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A non-commercial approach for the generation of transgenic *Leishmania tarentolae* and its application in antileishmanial drug discovery

TATIANA PINEDA†, YESENIA VALENCIA†, MARÍA F. FLÓREZ, SERGIO A. PULIDO, IVÁN D. VÉLEZ *and* SARA M. ROBLEDO*

PECET, Medical Research Institute, School of Medicine, University of Antioquia, Medellín, Colombia

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SUMMARY

Leishmaniasis is a parasitic infection caused by several species of the genus *Leishmania* that is considered as a neglected disease. Drug development process requires a robust and updated high-throughput technology to the evaluation of candidate compounds that imply the manipulation of the pathogenic species of the parasite in the laboratory. Therefore, it is restricted to trained personal and level II biosafety environments. However, it has been established the utility of *Leishmania tarentolae* as a model for *in vitro* screening of antileishmanial agents without the necessity of level II biosafety setups. In parallel the transfection of *Leishmania* parasites with reporter genes as the eGFP using non-commercial integration vectors like the pIRmcs3(−) has proved to be a powerful tool for the implementation of semi automatized high-throughput platforms for the evaluation of antileishmanial compounds. Here we report the generation of a new *L. tarentolae* strain overexpressing the eGFP gene harboured by the non-commercial vector pIR3(−). We also demonstrate its utility for the semi-automatized screening of antileshmanial compounds in intracellular forms of the *L. tarentolae* parasite.

Key words: *Leishmania tarentolae*, green fluorescent protein, transgenic parasites, pIRmsc3(−).

INTRODUCTION

Leishmaniasis is an infectious disease caused by several species of protozoan parasites belonging to *Leishmania* genus that are transmitted by female *Lutzomyia* and *Phlebotomus* sand flies. The infection is manifested in humans in three major clinical forms: (i) cutaneous leishmaniasis (CL) characterized by single or multiple lesion into the skin, mainly exposed areas of the body such as face, arms and legs; (ii) mucosal leishmaniasis (ML) manifested by the presence of lesions in mucosal membranes of naso-oro-pharyngeal cavity that could destroy total or partially the affected region; and (iii) visceral leishmaniasis (VL) that occurs with lesions in vital organs and tissues such as bone marrow, liver and spleen and it is characterized by the presence of fever, loss of body weight, anaemia and hepatosplenomegaly (World Health Organization, 2010).

This disease is endemic in 100 countries located in tropical and subtropical regions around the world. As estimated by The World Health Organization (WHO) there are 350 million people at risk of becoming infected, 11 million people infected and 1·3–2 million of new cases occur every year, of which 0·7–1·3 million correspond to CL (Alvar *et al*. 2012). Due to the absence of an effective vaccine against the infection, the management of the disease relays on treatment of cases and vector control. The pentavalent antimonial meglumine antimoniate (MA) or sodium stibogluconate, and more recently, miltefosine are the drugs commonly used to treat all clinical forms of leishmaniasis. Other drugs available as second and third options are pentamidine isethionate and amphotericin B (World Health Organization, 2010). All these medications, although effective, have drawbacks ranging from major adverse effects, associated with high doses and long-term treatments, and the high cost of the treatments (Den Boer *et al*. 2011). Additionally, the efficacy of these compounds is becoming lower, a fact that could be related with the emergence of more tolerant or even resistant parasites to these drugs, most likely due to incomplete use of treatments (World Health Organization, 2010). The need for optimal treatment with minor drawbacks has led the WHO to declare a priority to develop new and better drugs. However, a process of discovery of efficient drugs involves the availability of appropriate *in vitro* and *in vivo* experimental models for use in high-throughput assays.

Leishmania tarentolae is a *Leishmania* species which is non-pathogenic to humans and therefore is considered a safer experimental model only requiring biosafety level I for the *in vitro* manipulation of this parasite (U.S. Department of Health and

* Corresponding author: PECET, Medical Research Institute, School of Medicine, University of Antioquia, Medellín, Calle 70 # 52-21, Colombia. Phone: +574 2196503. Fax: +574 2196511. E-mail: sara.robledo@udea.edu.co

† These authors contributed equally to this work.

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Human Services, 2009). A previous study demonstrated that the sensitivity of axenic and intracellular amastigotes of wild-type *L. tarentolae* was compared with the sensitivity showed by *Leishmania* species causative of human leishmaniasis and validated the use of *L. tarentolae* in the screening of antileishmanial drugs (Taylor *et al*. 2010). However, the use of these parasites in high-throughput screening of drug candidates is limited due to the fact that the antileishmanial activity has to be determined by manual approaches like observation of Giemsa-stained parasites under optical microscopy, which is time consuming and require highly trained personnel.

