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Indoor transmission dominance and vector efficiency of *Anopheles gambiae* s.s. among sibling species of the *Anopheles gambiae* complex in southeastern Nigeria

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Abstract

This study investigates the ecological and epidemiological roles of sibling species within the *Anopheles gambiae* complex, focusing on their behaviors and transmission indices impacting malaria dynamics. Utilizing human-baited CDC light traps and Pyrethrum Spray Catch, anophelines were collected from selected structures. Species-specific analysis revealed that 66/551(12%) of *Anopheles gambiae* s.l. were *An. gambiae* s.s., 258/551(47%) *An. coluzzii*, and 227/551(41%) *An. arabiensis*. The highest proportions of *An. gambiae* s.s.(6/10), *An. coluzzii* (15/46), and *An. arabiensis* (21/59) fed on human blood indoors in Awba-ofemili [42/115(36.5%). *Plasmodium falciparum* sporozoite rates in *An. gambiae* s.s. collected indoors ranged from 12.5% in Achalla to 18.2% in Awba-ofemili. Infective bites/person/annnum (ib/p/a) which is used to measure the entomology inoculation rate (EIR) were more frequent indoors, with *An. gambiae* s.s. recording 3 infective bites/person/annum in Achalla and 6.01 ib/p/a in Awba-ofemili. *An. coluzzii* showed 3 ib/p/a in Ebenebe and Awba-ofemili, while *An. arabiensis* recorded 3 ib/p/a indoors in Awba ofemili. No infective bites were recorded outdoors. The finding highlights the significant roles of *An. gambiae* s.s., *An. coluzzii*, and *An. arabiensis* in malaria transmission in the study communities. The finding of *An. gambiae s.s., An. coluzzii* and *An. arabiensis* transmitting malaria in the study communities calls for urgent malaria intervention that will target all the primary malaria species as the density and diversity of *Anopheles* species is highly influenced by availability of humans sleeping without bed nets. This study provides details into malaria transmission pattern in southeastern Nigeria as it suggests that malaria control programs should prioritize high-risk locations like Awba-Ofemili, where all three sibling species were active indoors with high EIRs. Although no infective bites were recorded outdoors across any site or species, the outdoor biting pressure of *An. arabiensis* in Ebenebe warrants attention: this species recorded the highest outdoor human biting rate across all the study communities (2 bites/person/night) and its predominantly exophilic and zoophilic behaviour means that standard indoor-targeted interventions would offer limited protection against its biting activity. Therefore, tailored vector control strategies addressing species-specific and spatial transmission patterns, including measures capable of

protecting against outdoor-biting species, are essential to reducing malaria incidence and achieving elimination goals.

Keywords: malaria, molecular, ecology, mosquito control, *Anopheles,* sporozoite, *Plasmodium*
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INTRODUCTION

Malaria transmission in sub-Saharan Africa is a huge public health concern, basically because of the abundance of malaria vectors that are very susceptible to the *Plasmodium* parasite (WHO, 2023). *Anopheles gambiae* s.l. and *An. funestus* complex are the major malaria vectors in sub-Saharan responsible for transmitting the deadliest malaria parasite, *Plasmodium falciparum* (Kelly-Hope *et al*., 2009). These complexes consist of sibling species that are efficient in malaria transmission (Awolola *et al*., 2005).

Analysis of deoxyribonucleic acid (DNA) extracted from *Anopheles* mosquitoes in a given geographic location have aided researchers to amplify specific genetic markers that differentiate the sibling species within the complex using Polymerase Chain Reaction (PCR) and identifies the malaria parasite species in the *Anopheles* species using Enzyme-linked Immunosorbent Assay (ELISA). This process has enhanced the identification of individual species/parasite away from the complex, provides valuable ecological insights into their distribution dynamics and reveals their transmission potential and implications of the control in malaria transmission (Onyango *et al*., 2022). Molecular characterization procedures have revealed that sibling species within the *An. gambiae* s.l. such as *An. gambiae s.s*., *An. arabiensis, An. merus* and *An. coluzzii* exhibit a different ecological preference and behaviours (Coluzzi *et al*., 2002), for example in their host-seeking behaviours, resting site preference or breeding site preference.

The high inclination of these sibling species to the *Plasmodium* parasite is the sustaining factor in malaria transmission as these vectors acquire the parasite through blood meals taken from an infected person and subsequently transmit to a non-infected person during the next blood feeding (Santos *et al*., 2019). The prevalence of malaria seen in Africa and the efficacy of the malaria vectors in combination with the persistence of *Plasmodium* parasite within the population (Olayemi *et al*., 2011), contributes to the continued stay of malaria transmission in sub-Saharan Africa.

Anopheles gambiae s.s. is considered as one of the most efficient malaria vectors, particularly responsible for transmitting *Plasmodium falciparum* (Zoh *et al*., 2020) while *An. coluzzii* has been found to be in close association with the *An. gambiae* s.s. and it is also highly competent in transmitting malaria (Akogbeto *et al*., 2018). *An. arabiensis* is a very important malaria vector in arid areas characterized by its ability to adapt to various harsh ecological conditions and persistently transmit malaria parasites (Sy *et al*., 2023).

