Characterization and comparison of *leishmania*-like isolates from rodents, lizards and sand flies caught at Masinga location in Machakos district, Kenya

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SUMMARY

A laboratory based study was designed to characterize 43 cryo-preserved Leishmania-like flagellates. These Leishmania-like flagellates were originally obtained from non-human hosts that included spiny mice (Acomys subspinosus), plated lizards (Gerrosaurus major) and sand flies of the Genus Sergentomyia caught at Masinga location, Machakos District in Kenya. Morphological features and isoenzyme banding patterns of the flagellates were studied. The isoenzyme markers which were used for isoenzyme electrophoresis included Malate dehydrogenase (MDH), Phosphoglucomutase (PGM), Glucose phosphate isomerase (GPI), Glucose 6-phosphate dehydrogenase (G6PD), Malic enzyme (ME), 6 phosphogluconate dehydrogenase (6PGD) and Mannose phosphate isomerase (MPI). The isoenzyme banding patterns of the flagellates' lysates were compared with those of six WHO Leishmania reference strains and those of seven well characterized reference strains of Trypanosoma, Crithidia, Herpetomonas and Leptomonas species. The results showed that the morphological changes of the Leishmania-like flagellates in the growth medium were indistinguishable from those of Leishmania WHO reference strains used. The isoenzyme profiles of the flagellates were all distinguishable from the reference strains used except for isolate NLB-1236 from G. major which had an enzyme profile identical to that of L. tropica (NLB-305) in 6 enzymes (MDH, GPI, MPI, ME, PGM, and G6PD). The banding pattern of isolate NLB-1261 from A. subspinosus was indistinguishable from that of L. major (NLB-326) in 3 enzymes only (MDH, GPI and ME) while isolate NLB-1231 from A. subspinosus had an enzyme profile identical to those of L. tropica (NLB-305) and L. arabica (NLB-664) in six enzymes (MDH, GPI, ME, PGM, MPI, and 6PGD). More than 80% of the Leishmania-like flagellates had enzyme profiles indistinguishable from each other, in all the isoenzyme markers. The morphological traits of the flagellates suggested that they were Leishmania or strains closely related to Leishmania. Isoenzyme analysis suggested that Sergentomyia sand flies most likely feed on both lizards (reptiles) and rodents (mammals). There is need to carry further investigations on NLB-1236 (from plated lizards), NLB-1261 (from wild spiny mice) and NLB-1231 (from wild spiny mice).

[Afr J Health Sci. 2011; 18:6-13]

Introduction

Leishmania parasites *(Kinetoplastida; Trypanosomatidae)* are the smallest nucleated cells which multiply inside a digestive vacuole in the mammalian macrophage and also live in the gut of a sand fly which acts as a vector [1]. In humans, pathogenic *Leishmania* parasites may cause the deadly *visceral leishmaniasis (VL)*, *cutaneous leishmaniasis (CL)* or the disfiguring as well as stigmatizing muco-cutaneous leishmaniasis (MCL). WHO estimates that about 350 million people are at risk of Leishmania infection and majority of these people live in developing countries (http://www.who.int/ctd/html/leis.html). Apart from *Leishmania,* other trypanosomatids infecting warm-blooded animals include a variety of *Leishmania*-like parasites. Among the strategies of controlling leishmaniases in Kenya, the search for reservoir hosts [2] and identifi

cation of the vectors have been of paramount importance. VL reservoir hosts are domestic dogs in South America [3] and suspected reservoirs in West Pokot and Machakos Districts in Kenya [4]. *Sporadic L. donovani* infection has also been attributed to rodent reservoirs in Africa south of the Sahara [5]. Despite the occassional outbreaks of VL in Kitui and Machakos Districts of Kenya, the reservoir host has not yet been established [6]. In Kenya, CL reservoir hosts include rock hyraxes *(Procavia johnstoni)* and tree hyraxes *(Dendrohyrax auboreus)* [7] and wild rodents including Tatera robusta [8].

