In vitro sensitivity testing of *Leishmania* clinical field isolates: pre-conditioning of promastigotes enhances infectivity for macrophage host cells.

(WORKING TITLE)

In vitro pre-conditioning of *Leishmania* promastigotes

(AUTHORS)

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Abstract

Diagnostic material from patients with leishmaniasis is generally available as promastigotes and proper susceptibility testing to first-line drugs using the intracellular amastigote assay is frequently hampered by poor infectivity for the macrophage host cell. Several conditions were investigated to optimize the in vitro metacyclogenesis and cell-infectivity of L. donovani, L. guyanensis and L. braziliensis field strains obtained from patients receiving standard antimony medication. Triggering log-phase promastigotes to become ‘amastigote-like’ by increasing temperature or acidifying the culture medium was not successful. Adequate metacyclogenesis and highest levels of macrophage infection were obtained after pre-conditioning 5-day-old late log-phase promastigote cultures at 25°C to pH 5.4 for 24h in Schneider’s medium prior to infection. The susceptibility assay on primary peritoneal mouse macrophages included pentavalent antimony (SbV: sodium-stibogluconate), trivalent antimony (SbIII: potassium antimonyl tartrate), miltefosine and the experimental drug PX-6518. All strains were sensitive to miltefosine (IC_{50} <10µM) and PX-6518 (IC_{50} <2µg/ml), but showed a distinct susceptibility to either SbV and/or SbIII depending whether they were derived from ‘cured’, ‘relapse’ or ‘non-responder’ patients. Within the available set of Leishmania species and strains, simultaneous SbV-SbIII resistance was clearly associated with treatment failure, however, a larger set of isolates is still needed to judge the predictive value of SbV-SbIII susceptibility profiling on treatment outcome. In conclusion, the proposed conditioning protocol further contributes towards a more standardized laboratory model for drug sensitivity evaluation of field isolates.

Key words

in vitro susceptibility, metacyclogenesis, Sb-resistance, Leishmania
Introduction

As first-line treatment failure for all clinical forms of leishmaniasis is a growing problem, it is pivotal to monitor the efficacy of standard drugs and map the prevalence of drug-resistance in disease-endemic areas (7, 9, 24). Diagnostic field isolates are mostly provided to the laboratory as promastigotes, but it remains an experimental challenge to appropriately adopt them to the amastigote-macrophage model, still considered as the golden standard for susceptibility evaluation (23, 31). Infection of the macrophage is generally achieved with metacyclic promastigotes, but unfortunately is subject to large variability, hereby among several other factors affecting the outcome of the sensitivity test (8, 10). The latter strongly endorses the need for further standardisation of susceptibility testing of clinical field isolates.

In the normal course of events, infective metacyclic promastigotes are inoculated by the sandfly into the mammalian host where they rapidly penetrate susceptible cells, undergo intracellular transformation to the amastigote form and start dividing. Procyclic and metacyclic phases observed during in vitro culture appear to be similar to the stages occurring in the sandfly gut. Moreover, the infectivity for the vertebrate host steadily increases from the log- to stationary phase and is linked to the progressive increase of metacyclic promastigotes (5, 27). Metacyclics are identified as small, slender promastigotes (≤8µm x 1.2 -1.5µm) with a flagellum measuring at least twice the length of the cell body and they occur in greatest numbers in vitro during late stationary-phase. However, the level of metacyclogenesis may vary considerably depending on the species, strain and culture conditions (13). Since stationary-phase cultures appear to be a heterogeneous population, another relevant limitation is the incomplete transformation into actively dividing amastigotes after internalisation by the macrophage host cell. Not all metacyclics appear to have the capability to transform into amastigotes (19), thereby influencing again the outcome of the sensitivity testing. The latter is particularly relevant for drugs that specifically act on intracellular amastigotes, such as pentavalent antimonials (4) and the experimental antileishmania compound PX-6518 (22, 31).

