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Molecular characterization and *Plasmodium falciparum* transmission risks of *Anopheles mosquitoes* in Malete, Nigeria

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ABSTRACT. Introduction: Studies on malaria vector surveillance are useful for evidence-based control in specific communities. Such studies are lacking in Malete, a rapidly growing peri-urban community in Nigeria. **Objective**: To assess sibling species identity, human blood indices, and *Plasmodium falciparum* transmission risks by *Anopheles* mosquitoes, in Malete. **Methods**: I collected endophilic mosquitoes quarterly from inhabited houses using the pyrethrum spray catch technique. I identified the mosquitoes, and probed for the presence of human blood and *P. falciparum*, using standard PCR and ELISA methods, respectively. **Results**: *Anopheles* mosquitoes (90%) were the most abundant compared to *Culex* (10%) and *Mansonia* (0,5%). Specifically, *A. gambiae* (85%) were predominant over *A. coluzzii* (11%) and *A. arabiensis* (3%). The *Anopheles* sibling species had generally high human blood indices (≥0,82). However, *A. gambiae* man-biting rates (0,92-3,64) were higher than *A. coluzzii* (0-0,84) and *A. arabiensis* (0-0,27). *Plasmodium falciparum* sporozoite infection (3%) was found only in *A. gambiae*. **Conclusion**: While *P. falciparum* infection was 3%, long-lasting insecticidal nets should be deployed for control in Malete, particularly of *A. gambiae*.

Keywords: Mosquito, *Anopheles*, Malaria transmission, Malete, Kwara State, Nigeria.

RESUMEN. "Caracterización molecular y riesgos de transmisión de Plasmodium falciparum de mosquitos Anopheles en Malete, Nigeria". **Introducción:** Los estudios de vigilancia de vectores de malaria son útiles para el control basado en evidencia en comunidades específicas. Tales estudios faltan en Malete, una comunidad periurbana de rápido crecimiento en Nigeria. **Objetivo:** Evaluar la identidad de especies hermanas, los índices de sangre humana y los riesgos de transmisión de *Plasmodium falciparum* por mosquitos *Anopheles*, en Malete. **Métodos:** Trimestralmente recolecté mosquitos endófilos de casas habitadas, utilizando la técnica de captura por aspersión de piretro. Identifiqué los mosquitos y analicé la presencia de sangre humana y *P. falciparum* por métodos estándar de PCR y ELISA, respectivamente**. Resultados:** Los mosquitos *Anopheles* (90%) fueron, por mucho, más abundantes que *Culex* (10%) y *Mansonia* (0,5%). Específicamente, *A. gambiae* (85%) fue predominante sobre *A. coluzzii* (11%) y *A. arabiensis* (3%). Las especies hermanas de *Anopheles* tenían índices de sangre humana generalmente altos (≥0,82). Sin embargo, las tasas de picadura de *A. gambiae* (0,92-3,64) fueron más altas que las de *A. coluzzii* (0-0,84) y *A. arabiensis* (0-0,27). La infección por esporozoitos de *Plasmodium falciparum* (3%) solo se encontró en *A. gambiae*. **Conclusión:** Si bien la infección por *P. falciparum* fue del 3%, se podría evaluar mosquiteros insecticidas de larga duración para el control en Malete, particularmente de *A. gambiae.*

Palabras clave: Mosquito, *Anopheles*, Transmisión de la malaria, Malete, Estado de Kwara, Nigeria.

Global malaria cases reported across 85 endemic countries were 241 million in 2020, increasing from 227 million in 2019 (World Health Organization [WHO], 2021). Ninety five percent of these increases were recorded within countries located in the WHO African Region (WHO, 2021). Total estimated deaths resulting from the global malaria cases also increased from 558 000 in 2019 to 627 000 in 2020. Six countries accounted for 55% of all burdens with Nigeria alone bearing the highest (27%) estimated global share of the malaria cases reported (WHO, 2021). Malaria accounts

for 60% of outpatient visits, 30% of hospitalizations, 10% of low birth weight and 11% of maternal mortality in Nigeria (National Malaria Elimination Programme et al., [NMEP et al.], 2016).

