

# Prevalence of Sand Flies and *Leishmania donovani* Infection in a Natural Population of Female *Phlebotomus argentipes* in Bihar State, India

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## Abstract

Leishmaniasis is a vector-borne disease, and in the Indian subcontinent the female *Phlebotomus argentipes* is the vector for *Leishmania donovani*. However, data on the extent of sand fly infection rates in natural settings using molecular methods have not been extensively reported in India. In this study a PCR technique was applied targeting the 18S rRNA encoding region to determine the prevalence of *Leishmania* infection in female *P. argentipes* captured in the field. For this study, sand flies were collected from 897 houses selected from 50 villages endemic for visceral leishmaniasis (VL) in Muzaffarpur district, Bihar state, using CDC miniature light traps and mouth aspirators. A total of 14,585 sand flies were collected of which 449 were female *P. argentipes* divided into 132 pools. Molecular detection using PCR targeting the 18S rRNA gene was carried out for the identification of *P. argentipes* and *Leishmania*. The overall prevalence of infection was 4.90–17.37% for *L. donovani* in female *P. argentipes* in endemic regions of Bihar state. In this study no correlation was found between the presence of infected sand flies and the occurrence of clinical VL. This study provides the first report evaluating the prevalence of *Leishmania* infection in sand flies in a region endemic for VL in India. *Sergentomyia* species are the most common species of sand fly. Knowledge of the infection rate in female *P. argentipes* may help in predicting severity of disease and in vector elimination programs.

**Key Words:** *Leishmania donovani*—*Phlebotomus argentipes*—18S rRNA—Gene—PCR—Sand fly—Visceral leishmaniasis.

## Introduction

LEISHMANIASIS ARE AMONG THE WORLD'S MOST neglected diseases, affecting mostly developing countries. In all, 350 million people are considered to be at risk of leishmaniasis, and about 2 million new cases occur annually. Leishmaniasis is caused by at least 20 *Leishmania* species, and is transmitted by the female sand fly of the genus *Phlebotomus* in the Old World, and *Lutzomyia* in the New World. In India a total of 33,233 cases were reported in 2008, of which more than 84% were from Bihar state (WHO 2010). In the Indian subcontinent, transmission is anthroponotic, and for visceral leishmaniasis (VL) female *P. argentipes* is the vector, and *L. donovani* is the parasite (Desjeux 1996). Female sand flies need blood for their eggs to develop. They become infected with the *Leishmania* parasites when they suck blood from an infected person or animal (WHO 2010). Monitoring infection

in the vector population is an essential tool for surveillance of vector-borne diseases. There are two classical methods for the estimation of infection rates in the reservoir hosts or vectors: microscopic analysis and culture isolation. Both methods are laborious and not feasible for use with large samples. Molecular methods such as PCR are highly sensitive and easy to perform compared to optical microscopy. Using PCR for the detection of *Leishmania* has proven useful (Cabrera et al. 2002; Bhattarai et al. 2009; Azpurua et al. 2010). This technique would be expensive and highly labor-intensive if individual insects are analyzed, as the number of sand flies collected could be in the thousands. In previous studies of detecting infection rates in the vector population, insects were pooled together before performing PCR (Katholi and Unnasch 2006; Bhattarai et al. 2009). The aim of this study was to assess the prevalence of different species of sand flies, and to find out the infection rate of *L. donovani* in the natural population using

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PCR targeting the 18S rRNA of female *P. argentipes* in VL-endemic regions of Bihar state, India.

## Materials and Methods

### Study area

The study area consisted of 50 villages from the endemic regions of Muzaffarpur district, Bihar state, India, situated between 85.163570 and 85.276040 longitude and between 26.126200 and 26.240060 latitude (Fig. 1). For sand fly collection, a total of 897 houses were selected (141 houses with a VL case history, and 282 matched control houses, which included two houses without VL case histories adjacent to each VL-case house, and 474 random houses).

This study was approved by the ethics committee of Banaras Hindu University, Varanasi, India, and informed written consent was taken from the head of each household involved in the study.

