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ORIGINAL PAPER

The vector competence of *Phlebotomus perniciosus* for *Leishmania infantum* zymodemes of Tunisia

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Abstract

Experimental infections of *Phlebotomus (L.) perniciosus* from a colony established in Madrid (Spain) carried out with the *Leishmania (L.) infantum* zymodemes MON-1, MON-24, and MON-80 isolated in Tunisia are reported here. Laboratory-reared female sand flies were experimentally fed via membrane feeding device on a suspension of *L. infantum* promastigotes in defibrinated rabbit blood (10^7/ml). Engorged females were dissected at progressive time points postfeeding to observe the intravectorial cycle of different *L. infantum* zymodemes. Development in the sand fly midgut of *L. infantum* parasites to the infective metacyclic promastigotes and monitoring the forward progression of parasites to finally reach the stomodeal valve (SV) of the sand fly were assessed. All tested *L. infantum* zymodemes developed properly in *P. perniciosus*. Experimental feeding with suspensions of promastigotes of all zymodemes led to very heavy late-stage infections. MON-24 and MON-80 zymodemes colonized the (SV) of *P. perniciosus* earlier than zymodeme MON-1, 2 and 4 days, respectively. Metacyclic promastigotes were observed in all experimental infections. The study shows for the first time that colonized *P. perniciosus* is able to acquire, retain, and develop in its midgut the zymodemes MON-24 and MON-80 isolated in Tunisia and highlights the putative role of this sand fly species in the transmission of such zymodemes to mammalian hosts in this country. The ability of experimentally infected sand fly species to transmit by bite such zymodemes needs to be assessed.

Keywords *Phlebotomus (P.) perniciosus* · Vector competence · *Leishmania (L.) infantum* · MON-1, MON-24, MON-80 · Tunisia

Introduction

Leishmania (L.) infantum is a protozoan parasite (Kinetoplastida, Trypanosomatidae) responsible for both cutaneous (CL) and visceral (VL) human leishmaniasis. *Leishmania (L.) infantum* is frequently associated with humid and sub-humid bioclimatic areas in the old world. It is mainly distributed in the Mediterranean Europe, North Africa, Southwest Asia, and the People's Republic of China. Domestic dogs, wild canids, wild rabbits, hares, and domestic cats are the main reservoir hosts. *Phlebotomus* species belonging to the subgenus *Larroussius* are the incriminated vectors of *L. infantum* (Maroli et al. 2013), being *Phlebotomus (L.) perniciosus* the main vector in the western region of the Mediterranean basin. The infection of this sand fly species with various zymodemes of *L. infantum* has been reported in Granada (Spain), showing the diversity of zymodemes isolated from naturally infected *P. perniciosus* (Martín-Sánchez et al. 1994).

In Tunisia, southern Mediterranean basin, *L. infantum* zymodemes MON-1, MON-24, and MON-80 have been identified as responsible for both VL and sporadic CL human cases (Aoun et al. 2001, 2000; Belhadj et al. 2003; Haouas et al. 2007, 2012; Kallel et al. 2008). Natural infection of *P. perniciosus* with *L. infantum* MON-1 has been reported by Ben Ismail (1993). However, the vector competence of this sand fly species for different *L. infantum* zymodemes is currently under discussion. Although the infection of *Phlebotomus* (*L.*) *langeroni* with *L. infantum* has also been reported in an endemic area of sporadic CL in Tunisia (Guerbouj et al. 2007) the role

