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Standardization of the protocol for Leishmania spp. promastigote and amastigote-initiated infections in sand flies (Diptera, Phlebotominae) conducted in BSL3 facilities

- BSL3 protocol -

Charles University, Faculty of Science, Department of Parasitology, Prague, Czech Republic
MIVEGEC, Institute of Research for Development (IRD), Montpellier, France

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INTRODUCTION AND OBJECTIVES

Leishmania are dixenous parasites and causative agents of leishmaniases. In their natural life cycle, they alternate between intracellular amastigote stages present in vertebrate hosts and extracellular promastigote stages in female sand flies.

Under laboratory conditions, either *Leishmania* amastigotes or promastigotes can be used for the initiation of experimental infection in sand flies. While promastigotes can be cultivated *in vitro*, ingestion of promastigote forms by sand fly females is unnatural and does not mimic the natural infections. In contrast, amastigote-initiated infections are more natural and cultivation of amastigotes inside macrophages or macrophage-like cell lines is considered the best choice [1], although this process is relatively laborious and time consuming.

Within the INFRAVEC2 project, the team of Charles University (CUNI), Faculty of Science, Department of Parasitology, Prague, Czech Republic, established an experimental protocol for promastigote- and amastigote-initiated infections in sand flies. Protocol is adaptable for different sand fly and *Leishmania* species, as well as for different macrophage cell lines. Detailed description of the requirements and methodology necessary to conduct promastigote- and amastigote-initiated experimental infection in sand flies within Biosafety Level 3 (BSL3) facilities is given below.

For the purpose of standardization of this protocol and infection parameters/readouts in promastigote- and amastigote-initiated infections, we present the original data obtained from the experimental infection of *Phlebotomus perniciosus* with *Leishmania infantum* mCherry. Experimental infections were conducted by dr. Jorian Prudhomme (MIVEGEC) and dr. Slavica Vaselek (CUNI) in the BSL3 facilities of the insectarium (Vectopôle) at Institute of Research for Development (IRD), Montpellier, France under the supervision of dr. Anne-Laure Bañuls (MIVEGEC).

EQUIPMENT AND CONSUMABLES REQUIRED

1. *Facility and large equipment requirements:*
   - large-space incubator for maintenance of infected and uninfected sand flies; if available, room with controlled temperature and humidity can be used instead
   - incubator for *Leishmania* cultivation
   - CO_2 incubator for macrophage cultivation
   - separate room for sand fly infections; if not available, large-space incubator can be used
   - water bath with external circulation
   - cooled centrifuge for eppendorf tubes and for 50 mL tubes
   - stereomicroscope with upper light position (minimum 10x2 magnification)
   - inverted fluorescent microscope (minimum magnification 10x40)

2. *Small equipment and consumables requirements:*
   - glass feeders
   - mouth aspirator
   - chicken skin membrane
   - hemocytometer
   - sterile plastic (cultivation flasks, pipettes, tips, Petri dishes etc.),
   - fine sharp tweezers
   - entomological pins
   - nylon mesh cages
   - plastic trays
   - plastic containers used for infection feeding
   - cardboard boxes used for storing/maintaining infected sand flies prior to the dissection

3. *Chemicals requirements:*
   - Components for *Leishmania* medium cultivation:
     M199
     fetal bovine serum
     Basal Medium Eagle vitamins
     amikacin
     selective antibiotic (neomycin)
     sterile urine
   - Components for macrophage medium cultivation:
     RPMI-1640 medium
     fetal bovine serum
     L-glutamine-penicillin–streptomycin
     β-mercaptoethanol
   - Sterile saline (150 mM NaCl)

DESCRIPTION OF WORK

1. Sand fly colony maintenance
2. Cultivation of *Leishmania* parasites
3. Cultivation of mouse macrophage line
4. Infection of macrophages with *Leishmania* and transformation to amastigotes
5. Preparation for infections of sand flies with promastigotes and amastigotes
   5.1. Preparation for infections of sand flies with promastigotes
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6. Adaptation in the feeding process, post-feeding manipulation and dissection of infected sand flies in accordance with BSL3 facilities
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   6.2. Post-feeding manipulation and maintenance
   6.3. Sand fly dissection

