Fluorescent *Leishmania* species: Development of stable GFP expression and its application for *in vitro* and *in vivo* studies

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**Abstract**

Reporter genes have proved to be an excellent tool for studying disease progression. Recently, the green fluorescent protein (GFP) ability to quantitatively monitor gene expression has been demonstrated in different organisms. This report describes the use of *Leishmania* tarentolae (L. tarentolae) expression system (LEXSY) for high and stable levels of GFP production in different *Leishmania* species including *L. tarentolae*, *L. major* and *L. infantum*. The DNA expression cassette (pLEXSY-EGFP) was integrated into the chromosomal ssu locus of *Leishmania* strains through homologous recombination. Fluorescent microscopic image showed that GFP transgenes can be abundantly and stably expressed in promastigote and amastigote stages of parasites. Furthermore, flow cytometry analysis indicated a clear quantitative distinction between wild type and transgenic *Leishmania* strains at both promastigote and amastigote forms. Our data showed that the footpad lesions with GFP-transfected *L. major* are progressive over time by using fluorescence small-animal imaging system. Consequently, the utilization of stable GFP-transfected *Leishmania* species will be appropriate for *in vitro* and *in vivo* screening of anti-leishmanial drugs and vaccine development as well as understanding the biology of the host–parasite interactions at the cellular level.

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**1. Introduction**

*Leishmania* species are protozoan parasites of the genus trypanosomatidae family with a complex life cycle (promastigotes and amastigotes) that affect millions of people worldwide (Singh and Dube, 2004). Depending on the species of *Leishmania*, infection of humans may result in variety of symptoms collectively known as Leishmaniasis. The three major forms of Leishmaniasis caused by different species of *Leishmania* include cutaneous, mucocutaneous and visceral. Infection by species such as *L. major*, *L. tropica* and *L. mexicana* may cause localized cutaneous lesions that generally self-heal, resulting in lifelong immunity. Infection by *L. donovani*, *L. infantum* and *L. chagasi* may result in a chronic disseminating visceral disease in the liver and spleen that can become fatal unless treated with chemotherapy (Papadopoulou et al., 2003). Emerging technologies using fluorescence and bioluminescence imaging have been recently adapted for the study of host-*Leishmania* interactions to describe their molecular mechanisms in the cellular context (Lang et al., 2005, 2009). Fluorescent parasites can be obtained by labeling with a fluorescent dye or after transfection with genes encoding fluorescent proteins. Recently, the reporter genes have developed as a promising tool for studying disease progression. They typically encode a protein that has a readily measurable phenotype and is easily distinguishable over endogenous cellular background (Dube et al., 2009). Reporter genes are classified as intracellular and extracellular. Intracellular reporter gene products include chloramphenicol acetyltransferase (CAT), β-galactosidase, green fluorescent protein (GFP), firefly and bacterial luciferase and glucuronidase (D’Aiuto et al., 2008; Dube et al., 2009). Extracellular reporter gene products include human growth hormone (HGH) and secreted alkaline phosphatase (SEAP) (D’Aiuto et al., 2008; Dube et al., 2009). Advantages and disadvantages of various reporter genes have been studied for protozoan parasites as mentioned in Table 1. Among them, GFP is intrinsically fluorescent and allows easy imaging and quantification. GFP fluorescence activity can be detected with minimal handling using a fluorescence microscope, a fluorimeter, or a fluorescent activated cell sorter (FACS). Several mutant forms of native GFP have been generated that have different emission spectra and thus fluoresce at wavelengths longer than that of native GFP, for example enhanced green fluorescent protein (EGFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP) and cyan fluorescent protein (CFP) (Dube et al., 2009). Recently, new *Leishmania* expression systems have been designed for constitutive expression of target protein following
integration of the DNA expression cassette into the chromosomal 18S rRNA locus (ssu) making advantage of the high-level transcription rates by the host RNA polymerase I (Miblitz et al., 2000).

