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Research paper

Detection of *Leishmania infantum* DNA in phlebotomine sand flies from an area where canine leishmaniosis is endemic in southern Italy

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ABSTRACT

Phlebotomine sand flies (Diptera, Psychodidae) are vectors of *Leishmania* spp., among which *Leishmania infantum* is recognized as the main agent of human and canine leishmaniosis (CanL) in the Mediterranean area. In this study, females of *Phlebotomus* spp. (*P. perniciosus*, *P. neglectus* and *P. papatasi*) and *Sergentomyia minuta* were collected in a dog shelter of southern Italy, where CanL is endemic, and examined for *Leishmania* DNA. In total, 32 out of 56 of *Phlebotomus* spp. insects (57.1%) were found positive for *L. infantum* DNA by quantitative PCR (qPCR), with a mean parasite load of 1.9×10^3 promastigotes/ml among 23 positive *P. perniciosus* and 2.1×10^3 promastigotes/ml among five positive *P. neglectus*. Four *P. papatasi*, a species known to be refractory to *L. infantum* development, were also found positive. Among 216 *S. minuta* specimens examined, 25 (11.6%) scored positive for *Leishmania tarentolae* by conventional nested PCR; two (16.7%) of them were also positive for lizard blood, which is in agreement with the feeding preference of this phlebotomine species. Nine *S. minuta* (4.2%) were positive for *L. infantum* by qPCR, with a mean parasite load of 1.62×10^2 promastigotes/ml. The detection of *L. infantum* DNA in *S. minuta* may suggest that this species could acquire the protozoan, occasionally feeding on infected dogs. Further investigations need to clarify the potential role that *S. minuta* may have in the transmission of *L. infantum* to receptive mammal hosts.

1. Introduction

The leishmanioses are zoonotic or anthroponotic diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae) transmitted by phlebotomine sand flies of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) (Munstermann et al., 2004). *Leishmania infantum*, a major agent of canine and human leishmaniosis, is endemic throughout the Mediterranean basin. In Italy, this parasite has spread from traditional endemic areas of southern and insular regions to central and northern areas (Maroli et al., 2008; Otranto and Dantas-Torres, 2010). Italian phlebotomine species include *Phlebotomus ariasi*, *Phlebotomus mascittii*, *Phlebotomus neglectus*, *Phlebotomus papatasi*, *Phlebotomus perfiliewi*, *Phlebotomus perniciosus*, *Phlebotomus sergenti* and *Sergentomyia minuta* (Maroli et al., 1994; D'Urso et al., 2004). Of these, *P. perniciosus* and *P. perfiliewi* were conclusively incriminated as vectors of *L. infantum* in Italy, whereas *P. ariasi* and *P. neglectus* were so in neighboring countries (Maroli et al., 2013). *Sergentomyia minuta,* a species that feeds primarily on cold-blooded animals such as lizards (Lewis, 1987), was found infected by *Leishmania* (*Sauroleishmania*) *tarentolae*, in southern Italy (Maroli et al., 1988). This protozoan species appears to be widespread in Gekkonidae species of the Apulia region of Italy, mainly *Tarentola mauritanica* (Pozio et al., 1983). Although *Sergentomyia* spp. are responsible for the transmission of *Leishmania* (*Sauroleishmania*) spp., the presence of DNA of *Leishmania* spp. pathogenic to humans has been reported in sand flies of this genus (Berdjane-Brouk et al., 2012; Campino et al., 2013; Kanjanopas et al., 2013; Jaouadi et al., 2015; Maia et al., 2015; Bravo-Barriga et al., 2016).

In particular, *L. infantum* DNA has been molecularly detected in *Sergentomyia dubia*, *Sergentomyia magna* and *Sergentomyia schewtzi* in Senegal (Senghor et al., 2016). Furthermore, since *S. minuta* were collected in the same area endemic for *L. infantum* along with *P. perniciosus* and *P. neglectus* (Tarallo et al., 2010; Dantas-Torres et al., 2014; Bravo-Barriga et al., 2016), the possibility for *S. minuta* to acquire the pathogen during blood meal could be suspected, provided that they are attracted to and feed on dogs. Indeed, the occurrence of a high number

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of *S. minuta* collected during a 1-year entomological survey in an area endemic for *L. infantum* of southern Italy (Tarallo et al., 2010) represents the foundation for the investigation herein described.

Thus, the aim of this study was to detect and quantify the amount of *L. infantum* kinetoplast DNA (kDNA) in *S. minuta* and *Phlebotomus* spp. collected from a dog shelter where a high incidence of *L. infantum* infections was recorded during a sand fly season (Otranto et al., 2010).

2. Materials and methods

2.1. Study area and sand flies

Trapping of sand flies was performed from June through October 2009 using sticky traps from a dog shelter in Putignano (40°51′N, 17°07′E; province of Bari, Apulia region, Italy), where a 47.6% prevalence of canine *Leishmania* infection was recorded in a previous study (Otranto et al., 2010). All samples were identified using morphological keys provided by Killick-Kendrick et al. (1991) and Dantas-Torres et al. (2014). All specimens were also molecularly identified following the procedures described elsewhere (Latrofa et al., 2011, 2012).

