Comparative Study of Promastigote- and Amastigote-Initiated Infection of Leishmania infantum (Kinetoplastida: Trypanosomatidae) in Phlebotomus perniciosus (Diptera: Psychodidae) Conducted in Different Biosafety Level Laboratories

Authors: Vaselek, Slavica, Prudhomme, Jorian, Myskova, Jitka, Lestinova, Tereza, Spitzova, Tatiana, et al.

Source: Journal of Medical Entomology, 57(2) : 601-607

Published By: Entomological Society of America

URL: https://doi.org/10.1093/jme/tjz199

Journal of Medical Entomology, 57(2), 2020, 601–607
doi: 10.1093/jme/tjz199
Advance Access Publication Date: 8 November 2019
Research

Comparative Study of Promastigote- and Amastigote-Initiated Infection of *Leishmania infantum* (Kinetoplastida: Trypanosomatidae) in *Phlebotomus perniciosus* (Diptera: Psychodidae) Conducted in Different Biosafety Level Laboratories

Slavica Vaselek,[1,3,*] Jorian Prudhomme,[2,*] Jitka Myskova,[1] Tereza Lestinova,[1] Tatiana Spitzova,[1] Anne-Laure Bañuls,[2] and Petr Volf[1]

[1]Department of Parasitology, Faculty of Science, Charles University, Viničná 7, 128 43 Prague 2, Czech Republic, [2]UMR MIVEGEC (IRD - CNRS - University of Montpellier), IRD center, 911 avenue Agropolis, F34394 Montpellier, France, and [3]Corresponding author, e-mail: slavica.vaselek@gmail.com

*These authors contributed equally to this work.

Subject Editor: David Florin

Received 20 May 2019; Editorial decision 6 October 2019

Abstract

Sand flies (Diptera: Psychodidae) are natural vectors of *Leishmania*. For the initiation of sand fly experimental infections either *Leishmania* amastigotes or promastigotes can be used. In order to obtain comparable results, it is necessary to adjust and standardize procedures. During this study, we conducted promastigote- and amastigote-initiated infections of *Leishmania infantum* Nicolle, 1908 parasites in *Phlebotomus* (*Larroussius*) *perniciosus* Newstead, 1911 in two laboratories with different levels of biosafety protection. Protocol originally designed for a biosafety level 2 facility was modified for biosafety level 3 facility and infection parameters were compared. Particularly, specially designed plastic containers were used for blood feeding; feeders were placed outside the sand fly cage, on the top of the mesh; feeding was performed inside the climatic chamber; separation of engorged females was done in Petri dishes kept on ice; engorged females were kept in the cardboard containers until dissection. All experiments, conducted in both laboratories, resulted in fully developed late stage infections with high number of parasites and colonization of the stomodeal valve. We demonstrated that protocol originally designed for biosafety level 2 facilities can be successfully modified for other biosafety facilities, depending on the special requirements of the individual institution/laboratory.

Key Words: *Leishmania*, amastigote, promastigote, infection, sand fly

Leishmaniases are parasitic diseases transmitted to humans and other mammals by the bite of an infected female sand fly. Between 12 and 15 million people worldwide are infected, and 350 million are at constant risk of acquiring the disease (Alvar et al. 2012). Phlebotomine sand flies (Diptera: Psychodidae) are relatively small blood-sucking insects; out of 800 identified sand fly species, 98 are described as potential or proven vectors of some *Leishmania* species (reviewed by Maroli et al. 2013).

Life cycle of *Leishmania* parasites within sand flies is confined to the vector digestive tract. During the vectorial part of the life cycle, *Leishmania* undergo a numerous changes, going through several morphologically and functionally distinct forms in order to develop infective metacyclic stages. Development in the vector is initiated when female sand flies ingest blood containing macrophages infected with amastigotes—small, immotile, and rounded forms of the parasite. Within the bloodmeal surrounded by peritrophic matrix, amastigotes transform to motile and flagellated procyclic promastigotes, which replicate and transform to series of other promastigote forms (reviewed by Kamhawi 2006, Bates 2008, reviewed by Dostálová and Volf 2012).

Under laboratory conditions, either *Leishmania* amastigotes or promastigotes can be used for the initiation of experimental infection in sand flies. While promastigotes can be axenically cultivated in vitro relatively easily, their ingestion by sand fly females does not mimic the natural infections. In contrast, amastigote-initiated infections of sand flies are natural but usually require the sacrifice

of laboratory animals to obtain sufficient numbers of lesion-derived amastigotes. Therefore, cultivation of amastigotes in macrophage-cell lines is considered as the best choice (Sadlova et al. 2017), although this process is relatively laborious and time consuming.