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of this sand fly as vector of *L. infantum* remains unproved. Moreover, *L. infantum* DNA has been detected in *P. perniciosus* from the eastern central region of Tunisia (Chargui et al. 2013) and more recently in *P. perniciosus*, *Phlebotomus (L.) longicuspis*, *Phlebotomus (P.) papatasi*, *Phlebotomus (L.) perfiliewi*, and *Sergentomyia (S.) minuta* from central Tunisia, although no information about the involved zymodemes is available (Barhoumi et al. 2016). The geographical distribution in Tunisia of *L. infantum* zymodemes and its spreading throughout the center and the south of the country is variable (Haouas et al. 2012) and is probably related to the pattern of spread of *P. perniciosus* and *P. perfiliewi* (Barhoumi et al. 2014). It should be noted here that studies carried out so far in Tunisia on the vector competence of sand flies to transmit *L. infantum* have only been based on the molecular DNA identification of the parasite. In addition, there is no information about the presence of zymodemes MON-24 and MON-80 in Tunisian sand flies as well as on the reservoir hosts in which they can be found. Only the dog has been described as a reservoir of the MON-1 zymodeme. Despite the identification of MON-80 zymodeme in three dogs from Tunisia this matter is under discussion (Benikhlef et al. 2009; Haouas and Babba 2017).

For a better understanding of the natural history of leishmaniasis in Tunisia, further investigations on the transmission of such zymodemes have to be performed which could help in the prevention of the spread of this disease to new foci. In natural conditions, *Leishmania* transmission cycles are complex, involving a large number of mammals and phlebotomine sand fly species. Experimental research has proven to be very effective in studying the life cycle of *Leishmania*. For example, the xenodiagnosis is currently used to identify reservoir hosts of *Leishmania* being the more reliable approach for testing the infectivity of wild and domestic hosts to sand flies. In this way, hares (*Lepus granatensis*) and wild rabbits (*Oryctolagus cuniculus*) have been proposed as new reservoirs of *L. infantum* (Molina et al. 2012; Jiménez et al. 2014). This tool has also allowed demonstrating that dogs with leishmaniasis (symptomatic and asymptomatic) are equally infective to *P. perniciosus* (Molina et al. 1994). No less important is to point out here the importance of experimental infections in understanding parasite-vector interactions, which have become an important approach to studying the transmission of leishmaniasis. The purpose of this methodology is to follow the development of the parasite in the gut of a suspected sand fly species. In a natural vector, the parasite persists during the blood meal digestion and attaches to the microvilli of the midgut epithelial cells (Kamhawi 2006). The presence of infective forms of the parasite (metacyclic promastigotes) close the stomodeal valve (SV) of the sand fly, and its experimental transmission by the sand fly bite are crucial aspects for the characterization of the vector competence of a potential vector of leishmaniasis (Killick-Kendrick 1990). Indeed, this method has been widely used to investigate the sand fly competence. Thus, experimentally infected specimens of genus *Lutzomyia* are able to transmit *Leishmania mexicana amazonensis* to hamsters (Killick-Kendrick et al. 1977). Similarly, Falcão de Oliveira et al. (2017) demonstrated the capacity of *Lu. cruzi* to transmit *L. infantum* and *L. amazonensis*. Even more, Guimarães et al. (2016) reported the permissiveness of *Lu. migonei* for *L. infantum* and Maia et al. (2011) investigated the natural infective dose of viscerotropic and dermotropic *L. infantum* strains in two major vectors, *Lutzomyia longipalpis* and *P. perniciosus*.

In spite of the important epidemiological impact that the knowledge of the vector competence of *P. perniciosus* for the *L. infantum* zymodemes isolated in Tunisia can suppose, no studies have been conducted on this matter to date. In this context, the susceptibility of a Spanish *P. perniciosus* colony for the Tunisian *L. infantum* zymodemes MON-1, MON-24, and MON-80 is investigated in the present study.

Material and methods

Leishmania strains

Three *L. infantum* strains, MHOM/TN/2003/23S (zymodeme MON-1), MHOM/TN/2005/SFC51 (zymodeme MON-24), and MHOM/TN/2006/PLC8 (zymodeme MON-80) were tested in this study. The SFC51 and PLC8 strains were obtained from two cases of human CL and the 23S strain was obtained from a case of human VL. Promastigote cultures were maintained at 27 °C in NNN and RPMI media supplemented with 10% inactivated fetal calf serum (FCS, Lonza, Basilea) and a penicillin-streptomycin mixture (10,000 U/mL, Lonza, Basilea).

