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Succinctus

Experimental infection of *Phlebotomus perniciosus* and *Phlebotomus tobbi* with different *Leishmania tropica* strains

Slavica Vaselek, Petr Volf [*]

Department of Parasitology, Faculty of Science, Charles University, Viničná 7, 128 43 Prague 2, Czech Republic

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ABSTRACT

Cutaneous leishmaniasis due to *Leishmania tropica* is increasingly documented in Europe and the Middle East. Besides its specific vector, *Phlebotomus sergenti*, permissive *Phlebotomus* sand flies are suspected as potential vectors of *L. tropica*. We investigated the susceptibility of two widely distributed species, *Phlebotomus perniciosus* and *Phlebotomus tobbi*. Laboratory-reared sand flies were infected experimentally with *L. tropica* strains differing in lipophosphoglycan epitopes, geographical distribution and epidemiology. High infection rates, heavy parasite loads and fully developed late-stage infections including colonization of the stomodeal valve were observed in all parasite-vector combinations. Our findings demonstrate that *P. perniciosus* and *P. tobbi* are susceptible to different *L. tropica* strains and may play a role in their circulation in endemic foci of Europe, the Middle East and North Africa.

Leishmania (Kinetoplastida, Trypanosomatidae) are protozoan parasites with a digenetic life cycle, constituting an important global health problem with millions of people affected by cutaneous and visceral leishmaniases. *Leishmania tropica* is the main causative agent of cutaneous leishmaniases (CL) in the Old World. CL occurs in many countries of the Middle East and Africa (Alvar et al., 2012) while in Europe autochthonous cases were reported almost exclusively only from Crete (Antoniou et al., 2019). Currently, a great concern is posed by outbreaks of CL in the Middle East, connected with people displaced from their homes due to civil war (Saroufim et al., 2014; Sharara and Kanj, 2014). In addition, imported cases of CL caused by *L. tropica* are increasingly documented in Europe among the general and migrant populations (Söbirk et al., 2018).

Among *Leishmania* spp., *L. tropica* has several unique features. It has striking genetic heterogeneity, reflected by high isoenzymatic polymorphism (Pratlong et al., 1991) and variability in DNA markers including ITS1 (Schönian et al., 2001). From the epidemiological point of view, it is important that CL caused by *L. tropica* could be either an anthroponotic or a zoonotic disease. Anthroponotic transmission was documented in classical urban foci such as Kabul in Afghanistan, Aleppo in Syria, Shiraz and Mashad in Iran, Mosul in Iraq and Taza in Morocco. Rural foci with hyraxes involved in zoonotic transmission were reported mainly from Middle Eastern countries and Kenya (reviewed by Jacobson, 2003).

Diversity in genetic markers and epidemiology is also accompanied by differences in vector species. In most foci *L. tropica* is transmitted by its specific vector *Phlebotomus sergenti*. Various *P. sergenti* colonies were experimentally demonstrated to be susceptible to different strains of *L. tropica* (Kamhawi et al., 2000; Jancarova et al., 2015). However, in northern Israel, *Phlebotomus arabicus* is the proven vector of zoonotic *L. tropica* and strains with modified surface lipophosphoglycan (LPG), such as L810, are limited to this vector (Svobodova et al., 2006b). In Kenya, *Phlebotomus guggisbergi* appears to be the main vector while *L. tropica* parasites were isolated also from *Phlebotomus aculeatus* and *Phlebotomus saevus* (reviewed by Jacobson, 2003).

Recently, Bongiorno et al. (2019) investigated the ability of *Phlebotomus perniciosus* to support the growth and transmissibility of an Asian strain of *L. tropica* isolated from the skin lesion of an Afghan refugee. Although in 80% of infected sand flies parasites died after defecation of the bloodmeal, the rest developed late stage infections. More interestingly, the full parasite cycle was demonstrated by exposing naive hamsters to *P. perniciosus* females previously infected on chronic non-ulcerative lesions developed in hamsters injected with *L. tropica*. Hamsters bitten by infected sand flies developed lesions and their infectiousness was demonstrated by xenodiagnosis assays (Bongiorno et al., 2019).

Phlebotomus perniciosus is a natural vector of *Leishmania infantum* in the western part of the Mediterranean basin

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[*] Corresponding author.
E-mail address: volf@cesnet.cz (P. Volf).

