Biotechnology

Molecular characterization of *Anopheles gambiae* complex in communities affected by flood in Anambra East L.G.A, Southeastern Nigeria

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Abstract

Malaria is a serious public health disease caused by Plasmodium parasites transmitted by infected female *Anopheles* mosquitoes. This study was conducted between 2020 and 2023 to determine the molecular characterization of *Anopheles gambiae* complex in Anambra East L.GA. The specific objectives were to identify the sibling species of *An. gambiae* complex, determine their indoor resting density, man-biting rate, physiological states, and the distribution of the S (Savannah) and M (Mopti) molecular forms within the *An. gambiae sensu stricto (s.s).* in the area. Indoor biting and resting mosquitoes were collected using pyrethroid-based insecticide knock down (PKD). Indoor density and man biting rates were calculated using standard formulas. The sibling species were identified using polymerase chain reaction (PCR). *An. gambiae s.s* M and S forms were identified through Restriction Fragment Length Polymorphism (RFLP). Data obtained was analyzed using descriptive statistics. A total of 893 *An. gambiae* complex mosquitoes were collected. The indoor density of *An. gambiae* complex was calculated as 0.661 mosquitoes/room/night while man biting rate was 0.154 bite/man/night. Of the 893 *An. gambiae* complex collected indoors, 186 (20.83%) were unfed, 417 (46.70%) freshly fed, 212 (23.74%) half gravid while 78 (8.73%) were gravid. Of a total of 300 *An. gambiae* complex randomly selected and subjected to PCR, 278 (92.67%) were identified as *An. gambiae* s.s. while 22 (7.33%) were unidentified. All the *An. gambiae s.s* were identified as S forms. A total of 142 (51.10%) were from Aguleri while 136 (48.92%) were from Umuoba Anam. The finding of this study shows that *An. gambiae s.s* was the major malaria vector in the area. Integrated vector management targeted toward the vector species is recommended.

Keywords: malaria, vectors, *Anopheles gambiae*, molecular characterization, anambra east

Introduction

Malaria is a serious public health disease caused by Plasmodium parasites, transmitted by infected female *Anopheles* mosquito that feed on human blood (WHO, 2020) [27]. *Anopheles gambiae s.l.* is the main vector of malaria parasite in Africa. The vector breeds in stagnant water bodies around human habitations (Egbuche *et al* 2020; Onyido *et al*, 2016) [9, 24]. Many communities in the riverine areas in Nigeria were submerged by flood during the last quarter of the year 2022 (Leadership, 2022) [20]. As the flood abate, shallow stagnant water bodies formed in different parts of the communities and remained for several months due to saturation of the soil. This resulted in increased mosquitoes breeding and biting, exposing more people to the malaria disease due to increased human-vector-contact. This development is an important epidemiological factor in malaria transmission (Konlan *et al*., 2019; Oladepo *et al*., 2019) [18, 22].

As part of malaria vector control, use of Long-Lasting Insecticidal Nets (LLINs), Indoor Residual Spraying (IRS) and other insecticide products were recommended by World Health Organization (WHO, 2018) [29]. At present the LLINs, IRS and application of insecticides are widely in use (Heming *et al*., 2016) [14]. However, rapidly developing insecticide-resistant vectors to the available insecticides is jeopardizing the effectiveness of these strategies (WHO, 2016) [28]. Using these control tools requires proper identification of mosquito species in an area for accurate choice of insecticide to use. This is important because of numerous reports of insecticide resistance with mosquitoes to specific classes of insecticides (WHO, 2020; Chukwuekezie *et al*., 2020) [27, 3].

Some mosquito vectors are species complexes that are difficult to separate morphologically. For example, the main malaria vector, *An. gambiae* complex contain about seven or more sibling species that are morphologically indistinguishable (Ikpo *et al*, 2021; Irikannu *et al*, 2019; Coetzee, 2004; Coetzee *et al*., 2000) [15, 16, 4, 3]. Members of the complex show marked differences in their spatial distribution in Africa (Dzorgbe *et al*., 2017; Mattah *et al*., 2017; Labbo *et al*., 2016; Ebenezar *et al*., 2016) [6, 21, 19, 8]. The differences in their spatial distribution can also translate to differences in their vectoral efficiency, hence the need for accurate identification of members of *An. gambiae* complex in an area. The specific objectives of this study were to identify the sibling species of *An. gambiae* complex mosquitoes, determine their indoor resting density, man-biting rate, and the distribution of the S (Savannah) and M (Mopti) molecular forms within the *An. gambiae sensu*

stricto (s.s) in Anambra East L.G.A, Anambra State.

