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Identification of blood meals in field captured sand flies by a PCR-RFLP approach based on cytochrome b gene

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Abstract

Leishmaniasis is a vector-borne disease transmitted by phlebotominesand flies. Information about blood meal preferences in sand flies is essential to understand the epidemiology of the disease to adopt control measures. In previous studies, a polymerase chain reaction (PCR) of 359 bp fragment of the conserved gene cytochrome b (cyt b) and further sequencing were applied in the study of blood meal sources in sand flies collected in the area of a leishmaniasis outbreak in southwest Madrid, Spain, providing significant information about blood meal preferences in the focus.

In this work, a PCR-restriction fragment length polymorphism (RFLP) targeting a fragment of 359 bp of vertebrate cytb gene was developed. Restriction
endonucleases *Hae*III and *Hinf*I generated specific patterns consistent with the blood meal sources found in sandflies. The protocol has been validated with twenty-six engorged females collected in the field with CDC traps. Blood meals from nine vertebrates were identified based on PCR-cytb and sequencing - human, dog, cat, horse, hare, rabbit, sheep, goat and chicken - and mixed blood meals (sheep/human; sheep/goat) – and successfully distinguished by PCR-RFLP. Therefore, this approach is an efficient and reliable alternative method to be applied in entomological surveys.

Keywords: PCR-RFLP, sand fly, blood meal, cytochrome b
1. Introduction

Leishmaniasis is a vector-borne disease caused by a trypanosomatid protozoan of the genus *Leishmania* (WHO 2010). In Spain it is caused by *Leishmania infantum*, using the vectors *Phlebotomus perniciosus* and *Phlebotomus arsii* (Rioux et al. 1986). The dog is the main reservoir, although it has been demonstrated that other wild mammals can play an important role in the maintenance and transmission of the parasite (Molina et al. 2012, Del Río et al. 2013, Ruiz-Fons et al. 2013, Jiménez et al. 2014).

Although leishmaniasis in Spain is considered hypoendemic, some regions are more affected, like the Mediterranean coast and the center of Spain. Moreover, since 2010 an important increase of the disease in urban areas of southwest Madrid has been detected. Actually, one of the areas studied in this study belongs to this affected area, where the mean incidence rate reached 44.55 cases per 100,000 inhabitants in 2013 (data provided by Autonomous Community of Madrid). In case of Mediterranean area, other studied region, Minorca (Balearic Islands), were reported an emergence of canine leishmaniasis (Gil-Prieto et al. 2011, Alcover et al. 2013, Arce et al. 2013, Ballart et al. 2013).

The knowledge of possible reservoirs implicated in an area affected with leishmaniasis is essential when deciding which control measures are needed to take. In this sense, the study of blood meal sources of vectors plays an essential role. Accordingly, serological techniques as ELISA were applied as first approach in this way (De Colmenares et al. 1995, Bongiorno et al. 2003), although these methods have been replaced by molecular tools for their better accuracy (Mukabana et al. 2002, Haouas et al. 2007, Kent 2009, Maleki-Ravasan et al. 2009). Amplification by polymerase chain reaction (PCR) and subsequent sequencing is widely used to study
bloodmeal preferences in sand flies and other vectors as mosquitoes or ticks (Kirstein and Grey 1996, Oshagi et al. 2006, Danabalan et al. 2014). Thus, different molecular targets like cytochrome b (cytb), cytochrome oxidase I (COI) and prepronociceptin (PNOC) are being used to detect the origin of blood meals (Kocher et al. 1989, Haoaus et al. 2007, Abassi et al. 2009). On the other hand, other techniques as PCR- restriction fragment length polymorphism (RFLP) or PCR-reverse-line blotting (RLB) have been developed in order to study blood meal preferences in mosquitoes, tsetse flies and sand flies (Osaghi et al. 2006, Steuber et al. 2005, Maleki-Ravasan et al. 2009, Quaresma et al. 2012, Soares et al. 2014, Gebresilassie et al. 2015).