Sand flies and experimental infections

A colony of *P. perniciosus* established in Madrid (Spain) was used (Molina 1991). It was maintained in the insectary of the Laboratory of Medical Entomology, Institute of Health "Carlos III", Madrid, Spain at 27 °C, 90–100% relative humidity and 17:7 light:darkness photoperiod, according to the methodology described by Molina et al. (2017).

Promastigotes in stationary phase were washed, resuspended in PBS, and mixed with defibrinated rabbit blood (1×10^7 promastigotes/ml). Five to 7-day-old female sand flies (400 per experiment) were fed for at least 1 h on the infective mixture through a chicken skin membrane attached to a feeding device. Blood-engorged females were immediately separated and maintained on 30% sucrose diet under standard conditions. Dissections of blood-engorged females

were carried out at 2, 4, 7, and 9 days post-infection (DPI) in order to follow the course of the infection. The localization and intensity of infection (number of promastigotes) in the midgut were estimated using a phase contrast microscope. Individual dissected guts were placed in a drop of PBS on a slide and parasite number was estimated at × 400 magnification. The localization and intensity of infection (number of promastigotes) in the midgut were estimated using a phase contrast microscope. Intensity of infection was graded as being light or weak (< 100 parasites/gut), moderate (> 100 parasites/gut), heavy (> 1000 parasites/gut), and very heavy (> 10,000 parasites/gut) as described by Myskova et al. 2008. Infections were performed in duplicate for each *Leishmania* strain. The infection rate (IR) for each experiment was calculated (dissected sand flies/infected sand flies × 100). As well, the presence of metacyclic promastigotes close the (SV) was checked in all infections.

Statistical analysis

Data were analyzed using the SPSS 18.0. The χ^2 test was used to compare infection intensities (very heavy, heavy, moderate, and light) and the IRs of *P. perniciosus* with the different zymodemes of *L. infantum*. A statistically significant association between variables was considered to exist if the p value was < 0.05.

Results

Monitoring of the evolution of the infection within the sand fly gut was conducted at 2, 4, 7, and 9 DPI as scheduled. The number of dissections depended on the number of surviving females. The data presented on the development of three strains of *L. infantum* in the midgut of *P. perniciosus* are the mean of two replicated experiments. A detailed comparison of the two replicates showed very similar IRs in all infections ($p > 0.05$). As to the intensities of infections, the differences were not significant in the weak infection category for all replicates with all zymodemes ($p > 0.05$); however, the difference was significant for the moderate infection category ($p < 0.05$). For the heavy infection category, the differences were not significant with MON-24 and MON-1 zymodemes ($p > 0.05$) and significant with MON-80 zymodeme ($p < 0.05$). The comparing of the replicated infections in the very heavy category shows that the differences were significant with MON-80 and MON-24 zymodemes but not for MON-1 zymodeme. The total number of flies dissected in the two replicated experiments was 45, 46, and 57 for MON-1, MON-24, and MON-80 zymodemes, respectively. Among the dissected flies, 40, 29, and 54 were infected with MON-1, MON-24, and MON-80 zymodemes, respectively.

Intravectorial development of *L. infantum* zymodemes

The experimental infections of *P. perniciosus* with *L. infantum* zymodemes MON-1, MON-24, and MON-80 were done and controlled from 2 to 9 DPI. On the second DPI, the infection with MON-1 zymodeme was moderate or heavy in 100% of infected sand flies, and moderate, heavy, or very heavy for flies infected with MON-24 and MON-80 zymodemes (Fig. 3). The intravectorial development of MON-24 and MON-80 zymodemes revealed that in 11 and 27% of sand flies, respectively, the parasites had already migrated to the thoracic midgut and reached the SV (Fig. 1 and Fig. 2a). The IRs were 100% for both infections (Fig. 2b). Regarding MON-1 zymodeme parasites were still in the residual blood meal of the abdominal midgut in 100% of flies dissected and the IR was 100% (Fig. 2A and B).

