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Detection of Plasmodium Sporozoites and Blood-Meal Source in a Population of Anopheles Coustani Senso Lato in Kakamega County, Western Kenya

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Abstract:
Background
Re-emerging of malaria vectors in the highlands of Western Kenya pose a challenge to malaria eradication efforts. Anopheles coustanis.l is a sub-Saharan mosquito species implicated in transmission of malaria in many parts of Africa as a secondary vector. It is zoo-anthropophilic species that has been assumed to be of negligible importance which may not be the case. This study therefore aimed at getting the malaria vectorial system of the study area, has tried to determine the relative abundance of the vectors and a new outdoor malaria vector.
Methods
A cross sectional study was carried out in April to June, 2020 in Eluche location, Mumias East sub-County, Kakamega County, Kenya. Pyrethrum spray collections (PSC) and Centers for Disease Control (CDC) and prevention light traps were used for sampling mosquitoes. Mosquitoes were collected both indoors; between 0700h and 1100h using PSC and outdoors between 1800h and 0700h using CDC light traps. All mosquitoes were identified morphologically and female Anopheles' heads and thorax were analyzed using Polymerase Chain Reaction (PCR) for Plasmodium sporozoite detection and blood-meal source identification.
Results
A total of 376 female Anopheles mosquitoes were collected composed of: An. coustanis.l, 42.55%; An. funestus, 27.66%; An. maculipulpis, 25.00%; An. arabiensis, 4.26% and An. gambiaes.s, 0.53%. Malaria sporozoites were detected in only An. coustani (1.06%).
Conclusion
There is a possibility of Anopheles coustani mosquito involvement in malaria transmission in Mumias east, Kakamega County.

Keywords: *Anopheles coustani, malaria vectors, Plasmodium falciparum*

1. Background

Anopheles gambiae, An. arabiensis, An. funestus, An. Nili and *An. moucheti* are Africa's known primary malaria vectors (1,3). These vectors play a major role in malaria transmission and its sustenance (3–5). Studies have shown that *An. coustani, An. pharoensis, An. ziemnni, An. rivolorum*and *An. maculipulpis*are involved in malaria transmission as secondary vectors (3,5,6). However, scanty information exists on the involvement of *An. coustani*in disease transmission as

a secondary vector.(7–9). Secondary vectors are considered to have minimal or no importance in malaria transmission (10). In-depth knowledge on feeding and resting behavior of these vectors is key in malaria control strategies (11–13). To contribute to strategic malaria control program, it is vital to understand malaria transmission in terms of prevalence, incidence and mortality (14,15). Currently, long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are the base plans for vector elimination (7,16–18), which are however less effective against exophilic and exophagic vectors including *Anopheles coustani*.

Transmission of malaria has been attributed to vector presence, distribution, abundance, competence, capacity, (19,20) and of late shift in vector composition (21). Even though there is evidence of outdoor transmissions in some parts of sub-Saharan Africa, the available entomological scrutiny and monitoring tools focus on indoor mosquito populations which have changed in composition and feeding behavior (22). Vector abundance determines malaria transmission hence disease prevalence (19,23). In this relation therefore, this study tried to get the malaria vector species composition and the individual abundance.

Insecticide resistance especially of pyrethroids that are used for vector control is on the rise (24–26). Outdoor malaria transmission phenomenon has been reported (28,29). Recent studies have also documented shift in vector behavior from late night biting to early evening and morning biting (28,29). Increased use of LLINs, IRS and ITNs has seen vector behavioral change to zoophagic, exophagic and exophilic(21). Stable transmission of malaria in Western Kenya highlands is supported by the biome, both micro and macroclimates. Equally, human population pressure has seen changes in land use; valley bottom crop cultivation, deforestation and reclamation of swamps (30,31). In view of increasing concerns over residual malaria transmission, there is need to understand the behavior and feeding pattern of the secondary vectors. The current study evaluated the possible role of *An. coustani*in malaria parasite transmission in Mumias East Sub-County, Kakamega County.

Malaria vectors, mosquitoes, have potentially assorted feeding hosts necessitating the establishment of blood feeding habits. This is crucial in the understanding of transmission dynamics(32). The blood meal sources determine the feeding rates in turn the survival of the adults(33). Opportunistic transmissions in this area are majorly propagated by the human activity and lifestyle. The present study determined *Plasmodium* sporozoites and blood-meal source in a population of *Anopheles coustanisensola to* in Kakamega County, Western Kenya

2. Methods

2.1. Study Area and Design

A cross sectional survey was carried out at Eluche location in Mumias east Sub-County of Kakamega County in Western Kenya; approximately 25 KM South-west of Kakamega town, the County headquarters. The study site is located at 00.34120 E and 034.54727 N with an average elevation of 1315 meters above sea level. The terrain of the area is fairly undulating with swampy valleys slopping to the west. The area receives adequate bimodal rainfall with a monthly mean of 222 mm (34) . Human settlement is clustered and people stay outdoors up to very late in the evening in family social gatherings which could be enhancing opportunistic feeding. The living standards are low, evidenced by grass thatched houses and semi-permanent buildings which could be providing hiding places for the vectors. Agricultural activities in the area include sugarcane farming and mixed small scale farming (livestock; cattle, goat sheep, rabbit and poultry: horticulture; beans, kales, cabbages and indigenous vegetables and maize).

