc.) and ran at 120 volts for 60 minutes in a gel box (BIO-RAD). S N0551S Quick-Load Purple 100bp DNA Ladder (New England BioLabs, Inc.) was used. The bands were visualized by UV fluorescence light using a gel documentation system (BioSens SC750, USA).

2.8 Statistical Analysis

Excel was used to analyze the proportion of mosquito by species and habitat types, and to calculate the Simpson's Index. Simpson's Index of diversity (D) was calculated using the formula below to determine mosquito diversity in the communities.

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right) \quad (1)$$

Where; n = the total number of mosquitoes of a particular species, N = the total number of

mosquitoes of all species in a community and, ∑ = the sum of calculations [33].

3 Results

3.1 Mosquito larval habitats

In the two autonomous communities sampled, mosquito larva was collected from plastic containers, earthen pots, metallic containers and used car tyres, for Nsukka community as in Figure 3A below whereas, plastic containers and earthen pots contained the larvae in Obimo community as in Figure 3B. A total of 307 mosquito breeding habitats were sampled in Nsukka LGA. Plastic containers were the most abundant mosquito larvae habitat 73%(n= 224) and the least was the wooden container type.

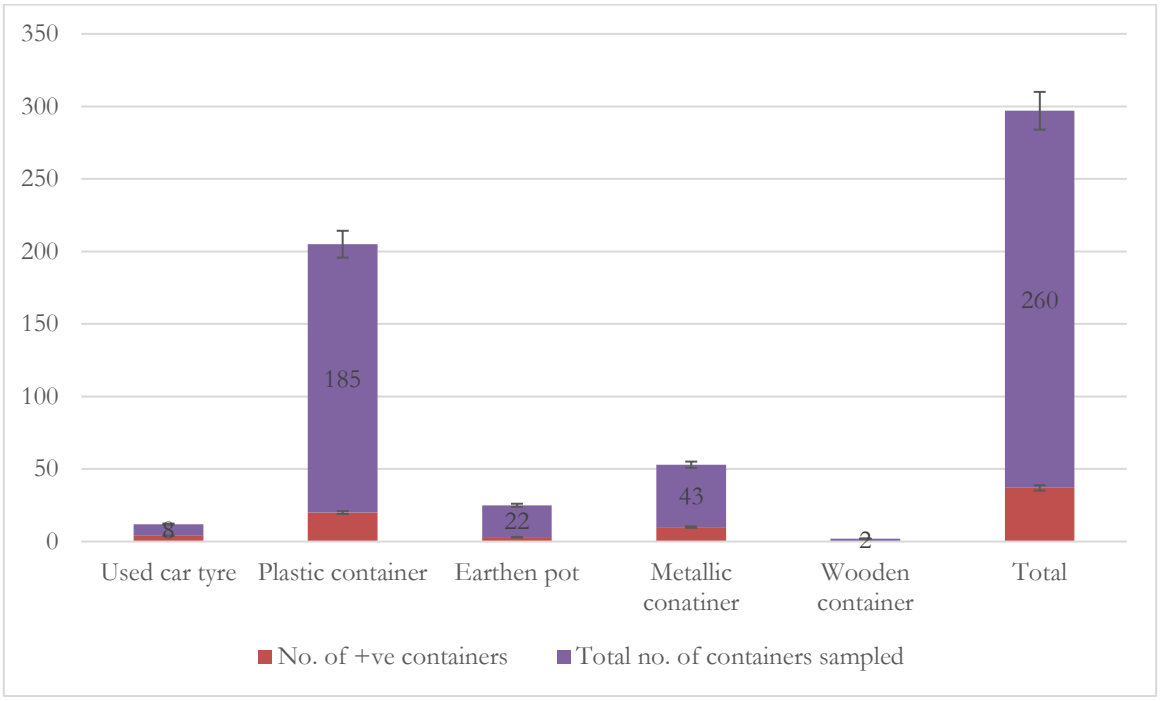


Figure 3A: *Mosquito larvae habitats in Nsukka autonomous community.*

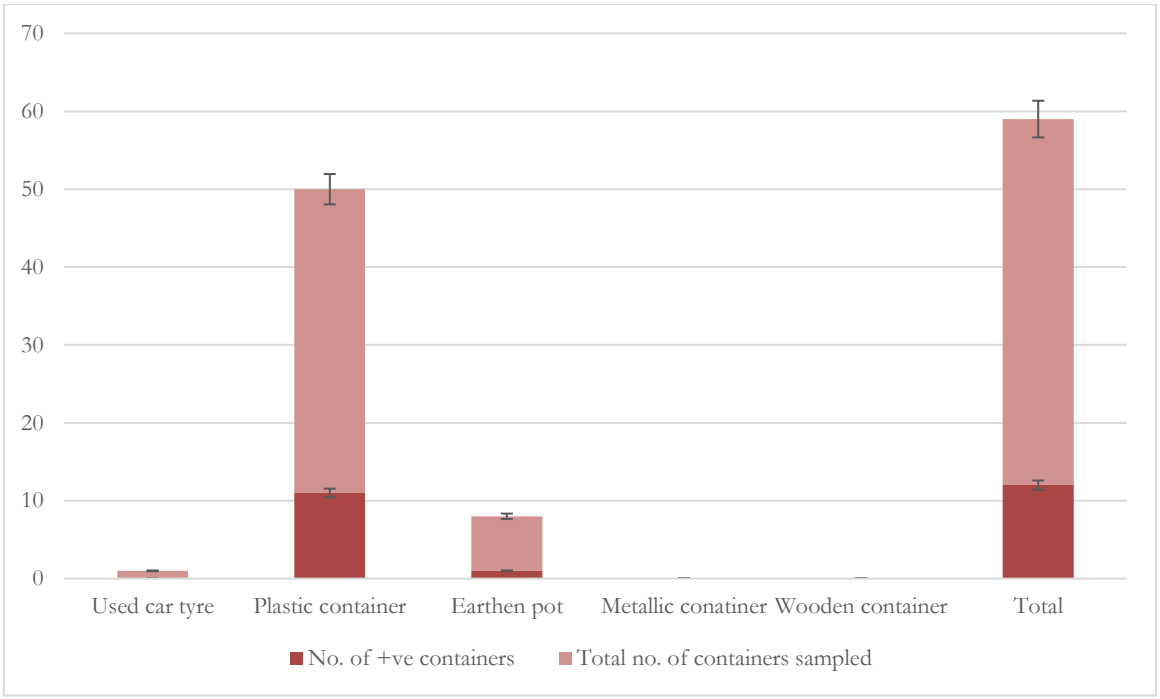


Figure 3B: *Mosquito larvae habitats in Obimo autonomous community.*

