

Identification of Blood Meal of Sand Flies in a Cutaneous Leishmaniasis Endemic Area, Volta Region-Ghana

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Abstract: *Background:* Vector incrimination is of utmost importance in the transmission of *Leishmania* species identified in the Volta region of Ghana. The feeding preference of sand flies provides valuable information about the vector-host interactions, including reservoir host. *Objective:* The main aim of this study was to identify the blood meal in collected sand flies and its source from three villages in a cutaneous leishmaniasis endemic area in the Ho Municipality. *Method:* Sand flies were collected using CDC light traps and sticky paper traps from Lume Atsiame, Dodome Dogblome and Dodome Awuiasu. They were morphologically identified by taxonomic keys. DNA was extracted from the individual sand flies using the potassium acetate extraction method. Polymerase chain reaction (PCR) amplification of cytochrome b gene (*cyt b*) fragment was carried out and subsequently DNA sequenced. *Results:* Of a total of three hundred and sixty-three (363) female sand flies that were analysed, eighty-four (84) representing 23.14% were positive for blood meal presence with an amplified DNA of the expected size, 359bp. Out of the number that was sequenced, the *cyt b* sequencing revealed that four (4) blood fed females fed on bloodmeal from human (*Homo sapiens*) and house mouse (*Mus musculus*). *Conclusion:* The blood meal presence was more predominant in *Sergentomyia ghesquierei* and least in *Sergentomyia schwetzi*. This may aid in the development of effective strategies to control cutaneous leishmaniasis in the endemic areas.

Keywords: Cytochrome B Gene, Feeding Preferences, Identification, Polymerase Chain Reaction, Sequencing

1. Introduction

Leishmaniasis, caused by the protozoan parasite *Leishmania* is transmitted by the bite of various species of sand flies. These sand flies are blood sucking insects of great medical importance. They require blood to supplement the essential proteins for development and production of their eggs during their gonotrophic cycle [1, 2]. The disease is caused by several species of intracellular parasites of the genus *Leishmania*, which presents three distinct clinical manifestations: cutaneous (CL), mucocutaneous (MCL) and visceral forms (VL) [30]. CL is the most common and self-healing and VL is the most severe.

Some species of the sand fly have preference for particular

or group of animal host species, while most others tend to be generalists due to availability of hosts [1]. Research into the source of blood meal of sand flies which feed on a wide range of mammalian hosts, including birds, dogs, domestic livestock, man and rodents is of great eco - epidemiological significance [3-5]. This is because they enable the correct identification of the vector feeding preferences [6] and contribute to the explanation of the natural transmission cycle in a given area [7]. It is worth noting that sand flies are known to have a low and weak flight pattern [8, 9] and as such will not travel too far to get their source of blood meal. Thus, one of the most important steps towards predicting and controlling this disease is by monitoring the sand flies in a region [10].

The identification of the source of blood meal of blood sucking insects were until recently conducted using mainly

serological techniques such as the enzyme-linked immune-sorbent assays, latex agglutination test and the precipitin test [3]. These techniques are now regarded to be time-consuming and with low level of sensitivity [11]. Currently, DNA based molecular techniques [12, 13] have thus gained popularity because they are sensitive and specific although the approach is laborious and quite expensive. Valinsky *et al.*, [14] reported that targets used for identifying the source of blood meal to the species level include polymerase chain reaction (PCR) and sequencing of the mitochondrial genes; cytochrome b (*cyt b*), cytochrome c oxidase I (COI), rRNA genes (12S, 16S, and 18S), and nuclear genes. These techniques are based on DNA sequence analysis of targets previously amplified by PCR [15]. These techniques have further been used for the molecular monitoring of leishmaniasis as well as for determining the feeding behaviour of sand flies [16]. As an alternative to DNA techniques, two approaches utilizing liquid chromatography-mass spectrometry (LC-MS/MS) and MALDI-TOF MS protein

profiling have been designed [17] to identify blood meals.

In Ghana, Kweku *et al.*, [18] reported in their study that the geographic range of leishmaniasis is limited by the sand fly vector and its feeding preferences. There is thus, limited research carried out on the blood meal source of sand flies in the cutaneous leishmaniasis focus in the Ho Municipality of the Volta Region, Ghana. Thus, in this study, PCR amplification of the mitochondrial cytochrome b (*cyt b*) gene was carried out followed by direct DNA sequencing to detect presence of blood meal and its source for the sand flies collected in the CL endemic area.