The major malaria vector control efforts of the Nigerian National Malaria Elimination Programme (NMEP) include the expansion of universal access to insecticide-treated materials through mass distribution of Long-lasting insecticidal bed-nets (LLINs), significant scaling up of Indoor Residual Spraying (IRS) of recommended insecticides, and expansion of malaria mosquito larval source management (Federal Ministry of Health [FMoH], 2014). Both methods have been very effective not only in reducing the malaria vector populations, but also preventing malaria morbidity (Thiaw et al., 2018). Some other improved vector control tools evaluated for deployment against malaria transmitting mosquitoes in the country include the insecticide-treated durable wall lining designed to outlast IRS (Obembe et al., 2018a; Obembe et al., 2019). All of these vector control measures aim to limit the transmission of the malaria parasites by reducing or eliminating human contact with the vector.

Vector control represents a major component of the global strategy for malaria prevention, control and elimination and remains highly effective, when comprehensively applied and sustained (Wilson et al., 2020). However, malaria parasite transmission potential and susceptibility to vector control measures vary according to malaria vector species, location and season (WHO, 2017). Therefore, effective vector control implementation must be based on adequate knowledge of local vector species in terms of vector sibling species composition, population density and malaria parasite infection rates (WHO, 2019). Assessments of these entomological indices after the implementation of the suitable vector control intervention will also serve as the basis for performance evaluation of such interventions (WHO, 2019).

Entomological indices assessments in specific localities therefore becomes necessary to guide the choice of appropriate intervention to be implemented for the control of the prevailing malaria transmitting mosquitoes in each specific area. Kwara State which belongs to the north central geopolitical zone of the country has the unique feature of being one of the links between the Northern and Southern parts of Nigeria. However, apart from data generated in a few locations (Obembe et al., 2019; Oduola et al., 2021), most parts of kwara have no published reports on malaria entomological indices that could inform the selection of appropriate malaria vector control strategies. Malete is one of such areas lacking malaria vector data but with significant human population increases due to proximity to the Kwara State University Campus. This study was conducted to provide information on relative abundance, sibling species composition and *Plasmodium falciparum* infection rates of *Anopheles* mosquitoes in Malete, Kwara State, Nigeria.

MATERIALS AND METHODS

Study area: Malete (84°2'01.3''N 4°27'59.3''E), a peri-urban community around the Kwara State University Campus. The community has witnessed significant transformation especially in terms of housing types and population structures due to the influx of students and entrepreneurs providing goods and services. However, houses without ceilings and window nets leaving eaves and window entry points for malaria mosquitoes are still available showing the sub-urban nature of the locality. Kwara State is within the Guinea savannah zone with average daily temperatures between 26°C and 32°C. The dry season lasts from October to February while the rainy season begins towards the end of March and ends in October with two peak periods in June and September. The annual mean rainfall is about 1352,0 mm (Alaaya et al*.,* 2013).

Mosquito collection and morphological identification: Quarterly mosquito collections were conducted in ten randomly selected houses willing to allow indoor mosquito collection from October 2014 to July 2015 using the pyrethrum spray catch technique described by WHO (2003). The same rooms and houses were used for mosquito collections all through the period of the study. Collected female *Anopheles* mosquito samples were preserved individually in 1,5ml Eppendorf tubes containing desiccated silica gel. Further analyses of the mosquitoes were carried out at the Molecular Biology Laboratory of the Department of Zoology, Kwara State University, Malete, Nigeria. Standard keys (Gillies & Coetzee, 1987) for *Anopheles* mosquitoes were used for the morphological identification of each collected female *Anopheles* mosquito under a stereo-microscope.