Materials and methods

Study area

This study was conducted in Anambra East Local Government Area (L.G.A), Anambra State, South-eastern Nigeria. The L.G.A is located between coordinates 6°15'46''N and 6°48'50''E. The area experience two seasons in a year-the dry seasons (November-March) and wet season (April-October). The L.G.A is made up of several communities which includes; Otuocha, Aguleri, Umuleri, Igbariam, Nsugbe, Nando and Umuoba Anam amongst others. The inhabitants of the area are largely of Igbo ethnic group, and mainly engage in trading, farming and fishing as their occupation, although a few are civil servants.

Advocacy visits

Advocacy visits were made to the communities before the commencement of the study. A letter of introduction obtained from the Head of Department of Parasitology and Entomology, Nnamdi Azikiwe University, Awka was presented to community leaders to obtain their permission to carry out the study in their communities. The study was conducted between 2020 and 2023.

Collection of indoor biting and resting mosquitoes

Indoor-biting and resting mosquitoes were collected from the communities using pyrethroid-based insecticide knock down (PKD) method (Onyido *et al*., 2016) [24]. The adult mosquitoes were collected from living rooms where people slept the previous night. Head count of selected household was done and the number of persons that slept in each room was recorded. In each room, the doors and windows were shut and a large white spread sheets laid on the floor. A pyrethroid-based insecticide aerosol (Raid®) was sprayed in the room and allowed to remain for 20 minutes before collection. At the end, the knocked down mosquitoes were collected from the white sheet using a pair of entomological forceps into a petri dish.

Physiological state of the *Anopheles gambiae* complex mosquitoes

The abdomen of all *An. gambiae* complex mosquitoes collected indoors were closely observed in other to identify those that were unfed, freshly fed, half gravid and gravid (Service, 1985) [26].

Indoor resting density of the *Anopheles gambiae* complex mosquitoes

The indoor resting density of the mosquitoes was calculated from the result of PKD as described by Ezihe *et al*., 2017 [10]. The room density was determined by the number of *An. gambiae* complex mosquitoes collected, divided by the total number of rooms sampled and the total number of nights the mosquitoes were collected. It is calculated thus as;

Indoor Resting Density (D) = (number of *Anopheles* females ÷ number of rooms) ÷ number of nights. The results were expressed as number of mosquitoes/room/night.

Man biting rate of the *Anopheles gambiae* complex mosquitoes

Man-biting rate was expressed as the number of bites a person receives from a specific vector species per night. This was determined from PKD collections as the total number of freshly fed *An. gambiae* complex females collected, divided by the total number of occupants who spent the night in the rooms, and the total number of nights of collection (Ezihe *et al*., 2017) [10]. It is calculated thus;

Man-biting rate (Mbr) = (number of freshly fed females ÷ total number of occupants) ÷ total number of nights. The results were expressed as mosquito bites/man/night.

Morphological identification of the mosquitoes

At the end of each collection period, all the mosquitoes collected were properly labeled and sent to the Entomology Laboratory of the Department of Parasitology and Entomology, Nnamdi Azikiwe University for proper identification of *An. gambiae* complex mosquitoes using morphological keys (Gillies, and Coetzee, 1987; Gillet, 1972) [13, 12].

Polymerase chain reaction assay

All *An. gambiae* complex mosquitoes identified morphologically were preserved in Eppendorf tubes using silica gel as preservative. The tubes were transported to the Laboratory of Nigerian Institute of Medical Research, Yaba, Lagos State, where the Polymerase Chain Reaction (PCR) assay was conducted following the method described by Scott *et al*. (1993) [25].

DNA extraction

The severed wings and legs of the mosquitoes were introduced into centrifuge tubes for Deoxyribonucleic acid (DNA) extraction. The DNA was extracted using Blood-Animal-Plant DNA preparation Kit manufactured by Jena Bioscience, Germany. The extraction was done by adding the severed specimens to a ZR Bashing Bead lysis tube. Then 750µl lysis solution was added. The set-up was fixed in a bead beater fitted with a 2ml tube holder and was processed at maximum speed for 10 minutes. The ZR Bashing Bead lysis tube was centrifuged at ≥10,000rpm for 1 minute and 400µl of the supernatant. It was transferred to Zymo-Spin IV Spin Filter (orange top) in a collection tube, centrifuged at 7,000rpm for 1 minute, 1200µl of Genome Lysis Buffer was then added to the filtrate in the collection tube and mixed. Eight hundred microlitres (800µl) of the mixture was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000rpm for 1 minute. The flow through the collection tube was discarded and the previous process was repeated. Two hundred microlitres (200µl) of DNA Pre-Wash Buffer was introduced to the Zymo-Spin IIC column in a new collection tube and was centrifuged at 10,000rpm for 1 minute. Five hundred microlitres (500µl) g-DNA Wash Buffer was added to the Zymo-Spin IIC column and centrifuged at 10,000rpm for 1 minute. The Zymo-Spin IIC column was transferred to a clean 1.5ml microcentrifuge tube and 50µl DNA Elution Buffer was