### Sand fly collection

Collection was done in April–June 2009. Insects were collected using Centers for Disease Control and Prevention light traps, and by mouth aspiration. One trap was installed inside each house in the evening and was left there for the whole night (approximately 12 h). The next day, in the early morning the sand flies were collected from the trap, and sand flies resting on the walls were collected by mouth aspiration for 15 min. The collected insects from each house were stored in a Petri dish with chloroform-soaked cotton balls. The collected insects were brought to the entomology laboratory at the Kala Azar Medical Research Centre (KAMRC), Muzaffarpur.

### Morphological identification

Field-collected insects were killed for identification after storage at  $-20^{\circ}\text{C}$  for 20 min. All the collected insects were differentiated morphologically as sand flies or other insects such as mosquitoes. Further, sand flies were differentiated using sand fly identification keys, such as morphology of the maxillae, and the hairs of the abdominal tergites and terminalia under a stereomicroscope, and identified as *P. argentipes*, *P. papatasi*, or *Sergentomyia* species (Lewis 1982). All sand flies were further differentiated as either male or female on the basis of morphology of the reproductive organs as assessed using the stereomicroscope. Only morphologically-confirmed female *P. argentipes* were selected for the detection of *Leishmania* infection, and they were pooled on the basis of the houses from which they were collected. The sand flies were fixed in 70% ethanol and stored at  $-20^{\circ}\text{C}$ .

### DNA extraction

DNA from each pool of female *P. argentipes* was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), following the manufacturer's instructions. DNA was eluted in molecular-biology-grade water, and quantified using a ND-2000 spectrophotometer (Thermo Scientific, Powai, Mumbai, India).

### Molecular analysis

Validation of each sand fly pool was done by species-specific PCR. Molecular discrimination of *P. argentipes* from other sand flies was also done using a species-specific PCR test targeting the 18S rRNA coding gene with forward (5'-