In previous studies, PCR of a 359 bp fragment of the conserved gene cytb and further sequencing were applied in the study of blood meal sources in sand flies collected in the area of a leishmaniasis outbreak occurring in southwest Madrid region, Spain. This method provided significant information about the role that hares and rabbits are playing as reservoirs, in the focus (Jiménez et al. 2013, 2014). The aim of this work was to develop a PCR-RFLP method targeting the fragment of 359 bp of vertebrate cytb gene. The protocol was validated in twenty six blood fed female sand flies collected in the field with CDC traps in three separate entomological surveys carried out during 2012, 2013 and 2014 in three different regions of Spain. Blood meal preferences in all sand flies were studied at first by PCR of the 359 bp fragment of cytb following sequencing. The developed cytbPCR-RFLP proved to be an effective and reliable approach in the identification of blood meals in sand flies, alternative to the PCR and sequencing method, to be used in entomological surveys.
2. Material and methods

2.1. Sand flies and DNA extraction

Female sand flies used in this study were collected with CDC traps in three separate entomological surveys carried out in the transmission period of 2012, 2013 and 2014 in three different regions from Spain: Fuenlabrada (southwest Madrid, center of Spain, n=17), Girona (northeast Catalonia, northeast of Spain, n= 7) and Minorca (Balearic Islands, n=2) (Table 1). In all cases sand flies were stored in ethanol 70º at 4ºC until processed.

Sand flies were washed individually in sterile distilled water using ELISA plates to eliminate ethanol. After that, wings and legs were removed, and head and genitalia were cleared and mounted in Hoyer medium for later morphological identification under the microscope.

DNA of sand flies was obtained from thorax and abdomen using the DNeasy® Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany). Quantification and purity of the DNA was determined by spectrophotometry with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). In all cases DNA samples were stored at -20 ºC until use.

2.2. PCR-cytband sequence analysis

Blood meal identification in sand flies was conducted by the amplification of a fragment of 359 bp of vertebrate cytb gene followed by sequencing as described before (Jiménez et al. 2013, 2014). Multiple sequence alignment was obtained by Clustal W method in Megalign program (DNASTAR, Lasergen v7.1®, Madison, WI) with homologous sequences of the 359 bp cytb gene fragment of different species of
vertebrates found in sand flies blood meals obtained using BLAST (http://blast.ncbi.nlm.nih.gov/) and retrieved from Genbank™. The following sequences were used: *Canis familiaris* (DQ309764.1); *Felis catus* (AY509646.1); *Homo sapiens* (KJ154500.1, KM102050.1, KM213520.1); *Lepus granatensis* (JF299042.1) and *L. timidus* (HM233015.1); *Oryctolagus cuniculus* (HG810791.1, HQ596486.1, FM205927.1); *Capra hircus* (FM205715.1, GU120393.1) *Equus caballus* (KF038165.1); *Gallus gallus* (KF981434.1) and *Ovis aries* (AJ971339.1, JX567811.1, KF977846.1). The predicted endonuclease restriction sites *Hae*III and *Hinf*I were analyzed with the bioinformatics program Seqbuilder (DNASTAR, Lasergen v7.1®, Madison, WI)(Fig. 1).

2.3. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism of cytb (*PCR-RFLP*cyt b)

Amplification of a fragment of 359 bp of cytb gene was performed as defined before with few modifications (Jiménez et al. 2013, 2014). Degenerated primers: cyt_bb1 (5´-CCA TCM AAC ATY TCA DCA TGA TGA AA-3´) and cyt_bb2 (5´-GCH CCT CAG AAT GAY ATT TGK CCT CA-3) were used (Svobodova et al. 2009). PCR was carried out with 70 ng of DNA in a final volume of 25 μl: 1x Buffer (Roche®, Basel, Switzerland), 1.5 mM MgCl₂ (Roche®, Basel, Switzerland), 100 μM dNTPs mixture (10 mM, Roche®, Basel, Switzerland), AmpliTaqGold (Roche®, Basel, Switzerland), 1 μl of BSA DNase Free (20 mg/ml, Roche®, Basel, Switzerland). Primers were used in a concentration of 0.5 μM. PCR amplification was performed as follows: one cycle of 9 minutes at 95°C, followed by 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 45 seconds; final elongation was at 72°C for 10 minutes (ThermocycleGeneAmp ® PCR
system 2700, Applied Biosystems, Foster, CA). Subsequently, 25 µL of each PCR product was digested with 3U of HaeIII and 5U of HinfI at 37°C overnight in 30 µl of reaction volume according to the manufacturer’s protocols (New England Biolabs, UK). The digested PCR products were then fractionated in 3% MS-8 agarose (Conda®, Spain) gel stained with Pronasafe Nucleic Acid Staining Solution (10 mg/ml) (Conda®, Spain) and specific band patterns were observed under UV light exposure.

