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SALIVARY GLAND BACTERIAL COMPOSITION OF FEMALE ANOPHELES MOSQUITOES IN BAMESSINGUE LOCALITY, MBOUDA, WEST REGION OF CAMEROON: A BASELINE INVESTIGATION

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ABSTRACT

The extent to which there is a causal relationship between *Plasmodium* infection and bacteraemia is unknown. It remains unclear whether such infections are attributable to female *Anopheles* mosquitoes' bite during a blood meal, other risk factors or are coincidental. As such, in taking a blood meal, the mosquito can introduce bacteria into the blood stream of man which if it is positive with *Plasmodium*, the victim suffers the risk of mixed infection with bacteria and parasite. This study was aimed at determining the salivary gland bacterial composition of the female *Anopheles* mosquitoes in Bamessingue locality, Mbouda, West Region of Cameroon so as to provide a baseline understanding about the relationship between female *Anopheles* mosquitoes' bite and bacteraemia of unknown origin. An experimental study was carried out at Standard Medical Diagnostic and Research Centre Mbouda. Six female *Anopheles* mosquitoes were collected from Bamessingue locality in Mbouda. They were anaesthetised by chloroform and dissected aseptically and their salivary gland extracts were extracted for culture. They were inoculated on blood agar, chocolate agar, MacConkey and Chapman agar and cultured at 35±2°C for 24 hours. Isolation and identification of bacteria strains was done using standardized methods. From the field collected female *Anopheles* mosquitoes, *Staphylococcus aureus* was proven to be the dominant bacteria isolated from the salivary gland of the female *Anopheles* mosquitoes, followed by *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and lastly *Klebsiella pneumoniae*. This is the first time *Staphylococcus aureus* is identified with the highest abundance from the salivary glands of female *Anopheles* mosquitoes and could be a potential cause of bacteraemia by the female *Anopheles* mosquito's bite during a blood meal. It is thus recommended that similar studies should be carried out in others regions in order to sort out the possible bacteria responsible for bacteraemia in their various localities.

KEYWORDS: Bacteraemia, Salivary gland, Female Anopheles Mosquito, Mbouda, West Region.

INTRODUCTION

Bloodstream infections are the main causes of hospital admissions and deaths in Sub-Saharan Africa (SSA).[1] Bacterial blood stream infections are the leading cause of morbidity and mortality in both high-income and low-income countries but the causative agents and risk factors differ.[2] The burden of bacteraemia is insufficiently studied in Africa where few healthcare facilities have the ability to identify invasive diseases as the prevalence of mortality associated to bacteraemia remains significantly high.[3] Bacteraemia and malaria are often difficult to differentiate clinically.[1] The overlapping clinical features in these co-infections may pose serious challenges especially in impoverished areas where the capacity for laboratory testing is limited and simultaneously, may be more severe or produce

additional sequelae compared to single infection.[4] However, it remains unclear whether such infections are attributable to female *Anopheles* mosquitoes' bite during a blood meal, other risk factors or are coincidental.

The extent to which there is a causal relationship between *Plasmodium* infection and bacteraemia is unknown, and there is no enough statistics regarding bacterial co-infection in patients with malaria. This has led to the use of antibiotics in combination to antimalarial drugs in severe malaria without any convincing evidence that this can reduce the mortality in hospital settings.[5] This indiscriminate use of antibiotics can contribute to increase in existing problem of antimicrobial resistance.[1] In taking a blood meal, the mosquito can introduce bacteria into the blood stream of man which if it is positive with *Plasmodium*, the victim suffers the risk of mixed infection with bacteria and parasite. Many studies such as that of Sharma *et al*.[6], Tchioffo *et al.*[7] and Berhanu *et al.*[8] have proven the presence of bacteria in the salivary gland of female *Anopheles* larvae and adult mosquitoes.

Mosquitoes feed on microorganisms such as algae, bacteria and even organic matter.[9] Bacteria are necessary for their development and also known to influence larval development. Some bacteria are transmitted to adults and make up the microbiota composition in adult mosquitoes.[10] A study by Sharma *et al.*[6] using a 16sRNA based real-time Polymerase Chain Reaction (PCR) analysis, in Dwarka, India showed that the salivary gland microbial composition is more diverse than the midgut of 3-4 day old sugar fed adult female *Anopheles culicifacies*. This study identified the salivary gland microbiota belonging to a total number of 17 different phyla predominated by phylum Proteobacteria, Firmicutes, Bacteriodetes, Tenericutes, and Actinomycetes.