Molecular characterization of mosquitoes: After morphological identification, mosquitoes confirmed as belonging to the *Anopheles gambiae* complex were analyzed further by species-specific Polymerase Chain Reaction (PCR) (Scott et al., 1993) and PCR-Restriction Fragment Length Polymorphism (RFLP) (Favia et al., 1997) for sibling species identification. Genomic DNA was extracted from each female *Anopheles* mosquito according to the standard method of Collins et al. (1987). The extracted DNA (1,4µl) from each *Anopheles* mosquito was added to the PCR master mix in a final reaction volume of 12,5µl containing ready-to-use Firepol® Solis Biodyne premix, primers and deionized water. The species-specific primers used were (Primer sequence 5' to 3'): 0,52ng primer Melas (TGA CCA ACC CAC TCC CTT GA), 0,53ng primer Gambiae (CTG GTT TGG TCG GCA CGT TT), 0,57ng primer Arabiensis (AAG TGT CCT TCT CCA TCC TA), 0,49ng primer Quadrimaculatus (CAG ACC AAG ATG GTT AGT AT) and 0,47ng primer UN (GTG TGC CCC TTC CTC GAT GT). The PCR amplification was carried out in a thermal cycler (Primus 96 PCR-system TECHNE TC-4000) with an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles each consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C and 40 seconds elongation at 72°C. The final elongation was carried out at 72°C for 5 minutes. The PCR product was digested using restriction enzyme Hha1. The Hha1 enzyme (0,2µl), cut buffer (0,6µl) and water (1,0µl) were added directly to the PCR product from each sample and digestion was carried out at 37°C for 1hr 30mins. The PCR products were electrophoresed in 1,5% agarose gel. The amplified fragment was then captured and photographed under a gel documentation machine.

Detection of *Plasmodium falciparum* infection in mosquitoes: *Plasmodium falciparum* circumsporozoite protein (CSP) Enzyme-linked Immunosorbent Assay (ELISA) analysis was conducted on the head-thorax of each female *Anopheles* mosquito sample according to the standard protocol described by Wirtz et al. (1987). Only the blood-fed and half-gravid female mosquitoes were used for the ELISA analysis. Non-blood-fed laboratory reared *A. gambiae* mosquitoes were used as negative controls while positive controls and monoclonal antibodies (mAbs) were provided by the Centers for Disease Control and Prevention (CDC), Atlanta USA. The head-thorax of each mosquito sample was homogenized in phosphate buffer solution. Each well of the ELISA plate was coated sequentially with 50µl capture mAb for 30 minutes incubation, aspirated and filled with 200µl blocking buffer (BB) for 1 hour incubation, and aspirated and filled with 50µl of mosquito triturate and positive control followed by 2 hours incubation. Fifty microliters of peroxidase-mAb was added for 1 hour incubation in the dark after aspirating and washing the wells twice with 200µl PBS-0,05% Tween 20. Wells were aspirated and washed three times with 200µl PBS-0,05% Tween 20 after which 100µl ABTS peroxidase substrate solution was added to each well for another 30 minutes incubation. The ELISA plates were read visually and with an ELISA reader. Samples were considered positive if absorbance values at 405nm exceed twice the mean of the negative control (Wirtz et al., 1987).

Identification of mosquito blood meal origin: Mosquito blood meal origin ELISA test was used to determine the presence of human blood in the blood-fed *Anopheles* samples following the standard procedure described by Beier et al. (1988). The capture and conjugated mAbs for blood meal origin identification were obtained from Kikergaard and Perry Laboratories incorporated, Gaithersburg, USA while human serum was ordered from Rockland immunochemicals, Gilbertsville, USA. The *Anopheles* mosquito abdomen remaining from specimen preserved over desiccated silica gel was homogenized in 1,5ml eppendorf tube containing 500µl of PBS. Male *Anopheles gambiae* were homogenized, each in 1,5ml eppendorf tube containing 50µl of PBS to serve as negative controls while human serum diluted in ratio 1:500 (2µl in 1000µl PBS) was used as positive control. Fifty microliters of respective controls and mosquito triturate were added to appropriate wells of the ELISA plate for incubation period of 1 hour. Wells were aspirated, washed with PBS-Tween 20 and filled sequentially with enzyme conjugated solution, ABTS peroxidase substrate and phosphatase substrate solution for the prescribed incubation periods (Beier et al., 1988). The ELISA plates were read visually and with ELISA plate reader. Samples were considered positive if absorbance values at 405nm exceed three times the mean of the negative controls.