Experimental infection of sand flies with most *Leishmania* strains are usually performed in laboratories with biosafety level 2 (BSL2), which are designed for work with agents associated with human diseases that pose a moderate health hazard. Biosafety level 3 (BSL3) laboratories are designed for experiments on microbes that can cause very serious or potentially lethal diseases and are recommended in some countries for *Leishmania* strains causing visceral leishmaniases. Main restriction factor that determine the use of BSL3 versus BSL2 laboratories is that not many institutions have a proper facilities and licensed BSL3 laboratories. Experiments conducted in different BSL laboratories, and by different research teams are differing in the techniques, procedures and quite often even in the infective dose, thus obtained results are difficult to reproduce and compare. In order to obtain comparable results, it is necessary to adjust and standardize these procedures among both, research teams and different facility requirements/biosafety levels.

In this study we evaluated infection parameters of *Leishmania infantum* Nicolle, 1908 parasites in the natural vector *Phlebotomus* (*Larroussius*) *perniciosus* Newstead, 1911, using both forms, promastigotes and amastigotes. The method described by Sadlova et al. (2017) for cell line macrophages was adopted to avoid sacrificing of laboratory animals. Experimental infections were conducted in two institutions with different levels of biosafety protection to adapt the protocol designed for BSL2 to BSL3 facilities, to standardize infection parameters/readouts for promastigote- and amastigote-initiated infection and to compare the results.

Materials and Methods

In order to standardize the protocol for promastigote- and amastigote-initiated infection designed for BSL2 facilities in BSL3 facilities, comparison of *L. infantum* infection in *P. perniciosus* was performed in parallel in BSL2 facilities of Charles University (CUNI), Faculty of Science, Department of Parasitology, Prague, Czech Republic and in BSL3 facilities of the Vectopôle, MIVEGEC, Institute of Research for Development (IRD), Montpellier, France. For the purpose of standardization, original BSL2 protocol was modified to meet the special requirements of the BSL3 facilities.

Sand Fly Colony

For all experimental infections, well-adapted laboratory colony of *P. perniciosus* (originating from Spain) was used. Colony was maintained in the insectary of the Charles University in Prague under standard conditions (26 ± 1°C with 60–70% relative humidity, a light: dark cycle of 14 h: 10 h and 50% sucrose solution). Details of the colony maintenance are described by Volf and Volfova (2011) and Lawyer et al. (2017). For experimental infection in IRD Montpellier, 500 *P. perniciosus* sand flies were shipped weekly (4 d before experiments). After reception, individuals were transferred in nylon mesh cages and maintained in climatic chamber under the same conditions as in CUNI.

Cultivation of Leishmania Parasites

Leishmania infantum mCherry (MHOM/TR/2000/OG-VL) promastigotes (transfected with red fluorescence protein) were cultivated in M199 medium (Sigma) containing 20% heat-inactivated fetal bovine serum (FBS) (Gibson), supplemented with 2% sterile urine, 1% Basal Medium Eagle vitamins (Sigma), 250 µg mL^{-1} amikacin (Amikin, Bristol-Myers Squibb) and 150 µg mL^{-1} selective antibiotic neomycin (Sigma). Each week parasites were passaged to the fresh medium, during all experiments, parasites with *in-vitro* passage number less than 10 were used. For experimental infection in IRD Montpellier, two vials containing frozen *L. infantum* mCherry (MHOM/TR/2000/OG-VL) were shipped on dry ice from CUNI, 15 d before the first experimental infection, allowing the IRD hosts to establish stable cultures of parasites.

Cultivation of Macrophages

Immortalized macrophage cell line J744 originating from BALB/c mice was used. J744 macrophages were cultured at 37°C with 5% CO_2 in complete RPMI-1640 medium (Sigma) containing 10% FBS, 1% penicillin–streptomycin (Sigma) and 0.05 mM of β-mercaptoethanol. Macrophages were seeded at about $2x10^4$ cells/mL in culture medium and were subcultured every 5 d. For experiments in IRD Montpellier, two vials containing frozen J744 macrophage cell lines were shipped on dry ice from CUNI, 15 d before the first experimental infection, allowing IRD to establish stable cultures of macrophages.