Numerous studies (Akpan *et al*., 2018; Awolola *et al*., 2007, Irikannu *et al*., 2019, Oringanje *et al*, 2011) have documented the transmission potentials of these sibling species and other *Anopheles* species. These studies have shown good evidences into the malaria transmission dynamics and vectorial capacities connected with specific sibling species. For example, the high anthropophily and endophily of *An. gambiae* s.s. and *An.*

coluzzii are considered mainly for sustaining malaria transmission in many sub-Saharan regions as the vectors deliberately increases contact with humans by biting and resting indoors. *Anopheles arabiensis* have demonstrated high exophagic and exophilic behaviour and mostly prefer to bite animals except when the animals are not available (Massebo *et al*., 2015). These studies employed diverse methodologies including entomological surveys, laboratory infections and molecular assays to assess the transmission potential of these species. By understanding the specific characteristics and behaviours of the *Anopheles* species, researchers are developing targeted interventions and strategies to mitigate malaria transmission.

Malaria remains a major public health burden in Anambra State, as in the rest of southeastern Nigeria with *Plasmodium falciparum* accounting for nearly all infections nationally (WHO, 2019). Awka North LGA lies within the rainforest belt characterized by eight-month rainy season (April to November) and a four-month dry season (December to March) (NIMET, 2019). This pronounced seasonality is epidemiologically important, since malaria transmission in southeastern Nigeria has previously been shown to track rainfall and humidity patterns, with vector abundance and biting activity rising sharply during the rainy months (Ezihe *et al*., 2017). The state has implemented several interventions like IRS supported by World Bank-Malaria Booster States from 2009-2014 to supplement Insecticidal Treated Nets (ITNs). The study area had specifically undergone two rounds of IRS prior to data collection, in addition to the routine LLIN coverage in the communities.

Despite the numerous bodies of information on the behavioural ecology of *An. gambiae* s.l., there remains a critical gap in the transmission-level data for southern Nigeria. In Awka North LGA where this study was carried out, Aribodor *et al*., (2016) characterized the species composition, abundance, indoor/outdoor biting behaviour following an indoor residual spraying exercise in some of the communities. The authors recorded zero infection in all dissected *An. gambiae* s.l. and *An. moucheti* providing the baseline entomological data without sporozoite or entomological inoculation rate data. Similarly, in the study of Awolola *et al*. (2018), of which one of the sites (Achalla) used in this study served as one of the national sites for pyrethroid resistance monitoring. The study established the presence of *An. gambiae* s.s population with low resistance intensity, but this study was focused more on insecticide resistance rather than malaria transmission indices. Neither study determined nor established the sporozoite rat or the EIR of *Anopheles* vectors in this part of Anambra State. This represents an important gap in the malaria transmission of which this present study seeks to address and by building directly on the entomological baseline established by Aribodor *et al*. (2016) and the resistance profile established by Awolola *et al*. (2018) for this part of Anambra State.

The study aims to investigate the ecological and epidemiological significance of these sibling species within the *Anopheles gambiae* complex, examining their behaviours and environmental conditions that impacts on their intensities and dynamics in malaria transmission. Additionally, the study intends to outline the implication of the presence of sibling species in malaria control measures in the study location.

MATERIALS AND METHODS

The study was conducted in Awka North Local Government Area of Anambra State. Awka North Local Government Area has geographical coordinates of 6^0 15’ 0” N,

7^0 10’ 0” E of the Equator. It covers an area of land of about 116.41km with a population of 112,192 inhabitants according to the Nigeria Population Commission (2006). The study area has been the subject of prior entomological and resistance surveillance (Aribodor *et al*., 2016; Awolola *et al*., 2018), as detailed above.

