Development of an enzyme-linked immunosorbent assay (ELISA) to identify host feeding preferences of *Phlebotomus* species (Diptera: Psychodidae) in endemic foci of visceral leishmaniasis in Nepal.

Burniston Ian*[1], Roy Lalita*[2], Picado Albert[1], Das Murari[2], Rijal Suman[2], Rogers Matthew[3], Coosemans Marc[4], Boelaert Marleen[4], Davies Clive[1], Cameron Mary[1]

[1] London School of Hygiene and Tropical Medicine, London, UK

[2] B.P. Koirala Institute of Health Sciences, Dharan, Nepal

[3] Imperial College of Science, Technology and Medicine, London, UK

[4] Institute of Tropical Medicine, Antwerp, Belgium

* Both authors contributed equally to this work

Word count abstract: 213

Word count text: 2486

Corresponding author

Mary Cameron (mary.cameron@lshtm.ac.uk)

Infectious and Tropical Diseases Department, London School of Hygiene and tropical Medicine, Keppel Street, London WC1E 7HT, UK

Tel: +44 (0)20 7927 2337 Fax: +44 (0)20 7927 2918

ABSTRACT

Anthroponotic visceral leishmaniasisis, 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 hours post-blood 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: ELISA, blood meal analysis, *Phlebotomus argentipes*, *Phlebotomus papatasi*, host preference, sandfly, *Leishmania donovani*.

INTRODUCTION

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 approximately 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) an established vector of cutaneous leishmaniasis (CL) 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). Defined knowledge on the preference of hosts of human pathogen vectors like *P. argentipes* can provide information on the degree of anthropophily, and 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. Firstly, sand flies ingest considerably less blood than mosquitoes. An engorged female *P. argentipes* sand fly ingests an average of 0.2 to 0.3µl per blood meal (Rogers, personal communication), whereas mosquitoes can take an average blood meal of 2-6µl (Clements 1992). However, ELISA seems to detect as little as 0.02µl of blood (Service et al. 1986). Secondly 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 dependant but is usually completed

93 within 36-72 hours (Dillon and Lane 1993, Secundino et al. 2005). The effective time
94 limit in sand flies to determine the blood meal source seems to be 24-48 hours post blood
95 meal ingestion (Gomes et al. 2001, Sant'Anna et al. 2008). Another limitation of the tests
96 commonly used is that they do not allow for identifying multiple feeds taken on the same
97 host (Lardeux et al. 2007), which plays an important role in the transmission of
98 *Leishmania* parasites.
99 The objectives of this study were (i) to develop an ELISA to determine the blood meal
100 source from *Phlebotomus spp*, (ii) compare its sensitivity and specificity with the
101 precipitin test, and (iii) describe the host preferences of *P. argentipes* and *P. papatasi* in
102 the Terai region, Nepal.
103
104 **MATERIALS AND METHODS**
105 **Sand flies**
106 To develop the ELISA test and compare its performance to the precipitin test, laboratory
107 reared *P. argentipes* (donated by Dr Gordon Hamilton, Keele University) were used.
108 Five-day-old adult sand flies were bloodfed on either a human volunteer or an
109 anaesthetized hamster. Bloodfed sand flies were separated and maintained at 90% relative
110 humidity, 28^{o}C in a netted cage and fed 70% sucrose *ad libitum* for 72 hours. Bloodfed *P.*
111 *argentipes* and *P. papatasi* were collected from 8 villages in the VL endemic region of
112 Terai, Nepal by CDC light trap and aspiration in households and cattle sheds from
113 September 2006 to October 2008. All wild sand flies collected in Nepal were identified to
114 species. The villages where sand flies were collected are rural or peri-urban settlements.
115 The majority of people live in mud houses and around 60% own domestic animals,

mainly goats and bovines, which are kept in the proximities of the households (Rijal, personal communication)

Sample preparation

Bloodfed *P. papatasi* and *P. argentipes* were squashed individually onto Whatman #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^{o}C in 800µl of phosphate buffered saline (PBS). The fly debris and filter paper were then removed from the tubes and the samples were centrifuged at 9000 rpm for 10 minutes. Blood from bovine, goat, dog, rat and chicken were also spotted on Whatman N°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 were added to the 96-well flat-bottomed PVC microtiter ELISA plate (maxisorb) and incubated for 2 hours 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 chicken serum albumin/carbonate-bicarbonate buffer (200mg chicken serum albumin (CSA) in 20 ml carbonate-bicarbonate buffer), and left to incubate for 1 hour at room temperature. Following washing 100 µl of HRP-labeled anti-species IgG (all antibodies from KPL) diluted in 1% CSA/PBS-Tween was added to each well. Anti-human, bovine, dog, rat, and 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 were added to

the antibody solution in order 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; while human, bovine, and rat sera were added to the anti-goat, dog, and chicken antibody solutions. The plate was incubated for one hour 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, UK). The absorbance was read after 15 minutes at 450 nm. Absorbance readings from the blank PBS wells were subtracted from the test values. Final readings of ≥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, UK); 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 15-20 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. In order 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 post-feeding times were analyzed using ELISA and precipitin tests. The 34 samples were distributed at different digestion stages: 10, 6, 7, 6 and 5 fed *P. argentipes* were squashed on filter paper at 1, 12, 24, 48 and 72 hours post-bloodfeeding 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 Chi-square 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, UK. 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.

