Journal of Microbiological Methods 166 (2019) 105732

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Assessment of *Leishmania* cell lines expressing high levels of beta-galactosidase as alternative tools for the evaluation of anti-leishmanial drug activity

Aline C. da Silva Santos[a,*], Danielle M.N. Moura[a], Thiago A.R. dos Santos[a], Osvaldo P. de Melo Neto[b], Valéria R.A. Pereira[a]

[a] Departamento de Imunologia, Instituto Aggeu Magalhães- FIOCRUZ, Recife, Pernambuco, Brazil
[b] Departamento de Microbiologia, Instituto Aggeu Magalhães- FIOCRUZ, Recife, Pernambuco, Brazil

ARTICLE INFO

Keywords:
Leishmania amazonensis
Leishmania infantum
β-Galactosidase
Drug screening
Chemotherapy

ABSTRACT

Leishmaniasis, caused by protozoa belonging to the genus *Leishmania*, is an important public health problem found in > 90 countries and with still limited options for treatment. Development of new anti-leishmanial drugs is an urgent need and the identification of new active compounds is a limiting factor that can be accelerated through large scale drug screening. This requires multiple steps and can be expensive and time consuming. Here, we propose an alternative approach for the colorimetric assessment of anti-*Leishmania* drug activity that can be easily scaled up. L. *amazonensis* and L. *infantum* cell lines were generated having the β-galactosidase (β-gal) gene integrated into their chromosomal 18S rRNA (ssu) locus. Both cell lines expressed high levels of β-gal and had their growth easily monitored and quantified colorimetrically. These two cell lines were then evaluated as tools to assess drug susceptibility and their use was validated through *in vitro* assays with Amphotericin B, which is routinely used against leishmaniasis. β-gal expression was also confirmed through flow-cytometry, another method of phenotypic detection. With these recombinant parasites, an alternative *in vitro* model of drug screening against cutaneous and visceral leishmaniasis is now available.

1. Introduction

Leishmaniasis is a neglected disease caused by protozoa belonging to the genus *Leishmania* that infect mammalian mononuclear phagocytic cells (Sacks and Sher, 2002). Depending on the *Leishmania* species and the host immune response, the disease is associated with distinct clinical forms: cutaneous, mucocutaneous and visceral leishmaniasis (Santos et al., 2018). This disease is transmitted by sandflies and it is found in > 90 countries, with 310 million people at risk of infection, and around 1.3 million new cases registered each year (WHO, 2015). Currently there is no vaccine available to be used against *Leishmania* infection in humans, and conventional treatments are toxic to patients, with long duration and severe adverse reactions, often leading to the interruption of treatment. The development of new anti-leishmanial drugs is therefore an urgent need (Bezerra de Menezes et al., 2015; den Boer et al., 2011; Jesus et al., 2017; Tiuman et al., 2011).

Identification of active compounds is one of the limiting factors in discovery and development of new drugs for leishmaniasis treatment, since drug screening requires multiple steps and it is time consuming (Dube et al., 2009). When any new compound is postulated to act directly on the parasite, a critical step is to evaluate its activity against *Leishmania in vitro* (Muylder et al., 2011). A classical method for the screening of new molecules against *Leishmania* promastigotes then is through parasite counting in a Neubauer's chamber, with flagellar motility used to determine viability after exposure to the drug. Although this is an inexpensive method to implement, it is known to be poorly reproducible, non-sensitive and laborious. In addition, flagellar motility may not be sufficient to determine whether the parasite is viable or not, and the procedure is time consuming when one is faced with several compounds to test (Gupta and Nishi, 2011; Muylder et al., 2011; Rodriguez and Tarleton, 2012). The use of a reporter gene technology in genetically modified parasites has been suggested as an alternative to facilitate drug screening, since it can produce quantitative data in a shorter time frame and it also reduces manual labor (Pulido et al., 2012; Rodriguez and Tarleton, 2012; Zulfiqar et al., 2017). Transfection of protozoa, such as *Plasmodium*, *Leishmania*, *Trypanosoma* and *Toxoplasma*, with constructs encoding reporter genes has revolutionized studies that sought to understand parasite-host

* Corresponding author.
E-mail address: aline.caroline.bm@gmail.com (A.C. da Silva Santos).

https://doi.org/10.1016/j.mimet.2019.105732
Received 12 April 2019; Received in revised form 23 September 2019; Accepted 28 September 2019
Available online 17 October 2019

