

Molecular detection of *Leishmania* parasites from whole bodies of sandflies collected in Nepal

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Abstract

Visceral leishmaniasis is endemic in the southern part of the Terai region of Nepal. Natural infections of *Phlebotomus* species with *Leishmania* parasites in these endemic areas were analyzed by a polymerase chain reaction (PCR) amplification based assay. A total of 401 *Phlebotomus argentipes* and 202 *P. papatasi* female sandflies were captured in the Dhanusa district from 2004 to 2006, and analyzed. It was found that 6.7% of *P. argentipes*, but no *P. papatasi* were positive for *Leishmania* parasites, suggesting that *P. argentipes* is a major vector in these areas. The infectivity of *P. argentipes* with *Leishmania* was consistent with infection rates reported from other areas of the world. This is the first report of naturally infected *Leishmania* parasites in sandflies collected from Nepal.

Keywords: *Phlebotomus*, infectivity, visceral leishmaniasis, Nepal

Introduction

Leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania*. The parasites are distributed worldwide, especially in tropical and subtropical areas, and affect at least 12 million people. More than 20 species of *Leishmania* have been described as causative agents of human leishmaniasis. (Desjeux 1996, Choi and Lerner 2001). *Leishmania* parasites are transmitted by female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. The spread of leishmaniasis depends on the distribution of vectors and reservoir animals. There are more than 500 sandfly species, but only 20 are of medical importance. In addition, it has been suggested that only restricted sandfly species can support the development of specific *Leishmania* parasites (Killick-Kendrick 1999). Thus, the detection and identification of *Leishmania* species within naturally infected sandflies is important for the prediction of the risk and expansion of the disease in endemic areas. In Nepal, visceral leishmaniasis (VL) is confined mainly to the southern parts of the Terai region, which borders the VL endemic districts of Bihar state in India. Around 5.5 million people live in the affected areas, and those people are classified as "at risk". A total of 25,704 cases with 530 deaths were reported between 1980 and 2004, and the fatality rate in reported cases varied between 0.23 and 13.6% (Ministry of Health, Nepal 2005/2006).

Phlebotomus argentipes are much more abundant than *P. papatasi* and *Sergentomyia* spp. in India (Dinesh et al. 2000, Mukherjee et al. 1997). *P. argentipes* is largely restricted to domestic and peri-domestic habitats and a limited number of studies have detected breeding sites in humid ground rich in organic content mainly in animal shelters (Palit et al. 2005). It is important to survey the infection rate of naturally infected sandflies in endemic areas. However, studies based on the demonstration of promastigotes in dissected sandflies by microscopy are

difficult to carry out, as the infection rate in the competent vectors is generally low and its estimation requires examination of a large number of specimens. Previous microscopical surveys on different species of sandfly in different regions showed infection rates ranging from 1.7 to 10.7% (Bettini et al. 1986; Maroli et al. 1994b). Recently, polymerase chain reaction (PCR)-based techniques have been adapted to detect *Leishmania* spp. in sandflies. By semi-nested PCR assay, Aransay et al. (2000) found a high rate of *L. infantum* infection (8.3%) in *P. papatasi* in Greece. Similarly in Argentina, Corbado-Launus et al. (2006) reported a 9.1% positive rate for *Leishmania* in *Lutzomyia neivai*. In this study we employed a PCR method to directly detect *Leishmania* parasites from individual sandflies collected from endemic areas of Nepal.

Material and methods

Study sites

Dhanusa district is located in the southern part of the Terai region in Nepal, 500 km from Kathmandu, possessing many ponds, a small dam and dispersed dwellings with domestic animals such as dogs, goats, cows, water buffaloes, hogs and poultry. Sandflies were collected at 5 Village Development Committees (VDC; Yagyabhumi, Bateswor, Umaprempur, Godar and Sinurjoda) of Dhanusa district (Fig 1). These 5 areas were chosen based on previously reported cases of leishmaniasis (Ministry of Health, Nepal 2005/6).

