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DETECTION OF LEISHMANIA INFECTION IN STRAY DOGS IN HUMAN LEISHMANIASIS ENDEMIC AREA IN MYMENSINGH DISTRICT WITH ITS POSSIBLE PUBLIC HEALTH SIGNIFICANCE IN BANGLADESH

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ABSTRACT

Background: Leishmaniasis is primarily caused by two species of *Leishmania* (*L. donovani* and *L. infantum*) of which clinical infection with *L. infantum* has been recognized in both humans and dogs as zoonotic disease with dogs as the main reservoir hosts in the Mediterranean, the Middle East, Asia and South America. Although *L. donovani* has been associated with both clinical and asymptomatic infection in humans but it is still associated with asymptomatic infection in dogs in Indian sub-continent without any evidence of zoonotic infection.

Objectives: The objective of this research was to investigate the potentiality of dog as reservoir host for visceral leishmaniasis in the human leishmaniasis endemic regions in Bangladesh.

Materials and Methods: A total of 20 stray dogs in the human VL endemic areas of Mymensingh district were captured for the detection VL during the period of November 2010 to May 2011. The dipstick test rK39 (Bios International; n = 20), Giemsa's stained impression smears of liver and spleen (n = 6) and PCR with the tissue of liver and spleen (n = 6) were tested as per manufacturer instructions and conventional standard methods.

Results: Out of 20 stray dogs examined, 4 (20.0%) were positive for *L. donovani* infection with rK39 strip test. Of the six randomly selected dogs tested with Modified Giemsa's stained of impression smears of spleen and liver showed 2 (33.33%) positive whereas PCR technique detected 5 (83.33%) positive for *L. donovani*. Results of PCR showed 145bp amplicon, specific for *L. donovani* infection in 83.33% stray dogs.

Conclusions: This study reveals that a high percentage of *L. donovani* asymptomatic carrier infections occur in dogs and evidence indicates that dogs and humans may potentially serve as a source of infection to sand fly vectors and accordingly dogs can be recognized as a probable animal reservoir for the Leishmania infection in the endemic region in Bangladesh. However, further studies are required to determine the ability of dogs to transmit the *L. donovani* to the vector sand fly in nature and its evidence on 'One Health' perspectives.

Keywords: Visceral leishmaniasis, Endemic region, Stray dogs, rK39 strip test, Giemsa's stained liver and spleen impression smears, PCR, Reservoir host, Mymensingh

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INTRODUCTION

Leishmaniasis is an obligate intracellular protozoan zoonotic disease, caused by *Leishmania* species and transmitted by vector host of sandflies. Visceral leishmaniasis (VL) was first noticed in Jessore district in 1824, reportedly killed around 75,000 people from 1824 to 1827,[1] when patient suffering from fevers characterized by relapses and progressive emaciation that were thought to be due to malaria failed to response to quinine; by 1862 the disease had spread to Burdwan, where it reached epidemic proportions and becomes known as 'Burdwan fever,' 'Kala-azar' or 'Black disease.' VL epidemic peaks were recorded in Bengal in the 1820s, 1860s, 1920s and 1940s.[2,3] The cause was unknown and it was not until the parasite, *L. donovani* was discovered in 1900 by Leishman and Donovan that the true nature of the disease became apparent.[3] The related VL causing species *L. infantum* was first described in children in Tunisia suffering from splenic anemia in 1908 and simultaneously found the parasite in dogs in Tunis. Since then, dogs have been implicated an important reservoir host for VL.[4] More than 1,000,000 VL cases were reported in former Bengal during 1931 to 1943.[5] The case numbers declined during DDT spraying campaign of the National Malaria Eradication Program and VL was thought to be eliminated after the 1970s. Between 1968 and 1980, only 59 cases were recorded in Bangladesh.[6] But since the 1980s, following the suppression of DDT spraying, there has been a resurgence of VL, with 73,467 cases reported between 1994 and 2004.[5] VL is now endemic in 45 districts with the Mymensingh district representing over 50% of the cases. In 2007, the estimated number of active cases was 136,500.[5] The estimated incidence of VL is 15.6/1,000 person-year in Fulbaria[7] and 27/10,000 population in Godagari, Rajshahi.[7] Currently, there are more than 20 different species of Leishmania pathogenic for humans but only two related species (*L. donovani* and *L. infantum*) and of the 500 known *Phlebotomine* species, only some 30 of them have been positively identified as vectors of the disease.[8,9] VL is widespread in the tropical and sub-tropical areas and endemic in 98 countries in Europe, Africa, Asia and America.[4] At least 12 million people reported to be infected worldwide and 400,000 new cases occur each year[8] and 20,000 to 30,000 will eventually die. The VL is a serious public health problem in the Indian subcontinent and an estimated 200 million people are at risk, which represents approximately 67% of the global VL burden.[10,11] Currently, the prevalence of this disease is estimated to be 40,000 to 45,000 cases with more than 40.6 million people are at risk of developing the disease in Bangladesh.[12] Out of 64 districts of Bangladesh, 45 are endemic for VL and 20 million people, around 18% of the total population are considered to be at risk for VL and most reported cases are from the Mymensingh district.[13] The disease in Indian subcontinent including Bangladesh is caused by a protozoa called *L. donovani* which is still considered to be anthroponotic and is transmitted by the bite of female sandfly, *Phlebotomus argentipes* is zoophilic in nature.[13,14] Parasitological, serological and molecular detection methods were extensively used to examine canine clinical or subclinical infection with *Leishmania* protozoa. Impression smears from the liver, spleen and blood was stained with Giemsa's to identify the presence of either the pro or amastigote stages of the parasites. Several molecular methods have successfully been used and evaluated in diagnosis of leishmania like PCR that allows a highly sensitive and specific (up to 100%) detection. Some studies have been conducted on the role of animal reservoir especially dogs in maintaining *L. donovani* in the

