Development of a Molecular Tool for the Identification of Leishmania Reservoir Hosts by Blood Meal Analysis in the Insect Vectors

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Abstract. The transmission of parasites of the genus *Leishmania* involves a large diversity of mammalian reservoir hosts. However, many of these are yet to be identified, mainly in isolated biotopes such as the Amazonian rain forest. Furthermore, the trophic preferences of insect vectors have major epidemiologic implications. In this study, we developed a molecular tool for the identification of blood meals of phlebotomine sand flies. This assay is based on specific amplification and sequencing of the blood meal–derived single copy prepronociceptin (*PNOC*) gene, which is used as a target in phylogenetic studies of mammals. Sand flies were identified simultaneously with the blood-meal identification, using molecular analysis of a ribosomal locus. After a systematic assessment of the sensitivity and specificity of polymerase chain reaction amplification of the *PNOC* gene using human fed sand flies, the assay was tested on wild-caught sand flies. This work has important implications for the discovery of new *Leishmania* reservoir hosts and for a better understanding of complex parasite life cycles.

INTRODUCTION

The identification of an animal reservoir host in a vector-borne disease transmission cycle is critical for the establishment of an efficient control strategy. The collection of wild animals for isolation of possible vector-borne pathogens is the primary method for reservoir discovery and identification. However, this strategy is time consuming and could be extremely difficult in many biotopes such as the Amazonian rain forest or other isolated areas. Blood-meal identification of hematophagous insects provides information on host-feeding preferences or host feeding patterns in natural conditions, and such information may provide indirect data on reservoir potential. Several blood-meal studies have been developed for various insect orders implicated in the transmission of parasitic and viral diseases such as *Culicidae*, *Glossinidae*, and *Psychodidae*. These studies have contributed to the knowledge of the life cycle of both vectors and the transmitted pathogens and the establishment of control strategies for these arthropods.

*Leishmaniasis* constitutes a group of diseases caused by flagellated parasitic protozoa belonging to the genus *Leishmania*, which are transmitted through the bite of naturally infected female phlebotomine sand flies. *Leishmaniasis* is endemic in 88 countries, including 72 developing countries, on four continents. The clinical manifestations depend on the immune status of the host and the causative parasite species involved and range from curable skin lesions to serious visceral or mucosal infections that may be fatal.

Under natural conditions, *Leishmania* transmission cycles can involve a large number of mammalian species. More than 62 mammal species belonging to 46 genera, 21 families, and 9 orders are thought to be involved. Among the 1,000 phlebotomine sand fly species found worldwide, ~70 are suspected to be *Leishmania* vectors, and 20 have been confirmed to be efficient vectors. Because of this complexity, it can be difficult to identify all of the mammalian reservoir hosts involved. For example, the role of wild and domestic hosts in the zoonotic cycle of *L. braziliensis*, the most pathogenic species in South America, is still poorly understood. The involvement of different vertebrate hosts in the transmission of anthroponotic *L. tropica* remains debatable. One of the possible approaches to clarify this last point could be to develop a systematic analysis of the host-feeding preference of the insect vector. By doing so, it is not only possible to provide significant information about many still unknown reservoir host species but also to quantitatively evaluate the host feeding pattern of each vector species on different mammals present in its vicinity and the dynamics of their interaction.

Insect blood-meal origin has been identified by a variety of methods that vary considerably in their ability to identify a blood-meal source at the species level. The most widely used approaches are based on antigen-antibody assays (i.e., precipitation test, gel diffusion test, and ELISA). These assays have the potential to identify blood-meal sources to the species level. However, they present technical limits such as the possibility of cross-reactivity between species, the requirement to produce specific antibodies to a wide range of potential hosts, and the inability to discover unpredicted reservoirs.

