Original Article

Blood Meal Identification in Field-Captured Sand flies: Comparison of PCR-RFLP and ELISA Assays

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Abstract

Background: We aimed to develop a PCR-RFLP assay based on available sequences of putative vertebrate hosts to identify blood meals ingested by field female sand fly in the northwest of Iran. In addition, the utility of PCR-RFLP was compared with ELISA as a standard method.

Methods: This experimental study was performed in the Insect Molecular Biology Laboratory of School of Public Health, Tehran University of Medical Sciences, Iran in 2006-2007. For PCR-RFLP a set of conserved vertebrate primers were used to amplify a part of the host mitochondrial cytochrome b (cyt b) gene followed by digestion of the PCR products by *Hae* III enzyme.

Results: The PCR-RFLP and ELISA assays revealed that 34% and 27% of field-collected sand flies had fed on humans, respectively. Additionally, PCR-RFLP assays could reveal specific host DNA as well as the components of mixed blood meals. Results of PCR-RFLP assay showed that the sand flies had fed on cow (54%), human (10%), dog (4%), human and cow (21%), dog and cow (14%), and human and dog (3%).

Conclusion: The results can provide a novel method for rapid diagnosis of blood meal taken by sandflies. The advantages and limitations of PCR and ELISA assays are discussed.

Keywords: *Leishmaniasis, sand flies, blood meal, PCR, ELISA, Iran*

Introduction

Leishmaniasis is caused by single-celled parasites of the genus *Leishmania* and is spread to humans through the bite of the sand fly. There are three main forms of leishmaniasis: cutaneous, mucocutaneous and visceral, each form is caused by different species of *Leishmania* (WHO 1990). Leishmaniasis affects over 12 million people distributed in 88 countries (Sakthianandeswaren et al. 2009). Every year, new cases amount to more than 2 million (Sakthianandeswaren et al. 2009). Nearly one tenth of the world population is at risk of infection. These figures have led to the WHO to consider leishmaniasis as one of the most serious diseases of the world. Visceral leishmaniasis (VL) or kala-azar is the most severe manifestation of the disease and results fatal when is not treated promptly. There are 500,000 new cases of kala-azar every year and it is endemic in Asia, Europe, and South America (WHO 2002).

The parasite is transmitted by sand flies, particularly members of the genera *Phlebotomus* spp. and *Lutzomyia* spp. which are found in a wide range of habitat, from desert to tropical rain forest. Sand flies take blood meals from a wide variety of hosts, including human, livestock, dogs and chickens (Lainson and Rangel 2005). The Human Blood Index (HBI, proportion of blood meals of a haemophagous insects population obtained from man) is relevant

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to epidemiological assessment and to the modification of measures to interrupt any Vector Borne Diseases transmission since the vectorial capacity of the vector varies as the square of the HBI (Macdonald 1957, Garrett-Jones 1964). Detailed knowledge of the feeding behavior of sand flies on their various vertebrate hosts is considered to be a prerequisite for a successful sand flies and leishmaniasis control program.

Identification of the blood meals of haematophagous insects to date has largely depended on serological techniques such as the precipitin test, latex agglutination test and the enzyme-linked immunosorbent assay (ELISA) (Boorman et al. 1977, Washino and Tempelis 1983, Beier et al. 1988, Gomes et al. 2001, Mwangangi et al. 2003). Although these methods have yielded important information on the identity of the vertebrate hosts of many blood-feeding arthropods, they are time-consuming and lack sensitivity. So, an alternative method may be desired in laboratories not set up to perform immunologic assays, or if samples are already in the form of extracted DNA. The PCR-based identification of arthropod blood meals provides a convenient alternative for laboratories using primarily DNA-based techniques, and may be necessary when the study design already requires the use of individual DNA extractions for multiple purposes such as species confirmation, determination of infection status for various pathogens, and vector population genetic studies. Furthermore, engorged specimens collected in the field may be preserved dry, stored for long periods of time, and tested at facilities that may be physically distant from the point of collection.

