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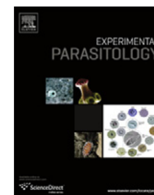

Fluorescent *Leishmania* species: Development of stable GFP expression and its application for *in vitro* and *in vivo* studies

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A B S T R A C T

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

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Table 1
The advantages and disadvantages of various reporter genes for protozoan parasites (Dube et al., 2009).

Reporter gene	Advantages	Disadvantages
β-gal	Simple, inexpensive, sensitive	Large size monomer, endogenous expression of β-gal by some mammalian cell types such as macrophages
β-lac	No endogenous expression by mammalian cells, rapid, sensitive, non-radioactive	Colorimetric assay, lysis of cells required, repeated measurements not possible
luc	Allows bioimaging, highest efficiency, absence of background activity by host cells	Limited due to inefficient entry of expensive substrate luciferin to cells, destructive sampling of tissues, short half-life, repeated measurements not possible
gfp	Low toxicity, easy imaging, no substrate required, no need for permeabilization and fixation of cells, no additional steps required mutants with altered spectral qualities available	Requires post-translational modification, low sensitivity

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 profile 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 μg/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 antisense primers correspond to *Eco*RI, *Bgl*II and *Xba*I, *Kpn*I, respectively.

EGFP1: 5′-AT **GAT ATC** A **AGA TCT** ATG GTG AGC AAG GGC-3′
EGFP2: 5′-GC **TCT AGA** TTA **GGT ACC** CTT 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 *Bgl*II and *Kpn*I and ligated into a similarly digested *Leishmania* expression vector pLEXSY-neo2 (EGE-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 *Swa*I for electroporation. The linearized expression cassettes with *Swa*I 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*) re-suspended in 400 μl of ice-cold electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 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 2x 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 episomal cassette was prepared. Briefly, *egfp* was cloned into the *Xba*I/ *Hind*III site of pGEM7Zf-L.d. rDNA promoter, a *Leishmania*-specific vector obtained from Dr. Barbara Papadopoulou (Laval University, Quebec, Canada), transformed in DH5 α *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. Episomal transfectants were passaged continuously under these selective conditions *in vitro* (M199 10% medium containing 400 μg/ml of drug). EGFP expressing cells was analyzed by Epi-fluorescent 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

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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 1×10^7 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 CTG GTT GAT TCT GCC AGT AG-3′
A1715: 5′-TAT TCG TTG TCA GAT GGC 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, 100 U/ml Gentamicin, 50 μM 2-β-mercaptoethanol, 2 mM L-glutamine, 1 mM pyruvate (Sigma), and 30% (vol/vol) L-929 cell-conditioned medium. The cells were transferred to a 6-well plate (Greiner, Germany) and incubated at 37 °C in 5% CO_2 for 7 days. The supernatant was refreshed with complete RPMI on day 3. After removal of the non-adherent cells, the adherent cells were collected with pre-cold PBS, transferred into the individual wells of a 24-well plate including sterile glass cover slips at 2×10^5 cells/well, washed after 4 h and then incubated overnight at 37 °C in 5% CO_2. The next day, the non-adherent cells were removed and the adherent cells were exposed to 2×10^6 stationary-phase promastigotes per well. The culture slides were incubated in 5% CO_2 for 4 h at 37 °C for *L. major*, *L. infantum* and *L. tarentolae*. The free promastigotes were then removed by replacing the overlying medium with RPMI 10% and the cells were incubated for the appropriate times (24, 48 and 72 h) in 5% CO_2 at 37 °C. At the end of the incubation period, EGFP expression was directly monitored using an Epi-fluorescence microscope (Nikon, E200, Japan). The initial infection ratios of parasites to macrophages for *L. major*, *L. infantum* and *L. tarentolae* were 10:1.

