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ANIMAL RESERVOIRS OF VISCERAL LEISHMANIASIS IN INDIA

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ABSTRACT: Visceral leishmaniasis (VL) is a disease that has both zoonotic and anthroponotic etiologies. In India, VL is endemic, considered to be anthroponotic, and caused by *Leishmania donovani*. Anthroponotic diseases are maintained by transmission from human to human and to a lesser extent from human to animals. Serum samples from 1,220 animals from 7 human VL endemic districts of Bihar, India, were tested for antibodies to a recombinant kinetoplast antigen (rK39 antigen) present in amastigotes of visceralizing *Leishmania* species, i.e., *L. donovani* complex. Additionally, PCR was used to examine samples positive by rK39 antigen serology. Antibodies to rK39 indicative of VL were detected in 33 of 1,220 animals. Thirty-one of 867 goats (*Capra hircus*), 1 of 161 cattle (*Bos indicus*), and 1 of 54 wild rats (*Rattus* sp.) were positive by rK39 serology. None of 106 chickens (*Gallus domesticus*), 26 sheep (*Ovis aries*), 3 water buffaloes (*Bubalus bubalus*), or 3 dogs (*Canis familiaris*) was positive by rK39 serology. *Leishmania donovani* DNA was detected by PCR in 20 rK39 positive blood samples from goats and 1 sample from a cow. The present study indicates that goats are potential animal reservoirs of human VL in India.

Leishmaniasis is a vector borne parasitic disease caused by hemoflagellate parasites in the genus *Leishmania*. Human leishmaniasis presents clinically as 3 different forms, consisting of a broad range of manifestations extending from cutaneous, to mucocutaneous, to visceral forms (Singh et al., 2011). The disease is endemic in 88 countries throughout the world, accounting for about 12 million clinical cases each year. Visceral leishmaniasis (VL), or kala-azar, has 2 epidemiological patterns. The first form is anthroponotic VL, with transmission of infection from humans to humans and to a lesser extent humans to animals. The second form is zoonotic VL, with transmission between animals to humans and to a lesser extent from humans to humans. The first type of VL is characteristic of *Leishmania donovani,* which is found in East Africa, Bangladesh, India, and Nepal (Desjeux, 2004). The zoonotic form is caused by *Leishmania infantum* (syn. *Leishmania chagasi*), which is endemic in the Mediterranean basin and South America (Singh, 2006). Canines and other mammals are the main reservoirs of zoonotic VL (Dereure et al., 2003). Epidemiological studies of VL worldwide have incriminated several animal species as reservoirs for zoonotic VL, including dogs, jackals, rodents, and foxes (Mukhtar et al., 2000; Reithinger et al., 2002; Chappuis et al., 2007). The importance of animals as reservoirs of anthroponotic VL is not well documented.

Approximately, 500,000 anthroponotic VL cases occur annually worldwide, of which more than 23,000 cases are in India (Desjeux, 2004; Singh, 2006). In India, the disease is endemic in the northeast, along the river Ganges, mainly in the states of West Bengal, Bihar, and eastern Uttar Pradesh. Sporadic cases have also been reported from Gujarat (western India), Tamil Nadu, Kerala (southern India), and sub-Himalayan parts of northern India, including Uttarakhand, Himachal Pradesh, Jammu, and Kashmir (Sharma et al., 2007).

The sand fly, *Phlebotomus argentipes*, is the only known vector for *L. donovani* on the Indian subcontinent. It feeds on both bovine and human blood, but it prefers cattle to human blood. However, the role of the animals in maintaining *L. donovani* parasites and being reservoirs of anthroponotic VL has not been fully investigated. Post kala-azar dermal leishmaniasis (PKDL) patients are considered to be the putative reservoirs for anthroponotic VL in India (Sanyal, 1985). In the present study, we aim to contest the belief that kala-azar in India is anthroponotic. Therefore, the objective of the study was to investigate and identify potential domestic animal reservoirs of VL infection in India.

