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Molecular detection of *Leishmania infantum* in naturally infected *Phlebotomus perniciosus* from Algarve Region, Portugal

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Abstract

Background & objectives: In Portugal, *Phlebotomus perniciosus* and *P. ariasi*, (Subgenus *Larroussius;* Diptera: Psychodidae) are the proven vectors of leishmaniasis caused by *Leishmania infantum*. The Algarve Region in southern Portugal has been considered an endemic focus of leishmaniasis since 1980s. The main objective of the present study was to validate a molecular approach to detect *Leishmania* infection in phlebotomines based on DNA extraction from the female sandfly whole body, minus genitalia, followed by PCR for application on epidemiological surveys.

Methods: In Algarve Region, from early May until early November 2006, sandflies were captured by CDC miniature light-traps. kDNA-PCR and ITS1-PCR were used to screen the presence of *Leishmania* DNA in female sandflies after species identification by entomological keys.

Results: A total of 474 sandflies were collected in 108 biotopes. One female of *P. perniciosus*, the predominant species, was found infected with *L. infantum* reflecting an overall infection rate of 0.47%.

Interpretation & conclusion: PCR associated with morphological characterization of the sandflies will be a powerful epidemiological tool for the determination of the number of phlebotomines infected with *Leishmania* spp in nature. In addition, the simultaneous occurrence of dogs and *P. perniciosus* infected with *L. infantum* shows that Algarve continues to be an endemic focus of canine leishmaniasis. Furthermore, as *P. sergenti* and *P. papatasi* which transmit *L. tropica* and *L. major*, respectively were present, the future introduction of these two *Leishmania* species in southern region of Portugal should not be neglected.

Key words *Leishmania infantum* – PCR – *Phlebotomus perniciosus* – Portugal

Introduction

Leishmaniasis caused by *Leishmania infantum* is an endemic zoonosis in the Mediterranean basin[1]. It is a serious public health and veterinary problem. Dogs are the major hosts for these parasites, and the main reservoir of human visceral infection. Endemicity of leishmaniasis is associated with the distribution and abundance of phlebotomine sandfly vectors. In Portugal, *Phlebotomus perniciosus* Newstead and *P. ariasi* Tonnoir belonging to the subgenus *Larroussius* (Diptera: Psychodidae) are the proven vectors of *L. infantum*[2]. The Algarve Region in southern Portugal was considered an endemic focus of human leishmaniasis in the 1980s. Forty, three infantile cases of visceral leishmaniasis were diagnosed in the Pediatric Service at the Faro District Hospital between 1980 and 1988, most of them were from Loulé Municipal-

ity[3]. In a canine leishmaniasis seroepidemiological survey performed in Loulé in the 1990s, a prevalence of 7% was found[4]. However, in the last two decades, visceral leishmaniasis in this region was only notified in immunocompromised patients suggesting that humans only develop this disease in particular conditions, such as immunosuppression.

In 1979, Pires[5] collected the first specimens of *P. perniciosus, P. ariasi* and *Sergentomyia minuta* Rondani in Algarve Region. Ten years later, Schrey *et al*[6] observed *P. perniciosus* infected with *Leishmania* promastigotes for the first time. In 1993, Alves-Pires *et al*[7] found one *P. perniciosus* female infected with *L. infantum*.

The main objective of the present study was to validate a molecular approach to detect *Leishmania* infection in phlebotomines by DNA extraction from the female sandfly whole body, minus genitalia, followed by PCR for application on epidemiological surveys.

