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Molecular characterization and malaria transmission potential of *Anopheles gambiae* complex in Awka, Anambra state, Nigeria

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Abstract

The molecular characterisation and malaria transmission potential of *Anopheles gambiae* complex mosquitoes in Awka, Anambra State were studied from June to September, 2018. Indoor and outdoor biting mosquitoes were collected by human bait and pyrethrum knockdown methods. Sporozoite index was determined with Enzyme-linked Immunosorbent Assays (ELISA). Molecular characterization of sibling species of *An. gambiae* complex was done with Polymerase Chain Reaction (PCR). A total of 211 *An. gambiae* complex mosquitoes were collected among which 80(38.0%) were outdoor biting adults while 131(62.0%) were indoor biting adults. There was a significant difference in the number of *An. gambiae* mosquitoes collected indoors and outdoors ($P < 0.05$, P value = 0.000). Out of 150 *An. gambiae* complex mosquitoes randomly selected, 124(82.67%) were *An. gambiae*s while 26(17.33%) could not be identified. Only 5(10%) out of 50 randomly selected were positive for sporozoites. These observations indicate that malaria transmission in Awka is mainly by *An. gambiae*s.

Keywords: Molecular. *Anopheles*, vectors, malaria, mosquitoes, sporozoites

1. Introduction

Mosquitoes are group of insects, which transmit many dreadful diseases causing serious health problems to humans. Mosquitoes serve as vectors to diseases such as malaria, filariasis and yellow fever, which affect hundreds of millions of people every year [1]. The *Anopheles gambiae* complex mosquitoes are important vectors of human malaria. A report has shown that they cause serious biting annoyance, noise nuisance, sleeplessness, allergic reactions and disease transmission through their bites on their victims [2].

An. gambiae complex mosquitoes are difficult to separate morphologically. The complex is made up of at least seven sibling species that look alike morphologically. The close resemblance among the members of the *An. gambiae* complex mosquitoes makes it very difficult even for trained eyes to identify and separate them under the microscope. The reported sibling species include *An. gambiae sensu stricto* (s.s.), *An. arabiensis*, *An. quadriannulatus* species A and *An. quadriannulatus* species B [3, 4]. Others are *An. melas* and *An. merus* [5, 6] and *An. bwambae* [7]. *An. arabiensis* and *An. gambiae sensu stricto* (s.s.) were reported to be the most common species. *An. arabiensis* and *An. melas* has been observed to transmit malaria parasites in the Savannah-forest area and around lagoons respectively [8, 9].

The marked differences in the vectorial efficiency of the species within the complex mean that correct identification is vital for focused effort in their disease control programmes. Identification has relied mainly on morphological technique which has limited application on the identification of sibling species. It is therefore pertinent that molecular characterization of the *An. gambiae* complex species in the study area should be done to provide baseline data on which members of the complex actively transmit malaria in the area so as to plan appropriate control strategies for the species. The objectives of the studies were to determine the abundance, molecular characterisation and malaria sporozoite rates of *An. gambiae* complex mosquitoes.

2. Materials and Methods

2.1 Description of Study Area

Awka is situated between Longitude 7° 04'E and Latitude 6°10'N. Awka is in the tropical rainforest zone of Nigeria and it experiences two distinct seasons in a year. Eight months of heavy tropical rains between March and October and is followed by four months of dryness (November - February). There is also a short spell of cold dry period (Harmattan) at the beginning of the dry season. The temperature range in Awka is generally 27-30 °C between June and December but rises to 32-34 °C between January and April, with the last few months of the dry season marked by intense heat. The relative humidity of the area is about 70% in the dry season reaching 80% during the wet season. The annual rainfall is between 2000-3000mm. The community is about 150m above sea level. Awka has a population of 189,049 inhabitants^[10]. The inhabitants of the area are mainly farmers. Others are civil servants, traders, and blacksmiths. The agricultural produce of the area include cassava, yam, maize, palm produce, plantain and coconuts.

2.2 Study Design

The study was a laboratory based molecular characterization and a field survey of the biting and resting habits of the adult *Anopheles* mosquitoes. The study was conducted from June to September, 2018.