The aim of this study was to investigate how promastigotes of different Leishmania species can be effectively triggered to differentiate into highly infective metacyclics.
Stimulation of metacyclogenesis has been obtained by culturing promastigotes at acidic pH, leading to a more homogeneous stationary-phase population of metacyclic-like promastigotes (33, 34). However, continuous growth in acidic conditions leads to early appearance of metacyclics and a lower final cell density (1, 6). Therefore, we hypothesized that promastigotes after growth at neutral pH could be more optimally conditioned by exposing them briefly to lower pH just prior to infection, leading to a higher final cell density and more reproducible intracellular infections. Different strains could even be synchronized to infect macrophages at a same time, which may be a practical advantage in laboratories that have to run sensitivity evaluations on a large number of field isolates in the frame of epidemiological studies.
Materials and methods

Culture media, products, reagents and animals

Adenosine, folic acid, D-biotin, hemin, NaHCO₃, potato starch, DMSO, Giemsa-stain, resazurin and trivalent antimony (SbIII: potassium antimonyl tartrate trihydrate) were purchased from Sigma, whereas MEM, RPMI-1640, Schneider’s medium, L-glutamine and fetal calf serum (FCS) were supplied by Invitrogen. Miltefosine (MIL) and pentavalent antimony (SbV: sodium stibogluconate) were kindly provided by WHO-TDR. The experimental antileishmania compound PX-6518 was available from previous work (22). Stock solutions of SbIII and SbV were prepared in pre-heated PBS at 37°C immediately before use. MIL, AMB and PX-6518 were dissolved in MilliQ-water and stored at 4°C.

Swiss mice were supplied by Janvier (France). Animal experiments were approved by the Ethical Committee of the University of Antwerp.

Parasite- and cell cultures

Parasites: field isolates of visceral (VL: L. donovani), cutaneous (CL: L. guyanensis) and mucocutaneous (MCL: L. braziliensis) clinical cases were obtained from the Institute of Tropical Medicine Antwerp (ITMA) (26, 32) as promastigote cryostabilates after primary isolation on 3N blood slopes with a saline/antibiotic overlay. Upon thawing, the strains were sub-cultured twice a week on 3N blood slopes for 2-3 weeks and adaptation to MEM medium consisted of at least 2 additional passages. In total, about 10 passages were generally performed before in vitro susceptibility testing was performed. Culture of the promastigotes was performed in MEM supplemented with 200 mM L-glutamine, 16.5 mM NaHCO₃, 10% heat-inactivated FCS (FCS), and of 40 mg/L adenine, 3 mg/L folic acid, 2 mg/L D-biotin and 2.5 mg/L hemin. The culture had an initial pH of 7.5 and was kept at 25°C under normal atmospheric conditions. All strains were derived from patients (VL: Nepal; CL and MCL: Peru) that had received standard antimony treatment and of which treatment outcome was adequately documented. For some strains, clones were also available (Table 1).
Cell culture: primary peritoneal mouse macrophages (PMM) were collected from Swiss mice two days after peritoneal stimulation with a 2% potato starch suspension. Cells were collected and grown in RPMI-1640 medium supplemented with 200 mM L-glutamine, 16.5 mM NaHCO₃ and 5% FCS, at 37°C under 5% CO₂.

Pre-conditioning of promastigotes

Temperature, differentiation time and acidification of the medium were used as variables for pre-conditioning of the promastigote cultures. The influence of temperature was evaluated by incubating the (M)CL and VL promastigotes at either 25°C, 34°C or 37°C. Conditioning time of the promastigotes varied from 24h to 72h. The following protocols were adopted for acidification:

1. Standard growth in MEM with spontaneous acidification and metacyclogenesis, the latter being largely dependent on inoculum size and speed of growth. For slow growing strains, the starting inoculum was adaptively increased.

