Development and validation of four *Leishmania* species constitutively expressing GFP protein. A model for drug discovery and disease pathogenesis studies

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(*Received 22 April 2013; revised 14 June and 8 July 2013; accepted 15 July 2013; first published online 20 November 2013*)

SUMMARY

Green fluorescent protein (GFP)-parasite transfectants have been widely used as a tool for studying disease pathogenesis in several protozoan models and their application in drug screening assays has increased rapidly. In the past decade, the expression of GFP has been established in several *Leishmania* species, mostly for *in vitro* studies. The current work reports generation of four transgenic parasites constitutively expressing GFP (*Leishmania mexicana, Leishmania aethiopica, Leishmania tropica* and *Leishmania major*) and their validation as a representative model of infection. This is the first report where stable expression of GFP has been achieved in *L. aethiopica* and *L. tropica*. Integration of GFP was accomplished through homologous recombination of the expression construct, pRib1.2*α*NEO*α*GFP downstream of the 18S rRNA promoter in all species. A homogeneous and high level expression of GFP was detected in both the promastigote and the intracellular amastigote stages. All transgenic species showed the same growth pattern, ability to infect mammalian host cells and sensitivity to reference drugs as their wild type counterparts. All four transgenic *Leishmania* are confirmed as models for *in vitro* and possibly *in vivo* infections and represent an ideal tool for medium throughput testing of compound libraries.

Key words: GFP, THP-1, *Leishmania aethiopica*, *Leishmania major*, *Leishmania mexicana*, *Leishmania tropica*, flow cytometry, intracellular amastigote.

INTRODUCTION

*Leishmania*sis is a vector-borne disease caused by protozoan haemoflagellate parasites of the genus *Leishmania*. Over 20 species of *Leishmania* both from the New and Old World are able to cause one or more types of leishmaniasis in humans. An estimated 350 million people in over 88 countries are at risk of infection, with an annual incidence of 500 000 cases for the lethal visceral form and 1·5 million for the cutaneous forms (WHO, 2010). No effective vaccines are available to date and current chemotherapy for leishmaniasis is reliant predominantly on pentavalent antimonials and miltefosine as first-line drugs, with amphotericin B and pentamidine following as second-line alternatives (Zucca and Savoia, 2011; Ejazi and Ali, 2013).

Although *Leishmania* species are morphologically indistinguishable and show little genetic and expression profile variability, species-specific parasite factors are known to cause differences in both virulence and pathogenicity in the host (Rogers *et al*. 2011). Differences associated with each species and strains are also a recognized cause of variable susceptibility to drugs and vaccines (Croft *et al*. 2002, 2006; Melby, 2002). This implies that investigations of the processes behind *Leishmania* infection as well as identification of novel treatments and/or vaccines must be based on models that include a variety of species. Such studies are slowed down by the necessity of constantly monitoring infection mostly via time-consuming and laborious procedures based on Giemsa staining and microscopic counting (Nwaka and Hudson, 2006).

In the present study, we established a fast and sensitive method to quantify infection of four *Leishmania* species in terminally differentiated THP-1 cells. This method is based on the development and validation of stable expression of GFP in Old and New World *Leishmania* species (*Leishmania mexicana, Leishmania aethiopica, Leishmania tropica* and *Leishmania major*) through homologous recombination in the 18 s rRNA locus. All four transgenic species were able to express stable level of GFP without any antibiotic pressure. Expression of GFP did not significantly impair viability or infection ratio *in vitro*. They were successfully used to set up a flow cytometry-based semi-automated *in vitro* assay for quantification of infected cells. Transgenic GFP parasites were generated for the first time in *L. tropica* and *L. aethiopica* and proved to be excellent models for further studies. Both species are able to cause a range of diseases (LCL, DCL and MCL by *L. aethiopica* and VL and CL by *L. tropica*) and the generation of such infection models will greatly

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doi:10.1017/S0031182013001777

contribute to further understanding of disease development as well as to the drug discovery process.

MATERIALS AND METHODS

Culturing of Leishmania *species and mammalian THP-1 cells*

Four *Leishmania* species, *L. mexicana* (MNYC/BZ/62/M379), *L. aethiopica* (MHOM/ET/72/L100), *L. tropica* (MHOM/SU/58/OD) and *L. major* (MHOM/SU/73/5ASKH) were maintained as promastigotes at 24 °C in Schneider (Gibco-Invitrogen) medium supplemented with 23% (v/v) heat-inactivated bovine serum and 1× penicillin-streptomycin-glutamine (Gibco-Invitrogen). For selection and transfection procedures, parasite species were maintained in the same medium above but supplemented with 700 μg mL^{-1} G418 (Sigma).

Human acute monocytic leukaemia (THP-1) cell line (ECACC, catalogue code: 88081201) was routinely cultured at a density of $2{\cdot}5\times10^5$ cells mL^{-1} in complete RPMI medium supplemented with 10% (v/v) heat-inactivated bovine serum and 1× penicillin-streptomycin-glutamine at 37 °C in a humidified CO_2 (5%) incubator.

GFP construct

The reporter gene, GFP, obtained as a vector construct, pRib1.2*α*Neo*α*GFP (a generous gift from Dr Papadopoulou, Infectious Diseases Research Center, CHUL Research Center, Laval University, Quebec, Canada), for genomic integration of GFP downstream of promoter of the 18S rRNA in all four species described above was used.

To facilitate genomic integration, the construct was linearized with restriction enzyme BstXI (Fermentas), phenol-chloroform isoamyl alcohol treated, precipitated in ethanol with 0·3 M sodium acetate and pellet resuspended in nuclease-free deionized distilled water.

Transfection of Leishmania *species with pRib1.2αNeoαGFP vector construct*

Transfections of *L. mexicana*, *L. aethiopica*, *L. tropica* and *L. major* were carried out as previously described (Robinson and Beverley, 2003). Clonal selection was carried out by limiting dilution procedures in 96-well plates isolating high GFP-expressing parasites, followed by expansion of desired clones in 24-well plates and subsequently in T-25 flasks.