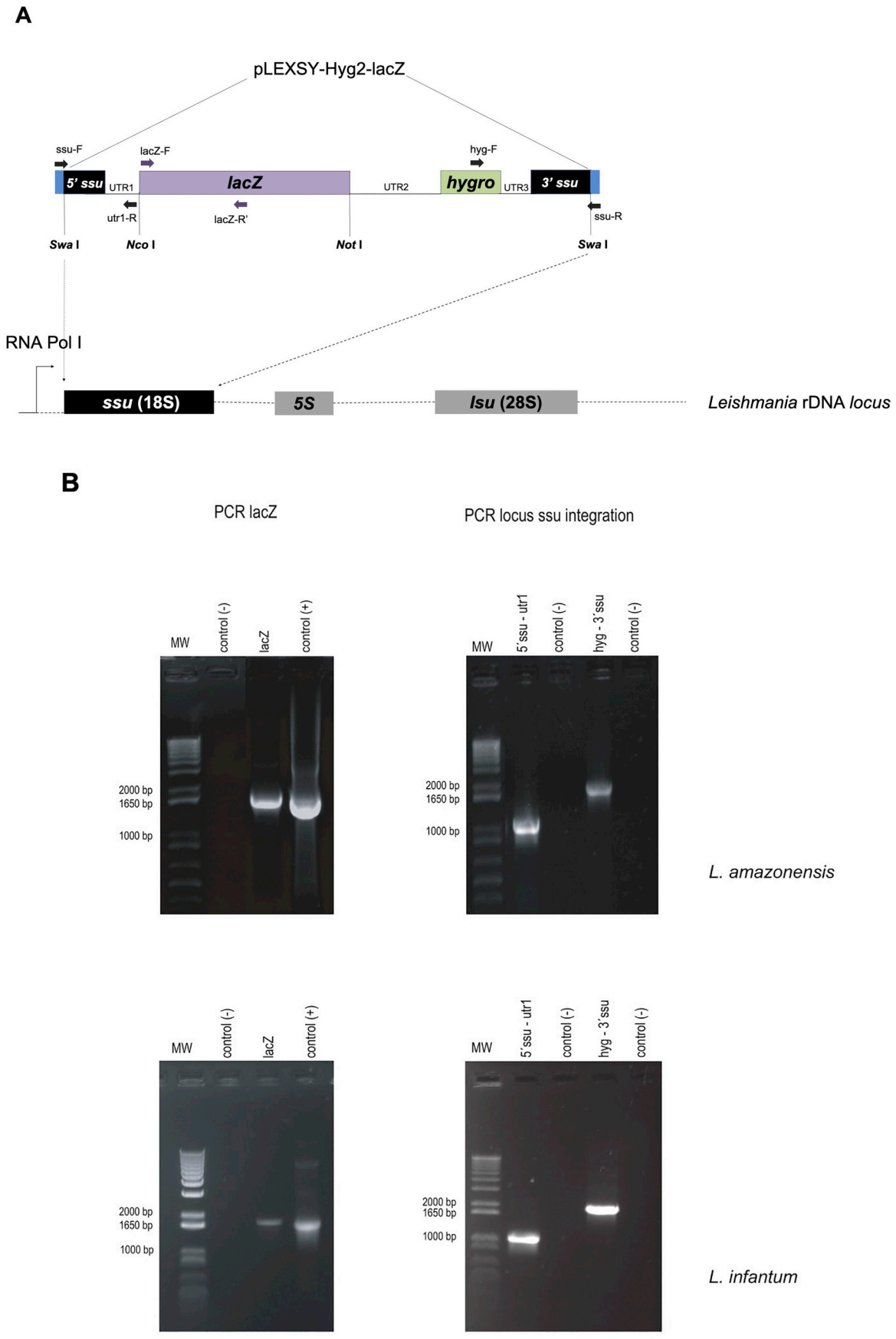

Fig. 1. (A) Scheme of the *lacZ* cassette organization and its integration into the *Leishmania* genome. (B) The panels on the left show the agarose gels showing the amplification of a specific band of ~1600 bp confirming the presence of the *lacZ* gene. Panels on the right confirm the integration of the pLEXSY-hygro2-*lacZ* cassette in the *ssu* locus, using the combination of *ssu* primers and internal pLEXSY-hyg2 primers, with the amplification of specific bands of 1100 bp (5'ssu-F, utr1-R) and 1800 bp (hyg−F, 3'ssu-R).

interactions, and the development of new methods for the screening of anti-parasitic compounds (Dube et al., 2009). Several reporter genes and expression vectors are available and have already been described in the literature, including some for use in *Leishmania*: green fluorescent protein/ enhanced green fluorescent protein (GFP / eGFP), infrared fluorescent protein (iRFP), β-galactosidase (β-gal), β-lactamase and luciferase genes (Calvo-Alvarez et al., 2015; De Rycker et al., 2013; Gupta and Nishi, 2011; Khraiwesh et al., 2016; Oliveira et al., 2016; Plovins et al., 1994; Pulido et al., 2012; Reimão et al., 2013; Rocha et al., 2013; Sadeghi et al., 2015; Zulfiqar et al., 2017). Drawbacks have been cited, however, due to variations in gene expression within transgenic cell populations, substrate costs and longer time periods to produce results (Gupta and Nishi, 2011; Okuno et al., 2003; Pulido et al., 2012; Seeber and Boothroyd, 1996; Vega et al., 2005). Here we report the development of an alternative model for colorimetric drug screening, using L. *amazonensis* or L. *infantum* with high, stable and constitutive levels of β-gal expression. With this approach, it is possible to easily and rapidly quantify *Leishmania* promastigotes and intracellular amastigotes in an *in vitro* screen for drugs to be used against cutaneous and visceral leishmaniasis.

2. Material and methods

2.1. Parasite and macrophage cultivation

L. *amazonensis* and *L. infantum* promastigotes (WHOM/00LTB0016 and MHOM/MA/67/ITMAP-263, respectively) were grown at 26 °C in Schneider's (Sigma, St. Louis, USA) medium with 10% fetal calf serum (FCS) (Cultilab, Campinas, Brazil). For transgenic parasite cultivation, Schneider's medium was supplemented with 2.5 μg/mL hemin (Sigma) and 50 μg/mL hygromycin (Invitrogen, Carlsbad, Canada).

Murine peritoneal macrophages were obtained according to da Silva et al., 2017. The experimental protocol used in this study was evaluated and approved (approval number 103/2016) by the Ethics Committee on Animal Use of the Aggeu Magalhães Institute/ Oswaldo Cruz Foundation (IAM/FIOCRUZ, Recife, Brazil), and was performed according to the principles defined by the Brazilian National Council for the Control of Animal Experimentation. Peritoneal macrophages and the RAW 264.7 cell line were used to generate intracellular amastigotes and were maintained in RPMI 1640 medium (Cultilab), supplemented with 100 mg/mL streptomycin (Cultilab), 100 U/mL penicillin (Cultilab) and 10% FCS, at 37 °C and 5% CO_2.

2.2. Cloning procedures and parasite transfection

The β-galactosidase (β-gal) gene from *Escherichia coli* (*lacZ*) was amplified through conventional PCR using as template the pSV-β-galactosidase plasmid (Promega, Madison, USA), with the enzyme Accuprime Taq DNA Polymerase™ (Thermo Fisher Scientific™, Waltham, USA), and primers flanked by sites for the restriction enzymes *Nco* I and *Not* I (*lacZ*_F – 5'-TCTCCATGGATGCTAGAGGATCCCGTCGTTTTACAACGTC-3′ and *lacZ*_R – 5'-CTCGCGGCCGCTTATTTTTGACACCAGACCAAC-3′). The PCR product (3075 bp) was cloned into pGEM®-T Easy (Promega) and sequenced, prior to subcloning between the *Nco* I and *Not* I sites of the pLEXSY-Hyg2 plasmid (Jena Bioscience, Jena, Germany). The final construction, pLEXSY-Hyg2-*lacZ*, was digested with *Swa* I for linearization and the expression cassette was electroporated into L. *amazonensis* and L. *infantum* promastigotes (500 μF, 450 V and pulse time 3–5 ms) using the Bio-Rad Gene Pulser Xcell™Electroporation System (BioRad Laboratories, Hercules, USA). Transfected cells were selected with 100 μg/mL hygromycin (Sigma).

To confirm gene integration, total genomic DNA was extracted from transfected *Leishmania* promastigotes with DNAzol Reagent (Thermo Fisher Scientific) and PCR reactions were performed using the combination of *Leishmania* ssu primers (ssu-F 5′-GATCTGGTTGATTCTGCCAGTAG-3′, ssu-R 5′-CTGCAGGTTCACCTACAGCTAC-3′) and internal pLEXSY-hyg2 primers (utr1-R 5′-TATTCGTTGTCAGATGGCGCAC-3′, hyg_F 5′-CCGATGGCTGTGTAGAAGTACTCG-3′) (Jena Bioscience). The presence of the *lacZ* gene was confirmed using the *lacZ*-F primer and an internal reverse primer *lacZ*-R' (5′-CGAAACCGCCAAGACTG-3′).