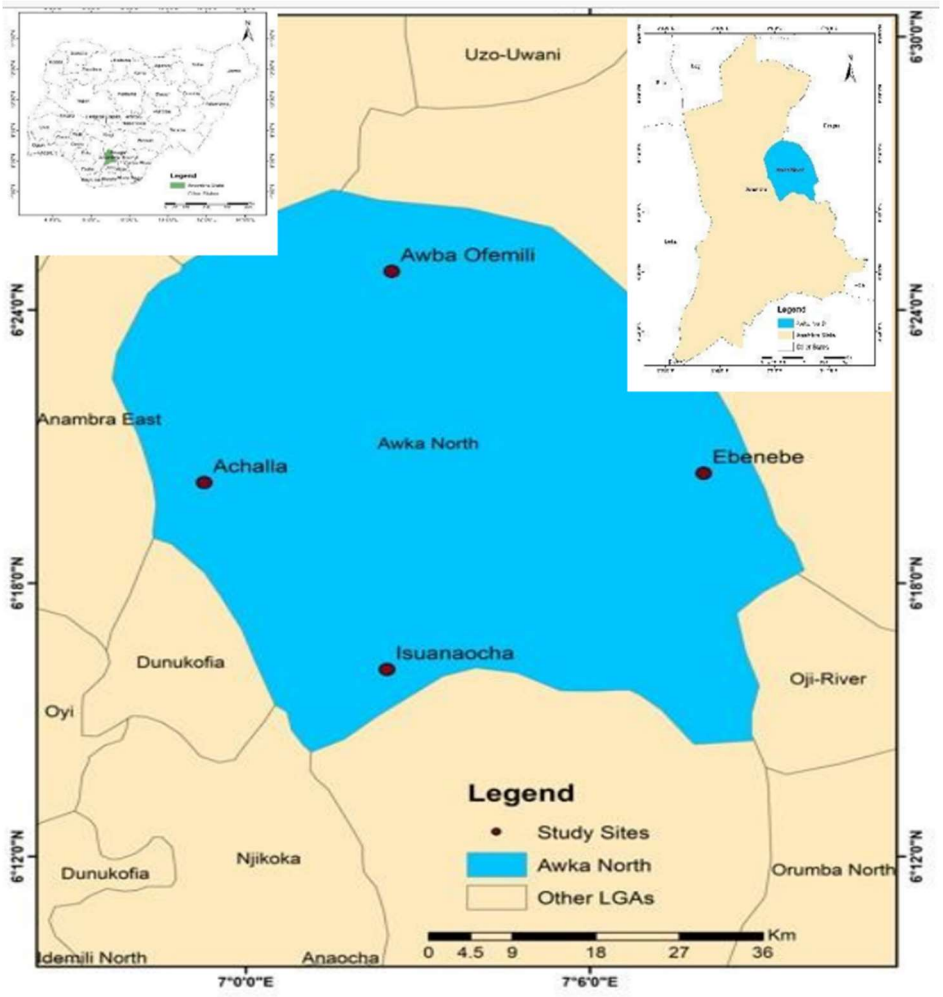

Figure 1: Map of Awka North Local Government Area showing the sites (using geographical coordinates of the communities with QGIS Geographic Information System, version 2.10.1 ‘Pisa’; QGIS Development Team).

This study was a longitudinal entomological collection were carried out over a one-year period, from August 2020 to July 2021, spanning both wet and dry seasons in the study communities. Mosquito collections were carried out monthly across the four study communities (Achalla, Ebenebe, Isuanaocha and Awba-ofemili) throughout this period. For the human-baited CDC light trap collections, one sentinel structure was selected per community (four in total), with paired indoor and outdoor light traps running concurrently at each structure on three consecutive nights monthly, giving a total of 144 indoor trap-nights and 144 outdoor trap-nights (4 structures * 3 nights* 12 months = 144 each), or a 288 trap-nights across the 12 months study. Pyrethrum spray

catch collections were carried out monthly across 40 sleeping rooms (ten rooms per community) over the same 12 months study period. These collections form the bench denominator for the human biting rate and entomological inoculation rate calculations.

A total of 44 houses (10 rooms for PSC and 1 room for human-baited CDC LT) were selected across the four study communities using stratified random sampling. Selection criteria included suitability of structure for mosquito collection, presence of children under-five in the household, household head consent and willingness of the family to partake for the full duration of survey. The PSC and human-baited CDC light trap structures were distinct as there was no overlapping sets of houses for the two collection methods. These 44 structures were maintained throughout the 12 months survey periods. Written consents were obtained from the heads of the household.

The longitudinal survey used the human-baited CDC light traps to trap mosquitoes in the four structures selected, to determine the endophagic and exophagic, biting rates of malaria mosquitoes and other entomological indices. The CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) positioned 1meter above the floor at the foot end of the bed where a person sleeps under an untreated bed-net. The light trap indoors and outdoors were set up at 18.00h with hourly collections to 06.00h.

The pyrethrum spray catch collection method of WHO (1975) was used to collect indoor resting anophelines from forty structures. The collection was between 06.00h-08.00h and recorded according to the time of collection.

Anopheles mosquitoes collected were morphologically identified with the keys of Coetzee (2020) at the Disease Surveillance and Epidemic Response laboratory of National Arbovirus and Vectors Research Centre (NAVRC), Enugu before they were sent for molecular bioassay at the molecular unit of the Molecular Entomology and Vector Control Research laboratory, Nigerian Institute of Medical Research (NIMR), Lagos.

Anopheles gambiae species determination

Deoxyribonucleic acid was extracted from all the *Anopheles gambiae* s.l. collected using extraction kits. The genomic DNA samples were utilized for species identification using the protocol of Scott (1993). Characterization of *An. gambiae* s.l. with PCR. The amplification of PCR was carried out using the *An. gambiae* species-specific multiplex PCR. PCR products were separated into agarose gel stained with ethidium bromide and visualized under an ultraviolet (UV) transilluminator. SINE 200 PCR (Santolamazza *et al*., 2008) protocol was used to differentiate *Anopheles gambiae* s.s*., An. coluzzii* and *An. arabiensis*. All PCR negative samples were repeated for confirmation.