Infection of Macrophages with Leishmania Promastigotes and Their Transformation to Amastigotes

Macrophage infection was performed 3 d prior to the infection feeding of sand flies. *Leishmania* promastigotes in a stationary phase of growing were washed two to three times in sterile saline solution and counted in hemocytometer. Three- to four-day-old macrophages were counted in hemocytometer and later on exposed to stationary-phase parasites at a ratio of eight promastigotes per one macrophage. Infected macrophages were cultivated in the same medium mixture as noninfected ones.

Preparation for Promastigote-Initiated Infection

For promastigote-initiated infection, *Leishmania* promastigotes from log-phase cultures (4 d postinoculation) were resuspended in heat-inactivated rabbit blood at concentration of 10^6 promastigotes per millilter.

Preparation of Parasites for Amastigote-Initiated Infection

For amastigote-initiated infections, *Leishmania* parasites were cocultivated with macrophage cell line J774 for 72 h. Noninternalized parasites were removed by washing with preheated culture medium. The macrophages were removed from the culture plates by extensive washing with cold saline solution, centrifuged at 300×g, 4°C for 10 min and resuspended in saline solution. Ten microliters of mixture were used for amastigotes per macrophage counting under fluorescent microscope. After counting, macrophages were resuspended in heat-inactivated rabbit blood for sand fly infections at the concentration of 10^6 amastigotes per mlilliter.

Feeding Process, Postfeeding Manipulation and Dissection of Sand Flies

Process of sand fly feeding, postfeeding manipulation and dissection varied between BSL2 and BSL3 laboratories. Even though the preparation of parasites and its dosage for experimental infection was the same, process of feeding was performed according to the special requirements of the laboratories and their level of protection (BSL2 vs BSL3) (Supp Table 1 [online only]).

Experimental Infections and Sand Fly Manipulation in BSL2 Facilities

Twenty-four hours prior to the infection, approximately 200 sand fly females (5–9 d old) were put in separate mesh cages and deprived of sugar. During the infection feeding, lower part of the glass feeder was placed directly inside the mesh cage through the sleeve, allowing sand fly direct access to the chicken skin membrane. Constant temperature of 37°C for heating the blood in the feeders was maintained by water bath with external circulation. Feeding was performed for 2 h at 26°C in a darkened room.

Twenty-four hours after blood feeding engorged females were separated using mouth aspirators into a separate mesh cage and kept under standard conditions. Briefly, the cage was placed in a plastic bag with moist cotton wool to maintain high humidity and kept at 26°C in a separate incubator. Day after blood feeding, small piece of cotton wool pad soaked by 50% sucrose solution was provided to females on Petri dish placed directly inside the cage.

For the dissection, females were aspirated from the mesh cages into a plastic pot and cooled on ice to anesthetize them. Guts of experimentally infected sand flies were dissected under a stereomicroscope in saline solution by fine tweezers and/or entomological pins. Females were dissected before defecation (early stage of infection at day 2), and after defecation (late stage of infection) at day 8 and 11 post-bloodmeal (PBM).

Abundance and localization of *Leishmania* parasites in the sand fly gut was examined by light and fluorescent microscopy. Parasite loads were graded as light (less than 100 parasites per gut), moderate/medium (100–1,000 parasites per gut), and heavy (more than 1,000 parasites per gut) (Myskova et al. 2015).

Experimental Infections and Sand Fly Manipulation in BSL3 Facilities

The use of mesh cages for sand fly infectious feeding within BSL2 facilities is a common practice in laboratories studying *Leishmania* experimental infections in sand flies. However, within BSL3 facilities, due to the work with more dangerous pathogens, it is necessary to reduce the risk of exposure and contamination. As opening the mesh cages and insertion/retrieval of feeders containing infectious material pose a significant risk of sand fly escape, for membrane feeding within BSL3 laboratory we specially designed cylindrical plastic containers with large opening (approximately 8 cm). Bottom of the container was closed with styrofoam cap while tight mesh was placed on the top (Picture 1). The net used for the purpose of feeding had a larger hole diameter (about 0.020 inches), compared with the standard nets used for sand fly maintenance cages (thickness 0.0078 ± 0.0015 inches), which allowed sand flies to feed though the mesh. During the infection, containers were set upright and feeders were placed on top of the mesh. Feeding of approximately 200 females per cage (5–9 d old) was performed inside the climatic chamber at a temperature of 26°C during 2 h. The blood in the feeders was maintained at constant temperature (37°C) by water bath with external circulation.