2.3. Colorimetric detection

Leishmania promastigotes were diluted to a starting concentration of 4×10^8 cells/mL followed by two-fold serial dilutions in a 96-well plate, in 100 μL of culture medium, down to approximately 70 parasites/well. This was followed by adding to each well 50 μL of a 500 μM Chlorophenol Red-β-D-Galactopyranoside (CPRG, Sigma) solution in phosphate-buffered saline (PBS), supplemented with 0.5% Nonidet P-40. Plates were then incubated at 23 °C for 20 min and read at 570 nm using the Multiskan FC apparatus (Thermo Fisher Scientific). Blank reaction wells received the same volume of culture medium and CPRG solution.

For the amastigote assay, RAW 264.7 and murine peritoneal macrophages were counted and adjusted to a concentration of 4×10^5 cells/mL followed by plating 200 μL per well in a 96-well plates. The cells were then maintained for 2 h at 37 °C and in 5% CO_2 for adhesion. Transfected promastigotes were added to the wells at different parasite/macrophage ratios: 5:1, 10:1, 15:1, 20:1, 30:1 and 50:1. The plates were then incubated at 37 °C and in 5% CO_2 for 6 h, followed by washing the wells three times with culture medium. After adding 200 μL of fresh medium to each well, the plates were once again incubated at 37 °C and 5% CO_2. After macrophage infection, at three different time points (24, 48 or 72 h), the wells were washed three times to remove any free parasite. The CPRG solution was then added to each well followed by incubation at 23 °C, for 20 min, with the absorbance read as described previously. Blank reaction wells consisted of the macrophages submitted to the same procedures, except the incubation with transfected promastigotes. All the experiments, using promastigotes or macrophage-derived amastigotes described in this study were performed in triplicates.

2.4. Amphotericin B assay

To validate the β-galactosidase assay for drug screening, a traditional 96-well plate assay with Amphotericin B (AmB) (Sigma) was performed. Promastigotes were first diluted to 1×10^6 cells/mL in Schneider's medium with 10% FCS and incubated in a 96-well plate with ten different concentrations of AmB (0.019–10 μg/mL), at 26 °C for 72 h. Cells without treatment were used as negative controls. Cell growth was assessed by counting in a Neubauer chamber or using the CPRG protocol as described above. IC_{50}/72 h was determined by regression analysis.

Intracellular amastigotes, generated by macrophage infection with *Leishmania* promastigotes at a 15:1 promastigote:macrophage ratio, were submitted to ten different AmB concentrations (0.0039 to 2 μg/mL). The parasite:macrophage ratio was established after the standardization shown in section 2.3. After 24 h of incubation, the wells were washed and the CPRG protocol performed as described above, with the IC_{50} determined by regression analysis. In order to compare the results of the CPRG protocol with a traditional intracellular amastigote assay protocol, macrophages were harvested and plated in a 24-well plate containing a 13 mm glass cover slip and allowed to adhere for 2 h, followed by infection with *Leishmania* promastigotes for 6 h. The wells were washed to remove non-internalized parasites, and treated with the different AmB concentrations. After 24 h of incubation, the cover slips were collected and stained using panoptic stain (kit Panótico Rápido - Laborclin, Curitiba, Brazil). The number of intracellular amastigotes found in sets of 100 macrophages were counted, with the IC_{50} determined by regression analysis comparing the decrease in the number of intracellular amastigotes in the treated samples with the untreated control.

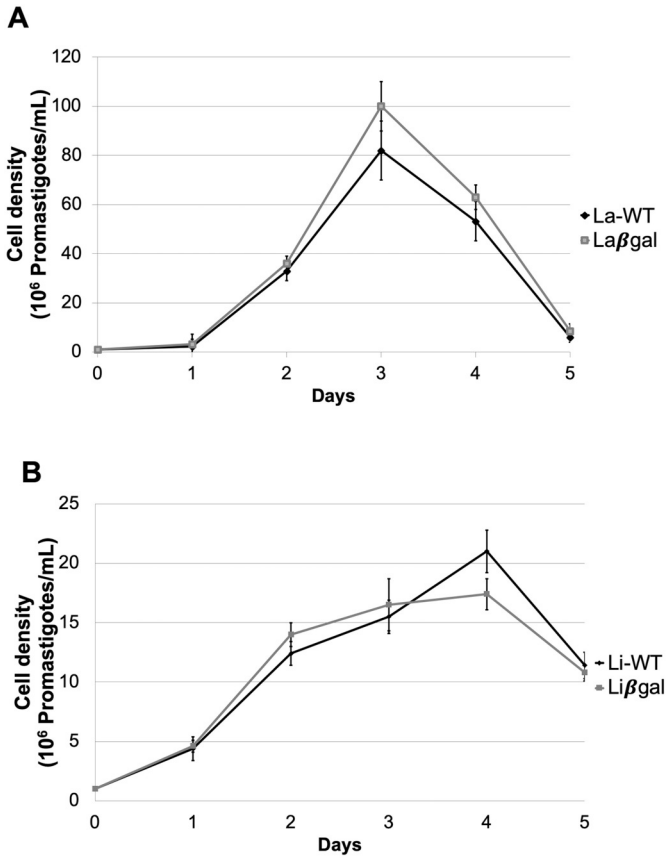

Fig. 2. Growth kinetics of wild-type and recombinant promastigotes. (A) *L. amazonensis* and (B) *L. infantum*. The experiment was carried out at logarithmic phase starting with 10^6 promastigotes/mL, and the studies were carried out in duplicate. Results were expressed by mean ± standard deviation. Curves were analyzed by ANOVA.

2.5. Flow cytometry

For the flow cytometry analysis, the Fluorescein Di-β-D-Galactopyranoside (FDG, Sigma) reagent was used as an alternative β-galactosidase substrate. The assay used an adaptation of the protocol described by Bachy et al. (1999) and Nolan et al. (1988). Briefly, 100 µL of *Leishmania* promastigote cultures at a concentration of 10^7 cells/mL were transferred to a polystyrene tube and incubated in a water bath at 37 °C for 5 min prior to mixing with 200 µL of a 2 mM FDG solution (in H_2O), pre-warmed to 37 °C, and incubated for 30 more minutes at the same temperature. The tubes were then placed on ice and mixed with 1.8 mL of cold PBS supplemented with 2% FCS. Cells were kept on ice and in the dark until analysis on the cytometer. A total of 20,000 events were analyzed per sample using a FACS Calibur cytometer and the numeric data processed with Cellquest Pro software (both from Becton Dickinson, San Jose, USA).

