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MORPHOLOGICAL IDENTIFICATION AND MOLECULAR CHARACTERISATION OF *ANOPHELES* MOSQUITOES FROM OYE LOCAL GOVERNMENT AREA OF EKITI STATE, NIGERIA

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Abstract

Malaria remains one of the most important parasitic diseases in sub-Saharan Africa. Effective control and possible elimination of the disease must take into cognizance proper identification and characterization of mosquito vector species. This study documents the abundance and spatial distribution of Anopheles mosquitoes in Oye Local Government Area of Ekiti state. Mosquito larvae were collected from natural breeding habitats from three randomly selected study sites and maintained to adult stage in the insectary. Indoor collections of adult mosquitoes using Pyrethrum Spray Catch (PSC) method was also carried out in the three communities. The mosquitoes were identified using morphological keys and subsequently subjected to Polymerase Chain Reaction (PCR) procedures for molecular characterization. Data obtained were analysed using SPSS version 20.0and confidence interval was set at 95%. A total of 1,364 mosquitoes were collected out of which 738 (54%) were Culex, 473 (35%) were Aedes and 153 (11%) were Anopheles. There was a significant difference in the number of mosquitoes collected across the three study sites ($P<0.05$) with OyeEgbo community having the highest (44%). Polymerase Chain Reaction (PCR) analysis revealled that all the sibling species were Anopheles gambiae s.s. Proper identification of malaria vectors and an understanding of their malaria transmission indices are critical for strategic planning in malaria control interventions.

1.0 Introduction

Malaria remains a public health challenge for most developing countries, with about 214 million cases and 438,000 deaths recorded in 2015 alone [1]. Over 90 percent of deaths in sub-Saharan Africa have been linked to the disease with 30 percent childhood mortality, 11percent maternal mortality, 60percent outpatient attendance and 30 percent hospital admissions in Nigeria alone [2]. The causative agent of the disease (*Plasmodium spp*.) is transmitted by female *Anopheles* mosquitoes while taking a blood meal from humans [3,4].

The Anopheline group comprise of diverse species with widespread distribution varying from one geographical location to another, with the primary subgroups been *Anophelesgambiae* and *Anophelesfunestus.* In sub-Saharan Africa, *Anopheles gambiae* and *Anopheles funestus*are the main andthe most efficient mosquito species responsible for transmitting the parasites [5, 6]. The sub-species in the *An. gambiae* complex, as well as those in the *An. funestus* group are morphologically indistinguishable, but vary in behaviour, host preference for feeding and breeding requirements (7,8). They also vary in biology and vectoral capacity from place to place (9, 10). Conventional means of identifying malaria vectors are usually based on morphological characteristics, but the limitations of this method makes it important that molecular (DNA-based)methods of identification are employed, in order to completely characterize the vectors. Members of the *An. gambiaes.l.* are in species complexes which comprises of: *An melas*, *An. merus*, *An arabiensis*, *An. quadriannulatus* (A) and *An.quadriannulatus* (B), *An.bwambe* and *An. gambiaes.s.* (Recently separated into two: *An. gambiaes.s.* (S form) and *An. coluzzii* (M form)). Morphological identification often reveals that the identified mosquito could be any of the members stated above since some members are ubiquitous. Therefore, PCR is often conducted using primers for all the members to ascertain which member of the complex is found in the area. However, it is possible to find more than one species in an area indicating multiple species transmission while each species often have different behaviour and different transmission dynamics.

Efficient control and possible elimination of the disease must take into cognizance proper identification of malaria vectors in order to discrimination between the major vectors, lesser vectors and non-vectors, so that scarce resources are not wasted on controlling lesser vectors [11]. Presently in Ekiti State, there is paucity of data on the *Anopheline* fauna in general and the malaria vectors in particular. This study provides baseline entomological data on the mosquito species present in Oye Local Government Area (LGA) of Ekiti state. This information will be essential in planning long – term malaria intervention programmes for the state.

2.0 Materials and methods

2.1 Study area

The study was carried out in three randomly selected communities: Oye-Egbo, Temidire (Marble Lodge) and Irare, in Oye LGA of Ekiti State. The communities are largely occupied by students of the Federal University, Oye-Ekiti, farmers and civil servants.

2.2 Collection and rearing of mosquito larvae

Mosquito larvae were collected from ditches, tyre tracks and standing water bodies within the study locations. The Global Positioning of the study communities were captured using GPS reader. The collection sites were then classified using the WHO larvae site characterization form. The larvae were scooped with the aid of long spoons into plastic bowls and transported to the insectary in the Department of Animal and Environmental Biology, Federal University, Oye-Ekiti where they were maintained till they

metamorphosised into adults. In the Insectary, the larvae were separated into different plastic containers and washed severally to remove dirts. The larvae were then transferred to 5 plastic containers with open top, covered with wire mesh and fed with yeast. Emerging adults from the plastic containers were collected with the aid of an aspirator and transferred into adult mosquito cages. They were left unfed and upon their death, were stored in well-labelled Eppendorf tubes containing desiccated silica gel preparatory for PCR analysis.