PCR-based identification of vertebrate host blood meals is a potentially convenient alternative, which has already been performed on several vectors including ticks (Pichon et al. 2003, Estrada-Peña et al. 2005), triatomine bugs (Bosseno et al. 2006, Pizarro et al. 2007) and mosquitoes. PCR based on primers designed from multiple alignments of the mitochondrial cytochrome *b* gene have identified avian and mammalian hosts of various species of mosquito (Ngo and Kramer 2003, Kent and Norris 2005, Molaei et al. 2006, Kent et al. 2006). PCR-RFLP cytochrome b analysis was also used to identify the origin of blood meals in the tick *Ixodes ricinus* (Kirstein and Gray 1996), tsetse flies (Steuber et al. 2005) and the mosquito *Anopheles stephensi* (Oshaghi et al. 2007a, and b). In their study, the cyt b sequences showed sufficient inter-specific polymorphism to distinguish between human, cow, sheep, chicken, and guinea pig hosts.

Until recently sand fly host identification by blood meal analysis had been limited to serological studies using ELISA (Gomez et al. 1998, Agrela et al. 2002, Bongiorno et al. 2003, Svobodová et al. 2003, Marassá et al. 2006, Rossi et al. 2008), counter immunoelectrophoresis (Morsy et al. 1993), agarose gel diffusion (Srinivasan and Panicker 1992), precipitin test (Tesh et al. 1971, Tesh et al. 1972, Javadian et al. 1977, Morrison et al. 1993, Nery et al. 2004, Afonso et al. 2005) and a more laborious histological technique (Guzman et al. 1994). The first PCR-based method using the prepronociceptin gene has been recently described (Haouas et al. 2007). In Iran, most studies for identification of sand fly blood meal have been based on ELISA (Javadian et al. 1977, Azizi et al. 2006, Rassi et al. 1999, 2005, Maleki 2007).

In the present study, we adapted a PCR protocol designed by Kent and Norris (2005) and developed this with sequence analysis of major vertebrate hosts in north of Iran in order to find putative restriction enzyme to develop host specific RFLP patterns for DNA sources in sand flies. Also the ELISA method was applied to compare the results of these two methods for blood meal identification.

Materials and Methods

Study area

The study was conducted in Germi district, Ardabil Province, in northwestern Iran. This region is 1,490 meters above the sea

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level. The total population of Germi was approximately 123,000 in 2002. The weather is hot (up to 40 °C) in summer and cold (less than −20 °C) in winter. The warm season is short (mid-May to mid-September). Annual rainfall is approximately 114 mm. The main occupations of the population are farming and raising animals. On the basis of available epidemiologic data obtained from the Ministry of Health (MOH), local health authorities, and medical centers in Germi district, villages with higher incidence of VL were selected for the study. Three primary villages (Kalansora, Shah-Tapeh-si, and Hamzeh-Khanloo) were selected and analyzed in an entomologic survey. Three secondary villages (Hasi-Kandy, Sarv-Aghaji, and Ghasem-Kandy) were also study periodically.

Sample collection

Sticky traps were used to collect sand flies from human and animal dwellings, rodent and fox burrows, under bridges, and on the shores of rivers. Traps were set at dusk and flies were collected at dawn. A total of 150–200 sticky traps were set each day in each village. Sample collection began in early July 2008 and continued until late September 2008 when sand fly activity was reduced sharply. Sampling was carried out every 3 d in the primary villages and 1-3 times in the secondary villages. Trapped sand flies removed from stick papers with needles, washed with absolute ethanol, and transferred into micro tubes filled with 96% ethanol. Tubes were kept frozen (-20 °C) until species identification and DNA extraction. Of the many samples collected in the region, 400 blood-fed females were randomly selected for detection of blood meal.

Identification of sand fly species

In the laboratory, samples were washed with detergent and double-distilled water, and heads and terminal abdomens of females were removed and mounted with Pouri solution on glass slides for diagnosis. For males, only heads were removed and mounted with Pouri solution on glass slides. Species were identified by using specific morphologic keys. Middle parts of female sand flies were placed in micro tubes and kept frozen (−20 °C) until DNA extraction.