Herein, we report the use of L. tarentolae expression system (LEXSY) for improved EGFP production in different Leishmania species such as L. tarentolae, L. major and L. infantum through homologous recombination. In this study, we have generated different transgenic Leishmania species stably expressing EGFP gene. These parasites produced significant fluorescent signals in vitro as well as real time visualization in vivo. The presence of viable Leishmania species in the amastigote form inside adherent mouse macrophages (Bone marrow-derived macrophages or J774A.1 mice cell line) could provide a more accurate approach in drug sensitivity profiling of an anti-leishmanial compound. Indeed, the transgenic species will allow parasite detection at high sensitivity and will be extremely useful tool for tracking infections in macrophages at the level of both in vitro and in vivo manipulations.

2. Materials and methods

2.1. Preparation and cultivation of three Leishmania strains

The L. tarentolae (ATCC 30267) and L. major (Friedlin, MHOM/IL/81) promastigote forms were grown at 26 °C in M199 medium (Sigma, Germany) supplemented with 5% heat-inactivated fetal calf serum (HI-FCS, Gibco, UK), 40 mM HEPES, 2 mM L-glutamine, 0.1 mM adenosine, 0.5 μM/ml hemin and 50 μg/ml gentamicin (Sigma, Germany). L. infantum (MCAN/98/LLM-877, kind gift from WHO collaborating center for leishmaniasis, Instituto de Salud Carlos III, Spain) was cultured in liquid complete media on Novy, McNeal and Nicolle (NNN) solid medium. Both logarithmic and stationary-phase promastigotes were used for evaluation of GFP expression.

2.2. Generation of fluorescent Leishmania parasites

The EGFP gene (~720 base pairs) was obtained from the pEGFP-N1 vector (Clonetech, Palo Alto, CA) containing the coding sequence of EGFP protein by using PCR technique. The forward and reverse primers for amplifying the EGFP (EGFP1 as sense primer & EGFP2 as anti-sense primer, respectively) were designed with the restriction sites as shown in bold. The restriction sites in sense and anti-sense primers correspond to EcoRI, BgIII and XbaI, respectively.

**EGFP1:** 5′-ATG CAT ATC A AGA TCT ATG GTG AGC AAG GCC-G3′

**EGFP2:** 5′-GC TCT AGA TTA GGT ACC CTG GTA CAG CTC GTC-3′

PCR reaction was performed by PCR thermal cycler (Techne) under standard conditions (94 °C, 1 min; 62 °C, 2 min and 72 °C, 1 min for 30 cycles) and the product was separated on a 0.8% agarose gel. The bands corresponding to the expected PCR product size was gel purified (QiAquick gel extraction kit protocol, Qiagen, Germany), digested with BglII and KpnI and ligated into a similarly digested Leishmania expression vector pLEXSY-neo2 (E3E-233, Jena bioscience, Germany). The ligation mixture was used to transform Escherichia coli DH5α strain. The plasmid DNA (pLEXSY-EGFP) was purified from recombinant colonies by an alkaline lysis method (Qiagen Plasmid Mid Kit) verified by restriction enzyme digestion and sequenced using the dideoxy chain termination method on an automated sequencer. Approximately 5 μg of the expression plasmid was digested with SwaI for electroporation. The linearized expression cassettes with SwaI containing the encoding region for EGFP (pLEXSY-EGFP) was integrated into the chromosomal 18S rRNA locus (ssu) of three Leishmania strains through homologous recombination.

For transfection, 4 × 10^7 log-phase parasites (L. tarentolae, L. major and L. infantum) were resuspended in 400 μl of ice-cold electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5) containing 5 μg of linearized pLEXSY or pLEXSY-EGFP, stored on ice for 10 min, and electroporated using Bio–Rad Gene Pulser Ecell under conditions of 500 μF, 450 V and pulse time ~5–6 ms. The electroporated promastigotes were then incubated for 24 h in M199 10% medium at 26 °C without any drug (Neomycin or G418, Sigma, Germany), and plated on solid media (2% of Noble agar and 2% M199 10% (vol/vol), Sigma, Germany) containing 50 μg/ml of G418. The growth of cells highly resistant to Neomycin was observed after 15–20 days. Clones were selected on Noble agar plates and further propagated in liquid M199 10% medium in the absence of G418. Expression of EGFP protein in promastigote stage of the recombinant Leishmania species was evaluated by Epifluorescent microscopy for 6 months (Nikon, E 200, ACT-1 software, Digital sight Camera, Japan).

