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Succinctus

Laboratory transmission of an Asian strain of *Leishmania tropica* by the bite of the southern European sand fly *Phlebotomus perniciosus*

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ABSTRACT

Imported cases of anthroponotic cutaneous leishmaniasis due to *Leishmania tropica* are increasingly documented in Europe. We investigated the ability of *Phlebotomus perniciosus*, a competent vector of *Leishmania infantum* widespread in southwestern Europe, to support the growth and transmissibility of an Asian strain of *L. tropica* recently isolated from a refugee. Parasite growth behavior was investigated in laboratory-reared sand flies fed artificially with promastigotes as well as in sand flies infected after biting on footpad lesions induced in hamsters by promastigote inoculation. The evolution of infection was checked by gut microscopy and quantitative real-time PCR, and it was found to be similar between promastigote- and amastigote-initiated infections. In 80% of infected sand flies, despite survival and flourishing growth of promastigotes after blood digestion and defecation, either the parasites died, or failed to migrate to the foregut and/or to mature into infective forms. However, in the remaining 20% *L. tropica* developed into abundant metacyclic promastigotes. The quantitative real-time PCR assay detected variable loads of gut promastigotes irrespective of morphological evidence of viability or progressive/final death. Parasite transmissibility was investigated by exposing naive hamsters to *P. perniciosus* previously infected on chronic lesions induced in hamsters which survived to take a second blood meal. Two months post exposure, lesions developed in skin sites bitten by sand flies confirmed to harbor metacyclic promastigotes; in the following months, the presence of viable and transmissible *L. tropica* parasites in lesions was demonstrated by xenodiagnosis assays. Our findings support the hypothesis that, in particular epidemiological situations, *P. perniciosus* may play the role of an occasional *L. tropica* vector.

The leishmaniases are a group of human and animal diseases caused by kinetoplastid protozoa of the genus *Leishmania* and transmitted by the bite of phlebotomine sand flies. At least 20 recognized *Leishmania* spp. are pathogenic to humans (Akhoundi et al., 2017), resulting in diverse clinical manifestations. Two main clinical forms are prevalent worldwide, visceral leishmaniasis (VL), a life-threatening condition that results from the dissemination of *Leishmania* in macrophage-rich tissues, and cutaneous leishmaniases (CL), a benign but disfiguring skin condition which has a tendency towards spontaneous resolution. CL agents widespread in the Old World are, among others, *Leishmania tropica* and *Leishmania infantum*, which differ in geographical distribution, epidemiology and, to a lesser extent, in clinical features. CL caused by *L. tropica* is assumed to be anthroponotic with some exceptions, e.g. in Israel (Svobodova et al., 2006). The disease may exhibit hyperendemic/epidemic trends in densely populated settlements of the Middle East (Rehman et al., 2018) and central Asia, and is endemic in smaller foci of southeastern Europe, northern Africa, Kenya, Ethiopia and India (WHO, 2010). CL caused by *L. infantum* is a zoonotic entity, using dogs as the main reservoir host. It has a typical sporadic pattern and represents the only CL form endemic in southwestern and parts of southeastern Europe.

Approximately 40 phlebotomine species belonging to the *Phlebotomus* genus are proven or suspected vectors of human leishmaniases in the Old World (Maroli et al., 2013). *Phlebotomus perniciosus* is an efficient vector of *L. infantum* in southwestern European countries such as Portugal, Spain, France and Italy (Alten et al., 2016). However this sand fly species was reported to be "permissive" for multiple *Leishmania* spp. under laboratory conditions (Volf and Peckova, 2007). In the above European countries, spot populations of the specific *L. tropica* vector, *Phlebotomus sergenti*, are also recorded (Depaquit et al., 2002) but their epidemiological significance remains poorly determined (Barón et al., 2013; Bongiorno, G., Lisi, O., Severini, F., Vaccalluzzo, V., Khoury, C., Di Muccio, T., Gradoni, L., Maroli, M., D'Urso, V., Gramiccia, M., 2014. Investigations on sand fly bionomics and *Leishmania* natural infections in eastern Sicily, Italy, with particular

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Southern Europe is experiencing an unprecedented human migration driven by poverty and civil war scenarios. Every year, tens of thousands of asylum seekers or 'economic immigrants' arrive at the Mediterranean coasts of Italy, Greece and Spain (www.unhcr.org), mainly from sub-Saharan Africa, but also from Syria, Iraq and Afghanistan where anthroponotic CL is highly prevalent. As a result, imported cases of *L. tropica* disease are increasingly documented in Europe in the migrant population (Di Muccio et al., 2015; Mockenhaupt et al., 2016; Söbirk et al., 2018). The objective of the present work was to investigate the potential role of *P. perniciosus* in the transmission of imported *L. tropica*.

