In vitro screening test using *Leishmania* promastigotes stably expressing mCherry protein

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Running title: mCherry fluorescence to test drug efficacy.

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ABSTRACT

Transgenic *Leishmania major* and *Leishmania donovani* axenic promastigotes constitutively expressing mCherry were used for in vitro antileishmanial drug screening. This method requires minimal sample manipulation and can be easily adapted to automatic drug test allowing primary high-throughput screenings without the need of expensive and sophisticated instruments.
Protozoan parasites of the genus *Leishmania* are the causative agents of a wide spectrum of human and animal diseases. The clinical manifestations of *Leishmania* infections range from lesions of the skin and mucous membranes to lethality, the latter caused by visceral species, including *L. donovani* (1). *Leishmania* species cause morbidity and mortality throughout large areas of the Old and New World. Leishmaniasis is an emerging disease with an annual incidence of 2 million cases and more than 12 million people are infected in over 80 endemic countries (2), causing 80,000 deaths per year and 350 million people are at risk of infection and disease. Increased prevalence of *Leishmania*-HIV co-infection is the major reason for the recent emergence of leishmaniasis in the Western World (3).

The highly toxic pentavalent antimonials (Sb(V)) have been the primary first-line treatment for all type of leishmaniasis in most parts of the world since their discovery in the 1940s. However the increasing frequency of relapses in leishmaniasis patients is forcing the use of other chemotherapeutical agents including amphotericin B, isethionate pentamidine, paromomycin and miltefosine (4, 5). Unfortunately, the majority of these antiparasitic drugs present severe side effects, there is not guarantee of cure and some of them are frequently accompanied by emergence of drug resistance (6-8).

Alternative treatments are needed, however advances in drug discovery approaches have not been satisfactory. Although the use of axenic promastigote cultures of *Leishmania* as a primary screening method has being validated (7-11), the traditional approaches employed to determine the drug effect in culture present several shortcomings. Microscopical counting is prone to error, time consuming and requires trained personnel; the use of tetrazolium salts such as MTT to
measure cell viability is labor-intensive and results are not always reliable (12). The more sensitive and less labor-intensive colorimetric resazurin-based method may present variable metabolic behavior under different cell culture conditions. Furthermore, it is not appropriate to perform kinetic experiments due to the significant cytotoxicity as a result of prolonged exposure with the reagent (13-15). Other disadvantages of these metabolic assays are the requirement to incubate the substrate with viable cells at 37 °C for a sufficient time to generate a measurable signal. This may lead to undesirable artifacts resulting from interactions between the reagent, the tested drug and the biochemistry of the cells (16). On the basis of these considerations there is a strong need to evolve appropriate screening technologies. The use of fluorescent reporter genes such as GFP (17-19) and the red versions RFP (20, 21) and mCherry (21, 22) have considerably facilitated the screening and test of antimicrobial agents in axenic amastigote and intracellular amastigote drug screening, allowing both in vitro as well as real time visualization in vivo. Although animal models are well established for large-scale primary drug screening, its practical use is limited due to high costs.

Here, we introduce a simple fluorometric method for primary drug screening using red fluorescent promastigotes. We generated *L. major* and *L. donovani* parasite cultures stably expressing the mCherry gene, whose product is a red monomeric fluorescent protein derived from the nowadays obsolete tetrameric *Discosoma* protein DsRED and presents an excitation maximum at 587 nm and an emission maximum at 610 nm. mCherry is constitutively expressed in the cytoplasm, presents high photostability, is resistant to photobleaching and does not require post-translational modifications as GFP protein does. It matures very rapidly allowing fluorescence detection shortly after the gene is expressed, being this appropriate to real time analysis (12, 23).
L. major promastigotes (MHOM/JL/80/Friedlin) and L. donovani promastigotes (MHOM/SD/62/1S-CL2D) were maintained at 27 °C in M199 medium supplemented with 10 % FBS (Atlanta Biolabs). mCherry gene (711 bp) was obtained from the pmCherry-N1 vector (Clonetech, Palo Alto, CA) by PCR amplification (LongAmp Taq, NEB). The PCR product was purified and cloned into pGEMT (Promega) to create the construct mCherry-pGEMT and further subcloned into the Leishmania expression vector pLEXSY-Hyg2 (Jena bioscience) (Figure 1A).

5 µg of the resulting expression plasmid mCherry-pLEXSY was digested with SwaI. The linearized expression cassette was electroporated as described previously into L. major and L. donovani log-phase cultures and transfected parasites were selected in presence of hygromicin B (Sigma) at a final concentration of 5 µg/mL (24). Integration into the chromosomal 18S RNA locus (ssu) was confirmed by PCR. A primer pair including one primer hybridizing within the expression cassette (P3) and other hybridizing to a ssu sequence not present on the plasmid were used (Figure 1A). Additional PCR reactions including mCherry specific primers (P2 and P3) were performed. A band of 1279 Kbp confirmed the integration of the gene and a band of 711 bp was also obtained for mCherry positive clones. Primer sequences available upon request.