On the fourth DPI, MON-24 and MON-80 zymodemes continued their successful development in the SV, with 100% of flies producing moderate, heavy or very heavy infections for both zymodemes (Fig. 2a and Fig. 3). The IRs were 69 and 82% for MON-24 and MON-80 zymodemes, respectively (Fig. 2b). MON-1 parasites migrated and colonized the SV of the sand fly females with a moderate or heavy infection and the IR was 85% (Fig. 2a, b, and Fig. 3).

On the seventh DPI, all studied *Leishmania* strains developed well and more than half of parasites were in the SV (Fig. 2a). The IRs were 100% for MON-80 and MON-1 zymodemes, and 48% for MON-24 zymodeme (Fig. 2b). Very heavy infections were observed in all experiences (Fig. 3).

Leishmania infantum strains continued to develop successfully in the sand fly midgut at 9 DPI, and led to a very heavy late stage infections in all experiences. For MON-24 zymodeme the number of flies producing a weak infection was high (75%) (Fig. 3). The IRs were above 75, 22, and 100% for MON-1, MON-24, and MON-80 zymodemes, respectively (Fig. 2b). Regarding MON-1 zymodeme, the parasites were localized in the SV in all infected flies (100%); for MON-24 zymodeme, the 25% of the parasites reached the SV, and for MON-80 zymodeme, the parasites were in the SV in more than half of the infected flies (91%) (Fig. 2a).

Comparison of *L. infantum* infection for the different zymodemes

The mean IR was 80% (88, 61, and 93% for MON-1, MON-24, and MON-80 zymodemes, respectively) (Table 1). The percentage of infected flies with MON-80 zymodeme was significantly higher than with MON-1 and MON-24 zymodemes.

