

Molecular and Serological Evidence of *Leishmania* Infection in Stray Dogs from Visceral Leishmaniasis–Endemic Areas of Bangladesh

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Abstract. Visceral leishmaniasis (VL), or kala-azar, is mainly caused by two closely related *Leishmania* species, *Leishmania infantum* and *Leishmania donovani*. *Leishmania infantum* is responsible for zoonotic VL, with dogs as the main reservoir host in the Mediterranean, the Middle East, Asia, and South America. In the Indian subcontinent, VL is caused by *L. donovani* and is considered anthroponotic, although the only known vector, the sand fly, is zoophilic in nature. The role of domestic and stray dogs in VL transmission is still unclear in this area. We screened 50 stray dogs from VL-endemic areas of Bangladesh for serological and molecular evidence of *Leishmania* infection. We detected anti-*Leishmania* antibodies in six (12%) dog serum samples using rK39 immunochromatographic tests. We observed *Leishmania* kinetoplast DNA in 10 (20%) buffy coat DNA samples by real-time polymerase chain reaction (PCR), five of which were positive based on internal transcribed spacer 1-PCR. A sequencing analysis of the amplified products confirmed that the parasitic DNA was derived from *L. donovani*. Our findings support the hypothesis that stray dogs are an animal reservoir for *L. donovani* in this endemic region. Further studies are required to determine the precise role of dogs in the epidemiology of VL in Bangladesh.

INTRODUCTION

Visceral leishmaniasis (VL), or kala-azar, is a fatal vector-borne parasitic disease caused by the *Leishmania donovani* complex (*Leishmania infantum* and *L. donovani*) of intracellular protozoan parasites. VL is a serious public health problem in the Indian subcontinent; an estimated 200 million people are at risk, which represents approximately 67% of the global VL burden.^{1,2} In Bangladesh, the current prevalence is estimated to be 40,000–45,000 cases with more than 40.6 million people at risk of developing the disease.^{3,4} The disease is prevalent in 45 districts of Bangladesh, and most reported cases are from the Mymensingh District.³

VL has two epidemiological patterns. Anthroponotic VL is transmitted via infection from humans to humans and to a lesser extent from humans to animals. Zoonotic VL is transmitted from animals to humans and to a lesser extent from humans to humans. *L. infantum* is responsible for zoonotic VL, with dogs as the main reservoir hosts, in the Mediterranean, the Middle East, Asia, and South America. In areas where zoonotic VL is endemic, the prevalence of *L. infantum* infection in dogs is often high, although many infections are asymptomatic.⁵ The transmission of VL caused by *L. donovani* is thought to be anthroponotic in the Indian subcontinent and eastern Africa.⁶ The importance of animal reservoirs in these regions is not well studied.

There are several reports of *L. donovani* infection in leishmaniasis symptomatic dogs in Sudan⁷ and in apparently healthy dogs in northwest Ethiopia.⁸ Infections in dogs with both *L. donovani* and *L. infantum* were reported in a village along the Albara River in eastern Sudan.⁹ Few studies have investigated the role of animal reservoirs in maintaining *L. donovani* in the Indian subcontinent. Recently, *Leishmania* amastigotes were detected in skin exudates of dogs in Sri Lanka¹⁰ and *Leishmania* DNA in cows, buffaloes, and

goats in Nepal.¹¹ In Himachal Pradesh, India, anti-*Leishmania* antibodies were detected in two of 31 dogs using the rK39 immunochromatographic test (ICT).¹² Furthermore, *Phlebotomus argentipes*, the only known vector for *L. donovani* in the Indian subcontinent, is zoophilic, which supports the hypothesis of a zoonotic *L. donovani* transmission cycle.

In Bangladesh, the stray dog population is quite high, although the precise population size is unknown. These dogs typically live in or next to human houses, and thereby can contribute to the domestic transmission of major zoonotic diseases, including leishmaniasis. However, there is a lack of information about the importance of animals as a VL reservoir in Bangladesh. Recently, antibodies against the *Leishmania* parasite were detected in cattle from an endemic area of Bangladesh, but no parasitic DNA was detected by polymerase chain reaction (PCR).¹³ In our recent study, *Leishmania* DNA was detected in one stray dog from VL-endemic areas of Bangladesh.¹⁴ For further verification, we investigated additional stray dog samples from the same endemic areas and detected anti-*Leishmania* antibodies and *Leishmania* DNA, lending support to the hypothesis that dog is an animal reservoir for *Leishmania* parasites in the endemic area.