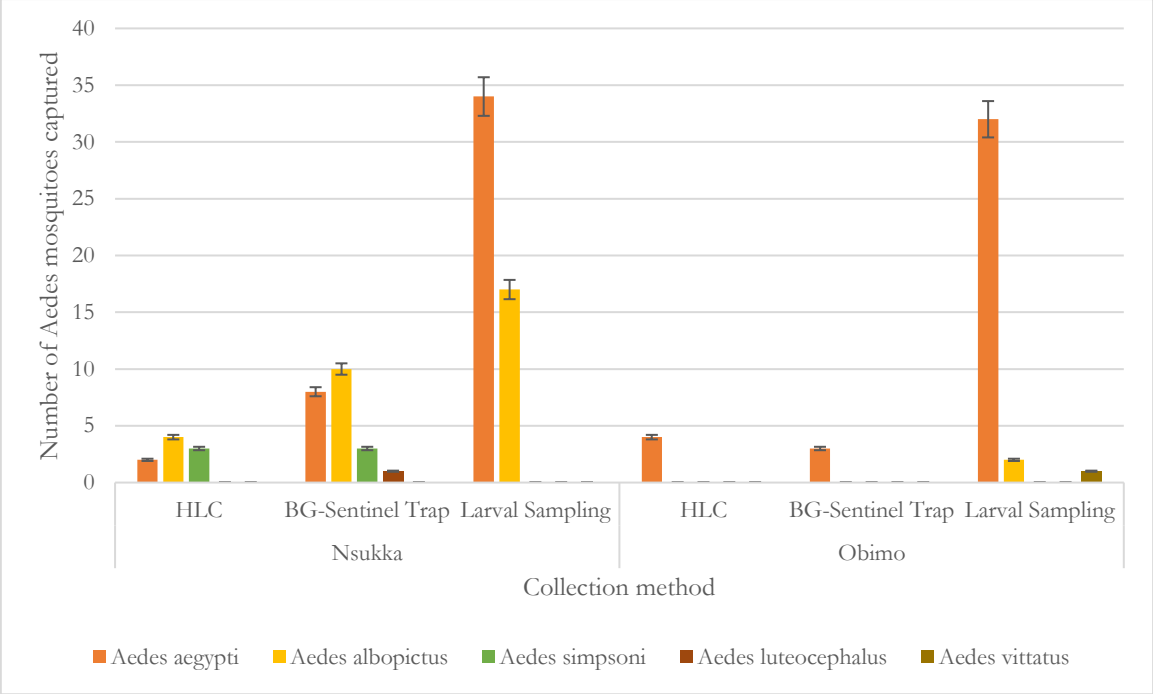


Figure 4: *Aedes mosquitoes collected using different sampling strategies in Nsukka LGA.*

Table 1: *Aedes mosquitoes species captured in the two communitites of Nsukka LGA.*

	Community		
	Nsukka	**Obimo**	**Total**
Mosquito Species	**N(%)**	**N(%)**	**N(%)**
Aedes aegypti	44(35.48)	39(31.45)	83(66.94)
Aedes albopictus	31(25)	2(1.61)	33(26.61)
Aedes simpsoni	6(4.84)	-	6(4.84)
Aedes luteocephalus	1(0.81)	-	1(0.81)
Aedes vittatus		1(0.81)	1(0.81)
Total	**82**	**42**	**124**
D	**0.9982**	**0.9965**	

D*=Simpson's index of diversity

3.2 Species diversity

The 3 sampling strategies for *Aedes* mosquitoes were successful. Mosquitoes were mainly collected from Ihe Owerre, Umudikwu, Mkpuru Ikwere, Amaukwa and surrounding areas for Nsukka community, and Ajouna Obimo and surrounding areas for Ajouna community as indicated in Figures 2A and 2B. The highest number of *Aedes* mosquitoes was collected using larval sampling, with just 10.48% (n=13) collected using human landing catches (HLC) and 20.16%(n=25) using BG-Sentinel traps. This is illustrated in Figure 4. Equal sampling efforts were considered during sampling although not all *Aedes* mosquitoes could probably be sampled in the sampling areas due to time and labour constraints.