3. Results and Discussion

A total of twenty-six blood fed sand flies captured in three different entomological surveys performed in different locations during the transmission period of 2012, 2013 and 2014 were selected for this study. Of these, seventeen fed female sand flies were captured in the active human leishmaniasis focus of Fuenlabrada (Madrid). Moreover, seven sand flies from Girona (Catalonia) and two from Minorca (Balearic Islands) were analyzed (Table 1).

Firstly, the analysis of blood preferences was carried out by the amplification of a fragment of 359bp of vertebrate cytb gene, further sequencing, and subsequent comparison with homologous sequences deposited in the GenBank™ using BLAST (http://blast.ncbi.nlm.nih.gov/). Regarding to the sand flies from Madrid, all were identified as *P. perniciosus*. Analysis of blood feeding preferences revealed that sand flies mainly fed on rabbit (n=9). Other sources of vertebrates found were hare (n=4), human (n=2), cat (n=1) and dog (n=1) (Table 1).

Moreover, sand flies from Girona were identified as *P. perniciosus* (n=2) and *P. ariasi* (n=3). From these, one *P. perniciosus* was fed on horse and two *P. ariasi*...
sheep. Additionally, two mixed blood meals—sheep/human and sheep/goat—were detected in both *P. perniciosus* (*n*=1) and *P. ariasi* (*n*=1), respectively. In the remaining two sand flies from Girona (species not identified, there were no genitalia) mixed blood meals—sheep/goat—were detected. The two *P. perniciosus* from Minorca were fed on chicken and goat, respectively (Table 1).

Analysis of multiple sequence alignment by CLUSTAL W method of homologous sequences of 359 bp of *cytb* was carried out searching for specific restriction sites in each sequence for a RFLP approach. Restriction endonucleases *Hae*III and *Hinf*I were able to distinguish a differential pattern of fragments in the targeted species according with the results obtained by the bioinformatic program Seqbuilder (Fig. 1). With the aim to confirm these bioinformatic analysis, PCR products of 359 bp *cyt b* gene obtained from fed sand flies previously described were digested with restriction endonucleases *Hae*III and *Hinf*I.

The RFLP band pattern produced by these two restriction enzymes used in combination is able to discriminate among the 359 bp *cytb* fragments of all the vertebrates analyzed (Fig. 2 and 3). In addition, the band pattern obtained using *Hae*III and *Hinf*I shows differences in the 359 bp *cytb* fragment even among closely related species of hares as *L. granatensis* and *L. timidus*, which appear in Spain as an introgression of the *cytb* gene (Alves et al. 2003, Melo-Ferreira et al. 2012) (Fig. 2A and 3A). In this way, hares have been demonstrated to be an important reservoir in the active focus of leishmaniasis in southwest of Madrid. In this sense, this PCR-RFLP *cyt b* method allows to differentiate this reservoir from the others found in the focus (Molina et al. 2012, Jiménez et al. 2013).

Moreover, digestion with restriction enzyme *Hae*III was not able to discriminate among two vertebrate sequences (Fig. 2D), this is the case of *E. caballus*
(KF038165.1, lane 3) and G. gallus (KF981434.1, lane 4) giving rise to 20, 75, 105 and 159 bp band pattern. On the other hand, HinfI enzyme solves this match by providing a different band pattern enable to distinguish these species (Fig. 3C). Otherwise, HinfI could not differentiate between sequences (Fig. 3A) as L. granatensis (JF299042.1, lane 6) and O. cuniculus (HQ596486.1, lane 1) appear with an identical band pattern of 25, 125 and 162 bp; or between L. timidus (HM233015.1, lane 8) and O. cuniculus (HG810791.1, lanes 2, 4, 7; FM205927.1, lane 3), which match in a band pattern of 125 and 234 bp (Table 1 and Fig. 3A). In these cases, HaeIII is able to distinguish these species (Fig. 2A). Consequently, it is necessary to use the two restriction enzymes in order to obtain conclusive results.

Unexpected bands appear in the gel visualization in some specimens. Partial digestion or contamination seems unlikely; consequently the most probable explanation of these bands could be the co-amplification of cytb pseudogenes, as reported before (Meyer et al. 1995 and Partis et al. 2000).