2. Materials and Methods

2.1. Study Area

The Ho town doubles as the capital of the Municipality and the Regional Capital of the Volta Region (Figure 1).

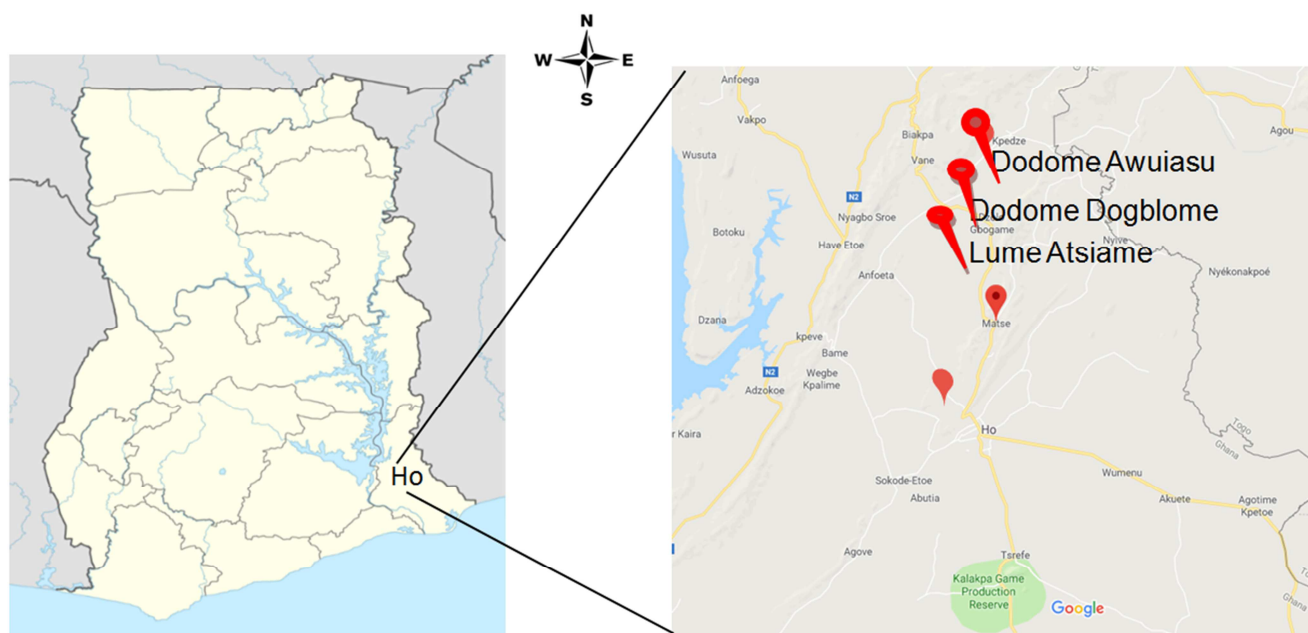


Figure 1. Map of Ghana showing the study areas.

It lies between latitudes 6°36'43"N and longitude 0°28'13"E. The Municipality is a moist semi-deciduous forest zone with villages dotted around. The vegetation of the Municipality is mainly forest and forest savannah. The population of the Municipality according to 2010 population and housing census stands at 177,281 with 83,819 males and 93,462 females as stated by the Ghana Statistical Services Report [19]. The study was carried out in three communities namely Lume Atsiamie (6.619918°N, 0.464762°E), Dodome Dogblome (6.760774°N, 0.51546°E) and Dodome Awuiasu (6.767553°N, 0.514789°E). The major economic activities of the population are agriculture, animal farming and trading.

2.2. Ethic Issue

Ethical clearance was obtained from the University of Cape

Coast Institutional Review Board (UCCIRB/CHAS/2017/32). The leaders of the endemic communities and family heads consented to this investigation before setting the traps for the sand flies' collection around the residence.

2.2.1. Sand Fly Collection

The collection of sand fly was carried out every day (from 6pm to 6am) in November and December 2017, January, February, March, April, May, June and August 2018. Siting of the CDC light and sticky traps were randomly selected for sand fly collections in the three endemic areas in the Ho Municipality (Figure 1). The selected communities represent areas in the Municipality where cases of CL had been reported [20].

A total of three (3) CDC light traps and 20 sticky traps were set from dusk to dawn (approximately 12hr) each day for the

collection of flies. The collected sand flies by the CDC light traps were freeze-killed at -20°C and sorted out into labelled 1.5ml eppendorf tubes containing silica gel for dry preservation. However, the collected sand flies by the sticky traps were preserved in 70% ethanol. The tubes were secured in sealed sample collection bags and transported to the entomology laboratory for morphological identification.