The adult female *Anopheles* mosquito salivary glands have been shown to be colonized by three dominant classes of bacteria; Gammaproteobacteria, Alphaproteobacteria, and Betaproteobacteria in a DNA-based Pyrosequencing analysis study on laboratory reared female *Anopheles* mosquitoes in 3 peri-urban areas (Ahala, Nkolondom, and Nkolbisson) of Yaoundé, Cameroon.[7] Results in malaria endemic areas of Ethiopia showed that Gram negative bacteria were found to be the dominant bacteria and also identified Pseudomonas as the dominant microbiota among all the species of *Anopheles* mosquitoes. The bacteria identified from salivary glands of laboratory reared female *Anopheles* mosquitoes in this study above were; *Klebsiella pneumoniae*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Kocuria rhizophila*, *Streptococcus thoraltensis*, *Methylobacterium lacunta*, *Lactococcus garviea*, *Pseudomonas* species, *Bacillus* species and *Acinetobacter* species.[8]

There could therefore be an association between the feeding habit of a mosquito and the microbial flora of its salivary gland. As such, in taking a blood meal, the mosquito can introduce bacteria into the blood stream of man which if it is positive with *Plasmodium*, the victim suffers the risk of mixed infection with bacteria and plasmodium parasite. This study was therefore aimed at determining the salivary gland bacterial composition of female *Anopheles* mosquitoes in Mbouda, West Region of Cameroon so as to provide a baseline understanding about the relationship between female *Anopheles* mosquitoes' bite during a blood meal and bacteraemia of unknown origin.

MATERIALS AND METHODS

This study was carried out in March 2020 in Bamessingue, a locality in Mbouda, located in the Bamboutos Division of the West Region of Cameroon (Figure 1). It is found along the Bamenda-Bafoussam highway, some 49.4 Km from the city of Bamenda and 30.4 Km from the city of Bafoussam. The area is characterized by poor drainage patterns with the presence of stagnant waters in gutters harboring various wastes derived from households and as such, constitutes a favourable breeding ground for mosquitoes.

