Identification of Bloodmeals in Wild Caught Blood Fed *Phlebotomus argentipes* (Diptera: Psychodidae) Using Cytochrome *b* PCR and Reverse Line Blotting in Bihar, India

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ABSTRACT Identification of the source of bloodmeals in vectors plays an important role in epidemiological studies by determining the host preferences of wild sand flies in natural habitat. The anthropophilic index is a crucial component in human leishmaniasis. Bloodmeal analysis can identify the reservoir hosts of vector borne diseases. The amplification of the mitochondrial cytochrome *b* gene, followed by reverse line blot analysis, helps to identify the bloodmeal ingested by the wild caught sand flies. In the current study, blood fed sand flies were collected from three different villages in Bihar, India, by using Centers for Disease Control mini traps with incandescent light. Traps were placed in five different sites in the villages. Whole genome DNA was extracted from the blood fed sand flies and was amplified for the cytochrome *b* region, followed by reverse line blot analysis. In total, 442 blood fed sand flies were analyzed out of which 288 (65%) were positive to cytochrome *b* polymerase chain reaction. Humans, cattle, buffalo, and goats were the major bloodmeals identified, followed by chickens. In some of the blood fed sand flies, multiple bloodmeals were identified. In the current study, sand flies mostly fed on humans, followed by cattle, buffalo, and goats. In this regard, it is necessary to also consider cattle, buffalo, and goats when addressing vector control in Bihar, India.

KEY WORDS bloodmeal identification, visceral leishmaniasis, sand fly, host, cytochrome *b* PCR

A clear understanding of the host preferences and their vectorial capacity for disease agents plays an important role in developing efficient control strategies for vector borne diseases. Phlebotomine sand flies are the vectors for cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). In India, *Phlebotomus argentipes* (Annandale & Brunetti) is the only proven vector for visceral leishmaniasis in reference. This form of leishmaniasis is the most severe and can be fatal if untreated (Alvar 2006). Visceral leishmaniasis occurs mostly in rural areas with poor sanitary conditions, malnutrition and other factors associated with poverty (Thakur 2000). Characterization of the feeding habits of sand flies is crucial for identifying the natural transmission cycles of leishmaniasis and for developing efficacious control strategies in vector control programs (Killick-Kendrick 1990, 1999).

Visceral leishmaniasis, also known as kala-azar, is caused by a protozoan *Leishmania donovani,* which is transmitted by *P. argentipes* in India. Epidemics of VL were reported in India, Bangladesh, parts of Sudan and Latin America (Khlabus 2007). In India, VL is largely confined to the eastern part of the country and occurs mainly in the state of Bihar and to some extent in Uttar Pradesh and West Bengal.

Female sand flies require blood to supply protein for maturation of their eggs. Transmission of VL occurs when a sand fly receives the parasite by feeding on an infected host during one bloodmeal and transmits the parasites during a subsequent one.

Many serological methods have been used to identify the blood sources of blood feeding arthropods (Washino and Tempelis 1983). The history of bloodmeal analysis in blood feeding in sand flies began with precipitin test and hemagglutination inhibition assays (Ogusuku et al. 1994) that were later replaced by enzyme linked immunosorbent assay (ELISA) (Gomez et al. 1998, 2001; Svobodova et al. 2003). In Maharashtra, India *P. argentipes* bloodmeals were identified using precipitin ring test and counter-current immune-electrophoresis (Dhanda and Gill 1982). In North Bihar, bloodmeal preference was established by using a modified ouchterlony gel diffusion technique (Mukhopadhyay and Chakravarty 1987) and in Pondicherry bloodmeals of wild caught sand flies were identified using agarose gel diffusion method (Srinivasan and Panicker 1992). In West Bengal, *P. argentipes* host preferences were investigated by testing their bloodmeals by gel diffusion technique (Ghosh et al. 1990). Palit et al. (2005) found it was more common to find

