Development of an Enzyme-Linked Immunosorbent Assay to Identify Host-Feeding Preferences of *Phlebotomus* Species (Diptera: Psychodidae) in Endemic Foci of Visceral Leishmaniasis in Nepal

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ABSTRACT Anthroponotic visceral leishmaniasis, transmitted by *Phlebotomus argentipes* Annandale & Brunetti (Diptera: Psychodidae) sand flies, is regarded as a major problem of public health importance in the Indian subcontinent. Understanding the feeding behavior of the vector can be used to investigate changes in human-vector contact during intervention programs. An enzyme-linked immunosorbent assay (ELISA) was modified to make it suitable to identify the origin of *P. argentipes* and *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) blood meals. The sensitivity and specificity of the precipitin ring test and ELISA were compared, as well as the stability of the tests across different stages of blood meal digestion. The ELISA was more sensitive and specific than the precipitin test for identifying the sources of blood meals. When using the ELISA method with a plate reader, it was possible to obtain 100% sensitivity and specificity. When comparing the techniques across digestion stages, it was found that there was a drop in sensitivity, 48 and 72 h postblood meal for precipitin and visually read ELISA, respectively. However, the sensitivity of the ELISA using a plate reader was not altered by the digestion time. The feeding habits of *P. argentipes* and *P. papatasi* from the Terai region of Nepal, determined by the ELISA developed, showed *P. papatasi* to be highly anthropophilic, and *P. argentipes* appeared to feed both on humans and animals, in particular bovines.

KEY WORDS blood meal analysis, *Phlebotomus argentipes*, *Phlebotomus papatasi*, sand fly, *Leishmania donovani*

Visceral leishmaniasis (VL), otherwise known as kala-azar, is a neglected vector-borne disease, and is often fatal if left untreated. This disease is caused by infection with a protozoan parasite of the *Leishmania* genus, transmitted by bites of phlebotomine sand flies. The estimated annual incidence of VL is 500,000, and the prevalence is ≈2.5 million cases worldwide. The majority of reported cases are from the Indian subcontinent, and VL is regarded as a major problem of public health importance in the area. In Nepal, the disease is endemic in the southern part of the Terai region, which lies adjacent to the Indian state of Bihar (Joshi et al. 2006).

On the Indian subcontinent, VL is exclusively transmitted by the bite of female *Phlebotomus argentipes* Annandale & Brunetti (Diptera: Psychodidae) infected with *Leishmania donovani* Laveran & Mesnil (Kinetoplastida: Trypanosomatidae). Unlike the zoonotic form of VL, caused by *Leishmania infantum* Nicolle (Kinetoplastida: Trypanosomatidae), there is no known animal reservoir of *L. donovani* (Dinesh et al. 2000). *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) is an established vector of cutaneous leishmaniasis in many regions of the Old World (Killick-Kendrick 1999), but has yet to be incriminated as a vector in Nepal (Pandey et al. 2008).

On the Indian subcontinent, *P. argentipes* are seasonal (Dinesh et al. 2001), mainly endophilic (Ghosh et al. 1982), endophagic (Shrestha 1994, Dinesh et al. 2001), and zoophilic (Mukhopadhyay and Chakravarty 1987, Palit et al. 2005). However, some studies report no host preference (Ghosh et al. 1982), stating that *P. argentipes* will feed opportunistically on both humans and cattle, whichever is most available and convenient (Palit et al. 2005). *P. papatasi* are also described as seasonal and endophilic (Srinivasan et al. 1993) on the Indian subcontinent, but in contrast to *P. argentipes*, they are considered to be mainly anthropophilic (Mukhopadhyay and Chakravarty 1987).

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Defined knowledge on the preference of hosts of human pathogen vectors like *P. argentipes* can provide information on the degree of anthropophily, and can be used to evaluate changes in human-vector contact during intervention programs relevant to bed net usage or insecticide-spraying campaigns (Afonso et al. 2005). The main techniques used for sand fly blood meal identification to date are the enzyme-linked imunosorbent assay (ELISA) (Service et al. 1986, Ngumbi et al. 1992, Srinivasan and Panicker 1992, Bongiorno et al. 2003, Svobodova et al. 2003) and precipitin tests (Dhiman et al. 1984, Morrison et al. 1993, Ogusuku et al. 1994, Afonso et al. 2005). Other serological tests such as counter immunoelectrophoresis (Morsy et al. 1993) and agarose gel diffusion (Srinivasan and Panicker 1992), as well as polymerase chain reaction (Sant'Anna et al. 2008), have also been applied. Despite the wide variety of techniques, the number of blood meal studies on *Phlebotomus spp* is limited.

