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Molecular Characterization of *Anopheles gambiae* Complex, Blood Meal Sources and Sporozoite Infection Status in Selected Communities in Central Nigeria

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Abstract

Mosquitoes constitute important vectors of diseases of public health importance. Thus, this study characterized Anopheles gambiae complex, blood meal sources and sporozoite infection rate in selected communities in Central Nigeria was carried out from September to October 2023. Forty (40) isolated deoxyribonucleic acid (DNA) samples of Anopheles gambiae sensu lato were molecularly processed using polymerase chain reaction (PCR) for sibling species identification while enzyme-linked immunosorbent assay (ELISA) was used to detect sources of blood meal and sporozoite infection in Anopheles mosquitoes. Out of the 40 Anopheles gambiae s. l. characterized, only 25 amplified, in which An. coluzzi had a high abundance 20 (80.0%) than An. gambiae 5 (20.0%). Udege had the highest abundance of An. coluzzii in comparison with the other two locations while An. gambiae sensu stricto was dominant in Nasarawa, and differences between the locations was significant (χ^2 = 11.2, df = 2, P = 0.003698). A significant difference (χ^2 = 54.56, df = 5, P < 0.05) among the Anopheles mosquito sources of blood meal was observed in which An. coluzzii preferred blood from humans as well as human/goat hostwhile An. gambiae s. s. sought blood from both human/goat and human/bovines hosts, respectively. No sporozoite was detected in the screened An. coluzzi and An. gambiae s. s. from the three study locations. In conclusion, the abundance of An. coluzzii and its preference for human blood is an indication that An. coluzzii is the primary vector of malaria in the study area. Hence, clearing of potential breeding sites as well as the use of insecticide treated nets should be encouraged in order to reduce malaria transmission.

Keywords: *Anopheles gambiae complex, Molecular characterization, Sporozoite, Blood meal sources, Central Nigeria*

1. Introduction

Mosquitoes are significant disease vectors that have an impact on public health. An efficient and successful mosquito control program requires mosquito surveillance (Ombugadu *et al.,* 2024a). An important source of information for assessing the possibility of mosquito-borne illness transmission is the dynamics of shifting mosquito populations (Njila *et al*., 2022; Ombugadu *et al.,* 2024b). *Anopheles gambiae sensu lato* and *Anopheles funestus* group are the predominant malaria vectors among the 37 *Anopheles* species that have been identified as such in Nigeria (World Health Organization [WHO], 2018). In the northern region of Nigeria, *An. arabiensis* is the most common species of the *An. gambiae* complex, while *An. melas* is found in the mangrove coastal zones (Madara & Ebuzoeme, 2023). According to WHO (2020), additional localized vectors include *An. nili*, *An. coustani*, *An. moucheti*, and *An. squamosus*.

The characterization and identification of malaria vectors have always been a major concern in vector control. Nowadays, deoxyribonucleic acid (DNA) amplification, mainly by polymerase chain reaction (PCR), coupled with DNA polymorphism analysis, is the method of choice for mosquito species molecular identification and characterization (OMS, 2022). *Anopheles gambiae s. l.* are the primary vectors of malaria in sub-Saharan Africa. *An. gambiae s. l.* is a complex of mosquitoes comprising more than eight sibling species (Tennessen *et al*., 2021). The anthropophilic and indoor-biting Afrotropical vectors *An. coluzzii* and *An. gambiae sensu stricto* previously known as M and S molecular forms of *An. gambiae,* respectively are sibling members of this complex and contribute to the high risk of malaria in Nigeria and neighbouring West and Central African countries (Irish *et al*., 2019; Ebhodaghe et al., 2024). In Burkina Faso, *An. coluzzii* co-existed with *An. gambiae s. s.* but preferred to breed in large, permanent, and vegetation-dense habitats (rice paddies), whereas *An. gambiae s. s.* preferred temporary puddles. Additional differences in the ecologies of *An. coluzzii* and *An. gambiae s. s.* have been described in Mali and Cameroon among countries in the West and Central African sub-regions (Ebhodaghe *et al.*, 2024). In rural areas in Nigeria, the typically indoor-biting vectors *An. coluzzii* and *An. gambiae s. s.* have been found to feed on humans in outdoor locations (PMI, 2022). *Anopheles arabiensis*, *An. coluzzii*, and *An. gambiae s. s.* were reported as the come *Anopheles gambiae* complex siblings in rural areas in Nigeria (Obembe *et al*., 2022; Adeogun *et al*., 2023; Ebhodaghe *et al*., 2024).