2. Artificial acidification of MEM with 1N HCl to pH 5.4 during late log-phase, i.e. 5-day old cultures.

3. Transfer of 5-day promastigote culture into Schneider’s medium at pH 5.4, supplemented with 20% FCS (11).

Intracellular drug sensitivity assays

PMM were seeded in 96-well microtiter plates at 3x10⁵ cells/well and left for adhesion and differentiation for 48 hours before infection with metacyclics at an infection ratio of 10/1. After 24 hours, non-internalized promastigotes were removed and the medium was replaced with medium containing two-fold drug dilutions starting from 77µg Sb⁵⁺/ml, 88 µg Sb⁴⁺/ml, 40 µM MIL and 2 µg/ml PX-6518. After 5 days incubation in 5% CO₂ at 37°C for VL and 34°C for CL and MCL, parasite burdens (=% infected macrophages X mean no. of amastigotes/macrophage) were microscopically assessed on Giemsa-stained preparations and compared to untreated-infected controls. At least 100 macrophages were counted and infection was judged adequate if 70-80% of macrophages were infected. The results are expressed as % reduction of total parasite burdens and IC₅₀-values were...
calculated (Statview®). At least 6 independent replicate tests were performed for each observation. To more easily compare the results obtained with Sb\textsuperscript{V} and Sb\textsuperscript{III} in the different experiment series, an activity index (AI) was calculated representing the ratio IC\textsubscript{50} field strain/IC\textsubscript{50} sensitive reference strain. Strains BPK 043 and PER 106 were chosen as Sb-sensitive reference strains (Table 1). An AI of ≥4 is defined as resistance (32).

**Morphological studies**

*Light microscopy:* morphological changes during pre-conditioning were monitored by preparing thin smears from promastigotes that were air dried, fixed in methanol and stained with Giemsa. Slides were examined at 1000x magnification under immersion (“Axiophot” Zeiss microscope equipped with DP70 Olympus camera). The length of the flagella and cell dimensions of 100 parasites per preparation were measured with the CellP program (Olympus, Soft Imaging System). Promastigotes with a cell body <8 µm and flagel/cell body ratio ≥2 are considered as metacyclic (2, 33).

*Flow-cytometry:* in view of the morphological differences between procyclic and metacyclic forms, flow-cytometric analysis can be applied for discrimination of both forms (28). Briefly, non-fluorimetric measurements were performed (Beckmann Coulter, Cell Lab Quanta) by suspending 10µl of promastigote culture in 1ml PBS and analysed for light-scatter. Dot plots of electronic volume (EV) and side-scatter (SS) of 10,000 events were analysed.
Results

Conditioning factors for promastigotes

Standard growth curves were established for each strain (data not shown). The VL strains entered into log-phase on day 2 and early stationary phase was reached within 4-5 days, after which spontaneous acidification of the medium became very prominent leading to a decrease in viable cell numbers by day 7. The CL and MCL strains reached early stationary phase after about 4-5 days, but at lower cell densities and with minimal acidification of the medium. One strain (PER 106) showed particular slow initial growth with parasite numbers still increasing until day 6. Since all cultures reached early stationary phase after about 4-6 days, day 5 was retained for artificial pre-conditioning.

To check the influence of elevated temperature and duration of conditioning, 5-day old promastigote cultures were adjusted to 37°C (VL) or 34°C (CL) for 24, 48 or 72h. The promastigote cell bodies became more rounded to oval, but with the flagellum still clearly present. These ‘rounded’ stages were not able to adequately infect macrophages. Using elevated temperatures at pH 5.4 produced similar results. However, when promastigotes were maintained at 25°C in acidic conditions for 24h, significantly higher levels (>70%) of infection were obtained for both VL (Fig. 1a) and CL and MCL strains (Fig. 1b). The VL strains showed declining levels of infection after longer incubation times. The (M)CL strains produced more variable results: *L. brasiliensis* produced adequate intracellular amastigote burdens after 24h and 48h conditioning, while the *L. guyanensis* strains showed an overall low infectivity (data not shown).