Despite the fact that engorged females are very sensitive and it is better not to handle them during the first 24 h postingestion, due to the safety regulations within BSL3 laboratory (Supp Table 1 [online only]) leaving infected blood-stained mesh freely exposed is prohibited, thus females were separated immediately after ingestion. Since mandatory equipment in BSL3 facilities considers the use of protectant mask for mouth and nose, use of mouth aspirators for separation of blood fed females is not applicable. For this purpose, immediately after feeding, plastic containers were placed on ice to anesthetize sand flies. Separation of engorged females was done with soft tweezers in Petri dishes kept on ice. Engorged females were transferred into the cardboard containers with a maximum of 30 individuals per container. Containers were made out of tick cylindrical cardboard (diameter 10 cm, length of cylinder 10 cm) which was closed with tick mesh on both sides (Picture 2). Containers with sand flies were enveloped by plastic bag, together with wet cotton pad which was placed inside to maintain high humidity and put in incubator (26°C). Twenty-four hours after blood feeding wool pad soaked by 50% sucrose solution was placed on the top mesh of the container.

Statistical Analysis

Statistical analyses of amastigote- and promastigote-initiated infection (intensity of infection and localization) in different BSL were done by Fisher´s exact test using R software version 3.1.0 (R Development Core Team 2018). The results obtained in CUNI and IRD were compared using Pearson's Chi-squared test using R software. A P-value of < 0.05 was considered significant. Statistical results were presented in legends of Figs 1 and 2.

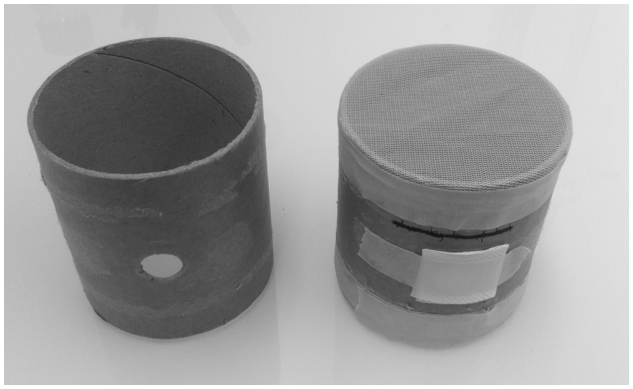
Results

Experimental Infections in BSL2 Facilities

Promastigote-initiated infection

In promastigote-initiated infection, a total of 169 blood fed sand fly females were dissected and examined for presence and localization of *Leishmania* parasites in the gut with a total infection rate of 82.8%.

Picture 1. Feeding pots—specially designed containers used during infection feeding of sand flies conducted in BSL3 facilities.



Picture 2. Resting pot—specially designed containers used for storing of blood fed females after infection feeding conducted in BSL3 facilities.