A *Leishmania* strain (MHOM/IT/2016/ISS3183) recently isolated in culture from the skin lesion of an Afghan refugee attending an Italian hospital in 2016, was used to infect laboratory-reared *P. perniciosus*. The parasite was identified as *L. tropica* by ribosomal internal transcribed spacer 1 -PCR Restriction Fragment Length Polymorphism (Schönian et al., 2003) and heat shock protein gene *hsp70* sequencing (Van der Auwera et al., 2016). The partial sequence (deposited in GenBank with accession number MK335938) was aligned with homologous sequences available in GenBank and 99% identity with sequences of *L. tropica* from the Middle East was confirmed. Two sand fly colonies which originated from Spain and Italy, respectively, were used alternatively in the experiments as they showed very similar *L. tropica* infection patterns. A preliminary study aimed to investigate the growth behavior of *L. tropica* in the unusual vector. Sand fly females were fed artificially through chicken-skin membrane with rabbit blood containing high (10^7/mL) or low ($2 \cdot 10^5$/mL) doses of log-phase promastigotes from recent cultures, i.e. a maximum of two in vitro passages after primary strain isolation. In vivo amastigote-initiated infections were also performed by inducing sand flies to feed on non-ulcerated footpad lesions developed in golden hamsters (*Mesocricetus auratus*) a few months after syringe infection with 0.1 mL of stationary-phase promastigotes from recent cultures, or on intact skin distal to the lesions in chronically-infected animals. Infection rates and developmental parasite stages in sand flies were checked by specimen dissection and gut microscopy. In this part of the study, individual sand fly gut material was re-collected from slides after microscopy observation, and stored at −20 °C pending estimation of *Leishmania* load by quantitative real-time (q) PCR targeting a kinetoplast DNA sequence (Mary et al., 2004; Seblova et al., 2015).

Promastigote-initiated infections were evaluated in 850 engorged females from four experiments (Fig. 1Aa). High and low dose promastigote sources produced similar infection patterns in sand flies. Starting from nearly 100% infected specimens on day 1 post blood meal (PBM), altogether viable parasites were recorded in 78% of specimens dissected over a 13-day period. In 80% of infected sand flies, despite survival of procyclic promastigotes, flourishing growth of nectomonad and attachment of leptomonad promastigotes to midgut epithelium after blood digestion and defecation, either the parasites died and were progressively digested in the midgut from day 6 PBM, or viable leptomonads failed to migrate to the foregut and/or to mature into infective forms. In the remaining sand flies, however, late stage infections with colonization of the stomodeal valve were observed starting from day 4 PBM (Fig. 1Ba), eventually resulting in abundant and highly motile metacyclic promastigotes on day 13 PBM (Fig. 1Ca). This condition was observed in approximately 20% of sand flies on average, but in variable proportions ranging 14–70% of specimens from different experiments and at different days PBM. The qPCR assay confirmed a variable parasite load in different experiments, with a range of 0.25×10^2 up to 3.5×10^6 parasites/gut irrespective of morphological evidence of viability or progressive/final death (Fig. 1Da).

