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Presence of Leishmania donovani DNA in wild-caught biting midges Culicoides sp. at a Leishmaniasis disease endemic area in Sri Lanka; An alternative vector for disease transmission

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Abstract

Biting midges, belonging to the Ceratopogonidae family, are a group of dipteran insects implicated as vectors for various parasites and viruses. In Sri Lanka, where leishmaniasis is endemic, biting midges are found in high densities, causing significant biting nuisance. This observation raises the possibility that these insects may serve as potential vectors for leishmaniasis in these areas. To investigate the presence of *Leishmania donovani* parasites within biting midge populations in a leishmaniasis-endemic area of Sri Lanka, a study was conducted in the Medawachchiya Medical Officer of Health area, Anuradhapura District. Biting midges were collected using cattle-baited net traps in December 2021. Morphological identification keys were used to classify the collected specimens, which were then surface sterilized and subjected to DNA extraction. Polymerase Chain Reaction (PCR) targeting the kinetoplast minicircle gene specific to *L. donovani* was employed to detect the presence of parasite DNA. The resulting PCR amplicons were visualized using gel electrophoresis. Out of the 42 biting midges collected, all were morphologically identified as *Culicoides imicola*. Interestingly, 4.76% (n=2) of the field-caught samples were positive for *L. donovani* DNA. These findings suggest that *L. donovani* circulates within biting midge populations. These insects may act as vectors for leishmaniasis transmission in the country. Therefore, it is strongly recommended to continue studying biting midges in Sri Lanka to enhance our understanding of their role in disease transmission.

Keywords: *Leishmania*, Parasites, Biting midges, DNA, Vector

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Introduction

Leishmaniasis is a vector-borne disease caused by the parasites in the *Leishmania* genus and transmitted to humans and animals through the bites of infected female phlebotomine sand flies, commonly known as sand flies. There are three main forms of the disease based on its clinical presentation, namely; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) also known as *kala-azar* (Vargas Martínez et al., 2011). So far, about 21 species of both sub-genera have been identified as causative agents of the disease. *L. donovani* complex (*L. donovani* and L. *infantum/*or *L*. *chagasi*) is mainly responsible for the VL (Barrett and Croft, 2012). Cutaneous leishmaniasis is caused by more than 15 species including *L. tropica*, *L. major* and species of *L. mexicana* complex (*L. mexicana*, *L. amazonensis* and *L. venezuelensis*). However, *L. donovani* is responsible for the CL in some areas of the world (Kumar et al., 2015; Ponce et al., 1991; Sharma et al., 2005). Mucocutaneous leishmaniasis, on the other hand, is mainly caused by species of the subgenus *Viannia* (*L. braziliensis; L. guyanensis; L. panamensis* and *L. peruviana*) (Vargas-Inchaustegui et al., 2008).

Sri Lanka is considered an endemic country for CL, a parasitic disease caused by *L. donovani*. The primary vector responsible for transmission in Sri Lanka is *Phlebotomus argentipes* (WHO, 2021). *Phlebotomus argentipes* sand fly is the primary vector for leishmaniasis transmission in Sri Lanka (Senanayake et al. 2011). Some studies have highlighted that along with the sand fly collections, a high density of midges (Ceratopogonidae family) have been noticed in the sites that had reported leishmaniasis cases (Wijerathna et al., 2021). Until recently, the sand flies remained the only proven vectors of *Leishmania spp*. However, the recent implementation of PCR techniques has led to increasing speculation about biting midges as alternative vectors for leishmaniasis (Seblova et al., 2014).

Several studies have reported the presence of *Leishmania* parasites in biting midges, indicating their potential role in disease transmission. For example, a study conducted in Spain found *Leishmania* DNA in biting midges captured in areas where leishmaniasis cases had occurred, suggesting their involvement in the transmission cycle (Molina et al., 2012; Songumpai et al., 2022). Similarly, a study in Italy detected *Leishmania* DNA in biting midges caught in areas endemic to leishmaniasis, further supporting their potential as secondary vectors (Pombi *et al.,* 2017). These findings strongly indicate that biting midges may contribute to leishmaniasis transmission in certain regions.