Highly variable infection rates were observed with spontaneous late-stationary phase VL promastigotes (65 to 93% with variation across the different test replicates). Transferring 5-day old cultures to either acidified MEM or Schneider’s medium (pH 5.4) resulted in enhanced infection of macrophages with infection rates of about >80%, irrespective of the conditioning medium used (Table 2) Surprisingly, the clone of BPK-206 showed lower infectivity after conditioning in MEM. The use of spontaneous late-stationary phase promastigotes of CL and MCL strains was very problematic since almost no infection of macrophages was obtained (infection rate <5%). Pre-conditioning of *L. brasiliensis* produced adequate infection rates (>80%) with a further improvement when Schneider’s medium was used (>90%). Conditioned promastigotes of *L. guyanensis*...
showed rather low and variable infectivity. The slow growing PER-106 strain failed to produce adequate infections both after conditioning in MEM (47.7% ± 12.3) and Schneider’s medium (42.2% ± 13.9), but was still much better compared to the spontaneous stationary-phase promastigotes. Strain PER-072 was adequately infective only after conditioning in Schneider’s medium (87.1% ± 7.6).

The process of induced metacyclogenesis was morphologically evaluated for *L. donovani* by comparing spontaneous late-stationary phase and pre-conditioned promastigotes using flow-cytometry (Fig. 2) and light microscopic measurements (Fig. 3). Late-stationary phase cultures clearly consisted of a mixed population of larger and smaller cells with slight shift to the left comparing to the log-phase cultures (Fig. 2a,b). After pre-conditioning for 24h, a clear shift to the left is noted (Fig 2c), indicative for larger proportion of smaller cells, *e.g.* metacyclics. Based on cell dimensions, strain BPK-206cl only had about 10% metacyclics in stationary-phase cultures, which increased to >50% after conditioning. Strain BPK-190 already contained about 50% metacyclics in late-stationary phase, which further increased to about 64% after conditioning (Fig. 3).

**In vitro susceptibility of field isolates**

Adopting the established conditioning protocol, the *in vitro* susceptibility was subsequently determined of a larger set of clinical field isolates of *L. donovani*, *L. guyanensis* and *L. braziliensis* (Table 1). The reference PX-6518 confirmed adequate transformation into intracellular amastigotes as demonstrated by IC₅₀ <1µg/ml for VL and IC₅₀ <2µg/ml for (M)CL. Since treatment outcome was also known for each isolate, information could be gained on the predictive value of the intracellular amastigote susceptibility test for Sb-resistance. In addition, miltefosine was included as a reference for which full drug susceptibility was observed (IC₅₀ <5µM for VL and <10µM for CL and MCL) since the patients had never been treated with the drug.

For *L. donovani*, BPK043 was included in each experiment as the sensitive reference (IC₅₀ Sb⁵⁺ = 13.3 µg/ml eq.; Sb⁰ = 6 µg/ml eq) and to allow calculation of the activity index (AI). Combining the AI-values for Sb⁵⁺ and Sb⁰, strains BPK177 and BPK190 clearly showed decreased sensitivity, which also fully corresponded to the clinical profile of non-responder or relapse. BPK206, derived from a cured patient, showed marginally
decreased sensitivity to SbV (AI = 3.6) but still fully sensitive to SbIII (SI = 1.8). Its clone had about the same profile (AI SbV = 5.5 / SbIII = 0.8). Strains BPK091 and BPK178 were derived from a cured patient and just fell within the sensitive range (AI <4). Similar results were obtained with L. guyanensis where all (except PER072) were sensitive to both SbV and SbIII, thereby reflecting the clinical outcome of definite cure. Since only clones were available of the L. braziliensis strains, the SbV – SbIII susceptibility outcome does not reflect the clinical outcome.
In vitro susceptibility testing on live Leishmania stages still remains the primary tool for epidemiological mapping of drug resistance, as molecular resistance markers have not yet been identified nor fully validated (17, 21, 25). Susceptibility evaluation of patient-derived diagnostic material is still hampered by the lack of reliable and reproducible in vitro models and prediction of treatment outcome is even more controversial (26, 32). While intracellular amastigotes in macrophages are still considered as the golden standard for all drugs, axenic amastigotes may also be appropriate for susceptibility evaluation to miltefosine and amphotericin B (31). The present study aimed to provide a standardized method for infection of macrophages with clinical isolates of visceral (L. donovani), cutaneous (L. guayensis) and mucocutaneous (L. braziliensis) species, and attempt to link in vitro Sb-susceptibility to clinical outcome. From the knowledge that macrophages can be adequately infected with either metacyclic promastigotes or amastigotes, both options were addressed.