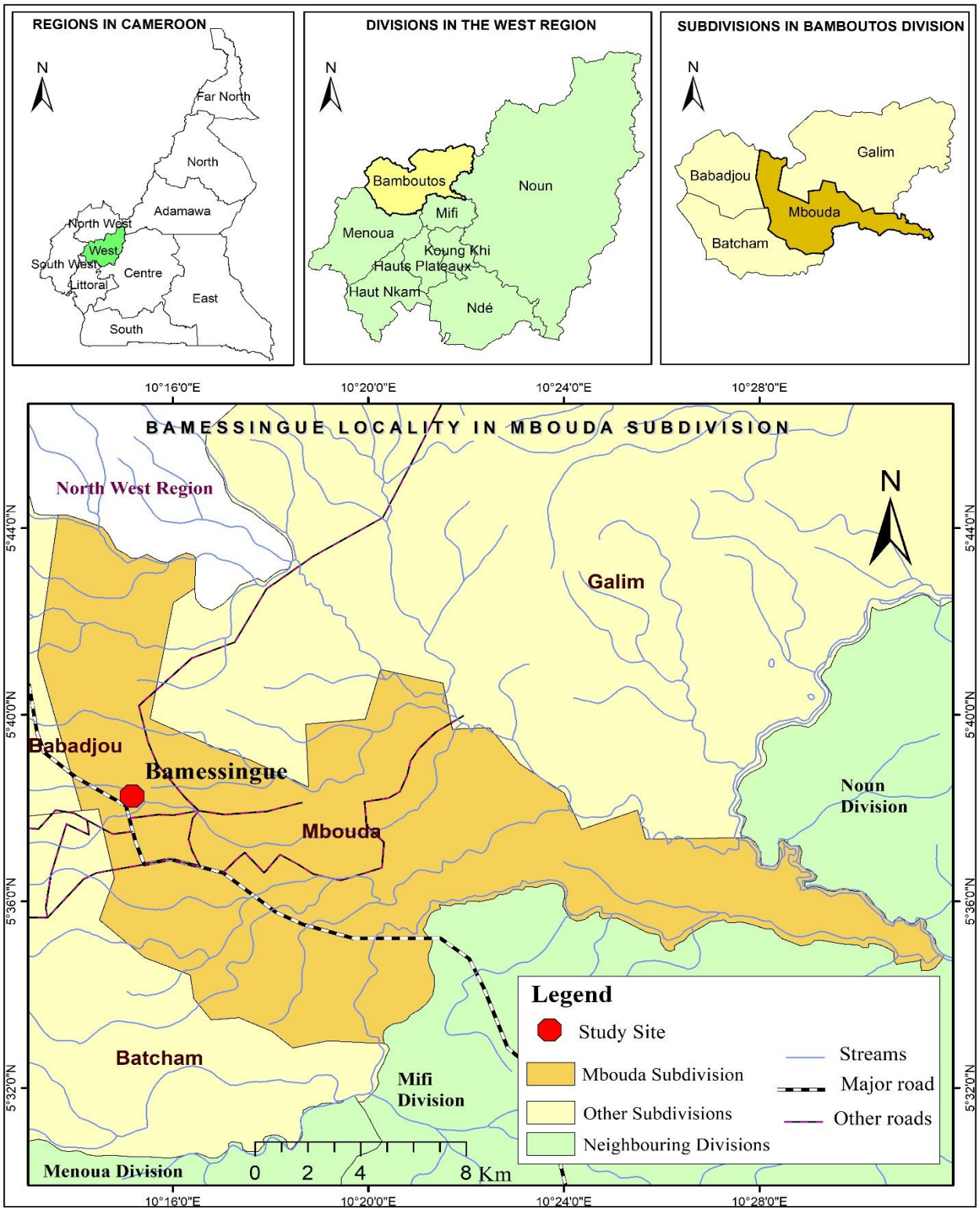

Figure 1: Location of Bamessingue locality in Mbouda.

An experimental study design was used, involving 6 conveniently sampled female anopheles mosquitoes collected via the human landing collection technique within the premises of the laboratory in Bamessingue locality from 3am to 6am. The mosquitoes were rendered inactive by anaesthetising with chloroform 5% spray. They were later cleaned in 70% alcohol (v/v), kept in a sealed sterile container to dry before dissection on sterile microscope slides for the extraction of the salivary gland extract for culture aerobically at 35 ± 2°C. Blood agar, chocolate agar, MacConkey agar and Chapman agar were prepared following the manufacturer's instructions. Catalase, Coagulase, Urease, Indole and Oxidase tests were used to identify the specie of the bacteria isolates. `

Throughout the dissection procedure, working area, dissecting needles and forceps were dipped and sprayed in every dissection using 70% ethanol. These materials had initially been autoclaved at 121^0C. Prior to the salivary gland dissection, each mosquito was surface sterilized by washing in 70% ethanol followed by rinsing. Each dissected salivary gland was squashed in sterile distilled water and incubated at room temperature for 3 minutes. A loop of each of these preparations containing a squashed salivary gland from each mosquito was inoculated on Blood agar, chocolate agar, MacConkey agar and Chapman agar for 24 hours at 35 ± 2°C.

Descriptive statistical analysis such as frequency tables, percentages and graphics were used to present research findings. All statistical analyses were done using SPSS version 21 (IBM SPSS Statistics, IBM Corporation, Chicago, IL). The Chi-square analysis which tested the relationship between categorical variables was used in the analysis of the data. $p<0.05$ was considered statistically significant.

RESULTS

Salivary gland microbiota composition from field collected female *Anopheles* mosquitoes

Table 1 below presents the bacterial growth based on the culture media used. Out of the 6 sampled female *Anopheles* mosquito salivary gland contents cultured on Blood agar, Chocolate agar, MacConkey agar and Chapman agar, Gram positive cocci isolation constituted 66.67%, followed by Gram negative bacilli with 29.63%. Only 3.70% of the total isolate was Gram negative cocobacilli. This is shown in figure 2 below.