PCR analysis of genomic integration of pRib1.2αNeoαGFP construct in 18S ribosomal RNA locus

Genomic DNA was isolated from promastigotes of all four species expressing GFP using the DNAeasy® Blood and Tissue kit (Qiagen), according to manufacturer's protocol. Primers were designed for full length GFP amplification (5′-GFP 829 – GTGAGCAAGGGC GAGGAGCT and 3′-GFP 1543 – ACTTGTACAGCTCGTCCATGC) and to assess site-specific homologous recombination of the reporter gene (5′−18SRibo. – GCATTGCTTCGCGTGTGAGT and 3′-sequence primer, as above) at the 18S ribosomal locus of all four species. Amplification was achieved using the REDtaq® ReadyMix™ PCR reaction mix (Sigma) under the following conditions: 94 °C 2 min, 30 cycles of 94 °C 30 s, 64 °C 30 s and 72 °C 2 min followed by an extended 72 °C 10 min elongation cycle. Amplification conditions to assess site-specific genomic integration of GFP at 18S ribosomal locus were 94 °C 2 min, 30 cycles of 94 °C 30 s, 50 °C 30 s and 72 °C 2 min followed by an extended 72 °C 10 min elongation cycle.

Western blot analysis of GFP expression

Approximately 4×10^6 parasites mL^{-1} were harvested from log phase promastigotes at 1000 ***g*** for 10 min. Parasite pellets for each of the four species were washed twice in ice-cold PBS and resuspended in cells lysis buffer (930 mg EDTA, 4350 mg NaCl, 3028·5 mg Tris, 2·5 mL IGEPAL® CA-630 and 497·5 mL H_2O). Parasites were snap-frozen in liquid nitrogen and immediately thawed at 80 °C for 3 cycles to lyse the cells. Lysates were centrifuged at a maximum speed (12 500 ***g***) for 10 min to remove cell debris. Protein concentration was determined using the Bradford reagent (Sigma) according to manufacturer's instructions.

Approximately 40 μg of total proteins were separated on precast SDS-Tris-Glycine (Pierce) gels and transferred to HYBOND (Amersham) membranes by electroblotting. Membranes were blocked overnight at 4 °C in blocking solution (PBS, 0·01% (w/v) Tween and 5% (v/v) milk). Subsequently, membranes were incubated with rabbit anti-GFP immunoglobulin (1 : 1000 dilution) at 37 °C for 1 h followed by three 5-min washes each in PBS with 0·01% (w/v) Tween. A secondary ECL™ peroxidase labelled anti-rabbit immunoglobulin was applied to the membranes incubated under the same conditions as the primary antibody and washed as above to remove unbound excess antibody. Detection was performed using The ECL™ Plus Western Blotting Detection Reagents (GE Healthcare).

Fluorescent microscopy of transgenic Leishmania *species*

A total of 5 μL of log phase transgenic promastigotes or infected THP-1 cells were independently spotted onto glass microscopy slides. Log phase

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promastigotes were previously fixed with a 0·02% (v/v) formaldehyde solution. Slides were allowed to stand still for 2 min before microscopy analysis. Images were acquired using the Nikon ECLIPSE 90*i* overhead epifluorescent microscope attached to a Nikon digital camera (DS-Qi1Nc) and a computer running Nikon NIS-Elements Advanced Research software. The principal objective used for fluorescent imaging was an oil immersion CFI Plan Apochromat VC 60X N2 (NA1·4, WD 0·13 mm).

Infection of THP-1 cells with transgenic GFP-expressing Leishmania *parasites*

Stationary phase metacyclic promastigotes were isolated via peanut agglutination from transgenic GFP-expressing parasites of all four species as previously reported (Ready and Smith, 1988) and used to infect retinoic acid-treated THP-1 cells at a ratio of 10 : 1.

Percentage of infection was monitored over a period of 96 h by microscopic counting of infected THP-1 cells through visualization of GFP fluorescence combined with flow cytometry analysis.

Antileishmanial activity assay

Promastigote stages: Stationary phase promastigotes of wild-type and GFP-expressing species were seeded in 96-well plates at a final concentration of 1×10^6 parasites mL^{-1} in triplicate, and treated with appropriate concentrations of amphotericin B (0·0169–1·08 μM) and miltefosine (6·25–400 μM). Plates were incubated for 24 h at 24 °C and inhibition of promastigote growth was determined by MTS assay as described previously (Getti *et al*. 2009).

Intracellular amastigote stages: Infection of retinoic acid-treated THP-1 cells was prepared as described above. Following 24 h infections with all four transgenic GFP-expressing *Leishmania* species, THP-1 cultures of infected and uninfected controls were centrifuged at 100 ***g*** for 5 min and washed twice in pre warmed complete RPMI medium to remove extracellular promastigotes. The remaining cells were then transferred to a 96-well plate at a final concentration of $2{\cdot}5 \times 10^5$ cells mL^{-1} and treated with amphotericin B (0·039–5 μM) and miltefosine (0·0312–80 μM). Plates were incubated at 37 °C in a CO_2, humidified incubator until analysis. Drug activity was detected at 24 and 72 h after treatment by assessing the decrease in the percentage of THP-1 cells infected with fluorescent parasites via flow cytometry (C6 Accuri, BD Bioscience).

Flow cytometry analysis of GFP-expressing promastigotes and intracellular amastigotes

GFP-expressing promastigotes from all four transgenic *Leishmania* species were analysed on the Accuri C6 flow cytometer for GFP expression using a solid state blue laser with a 488 nm excitation spectrum and a detector of FL1 path with filter 530/30 nm. A minimum of 10 000 gated events was acquired from the promastigote population and data analysed using the Accuri C6 software.