Sporozoite infection rate

Plasmodium parasites were detected for each *Anopheles gambiae* species stored on desiccant using an enzyme-linked immunosorbent assay (ELISA) technique (Wirtz *et al*., 1987). ELISA tests for *Plasmodium* species were carried out on all *An. gambiae* s.l. mosquitoes that were successfully amplified by species-specific PCR. The head and thorax of each female *Anopheles* mosquito were crushed in phosphate-buffered saline and tested for circumsporozoite antigen using an ELISA assay (Burkot *et al*., 1984)**.**

Human biting rate (HBR)

The human biting rates were estimated with human-baited CDC LT collected *Anopheles* mosquitoes that fed on humans. It

was obtained by calculating the number of mosquitoes collected per human-baited CDC LT per night per number of people protected by each light trap used. For the human-baited CDC light trap collections, species-specific HBR was calculated as: HBR = N/T, where N = number of female *Anopheles* of a given species collected by the traps and T is the total nights of trapping, calculated as the number of traps multiplied by the number of nights of collection (one trap per site x 36 nights per site over the 12 months study at 3 nights per month). For the pyrethrum spray catch collections, HBR was calculated separately as HBR = F/W, where F is the number of freshly blood-fed *Anopheles* collected and W is the total person per number of days PSC was carried out and calculated as the number of human occupants who slept in the house the night before multiplied by the number of nights of collection. The species-specific HBR values obtained by each method were used to calculate the entomological inoculation rate.

Determination of blood-meal source

Human Blood Index (HBI) analysis was carried out on mosquitoes collected using PSC and CDC light trap methods according to [19]. Each blood meal sample was tested against human and goat/sheep blood meal antigens. The results of all ELISA assays were scored visually before using the ELISA plate reader. All negative tests were re-confirmed (Beier, 1998).

Entomological inoculation rate (EIR)

EIR is a widely used entomological indicator for assessing the potential risk of transmission of vector-borne diseases. It represents the average number of infective bites an individual receives within a year and is typically calculated using the human biting rate and the sporozoite rate (PMI, 2021).

EIR = HBR X Sporozoite Rate

Analysis of data

The data generated were analyzed using statistical package for social sciences (SPSS), version 20 and microsoft excel. Descriptive statistics (frequencies and percentages) were used to summarize the species composition, blood-meal source, human biting rate, sporozoite rate and EIR across communities, collection methods and collection locations (indoor and outdoor). Chi square was employed to assess differences in the blood-feeding preference of *Anopheles* mosquitoes between human and goat, with statistical significance at $p<0.05$.

RESULTS

Molecular identification of members of the An. gambiae complex

A total of 800 *An. gambiae* s. l. mosquitoes collected using PSC and human-baited CDC LT were subjected to species-specific PCR assays (Table 1). Of the total samples analyzed, 551 (69%) mosquito samples amplified while 249 (31%) failed to amplify (Table 2). Results showed that 66 (12%) were identified as *An. gambiae* s.s., 258 (47%) were *An. coluzzii*, 227 (41%) were *An. arabiensis*.

Table 1: PCR Identification of sibling species of *Anopheles gambiae* s. l.

***Anopheles gambiae* s.l. analysed**	**Communities**				**Number of species-specific analysed (%)**
	Achalla	Ebenebe	Isuanaocha	Awba-ofemili	
An. gambiae s. s.	15	9	22	20	66 (12)
An. coluzzii	73	38	61	86	258(47)
An. arabiensis	52	72	47	56	227(41)
Total amplified	140	119	130	162	551(69)
Unamplified	60	81	70	70	249(31)
Number analysed	200	200	200	200	800(100)

A total of 275 *Anopheles gambiae* s.l. mosquitoes were analyzed for blood meal sources, with 197 (71.6%) feeding on humans (Figure 2) and 78 (28.4%) showing preference for goat bloodmeal (Figure 3). Among the species identified, *An. coluzzii* had the highest number of human-fed mosquitoes (91/197; 46.2%), followed by *An. arabiensis* (90/197; 45.7%), while *An. gambiae* s.s. accounted for only 16/197 (8.1%) of the human-fed mosquitoes (Figure 2). In contrast, for those that fed on goat blood (figure 3), *An. coluzzii* (44/78; 56.4%) was still the most predominant, followed by *An. arabiensis* (31/78; 39.7%), and *An. gambiae* s.s. (3/78; 3.8%).

A Chi-square test was conducted to compare the feeding preference between humans and goats. The analyzed result showed a statistically significant difference ($\chi 2=51.49$, $p<0.0001$), indicating that mosquitoes had a significantly higher preference for human blood compared to goat blood