Data Analysis: Sporozoite rates (SPR) were then determined as number of *Anopheles* mosquitoes found with *Plasmodium falciparum* (pf) circumsporozoite divided by total number of *Anopheles* mosquitoes analysed multiplied by 100 (WHO, 2003). Human blood index (HBI) was determined as number of female *Anopheles* mosquitoes found with human blood divided by total numbers of *Anopheles* mosquitoes analyzed (WHO, 2003). Man-biting rate was also determined as numbers of all blood-fed *Anopheles* mosquitoes divided by the number of occupants in the rooms multiplied by the HBI (Shililu et al., 1998).

RESULTS

Relative abundance of different endophilic mosquitoes collected in the community is presented in Figure 1. *Anopheles* mosquitoes (190) were the most abundant compared to *Culex* (21) and *Mansonia* (1). Higher numbers of *Anopheles* mosquitoes were collected in October (76) and April (62) than in January (24) and July (28). Conversely, the highest number of *Culex* mosquitoes was collected in January (10) compared to lower number in April (2). Only one *Mansonia* mosquito was encountered in the community in the month of January.

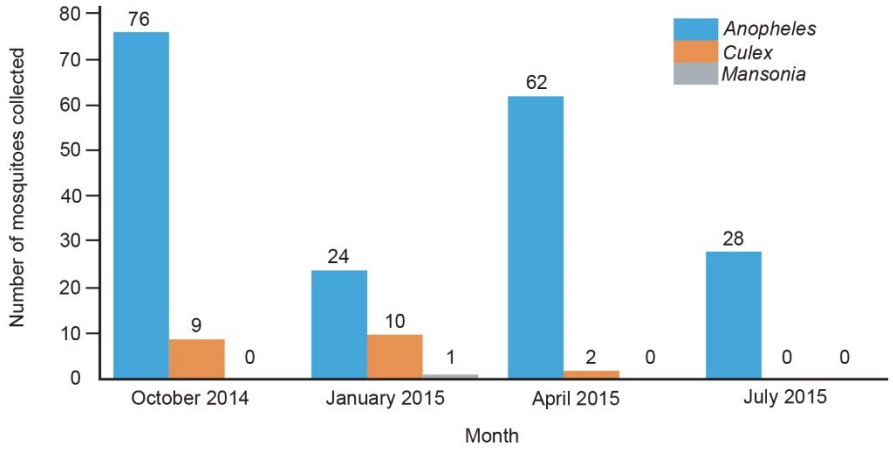

Fig. 1. Relative abundance of mosquitoes collected in Malete.

Molecular identities of *Anopheles* mosquitoes collected in the community are presented in Table 1. Predominance of *A. gambiae* (85,5%) over *A. arabiensis* (3,2%) and *A. coluzzii* (11,3%) sibling species were recorded all through the period of mosquito collection in the community. Highest numbers of *A. gambiae* were found in October (54) and April (60) compared to January (17) and July (28). Few *A. arabiensis* were encountered only in October (2) and January (4). Highest occurrence of *A. coluzzii* was recorded in October (16) compared to fewer numbers in January (3) and April (2).