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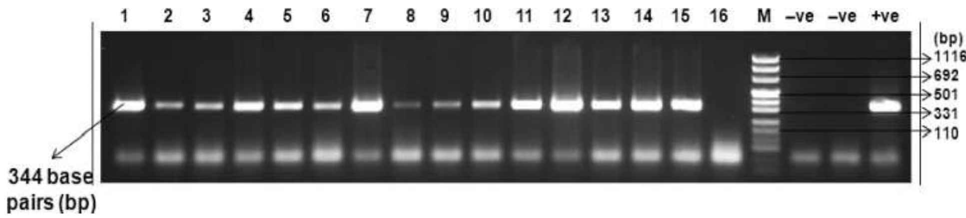

Fig. 1. Gel image of cyt *b* PCR targeting DNA extracted from wild caught blood fed sand flies. Lanes 1 to 16 PCR product of blood fed sand fly amplifed for cyt *b* region. M is DNA ladder. –ve, negative control (pure water). +ve, positive control (cow).

multiple bloodmeals in *P. argentipes* than in *P. papatasi* Scopoli.

Serological approaches are limited by the need for relatively fresh blood and the availability of species specific antibodies against each potential animal host (Blackwell et al. 1994). These shortcomings were largely solved by the introduction of polymerase chain reaction (PCR). PCR based molecular approaches have made bloodmeal identification easier and considerably improved the sensitivity of the tests (Mukabana et al. 2002).

Different methods such as amplification of the nuclear or mitochondrial DNA followed by restriction digestion for species identification, terminal restriction length polymorphisms, heteroduplex mobility assays, and sequencing were used in the bloodmeal analyses. However, such methods require relatively large amounts of template DNA and they cannot identify multiple bloodmeals in a single insect. Recently, bloodmeal identification based on amplification of the mitochondrial cytochrome *b* (cyt *b*) gene followed by reverse line blot (RLB) analysis was used to identify bloodmeals in *Phlebotomine* sand flies (Abbasi et al. 2008). Using this method bloodmeals were identified up to 96 h after ingestion and multiple bloodmeals were identified from single insects with minimal amounts of DNA. There are no recent studies involving identification of bloodmeal sources of wild caught sand flies in Bihar, India. Bloodmeal source identification in wild caught sand flies plays an important role in vector control. Here we report the identification of bloodmeals from wild caught sand flies from the Saran District of Bihar, India, using cyt *b* PCR and RLB.

Materials and Methods

Collection of Bloodfed Sand Flies. Blood fed sand flies were collected from trapping conducted in Bihar, India, by Poché et al. 2011. The collection of blood fed sand flies continued until 1 December 2010 for the current study. Three villages Rasulpur, Mohammadpur, and Mahesia, located in the Saran District northwest of the capital city, Patna (Poché et al. 2011), were selected for the study. The weather is hot and humid in summer (35–40°C) followed by cool and humid in winter (4–10°C). In each village, 21 Centers for Disease Control (CDC) mini light traps with incandescent lights (Bioquip Products, Rancho Dominguez, CA) were used to collect sand flies. All the traps were randomly placed: five traps in houses, five in cattle sheds, five in combined dwellings (structures shared by humans and life stock), five in vegetation (banana, bamboo, papaya, citrus, palm, neem, etc.), and one in a poultry house (Poché et al. 2011). All the traps were labeled with a unique identification number and the GPS coordinates were taken. Traps were set every Wednesday from 2 December 2009 through 1 December 2010. All the traps were activated for 12 h from 18:00 until 06:00 the following morning. *P. argentipes* were identified based on keys provided by the U.S Department of Defense, Walter Reed Army Institute of Research, and Hebrew University sand fly experts (Poché et al. 2011). After identification, the blood fed sand flies were separated and placed individually in dry 1.5 ml centrifuge tubes and stored in a −20°C freezer until further analysis. The sand flies' heads were separated from the bodies and the abdomens and thoraxes were used for DNA extraction. Heads were mounted on microscope slides to facilitate identification.