There are several *Leishmania* species expressing reporter genes, either temporarily (using episomal vectors) or stable (integration of the gene in the genome). The most commonly genes used as reporters in *Leishmania* species are the *β*-galactosidase in *L. amazonensis* (Okuno *et al*. 2003), *β*-lactamase in *L. major* and *L. amazonensis* (Buckner and Wilson, 2005), the luciferase gene in *L. amazonensis* (Ashutosh *et al*. 2005), *L. donovani* (Lang *et al*. 2005), *L. infantum* (Roy *et al*. 2000), *L. major* (Sereno *et al*. 2001) and *L. panamensis* (Henao *et al*. 2004); and lastly, the green fluorescent protein gene (GFP) in *L. major, L. amazonensis, L. donovani, L. infantum, L. braziliensis, L. panamensis* and *L. mexicana* (Kamau *et al*. 2001; Chan *et al*. 2003; Okuno *et al*. 2003; Singh and Dube, 2004; Singh *et al*. 2009; Varela *et al*. 2009; Bolhassani *et al*. 2011; Pulido *et al*. 2012) Seemingly an *L. tarentolae* strain expressing the GFP has been previously generated using the commercial system pLEXY (Bolhassani *et al*. 2011), which showed to be very efficient in generating different fluorescent *Leishmania* strains with stable, homogeneous and sustainable fluorescent phenotype due to the incorporation of the GFP gene into the genome of the parasite by homologous recombination; unfortunately, the pLEXSY-GFP expression systems, owned by Jena Biosciences GmbH (Jena, Germany), are expensive and difficult to access. An alternative system for the generation of stable transgenic parasites expressing a reporter gene is the pIRmsc3(−) vector, which also allows the integration of the reporter gene (i.e. eGFP) into the 18S rRNA locus by homologous recombination (Hoyer *et al*. 2004). We previously showed that this system enables a high transcriptional rate of the transfected reporter gene eGFP and thus, the production of functional protein within the parasite occurs in high levels in the absence of selective pressure (Pulido *et al*. 2012). The system was successfully implemented in parasites from several *Leishmania* species producing highly stable and homogeneous fluorescent populations of parasites. Those parasites have proved to be useful for the evaluation of antileishmanial activity of drugs even in the intracellular amastigotes without any background that could lead to false results. Nevertheless, to the date the use of the pIRmsc3(−) for the generation of transgenic *L. tarentolae* parasites has not been reported, although it may represent a robust option to the commercially available transfection systems. In this study, we demonstrate the applicability of the pIRmcs3(−) for the generation of *L. tarentolae* strains expressing the eGFP, which represent a safe and powerful tool for the establishment of semi automatized protocols in academic institutions for the evaluation of antileishmanial candidates in level I biosafety laboratories.

MATERIALS AND METHODS

Parasites and cells

The *L. tarentolae* strain LEM125, isolated from the lizard of the Gekkonidae family was courtesy from Dr J. Clos (Howard Hughed Medical Institute, University of California, LA, USA). Parasites were kept in biphasic media Novy–MacNeal–Nicholle (NNN) at 25 °C, pH 6·9. Genetically modified parasites were grown in the Schneider medium (Sigma-Aldrich, St. Louis MO, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco Life Technologies, Grand Island, NY, USA). During selection phases of the transfected parasites, 70 μg mL^{-1} of the antibiotic Nourseothricin (NTC) (Jena Biosciences, Jena, Germany) was added to the media; this concentration of NTC corresponds to the effective concentration 50 (EC_{50}) for the *L. tarentolae*-WT strain, determined previously (data not shown).

Cells of the human promonocytic cell line U937 (CRL1593·2™) (American Type Culture Collection – ATCC, Manassas, VA, USA) were kept under standard conditions, 37 °C, 5% CO_2 in complete medium composed by RPMI 1640 media (Sigma-Aldrich), 10% FBS and 1% of antibiotic solution (penicillin 100 U mL^{-1} and streptomicyn 0·1 mg mL^{-1}) (Gibco).

Compounds

Susceptibility assays were performed using conventional antileshmanial agents: MA and pentamidine isethionate (Sanofi, Aventis. Bogota, Colombia), miltefosine (Aeterna Zentaris Inc., Summerville, SC, USA) and amphotericin B (Sigma-Aldrich). In addition, the N-iodomethyl-N,N-dimethyl-N-(6,6-diphenyl-5-hexen-1-yl)ammonium iodide (C6) a new compound proved to have antileishmanial properties in several species of *Leishmania* was also tested [Rios *et al*. (2015) Patent US20140194640 A1].