2.3 Indoor collection of adult mosquitoes

In each of the participating communities, one house was randomly selected for indoor adult mosquito collection and this was carried out between 06.00-08.00hrs using Pyrethrum Spray Catch (PSC) method. Members of the household were asked to exit the house and the floor of each room was lined with sheets of large white cloth for easy recognition of adult mosquitoes that will fall to the ground after spraying. Each room in the household was then sprayed with RAID insecticide and the doors were closed for 15 minutes. The mosquitoes were sorted, differentiated according to species and documented using appropriate standard tools. All *Anopheles* mosquitoes were preserved individually in labelled desiccated silica gel, in eppendorf tubes layered with cotton wool to keep them dry and afterwards, transported to the laboratory for PCR procedures.

2.4 Mosquito Identification

Morphological Identification

Morphological identification of the *Anopheles* mosquitoes was carried out under a microscope using morphological keys [12, 13]. The identification focused on dark spot at the upper margins of the wings which is common to all *Anopheles* species. Speckles on the legs, third pre-apical dark area on vein 1 with a pale interruption and tersi 1- 4 with conspicuous pale bands are features for *Anopheles gambiae.* A pale spot on second dark area, a light spot between the two dark spots on vein 6, two dark spots on vein 6, and absence of fringes on vein 6, are features of *Anopheles funestus*.

2.5 Molecular Characterisation using PCR Techniques

Molecular analysis for DNA extracted from *An.gambiae*was carried out in the Molecular Entomology Laboratory in the Department of Public Health at Nigerian Institute of Medical Research (NIMR), Yaba, Lagos.DNA was extracted from a leg and wing of each specimen following the method of [14]. The specimen was placed in 1.5 ml Eppendorf tubes and grinded in 100 µl extraction buffer using a pestle. The mixture was incubated at 70°C for 30 minutes in a dry bath and centrifuged at 16,000 rpm using a Fresco Biofuge (Haraeus, Kendro Laboratory Products Germany).

2.6 Preparation of Agarose gel and loading of PCR products

Agarose gel (2.5%) was prepared by adding 2.0g agarose powder to 200ml of Tris Base EDTA (x1 TBE) buffer (pH, 7.8), stirred with a glass rod and the mixture heated to boiling and allowed to cool for a few minutes after which 3µl of ethidium bromide was stirred evenly into the gel. The gel was poured into a prepared tray containing combs. On solidification, the combs were removed and the gel was cut into size and placed into the electrophoresis tank containing the x1 TBE running buffer. The PCR product mixed with 1µl of the loading dye was loaded into each well. The gel was run at a constant

voltage of 100 V and not more than 120-150 mA. The amplified products were visualized using an Ultraviolet (UV) transilluminator.

2.7 PCR amplification of DNA extracted from *An.gambiaes.l*

One microlitre of the DNA extracted from a single mosquito was added to the PCR master mix in a final reaction volume of 12.5µl that contained 10X PCR buffer (Takara Bio Inc. Japan), 2.5mM of each dNTPs,25mM $MgCl_2$, species specific primer (Primer sequence 5' to 3') namely: 0.53ng primer *gambiae* [CTG GTT TGG TGG GCA CGT TT], de-ionised water and 0.5N DNA polymerase TaKaRaTaq (enzyme) (Takara Bio Inc.Japan). PCR amplification was carried out in a thermal cycler (Primer 96 PCR-system MWG Genomic Technology) with an initial denaturation step at 94°C for 1 minute, followed by 30 cycles, each consisting of 30 seconds denaturation at 94 °C for 30 seconds annealing at 50°C and 30 seconds extension at 72°C. The final extension was carried out at 72°C for 10 minutes.

2.8 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) identification of molecular forms of *An.gambiae*

After amplification, the PCR product was digested using restriction enzyme Hha I. One unit of Hha I x SuRE/Cut Buffer L (Roche diagnostic, Basel, Switzerland) was directly added to the PCR product and digestion carried out at 37°C for 3½hours.Digested fragments were then separated through a 2% agarose gel stained with ethidium bromide. Diagnostic bands were viewed under an ultraviolet light transilluminator and photographed using a photo documentation system.