In Kenya, morphologically similar Leishmania and Leishmania-like flagellates from mammals, reptiles and sand flies have been observed in leishmaniasis endemic foci [4, 8]. Such, are the pathogenic *L. donovani, L. major or L. aethiopica*; non-pathogenic *L. adleri* or potentially pathogenic monoxenous insect flagellates of *Crithidia, Herpetomonas and Leptomonas* species [8]. Infections emanating from trypanosomatid flagellates are now considered as opportunistic infections in acquired immune deficiency syndrome (AIDS) patients [9, 10, 11]. Increasing numbers of immuno-suppressed patients such as those with AIDS are found to suffer from VL with no known history of leishmaniasis [10]. L. infantum which causes CL and VL has been isolated from the blood of an immuno-suppressed AIDS patient [9, 12]. It is on this background that the current study was designed to characterize the *Leishmania-like* flagellates obtained from VL-prone areas of Kenya with an aim of establishing the likely vectors and reservoir hosts of *Leishmania* and *Leishmania-like* fllagellates in Machakos District. This would assist in designing effective strategies of controlling leishmaniasis in Kenya.

Materials and methods

In-vitro cultivation of *Leishmania-like* flagellates: Both the flagellates and the WHO Leishmania reference strains were retrieved from liquid nitrogen where they had been cryopreserved. A total of 43 unidentified flagellates and 16 reference strains (Table 1) were used. The organisms were cultured in 25cm3 culture flasks containing NNN/Schneider's Drosophila medium with an overlay of 20% heat-inactivated foetal Bovine Serum (FBS), Streptomycin (250µg/ml), Gentamycin (250µg/ml), Penicillin (250U/ml) and 5-Fluorocytosine (500µg/ml) [13,14]. Cultures were incubated at 25°C. The organisms were observed dailyand their developmental changes were noted until they attained the stationery promastigotes phase. The stationary phase promastigotes were harvested and centrifugally washed three times at 3000g at 4°C for 10 minutes and their concentration adjusted to 1 x 106 promastigotes per ml of phosphate buffered saline (PBS). The lysates were then made for use in electrophoresis assays.

Table 1: Shows the WHO references and other well characterized reference strains used.

STRAIN CODE	PARASITE IDENTITY
(a) WHO REFERENCE STRAINS	
MHOM/SU/58/STRAIN OD/NLB-305	*L. tropica*
MHOM/IL/67/JERICHO-11/NLB-326	*L. major*
MHOM/ET/72/L.100/ NLB-310	*L. aethiopica*
MHOM/KE/82/LRC-L445/NLB-065	*L. donovani*
MRHO/SA/83/NLB-664	*L. arabica*
IPHL/KE/LRC-L447/ NLB-144	*L. major*
(b) OTHER REFERENCE STRAINS USED	
LRC-L466/LN-277/ NLB-327	*Crithidia fasciculate*
ATCC 30260/NLB-341	*Herpetomonas muscarum muscarum*
ATCC 30209/LN295/ NLB-340	*H. megaseliae*
ATCC 30220/LN294/ NLB-339	*Leptomonas seymouri*
SND-FLY 3523/NLB 202/C.25	*L. adleri*
SND-FLY 3364/LRC-L454/NLB-203	*Crithidia Sp.*
SND-FLY3090/NLB-148	*L. adleri*
LN-474/NLB-508	*Trypanosoma microti*
LN-475/NLB-509	*T. evotomys*
NLB-051	*L. donovani*

Electrophoresis: The isolates were characterised by using cellulose acetate electrophoresis (CAE) using published methods [15,16]. The enzymes examined were Malate dehydrogenase (MDH, E.C. 1.1.1.37); Malic enzyme (ME, E.C. 1.1.1.40); 6-phosphogluconate dehydrogenase (6PGD, E.C.1.1.1.44); Glucose 6-phosphate dehydrogenase (G6PD, E.C.1.1.1.49); Phosphoglucomutase (PGM, E.C. 2.7.5.1); Mannose phosphate isomerase (MPI, E.C.5.3.1.8) and Glucose phosphate isomerase (GPI, E.C. 5.3.1.9). The unidentified *Leishmania-like flagellates* were first compared with each other before comparing them with the reference strains. The comparison was based on the similarities of the zymogram banding patterns formed. Isolates which formed similar isoenzyme bands (same distance from the point of application) on the cellulose acetate (CA) plate were taken to be identical. Likewise, any Leishmania-like flagellate and reference strain whose isoenzyme banding patterns were similar were considered to be identical.