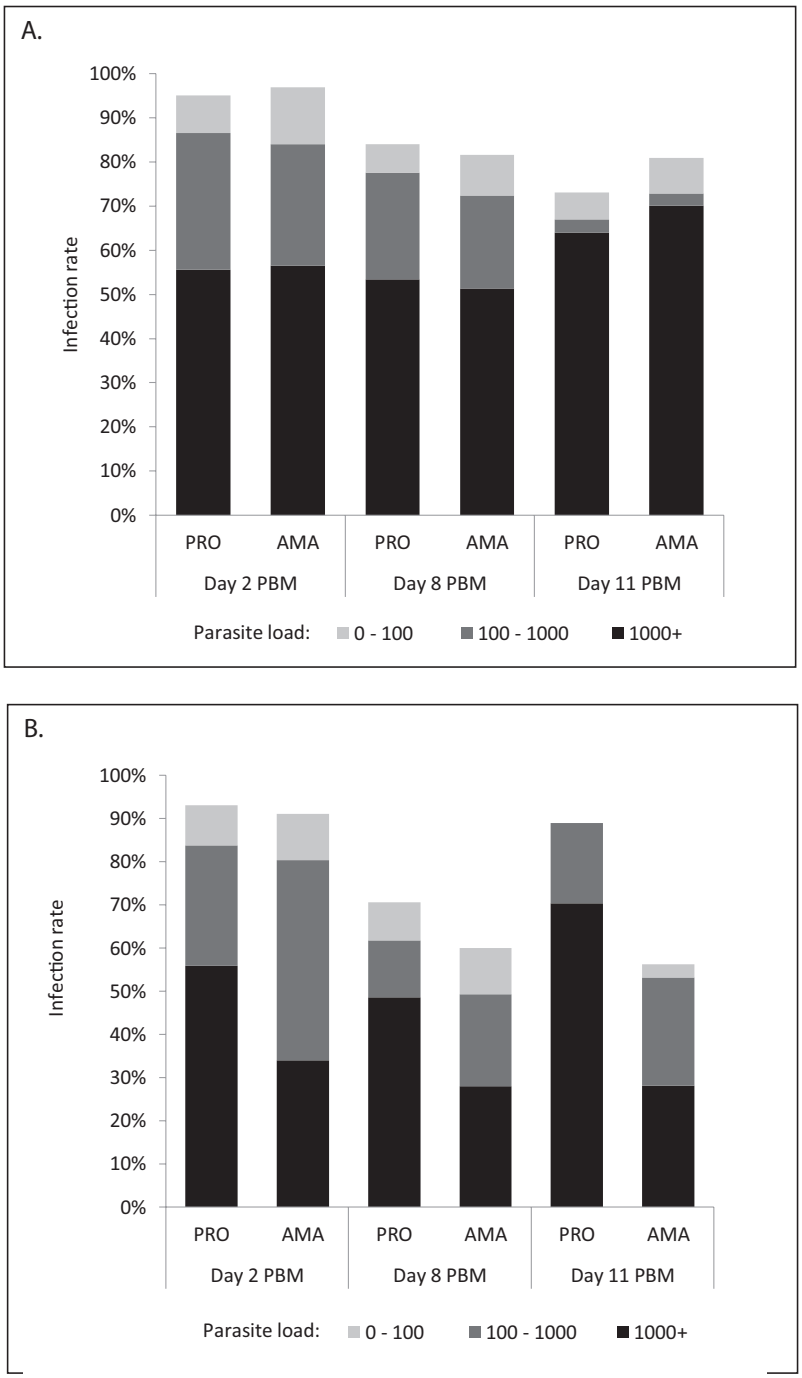


Fig. 1. Infection rate and parasite loads: A – BSL2: B – BSL3. (PRO – promastigotes; AMA – amastigotes). Comparison of parasite loads among promastigote- and amastigote-initiated infection was evaluated by Fisher exact test on day 2 PBM (BSL2: $P = 0.843$, df = 3; BSL3: $P = 0.164$, df = 3), 8 PBM (BSL2: $P = 0.6927$, df = 3; BSL3: $P = 0.088$, df = 3) and day 11 PBM (BSL2: $P = 0.3506$, df = 3; BSL3: $P = 0.004$, df = 3). Differences between results obtained in CUNI and IRD were evaluated using Pearson's Chi-squared test (all days and all experiments together): $P = 0.046$, df = 3.

Infection rates and intensities of infection

On the day 2 PBM, 60 sand flies were dissected with 95% infection rate. High-intensity infections were detected in 56.6% of females, medium in 30%, and low infections in 8.3% (Fig. 1A). On day 8 PBM, 71 sand flies were dissected with infection rate of 83%. High intensity infections were detected in 56.3%, medium in 21.1%, and low infection in 5.6% of blood fed females. Finally, on day 11 PBM, 38 sand flies were dissected with infection rate of 63.1%. High, moderate, and low intensity infections were recorded in 55.2%, 2.6%, and 5.2% of dissected females, respectively (Fig. 1A).

Localization of parasites in sand fly gut

On day 2 PBM, all parasites were localized inside the endoperitrophic space surrounded by the peritrophic matrix (Fig. 2A). On day 8 PBM, colonization of the stomodeal valve was observed in 76.2% of infected females (Fig. 2A). Similarly, on day 11 PBM, parasites reached the stomodeal valve in 75% of infected females (Fig. 2A).