The same parameters as above were used to analyse THP-1 cells infected with GFP-expressing *Leishmania* species, independently over a time course of 96 h (at 4, 24, 48, 72 and 96 h time points). Gates were set based on size and granularity of infected THP-1 cells. A minimum of 10 000 gated events was acquired for the total population of viable THP-1 cells.

To determine inhibition of antileishmanial drugs on the amastigote stage of GFP-expressing *Leishmania* species in retinoic acid-treated THP-1 cells as a measure of decrease in fluorescent intensity, the same parameters as above were used. A minimum of 10 000 gated events was acquired for the total population of viable THP-1 cells with a cell density of 1×10^6 cells mL^{-1}.

Data and statistical analyses

All numerical data was analysed using GraphPad Prism 4. IC_{50} values were extrapolated from dose–response curves using the sigmoidal–dose response (variable) model.

To establish statistical significance, the unpaired student *t*-test validated by the Mann–Whitney *U* test was applied using GraphPad Prism 4.

RESULTS

GFP expression in Leishmania *species*

Successful transfection and expression of GFP in *L. mexicana* and *L. major* has been demonstrated previously (Bolhassani *et al*. 2011) using various expression cassettes and has been reproduced in this study as controls for subsequent experiments. Fluorescence of GFP was observed throughout the cytoplasm and flagella in viable extracellular promastigotes (Fig. 1B and C) and throughout the cytosol in intracellular amastigotes (Fig. 1E and F). Clonal selection procedure enabled isolation of a homogeneous population of several clones expressing GFP for each of the species. In this study, four represented clones are shown for each species except *L. major* for which a single clone was isolated. Flow cytometry and fluorescent microscopy analyses demonstrated a distinct and high level of GFP fluorescence both for the promastigotes and the intracellular amastigotes (*L. aethiopica* and *L. tropica* shown in Fig. 2).

The presence of GFP in all four species (*L. mexicana, L. major, L. aethiopica* and *L. tropica*) was further validated by Western blot analysis. Green fluorescent protein was detectable in whole cell

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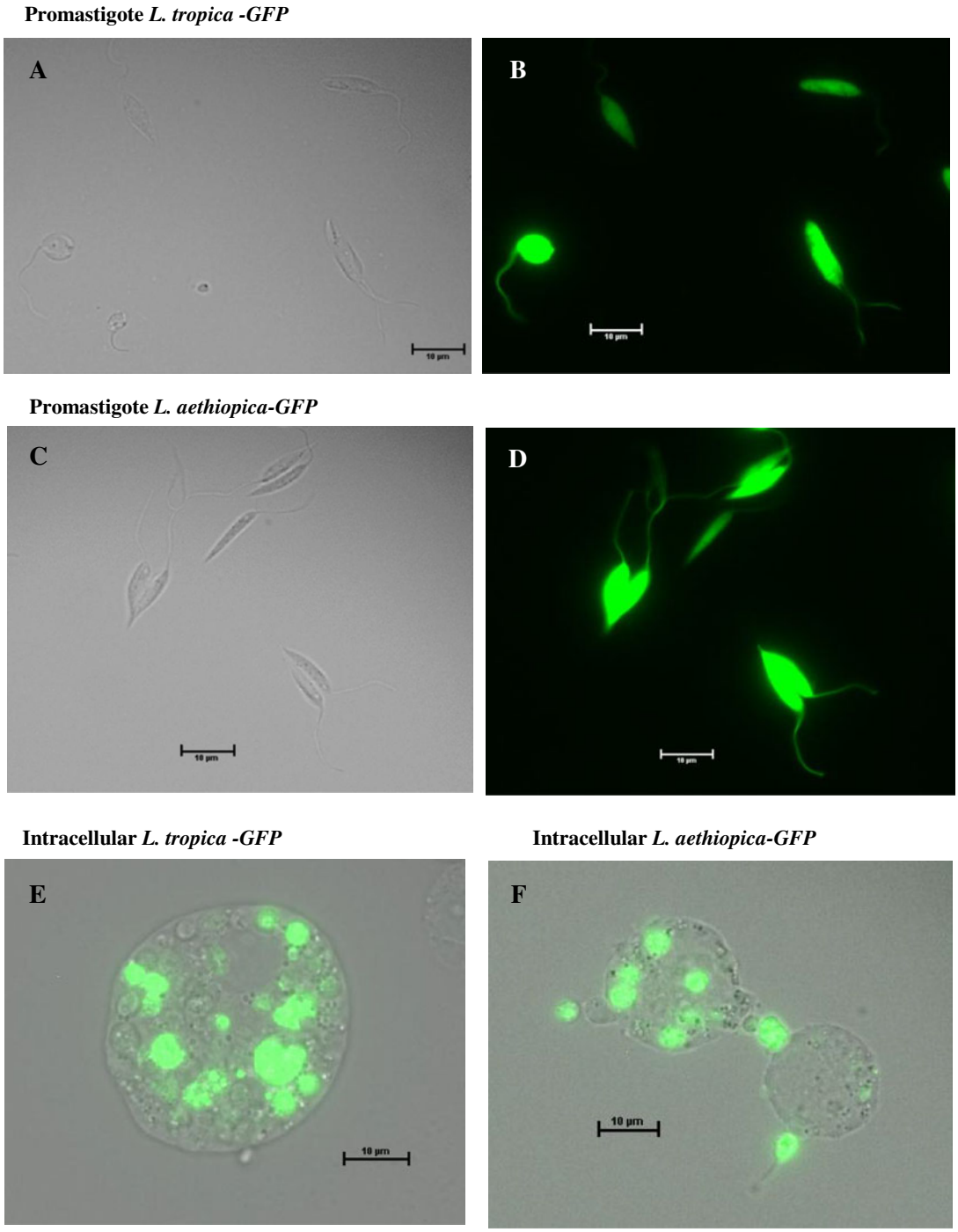

Fig. 1. Detection of GFP expression in transgenic parasites by epi-fluorescence microscopy; fluorescent microscopic images show expression of GFP in transfected *L. tropica* (before (A) and after (B) glinting of fluorescence) and in *L. aethiopica* (before (C) and after (D) glinting of fluorescence) during logarithmic phase of growth. Bright field images (A and C) for each species are shown to represent same field view. Bright field images of infected THP-1 cells were overlapped with corresponding FITC images; intracellular parasites were clearly visible 48 h following infection with (E) GFP-expressing *L. tropica-GFP* and (F) *L. aethiopica-GFP*. All images were captured under a 60× oil immersion lens.

lysates of transgenic parasites using polyclonal rabbit antiserum. An expected dominant band of 27 kDa protein was visible in all transgenic clones while no protein of this molecular weight was detected in the negative controls, i.e. whole cell lysates prepared from wild-type promastigotes of all four species (Fig. 3).