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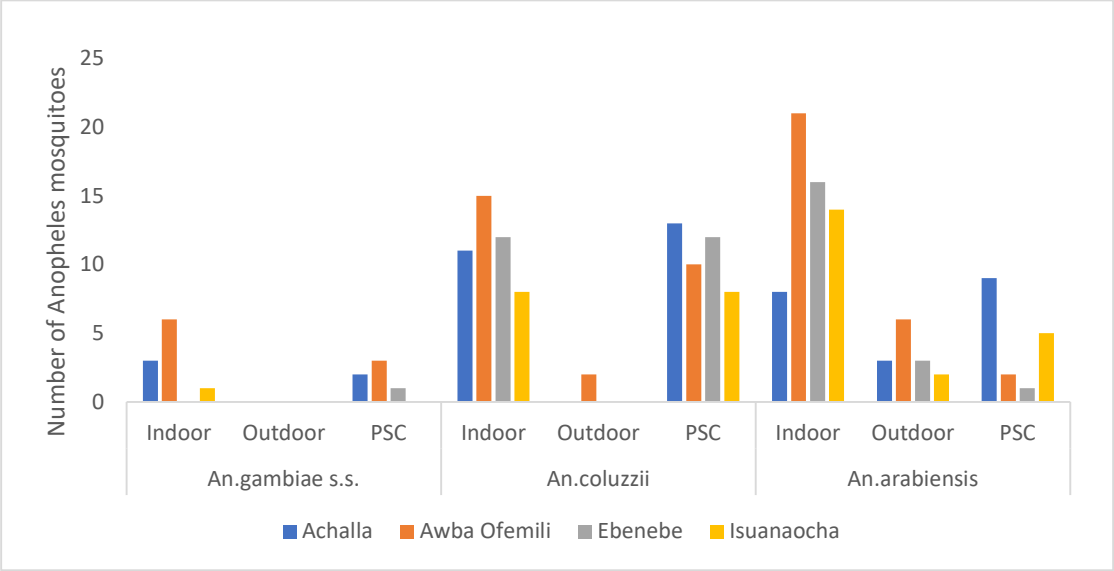

Figure 2: The blood meal sources of *An. gambiae s.s., An. coluzzii*, and *An. arabiensis* from humans, collected using PSC and human-baited CDC light trap across the sites.

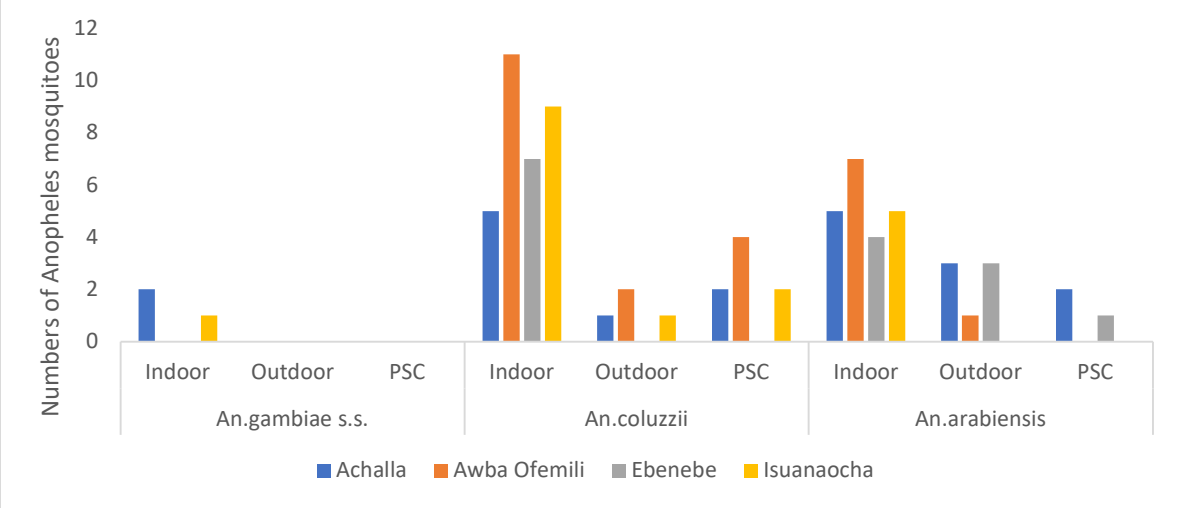

Figure 3: Blood meal sources of *An. gambiae s.s., An. coluzzii*, and *An. arabiensis* from goat, collected using PSC and human-baited CDC light trap across the sites.

Human biting rate (HBR) of An. gambiae species

Using the formulae described in the Methods section, the species-specific human biting rate (HBR) for *Anopheles* mosquitoes was calculated from the 551 PCR-confirmed specimens. From the mosquitoes collected using human-baited CDC LT, human biting was more pronounced indoors than outdoors. *An. gambiae* s.s. had an average indoor biting of (1 bite/person/night) in all the communities. Indoor biting of *An. coluzzii* was highest indoors in Awba-ofemili with 2 bites/person/night. Outdoor biting of *An. coluzzii* in Awba-ofemili and Achalla was observed as 1 bite/person/night. *Anopheles arabiensis* recorded the lowest human biting rate indoors and more biting outdoors. For indoor biting, *An. arabiensis* had an average of 1 bite/person/night in each of the communities while outdoor biting of *An. arabiensis* was highest in Ebenebe with 2 bites/person/night and 1bite/person/night in Isuanaocha, Achalla and Awba-ofemili respectively (Table 1).