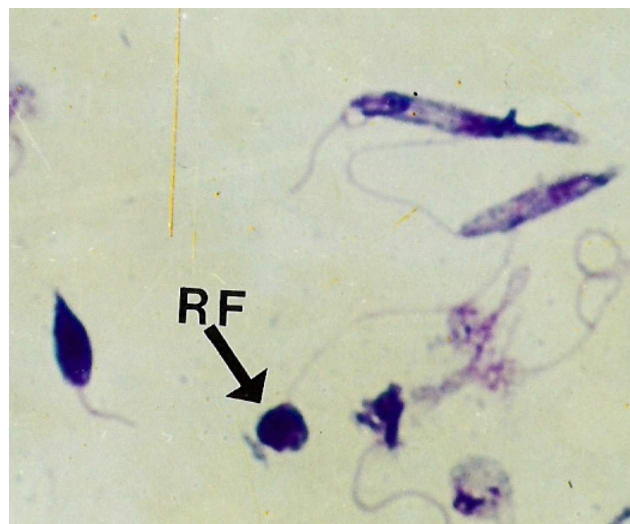
Results

Morphological and in-vitro growth characteristics: The *Leishmania-like flagellates* took an average of 9 days to grow up to stationary phase promastigote stage. In the first 4 days, the flagellates were short, broad with short flagellum and had a characteristic fast movement. At 7 days post-culture, flagellates were slightly longer with relatively long flagellum, a stage in which they occasionally formed rosettes by their anterior ends.

Plate 1: Slender form (indicated as SF by the arrow) observed after in vitro growth of isolate NLB-1148 from A. subspinosus liver

After 8 to 10 days post-culture, the flagellates changed to thin slender forms with a very long flagellum (Plate 1). The morphological growth characteristics of the Leishmania-like flagellates in biphasic growth medium were indistinguishable from those of *Leishmania* reference strains used. *Flagellates* NLB-1137 and NLB-1243 had distinct round forms with a long flagellum prior to their slender forms (Plate 2). All the unidentified *flagellates* plus the reference strains had ante nuclear kinetoplast, flagella which emerged from their anterior ends and all of them divided mitotically along the anterior axis.

Plate 2: The round form (indicated as RF by the arrow) observed after in vitro growth of isolate NLB-1243 from A. subspinosus spleen.



Isoenzyme analysis: From a total of 27 electrophoretic runs, all the *Leishmania-like flagellates* had isoenzyme banding patterns distinguishable from those of reference strains used except three *Leishmania-like flagellates* namely, NLB-1236 from *G. major* which had isoenzyme patterns indistinguishable from *L. tropica* (NLB 305) in 6 enzymes (MDH, GPI, MPI, ME, PGM and G6PD) (Plate 3); NLB-1231 from *A. subspinosus* (liver) which had isoenzyme banding patterns indistinguishable from both *L. arabica* (NLB-664) and L. tropica (NLB-305) in 6 enzymes (MDH, MPI, GPI, ME, PGM and 6PGD) (Plate 4) and NLB-1261 obtained from *A. subspinosus* (bone-marrow) which had isoenzyme profiles identical to those of *L. major* (NLB-326) in 3 enzymes (MDH, ME and GPI). The isoenzyme banding patterns of the Leishmania-like flagellates obtained from sandfly of the Genus Sergentomyia were indistinguishable from those of *Leishmania-like flagellates* obtained from wild spiny