1. Sand fly colony maintenance

A viable sand fly colony is a prerequisite for research of experimental infection. Most detailed instructions for establishment and maintenance of sand fly colonies were given by Volf and Volfova in "Establishment and maintenance of sand fly colonies" [2] and by Lawyer et al. in "Laboratory colonization and mass rearing of phlebotomine sand flies (Diptera, Psychodidae)" [3]. If viable colony is not available in the institution where experiments are performed, non-infected adults sand flies can be shipped from another institution. For experimental infection in the Vectopôle of IRD Montpellier, 500 *P. perniciosus* sand flies were shipped weekly (4 days before experiments) by CUNI. After reception, individuals were transferred in nylon mesh cages and maintained in climatic chamber under the same conditions as in CUNI.

2. Cultivation of Leishmania parasites

Leishmania promastigotes (transfected with fluorescence protein – GFP, RFP etc.) 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). Lower concentration of the fetal bovine serum is recommended for *L. major* and *L. mexicana* and higher concentration for *L. donovani* and *L. infantum*. It is preferable to passage parasites weekly and for the experiments use only parasites with passage number less than 10.

Leishmania strains can be shipped from another institution on dry ice and be unfrozen/cultivated at least two weeks prior to the experimental infection in order to establish stable cultures of parasites. For experimental infection in IRD Montpellier, 2 vials containing frozen *L. infantum* mCherry transfected with red fluorescence protein (MHOM/TR/2000/OG-VL) were shipped on dry ice from CUNI, 15 days before the first experimental infection, allowing MIVEGEC to establish stable cultures of parasites in BLS2 laboratory.

3. Cultivation of mouse immortalized macrophage line

Obtaining amastigotes derived from organs of infected animals or from bone-marrow derived cells requires frequent animal sacrifice. In order to avoid consumption of animals, designed protocol is applied on an immortalized macrophage cell line (J744). These cells were cultured

at 37°C with 5% CO2 in complete RPMI-1640 medium (Sigma) containing 10% FBS, 1% penicillin–streptomycin (Sigma) and 0.05 mM of β-mercapto-ethanol. Macrophages were seeded at about $2x10^4$ cells/mL in culture medium and were subcultured every five days.
Macrophage cell lines can be shipped from another institution on dry ice and be unfrozen/cultivated at least two weeks prior to the experimental infection in order to establish stable cultures of macrophages. For experiments at IRD Montpellier, 2 vials containing frozen J744 macrophage cell lines were shipped on dry ice from CUNI, 15 days before the first experimental infection, allowing MIVEGEC to establish stable cultures of macrophages.

4. Infection of macrophages with Leishmania and transformation to amastigotes

For experiments in IRD macrophage infection was performed 3 days prior to the infection feeding of sand flies. Instructions for different *Leishmania* species are given in the table below.

Leishmania species	Washing of non-internalized promastigotes (days after macrophage infection)	Sand fly infections (days after macrophage infection)
Leishmania infantum [MHOM/TR/2000/OG-VL *mCherry*]	Day 1	Day 2
Leishmania major [WHOM/IR/-/173DsRed]	Day 3	Day 5
Leishmania donovani [MHOM/ET/2010/DM-1033/GR374 GFP] [MHOM/ET/2009/AM459 RFP]	Day 2	Day 3
Leishmania mexicana [MNYC/BZ/62/M379 GFP]	Day 1	Day 2

Macrophage infection was performed 3 days prior to the infection feeding of sand flies. *Leishmania* promastigotes in stationary phase of growing were washed 2-3 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 non-infected ones.

5. Preparation for infections of sand flies with promastigotes and amastigotes

5.1. Preparation for infections of sand flies with promastigotes

For promastigote-initiated infections, *Leishmania* promastigotes from log-phase cultures (4 days post-inoculation) were resuspended in heat-inactivated rabbit blood at concentration of 10^6 promastigotes per mL.