Material & Methods

In Algarve Region, from early May until early November 2006, Centers for Disease Control and Prevention (CDC) miniature light-traps were set out from sunset to sunrise in 108 biotopes. Collections were made in the counties of Lagos, Portimão and Silves, in the southwest area of Algarve ('Barlavento') as well as in Faro, Olhão, Loulé and São Brás de Alportel from southeast area ('Sotavento') (Fig. 1). Collecting places were in cottage houses with yard and garden. Dogs, cats and poultry were present in all of them. All CDC light-traps were placed outside the houses. Sandflies collected were preserved in 70% ethanol and sent to the laboratory of the Institute of Tropical Medicine and Hygiene, Lisbon, 300 km away from Algarve, and maintained at room temperature (22–26°C) up to six months for further analysis. Genitalia of individual sandfly female and complete male specimens were used for morphological identification of the invertebrate host according to Pires[5]. After species identification, the rest of the body from each

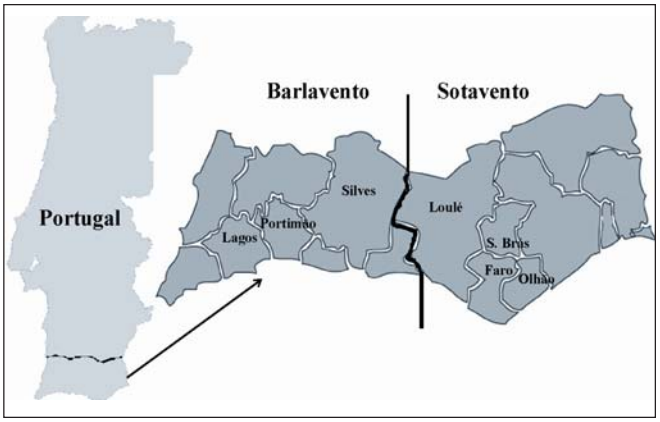

Fig. 1: Map of Algarve Region with the counties where sandfly collections were made.

female was used for molecular analysis. The female's body (minus genitalia) conserved in lysis buffer [200 µl from PCR kit (PCR-Template Preparation kit, Roche Diagnostics GmbH, Germany)] was smashed and incubated with the buffer and proteinase K (40 µl). The DNA was then extracted according to the instructions of the manufacturer. The PCR amplification for detection of *L. infantum* in *P. perniciosus* and *P. ariasi* females was performed using specific primers for two molecular markers: internal transcribed spacer 1 (ITS1) of the ribosomal operon of *Leishmania*[8] and kinetoplastid minicircle DNA (kDNA) sequence of *L. donovani* complex[9]. Amplification of 314 base pair (bp) and 447 bp PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide. The ITS1 primers were also used to screen *L. tropica* DNA in *P. sergenti* (Parrot) females. A positive control (*L. infantum* and *L. tropica* DNA) and a negative control without DNA template were included.

PCR amplified product was sequenced on both strands using kit BigDyeTerminator v1.1 Applied Biosystem, ABI PRISM 3700 DNA Analyzer (Stabvida® Sequence Service, Portugal). After purification the amplified PCR-ITS1 product was digested with *Hae*III[8] and analyzed in 3% agarose gel. To exclude the possibility of PCR inhibition and to control the extraction procedure, ITS2 region from phlebotomine genome was amplified by PCR using primers based on conserved sequences of the 5.8 S

and 28 S coding regions[10]. All extracted sandfly DNA samples were amplified (459 bp) confirming that no PCR inhibitors were present.

Results & Discussion

A total of 474 sandflies were collected between 14 May and 5 November 2006. The prevalence of species was 90.51% for *P. perniciosus*, 6.33% for *S. minuta* and 2.11% for *P. sergenti*. Few specimens of *P. ariasi* (0.84%) and *P. papatasi* (0.21%) were also collected (Table 1). The highest phlebotomine density was observed in July with collection of specimens from all species (Table 2). Although the light-traps were set out in November, only one *P. ariasi* was collected in this month probably because of its humidity preferences.

In one *P. perniciosus* female sample, collected in July in a cottage´s garden in Silves county (Fig. 2), *Leishmania* DNA was detected. Gene sequence of the kDNA product was compared with the *Leishmania* sequences available in the GeneBank showing a homology of about 98% with *L. infantum.* After *Hae*III digestion of ITS1 PCR product, the three characteristic *L. infantum* restriction fragments (184 bp, 72 bp and 55 bp) were observed.