A total of 124 *Aedes* mosquitoes that were collected from Nsukka LGA including 31% (n=38) adult mosquitoes and 69.35%(n=86) just-reared to adult from larvae collected during sampling. More *Aedes* mosquitoes were collected from Nsukka community than Obimo community, representing 66.13% (n =82) and 33.87% (n = 42) respectively. The *Aedes* mosquito species captured in Nsukka LGA were: *Aedes aegypti*, *Aedes albopictus*, *Aedes simpsoni*, *Aedes luteocephalus* and *Aedes vittatus*. The *Aedes aegypti* recorded the highest number of *Aedes* mosquitoes

sampled (66.94%; n = 83) of all the species, and occuring predominatly in all sites, followed by *Aedes albopictus*, *Aedes simpsoni* , *Aedes luteocephalus* and *Aedes vittatus*, in that order. 70.73% (n=58) of all the collections in Nsukka community were female mosquitoes as shown in Table 1.

Since Nsukka autonomous community has a higher value of Simpson's index of diversity (D= 0.9982) compared to Obimo (D= 0.9965), this implys that Nsukka comminity has a higher *Aedes* species diversity. The highest number of females collected belonged to *Aedes albopictus* (83.87%, n=31) although *Aedes aegypti* species had the highest overall proportion of female mosquitoes. The dengue vectors, *Aedes aegypti* (67%, n =83) and *Aedes albopictus* (26%, n=33) species represented the highest collections from all the sampled sites of Nsukka LGA captured during the study. *Aedes leteocephalus* (≤1%, n=1) and *Ae. vittatus* (≤1%, n=1), were only captured in Nsukka and Obimo communities respectively, and they represent the lowest and the least abundant species in Nsukka LGA. These results are depicted in the Figure 5.