DNA vectors and transfection experiments

The vector pIRmsc3(−) was kindly donated by Joachim Clos from the Bernard Nocht Institute for Tropical Medicine (Hamburg, Germany). The construction of the pIRmsc3(−)-eGFP vector was done as described (Pulido *et al*. 2012). In brief, the eGFP

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was amplified by PCR from the p6·5-eGFP (Chan *et al.* 2003) using the primers GFP5BglFw (5′-GGAGATCTATGGTGAGCAAGGGCGA GGA-3′) and GFP3NdeRv (5′-GGCATATGTTA CTTGTACAGCTCGTCCA-3′) in the Pwo master mix (Roche Diagnostics Corporation, Indianapolis, IN, USA). The PCR conditions were: 95 °C 1 min, 55 °C 45 s, 72 °C 45 s (35 cycles). The PCR product and the pIRmsc3(−) were digested with *BglII* and *NdeI* enzymes (NEB); the digested products were ligated in the presence of T4 ligase (NEB) and finally transformed in DH5α cells (Invitrogen). Positive clones containing the pIR3msc(−)-eGFP construct were verified for PCR using GFP5BglFw and GFP3NdeRv primers and *BglII-NdeI* digestion; plasmids of positive clones were purified and sequenced by the Sanger method.

For the transfection of the *L. tarentolae* parasites with the pIRmsc3(−)-eGFP construct 400 μL of a 10×10^8 cells mL^{-1} solution in Cytomix electroporation buffer (120 KCl, 0·15 $CaCl_2$, 10 K_2HPO_4, 25 mM HEPES, 2 EDTA, 5 $MgCl_2$ pH 7·6) were mixed in an electroporation cuvette with 2 μg of pure *SwaI*-linearized pIR3(−)-eGFP vector and then incubated 4 min at 4 °C followed by one pulse of 1500 V 25 μF. After electroporation, the parasites were incubated 5 min on ice and transferred to 5 mL of Schneider medium (Sigma-Aldrich) with 20% FBS (Gibco) and incubated at 25 °C for 24 h in the absence of selective pressure. Twenty-four hours post-electroporation 70 μg mL^{-1} of NTC antibiotic was added to the media and incubated for 4 days at 25 °C. The media was exchanged every 48 h for new media containing NTC (Jena) until not alive cells were detected in the mock culture (electrophoresed parasites in the absence of exogenous DNA) by microscopic observation.

Bioinformatic analysis

Since the pIR3(−) is a variation of the patented vector pIRSAT1 (Beverley, 2000) we used the published sequence of the pIRSAT1 for the identification of the recombination arms and the regulatory sequences also present in the pIRmsc3(−). The sequences of the ssu-RNA region of *L. major* and the intergenic regions of the dihydrofolate reductase-thymidylate synthase of *L. major* (IR-DHFR-TS), cysteine proteinase 2 of *L. pifanoi* (IR-CYS2) and Galf-transferase of *L. donovani* (IR-LPG1) were isolated from the vector sequence and used for the manual localization of the homologous sequences in the *L. tarentolae* genome using the BLAST tool of the TriTrypDB database (http://tritrypdb.org/tritrypdb) (Aslett *et al.* 2010). The located sequences were annealed with its homologous sequences in the pIRSAT1 vector using the Needle EMBOSS alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle) (Rice *et al.* 2000).

Determination of the integration of the pIR3 (−)-eGFP construct into the 18S ssu-rRNA locus of L. tarentolae

For the confirmation of the integration cassette we used conventional PCR using the primers 18S5′Fw (5′-ATCTGCGCATGGCTCATTACA-3′) that anneals in upstream the recombination locus inside the chromosome and 18S3′Rv (5′-CCAGCTGCA GGTTCACCTACA-3′), which anneals in the 5′ region of the eGFP gene; these primers were designed for the amplification of a 2·7 kb fragment flanking the 5′ region of the integration cassette. Additionally, we used the primers eGFP3′Fw (5′-CGGCATGGA CGAGCTGTACAA-3′), which anneals in the 3′ region of the eGFP gene and eGFP5′Rv (5′-GC TCCTCGCCCTTGCTCA-3′), which anneals downstream the recombination locus inside the chromosome; in this case, these primers were designed for the amplification of a 3·2 kb fragment flanking the 3′ region of the integration cassette (Pulido *et al.* 2012). The PCR products were verified by DNA agarose electrophoresis. The amplified fragments were further cloned into the pTZ vector (Thermo Scientific, Waltham, MA, USA) for further sequencing confirmation (Macrogen, Seoul, Korea).