Indian subcontinent[15-17] including Bangladesh.[18,19] However, more studies are required to determine the precise role of dogs in the epidemiology of human VL endemic areas in Bangladesh.

MATERIALS AND METHODS

A total of 20 stray dogs (12 female and 8 males) were captured at the Bangladesh Agricultural University (BAU) campus and Trishal Upazila under Mymensingh district during the seven months period from November 2010 to May 2011. Methods for stray dog capture and sample collection were approved by the Mymensingh Municipality Bureau.

Collection of blood samples

After capturing the dogs, blood was collected from radial vein and immediately rk39 test was done. Smear was taken from blood on glass slide. Then blood was taken into the EDTA containing tubes and preserved in ultra-low freeze. After collection of blood, the dogs were euthanized with magnesium sulfate of 50% solution**.**

Collection different organs

The liver and spleen of sacrificed dogs were examined for any gross lesions and then small pieces of liver and spleen were collected in falcon tubes, each for two pieces. Femoral bones were collected after skinning and wrapping with aluminum foil. All samples were frozen after collection.

rk39 strip test

The dipstick test (CTK Biotech, Inc., Seattle, WA) was performed according to the manufacturer's instructions. Briefly, 20 µml (1 drop) blood or serum was used in this test. 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*). The results were positive if two distinct red or pink lines appeared (one in the test region and another in the control region), they were negative when no red or pink lines appeared in the test region, and they were invalid if the control line failed to appear.

Giemsa's staining of the impression smears samples

Thin blood smears and impression smears of liver and spleen were made on glass slides, dried in air and fixed in absolute methanol (acetone free) for 5 minutes. The slides were placed in Coplin jar containing working Giemsa's solution. Then these were allowed to stain for 45 to 60 minutes. Slides were washed under tap water for 30 seconds. Finally slides were dried in the air and examined under a microscope.

Molecular technique for the detection of *Leishmania donovani*

DNA extraction from the specimen of liver

Wizard® Genomic DNA Purification Kit was used to extract the DNA using manufacturer instructions.

Quantification of DNA concentration

One of the important variables for PCR amplification is the concentration of DNA.

Because different DNA extraction methods produced DNA of widely different purity, it is necessary to optimize the amount of DNA used in PCR assay to achieve reproducibility and strong signal. DNA may result in smears or in a lack of clearly defined bands in the gel; on the other hand, too little DNA gives non-reproducible patterns. Thus, it is necessary to optimize the DNA concentration. For quantification of DNA concentration, the spectrophotometer's wavelength was set at 260 nm after the spectrophotometer UV lamp was warmed up. A square cuvette (the "zero" or "blank" cuvette) was filled with 2 ml sterile distilled water and place in the cuvette chamber and the absorbance reading was adjusted to zero for standardization. The test samples were prepared by taking 2µl of each DNA sample in the cuvette containing 2 ml sterile distilled water and thorough mixing by pipetting. After recording the absorbance reading, the cuvette was rinsed out with sterile water, tamped out on a paper wipe, and absorbance readings of extracted DNA samples of the *Leishmania donovani* were recorded (Table 1).