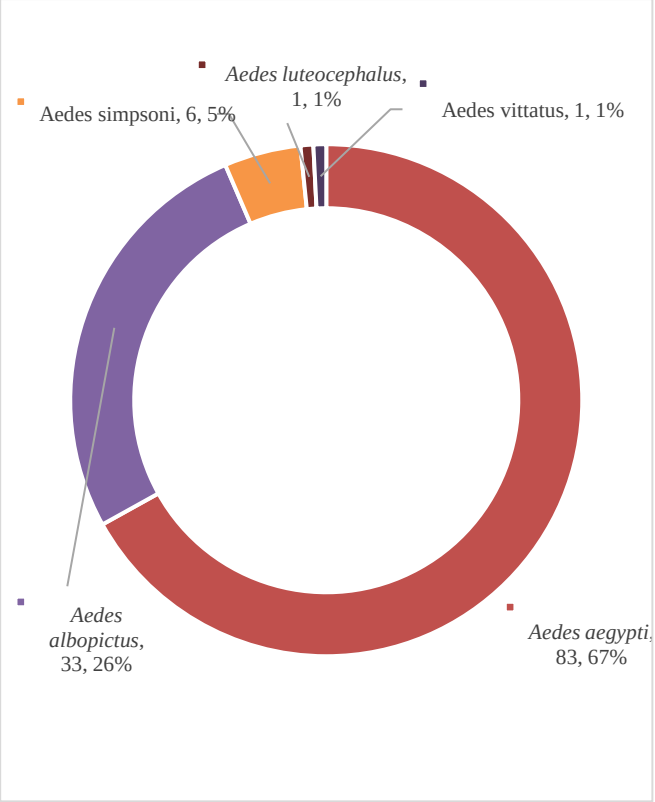
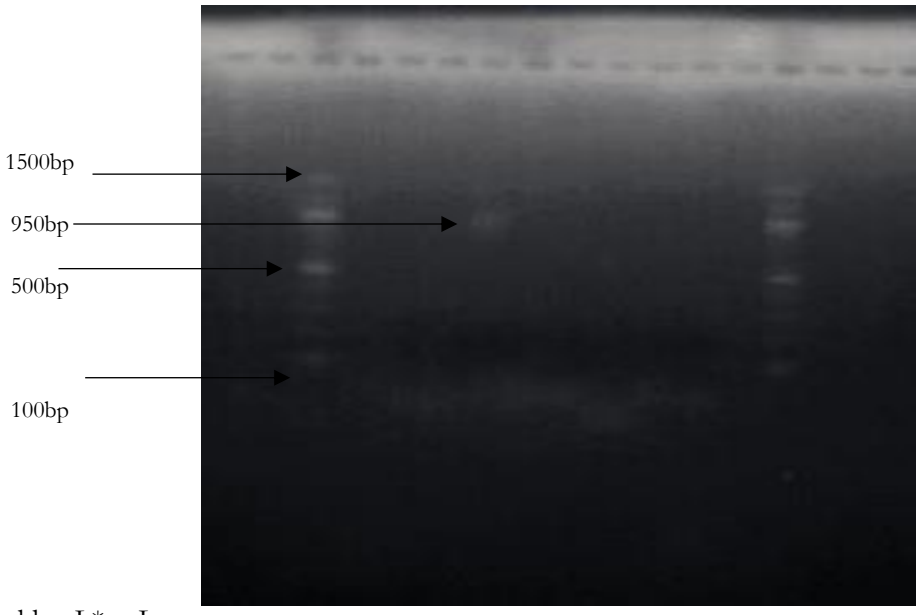


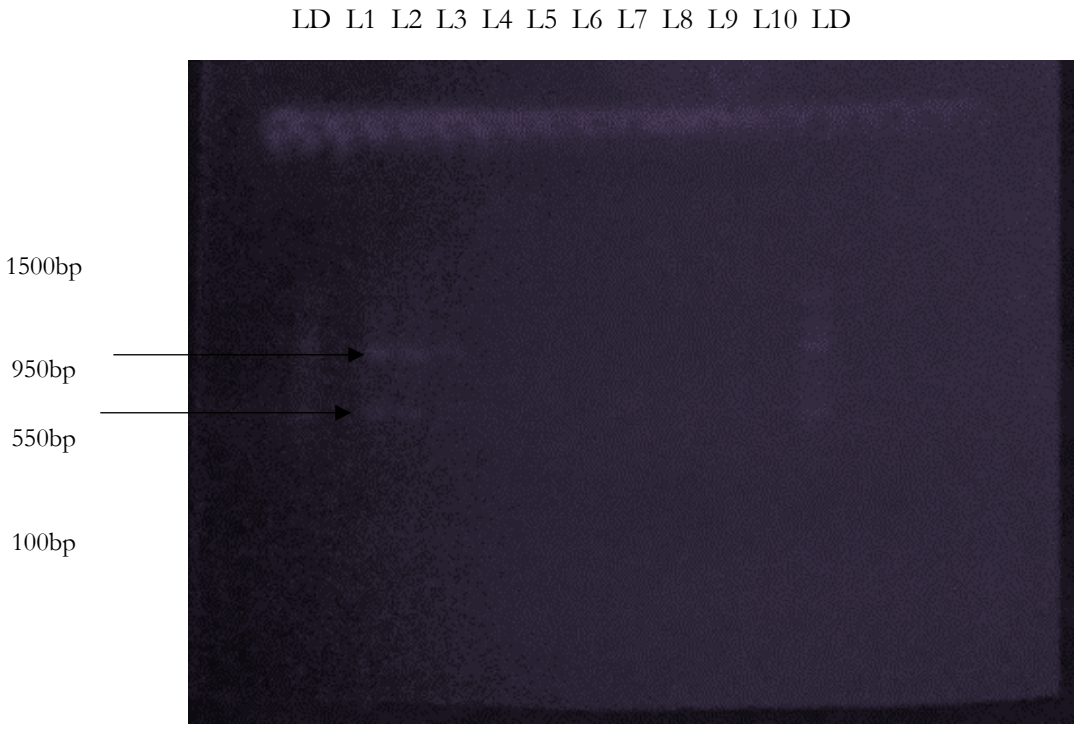
Figure 5: *Aedes species composition from Nsukka LGA*