Furthermore, experimental studies have demonstrated the ability of biting midges to acquire and transmit *Leishmania* parasites. In laboratory settings, biting midges have been shown to become infected with *Leishmania* parasites after feeding on infected hosts. Subsequently, they can transmit the parasites to uninfected hosts during subsequent blood meals (Molina et al., 2012). These experimental findings highlight the vector competence of biting midges for Leishmania transmission.

Although the role of biting midges in leishmaniasis transmission is not as well-established as that of sand flies. Biting midges are known to be widespread and abundant in various regions worldwide, including areas where leishmaniasis is endemic (Panahi et al., 2020). Their small size and ability to bite humans and animals make them capable of transmitting infectious agents, including *Leishmania* parasites (Dougall et al., 2011; Panahi et al., 2020; Sunantaraporn et al.,

2021). Additionally, the feeding habits of biting midges, which involve multiple blood meals from different hosts, enhance the likelihood of parasite transmission between infected and uninfected individuals (Dougall et al., 2011; Panahi et al., 2020; Sunantaraporn et al., 2021). Therefore, the present study was focused on detecting the *L. donovani* parasites in the wild-caught midges in Sri Lanka. This study is the first attempt to explore the presence of *Leishmania* parasite species in biting midges.

Materials and Method

Selection of the study site

Anuradhapura District reports the highest incidence of leishmaniasis in Sri Lanka for the past several years (Galgamuwa et al., 2018; Hewawasam et al., 2020). Madawachchiya Medical Officer of Health (MOH) area in the District of Anuradhapura reported the highest number of leishmaniasis cases over the last few years. Therefore, the Madawachchiya MOH area in the Anuradhapura District was selected for the present study (Figure 1).