DNA Extraction and Amplification. DNA from thorax and abdomen was extracted using a whole tissue DNA extraction kit from Epicenter Biotechnologies (Epicenter, Madison, WI). The DNA was eluted in 70 μl of TE buffer and stored in a −80°C freezer until further processing. A 344 bp sequence (Fig. 1) of the conserved region of the mitochondria cyt *b* gene was amplified using bio-tinilated universal primers designed by Abbasi et al. (2008). The cyt *b* region was amplified in a total reaction of 50 μl consisting of 25 μl Hot start taq Master mix (1.5 mM $MgCl_2$, 200 μM each dNTP and 75 nM KCl, 10 MM Tris HCl pH 8.8) (Qiagen, Valencia CA) and 0.5 μM of each primer and 5 μl of genomic DNA. The thermo cyclic conditions consisted of 95°C for 15 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by elongation step at 72°C for 10 min. Cow blood is used as positive control and sterile water as negative control. The amplified PCR products were used as probes in RLB hybridization reactions followed by chromogenic detection. We followed the methods of Abbasi et al. (2008) for immobilization, hybridization, and detection.

Species-Specific Probes, Immobilization, Hybridization, and Detection. The villages of Bihar, where the current research was performed, consist of various animals including: cattle, buffalo, goats, dogs, chickens, and rats. Village residents mainly use cattle for

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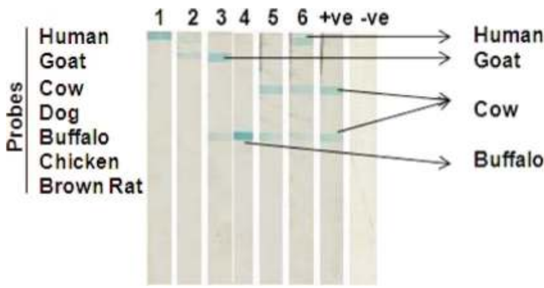

Fig. 2. Reverse line blot results of cyt *b* PCR products from field caught blood fed sand flies. Sample number 1 shows the presence of human blood. Sample 2 shows the presence of both human and goat. Sample 3 shows the presence of both goat and buffalo. Sample 4 shows the presence of buffalo. Sample 5 shows the presence of cow blood. Sample 6 shows the presence of both human and cow. +ve sample is cow blood. –ve sample with no PCR product.

milk and agricultural purposes. Goats and chickens are mainly used as a source of meat. Rats are common house hold and agricultural pests. The dogs seen in these villages are mostly feral while some are domestic. In this regard, specific 5′-amino linked oligonucleotide probes for human, cow, buffalo (Gazelle probe matches, when sequenced there is 99.9% percent match with buffalo cyt *b* sequence), goat, canid species (matches for dog), brown rat, and a general avian probe two (matches for chicken) developed by Abbasi et al. (2008) were used in the current study. The above probes were covalently linked to a nylon membrane through the formation of amide bonds between the carboxyl groups on the nylon membrane and amino groups linked to the oligonucleotides using a manifold blotter apparatus (Immunetics, Cambridge, MA). The nylon membrane sheets with the above mentioned probes were cut at a right angle to the direction of the blot so that each strip contained a section of each probe. Hybridization of the denatured biotinylated PCR products took place at 46°C for 1 hr. The procedure for immobilization, hybridization, and chromogenic detection using Streptavidin-horse radish peroxidase (Jackson Immuno Research Laboratories Inc., PA) and 3,3′,5,5′- Tetramethylbenzidine were done according to Abbasi et al. (2008) (Fig. 2).

Results

In total, 442 blood fed sand flies were trapped from March 2010 to November 2010. Sand fly numbers were very low in December, January, and February and there were no blood fed sand flies trapped. Out of 442 blood fed sand flies, 288 (65%) were positive to cyt *b* PCR and were used for bloodmeal identification using RLB. All the bloodmeals of samples that were positive to PCR were identified using RLB. Human, cow, buffalo, goat, and chicken were the individual bloodmeals identified by cyt *b* PCR and RLB. In the current study we did not identify dog or rat bloodmeals in sand flies. Multiple bloodmeals from a single blood fed sand fly were also identified using the above method. In the current study, multiple bloodmeals such as human-cow, human-buffalo, human-goat, cow-goat, buffalo-goat, and human-chicken were identified through the bloodmeal analysis (Fig. 2). A cow sample has two bands in the reverse line blot consisting of cow and buffalo, where as a buffalo sample has only one buffalo band. When the cow sample was sequenced it matched 99.9% with the cyt *b* sequence of cow and buffalo. The whole blood of cow which is taken as positive control also has two bands of cow and buffalo. Because of this limitation of the cow and buffalo probes, we were unable to determine multiple bloodmeals that may have contained both cow and buffalo bloodmeals.