Table 1. Absorbance of reading and concentration of six DNA samples

Sample No.	Absorbance (at 260 nm)	DNA conc. (ng/µl)	Sample No.	Absorbance (at 260 nm)	DNA conc. (ng/µl)
1	0.003	150	4	0.002	100
2	0.003	150	5	0.003	150
3	0.003	150	6	0.002	100

Polymerase Chain Reaction (PCR) protocol

PCR is a highly sensitive and specific technique used for DNA amplification with specific primers.

Primer used for PCR

The pair of oligonucleotide primers that was used for PCR amplification of *Leishmania donovani* listed in Table 2.

Table 1. Primers and their sequences used for detection of *Leishmania donovani* in this study

Primer name	Sequence	Product size	Reference	Species
RV_1	5'-CTTTTCTGGTCCCGCGGGTAGG-3'	145bp	[20]	*L. donovani*
RV_2	5'-CCACCTGGCCTATTTTACACCA -3'			

Master mix preparation to perform PCR

PCR reactions were performed on each DNA sample in a 25 µl reaction mix containing Nuclease free H_2O (6.5 µl), 2x PCR master mix (12.5 µl), 0.5 µl reverse and 0.5 µl forward primer and 5 µl DNA template.

Procedure

Ice was taken in a plastic bag and put into a bucket with lick cork sheet. Small holes were made without touching the ice. The tubes of primers were placed on the hole**.** All the ingredients mentioned above were put into the ice. Eight PCR tubes were placed in the ice and labeled them, 20µl of master mix was dispensed to the each PCR tubes and 5µl of extracted DNA from 6 dog's liver were added to the respective tubes. 5µl of positive control DNA was added into the eight tubes. Then the PCR tubes were placed into the thermal cycler.

A total of 45 cycles of DNA amplification reaction for *L. donovani* was carried out. The thermocycler used for *L. donovani* comprised of initial denaturation for two minutes at 94°C. The condition of PCR amplifications were denaturation for 60 sec at 94°C, primer annealing for 90 sec at 62°C and extension for 30 sec at 70°C and final extension for 10 min at 70°C.

Electrophoresis

PCR products were analyzed by 2% agarose gel, stained with ethidium bromide and examined against UV light using an image documentation system. The positive samples were recorded based on the appearance of expected size of band in the gel.

RESULTS AND DISCUSSION

All the Leishmania positive dogs were examined physically and these dogs had ill-health, very rough hair coats and alopecic **(Photo 1)**. In addition, depression, loss of condition particularly decreased muscle mass over shoulders, hips and spine, serosanguinous nasal discharges, splenomegaly and generalized lymphadenopathy were recorded. Two dogs had fever and two had diarrhea, dry brittle hair coat and long brittle nails.

Three methods, rK39 strip test, microscopic examination of Giemsa's stained impression smears and PCR were used to detect the *L. donovani* infection in 20 stray dogs. Four dogs (20%) were found positive for *L. donovani* antibodies with rK39 strip test **(Table 3).** Randomly selected six dogs were euthanized for the collection of different internal organs for impression smears and PCR tests (**Table 3**).

Table 3. Evaluation of three tests to detect Leishmania infection in stray dogs

S/N	Test used	No. of dogs	Positive No. (%)	S/N	Test used	No. of dogs	Positive No. (%)	S/N	Test used used	No. of dogs	Positive No. (%)
1	rK39 Strip	20	4 (20.0)	2	GSIS	06	2 (33.33)	3	PCR	06	5 (83.33)

rK39 test (Kalazar DetectTM Rapid Test; Bios International, Inc, Seattle, WA)
GSIS = Giemsa's stained impression smears of liver and spleen

Microscopic examination of Giemsa's stained impression smears

Leishmanial amastigote were detected in the spleen **(Photo 4)** and liver **(Photo 5)** impression smears of two dogs stained with Modified Giemsa's stain.