2.3 Advocacy visits and Community Sensitisation

Advocacy visits was made with an introductory letter from the Head of the Department of Parasitology and Entomology to the traditional ruler and the opinion leaders of the community for permission to carry out the project in their domain. The community members were also sensitized through meetings organized with the aid of their community leaders. The project intent and their significance were explained to them and their consent was obtained for the use of their environment for the study.

2.4 Collection of Adult Mosquitoes

Mosquitoes were collected through the assistance of volunteer workers. The volunteer workers were properly instructed on the techniques of mosquito collections. Each volunteer worker was vaccinated with yellow fever vaccine for at least 10 days before the study. Also, all their health matters were taken care of during the study. Mosquito collected was subdivided into outdoor and indoor biting mosquitoes collection.

2.4.1 Collection of Outdoor-biting Mosquitoes

Outdoor-biting mosquitoes were collected using human-bait collection method (HBC). The collections were done all-night from 6.00pm-6.00am (local time). The collections were done in selected premises of inhabited houses in the community. The volunteer collectors sat on kitchen stools, exposed their legs and hands for mosquito bites by rolling up their trousers and shirt sleeves to knee and elbow levels respectively. Mosquitoes alighting on the volunteers were collected with the aid of test tube vials and torchlight. On collection of each mosquito, the vial was quickly covered with a ball of cotton wool to avoid escape of the mosquito. The time of collection of each mosquito was properly recorded. Mosquito collections were collated at 15 minutes interval.

2.4.2 Collection of Indoor-biting and Resting Adult Mosquitoes

Indoor-biting and resting adult mosquitoes were collected from the communities using pyrethroid-based insecticide knock down (PKD) method. The adult mosquitoes were collected from living rooms where people slept the previous night. Head count of each selected household was done and the number of persons that slept in each room was noted. In each room, the doors and windows were shut and white spread sheets laid from wall to wall, covering furniture and other non-movable items in the rooms. A pyrethroid-based insecticide aerosol (Baygon®) was sprayed in the room and allowed to remain for 20 minutes before collection. Cracks or any observed escape routes from the walls, doors and windows were closed with old newspapers to prevent escape of mosquitoes through them. At the end of the time interval after spraying, the white spread sheets were folded and were taken outside the room. The knocked down mosquitoes were collected using a pair of entomological forceps into a damped petri dish.

2.4.3 Determination of Physiological State of the Mosquitoes Collected Indoors

The physiological states of female mosquitoes collected indoors were determined in order to observe mosquitoes that had blood meal and those that had not fed. The mosquitoes were grouped into four categories; unfed, freshly fed, half gravid and gravid^[11].

2.4.4 Determination of Indoor Resting Density of Mosquitoes

The indoor resting density of the mosquitoes in the study area was calculated from the result of PKD^[12]. The room density was determined by the number of *Anopheles* mosquitoes collected divided by the total number of rooms sampled and the total number of night 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.

2.4.5 Determination of Man biting Rate of Mosquitoes Collected

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 *Anopheles* females of a species collected divided by the total number of occupants who spent the night in the rooms and then the total number of nights that were used for the collection^[12]. 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.

2.5 Morphological Identification

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 identification. The morphological identifications were later confirmed at the Laboratory of National Arbovirus and Vectors Research Centre, Enugu. The mosquitoes were identified using the gross morphology of the species especially the body colour,

patches of scales on the palps, antennae, proboscis, patches of pale and black scales on the wings and legs and the terminal abdominal segments using standard keys [13, 14].

2.6 Preservation of Mosquitoes for Molecular Characterisation

After morphological identification, all the outdoor and indoor biting adult *Anopheles* mosquitoes belonging to *An. gambiae* complex were preserved in Ependorf tubes for molecular studies. An Ependorf tube was used to preserve a single adult mosquito. Each Ependorf tube was 70% filled with silica gel in its solid form. The silica gel served as a preservative to prevent the mosquito from decaying during transportation. A ball of cotton wool was placed in the tube to separate the preservative from the adult mosquito. Each adult mosquito was placed on the cotton ball and the Ependorf tube was covered. All the tubes containing the preserved mosquitoes were transported to the Laboratory of Nigerian Institute of Medical Research, Yaba, Lagos State, for Polymerase Chain Reaction (PCR) studies.