MATERIALS AND METHODS

Testing of naturally exposed animals

Blood samples were collected in 2008 and 2009 from 1,166 domesticated animals and 54 wild rats from 7 districts of Bihar (25°11′N, 85°32′E) where anthroponotic VL is endemic (Table I; Fig. 1). Samples from domestic animals were obtained from individual animals at slaughterhouses, veterinary clinics, and from privately owned animals. The rats were captured using live-traps. One hundred blood samples were collected from animals at slaughter houses in Delhi (28°39′N, 77°12′E), a non-endemic region, and served as controls. All blood samples were collected in 2 tubes, 1 with anticoagulant (EDTA), and 1 without anticoagulant. The samples were transported cold to the Division of Clinical Microbiology, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, where all testing was conducted.

Serologic examination

The sera were tested for anti-leishmania antibodies to kinesin protein using the commercially available immunochromatographic dipstick assay, licensed for animal and human use (InBios International, Seattle, Washington) for qualitative antibody detection (Scalone et al., 2002). The test uses a recombinant protein (rK39) found on amastigotes in all visceralizing species as a test antigen (Burns et al., 1993). It is currently considered the best field diagnostic tool available for detecting VL in remote locations (Chappuis et al., 2007). Positive control sera to VL were obtained from humans (serologically and/or culture-proven) from our laboratory. The test was performed according to the manufacturer's instructions; briefly, the dipstick was placed in 20 µl of serum followed by 2–3 drops of the chase buffer, and after 10 min the results were read. Samples were considered positive when 2 distinct red lines, 1 in the positive test region and another in the control region, were present after 10 min.

DNA extraction and PCR amplification

Confirmation of serologic results was sought by PCR testing. For this procedure, DNA was extracted from buffy coat using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The yield of the extracted DNA was then measured at 260 nm using a spectrophotometer, and the purity was calculated using the 260/280 nm ratio. All DNA samples were stored at –20 C until further analysis. The whole ribosomal ITS region was amplified with the following specific *Leishmania* primers: 5′-CTGGATCATTTTCCGATG-3′ and 5′-ACACTCAGGTCTGTAAAC-3′ (El-Tai et al., 2001), which amplifies a 1020 bp region in the genomic DNA. A conventional PCR was performed in 25 µl reaction volumes containing 100 ng of genomic DNA, 10 pmol

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TABLE I. Leishmanial infection in different animal species from 7 districts of Bihar, India.

District	Cattle* (*Bos primigenius indicus*)	Goats (*Capra hircus aegagrus*)	Buffalo (*Bubalus bubalis*)	Rats (*Rattus rattus*)	Chicken (*Gallus gallus domesticus*)	Dogs (*Canis familiaris*)	Sheep (*Ovis orientalis vignei*)	Total
Khagaria	52	410 (19)	0	20 (1)	20	0	0	502 (20)
Begusarai	40	20 (1)	0	15	15	0	0	90 (1)
Vaishali	41	31 (3)	0	7	64	0	0	143 (3)
Muzaffarpur	28 (1)	89 (5)	3	2	7	0	0	129 (6)
Patna	0	242	0	0	0	3	10	255 (0)
Nalanda	0	52	0	0	0	0	16	52 (0)
Saran (Chhapra)	0	23 (3)	0	10	0	0	16	49 (3)
Tested (N =)	161	867	3	54	106	3	26	1,220
Positive (%)	1	31 (3.6)	0	1	0	0	0	33 (2.7)

* Cattle category includes cows, oxen, and calves.

each of the gene-specific forward and reverse primers, 10 μM of each dNTP, 2 mM $MgCl_2$, and 5 units of *Pfu Taq* DNA polymerase (Bangalore Genei, Bangalore, India). The conditions for PCR were as follows: 95 C for 2 min, then 34 cycles of 95 C for 20 sec, 53 C for 30 sec, and 72 C for 1 min. Final extension was carried out for 1 min at 72 C. PCR products were separated on 1.5% agarose gel stained with ethidium bromide (Fig. 2). To confirm the amplified DNA corresponded to *Leishmania* spp., 10 amplified products were randomly selected and were subjected to gel purification using QIAmp gel extraction kits (Qiagen). Of 10 PCR products sequenced, 9 were collected from goats and 1 was collected from a rat. The purified products were sequenced directly with BigDye Terminator Mix version 3.1 (Applied Biosystems, Foster City, California), according to the manufacturer's instructions, and were then analyzed on an ABI-3100 Genetic Analyzer (Applied Biosystems). The sequences were compared with those of *Leishmania* spp. and other trypanosomatids from GenBank using Blast software tool. The PCR for this study was standardized using DNA from culture grown *L. donovani* promastigotes.