As products of metabolism reduce the pH in promastigote cultures, spontaneous acidification in the stationary phase has been used by most investigators to obtain infective metacyclic forms (6, 12). However, considerable variation in the efficiency of metacyclogenesis between different species and strains has been observed (13, 15). For example, rapidly dividing strains will show enhanced metacyclogenesis compared to slow growing strains where no adequate acidification of the medium can be attained. In our study, this was particularly the case for the faster growing VL strains compared to the slower growing (M)CL strains. Even within VL, BPK190 entered into stationary phase one day earlier than BPK206; thereafter, parasite numbers and viability started to decline affecting infectivity for macrophages. Moreover, the rate of spontaneous metacyclogenesis can still vary a lot during different sub-cultivation cycles, for example for BPK206cl being quite low (<20%) during an early passage (Fig. 3) and improving a lot (>70%) in a next passage (Table 2). Therefore, the use of spontaneously acidified stationary-phase cultures is not practical as it requires daily monitoring for acidification and metacyclogenesis state and may vary with every species, isolate and even sub-passage.
A first option for improvement was to trigger log-phase promastigotes to transform into
amastigotes by adopting the conditioning factors that had been used for the generation of
axenic amastigotes (14, 16, 18, 29). Acidification of the growth medium to pH 5.4 or
temperature elevation from 26°C to 34°C or 37°C was sufficient to induce promastigote
transformation (19, 30). The combined effect of temperature and acidity is more effective
to initiate promastigote transformation to axenic amastigotes (3, 34). However, the
‘rounded’ stages obtained in our study were poorly infective for macrophages,
particularly after conditioning at high temperature (37°C or 34°C) (Fig. 1). It has also
been suggested that a stepwise adaptation to temperature would ameliorate amastigote
transformation (14), but this method would be far too time-consuming to adopt for field
isolates. Moreover, a selection process would occur favoring those organisms that are
best adapted for in vitro transformation and growth.

The second option was to condition the process of promastigote metacyclogenesis, which
is mainly triggered by a drop of external pH altering the timing of in vitro events by
causing first differentiation into non-dividing metacyclics and subsequent entry into
stationary phase earlier than might occur otherwise (6). Several authors indeed cultured
promastigotes at lower pH (1, 33) with generation of more homogeneous populations of
metacyclics for multiple species (33). However, it was also observed that lowering the pH
induced a more early entry into stationary phase with lower final viable cell densities (6).
In this study, cultures were grown first in conventional conditions taking into account that
a certain synchrony could already be promoted by adapting the infection inoculum, i.e. a
small inoculum for fast growing strains and a larger inoculum for slow growing strains.
This way, all VL en CL strains reached early stationary phase after about 5 days which
was withheld as the most appropriate time to start conditioning. In cases where stationary
phase was not yet attained, for example BPK-206, enforced acidification resulted in a
marked increase of metacyclics, while for those already in stationary phase (BPK 190), a
further increase could be observed (Fig. 3). Other than the enforced drop to pH 5.4, the
duration of conditioning was also critical with 24h being most appropriate for L.
donovani and L. braziliensis. The external acid environment was apparently not sufficient
to trigger adequate differentiation of L. guyanensis, underlining the need for at least a
minimal growth rate for successful pre-conditioning. Besides the drop of external pH, the
culture medium may also influence developmental changes (15). In our study, acidified MEM and Schneiders medium (pH 5.4) were compared and were able to trigger differentiation in an almost similar way with >80% infection rates for all species except *L. guyanensis* (Table 1).