2.7 Molecular Identification of Sibling Species of *Anopheles gambiae* complex Using Polymerase Chain Reaction (PCR)

2.7.1 Step 1: DNA Extraction

The wings and legs of each mosquito were severed using a scalpel and were put 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 to the tube. The set-up was secured in a bead beater fitted with a 2ml tube holder assembly and was processed at maximum speed for 10 minutes. The ZR Bashing Bead lysis tube was centrifuged at $\geq 10,000$ rpm for 1 minute and 400µl of the supernatant 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 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 added 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.

2.7.2 Step 2: Preparation of 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. The total mixture obtained is called the master mix which was used for PCR in step 3.

Table 1: Master Mix for *An. gambiae* complex

Mosquitoes	Reagent	X1 (µl)
<i>An. gambiae</i> complex	Pre-mix	4.0
	ddH ₂ O	5.25
	ME	0.5
	AR	0.5
	GA	0.5
	UN	0.5
	OD	0.25
	DNA	1.0
	Total	12.5

2.7.3 Step 3: PCR Procedure for *An. gambiae* complex

Twelve and half microlitres (12.5 µl) of PCR master mix of each adult mosquito was added into each of the 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 chosen on the machine. The PCR conditions for *An. gambiae* complex chosen were; Initial Denaturation @ 95°C – 2 mins, Denaturation @ 95°C – 30sec, Annealing @ 55°C – 30sec, Extension @ 72°C – 40sec, Final extension @ 72°C – 7mins. All the conditions were set to run for 30 cycles. After subjecting to polymerase chain reaction, 1.5% agarose gel was prepared by weighing 1.5g of agarose powder in 100ml of Trisborate ethylene-di-amino tetraacetic acid (TBE) buffer and boiled in microwave until the solution was clear. This was brought out and allowed to cool for few minutes until no steam was observed and 10µl of ethidium bromide was added. The gel was poured into a trough and allowed to solidify. Ten (10µl) of the microlitres µl of DNA ladder, negative control and PCR product was then added into each well for electrophoresis and gel was viewed using gel documentation machine.

2.7.4 Step 4: 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 as shown in table 2 below.

Table 2: DNA ladder showing the molecular weights of sibling species of *An. Gambiae* complex

Anopheles mosquitoes Molecular weights	
<i>Anopheles merus</i>	464 base pair
<i>Anopheles gambiae</i>	390 base pair
<i>Anopheles arabiensis</i>	315 base pair
<i>Anopheles quadriannulatus</i>	153 base pair

2.8 Determination of the Sporozoite Rate of Female *Anopheles gambiae* complex Collected Indoors

The Sporozoite index of the *Anopheles* mosquitoes was determined with Enzyme-linked Immunosorbent Assays (ELISA) in the Laboratory of Nigerian Institute of Medical Research, Yaba, Lagos State. The head and thorax of each of the mosquito samples were cut off using entomological dissecting blade. Each head and thorax of the mosquito was placed in labeled 1.5ml micro-centrifuge grinding tubes. Fifty microlitres (50µl) of grinding buffer was added into each centrifuge tube containing either the head or the thorax and properly grounded. After grinding, the grinding pestles were rinsed with 100µl of the grinding solution into the tubes

containing the mosquito triturates. The assay plates were coated with fifty microlitres (50µl) capture of monoclonal antibodies (mAb) and was incubated for 30 minutes. Using an aspirator, the wells of the assay plates were filled with 200µl of blocking buffer (BB) and again incubated for 1 hour. Fifty microlitres (50µl) of mosquito triturate and positive control were added to the wells and incubated for another 2 hours. The wells were washed twice using 200µl of phosphate buffered saline (PBS-0.05% Tween 20), 50µl peroxidase-monoclonal Antibodies (mixed 1:1) was added, incubated in the dark for 1 hour and was washed three times with phosphate buffered saline (PBS-0.05% Tween 20). Then 100µl of substrate was added and absorbence read using its characteristics colour change.

2.9 Data Analysis

Data collected from the study were analysed using the Statistical Package for Social Sciences (SPSS) version 2.10. Analysis of variance (ANOVA) at 5% significant level was used to test the differences between indoor and outdoor *An. gambiae* populations.