Sandflies were captured with mouth aspirators, test tubes and the help of battery-operated torches in the evening (3 hrs) and early in the morning (3 hrs) from two different places: inside houses, and in animal shelters, in August 2004, June 2005 and July 2006. On the day, the collected sandflies were brought to the field laboratory, anaesthetized with chloroform, and morphologically

examined for species identification using criteria given by Lewis (Lewis 1987). The sandflies were fixed in 70% ethanol and transported to Japan for PCR analysis.

Leishmania donovani

Leishmania donovani (D10 isolate) was maintained as previously reported [Pandey et al 2007].

DNA extraction

Prior to DNA extraction, legs and wings of each sandfly were removed under a binocular microscope and individual female sandflies were macerated in a micro tube using a plastic pestle. DNA was extracted independently from each sandfly using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA). Finally DNA was resuspended in 100 µl of Tris-EDTA buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). In the absence of laboratory reared female sandflies, field-caught male sandflies were used as a control, as they do not serve as vectors for *Leishmania* parasites.

PCR amplification

Leishmania parasites were detected by PCR amplification of a variable region of the minicircle kinetoplast DNA as previously described (Aransay et al. 2000). Copy number of minicircle kinetoplast DNA is more than 10⁴ per parasite, maximizing the possibility of detection (Smyth et al 1992; Salotra et al 2001). PCR analysis consisted of two steps. First round PCR amplification was carried out in a total of 10 µl reaction mixture containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 250 µM deoxynucleotide triphosphates, 1.5 mM MgCl₂, 1 unit of *TaKaRaTaq*TM DNA polymerase (TaKaRa, Japan), 1 µM LINR4 primer (5'-GGGGTTGGTGAAAATAGGG-3'), 0.2 µM LIN17 primer (5'-TTTGAACGGGATTTCTG-3') and 1 µl of DNA solution. The mixture was incubated at 94°C for 5 min followed by 17 cycles, each consisting of 30 sec at 94°C, 30 sec at 52°C and 1 min at 72°C. After the last cycle, the extension was continued for a further 5 min. The second round semi-nested PCR was carried out in a 20 µl reaction mixture volume as described for the first round by adding 1 µM LIN19 primer (5'-CAGAACGCCCTACCCG-3') and 1 µl of first PCR product for 33 cycles with the final extension at 72°C for 10 min. Ten µl of the PCR-amplified products was subjected to a 2% agarose gel electrophoresis (LO3; TaKaRa), stained with ethidium bromide (0.5 µg/ml), and visualized under ultraviolet transillumination.

Sequencing

DNA fragments were recovered from agarose gels after electrophoresis and purified using QIAquick gel extraction kit (Qiagen). Purified DNA fragments were directly sequenced using an ABI Prism BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystem, USA) with the LIN 19 primer according to the manufacturer's instruction.

Results

Sensitivity of the PCR assay

To establish a reliable detection system for *Leishmania* DNA, we initially tested the specificity and sensitivity of the PCR method with primers specific for *Leishmania* minicircle kinetoplast DNA. We could detect parasite DNA from a sample prepared from a (theoretically) single *L. donovani* culture parasite (data not shown). We subsequently tested PCR sensitivity against samples prepared from different numbers of culture parasites mixed with a single male sandfly. We found that a single parasite could be detected when mixed with the body of male sandfly (Fig. 2).

Identification of *Leishmania* parasites from sandflies collected in Nepal

A total of 671 female sandfly specimens were collected between 2004 and 2006 in 5 different sites in Nepal (Table 1). Four hundred and one were *P. argentipes* (59.8%), 202 were *P. papatasi* (30.1%), and 68 were *Sergentomyia* spp. (10.1%). Twenty seven out of 401 *P. argentipes* showed positive bands (720 bp) by *Leishmania* spp. specific PCR assay, indicating that at least 6.7% were naturally infected with *Leishmania* parasites (Table 2). However, parasites were not detected from *P. papatasi* (0/202) in this survey. Thus, *P. argentipes* appears to be the predominant vector for *Leishmania* parasites in these areas. All of the visualized bands showed the same size as standard *L. donovani* originating from Nepal (Fig. 3; Pandey et al, 2007). The PCR products were directly sequenced and compared with known sequences for species-identification within genus *Leishmania*. To this end, 15 samples were identified as *L. donovani* based on the strong homology with reported *L. donovani* kDNA sequence (GenBank accession number Y11401.1; Basselin et al. 1998). The remaining samples could be categorized only as *Leishmania* spp. based on the positive amplification with the *Leishmania*-specific primer set. However, six parasites showed sequences distinct from known *Leishmania* spp. and reliable sequences were not obtained from the remaining six samples.