LD L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 LD



LD*= Ladder, L* = Lane

Figure 6: *Gel electrophoresis image showing the observed bands after an allele specific amplification for species identification of samples from Nsukka LGA. A distinct band at ~950 bp confirms the presence of Aedes albopictus upon (L4). Other samples didn't produce clear bands (L1, L2, L3, L5, L6, L7, L8, L9 and L10).*



LD*= Ladder, L* = Lane

Figure 7: *Gel electrophoresis image showing the observed bands after an allele specific amplification for species identification of Aedes aegypti at~550 bp (L2 and L3) and Aedes albopictus at ~950 bp upon (L2, L3 and L4).*

3.3 Species identification

Species identification using a PCR was strictly carried out on the two principal vectors of dengue fever, *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus*. The PCR allele-specific amplification for species identification produced a clear band at ~950 bp upon viewing the gel confirming *Aedes albopictus* species as shown in Figure 6. Also, 2 distinct fragments of ~550 bp and ~950 bp were observed in another gel, confirming the presence of *Aedes aegypti* and *Aedes albopictus* respectively, as had been morphologically identified. The gel results are shown in the Figure 7 confirming the presence of dengue vectors.

4 Discussion

The most recent outbreak and re-emergence of arboviral diseases such as dengue fever and Zika is closely associated with *Aedes* species [5]. These mosquitoes and hence arboviruses, continue to spread across the world even into areas that they never existed before [3], [34]. Nigeria, an endemic country for dengue fever, is currently experiencing several outbreaks of yellow fever [35], [36]. Despite these threats, entomological studies continue to rely on standard morphological keys for vector identification, at the expense of molecular methods like the PCR [24], [26], [37]. This study attempted to identify the major dengue fever vectors from Nsukka LGA, their breeding habitats and a simple PCR based method for species identification. This study reports that, tyres, plastic containers, earthen pots, and metallic containers that collected water around homesteads were found to be the breeding grounds for *Aedes* species. This has been reported in other studies [24], [38], [39], as susceptible habitats in harbouring mosquito larvae, particularly those of *Aedes* species. This was evident with the high presence of *Aedes* mosquito species collected. Similar findings [26] indicate the same type of breeding by the exophagic *Aedes* mosquitoes in Nigeria. The increasing habit of discarding and littering containers especially by the Nigerian urban dwellers has tremendously increased chances of breeding by mosquitoes [16], [37], [40]. This study also discovered that most of the species

sampled belonged to *Aedes aegypti* and *Aedes albopictus*, and occur in both communities of Nsukka LGA. However, the morphologically identified *Aedes simpsoni* mosquitoes were collected only from Nsukka autonomous community. According to the previous studies, *Aedes simpsoni* was most recently cited in other parts of Enugu State, Anambra and Ondo States of Nigeria [26], [41] and some parts of West Africa [42]. Nsukka community had a greater species diversity compared to Obimo community. This can be attributed to high human activities such as, improper disposal of containers, presence of old tyres that harbour stagnant water and enhance the breeding and improper drainage system of the area, especially during rainy seasons. PCR findings of this study give further evidence about the presence of potential dengue vectors, *Aedes aegypti* and *Aedes albopictus* which are respectively, principal and secondary vectors of dengue fever viruses and several other arboviruses [4], [5]. Similar bands at ~550 bp and ~950 bp for *Aedes aegypti* and *Aedes albopictus* were respectively reported in other studies [43], [44]. This therefore indicates a high potential for maintenance and transmission of mosquito borne viruses in Nsukka LGA.