Analysis of blood meals in sand flies

Four hundred blood fed female sand flies were randomly selected for blood meal identification. Sand flies were selected on the basis of location and capture sites to obtain a representative sample of sand flies in a region. Samples were divided randomly into two groups of 200 specimens; each group was analyzed by PCR-RFLP or ELISA method.

Extraction of DNA from blood meals in sand flies

DNA extraction from blood-fed female, and male, unfed female sand flies and water (used as a negative controls), and human and cow (used as a positive controls) was conducted according to the procedure of Steiner et al. (1995). Samples were individually disrupted by mechanical homogenization in buffer containing 10 mM Tris-HCl, pH 8.0, 312.5 mM EDTA, 1% (w/v) sodium lauryl sarcosine, and 1% polyvinyl pyrolidone. Homogenates were heated to 90 °C for 20 min and chilled on ice for 5 min. Samples were centrifuged at 13,000 RPM for 5 min at room temperature. The supernatant was removed and diluted 20-fold in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

PCR amplification of the mtDNA cyt b gene

Two regions of the mtDNA cyt b gene were amplified for host blood meal identification of the blood-fed female specimens. For identification of human blood meals a portion (358 bp) of the cyt b gene was amplified and digested with *Xho*I enzyme as previously explained by Oshaghi et al (2006a). The sequence of the primers used were 5′-CCATCCAACATCTCAGCATGATGAAA-3′ (forward) and 5′-CCCCTCAG AATGATATTTGTCCTCA-3′ (reverse) (Kocher et al. 1989, Boakye et al. 1999). The PCR amplifications were per-

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formed in 25 µL of a solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl 2, 0.001% gelatin, 200 mM deoxynucleotide triphosphates, 10 pmol of each primer, 1 unit of *Taq* DNA polymerase (Cinagene, Tehran, Iran), and 2.5 µl of DNA template solution. Samples were incubated at 95 °C for 3.5 min; followed by 36 cycles at 95 °C for 30 s, 58 °C for 50 s, and 72 °C for 40 s; and 72 °C for 5 min. To discriminate animal host blood meals, a second region of the mtDNA *cytB* gene was amplified by using the protocol of Kent and Norris (2005). The sequences of forward and reverse primers were respectively 5′-TGAGGACAAATATCATTCTGAGG-3′ (UNFOR403) and 5′-GGTTGTCCTCCAATTCATGTTA-3′ (UNREV1025), respectively. Primers amplified a 623-basepair region of the *cytB* gene of vertebrate mtDNA. The PCR amplifications were performed in 25 µL of a solution containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl 2, 0.01% gelatin, 1.0 mM deoxynucleotide triphosphates, 0.5 units of *Taq* polymerase, 50 pmol of each primer, and 2.5 µL of extracted DNA. Samples were incubated at 95 °C for 5 min; followed by 35 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and 72 °C for 7 min. Products were visualized by electrophoresis on 2% agarose gels stained with ethidium bromide. Electrophoresis was conducted using a GeneRuler 100-basepair molecular mass marker (Cinagene).

Sequence analysis for selection of restriction enzymes

Available sequences of the 623 bp for human and probable vertebrate hosts (cow, goat, horse, ass, dog, and other Canidae) in the study area were obtained from Gene Bank and checked for species-specific restriction enzyme sites for each host DNA using the Nebcutter program (http:/tools.neb.com/nebcutter. Twenty eight sequence analyses showed that *Hae* III did not have a restriction site on human PCR products but it has various specific sites in PCR products for other vertebrates. This enzyme was selected for discrimination of the blood meal sources within sand flies. Digestion of PCR products was performed in 25 µL of a solution containing 15 µL of PCR product mixed with 2.5 µL of enzyme buffers and 5 units of the restriction enzyme overlaid with two drops of mineral oil. The mixture was incubated at the temperature recommended by enzyme suppliers. An aliquot (14 µL) of the digestion product was mixed with 6 mL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), loaded onto a 2.5% agarose gel, and subjected to electrophoresis. Gels were stained with ethidium bromide (2 mg/mL) and the RFLP profiles were visualized under ultraviolet light.