2.2.2. Morphological Identification

Taxonomical identification of the collected sand flies was carried out on the basis of the morphological criteria [21]. The identification was based on three morphology features - the cibarium, pharynx and spermatheca. *Phlebotomus* species had a cibarium which laced cibarial teeth and a pigmented patch whilst *Sergentomyia* species had both the cibarial teeth and pigment patch present.

2.3. DNA Extraction

DNA was extracted from each sand fly using the potassium acetate extraction method as described by Aransay *et al.*, [22] with slight modifications. The individual sand fly was grinded with disposable pestle in 1.5 mL labelled tubes. One hundred and fifty microliters (150 µL) of extraction buffer, which was made up of 1% sodium dodecyl sulphate [SDS], 25 mM NaCl and 25 mM EDTA was added, and tubes were placed in a water bath at 65°C for 30 minutes. Following the addition of 100 µL of 3 M potassium acetate (pH 7.2), the homogenates were incubated on ice for 30 minutes and then centrifuged for 15 minutes at 13,000 rpm. The supernatant was recovered, and DNA was precipitated by the addition of 600 µL of 100% ethanol. The homogenate was placed in the freezer at 30 minutes for 1 hour, then centrifuged at 13000 rpm for 25 minutes, after which the ethanol was discarded and the resulting pellet air dried for 10 minutes. The DNA pellets was re-suspended in 50 µL of 0.5X Tris-EDTA (TE) (pH 8.0). Five-microliter (5 µL) portions of the DNA extracted were used for PCR amplification.

2.4. Identification of Blood Meal in Sand Flies by PCR

The identification of the blood meal in the individual sand fly was based on PCR amplification of cytochrome b gene (*cyt b*) carried in the mitochondrial DNA (mt DNA) of mammalian hosts. A 359 bp fragment of the *cyt b* gene was amplified using previously described primers [23]. DNA extracts from individual sand fly were used as template and the primers *cyt b* forward: 5'-CCA TCC AAC ATC TCA GCA TGA AA-3' and *cyt b* reverse 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3' (Macrogene, Korea) were designed for the amplification of 359 bp of the conserved region of the *cyt b* gene. The 359 bp *cyt b* segment was amplified using 0.125 µL of One Taq DNA polymerase [1.25 U/50 µL] (New England Biolabs, Inc.) in a total reaction volume of 25 µL consisting of 5 µL of buffer (containing 1.8 mM of MgCl) [5X], 0.5 µL of 10 mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 µL of each of primer [10 µM], 5 µL of DNA and sterilized water to make up the final volume. The thermal cycling conditions used were: an initial denaturation at 95°C for 10 mins, amplification was

performed with 40 cycles consisting of denaturation at 94°C for 30s, annealing at 52°C for 30s, extension at 68°C for 45s, followed by a final extension at 68°C for 5 mins, and 4°C as holding temperature. For each PCR, a negative control containing sterilized distilled water separately and a male sand fly separately instead of DNA was included. The positive control was positive blood fed mosquito. The reaction was carried out in the BIO RAD System Peltier Thermal Cycler. The amplified PCR products were subjected to electrophoresis on a 2% agarose gel in 1X TAE buffer stained with ethidium bromide (10mg/mL) at 80 V for 1 hour, with a 100 bp/ 200bp DNA Ladder provided as molecular weight size standard. The final amplicons were visualized under ultraviolet light and subsequently photographed.

2.5. Sequence and Phylogenetic Analysis

The PCR amplified products obtained was sequenced by Sanger's method in both forward as well as reverse directions and sequencing results were obtained from Applied Biosystems (ABI): ABI 3500XL Genetic Analyser, POP7TM and Brilliant DyeTM Terminator v3.1 (NimaGen BV, Nijmegen). The same primers used for the PCR reactions was used. The nucleotide sequences from both strands were aligned using the DNASTAR (Lasergene) software. Sequences was then compared with the GenBank database using NCBI (National Centre for Biotechnology information) BLASTN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), aligned and analysed in MEGA (Molecular Evolutionary Genetics Analysis) version 7. Phylogenetic tree was constructed using the Neighbour Joining (NJ) method.