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 hours post-blood meal for precipitin and visually read ELISA, respectively.

Host preference of *P. argentipes* and *P. papatasi* in the Terai

One hundred and forty seven *P. argentipes* were analyzed. As shown in Figure 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. *Phlebotomus 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 4 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.

205 Eighty-eight *P. papatasi* were analyzed. Overall, 84.1% fed on human blood, 1.1% on
206 dog, 0.6% on bovine, 0.6% on chicken, and 13.6% were unidentified (Figure 1). Two
207 mixed blood meals were discovered; one human/bovine mix and one human/chicken mix.
208 These two mixed feeds were the only samples with either bovine or chicken blood meals.
209 Like *P. argentipes, P. papatasi* did not have an equal preference for each host species
210 ($P<0.05$), and no blood meals were taken from goats or rats by either species.
211 In total, the ELISA protocol enabled the identification of 87.7% of blood meal sources of
212 sand flies collected from the Terai region, Nepal.
213

214 **DISCUSSION**

215 The modified ELISA protocol described had 100% specificity and sensitivity, when
216 laboratory samples were analyzed, gave consistently better results than the precipitin test,
217 and was able to identify 87.7% of the samples collected in the field. The percentage of
218 unidentified blood meals was similar to that reported by previous studies using either
219 phlebotomine sand flies (Bongiorno et al. 2003) or anopheline mosquitoes (Lardeux et al.
220 2007). The inability to identify blood meals could be due to: (1) the sand fly either being
221 unfed, or partially fed, (2) the blood meal source being of untested host origin, and/or (3)
222 the blood meal being fully digested (Blackwell et al. 1994). However, the ELISA was
223 able to identify blood meals up to 72 hours after feeding in laboratory reared sand flies
224 unlike previous protocols (Gomes et al. 2001, Sant'Anna et al. 2008), and was used
225 successfully to determine the host preference of wild *Phlebotomus* (for which the time of
226 digestion was unknown).

227 The number of studies investigating blood meal sources of *Phlebotomus spp* is limited,
228 especially in Nepal. Data from our study reports that in VL foci in the Terai, *P.*
229 *argentipes* is somewhat anthropophilic, but will still take a significant proportion of
230 blood meals from bovines (17%). In a previous study, *P. argentipes* from Nepal had a
231 higher preference for bovines (59%) (Lawyer 1992). The fact that most of the sand flies
232 analyzed in our study were collected inside houses may explain the difference observed,
233 as the site of collection may bias the catch. Interestingly, 75% of the *P. argentipes*
234 collected in cattle sheds had fed on humans. This figure is based on a small number of
235 sand flies (n=4) and needs to be interpreted carefully. Similar studies in India have also
236 reported that a significant percentage (15-20%) of bloodfed sand flies collected in cattle
237 sheds neighboring houses had fed on humans (Ghosh et al. 1990, Basak et al. 1995). This
238 suggests some movement of sand flies after feeding, so demonstrating that one cannot
239 assume that bloodfed flies collected inside a house necessarily came from hosts inside the
240 same house. This finding would also favor the use of IRS covering cattle sheds as well as
241 households to have an impact on *L. donovani* vectors.
242 *Phlebotomus papatasi* was shown to be highly anthropophilic in the Terai, and thus may
243 be a potential vector of CL in the area. In many regions of the Old World, where *P.*
244 *papatasi* is the dominant species of sand fly, the close association between humans and
245 the vector has produced an effective cycle of transmission of CL. Therefore, further
246 incrimination studies, and monitoring of disease cases in Nepal is recommended.
247 In conclusion, the ELISA protocol described is a significant improvement on existing
248 protocols. It may be used to identify blood meal sources of wild sand flies, thus
249 permitting a vast range of further studies aiming either to incriminate sand flies, or to

investigate human-sand fly contact following intervention programs.

ACKNOWLEGMENTS

The authors would like to thank Shahida Begum from LSHTM (UK), Gordon Hamilton from Keele University (UK), James Cook and Emma Weeks from Rothamsted Research (UK), and Anna Alba from CReSA (Spain) for their assistance in developing the ELISA. The work was financially supported by EU-funded INCO-DEV KALANET project (EU contract no 015374).

