NOTE *Parasitology*

PCR-Based Detection of *Leishmania donovani* DNA in a Stray Dog from a Visceral Leishmaniasis Endemic Focus in Bangladesh

Mohammad Zahangir ALAM[1,2], Md. Golam YASIN[2], Hirotomo KATO[1], Tatsuya SAKURAI[1] and Ken KATAKURA[1]*

[1]*Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060–0818, Japan*
[2]*Department of Parasitology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh–2202, Bangladesh*

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ABSTRACT. Although *Phlebotomus argentipes* as the only known vector of visceral leishmaniasis (VL) is zoophilic in nature, VL is considered to be anthroponotic in the Indian subcontinent. Peripheral blood samples from 85 stray dogs were examined for any molecular evidence of *Leishmania* infection in VL endemic areas of Bangladesh. Parasite DNA was detected in a blood sample from 1 of 85 (1.2%) stray dogs using ITS1-PCR, and PCR sequencing of the rRNA-ITS and cytochrome *b* gene confirmed that the parasitic DNA was *Leishmania donovani*. The results support the assumption that dogs are a probable animal reservoir for the *Leishmania* parasite in Bangladesh. It will be important to investigate the possible epidemiological role of dogs in domestic foci of VL endemic areas in Bangladesh.
KEY WORDS: Bangladesh, canine, *Leishmania donovani*, PCR, visceral leishmaniasis.

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Visceral leishmaniasis (kala-azar) is a fatal vector-borne parasitic disease of major public health importance in rural communities in Bangladesh. About 200 million people are estimated to be at risk of developing VL in the Indian subcontinent, which harbors an estimated 67% of the global VL disease burden [5, 20]. The current prevalence of VL in Bangladesh is estimated to be 40,000–45,000 cases [3] with more than 40.6 million people at risk of developing the disease [17].

VL is caused by *Leishmania donovani* complex, *L. infantum* or *L. donovani*, which differ in their disease pathology, vectors and reservoir hosts [14]. *L. infantum* is found in Mediterranean countries, the Middle East, Asia, and South America, and is responsible for zoonotic VL, with dogs as the main reservoir host. Transmission of VL caused by *L. donovani* is thought to be anthroponotic in the Indian subcontinent and East Africa. *Phlebotomus argentipes*, the only known vector for this parasite in the Indian subcontinent, is zoophilic in nature. Recently, *Leishmania* amastigotes from skin exudates of dogs in Sri Lanka [16], and *Leishmania* DNA from several domestic animals, including goats, cows and buffaloes, was detected in Nepal [4]. In Himachal Pradesh, India, two dogs out of 31 were positive for anti-*Leishmania* antibodies using a rK39 immunochromatographic rapid test [19]. These findings strengthen the idea of an animal reservoir for *L. donovani* in Bangladesh. A recent study [1] detected antibodies against the *Leishmania* parasite in cattle from an endemic area of Bangladesh, but no parasitic DNA was found by PCR. This result suggests that cattle do not play a role as a reservoir host, but further studies on other domestic animals need to be conducted. Therefore, the present study aimed to investigate the presence of *Leishmania* DNA by PCR using blood from stray dogs in VL endemic areas of Mymensingh district in Bangladesh.

The animals screened were stray dogs from Trishal and Fulbaria upazila (subdistricts) of Mymensingh district in Bangladesh (Fig.1), where VL is endemic [2]. To facilitate the fieldwork, collaborations were established with locally based partners; Bangladesh Agricultural University and Mymensingh Municipality Bureau. Although the capture of stray dogs was approved by Mymensingh Municipality Bureau, at present, there is no established central ethical committee on animal experimentation in Bangladesh. In this study, peripheral blood samples from 85 stray dogs without obvious signs of leishmaniasis were collected during the period of May to August 2011. For the collection of blood samples, field visits to the target area were made with a veterinary surgeon. Venous blood collected from the saphenous vein was spotted on filter paper (Whatman no. 3) for subsequent PCR. Filter papers were air dried for 3 hr. To avoid contamination, each sample was transferred separately into a small plastic bag. The samples were stored at 4°C. DNA was extracted from the whole blood spotted on filter paper by a phenol-chloroform method as described previously [9, 18]. A negative DNA extraction control was used to control for possible contamination during DNA extraction process. Internal transcribed spacer 1 (ITS 1) was amplified from the DNA extracted from filter papers using primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') and PCR conditions as described previously [18]. PCR products were separated using a 2% (w/v) agarose gel stained with RedSafe solution and visualized using UV light (Fig. 2A). Restric-

*CORRESPONDENCE TO: KATAKURA, K., Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060–0818, Japan.
e-mail: kenkata@vetmed.hokudai.ac.jp