5 Conclusion

This study has shown that arbovirus vectors are well established throughout Nsukka LGA and a simple PCR assay can be used to discriminate dengue vectors. The presence of these potential dengue vectors and their close proximity to humans pose a high risk of arbovirus transmission in the area, therefore, continuous risk assessment should continue being in place to protect the general public. There is also need for immediate preventive measures including community health education in dealing with the main 'breeding containers' which hold water for a long time around homesteads and create the enabling environment for breeding mosquitoes. Integrated vector management can now target the identified key containers in applying larvicides. Designing universal primers that target all the entire *Aedes* species may be useful.

6 Declarations

6.1 Study Limitations

The gel documentation system (BioSens SC750, USA) at the laboratory faulted during the analysis and could not directly print out the results upon visualization. Gel results had to be taken by a mobile phone.

6.2 Acknowledgements

The authors appreciate the staff of National Arbovirus and Vectors Research Centre, Enugu, Federal Ministry of Health, Nigeria, who helped in the laboratory aspects of this work. HRHs the leaders of Obimo and Nsukka autonomous communities are also appreciated.

6.3 Funding Source

The authors are grateful to Malaria Consortium for the Dr Sylvia Meek Scholarship Fund that financially supported this study.

6.4 Informed Consent

HRHs the ccommunity heads of Obimo and Nsukka autonomous communities consented to this study. Further verbal consent was obtained from heads of households during sampling and installation of mosquito traps in their premises. For human landing catches, a modified method in which the collector, a Research Entomologist from the NAVRC was protected from mosquito bites, was used.

6.5 Competing Interests

The authors declared that no competing interests exist regarding the publication of this research.

How to Cite this Article:

A. Watentena, I. C. Okoye, I. E. Onah, O. C. Ogbonnaya, and E. Ogudu, "A Simple PCR Assay for Discrimination of Dengue Vectors from Nsukka LGA, Nigeria", *Int. Ann. Sci.*, vol. 10, no. 1, pp. 67-77, Oct. 2020. https://doi.org/10.21467/ias.10.1.67-77

References

[1] Malaria Consortium, "Vector control: the untapped potential for neglected tropical diseases," no. September. Leonard Street, London EC2A4LT, United Kingdom, pp. 1–8, 2017.

[2] J. A. Patz, A. K. Githeko, J. P. McCarty, S. Hussein, U. Confalonieri, and N. de Wet, "Climate change and infectious diseases," World Health Organization, 2003,