Table 1. Numbers of sandfly species collected in Algarve Region, Portugal and their relative abundance (%)

Species	Male	Female	Total	%
P. perniciosus	236	193	429	90.51
P. sergenti	4	6	10	2.11
P. ariasi	1	3	4	0.84
P. papatasi	1	0	1	0.21
S. minuta	19	11	30	6.33
Total	261	213	474	100

Fig. 2: The biotope where the infected sandfly was collected.

Table 2. Phlebotomine densities per hight trap in Algarve Region, Portugal, between May and November 2006

Species	May	Jun	Jul	Aug	Sep	Oct	Nov
P. perniciosus	0.36	1.68	13.45	2.17	4.93	1.09	0
P. ariasi	0.09	0.05	0.05	0	0	0	1
P. sergenti	0	0.32	0.15	0.08	0	0	0
P. papatasi	0	0	0.05	0	0	0	0
S. minuta	0	0	1.05	0.25	0.33	0	0
Total*	0.45	2.05	14.75	2.5	5.26	1.09	1

*Sandfly density = Total number of sandflies/CDC light-trap (month).

The *L. infantum* detection in one female reflects an overall infection rate of 0.47% (1/213) and an infection rate of *P. perniciosus* of 0.52% (1/193) in Algarve Region. This result was similar to the infection rate of 0.35% obtained by Alves-Pires *et al*[7] by microscopic dissection of 287 *P. perniciosus* females collected between June and October 1993. In both studies, CDC light-traps were used for sandfly collections. On the other hand, Schrey *et al*[6] found a *P. perniciosus* infection rate of about 5% between June and August 1986 in Barlavento and Sotavento subregions of Algarve. In their study, from a total of 29 *P. perniciosus* found infected, 22 were collected by aspiration. The differences between results could have been attributed to the active way of collection used in the study, which have probably enhanced the possibility of getting *Leishmania* infected specimens.

In the present study, it was observed by microscopical observation that the infected sandfly was gravid allowing us to hypothesize that it was not only a parasite DNA carrier but a competent vector. Although PCR only detects the number of positive specimens, e.g. *Leishmania* carrier status of sandflies, it has several advantages such as: (i) high sensitivity and specificity, (ii) can be used in ethanol-fixed sandflies, and (iii) allows processing of a large number of specimens with limited effort overcoming the laborious dissection method. Since in this study on-site sandfly dissection was not possible to be performed, the only way of acquiring data on the *Leishmania* presence in phlebotomine populations was to use ethanol preserved flies. It could be suggested that in the future field studies conducted in areas where it is difficult to perform sandfly dissection and identification, specimens can be collected and preserved at room temperature for months until morphological species identification and molecular analysis could be performed in the laboratories. Nevertheless, molecular diagnosis in extensive studies would be complicated due to its relative high cost (DNA extraction kit and Taq polymerase), particularly when each sandfly female is analyzed separately, as done in this study. To overcome this economic issue, recent studies were carried out by PCR analysis of sandfly pools. However, the use of pools reduces the possibility of analyzing important information at a household level, such as the ecology of trap placement, since sandflies collected in the field do not produce homogeneous sample sizes[11].

The simultaneous occurrence of dogs, 38 infected out of 132 (data not showed), and *P. perniciosus* infected with *L. infantum* in the Algarve Region shows: (i) the zoophylic preferences of this sandfly species, (ii) that the region continues to be an endemic focus for canine leishmaniasis, and (iii) the potential transmission of infection to humans. The gap between the number of human and canine leishmaniasis cases observed in this region emphasizes the fact that human leishmaniasis can be considered accidental or an opportunistic infection. On the other hand, climate changes would enhance the number of days favourable for transmission of parasites, due to higher density and activity of sandflies during a longer period, which will increase the incidence of leishmaniasis not only in animals but also in humans[1].