Chromosomal integration of GFP in the 18S rRNA locus

Integration of the complete GFP gene was confirmed by PCR amplification in the genome of each of the transfected species mediated via site-specific integration (Fig. 4). Transfection of *L. mexicana*,

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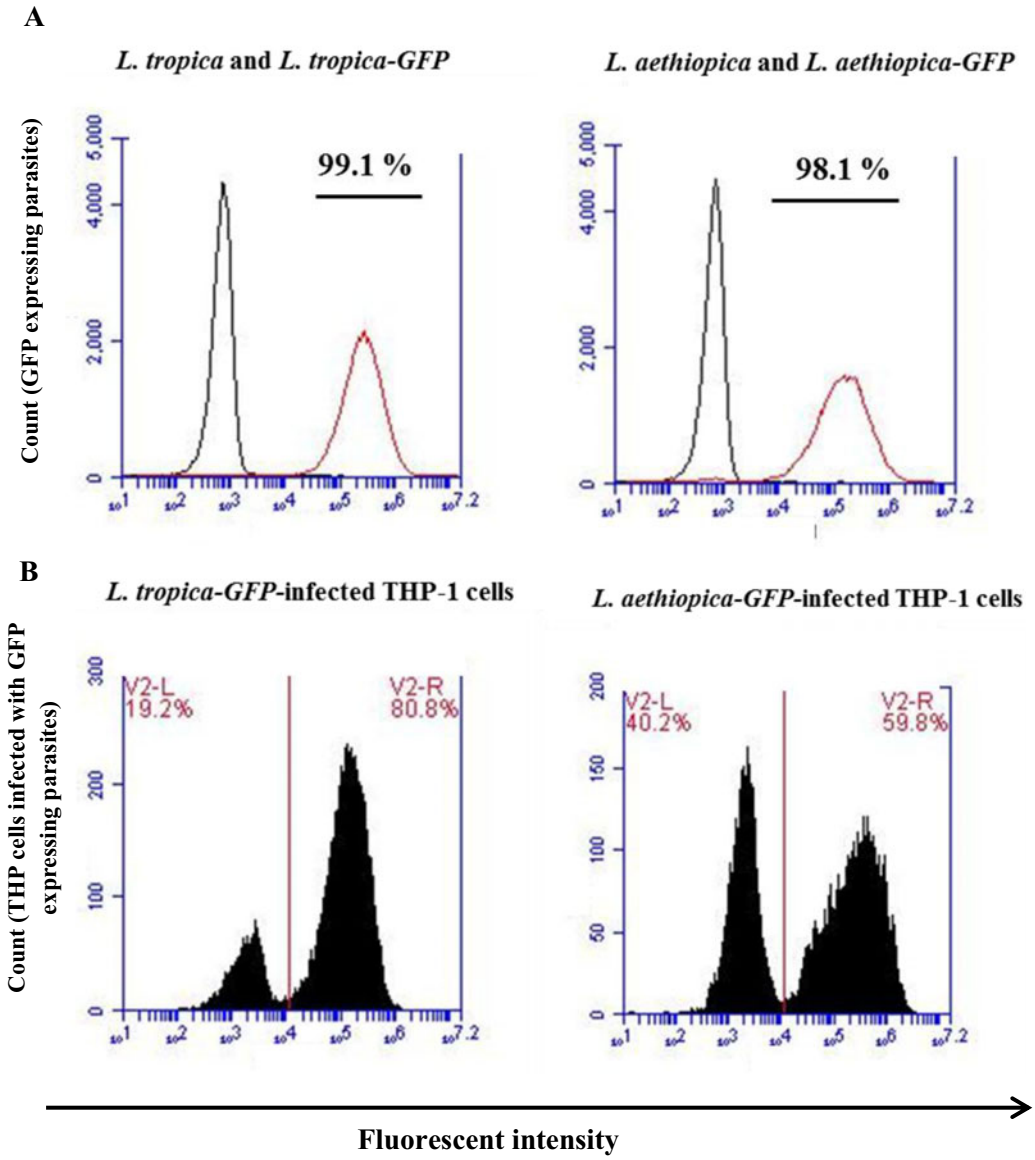

Fig. 2. Flow cytometry analysis of GFP expressing parasites. (A) Extracellular promastigotes of *L. tropica-GFP* and *L. aethiopica -GFP* at logarithmic phase of growth. The figure shows two samples overlapping. Black histogram represents non-transfected parasites and red histogram show homogeneous population of transfected parasites. Over 98% of transfected parasites show high expression of GFP; (B) THP-1 cells following 24 h infection with *L. tropica-GFP* and *L. aethiopica -GFP*. The first peak represents THP-1 cells not affected by presence of GFP expressing parasites, therefore considered uninfected; the second peak represents THP-1 cells showing high degree of fluorescence therefore considered infected with GFP-expressing parasites, the two populations are clearly distinguishable.

L. major, L. aethiopica and *L. tropica* with linearized pRib1.2αNEOαGFP enabled chromosomal integration of the complete GFP gene at the 18S rRNA locus downstream of the DNA Pol I-driven promoter.