Furthermore, the L. infantum EGFP-containing epimastigote stage was prepared. Briefly, egfp was cloned into the XbaI/HindIII site of pGEM7Zf-L.d. rDNA promoter, a Leishmania–specific vector obtained from Dr. Barbara Papadopoulou (Laval University, Quebec, Canada), transformed in DH5α E. coli. The construct was used to transfect L. infantum promastigotes by electroporation. Then, the electroporated parasites were selected for resistance to G418 at 10 μg/ml. The growth of cells highly resistant to G418 was observed after 15–20 days. Epimastigotes were passaged continuously under these selective conditions in vitro (M199 10% medium containing 400 μg/ml of drug). EGFP expressing cells was analyzed by Epifluorescent microscopy.

2.3. Confirmation of genomic integration by diagnostic PCR

Integration of the expression cassette into the ssu locus was confirmed by diagnostic PCR using genomic DNA of wild type and transgenic strains of L. tarentolae, L. major and L. infantum as template. For this purpose, a primer pair including one primer
hybridizing within the expression cassette and one primer hybridizing to a ssu sequence not present on the plasmid were used. We prepared genomic DNA from 1 x 10^7 cells with a commercial kit (Genomic DNA Extraction Mini Kit; Real Biotech Corp, Taiwan). Diagnostic PCR analysis was performed by PCR thermal cycler (Techne) under standard conditions (annealing temperature 60 °C) with ssu forward primer F3001 (Jena bioscience, Germany) and aprt reverse primer A1715 (hybridizing within the 5’ UTR of the target gene, Jena bioscience, Germany) designed as following:

F3001: 5’-GAT CGT GTT GAT TCT GCC AGT AG-3’  
A1715: 5’-TGA TCG TTT TCA GAT GGA GCA C-3’

Furthermore, additional diagnostic PCR reactions including EGFP-specific primers (EGFP1 and EGFP2 as forward and reverse primers, respectively) were performed by using genomic DNA of transgenic strains as template.

2.4. In vitro macrophage infection using transfected parasites

Bone marrow-derived macrophages were established as described (Daneshvar et al., 2003). Briefly, the femurs and tibias of naive BALB/c mice were flushed out with 5 ml of ice-cold RPMI (Sigma, Germany). The cells were collected and centrifuged at 1700 rpm for 15 min at 4 °C. The pellets were subsequently re-suspended in complete RPMI supplemented with 10% (vol/vol) HI-FCS, 1700 rpm for 15 min at 4 °C (Sigma, Germany). The cells were collected and centrifuged at 4 °C.

2.5. Flow cytometry analysis

Wild type and GFP expressing promastigote forms of parasites (L. tarentolae, L. major and L. infantum) were analyzed for EGFP expression using flow cytometry. Parasites at two different growth phases (logarithmic and stationary phases) were centrifuged at 3000 rpm for 15 min, washed and then re-suspended at 10^6 cell/ml in PBS and stored on ice. Cells were analyzed on a FACScalibur flow cytometer (BD: Becton Dickinson, Franklin Lakes, NJ) equipped with a 15 mV, 488 nm, air-cooled argon ion laser. 50,000 events were recorded and EGFP expression in transgenic Leishmania species was measured in comparison with wild type (WT) parasites.