Photo 1. A stray dog positive for *L. donovani* infection detected by rK39 test, Giemsa's stained impression smear and PCR

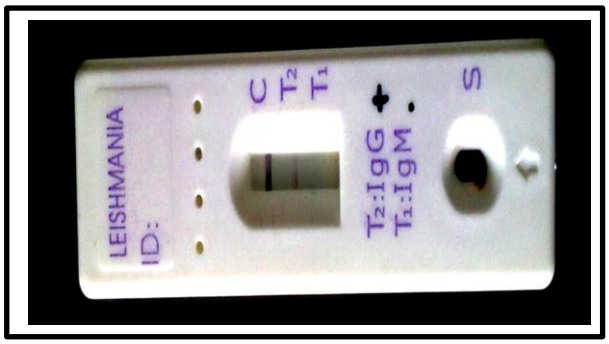

Photo 2.The rK39 strip test positive test result showing two bands (Control line C and Positive line T2)

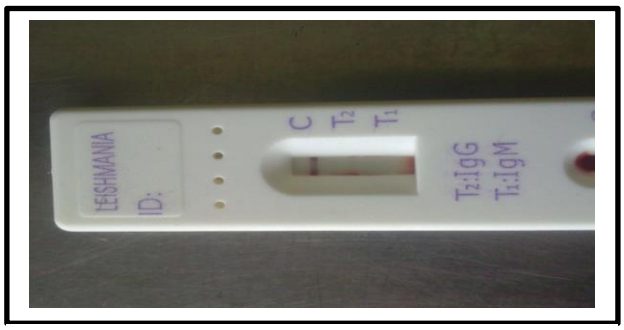

Photo 3. Negative result of rK39 strip test showing only one band (Control line C)

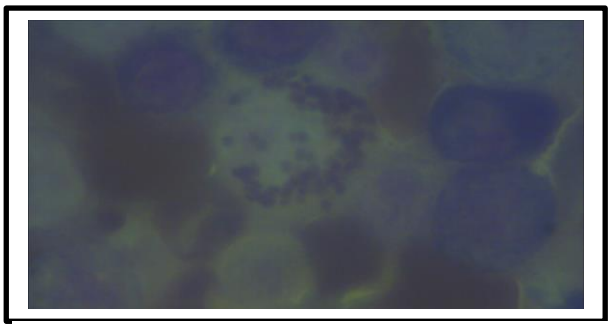

Photo 4. Spleen impression smears of stray dog showing dark blue colored amastigote (arrow) inside the monocyte. MG stained, 3000 ×

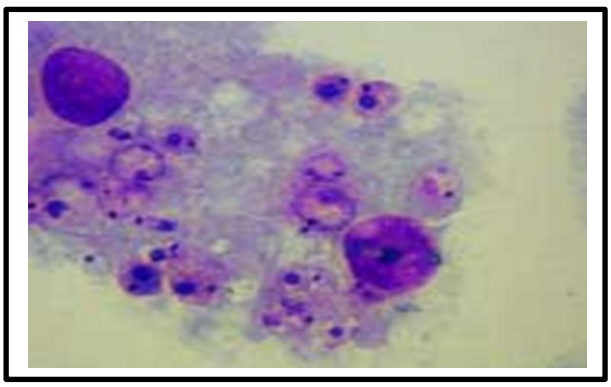

Photo 5. Liver impression smears of stray dog showing 2 to 4 sperm shaped purple color promastigote of *Leishmania* sp. Modified Giemsa's stained, 3000 ×

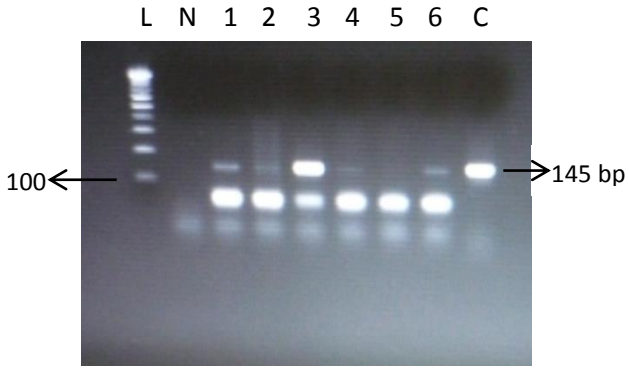

Photo 6. Agarose gel electrophoresis of PCR products that is specific for *L. donovani*. Lane-1,2,3,4,5 and 6 containing PCR product from dog's liver. Lane-L containing 100bp DNA ladder, Lane-N containing negative control and Lane-C containing 145bp positive sample

Application of PCR

The PCR was done for the determination of VL from specimen of canine liver. Species specificity PCR amplification using RV_1 and RV_2 primers amplified 145bp long product. Samples of five dogs showed the 145bp long band indicating 83.33% carrier state of the protozoa. These are 1,2,3,4, 5 and 6 (**Photo 6**).