From sample S1 and S3, 100% of the total isolates were Gram positive cocci. S2 recorded 66.67% of Gram positive cocci and 33.33% of Gram negative bacilli. S4 recorded a 50% score each for Gram negative bacilli and Gram positive cocci. S5 on the other hand had 50% Gram positive cocci, 33.33% Gram negative bacilli and 16.67% Gram negative coccobacilli isolated from the 4 culture media used. From S6, 57.14% were Gram positive cocci while 42.86% were Gram negative bacilli.

Table 1: Salivary gland microbiota composition from field collected female *Anopheles* mosquitoes.

Specimen	Blood agar	Chocolate agar	MacConkey agar	Chapman
S1	Gram positive cocci	Gram positive cocci	-	Gram positive cocci
S2	Gram positive cocci	Gram positive cocci	-	Gram negative bacilli
S3	Gram positive cocci	Gram positive cocci	-	Gram positive cocci
S4	Gram positive cocci	Gram negative bacilli and Gram-positive cocci	-	Gram negative bacilli and Gram-positive cocci
S5	Gram positive cocci	Gram positive cocci	Gram negative coccobacilli Gram negative bacilli	Gram positive cocci Gram negative bacilli
S6	Gram positive cocci	Gram negative bacilli and Gram-positive cocci	Gram negative bacilli and Gram-positive cocci	Gram negative bacilli and Gram-positive cocci

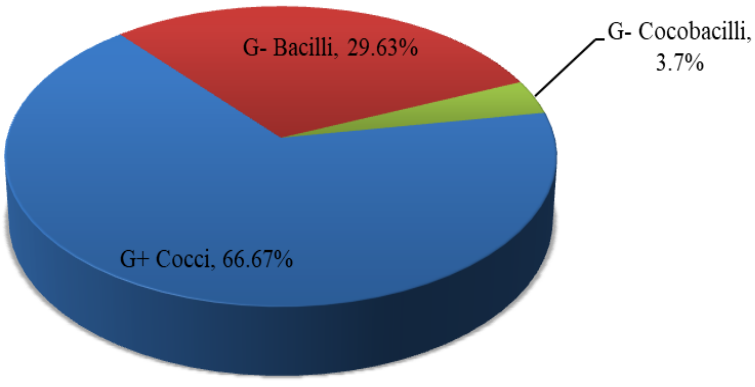

Figure 2: Salivary gland microbiota composition from field collected Female Anopheles mosquitoes.

Different groups of bacteria in the salivary gland extract of the female *Anopheles* mosquito – biochemical identification of bacteria

Only Gram positive cocci were isolated from the salivary gland of these mosquitoes. The two lone cocci isolated and confirmed from Gram staining and catalase tests were of the Genera *Staphylococcus*, specifically *Staphylococcus aureus* and *Staphylococcus epidermidis. Staphylococcus aureus* constituted 88.89% of all the

cocci isolated while *Staphylococcus epidermidis* lasted with 11.11%.

Only *Escherichia coli*, and *Pseudomonas aeruginosa*, were the isolated Gram negative bacilli from the 6 salivary glands sampled. *E. coli* was the highest occurring bacilli with 75% while *P. aeruginosa* came with 25%. These were confirmed from Gram stain, catalase test (which are all positive) and selective growth patterns on Chapman. *P. aeruginosa* distinctively produces white non-raised single colonies on Chapman.

Klebsiella pneumoniae was the only isolated Gram negative cocobacillus in this study. It was isolated from being catalase positive and Gram of short cocobacilli observed.

Biochemical identification of bacteria based on the sample

The highest isolate based on the number of colonies counted from all the samples was *S. aureus*. It dominated in all samples from S1 to S6. The second most observed bacteria was *E. coli*, isolated from S4, S5 and 56 with the highest occurrence noticed in S4 and S6. *S. epidermidis* was only found in S1 and S6 while *K. pneumoniae* could only be isolated from S5. This is shown in figure 3 below.