Amastigote-initiated infections were evaluated through 15 experiments on two distinct groups of sand flies: a total of 792 females took a blood meal on cutaneous lesions (Fig. 1Ab), and 161 females fed on intact skin distal from the lesions, for a period from 1.5 months up to 11 months after hamster footpad infection by syringe. Infectiousness to sand flies increased with the progression of lesions, resulting in infection rates from approximately 26% through 70% detected in specimens fed on early or late lesions, respectively, and from approximately 10% through 40% in specimens fed on the respective intact skin distal to the lesions. At gut dissection, the *L. tropica* infection evolution was comparable to that observed in promastigote-initiated infections - similar to what was recently reported by Sadlova et al. (2017) for the *Leishmania donovani*/*Phlebotomus argentipes* pair – although metacyclic promastigotes appeared a little later, i.e. from day 7 PBM (Fig. 1Bb). Among all specimens initially detected as promastigote-positive, approximately 20% developed abundant metacyclic promastigotes over 16 days PBM (Fig. 1Cb). The qPCR parasite load was in a range of 3.5×10^3–5×10^6 parasites/gut in sand flies engorged on a lesioned footpad, and 5×10^3–2×10^7 in specimens engorged on intact skin, again without distinguishing between viable or dead promastigotes (Fig. 1Db).

The second part of the study aimed to verify the ability of *L. tropica*-infected *P. perniciosus* to transmit parasites to naïve young hamsters, known to be susceptible to *L. tropica* strains from Afghanistan (Bastien and Killick-Kendrick, 1992). Chronic cutaneous lesions, artificially induced in hamsters as described above, represented the source of parasites. Infectiousness of the hamsters' lesions to sand flies was followed up through consecutive xenodiagnosis assays, as long as approximately 70% infection was confirmed in the insects examined at 2–5 days PBM. A massive sand fly infection was then performed to allow a sufficient number of potentially infectious females to take a second blood meal, owing to the elevated mortality usually recorded in laboratory-reared *P. perniciosus* after the first blood digestion. Naïve hamsters were exposed to the bites, of which the sites were recorded, and any sand fly having the second blood meal was dissected to determine the transmissible infection status. Wherever cutaneous lesions developed in potential sites of infection, invasive diagnostic biopsies on the hamsters were avoided, and were replaced by xenodiagnosis assays using naïve *P. perniciosus* sand flies in order to simultaneously detect the presence of *L. tropica* in lesions as well as its viability and transmissibility to vectors.

Two clusters of potentially infectious sand flies were produced from a total of 691 females engorged on chronic lesions developed in footpads of six infected hamsters, and found infected at a rate of approximately 70% (Fig. 2A). From day 8 through day 19 PBM, a total of 59 females (8.5%) had a second blood meal on the same two naïve hamsters in five acts of transmission. Bites were mainly noted on front paws and snouts of the animals. At the sand fly dissections performed immediately after the bites, 22/59 specimens (37.3%) were detected positive for parasites, of which 9/22 (41.0%) harbored abundant metacyclic promastigotes (Supplementary Movie S1). Two months post exposure (PE), small nodular lesions developed in bitten sites of both hamsters (Fig. 2Ba and b). Starting from month 3 PE, the presence of viable and transmissible *L. tropica* parasites in lesions of both hamsters was demonstrated by consecutive xenodiagnosis assays. Interestingly, naïve sand flies used in the assays tended to bite on the lesions rather than on intact skin (Fig. 2Ca and b). At 4.5 months PE, the hamsters' infectiousness rate was found to be as high as 41–43%.

Supplementary Movie S1 Fresh preparation of a gut isolated through the dissection of a *Phlebotomus perniciosus* female infected by *Leishmania tropica*. The movie shows the gut isolated through the dissection of a *Phlebotomus perniciosus* female previously infected on a hamster's lesion caused by *Leishmania tropica*, and which took a second bloodmeal on a naŸve hamster. Starting from the hindgut, recognizable by the insertion of Malpighian tubules, large masses of motile parasites are seen mixed with blood in the midgut and part of the foregut. A huge number of extremely motile metacyclic promastigotes are emerging from the stomodeal valve cut and spreading throughout the dissection medium.