Visceral leishmaniasis is one of the most important ancient diseases of humans but it is first noticed in 1824 in Jessore district in Indian sub-continent and currently out of 64 districts of Bangladesh, 45 are endemic for VL and 20 million people, around 18% of the total population are considered to be at risk for VL.[13] A national VL elimination program was initiated in 2008 in Bangladesh after the signing of a memorandum of understanding between the Government of Bangladesh, India and Nepal in 2005 for the elimination of VL from these countries by 2015. It has shown that VL burden has declined but is still far from the target of VL elimination with lot of challenges including insufficient human resources, funds and logistics.[21] It appears from the recent reports (**Table 4**) that 82% humans have shown to be positive to VL with rK39 test in Panchaghar district[22] and 45.12% clinical VL and 38.14% PKDL (post-kala-azar dermal leishmaniasis) in Fulbaria, Mymensingh district.[13] An overall 6.12% mortality has been reported caused by VL in humans in the two endemic areas of Godagari, Rajsahi and Fulbaria, Mymensingh in Bangladesh.[23] The current burden of human VL in Bangladesh, India and Nepal is 20 times higher than the elimination target in 2015.[24] Vector sand-fly may obtain the leishmania directly from the infected skin or by ingesting the parasite from the circulating blood of the reservoir human hosts. A major challenge towards VL elimination is the rising incidence of PKDL which occurs after the VL treatment and act as an infection reservoir, threatens the VL elimination initiative. PKDL is a skin disorder which usually develops in 10 to 20% in clinical cases and about 60% of patients with VL after treatment.[13] In 2015 the WHO classified VL as a neglected tropical disease (NTD) due to relatively minimal granted attention from public, resulting in high mortality rates (> 20,000 in 2015) and endemic spreading in poverty-stricken regions around the world.[25]

Transmission of VL caused by *L. donovani* is still considered to be anthroponotic in the Indian subcontinent although the only known vector, *Phlebotomus argentipes* is zoophilic in nature.[14,19] The recent studies on the prevalence of leishmanisis in ruminant species (cattle, goats) reveals that there is no evidence of *L. donovani* DNA in seropositive and sero-negative cattle and goat samples used in PCR.[14,18] The absence of *L. donovani* DNA is suggestive of no role of cattle and goats as reservoir of VL in this endemic focus in Bangladesh (**Table 4**).

The presence of *L. donovani* infection in canine animals (dogs, golden jackals) with clinical signs and lesions in Bangladesh (**Table 4**) indicate that these animals play a role in the maintenance and reservoir of Leishmania protozoa in the VL endemic areas.

Dogs are recognized as the primary reservoir host for *L. infuntum*[29] and there are also reports of canine infection with *L. donovani* in Indian subcontinent and East Africa,[30] Sudan[31] and other species of animals.[27] We detected 20% positive for *L. donovani* infection in street dogs with rK39 strip test, 33.33% with Modified Giemsa's stained impression smears of liver and spleen and 83.33% with PCR along with clinical signs and lesions (**Table 4**) which indicates

Table 4. Comparison of the findings on the recent reports on *Leishmania donovani* infection in human and animals in Bangladesh