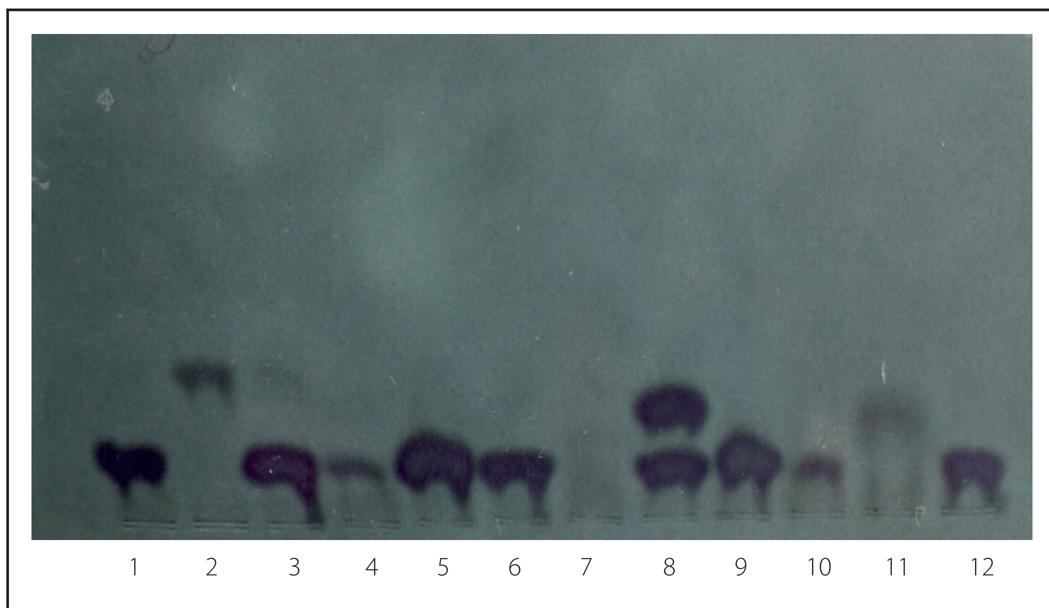


Plate 3: Isoenzyme banding patterns for enzyme GPI showing that isolate NLB-1236 from G. major was indistinguishable from L. tropica. Key: 1, NLB-1236 from G. major; 2 and 5, NLB-1137 and NLB-1528 respectively from S. garnhami; 3, L. tropica Ref. (NLB-305); 4, 6 and 9, NLB-1148, NLB-1200 and NLB-1255 respectively all from A. subspinosus liver; 5, NLB-1528 from S. garnhami, 7, NLB-1261 from A. subspinosus bone marrow; 8, L. major Ref. (NLB-326); 10, NLB-1169 from A. subspinosus skin; 11, NLB-1243 from A. subspinosus spleen; 12, NLB-1511 from S. graingeri

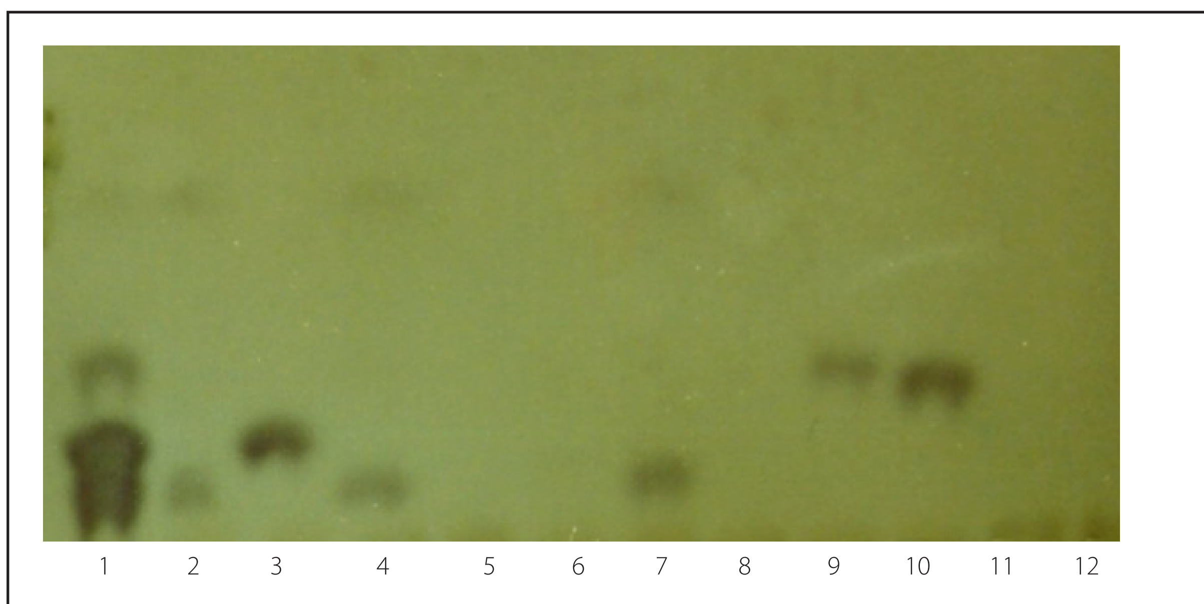
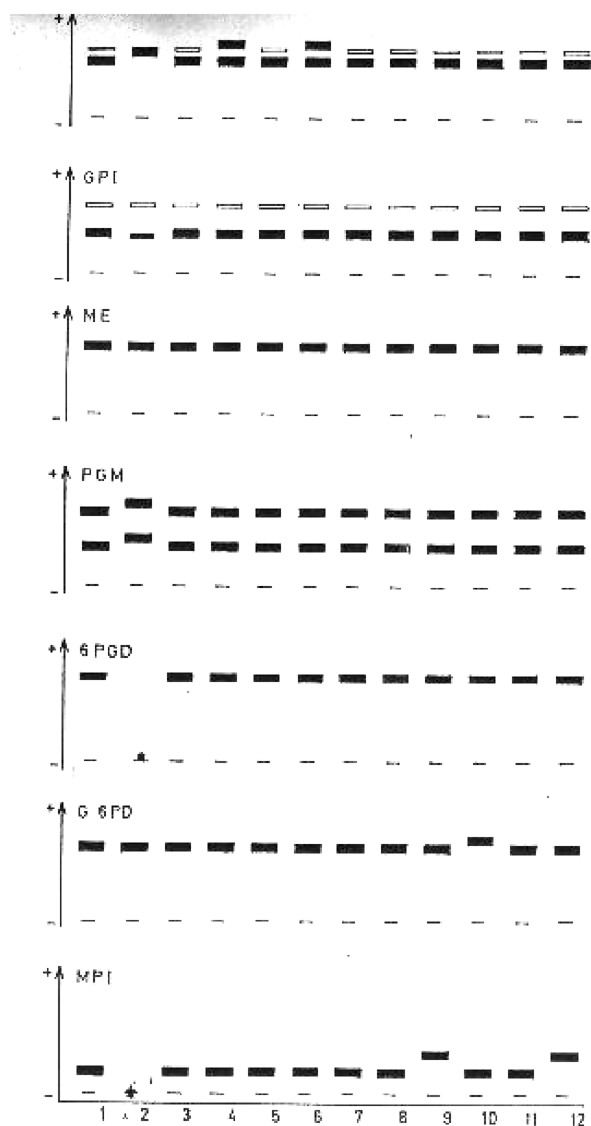