Sporozoite rate determination of the An. gambiae species

Out of the 800 *An. gambiae s.l.* collected, 551 were analyzed using ELISA to detect *Plasmodium falciparum* sporozoites. The sporozoite rates varied across species, locations and collection methods. *An. gambiae s.s.* had the highest sporozoite rate indoors, ranging from (1/8, 12.5%) in Achalla and (2/11, 18.2%) in Awba-Ofemili and no sporozoites detected in the collections from Ebenebe and Isuanaocha. No sporozoites were detected in *An. gambiae s.s.* collected outdoors or through PSC. For *An. coluzzii*, indoor sporozoite rates ranged from (1/54, 1.9%) in Awba-Ofemili and (1/21, 4.8%) in Ebenebe and no sporozoite were recorded in Achalla and Isuanaocha. No sporozoites were detected in outdoor collections, while PSC collections recorded a rate of (1/30, 3.3%) in Awba-Ofemili and 0% elsewhere. *An. arabiensis* recorded a (1/16, 6.3%) sporozoite rate indoors in Awba-Ofemili, while no sporozoites were detected in outdoor collections. However, PSC collections showed a (1/10, 10%) sporozoite rate in these species (Table 1). Overall, *An. gambiae s.s.*

had the highest sporozoite rate (2/11, 18.2%) in Awba-Ofemili, while *An. arabiensis* recorded the highest PSC sporozoite rate (1/10, 10%). No sporozoites were detected in mosquitoes collected outdoors across all species. Given the small denominators involved, particularly outdoors and PSC subsamples, single positive finding should be interpreted carefully as a single additional positive.

EIRs were recorded indoors in three of the four sites (Achalla, Ebenebe and Awba-ofemili) among the mosquito species. As seen in table (1), there were more infectious bites recorded indoors among *An. gambiae* s.s. (3 infective bites/person/annum in Achalla and 6.01 infective bites/person/annum in Awba-ofemili). The indoor EIR recorded with *An. coluzzii* was seen at Ebenene (3 infectious bites/person/month) and Awba-ofemili (3 infective bites/person/annum). The infective bite with *An. arabiensis* indoors was seen at Awba-ofemili with 3 infectious bites/person/annum). There was no infective bite recorded outdoor observed across the sites.

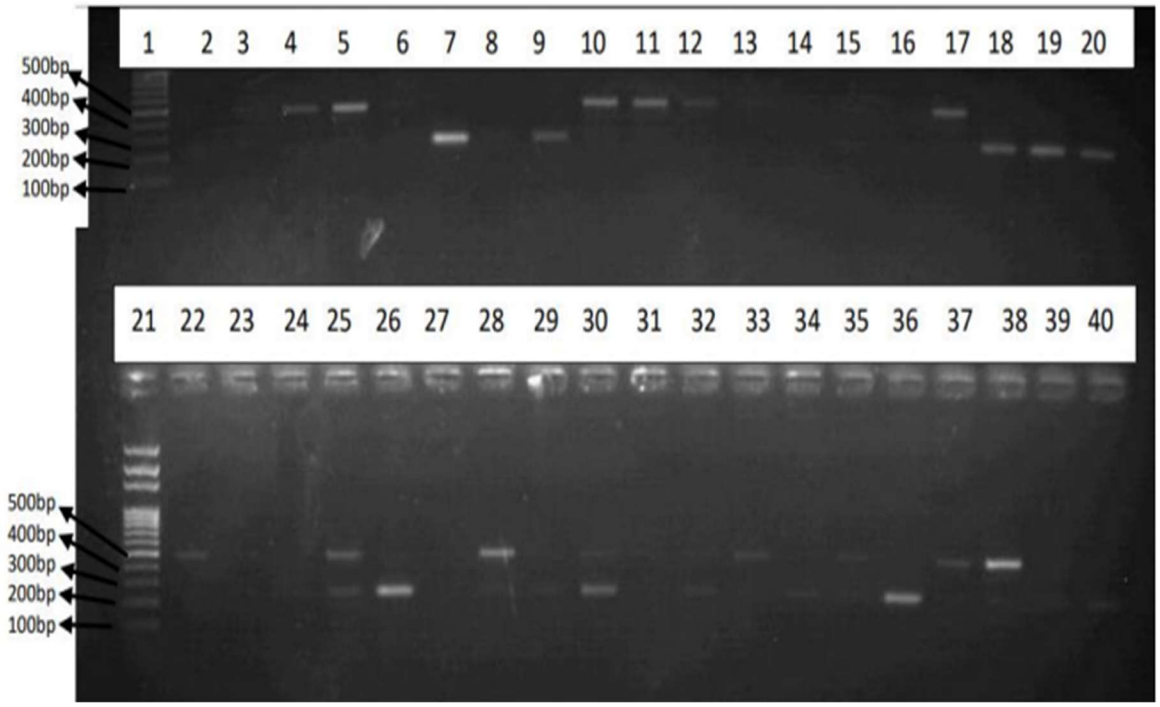

Plate 1: Molecular identification of *Anopheles gambiae* s.l. sibling species using SINE 200 PCR by Santolamazza *et al*., (2008). The protocol is used to differentiate *Anopheles gambiae* s.s., *An. coluzzii* and *An. arabiensis*

Table 2: Entomological inoculation rate (EIR) of the mosquitoes across the different sites

An. gambiae s.s.