3. Results

3.1. PCR Results

A total of 727 sand flies was captured during a nine (9) months sampling period from the three study communities. Females made up 76.07% (553/727) of the sand flies collected whereas males made up 23.93% (174/727). Males are not involved in the transmission cycle of the parasite since male sand flies are not blood feeders and were thus excluded from the study. Of the 553 female sand flies captured, 363 representing 65.64% were morphologically identified successfully by observing under an optical microscope. A total of 69.97% (254/363) were captured at Dodome Dogblome, 23.69% (86/363) at Lume Atsiamé and 6.33% (23/363) at Dodome Awuiasu. It is worth mentioning that only two sand fly species were identified: *Phlebotomus* (3.31%) and *Sergentomyia* (96.69%). The morphologically identified species are namely *Phlebotomus rhodaini*, *Sergentomyia antennata*, *Sergentomyia africana*, *Sergentomyia buxtoni*, *Sergentomyia collarti*, *Sergentomyia dureini*, *Sergentomyia ghesquierei*, *Sergentomyia hamoni*, *Sergentomyia ingrami*, *Sergentomyia schwetzi* and *Sergentomyia similima*. *Sergentomyia africana* was the most abundant species (n=95; 26.17%) and *Sergentomyia collarti* was the least identified (n=1; 0.28%).

The female sand flies were grouped as non-engorged and engorged. The non-engorged totalled 65.56% (238/363) while the engorged totalled 34.43% (125/363). Majority of the engorged sand flies were collected at Dodome Dogblome. After amplification of the cytochrome b gene by PCR on all the morphologically identified female sand flies, 67.2% (84/125) of the engorged female sand flies showed presence of blood meal by having the DNA product of 359 bp. The remaining 32.8% (41/125) were negative despite the presence of blood in their abdomen. The species of sand fly that showed

the presence of blood meal (Figures 2 and 3) included *Phlebotomus rodhaini*, *Sergentomyia ghesquierei*, *Sergentomyia similima*, *Sergentomyia africana*, *Sergentomyia antennata*, *Sergentomyia ingrami*, *Sergentomyia hamoni* and *Sergentomyia schwetzi* (Table 1). Since there was no yield in PCR products of negative control, this implied that only host DNA was amplified.

The cytochrome b gene for blood meal was analysed with AY509658.1, HQ180173.1, LC088152.1 and JX266260 from the GenBank.

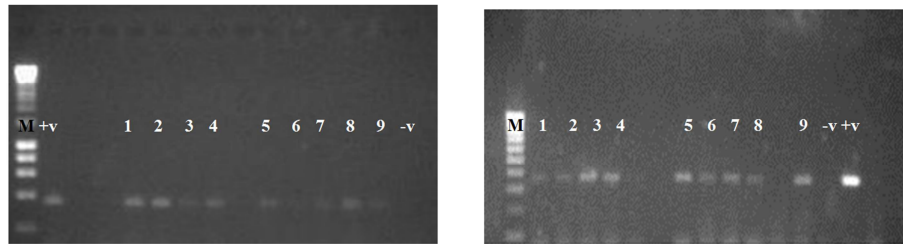


Figure 2. (LEFT) Agarose gel electrophoresis of PCR products of *Phlebotomus rodhaini* Lane M: Molecular weight marker (200bp) and (RIGHT). *Sergentomyia similima* Lane M: Molecular weight marker (100bp) after amplification of *cyt b* gene (359bp). +v, positive control -v, negative control.

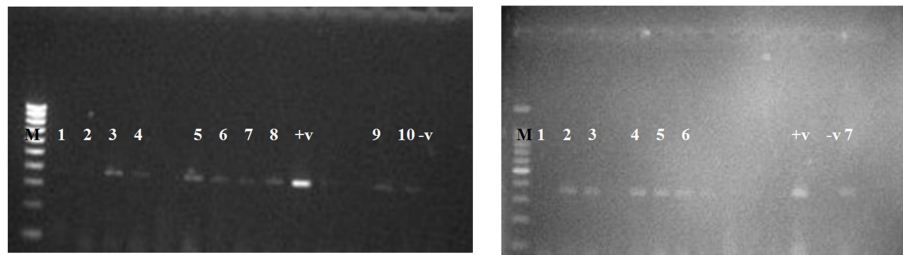


Figure 3. (LEFT) Agarose gel electrophoresis of PCR products of *Sergentomyia ghesquierei* (RIGHT) *Sergentomyia africana* after amplification of *cyt b* gene (359bp). Lane M: Molecular weight marker (100bp). +v, positive control of mosquito -v, negative control.