Serological analysis

Analysis was performed by ELISA as described by Edrissian et al. (1985) the abdomen of blood fed sand flies was dissected, placed in the well of a micro-ELISA plate (Nunc, Roskilde, Denmark), squashed with a glass rod, and eluted with 50 µL of distilled water for 2 h at room temperature. Fifty micro liters of coating buffer (carbonate bicarbonate, pH 9.6) was then added to each well. Plates were washed three times with phosphate-buffered saline, Tween 20, pH 7.2. Fifty micro liters of diluted goat anti-human IgG conjugated to alkaline phosphates were added onto each well, incubated for 2 h at 37 °C, washed three times with phosphate-buffered saline, Tween 20, pH 7.2. One hundred microliters of substrate solution (1 mg/mL of *p*-nitrophenyl phosphate [Sigma, St. Louis, MO] in 10% diethanolamine buffer, pH 9.8, containing 0.5 mmol MgCl 2 and 0.02% NaN 3) was added to each well, and incubated in a dark chamber for 30 min at room temperature. Two wells that did not contain blood were used as negative controls and two wells that contained human blood were used as positive controls. Results were visually assessed, and absorbance was measured with an ELISA reader at 405 nm approximately 30 min after addition of substrate solution. The test well result was considered positive if it a yellow color was observed.

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Results

PCR-RFLP

DNA isolated from the blood-fed sand flies, positive controls (blood from a human and a cow), and negative controls (water, unfed female sand fly, and a male sand fly) were used as a template in a PCR. Most host DNAs were amplified, and negative controls yielded no PCR product. This result implied that only host, but not sand fly, DNA patterns were detected in amplified specimens. DNA sequence analysis showed that the two regions of the mtDNA *cyt b* gene digested with either *Xho* I or *Hae* III could distinguish human DNA in blood of blood-fed sand flies from the DNA of blood of other vertebrates. *Xho* I digested only the 358-basepair PCR product of human DNA and produced two bands (215 bp and 143 bp) whereas it had no restriction site in the DNA of blood of other vertebrates. This finding was confirmed by results of digestion with *Hae* III on the 623-basepair region of the cyt b gene. This enzyme did not digest the 623 bp fragment of the cyt b gene in DNA from human blood, but it digested the equivalent DNA fragment from other vertebrates and could distinguish DNAs from cow, ass, goat, horse, dog, and other Canidae from each other. For example, *Hae* III produced two fragments of 345 bp and 304 bp from cow DNA, two fragments of 552 bp and 70 bp from Canidae DNA, and two fragments of 170 bp and 453 bp from goat DNA (Fig. 1). Based on PCR-RFLP analysis rates of blood meal sources was found to be 54% for cows, 19% mix of humans and cows, 14% mix of dogs and cows, 8% for humans, 3% mix of humans and dogs, and 2% for dogs. On overall, 34% of the female sand flies had human blood or mix of human and an animal blood. Except for a few specimens (n=4) that contained *P.* (*Adlerius*) spp., all blood-fed specimens were *P. perfiliewi*. Details of PCR-RFLP analysis are shown in Table 1.

ELISA

Serologic analysis by ELISA on 200 blood-fed sand flies showed that 54 (27%) specimens fed on humans (Table 2). Fifty one of 54 human blood-fed samples were of *P. perfeliewi.* The three other seropositive samples in human blood were *P.* (*Adlerius*) spp. Approximately 61% of these blood-fed sand flies were found in either human or animal shelters, which suggested that they were highly endophilic.

Generally, based on PCR-RFLP and ELISA assays, it seems that sand flies, particularly *P. perfiliewi*, although have close relationship with human dwellings in the study area, but are more zoophilic than anthropophilic (73-66% versus 27-34%), and in a descending order, they prefer to feed on cows, humans, and dogs.

Table 1. Host blood meals ingested by female sand flies in Germi, northwest of Iran detected by PCR-RFLP analysis of a 623 bp fragment of the mitochondrial DNA *cytochrome b* (Cyt b) gene.