2.2. DNA extraction, parasite identification and blood-meal determination

Genomic DNA was extracted from individual insects using DNeasy Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany) in accordance to the manufacturer's instructions. The detection of *L. infantum* kDNA minicircle was achieved using primers, probes and protocol described elsewhere (Francino et al., 2006; Dantas-Torres et al., 2011). A 10-fold dilution series of standard DNA from promastigotes (log phase concentration, 1.7×10^6 promastigotes/ml) of *L. infantum* zymodeme MON-1 (strain MHOM/TN/80/IPT1) were used for the quantification DNA and the detection limits of the qPCR was assessed using serial dilutions from 1.7×10^1 to 1.7×10^{-3} promastigotes/ml.

All sand fly specimens were also tested for detection of *L. tarentolae* DNA by nested-PCR using primers targeting the internal transcribed spacer 1 (ITS-1) and small subunit of ribosomal DNA (SSU rRNA) region, followed by restriction fragment length polymorphisms (PCR-RFLP) using *Hae* III and *Rsa* I, respectively (Schönian et al., 2003; Rossi et al., 2008). The digested samples were examined on 3% agarose gel stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). A control *L. tarentolae* DNA (RTAR/IT/81/ISS21-G.6c) has been included in the analysis.

Conventional PCR (cPCR) was carried out for the blood-meal determination using primers targeting the vertebrate host mitochondrial cytochrome *b* (350 bp) and 16S rRNA gene (600 bp) (Whiting et al., 2003; Kent and Norris, 2005). PCR products were purified and sequenced, in both directions using the same primers as for nested and cPCR, employing the Big Dye Terminator v.3.1 chemistry in a 3130 genetic analyzer (Applied Biosystems, California, USA) in an automated sequencer (ABI-PRISM 377).

Gene sequences were aligned using ClustalW program (Larkin et al., 2007) and compared with those available in GenBank using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

Fifty-six females of *Phlebotomus* spp. (38 *P. perniciosus*, 10 *P. neglectus*, and eight *P. papatasi*) and 216 females of *S. minuta* were collected and examined. In addition, 929 males (i.e., 391 *P. perniciosus*, 188 *P. neglectus*, eight *P. papatasi* and 342 *S. minuta*) were also collected. A total of 32 *Phlebotomus* spp. (57.1%) and nine *S. minuta* (4.2%) were found positive for *L. infantum* DNA, respectively, with a mean parasite load ranging from 1.6×10^2 to 2.6×10^3 promastigotes/ml (Table 1). Of the 11 *Phlebotomus* spp. and 12 *S. minuta* females found blood engorged, 10 (91%) and 1 (8.3%), respectively, resulted positive for *L. infantum* (Table 1), with a parasite load ranging from 1.9×10 to 1.9×10^4 promastigotes/ml. The number of *L. infantum*-positive specimens by species and the corresponding mean parasite load are reported in Table 2 according to each month of collection. The majority (n = 32; 13.3%) of the *L. infantum* positive sand flies was collected in July and August, when the greatest number of specimens was caught (Table 2). The mean parasite load in specimens was consistent throughout the observation period ranging from 9.9×10^2 to 3.4×10^3 promastigotes/ml (Table 2).

Amongst 216 specimens of *S. minuta*, 25 (11.6%) scored positive for *L. tarentolae*, of which only two were blood engorged (Table 1). The majority of *S. minuta* positive for *L. tarentolae* was collected in July and August (n = 23; 11.5%), whilst only two out of nine specimens collected in October scored positive for this pathogen. Importantly, no co-infection with both protozoan species was recorded in the tested samples.

At the blood meal identification, out of 272 specimens examined, only three *S. minuta* (1.1%) scored positive for lizard DNA and no mammalian (e.g. canine or human) blood was detected in other blood-fed or -unfed specimens. Sequences of *L. tarentolae* and blood meal source showed a high nucleotide identity (98–100%) to those available in the GenBank database (Accession numbers LC086293, KC205986, AF206592).

4. Discussion

This study reports on the presence of *L. infantum* kDNA in *P. neglectus*, *P. perniciosus*, *P. papatasi* and *S. minuta* caught in a dog shelter from southern Italy, highly endemic for *L. infantum*. Nevertheless, the detection of *Leishmania* DNA alone does not prove vectorial competence of sand flies (Killick-Kendrick, 1990; Lane, 1993). For example, *Leishmania donovani* DNA was detected in *P. papatasi* and *Sergentomyia babu* in India (Mukherjee et al., 1997), and *L. infantum* DNA in *P. papatasi* in Greece (Aransay et al., 2000) and in Iran (Yavar et al., 2013). However, *L. donovani*, *L. infantum* and *Leishmania tropica* do not develop in *P. papatasi* as these parasites are eliminated after blood digestion and defecation (Pimenta et al., 1994). Nonetheless, natural *L. infantum*/*Leishmania major* hybrids may develop late-stage infections in *P. papatasi*, which indicates that these hybrid strains may circulate by this sand fly vector, increasing the risk of their spreading into new foci throughout the broad range of vector distribution (Volf et al., 2007).