Subsequently, to measure the fluorescent activity of intracellular amastigotes and *in vitro* studies on the differences of GFP expression in both episomal and integrative forms of *L. infantum*, J774A.1 mouse (BALB/c) macrophage cell line (TIB-67) was cultured in complete RPMI medium (Gibco, Germany) supplemented with 10% HI-FCS (Gibco) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Briefly, 10^5 cells/well in 6-well culture plates (Greiner Bio-One, Germany) were infected with episomal and integrative GFP-expressing stationary phase promastigotes of *L. infantum* in a ratio of 10:1 (parasites/ macrophage) and incubated at 37 °C in 5% CO_2 for 4 h. After time interval, infected macrophages were harvested by washing with pre-cold PBS (pH 7.2), and monitored using a Epi-fluorescence microscope (Nikon, E200, Japan). We applied episomal GFP-expressing stationary phase promastigotes of *L. infantum* grown without drug pressure and with 400 μg of G418 (Sigma, Germany).

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 protran 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 2×10^8 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 *Bgl*II and *Kpn*I 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 kbp 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* (97.65% 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*, 25.09% 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 EGFP-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* transfectants *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 footpads 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

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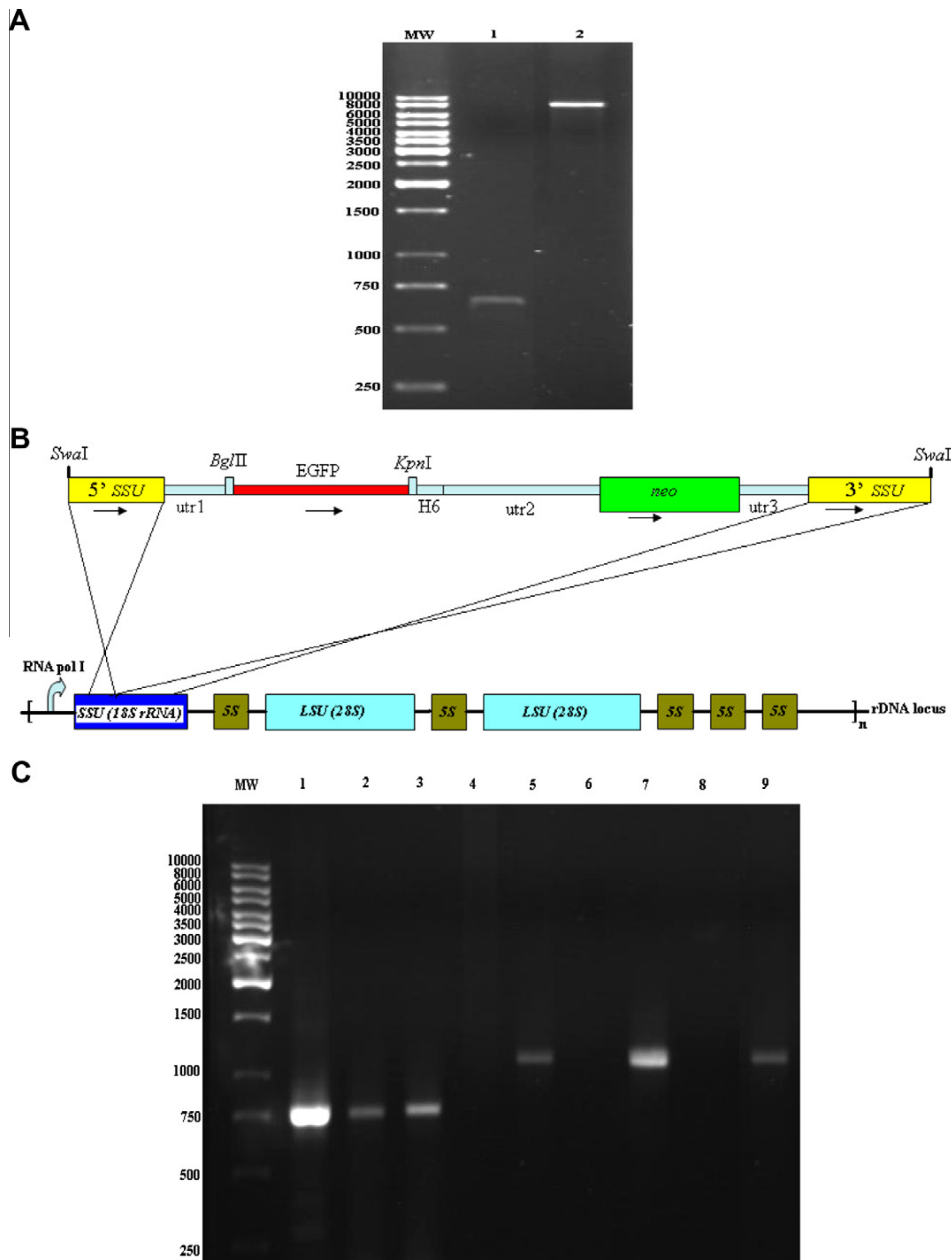