Out of 288 blood fed sand flies positive to PCR, the results after immobilization, hybridization and chromogenic detection revealed the following number of individual bloodmeals: 119 (41%) were human, 60 (20%) were cattle (26 cow and 34 buffalo), 8 (2%) were goat, and 1 (0.3%) was chicken. Multiple bloodmeals were also identified that include: 51 (17%) human-cow, 29 (10%) human-buffalo, 15 (5%) human-Goat, 3 (1%) buffalo-goat, and 1 (0.3%) each of human-chicken and cow-goat (Fig. 3).

In the months of June, August, September, October, and November, humans were the dominant hosts. In cattle sheds, the dominant hosts were human and cattle, which were nearly equal in number. In combined dwellings and houses, sand flies preferably fed on humans. In vegetation, the dominant bloodmeal identified was human, followed by cattle (Figs. 4 and 5).

The number of blood fed sand flies varied from March to November. The highest numbers of blood fed flies were captured in June with 63 being trapped (21%) followed by 47 in August (16%), 38 in September (13%), and 32 in October (11%) (Fig. 6). The highest numbers of source identified blood fed flies (90) were trapped, in combined dwellings (31%), followed by the 74 in vegetation (26%), 70 in cattle sheds (24%), 43 in houses (15%), and 11 in poultry (4%) from March to November in 2010 (Fig. 7).

The PCR products in 15 of the samples identified through RLB were also sequenced to check the credibility of cyt *b* PCR and RLB analysis. The results of sequencing also matched with the RLB results. The sequences obtained matched 99.9% with source identified cyt *b* sequences. Hence, RLB is the effective method and helps in reducing the cost of sequencing of each and every blood fed sand fly. Cyt *b* PCR followed by RLB requires less time than sequencing and is easy and effective way to identify the bloodmeal source from blood fed sand flies.

Discussion

Blood fed sand flies were not trapped during December 2009, January, February, and December 2010 because of cold temperatures (Poché et al. 2011). By using cyt *b* PCR and reverse line blot, bloodmeal source can also be identified from the blood fed sand flies having minute quantities of DNA (>0.1 pg). The

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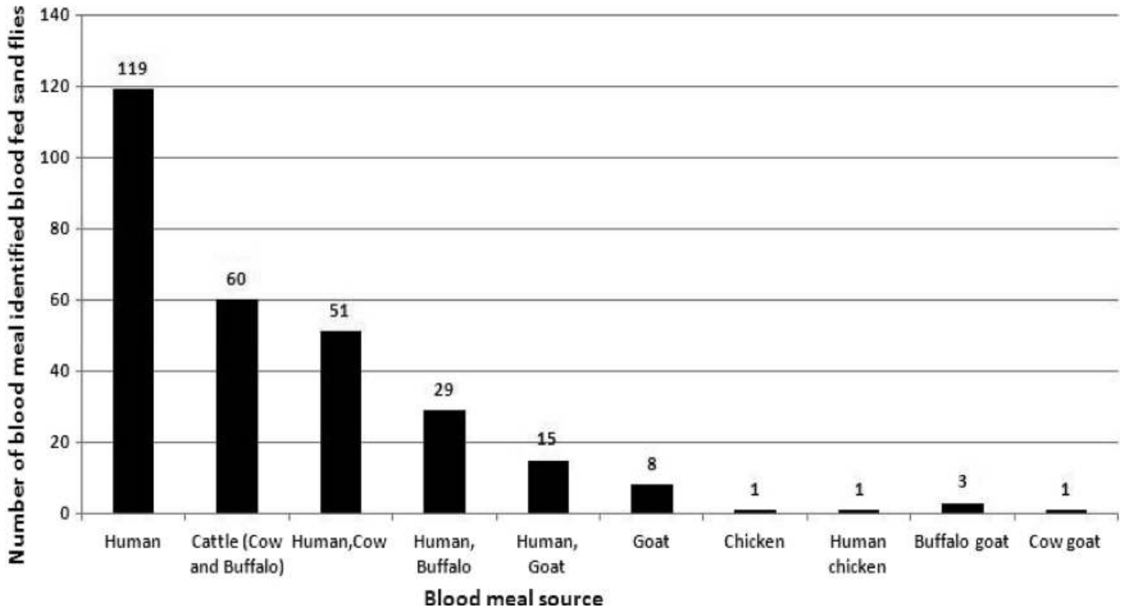