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(https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps) and a permissive vector susceptible to other *Leishmania* spp. (reviewed by Volf and Myskova, 2007). Its sister species in the subgenus *Larroussius*, *Phlebotomus tobbi*, is widespread in the Middle East and eastern part of the Mediterranean basin (https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps). It is a proven vector of *L. infantum/Leishmania donovani* hybrids in southern Turkey (Svobodova et al., 2009) and susceptible to both of these *Leishmania* spp. (Seblova et al., 2015).

Here we aimed to determine the susceptibility of *P. perniciosus* and *P. tobbi* to two very different strains of *L. tropica*: strain L810 (IARA/IL/2001/LCR-L810 Amnunfly1), isolated from naturally infected *P. arabicus* in a zoonotic focus in northern Israel and strain SU23 (MHOM/TR/1998/SU23) isolated from a CL patient in an urban focus in Sanliurfa, Turkey. These two strains differ also in the terminal carbohydrates of their LPG: while SU23 has typical *L. tropica* LPG with terminal glucose, the terminal sugars of the LPG of L810 are mostly galactose which causes their inability to develop in *P. sergenti* (Svobodova et al., 2006b). Infectivity of SU23 was demonstrated for mice and rats (Svobodova et al., 2003) while L810 was experimentally transmitted to hyraxes by the bite of *P. arabicus* (Svobodova et al., 2006a). After passage through the mammalian host, the strains were cryopreserved. *Leishmania* promastigotes were cultivated in M199 medium (Sigma) containing 20% heat-inactivated fetal bovine serum (Gibco), supplemented with 2% sterile urine, 1% Basal Medium Eagle vitamins (Sigma) and 250 $\mu g\ mL^{-1}$ amikacin (Amikin, Bristol-Myers Squibb, UK). For all sand fly infections, parasites with in vitro passage numbers less than 10 were used.

Four well established insectary-reared sand fly colonies were used. *Phlebotomus tobbi* originating from females caught in Cukurova, Turkey (the only laboratory colony of this species in the world (Lawyer et al., 2017)) and the colony of *P. perniciosus* originating in Murcia, Spain, were used as test species. *Phlebotomus sergenti* and *P. arabicus* colonies originated in the same localities as *Leishmania* isolates: *P. arabicus* from Amnun, northern Israel (origin of L810), *P. sergenti* from Sanliurfa, Turkey (origin of SU23). Based on their previously confirmed susceptibility to respective isolates (Svobodova et al., 2006b), they were used as positive controls. All sand fly colonies were maintained under standard conditions (at 26 °C; fed on 50% sucrose; with a 14:10 h light:dark photoperiod), as described previously by Volf and Volfova (2011).

Twenty-four hours prior to the infectious meal, approximately 200 sand fly females (5–9 days old) from each species were separated and deprived of sucrose food. Promastigotes from log-phase cultures were resuspended in heat-inactivated rabbit blood at a concentration of 10^6 promastigotes per mL and offered to sand flies through chicken skin. Engorged females were separated, provided with sucrose and maintained under standard conditions (Volf and Volfova, 2011). Dissections were performed before defecation (early stage of infection) on day 2 post blood meal (PBM) and after defecation (late stage of infection) on day 8 PBM. Abundance and localization of *Leishmania* parasites in the sand fly gut were examined by light microscopy as described previously by Myskova et al. (2015); briefly, parasite loads were graded as light (less than 100 parasites per gut), moderate/medium (100–1000 parasites per gut) and heavy (more than 1000 parasites per gut).

Experimental infection of *P. perniciosus* was repeated four times per *L. tropica* strain. *Phlebotomus tobbi* females are generally reluctant to feed through any type of membrane and have high post-blood meal mortality, therefore experimental feeding of *P. tobbi* was performed six times with SU23 and eight times with the L810 strain.

First, we infected *P. perniciosus* and *P. tobbi* with the antroponotic *L. tropica* SU23, and the natural vector *P. sergenti* served as a positive control. Dissections conducted during the early stage of infection, on day 2 PBM, revealed similar infection rates (91–97%) among all three sand fly species tested (Fig. 1A). Procyclic promastigotes were located inside the endoperitrophic space, within the bloodmeal surrounded by the peritrophic matrix. On day 8 PBM, fully developed late-stage infections were found in all three sand fly species and the majority of dissected females harboured heavy parasites loads. Relatively high infection rates, 91% and 89%, were found in *P. perniciosus* and *P. tobbi,* respectively (Fig. 1A). Infection rates in *P. sergenti* were lower (67%) which correlates well with previous studies on this species (Svobodova et al., 2006b; Jancarova et al., 2015). In almost all infected females of the three sand fly species, promastigotes colonized the thoracic midgut (92–97%), and in most of them promastigotes colonized also the stomodeal valve (79%, 75% and 71% for *P. perniciosus, P. tobbi* and *P. sergenti*, respectively), see Fig. 2A.