RESULTS

Leishmanial antibodies were detected in 33 (2.7%) of 1,220 blood samples from endemic areas using the rK39 test (Table I; Fig 1.). Examination of the 33 seropositive samples using PCR demonstrated *Leishmania* spp. DNA in 20 (60.6%) samples (Fig. 2), of which 19 were from goats and 1 from a rat. The lone seropositive cow sample could not be tested by PCR because the storage tube was broken. The 10 positive samples (9 goats and 1 rat) that were sequenced produced a *L. donovani* specific sequence.

FIGURE 1. Map of Bihar state of India, showing the endemicity of human visceral leishmaniasis. Each black spot represents 1 infected animal and indicates clustering of animal reservoirs in the respective districts.

DISCUSSION

Kala-azar is presumed to be anthroponotic in India, and previous attempts to identify an animal reservoir host, albeit using poorly sensitive methods, have failed. A few cases of visceral leishmaniasis in different animal species have been reported from India (Srivastava and Chakravarty, 1984), but none has been confirmed to be zoonotic VL (Bhattacharya and Ghosh, 1983; Prasad, 1999). Although sporadic cases of zoonotic cutaneous leishmaniasis are reported (Prasad, 1999), they remain restricted to Rajasthan state, which is about 628 miles from Bihar (Srivastava et al., 1987; Ahuja et al., 1993; Sharma et al., 2003). A recent outbreak of localized cutaneous leishmaniasis in Himachal Pradesh incriminated a local sand fly vector and dogs

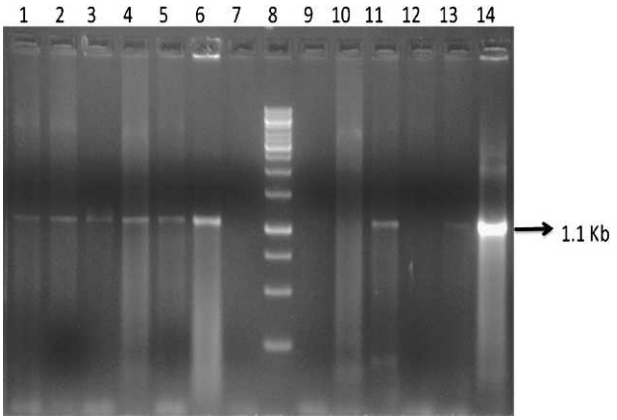

FIGURE 2. Polymerase chain reaction (PCR) from the clinical samples from serologically positive animals was performed followed by gel electrophoresis on 1.5% agarose and stained with ethidium bromide. Lanes 1–6 are PCR amplified products of DNA extracted from the buffy coat of animals; lane 8 is a 1 Kb MW DNA marker; lanes 9–13 are PCR amplified products of DNA extracted from the buffy coat of animals; lane 7 is negative control (DNA from the buffy coat of an animal from non-endemic region used as internal control); and lane 14 is a positive control (DNA extracted from cultured *L. donovani* promastigotes) used as internal control in the PCR.

as reservoirs (Sharma et al., 2005). Interestingly, Kellick-Kendrick made a reference to bovine cutaneous leishmaniasis in a kala-azar endemic area of Assam and indicated the possibility that both zoonotic and anthroponotic transmission of VL occurred in India (Killick-Kendrick, 1990). Unfortunately, owing to the limited sensitivity of microscopy, histopathology, and culture based parasitological diagnostic methods, which were used in these studies, knowledge of animal reservoirs of VL in India has remained incomplete, and scientists have settled on a dictum that Indian visceral leishmaniasis is strictly an anthroponotic disease and that PKDL patients act as reservoirs (Singh et al., 2011).