Fig. 3. Types of bloodmeal sources identified in 288 blood fed sand flies through cyt *b* PCR and reverse line blotting in three villages of Bihar, India.

cyt *b* PCR followed by RLB analysis is a rapid and sensitive technique to identify bloodmeals of wild caught sand flies. This technique can easily differentiate between two bloodmeal sources in a single insect (Abbasi et al. 2008).

The current study is part of a vector control project in Bihar, India. The current study clearly demonstrates that *P. argentipes* in nature is anthropophilic, feeding on humans. Cattle and goats also provide a significant source of blood for *P. argentipes* in peridomestic village habitats. From the current study, multiple bloodmeals from a single sand fly were also prevalent. Sand flies are also capable of taking bloodmeals from two or more people. The tendency for feeding on more than one human has important implications for disease transmission. Further investigations should be performed to identify multiple persons in a single bloodmeal.

Bloodmeals of 725 *P. argentipes* collected from six districts of North Bihar found that nearly 68% of *P. argentipes* preferentially fed on bovine blood (Mukhopadhyay and Chakravarthy 1987). In West Bengal, India, in two kala-azar endemic districts, it appeared that host preference of *P. argentipes* biotopes varied widely and were mainly zoophilic. (Palit et al. 2005).

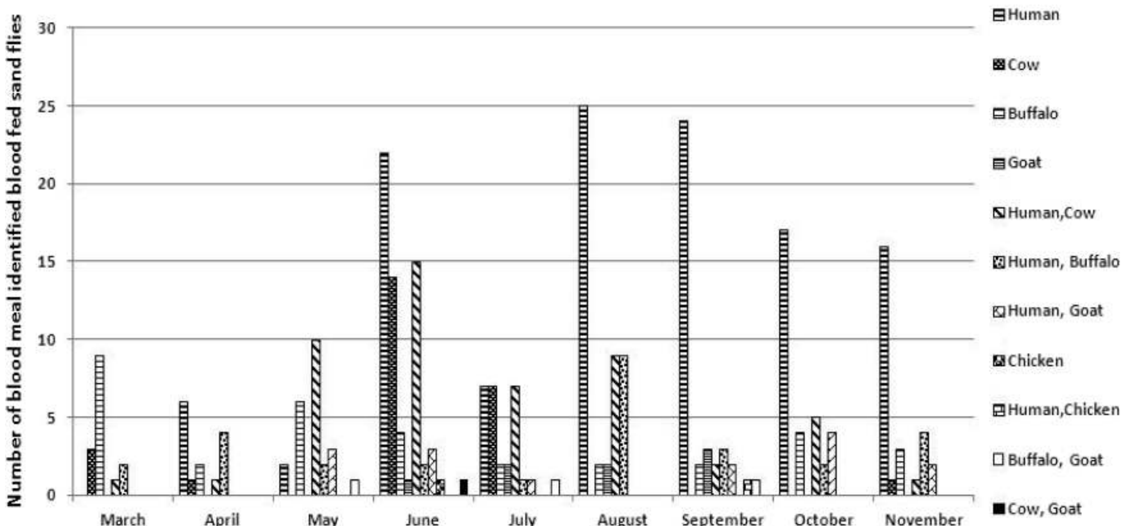

Fig. 4. Bloodmeal source variation in blood fed sand flies captured in different months of a year in three villages of Bihar.