Moreover, when analyzing blood meals of female sand flies from Fuenlabrada fed on rabbits and hares, we found some nucleotide polymorphism between sequences of the same species. Some of these differences don’t affect the restriction sites and in BLAST analysis homologous sequences JF299042.1 and HG810791.1 matching cytb sequences corresponding to L. granatensis and O. cuniculus present the maximum similarity situated between 97-99% (b labelled in Table 1). On the other hand, some nucleotide differences affect the restriction sites like in rabbit and goat sequences. Thus, 359 bp cytb sequences that match with GenbankTM homologous sequences O. cuniculus (HG810791.1) and C. hircus (GU120393.1) present one more restriction site with HaeIII, located at position nt=76 and nt=235, respectively (c labelled in Table 1, Fig. 1). In the case of restriction enzyme HinfI, the homologous
sequence in Genbank™ HQ596486.1 shows one more restriction site at position nt=165, which is not present in the other sequences of rabbits (labelled in Table 1, Fig. 1). In case of homologous Genbank™ sequence DQ309764.1, corresponding to C. familiaris, HinfI cuts in two sites according to bioinformatics study, however gel visualization did not show any band pattern (Fig. 2D and 3A). These differences are visible in the band fragment pattern and might possibly be due to polymorphism within the species. This intra-specific variation in cytb gene has been previously reported (Meyer et al. 1995, Branco et al. 2000).

Study of mixed blood meals by sequencing can be difficult in some cases due to the apparition of background peaks that complicate the identification of possible mixed blood meals. Similarly, although the ELISA and PCR-RLB techniques may allow identifying mixed blood meals by testing different antigens and probes, respectively (De Colmenares et al. 1995, Rossi et al. 2008, Abassi et al. 2009) both methods are more complex than PCR-RFLP. Moreover, in ELISA based methods frequently cross-reactions were observed between close species (De Colmenares et al. 1995).

On the other hand, in PCR-RLB the design and application of species-specific probes for a large number of different hosts is difficult to optimize and several problems with cross-hybridization occur (Abassi et al. 2009).

As an alternative, PCR-RFLP methodology shows a simple analyzable band pattern. In this study, mixed blood meals from four sand flies were distinguished by cytb-RFLP protocol, all of them from Girona (Table 1). Both restriction enzymes, HaeIII and HinfI, generate a differential pattern that allows distinguishing the mixed blood meal from human and sheep, (75, 125, 159 / 20, 105, 234 bp) and (64, 295 / 162, 197bp), respectively found in P. perniciosus (Fig. 2B and Fig 3A, 3B). In case of goat/sheep mixed blood meals found in one P. ariasi and two unclassified sand flies,
the restriction enzyme study at first indicated that only \textit{Hinf}I was able to discriminate between the two species (Fig. 1 and Table 1), although band pattern visualization after digestion with both enzymes showed that the two restriction enzymes could differentiate this mixed blood meal (Fig. 2C and Fig. 3B). This discrepancy among the bioinformatic study and band pattern visualization may possibly be caused by polymorphism in the nucleotide sequence, as explained before.

Accordingly, the \textit{cytb}-RFLP presented here supportsthe results previously obtained by PCR following by sequencing of the 359 bp fragment of \textit{cytb}\cite{Jiménez2013, Jiménez2014}. Moreover, this method is less time consuming and could be an alternative to sequencing.

In conclusion, this PCR-RFLP\textit{cytb} method showstobe highly effective for the analysis of blood meal preferences and the differentiation of mixed blood meals in phlebotomine sand flies, caught in Spain. Although, this method should be evaluated and validated by investigators in the specific regions of study in order to apply it in the identification of sand fly blood meal preferences.

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\section*{Conflict of interest}
The authors declare no conflicts of interest.

References


Table 1. Sandfly description and corresponding blood meal identification by PCR-sequencing and cyt-b-RFLP.