The districts we investigated are known to have active transmission of kala-azar. The immunochromatographic dipstick test used in the study is based on a 39–amino acid–repeat recombinant antigen prepared from *L. chagasi* to screen sera from domestic animals for the presence of antibodies to VL. The rK39 dipstick tests have been shown to be highly sensitive and specific for the detection of antibodies to visceralizing *Leishmania* spp. (*L. donovani*, *L. chagasi*, and *L. infantum*) that cause VL in animals, including humans, *L. chagasi*, (Bern et al., 2000; Scalone et al., 2002). In contrast, it does not show detectable antibodies in cutaneous or mucocutaneous leishmaniasis (Singh and Sivakumar, 2003). Recently, comparative evaluation of serological tests in the diagnosis of human visceral leishmaniasis in India has reported rK39 ICT (Kalazar Detect™ InBios International Inc.) to be 98.9% sensitive and 97.0% specific (Sundar et al., 2007). Its usefulness has also been validated in serodiagnosis of zoonotic visceral leishmaniasis (Toz et al., 2004), although its sensitivity and specificity has not yet been evaluated in India for animal leishmaniasis. There are few reports that demonstrate low sensitivity and specificity of dipstick for diagnosis of asymptomatic dogs compared with suspected clinical human cases (Reithinger et al., 2002; Mettler et al., 2005; Lemos et al., 2008). Therefore, in addition, we carried out PCR tests to confirm the infection in seropositive animals. PCR tests have consistently been shown to be a better tool than microscopy, culture, or serologic tests, particularly in samples with low parasite loads for VL diagnosis (Reithinger et al., 2002). One may argue that detection of antibodies and the parasite DNA indicate persistent exposure of the animal to *Leishmania* spp.; it is noteworthy that the kinesin protein (K39) is predominantly expressed by amastigote stages of the parasite and not during the promastigote stage of the parasite. Therefore, it is not likely that antibodies to K39 would be produced unless amastigote multiplication was occurring inside the host cells (Badaro et al., 1996). Additionally, confirmation by DNA amplification of the ITS region and sequencing of the PCR amplicons further indicate current, or recent, infection. It should be noted that DNA of dead parasites will persist in a host cell only for no more than 24 hr (Prina et al., 2007). Moreover, not all exposed animals were found positive by serology or PCR.

Vector species and their host feeding preference behavior is an important factor that influences the parasite life cycle and its reservoirs. In the VL endemic area of Bihar, the vector, *P. argentipes*, feeds on both bovids and humans, but with a preference for cattle. These animals in endemic areas must be frequently bitten by sand flies infected with *L. donovani*, but nothing is known about their susceptibility to infection. In India, kala-azar is a disease found among economically deprived populations consisting mostly of herdsmen. The presence of anti-rK39 antibodies with supporting PCR mainly in goats, but not cattle, suggests that even though all domestic animals are bitten by the sand fly, only goats had serological and molecular evidence of intracellular multiplication of the parasite. This suggests that goats are very likely involved in the maintenance of human VL in Bihar. These animals are usually kept close to human houses, or sometimes inside the house, which are most often mud huts (Singh et al., 2000). The Khagaria region had the majority of positive samples in our study (Table I) and also is highly endemic for human VL (Croft et al., 2006). Hence, our animal findings are consistent with the human epidemiology of kala-azar. Nevertheless, further studies are needed to determine whether this high prevalence in domestic animals occurs in other regions too and whether goats can serve as hosts capable of transmitting the infection to permissive sand flies, fulfilling Koch's postulates.

To the best of our knowledge, there are no prior reports of leishmanial infection of goats from India; however, *Leishmania* sp. has been isolated from a naturally infected domestic goat from West Pokot, Kenya (Williams et al., 1991). We are continuing our attempts to isolate viable *Leishmania* from naturally exposed goats and other animals in India and characterize the isolates. We believe that the involvement of goats in the maintenance and transmission of VL has been overlooked by public health and veterinary researchers and that this component of the epizootiology of VL needs critical examination to update prevention and treatment programs in areas endemic for kala-azar.

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