2.2. Household Survey

Two hundred and twenty houses were randomly selected based on the presence of aquatic habitats, house accessibility and characteristic; grass thatched, iron roofed, screened, unscreened, new or old. Distance between the houses with similar characteristics ranged from 50 to 100 meters apart. Written consent was issued for signing by the household head or representative after giving a go ahead during survey for sampling. Where consent was not granted, the next house was taken for sampling.

2.3. Mosquito Sampling and Processing

Standard Centers for Disease Control and prevention, (CDC) light-traps were used to trap mosquitoes. Two light traps were used per house, one indoor and another outdoor. Indoor traps were placed one and a half meters above the ground within sleeping areas for both animals and people between 19:00 and 06:00 hrs. Outdoor traps on the other hand were equally placed one and a half meters above the ground at areas secluded for animals to sleep for equal duration. Mosquitoes resting indoors were sampled using pyrethrum spray catch (PSC) method between 07:00h to 11:00h each day (35). Before spraying, all food stuff was covered. All openings to the houses including doors, windows and eaves were well sealed to avoid escape of mosquitoes. The roofs and walls were sprayed with 0.025% pyrethrum emulsifiable concentrate with 0.1% piperonylbut oxide in kerosene. All dead and unconscious mosquitoes from each house were collected after ten minutes and put in collection bottles (one per house), labelled, packed in a cool box and transported to Masinde Muliro University of Science and Technology, biotechnology laboratory where sorting was done and recorded. Each day's anopheline collection was identified morphologically to species and sex level and kept in 1.5 ml Eppendorf tubes containing 70% isopropanol for further analysis. Malaria vector species complexes were identified by polymerase chain reaction (36).

2.3.1. Determination of Plasmodium Falciparum Sporozoites

Each head and thorax of the 376 anopheline malaria vectors was separated from the abdomen and put in Eppendorf tube and processed for circumsporozoite protein (CS) and tested for presence of *P. falciparum* (CS) antigens according to (37)&Beier*et al.* 1990(38).

2.4. Identification of Blood Meal Sources

The abdomens of engorged female *Anopheles coustani* mosquitoes were separated from the rest of the body and placed into sterile 1.5 mL micro centrifuge tubes (39). Each abdomen was squashed in 300µL cell lysis buffer using a hand held battery operated homogenizer then incubated at 65°C for 15 minutes. 100 µL of protein precipitation buffer was added, followed by vortexing for 30 seconds at room temperature before placing on ice for 5 minutes, then centrifuging at 25 000 relative centrifugal force for another 5 minutes. The pellets were discarded and the supernatants were placed in fresh tubes with 300 µL isopropanol, and then mixed by inverting 100 times and centrifuging at 25000 relative centrifugal force for 30 minutes. The supernatants were removed and 300 µL of 70% cold ethanol was added to each tube. The tubes were inverted 50 times then centrifuged for 15 minutes. The ethanol was pipetted off and the tubes were inverted on paper towels overnight to air dry. The DNA pellets were then eluted with 50 µL of nuclease-free water and stored in a refrigerator at 4°C for 3 hours before use. Three sets of PCR primers (table 3) targeting vertebrate CO1, cytochrome b (cyt b), 16S ribosomal (r) RNA genes were used to identify blood meal sources in separate 10 µL PCR reactions containing µL of nuclease-free PCR water, 0.5 µM concentration of each primer,2 µL of 5 × Hot FIRE Pol Eva Green HRM Mix (Solis Biodyne, Tartu, Estonia), and 1 µL of DNA as a template, using the same conditions(40)

2.5. Data Management and Analysis

Data were entered in spread sheets and cleaned using Microsoft Excel, 2007. Statistical analyses were performed using SPSS version 23 and Graph pad Prism version 6 statistical software. One way Analysis of Variance (ANOVA) was used to obtain variation in mean densities between species and collections (indoor and outdoor). The statistical analysis for number and proportions of *Anopheles* species was performed using the Fisher's exact test. P value was set at 0.05 and 95% confidence interval.