Then we studied the development of zoonotic *L. tropica* L810 from Israel, and *P. arabicus* was used as a control. On day 2 PBM, high infection rates (93–100%) with a majority of heavy infections (65–71%) were observed in all tested sand fly species (Fig. 1B), all parasites being localized in the endoperitrophic space. On day 8 PBM, very high infection rates were observed in all species tested (90%, 95% and 92% for *P. perniciosus, P. tobbi* and *P. arabicus,* respectively) and heavy parasite loads dominated (57%-68% for all three species (Fig. 1B). Very high proportions of *Leishmania* infections colonized the thoracic midgut (>95% of infected females in all species); colonization of the stomodeal valve with numerous metacyclic promastigotes was observed in 81%, 86% and 94% for *P. perniciosus, P. tobbi* and *P. arabicus,* respectively (Fig. 2B and 3).

In summary, we dissected 840 experimentally infected sand flies (354*P. perniciosus*, 129*P. tobbi*, 190*P. sergenti* and 167*P. arabicus*); infection rates and localization of infections in *P. tobbi* and *P. perniciosus* were fully comparable to natural vectors used as positive controls. Both *L. tropica* strains tested, SU23 and L810, grew well in all sand fly species; high infection rates, heavy parasite loads and fully developed late-stage infections with colonization of the stomodeal valve and highly motile metacyclic promastigotes were observed in all parasite-vector combinations (Fig. 3).

To our knowledge this is the first experimental study showing high vector competence of *P. tobbi* for *L. tropica.* In addition, we confirmed that *P. perniciosus* colonies of European origin are susceptible to two very different *L. tropica* strains. The developmental parameters of *L. tropica* in *P. perniciosus* demonstrated in our study differ from those reported by Bongiorno et al. (2019), who reported late stage infections with abundant metacyclic promastigotes in only 20% of infected *P. perniciosus* females. Higher percentages of late stage infections found in our study (91% for SU23 and 90% for L810) as well as more frequent colonization of the stomodeal valve (79% for SU23 and 81% for L810) are most probably caused by the diversity of *L. tropica* strains. While Bongiorno et al. (2019) used a *Leishmania* isolate from an Afghan refugee, in our study both parasite strains were isolated in the Middle East. On the other hand, it is unlikely that differences observed in infectivity to *P. perniciosus* were due to experimental conditions or different sand fly colonies. In the experiments described by Bongiorno et al. (2019), two *P. perniciosus* colonies, originating from Spain and Italy, were used alternatively in the experiments and showed very similar *L. tropica* infection patterns. Moreover, the colony of Spanish origin was identical to the one used in our study.

Our finding suggests that *P. tobbi* could play a role in the transmission of *L. tropica* in the Middle East, Turkey and south-eastern Europe, and that *P. perniciosus* may serve as a vector of this parasite

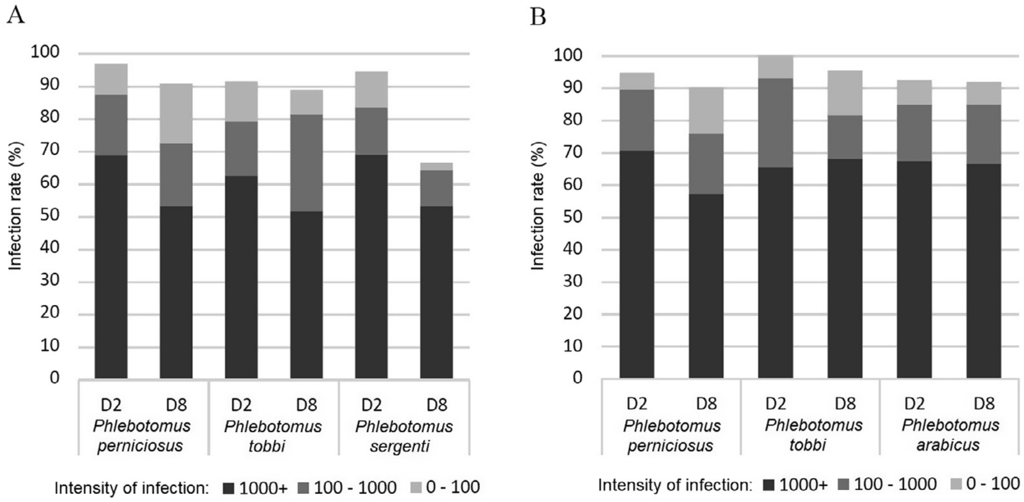

Fig. 1. Infection rates and intensities of *Leishmania tropica* SU23 (A) and L810 (B) strains in *Phlebotomus perniciosus* and *Phlebotomus tobbi* on day 2 (D2) and day 8 (D8) p.i. Natural vectors (*Phlebotomus sergenti* and *Phlebotomus arabicus*, respectively) were used as positive controls.