Plate 4: Isoenzyme banding patterns for enzyme MPI showing that NLB-1231 (lane 2) from A. subspinosus (liver) was indistinguishable from L. tropica (lane 4) and L. Arabica (lane 7). Key: 1, L. major Ref (NLB-326); 2, NLB-1231; 3, NLB-1261 from A. subspinosus bone marrow; 4, L. tropica Ref (NLB-305); 5, NLB-1243 from A. subspinosus spleen; 6, NLB-1202 from A subspinosus liver; 7, L. arabica Ref (NLB-664); 8, NLB-1200 from A. subspinosus liver 9, NLB-1137 from S. garnhami; 10, L. aethiopica Ref (NLB-310); 11, NLB-1508 from S. bedfordi; 12, NLB-1236 from G. major.

mice *(Acomys subspinosus)* and also indistinguishable from Leishmania-like flagellates NLB-1236 which was obtained from plated lizards *(Gerrhosaurus major)* (Fig 1).



**Isoenzyme band did not develop.*

Fig. 1: Diagrammatic representation of isoenzyme banding patterns for 7 enzymes obtained by electrophoresis of isolates from rodents (Acomys spp), lizards (Gerrhosaurus spp) and sandflies (Sergentomyia spp) caught at different locations. Key:- 1, 3, NLB-1236 (Lizards); 2, NLB-1237 (S. garnahami); 4, 6, NLB-1508 (S. bedfordi); 5, 7, NLB-1148 (A. subspinosus-liver); 8, 11, NLB-1511 (S. graigeri); 9, 12, NLB-1528 (S. garnahami); 10, NLB-1304 (A. subspinosus-spleen).

Discussion

Previously, Leishmania characterisation was based on morphological features [8], geographical and ecological distribution [17]; clinical manifestation of the parasites in patients [18]; behavioural characters of the parasites in susceptible mice [19, 20] or in sand flies [21, 22]. Biomedical methods which are powerful tools in the field of *Leishmania taxonomy* and identification are also available [23, 24]. Such methods include the use of deoxyribonucleic acid (DNA) probes as diagnostic tools in the polymerase chain reaction (PCR) technology [25]; the use of monoclonal antibody based enzyme linked immunosorbent assay (ELISA); DNA hybridisation [26] and isoenzyme electrophoretic studies [6].