3. Results

3.1. Anopheles Species Composition

Anopheles mosquito composition and abundance is presented in Table 1. A total of 376 anophelines were collected using CDC light traps; Outdoor collections comprised 67.6% (254) anophelines and indoor collections had 32.4% (122). There was significantly high abundance of *An. coustani [n*=160, (63.0%), *P<0.0001*] followed by *An. maculipalpis*[n=94, (37.0%), *P*<0.0001] in outdoor collections compared to indoor one. There was significantly high abundance of *An. funestus,* [n=104, (85.2%), *P*<0.0001] followed by *An. arabiensis,* [n=16, (13.1%), *P*<0.0001] in the indoor collections in comparison to outdoor. However, there was insignificant abundance of *An. gambiae* [n=2, (1.6%) *P*=0.324] in indoor collection compared to outdoor collection.

3.2. Plasmodium Sporozoites Detection

Plasmodium sporozoites detection analysis results are presented in table 2**.** All the 376 anopheline mosquitoes captured were tested for presence of *Plasmodium* sporozoites. Only four *Anopheles coustani* tested positive with an infectivity rate of 2.5%. These results indicate that *An. coustani*could be responsible for outdoor malaria transmission in this area. All the *An. coustani*used in this study were caught outdoors.

4. Blood-meal Source Analysis

Anopheles coustani mosquito blood-meal source is presented in figure 1. A total of 86 blood fed *An. coustani*were analyzed for blood-meal source. A total of five different blood-meal sources were identified; human, bovine, goat, rabbit and human-bovine combination. Three different PCR primers were used, cytochrome (cyt b), 16 s ribosomal (r) RNA genes for vertebrate COI to identify blood-meal sources. Human preference was the second as shown in all the three primers.

5. Discussion

Malaria infections remain the major cause of morbidity and mortality (41). Despite intensive studies on involvement of primary vectors (10), petite information exists on the role of secondary vectors on the disease transmission in western Kenya. Presently, there are no proper interventions that specifically target outdoor biting mosquitoes. Studies have confirmed the importance of secondary vectors in the transmission of malaria parasite in some parts of African Continent (3,4,10,42). The present study evaluated the role of secondary vector in the transmission of malaria in Eluche, Mumias east, Kakamega County.

The study observed that *An. coustani* had the highest proportion of the total *Anopheles* female mosquitoes collected. *Anopheles arabiensis, An. gambiae, An. funestus, An. maculipulpis*and *An. coustani*were the five species collected. However, three of these, *An. arabiensis, An. gambiae and An. funestus*werecollectedindoors*;* while *An. maculipulpis*and *An. Coustani*werecollectedout doors*.* This implies that the inhabitants of the area are exposed to both outdoor and indoor malaria transmission. Outdoor and indoor mosquito densities varied considerably in the region as per the result. There was a higher number of mosquitoes collected outdoors as compared to those collected indoors. This could be attributed to agricultural activities where swamps have been reclaimed creating breeding habitats(10,43). Never the less cattle and

other big livestock in the region spend the nights in the open fields which could be providing opportunistic hosts to the exophagic*An. coustani*mosquitoes. The very high abundance of the vector and detection of *Plasmodium* coupled with probable opportunistic feeding behavior enhances support of the role of the vector in malaria transmission (42).

Animal husbandry is an economic activity in this area besides crop farming. Livestock are kept for food, source of income, animal products, traditional activities like payment of dowries and land cultivation (44). From our results, mosquitoes potentially feed on diverse hosts including bovine, human, goat and rabbit which could be encouraging large mosquito population sustenance. At least five individuals were found to have a combination of human and bovine blood. This could be as a result of the individuals getting to complete incomplete blood-meals(39,45). This therefore calls for establishment of the mosquito blood-feeding patterns hence optimize vector control strategies. It was of necessity to establish the mosquito feeding patterns as mosquitoes feed potentially on diverse hosts (39). This study concurs with previous studies which showed high composition of *Anopheles coustani*in outdoor collections (10,42). It is also a revelation of *A. coustani*being in sympatry with *An. maculipulpis*as both contain high compositions. It however refutes some preceding studies where *An. coustani*were caught indoors while in this, non was caught indoors (10,39,46,47). From our results it is evident that Mumias East Sub-county of Kakamega County experiences both indoor and outdoor malaria transmission. Indoor transmission is propagated by *An. funestuss.s, An. arabiensiss.s*and *An., gambiaes.s.* Outdoor transmission on the other hand is perpetuated by *An. coustani.*

This study was limited by the fact that it was a cross sectional study and consequently did not collect mosquitoes based on seasonality. It also did not look into vector species densities and distribution in relation to inoculation rates. The area of coverage was also relatively small due to financial constrains leaving our results being speculative rather than conclusive.