pp. 103–132.
[3] A. Watentena, I. Okoye, and I. Onah, "Dengue Reemergence: The Challenges Ahead," *Int. Ann. Sci.*, vol. 9, no. 1 SE-Review Article, Jun. 2020, doi: 10.21467/ias.9.1.132-140.
[4] B. N. Patrick *et al.*, "Distribution and diversity of mosquitoes and the role of Aedes in the transmission of arboviruses in selected districts of Tanzania," *Int. J. Mosq. Res.*, vol. 5, no. 1, pp. 53–60, 2018.
[5] L. Braack, A. P. Gouveia De Almeida, A. J. Cornel, R. Swanepoel, and C. De Jager, "Mosquito-borne arboviruses of African origin: Review of key viruses and vectors," *Parasites and Vectors*, vol. 11, no. 1, 2018, doi: 10.1186/s13071-017-2559-9.
[6] C. Y. Kow, L. L. Koon, and P. F. Yin, "Detection of dengue viruses in field caught male Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in Singapore by type-specific PCR," *J. Med. Entomol.*, vol. 38, no. 4, pp. 475–479, 2001, doi: 10.1603/0022-2585-38.4.475.
[7] J. A. Jackman and J. K. A. J. and J. K. Olson, "Mosquitoes and the diseases they transmit," *Agric. Commun. Texas A&M Univ. Syst. Ext. Publ.*, vol. B-6119, pp. 1–8, 2011, doi: 10.1007/s10531-005-2089-1.
[8] WHO/WMO, *Health and climate*, WMO-No. 10. 20 Avenue Appia, 1211 Geneva 27, Switzerland: WHO Press, 2012.
[9] M. Kashif Zahoor *et al.*, *Dengue fever: a general perspective*. IntechOpen, 2019.
[10] M. L. Phillips, "Dengue reborn," *Environ. Health Perspect.*, vol. 116, no. 9, pp. 382–388, 2008, doi: 10.1289/ehp.116-a382.
[11] World Health Organization, "Global strategy for dengue prevention and control 2012-2020," Geneva, 2012.
[12] M. S. A. Alshehri, "Dengue fever outburst and its relationship with climatic factors," *World Appl. Sci. J.*, vol. 22, no. 4, pp. 506–515, 2013, doi: 10.5829/idosi.wasj.2013.22.04.443.
[13] Global Malaria Programme, "Tailoring malaria interventions in the COVID-19 response," Geneva, 2020.
[14] G. Benelli, "Managing mosquitoes and ticks in a rapidly changing world – Facts and trends," *Saudi J. Biol. Sci.*, vol. 26, no. 5, pp. 921–929, 2019, doi: 10.1016/j.sjbs.2018.06.007.
[15] A. Amarasinghe, J. N. Kuritsky, G. William Letson, and H. S. Margolis, "Dengue virus infection in Africa," *Emerg. Infect. Dis.*, vol. 17, no. 8, pp. 1349–1354, 2011, doi: 10.3201/eid1708.101515.
[16] F. Were, "The dengue situation in Africa," *Paediatr. Int. Child Health*, vol. 32, no. Sup1, pp. 18–21, 2012, doi: 10.1179/2046904712Z.00000000048.
[17] J. Soghigian, T. G. Andreadis, and T. P. Livdahl, "From ground pools to treeholes: convergent evolution of habitat and phenotype in Aedes mosquitoes," *BMC Evol. Biol.*, vol. 17, no. 1, pp. 1–13, 2017, doi: 10.1186/s12862-017-1092-y.
[18] D. Guha-Sapir and B. Schimmer, "Dengue fever: New paradigms for a changing epidemiology," *Emerg. Themes Epidemiol.*, vol. 2, no. 1, pp. 1–10, 2005, doi: 10.1186/1742-7622-2-1.
[19] M. Q. Benedict, R. S. Levine, W. A. Hawley, and L. P. Lounibos, "Spread of the Tiger: global risk invasion by the mosquito Aedes albopictus," *Vector Borne Zoonotic Dis.*, vol. 7, no. 1, pp. 76–85, 2007, doi: 10.1055/s-0029-1237430.Imprinting.
[20] G. Rezza, "Aedes albopictus and the reemergence of dengue," *BMC Public Health*, vol. 12, no. 1, pp. 2–4, 2012, doi: 10.1186/1471-2458-12-72.
[21] WHO, "Dengue," 20 Avenue Appia, 1211 Geneva 27, Switzerland, 2014.
[22] K. J. Linthicum, P. G. Da Vies, and A. Kairo, "Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya," *J. Hyg. (Lond).*, vol. 95, no. 1, pp. 197–209, 1985, doi: 10.1017/S0022172400062434.
[23] B. J. Trewin *et al.*, "The elimination of the dengue vector, Aedes aegypti, from Brisbane, Australia: The role of surveillance, larval habitat removal and policy," *PLoS Negl. Trop. Dis.*, vol. 11, no. 8, pp. 1–23, 2017, doi: 10.1371/journal.pntd.0005848.
[24] G. E. Odo, E. J. Agwu, and A. S. Haruna, "Culicid forms distribution and breeding sites in Nsukka ecological zone of South Eastern Nigeria," *J. Parasitol. Vector Biol.*, vol. 7, no. 5, pp. 94–100, 2015, doi: 10.5897/JPVB2015.0193.
[25] M. N. Ezike *et al.*, "A preliminary study of mosquito vectors of human diseases and infection rates in Oru West, Imo State. Nigeria," *Glob. Res. J. Sci.*, vol. 5, no. 1, pp. 1–10, 2017.
[26] C. O. Chimaeze *et al.*, "Diversity and distribution of Aedes mosquitoes in Nigeria," *New York Sci. J.*, vol. 11, no. 2, 2018.
[27] S. B. Kumar, M. R. Prusty, A. Rath, S. K. Kar, and R. K. Hazra, "A simple, rapid and very efficient protocol for DNA isolation from mosquito species Sushanta." Regional Medical Research Centre, Nalco Square, Chandrasekharpur, Bhubaneswar 751023, India, pp. 3–8, 2010.
[28] K. S. Walter, J. E. Brown, and J. R. Powell, "Microhabitat partitioning of Aedes simpsoni (Diptera: Culicidae)," *J. Med. Entomol.*, vol. 51, no. 3, pp. 596–604, 2014, doi: 10.1603/me13097.
[29] N. Becker *et al.*, "Medical importance of mosquitoes," in *Mosquitoes and their control*, Springer Berlin Heidelberg, 2010, pp. 25–42.
[30] FRNOG, "Legal notice on publication of the details of the breakdown of the national and state provisional totals 2006 census," 2007. .
[31] Climate Data, "Climate of Nsukka," 2020. [Online]. Available: https://en.climate-data.org/location/404904/. [Accessed: 10-Jul-2020].
[32] J. D. Gillett, *Common African mosquitos and their medical importance*. William Heinemann, London, UK, 1972.
[33] E. H. Simpson, "Measurement of diversity," *Nature*, vol. 163, no. 4148, p. 688, 1949, doi: 10.1038/163688a0.
[34] S. C. Weaver and W. K. Reisen, "Present and future arboviral threats," *Antiviral Res.*, vol. 85, no. 2, pp. 328–345, 2010, doi: 10.1016/j.antiviral.2009.10.008.
[35] NCDC, "Response to yellow fever cases in Edo State," *Press Release*, 24-Nov-2018. [Online]. Available: https://ncdc.gov.ng/news/157/response-to-yellow-fever-cases-in-edo-state.
[36] A. B. Onoja, J. A. Adeniji, and O. D. Olaleye, "High rate of unrecognized dengue virus infection in parts of the rainforest region of Nigeria," *Acta Trop.*, vol. 160, pp. 39–43, 2016, doi: 10.1016/j.actatropica.2016.04.007.
[37] C. K. Ezihe *et al.*, "Mosquito species associated with