Furthermore, infected bone marrow-derived macrophages by three EGFP-labeled Leishmania species were also analyzed by flow cytometry using forward (FCS) vs side scatter (SCC) to gate the macrophage population and a FL1 histogram to quantify fluorescence of cells at different time periods (24, 48 and 72 h). Cell debris and extracellular parasite, were excluded from the analysis on the basis of forward- and side-scatter characteristics. 50,000 events were acquired for each analysis and frequency of infected macrophages was obtained on histograms using FlowJo software (TreeStar, Inc., USA, version 7.5.3). Additionally, J774A.1 macrophage cells, infected with stationary phase promastigotes of L. infantum, harboring fluorescent amastigotes in integrative and episomal GFP forms were analyzed on a FACScalibur flow cytometer (BD: Becton Dickinson, Franklin Lakes, NJ).

2.6. Western blot analysis

Promastigote forms were harvested by centrifugation at 3000 rpm for 15 min and washed in PBS. The pellets were immediately lysed in 2× SDS–PAGE sample buffer (4.5 mM Tris–HCl, pH 6.8, 10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol, 0.05% w/v bromophenol blue) on ice and then boiled for 5 min. Samples from both wild type and transgenic L. major, L. infantum and L. tarentolae were separated by SDS–PAGE in a 15% (w/v) polyacrylamide gel (SDS gel apparatus; Bio-Rad). For western blot analysis, the resolved proteins were transferred onto nitrocellulose transfer membrane (Schleicher and Schuell Bioscience, Dassel, Germany) using a Bio-Rad wet blotting system. The membrane was pre-equilibrated with TBST solution (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) containing 2.5% bovin serum albumin (BSA) for overnight and then reacted with anti-GFP antibody (1:5000 v/v; polyclonal antibody to GFP-HRP; Acris antibodies GmbH) for 2 h at room temperature. The immunoreactive protein bands were visualized using peroxidase substrate named 3,3’-Diaminobenzidine (DAB, Sigma, Germany).

2.7. RNA extraction and reverse-transcription PCR

RNA samples were extracted from promastigotes using RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. RNA integrity was determined using formaldehyde agarose (FA) gel electrophoresis. Purity of RNA samples was determined by NanoDrop (ND-1000) spectrophotometer. Both 260/280 and 260/230 ratios for all samples were between 1.9–2.1 and >2, respectively. The cDNA synthesis was performed using Omniscript Reverse Transcriptase kit (Qiagen) from 1 μg of RNA. For EGFP detection, PCR analysis was performed by PCR thermal cycler (Techne) under standard conditions (annealing temperature 62 °C) as described above.

2.8. Detection of EGFP-transfected L. major promastigotes in BALB/c mice model

The right hind footpad of 6–8 week old female BALB/c mice was infected with 2 x 10^6 of either WT or EGFP-transfected late-stationary-phase L. major promastigotes suspended in 50 μl of PBS. The infected footpad was imaged at different time intervals,
beginning at day 7, with the KODAK Image Station 4000 Digital Imaging System. Briefly, mice were treated with a depilatory substance (Nair) to remove hair from their legs and feet to reduce background autofluorescence, as described previously (Mehta et al., 2008). After that, they were temporarily anesthetized with a mixture of Xylazine 2% (7.5 µl), Ketamine 10% (30 µl) and saline solution (260 µl) per mice intraperitoneally and then imaged at 7th, 28th, 52nd, 59th and 66th days post-infection. In addition, at day 66, the infected lymph node was also demonstrated using the same imaging system. Pixel counting and measurement of the lesions were performed using KODAK molecular image software version 5.3. Measurements were reported as “sum green intensity”, a quantitative measurement defined as the number of green pixels in a given area multiplied by the average intensity of each pixel.

3. Results

3.1. Development of different EGFP – expressing transgenic Leishmania species

The 720 bp fragment encoding the EGFP was cloned into the BgIII and KpnI sites of pLEXSY-neo2 in which neo gene is used as selection marker (Fig 1A). The recombinant plasmid (pLEXSY-EGFP) was prepared in large scale with high purity, linearized and electroporated into parasites. The linearized expression cassette (pLEXSY-EGFP) was integrated into the chromosomal ssu locus of three Leishmania species through homologous recombination (Fig 1B). The transformants were selected by plating on solid medium containing G418. Integration of the expression constructs into the ssu locus was confirmed by genomic PCR which yielded a ~1 kb fragment. Amplification of this fragment was only possible if integration of the DNA cassette into a 18S rRNA gene locus occurred. A band corresponding to the expected PCR product size for EGFP (~720 bp) was also obtained for EGFP positive clones (Fig 1C).