2.6. Statistical analysis

Linear regression and significance analysis were carried out using the GraphPad Prism 7.0 software (GraphPad software, San Diego, USA). The data comparing differences between two groups were assessed using Student's *t*-test or ANOVA ($p < .05$).

3. Results and discussion

Generation of L. *amazonensis* (Laβgal) and L. *infantum* (Liβgal) cell lines expressing β-galactosidase

To generate L. *amazonensis* and *L. infantum* cell lines constitutively expressing β-galactosidase (β-gal), we first cloned the *lacZ* gene open reading frame into the *Leishmania* expression vector (pLEXSY-Hyg2) and transfected the expression cassette in wild-type strains of both L. *amazonensis* and *L. infantum*. This vector allows the integration of the targeted gene into a *Leishmania* 18S rRNA locus (*ssu*) through homologous recombination (Fig. 1A). After transfection, but prior to clonal selection, the transfected parasites were initially tested regarding the expression of β-gal and the viability of its colorimetric detection. According to the literature, the concentration of the colorimetric CPRG reagent used for β-gal detection may vary from 100 µM to 100 mM (Andriani et al., 2011; Buckner et al., 1996; Okuno et al., 2003). Here, 500 µM proved to be the most appropriate concentration for assaying β-gal in both L. *amazonensis* and *L. infantum*, confirming the efficient expression of the enzyme in both species (data not shown). The same concentration of CPRG was adjusted for all experiments described below, reducing the costs of the test.

After clonal selection with hygromycin, the integration of the expression cassette into the *ssu* locus was first confirmed through a set of PCRs. A unique and expected product of approximately 1600 bp, corresponding to part of the *lacZ* gene, was observed. The correct integration was confirmed through PCR using primers annealing to the 18S rRNA locus. Fragments of 1100 and 1800 bp were thus amplified, corresponding to the correct integration into the 18S locus (Fig. 1B). In order to identify any significant changes in the transfectant cells, we next evaluated the growth kinetics, morphology and motility of both transgenic promastigote species. These features were then compared to the wild-type *Leishmania* controls. As shown in Fig. 2, slight decreases in velocity of growth were observed when the two strains constitutively expressing β-gal (Laβ-gal and Liβ-gal) were compared with the corresponding non-transfected controls (La-WT and Li-WT). These however were not statistically significant, thus demonstrating that the expression of β-gal did not impact on the *Leishmania* growth. In addition, no variations in morphology and motility were detected by light microscopy (data not shown).

3.1. Assessment of β-galactosidase expression in transgenic promastigotes

A substantial expression of β-galactosidase was expected due to the use of the expression vector pLEXSY, which mediates the integration of the targeted gene into the chromosomal 18S rRNA (*ssu*) locus, transcribed by the endogenous RNA polymerase I under the control of an rRNA promoter. Since the gene is integrated into the *Leishmania* genome, instead of being localized within a variable copy number plasmid as in an episomal transfection, little or no variation in expression is expected by the transfectants (Reimão et al., 2013; Roy et al., 2000). By testing various dilutions of parasites per well, we were able to better quantify expression as well as define the detection limit for the colorimetric method based on the β- gal assay. Fig. 3 (A and B) shows the absorbance curves produced according to the number of transgenic parasites after incubation with the CPRG solution. The assay was able to detect parasites down to the last dilution, or ~75 promastigotes/mL. Counting such a low number of parasites by traditional approaches with the Neubauer chamber would not be feasible. In our hands, a linear correlation between absorbance and cell density for the colorimetric assay was only seen between $\sim 2 \times 10^4$ to 2×10^7 promastigotes/mL. This would be the best density range for the colorimetric detection to be used with the *in vitro* assays. Further increases or decreases in promastigote density in less or more diluted samples are not reflected by equivalent changes in absorbance. Fig. 3 also shows that both species produced similar results. The substantial amounts of enzyme produced allows the test to be finalized after a short period of incubation (only 20 min). This contrasts with what is commonly seen in other assays that also use β-gal as reporter, where longer incubation periods (usually over three hours) are required for complete reaction (Okuno et al., 2003; Seeber and Boothroyd, 1996; Vega et al., 2005).