5.2. Preparation for infections of sand flies with amastigotes

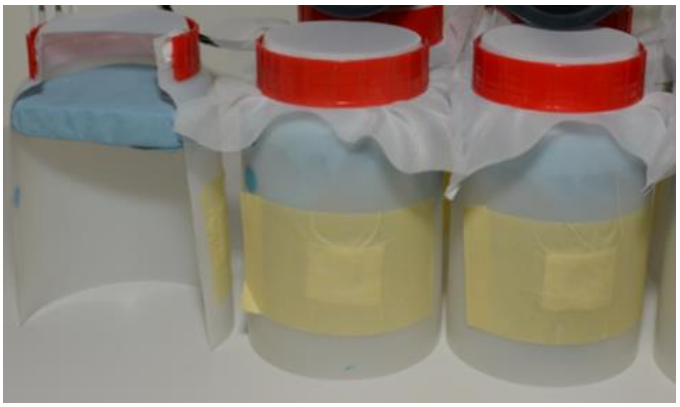
For amastigote-initiated infections, *Leishmania* parasites were co-cultivated with macrophages line J774 for 72 h. Non-internalized parasites were removed by washing with pre-heated culture medium. The macrophages were removed from the culture plates by extensive washing with cold saline solution, centrifuged at 300×g, at 4°C for 10 min and resuspended in saline solution. 10 µL of solution was 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 mL.

6. *Adaptation in the feeding process, post-feeding manipulation and dissection of infected sand flies in accordance with BSL3 facilities*

Process of blood feeding, post feeding manipulation and dissection of sand flies varied between BSL2 and BSL3 laboratories. Even though 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 *versus* BSL3).

6.1. Blood feeding

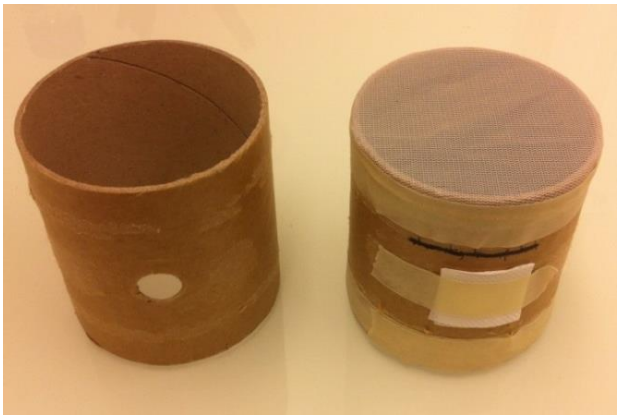
24 hours prior to the infection, females were deprived of sugar. On the day of infection, approximately 200 females (5–9 days old) were placed in specially designed plastic containers used for blood feeding. For this purpose, cylindrical plastic containers with large opening (approximately 8 cm) were used. Bottom of the container was closed with styrofoam cap while tight mesh was placed on the top (Picture 1). During the infection, containers were set upright and feeders were placed on the top of the mesh. Feeding was performed inside the climatic chamber at 26°C during 2 hours. The blood in the feeders was maintained at constant temperature (37°C) by water bath with external circulation.



Picture 1. Plastic containers used for sand fly feeding in BSL3 facilities

6.2. Post-feeding manipulation and maintenance

Since mandatory equipment in BSL3 facilities requires 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, 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). 24 hours after blood feeding, a wool pad soaked by 50% sucrose solution was placed on the top mesh of the container.



Picture 2. Cardboard containers used for blood-fed female storage during experiments in BSL3 facilities

6.3.Sand fly dissection

For dissection, cardboard boxes containing maximum 30 females were placed in plastic bags and put in ice to anesthetize insects. 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 post blood meal (PBM). Additional dissection may be performed, e.g. on day 11 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 (100–1000 parasites per gut) and heavy (more than 1000 parasites per gut). For experimental infection in the IRD Vectopôle, infection rates and intensity of infection were studied on days 2, 8 and 11 post blood meal.

RESULTS

- *Leishmania infantum* mCherry promastigote and amastigote initiated infections in *Phlebotomus perniciosus* conducted in BSL3 facilities -

In BSL2 facilities, glass feeders were placed directly inside the mesh cages allowing sand flies direct access to the chicken skin membrane. In BSL3 facilities glass feeders were placed on the mesh on the top of the feeding containers. Presence of the mesh did not significantly affect the feeding rate since majority of females took a blood meal.

1. Promastigote initiated infections

For promastigote-initiated infections, in total 138 sand flies were dissected and examined for abundance and localization of *Leishmania* parasites with a total infection rate of 81.15%.