added directly to the column matrix. It was then centrifuged at 10,000rpm for 30 seconds and the DNA was eluted.

Master mix

A master mix for *An. gambiae* complex mosquitoes was prepared by mixing the primers and other reagents in the order listed in Table 1. Sequence of primers are also shown (Table 2).

Table 1: Master Mix for *Anopheles gambiae* complex

Reagent	XI (µl)
Pre-mix	40
ddH_2O	5.25
ME	0.5
AR	0.5
GA	0.5
UN	0.5
QD	0.25
DNA	1.0
Total	12.5

Table 2: The sequence of primers used in the polymerase chain reaction

Mosquito	Primers	Sequence (5' to 3')
An. gambiae complex	ME	TGACCAACCCACTCCCTTGA
	AR	AAGTGTCCTTCTCCATCCTA
	QD	CAGACCAAGATGGTTAGTAT
	UN	GTGTGCCCCTTCCTCGATGT
	GA	CTGGTTTGGTCGGCACGTTT

Twelve and half microlitres (12.5 µl) of PCR master mix of each mosquito was introduced into two hundred microlitres (200µl) tube. One microlitre (1µl) of DNA was added into each tube. Each of the tube was loaded in the PCR machine and an appropriate programme and PCR condition was selected on the machine. The PCR conditions for *An. gambiae* complex selected were; Initial Denaturation @ 95ºC – 2 mins, Denaturation @ 95ºC – 30sec, Annealing @ 55ºC – 30sec, Extension @ 72ºC – 40sec, Final extension @ 72ºC –7mins. They ran for 30 cycles.

Preparation agarose gel

Agarose gel (1.5%) was prepared by weighing 1.5g of agarose powder in 100ml of Trisborate ethylene-di-amino tetraacetic acid (TBE) buffer. This was boiled in microwave until the solution was clear. It was allowed to cool for few minutes until no steam was observed. Ten microlitres (10µl) of ethidium bromide was added. The gel was poured into a trough and allowed to solidify. Then, 10µl of DNA ladder, positive and negative control, and the PCR product were added into the well for electrophoresis. The gel was viewed using gel documentation machine.

Interpretation of gel bands

The gel picture was taken under UV light using gel documentation machine and was read using the molecular weights of the *An. gambiae* sibling species. The molecular weight band size of the expected sibling species was; *An. gambiae s.s.* 390bp, *An. arabiensis* 315bp, *An. melas* 464bp, and *An. quadriannulatus* 153bp.

Restriction fragment length polymorphism on *Anopheles gambiae s.s.*

Restriction fragment length polymorphism (RFLP) was done by placing 16ul of PCR product into a clean 1.5ml micro-centrifuge tube and adding 2ul of 10x restriction buffer to the tube. A measure 1ul of Hahl (20 units) restriction enzyme was added to the same tube. It was thoroughly mixed and centrifuge for 10 seconds to obtain the mixture at the bottom of the tube. This was incubated at 37 ºC for 3 hours. The digest product was run on 2% agarose gel at 120 volts for 1 hour 30 minutes to determine the different size patterns (Fanello *et al*., 2002) [11].

Data analysis

Data obtained from this study was analyzed using descriptive statistics, tables, and bar charts.

Results

A total of 893 *An. gambiae* complex mosquitoes were collected in the study. The indoor density of *An. gambiae* complex was calculated as 0.661 mosquitoes/room/night while man biting rate was 0.154 bite/man/night in the study area (Table 3).

Table 3: Indoor Resting Density and Man-biting rate of *Anopheles gambiae* complex collected in Anambra East L.G.A, Anambra State

Mosquito species collected indoors	No. of females collected	No. freshly fed females	Indoor Resting Density (D) (No. of mosquitoes /room/night)	Man Biting Rate (MBR) (No. of bite/man/night)
Anopheles gambiae complex	893	417	0.661	0.154

The physiological states of the indoor *An. gambiae* complex mosquitoes were determined. Of the 893 the adult mosquitoes collected indoors, 186 (20.83%) were unfed, 417 (46.70%) freshly fed, 212 (23.74%) half gravid while 78 (8.73%) were gravid (Fig. 1).