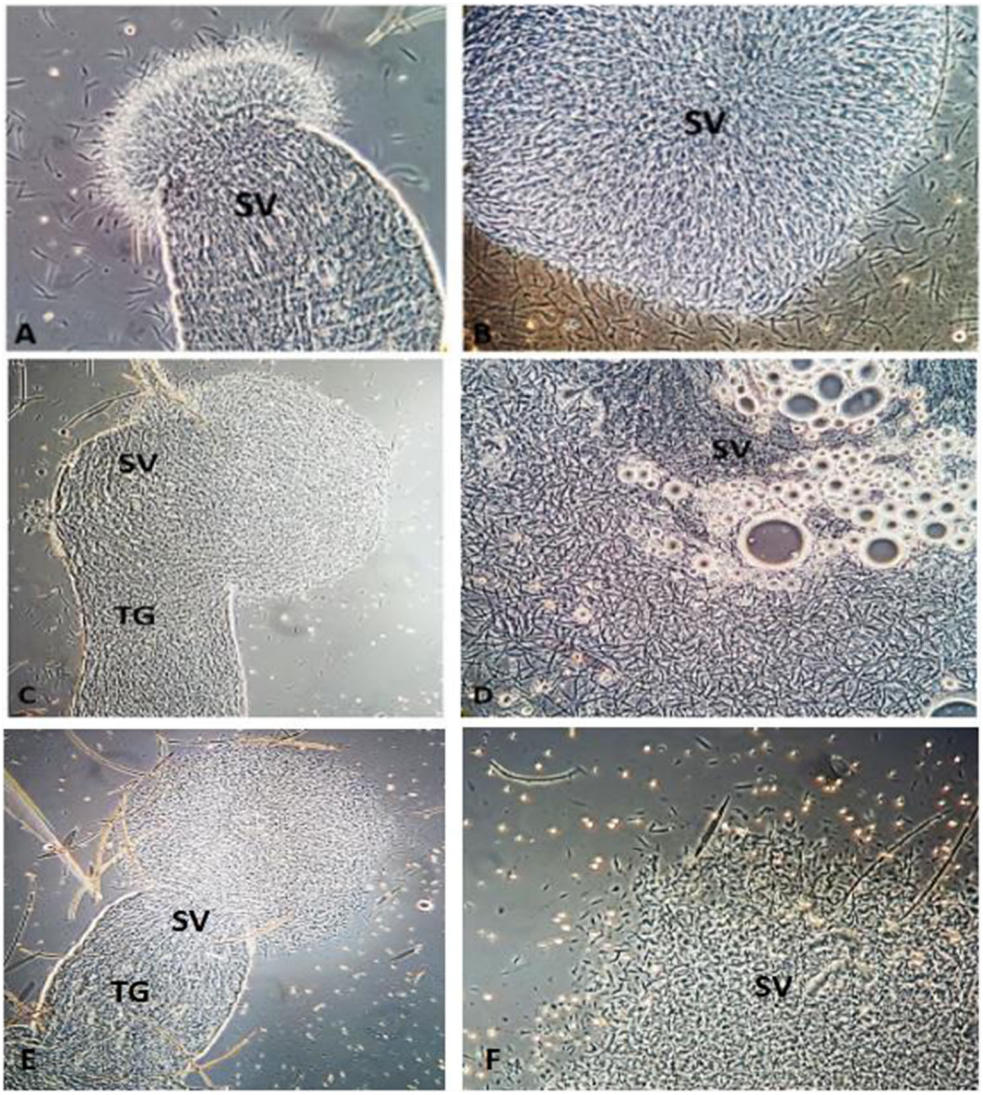

Fig. 1 Development of *L. infantum* in the gut of *P. perniciosus*. Location of parasites in the SV and the thoracic midgut (TG). **a**, **b** Development of *L. infantum* zymodeme MON-80 (× 25 and × 40) 2 DPI. **c**, **d** *L. infantum* zymodeme MON-24 (× 16 and × 40) 2 DPI. **e**, **f** *L. infantum* zymodeme MON-1 (× 16 and × 25) 4 DPI

Furthermore, the IR with MON-1 zymodeme was significantly higher than with MON-24 zymodeme (Table 1). In all experimental infections there was no correlation between sand fly mortality and the zymodemes.

Globally, a very heavy late stage infection with more than 10,000 parasites in the SV was noticed for all infections. Consequently, very heavy infections were observed from 2 to 9 DPI in MON-80 and MON-24 zymodemes and from 7 to 9 DPI in MON-1 zymodeme.

In all experiments with MON-80 and MON-24 zymodemes, the parasites reached the SV from 2 to 9 DPI. On the other hand the parasites of MON-1 zymodeme reached the SV from 4 to 9 DPI, but after 9 days, 100% of the parasites were in the SV in all infected flies (Fig. 2a). Metacyclic promastigotes were observed in the SV of sand fly females in the different infections. Indeed, it was noticeable that with MON-80, zymodeme were observed more metacyclic promastigotes than with MON-1 and MON-24 zymodemes but the number was not quantified.

Discussion

Leishmania infantum is responsible for both VL and CL in Tunisia (Bouratbine et al. 1998; Chaara et al. 2014) and *P. perniciosus* the proven vector of the zymodeme MON-1 of *L. infantum* while no information is available about the vector competence of this sand fly species for MON-80 and MON-24 zymodemes. The lack of information about the intravectorial cycle of these *L. infantum* zymodemes in Tunisia makes difficult to predict and to control the disease. According to the criteria of Killick-Kendrick (1990) on the incrimination of a sand fly species as a vector of leishmaniasis, data are already available on the anthropophilia of and the detection of *L. infantum* DNA in *P. perniciosus* collected in leishmaniasis foci of Tunisia (Guerbouj et al. 2007; Barhoumi et al. 2016). The current study provides the experimental evidence that the zymodemes MON-24 and MON-80 are able to complete the metacyclogenesis in *P. perniciosus*. The parasites