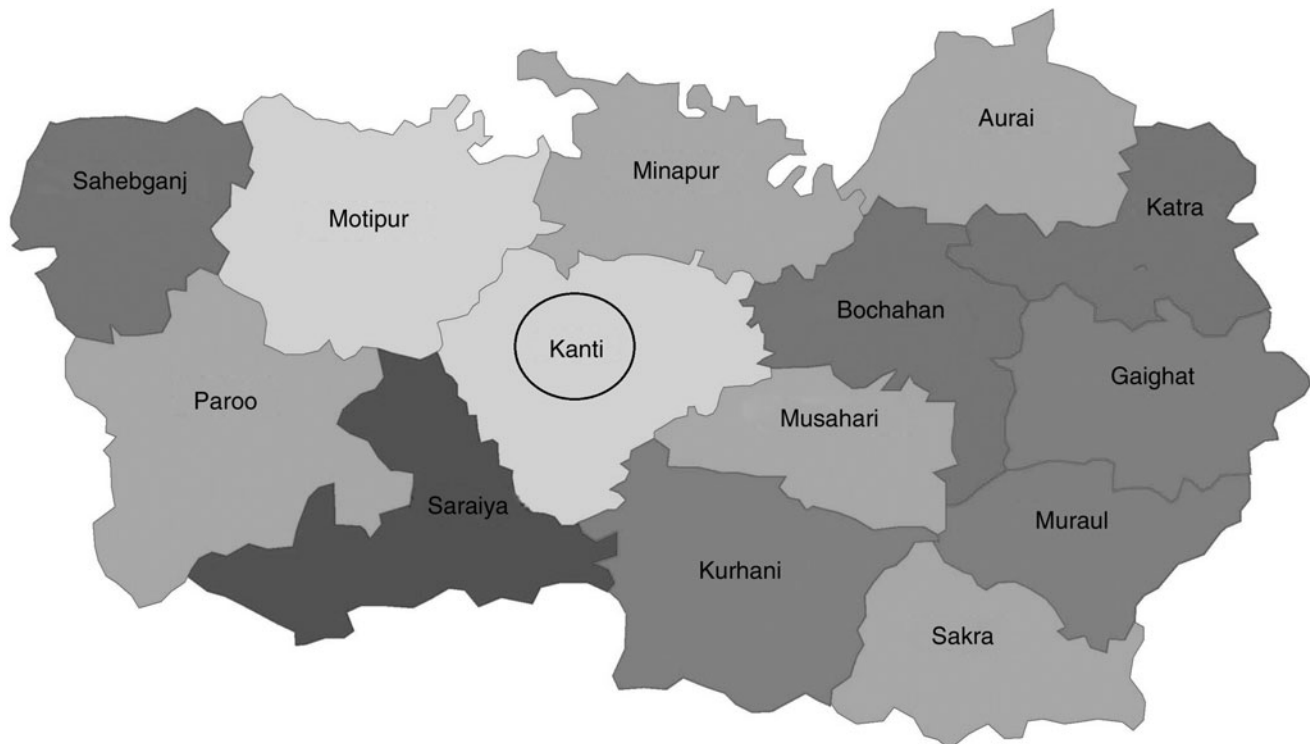


FIG. 1. Map of the Muzaffarpur district. The black circle shows the area where sand flies were collected for the present study.

TCGAATCTATGGGTGGTGGT-3') and reverse (5'-CACAAATCCAACCACGAAG-3') primers that specifically amplified a 399-bp conserved region of phlebotomine sand flies. The reaction was carried out in a volume of 25  $\mu$ L using the pair of primers (10 pM each) and the Hot Start Taq kit (Qiagen India, New Dehli, India), supplemented with 10 $\times$  buffer, MgCl<sub>2</sub>, and 100 ng of sand fly DNA. An initial denaturation of 15 min at 95°C, followed by PCR amplification for 35 cycles of denaturation (95°C for 30 sec), annealing (57°C for 30 sec), and polymerization (72°C for 40 sec), with final extension of 10 min at 72°C. To discriminate *P. argentipes* from other species, the amplified PCR product was subjected to restriction digestion using a Hae III restriction enzyme (recognition site GGCC; New England Bio Labs, Hitchin, Herts, U.K.) in the supplied reaction buffer supplemented with 1 mg/mL<sup>-1</sup> BSA at 37°C for 2 h. The whole reaction was electrophoresed on a 1.5% (w/v) agarose gel and stained with ethidium bromide (Surendran et al. 2005).

A highly sensitive primer specific for the 18S rRNA coding gene of *Leishmania* was used to identify infection in female *P. argentipes* (Deborggraevae et al. 2008). The nuclear sequence of the small subunit of the rRNA gene in *Leishmania* is found as 20–40 copies per cell (van Eys et al. 1992; Meredith et al. 1993; Costa et al. 1996; Osman et al. 1997). Pools of *P. argentipes* were used for the study of natural infection of *Leishmania* using PCR specific for the 18S rRNA gene of *L. donovani*. A forward primer (5'-CGTAGTTGAACTGTGGGCTGTGC-3') and a reverse primer (5'-ACTCCCGTGTTCCTTGTTCCTT-GAA-3') were used to amplify a 115-bp sequence within the 18S rRNA gene of *L. donovani*. The reaction was carried out in a volume of 25  $\mu$ L using the pair of primers (20 pM each), and a Hot Start Taq kit supplemented with 10 $\times$  buffer, MgCl<sub>2</sub>, and 200 ng of template. An initial denaturation was done for 15 min at 95°C, followed by PCR amplification for 45 cycles of denaturation (95°C for 30 sec), annealing (60°C for 30 sec), and polymerization (72°C for 45 sec), with a final extension of 5 min at 72°C (Looker et al. 1988; Deborggraevae et al. 2008; Bhattarai et al. 2009). The DNA of reference strain *L. donovani*, LEM138 (MHOM/IN/00/DEVI), was used as a positive control, and molecular-biology-grade water was used as the negative control. Amplified products were resolved on 1.5% ethidium bromide-stained agarose gels.