The *L. infantum* PCR positivity in *P. papatasi* may be a result of recent blood feeding as this species may become infected after feeding on dogs with canine leishmaniosis (CanL), with the insect infection rate dropping from 96% after day 1 to 4% after day 7 post blood meal, as an effect of its digestion (Adler and Theodor, 1932).

The finding of *L. infantum* DNA in *P. perniciosus* and *P. neglectus* specimens was expected – although not at such a high rate – since these sand fly species are recognized as vectors of *L. infantum* in Apulia, Italy (Maroli et al., 1988), in Corfu, Greece (Léger et al., 1988) and in Albania (Velo et al., 2017), respectively. In southern Portugal, *S. minuta* was found positive for *Leishmania* sp. DNA phylogenetically related to putative agents of human and canine leishmaniosis in China (Maia et al., 2015).

The positivity of *S. minuta* for *L. infantum* DNA herein scored (4.2%) and the high number of specimens collected in an area endemic for CanL might suggest its role in the circulation of the protozoan (Maia and Depaquit, 2016). Accordingly, the overall high rate of any sand flies positive for *L. infantum* (15.1%) could be due to the high prevalence of infected dogs in that confined area (Otranto et al., 2010). Previous studies reported a percentage of sand fly species positive to *L. infantum* ranging from 2.9% to 50% (Aransay et al., 2000; Gómez-Saladín et al., 2005; Ergunay et al., 2014; González et al., 2017). The finding of *L. infantum* in *Phlebotomus* spp. and *S. minuta* herein collected from July to August with a similar parasite load (i.e., up to 9.6×10^2 and 1.3×10^2 promastigotes/ml, respectively) suggests that, in

Table 1
Number of phlebotomine sand fly species positive for *Leishmania infantum* by qPCR and for *Leishmania tarentolae* by nested-PCR. The positivity for engorged females is also reported. The mean, minimum, maximum and standard deviation (sd) values of promastigotes/ml of *L. infantum* (Starting Quantity, SQ) is reported.

Species	*Leishmania infantum*					*Leishmania tarentolae*	
	Pos (Tot)	Pos (Engorged)	SQ			Pos (Tot)	Pos (Engorged)
			Mean	Min/Max	sd		
P. perniciosus	23 (38)	7 (8)	1.9×10^3	$1.9 \times 10/9.8 \times 10^3$	3.6×10^3	–	–
P. neglecuts	5 (10)	1 (1)	2.1×10^3	$2.4 \times 10^2/1.7 \times 10^4$	3.6×10^3	–	–
P. papatasi	4 (8)	2 (2)	2.6×10^3	$4.6 \times 10^3/1.9 \times 10^4$	4.6×10^3	–	–
S. minuta	9 (216)	1 (12)	1.6×10^2	$2.4 \times 10/5.6 \times 10^2$	1.7×10^2	25 (216)	2 (12)

Table 2
Number of phlebotomine sand fly species positive for *Leishmania infantum* by qPCR, divided according to months of collection. The parasite load (Starting Quantity (SQ) mean value of promastigotes/ml) is also reported.

Species	June		July		August		September		October	
	Pos (Tot)	SQ mean	Pos (Tot)	SQ mean	Pos (Tot)	SQ mean	Pos (Tot)	SQ mean	Pos (Tot)	SQ mean
P. perniciosus	1 (1)	6.4×10^3	11 (17)	2.6×10^3	7 (11)	9.6×10^2	1 (5)	2×10^3	3 (4)	3.4×10^3
P. neglecuts	1 (1)	1.2×10^3	2 (3)	3.4×10^3	2 (4)	1.9×10^3	–	–	–	–
P. papatasi	1 (1)	1.9×10^3	3 (6)	7.6×10^3	–	–	–	–	–	–
S. minuta	1 (1)	5.6×10^2	3 (109[a])	1.3×10^2	4 (90[a])	1×10^2	–	–	1 (9[a])	9×10
Total	4 (4)	3×10^3	19 (135)	3.4×10^3	13 (106)	9.9×10^2	1 (12)	2×10^3	4 (15)	1.7×10^3

[a] Includes specimens positive for *Leishmania tarentolae*.

endemic areas, *S. minuta* may feed on infected dogs.

Accordingly, the detection of lizard blood (present study) as well as of human blood (Maia et al., 2015) in *S. minuta* is consistent with the sporadic/opportunistic anthropophilic feeding behaviour of this sand fly species. Though no scientific evidence is yet available about the role of *S. minuta* as vector of *Leishmania* spp. pathogenic to humans, experimental studies indicate the potential of *L. tarentolae* to infect/develop in human phagocytic cells (Taylor et al., 2010; Novo et al., 2015).

The detection of *L. infantum* and *L. tarentolae* DNAs in *S. minuta* suggests that studies are needed to determine the role played, if any, by this sand fly species in the transmission of these agents to humans.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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