More recently, molecular-based assays have been developed to detect and identify blood-meal sources of some insect vectors with a higher degree of sensitivity and specificity. However, most of the studies have focused on the detection and the quantification of human blood for epidemiologic or forensic purposes, where they have used hyper-variable genetic markers such as minisatellites, microsatellites, or human leukocyte antigen genetic (HLA) loci. Only a few studies concerning host preference patterns have been developed to detect and identify possible vertebrate species. Most of these were based on the analysis of the mitochondrial cytochrome B (*cytb*) gene, which has been widely used in vertebrate phylogenetic studies and for which a large set of data is available. Restriction analysis of *cytb* polymerase chain reaction (PCR) products (PCR restriction fragment length polymorphism [RFLP]) has been used for *Anopheles* blood-meal identification, allowing differentiation of human blood meals from those of other mammals and birds.
heteroduplex assay (PCR-HDA) was developed for studying the host preferences of both Culicidae and Glossinidae and has been successfully used to evaluate trophic preferences of mosquitoes to aviary-bird reservoirs\textsuperscript{21} and of tsetse flies to humans and domestic and wild animals.\textsuperscript{2,4} However, PCR-HDA has the disadvantage of a limited number of reference patterns to be used for the analysis and the difficulty of the visual interpretation of PCR-HDA gels.\textsuperscript{4,21}

Regarding phlebotomine sand flies, the topic of this work, the study of the host-feeding preferences and host feeding patterns among a large number of vertebrate species has thus far been based only on immunologic assays. Immunoprecipitation and ELISA have been largely used to study different Leishmania foci. In total, immunologic assays have successfully identified 13 different mammal and bird species. However, as previously stated, this technique has several inherent limitations such as the inability to detect unexpected animal species.\textsuperscript{20}

In this study, we developed a molecular tool for sand fly blood-meal identification. This assay is based on specific amplification and sequencing analysis of the blood meal–derived single copy prepronociceptin (PNOC) gene, which is used as a target in mammalian phylogenetic studies, and sequences of > 64 mammalian species are already published on GenBank.\textsuperscript{31,32} Molecular identification of the sand flies to the species level was carried simultaneously. It was based on the amplification and the sequencing of a ribosomal locus. After a systematic assessment of the sensitivity and the specificity of the technique, the method was tested on wild-caught sand flies from a natural Leishmania focus.

**MATERIALS AND METHODS**

**Sample collection.** Laboratory-reared phlebotomine sand flies (*Phlebotomus duboscqi*) were fed on humans and held at 28°C and 80% relative humidity for different amounts of time after engorgement to study the time-course of blood-meal digestion in the mid-gut of the insect.

Wild-caught blood-fed phlebotomine sand flies were collected using home-made CDC miniature light traps between July and August 2006 in three rural sites in central Tunisia where domestic animals (i.e., cattle, horses, dogs, cats, rabbits, and chicken), wild animals (i.e., rodents and reptiles), and humans are present. Traps were set before sunset and collected the next morning. For the molecular identification of blood-meal origin and morphologic identification of sand flies, they were kept in 70% ethanol and stored at −20°C.

**DNA extraction, amplification, sequencing, and analysis.**

Genomic DNA from human blood samples or blood-fed phlebotomine sand flies was extracted using the QiaAmp blood DNA mini Kit (Qiagen, Hilden, Germany) and eluted in 100 μL of Tris-EDTA buffer. The reaction was performed in a final volume of 50 μL containing 1 μL of sample or control DNA, 1× PCR buffer, 3 mmol/L MgCl\textsubscript{2}, 400 μmol/L of each deoxynucleotide, 2 units of Taq DNA polymerase, and 30 pmol of each primer. Five microliters of the eluted DNA was used as a template for PCR amplification of mammal PNOC, human AluYb8 element, and phlebotomine 12S ribosomal RNA small subunit DNA using the following primers\textsuperscript{32–34}:

- PNOC-F: 5’-GCATCCTTGAGTGGAAGAGAA-3’,
- PNOC-R: 5’-TGCCCTCTAAACTACCTGAAAC-3’,
- Alu-F 5’-CGAGGCGGGTTAGCATCGAGGT-3’,
- Alu-R: 5’-TC-TGTCGCCAGGCCGACT-3’,
- 12S-F: 5’-AAACTAGGATTAGATTACCC-3’ and 12S-R: 5’-AATGAGAGCGAGCGCGATGT-3’. The optimized PCR conditions were as follows: 5 μL of 10× buffer, deoxynucleoside triphosphates at a concentration of 400 μmol/L each, 3 mmol/L MgCl\textsubscript{2}, 30 pmol of each primer, and 2 units of Taq DNA polymerase (Goldstar; Eurogentec), for a total volume of 50 μL including 5 μL of sample DNA. All reactions were cycled in an MJ Research PTC-100 thermostycler (MJ Research Inc., Watertown, US), and two negative controls, distilled H\textsubscript{2}O, and DNA of male sand flies (which are not blood-feeders), were run simultaneously to detect possible contaminations during both the extraction and amplification steps. To evaluate the possibility of PCR inhibition and to test the fidelity of the extraction, all extracted sand fly DNA (laboratory-reared and field collected) was amplified using a sand fly housekeeping gene (12S rRNA gene).