#### DNA sequencing

Identification of *P. argentipes* and *Leishmania* were confirmed by sequencing of amplified PCR products. A few specimens were randomly selected from both groups. The PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen India). Purified fragments were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). The

cycle sequencing reaction was stopped according to the manufacturer's instructions, and then analyzed with an ABI 3130 genetic analyzer.

The *P. argentipes*-specific PCR products of 399 bp were aligned and compared with the available nucleotide sequence of the *P. argentipes* 18S rRNA gene, clone ARG1 (GenBank accession no. AJ244359.1) (Surendran et al. 2005). The sequence of *Leishmania*-specific PCR products were aligned and compared with the 18S rRNA sequence of *L. donovani* (GenBank accession no. X07773.1) (Deborggraevae et al. 2008).

#### Statistical analysis

For calculating the prevalence of the infection rate of *Leishmania* parasites in the unequal pools of sand flies, the minimum-maximum infection rate was used. For the minimum infection rate, a pool was considered positive if the pool contained a single infected insect, while for the maximum infection rate, all of the insects were considered to be infected (Katholi and Unnasch 2006).

## Results

#### Sand fly collection

After morphological identification, sand flies were found in 880 out of a total of 897 houses (98%). *P. argentipes* were detected in 319 houses (Table 1). A total of 14,585 sand flies were collected and their distribution is given in Table 2. For the detection of the *Leishmania* parasite, only female *P. argentipes* were selected, and were found in 132 houses (14.7%) from 33 of 50 villages in the study area. The total number of female *P. argentipes* collected was 449, which were divided into 132 pools, with each pool representing all the female *P. argentipes* collected from a single house. The number of female *P. argentipes* in each pool varied from 1 to 22. The total number of pooled samples per village, the total number of sand flies in that pool, the number of pools found to be positive, the total number of sand flies in the positive pool, the percentage of positive samples, and the prevalence of infection (%) are given in Table 3.

#### Molecular identification of sand flies

Microscopically-identified female *P. argentipes* were confirmed using PCR. Using species-specific PCR targeting the 18S rRNA region of phlebotomine sand flies, we amplified a 399-bp region that was further digested with restriction endonuclease to differentiate *P. argentipes* from other phlebotomine species. The two Hae III cutting sites at positions 27 and 158 in the conserved region of *P. argentipes* generated three bands of 27 bp (not visible after electrophoresis), of 131 bp and 241 bp, respectively, while the single site in *P. papatasi* or

TABLE 1. THE TOTAL NUMBERS OF HOUSES POSITIVE FOR DIFFERENT TYPES OF SAND FLIES

Sand fly species	Male n (%)	Unfed female n (%)	Fed female n (%)	Gravid female n (%)	Total houses
<i>Sergentomyia</i> spp.	716 (79.8)	127 (14.2)	135 (15.1)	306 (34.1)	880
<i>P. argentipes</i>	276 (30.8)	122 (13.6)	72 (8.0)	27 (3.0)	319
<i>P. papatasi</i>	33 (3.7)	3 (0.3)	11 (1.2)	7 (0.8)	28

TABLE 2. THE TOTAL NUMBER OF SAND FLIES COLLECTED, INCLUDING MALES AND FEMALES, OF ALL THREE SPECIES

Sand fly species	Male n (%)	Unfed female n (%)	Fed female n (%)	Gravid female n (%)	Total
<i>Sergentomyia</i> spp.	5047 (34.6)	5743 (39.4)	931 (6.4)	1060 (7.2)	12781 (87.6)
<i>P. argentipes</i>	1287 (8.8)	307 (2.1)	104 (0.7)	38 (0.3)	1736 (11.9)
<i>P. papatasi</i>	43 (0.3)	3 (0.02)	14 (0.09)	8 (0.05)	68 (0.5)
Total	6377 (43.7)	6053 (41.5)	1049 (7.2)	1106 (7.6)	14,585

*Sergentomyia* species at position 27 generated two bands of 27 bp and 372 bp (Fig. 2).