Fig. 1. (A) Directional cloning of EGFP into pLEXSY-neo2 expression vector; the bands corresponding to the digested PCR product of EGFP with *Bgl*II and *Kpn*I (Lane 1) and ligated into a similarly digested *Leishmania* expression vector pLEXSY-neo2 (Lane 2). DNA molecular weight marker (1 kbp, Fermentase) was shown in lane MW. (B) Integration of pLEXSY-EGFP expression construct into the *Leishmania* rDNA locus following linearization with *Swa*I. 5′ and 3′ *SSU* integration sequences are regions for homologous recombination into the multiple host *SSU* locus (*18S rRNA* gene). This system allows high-level transcription of EGFP gene under the control of the most efficient host RNA polymerase I. Utr 1–3 are obtained from *cam*, *dhfr* or *aprt* operons and provide efficient splicing and polyadenylation signals. *neo* is used as a marker gene for selection. The arrow demonstrates the start of transcription from the rRNA genes. (C) Confirmation of genomic integration by diagnostic PCR; the expected PCR product size for EGFP (∼720 bp) was obtained for EGFP positive clones of *L. tarentolae* (lane 1), *L. major* (lane 2), *L. infantum* (lane 3). Integration of pLEXSY-GFP expression cassette into the *ssu* locus of *L. tarentolae* (lane 5), *L. major* (lane 7) and *L. infantum* (lane 9) appeared as ∼1 kbp fragment not obtained in genomic DNA from the wild type strains, respectively (Lane 4, 6, 8). DNA molecular weight marker (1 kbp, Fermentase) was shown in lane MW.

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).

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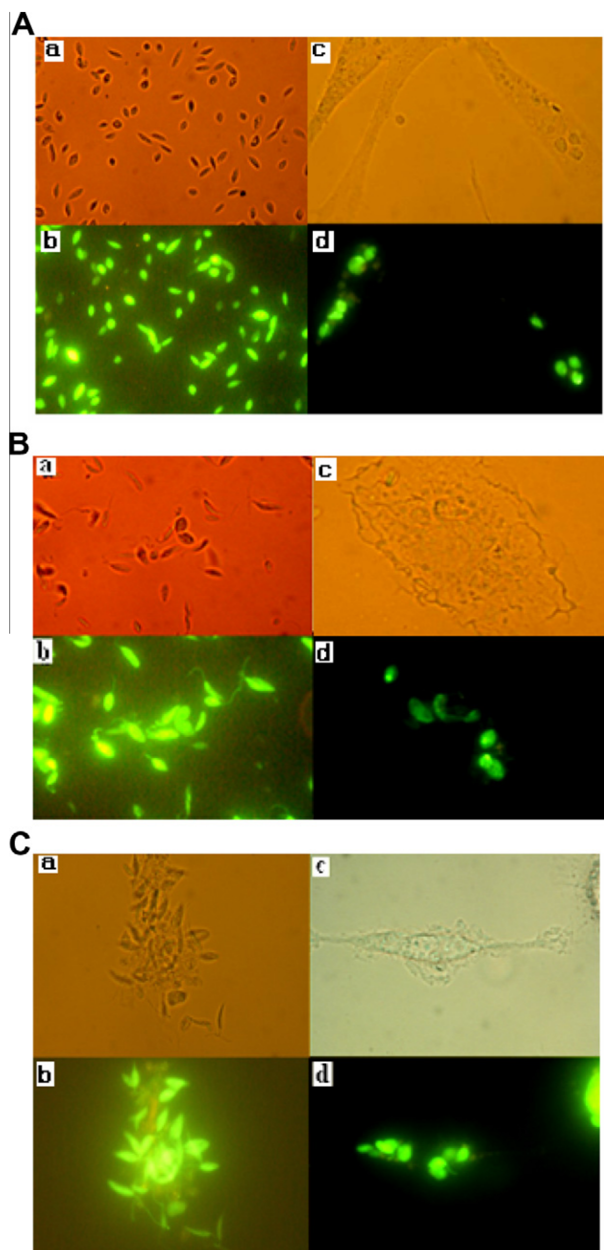