The transmission of mosquito-borne pathogens happens due to the feeding habit of mosquitoes. Each species of *Anopheles* has its own blood-feeding pattern and host preference (Becker et al., 2012). The correct identification of the preferred host for malaria vectors determines the major hosts in the support of the sustainability of vector population (Altahir *et al*., 2022). In the Bure district of Ethiopia, Adugna *et al*. (2021) observed that all Anopheles mosquitoes tested for a blood meal source had mixed- rather than single-source blood meals. All *Anopheles* species, with the exception of *An. squamosus*, had frequencies for humans that were marginally higher than those for cattle. This indicated that, for each pair of vertebrate hosts, humans were a marginally preferred blood meal source over cattle. In Benue's north central region of Nigeria, human blood meal sources ranged from 97% to 100%, clearly indicating a high level of human-vector contact (Aju-Ameh *et al*., 2016).

Plasmodium sporozoite proportions can be estimated by detecting sporozoites under a microscope, using Circumsporozoite-ELISA (CS-ELISA) or polymerase chain reaction (PCR) (Marie *et al*., 2013) from mosquito samples. The presence of sporozoites in the salivary gland of mosquito thus defines mosquito infectiousness. These sporozoites are injected to human body through the bites of infected female *Anopheles* mosquito, thereby establish malaria parasites which is a major public problem in the tropics. Brugman *et al*. (2018) demonstrated that, *Anopheles* mosquitoes expel malaria sporozoites while feeding. The quantification of malaria sporozoites is of utmost importance, this would aid in predicting the forces of infection transmission and enhance field surveillance activities for a successful malaria elimination (Nassai *et al*., 2023).

There is still dearth of information on *An. gambiae* complex siblings in some parts of Nigeria. To this end, this study investigated *An. gambiae* complex siblings, blood meal sources and sporozoite burden in three selected communities in Nasarawa Local Government Area (LGA) of Nasarawa State, Central Nigeria, hence, adding up to existing entomological surveillance data of the national malaria elimination programme so as to bring about effective control measures on malaria transmission in the country.

2. MATERIALS AND METHODS

2.1. Study Area

The study was conducted in Loko, Udege and Nasarawa communities in Nasarawa LGA of Nasarawa State, Central Nigeria. Nasarawa LGA is located on latitude 7.7013° N, longitude 8.5345°E, and it is comprised of 15 electoral wards with headquarters in Nasarawa town. The Local Government Area has a land mass of 5,704 km^2 and a population of 189,835 as at the 2006 census. Fishing and farming are the main occupations of the residents of Nasarawa LGA. Nasarawa LGA has a significantly high temperature (36°C).

2.2. Selection of Houses

A total of 60 houses were surveyed after informed consents were granted by the head of households. Random sampling technique was used in the selection of houses in each of the study area (Ombugadu *et al.*, 2022). The number of houses selected in each community was based on the population of households found. Of the 60 houses surveyed, 28 houses were selected from Nasarawa Central (with 7 electoral wards), 20 houses from Udege Development Area (5 electoral wards) and 14 houses from Loko Development Area (with 3 electoral wards), respectively. The interval apart between the selected houses was at least 200 m. Occupants of the selected houses were informed to keep doors and windows closed until after it was sampled.