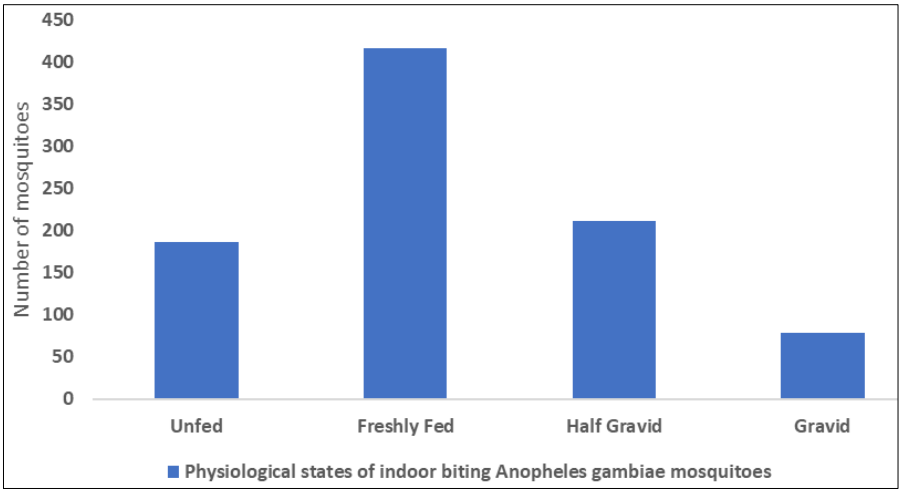

Fig 1: Physiological states of indoor biting *Anopheles gambiae* complex mosquitoes in the study area

A total of 300 *An. gambiae* complex mosquitoes were randomly selected and subjected to PCR. A greater number, 278 (92.67%) were identified as *An. gambiae s.s*. while 22 (7.33%) were unidentified (Fig. 2).

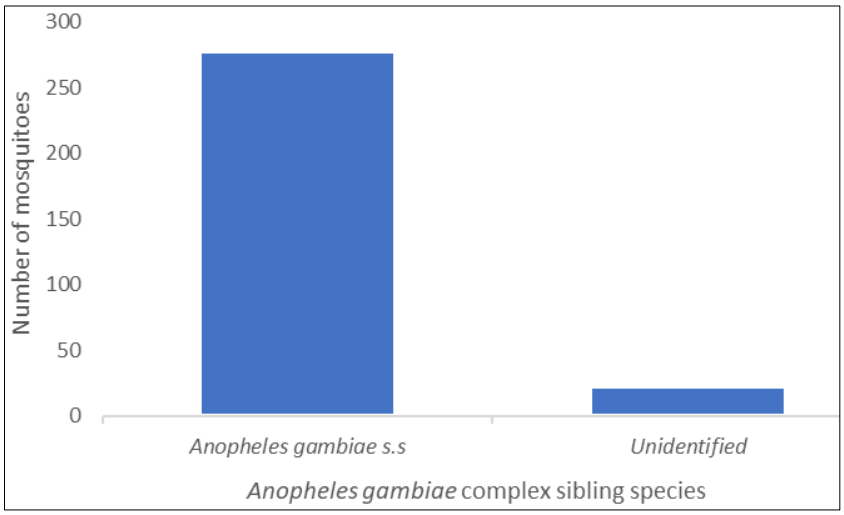

Fig 2: Percentage distribution of identified and unidentified mosquito species in the study area

The RFLP was conducted on the 278(92.67%) identified *An. gambiae s.s.* All the *An. gambiae s.s* were identified as *An. gambiae* S forms. A total of 142 (51.10%) were from Aguleri while 136 (48.92%) were from Umuoba Anam. (Table 4).