#### Detection of *Leishmania* infection in sand flies

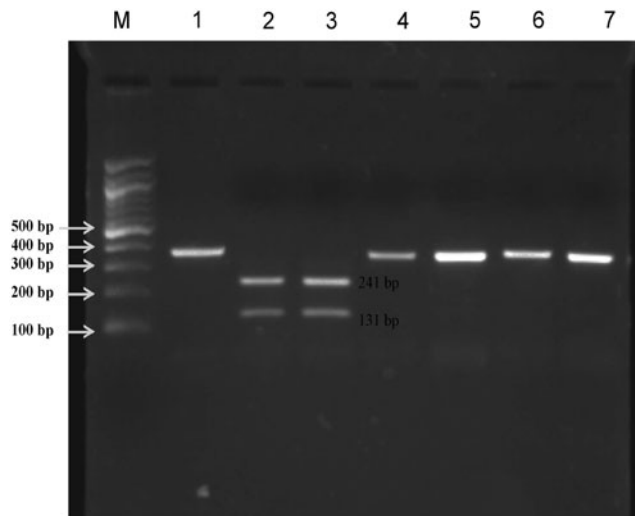
The pools containing *P. argentipes* collected from 132 houses were used to determine natural infection by *L. donovani*. This 18S rRNA gene-specific PCR study confirms that only 22 pools, containing a total of 78 sand flies, were infected with *L. donovani* (Fig. 3). According to the minimum and maximum infection rates, the prevalence of infection of *L. donovani* in female *P. argentipes* was 4.90–17.37%, with a minimum infection rate of 22, and a maximum infection rate of 78, out of a total of 449 sand flies considered to be infected.

#### Discussion

The success of vector control programs depends on the vectorial capacity and the understanding of disease transmission by the various vectors. We observed a prevalence rate of sand flies in 98% of houses, with *Sergentomyia* as the most prevalent genus, followed by *P. argentipes*. In India, other studies have also done of the distribution of sand flies, but in different endemic regions of Bihar state (Kumar et al. 2009). The prevalence of *L. donovani* infection in the female *P. argentipes* population in India has not been studied previously, although the presence of *Leishmania* DNA in *Phlebotomus* and *Sergentomyia* sand flies has previously been reported (Mukherjee et al. 1997). In this study a species-specific primer-

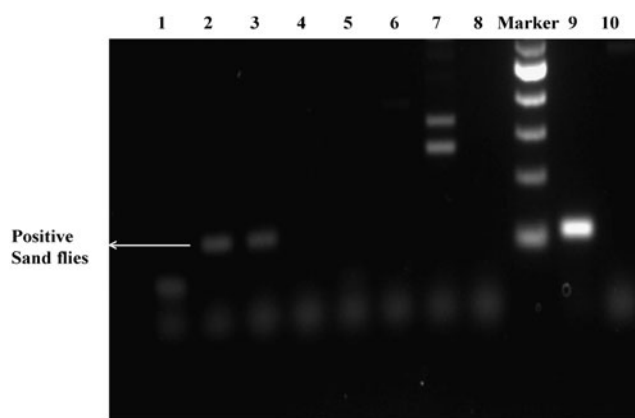
TABLE 3. VILLAGE-WIDE INFECTION RATES OF *LEISHMANIA DONOVANI* IN FEMALE *PHLEBOTOMUS ARGENTIPES*