Fig. 2. EGFP detection by Epi-fluorescent microscopy; Fluorescent microscopic images show expression of EGFP in transfected *L. tarentolae* (A), *L. major* (B), and *L. infantum* (C) promastigotes (before and after glinting of fluorescence: a & b; left) and intracellular amastigotes expressing EGFP (before 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.

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 heterogenous 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 mouse bone marrow-derived 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. Clealy, 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-

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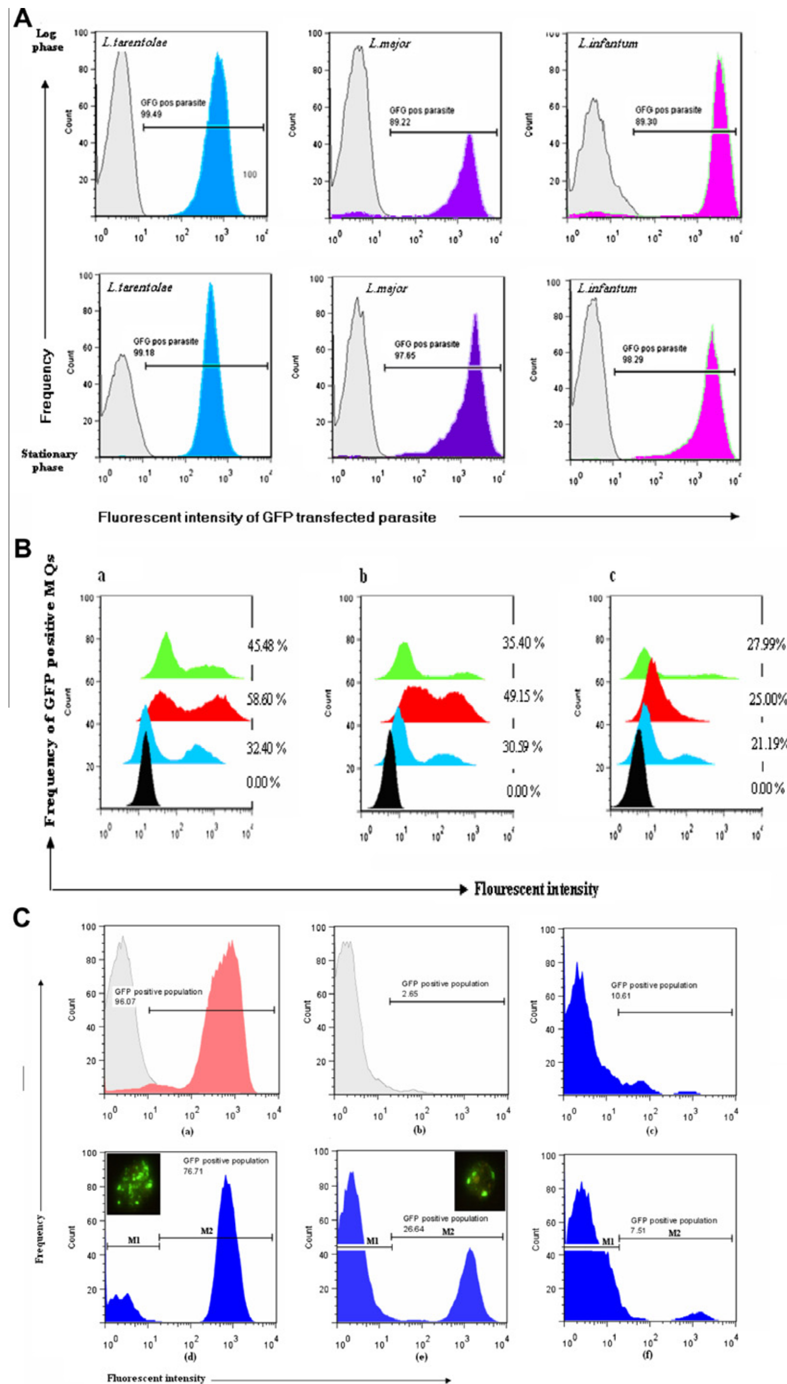