TABLE 1

Molecular identities of *Anopheles* mosquitoes collected in Malete

Month	*A. Gambiae* N (%)	*A. Arabiensis* N (%)	*A. Coluzzii* N (%)
October	54(75)	2(2,8)	16(22,2)
January	17(70,8)	4(16,7)	3(12,5)
April	60(96,8)	0(0)	2(3,2)
July	28(100)	0(0)	0(0)
Total	159(85,5)	6(3,2)	21(11,3)

Human blood indices of all the *Anopheles* mosquito sibling species were generally high (0,82-1,0) in all the months when mosquitoes were found in the community (Table 2). *A. gambiae* had higher man-biting rates (0,92-3,64) compared to *A. arabiensis* (0-0,27) and *A. coluzzii* (0-0,84) (Table 3). Higher *A. coluzzii* man-biting rates were recorded in October (0,84) compared to other months. Equally, *A. gambiae* man-biting rates were higher in October (2,79) and April (3,64) compared to other months. *A. arabiensis* had the lowest man-biting rates recorded in January (0,27) and October (0,11), respectively (Table 3).

TABLE 2

Human blood indices of female *Anopheles* mosquitoes collected in Malete

Month	*A. gambiae*			*A. arabiensis*			*A. coluzzii*		
	No. with human blood	Total No. analysed	HBI	No. with human blood	Total No. analysed	HBI	No. with human blood	Total No. analysed	HBI
October	50	54	0,93	2	2	1,0	15	16	0,94
January	14	17	0,82	4	4	1,0	3	3	1,00
April	58	60	0,97	0	0	0,0	2	2	1,00
July	28	28	1,00	0	0	0,0	0	0	0,00

Human blood Index (HBI)=number of mosquitoes with human blood/total number of mosquitoes analysed.

TABLE 3

Man-biting rates of female *Anopheles* mosquitoes collected in Malete

Month	No. of sleepers	*A. Gambiae*			*A. arabiensis*			*A. coluzzii*		
		No. fed	HBI	MBR	No. fed	HBI	MBR	No. fed	HBI	MBR
October	18	54	0,93	2,79	2	1,00	0,11	16	0,94	0,84
January	15	17	0,82	0,92	4	1,00	0,27	3	1,00	0,20
April	16	60	0,97	3,64	0	0,00	0,00	2	1,00	0,13
July	19	28	1,00	1,47	0	0,00	0,00	0	0,00	0,00

Man-biting rates (MBR)= number of blood-fed mosquitoes/number of sleepers x HBI.

Plasmodium falciparum sporozoite infection was found only in a few of the *A. gambiae* mosquito samples collected in October and April when the highest numbers of this sibling species was recorded (Table 4). Sporozoite infection rate of the *A. gambiae* mosquitoes was higher in April (3,2%) than in October (2,8%). Overall sporozoite rates of *A. gambiae* mosquitoes was 2,9% (Table 4).

TABLE 4

Plasmodium falciparum sporozoite infection rates of *Anopheles* mosquitoes in the community

Month	Total No. of female A*nopheles*	No. (%) of *Anopheles* positive for *P. falciparum* sporozoites			Sporozoite rates of mosquitoes (%)
		A. gambiae	*A. arabiensis*	*A. coluzzii*	
October	72	2(2,8)	0(0,0)	0(0,0)	2,8
January	24	0(0,0)	0(0,0)	0(0,0)	0,0
April	62	2(3,2)	0(0,0)	0(0,0)	3,2
July	28	0(0,0)	0(0,0)	0(0,0)	0.0

DISCUSSION

This study elucidates the sibling species identities and *Plasmodium falciparum* transmission risk indices of *Anopheles* mosquitoes in Malete, a peri-urban area currently undergoing rapid development due to proximity to the Kwara State University Campus in Kwara State, Nigeria. In the rainy season within the same year, higher numbers of *Anopheles* mosquitoes were found in April and October (compared to July). Similar lower numbers of mosquitoes collected in July have been reported in other communities in Kwara and attributed to high rainfall frequency, which destabilizes available mosquito larval breeding sites and reduces the numbers of emerging adult mosquitoes in the mid-rainy season (Obembe et al., 2018a).