The methods of blood meal analysis in sand flies often derive from those used for mosquitoes. The applicability of these methods to sand flies is hindered by several features associated with their blood feeding. First, sand flies ingest considerably less blood than mosquitoes. An engorged female *P. argentipes* sand fly ingests an average of 0.2–0.3 μl per blood meal (M. R., unpublished data), whereas mosquitoes can take an average blood meal of 2–6 μl (Clements 1992). However, ELISA seems to detect as little as 0.02 μl of blood (Service et al. 1986). Second, the rapid digestion of blood by mosquitoes and sand flies can severely affect the efficacy of the blood meal test. The digestion time of the blood meal is temperature dependent, but is usually completed within 36–72 h (Dillon and Lane 1993, Secundino et al. 2005). The effective time limit in sand flies to determine the blood meal source seems to be 24–48 h post-blood meal ingestion (Gomes et al. 2001, Sant'Anna et al. 2008). Another limitation of the tests commonly used is that they do not allow for identifying multiple feeds taken on the same host (Lardeux et al. 2007), which plays an important role in the transmission of *Leishmania* parasites.

The objectives of this study were as follows: 1) to develop an ELISA to determine the blood meal source from *Phlebotomus spp;* 2) to compare its sensitivity and specificity with the precipitin test; and 3) to describe the host preferences of *P. argentipes* and *P. papatasi* in the Terai region, Nepal.

Materials and Methods

Sand Flies. To develop the ELISA test and compare its performance with the precipitin test, laboratory-reared *P. argentipes* (donated by Dr. Gordon Hamilton, Keele University) were used. Five-day-old adult sand flies were blood fed on either a human volunteer or an anesthetized hamster. Blood-fed sand flies were separated and maintained at 90% RH, 28°C in a netted cage and fed 70% sucrose ad libitum for 72 h. Blood-fed *P. argentipes* and *P. papatasi* were collected from eight villages in the VL endemic region of Terai, Nepal, by Centers for Disease Control and Prevention light trap and aspiration in households and cattle sheds from September 2006 to October 2008. All wild sand flies collected in Nepal were identified to species. The villages in which sand flies were collected are rural or periurban settlements. The majority of people live in mud houses, and approximately 60% own domestic animals, mainly goats and bovines, which are kept in the proximities of the households (S. R., unpublished data).

Sample Preparation. Blood-fed *P. papatasi* and *P. argentipes* were squashed individually onto Whatman number 1 filter paper, labeled, and stored at 4°C in sealed bags containing silica gel until analyzed. To test for blood meal origin, the samples squashed on filter paper were cut out, placed in individual Eppendorf containers, and eluted out overnight at 4°C in 800 μl of phosphate-buffered saline (PBS). The fly debris and filter paper were then removed from the tubes, and the samples were centrifuged at 9000 $\times$ *g* for 10 min. Blood from bovine, goat, dog, rat, and chicken was also spotted on Whatman number 1 filter paper and processed, as described above. These bloodspots were used as positive and negative controls for the ELISA and precipitin tests.

ELISA. One hundred microliters of the eluted sand fly blood meal samples was added to the 96-well flat-bottomed polyvinylchloride (PVC) microtiter ELISA plate (maxisorb) and incubated for 2 h at room temperature. Wells were washed three times with PBS-Tween wash buffer (PBS, pH 7.4, containing 0.05% Tween 20). The plate was blocked using 200 μl of chicken serum albumin/carbonate-bicarbonate buffer (200 mg of chicken serum albumin in 20 ml of carbonate-bicarbonate buffer), and left to incubate for 1 h at room temperature. After washing 100 μl of horseradish peroxidase-labeled anti-species immunoglobulin G (all antibodies from Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted in 1% chicken serum albumin/PBS-Tween was added to each well. Anti-human, anti-bovine, anti-dog, anti-rat, and anti-chicken antibody solutions were used at a concentration of 0.25 μg/ml, and 0.5 μg/ml for anti-goat antibodies. Twenty microliters of each heterologous serum was added to the antibody solution to cut down cross-reactivity (Service et al. 1986). Bovine, dog, and rat sera were added to the anti-human antibody solution; human and rat sera were added to the anti-bovine antibody solution; human, bovine, and dog sera were added to the anti-rat antibody solution; whereas human, bovine, and rat sera were added to the anti-goat, anti-dog, and anti-chicken antibody solutions. The plate was incubated for 1 h at room temperature. The wells were washed another three times, and the assay was developed by adding 200 μl of the substrate solution (10 mg of θ-phenylenediamine, 2% H_2O_2 in 25 ml of phosphate-citrate buffer) to each well. Results were obtained both visually by noting a color change, and with a microplate reader (Thermomax, Molecular Devices, Workingham, United Kingdom). The absorbance was read after 15 min at 450 nm. Absorbance readings from the blank PBS wells were subtracted from the test values. Final readings of $\geq$0.1 were regarded as positive. Control samples of

known origin from human, bovine, goat, dog, rat, and chicken were blindly tested.