3.2. EGFP expression in promastigote and amastigote forms of Leishmania species

The transfectants of three Leishmania species in promastigote stage were tested for EGFP expression by Epi-fluorescent microscopy. The expression of EGFP was readily evident from the intense green fluorescence of the parasites (Fig 2). These promastigotes were used to infect bone marrow-derived macrophages at a ratio of 10:1. At the end of the incubation period (24, 48 and 72 h), EGFP expression was directly monitored. Fluorescent microscopic image indicated that the intracellular amastigotes expressing EGFP from the integrated vector can be visualized directly without the need for any additional preparation as shown for L. tarentolae (Fig 2A(c and d)), L. major (Fig 2B(c and d)) and L. infantum (Fig 2C(c and d)) at 48 h after parasite infection.

3.3. Monitoring of EGFP expression in promastigotes and amastigotes by flow cytometry

EGFP expression in transgenic promastigotes was monitored by flow cytometry. High expression levels of EGFP were observed at both logarithmic and stationary phases and compared with wild type in each species. Analysis by fluorescence-activated cell sorting (FACS) indicated a clear quantitative separation between transfected and wild type parasites as shown in Fig 3A. The percentage of GFP-expressing parasites was increased in stationary phases of L. major and L. infantum (97.85% and 98.29%, respectively) as compared to logarithmic phases of these parasites (89.22% and 89.30%, respectively) without any changes in mean fluorescent intensities (MFI). EGFP expression was similar in L. tarentolae at both phases. Additionally, the percentage of GFP-expressing L. tarentolae parasites was higher than that in two other parasites in logarithmic (99.49%) and stationary (99.18%) phases. The stability of GFP expression was monitored over a period of 6 months post-electroporation and it was observed that there was no decrease in fluorescence intensity during this period in the absence of G418 (data not shown). Therefore, L. tarentolae, L. major and L. infantum transfectants can stably express and maintain the reporter green fluorescent gene without any drug pressure even for more than 6 months.

Furthermore, all three transfected Leishmania species were infective to bone marrow-derived macrophages. The frequency of EGFP positive cells after 24, 48 and 72 h were determined by FACS analysis using the appropriate gating as shown in Fig 3B. The percentage of infected macrophages for each transfected Leishmania species is shown at 24, 48 and 72 h post-infection. L. infantum has the highest infection rate (58.60%) in comparison with L. major (45.48%) and L. tarentolae (32.40%) after 24 h. It is noteworthy that the rate of infection declines in all three species after 72 h (L. major, 27.99%; L. infantum, 25.08% and L. tarentolae, 21.19%).

Furthermore, Fig. 3C shows the results of J774A.1 macrophage cells, infected with stationary phase promastigotes of L. infantum expressing GFP in the form of integrated and episomal (in presence or absence of 400 µg of G418). There is significant difference in the level of GFP expression. The highest percentage of GFP expressing cells is belongs to integrated GFP (76.71%; d). Our data indicated that the episomal GFP expressing Leishmania required the constant drug pressure for its expression continuously (26.64%, e) and it was diminished without drug pressure (7.51%, f). These results were confirmed by Epi-fluorescent microscopy as shown in Fig. 3C(d and e).

3.4. Confirmation of EGFP expression by Western blotting and RT-PCR

EGFP expression was also detectable in cell extracts of transgenic parasites in comparison with wild type using anti-GFP antibody. As it is shown in supplement A, a dominant band of ~27 kDa that was immunoreactive with anti-GFP antibody detected in all three Leishmania transgenic parasites (L. tarentolae, L. major and L. infantum).