In our study, we simulated what is believed to be the probable anthroponotic mode of *L. tropica* infection of competent vectors, i.e. through their bite on human skin presenting chronic CL lesions. In the in vivo infection experiments, *P. perniciosus* females have most probably ingested "pathophysiological" doses of amastigotes from the susceptible rodent model showing this type of lesion. *Phlebotomus perniciosus* showed a great capacity to become infected by and permit initial massive growth of *L. tropica*, and we took advantage of that for xenodiagnosis purposes. On the other hand, the proportion of sand flies showing parasite maturation, consisting of foregut promastigote migration, infection of the stomodeal valve and massive production of metacyclic promastigotes (Bates, 2007),

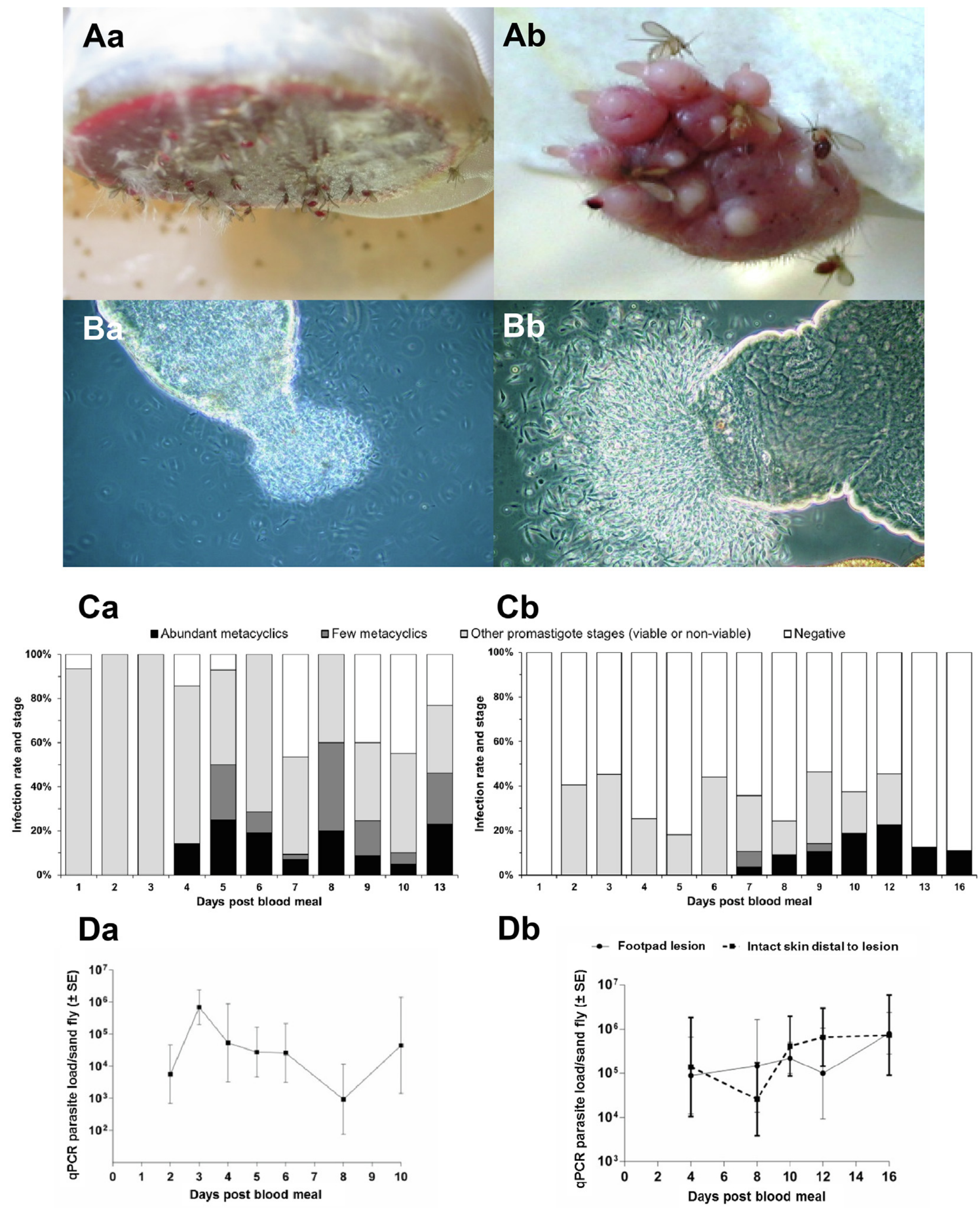

Fig. 1. Promastigote- (Aa, Ba, Ca and Da) and in vivo amastigote- (Ab, Bb, Cb and Db) initiated *Leishmania tropica* infections of laboratory-reared *Phlebotomus perniciosus*. (Aa) Chicken skin membrane feeding. (Ba) Late-stage promastigotes emerging from the stomodeal valve gut dissection. (Ca) Infection rate and developmental stages of promastigotes. (Da) Trend of parasite DNA mean loads determined by quantitative real-time (q) PCR. (Ab) Hamster's infected footpad exposed to sand fly bites. (Bb) Late-stage promastigotes emerging from the stomodeal valve gut dissection. (Cb) Infection rate and developmental stages of promastigotes. (Db) Trend of parasite DNA mean loads determined by qPCR.