Precipitin Ring Test. One hundred microliters of antiserum (diluted 1/10 with PBS) was transferred into a small, narrow precipitin tube (1.0-ml round-base clear polystyrene tube; Thermo Life Sciences, Basingstoke, United Kingdom); care was taken to avoid the creation of bubbles by slowly releasing the liquid against the side of the tube. Then 100 μl of the blood meal elute was carefully added to the antiserum without mixing the two solutions. After 15- to 20-min incubation at room temperature, the tube was inspected for a cloudy band of precipitate at the blood meal elute:antiserum interface, indicating a positive reaction.

Comparison of the ELISA and Precipitin Tests. Forty human blood-fed *P. argentipes,* eight hamster-fed *P. argentipes*, nine bovine bloodspots, three human bloodspots, and three blank buffer solutions were used to compare the sensitivity and specificity of the anti-human ELISA and precipitin tests. The ELISA results were read visually (63 samples) and with a plate reader (39 samples). All precipitin tests (85 samples; 63 were analyzed with human antiserum and 22 with bovine antiserum) were visually read by the same person. The sensitivity and specificity were calculated as defined by Beier and Koros (1991) and presented as percentages. To determine the effect of blood meal digestion on the sensitivity of ELISA and precipitin tests, 34 laboratory-reared, human-fed *P. argentipes* samples at different postfeeding times were analyzed using ELISA and precipitin tests. The 34 samples were distributed at different digestion stages, as follows: 10, 6, 7, 6, and 5 fed *P. argentipes* were squashed on filter paper at 1, 12, 24, 48, and 72 h postbloodfeeding, respectively. Positive controls of human bloodspots and negative controls of hamster-fed *P. argentipes* and bovine bloodspots were tested alongside. Two replicates of the ELISAs were done, but only one replicate of the precipitin test was undertaken.

Host Preference of *P. argentipes* and *P. papatasi* in the Terai. A χ^2 test was used to assess the feeding preference of *P. argentipes* and *P. papatasi* sand flies from the Terai based on the ELISA blood meal analysis.

Ethical Considerations. Ethical clearance to conduct this study was obtained from the Ethical Committee of the B.P. Koirala Institute of Health Sciences (Dharan, Nepal) and the corresponding bodies at the Institute of Tropical Medicine (Antwerp, Belgium) and the London School of Hygiene and Tropical Medicine (London, United Kingdom). Informed consent was obtained from the head of the households where sand flies were collected.

Results

Comparison of ELISA and Precipitin Test. Both the precipitin test and the ELISA had 100% specificity. However, the sensitivity varied from 100 to 97.7% for the anti-human ELISA, when results were read with a plate reader or visually, respectively, and was 94.1% for the precipitin test.

Table 1. Sensitivity of the anti-human precipitin and ELISA tests to identify the blood meal of laboratory-reared *P. argentipes* at different times postfeeding on a human host

Test	% time after feeding (hr)				
	1 (10)	12 (6)	24 (7)	48 (6)	72 (5)
Precipitin test	100	100	100	80	60
ELISA: visual	100	100	100	100	80
ELISA: plate reader	100	100	100	100	100

In parentheses are the number of blood meals tested.

As shown in Table 1, the sensitivity of the ELISA using a plate reader was not altered by the digestion time. However, there was a drop in sensitivity 48 and 72 h postblood meal for precipitin and visually read ELISA, respectively.

Host Preference of *P. argentipes* and *P. papatasi* in the Terai. A total of 147 *P. argentipes* was analyzed. As shown in Fig. 1, 69.0% fed on human blood, 17.0% on bovine, 2.0% on dog, 0.3% on chicken, and 11.6% were unidentified. Blood meals were counted as 0.5 when flies contained blood of two different hosts. *P. argentipes* did not have an equal preference for each host species ($P < 0.05$). The site of collection was recorded for 72 of 147 sand flies captured: 68 were collected in houses and four were collected in cattle sheds. In the house collections, 79.4% fed exclusively on human, 4.4% on dog, and 14.7% were unidentified. One (1.5%) human/chicken mixed feed was found, accounting for the only source of chicken blood in this group of *P. argentipes.* Of the four sand flies collected in cattle sheds, three fed on humans, but the origin of the blood in one of them could not be identified.

Eighty-eight *P. papatasi* were analyzed. Overall, 84.1% fed on human blood, 1.1% on dog, 0.6% on bovine, 0.6% on chicken, and 13.6% were unidentified (Fig. 1). Two mixed blood meals were discovered: one human/bovine mix and one human/chicken mix. These two mixed feeds were the only samples with either bovine or chicken blood meals. Like *P. argentipes, P. papatasi* did not have an equal preference for each host species ($P < 0.05$), and no blood meals were taken from goats or rats by either species.