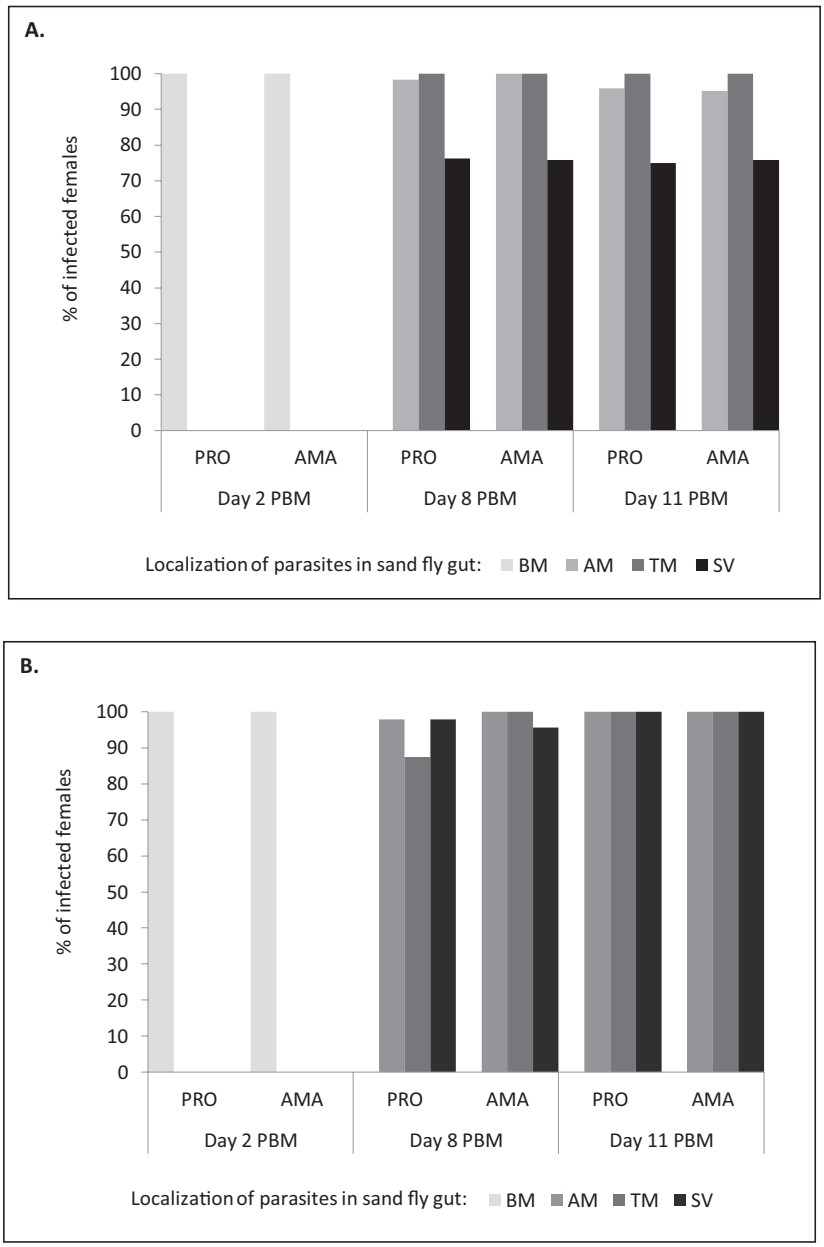


Fig. 2. Localization of parasites in sand fly gut: A– BSL2, B – BSL3D. (BM – bloodmeal; AM – abdominal midgut;TM – thoracic midgut; AM – abdominal midgut). Comparison of parasite localization inside the sand fly gut among promastigote- and amastigote-initiated infection was evaluated by Fisher exact test on day 2 PBM (BSL2: *p*-value = 1, df = 2; BSL3: *p*-value = 1, df = 2), 8 PBM (BSL2: *p*-value = 1, df = 2; BSL3: *p*-value = 1, df = 2) and day 11 PBM (BSL2: *p*-value = 1, df = 2; BSL3: *p*-value = 1, df = 2). Differences between results obtained in CUNI and IRD were evaluated using Pearson's Chi-squared test (all days and all experiments together): $P < 0.05$, df = 5.

Amastigote-initiated infection

In amastigote-initiated infections, 184 females were dissected and examined for presence and localization of *Leishmania* parasites in the gut. Total infection rate was 85.3%, similar to promastigote-initiated infections.

Infection rates and intensities of infection

On day 2 PBM, 62 females were dissected with infection rate of 96.7%. High-intensity infection was observed in 56.4%, medium in 27.4%, and low infection in 12.9% of dissected females (Fig. 1A). On day 8 PBM, 80 females were dissected with infection rate of 77.5%. High-intensity infections were found in 48.7%, medium in 20%, and low in 8.7% of dissected females. Finally, on day 11 PBM, 42 sand flies were dissected, and the infection rate was 78.5%. High, moderate, and low intensity infections were detected in 61.9%, 9.5%, and 7.1% of dissected females, respectively (Fig. 1A).