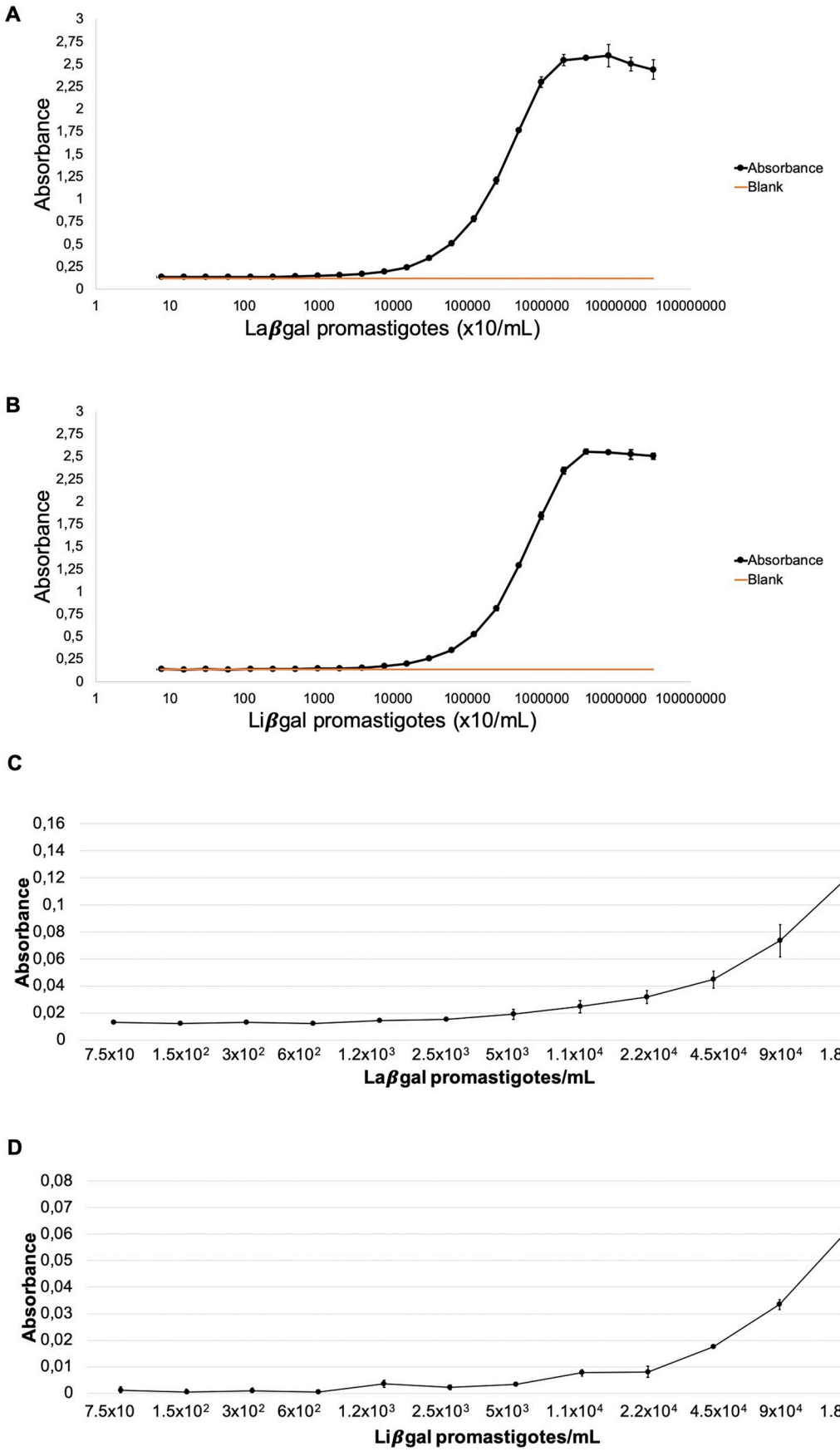

Fig. 3. Colorimetric detection of transgenic *Leishmania* promastigotes expressing β-galactosidase. Graphic representation plotting the number of Laβgal (A) or Liβgal (B) promastigotes *versus* the absorbance detected in each well after the reaction with the CPRG solution. La = *L. amazonensis*; Li = *L. infantum*. The orange line corresponds to the blank value. The absorbance values of the beginning of curves A and B (corresponding to ~ 75 to 1.8×10^5) are respectively enlarged in C and D. In these absorbance values, the blank is already discounted. All experiments were done in triplicate and independently. Results are expressed as mean ± standard deviation.

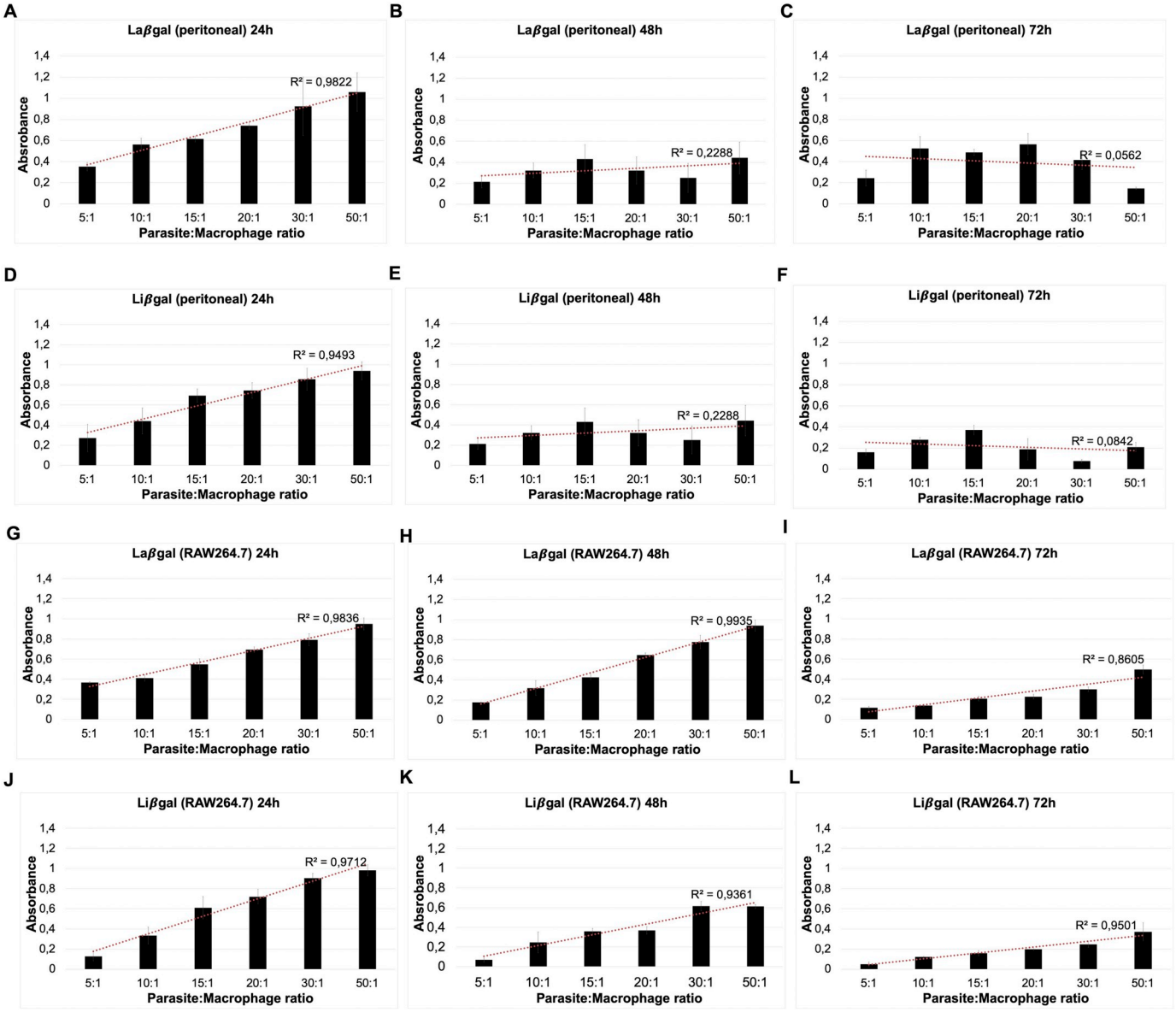

Fig. 4. Colorimetric detection of amastigotes expressing β-galactosidase. The graphic representations show that the correlation between absorbance and number of parasites was linear. In all, the absorbance of macrophages without infection, used as blank reactions, was subtracted from the values from each assay. All experiments were done in triplicate. Results are expressed by mean ± standard deviation. R^2 values are shown on each bar graph. A-C; G-I: *L. amazonensis*; D-F; J-L: *L. infantum*.

Table 1
IC_{50} (μM) values for Amphotericin B against Laβgal and Liβgal promastigotes and intracellular amastigotes. Promastigotes of both species were counted in Neubauer chamber and tested in the same experiment with the CPRG solution for the colorimetric assay. Due to the need for staining with the counting protocol, macrophage infection was analyzed by counting using optical microscopy and by the colorimetric protocol in independents experiments. Both methodologies, traditional and colorimetric, were used to determine IC_{50} (at 72 h for promastigotes and at 24 h for amastigotes). (*) significant difference using the Student's t-test ($p < .05$).