Growth and infectivity of GFP-expressing Leishmania *species*

Promastigotes of transgenic GFP-expressing species were monitored for growth to determine infective metacyclic stationary phase. The growth cycle of transgenic species are comparable to each other in that stationary phase is reached at day 5 for all species (Fig. 5A and supplementary Fig. 3A – supplementary material available online only).

The metacyclic form of all four stable GFP-expressing species, isolated from late stationary phase promastigotes were able to infect terminally differentiated THP-1 cells. Infection was monitored at 24, 48, 72 and 96 h through both fluorescent microscopy and flow cytometry (Fig. 5B and supplementary Fig. 5B).

As previously reported (Singh *et al*. 2009), flow cytometry analysis enabled distinct separation of uninfected macrophage from an infected macrophage population based on differences in fluorescence

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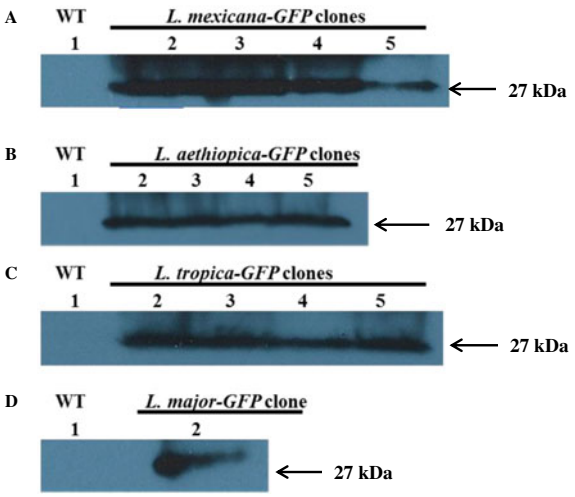

Fig. 3. Western blot analysis of GFP expression in represented clones of (A) *L. mexicana-GFP* (clones 2, 3, 4 and 5); (B)*L. aethiopica-GFP* (clones 2, 3, 4 and 5); (C) *L. tropica-GFP* (clones 2, 3, 4 and 5); and (D) *L. major-GFP* (clone 2). Arrows on the right-hand side indicates the molecular weight of GFP (27 kDa).

levels between the two populations (Fig. 2B and supplementary Fig. 2B – supplementary material available online only). The percentage of infection in THP-1 cells calculated by either method followed a similar trend; percentage of infected cells peaked between 24 and 48 h and a significant decrease in infection ratio was detected after 48 h during infection with all four species (Fig. 5B and supplementary Fig. 5B). These results suggested that 24 h following infection is the best time point for assessing antileishmanial activity of reference drugs in this model.

GFP-expressing Leishmania *species are susceptible to standard antileishmanial drugs*

The evaluation of standard antileishmanial drugs, amphotericin B and miltefosine was carried out on both promastigote and intracellular amastigote stages of all four *Leishmania* species.

Half-maximal inhibitory constants were obtained for both amphotericin B and miltefosine following MTS assay on promastigotes. The IC_{50} values of transgenic GFP-expressing *Leishmania* spp. are comparable to those obtained for wild-type promastigotes (Table 1), suggesting introduction of GFP in all four *Leishmania* spp. retained their sensitivity to the standard antileishmanial drugs at the promastigote stage level. There were no significant differences in IC_{50} values between wild-type and transgenic parasites for all species ($P > 0{\cdot}1$). Specifically, wild-type *L. aethiopica* IC_{50} following treatment with miltefosine appears to be 1·24-fold different to the GFP-expressing strain, nevertheless this difference was not statistically significant ($P = 0{\cdot}2$). Likewise, IC_{50} values obtained for wild-type *L. major* when tested with amphotericin B or miltefosine demonstrated a 1·62- and 2·18-fold difference in sensitivity, respectively, compared with GFP-expressing strain. However, these differences were not statistically significant ($P = 0{\cdot}2$ and $P = 0{\cdot}1$ for amphotericin B and miltefosine-treated parasites, respectively).

Intracellular GFP-expressing amastigote forms of *L. mexicana, L. major, L. aethiopica* and *L. tropica* resident in THP-1 macrophages were susceptible to inhibition by amphotericin B and miltefosine over a 24 and 72 h treatment period following 48 and 96 h post-infection, respectively. IC_{50} data are summarized in Table 1. Susceptibility was comparable with published data (Escobar *et al*. 2002; Utaile *et al*. 2013), confirming that introduction of GFP in all four species did not alter sensitivity to drugs during the intracellular stage of the parasite's life cycle.

DISCUSSION

Development of genetically modified *Leishmania species* transfected with the reporter gene GFP has proved a powerful tool in *Leishmania* research.

Stable and constitutive expression of GFP in *Leishmania* species has been shown by targeted integration of exogenous genes in the transcriptionally active 18S ribosomal locus through homologous recombination (Mißlitz *et al*. 2000). The presence of several copies of the 18S rRNA gene tandemly arrayed in the *Leishmania* parasite genome is advantageous in that introduction of transgenes into the chromosome has no detrimental effects on parasite survival (Mißlitz *et al*. 2000). This strategy enabled uniform and high-level constitutive expression with no need for selective pressure and can offer a possibility for *in vivo* work, ideal for testing in intracellular amastigote forms. Therefore the 18S rRNA locus was chosen as the targeted integration site for transgene expression of GFP in this report. Homologous recombination and recombination rate in the *Leishmania* genome is dependent on several parameters such as length of the homologous region, level of sequence homology between donor and acceptor regions and copy number of the target genes (Papadopoulou and Dumas, 1997; Pulido *et al*. 2012). Based on the above criteria, the current study used pRib1.2αNEOαGFP as the vector for transfection of four *Leishmania* species. This construct was successfully used for stable integration of GFP in the 18S rRNA locus of the *Leishmania donovani* genome and no demonstrable effect of GFP expression on parasite morphology and infectivity was reported (Singh *et al*. 2009). Similarly, the current study has shown no effects of the GFP transgene on morphology and infectivity in *L. mexicana, L. aethiopica, L. tropica* and *L. major*. Transgenic parasites exhibited bright green fluorescence throughout the cytoplasm (Fig. 1A and supplementary Fig. 1A – supplementary material available online only) and