Localization of parasites in sand fly gut

On the day 2 PBM, all parasites were localized inside the endoperitrophic space surrounded by the peritrophic matrix (Fig. 2A). On day 8 PBM, parasites were found in thoracic and abdominal midgut in all dissected specimens (100%) while stomodeal valve was colonized in 75.8%. On day 11 PBM, parasites colonized stomodeal valve in 75.7% (Fig. 2A).

Experimental Infections in BSL3 Facilities

In BSL3 facilities, glass feeders were placed on the mesh on the top of the feeding containers as compared to the BSL2 facilities where the glass feeders were placed directly inside the mesh cages allowing sand flies direct access to the chick skin membrane. Presence of the mesh did not seem to affect the feeding rate since majority of females took a bloodmeal (≈70% in BSL3; ≈75% in BSL2).

Promastigote-initiated infection

For promastigote-initiated infection, in total 138 sand flies were dissected and examined for abundance and localization of *Leishmania* parasites with total infection rate of 81.1%.

Infection rates and intensities of infection

Out of 43 sand flies dissected on day 2 PBM, 40 (93%) were infected. High-intensity infections were detected in 55.9%, medium in 27.9%, and low infections in 9.3% of dissected females. On day 8 PBM, 68 sand flies were dissected with infection rate of 70.5%. High, moderate, and low intensity infections were detected in 48.5%, 13.2%, and 8.8%, respectively. Finally, on day 11 PBM, 27 sand flies were dissected with infection rate of 88.8%. High, moderate, and low intensity infections were detected in 70.3%, 18.5%, and 0% of dissected females (Fig. 1B).

Localization of parasites in sand fly gut

On day 2 PBM, all parasites were localized inside the endoperitrophic space surrounded by the peritrophic matrix. On day 8 PBM, colonization of the stomodeal valve was observed in 97.9% of infected females while on day 11 PBM, parasites were present on stomodeal valve in all examined specimens (100%) (Fig. 2B).

Amastigote initiated infection

For amastigote-initiated infection, a total of 163 females were dissected with a total infection rate of 69.9%.

Infection rates and intensities of infection

Total of 56 specimens were dissected on day 2 PBM, with infection rate of 91%. High intensity infection was observed in 33.9%, medium in 46.4%, and low infection in 10.7%. On day 8 PBM, 75 sand flies were dissected with infection rate of 60%. High, moderate, and low intensity infections were detected in 28%, 21.3%, and 10.6%, respectively. Finally, on day 11 PBM, 34 sand flies were dissected and the infection rate was 56.2%. High, moderate, and low intensity infections were detected in 28.1%, 25%, and 3.1%, respectively (Fig. 1B).

Localization of parasites in sand fly gut

On day 2 PBM, all parasites were localized inside the endoperitrophic space surrounded by the peritrophic matrix. On day 8 PBM, parasites were found high percent on stomodeal valve (95.5%). On day 11 PBM, parasites were always present in all three sections of the midgut (100% in each) (Fig. 2B).

Discussion

Phlebotomus perniciosus is a natural vector of *L. infantum* in western part of Mediterranean (rewieved by Maroli et al. 2013) and known as sand fly permissive for various *Leishmania* species (Volf and Myskova 2007, Bongiorno et al. 2019). Here, we confirmed high susceptibility of *P. perniciosus* for *L. infantum*, all experiments conducted in BSL2 and BSL3 facilities resulted in fully developed late stage infections with high number of parasites and colonization of the stomodeal valve.

Process of sand fly feeding and post feeding manipulation varied between BSL2 and BSL3 laboratories to meet the special requirements of biosafety laboratory level. Particularly, within BSL3 laboratory, specially designed plastic containers were used for blood feeding; feeders were placed outside on the top of the mesh; feeding was performed inside the climatic chamber; separation of engorged females was done with soft tweezers in Petri dishes kept on ice; engorged females were kept in the cardboard containers until dissection.

During the early stage infection, on day 2 PBM, promastigote- and amastigote- initiated infections conducted in both laboratories had similar infection rates (Fig. 1A and B) and all parasites were localized inside the bloodmeal enclosed by the peritrophic matrix (Fig. 2A and B). Nevertheless, statistically significant differences were observed between amastigote- and promastigote-initiated infections and/or BSL2 and BSL3 facilities in infection rates during late-stage infections.