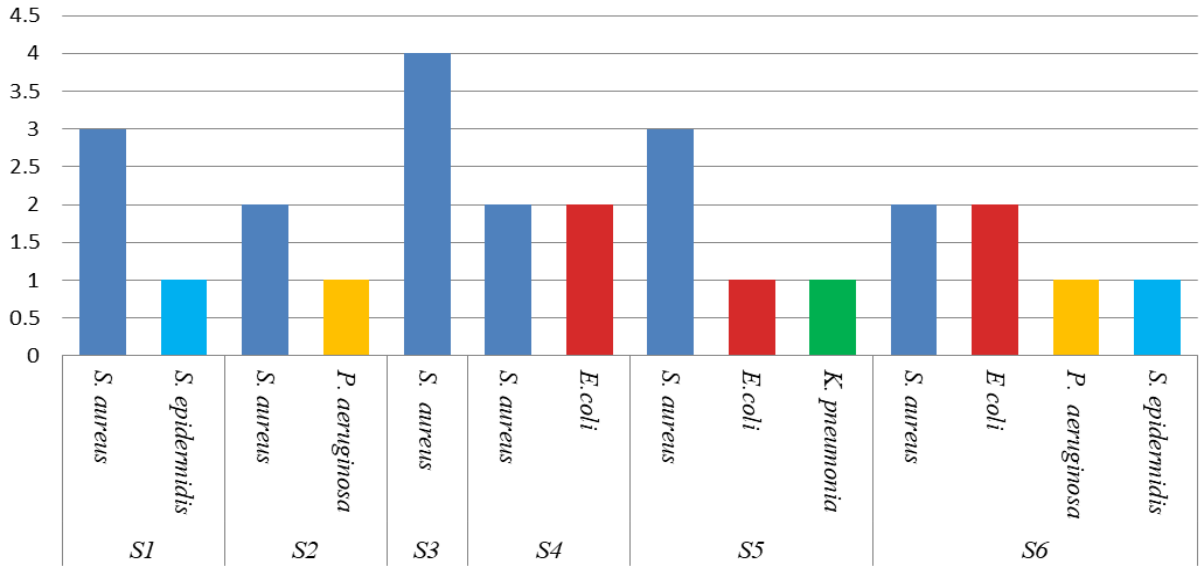

Figure 3: Biochemical identification of bacteria based on the sample.

In table 2 below, *Staphylococcus aureus* was the most prevalent germ isolated from all the salivary glands of the 5 mosquitoes. *Escherichia coli* on the other hand was isolated from 3 salivary glands, while *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were isolated from 2 mosquito salivary glands each. *Klebsiella pneumoniae* however was only identified and isolated from one mosquito salivary gland.

Table 2: Identification of isolates based on the number of salivary glands from which each was isolated.

Microbe	Number of isolates	Number of salivary glands
Staphylococcus aureus	16	6
Escherichia coli	6	3
Staphylococcus epidermidis	2	2
Pseudomonas aeruginosa	2	2
Klebsiella pneumoniae	1	1

Differentiation of salivary gland bacteria content based on whether the female mosquito had had a blood meal or not

With respect to blood meal, only *Staphylococcus aureus* isolate showed a significant difference from mosquitoes from whom had taken a blood meal before ($X^2 = 27.08$, $p = 0.001$). Mosquitoes that had been on a blood meal (S1, S2, S3 and S4) had 87.5% increase in *Staphylococcus aureus* isolates, compared to S5 and S6. *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* isolates had no significant differences based on whether the mosquito had just taken a blood meal or not. This is shown in table 3 below.

Table 3: Differentiation of salivary gland bacteria content based on whether the female mosquito had had a blood meal or not.

Microbe	Blood Meal	Number of isolates	X^2, *p* value
Staphylococcus aureus	Yes	14	27.08
	No	2	
	Total	**16**	**0.001***
Staphylococcus epidermidis	Yes	1	17.34
	No	1	
	Total	**2**	1.00
Pseudomonas aeruginosa	Yes	1	17.34
	No	1	
	Total	**2**	1.00
Escherichia coli	Yes	3	21.20
	No	3	
	Total	**6**	1.00
Klebsiella pneumoniae	Yes	0	7.05
	No	1	
	Total	**1**	1.00