Village	Number of pool	Number of flies	Positive pool	Number of flies in positive pool	Percentage Infection	Minimum infection rate	Maximum infection rate
S.C. Devanand	5	17	0	0	0	0	0
Veerpur East	6	9	0	0	0	0	0
Veerpur Mid	9	58	1	15	0.76	0.22	3.34
Veerpur West	2	4	0	0	0	0	0
Premi Chapra	4	44	0	0	0	0	0
Bahuara	3	3	1	1	0.76	0.22	0.22
Sadiquepur	1	1	0	0	0	0	0
Bhimalpur	4	13	0	0	0	0	0
Y. Math	1	1	0	0	0	0	0
Bishunpur TP	2	2	0	0	0	0	0
B. Mahanand	3	4	3	4	2.27	0.67	0.89
B.M. Kachahari	7	28	2	3	1.52	0.45	0.67
B.A. Bhumihar	5	13	2	9	1.52	0.45	2.00
B.A. Shekhtolla (E)	5	5	1	1	0.76	0.22	0.22
B.A. Shekhtolla (W)	4	15	0	0	0	0	0
B. Thikahan	7	28	3	6	1.51	0.45	1.34
B. Noonfar	3	4	1	1	0.76	0.22	0.22
B.G. Bishunpur	6	11	0	0	0	0	0
Jhitkahi	6	25	2	13	1.52	0.45	2.9
Madhuban	3	5	0	0	0	0	0
Gopalpur	2	5	1	2	0.76	0.22	0.45
Maharatha	11	69	2	14	1.52	0.45	3.12
Bathnaha	2	6	0	0	0	0	0
Sin. Phulkahan	7	20	1	6	0.76	0.22	1.34
Maisahan	2	2	0	0	0	0	0
Salona Bheryahi	5	18	0	0	0	0	0
G. Phulkahan	2	5	0	0	0	0	0
M. Chhapra	5	8	1	1	0.76	0.22	0.22
Panapur Kariyat	1	1	0	0	0	0	0
Godai jamal	1	1	0	0	0	0	0
Vishwanathpur	4	14	0	0	0	0	0
Raksha South	2	3	1	2	0.76	0.22	0.45
Raksha Deah	2	7	0	0	0	0	0
Total	132	449	22	78	16.67	4.90	17.37



**FIG. 2.** PCR-restriction endonuclease-digested patterns of 399-bp amplified products of the 18S rRNA gene fragment digested with Hae III (lane 1, undigested PCR product; lanes 2 and 3, Hae III restriction digestion patterns of *P. argentipes* [241-bp and 131-bp fragments]; lanes 4 and 5, *P. papatasi*; lanes 6 and 7, *Sergentomyia* species [only one band of 372 bp]; lane M, for 100-bp marker).

based diagnostic PCR was used to detect parasitic nucleic acids in sand fly (Deborggraeve et al. 2008). Another advantage of PCR is that this technique is highly specific and reproducible due to the use of specific primers for conserved regions of *P. argentipes* and *L. donovani*, whereas in microscopic dissection studies, there is the possibility of an observer mistaking for other flagellated parasites for *L. donovani* (Srivastava et al. 2010). In recent years, different methods have been developed to screen pools or clusters of insects, which can efficiently estimate the infection potential of disease spread by a vector species (Yameogo et al. 1999; Martin-Sanchez et al. 2006; Bhattarai et al. 2009; Severinsson, et al. 2010). The rates of sand fly infection varied in accordance with the transmission of the disease, as observed in other studies reported from Panama



**FIG. 3.** Detection of the *L. donovani* 18S rRNA gene in female *P. argentipes* showing amplification of 115 bp (lane 9, positive control; lane 10, negative control; lanes 1, 4, 5, 6, 7, and 8, uninfected samples; lanes 2 and 3, infected samples; lane M, 100-bp marker).

(Azpurua et al. 2010), Athens (Aransay et al. 2000), and Turkey (Svobodova et al. 2009). The prevalence of infection with *Leishmania* in the vector female *P. argentipes* may be exploited as a tool for the surveillance of these infections, and for measuring the success of control programs.

To the best of our knowledge, this is the first report of a molecular-based study in a VL-endemic region of India. Because of the lack of firm evidence and supporting literature regarding the prevalence of infection of sand flies in VL-endemic areas of India, we expected that the prevalence of infection in these sand flies would be low. Large numbers of insects were examined to obtain an accurate estimate of infection levels. The high infection rates observed in this study indicate that a similar type of study can be performed on individual sand flies, which could provide vital information about vector control strategies to help control or eliminate VL in the Indian sub-continent.

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### Author Disclosure Statement

No competing financial interests exist.

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