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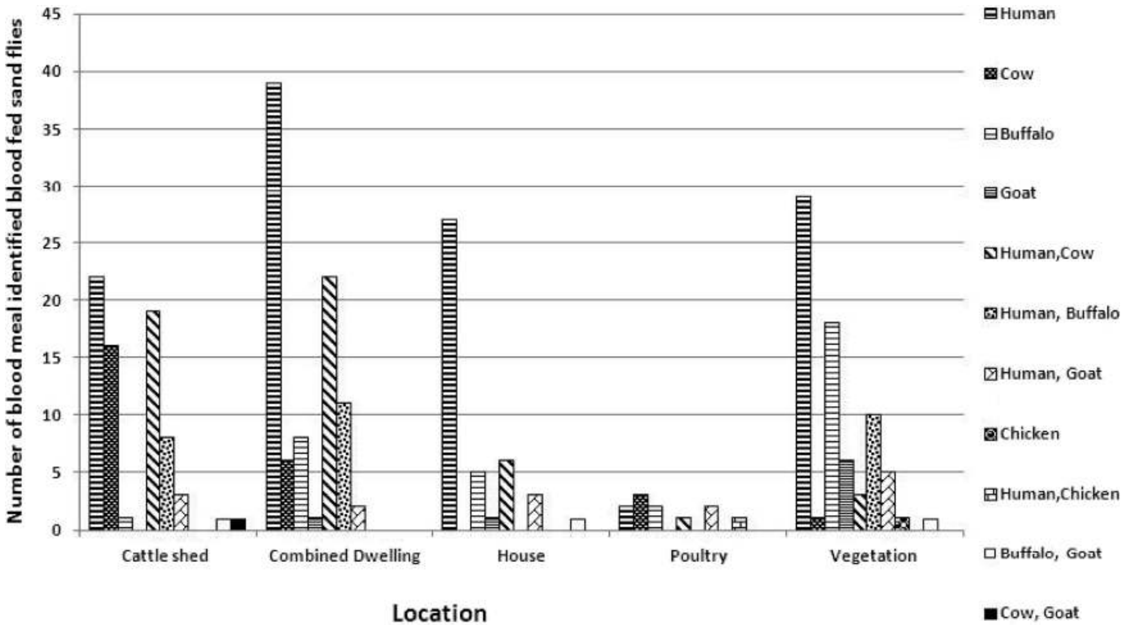

Fig. 5. Bloodmeal source variation in blood fed sand flies captured in different trap locations in three villages of Bihar.

Identification of host preferences is important in the control and management of haematophagous arthropods. The *P. argentipes* species is the only known vector for VL on the Indian sub continent. It causes great damage by transmitting the parasite to humans. In the current study, blood fed sand flies were collected weekly for a year. Nearly 57% ($n = 228$) of the blood fed sand flies came in contact with the cattle and goats. In villages in Bihar, goats are kept as a source of meat and in many of the villages the goat population exceeds that of cattle. In this regard, cattle and goats play an important role in the control of *P. argentipes.* Treating cattle and goats with systemic insecticides helps in the control of blood sucking insects like sand flies. When sand flies ingest the blood from cattle, they will ingest the blood with insecticide and adult sand flies die before oviposition, because of the insecticidal action (Poché et al. 2011).

In total, 442 blood fed sand flies were collected in the study, only 65% (288) of the total blood fed flies were positive to the PCR reaction. The insects that were negative to PCR may have had dried blood