The final step for confirming of EGFP expression was cDNA samples amplification by GFP-specific primers. All transfected parasites showed a dominant band of EGFP (~720 bp) in comparison with wild type as shown in supplement B.

3.5. EGFP-transfected L. major promastigotes were detected by using fluorescence imaging system

In this experiment, we decided to detect EGFP-labeled L. major transfecteds in vivo using imaging system. The mice were examined periodically for almost two months. As shown in Fig 4A (right panel), GFP fluorescence which was initially localized to the site of the inoculation, subsequently spread to a wider area at days 59 and 66 post infection. The increasing thickness of the infected footpad with wild type L. major was simultaneously demonstrated toward the end of the 5th week (Fig 4A, left panel). The fluorescence imaging system gives a precise two-dimensional image of the extent of infection, independent of the inflammatory response. It is also possible to clearly detect the infected lymph node with fluorescent parasites at day 66 post infection as shown in Fig 4A (right panel). In addition, there is an, in vivo correlation between fluorescence and footpad thickness as determined over time (Fig 4B). During the period under study, the sum green intensity (pixel) from the imaging studies was increased similarly to the thickness of infected footpad. Therefore, imaging system could be applied as a
semi-quantitative parameter which correlates to the number of parasites in vivo.

4. Discussion

Various reporter genes are currently available for protozoan parasites. All of them have different degrees of sensitivity. The advantages and disadvantages of main reporter genes have been extensively studied for protozoan parasites (Table 1, Dube et al., 2009). GFP-based assays offer several advantages over other non-reporter- or reporter-gene-based assays, including greater simplicity, easier kinetic monitoring, low cost and enhanced biosafety (Singh et al., 2009). Expression of GFP has been reported in several Leishmania species, and other parasites including Plasmodium species, Trypanosoma, Toxoplasma and Entamoeba (Dube et al., 2009).
Various recombinant Leishmania species carrying a reporter gene either as an episomal copy or after its integration in a defined locus, generally the rDNA locus is currently available. They include episomal/integrative GFP and luciferase-expressing L. donovani; integrative luciferase/β-lactamase and episomal GFP/EGFP/β-galactosidase-expressing L. amazonensis; episomal/integrative luciferase and integrative GFP/β-lactamase/β-galactosidase-expressing L. major; and episomal/integrative GFP and integrative β-galactosidase-expressing L. mexicana (Dube et al., 2009; Roy et al., 2000; Chan et al., 2003). The use of the episomal vector carrying a given gene has been limited for two reasons. First, gene expression is extremely heterogeneous in populations of transfected parasites, due to wide variation in copy number of plasmids per cell; second, the host loses the plasmid in the absence of antibiotic resistance marker. Permanent transfection by integration of DNA cassettes into the genome overcomes these problems (Miblitz et al., 2000).

Recently, the pLEXSY vectors have been used for the constitutive expression of target proteins either with or without secretory signal peptide (Basak et al., 2008). In current study, directional cloning of EGFP into pLEXSY-neo2 was performed and confirmed by PCR and restriction analysis of the recombinant plasmid (pLEXSY-EGFP). Promastigotes of three Leishmania species (L. tarentolae, L. major and L. infantum) were transfected by electroporation using the linearized pLEXSY-EGFP construct. Genomic integration was confirmed by diagnostic PCR using genomic DNA of transgenic species as template.

Currently, L. tarentolae has been used as a host for high level recombinant protein expression by integration of the expression cassette into the small ribosomal subunit rRNA gene (Breitling et al., 2002; Fritsche et al., 2007; Basile and Peticca, 2009). Also, L. tarentolae represents a completely safe live vector, as previously reported (Breton et al., 2007; Mizbani et al., 2009). In this study, we have established conditions for transfection of L. tarentolae, L. major and L. infantum stably expressing the EGFP gene. These EGFP expressing parasites were analyzed in both life cycles including promastigote form and amastigote-loaded macrophages. EGFP expression in live Leishmania strains was evaluated and confirmed by Epi-fluorescence microscopy, flow cytometry, western blotting, RT-PCR and external illuminator imaging system.