Collection site	Sand fly species	Blood sources	n (%)
Human dwelling	*P. perfiliewi*	Human and Cow	4 (2)
	P. (*Adlerius*) spp.	Human	4 (2)
Animal dwelling	*P. perfiliewi*	Human	16 (8)
		Dog	4 (2)
		Cow	108 (54)
		Human and Cow	36 (19)
		Human and Dog	6 (3)
		Dog and Cow	28 (14)
Artificial	*P. perfiliewi*	Dog	4 (2)
Total	–	–	200 (100)

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Table 2. Results of ELISA assay against human antibody for blood meal identification of fed female sand flies in Germi, northwest of Iran.

Capture site	Sand fly species	No. of blood fed (%)	No. of Human blood (%)
Human	*P. perfiliewi*	38 (14)	9 (23)
	P. (Adlerius) spp. *	11 (4)	3 (28)
Animal	*P. perfiliewi*	46 (17)	21 (46)
Artificial	*P. perfiliewi*	70 (36)	21 (30)
	P. (Adlerius) spp. *	24 (9)	0
	P. papatasi	3 (1)	0
	P. kandelaki	8 (4)	0
Total	-	200 (100)	54 (27)

*Males of *P. brevis*, *P. halepensis*, and *P. longiductus* of the *Adlerius* subgenus are morphologically indistinguishable.

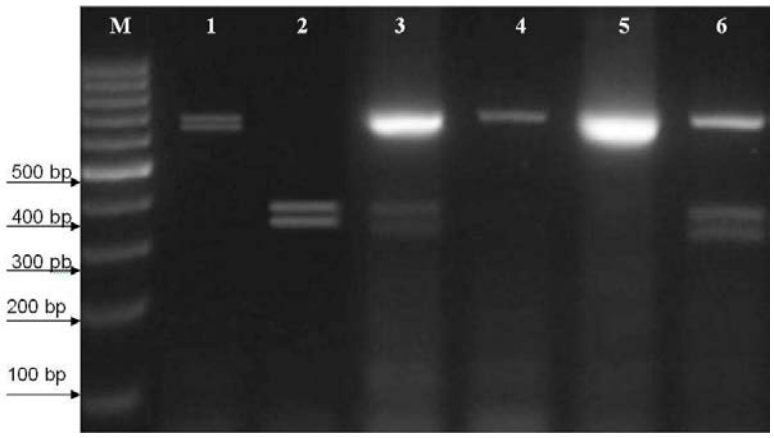

Fig. 1. Electrophoresis of mitochondrial DNA cytochrome B gene fragments from blood meals of sand flies digested with *Hae*III. Partial fragments (623 bp) of the *cytochrome B* gene from vertebrate hosts (human, cow, dog) of sand flies were amplified by a polymerase chain reaction and the products were digested with *Hae*III. Lane 1, mixture of human and dog blood; Lane 2, cow blood; Lane 3, mixture of cow and dog blood; Lane 4, human blood; Lane 5, dog blood; Lane 6, mixture of human and cow blood; lane M, molecular weight marker (100 bp Cinnagen, Iran)

Discussion

In this study first we have adapted a PCR method developed by Kent and Norris (2005) and then we developed the technique by restriction fragment length polymorphisms (PCR-RFLP) to identify blood meals from wild-collected sand flies in an endemic area of zoonotic visceral leishmaniasis in northwest Iran where *L. infantum* is transmitted. We also evaluated utility of PCR-RFLP with ELISA as a gold standard method. In our study successful amplification was obtained from genomic DNA of host blood meal of sand flies fed on human, cow, and dogs. The RFLP profiles for these vertebrates were specific and easily they could be distinguished from each other. Theoretically, based on sequence data available in Gene bank, the *Hae*III enzyme could provide diagnostic profiles for other vertebrate hosts such as ass, goat, horse, and other Canidae, however, we did not have DNA sources for these ones to confirm their diagnostic profiles.