2.3. Mosquito Samples Collection

During the late rainy season in September and October of 2023, mosquitoes were collected indoors in the research area. Throughout the study period, two visits were made to each household. Adult mosquitoes were trapped in the morning between 0600 and 0900 hours using a battery-operated Prokopack Aspirator (Ombugadu *et al*., 2022).

2.4. Morphological Identification and Preservation of Adult Mosquito

Mosquitoes were sorted out and morphologically identified based on their features with the aid of a dissecting microscope and identification keys by Gillies and Coetzee (1987) and Buttachon *et al.* (2022). Each *Anopheles gambiae s. l.* identified was preserved in an Eppendorf tube containing silica gel and taken to The Molecular Entomology and Vector Control Research Laboratory of the Nigerian Institute for Medical Research (NIMR), Yaba, Lagos State, Nigeria, for further molecular analysis.

2.5. Molecular Characterization of An. gambiae Complex Siblings

DNA was extracted from forty (40) *Anopheles gambiae s. l.* following the protocol by Collins *et al.* (1984) and processed at Nigerian Institute of Medical Research (NIMR), Yaba, Lagos State, Nigeria, using species-specific Polymerase Chain Reaction (PCR) as described by Scott *et al.* (1993). The *Anopheles gambiae* complex discrimination was done according to Wilkins *et al*. (2006) based on species-specific single nucleotide polymorphisms (SNPs) in the intergenic spacer region (IGS). The PCR cycling condition for amplification of the gene were 95°C/5min x 1 cycle; (95°C/30sec, 59.2°C/30sec, 72°C/30sec) x 30 cycles; 72°C/5min x l cycle, and 4°C holding for infinite time. Primers that were designed from the DNA sequence of the intergenic spacer (IGS) region of *An. gambiae s. l.* were added simultaneously to elucidate the ribosomal DNA type which include AR-3T 5'-GTGTTAAGTGTCCTTCTCCGTC-3', GA-3T 5'-CTTACTGGTTTGGTCGGCATGT-3', ME-3T 5'-CAACCCACTCCCTTGACGATG-3', QD-3T 5'-GCATGTCCACCAACGTAAATCC-3', IMP-S1 5'-CCAGACCAAGATGGTTCGCTG-3', IMP-M1 5'-TAGCCAGCTCTTGTCCACTAGTTTT-3' and the Intentional Mismatch Primers (IMPs) IMP-UN 5'-GCTGCGAGTTGTAGAGATGCG-3' (Wilkins *et al*., 2006). The PCR product (amplicons) was loaded on a 1.5% agarose gel stained with ethidium bromide (EtBr).

2.6. Blood Meal Enzyme-Linked Immunosorbent Assay

Blood meal enzyme-linked immunosorbent assay (ELISA) protocol as described by Beier *et al*. (1988) was adopted for the twenty-five (25) mosquitoes screened for sources of blood meal. Individual mosquito for testing was prepared by grinding in 1.5 ml Eppendorf tube using 100 µl phosphate buffer saline (PBS) at pH 7.4. Unfed reared laboratory mosquito (*Kisumu* strain) was used as negative control while human, goat and bovine sera (Rockland antibodies & assays) were used as positive control. Affinity purified and peroxidase-labeled antibodies were obtained from Seracare (KPL).

Fifty (50) µl of mosquito triturate and respective controls were added to the wells of a microtiter plate, including a positive control consisting of 10 µl of human serum and 500 µl of PBS. The plates were incubated for 1 hour then washed twice with 200 µl of PBS-Tween 20 solution (prepared by adding 500 µl of Tween 20 to 1L of PBS). Fifty µl of prepared enzyme conjugate solution was added and incubated for 1 hour. The enzyme conjugate solution was prepared by mixing Affinity-purified antibody to human IgG (H+L) at 1:500 dilution (primary antibody), Peroxidase-labeled affinity-purified antibody to human IgG (H+L) at 1:1000 dilution (secondary antibody) and 1 µl of each antibody was mixed with 500 µl

of PBS. The plates were washed three times with 200 µl of PBS-Tween 20 solution followed by addition of 100 µl of ABTS Peroxidase substrate to each well by mixing solution A and B (5 ml + 5 ml per plate). The plates were incubated for 30 minutes, and the absorbance was read at 414 nm. Samples were considered positive if absorbance values exceeded the mean plus the standard deviation of all replicates of the negative control.