The PCR products were purified using the Qiaquick PCR purification Kit (Qiagen) and directly sequenced in both directions using the MWG-Biotech sequencing service (Ebersberg, Germany).

Sequences were edited using Sequencher software (GeneCodes Corp., Ann Arbor, MI). Prepronociceptin gene sequences were blasted using Blastn algorithm against the “non-redundant” GenBank sequence database. For identification of sand flies, sequencing products were compared with a personal database. The GenBank accession numbers of phlebotomine 12S ribosomal RNA sequences are from EF613321 to EF613327.

**RESULTS**

**Evaluation of technical sensitivity on blood samples and laboratory-reared sand flies.** To determine the sensitivity of the PCR amplification of the PNOC gene, 5-fold decreasing dilutions of optical density-quantified human DNA were used to test detection threshold. A 330-bp PCR product was successfully detected from an estimated human genomic DNA quantity < 10 pg (Figure 1). This amount of DNA represents the statistical equivalent of only three human haploid genomes in the reaction mixture.

To study the time-course of detection of sand fly blood meals, amplification reaction of the PNOC gene was carried out on laboratory-reared sand flies fed on human blood. After feeding, insects were killed at different times (0, 4, 8, 12, 16, 20, 24, 37, and 48 hours) after engorgement. DNA was

![Figure 1](image-url)  
**Figure 1.** Analytical sensitivity of PNOC gene PCR amplification (333 bp). Lanes 1–6, 5-fold dilutions of human genomic DNA from 3 × 10\textsuperscript{4} to 10 pg of human DNA/reaction; Lane 7, negative control. MW, molecular weight marker (100-bp DNA ladder).
Four Time-course of detection of DNA in sand fly blood meals by PNOC gene amplification. After feeding on human blood, P. duboscqi were killed by freezing at various times. Lane MW, molecular weight (100-bp DNA ladder); C+, positive control (purified DNA from human blood); Lanes 0h to 48h, time post-feeding in hours; C−, negative control (no template); UF−, negative control (unfed sand fly).

Successfully amplified in individual sand flies until 24 hours after engorgement (Figure 2). No amplification signal was detected at 37 and 48 hours after feeding.

The decline of the mammal DNA amplification signal from fed flies between 24 and 37 hours may be explained by two hypotheses: 1) the increasing insensitivity of PNOC gene amplification for detecting a small amount of mammalian DNA in a progressive degradation process, and 2) the rapid degradation of mammal DNA caused by a sudden increase of the catalytic enzyme activity in the phlebotomine mid gut.

To verify these two hypotheses, we compared the detection threshold of the single copy PNOC gene amplification versus elements of the AluYb8 subfamily. AluYb8 are human multicopy transposable elements subfamily repeated > 5,000 times in the genome. A range from 2 \times 10^3 to 0.1 pg of human DNA per PCR reaction was used to amplify the AluYb8 sequence. A 226-bp PCR product was successfully amplified from 0.1 pg of human DNA, which corresponds to just 0.03 human equivalent haploid genome (Figure 3). This result is comparable to previous studies testing the same target.

Studying the effect of blood digestion level on the DNA detection in wild-caught phlebotomine sand flies. We assessed our approach using wild-caught sand flies collected in natural conditions in a Tunisian Leishmania focus. A total of 1,295 wild-caught phlebotomine sand flies were collected (930 males and 365 females), of which 97 females were blood fed.