The predominance of *A. gambiae* mosquito sibling species in this study agrees with its ancestral status across Sub-saharan Africa (Lehmann & Diabate, 2008) due to faster larval development ability especially when exposed to competitors (Gimonneau et al*.,* 2014) and better use of the temporary breeding sites compared to *A. coluzzii*. Earlier studies with almost pure collections of *A. gambiae* in Guinea Savannah region where the present study site belongs have been reported (Awolola et al*.*, 2005). Low occurrence of *Anopheles arabiensis* and *A. coluzzii* compared to *A. gambiae* prevalence have been found in other rural and peri-urban communities in Kwara state (Obembe et al., 2018a; 2018b; 2019). The few numbers of *A. arabiensis* mosquitoes found in the present study were collected in January and October. *A. arabiensis* preference for arid conditions (Gillies & Coetzee, 1987) probably explains its highest occurrence during the driest season (January) compared to fewer numbers in October. Low occurrence of *A. coluzzii* is also attributable to the unavailability of its preferred relatively permanent water bodies such as irrigated rice fields (Gimonneau et al., 2014) needed to support its larval development around the study community. Nevertheless, a few other studies have found the preponderance of either *A. arabiensis* (Obembe et al., 2022; Oduola et al., 2016) or *A. coluzzii* (Oduola et al., 2021) compared to lower occurrence of *A. gambiae* in specific communities in Kwara state. This differential vector species compositions across communities within the same state attests to the importance of initial mosquito vector surveillance and molecular identification in order to guide the deployment of appropriate control efforts. The high *A. arabiensis* occurrence reported were attracted to cattle reared within the community (Obembe et al., 2022; Oduola et al., 2016;) while *A. coluzzii* prevalence

was attributed to rice field larval breeding site proximity to the study community (Oduola et al., 2021).

High human blood indices of the three *Anopheles* mosquito sibling species found in this study conforms with the strong anthropophagic tendencies of *A. gambiae* and *A. coluzzii* (Awolola et al*.,* 2005) and absence of vertebrate animal host alternatives for *A. arabiensis*. Accordingly, *A. gambiae* and *A. coluzzii* with higher numbers and stronger anthropophagic behaviours had higher man-biting rates compared to *A. arabiensis* with lower numbers and stronger animal blood feeding affinity (Killeen et al., 2016; Mayagaya et al., 2015).

October and April are perhaps the months to note for significant adult *Anopheles* mosquito availability and man-biting activities in Kwara State. The highest numbers and man-biting rates of *Anopheles* mosquitoes were recorded in this study and others conducted in Kwara state (Obembe et al., 2018a; 2018b; Oduola et al., 2016; 2021) during April and October. *Plasmodium falciparum* infection of *A. gambiae* was highest in April and October, the months with the highest numbers of mosquito collections.

Plasmodium falciparum sporozoite infection found in *A. gambiae* mosquitoes in this study suggests the species as the major vector of malaria in the community. However, this may not exclude *A. coluzzii* and *A. arabiensis* species from *P. falciparum* transmission in the community especially since this study is limited by the implementation of quarterly and not monthly mosquito collections in the community. Regular vector surveillance should be conducted in this community to understand the temporal dynamics of malaria parasite transmission for judicious timing and deployment of appropriate control measures.

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ETHICAL, CONFLICT OF INTEREST AND FINANCIAL STATEMENTS

The author declares that all pertinent ethical and legal requirements have been fully complied with, both during the study and in the production of the manuscript; that there are no conflicts of interest of any kind; that all financial sources are fully and clearly stated in the acknowledgements section; and that he fully agreed with the final edited version of the article. A signed document has been filed in the journal archives.

The statement of each author's contribution to the manuscript is as follows: A.O.: Study design, data collection and analysis. A.O.: Data collection. A.O: preparation and final approval of the manuscript.

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