Discovery of Phlebotomus Species of Sand Flies in Mwea Irrigation Scheme, Kirinyaga County, Kenya: A Possible Leishmaniases focus

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INTRODUCTION

Phlebotomus sand fly species are the only proven vectors of visceral leishmaniasis (VL) but knowledge of their distribution and diversity in Kenya is partial. Fourteen sand fly species out of the known 47 reported in Kenya belong to the genus Phlebotomus Leow, whereas 33 are Sergentomyia Franca and Parrot species [1]. It was found out that a majority of P. martini occurs in Baringo (52.3%), Nakuru (8.6%), Marsabit (37.2%) [2], nothing has been documented on its occurrence in Mwea sub-Country.

Mwea irrigation scheme is an area that is predominantly malaria and schistosomiasis endemic and not known to harbor sand flies. It was intriguing to confirm whether the vectors trapped were Phlebotomus and fly species through morphological features and molecular techniques.

Presence of P. Martini in Mwea sub-County for the first time could indicate a possible leishmaniases disease focus. However, no cases of leishmaniases transmission have been reported and this finding calls for more studies on vector surveillance and disease transmission in Mwea irrigation scheme.

MATERIALS AND METHODS

Study site

The studies were conducted in the Mwea rice irrigation scheme in Kirinyaga County, Kenya, 100 km northeast of Nairobi, Kenya. Mwea rice scheme covers an area of approximately 13,600 ha with a population of 150,000 people in 2,500 households [3]. Mwea sub-County is situated on the east of Mount Kenya at an altitude of 1100 to 1350 metres above sea level. The irrigation scheme is also a settlement scheme that produces 75-90% of the rice that is consumed in Kenya (National Irrigation Board Report). Water from the two major rivers, Nyamindi and Thiba is used to irrigate the rice-fields in a network of primary and secondary feeder canals. The villages are discrete units made up of homesteads/compounds with varying numbers of households. The majority of families keep domestic animals such as cattle, goats, sheep, donkeys and chicken among others [4]. The most common activities of the people of Mwea are connected with

**BACKGROUND AND OBJECTIVES:** During our study that was evaluating control of mosquitoes and sand fly vectors of Mwea Irrigation Scheme, Kirinyaga County, Kenya, we came across a number of phlebotomine sand flies. A faunistic study of some Kenyan phlebotomine sand flies (Diptera: Psychodidae) was carried out to find out the species which were likely to be present in Mwea Irrigation Scheme.

**METHODS:** Morphological and molecular vector species identification was done using taxonomic keys and cytochrome c oxidase subunit 1 (COI) gene amplification followed by Sanger sequencing and phylogenetic tree was constructed using the neighbor-joining method.

**RESULTS:** A total of 251 sand flies were collected consisting of Phlebotomus martini Parrot 4.8% and P. rodhaini Parrot 0.8%. Majority of the sand flies collected were from Sergentomyia species (94.4%). High Resolution Melting (HRM) profiles of some male sand flies had same peak as that of P. martini controls and closely related in phylogenetic tree analysis.

**CONCLUSION:** The finding of these Phlebotomus species in Mwea is an indication that they can breed in non-arid/semi-arid urbanized areas and can be synanthropic unlike previously documented. PCR-High Resolution Melting profiles and phylogenetic analyses indicated that samples of sand flies were related to P. martini sequences (accession number JX105040).

**ABBREVIATIONS**

HRM: High Resolution Melting; VL: Visceral Leishmaniasis; CDC: Centre for Disease Control; KEMRI: Kenya Medical Research Institute; DNA: Deoxyribonucleic Acid; PCR: Polymerase Chain Reaction; CO1: Cytochrome Oxidase subunit1
Morphological identification of sand flies

In this study, in order to trap sand flies, miniature SSM CDC Light traps (John W. Hock, Gainesville, Florida) were positioned in outside 30 homesteads on a daily basis at 18:00hrs and retrieved at 06:00hrs the following morning for two consecutive days. The sand flies were aspirated, counted, recorded on excel data sheets. They were put in small vials with desiccant pebbles to provide dry condition and transported to KEMRI laboratory where they were dissected and mounted on micro-slides using chloral hydrate gum according to the method of Minter (1963). They were allowed to dry for one day at room temperature before being identified under the compound microscope using identification keys of Abonnenc [5].