2.9 Statistical analysis

Data obtained were entered using Microsoft Excel and analyzed using Statistical package for Social Sciences, SPSS Version 20.0 (SPSS Inc. Chicago, Illinois, USA). Descriptive statistics was used to describe important variables and chi-square was used to test for significance. P-value was set at 0.05.

3.0 Results

3.1 Identification of *Anopheles* mosquito larvae breeding sites

The *Anopheles* mosquito larvae breeding sites identified in the study areas included; puddles, ditches, and tyre tracks. In Oye Igbo, breeding sites were made up of ditches, puddles and tyre tracks. In Irare, the breeding sites were made up of tyre tracks and puddles while in Temidire (marble lodge), they were mainly puddles (Table 1).

3.2 Species abundance and distribution across the study area

A total of 1,364 mosquitoes were collected for the study and distinguished morphologically. Of these, 738(54%) were *Culex* species, 473(35%) were *Aedes* species and 153(11%) were *Anopheles* species. There was a significant difference ($P<0.05$) in the distribution of mosquitoes collected across the three study sites. Mosquitoes collected from Oye-Egbo were made up of 379(63%) *Culex*, 173(29%) *Aedes* and 47(8%) *Anopheles* species.Temidire (Marble Lodge) had 167(41%) *Culex* species, 198(48%) *Aedes* and 44(11%) *Anopheles* species.Irare community had 192(54%) *Culex*, 102 (29%) *Aedes* and 62 (17%) *Anopheles* species (Fig. 1).

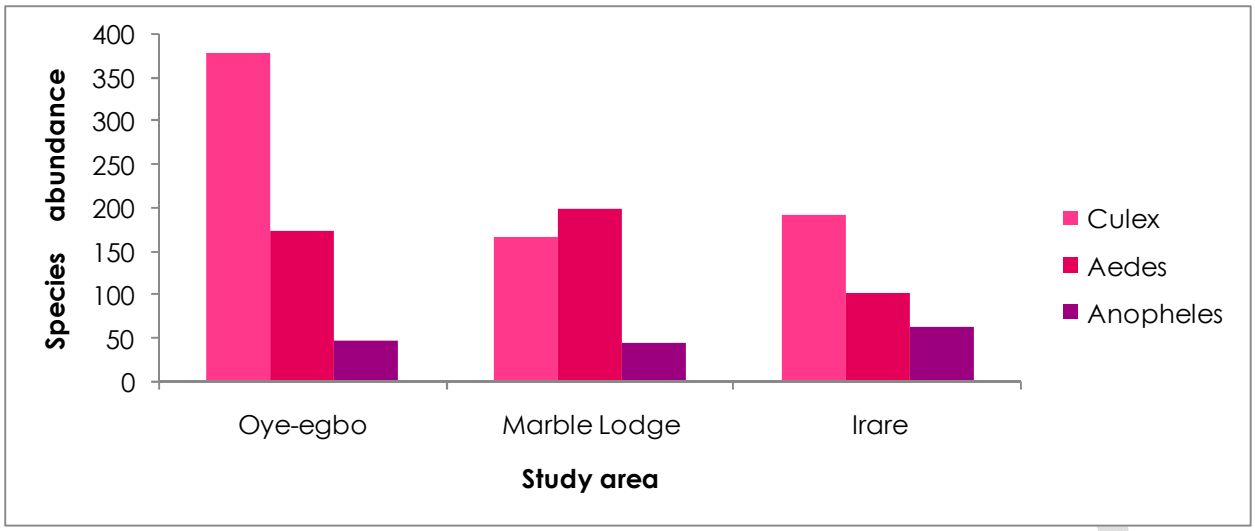

Figure 1: *Anopheles* species abundance and distribution across the study sites

3.3 **Molecular Identification of *Anopheles* species in the study area**

The molecular identification of the members of the *Anopheles gambiae* group by PCR revealled that all the sibling species were *Anopheles gambiaes.s.* (Plate 1).