Fig. 3. (A) Fluorescence-activated cell sorting analysis of EGFP transfected parasites at two different phases; first row shows logarithmic phase and second row shows the stationary phase of *Leishmania* strains. Gray histograms indicate untransfected parasites and colorful histograms show transfected 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.).

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 observa-

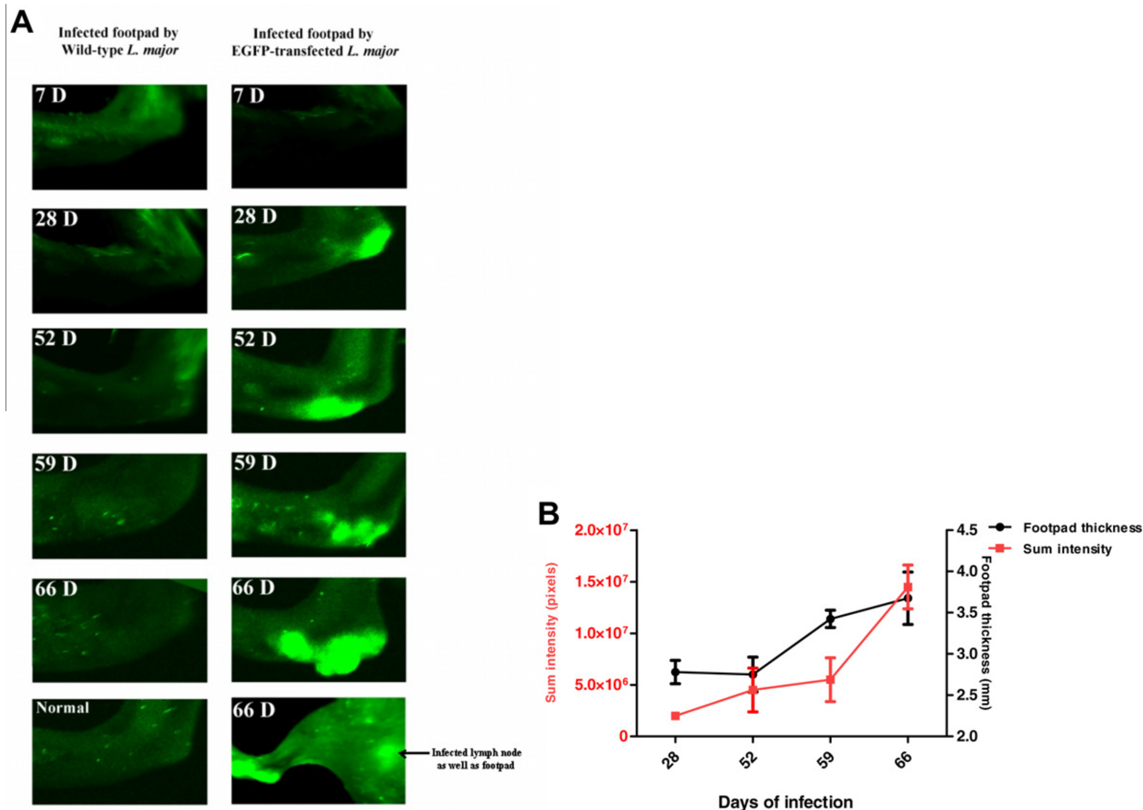

Fig. 4. (A) Imaging of BALB/c mouse footpad at different time intervals after inoculation with 2×10^8 EGFP-transfected *L. major* promastigotes. The right images demonstrate the infected footpad with EGFP transfected *L. major* at 7, 28, 52, 59 and 66 days post infection, while the left images show mouse footpad infected with wild type *L. major*. In addition, the infected lymph node with EGFP transfected *L. major* at day 66 is shown by arrow on right side. Four mice were used in this experiment and one mouse was represented in all photographs as a sample. (B) Graph of the mean footpad thickness measurement in mm (right axis) with standard error over the course of the *Leishmania* infection (solid circles). On the same graph, the mean of the sum green intensity (pixel) (solid rectangles) with standard error from images taken at different time points is plotted as well (left axis). GFP fluorescence, indicating infection, was visualized at different periods starting from day 28 to 66.

tion 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 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 leishmaniasis 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 transfectants 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

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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

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2010.12.006.

References

Basak, A., Shervani, N.J., Mbikay, M., Kolajova, M., 2008. Recombinant proprotein convertase 4 (PC4) from *Leishmania tarentolae* expression system: purification, biochemical study and inhibitor design. Protein Expression and Purification 60, 117–126.