In summary, the following pre-conditioning protocol is proposed (Fig. 4): promastigotes are cultured in conventional MEM medium until day 5 and then transferred into Schneiders medium (pH 5.4) for 24h at 25°C prior to infection of macrophages at a 10/1 infection ratio. Infected macrophages are washed 12 to 24h later to remove non-internalized promastigotes. In the susceptibility protocol, compounds are added at this time point and evaluation of parasite burdens is done five days later.

Ideally, in *vitro* models should not only consider internalisation of metacyclics in the host cell, but also subsequent transformation and multiplication of the amastigotes (23). For this reason, the experimental drug PX-6518 was included as internal reference because of its exclusive action on dividing intracellular amastigotes (22, 31). Next and to challenge the proposed pre-conditioning protocol (Fig. 4), susceptibility testing was performed on a larger panel of clinical field isolates to both SbV and SbIII. Since treatment outcome for each isolate was known (Table 2), a preliminary assessment of the predictive value may also become possible, despite the reservations of some investigators (26). The three phenotypes previously reported were also observed in our study (26, 32): 11 isolates were SbV sensitive/SbIII sensitive (5S3S), 4 isolates were SbV resistant/SbIII sensitive (5R3S) and 3 isolates were resistant to both (5R3R). Combination of the AI-values for both SbV and SbIII appeared necessary to fully correspond with the listed clinical profile of the listed species: AI-values \( \geq 4 \) for both SbV and SbIII were always linked to ‘relapse’ or ‘non-responder’, AI \( \geq 4 \) for SbV only could still be linked to ‘cure’. Although strains BPK091 and BPK178 were both derived from a cured patient, the AI values fell only just within the sensitive range, and it could be speculated that resistance and clinical failure may indeed ultimately develop. However, in view of the relatively low number of clinical isolates that was evaluated, particularly of *L. guyanensis* and *L. braziliensis*, more extensive studies with many more *Leishmania* species and strains are needed before a proper judgment can be made on the predictive value of *in vitro* susceptibility tests for treatment failure.
In conclusion, the proposed conditioning protocol allows a more reproducible infection of macrophages and enhances the validity of *in vitro* susceptibility testing. Using AI-values for phenotypic characterization (26, 32) may allow a better comparison of results between different series of experiments and between different laboratories. Hence, both tools are recommended for *in vitro* susceptibility testing and resistance profiling of clinical field isolates.
Acknowledgements

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References


**Fig 1:** Total macrophage amastigote burdens after transferring log-phase promastigotes to acidic conditions at either 37°C (VL) or 34 °C (CL, MCL) and 25°C (VL, CL, MCL). The conditioned promastigotes were subsequently used to infect macrophages and parasite burdens were determined on Giemsa-stained slides.

<table>
<thead>
<tr>
<th>Strain Type</th>
<th>% Infected Macrophage</th>
<th># Amastigotes/φ</th>
</tr>
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<tr>
<td><strong>Visceral</strong></td>
<td></td>
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<tr>
<td>Mean % infected macrophages</td>
<td>78.4 ± 14.1</td>
<td>50.6 ± 30.4</td>
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<tr>
<td>Mean # amastigotes/φ</td>
<td>2.3 ± 1.2</td>
<td>37.4 ± 0.9</td>
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<tr>
<td><strong>Cutaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean % infected macrophages</td>
<td>69.9 ± 27.3</td>
<td>46.2 ± 28.7</td>
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<tr>
<td>Mean # amastigotes/φ</td>
<td>4.0 ± 3.0</td>
<td>1.3 ± 1.1</td>
</tr>
</tbody>
</table>