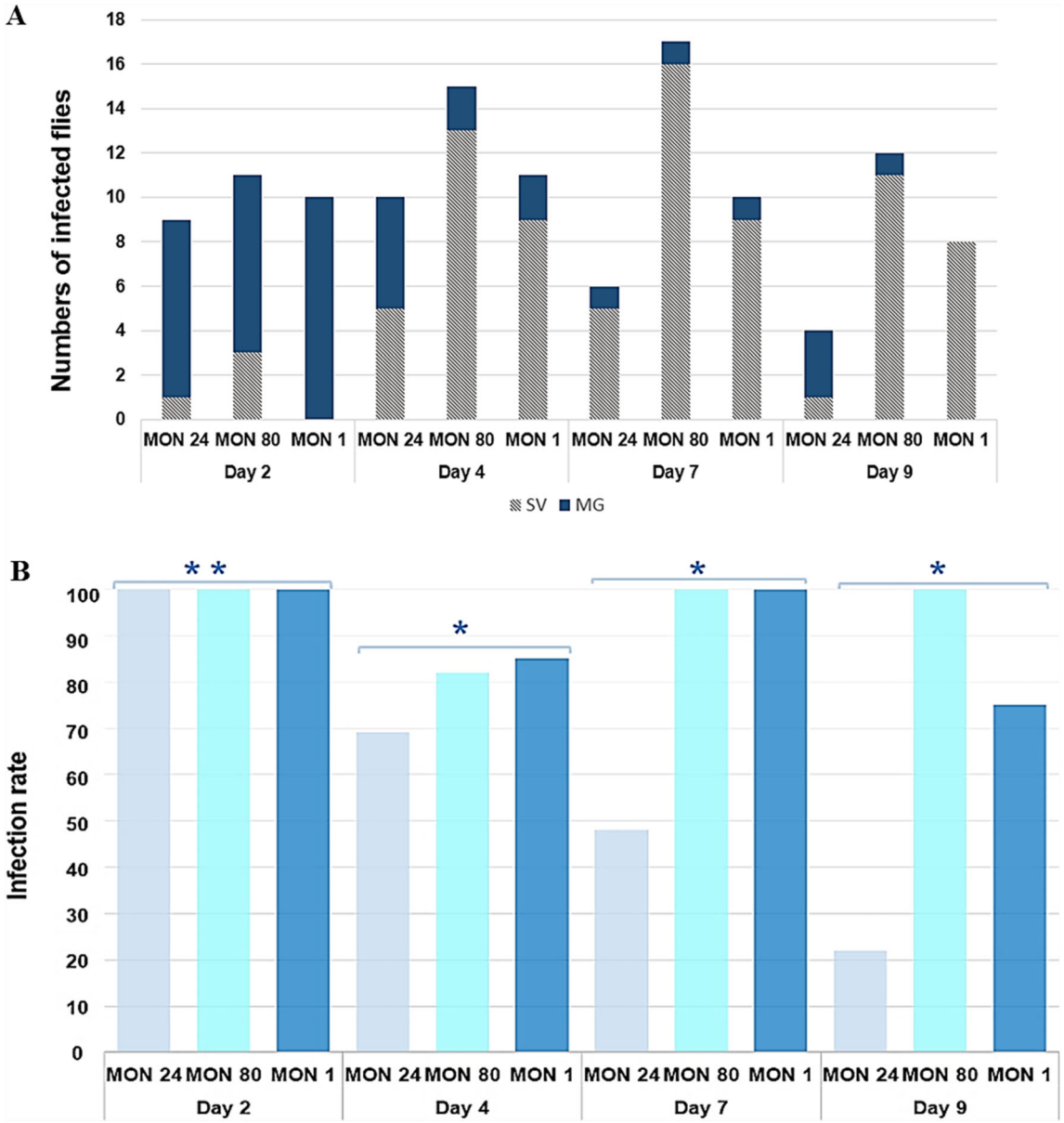

Fig. 2 Localization of *L. infantum* strains inside *P. perniciosus* (**a**) and comparison of the IRs between the different infections and during the different DPI (**b**). Differences between experiences were evaluated (**) no significants ($p > 0.05$) and (*) significant ($p < 0.05$)

persist in the sand fly midgut during blood digestion and after the defecation process, colonize the SV of the sand fly, and produce heavy late stage infections with metacyclic promastigotes which is a prerequisite for successful transmission of parasites by the sand fly bite (Dostálová and Volf 2012).