refuse dumps within Enugu Municipal, Enugu State, Nigeria," *Int. J. Mosq. Res.*, no. February, 2017, doi: 10.5376/jmr.2017.07.0006.

[38] D. N. Aribodor, O. B. Aribodor, E. C. I., and A. E. Onyido, "Survey of open larval habitats of mosquitoes in four communities in Awka South Local Government Area, Anambra State, Nigeria," *Biosci.*, vol. 1, no. 2, pp. 132–139, 2013.

[39] O. A. E. *et al.*, "Anthropophilic mosquito species prevalence in Nibo Community, Awka South Local Government Area, Anambra State, Southeastern Nigeria," *Ewemen J. Epidemiol. Clin. Med.*, vol. 2, no. 1, pp. 14–20, 2016.

[40] G. R. A. Okogun, J. C. Anosike, A. N. Okere, and B. E. B. Nwoke, "Ecology of mosquitoes of Midwestern Nigeria," *J. Vector Borne Dis.*, vol. 42, no. 1, pp. 1–8, 2005.

[41] U. C. Chukwuekezie, O. C. Nwangwu *et al.*, "A Cross sectional survey of yellow fever and dengue virus vectors in four communities of Ayamelum Local Government Area (LGA), Anambra State, Southeast Nigeria," *New York Sci. J.*, vol. 9, no. 3, 2018, doi: 10.7537/marsnys09031616.

[42] K. L. Bennett *et al.*, "Molecular differentiation of the African yellow fever vector Aedes bromeliae (Diptera: Culicidae) from its sympatric non-vector sister species, Aedes lilii," *PLoS Negl. Trop. Dis.*, vol. 9, no. 12, pp. 1–19, 2015, doi: 10.1371/journal.pntd.0004250.

[43] Y. Higa, T. Toma, Y. Tsuda, and I. Miyagi, "A multiplex PCR-based molecular identification of five morphologically related, medically important subgenus stegomyia mosquitoes from the genus Aedes (Diptera: Culicidae) found in the Ryukyu Archipelago, Japan," *Jpn. J. Infect. Dis.*, vol. 63, no. 5, pp. 312–316, 2010.

[44] H. Iwashita *et al.*, "Mosquito arbovirus survey in selected areas of Kenya: detection of insect-specific virus," *Trop. Med. Health*, vol. 46, no. 1, pp. 1–15, 2018, doi: 10.1186/s41182-018-0095-8.