* p<0.05

**Fig 2:** Flow-cytometric analysis of *L. donovani* strain BPK190. log-phase culture (A); stationary-phase promastigotes (7-day old cultures) (B) and after 24 hours pre-conditioning of a 5-day old culture in Schneider’s medium at pH 5.4 (C).
**Fig 3:** Microscopic enumeration of metacyclic promastigotes in spontaneous stationary phase (day 7) and after induction on day 5 in Schneider’s medium at pH 5.4 for 24h. Morphologic criterion for metacyclics = cell body <8µ and flagel-body ratio >2.

![Bar chart showing % metacyclic promastigotes](chart.png)

**Fig 4:** Flow-chart of promastigote pre-conditioning and susceptibility testing of *Leishmania* field isolates.

<table>
<thead>
<tr>
<th>Promastigote conditioning</th>
<th>Susceptibility testing</th>
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<tr>
<td>1 2 3 4 5 6</td>
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<tr>
<td>Promastigote culture at 25°C</td>
<td>Intracellular amastigote susceptibility</td>
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<tr>
<td>Selection of infection inoculum</td>
<td>IC₅₀ determination</td>
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<tr>
<td>Medium change and exposure to reference drug</td>
<td>Infection of macrophages at 10 promastigotes/cell</td>
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<tr>
<td>Transfer to Schneider’s medium (pH 5.4, 25°C)</td>
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<tr>
<td>Species</td>
<td>Strain</td>
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<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>L. donovani</td>
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<tr>
<td></td>
<td>BPK 091</td>
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<td></td>
<td>BPK 178</td>
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<td>BPK 206</td>
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<tr>
<td></td>
<td>BPK 206 cl</td>
</tr>
<tr>
<td>L. guyanensis</td>
<td>BPK 274</td>
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<td></td>
<td>BPK 294</td>
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<td></td>
<td>BPK 298</td>
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<tr>
<td>L. braziliensis</td>
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<td></td>
<td>PER 054</td>
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<td></td>
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Table 1: Susceptibility evaluation of clinical field isolates against antimony (Sb\(^V\), Sb\(^{III}\)), miltefosine and the experimental drug PX-6518. Activity index >4 is indicative for resistance.

Patients were treated with sodium-stibogluconate at 20 mg/kg i.m. for 1 month. Clinical follow-up was performed at 1, 3, 6 and 12 months after start of treatment. 'Non-responders' were cases with positive parasitology after the full 30 days treatment course; 'definite cure' was defined as no signs and symptoms of relapse at 12 months follow-up; 'relapse' was defined as initial cure but with reappearance of clinical symptoms and positive parasitology during follow-up (26, 32). Several strains are the same as described in references 26 and 32.
Table 2: Effect of different conditioning protocols for enhanced metacyclogenesis on promastigote infectivity for primary peritoneal mouse macrophages. Stationary-phase cultures showing spontaneous acidification were compared to 5-day old promastigote cultures adjusted for 24h to MEM at pH 5.4 or Schneider’s medium at pH 5.4.

<table>
<thead>
<tr>
<th>strain</th>
<th>% infected macrophages*</th>
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<tbody>
<tr>
<td></td>
<td>MEM spontaneous</td>
</tr>
<tr>
<td></td>
<td>mean</td>
</tr>
<tr>
<td>BPK 190</td>
<td>65.4</td>
</tr>
<tr>
<td>BPK 190 cl</td>
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<td>BPK 206</td>
<td>93.3</td>
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<tr>
<td>BPK 206 cl</td>
<td>70.7</td>
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<tr>
<td>PER 002 cl</td>
<td>&lt;5</td>
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<tr>
<td>PER 005 cl</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PER 072</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PER 106</td>
<td>&lt;5</td>
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* level of adequate infection set at >70%