Basile, G., Peticca, M., 2009. Recombinant protein expression in *Leishmania* tarentolae. Molecular Biotechnology 43, 273–278.

Boucher, N., McNicoll, F., Dumas, C., Papadopoulou, B., 2002. RNA polymerase I-mediated transcription of a reporter gene integrated into different loci of *Leishmania*. Molecular & Biochemical Parasitology 119, 153–158.

Breitling, R., Klingner, S., Callewaert, N., Pietrucha, R., Geyer, A., Ehrlich, G., Hartung, R., Muller, A., Contreras, R., Beverley, S.M., Alexandrov, K., 2002. Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. Protein Expression and Purification 25, 209–218.

Breton, M., Zhao, C., Ouellette, M., Tremblay, M.J., Papadopoulou, B., 2007. A recombinant non-pathogenic *Leishmania* vaccine expressing human immunodeficiency virus 1 (HIV-1) Gag elicits cell-mediated immunity in mice and decreases HIV-1 replication in human tonsillar tissue following exposure to HIV-1 infection. Journal of General Virology 88, 217–225.

Chan, M.M.Y., Bulinski, J.C., Chang, K.P., Fong, D., 2003. A microplate assay for *Leishmania amazonensis* promastigotes expressing multimeric green fluorescent protein. Parasitology Research 89 (4), 266–271.

Croan, D.G., Morrison, D.A., Ellis, J.T., 1997. Evolution of the genus *Leishmania* revealed by comparison of DNA and RNA polymerase gene sequences. Molecular & Biochemical Parasitology 89, 149–159.

D'Aiuto, L., Robison, C.S., Gigante, M., Nwanegbo, E., Shaffer, B., Sukhwani, M., Castro, C.A., Chaillet, J.R., 2008. Human IL-12p40 as a reporter gene for high throughput screening of engineered mouse embryonic stem cells. BMC Biotechnology 80, 52–66.

Daneshvar, H., Coombs, G.H., Hagan, P., Phillips, S., 2003. *Leishmania mexicana* and *Leishmania major*: attenuation of wild-type parasites and vaccination with the attenuated lines. The Journal of Infectious Diseases 187, 1662–1668.

Dube, A., Gupta, R., Singh, N., 2009. Reporter genes facilitating discovery of drugs targeting protozoan parasites. Trends in Parasitology 25 (9), 432–439.