MATERIALS AND METHODS

Sample collection and preparation. In May 2012, 50 stray dogs (30 males and 20 females) were captured in Trishal and Fulbaria upazila (subdistricts) of the Mymensingh district in Bangladesh, which are two endemic areas for VL (Figure 1). Captured dogs had no obvious clinical signs of leishmaniasis, but most were emaciated, with slight skin lesions. From the saphenous/cephalic vein, 5 mL venous blood was collected in tubes containing disodium ethylenediaminetetraacetate (Na₂EDTA). All tubes were immediately placed in a chilled ice box and stored until processing. The blood samples were centrifuged at 875 × g for 10 minutes at 4°C. The serum samples were stored at 4°C, and buffy coat samples were stored in lysis buffer for DNA extraction. Methods for stray dog capture and sample collection were approved by the Mymensingh Municipality Bureau and were described previously.¹⁴

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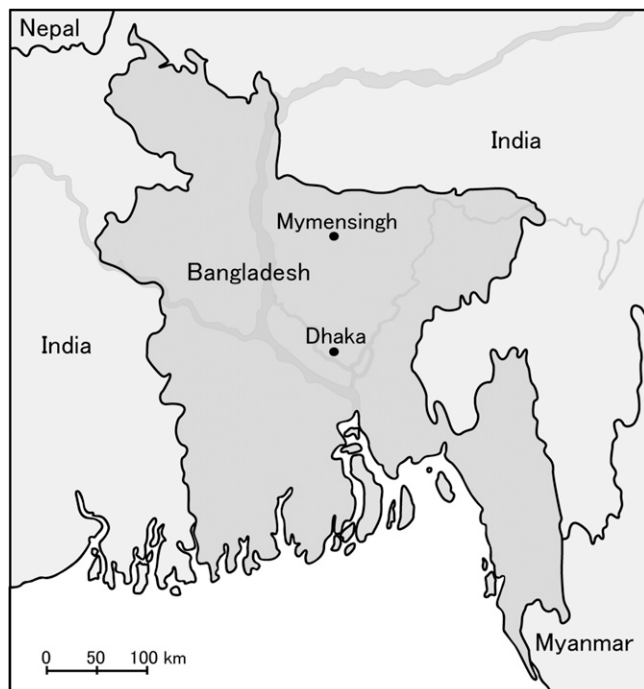


FIGURE 1. Map of Bangladesh. Dog samples were collected in the Mymensingh District.

rK39 dipstick test. Of serum sample, 20 μ L was used for the rK39 ICT (Kalazar Detect™ Rapid Test; In Bios International, Inc., Seattle, WA) according to the manufacturer's instructions. This test qualitatively detects anti-*Leishmania* circulating antibodies against a 39-amino-acid repeat that is conserved among viscerotropic *Leishmania* species (*L. donovani*, *L. infantum*, and *Leishmania chagasi*).^{12,15} The presence of a red line in the test area indicated a positive result according to the manufacturer's instructions. Sera of uninfected dogs ($N = 3$) from a nonendemic region were tested as negative controls for the dipstick test.

DNA extraction. DNA was extracted from 20 μ L of blood buffy coat using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Extracted DNA samples were kept at -20°C until further analysis.

ITS1-PCR assay. An internal transcribed spacer 1-PCR (ITS1-PCR) assay was performed to amplify the ribosomal ITS1 region using the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') as previously described.¹⁶ The amplification conditions were as follows: initial heating at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 6 minutes. PCR products were resolved by 2% agarose gel electrophoresis in $1\times$ Tris-Borate-EDTA buffer and visualized using ultraviolet light after staining with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnum, Korea). A positive control with *L. donovani* (strain MHOM/BD/2006/BD25) genomic DNA at 10 ng/ μ L and negative controls with DNA extracted from uninfected dogs ($N = 3$) from a nonendemic region and no-DNA (water) were included.