The green fluorescent protein has been expressed episomally in a variety of protozoan parasites, including Leishmania and trypanosomes and it has been more recently integrated into the ribosomal RNA (rRNA) locus of L. mexicana, L. major and L. donovani by homologous recombination (Boucher et al., 2002; Dube et al., 2009). The aim of the present study was to stably express reporter gene (EGFP) by using Leishmania tarentolae expression system (LEXSY) in the promastigote and amastigote stages of three Leishmania species in order to develop a method that allows easy and sensitive detection of infected cells both in culture and in infected hosts. Clearly, the choice of suitable UTRs is crucial for construction of an efficient expression cassette suitable for the large-scale recombinant proteins production. Furthermore, there is low diversity between the 18S rRNA sequences of Leishmania species (Breitling et al., 2002; Basile and Peticca, 2009; Mureev et al., 2007; Croan et al., 1997; Field et al., 1991). We showed here that EGFP transgenes can be abundantly and stably expressed in promastigotes and amastigotes of three Leishmania species in the absence of G418. Our studies indicated that the promastigotes and amastigotes expressing GFP from the integrated plasmid could be detected directly without the need for additional preparation. The fluorescence intensity pattern showed a bell-shaped distribution for L. tarentolae indicating a homogenous level of expression within the population. Similar results were obtained for L. major and L. infantum at lower frequency than that for L. tarentolae. Previously, the role of several parameters governing the efficiency of gene targeting mediated by homologous recombination was investigated in Leishmania species (Papadopoulou and Dumas, 1997; Croan et al., 1997; Dujardin, 2009). In fact, the frequency of homologous recombination between an introduced vector and chromosomal DNA sequences is influenced by many factors including the amount and nature of homologous sequences, the genetic locus and the copy number of the target and design of the vector (Papadopoulou and Dumas, 1997). Different studies have shown the suitability of transgenic L. donovani or L. infantum promastigotes that constitutively express GFP in their cytoplasm as target cells for in vitro screening of anti-leishmanial drugs (Singh and Dube, 2004; Monte-Alegre et al., 2006; Kamau et al., 2001). Also, trans-

Fig. 2. EGFP detection by Epi-fluorescent microscopy; Fluorescent microscopic images show expression of EGFP in transfected L. tarentolae (A), L. major (B), and L. infantum (C) promastigotes (before and after glinting of fluorescence: a & b; left) and intracellular amastigotes expressing EGFP (before and after glinting of fluorescence: c and d, right) in bone marrow-derived macrophages 48 h post-infection with L. tarentolae (A), L. major (B), and L. infantum (C) strains. High amounts of EGFP were observed in both life cycle-stages.
genic Leishmania expressing GFP have opened the way for a flow cytometry (FACS)-based method to assess the killing of Leishmania parasites inside their macrophage host (Kram et al., 2008). This technique is tremendously helpful because of its ability to discriminate between live and dead cells and could display the low frequency of host cells that harboring a limited number of amastigotes. In this study, GFP-expressing promastigotes were used to infect macrophage cells and the infectivity rate was analyzed by flow cytometry after 24, 48 and 72 h. Flow cytometric results indicated that infection rates gradually diminished over time. This observation...

Fig. 3. (A) Fluorescence-activated cell sorting analysis of EGFP transfected parasites at two different phases; first row shows logarithmic phase and second row shows the stationary phase of Leishmania strains. Gray histograms indicate untransfected parasites and colorful histograms show transfected parasites. (B) Representative histograms of macrophages infected with different Leishmania species expressing EGFP at (a) 24, (b) 48 and (c) 72 h. The infectivity rate of L. major (green), L. infantum (red) and L. tarentolae (blue) are 1.64, 2.32 and 1.5, respectively. The black histogram represents un-infected macrophages (control). (C) Representation of macrophages un-infected and infected with GFP-promastigotes. (a) FACS histogram of wild type (gray) and GFP transfectants (red) of L. infantum promastigotes; (b) Wild type un-infected J774A.1 macrophages; (c) J774A.1 macrophages transfected with wild type L. infantum as control; (d) J774A.1 macrophage cells, infected with stationary phase promastigotes of L. infantum, harboring fluorescent amastigotes in integrative GFP form, (e) episomal GFP form in presence of 400 μg of drug and (f) episomal GFP form without drug. M1 and M2 denote uninfected macrophage population and infected macrophages, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