SN	District (period)	Host	No. of population	Samples used	Test used	Positive No. (%)	Clinical findings No. (%) +ve	References
1.	BAU Cam-(BAUC), Mymensingh	Golden Jackals	05 Organs	Blood HP	GSIS + PCR	+ 05 +ve	-	26Pus
2.	Trishal, Mymensingh (M)	Cattle	138	Blood Buffy coat	ELISA DAT PCR	13 (9.4) 04 (30.8)* 0	-	18
3.	Trishal, Fulbaria, M	Stray Dogs	85	Blood	ITS1-PCR	1 (1.2)	-	19
4.	Trishal & Fulbaria Mymensingh	Stray Dogs	50	Serum	rK39 RT-PCR ITS1-PCR	06 (12.0) 10 (20.0) 05 ()	-	27
5.	Trishal, Mymensingh	Dogs	103	Radial vein blood	Test-1 Test-12 Total	36 (35.0)	Fever = 12 (43.0) SL = 30 (45.0) ELN = 30 (44.0)	28
6.	Trishal & Fulbaria, M	Cattle Goat	258 122	Blood Buffy coat	rK39 DNeasy	+ve -ve	-	14
7.	BAUC, Trishal, Mymensingh	Stray dogs	20 06 06	Blood Organs	rK39 GSIS PCR	4 (20.00) 2 (33.33) 5 (83.33)	Fever, SL, alopecic, splenomegaly	PS
8.	Godagari, R Trishal, M	Humans	51094	Clinical	Mortality	12/196 (6.12)	VL symptoms and Lesions	23
9.	Fulbaria, Mymensingh	Humans	215	Interview question-naire	Clinical exami-nation	97 (45.12) ♂: 51.22 ♀: 36.96	PKDL = 38.14 CS+ CS+	13
10.	Debigonj, Panchaghar	Humans	700	Signs Blood	Findings rK39 ICT	51 (7.2) ± 42 (82) +ve	CS- CS +	22

1= rK39 ICT (Kalazar DetectTM Rapid Test; Bios International, Inc, Seattle, WA)
2= Kalazar Detect TN2 (Bios International Inc, Seattle, WA)
ELISA = Recombi LISA Leishmania Ab Test (CTK Biotech Inc, San Siego, USA)
DAT antigen (Koninklijk Institute voor de Tropen, Amsterdam, The Netherlands)
Ln PCR (QIAamp® DNA blood mini kit (QIAGEN, Dusseldorf, Germany)
DNeasy = DNeasy blood & tissue kit (Qiagen, Germany)
GSIS = Giemsa's stained impression smears
HP = Histopathology SL = Skin lesions CS = Clinical signs PS = Present study
ELN = Enlargement of lymph nodes PKDL = Post-kala-azar dermal leishmaniasis

active *L. donovani* infection in stray dogs in endemic areas in Bangladesh. However, the prevalence of anti-leishmania antibodies and L. donovani DNA in blood and tissue samples obtained from stray dogs from endemic areas corroborates the reports of Sri Lanka,[15] Sudan[30]

and India.[17] Review of inland reports also revealed that *L. donovani* DNA detected in a single (1.2%) dog among 85 stray dogs using DNA extracted from whole blood spotted on filter paper,[19] whereas 20% and 10% of stray dogs were found positive based on real time PCR and PCR using buffy coat DNA, respectively.[27]

After a Leishmania infected sand fly bites a definitive host, promastigotes (flagellated forms) are phagocytized by dermal macrophages and transformed into round-shaped amastigotes, which replicate in macrophages leading to cell destruction and progressive infection of more phagocytes. Once an infection is established, Leishmania tends to localize in all tissues in which monocytic-macrophagic cells exist in higher number such as the liver, spleen, lymph nodes, bone marrow, gastro-intestinal tract and skin. In addition to detection of Leishmania in the reticulo-endothelial system on impression smears and DNA test, the isolation and identification of the viable Leishmania from naturally exposed canine animals will be required to confirm their role in the maintenance (reservoir) and transmission of VL to humans and animals. Therefore, further studies with a large sample size are required to demonstrate the existence of parasites in tissue specimens with more sensitive techniques and to isolate viable Leishmania from different internal organs of naturally exposed dogs.

CONCLUSIONS

The human VL is still very alarming especially endemic districts in Bangladesh and proper surveillance, rapid diagnosis, importance of canine reservoir and complete treatment course are very urgent for elimination of the disease from Bangladesh. The presence of anti-rK39 Leishmania antibodies and Leishmania DNA and protozoa in the liver and spleen in stray dogs in the VL endemic district of Mymensingh indicate the active role of dogs as a reservoir of the vector-borne protozoa and transmit the infection from dog to humans and humans to dogs. The isolation of viable Leishmania infection in dogs and the ability of dogs to transmit the protozoa to the vector sand fly in nature are needed to confirm the potential role of dogs in VL epidemiology in Bangladesh.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors

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