Very recently, Pruzinova et al. 2018 reported that *Leishmania* mortality in sand fly blood meal is not species-specific and does not result from direct effect of proteinases. Also observe that refractoriness of *P. papatasi* to *L. donovani* is due to the insufficiency of *L. donovani* to bind to the sand fly midgut. In our study were observed promastigotes in the SV within 48 h at least, from 2 to 9 DPI, very heavy infections and were high IRs in almost all experiments. This can be explained by the fact that promastigotes of *L. infantum* bind to *P. perniciosus* midgut very efficiently favoring their persistence during the defecation process. This finding is a proof of the ability of *P. perniciosus* to maintain the strains studied.

The comparison of IRs of *P. perniciosus* obtained with MON-1, MON-24, and MON-80 zymodemes revealed significant differences. Indeed, MON-80 zymodeme developed the highest IR (93%) followed by MON-1 zymodeme (88%) and MON-24 zymodeme (61%). The differences observed in the infection intensity between the three *L. infantum* zymodemes were statistically significant. The IRs reached a high level with all tested zymodemes (an average of 80% of the studied females were positive on all DPI), and all strains colonized the

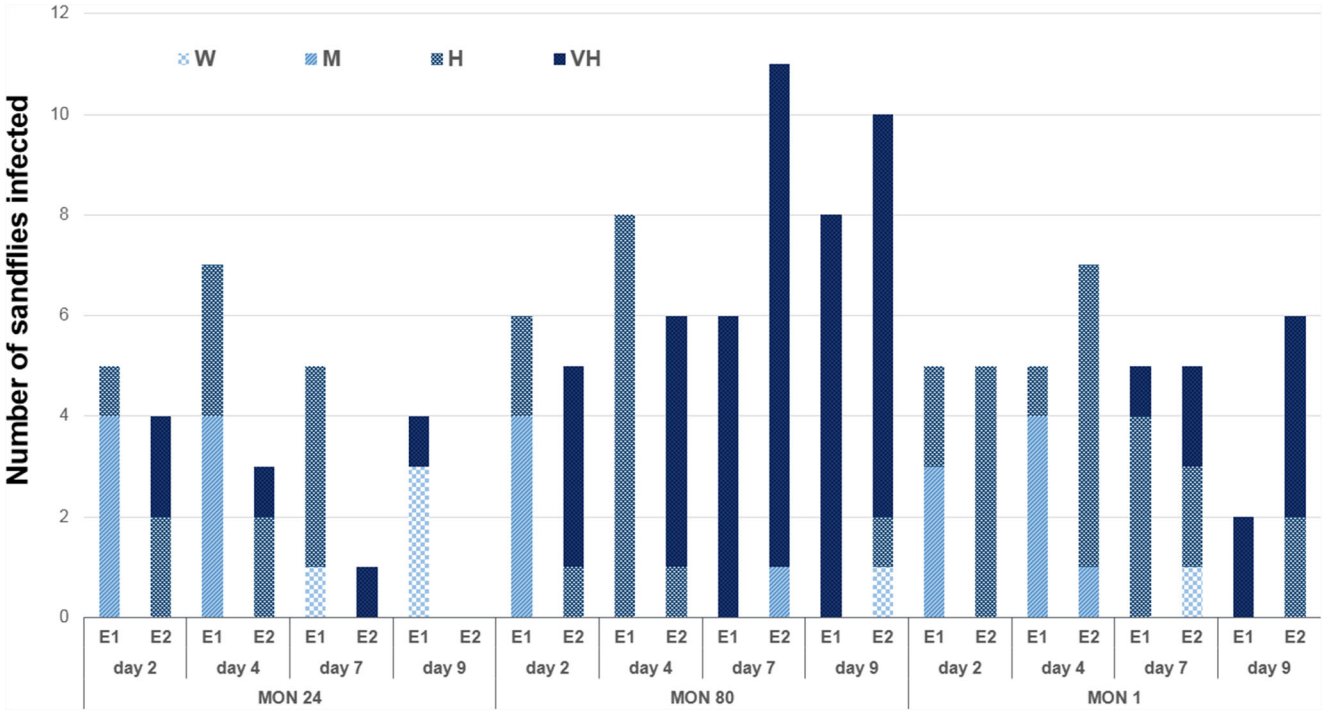

Fig. 3 Comparison of the infection level between *L. infantum* strains during post-infection control period in the two replicates. Infection levels: light or weak (< 100 parasites/gut), moderate (> 100 parasites/gut), heavy (> 1000 parasites/gut), and very heavy (>10,000 parasites/gut). Levels were calculated after 2, 4, 7, and 9 DPI. Two replicates: experiment 1 (E1) and experiment 2 (E2)

SV of the sand flies. A very heavy late infection stage was observed with all *L. infantum* zymodemes.

Concerning the progression of the infection, MON-24 and MON-80 zymodemes colonized sooner the SV within 2 DPI while MON-1 reached the SV after 4 DPI. According to the different stages of parasite development inside the sand flies described by Dostálová and Volf (2012), the promastigotes forward migrate and colonize the SV for further differentiation on infective metacyclic forms. The results obtained in the present work could be