tation is due to high ratio of parasites to macrophage (10:1) which cause macrophages lysis leading to fewer cells to be infected. For our in vitro studies on the differences of GFP expression in both episomal and integrative forms, J774.1 macrophage cells were infected with stationary phase GFP-promastigotes of *L. infantum* at a ratio of 10:1 (parasites/macrophage). The data indicated that the parasite with episomal expression of GFP require the constant drug pressure for its continuous expression. Additionally, the percentage of GFP-expressing cells using *L. infantum* with integrative GFP has higher level of expression than that in GFP-episomal expressing cells (76.71% vs 26.64%, respectively). Therefore, *Leishmania* strains with episomal expression of GFP have limitation to the parasite with episomal expression of GFP require the constant drug pressure for its continuous expression. Additionally, the percentage of GFP-expressing cells using *L. infantum* with integrative GFP has higher level of expression than that in GFP-episomal expressing cells (76.71% vs 26.64%, respectively). Therefore, *Leishmania* strains with episomal expression of GFP have limitation to be used in ex vivo or in vivo systems. This result was previously confirmed by Singh et al. (2009).

Subsequently, the application of GFP fluorescence for in vivo imaging provided another approach to have a dynamic follow up of parasite propagation in infected footpad. It has been shown by others that fluorescence measurements are more precise and sensitive than the standard caliper-based method of following *Leishmania* infection in vivo and could show the extent of infection independent of inflammatory response (Mehta et al., 2008). The ease of detecting parasites using GFP led to follow their migration of the parasites to other tissues. We could observe migration of *L. major* to the lymph nodes of infected mice at day 66.

Different studies have shown that the real-time bioluminescent assay is not only sensitive but also more rapid than culture-base techniques, allowing to monitor parasite-load before any clinical signs of leishmanianis are detectable. In short, the luciferase imaging study is useful to monitor the efficacy of anti-leishmanial drugs on live cell culture and to trace leishmanial infection in animal models (Lang et al., 2005).

Furthermore, in comparison with episomally GFP-expressing *Leishmania* (Mehta et al., 2008), we can take advantages of these stably fluorescent parasites for vaccine development including in vivo real time whole body fluorescence imaging to follow the progression of *Leishmania* infection in parasitized tissue of different animal models such as mouse, hamster and dog.

For in vivo testing of vaccine, several animal species have served as experimental host for visceral leishmaniasis (VL). Important among them are BALB/c mice and Syrian golden hamster (primary tests), dogs (secondary tests) and monkeys as tertiary screens (Garg and Dube, 2006). In a study, a constitutive and enhanced expression of GFP in promastigote and amastigote stages was achieved for ~12 months without any need for drug pressure. These transfecants were highly infective to macrophage cell lines as well as to hamsters, as observed by fluorescence microscopy and flow cytometry (FACS). The GFP-transfectants were found to be suitable for FACS-based ex vivo screening assays. They were also infective to hamsters up to day 60 post-infection (Singh et al., 2009). Furthermore, it has been shown recently, that in order to visualize the bite site in vivo, an RFP-expressing strain of *L. major* (*L. major*-RFP) were employed in mice model. In this study, in vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies (Peters et al., 2008). Surely this system can help to investigate the diversity of potential natural reservoir of *Leishmania*.

In summary, our data show that parasites with integrated GFP marker gene express high amounts of GFP in both life-cycle
stages. The utilization of this DNA cassette will be appropriate for studies of long-term expression of transgenes during infection detection.

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Appendix A. Supplementary data


References