<table>
<thead>
<tr>
<th>Sandfly identification</th>
<th>Sand fly origin/ year</th>
<th>PCR cyt-b-sequencing</th>
<th>Genbank™ homologous sequence (% Similarity)</th>
<th>cyt-b-RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HaeIII (bp)</td>
</tr>
<tr>
<td><em>P. perniciosus</em></td>
<td>Madrid/ 2013</td>
<td>Oryctolagus cuniculus</td>
<td>HG810791.1^{b,c} (97%)</td>
<td>30, 45, 131, 153</td>
</tr>
<tr>
<td></td>
<td>Madrid/ 2013</td>
<td>Lepus timidus</td>
<td>FJ299042.1^{b} (99%)</td>
<td>75, 284</td>
</tr>
<tr>
<td><em>P. ariasi</em></td>
<td>Girona/ 2012</td>
<td>Ovis aries</td>
<td>JX567811.1^{c} (99%)</td>
<td>54, 75, 230</td>
</tr>
<tr>
<td><em>P. ariasi</em></td>
<td>Girona/ 2012</td>
<td>Ovis aries/ Caprahircus</td>
<td>AJ711339.1^{c} (96%)/GU120393.1^{c} (96%)</td>
<td>75, 25, 125</td>
</tr>
<tr>
<td><em>N. simus</em></td>
<td>Girona/ 2012</td>
<td>Ovis aries/ Caprahircus</td>
<td>AJ711339.1^{c} (96%)/GU120393.1^{c} (96%)</td>
<td>75, 125, 159</td>
</tr>
<tr>
<td><em>P. perniciosus</em></td>
<td>Minorca/ 2013</td>
<td>Caprahircus</td>
<td>FM205715.1^{c} (99%)</td>
<td>54, 75, 230</td>
</tr>
<tr>
<td><em>P. perniciosus</em></td>
<td>Minorca/ 2013</td>
<td>Gallus gallus</td>
<td>KF981434.1^{c} (99%)</td>
<td>20, 75, 105, 159</td>
</tr>
</tbody>
</table>
Fig. 1. Alignment of 359 bp cyt b fragment of the different species of vertebrates, remarking restriction sites of *Hae*III (‘GGCC’) (continuous line) and *Hinfl* (‘GANTC’) (broken line). The following sequences were used: *C. familiaris* (DQ309764.1); *F. catus* (AY509646.1); *H. sapiens* (KJ154700.1, KM102050.1, KM213520.1); *L. granatensis* (JF299042.1) and *L. timidus* (HM233015.1); *O. cuniculus* (HG810791.1, HQ596486.1, FM205927.1); *C. hircus* (FM205715.1, GU120393.1) *E. caballus*
(KF038165.1); *G. gallus* (KF981434.1) and *O. aries* (AJ971339.1, JX567811.1, KF977846.1).

**Fig. 2.** *Cytb* PCR products digested with restriction enzyme *HaeIII*.

**A:** Lane *M1*, 100 bp molecular weight marker (Biotools), *lane 1 O. cuniculus* (HQ596486.1; 45, 131, 183 bp), *lanes 2, 4 and 5 O. cuniculus* (HG810791.1; 30, 45, 131, 153 bp), *lane 3 O. cuniculus* (FM205927.1; 45, 131, 183 bp), *lane 6 H. sapiens* (20, 105, 234 bp), *lane 7 L. granatensis* (131, 228 bp), *lane 8 L. timidus* (75, 284 bp), *lane M2 50 bp molecular weight marker* (Takara).

**B:** Lane *M* 100 bp molecular weight marker (Biotools), *lane 1 O. aries/H. sapiens*, *lane 2 H. sapiens*, *lane 2 O. aries*.

**C:** Lane *M1*, 100 bp molecular weight marker (Biotools), *lane 1, 2 and 3 mixed blood meal O. aries/C. hircus*, *lane 4 C. hircus* (54, 75, 230), *lane 5 O. aries* (75, 125, 159 bp), *lane M2 50 bp molecular weight marker* (Takara).

**D:** Lane *1 F. catus* (11, 20, 74, 254 bp), *lane 2 C.*
familiaris(359 bp), lanes 3 E. caballus(20, 75, 105, 159 bp), lane 4 G. gallus(20, 75, 105, 159 bp), lane M 100 bp molecular weight marker (Biotools).

Fig. 3. Cytb PCR products digested with restriction enzyme HinfI.

A: Lane M 100 bp molecular weight marker (Biotools), lane 1 O. cuniculus(HQ596486.1; 72, 125, 162 bp), lanes 2, 4 and 7 O. cuniculus(HG810791.1; 124, 235 bp), lane 3 O. cuniculus(FM205927.1; 124, 235 bp), lane 5 H. sapiens (162, 197 bp), lane 6 L. granatensis(72, 135, 162 bp), lane 8 L. timidus(124, 235 bp), lane 9 F. catus(45, 79, 117, 117 bp), lane 10 C. familiaris(9, 55, 295 bp). B: Lane M 100 bp molecular weight marker (Biotools), lane 1 to 3 mixed blood meal O. aries/C. hircus, lane 4 O. aries(64, 295 bp), lane 5 C. hircus(163, 196 bp).
bp). Lane M 100bp molecular weight marker (Biotools), lane 1 E. caballus (45, 80, 234 bp), lane 2 G. gallus (10, 162, 187 bp).