Molecular identification of sand flies

DNA was extracted from the remaining parts of the specimens using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendation. The insect was macerated first and briefly 180µl of buffer ATL was added to 1.5 ml microcentrifuge tubes containing each specimen and the tissues disrupted using micro pestles before addition of 20µl of proteinase K. The mixture was vortexed and incubated at 56°C for 1 hour and 30 minutes followed by vortexing for 15 seconds, addition of 200µl of buffer AL, mixing by vortexing, addition of 200µl of 96%-100% ethanol and vortexing again. The mixture from the previous step was pipetted into the DNeasy mini spin column placed in 2ml collection tube, centrifuged at 8000rpm for 1 minute and the flow-through and the collection tube discarded. The DNeasy mini spin was transferred into a new 2ml collection tube, 500µl of buffer AW1 added, centrifuged at 8000rpm for 1 minute and the flow-through discarded as in the previous step. The DNeasy mini spin column was transferred into a new 2ml collection tube followed by addition of 500µl of buffer AW2, centrifugation at 14000rpm during the pre-elution step for DNA and animal tissues (according to the protocol), for 3 minutes to dry the membrane and discarding the flow-through and the collection tube. The DNeasy mini spin column was placed in a clean 1.5ml microcentrifuge tube and 100µl of buffer AE pipette directly onto the DNeasy membrane. This was incubated at room temperature for 1 minute followed by centrifugation at 8000rpm to elute the DNA.

Molecular identification was done by polymerase chain reaction (PCR) of the mitochondrial cytochrome oxidase subunit 1 (COI) genomic marker. The COI gene was amplified using DNA barcoding primers; forward LCO1490 (5’-GGTCAACAATTGATATATTGG-3’) and reverse, HCO2198 (5’-TAAACTTCAAGGGTGAACCAAAAAATCA-3’) (Kumar, Srinivasan & Jambulingam, 2012). In a final volume of 15µl, the following was added: 7.5µl of 2x My Taq mix, 0.75µl of primer, 2µl of DNA extracted from the specimens (template) and 4 µl of PCR grade water. For the concentrations, we used ready reagents as follows: My Taq mix is a ready to use (2x) in concentration, primer stock 100U ml; http://www.bioline.com/uk/downloads/dl/file/id/2741/mytaqmixproductmanual.pdf. Thermal cycling conditions included an initial denaturation step of 15minutes at 95°C followed by 40 cycles of 95°C for 1 minute (denaturation), 52°C for 30 seconds (annealing) and 72°C for 40 seconds (extension) and a final extension of 72°C for 10minutes.

Further, the region was amplified using Uninimivar JF primers; forward primer, Uninimivar JF F (5’-ACCAATGACTAAGGATATTGGCA-3’) and reverse primer Uninimivar JF R (5’- AAAATTATAATAAAAAWGATGACC-3’), and the established 173bp PCR products resolved using high resolution melt (HRM) analysis (Ajamma et al., 2016) in Rotar gene Q HRM real time PCR thermal cycler (QIAGEN, Hannover, Germany). The PCR mixture consisted of 2µl of 5x Hot FirepolEvagreen HRM mix (Solis BioDyne, Tartu, Estonia), 0.25µl of each primer, 1 µl of DNA template and PCR grade water in a final volume of 10µl. The thermal cycling conditions involved an initial denaturation at 95°C for 15minutes followed by 40 cycles of denaturation at 95°C for 30seconds, annealing at 52°C for 45 seconds and extension at 72°C for 45 seconds and a final extension at 72°C for 10minutes. Without stopping the reaction, the PCR products were denatured at 95°C for 1 minute, held at 40°C for another minute and melted by gradually raising the temperature from 65°C to 95°C by 0.1°C in each step, waiting for 90 seconds of pre-melt conditioning on first step and 2 seconds for each step afterwards. In both the amplifications, PCR grade water was used as the negative control while positive controls included DNA from known (molecularly identified) sand fly species. Sand fly species identification was done by comparing the HRM profiles generated to those of the reference control species. Representative samples of identified sand fly species with unique HRM curves were identified by purification, of about 650bp PCR products established in the LCO/HCO amplification above, using ExoSAP-IT (USB Corporation, Cleveland, OH) and submitted for sequencing using the Sanger method. To confirm the identity of PCR-HRM differentiated sand flies, the generated DNA sequences were edited using Geneious and queried against the Genbank database using the Basic Local Alignment Search. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from the identifiers was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches. The evolutionary distances were computed using the Poison correction method and in the units of the number of amino acids. The evolutionary analyses were conducted in MEGA7 (www.megasoftware.net).