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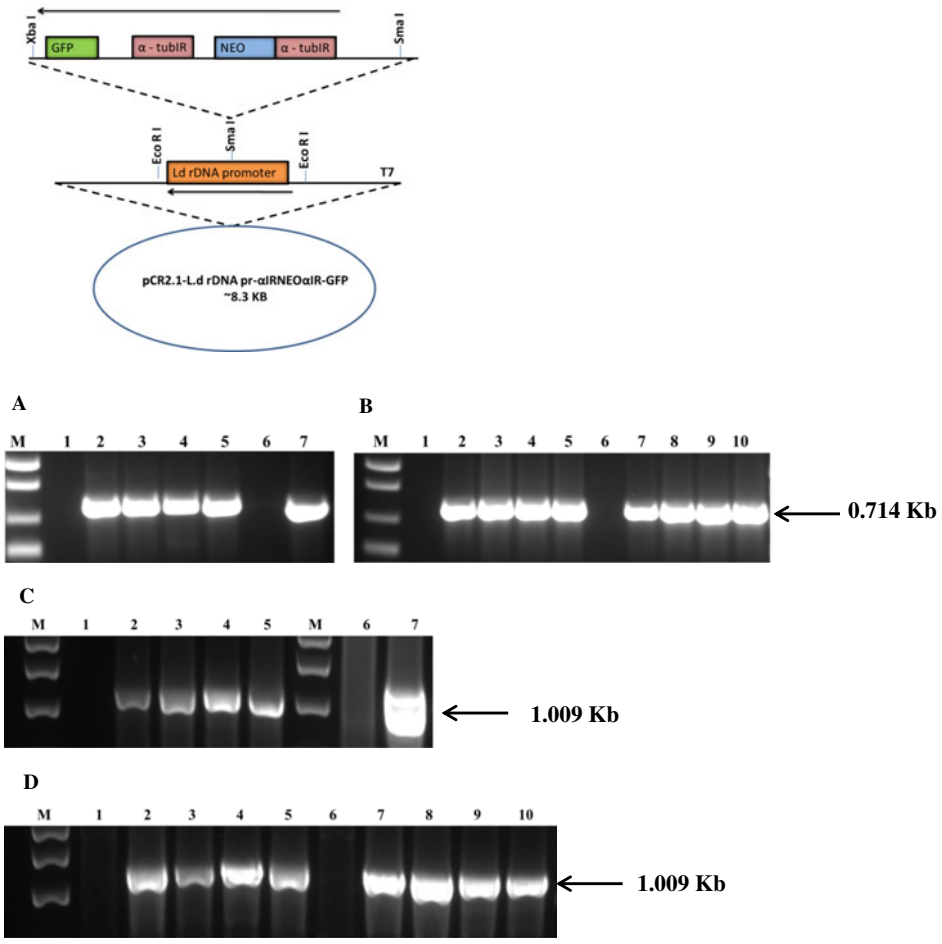

Fig. 4. *Top*: A schematic representation of GFP integration mediated by the pRib1.2αNeoαGFP vector construct at the 18S rRNA locus downstream of the DNA Pol I-driven promoter in the parasite genome. *Bottom*: PCR analysis to confirm integration of the full length *GFP* gene (panel A and B) present at the 18 s ribosomal locus (panel C and D) of *Leishmania species*. In panel A, lane M: 1 kB plus marker; lane 1: *L. mexicana*; lanes 2–5: *L. mexicana-GFP* clones; lane 6: *L. major* and lane 7: *L. major*-GFP clone. In panel B, lane M: 1 kB plus marker; lane 1: *L. aethiopica*; lanes 2–5: *L. aethiopica*-GFP clones; lane 6: *L. tropica*; and lanes 7–10: *L. tropica*-GFP clones. In panel C, lane M: 1 kB marker; lane 1: *L. mexicana*; lanes 2–5: *L. mexicana*-GFP clones; second lane M: 1 kB marker, lane 6: *L. major* and lane 7: *L. major*-GFP clone. In panel D, lane M: 1 kB marker; lane 1: *L. aethiopica*; lanes 2–5 *L. aethiopica*-GFP clones; lane 6: *L. tropica* and lanes 7–10: *L. tropica*-GFP clones.

were highly motile suggesting transgene expression did not affect motility. Furthermore, relative fluorescence measurements via flow cytometry data confirmed high level and homogeneous expression of GFP throughout parasite populations in all species (Fig. 2A and supplementary Fig. 2A) as well as a clear quantitative separation between transfected and wild type parasites with a percentage of GFP-expressing parasites ranging from 94·4% (*L. major*, supplementary Fig. 2A) to 99·1 (*L. tropica*, Fig. 2A). This mode of constitutive expression is under controlled regulation and not dependent on continuous drug selection pressure for high level of fluorescence expression (Pulido *et al*. 2012).