Infection rates and intensities of infection: Out of 43 sand flies dissected on day 2 PBM, 40 (93.02%) 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 an infection rate of 70.59%. High, moderate and low intensity infections were detected in 48.52%, 13.23% and 8.82%, respectively. Finally, on day 11 PBM, 27 sand flies were dissected with an infection rate of 88.88%. High, moderate and low intensity infections were detected in 70.37%, 18.51% and 0% of dissected females (Figure 1).

Localization of parasites in the sand fly gut: On day 2 PBM, all parasites were localized inside the endoperitrophic space surrounded by the peritrophic matrix (Figure 2). On day 8 PBM, colonization of the stomodeal valve was observed in 97.92% of infected females while parasites were also present in thoracic midgut (87.5%) and abdominal midgut (97.92%). On day 11 PBM, parasites were present in thoracic midgut (100%), abdominal midgut (100%) and stomodeal valve (100%) (Figure 2).

2. Amastigote initiated infections

For amastigote-initiated infections, a total of 163 females were dissected with a total infection rate of 69.94%.

Infection rates and intensities of infection: A total of 56 specimens were dissected on day 2 PBM, with an infection rate of 91.08%. High intensity infection was observed in 33.92%, medium in 46.42% and low infection in 10.72%. On day 8 PBM, 75 sand flies were dissected with an infection rate of 60%. High, moderate and low intensity infections were detected in 28%, 21.34% and 10.67%, respectively. Finally, on day 11 PBM, 34 sand flies were dissected and the infection rate was 56.25%. High, moderate and low intensity infections were detected in 28.13%, 25% and 3.13%, respectively (Figure 1).

Localization of parasites in the sand fly gut: On day 2 PBM, all parasites were localized inside the endoperitrophic space surrounded by the peritrophic matrix (Figure 2). On day 8 PBM parasites were found mainly in thoracic midgut (100%) and abdominal midgut (100%) but also in stomodeal valve (95.56%). On day 11 PBM, parasites were also present in thoracic midgut (100%), abdominal midgut (100%) and stomodeal valve (100%) (Figure 2).

In conclusion, both, promastigote- and amastigote-initiated infections of *L. infantum* mCherry in *P. perniciosus* led to mature infections accompanied by high parasite loads on days 8 and 11 (day 8 PBM – 70.59% in promastigote- and 60% in amastigote-initiated infections; day 11 PBM – 88.88% in promastigote- and 56.25% in amastigote-initiated infections). Parasites colonized

the stomodeal valve with similarly high frequency (day 8 PBM – 97.92% in promastigote- and 95.56% in amastigote-initiated infections; day 11 PBM – 100% in promastigote- and 100% in amastigote-initiated infections) (Figures 1 and 2).