mCherry expression in both Leishmania species was tested by epi-fluorescent microscopy (Nikon 90/NikonAZ 100). Cells were PBS washed, fixed in 4 % formaldehyde and subsequently stained with NucBlue Fixed Cell Stain (Life) to visualize the nucleus and kinetoplast (magnification 60x). Transgenic parasites showed normal DNA content and bright cytoplasmic red fluorescence, indicating that the fixation step did not quench mCherry fluorescence, making this cell line extremely valuable (Figure 1B). The high fluorescence detected was maintained in the axenic amastigote stage (data not shown). mCherry expression was also analyzed in wild type (WT) and transgenic promastigotes in a Beckman Coulter FC500 MPL flow cytometer.
Morphometric profiles of transfected parasites were comparable to the WT ones (data not shown). To examine parasite fitness, the growth rate of transfected cultures was measured and compared to WT strains. Parasites were seeded in triplicate (5×10^5 cells/mL) and the concentration of *L. major* and *L. donovani* WT (LmWT and LdWT) and mcherry-pLEXSY *L. major* and *L. donovani* (LmCherry and LdmCherry) cultures was assessed daily by direct microscopic counting until parasites reached stationary phase. No differences were observed in parasite concentration (Figure 1C). Additionally, to evaluate culture fluorescence in relation to parasite concentration, the fluorescence of each culture was measured (518 ex/605 em) daily during 4 days and subsequently plotted versus parasite concentration (Figure 1D). The background fluorescence was subtracted from each sample. Results demonstrated that both strains presented a linear relationship between promastigote number (parasites/mL) and the quantified fluorescence (arbitrary units of fluorescence -AUF-), with correlation coefficients > 0.99. While the linear response for *L. major* was between 5×10^5-3×10^7 parasites/mL, *L. donovani* showed a wider range, between 5×10^5-6×10^7 parasites/mL.

The antileishmanial effect of the reference compounds miltefosine (Sigma), amphotericin B (Sigma) and isethionate pentamidine (Sigma) was evaluated in transfected parasites and compared with WT cultures. EC50 was defined as the concentration of drug required to reduce by 50 % the initial parasite concentration. Culture viability was assessed by using the fluorometric resazurin-based reference method CellTiter-Blue (Promega) (25, 26). Briefly, 1×10^6 cells/mL were seeded in 96-well plate and incubated in presence of increasing drug concentration during 72 hours at 27 °C, along with appropriate solvent controls. Afterwards, 20 µL CellTiter-Blue reagent was added to 100 µL of culture. After 4 hours at 37 °C, fluorescence was measured (555 ex/580 em) using a Typhoon FLA 9500 (GE Healthcare) and analyzed with ImageQuant TL.
software (GE Healthcare). Table 1 shows the EC_{50} values obtained. For the three compounds tested no differences in antileishmanial activities were detected, indicating that transfection and constitutive mCherry expression did not affect drug efficacy. Finally, we evaluated the use of mCherry fluorescence quantification in order to assess cell viability in transgenic *Leishmania* promastigotes. LmCherry and LdCherry cultures (1x10^6 cells/mL, 200 μL/well in a 96-well plate) were incubated in presence of increasing drug concentration. After the incubation period (72 hours at 27 °C) fluorescence was quantified (518 ex/605 em) and EC_{50} values were determined (Table 1). Data reported here shows good accordance between EC_{50} values obtained with resazurin-based method and direct measurement of fluorescent cultures. These results underscore the value of mCherry fluorescent promastigotes as a tool for testing potential chemotherapeutical agents, ruling out the necessity of time-consuming, prone to error tests that normally require several manipulation steps. The streamlined strategy proposed in this work does not involve any manipulation other than the addition of the drug to be tested. To our knowledge, this is the first report where mCherry transfected *L. donovani* promastigotes are used as a tool to perform primary drug screenings. Previous work using *Leishmania major* (21) and *Leishmania infantum chagasi* (22) introduced the use of mCherry as a reporter gene to monitor *in vitro* as well as *in vivo* infections. The aim of this work is to validate an alternative method for primary drug screening on promastigotes of different *Leishmania* species. The proposed approach is inexpensive, does not require the use of host cell-based assays or animal models. Our results confirm this approach is rapid and reliable and it is in good accordance with results obtained by the standard resazurin-based assay. Although the promastigote form is not the infective stage and differences in susceptibility to drugs have being previously addressed, the implementation of a low-cost method with minimal manipulation steps is highly valuable in
reducing the extent of drug libraries. Remarkably, this method only requires a microplate reader in order to be implemented and can be easily adapted to automatic drug test allowing primary high-throughput screenings without the need of expensive and sophisticated instruments. This last feature is particularly appealing to laboratories with limited resources.

ACKNOWLEDGMENTS

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Figure 1

Figure 1. (A) Integration of mCherry on E. coli expression cassette into the L. monocytogenes DNA locus proving integration with host. The thick arrow indicates the start of transcription from the RNA genic. 

Figure 2. (B) 10% SDS-PAGE analysis of the proteins expressed from mCherry positive clones of E. coli. (C) A 2% agarose gel showing the size of DNA fragments obtained from mCherry positive clones of E. coli. (D) Integration of mCherry on E. coli expression cassette into the L. monocytogenes DNA locus proving integration with host.
Table 1: EC$_{50}$ of reference antileishmanial drugs obtained with CellTiter-Blue test ($555_{ex}/580_{em}$) or by mCherry quantification ($518_{ex}/605_{em}$) on WT and transfected parasites.

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<th>CellTiter-Blue test</th>
<th>mCherry Fluorescence</th>
<th>CellTiter-Blue test</th>
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<td>Miltefosine ($\mu$M)</td>
<td>11.47± 0.86</td>
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<td>Amphotericin B (nM)</td>
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<td>Isethionate Pentamidine ($\mu$g/mL)</td>
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<td>LmWT</td>
<td>10.54± 0.43</td>
<td>12.99± 0.77</td>
<td>14.44± 4.80</td>
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