In total, the ELISA protocol enabled the identification of 87.7% of blood meal sources of sand flies collected from the Terai region, Nepal.

Discussion

The modified ELISA protocol described had 100% specificity and sensitivity, when laboratory samples were analyzed, gave consistently better results than the precipitin test, and was able to identify 87.7% of the samples collected in the field. The percentage of unidentified blood meals was similar to that reported by previous studies using either phlebotomine sand flies (Bongiorno et al. 2003) or anopheline mosquitoes (Lardeux et al. 2007). The inability to identify blood meals could be the result of the following: 1) the sand

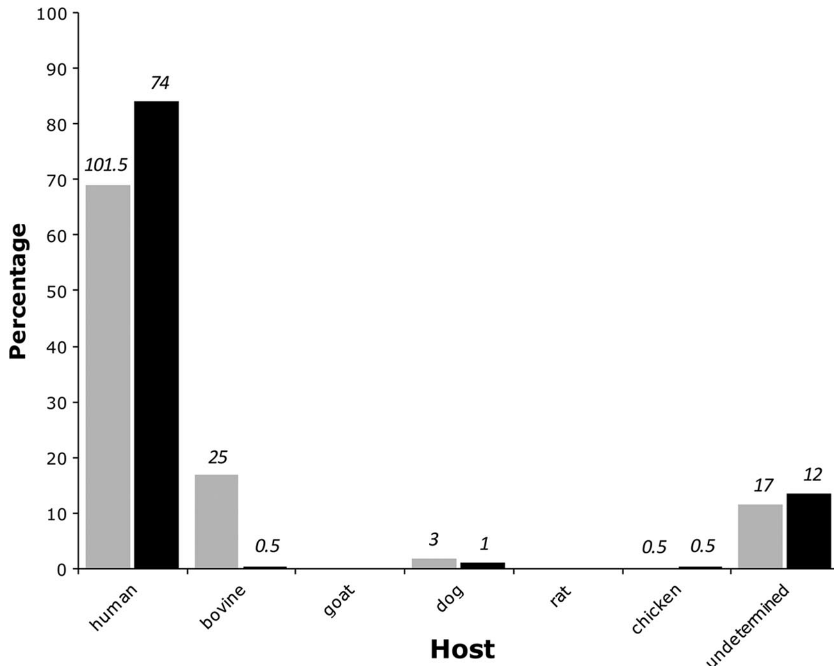

Fig. 1. Host-feeding preference of *P. argentipes* (▨) and *P. papatasi* (■) in the Terai region, Nepal. The histogram presents the percentage of samples per *Phlebotomus* species and host. The total number of samples per host is noted on the top of the bars (mixed blood meals were counted as 0.5 for each of the two hosts involved).

fly either being unfed or partially fed; 2) the blood meal source being of untested host origin; and/or 3) the blood meal being fully digested (Blackwell et al. 1994). However, the ELISA was able to identify blood meals up to 72 h after feeding in laboratory-reared sand flies, unlike previous protocols (Gomes et al. 2001, Sant'Anna et al. 2008), and was used successfully to determine the host preference of wild *Phlebotomus* (for which the time of digestion was unknown).

The number of studies investigating blood meal sources of *Phlebotomus spp* is limited, especially in Nepal. Data from our study report that in VL foci in the Terai, *P. argentipes* is somewhat anthropophilic, but will still take a significant proportion of blood meals from bovines (17%). In a previous study, *P. argentipes* from Nepal had a higher preference for bovines (59%) (Lawyer 1992). The fact that most of the sand flies analyzed in our study were collected inside houses may explain the difference observed, as the site of collection may bias the catch. Interestingly, 75% of the *P. argentipes* collected in cattle sheds had fed on humans. This figure is based on a small number of sand flies ($n = 4$) and needs to be interpreted carefully. Similar studies in India have also reported that a significant percentage (15–20%) of blood-fed sand flies collected in cattle sheds neighboring houses had fed on humans (Ghosh et al. 1990, Basak et al. 1995). This suggests some movement of sand flies after feeding, demonstrating that one cannot assume that blood-fed flies collected inside a house necessarily came from hosts inside the same house. This finding would also favor the use of indoor residual spraying covering cattle sheds as well as households to have an impact on *L. donovani* vectors.

P. papatasi was shown to be highly anthropophilic in the Terai, and thus may be a potential vector of confidence limits in the area. In many regions of the Old World, where *P. papatasi* is the dominant species of sand fly, the close association between humans and the vector has produced an effective cycle of transmission of confidence limits. Therefore, further incrimination studies and monitoring of disease cases in Nepal are recommended.

In conclusion, the ELISA protocol described is a significant improvement on existing protocols. It may be used to identify blood meal sources of wild sand flies, thus permitting a vast range of further studies aiming either to incriminate sand flies, or to investigate human-sand fly contact after intervention programs.

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