	Promastigotes		Amastigotes (RAW macrophages)	
	IC_{50} 72 h Count	**IC_{50} 72 h CPRG**	IC_{50} 24 h **Count**	IC_{50} 24 h **CPRG**
Laβgal	0.017 ± 0.00008	0.033 ± 0.00064*	0.125 ± 0.056	0.1303 ± 0.023
Liβgal	0.031 ± 0.0082	0.037 ± 0.0011	0.056 ± 0.0007	0.036 ± 0.0042*

The results shown here reinforce the main advantage of using reporter genes, which is the generation of large quantities of results that are easily to interpret (only absorbance) and in a short timeframe (Rodriguez and Tarleton, 2012).

3.2. Intracellular amastigotes and drug screening assay

Since amastigotes are the *Leishmania* intracellular life forms found within the macrophages of the vertebrate host (Gupta and Nishi, 2011), we decided to test the suitability of the colorimetric assay with them.

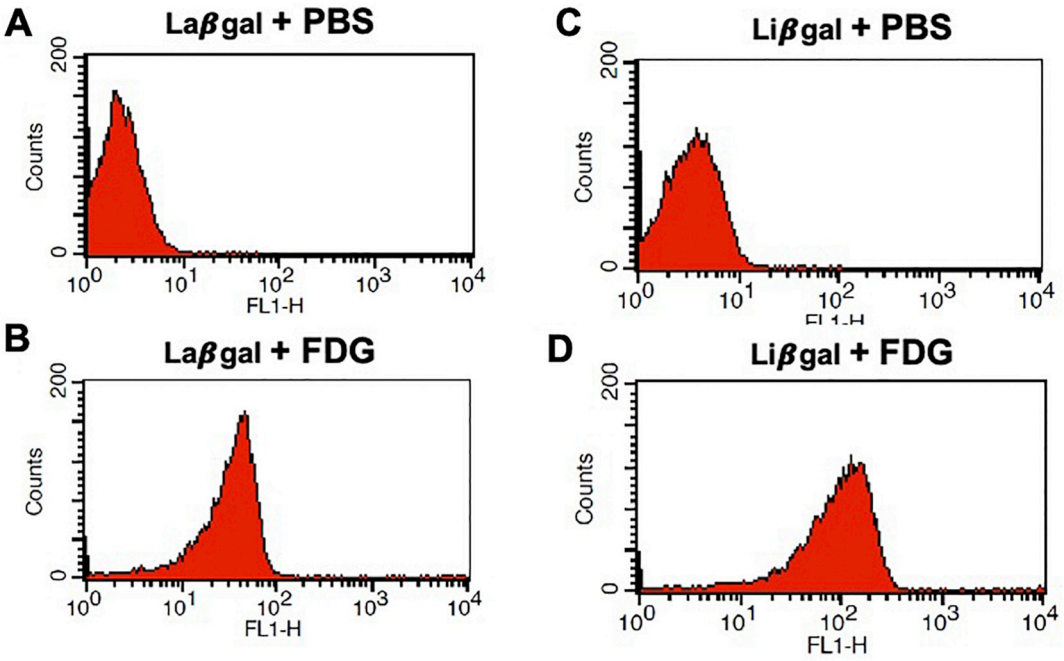

Fig. 5. Representative histograms for *Leishmania amazonensis* and L. *infantum* expressing β-galactosidase. (A and B) Histograms of Laβgal with PBS and FDG, respectively. (C and D) Histograms of Liβgal with PBS and FDG.

We evaluated two types of macrophages widely used in drug testing to generate amastigotes: peritoneal macrophages and the RAW 264.7 cell line. We noticed during our research that peritoneal macrophages are easier to be infected by *Leishmania*. On the other hand, cell line macrophages remain viable for longer in culture and, in addition, do not require animal use (Maia et al., 2007; Sereno et al., 2007). Here, variations in macrophage type and parasite:macrophage ratio were performed to optimize amastigote generation and to validate the assay. The idea of evaluating different parasite:macrophage ratios, at various time points, also emerged from the need to evaluate whether the method could detect distinct amounts of parasites present in three different incubation times, as commonly used in screening assays. To confirm that the known basal β-gal production by macrophages, related to cell senescence (Dube et al., 2009; Hall et al., 2017), did not interfere with the assay, macrophages without infection and under the same conditions were used as blank in all experiments. As shown in Fig. 4, the colorimetric method was efficient for amastigote detection in all parasite:macrophage ratios and in the three time periods tested. Both types of macrophages proved to be useful for this experiment, and those from the RAW cell line maintained the infection for a longer time period, as expected (compare Fig. 4 C and F with I and L). Absorbance from wells with infected macrophages was detected throughout the timeframe tested, with a good correlation between absorbance and number of parasites (Fig. 4). After 48 and 72 h, there was a reduction in parasite number in RAW macrophages infected with Liβgal in comparison to infection with Laβgal, possibly due to a greater killing of the *Leishmania* by the macrophages (compare Fig. 4 K and L). In the same periods, there was also a loss of linear correlation in the peritoneal macrophage assay, likely due to greater macrophage detachment from the plates (compare Fig. 4 B, C and E, F). Thus, 24 h of treatment and the use of the RAW 264.7 lineage were the conditions shown to be more adequate for anti-amastigote assays.

3.3. Validation of the colorimetric assay with Amphotericin B

To demonstrate that the transgenic L. *amazonensis* and L. *infantum* cells could be used in drug trials, they were tested against Amphotericin B, one of the standard drugs used in the leishmaniasis treatment (Batista et al., 2019). IC_{50} values were analyzed by *t*-test ($p < .05$) and can be observed in Table 1.

For Liβgal promastigotes, there was no significant difference between IC_{50} values derived from the two methods ($p = .3710$). On the other hand, for Laβgal there was a significant difference ($p = .0020$), which may be related to a method limitation caused by the counting in the Neubauer chamber. As seen in Fig. 2, *L. amazonensis* has a faster and more efficient growth in culture, resulting in the need for dilution before counting. This process of dilution could lead to errors in results, we thus consider the colorimetric method more reliable (Rocha et al., 2013).

We also evaluated the AmB effect on intracellular amastigotes, using the RAW cell line, which proved to be more suitable for the assay. After the IC_{50} calculation, no significant difference was found between values for Laβgal amastigotes ($p = .9209$). In contrast, for Liβgal IC_{50} values there was a small, but significant difference ($p = .0213$).