Discussion

In this report, we used a PCR-based system for the detection of *L. donovani* parasites in sandflies without the need for dissection. In previous studies, PCR identification assays were carried out on parasites collected from sandflies after dissection (Aransay et al. 2000, Kato et al. 2005). In order to avoid this technically demanding and time-consuming dissection step we explored a novel strategy to amplify parasite DNA from the whole bodies of collected sandflies. The use of male sandfly mixtures spiked with different concentrations of *L. donovani* promastigotes permitted the sensitivity of the detection system to be evaluated. We have demonstrated that a single *Leishmania* spp. parasite may be detected by this technique, even in the presence of excess phlebotomine DNA.

The results presented here constitute the first natural infection analysis using a DNA-based approach to identify phlebotomines involved in the transmission of VL in Nepal. *Leishmania* DNA was detected only from *P. argentipes*, but not from *P. papatasi*, suggesting that *P. argentipes* is a major vector for *L. donovani* parasite in the survey areas in Nepal. Although we could not detect

Leishmania DNA from *P. papatasi*, it is reported to be a vector for *L. infantum* (Aransay et al 2000) and *L. major* (Ready et al 1998) in other regions.

A prevalence of 6.7% for *P. argentipes* infection represents a relatively high index compared with other studies that use traditional dissection method (Table 3). We consider that this is because of the high sensitivity of PCR-based detection systems. The occurrence of laboratory contamination can be ruled out because of the lack of the amplification from negative control samples. However, it should be noted that *Leishmania* DNA-positive sandflies may include not only those carrying live promastigotes, but also those carrying DNA fragments of *Leishmania* resulting from non-established (abortive) infections or blood meals containing *Leishmania* DNA fragments (Gradoni, 2002).

More information regarding the distribution of this parasite in the phlebotomine fauna is essential in order to understand leishmaniasis epidemiology and to develop effective control measures against the disease. The PCR-based detection technique described here, which by-passes the need for sandfly dissection, provides a valuable tool for the analysis of large numbers of sandfly samples for endemic areas, and will greatly aid future epidemiological studies.

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Figure Legends

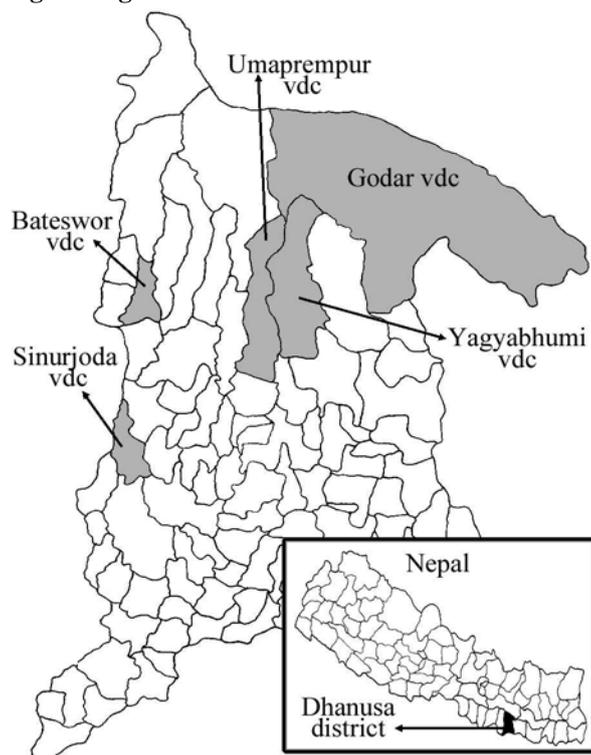


Figure 1. Collection sites of sandflies in Nepal.