	Indoor			Outdoor			PSC		
Location	HBR	SPR	EIR= HBR*SPR	HBR	SPR	EIR= HBR*SPR	HBR	SPR	EIR= HBR*SPR
Achalla	0.22	12.5	3	0.08	0	0	0.19	0	0
Ebenebe	0.14	0	0	0	0	0	0.08	0	0
Isuanaocha	0.47	0	0	0.06	0	0	0.17	0	0
Awba-ofemili	0.31	18.2	6.01	0.08	0	0	0.06	0	0

An. coluzzii

	Indoor			Outdoor			PSC		
Location	HBR	SPR	EIR= HBR*SPR	HBR	SPR	EIR= HBR*SPR	HBR	SPR	EIR= HBR*SPR
Achalla	1.08	0	0	0.19	0	0	0	0	0
Ebenebe	0.58	4.8	3	0.08	0	0	0	0	0
Isuanaocha	0.92	0	0	0.17	0	0	0	0	0
Awba-ofemili	1.5	1.9	3	0.06	0	0	0.03	3.3	0.01

An. arabiensis

	Indoor			Outdoor			PSC		
Location	HBR	SPR	EIR= HBR*SPR	HBR	SPR	EIR= HBR*SPR	HBR	SPR	EIR= HBR*SPR
Achalla	0.25	0	0	0.86	0	0	2	0	0
Ebenebe	0.39	0	0	1.33	0	0	1.67	10	0.17
Isuanaocha	0.22	0	0	0.92	0	0	1	0	0
Awba-ofemili	0.44	6.3	3	0.61	0	0	3	0	0

DISCUSSION

In the southeast Nigeria, *Anopheles gambiae* s.s. have been identified as the major malaria vector as seen in the studies of (Irikannu *et al*., 2019; Awolola *et al*., 2018, Chukwuekezie *et al*., 2020, Obi *et al*., 2021 in Anambra state. The molecular characterization of the *An. gambiae* s.l. in this study was useful in defining the ecological and behavioural distribution across the study sites as the study found three main *Anopheles* species in the study communities. Based on species abundance, *An. coluzzii* (47%) was the predominant species trailed by *An. arabiensis* (41%) and *An. gambiae* s.s. (12%). The surprising finding of the high percentage of *An. arabiensis* in the south is in line with the findings of Amawulu *et al*., (2012) in Bayelsa State, Nigeria. The study indicated that the progressive adaptation of *An. arabiensis* to more humid area highlights the limitation of the exclusive reliance on distributional data or boxing the species to the sahel regions or even the use of spatial imaging to predict species presence in any location. The observed change in the *Anopheles* species found in the study which differs from other findings about the non-cohabitation of the *Anopheles gambiae* species could be credited to ecological factors like vegetative cover, availability of breeding sites even as *An. coluzzii*, *An. gambiae* and *An. arabiensis* have

been found co-breeding in a rice farm and other breeding habitats as seen in the findings of Obi *et al*., 2021, Amawulu *et al*, 2012).

The sporozoite rate result found that *An. gambiae* s.s. indoors had a higher sporozite rate (12.5%) in Achalla and Awba-ofemili (18.2%) than *An. coluzzii* and *An. arabiensis.* The study of Irikannu *et al*., (2019) in Awka South found that *An. gambiae* s.s. could be the main vector in their study area. *An. coluzzii* had a lower sporozoite rate of 4.8% and 1.9% indoors from Ebenebe and Awba-ofemili respectively while *An. arabiensis* recorded sporozite rate of 6.3% indoors in Awba-ofemili. The study of Ekoko *et al*., (2019) in a rice farm area of Cameroun observed the same pattern of result where *An. gambiae* s.s. had the highest infectivity rate. Obviously, the sporozoite rate is one of the numerous factors contributing to malaria transmission as seen in the study of PMI (2021) and Samdi, (2012). However, the finding in this study on infectivity rate is not in conformity with the study of Msugh-Ter *et al*., (2014) in Markurdi where a very high sporozoite rate of 31.5% for *An. gambiae* s.l. was observed.

The human blood index of *An. gambiae* s.l. collected in the study shows that there was a higher level of *Anopheles* mosquitoes and human contact in the study area than with animals (goat) as seen in the study of Logue *et al*., (2016). More of the *Anopheles* species collected indoors using human-baited CDC LT had blood meal sourced from humans than those collected using PSC. The finding has an important implication for malaria control strategy, suggesting that ITNs and IRS are effective in reducing malaria transmission by targeting the indoor biting *Anopheles* mosquitoes that have a high biting preference for human blood. *An. arabiensis* had more human blood meal from outdoors and had blood meal from goats. This is in line with the studies of Akogbeto, (1995) and Ijumba *et al*., (1990) that *An. arabiensis* tends to exophilic behaviour. Some level of zoophilism were exhibited by *An. arabiensis* as they had more bloodmeal from goats (Mahande *et al* 2007). Similar findings on the zoophilic tendencies of *An. arabiensis* have been reported by (Sy *et al*., 2023, Mutero *et al*., 2004). *An. coluzzii* was observed to exhibit some level of zoophilism, with a notable proportion of blood meals from goats. The present study did not collect data on household structure layout, the proximity of livestock pen to human sleeping areas or the frequency of human contact with animal shelter, so no specific attribute can be aligned to this observation from the study. Although the finding is however consistent with the phenomenon of passive zooprophylaxis and livestock-associated host harbouring in the study of Bogh *et al*., (2002). This specific factor driving the observed goat blood meal frequency in *An. coluzzii* in this study requires more investigation