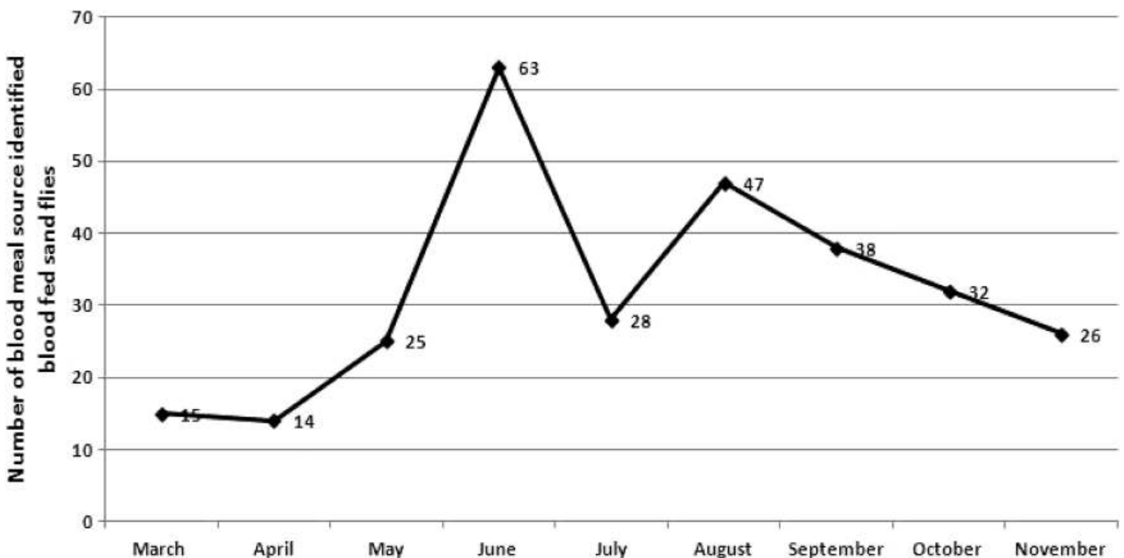

Fig. 6. Monthly variation of bloodmeal source identified sand flies captured during March to November 2010.

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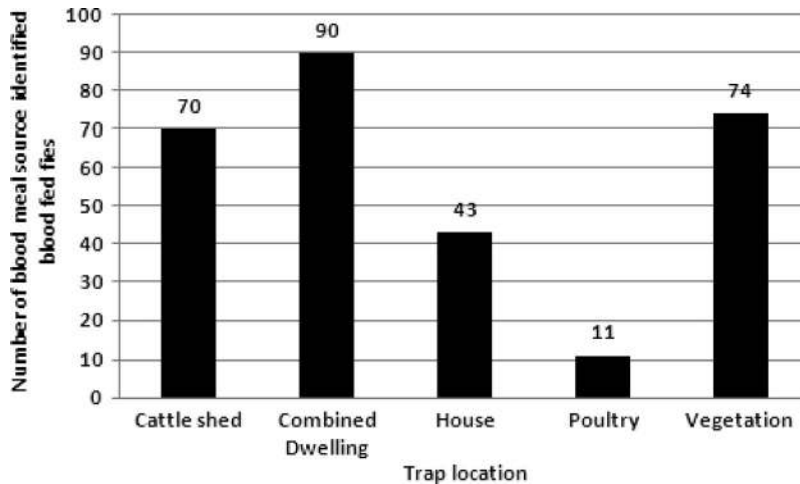

Fig. 7. Total number of bloodmeal source identified sand flies captured in different trap locations in three villages of Bihar.

in which DNA could not be extracted or <0.1 pg DNA was present in the ingested blood (Abbasi et al. 2008). Bloodmeals of all the insects positive to PCR reaction were identified.

The highest numbers of blood fed sand flies were caught during June, August, September, October, and November of 2009 and the host was mainly human in individual and multiple bloodmeals. The above months coincided with the months having the highest number of kala-azar incidences in Saran District in the year 2007 (Kumar et al. 2009). The villages where we collected sand flies were in the Saran District. From our results, it is evident that incidence of VL and the peak in blood feeding on humans coincide with each other.

Because of the high numbers of bloodmeals from live stock, treating cattle and goats will help in the management of sand fly populations in a very effective way. If the sand fly population is decreased, the rate of disease transmission and incidence of kala-azar will decrease. Further studies, involving the treatment of cattle and goats with systemic insecticides, in conjunction with the monitoring of sand fly populations, analysis of their bloodmeals, and the kala-azar incidence will aid in the management of vector populations in kala-azar endemic regions. The results of this study indicate that using PCR and RLB method is an invaluable tool in determining vector bloodmeal in wild caught sand flies.

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