Standardization of the protocol for Leishmania spp. promastigote and amastigote-initiated infections in sand flies (Diptera, Phlebotominae) conducted in BSL2 facilities

- BSL2 protocol -

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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 descriptions of the requirements and methodology necessary to conduct promastigote- and amastigote-initiated experimental infection in sand flies within Biosafety Level 2 (BSL2) facilities are 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 in BSL2 facilities at CUNI, laboratory of prof. Petr Volf, by dr. Slavica Vaselek, dr. Jitka Myskova (both CUNI) and dr. Jorian Prudhomme (MIVEGEC unit, Institute of Research for Development (IRD), Montpellier, France).

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 macrophages cultivation
   - separate room for sand fly infection; 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

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

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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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   6.3. Sand fly dissection

1. Sand fly colony maintenance

A viable sand fly colony is a prerequisite for research on experimental infections. 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 a viable colony is not available in the institution where experiments are performed, non-infected adults can be shipped from another institution. For experimental infection in CUNI, 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°C±1°C with 60-70% relative humidity, a light: dark cycle of 14 h: 10 h and 50% sucrose solution).

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 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 CUNI, *L. infantum* mCherry transfected with red fluorescence protein (MHOM/TR/2000/OG-VL), deposited in cryobank in CUNI, were unfrozen two weeks prior to the experiments and cultivated as described above.

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 use of animals, a protocol is designed for 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. For experimental infection at CUNI, J744 macrophage cell lines deposited in cryobank in CUNI, were unfrozen two weeks prior to the experiments and cultivated as described above.

4. Infection of macrophages with Leishmania promastigotes and their transformation to amastigotes

For experiments at CUNI, 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/-/173 DsRed]	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

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 a 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 infection of sand flies with promastigotes

For promastigote-initiated infection, *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. *Feeding process, post-feeding manipulation and dissection of sand flies in BSL2 facilities*

Process of sand fly feeding, post feeding manipulation and dissection varied between BSL2 and BSL3 laboratories. Even though preparation of parasites and dosage for experimental infection were 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

Twenty-four hours prior to the infection, approximately 200 sand fly females (5–9 days 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 hours at 26°C in a darkened room.

6.2. Post-feeding manipulation and maintenance

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. The 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.

6.3. Sand fly dissection

For 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 post blood meal (PBM). If necessary, 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 infections at CUNI, 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 infection in *Phlebotomus perniciosus* conducted in BSL2 facilities -

1. Promastigote initiated infections

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 82.84%.

Infection rates and intensities of infection: On day 2 PBM, 60 sand flies were dissected with 95% infection rate. High intensity infections were detected in 56.66% of females, medium in 30% and low infections in 8.34% (Figure 1). On day 8 PBM, 71 sand flies were dissected with infection rate of 83.09%. High intensity infections were detected in 56.33%, medium in 21.13% and low infection in 5.64% of blood fed females. Finally, on day 11 PBM, 38 sand flies were dissected with an infection rate of 63.16%. High, moderate and low intensity infections were recorded in 55.27%, 2.64% and 5.27% of dissected females, 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 colonization of the stomodeal valve was observed in 76.28% of infected females while parasites were also present in thoracic midgut (100%) and abdominal midgut (98.30%). On day 11 PBM, parasites were present in abdominal midgut (95.83%), thoracic midgut (100%), and stomodeal valve (75%) (Figure 2).

2. Amastigote initiated infections

In amastigote-initiated infections, 184 females were dissected and examined for presence and localization of *Leishmania* parasites in the gut. The total infection rate was 85.32%, similar to promastigote-initiated infections.

Infection rates and intensities of infection: On day 2 PBM, 62 females were dissected with an infection rate of 96.78%. High intensity infection was observed in 56.45%, medium in 27.42% and low infection in 12.9% of dissected females (Figure 1). On day 8 PBM, 80 females were dissected with infection rate of 77.5%. High intensity infections were found in 48.75%, medium in 20% and low in 8.75% of dissected females. Finally, on day 11 PBM, 42 sand flies were dissected, and the infection rate was 78.57%. High, moderate and low intensity infections were detected in 61.9%, 9.52% and 7.14% of dissected females, 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 in thoracic midgut and abdominal midgut in all dissected specimens (100%) while stomodeal valve was colonized in 75.8%. On day 11 PBM, parasites were also present in thoracic and abdominal midgut (100%), while stomodeal valve was colonized in 75.75% (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 PBM (day 8 PBM – 83.09% in promastigote- and 77.5% in amastigote-initiated infection; day 11 PBM – 63.16% in promastigote- and 78.57% in amastigote-initiated infection). Parasites colonized the stomodeal valve with similarly high frequency (day 8 PBM – 76.28% in promastigote- and 75.8% in amastigote-initiated infection; day 11 PBM – 75% in promastigote- and 75.75% in amastigote-initiated infection) (Figures 1 and 2).