By monitoring the blood-meal color in the mid-gut of laboratory-reared sand flies and following the classification proposed by Svobodova and others, we classified fed flies into three digestion levels. 1) The first level of digestion was observed between 0 and 18 hours after feeding when the female sand fly mid-gut contained fresh blood that is bright red in color. 2) The second level of digestion was observed during 18–24 hours after feeding and was characterized by a mid-gut containing partially digested dark red blood. 3) Finally, the third level of blood-meal digestion was characterized by extensively digested blood, brown in color, occurring between 24 and 72 hours after the blood meal (time of defecation).

According to this classification, wild-caught phlebotomine sand flies were organized as described in Table 1.

DNA was extracted from 97 individual blood-fed sand flies and used as a template in the mammal-specific PNOC gene PCR amplification. The percentage of detectable blood-meal DNA by PNOC gene amplification clearly indicates a decrease related to progressive blood digestion. The percentage of positive PCRs varied from 100% to 86% and 66% for blood digestion levels 1, 2, and 3, respectively. Overall, 79% of the blood-fed specimens showed positive results in the blood-meal PCR assay (Table 1).

All positive PCR products amplifying the PNOC gene were

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**Table 1**

DNA detection sensitivity of the PNOC gene in blood meals versus level of digestion in 97 wild-caught sand flies

<table>
<thead>
<tr>
<th>Digestion level (equivalence in hours post-feeding)</th>
<th>Positive (%)</th>
<th>Negative (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (0–18 hours post-feeding)</td>
<td>N = 2 (100%)</td>
<td>N = 0 (00%)</td>
<td>N = 2 (2%)</td>
</tr>
<tr>
<td>2 (18–24 hours post-feeding)</td>
<td>N = 54 (86%)</td>
<td>N = 9 (14%)</td>
<td>N = 63 (65%)</td>
</tr>
<tr>
<td>3 (24–72 hours post-feeding)</td>
<td>N = 21 (66%)</td>
<td>N = 11 (34%)</td>
<td>N = 32 (33%)</td>
</tr>
<tr>
<td>Total</td>
<td>N = 77 (79%)</td>
<td>N = 20 (21%)</td>
<td>N = 97 (100%)</td>
</tr>
</tbody>
</table>

PCR amplification results (negative/positive) are displayed for advanced levels of digestion (level 1 = fresh blood-fed, level 2 = dark blood-fed, level 3 = brown blood-fed) as previously described by Svobodova and others.
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sequenced. Edited sequences were compared with the GenBank database and were successfully identified to species level with a match of 100%. Among the identified blood-meal samples, the majority were from cattle (Bos Taurus, N = 71, 92%), humans (Homo sapiens, N = 4, 5%), and horses (Equus caballus, N = 2, 3%). No superposed fluorograms were detected to suggest a mixed blood meal.

**Phlebotomine sand fly species identification.** The sand flies used for blood-meal identification were identified to species level using genomic DNA. A 360-bp region of the 12S ribosomal locus was amplified, sequenced, and compared with a personal molecular database created from morphologically identified sand fly species belonging to the genus Phlebotomus. Four sand fly species were identified: 86 were P. perniciosus (proven vector of Leishmania infantum), 7 were P. papatasi (proven vector of Leishmania major), 2 specimens were P. longicuspis (suspected vector of L. infantum), and 2 were identified as P. chabaudi (suspected vector of L. tropica).

**DISCUSSION**

The molecular approach presented in this work is the first one developed for phlebotomine sand fly species, which are vectors of leishmaniasis throughout the world. The main purpose of this work was to develop and evaluate the technical feasibility of this molecular technique and to assess its viability in field conditions.

The most significant aspect of the method is its sensitivity to detect minuscule amounts of host DNA in the sand fly blood meal. The overall sensitivity of PNOC gene detection in wild-caught sand flies reached 79%. Immunologic assays are the only comparable approaches developed thus far. Studies based on various techniques (i.e., ELISA, precipitin test, and gel diffusion test) have shown a wide range of sensitivity between 31% and 96%, with an average near 60%.3,7,21,22,23 Many factors may explain these discrepancies but the most likely is the difference in the digestion stages between the sand flies studied.36