3.2. Sequence Analysis Results

The samples that showed presence of blood meal having the DNA product of 359 bp, was sent for sequencing. Only seven was able to be sequenced representing 8.3%. Unfortunately, of the seven, three did not show good quality sequence data. However, in order to have a fair idea of the source of blood meal of these four resulting sand flies (one *Phlebotomus rodhaini* and three *Sergentomyia similima* species) that showed sequencing results, the nucleotide sequence data obtained was edited and then subjected for BLAST with NCBI nucleotide sequence data library. The identity of the source of

blood meal for these four species was assessed based on retrieved *cyt b* sequences, considering the closest database matches at species level of vertebrate hosts.

After multiple alignments of experimentally generated sequences and those obtained from the BLAST and in addition to reference sequences obtained from the GenBank, a Neighbour-Joining phylogenetic tree was constructed using MEGA 7 program (Figure 4). The phylogenetic tree analysis showed that the resulting four species possess sequences in close relation to that of *Mus musculus* (house mouse) and *Homo sapiens* (human).

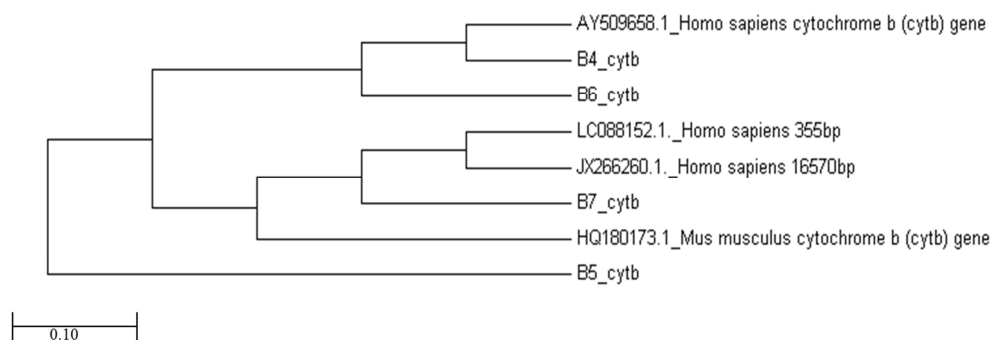


Figure 4. Neighbour joining tree using *cyt b* gene among four sand fly species. AY509658.1, LCO088152.1, JX266260.1 and HQ180173.1 are the reference sequence. B4 (*Phlebotomus rodhaini*), B5-B7 (*Sergentomyia similima*) are the sequences of this studies.

Table 1. The percentage (%) of blood meal present in each morphologically identified sand fly species.

Sand fly species	No. of sand flies identified (%)	Blood meal identification (%)
<i>Sergentomyia africana</i>	95 (26.17)	10 (2.75)
<i>Sergentomyia. similima</i>	93 (25.62)	20 (5.51)
<i>Sergentomyia ghesquierei</i>	83 (22.86)	27 (7.44)
<i>Sergentomyia schwetzi</i>	32 (8.82)	3 (0.83)
<i>Sergentomyia ingrami</i>	19 (5.23)	5 (1.38)
<i>Phlebotomus rodhaini</i>	12 (3.31)	8 (2.20)
<i>Sergentomyia antennata</i>	11 (3.03)	6 (1.65)
<i>Sergentomyia hamoni</i>	8 (2.20)	5 (1.38)
	363	84 (23.14)

4. Discussion

The analysis of source of blood meal provides valuable information about the feeding behaviour of each sand fly species and the role of a particular host in *Leishmania* foci [24]. It also provides which control strategies may be effectively implemented [25]. In the effort to achieving the objective of this study, which was to acquire basic data on the identification of blood meal in sand fly and its source, a molecular approach was used that involved amplification of potential host gene and subsequent sequencing [7]. This choice was based on the higher sensitivity of molecular methods in this endeavour. Hadj-Henni *et al.*, [26], stated in their study that *cyt b* is a good marker as it increases the accuracy of blood meal identification of female sand flies containing blood in their gut.