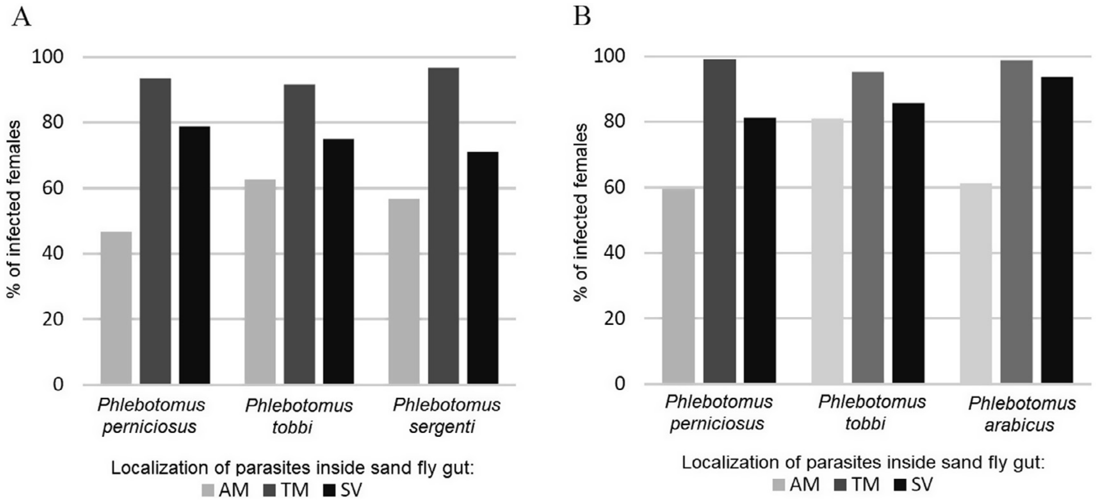

Fig. 2. Localization of *Leishmania tropica* SU23 (A) and L810 (B) strain parasites inside the sand fly gut on day 8p.i. AM, abdominal midgut; TM, thoracic midgut; SV, stomodeal valve.

in western parts of the Mediterranean basin including the Magreb. However, there are other permissive species belonging to the subgenera *Larroussius* and *Adlerius* which are potentially involved in transmission of *L. tropica* in various regions. *Phlebotomus* (*Adlerius*) *halepensis*, a species recorded in Turkey and the Middle East, was experimentally demonstrated to be highly susceptible to this parasite (Sádlová et al., 2003). We expect that permissive vectors such as *P. perniciosus*, *P. tobbi* and *P. halepensis* could be responsible for the establishment of new leishmaniases foci, as was described previously for the New World vector *Lutzomyia longipalpis* and its role in the transmission of *L. infantum* imported to Latin America in historical times (reviewed by Dvorak et al., 2018).

Accelerated movements of humans, together with changes in climate, may lead to an increased risk of the spread of leishmaniases (Alvar et al., 2012; Antoniou et al., 2019). In Europe, *P. tobbi, P. perniciosus* and other *Larroussius* spp. have much wider distributions than *P. sergenti* (https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps), a specific vector considered in predicted risk for establishment of *Leishmania tropica* in southern Europe (Barón et al., 2013). In classical urban foci of *L. tropica*, sand flies of the subgenus *Larroussius* are usually not abundant, for example in Sanliurfa, Turkey, their relative abundance was lower than 1% (Volf et al., 2002). On the other hand, in rural foci with zoonotic transmission, *Larroussius* spp. are common, for example in four endemic villages in northern Israel their relative abundance exceeded 40% (Jacobson et al., 2003) and in Kenya, *P.* (*Larroussius*) *guggisbergi* appeared to be the main vector (reviewed by Jacobson, 2003). Taking into account the impact of the persistent human migrations from *L. tropica* endemic countries to southern Europe, where various *Larroussius* spp. are widespread, we cannot rule out the possibility of autochthonous infections. In contrast with most leishmaniases, CL caused by *L. tropica* is usually anthroponotic, its circulation does not require an animal reservoir and therefore may become a higher risk for humans exposed to bites of these sand flies.