The findings of higher indoor human biting by *An. gambiae* s.s. and *An. coluzzii* than *An. arabiensis* is in line with the previous findings of PMI (2022) in Nigeria that *An. gambiae* and *An. coluzzii* show more anthropophilic and endophilic behaviour than *An. arabiensis*. The higher indoor biting of *An. coluzzii* observed in Awba-ofemili is probable to be the high abundance of this species as well as the presence of conducive indoor resting sites. As already stated above, this pattern of predominantly outdoor biting and zoophilic feeding in *An. arabiensis* is well established in the literature and has direct implications for vector control in the study communities, as indoor-based interventions such as LLINs and IRS would be expected to have a comparatively limited effect on this species relative to *An. gambiae* s.s. and *An. coluzzii*

The HBR of the *Anopheles* species differs slightly across the sites as communities like Achalla and Awba- ofemili where heavy rice cultivation is going on had more HBR than the other communities. Similar trend in biting rate was observed in the study of Fondjo *et al*., (2023) where rice paddies were recognized as a very good breeding habitats contributing factor to an increase in human biting by the anophelines in these communities. Biting in the communities became intense during the rainy season which is also the timing for rice farming. In the month of November, biting peaked in Awba-ofemili and this coincides with the aftermath of flooding that has been an annual occurrence in the location. Biting was seen to peak in the month of July at Isuanaocha which was the peak of rainfall in the study site.

The EIR observed in this study varied across locations and species as *An. gambiae* s.s. recorded an overall EIR of 9.01ib/p/a across the study sites. *An. coluzzii* and *An. arabiensis* recorded an overall EIR of 6.10 ib/p/a and 3.02 ib/p/a respectively across the sites the EIR recorded in this study contrasts with the findings of Ogbuefi and Aribodor (2023) in Njikoka, Awka North and Awka South LGA, respectively, where *An. gambiae* s.s. was identified, with no sporozoite-positive mosquitoes detected and the EIR could not be determined. The difference in absolute EIR can be interpreted by influence in the methodological and ecological factors beyond the transmission intensity.

The EIR of 12.11 ib/p/a across the *Anopheles* species (*An. gambiae* s.s., *An. coluzzii* and *An. arabiensis*) collected in Awba-ofemili was high compared to other communities because of the rice cultivation in the wetland area bounding the community to other states. This finding of higher EIR of 12.11 infective bites/person/year in the wetland area of the study area was also observed in the finding of Amawulu *et al*., 2016, Noutcha and Amawulu, (2010) and Noutcha, (2007). This is worth noting that the EIR seen in this study was only from *Anopheles* species collected indoors as none showed positive for *Plasmodium falciparum* from the *Anopheles* species collected outdoors. This is in line with the previous reports of Atieli *et al*., (2011) in Western Kenya and PMI (2019) in Nigeria. There was no recorded EIR in Isuanaocha. The study did not collect data on urbanization level, insecticide access or household wealth in the study and cannot attribute this finding to any specific cause. One possible hypothesis requiring direct check in the future work is the possibility of urbanization and associated amenities in Isuanaocha relative to other communities could be associated with greater access to malaria control tool. The suggestion of this hypothesis is in consonance with the observation of Ezihe *et al*. (2021).

One profound limitation was a notable proportion of the *An. gambiae* s.l. mosquitoes (249/800, 31%) failing to amplify by species-specific PCR and were excluded from other analyses. This failure rate is higher than is typically reported for fresh or properly preserved mosquito samples and attribute it primarily to prolonged sample preservation prior to molecular analysis.

The finding of *An. gambiae s.s., An. coluzzii* and *An. arabiensis* transmitting malaria in the study communities calls for urgent malaria intervention that will target all the primary malaria species as the density and diversity of *Anopheles* species is highly influenced by availability of humans sleeping without bed nets. This study provides details into malaria transmission pattern in southeastern Nigeria as it suggests that malaria control programs should prioritize high-risk locations like Awba-Ofemili, where all three sibling species were active indoors and EIRs highest. Although no infective bites were recorded outdoors across any site or

species, the outdoor biting pressure of *An. arabiensis* in Ebenebe warrants attention: this species recorded the highest outdoor human biting rate across all the study communities (2 bites/person/night), and its predominantly exophilic and zoophilic behaviour means that standard indoor-targeted interventions would offer limited protection against its biting activity.

Tailored vector control strategies addressing species-specific and spatial transmission patterns, including measures capable of protecting against outdoor-biting species, are essential to reducing malaria incidence and achieving elimination goals.

CONCLUSION

This study demonstrates that malaria transmission in Awka North LGA is sustained by three primary sibling species of the Anopheles gambiae complex, with *An. coluzzii* being the most abundant while *An. gambiae* s.s. exhibited the highest sporozoite rates and transmission potential. Indoor malaria transmission remained predominant, particularly in Awba-ofemili, where the highest human biting and entomological inoculation rates were recorded, pointing to a noticeable spatial variation in transmission intensity. The detection of *An. arabiensis* alongside its predominantly outdoor biting behaviour suggests that reliance on indoor interventions alone may not be sufficient for sustained malaria control. These findings strengthen the need for integrated, species-specific vector control strategies that combine conventional indoor interventions with complementary measures targeting outdoor transmission in high-risk communities.

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