In addition, our results reinforce that *P. perniciosus* can be considered the principal vector of leishmaniasis in this region not only due to the fact that it was the only species found infected with *L. infantum* but also to its high abundance and distribution. The detection of an infected *P. perniciosus* in Silves means that vectorial infection by *L. infantum* still occurs in the southwest part of Algarve Region (Barlavento). On the other hand, *P. sergenti* and *P. papatasi*, the vectors of *L. tropica* and *L. major*, respectively have also been identified in Algarve Region as well as in other geographic areas of Portugal[5]. No *L. tropica* DNA was amplified from *P. sergenti* specimens collected in this study. Despite human cases of *L. tropica* and *L. major* infections are not known to exist in Portugal, *L. major/L. infantum* hybrids have already been identified in autochthonous human leishmaniasis cases[12]. The risk of potential introduction of these new *Leishmania* species from travellers or immigrants from North Africa and from the Indian subcontinent should not be neglected, since their vectors exist in the country, emphasizing the need of

going on with systematic epidemiologic surveys on *Leishmania* reservoir hosts and vectors.

From this study it can be concluded that PCR for *Leishmania* identification in the sandfly associated with sandfly´s morphological characterization will be a powerful tool for the knowledge of leishmaniasis epidemiology and for the development and implementation of programmes to prevent and control this neglected disease.

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References

1. Dujardin J, Campino L, Cañavate C, Dedet J, Gradoni L, Soteriadou K, *et al*. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. *Emerg Infect Dis* 2008; *14:* 1013–8.
2. Pires C. *Phlebotomus* of Portugal. I—Natural infestation of *Phlebotomus ariasi* Tonnoir, 1921 and *Phlebotomus perniciosus* Newstead, 1911, by *Leishmania* in the zoonotic focus of Arrabida (Portugal). *Ann Parasitol Hum Comp* 1984; *59:* 521–4.
3. Vicente A. Kala-azar infantil. Dados epidemiológicos e incidência do kala-azar no Internamento de Pediatria do Hospital de Faro. *Rev Port D Infect* 1990; *13:* 173–82.
4. Campino L, Pratlong F, Abranches P, Rioux JA, Santos-Gomes G, Alves-Pires C, *et al*. Leishmaniasis in Portugal: enzyme polymorphism of *Leishmania infantum* based on the identification of 213 strains. *Trop Med Int Health* 2006; *11:* 1708–14.
5. Pires C. Contribuição para o conhecimento da distribuição e bioecologia dos flebótomos em Portugal (Díptera, Psychodidae). *B Soc Port Ciên Nat* 1979; *19:* 197–210.
6. Schrey C, Pires C, Macvean D. Distribution of phlebotomine sandflies and the rate of their infection with *Leishmania* promastigotes in the Algarve, Portugal. *Med Vet Entomol* 1989; *3:* 125–30.
7. Alves-Pires C, Campino L, Afonso MO, Santos-Gomes G, Dedet J, Pratlong F. Les Phlébotomes du Portugal X-Infestation naturelle de *Phlebotomus perniciosus* par *Leishmania infantum* MON-1 en Algarve. *Parasite* 2001; *8:* 374–5.
8. Schonian G, Nazereddin A, Dinse N, Schweynoch C, Shallig H, Presber W, *et al*. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis* 2003; *47:* 349–58.
9. Cortes S, Rolão N, Ramada J, Campino L. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *Leishmania donovani* s.l. specific kinetoplastid primers. *Trans R Soc Trop Med Hyg* 2004; *98:* 12–7.
10. Collins F, Paskewitz S. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Mol Biol* 1996; *5:* 1–9.
11. Bhattarai N, Das M, Rijal S, Van der Auwera G, Picado A, Khanal B, *et al*. Natural infection of *Phlebotomus argentipes* with *Leishmania* and other trypanosomatids in a visceral leishmaniasis endemic region of Nepal. *Trans R Soc Trop Med Hyg* 2009; *103:*1087–92.
12. Ravel C, Cortes S, Pratlong F, Morio F, Dedet J, Campino L. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*. *Int J Parasitol* 2006; *36:* 1383–8.

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