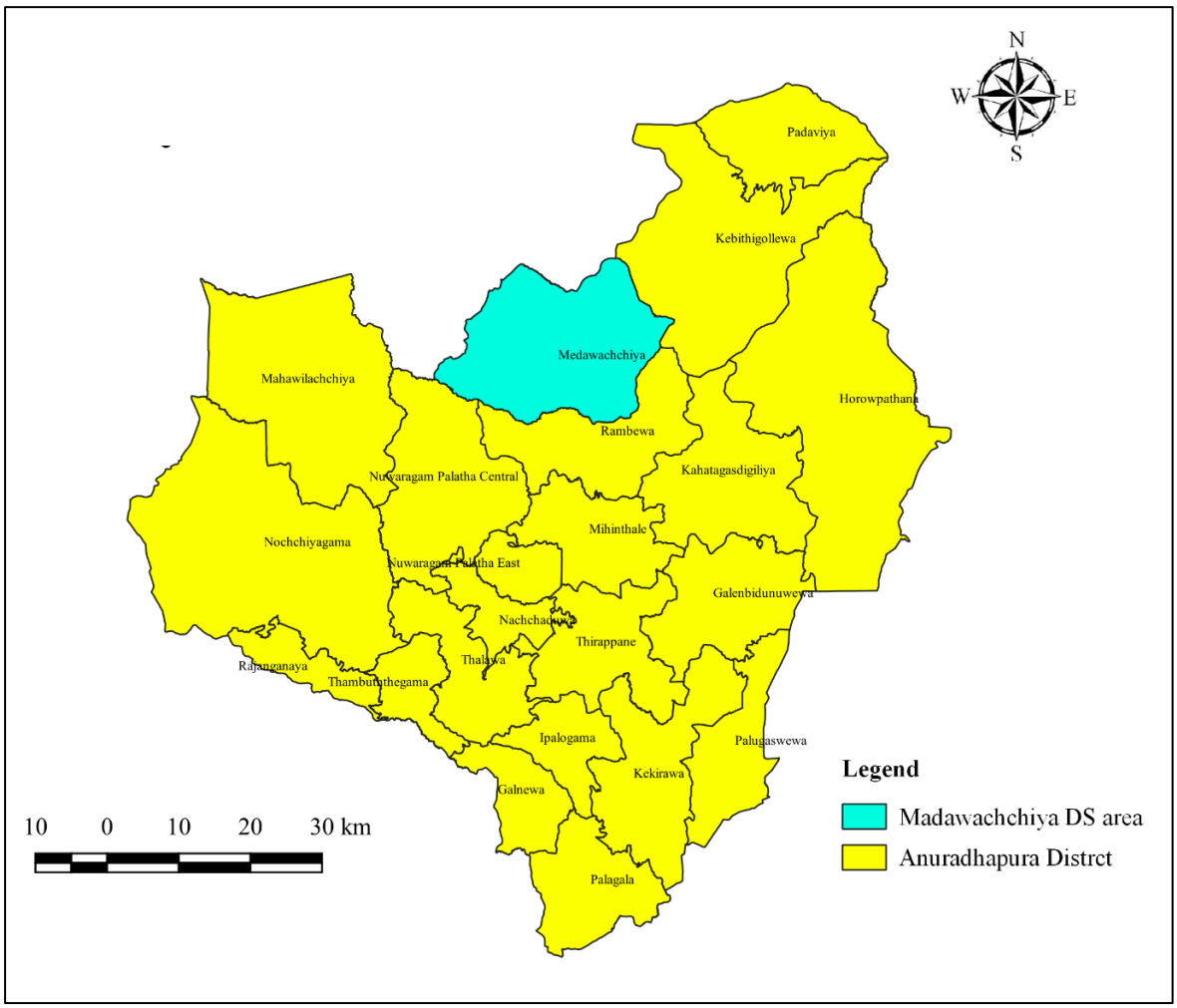

Figure 1 Madawachchiya MOH area in Anuradhapura District of Sri Lanka selected for entomological surveys.

Entomological investigation

Biting midges were collected using cattle-baited net traps in December 2021. Traps were placed at four different locations on a rotational basis for four weeks (one survey in one selected site per

month). The cattle-baited net traps were placed in the sites identified during the preliminary phase of the study. The trap made of a white cotton drill (3 m x 3 m x 1.5 m) with net windows (2 m x 1 m) on the sides was erected using a solid center pole of two meters in height and four side sticks, each of the same height (1.5 m). The trap was set at least 100 m away from the human dwellings and away from the places where cattle are usually tethered or herded during the night. A 15-25 cm gap was allowed between the lower edge of the net and the ground, enabling the sand flies to enter the trap. The cattle were introduced into the trap just before sunset and tethered to the pole fixed to the middle of the trap. The collection of sand flies from each trap was done from 8.00 to 11.00 pm and 5.00 to 6.00 a.m. the following morning. The collected sand flies were carefully transferred to paper cups covered with an organdie net (mesh size = 335 microns).

Morphological identification of biting midges

The samplings were performed for one night each in all four selected sites. Collected biting midges were anesthetized with 70% Chloroform and preserved in 70% v/v ethanol. Preserved specimens were introduced to microcentrifuge tubes with 70 ethanol and transferred safely to the Molecular Parasitology Laboratory at the Department of Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka. The specimens were morphologically identified, referring to the morphological taxonomic keys described by previous researchers (Campbell & Pelham-Clinton, 1960; Remm, 1988).

Genomic DNA extraction

The specimens were surface sterilized using 70% ethanol and the DNA was extracted from the fly using MightyPrep reagent for DNA (Takara, Japan) according to the manufacturer's instruction. *Leishmania donovani* specific primers targeting kinetoplast minicircle gene sequence (385 bp) described previously were used for amplification (Sreenivas et al., 2004). The PCR was conducted in 20 μL of total volume containing 1 μL of DNA as the template, 2 μL of CoralLoad Buffer (QIAGEN) with 15 mM $MgCl_2$ and loading dye, 1.6 μL of 0.2 mM dNTP mixture, 0.06 μL of 0.3 μM forward and reverse primers, 0.41 μL of 2.5 U MightyAmp DNA Polymerase (Takara, Japan), and 15.18 μL sterile nuclease free water. Amplification was performed in a thermal cycler (SimpliAmp™, Applied Biosystems) programmed for an initial denaturation step of 94°C for 5 min and 40 cycles of denaturation at 94°C for 30 seconds, annealing at 47°C for 30 seconds, and extension at 72°C for 24 seconds. Nuclease-free, PCR-grade water was used as the negative control, and DNA isolated from confirmed sample of *L. donovani* was used as the positive control.