3.4. β-galactosidase detection by flow cytometry

Another alternative for β-gal detection is the use of the FDG fluorimetric reagent as a substrate, followed by analysis through flow cytometry. Here, transgenic and wild-type promastigotes were also evaluated after incubation with FDG. Cytometry histograms are shown in Fig. 5. In the analysis of transgenic parasites with FDG, the displacement of histograms occurred as expected. The use of FDG may not be the best option to test several compounds during screening assays, due to its high cost and low stability (Nir et al., 1990; Plovins et al., 1994). Indeed, considering the particularities pointed out for the two types of β-gal detection methods tested here (colorimetric and fluorimetric), the colorimetric method seems to be most suitable in the laboratory routine for the screening of new anti-leishmanial compounds. However, as an independent assay, confirmatory or not, the fluorometric assay proved to be a valid alternative to quantify β-gal expression in both L. *amazonensis* and L. *infantum* transgenic cell lines.

4. Conclusions

Here we have generated transgenic cell lines of L. *amazonensis* and

L. infantum that constitutively express β-galactosidase. Colorimetric assays using these transgenic cells proved to be a fast and accurate alternative to the traditional method of cell counting, which is laborious and with questionable reproducibility. These cell lines, having also been demonstrated to be suitable for use in drug test trials, can be applied to the laboratory routine for research and development of new compounds with leishmanicidal activity against both parasite forms, promastigotes and intracellular amastigotes. In all, they constitute a simple, fast and accurate tool to be used in anti-leishmanial drug screening.

Declaration of Competing Interest

None.

Acknowledgments

We thank the Brazilian National Research Council (CNPq) and the Research Foundation of Pernambuco State (FACEPE) for financial support (BCT-0022-2.11/18) and Oswaldo Cruz Foundation (FIOCRUZ) for the use of its facilities.

References

Andriani, G., Chessler, A.D.C., Courtemanche, G., Burleigh, B.a., Rodriguez, A., 2011. Activity in vivo of anti-trypanosoma cruzi compounds selected from a high throughput screening. PLoS Negl. Trop. Dis. 5, 1–6. https://doi.org/10.1371/journal.pntd.0001298.

Bachy, M., Bonnin-Rivalland, A., Tilliet, V., Trannoy, E., 1999. Beta galactosidase release as an alternative to chromium release in cytotoxic T-cell assays. J. Immunol. Methods 230, 37–46. https://doi.org/10.1016/S0022-1759(99)00118-0.

Batista, S., Vandresen, F., Falzirolli, H., Britta, E., Almeida, S.A., Oliveira, D.N., Catharino, R.R., Gonçalves, M.A., Ramalho, T.C., La, F.A., Nakamura, C.V., Cleuza, C., 2019. Synthesis and comparison of antileishmanial and cytotoxic activities of S - (−) -limonene benzaldehyde thiosemicarbazones with their R - (+) -analogues. J. Mol. Struct. 1179, 252–262. https://doi.org/10.1016/j.molstruc.2018.11.017.

Bezerra de Menezes, J.P., Guedes, C.E.S., Petersen, A.L. de O.A., Fraga, D.B.M., Veras, P.S.T., 2015. Advances in development of new treatment for Leishmaniasis. Biomed. Res. Int. 2015, 1–12. https://doi.org/10.1155/2015/815023.

den Boer, M., Argaw, D., Jannin, J., Alvar, J., 2011. Leishmaniasis impact and treatment access. Clin. Microbiol. Infect. 17, 1471–1477. https://doi.org/10.1111/j.1469-0691.2011.03635.x.

Buckner, F.S., Verlinde, C.L.M.J., Flamme, A.C.L. a, Voorhis, W.C.V. a N., 1996. Efficient technique for screening drugs for activity against Trypanosoma cruzi using parasites expressing b-galactosidase. Antimicrob. Agents Chemother. 40, 2592–2597. https://doi.org/10.1128/AAC.40.11.2592.

Calvo-Alvarez, E., Stamatakis, K., Punzon, C., Alvarez-Velilla, R., Tejeria, A., Escudero-Martinez, J.M., Perez-Pertejo, Y., Fresno, M., Balana-Fouce, R., Reguera, R.M., 2015. Infrared fluorescent imaging as a potent tool for in vitro, ex vivo and in vivo models of visceral Leishmaniasis. PLoS Negl. Trop. Dis. 9, 1–19. https://doi.org/10.1371/journal.pntd.0003666.

De Rycker, M., Hallyburton, I., Thomas, J., Campbell, L., Wyllie, S., Joshi, D., Cameron, S., Gilbert, I.H., Wyatt, P.G., Frearson, J.A., Fairlamb, A.H., Gray, D.W., 2013. Comparison of a high-throughput high-content intracellular Leishmania donovani assay with an axenic amastigote assay. Antimicrob. Agents Chemother. 57, 2913–2922. https://doi.org/10.1128/AAC.02398-12.

Dube, A., Gupta, R., Singh, N., 2009. Reporter genes facilitating discovery of drugs targeting protozoan parasites. Trends Parasitol. 25, 432–439. https://doi.org/10.1016/j.pt.2009.06.006.

Gupta, S., Nishi, 2011. Visceral leishmaniasis: experimental models for drug discovery. Indian J Med Res 133, 27–39. https://doi.org/IndianJMedRes_2011_133_1_27_76700 [pii].

Hall, B.M., Balan, V., Gleiberman, A.S., Strom, E., Krasnov, P., Virtuoso, P., Rydkina, E., Vujcic, S., Balan, K., Gitlin, I.I., Leonova, K.I., Consiglio, C.R., Gollnick, S.O., Chernova, O.B., Gudkov, A.V., 2017. p16 (Ink4a) and senescence - associated β - galactosidase can be induced in macrophages as part of a reversible response to physiological stimuli. Aging (Albany NY) 9, 1867–1884. https://doi.org/10.18632/aging.101268.

Jesus, A., Fragoso, T.N., Yamamoto, E.S., Laurenti, D., Lago, G., Gomes, G.S., Silva, M.S., Ferreira, A.F., Passero, L.F.D., 2017. Therapeutic effect of ursolic acid in experimental visceral leishmaniasis. Int. J. Parasitol. Drugs Drug Resist. 7, 1–11. https://doi.org/10.1016/j.ijpddr.2016.12.002.

Khraiwesh, M., Leed, S., Roncal, N., Johnson, J., Sciotti, R., Smith, P., Read, L., Paris, R., Hudson, T., Hickman, M., Grogl, M., 2016. Antileishmanial activity of compounds derived from the medicines for malaria venture open access box against intracellular leishmania major amastigotes. Am. J. Trop. Med. Hyg. 94, 340–347. https://doi.org/10.4269/ajtmh.15-0448.