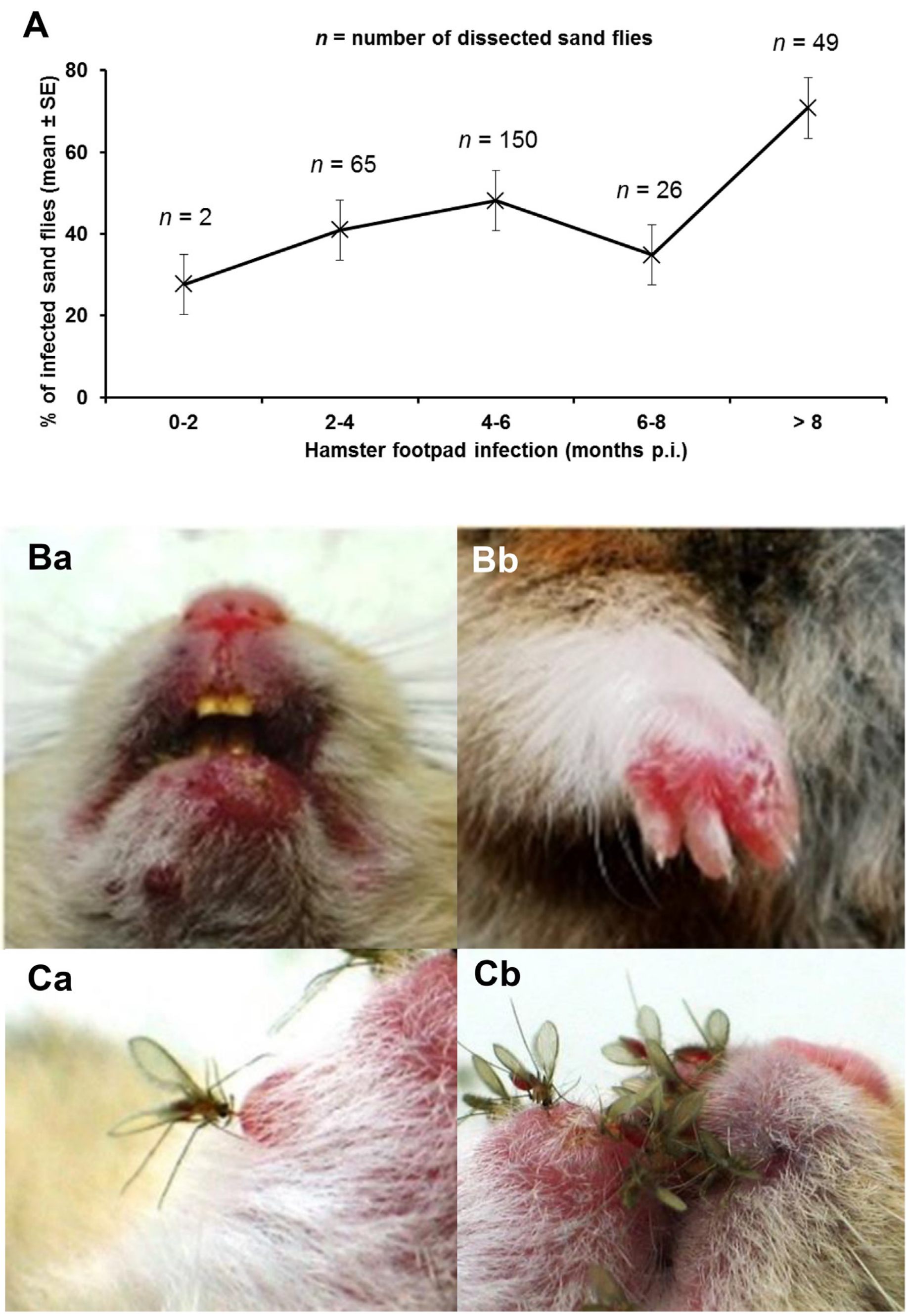

Fig. 2. Experimental transmission of *Leishmania tropica* to hamsters by the bite of potentially infected laboratory-reared *Phlebotomus perniciosus* at the second bloodmeal. (A) Trend of infectiousness to the vector associated with the clinical progression of hamsters' footpad lesions. A massive sand fly infection was performed by exposing lesions older than 8 months to the sand fly bites. Lesions developed in the hamster's snout (Ba) and front paw (Bb). Xenodiagnosis assay was performed to detect presence, viability and transmissibility of parasites. Sand flies tended to bite on the lesions, both small and isolated (Ca) and larger and diffuse ones (Cb).

averaged approximately 20% among those initially infected. This is much lower than the infection maturation rate (96%) found in the specific vector *P. sergenti* infected with an Afghan strain of *L. tropica* (Killick-Kendrick et al., 1995), but considerably higher than the small infection rates – consistently less than 6% – detected by the same team in a refractory vector such as *Phlebotomus papatasi* (Killick-Kendrick et al., 1994). It is noteworthy that successful development of *L. infantum* in experimentally-infected *P. perniciosus* was consistently above 90% as reported in experimental infection studies performed over the past three decades (e.g. Pozio et al., 1985; Seblova et al., 2015; Martín-Martín et al., 2015).

The incrimination of phlebotomine species as vectors of an anthroponotic *Leishmania* life cycle should be based on a series of widely accepted criteria (Killick-Kendrick, 1990; WHO, 2010): (i) the vector must feed on humans; (ii) the vector must be infected in nature with the same *Leishmania* spp. as occur in humans, and this must be ascertained by comparison of isolates using biochemical or molecular methods; (iii) the vector must support the complete development of the parasite after the infecting blood meal has been digested, and (iv) the vector must be able to transmit the parasite by bite to a susceptible host while taking a blood meal. This last point is often neglected due to the technical difficulty of performing transmission experiments with sand flies. It should also be emphasized that the exclusive use of PCR-based methods cannot be sufficient to meet the requirement of criterion iii, as we have confirmed that current molecular assays are unable to distinguish viable from deadly parasites, and early non-infective from mature infective stages (see Seblova et al., 2014).