Dissections conducted in BSL2 facilities during late stage infection (days 8 and 11 PBM) revealed similar results regarding the infection rates and colonization of the stomodeal valve among promastigote- and amastigote- initiated infections (Figs. 1A and 2A). In BSL3 facilities, we obtained similar results among promastigote- and amastigote- initiated infections for colonization but different results for the infection rates with a higher value for promastigotes (Figs. 1B and 2B). When we compare the data between BSL2 and BSL3, experiments conducted in BSL3 facilities revealed lower infection rates during days 8 and 11 PBM (Fig. 1A and B), however, colonization of the stomodeal valve was relatively frequent in all experiments conducted in BSL3 facilities (Fig. 2B).

Lower infection rates were observed in both, promastigote and amastigote-initiated infection in BSL3 facilities. In addition, slightly higher postfeeding mortality of females (data not shown) was noted during the first 4 d postfeeding. Higher mortality rate can be caused by the travel-induced stress in combination with immediate manipulation of engorged females after feeding and suboptimal conditions in BSL3, particularly by lower atmospheric pressure in BSL3 which poses higher danger of desiccation.

In conclusion, all experiments in BSL2 and BSL3 facilities led to mature infections of *L. infantum* in *P. perniciosus*, accompanied by high parasite load and colonization of the stomodeal valve (Figs. 1 and 2). We demonstrated that protocol originally designed for BSL2 facilities can be successfully implemented in its original state or with modifications in different laboratories within BSL2 and BSL3 facilities, depending on the special requirements of the individual institution/laboratory.

Supplementary Material

Supplementary data are available at Journal of Medical Entomology online.

Supplementary Table 1. General set of rules and requirement for Biosafety level 2 and 3 laboratories

Acknowledgments

This work was supported by European Commission, Horizon 2020 Infrastructure Infravec2 project (https://infravec2.eu/) and ERD Funds, project CePaViP CZ.02.1.01/0.0/0.0/16_019/0000759). The authors would like to acknowledge the technical/research platform dedicated on vectors at the IRD for providing technical support. This platform is a member of the

Vectopole Sud network and of the LabEX CEMEB (Centre Méditerranéen de l'Environnement et de la Biodiversité) in Montpellier.

References Cited

Alvar, J., I. D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, and M. den Boer; WHO Leishmaniasis Control Team. **2012**. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 7: e35671.

Bates, P. A. 2008. *Leishmania* sand fly interaction: progress and challenges. Curr. Opin. Microbiol. 11: 340–344.

Bongiorno, G., T. Di Muccio, R. Bianchi, M. Gramiccia, and L. Gradoni. 2019. Laboratory transmission of an Asian strain of Leishmania tropica by the bite of the southern European sand fly *Phlebotomus perniciosus*. Int. J. Parasitol. 49: 417–421.

Dostálová, A., and P. Volf. 2012. Leishmania development in sand flies: parasite-vector interactions overview. Parasit. Vectors. 5: 276.

Kamhawi, S. 2006. Phlebotomine sand flies and *Leishmania* parasites: friends or foes? Trends Parasitol. 22: 439–445.

Lawyer, P., M. Killick-Kendrick, T. Rowland, E. Rowton, and P. Volf. 2017. Laboratory colonization and mass rearing of phlebotomine sand flies (Diptera, Psychodidae). Parasite. 24: 42.

Maroli, M., M. D. Feliciangeli, L. Bichaud, R. N. Charrel, and L. Gradoni. 2013. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Med. Vet. Entomol. 27: 123–147.

Myskova, J., J. Votypka, and P. Volf. 2015. *Leishmania* in sand flies: comparison of quantitative polymerase chain reaction with other techniques to determine the intensity of infection. J. Med. Entomol. 45: 133–138.

R Development Core Team. 2018. R: a language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. http://www.r-project.org/.

Sadlova, J., J. Myskova, T. Lestinova, J. Votypka, M. Yeo, and P. Volf. 2017. *Leishmania donovani* development in *Phlebotomus argentipes*: comparison of promastigote- and amastigote-initiated infections. Parasitology. 144: 403–410.

Volf, P., and J. Myskova. 2007. Sand flies and *Leishmania*: specific versus permissive vectors. Trends Parasitol. 23: 91–92.

Volf, P., and V. Volfova. 2011. Establishment and maintenance of sand fly colonies. J. Vector Ecol. 36 (Suppl 1): S1–S9.