RESULTS

A total of 251 sand flies comprising of two genera (Phlebotomus and Sergentomyia) were caught and identified to species level. The collection comprised of two Phlebotomus species and six Sergentomyia species as shown in Table 1. The two Phlebotomus species that were caught were P. martini, (4.8%) and P. rodhaini, (0.8%) while Sergentomyia species were S. schwetzii Adler, Theodor and Parrot (6.0%), S. bedfordi
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Figure 1 HRM profiles of sand fly samples A, B and C compared with P. martini positive control.

<table>
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<tr>
<th>Well no</th>
<th>s#</th>
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</thead>
<tbody>
<tr>
<td>L</td>
<td>1Kb DNA ladder</td>
</tr>
<tr>
<td>1</td>
<td>males</td>
</tr>
<tr>
<td>2</td>
<td>males</td>
</tr>
<tr>
<td>3</td>
<td>females mwea</td>
</tr>
<tr>
<td>4</td>
<td>P. martini +ve control</td>
</tr>
<tr>
<td>5</td>
<td>ntc</td>
</tr>
</tbody>
</table>

Master mix
- H2O: 4
- 2X MyTaq Mix: 7.5
- LCO 1490: 0.75
- HCO 2193: 0.75
- Template: 2

Conditions
- 95°C: 15 min
- 55°C: 60 sec
- 55°C: 50 sec
- 72°C: 40 sec
- 72°C: 10 min
- 4°C: —

Figure 2 Electrophoretic analysis of PCR product (1kb).

Newstead, (3.2%), S. squamipleuris New stead, (27.9%), S. clydei Sinton, (0.4%), S. antennata New stead, (0.4%) and S. inermis Abonnenc (56.6%). Female sand flies were three times the number of males caught during the study. Out of the total number of sand flies caught, majority were S. inermis, Abonnenc followed by S. squamipleuris, Newstead. The results are summarized in the Table 1 and Figure 1. Sand fly species identification was done by comparing the High Resolution Melting (HRM) profiles generated to those of the reference control species (Figure 2). HRM analysis is a post PCR technique that measures the rate of sDNA to ssDNA with increasing temperature. It is applicable when detecting differences in the nucleotide composition of a specific real-time PCR product. PCR-HRM assay results demonstrated that sand fly samples B had a HRM profile peak similar to P. martini +ve control, whereas for C and A had profiles peak slightly outside that of P. martini +ve control, suggesting that B sand fly
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**Figure 3 Ultrametric phylogenetic tree for P. martini and Mwea sand fly samples A, B, C.**

Determination of the relationship of sand fly samples and *Phlebotomus martini* sand flies in Mwea site using phylogenetic tree. PCR-HRM assay results demonstrated that sand fly samples B had a HRM profile peak similar to *P. martini* +ve control, whereas for C and A had profiles peak slightly outside that of *P. martini* +ve control, suggesting that B sand fly samples were more related to *P. martini* +ve control and can be used to identify species. Multiple alignments of the sequences were done using CLUSTAL W and phylogenetic tree was constructed using The Neighbor-Joining method. This tree suggests that samples B for sandflies were genetically closely related to *P. martini* than samples A and C (Figure 3).