1	2	3	4	5	6	7	8	9	10	11	12	13	14

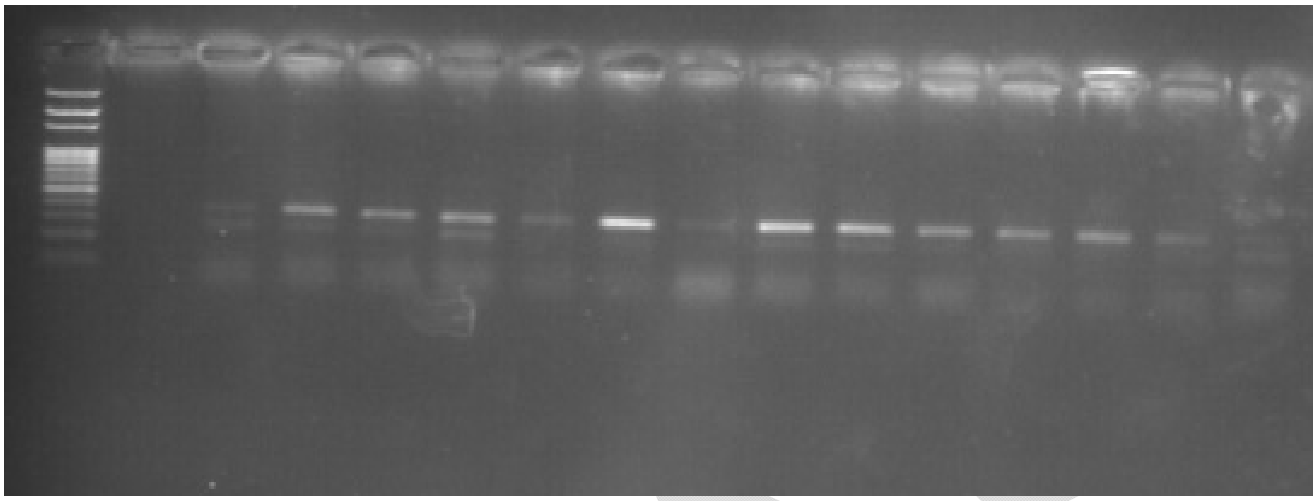

Plate 1: PCR Characterization of members of the *Anopheles gambiae* group

Table 1: *Anopheles* Mosquito Larvae breeding sites

Study area	Breeding sites	pH	Temperature (℃)	Presence of Vegetation	Origin of the water	Characteristics of the water
Oye-Egbo	Site 1 (Ditch)	7.94	25.0	Emergent	Rain	Clear
	Site 2 (Puddle)	8.11	29.0	Submerged	Rain	polluted
	Site 3 (Tire-track)	6.51	29.1	Floating	Rain	Polluted
Marble Lodge	Site 1 (Puddle)	6.31	25.6	Submerged	Rain	Turbid
	Site 2 (Puddle)	7.41	28.0	Submerged	Rain	Dark
	Site 3 (Puddle)	6.49	23.0	Floating	Rain	Clear
Irare	Site 1 (Tire tracks)	6.65	28.0	Submerged	Rain	Turbid
	Site 2 (Puddle)	7.90	29.0	Emergent	Rain	Clear
	Site 3 (Tire tracks)	6.56	25.2	Emergent	Rain	Turbid

Labels:
Lane 1 and 16: 1 kb standard ladder
Lane 2 and 3 : Negative control (Master Mix without DNA)
Lane 4: *Anopheles gambiae* positive control
Lane 5 - 15: Sample templates, *Anopheles gambiae s.s.*

3.4 Discussion

This study documents the abundance and distribution of mosquitospecies in Oye Local Government Area of Ekiti state. Majority of the identified larval habitats in the study area were puddles, with rain water sources. There is plethora of evidence on the preference of *Anopheles species* to such open, sunlit, man - made habitats because of the absence of predation on larvae and less competition for resources [15,16]. With the identified link between rainy season and abundance of mosquito breeding sites, it is necessary to identify and destroy breeding sites immediately rainy season commences by filling them up with sand. Morphological identification of the mosquitoes revealed that *Culex species* were the most abundant, followed by *Aedes* and *Anopheles*. This corroborates with the findings of [3, 10, 17] in other southwestern states of the country, who reported Culex species as the most abundant mosquito species. In addition,the abundance of Culex species recorded in this study is similar to the findings of [7]. Although *Culex* and*Aedes* species are non – malaria vectors, with the advent of re-emerging tropical diseases like Zika, there is need to intensify control efforts of these non – malaria vectors. In this study, all the *Anopheles* species collected were *An. gambiae s.s.,* with Irare community having the highest number. Our findings corroborate with that of [10, 18] who reported *An. gambiae s.s.,* as the most abundant species in southwest Nigeria. *Anopheles.gambiaes.s.* is the most efficient and deadly malaria vector in sub – Saharan Africa. It has been reported to be widely distributed across sub-Saharan Africa and particularly in Nigeria [19]. These vectors have marked preference for human environments and they rapidly adapt to changes in the environment induced by human habitation and agricultural development [20, 21, 22]. Since the Federal University, Oye-Ekiti is located within the study area; the over four thousand students residing within the communities are at a high risk of malaria transmission. There is therefore a need for massive enlightenment programmes on malaria prevention and control in the community. This study provides useful information for developing effective mosquito control strategies and vector surveillance programmes in Oye-Ekiti in particular and Ekiti state, Nigeria as a whole. There is however the need to scale up this delineation and characterization study to other parts of Ekiti state for effective mosquito management.

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