

The Development of *Leishmania tropica* in Sand Flies (Diptera: Psychodidae): A Comparison of Colonies Differing in Geographical Origin and a Gregarine Coinfection

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ABSTRACT *Phlebotomus sergenti* Parrot, 1917 is the main vector of *Leishmania tropica*; however, its broad geographical range and molecular heterogeneity suggest possible variability in vector competence. We infected laboratory-reared *P. sergenti* originating from Turkey and Israel to compare their susceptibility to *L. tropica*. In both tested groups, heavy late-stage infections with the presence of metacyclic forms and colonization of the stomodeal valve were observed. The similar development of *Leishmania* in both sand fly colonies indicates that the different geographical origin of *P. sergenti* is not reflected by a different vector competence to *L. tropica*. Additionally, we tested the effect of the gregarine *Psychodiella sergenti* on *L. tropica* coinfections; no apparent differences were found between *P. sergenti* infected or not infected by gregarines.

KEY WORDS *Phlebotomus sergenti*, *Leishmania tropica*, vector competence, coinfection, gregarine

Leishmaniasis are vector-borne diseases with a wide range of clinical outcomes. Their causative agents are parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) transmitted by the bite of phlebotomine sand flies (Diptera: Psychodidae). *Leishmania tropica* causes cutaneous leishmaniasis in many countries around the Mediterranean basin, the Middle East, Central Asia, and East Africa. The primary specific vector is *Phlebotomus sergenti* Parrot, 1917 (Kamhawi et al. 2000, Volf and Myskova 2007), although other sand fly species have been shown to transmit *L. tropica* in Ethiopia (Gebre-Michael et al. 2004) and northern Israel (Jacobson et al. 2003, Svobodova et al. 2006).

The geographical range of *P. sergenti* is very broad and more widespread than the distribution of *L. tropica*, suggesting some degree of intraspecific variability that may potentially affect the vector competence of different populations of this species (Depaquit et al. 2002). Sequencing of the internal transcribed spacer 2 (ITS2) of 12 populations from 10 countries revealed two principal branches of distinct geographical origin: 1) a more north-east area (Cyprus, Pakistan, Syria, and Turkey) and 2) a more south-west area (Israel, Egypt, Morocco, Sicily; Depaquit et al. 2002). These two branches were confirmed by subsequent studies using random-amplified polymorphic DNA and geometric morphometrics (Dvorak et al. 2006, 2011).

To study the possible consequences of the molecular heterogeneity of *P. sergenti* on the vector competence of *L. tropica*, we established two *P. sergenti* colonies of

different geographical origin, one from Turkey (the north-east branch) and the second from Israel (the south-west branch), and experimentally tested their susceptibility to *L. tropica*. As the Turkish colony was naturally infected by the gregarine *Psychodiella sergenti* (Apicomplexa: Eugregarinorida), and the egg-washing procedure by Poinar and Thomas (1984) commonly used to clean gregarines from sand fly colonies is not sufficiently effective in *P. sergenti* (Lantova and Volf 2012), we have now compared the development of *L. tropica* in two groups of Israeli *P. sergenti*, one being infected experimentally by gregarines.

Materials and Methods

Sand Flies and Parasites. Three laboratory colonies of *P. sergenti* were used—1) TU originating from Sanliurfa, Turkey; 2) IS originating from Amnun, Israel; and 3) ISG derived from IS females artificially infected by *Ps. sergenti* as described by Lantova et al. (2010). Sand flies were maintained under standard conditions as previously described by Volf and Volfova (2011). *Leishmania tropica* SU23 (MHOM/TR/98/HM) was maintained at 23°C on M199 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% foetal calf serum (Gibco, Life Technologies, Carlsbad, CA), 1% BME vitamins (Sigma-Aldrich), 2% filtered human urine, amikacin (250 mg/ml), and gentamicin (80 mg/ml).

Experimental Infection. Sand fly females (4–7 d old) were fed through a chick-skin membrane on heat-inactivated rabbit blood containing 1×10^6 promastigotes/ml. This infective dose corresponds to <1,000 parasites per female, as bloodmeal volumes taken by

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various sand fly species ranged from 0.53 to 0.91 μ l (Pruzina et al. 2015). Moreover, promastigote-initiated infections are fully comparable with amastigote-initiated infections (Freitas et al. 2012). Blood-fed females were maintained at 26°C and dissected on days 2 and 7–10 postinfection (p.i.), and their guts were microscopically checked for the presence and localization of *Leishmania* promastigotes. Intensities of infection were graded into three categories according to Myskova et al. (2008)—weak (1–100 promastigotes per gut), moderate (100–1,000 promastigotes per gut), and heavy (>1,000 promastigotes per gut). The experimental infection was repeated two (TU \times IS) or three times (IS \times ISG). Data were statistically evaluated by means of the χ^2 test using STATISTICA 12.0 software (StatSoft).

Results and Discussion

In the first series of experiments, the development of *L. tropica* was compared in *P. sergenti* TU and IS. Fig. 1A summarizes the data of two independent experiments. Parasites developed well in the females of both colonies tested. In early-stage infections (day 2 p.i.), all dissected females were infected, with a slightly higher proportion of heavy infections found in TU females ($\chi^2=6$; df=2; $P=0.05$; Fig. 1A). Nevertheless, on days 7–10 p.i., the infection rates and intensities of infection were the same in TU and IS females ($\chi^2=0.316$; df=1; $P=0.574$ and $\chi^2=4.747$; df=3; $P=0.191$; respectively). In both tested groups, heavy late-stage infections with anterior migration of *Leishmania* promastigotes, presence of metacyclic forms, and colonization of the stomodeal valve were observed from day 7 (Fig. 1A).