Real-time PCR. A quantitative real-time PCR assay based on the amplification of kinetoplast minicircle DNA (kDNA)

was performed using the LightCycler® Nano system (Roche Diagnostics, Tokyo, Japan) with the primers RV1 (5'-CTTTTCTGGTCCCTCCGGGTAGG-3') and RV2 (5'-CCACCCGGCCCTATTTTACACCAA-3').¹⁷ The 20 μ L reaction mixture contained $1\times$ FastStart Essential DNA Green Master (Roche, Mannheim, Germany), 0.25 μM of each primer, and 2 μ L of buffy coat DNA. The reaction conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The standard curve was established using *L. donovani* DNA extracted from 1.4×10^8 parasites from culture. Aliquots from serial dilutions (1 μ L), ranging from 0.005 to 500 pg of parasite DNA, were added to the reaction tubes. The assay included negative controls with DNA of uninfected dogs from a nonendemic region ($N = 3$) and water.

Sequencing. The PCR products from the agarose gel were excised with a sterile gel cutter and purified using the NucleoSpin Extract II Kit (Clontech Laboratories Inc., MACHEREY-NAGEL, Düren, Germany). Sequencing reactions were performed with the BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Direct cycle sequencing was performed using the ABI 310 Genetic Analyzer (Applied Biosystems). After generating a multiple alignment with a program BioEdit,¹⁸ the consensus sequences were compared with those in the National Center for Biotechnology Information database using BLASTn. The obtained DDBJ/EMBL/GenBank accession no. was LC123922.

Statistical analyses. A Fisher's exact test was used to determine statistical differences between the numbers of male and female dogs that were positive for *Leishmania* infection based on each of the three different diagnostic methods, that is, rK39 ICT, ITS1-PCR, and real-time PCR (Table 1). Analyses were conducted using an online Fisher's exact test calculator (<http://www.socscistatistics.com/tests/fisher/Default2.aspx>). The level of agreement between the diagnostic techniques was evaluated using kappa statistics with 95% confidence intervals (CIs; <http://graphpad.com/quickcalcs/kappa1.cfm>). Kappa values (κ) of 0.20–0.60 indicate fair to moderate agreement and values of 0.60–0.80 indicate substantial agreement between observations.¹⁹

RESULTS

rK39 dipstick test. We detected anti-*Leishmania* antibodies in six of 50 (12%) dog serum samples (Figure 2). We observed moderately strong bands in the test line region for four samples (e.g., dog IDs 27 and 28 in Figure 2), but faint bands in the rK39 dipstick test for two samples (data not shown).

TABLE 1

Infection rates for male and female dogs based on the three diagnostic methods

Diagnostic methods	Positive dog samples (%)		P value
	Male	Female	
rK39 ICT	5 (16.7)	1 (5)	0.38
ITS1-PCR	4 (13.3)	1 (5)	0.63
Real-time PCR	8 (26.7)	2 (10)	0.28

ICT = immunochromatographic test; ITS1 = internal transcribed spacer 1; PCR = polymerase chain reaction.

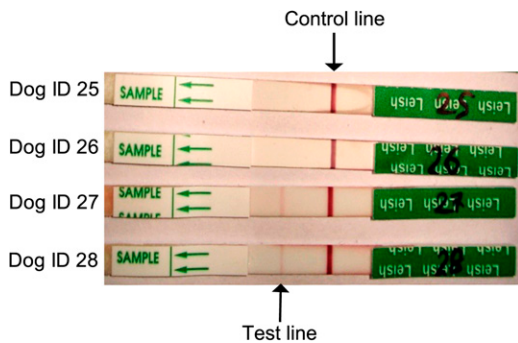


FIGURE 2. rK39 immunochromatographic strip test results. Strips with only the control band (dog IDs 25 and 26) represent negative results, whereas strips with both a control band and a positive test band (dog IDs 27 and 28) reflect positive results.

The dipstick test showed negative results for the control dogs sera ($N = 3$) from a nonendemic region.