2.7. Screening of Malaria Vectors for *Plasmodium* Infective Stage

Enzyme-linked immunosorbent assay (ELISA) was developed to detect *Plasmodium falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite (CS) proteins in malaria-infected mosquitoes. A total of forty (40) stored dried mosquitoes were screened for sporozoite. In order to achieve success in the CS-ELISA assay, the anti-sporozoite monoclonal antibodies (Mabs) was first put in the 96-well plate and adsorbed to the plate. After which, blocking buffer was added to prevent non-specific binding and then mosquito triturate added to the wells of the plate. Thereafter, peroxidase-linked anti-sporozoite Mab and ABTS substrate, respectively, were added to the wells (BEI Resources, 2020).

A working solution of monoclonal antibodies (mAbs) capture was prepared by adding 5 ml of phosphate buffered saline (PBS) to the 40 µl of reconstituted capture mAb (stock) for a plate. The mixture was vortexed gently and 50 µl of mAb solution made was added to each well of the ELISA plate. The plate was covered and incubated for 30 minutes at room temperature. Thereafter, well contents aspirated and plate banged upside down on paper towel 5 times, holding sides only. The wells were filled with 200 µl blocking buffer (BB) and the plate was covered and incubated for 1 hour at room temperature. The well contents aspirated and plate banged upside down on paper towel 5 times holding sides only. Samples and controls were loaded into the plate and covered and incubated for 2 hours at room temperature. Peroxidase substrate was prepared by mixing Substrate A and Substrate B at a 1:1 ratio. A full 96-well plate was 5 ml of Substrate A + 5ml of Substrate B. A working solution of mAb conjugate for *Plasmodium falciparum* (Pf) was prepared by adding BB to 10 µl of reconstituted capture mAb. Enzyme activity was checked by mixing 5 µl of the mAb conjugate with 100 µl of the substrate in a separate tube and vortexed gently. A rapid color change indicated that the peroxidase enzyme and the substrate were functional. Well contents properly aspirated and plate banged upside down on paper towel 5 times holding sides only. The wells were washed two (2) times with 200 µl of PBS-Tween, aspirating and banging plate 5 times with each wash. A 50 µl of peroxidase conjugate solution was added to each well and the plate covered and incubated for one (1) hour. Well contents aspirated and plate banged upside down on paper towel 5 times holding sides only. The wells were washed 3 times with 200 µl of PBS-Tween, aspirating and banging plate 5 times with each wash and a 100 µl volume of substrate solution was added per well. Plate covered and incubated for thirty (30) minutes. Plate was handled carefully to avoid splashing. Plates were read at 405 – 414 nm (Rogier et al., 2017). Samples whose absorbance values were above the cut-off (twice the mean absorbance value of the negative samples) were labelled positive for *P. falciparum* infection.

2.8. Statistical Analysis

Minitap Statistical Package version 21.1.3 was used to analyze the data obtained. Pearson's Chi-square (χ^2) test was used to compare the proportion between anopheline siblings, locations as well as in relation to blood meal sources. The level of significance was set at $P < 0.05$.