3. Results

A total of 211 adult *An. gambiae* mosquitoes were collected from the study. Of this number 80(38%) were collected outdoors while 131(62%) were collected indoors. There was a significant difference in the number of *An. gambiae* mosquitoes collected indoors and outdoors. (P<0.05), (P value=0.000). Of the 131(62%) mosquitoes collected indoors 4(3.0%) were unfed, 11(8.4%) were freshly fed, 87(66.4%) were half gravid and 29(22.1%) were gravid (Fig 1). Room density and man-biting rates of the mosquitoes were 0.30 mosquitoes/room/night and a man-biting rate of 0.017 bites/man/night (Table 3).The gel image of *Anopheles gambiae* complex is also shown below (Fig 2).

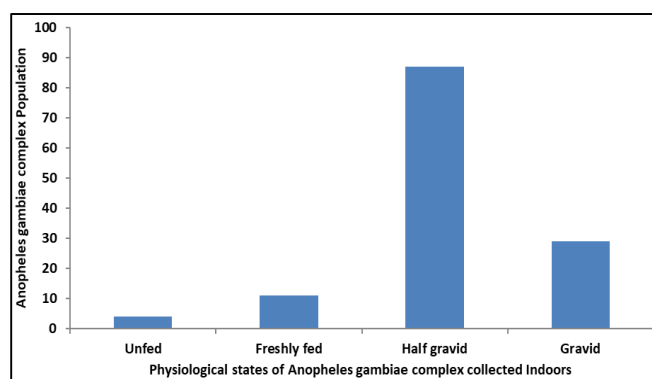


Fig 1: Abdominal Grading of Indoor-biting Adult Mosquitoes Collected using Pyrethroid Knock down (PKC) to determine their Physiological State

Table 3: Indoor Resting Density and Man-biting rate of *Anopheles gambiae* mosquitoes collected at Awka, Anambra State.

Mosquitoes species collected indoors	No. of females collected	No. freshly fed females	Indoor Resting Density (IRD) No. of mosquitoes /room/night	Man Biting Rate (MBR) No. of bite/man/night
<i>An. gambiae</i>	131	11	0.30	0.017

Of the 150 *An. gambiae* complex mosquitoes that were subjected to PCR, 124(82.67%) were amplified and identified

while 26(17.33%) were unamplified and could not be identified. All the amplified, 124(82.67%), were identified as *An. gambiae.s.* (Fig 3).

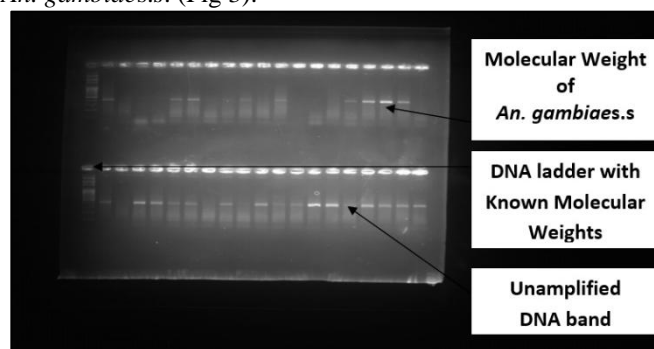


Fig 2: Gel image of *Anopheles gambiae* complex

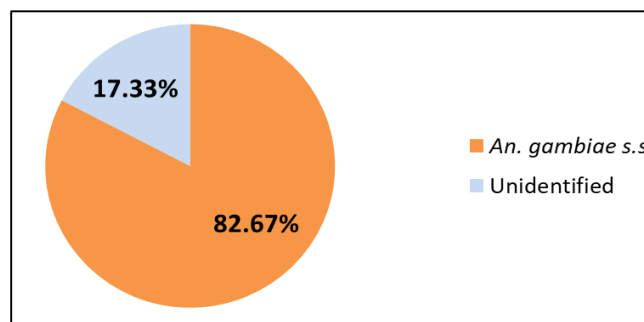


Fig 3: Percentage distribution of the identified and unidentified *Anopheles gambiae* complex mosquitoes

Of the 50 bloodfed *An. gambiae* mosquitoes subjected to Enzyme-linked immunosorbent assays (ELISA) for analysis of sporozoite rate, only 5(10.0%) were positive for sporozoites and 45(90.0%) were negative (Fig 4).