To assess this parameter, we tested the ability to detect human DNA in laboratory-reared P. duboscqi killed at different times after feeding. The human PNOC gene sequence was easily detected in the blood meal up to 24 hours after feeding, but it became abruptly undetectable after this time. This result was similar to previous published results that were applied to Luizomyia sand flies tested with an ELISA assay from which blood meals were detectable up to 24 hours after blood engorgement.39 Similar studies on other hematophagous arthropods have shown various lengths of time for the identification of the host blood meal, ranging from 24 hours to a few months depending on the species, digestive physiology, and host blood-meal origin.2,7,22,23

The sensitivity depends on both the quantity of target DNA and the progressive degradation of the host DNA. Sand flies vary in size within and between species, and blood meal volume varies from 0.5 to 1 µL.40 This blood-meal quantity is noticeably lower than many other insects such as tsetse flies (37 µL),41 ticks, which engorge ~ 100 times their unfed body weight, and anopheline mosquitoes, which are able to concentrate erythrocytes during the blood feeding process.42-44

Also, avian blood meals were shown to be identifiable for a longer period because large amounts of DNA are present in their nucleated erythrocytes.21

The second factor influencing the sensitivity of host DNA detection is the degradation of the DNA resulting from various digestive processes of hematophagous arthropods.45 Except for particular physiologic models, such as tick larvae in which undigested blood can be stored inside endosomes allowing its detection months later,7 the major mechanism affecting blood-meal detection is the degradation activity of various enzymes such as proteases.7 It has been shown that, in sand flies, there is a peak of protease activity between 24 and 48 hours after feeding, depending on the sand fly species.46 If associated with DNase activity, this enzymatic may explain the rapid decline in human DNA detectability 24 hours after feeding as shown in our study. Moreover, recent analyses have shown that both blood-meal origin and Leishmania infection affect the proteolytic activity of some phlebotomine species. In field conditions, dissimilarity of protease kinetics depending on blood-meal origin may favor the identification of some host species over others.45 Additionally, the infection of a blood meal with Leishmania parasites decreases the enzymatic activity in the midgut and could allow the detection of host DNA for longer periods.48 Finally, studies of the gonotrophic cycle (i.e., period between blood feeding and oviposition) of some mosquitoes such as Anopheles gambiae have shown that the rate of blood digestion is temperature dependent, the increase of which accelerates the digestion of the blood meal and could reduce the sensitivity of host DNA detection.49 Therefore, if we hypothesize that the sample described by Svobodova and others47 was comparable with ours, the global sensitivity of our technique seems noticeably higher (79% versus 53%) than that observed using the ELISA technique.

As previously stated, immunologic assays present other limits such as the requirement of various antisera against all potential hosts, the possibility of interspecific cross-reaction, and the inability to identify an unpredicted blood meal if a suitable antibody is not available.2,16 These features cannot be evaluated in this study because we used a small number of samples for technical validation. However, we assume that the availability of a large database of PNOC gene sequences will allow the identification of a large variety of mammalian species.

One other interest of our approach was the simultaneous identification of both blood-meal origin and sand fly species from extracted DNA. As far as we know, this double identification has not been developed previously in hematophagous insects. Leishmania life cycles are complex, frequently involving different reservoir hosts and vectors in the same focus, and a quantitative analysis of vector species and host preferences is an efficient way to best understand the dynamics of such a focus. For example, in West Bengal (India), a recent work on host preferences of the kala-azar vector, P. argentipes, clearly showed that host preference varied widely in different biotopes and evaluated the occurrence of multiple blood meals, indicating a greater probability of transmitting the disease in favorable eco-epidemiologic situations.50 Of course, the identification of sand fly blood-meal sources cannot incriminate a reservoir host. This depends on detection of the parasite in a host and a quantitative study of host, vector, and parasite population dynamics. Complementary studies
such as the detection of Leishmania parasites in the sand fly blood meal must be done to prove their role as a vector.

Another potential advantage of the technique presented here is the possibility of the detection and the molecular identification of the parasite within the DNA extracted from the whole insect. However, the low percentage of infected sand flies in natural conditions requires the examination of hundreds of insects, and we did not test this point in this preliminary study.

In conclusion, the developed molecular approach has shown good sensitivity in detecting the phlebotomine sand flies blood meal and in assessing insect host preference in natural conditions. The simultaneous molecular identification of insect species was also developed for the first time and seems to be an effective tool for large-scale studies.

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