3. RESULTS

3.1. Characterization of *An. gambiae* Complex in Nasarawa LGA

Plate 1 shows the *Anopheles gambiae* siblings present in the study area. Out of the 40 *Anopheles gambiae* screened, only 25 amplified in which *An. coluzzi* was more abundant 20 (80.0%) than *An. gambiae sensu stricto* 5 (20.0%) as shown in Table 1. Udege had the highest abundance of *Anopheles coluzzii* (32.0%) followed by Loko (28.0%) while Nasarawa had the least abundance (20.0%). *Anopheles gambiae s. s.* was most abundant in Nasarawa (12.0%) followed by Udege (8.0%) but absent (0.0%) in Loko community, and variations of *An. gambiae s. s.* in relation to locations showed a significant difference ($\chi^2 = 11.2$, df = 2, P = 0.003698).

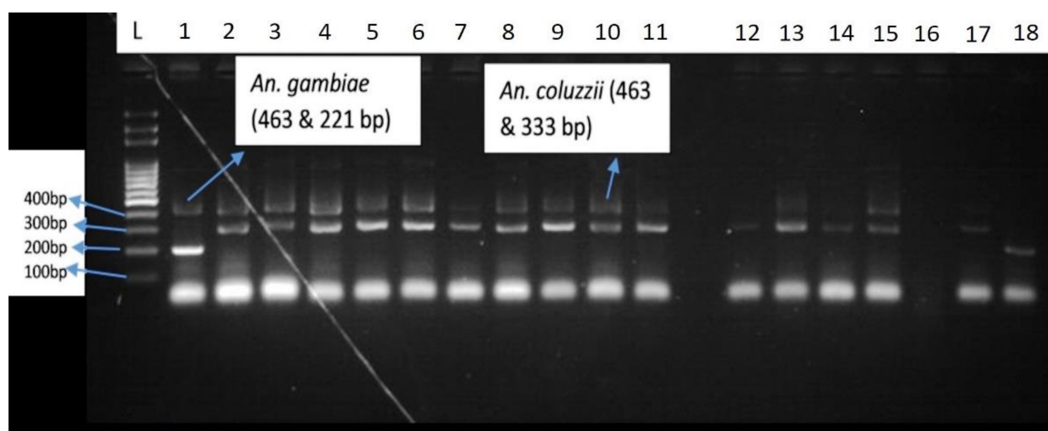

Plate 1. Ethidium Bromide stained Agarose Gel Image of *An. gambiae s. s.* and *An. coluzzii* in Nasarawa LGA, Nasarawa State, Nigeria

Table 1. *Checklist of An. gambiae Siblings in Nasarawa LGA, Nasarawa State*

Location	No. Examined	Species	
		An. coluzzi	***An. gambiae s. s.***
Nasarawa	8	5 (20.0)	3 (12.0)
Udege	14	8 (32.0)	2 (8.0)
Loko	18	7 (28.0)	0 (0.0)
Total	**40**	**20 (80.0)**	**5 (20.0)**

3.2. *Anopheles Species* Specific Blood Meal Host

The most preferred source of blood meal by *Anopheles coluzzii* was the human host followed by the combination of human/goat hosts while goat and bovines host blood, respectively, were the least preferred (Table 2). *Anopheles gambiae s. s.* showed a high blood feeding preference from both human/goat hosts as well as human/bovine hosts as shown in Table 2. Therefore, there was a significant difference ($\chi^2 = 54.56$, df = 5, $P < 0.05$) in relation to the *Anopheles* mosquitoes sources of blood meal.

Table 2. *Anopheles Species Blood Meal Hosts*

Species	No. Examined	Human (H)	Goat (G)	Bovine (B)	H/G	H/B	H/G/B
An. coluzzi	20	6 (30.0)	0 (0.0)	0 (0.0)	6(30.0)	4 (20.0)	4 (20.0)
An. gambiae s. s.	5	0 (0.0)	0 (0.0)	0 (0.0)	2(40.0)	2 (40.0)	1 (20.0)
Total (%)	**25**	**6 (24.0)**	**0 (0.00)**	**0 (0.00)**	**8(32.0)**	**6 (24.0)**	**5 (20.0)**

Key: H/G = Human/Goat, H/B = Human/Bovine, H/G/B = Human/Goat/Bovine

3.3. Prevalence of Sporozoite in *An. gambiae* Siblings

The main malaria vectors in this study, *An. coluzzi* and *An. gambiae s. s.* had no (0%) *Plasmodium* infective stage in them from the three pooled locations screened (Table 3).