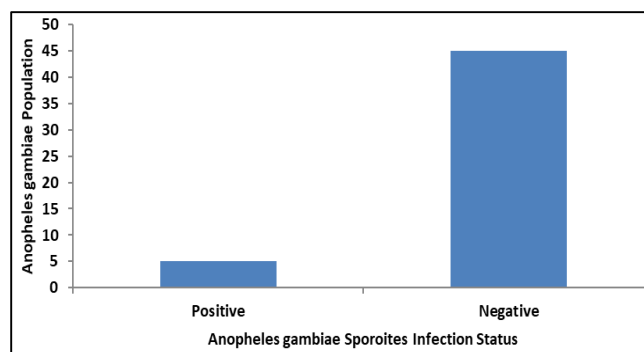


Fig 4: Malaria sporozoites rate of the bloodfed *Anopheles gambiae* by Enzyme-linked Immunosorbent Assay (ELISA)

4. Discussion

Of the 211 *An. gambiae* mosquitoes collected in the study, a greater number 131 were collected indoors, and 127 (97%) of the indoor collections were either freshly fed, half gravid or gravid at the time of collection. High number of bloodfed *An. gambiae* mosquitoes has also been reported in other studies. Ezihe *et al.* [12] Observed that 398 (74.4%) of *An. gambiae* mosquitoes collected indoors in their study in Enugu State were bloodfed. Also, similar observations were made in Abeokuta where 306 (84%) of *An. gambiae* collected indoors were either freshly fed or gravid [15]. These results indicate that a large number of the mosquitoes have had contact with human host and as such, there could be chances of the

infected mosquitoes being able to transmit the malaria parasites which are endemic in the area [16]. The high percentage of bloodfed *An. gambiae* complex mosquitoes collected indoors clearly shows that *An. gambiae* mosquitoes are both endophagic and endophilic as many with different physiological states were also caught while resting on the inside surfaces of walls in human dwellings.

Only 10% of *An. gambiae* complex mosquitoes subjected to Enzyme-linked immunosorbent assays (ELISA) for analysis of sporozoite rate were positive for malaria sporozoites. The result here is in agreement with 10.3% sporozoite rate observed in *An. gambiae*s. Lin Plateau State, Nigeria [17]. But the observation is higher than 4.7% reported in Ghana [18], but much lower than 35.5% of *P. falciparum* sporozoites rate in *An. gambiae* reported in Lagos, Nigeria [19]. The observations indicate that infection of *An. gambiae* complex mosquitoes with malaria sporozoites vary by location and the variation may be depended of parasite, host and vector factors. Also, all the mosquitoes of the complex identified with PCR were all *An. gambiae*s. This observation was a significant revelation that the *An. gambiae*s was either the only member of the complex or the key member biting and transmitting malaria parasites in the area.

In this study, *An. gambiae* has a room density of 0.30 mosquitoes/room/night and man biting rate of 0.017 bites/man/night. These observations were lower than the findings in Enugu State, where *An. gambiae* had a room density of 0.66 mosquitoes/room/night and a man biting rate of 3.9 mosquitoes/man/night [12]. It was also far below the results in Bayelsa State, where *An. gambiae* had a man-biting rate of 8.7 bites/man/night and room density of 20.5 mosquitoes/room/night [20]. This suggest that *An. gambiae* mosquitoes are biting less in the study area which may justify the lower infection rates of malaria sporozoites observed in this study when compared with the observation of a higher sporozoitesrate in Lagos [19].

5. Conclusion

The availability of *An. gambiae* mosquitoes in the study area has shown that the inhabitants were exposed to the bites and nuisance of these mosquitoes and possibly disease transmission. Also, malaria transmission in Awka area of Anambra State could be by *An. gambiae*s. Other species may be playing minor role. If the malaria vectors are not controlled, the effect will be disastrous. For one thing, when the community members get sick, loss of productive time from work will increase, money will be spent on treatment, caregiving will resume with its associated costs and government expenditure will increase. Further studies need to be carried out to determine the current malaria prevalence rate in the study area. Also, the community members need health education on the vector ecology and diseases. Integrated vector control which must include physical, chemical and biological methods, should be employed to effectively control the mosquito vectors.

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