Table 4: Distribution molecular forms of *Anopheles gambiae s.s.* in the study area

Anopheles gambiae molecular forms	Aguleri	Umuoba Anam	Total (%)
An. gambiae S form	142	136	278 (100.0)
An. gambiae M form	0	0	0 (0.00)
Total	142 (51.10)	136 (48.92)	278 (100.0%)

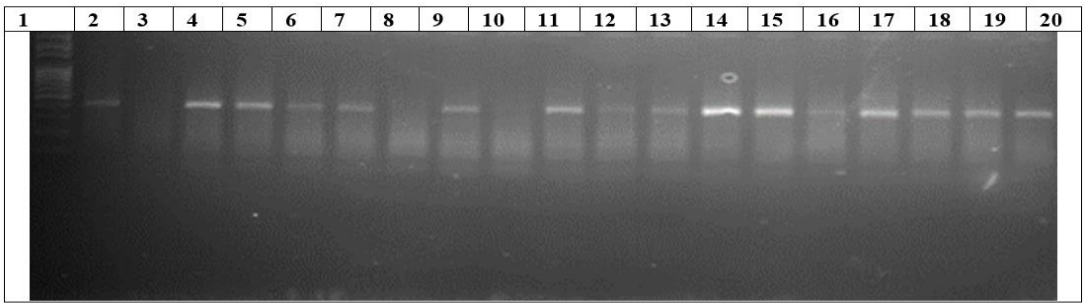

Fig 3: *Anopheles gambiae* complex fragments amplified by PCR: Well 1 represents 100 bp DNA ladder. Well 2 is a positive control. Well 3 is the negative control. Well 4-7,9,11-20 are *An. gambiae s.s* at 390bp. Well 8 and 10 are unamplified.

Discussion

In this study, *An. gambiae s.s* was the only sibling species of the *An. gambiae* complex that was recorded in the study area. Similar results have been recorded in some studies conducted in Awka Anambra State, and the three senatorial districts in Enugu State, both in Southeastern Nigeria, where *An. gambiae* s.s was the only sibling species reported (Ikpo *et al*. 2021; Irikannu *et al*., 2019) [15, 16]. Also, *An. gambiae s.s* was reported in other studies in Akure, Southwestern Nigeria (Akeju *et al*., 2022) [2] and in Kamuli District, Uganda (Kabbale, 2016) [17]. In this study, only S molecular form of *An. gambiae s.s* was recorded. Ikpo *et al*. (2021) [15] made same observation in Enugu, Nigeria. Onyabe *et al.* (2003) [23] earlier reported that S molecular form of *An. gambiae s.s.* had a wider distribution across Nigeria compared to the M form. But both molecular forms occur throughout the country with no apparent relationship to the ecological transition throughout the country (Onyabe *et al*., 2003) [23]. These findings pinpoint the S molecular form of *An. gambiae* s.s. as the major vector of malaria parasites in the study area.

This study revealed that 46.70% of the *An. gambiae* complex mosquitoes were freshly fed. The observation was lower than Abubakar *et al.* (2023) [1], who reported 75.25% and Ezihe *et al*. (2017) [10] who recorded 74.4% of freshly fed *An. gambiae* complex in Dutse and Enugu, Nigeria respectively. Ebenezer *et al*. (2013) [7] reported a much higher value, 78.3% in Bayelsa, while Irikannu *et al* (2019) [16] recorded just 8.4% freshly fed in Awka, Nigeria. The indoor density of *An. gambiae* complex was 0.661 mosquitoes/room/night, while man biting rate was 0.154 bite/man/night. The observations compare favourably to another study in Enugu where *An. gambiae* complex had a room density of 0.66 mosquitoes/room/night and a man biting rate of 3.9 mosquitoes/man/night (Ezihe *et al*., 2017) [10]. But the values were far below a study in Bayelsa State, where man-biting rate was 8.7 bites/man/night and room density, 20.5 mosquitoes/room/night (Ebenezer *et al*., 2013) [7]. On the contrary, another study in Awka recorded a room density of 0.30 mosquitoes/room/night and man biting rate of 0.017 bites/man/night by the same malaria vector species. The current observation on the mosquito species feeding pattern and indoor density confirms that the malaria vector is biting indiscriminately in the study area, which is an important epidemiological factor in transmission of malaria parasites from human to human.

Conclusion

The findings of this study show that *An. gambiae s.s* was the major malaria vector in the area. Most of the *An. gambiae* complex mosquitoes collected indoor were freshly fed, indicating the high level of human-vector contact occurring in the communities. This may lead to continued malaria endemicity in the area if left unchecked. Knowing which malaria vector species exists in a particular location, and understanding their insecticide susceptibility status lead to cost effective vector control programmes. Therefore, a vector management approach, targeted toward *An. gambiae* s.s. is recommended in the study area. This should involve chemical and biological vector control methods, accompanied with health education to the inhabitants of the riverine communities, on how to rid their environment of mosquito breeding sites.

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