Table 3. *Prevalence of Sporozoite in Two An. gambiae Complex Siblings*

Location	No. Examined	Species	
		An. coluzzi	***An. gambiae s. s.***
Nasarawa	8	0	0
Udege	14	0	0
Loko	18	0	0
Total (%)	**40**	**0 (0.0)**	**0 (0.0)**

4. DISCUSSION

This research revealed that two *Anopheles gambiae* siblings are present in the study area in which *Anopheles coluzzii* was dominant 20 (80.0%) over *Anopheles gambiae s. s.* 5 (20.0%). The high abundance of *Anopheles coluzzii* in this study shows that it is the primary malaria vector in the surveyed communities. The predominance of *An. culuzzi* in the area is due to their global spread and ability to adapt to distinct ecological niches. This agrees with previous studies by Hanemaaijer *et al.* (2019),

Ukonze *et al.* (2023) and Egedegbe *et al*. (2023) who reported *Anopheles culuzzii* as the dominant *Anopheles* species in Burkina Faso, Anambra state and Ughelli North LGA, Delta State, Nigeria, respectively. On the contrary, Atting and Akpan (2016) and Adamu *et al.* (2023) reported *Anopheles gambiae s. s.* to be the most dominant *Anopheles* species collected in Oyo and in two Area council of the Federal Capital Territory, Abuja, Nigeria, respectively.

In this study, *Anopheles gambiae* siblings were biased towards blood meal from both human and goat hosts which could be attributed to the practice of keeping goats at home by the inhabitants of the sampled communities. Similar findings were reported by Ndenga *et al.* (2016) and Abubakar *et al.* (2023) who reported human blood as the most preferred blood meal by *Anopheles* mosquitoes. Orsborne *et al*. (2020) also reported similar findings in Ghana. On the other hand, the finding of Altahir *et al.* (2022) reported that the blood meals of bovines was the most preferred followed by human, dogs and goat. Also, Kent *et al.* (2006) opined in a study on feeding and indoor resting behaviour of the mosquito *Anopheles longipalpis* in an area of hyperendemic malaria transmission in southern Zambia that *An. longipalpis* significantly imbibed greater proportion of cattle blood meal than expected and fewer blood meals than expected from goats and humans. According to Mayagaya et al. (2015) and Ogola *et al*. (2017), mosquitoes' propensity for feeding can be greatly influenced by the availability of alternative hosts such as goats and cows.

The absence of sporozoites in the *Anopheles gambiae* complex screened in this study could possibly be attributed to their nulliparous status, and also a very low malaria transmission in the area due late wet season period which may account for a very high chance of the nulliparous *Anopheles* mosquitoes not likely to bite the few number of infected persons at such period. This is in agreement with published work by Brugman *et al.* (2018) which has shown that sporozoites can only be found in female mosquitos that have lived long enough for the parasites to complete their sporogonic development and migrate from oocysts on the midgut wall to the salivary glands for onward transmission. However, this contradicts the findings of Oyewole *et al*. (2010) who reported that the sporozoite rate for *An. gambiae s. s.* ranged from 3.0% to 6.0% in Badagry, Lagos, Nigeria.

5. CONCLUSION

Result from this study reveals the abundance of *Anopheles coluzzii* and its preference for both human and goat blood meals which implies a high chance for zoonotic-borne infections at the long run. The findings in this research calls for constant clearing of potential breeding sites as well as the use of insecticide treated bed nets by the inhabitants of Nasarawa LGA, Nasarawa State. Interruption of vector-human contact through indoor residual spraying (IRS) and larval source management is of priority in breaking the transmission chain of malaria infection in the area.

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