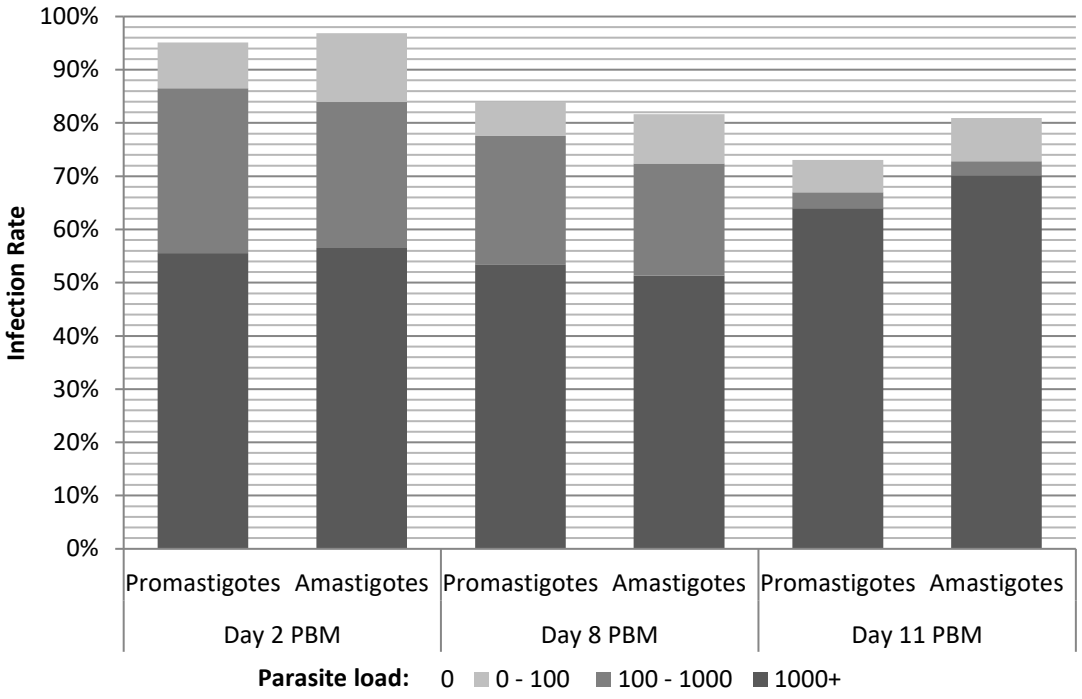

Figure 1. Infection rates and intensities of infection

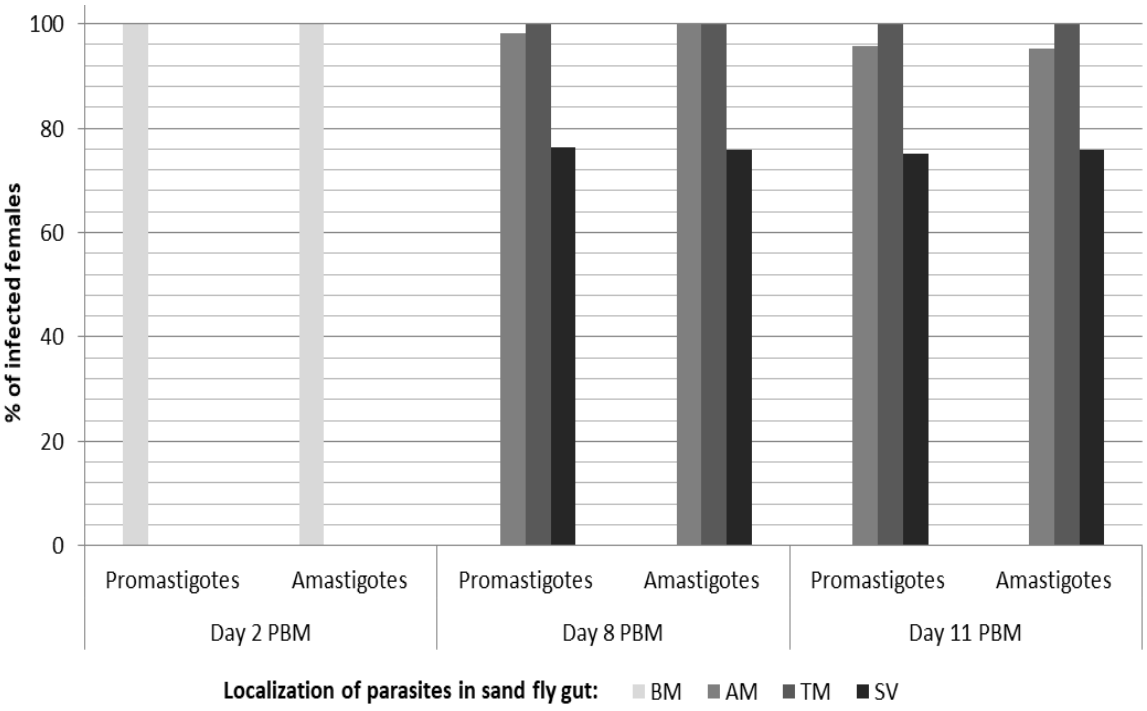

Figure 2. Localization of parasites in the sand fly gut (BM - blood meal; AM - abdominal midgut, TM - thoracic midgut, SV - stomodeal valve)

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 this protocol, designed in the scope of the INFRAVEC2 project, can be successfully implemented, in its original state or with modifications, in different laboratories with Biosafety Level 2, depending on the special requirements of the individual institution/laboratory.

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