ITS1-PCR and sequencing results. Five (10%) of 50 dog samples were positive for *Leishmania* DNA by ITS1-PCR. Sequencing analysis of the amplified products revealed 100% similarity to *L. donovani* DNA sequences previously deposited in GenBank (accession nos. KT273408, KR858307).

Real-time PCR. We obtained positive real-time PCR results for *Leishmania* kDNA amplification for 10 of 50 (20%) dog samples. The concentrations of parasite DNA were 0.005–4.344 pg, equivalent to 0.02–21.72 parasites.

Comparison of three diagnostic methods. Table 2 provides a summary of rK39 ICT, ITS1-PCR, and real-time PCR results. Three dogs showed positive results by all the three diagnostic methods used in this study. Six dogs were serologically positive by rK39 ICT, in which *Leishmania* DNA could be detected by ITS1-PCR and/or real-time PCR in five dog samples. Of 10 samples that were positive by real-time PCR, only five were positive based on ITS1-PCR. A moderate agreement was obtained between rK39 ICT and ITS1-PCR results ($\kappa = 0.50$, 95% CI = 0.10–0.87) and between rK39 ICT and real-time PCR results ($\kappa = 0.56$, 95% CI = 0.25–0.87). A substantial agreement was found between ITS1-PCR and real-time PCR results ($\kappa = 0.62$, 95% CI = 0.32–0.91). We did not observe significant differences in infection rates between male and female dogs for any of the three diagnostic tests (Table 1).

DISCUSSION

We investigated the presence of *Leishmania* infection in stray dogs and found evidence that dogs play a role in the maintenance of *Leishmania* parasites in the VL-endemic

areas of Bangladesh. Knowledge of reservoir hosts and their potential role in disease transmission is a prerequisite for understanding VL epidemiology and designing appropriate control strategies. Although VL in the Indian subcontinent is still thought to be anthroponotic, there is a good circumstantial evidence for a residual zoonotic reservoir. Disease emergence from stray dogs and other canids is of great concern, but the status of canine VL in Bangladesh is unclear.

In VL zoonotic foci, where dogs are the primary reservoir hosts, the disease is caused by *L. infantum*.²⁰ However, there are also reports of canine infection with *L. donovani*,^{7,21} the causative agent of human VL in the Indian subcontinent and East Africa. It has been reported that the domestic dog may be an important reservoir host of *L. donovani* in eastern Sudan.²² Some recent studies also reported reservoir hosts for *Leishmania* parasites other than dogs, such as red foxes in central Greece,²³ cats in the western provinces of Turkey,²⁴ and Brazilian bats.²⁵ In India, *L. donovani* DNA was recently detected in goats.²⁶

Our observations of anti-*Leishmania* antibodies and *Leishmania* DNA in blood samples obtained from stray dogs corroborate the findings of previous studies in Sri Lanka,¹⁰ Sudan,² and India.¹² In Bangladesh, cattle that are seropositive for leishmaniasis have been found, but there is no evidence of *Leishmania* DNA,¹³ suggesting that cattle do not play a role as reservoir hosts. In a recent study, *Leishmania* DNA was detected in a single (1.2%) dog among 85 stray dogs using DNA extracted from whole blood spotted on filter paper.¹⁴ We found that 20% and 10% of stray dogs were positive based on real-time PCR and PCR using buffy coat DNA, respectively, despite sampling from the same VL-endemic foci. The higher positive rate in our study probably reflects a higher assay sensitivity using buffy coat DNA than whole-blood preparations, as demonstrated in previous studies.^{27,28}