In this study, the sequences of developmental forms of the *Leishmania-like flagellates* observed were similar to those of Leishmania references used. The short and broad forms which gave rise to elongated forms, resembled *Leishmania nectomonads* and *haptomonads* respectively as previously described by Lawyer *et al*. [27]. Similarly, the round forms with a long flagellum and the slender forms with a long flagellum resembled Leishmania paramastigotes and metacyclic promastigotes respectively [27]. The flagellates could not have been trypanosomes because none of the flagellates had the typical undulating membrane. Similarly, the stationary phase promastigotes observed were distinguishable from those of Crithidia whose promastigotes have a broad posterior end and a flagellum emerging from a pocket-like structure (choanomastigotes) as described by Manson-Barr and Bell [28]. No opisthomastigotes with post-nuclear kinetoplast were observed in all the developmental stages of isolates. This suggested that the Leishmania-like flagellates were not likely to be those of Herpetomonas species which have the characteristic opisthomastigote stage [3]. Earlier reports indicated that rodents and canids of Machakos District have a high prevalence rate of trypanosomes of subgenus Herpetosoma [3, 29]. The Leishmania-like flagellates from rodents (A. subspinosus), lizards (G. major) and Sergentomyia sandflies that were characterized were therefore not those of trypanosomes of subgenus Herpetosoma.

Isoenzyme electrophoretic studies have also been used in distinguishing between closely related species of parasites, species of insects and also genetic analysis of population structures [16, 30, 31, 32]. However, the diagnosis of leishmaniasis requires an integration of

techniques [33, 34, 35]. In this study, the in-vitro growth characteristics and the isoenzyme analysis were used to characterize the trypanosomatid flagellates isolated from Sergentomyia sand flies and vertebrate hosts caught in Machakos District. The *Leishmania-like flagellates* from visceral organs (spleen, liver) and bone marrow tissue of Acomys subspinosus caught at different location of Machakos District (Masinga area) had isoenzymes profiles indistinguishable from each other (CAE plate not shown). This observation suggested that Acomys subspinosus mice were harbouring a common flagellate that was infecting its visceral organs. Such behaviour of visceralization is shown by Leishmania donovani which infects the visceral organs of human beings causing visceral leishmaniasis [36]. Having indistinguishable isoenzyme profiles also suggests two things; first, that the Acomys subspinosus species from different locations had a common source of the *Leishmania-like flagellates* and second, that the *Leishmania-like flagellates* are prevalent in the entire area since the locations from which the flagellates were got are distant away from each other.

In this study, *Leishmania-like flagellates* from spiny mice *(A. subspinosus)*, Sergentomyia sand flies and plated lizards *(G. major)* showed indistinguishable isoenzyme profiles. Though the Sergentomyia sand flies were previously classified as reptile feeders, they have been found to feed on both mammals and man [3, 37]. Sergentomyia sand flies could therefore be the most likely vectors of these *Leishmania-like flagellates* described here. It is also possible that spiny mice *(A. subspinosus)* and the plated lizards *(G. major)* could be the reservoir hosts of the Leishmania-like flagellates. The lizard flagellates (NLB-1236) described here has since been identified as Sauroleishmania (RGER/KE/89/NLB-1236) and has been shown to have an ability to cause *L. major-like* cutaneous lesions and visceral leishmaniasis in BALB/c mice [38]

Conclusion

Based on morphological features and in vitro growth characteristics, the *Leishmania-like flagellates* were indistinguishable from Leishmania reference strains used. The isoenzyme analysis indicated that the majority of the *Leishmania-like flagellates* were distinguishable from the *Leishmania, Trypanosoma, Crithidia, Herpetomonas* and *Leptomonas* references used. Exception to this however were NLB-1236 (from plated lizards), NLB-1261(from spiny mice) and NLB-1231 (from spiny mice) whose isoenzyme banding patterns were identical to *L. tropica, L. major and L. arabica/L. tropica* respectively. These observations require further clarification using PCR- technology. The CAE isoenzyme analysis seems to indicate that Sergentomyia sand flies feed on both lizards and spiny mice (rodents). The role of Sergentomyia sand flies, plated lizards and spiny mice in the spread of Leishmania or a closely related strain of Leishmania, requires further investigation since NLB-1236 flagellates originally obtained from plated lizards has since been shown to cause Leishmania-like lesions in BALB/c mice.

Acknowledgements

We would like to thank the staff of the Leishmania laboratory at Kenya Medical Research Institute (KEMRI), Nairobi for cryo-preserving the Leishmania-like flagellates in liquid nitrogen. This work was published with the approval of the Director, KEMRI-Nairobi, Kenya. This study received financial support from KEMRI and Moi University, Department of Zoology, Eldoret, Kenya.

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