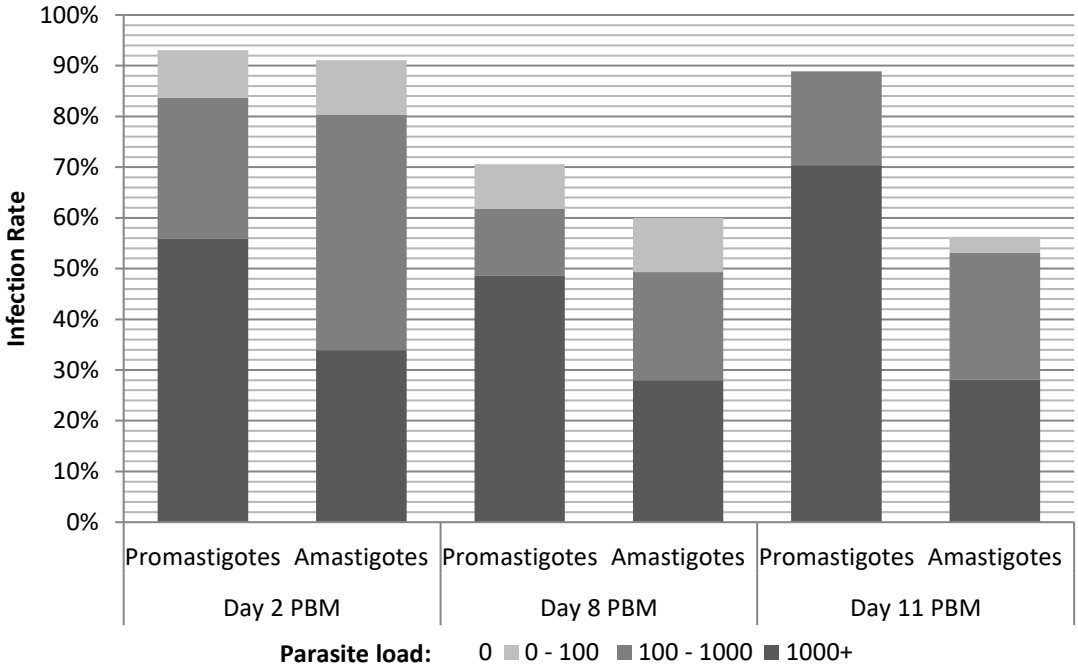


Figure 1. Infection rates and intensities of infection

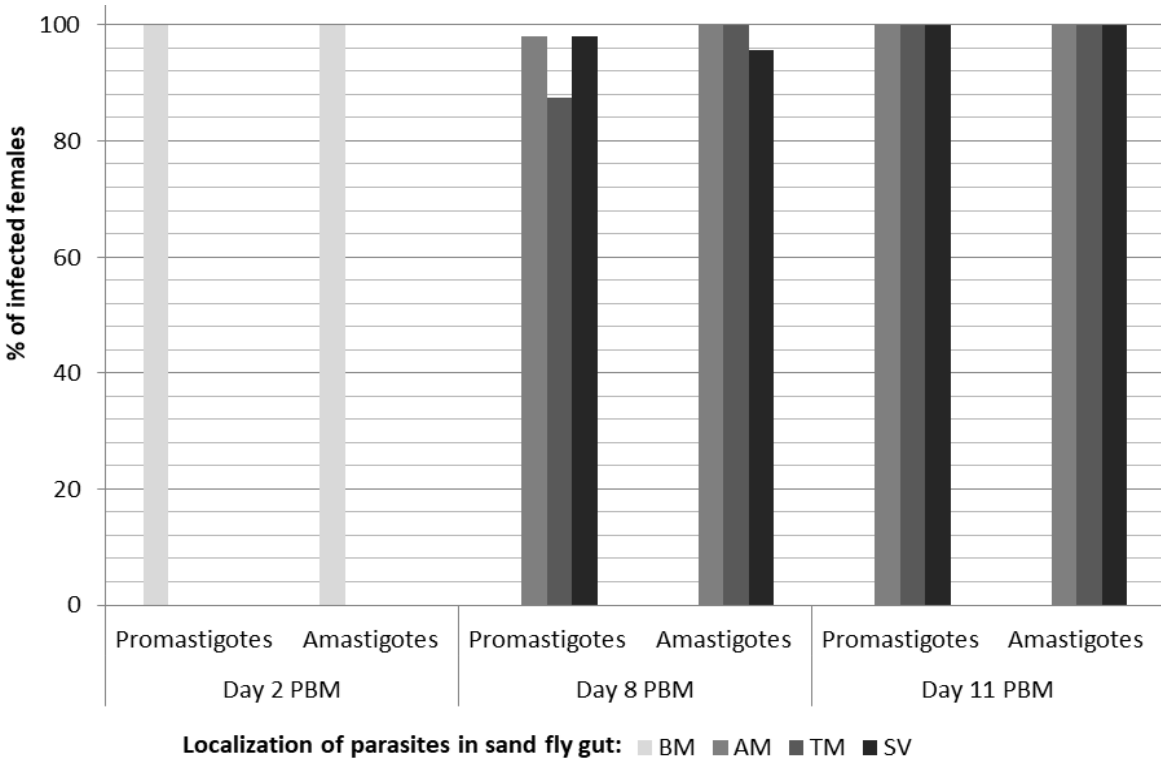


Figure 2. Localization of parasites in sand fly gut (BM - blood meal; AM - abdominal midgut, TM - thoracic midgut, SV - stomodeal valve)

SUMMARY AND CONCLUDING REMARKS

All experiments led to mature infections of *Leishmania infantum* in *Phlebotomus perniciosus*, accompanied by high parasite load and colonization of the stomodeal valve of sand fly females. Such late-stage infections are typical for natural parasite-vector pairs and colonization of the stomodeal valve is a prerequisite for eventual transmission into the vertebrate host.

The results show that the protocol of experimental infections, designed by the CUNI team within the INFRAVEC2 project, can be successfully adopted with certain modifications to BSL3, depending on requirements of individual institutions/laboratories.

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