**DISCUSSION**

The trapping of sand flies in Mwea irrigation scheme was carried out during the dry and wet season between January 2014 and January 2015. Kirinyaga County is at the border line of the Kenya highlands and the semi-arid regions. The collection of 12 (twelve) *Phlebotomus martini* and 2 (two) *P. rodhaini* of both sexes in Mwea Irrigation Scheme was unexpected because there were no termite mounds which act as the breeding and resting sites of the species, and moreover, the region is at the borderline between the highlands of Kenya and the semi-arid regions. The influence of climate variables on the distribution and activity of sand flies has been repeatedly reported [6-8]. In contrast with other reports, in the current study, climate variables did not affect the probability of finding *P. martini* and *P. rodhaini*, probably due to the short period of time when captures were performed and the homogenous geographical conditions of most trapping sites. Termite mounds have been incriminated as the ideal habitats for *P. martini*, *P. celiae*, and *P. vansomerenae*, in East Africa and their distribution is associated with the vectors of leishmaniasis, it now seems from the findings that the habitat of these, and *P. rodhaini* sand flies is dependent on additional environmental factors and can be found in areas lacking termite mounds [9]. The first record of natural infection of *Leishmania donovani* parasites

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**Table 1: Phlebotomine sand flies caught in households in Mwea Irrigation Scheme households.**

<table>
<thead>
<tr>
<th>Number</th>
<th>Sand fly species</th>
<th>Males</th>
<th>Females</th>
<th>Totals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>P. martini</em></td>
<td>8</td>
<td>4</td>
<td>12 (4.8)</td>
</tr>
<tr>
<td>2</td>
<td><em>P. rodhaini</em></td>
<td>1</td>
<td>1</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>3</td>
<td><em>S. antenata</em></td>
<td>1</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>4</td>
<td><em>S. bedfordi</em></td>
<td>4</td>
<td>4</td>
<td>8 (3.2)</td>
</tr>
<tr>
<td>5</td>
<td><em>S. clyde</em></td>
<td>0</td>
<td>1</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>6</td>
<td><em>S. schwetzi</em></td>
<td>3</td>
<td>12</td>
<td>15 (6.0)</td>
</tr>
<tr>
<td>7</td>
<td><em>S. squamipleuris</em></td>
<td>10</td>
<td>60</td>
<td>70 (27.9)</td>
</tr>
<tr>
<td>8</td>
<td><em>S. inermis</em></td>
<td>30</td>
<td>112</td>
<td>142 (56.7)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>57</td>
<td>194</td>
<td>251 (100)</td>
</tr>
</tbody>
</table>

**Abbreviations:** *P.* = *Phlebotomus*; *S.* = *Sergentomyia*
in *P. rodhaini* and any *Anaphlebotomus* sand flies was reported by Elnaiem et al., [10]. Despite its wide distribution in most leishmaniasis endemic foci, *P. rodhaini* is considered a rare species and therefore it was ignored as a probable vector of *Leishmania* parasites. The use of rodent baited traps was shown to increase the collection of *P. rodhaini* by more than 40 fold [11], supported the findings of Hoogstraal & Heyneman [11], who reported that *P. rodhaini* is more readily collected by rodent baited traps. This was not achieved in this study because only light traps were used. The presence of *P. martini* in Mwea Irrigation Scheme is a health issue since it is a vector of *L. donovani* in the endemic areas of Kenya.

**CONCLUSION**

There are no termite mounds in the study site. This was taken to indicate that there are other breeding sites that are still unknown in Kenya for *P. martini*. Leishmaniasis should therefore be considered a risk not only for the habitual inhabitants of the scheme, but also for the large numbers of people visiting the area, in case of the introduction of the parasite, coinciding with the period of vector activity. Our account of a sand fly, *P. martini* with a proven or capacity to transmit *L. donovani* and *P. rodhaini*, a probable vector in the Mwea non-endemic area, together with the arrival of *L. donovani* infected persons and animals, would favor the possibility of autochthonous transmission in the area.

On the other hand, medical personnel who work in the Health facilities need to be sensitized on the kala-azar symptoms and diagnosis so as to differentiate malaria and kala-azar since the two diseases symptoms are similar.

**ACKNOWLEDGEMENTS**

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**REFERENCES**