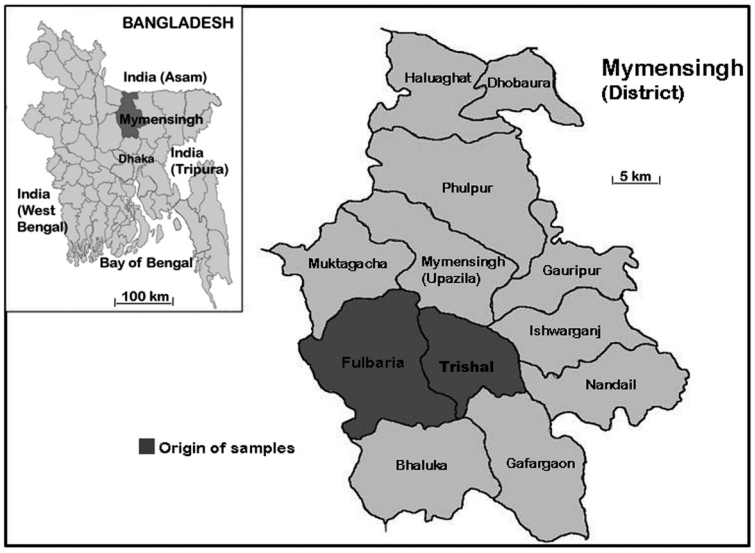

Fig. 1. Map of Mymensingh district, Bangladesh showing the area of collection of samples from dogs for the present study.

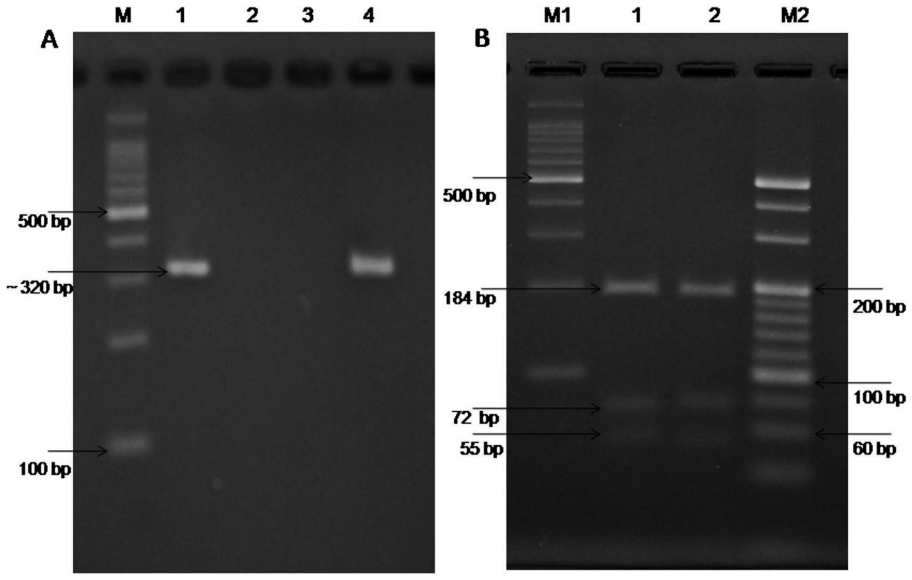

Fig. 2. PCR amplification products of blood samples spotted on filter papers. **A)** The PCR amplification products of the ~320 bp ITS1 region were separated on a 2% agarose gel; lane M, 100 bp marker; lane 1, positive PCR control, *L. infantum* (MHOM/ES/93/PM1); lane 2, negative DNA extraction control; lane 3, negative PCR control; lane 4, dog sample. **B)** Restriction analysis patterns of the amplified ITS1 fragments digested with *Hae*III; lane M1, 100 bp marker; lane 1, *L. infantum* (MHOM/ES/93/PM1) reference strain; lane 2, digested PCR product from dog samples; lane M2, 20 bp marker.