6. Conclusion

The present study detected *Plasmodium*sporozoites in the captured *Anopheles coustani* mosquitoes. This means that *Anopheles coustani*could be playing a role in outdoor malaria transmission in this region and possibly the entire county. There is therefore need to evaluate the feeding patterns and social behavior of *Anopheles coustani* in highlands of Western Kenya for proper malaria vector management practices. Intervention programs should also include outdoor vector control strategies besides change of living routine.

7. Abbreviations

PSC : Pyrethrum spray collection
CDC : Centre for disease control
PCR : Polymerase chain reaction
ANOVA : Analysis of variance.

8. Declarations

8.1. Ethics Approval and Consent to Participate

Ethics approval and participation consent was granted by MasindeMuliro University of Science and technology institutional review committee, (MMUST/IERC/207/2021) and National Commission for Science, Technology and Innovation (NACOSTI/P/21/12188).

8.2. Availability of Data and Materials

The data used to support findings in this study are available from the corresponding author upon request.

8.3. Competing Interests

The authors declare that there are no conflicts of interest.

8.4. Funding

This research article did not receive any external funding.

8.5. Author's Contributions

All the authors contributed equally to the entire production of this work.

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Appendix

Malaria Vector Species	Collection Type		P value
	Outdoor n=254 (%)	**Indoor n=122 (%)**	
An. coustani	160 (63.0)	0 (0.0)	<0.0001
An. maculpalpis	94 (37.0)	0 (0.0)	<0.0001
An arabiensis	0 (0.0)	16 (13.1)	<0.0001
An. funestus	0 (0.0)	104 (85.2)	<0.0001
An. gambiae	0 (0.0)	2 (1.6)	>0.324

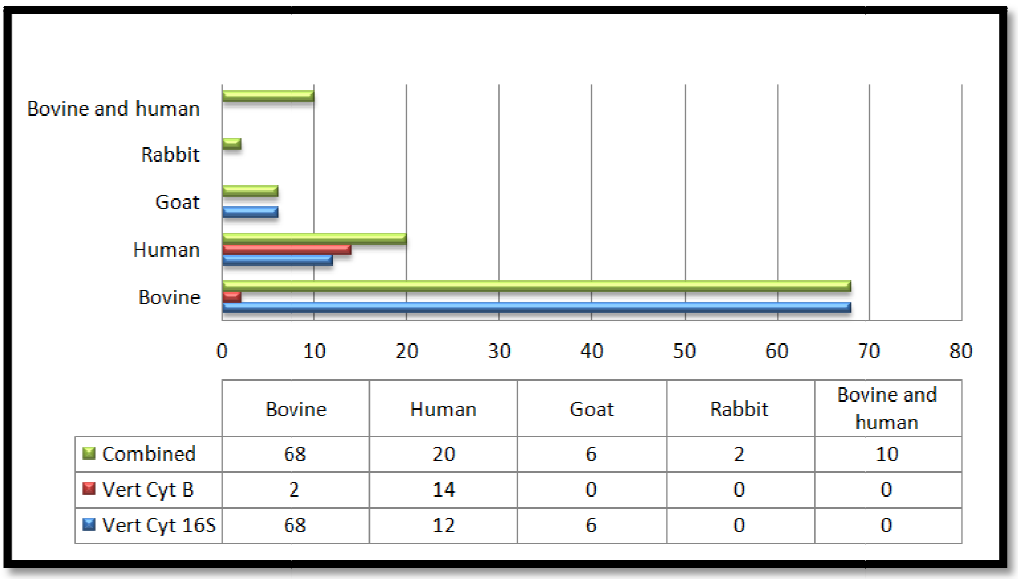
Table 1: Anopheles Species Composition

P value, significance at alpha 0.05; n, number and proportions of Anopheles species. Statistical analysis was performed using Fisher's exact test.

	AnophelineMosquitoes					P value
	An.arabiensis n (%)	*An.cosutani* n (%)	*An.funestus* n (%)	*An.gambiae* n (%)	*An.maculipalpis* n (%)	
Sporozoite distribution	0 (0.0)	4 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	-

Table 2 Plasmodium Sporozoites Detection Analysis

Key: N; Number of Mosquitoes Detected with Plasmodium Sporozoites, (%) Proportions of Mosquitoes Detected with Plasmodium Sporozoites, An., Anopheles, P, Significance Level of Alpha at 0.05



	Bovine	Human	Goat	Rabbit	Bovine and human
Combined	68	20	6	2	10
Vert Cyt B	2	14	0	0	0
Vert Cyt 16S	68	12	6	0	0

Figure 1: Individual Blood-Meal Source Analysis