Agarose gel electrophoresis and interpretation of results

The 2% agarose (Agarose S) gels prepared with 1 X TAE was stained with ethidium bromide (0.5 μg/ml). A volume of 4 μL of the amplified PCR products were loaded alongside Promega 100 bp lambda marker. Gel electrophoresis was carried out at 100 V for 25 minutes to achieve a good separation of the amplified products. Following the gel electrophoresis, the migrated DNA was visualized and photographed under UV illumination. The band size of 385bp was considered positive for *L. donovani* DNA.

Results

Identification of field-caught biting midges

A total of 42 biting midges were collected. All biting midges were females. The collection consisted of a single species similar to *Culicoides imicola* in morphology. The taxonomic description includes the following features to confirm the species identification. The lateral view of a wild-caught *C. imicola* is given in Figure 2.

Figure 2 Lateral view of an adult female Culicoides imicola

Molecular-based detection of *L. donovani* in wild-caught biting midges

Gel electrophoresis and subsequent UV visualization showed that out of the collected biting midge samples, 4.76% (n=2) were positive for *L. donovani* DNA. All the positive samples showed a band at 385bp (Figure 3). This finding suggests that L. *donovani* parasites were present within the biting midge population in the study area. The detection of *L. donovani* DNA in the biting midge samples indicates a parasite circulation rate of 4.76% within the wild biting midge population.

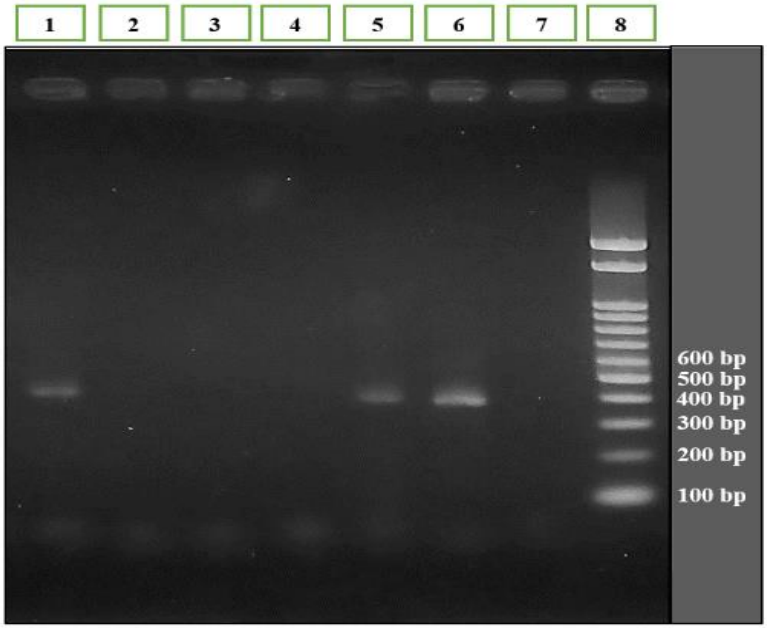

Figure 3 PCR products amplified with Leishmania donovani specific primers separated by agarose gel electrophoresis. ***Lane 1:*** *Positive sample,* ***Lanes 2-4:*** *Negative samples,* ***Lane 5:*** *Positive sample,* ***Lane 6:*** *Positive control,* ***Lane 7:*** *Negative control,* ***Lane 8:*** *100 bp ladder*

Discussion

Phlebotomine sand flies have long been implicated as the natural vectors responsible for transmitting *Leishmania* parasites in various geographic regions worldwide, including the subgenera Viannia, Leishmania, and Sauroleishmania (Cecílio et al., 2022). Some species of phlebotomines have been recognized as potential vectors (Srisuton et al., 2019). A previous study has identified the natural infection of *L. macropodum*, a parasite infecting kangaroos, in day-feeding midges (Dougall et al., 2011).