explained by the fact that dermotropic zymodemes of *L. infantum* develop faster to reach the SV of *P. perniciosus* compared to the viscerotropic ones. These findings are in agreement with those of Maia et al. (2011), who demonstrated the capacity of *P. perniciosus* to harbor more *L. infantum* dermotropic strains from Turkey than viscerotropic strains from Portugal. Guimarães et al. (2016) also reported the capacity of dermotropic *L. infantum* strains to develop slightly faster than the viscerotropic ones inside *Lu. migonei*.

Regarding the presence of metacyclic promastigotes observed in the SV of sand fly females in all infections demonstrates that they may be infective for the vertebrate host by the sand fly bite.

Conclusion

This study shows for the first time that *P. perniciosus* is a potential vector of the *L. infantum* zymodemes MON-24 and MON-80. Such a finding should be confirmed by assessing the ability of experimentally infected *P. perniciosus* to transmit the zymodemes to mammalian hosts which is a definitive test to determine the role played by this sand fly species in the

Table 1 Comparison of the infection rates (IR) between the different *L. infantum* zymodemes. Two replicates: experiment 1 (E1) and experiment 2 (E2)

Strains	Experiment	No. of fed sand flies/ no. of tested sand flies (%)	IR (%)	Mean IR (%)
L. infantum MON-80	E1	90/200 (45)	91	93
	E2	98/200 (49)	96	
L. infantum MON-24	E1	119/200 (59.5)	65	61
	E2	52/200 (26)	57	
L. infantum MON-1	E1	37/200 (18.5)	89	88
	E2	130/200 (65)	88	

transmission of MON-24 and MON-80 zymodemes in Tunisia.

Knowledge of this valuable epidemiological information is essential for the implementation of more accurate and effective vector control strategies in the context of leishmaniasis control programmes in endemic areas of Tunisia and bordering regions of the Mediterranean basin.

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Compliance with ethical standards

Not applicable

Conflict of interest The authors declare no conflicts of interest.

Abbreviations *CL*, Cutaneous leishmaniasis; *DPI*, Days post infection; *IR*, Infection rate; *SV*, Stomodeal valve; *VL*, Visceral leishmaniasis

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