Suitability of *L. mexicana*, *L. major*, *L. aethiopica* and *L. tropica* GFP-expressing parasites as tools for drug screening and infection studies was confirmed via a number of tests. All transfectant clones expressed high levels of fluorescence and could be easily visualized intra- and extra-cellularly under the microscope (Fig. 1) and via flow cytometry (Fig. 2). All four parasite species were able to infect retinoic acid-treated THP-1 cells *in vitro* and infected cells could be detected up to 96 h post infection (Fig. 5B). These transgenic species proved ideal for setting up of flow cytometry-based assays in intracellular parasites, as the population of infected cells was clearly quantifiable over a period of 4 days. Data obtained from flow cytometry-based counting in at least four repeated experiments were extremely accurate as shown by the very small standard error (Fig. 5B). This observation is in line with published data that underline the high sensitivity and accuracy of the flow cytometry method compared with manual fluorescent microscopy counting (Pulido *et al*. 2012). Transgenic promastigotes were assessed for sensitivity to amphotericin B and miltefosine in a concentration-dependent manner and showed IC_{50}

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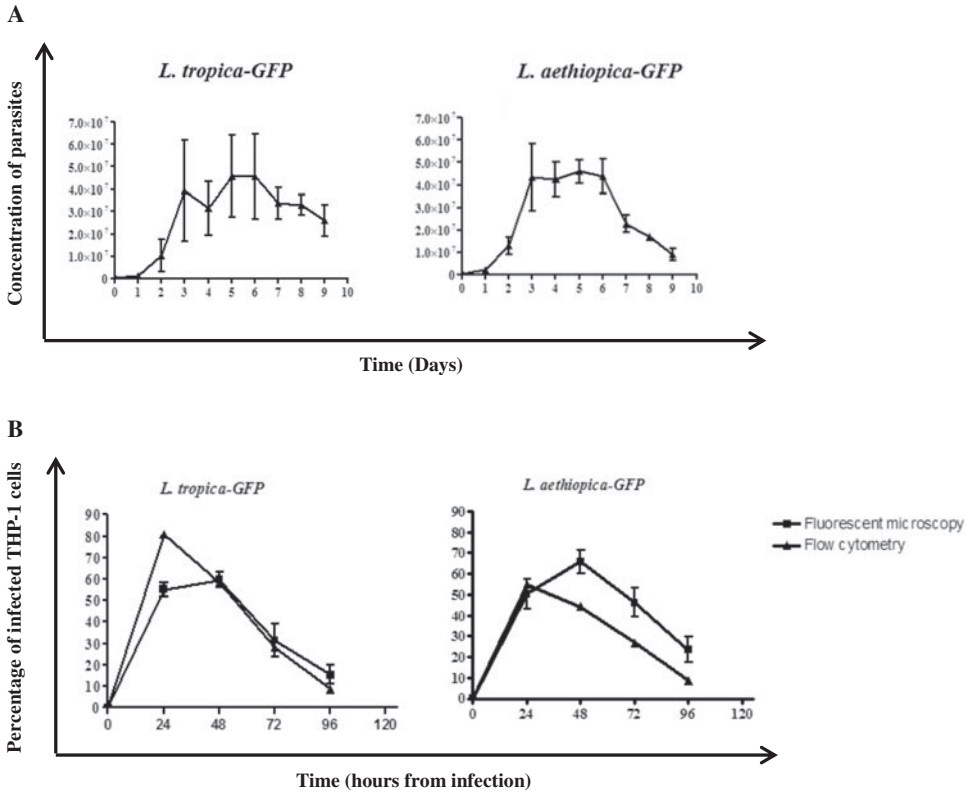

Fig. 5. Extracellular and intracellular growth of GFP-expressing parasites. (A) Growth curve of GFP-expressing *L. tropica* and *L. aethiopica* promastigotes carried over 8 days. Results are the mean (±S.E.M.) of three independent experiments. (B) Time course of infection in THP-1 cells of GFP-expressing *Leishmania* species over a period of 96 h, determined by fluorescence microscopy (results are the mean [±S.E.M.] of three independent experiments for GFP-expressing *L. tropica* and four independent experiments for GFP-expressing *L. aethiopica*) and flow cytometry (results are the mean [±S.E.M.] of three independent experiments; where not shown, error bars fall within the symbols).

values comparable to those of the wild types (Table 1). This suggests that introduction of the GFP gene in the *Leishmania* genome of all four species did not alter their sensitivity profiles to the reference drugs and supports the use of such parasites for drug screening. The IC_{50}s obtained via the semi-automatized medium throughput flow cytometry assay of THP-1 infected cells (Table 1) were also consistent with those obtained from previous studies.

Several molecular approaches have been used to express GFP in a variety of *Leishmania* strains. Antibiotic-induced expression of GFP was achieved in *L. major, Leishmania amazonensis, L. donovani* and *Leishmania infantum* by the transfection approach involving expression of GFP as an episomal transgene (Sean Ha *et al*. 1996; Kamau *et al*. 2001; Chan *et al*. 2003; Okuno *et al*. 2003; Singh and Dube, 2004). These fluorescent *Leishmania* species were amenable to *in vitro* drug testing assays in the promastigote stage. *Leishmania donovani* could also be used for testing drugs in the intracellular amastigote stage; however, due to the high background noise automated screening was impossible (Dube *et al*. 2009). Moreover episomal expression of GFP in *Leishmania* species is transient, resulting in a heterogeneous population of fluorescent promastigotes (Chan *et al*. 2003; Varela *et al*. 2009). Finally, continuous drug pressure is required for parasites to retain and express the GFP gene, as this cannot be maintained during infection; the parasite loses the gene during the intracellular stages of its life cycle both *in vitro* and *in vivo*.

Recent efforts have focused on the generation of stable recombinant *Leishmania* species constitutively expressing GFP through integration of the transgene in the *Leishmania* genome of various species. Integration of GFP has been stably carried out in various locations: in 18 s rRNA locus of *L. donovani, L. mexicana, L. panamensis, L. braziliensis, L. amazonensis* and *L. infantum* (Boucher *et al*. 2002; Dube *et al*. 2009; Singh *et al*. 2009; Pulido *et al*. 2012); in the *ssu* locus of *L. major, L. infantum* and *L. tarentolae* (Bolhassani *et al*. 2011). Based on the above studies, all of the *Leishmania* species displayed homogeneous and constitutive expression of GFP and fluorescence persisted for long periods of time. These parasite species were able to infect macrophages *in vitro* and were able to maintain fluorescence during the intracellular stages. Furthermore, parasites were infective *in vivo* and fluorescence could be detected a month after infection (Singh *et al*. 2009; Bolhassani *et al*. 2011). On the basis of such promising results our efforts have been focusing towards the development of *L. mexicana,*