In order to confirm a species as a vector for leishmaniasis transmission, it should fulfil two essential criteria, namely; this species should obtain a blood meal, the macrophages with *Leishmania* amastigotes ingested through the blood meal should transform to the infective promastigote stage (Dostálová and Volf, 2012; Wijerathna et al., 2021) and phosphoglycans on promastigote surface must bind with receptors in the midgut, otherwise, the promastigotes will pass with excretions (Dostálová & Volf, 2012).

This study identified engorged biting midges and sand fly collection in a leishmaniasis disease endemic area in Sri Lanka. However, the present study did not characterize the blood meal source. Recently, some West Bengal, India investigators demonstrated that three Culicoides species (*C. peregrinus*, *C. oxystoma*, and *C. huffi* were found feeding opportunistically on humans, implicating them as the putative vectors for the anthropozoonotic transmission of pathogens, including *Leishmania* parasites (Dougall et al., 2011; Kar et al., 2022). This finding also implies that Culicoides species, suspected vectors of leishmaniasis in our country, could also feed opportunistically on humans. Therefore, a more extensive collection of engorged specimens, especially from different affected sites, is needed for further blood source analysis to assess this speculation.

The present study identified *C. imicola* at sites endemic for leishmaniasis disease transmission. Interestingly, of the engorged females, about 4.76% were positive for *L. donovani* parasite DNA. Therefore, circulation of *L. donovani* parasites appear to occur within the biting midges. This fulfils a single vector incrimination criterion for the biting midges captured locally. However, the study does not support the possibility of parasite development into the infective stage.

Previous studies have shown that *C. sonorensis* biting midges can establish high proportions of metacyclic stages in *L. martiniquensis* and *L. orientalis*. These infected midges were then capable of transmitting the parasites and causing disease in BALB/c mice, thereby supporting the vector competence of Culicoides biting midges (Becvar et al., 2021). Some molecular screening of *C. mahasarakhamense* specimens collected near the residence of a patient with visceral leishmaniasis in Lamphun Province, northern Thailand, revealed positive results for *L. martiniquensis*. Further, a study published in 2022 in Thailand has proposed that *Leishmania* species can develop in biting midge *C. sonorensis*, suggesting the possible role of Culicoides in disease transmission along with the first molecular evidence of *L. martiniquensis* in different species of biting midges namely; *C. mahasarakhamens*, *C. peregrinus*, *C. oxystoma*, *C. huffi*, *C. fordae*, and *C. fulvus*; and *L. orientalis* in *C. peregrinus*, and *C. oxystoma* (Songumpai et al., 2022). Together with the present finding, these findings suggest that biting midges may play a role in the autochthonous transmission of leishmaniasis.

Conclusion

In conclusion, the present study conducted in a leishmaniasis endemic area of Sri Lanka provides evidence that biting midges, specifically a species similar to *Culicoides imicola*, may be potential vectors for *Leishmania donovani* parasites. The detection of *L. donovani* DNA within the biting midge population indicates a parasite circulation rate of 4.76%. These findings fulfil the first criterion of leishmaniasis vector incrimination according to the World Health Organisation (WHO) guidelines. However, further studies to assess the growth of the parasite within the biting midges and to assess the transmission using animal models are needed to confirm the vector status. Further research is also needed to determine the epidemiological significance of biting midges in leishmaniasis transmission and to develop appropriate control strategies.

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Conflict of interest

The authors declare no conflict of interest. All authors read and approved the manuscript.

Ethical approval

Ethical clearance of the study was obtained from the institutional ethics review committee at the Faculty of Applied Science, Rajarata University of Sri Lanka (ERC/04/021).

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