Despite criteria i, iii and iv being met, it stands to reason that, at present, *P. perniciosus* cannot be considered to have a substantial epidemiological role in supporting *L. tropica* transmission in natural foci of leishmaniasis of southwestern Europe, primarily because this species has never been found infected with this parasite in nature (criterion ii), nor have autochthonous human cases of *L. tropica* been reported in places where *P. perniciosus* is the prevalent vector. It is important to note that the role of this sand fly species as an efficient vector of *L. infantum* was shown by several reports of natural infections with this parasite in territories where VL and CL are caused by *L. infantum*, and which date from the early 1980s until recently (e.g. Bettini et al., 1986; González et al., 2017). On the other hand, the unprecedented human migration from *L. tropica*-endemic countries occurring in coastal Mediterranean territories of Europe, mainly during the warm season, cannot rule out completely the possibility of sporadic episodes of transmission, which could start from non-medically assisted individuals affected by chronic *L. tropica* infections. In territories already endemic for *L. infantum* CL, physicians and laboratories have good clinical and diagnostic experience to diagnose this condition, however they are not always in the position to distinguish autochthonous *L. infantum* from introduced *L. tropica* CL cases because lesions, both in early and chronic stages, are clinically similar between the two conditions and, most importantly, parasite typing is not frequently performed (Di Muccio et al., 2015).

In conclusion, our findings support the hypothesis that, in particular epidemiological situations, *P. perniciosus* may play the role of an occasional *L. tropica* vector, but most likely it cannot sustain introduction and endemic establishment of this parasite in southern European territories.

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