Results of ELISA were almost similar with PCR-RFLP (27% versus 34%). ELISA method has provided countless valuable data

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over the years for blood meal identification (Gomez et al. 1998, Agrela et al. 2002, Bongiorno et al. 2003, Svobodová et al. 2003, Marassá et al. 2006, Rossi et al. 2008). In sand flies with 0.5 µg engorged meal, the ELISA technique enables us to identify only one patent feed (Lehane 2005). However, ELISA methods are time-consuming and lack sensitivity. In addition, one of the major constraints of ELISA is that sand flies are diminutive insects, only able to ingest very small quantities of blood (Rogers et al. 2002). Due to low volume of engorged blood meal in sand flies it is impossible to check more than one antigen source. Another constrain is that sand flies have multiple feeding habituate that has major consequence on the epidemiology of Zoonotic diseases such as zoonotic visceral leishmaniasis (ZVL) and zoonotic cutaneous leishmaniasis (ZCL). These constrains direct researchers to use more sensitive and accurate methods such as PCR-RFLP to identify sand fly hosts for the study of vector–vertebrate host associations, as well as to improve current control interventions targeting sand flies vectors.

PCR-RFLP assay has more advantage than ELISA since it possesses the unique ability to analyze components of a multi species DNA sample. In this study, 36% of blood-fed field specimens contained multiple blood meals. This host-feeding behavior can influence pathogen transmission through increased frequency of vector-human contact, or possibly reduce vector-human contact if some blood meals are taken from alternative mammalian hosts. Since this method could identify multiple blood meals it will be extremely useful for entomologically based projects involving blood-feeding behavior and vectorial capacity of sand flies in endemic areas of ZVL and ZCL.

mtDNA Cytochrome B gene has been used to resolve vertebrate evolutionary questions as well as served as a target for molecular diagnostics (Ngo 2003). *CytB* has a proven utility for identifying arthropod blood meals due to high copy number as a mitochondrial gene and sufficient genetic variation at the primary sequence level among vertebrate taxa for reliable identification. In addition to mtDNA *cytB* gene, other markers that have been used to identify blood meals from arthropods include vertebrate 18S ribosomal DNA (Pichon 2003) the hypervariable region 2 of mitochondrial DNA (Lord 1998) and TC-11 and VWA (HUMVWFA31/A, a repeat polymorphism in the von Will brand factor gene) loci (Mukabana 2002).

PCR-based methods have been used for diagnosis of infectious diseases, including *Leishmania* detection in human patients (Dweik et al. 2007, Foulet et al. 2007, Kumar et al. 2007), infected dogs (de Andrade et al. 2006, Gomes et al. 2007, Solano-Gallego et al. 2007) and phlebotomine sand flies (Cabrera et al. 2002, Paiva et al. 2006, Myskova et al. 2008, Ranasinghe et al. 2008, Oshaghi et al. 2009). DNA prepared from whole body of *Phlebotomus* sand flies not only can be used for blood meal identification but also can be used for parasite detection/identification by PCR (Sant'Anna et al. 2008). This simple methodology could be very useful in epidemiological studies in endemic areas for leishmaniasis as specimens suspected to contain parasites.

In this study, sand fly of *P. perfiliewi* was the most predominant species and has a 27-34% tendency to human blood in the region. Previous studies on *P. perfiliewi*, have suggested nearly identical tendency to human blood (Rassi 1999). It seems that this sand fly is not highly anthropophilic and preferred cows or other animals. In rural environment where large domestic mammalian species occur in abundance, this may reduce *P. perfiliewi* vectorial capacity.

In conclusion, blood meal identification in field-caught sand flies can confirm a strong association between sand flies and reservoir hosts such as dogs in rural areas and help to improve understanding the role of domestic animals in transmission of *L. infantum* in endemic foci. The greater sensitivity of the PCR-

RFLP method in comparison with ELISA reported here means that information can be obtained from specimens that have ingested relatively small amounts of blood of one or multiple hosts which could be used in vector incrimination and reservoir determination of vector borne diseases such as ZVL and ZCL.

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