We detected some discrepancies among the results of the three diagnostic methods used in this study. We obtained the highest positive rate (20%) using kDNA-based real-time PCR, consistent with several previous studies showing that kDNA-based PCR is more sensitive than serological and ITS1-based PCR.^{29,30} kDNA is considered the most sensitive target for leishmaniasis diagnosis, because it contains ~10,000 minicircles per parasite.³¹ Samples that were positive based on PCR and/or real-time PCR, but negative based on rK39 ICT, might have a low infection burden and, therefore, lower levels of anti-*Leishmania* antibodies, consistent with previous studies^{32,33} in which some seronegative dogs were PCR positive. In our study, we observed one serologically positive dog that was negative for *Leishmania* DNA. This might be attributable to a past infection that was controlled via an immune response, as discussed elsewhere.³⁴ However, the possibility of false-positive results of each diagnostic test should also be considered, which might have led to the discrepancies among the diagnosing tests. For example, Mohammadiha and others reported that 3.6% (1/28) and 10.7% (3/28) of dogs from *Leishmania* non-endemic areas were positive by real-time PCR and ITS-based PCR, respectively.³⁰ The specificity of rK39 ICT with sera of dogs from nonendemic regions ranged from 94% to 100% according to some previous studies and a few false-positive reactions were also reported in dogs infected with *Ehrlichia canis*, *Trypanosoma cruzi*, or *Neospora caninum*.^{35–38}

It is important to isolate viable *Leishmania* from naturally exposed animals to clarify their role in the maintenance and

TABLE 2
Summary of the results of three different diagnostic tests

rK39 ICT	ITS1-PCR	Real-time PCR	No. of dogs
+	+	+	3
+	+	–	0
+	–	+	2
+	–	–	1
–	+	+	2
–	–	+	3
–	–	–	39

ICT = immunochromatographic test; ITS1 = internal transcribed spacer 1; PCR = polymerase chain reaction; + = positive; – = negative.

transmission of VL. After a *Leishmania*-infected sand fly bites a mammalian host, promastigotes (flagellated forms) are phagocytized by dermal macrophages and transformed into round-shaped amastigotes, which replicate in macrophages, leading to cell destruction and the progressive infection of more phagocytes.³⁹ Once an infection is established, *Leishmania* tends to localize in all tissues in which monocytic-macrophagic cells exist in high numbers, such as the liver, spleen, lymph nodes, bone marrow, gastrointestinal tract, and skin.⁴⁰ Several strains of *L. donovani*, *L. infantum*, and *Leishmania archibaldi* were isolated from lymph node cultures of dog samples in eastern Sudan.⁹ In the United States and Canada, *L. infantum* zymodeme MON1 was isolated from tissue specimen cultures of dogs.⁴¹ As part of a preliminary study, we attempted to detect *Leishmania* amastigotes in the spleen, liver, and lymph nodes of serologically positive dogs; however, we did not observe the parasites in the hematoxylin/eosin-stained tissue sections (data not shown), probably owing to the low number of parasites in the reservoir host. Further studies with an increased sample size are required to demonstrate the existence of parasites in tissue specimens with more sensitive tools and to isolate viable *Leishmania* from naturally exposed dogs.

We observed a higher infection rate in male dogs than in females, in agreement with the results of previous studies.^{42,43} Traditionally, canine leishmaniasis is transmitted directly from sand flies to dogs, but dog to dog transmission of *L. infantum* via direct contamination with blood and secretions was recently detected in the United States and Canada.⁴¹ The possible interaction between dogs and sand flies is an important issue with respect to the transmission of VL to humans. New and Old World sand fly species have varying degrees of host preferences, and hence are opportunistic feeders.^{44,45} In eastern Sudan, *Phlebotomus orientalis* and other sand flies are more attracted to dogs than to the mongoose, genet, and Nile rat.²² Although there is a lack of information about the host preference of *P. argentipes*, the only known vector of *L. donovani* in Bangladesh, the feeding behavior of *P. argentipes* is mainly zoophilic⁴⁶ and animals act as the preferred blood meal source.⁴⁷ Hence, we recommend that further studies should examine the host preferences of *P. argentipes* to dogs and other animals in the study area.

In conclusion, we confirmed the presence of anti-rK39 antibodies and *Leishmania* DNA in several stray dogs in the VL-endemic focus of Bangladesh. Although the number of animals examined was not adequate to incriminate dogs as a reservoir, our findings imply that dogs are probable animal reservoirs for VL transmission in this endemic focus. However, detailed analyses of *Leishmania* infection in dogs and the ability of dogs to transmit the parasite to the vector sand fly in nature are needed to reveal the potential role of dogs in VL epidemiology in Bangladesh.

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