***- significant at 0.05 significance level**

DISCUSSION

The salivary gland bacterial composition comprised of Gram positive cocci, Gram negative bacilli and Gram negative coccobacilli but the composition of isolates in this study revealed that Gram positive cocci dominate the salivary gland contents of field collected female *Anopheles* mosquitoes which had not yet been proven from previous studies. This contradicts results obtained from a culture-based study on laboratory reared and field collected female *Anopheles* mosquitoes by Berhanu *et al*.[8] in Edo Kontola village in South Central Ethiopia, where Gram negative bacteria were found to be the dominant bacteria. Also, the salivary gland bacterial composition from the female *Anopheles* mosquitoes had a narrow spectrum of 5 bacteria as compared to a broad spectrum of 36 bacteria genera identified using the 16sRNA-based real-time polymerase chain reaction (PCR) analysis on 3-4 day old sugar fed adult female Anopheles culicifacies mosquitoes by Sharma *et al*.[6] in Dwarka, India. The bacterial composition in the salivary glands of female Anopheles mosquitoes could vary according to the breeding site, indicating that some bacteria are acquired from the environment.[7] This is the reason for the discrepancy between the salivary gland bacterial composition in this study and those from previous studies which were carried out in different environments.

The different groups of bacteria isolated from all the salivary glands were cocci (*Staphylococcus aureus* and *Staphylococcus epidermidis*), bacilli (*Escherichia coli* and *Pseudomonas aeruginosa*) and coccobacilli (*Klebsiella pneumonia*). The dominant bacteria identified from all the female *Anopheles* mosquitoes in this study was *Staphylococcus aureus* which had not yet been proven from any previous study. This is the first time *Staphylococcus aureus* is been identified with the highest abundance from the salivary glands of female *Anopheles* mosquitoes. This contradicts results obtained by Berhanu *et al*.[8] whereby *Staphylococcus aureus* was identified among those with the lowest abundance. Also, it contradicts results from a DNA-based Pyrosequencing analysis on laboratory reared female *Anopheles* mosquitoes by Tchioffo *et al*.[7] in 3 peri-urban areas of Yaoundé, Cameroon, where by Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria were the dominant classes of bacteria with the genus *Escherichia* identified among those with a lower abundance. This previous study contradicts our study as *Escherichia coli* was second in abundance. Moreover, the presence of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus epidermidis* in this present study corroborates with the results obtained by Berhanu *et al*.[8] in which Pseudomonas was found to be the dominant bacteria identified from all the species of Anopheles mosquitoes. Moreover, the *Staphylococcus aureus* isolates were more statistically significant as compared to those of *Escherichia coli.* This could be due to the fact that the female *Anopheles* mosquitoes had taken a blood meal from humans whom their blood already contained *Staphylococcus aureus* because of the strong association between *Staphylococcus aureus* isolates and blood meal. This association could also be responsible for the reverse transfer of the bacteria from the female Anopheles mosquito's salivary gland back into the blood stream of man when taking a blood meal alongside with the Plasmodium parasite. This hypothesis correlates with the results obtained from a culture-based study from 396 patients attending the University Teaching Hospital, Yaoundé, Cameroon by Kamga et al.[11] whereby 112 (28.3%) patients had bacteraemia with *Staphylococcus aureus* (20.9%) as one of the leading causes of bacteraemia. This could be the reason why malaria has always been regarded to strongly predispose individuals to bacteraemia.

CONCLUSION

The different groups of bacteria in the salivary gland extracts of the female *Anopheles* mosquitoes were cocci (*Staphylococcus aureus* and *Staphylococcus epidermidis*), bacilli (*Escherichia coli* and *Pseudomonas aeruginosa*) and coccobacilli (*Klebsiella pneumoniae*). *Staphylococcus aureus* was the dominant bacteria identified and was closely linked to blood meal and therefore could be regarded as a potential cause of bacteraemia by the female *Anopheles* mosquito's bite during a blood meal.

Study Limitations

The study analyzed only six female *Anopheles* mosquitoes, which may not capture the full diversity and variability of bacterial communities in the area. Specimens were collected from a single locality (Bamessingue) and during a specific period (March 2020). Bacterial composition may vary with season, breeding site conditions, and ecological factors, which were not assessed in this baseline investigation. As a baseline investigation, the results provide a starting point but not sufficient evidence to infer causal relationships or to make broader ecological or epidemiological conclusions.

Conflict of Interest

The authors declare that they have no competing interests.

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