tion fragment length polymorphism (RFLP) analysis was performed with the positive PCR product using restriction enzyme *Hae*III. The restriction products were then separated using a 3% agarose gel to identify the species of *Leishmania*. To confirm the species of *Leishmania,* the rRNA ITS region was sequenced after amplification using nested PCR with a pair of outer primers (RI1, 5'- GCTGTAGGTGAACCTGCAGCAGCT-3', and RI2, 5'- GCGGGTAGTCCTGCCAAACA-3') [6] and then with a pair of inner primers (L.ITS-S, 5'- ATCATTTTCCGATGATTACA-3', and L.ITS-R, 5'- CTGTAAACAAAGGTTGTCGG-3'). To further determine the species of *L. donovani*, the cytochrome *b* gene was sequenced after amplification using a pair of specific primers (L.cyt-AS, 5'- GCGGAGAGRARGAAAAGGC-3';

and L.cyt-AR, 5'-CCACTCATAAATATACTATA-3'), and the PCR product was subjected to nested PCR with a set of inner primers (L.cyt-S, 5'-GGTGTAGGTTTTAGTY-TAGG-3'; and L.cyt-R, 5'-CTACAATAAACAAATCATA-ATATRCAATT-3') [12, 13]. These products were then sequenced from both forward and reverse sides using the inner primers. Direct cycle sequencing of PCR-amplified fragments was performed using an automated sequencer ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Automated sequencing reactions were carried out with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the use of PCR conditions recommended by the manufacturer. The sequences obtained were processed and aligned using the multiple alignment program BioEdit [10] and edited manually.

One (1.2%) of 85 blood samples was positive for parasite DNA by ITS1-PCR. The sample was considered as positive when a PCR fragment of ~320 bp could be detected (Fig. 2A).The *Leishmania* isolate was identified as belonging to the *L. donovani* complex by ITS1 PCR-RFLP analysis. The restriction products were 184, 72 and 55 bp in size and corresponded with those observed for the *L. donovani* complex (Fig. 2B). To substantiate the species confirmation of *Leishmania donovani* complex, rRNA ITS and cytochrome *b* gene products from the dog were sequenced. The sequences were submitted to DDBJ (accession nos AB725909 (rRNA ITS) and AB725910 (cyt *b*)). The sequences were compared with those in the NCBI database using BLASTn (Basic Local Alignment Tool for Nucleotide). Species identification was determined from the best-scoring reference sequence of the BLAST output. The molecular identities of the both gene amplicons (977 bp of rRNA ITS and 702 bp of cyt *b*) showed 100% similarity with *L. donovani* DNA sequences deposited at GenBank (accession nos AJ634378, AM901448 (rRNA ITS) and EF579911, EF579896 (cyt *b*)) and 99% similarity with *L. infantum* DNA sequences (accession nos AJ634371, AJ634370 (rRNA ITS) and EF579913, EF579895 (cyt *b*)).

Knowledge of reservoir hosts is an important pre-requisite for understanding the epidemiology of and designing control programs for VL. In areas where zoonotic VL is endemic, the prevalence of *L. infantum* infection in dogs is often high, although many of them are asymptomatic [7]. Dogs can remain infected by the *Leishmania* parasite without displaying apparent clinical signs of VL for years, even for their entire life [15]. Dogs are usually in or next to human homes, and thereby can contribute to the domestic transmission cycle of major zoonotic diseases including leishmaniasis [7]. There are no official figures on the number of stray dogs in Bangladesh, but officials estimate there could be more than 2.5 million. Chances of disease emergence from stray dogs and foxes are of great concern in Bangladesh, but information on the status of canine VL in the country is lacking. It is worth mentioning that our study is the first report to investigate stray dogs for *Leishmania* parasite infection in VL endemic areas of Bangladesh. *Leishmania* DNA was detected in one sample taken from a stray dog in Fulbaria upazila using ITS1-PCR approach, as described previously [18], and sequencing analysis of the rRNA-ITS region and cytochrome *b* gene confirmed that this stray dog was infected with the *L. donovani* parasite. The presence of *L. donovani* DNA in the blood sample from a stray dog in our study supports the findings of a previous study in Sri Lanka [16], Sudan [11], and India [19]. In a more recent study in Nepal, *Leishmania* DNA was found in several domestic animals such as goats, cows and buffaloes from an endemic area several months after the active transmission season [4], but there was no evidence of *Leishmania* DNA in domestic cattle in Bangladesh, although the cattle were seropositive for leishmaniasis [1]. However, other domestic animals, such as goats, buffaloes and wild animal (foxes) remain to be investigated. In Asia and other parts of the world, dogs are the primary reservoir hosts of *L. infantum*, although canine infection is possible with *L. donovani* [8], the causative agent of human VL in the Indian subcontinent and East Africa. It has been documented in eastern Sudan that domestic dogs are an important reservoir host of *L. donovani* [11]. Results from our study suggest that dogs are a probable animal reservoir for *L. donovani* in this endemic focus. In order to clarify the existence of a potential transmission cycle with dogs as the reservoir, it is necessary to prove that these animals can transmit the parasite to the vector in nature.

It was concluded that the presence of *Leishmania* DNA in a single dog, is insufficient to incriminate dogs as a reservoir, and more studies are needed to reveal the exact role of dogs in *L. donovani* infection.

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