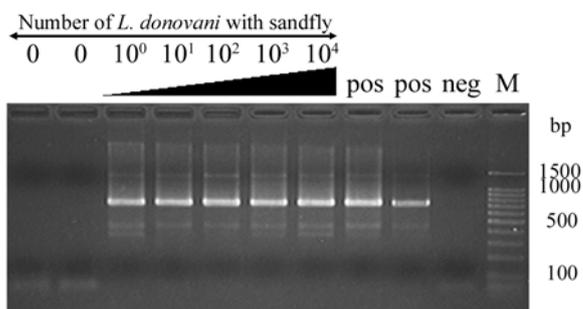


Figure 2. Sensitivity of PCR from DNA extracted from different numbers of *Leishmania donovani* parasite mixed with the whole bodies of male sandflies. PCR amplification was carried out on DNA was extracted from male sandflies crushed in the presence of 0, 1, 10, 100, 1000, 10000 *L. donovani* parasites . M, 100 bp DNA ladder marker; pos, DNA extracted from *L. donovani* cultured parasite as a positive template; neg, distilled water as a negative template solution.

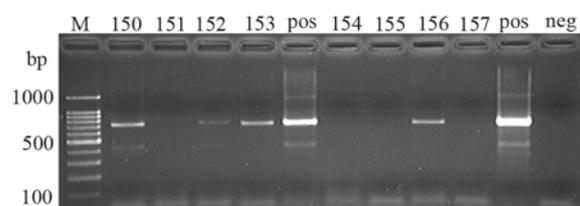


Figure 3. Representative PCR-positive samples from infected and noninfected sandflies with *Leishmania* spp. M, 100 bp DNA ladder marker; PCR from field-isolated *P. argentipes* (150, 152, 153 and 156 are positive; 151, 154, 155 and 157 are negative); pos, DNA extracted from *L. donovani* cultured parasite as a positive template; neg, distilled water as a negative template solution.

Table 1. Female sandflies collected each year

Year	<i>Phlebotomus argentipes</i>	<i>P. papatasi</i>	<i>Sergentomyia</i> spp.	Total
2004	22	16	7	45
2005	2	123	23	148
2006	377	63	38	478
Total	401	202	68	671

Table 2. Infective rate of sandflies with *Leishmania* parasites in different villages

Year	<i>Phlebotomus argentipes</i>	<i>P. papatasi</i>	<i>Sergentomyia</i> spp.
Bateswor	9/168 (5.4%)	0/43 (0%)	0/16 (0%)
Godar	0/2 (0%)	0/55 (0%)	0/19 (0%)
Sinurjoda	3/29 (10.3%)	0/20 (0%)	0/1 (0%)
Umaprempur	n.a.	0/68 (0%)	0/4 (0%)
Yagyabhumi	15/202 (7.4%)	0/16 (0%)	0/28 (0%)
Total	27/401 (6.7%)	0/202 (0%)	0/68 (0%)

n.a. indicates that no sandfly was collected.

Table 3. Comparison of *Leishmania* spp. infections in sandfly

Infection rate	<i>Leishmania</i> spp.	Sandfly spp.	Country	Reference	method
27/402 (6.7%)	<i>Leishmania</i> spp. ^a	<i>P. argentipes</i>	Nepal	Present study	PCR assay from sandfly without dissection
14/435 (3.4%)	<i>L. donovani</i>	<i>P. argentipes</i>	India	Kumar et al 2005	dot-immunoblot assay
9/572 (1.6%)	<i>L. donovani</i>	<i>P. orientalis</i>	Sudan	Hassan et al 2004	PCR assay from dissected sandfly

^a Among 27 samples, 15 were confirmed as *L. donovani* by nucleotide sequencing. The remaining 12 were uncharacterized.