Maia, C., Rol, N., Nunes, M., Gonc, L., Campino, L., 2007. Infectivity of five different types of macrophages by Leishmania infantum. Acta Trop. 103, 150–155. https://doi.org/10.1016/j.actatropica.2007.06.001.

Nir, R., Yisraeli, Y., Lamed, R., Sahar, E., 1990. Flow cytometry sorting of viable bacteria and yeasts according to b-galactosidase activity. Appl. Environ. Microbiol. 56, 3861–3866.

Nolan, G.P., Fiering, S., Nicolas, J.F., Herzenberg, L.A., 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-D-galactosidase activity after transduction of Escherichia coli lacZ. Proc. Natl. Acad. Sci. U. S. A. 85, 2603–2607. https://doi.org/10.1073/pnas.85.8.2603.

Okuno, T., Goto, Y., Matsumoto, Yoshitsugu, Otsuka, H., Matsumoto, Yasunobu, 2003. Applications of recombinant Leishmania amazonensis expressing egfp or the beta-galactosidase gene for drug screening and histopathological analysis. Exp. Anim. 52, 109–118. https://doi.org/10.1538/expanim.52.109.

Oliveira, J.C., da Silva, A.C., Oliveira, R.A. dos S., Pereira, V.R.A., Gil, L.H.V.G., 2016. In vivo near-infrared fluorescence imaging of Leishmania amazonensis expressing infrared fluorescence protein (iRFP) for real-time monitoring of cutaneous leishmaniasis in mice. J. Microbiol. Methods 130, 189–195. https://doi.org/10.1016/j.mimet.2016.08.003.

Plovins, A., Alvarez, A.M., Ibañez, M., Molina, M., Nombela, C., 1994. Use of fluorescein-di-beta-D-galactopyranoside (FDG) and C12-FDG as substrates for beta-galactosidase detection by flow cytometry in animal, bacterial,and yeast cells. Appl. Environ. Microbiol. 60, 4638–4641.

Pulido, S. a, Muñoz, D.L., Restrepo, A.M., Mesa, C.V., Alzate, J.F., Vélez, I.D., Robledo, S.M., 2012. Improvement of the green fluorescent protein reporter system in Leishmania spp. for the in vitro and in vivo screening of antileishmanial drugs. Acta Trop. 122, 36–45. https://doi.org/10.1016/j.actatropica.2011.11.015.

Reimão, J.Q., Trinconi, C.T., Yokoyama-Yasunaka, J.K., Miguel, D.C., Kalil, S.P., Uliana, S.R.B., 2013. Parasite burden in Leishmania (Leishmania) amazonensis-infected mice: validation of luciferase as a quantitative tool. J. Microbiol. Methods 93, 95–101. https://doi.org/10.1016/j.mimet.2013.02.007.

Rocha, M.N., Corrêa, C.M., Melo, M.N., Beverley, S.M., Martins-Filho, O.A., Madureira, A.P., Soares, R.P., 2013. An alternative in vitro drug screening test using Leishmania amazonensis transfected with red fluorescent protein. Diagn. Microbiol. Infect. Dis. 75, 282–291. https://doi.org/10.1016/j.diagmicrobio.2012.11.018.

Rodriguez, A., Tarleton, R.L., 2012. Transgenic parasites accelerate drug discovery. Trends Parasitol. 28, 90–92. https://doi.org/10.1016/j.pt.2011.12.003.

Roy, G., Dumas, C., Sereno, D., Wu, Y., Singh, A.K., Tremblay, M.J., Ouellette, M., Olivier, M., Papadopoulou, B., 2000. Episomal and stable expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol. Biochem. Parasitol. 110, 195–206. https://doi.org/10.1016/S0166-6851(00)00270-X.

Sacks, D., Sher, A., 2002. Evasion of innate immunity by parasitic protozoa. Nat. Immunol. 3, 1041–1047. https://doi.org/10.1038/ni1102-1041.

Sadeghi, S., Seyed, N., Etemadzadeh, M.H., Abediankenari, S., Rafati, S., Taheri, T., 2015. In vitro infectivity assessment by drug susceptibility comparison of recombinant Leishmania major expressing enhanced green fluorescent protein or EGFP-luciferase fused genes with wild-type parasite. Korean J. Parasitol. 53, 385–394. https://doi.org/10.3347/kjp.2015.53.4.385.

Santos, I.B., Silva, D.A.M., Paz, F.A.C.R., Garcia, D.M., Carmona, A.K., Teixeira, D., Longo-maugéri, I.M., Katz, S., Barbiéri, C.L., Muxel, S.M., 2018. Leishmanicidal and Immunomodulatory Activities of the Palladacycle Complex DPPE 1. 1, a Potential Candidate for Treatment of Cutaneous Leishmaniasis. Front Microbiol. 9, 1–9. https://doi.org/10.3389/fmicb.2018.01427.

Seeber, F., Boothroyd, J.C., 1996. Escherichia coli β-galactosidase as an in vitro and in vivo reporter enzyme and stable transfection marker in the intracellular protozoan parasite toxoplasma gondii. Gene 169, 39–45. https://doi.org/10.1016/0378-1119(95)00786-5.

Sereno, D., Cordeiro da Silva, A., Mathieu-Daude, F., Ouaissi, A., 2007. Advances and perspectives in Leishmania cell based drug-screening procedures. Parasitol. Int. 56, 3–7. https://doi.org/10.1016/j.parint.2006.09.001.

da Silva, A.C., dos Santos, T.A.R., da Silva, I.V.B., de Oliveira, M.V.G., Moreira, D.R.M., Leite, A.C.L., Pereira, V.R.A., 2017. Aryl thiosemicarbazones: in vitro and immunomodulatory activities against L. amazonensis. Exp. Parasitol. 177, 57–65. https://doi.org/10.1016/j.exppara.2017.04.003.

Tiuman, T.S., Santos, A.O., Ueda-Nakamura, T., Filho, B.P.D., Nakamura, C.V., 2011. Recent advances in leishmaniasis treatment. Int. J. Infect. Dis. 15, e525–e532. https://doi.org/10.1016/j.ijid.2011.03.021.

Vega, C., Rolón, M., Martínez-Fernández, A.R., Escario, J.A., Gómez-Barrio, A., 2005. A new pharmacological screening assay with Trypanosoma cruzi epimastigotes expressing b-galactosidase. Parasitol. Res. 95, 296–298. https://doi.org/10.1007/s00436-005-1300-3.

WHO, 2015. Leishmaniasis Fact Sheet.

Zulfiqar, B., Shelper, T.B., Avery, V.M., 2017. Leishmaniasis drug discovery : recent progress and challenges in assay development. Drug Discov. Today 22, 1516–1531. https://doi.org/10.1016/j.drudis.2017.06.004.