Of the morphologically identified number of female sand flies collected, 96.69% was of the genus *Sergentomyia* with 3.31% of the genus *Phlebotomus* (Table 1). This observation was consistent with studies carried out by Boakyee *et al.*, [27], Mosore, [28] and Nzeli *et al.*, [29] in the endemic area where *Sergentomyia* species was captured in abundance. Nevertheless, *Sergentomyia* is believed to transmit lizard *Leishmania* species whereas *Phlebotomus* transmit human pathogenic species of *Leishmania* [30]. However, studies in Ghana and elsewhere have suggested the possible involvement of *Sergentomyia minuta*, *Sergentomyia ingrami*, *Sergentomyia hamoni* and *Sergentomyia darlingi* as vectors of leishmaniasis [29, 31-33]. This reiterating the fact that some *Sergentomyia* species disclose periodic or opportunistic anthropophilic feeding behaviour. In this study, of the eleven sand fly species identified morphologically, presence of blood meal was observed in eight of the species (Table 1) with *Sergentomyia ghesquierei* species recording the most presence of blood meal.

Kent and Norris, [34] reported in their study that blood meal identification through successful molecular analysis depend on the amount of blood ingested and the period of blood digestion in the midgut of the insect. In the current study, although 34.43% (125/363) of the sand flies was blood fed, which was obvious during the morphological identification, however, only eighty-four of such sand flies tested positive by PCR. The fact that PCR could not detect the presence of blood meal in the sand fly could be attributed to the display of inter

and intraspecific variation in sand flies' sizes [3]. As a result, the volume of blood they ingest during blood feeding could be low, ranging from 0.1 to 1.0 L, thus escaping detectable threshold by this method [3, 35].

In order to zero-in on the particular hosts, in the endemic communities that these sand flies, may have been feeding on among the wide range of potential mammalian hosts, it was important to obtain DNA sequence data. Unfortunately, the quality of most of the DNA sequences of the amplified product were not good enough for further analysis. However, with the four sequences obtained, the BLAST analysis provided a narrow range of potential host. The analysis by the phylogenetic tree (Figure 4) showed close relatedness of the sequences to *Mus musculus* (house mouse) and *Homo sapiens* (human). This study using PCR and sequencing have observed that the source of blood meal could probably be from *Mus musculus* (house mouse) and *Homo sapiens* (human) though, further studies have to be carried out to ascertain this. Nonetheless, elsewhere in Africa, studies carried out by Bennai *et al.*, [36], Berdjane-Brouk *et al.*, [31], Tateng *et al.*, [37] and Yared *et al.*, [38], reported source of blood meal in *Sergentomyia darlingi*, *Sergentomyia minuta*, *Sergentomyia similima* and *Sergentomyia schwetzi* respectively to be *Homo sapiens* thus sending information across that some of these species of sand flies feed on human blood.

The sequences B4, B5, B6 and B7 (Figure 4) are closely related to both house mouse and human. With more improved DNA sequence quality, the relatedness of these sequence will be better understood. It is worth noting that the B4 is blood meal from *Phlebotomus rhodaini* while B5, B6 and B7 are blood meal from *Sergentomyia similima*. Despite the wide distribution of *Phlebotomus rodhaini* in most leishmaniasis endemic foci, it is considered a rare species and therefore it is ignored as a possible vector of leishmaniasis parasites [28]. Nevertheless, Anderson *et al.*, [39] reports in their study that though *Phlebotomus rodhaini* was collected in small numbers it is a known vector for *Leishmania major* elsewhere in West Africa specifically Mali.

The findings of this study although may not be fully appreciated for the comprehensive identification of the potential hosts and therefore the basic epidemiological information for vector control programs [3], it set the agenda that, it is a worthy research path to take and that the molecular approach as indicated by Ernieenor Faraliana *et al.*, [40] is the most straightforward and specific method to identify blood meals. Furthermore, this approach is ideal since primers may be improved based on initial data and later employed to amplify conserved homologous DNA fragments from diverse potential of vertebrate blood sources within the locality [41].

5. Conclusion

Conclusively, it can be said that sand flies are opportunistic feeders and their host preference is subject to how available and how abundant the blood meal source is. In this study,

Dodome Dogblome recorded the highest number of sand flies. *Sergentomyia africana* was the most abundant species identified whilst *Sergentomyia collarti* was the least identified. The most presence of blood meal was recorded in *Sergentomyia ghesquierei* and the least in *Sergentomyia schwetzi*. *Phlebotomus rodhaini* which is considered as one of the implicating vectors in the endemic area had eight out of twelve of its species showing presence of blood meal after amplification of *cyt b* gene (359bp). It can also be said that four (4) blood fed females fed on blood meal from human (*Homo sapiens*) and house mouse (*Mus musculus*).

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Conflicts of Interest

The authors declare that they have no competing interests.

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