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TABLE AND FIGURE CAPTIONS

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

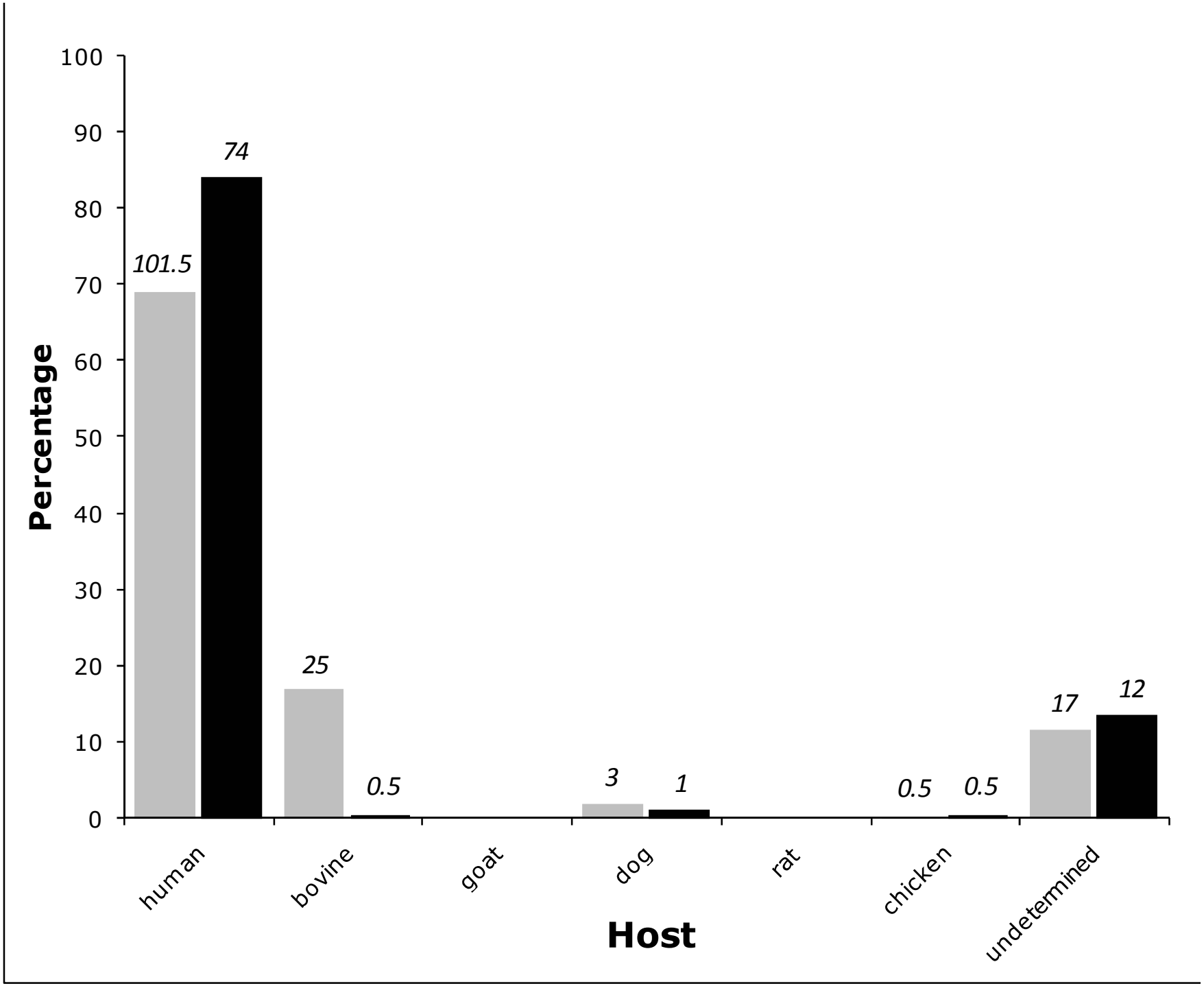
Figure 1. Host feeding preference of *P. argentipes* (grey) and *P. papatasi* (black) in the Terai region, Nepal. The histogram presents the percentage of samples per *Phlebotomus* species and host. The total number of samples per host is noted on the top of the bars (Mixed blood meals were counted as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

TABLE

Table 1

	Time after feeding (in hours)				
	1	12	24	48	72
Test	(10)	(6)	(7)	(6)	(5)
Precipitin test	100%	100%	100%	80%	60%
ELISA - visual	100%	100%	100%	100%	80%
ELISA - plate reader	100%	100%	100%	100%	100%

In brackets the number of blood meals tested.


100
90
80
70
60
50
40
30
20
10
0
Percentage
101.5
74
25
0.5
3
1
0.5
0.5
17
12
human
bovine
goat
dog
rat
chicken
undetermined
Host