Dujardin, J.C., 2009. Structure, dynamics and function of *Leishmania* genome: resolving the puzzle of infection, genetics and evolution? Infection, Genetics and evolution 9, 290–297.

Field, K.G., Landfear, S.M., Giovannoni, S.J., 1991. 18S rRNA sequences of *Leishmania enriettii* promastigote and amastigote. International Journal for Parasitology 21 (4), 483–485.

Fritsche, C., Sitz, M., Weiland, N., Breitling, R., Pohl, H.D., 2007. Characterization of the growth behavior of *Leishmania tarentolae* – a new expression system for recombinant proteins. Journal of Basic Microbiology 47, 384–393.

Garg, R., Dube, A., 2006. Animal models for vaccine studies for visceral leishmaniasis. Indian Journal of Medical Research 123, 439–454.

Kamau, S.W., Grimm, F., Hehl, A.B., 2001. Expression of green fluorescent protein as a marker for effects of anti-leishmanial compounds *in vitro*. Antimicrobial Agents and Chemotherapy 45 (12), 3654–3656.

Kram, D., Thale, C., Kolodziej, H., Kiderlen, A.F., 2008. Intracellular parasite kill: flow cytometry and NO detection for rapid discrimination between anti-leishmanial activity and macrophage activation. Journal of Immunological Methods 333, 79–88.

Lang, T., Goyard, S., Lebastard, M., Milon, G., 2005. Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice. Cellular Microbiology 7 (3), 383–392.

Lang, T., Lecoeur, H., Prina, E., 2009. Imaging *Leishmania* development in their host cells. Trends in Parasitology 25 (10), 464–473.

Mehta, S.R., Huang, R., Yang, M., Zhang, X.Q., Kolli, B., Chang, K.P., Hoffman, R.M., Goto, Y., Badaro, R., Schooley, R.T., 2008. Real-time *in vivo* green fluorescent protein imaging of a murine leishmaniasis model as a new tool for *Leishmania* vaccine and drug discovery. Clinical and Vaccine Immunology 15 (12), 1764–1770.

Miblitz, A., Mottram, J.C., Overath, P., Aebischer, T., 2000. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes. Molecular & Biochemical Parasitology 107, 251–261.

Mizbani, A., Taheri, T., Zahedifard, F., Taslimi, Y., Azizi, H., Azadmanesh, K., Papadopoulou, B., Rafati, S., 2009. Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. Vaccine 28 (1), 53–62.

Monte-Alegre, A., Quaissi, A., Sereno, D., 2006. *Leishmania* amastigotes as targets for drug screening. Kinetoplastid Biology and Disease 1, 3.

Mureev, S., Kushnir, S., Kolesnikov, A.A., Breitling, R., Alexandrov, K., 2007. Construction and analysis of *Leishmania tarentolae* transgenic strains free of selection markers. Molecular & Biochemical Parasitology 155, 71–83.

Papadopoulou, B., Dumas, C., 1997. Parameters controlling the rate of gene targeting frequency in the protozoan parasite *Leishmania*. Nucleic Acids Research 25 (21), 4278–4286.

Papadopoulou, B., Huang, X.F., Boucher, N., McNicoll, F., 2003. Stage-specific regulation of gene expression in *Leishmania*. ASM News 69 (6), 282–288.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M.P., Germain, R.N., Sacks, D., 2008. *In vivo* imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321 (5891), 970–974.

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. Molecular and Biochemical Parasitology 110, 195–206.

Singh, N., Dube, A., 2004. Fluorescent *Leishmania*: application to anti-leishmanial drug testing. The American journal of tropical medicine and hygiene 71 (4), 400–402.

Singh, N., Gupta, R., Jaiswal, A.K., Sundar, S., Dube, A., 2009. Transgenic *Leishmania donovani* clinical isolates expressing green fluorescent protein constitutively for rapid and reliable *ex vivo* drug screening. Journal of Antimicrobial Chemotherapy 64, 370–374.

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