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Table 1. IC_{50} values of antileishmanial reference drugs against extracellular promastigote stage of *L. mexicana-GFP, L. aethiopica-GFP, L. tropica-GFP* and *L. major-GFP* and the correspondent wild type. Unpaired *t*-test coupled with Mann–Whitney *U* test of the IC_{50} between each of the wild type parasites and their transgenic counterpart showed no significant difference ($P > 0{\cdot}1$). Lower part, IC_{50} values of antileishmanial reference drugs against intracellular amastigotes stage of *L. mexicana-GFP, L. aethiopica-GFP, L. tropica-GFP* and *L. major-GFP*. Extracellular parasites were treated for 24 h; Intracellular parasites were treated for 24 and 72 h post infection. Results are the mean (± S.E.M.) of three independent experiments

Wild type and GFP-expressing *Leishmania* promastigotes	Amphotericin B (mM)	Miltefosine (mM)		
Wild type parasites				
L. mexicana	0.184 ± 0.0624	19.65 ± 6.49		
L. aethiopica	0.129 ± 0.0217	40.13 ± 4.99		
L. tropica	0.117 ± 0.0132	26.87 ± 0.42		
L. major	0.086 ± 0.0096	23.38 ± 1.07		
GFP-expressing parasites				
L. mexicana-GFP	0.227 ± 0.0548	10.70 ± 3.16		
L. aethiopica-GFP	0.124 ± 0.0137	32.25 ± 1.99		
L. tropica-GFP	0.118 ± 0.0182	21.63 ± 12.49		
L. major-GFP	0.139 ± 0.0278	10.74 ± 6.20		
THP-1 cells infected with GFP-expressing parasites	24 h	72 h	24 h	72 h
L. mexicana-GFP	0.614 ± 0.0506	0.474 ± 0.0413	7.78 ± 1.202	0.602 ± 0.0293
L. aethiopica-GFP	0.442 ± 0.0507	0.148 ± 0.0367	1.73 ± 0.511	1.006 ± 0.212
L. tropica-GFP	0.239 ± 0.0093	0.117 ± 0.0264	1.36 ± 0.122	0.781 ± 0.203
L. major-GFP	0.291 ± 0.0297	0.583 ± 0.399	8.12 ± 1.451	5.43 ± 2.405

L. major, L. aethiopica and *L. tropica* transgenic parasites in order to produce a larger repertoire of GFP-expressing *Leishmania* species covering the whole range of disease manifestations. Production of stable GFP transfectants of both *L. mexicana* and *L. major* has been previously reported and we have replicated it as controls and also validated such transfectants for flow cytometry-based drug assays. Clones of *L. tropica* and *L. aethiopica* constitutively expressing GFP have been produced for the first time. *Leishmania tropica* is mainly responsible for cutaneous manifestation but it is also known to spread to internal organs and cause visceral leishmaniasis. *Leishmania aethiopica* causes a spectrum of disease manifestations ranging from localized, self-healing lesions (local cutaneous leishmaniasis; LCL) to disfiguring mucocutaneous lesions (mucocutaneous leishmaniasis; MCL) to diffuse, persistent lesions (diffuse cutaneous leishmaniasis; DCL). *Leishmania aethiopica* also shows a high degree of resistance to first-line treatments such as antimonials which adds to its importance as model for drug discovery.

Current chemotherapeutic options are limited by toxicity, long treatment regimes and cost as well as intrinsic (in the case of *L. aethiopica*) and emerging parasite resistance (Zucca and Savoia, 2011; Ejazi and Ali, 2013). Research towards new therapeutic approaches for leishmaniasis is therefore imperative. Development of new treatments based on rational drug design of identified novel molecular targets is a widely used tool in the drug discovery process (Alam *et al*. 2009). Nevertheless its application to leishmaniasis drug discovery is limited by the incomplete understanding of the complexities of the process of *Leishmania* survival. Therefore detection of new classes of compounds through screening of compound libraries in whole organisms remains the most exhaustive approach for selection of active compounds. An ideal screening procedure should involve a range of species and should be based on the intracellular stage of the parasite life cycle as this is the medically relevant one. Such screening should also be transferable to *in vivo* studies which are necessary as part of the drug development process. Green fluorescent protein is a reporter gene product renowned for its intrinsic fluorescent property which makes it ideal for both imaging and quantification by *in vitro* cell viability assays (Tsien, 1998; Kain, 1999).

Our findings support the use of genomic integration approaches to generate transgenic GFP-expressing *Leishmania* species suitable for investigation of the infection process as well as for flow cytometry-based drug assay. Such an assay is faster and cheaper than those currently available; moreover it is based on the medically relevant stages of the parasite life cycle. Flow cytometry analysis proved to be fast, highly sensitive and accurate in obtaining IC_{50} data of reference drugs tested on the intracellular stage compared with the microscopy-based method of

https://doi.org/10.1017/S0031182013001777 Published online by Cambridge University Press

determination. Moreover, such parasites have the potential to be used for *in vivo* infection as a model for drug testing as well as for increasing our understanding of disease pathogenesis.

The above study succeeded in generating, to our knowledge for the first time, transgenic *L. aethiopica* and *L. tropica* parasites constitutively expressing GFP. Such strains as well as transgenic *L. major* and *L. mexicana* were successfully validated as representing the same characteristic as their wild type counterparts. Specifically, because such parasites maintain infectivity and drug susceptibility comparable to their wild type correspondents they are an ideal model both for infection study and drug assays.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/S0031182013001777.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr Simon Richardson for his valuable suggestions during sample preparation and fluorescence microscopy, Rajeev Raj for assistance in producing the images of GFP-expressing parasites and Sam Ingram for technical assistance.

FINANCIAL SUPPORT

This work received financial support from the University of Greenwich and Greenwich Research & Enterprise (GRE).

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