Next, we performed an additional series of experiments comparing IS and ISG females. In mosquitoes, the gregarine *Ascogregarina culicis* has been implicated in maintenance of the chikungunya virus (Mourya et al. 2003). Here, we investigated if the gregarine *Ps. sergenti* has any effect on the development of *L. tropica* in *P. sergenti*. Figure 1B summarizes the data of three independent experiments. No significant differences in infection parameters were detected between IS and ISG females on any day p.i. On day 2, infection rates were 100% and were of similar intensities, with heavy infections observed in a majority of females of both colonies ($\chi^2=0.870$; df=2; $P=0.647$; Fig 1B). In the late development stage (day 7–10 p.i.), the infection rate was >60% in both *P. sergenti* groups (76% and 64% for IS and ISG, respectively; $\chi^2=1.155$; df=1; $P=0.283$), intensities of infection were comparable ($\chi^2=1.407$; df=3; $P=0.704$), and heavy late-stage infections with colonization of the stomodeal valve were observed in ~50% of infected females (Fig. 1B). Thus, we conclude that coinfection by the gregarine *Ps. sergenti* did not have any apparent effect on the development of *L. tropica* in *P. sergenti* in our experimental settings.

In summary, we demonstrate the ability of *L. tropica* to develop equally well in two *P. sergenti* colonies that represent the two different branches previously postulated by Depaquit et al. (2002). Our results support recent findings on the similarity of cytochrome b sequences in specimens from Turkey and Israel (Dvorak et al. 2011) and also correspond with the ability of *P. sergenti* colonies originating from Turkey and Israel to cross breed with no negative effect on their offspring (Dvorak et al. 2006). All these findings question the existence of a *P. sergenti* species complex. It seems that the different geographical origin of

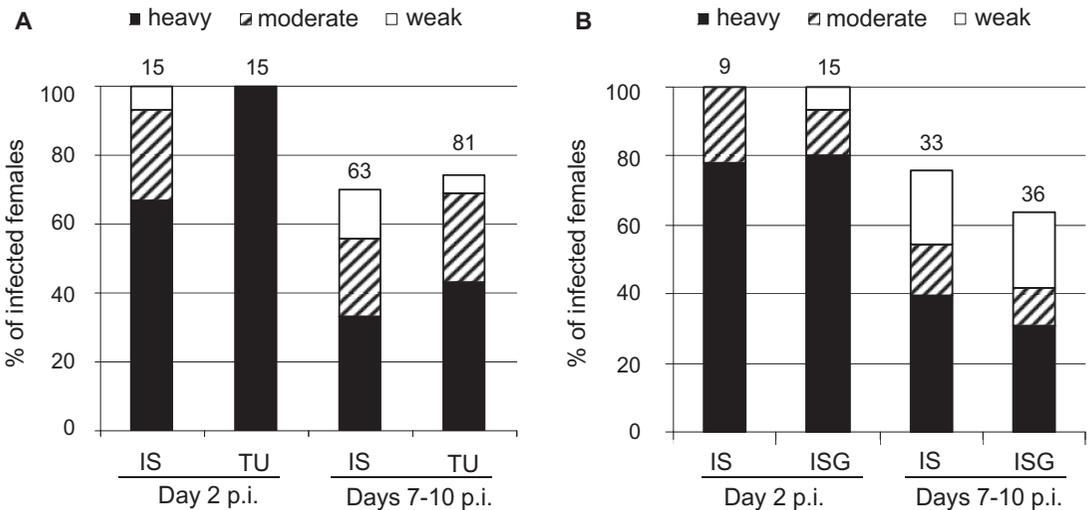


Fig. 1. The development of *L. tropica* in *P. sergenti*: (A) comparison of two *P. sergenti* colonies originating from Turkey (TU) and Israel (IS); (B) comparison of a colony infected by the gregarine *Ps. sergenti* (ISG) and a noninfected control (IS). Intensities of the leishmania infection were estimated as light (<100 promastigotes per gut)—white bar, moderate (100–1,000 promastigotes per gut)—striped bar, and heavy (>1000 promastigotes per gut)—black bar. Numbers above each bar indicate the number of dissected females.

P. sergenti tested here is not reflected by different susceptibility to *L. tropica*. Current results are consistent with the previously described vector competence of various *P. sergenti* populations for *L. tropica* (Svobodova et al. 2006, Kamhawi 2006, Maroli et al. 2013).

This finding on *P. sergenti* corresponds with results on *Leishmania donovani* vectors: two populations of *Phlebotomus orientalis* Parrot, 1936 from endemic and nonendemic areas in Ethiopia were equally susceptible to *L. donovani*, and the authors (Seblova et al. 2013) concluded that factors other than the vector competence of *P. orientalis* play a role in the epidemiology of *L. donovani* in Ethiopia. Similarly, differences in the distribution of *L. tropica* and its main vector *P. sergenti* may be rather attributed to factors other than the different vector competence of various *P. sergenti* populations.

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