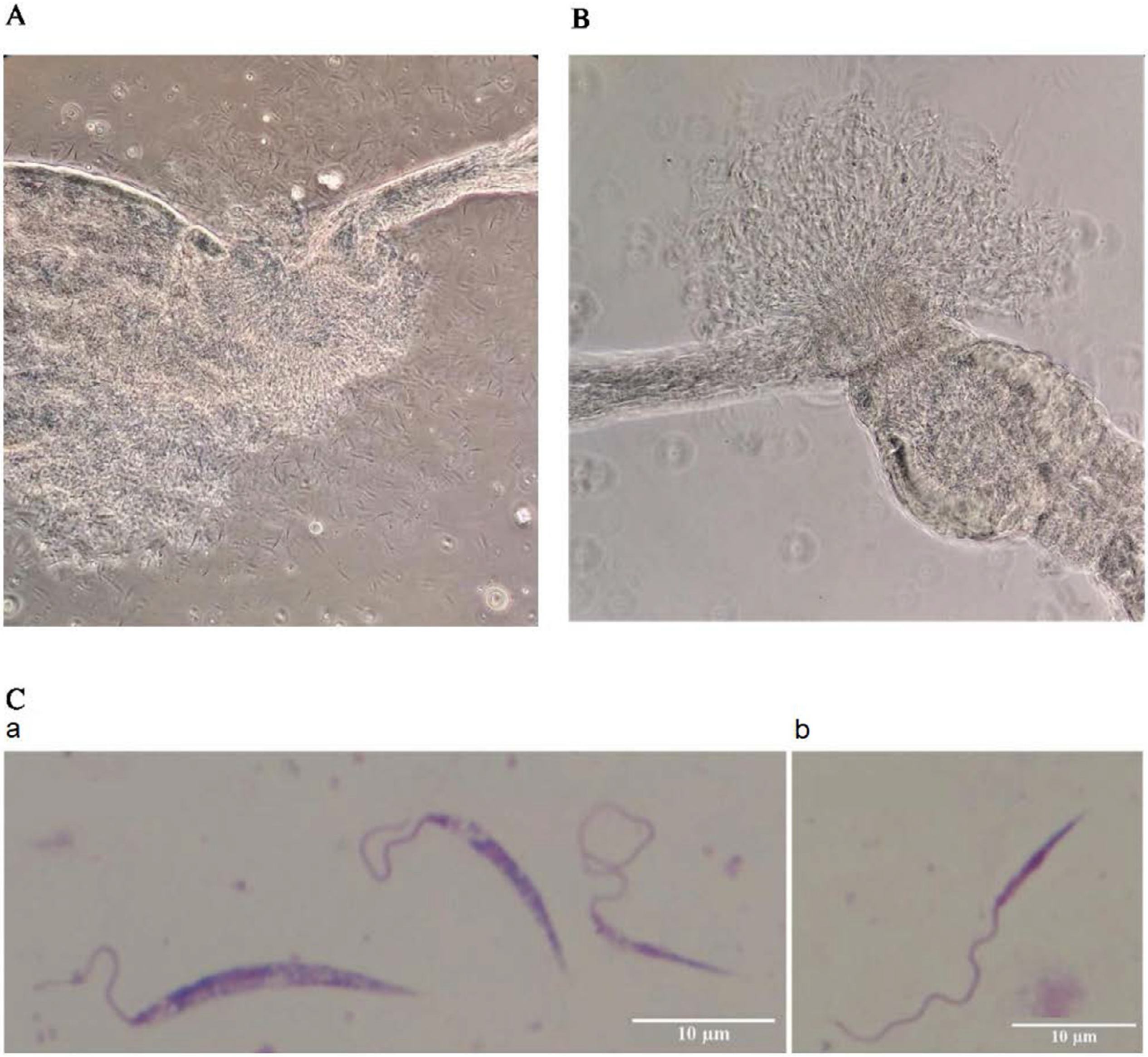

Fig. 3. Colonization of the stomodeal valve with a heavy load of promastigotes. (A) *Phlebotomus tobbi* infected with the SU23 *Leishmania tropica* strain. (B) *Phlebotomus perniciosus* infected with L810 *L. tropica* strain. (C) Different morphological forms (a) and metacyclic promastigote (b) of *L. tropica* during late stage infection (L810 in *P. perniciosus*; Giemsa stained, 100× magnification).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.05.009.

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