Fluorescence microscopy and flow cytometry analysis

After the selection of the transfected parasites with NTC the expression of the eGFP in the parasites was tested by fluorescence microscopy as follow: 30 μL of the cultured parasites were spread in a glass slide and air dried; the images of the fluorescent parasites were acquired in a Nikon eclipse 80i microscope with a green fluorescence filter b-2ec. The transfected parasites were transferred to a biphasic NNN medium without selective pressure after confirmation of the integration of the eGFP in the ssu-rRNA locus. For flow cytometry analysis the parasites were recovered from the NNN medium and washed with phosphate-buffered saline (PBS). The cells were finally resuspended in 500 μL of PBS and analysed in a flow cytometer equipped with an argon laser beam (Cytomics FC 500MPL, Beckman Coulter, Pasadena, CA, USA) with an excitation wavelength of 488 and 525 nm emission. Analysis of GFP-expressing promastigotes was performed at least in 10 000 gated events and numeric data were processed with WinMDI and CXP software (Beckman-Coulter) as described (Pulido *et al.* 2012).

Parasitological characterization of the L. tarentolae-*EGFP strain*

Growth curves were made in the Schneider medium 10% FBS with a starting cell concentration of 50 000 cells mL^{-1} in 24-well plates in 1 mL of Schneider medium (Sigma-Aldrich) and incubated at 25 °C for 20 days. Microscopic counting of the cells was

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performed in a Neubauer chamber every day and the counts were analysed with the GraphPad Prism 6·0 (Bellavista, Aljaraque, Huelva, Spain).

Determination of the *in vitro* infectivity of the *L. tarentolae*-EGFP and *L. tarentolae*-WT strains over the U937 cell line was performed as follows. Briefly, U937 cells in the second culture day in complete RPMI 1640 medium were washed with PBS and resuspended at 3×10^5 cells mL^{-1} in complete RPMI 1640 medium containing 0·1 $\mu g\ mL^{-1}$ of PMA (Phorbol 12-myristate 13-acetate) (Sigma-Aldrich). Approximately 1 mL of the cell suspension was dispensed in 24-well culture plates containing a 12 mm diameter glass slide. The plates were incubated at 37 °C, 5% CO_2 for 72 h. Finally, the cells were infected with stationary phase promastigotes of the *L. tarentolae*-EGFP or *L. tarentolae*-WT strains at 5:1, 10:1, 20:1 and 40:1 parasite/cell ratio. After 2 h incubation at 34 °C in 5% CO_2 the extracellular promastigotes were washed away with 1 mL of room temperature RPMI 1640 and the infected cells were incubated for 24 h at 37 °C, 5% CO_2. For the microscopic analysis of the infected cells, the medium was withdrawn and the wells were washed with room temperature PBS, then the cells were fixed in methanol and Giemsa stain (Merck S.A, Bogota, Colombia). Microscopic analysis was performed in a light microscope with a 1000× objective (Robledo *et al.* 1999). In a separate experiment, the cells infected with the *L. tarentolae*-EGFP strain were detached from the wells with trypsin/EDTA solution, washed with PBS and resuspended in 500 μL PBS for analysis by flow cytometry as described above (Pulido *et al.* 2012). About 10 000 events were counted from each well. The percentage of infected cells was determined by the dot plot analysis, while the parasitic load was calculated by histogram analysis of the fluorescence mean intensities.

Determination of the sensitivity of the L. tarentolae-*EGFP strain to antileishmanial compounds*

The evaluation of the sensitivity to conventional antileishmanial compounds of the *L. tarentolae*-EGFP strain in comparison with the *L. tarentolae*-WT strain was performed over intracellular amastigotes of both strains as described elsewhere (Varela *et al.* 2009; Pulido *et al.* 2012). The intracellular amastigotes were obtained by infection of U9367 cell with stationary phase promastigotes of the corresponding of the *L. tarentolae* strain as described above and 24 h after infection the medium was replaced with RPMI 1640 medium containing the respective antileishmanial compound (meglumine antimoniate, miltefosine, pentamidine isethionate, amphoterine B or C6). The evaluated concentrations for each compound were: 50, 12·5, 3·1256 and 0·781 $\mu g\ mL^{-1}$ meglumine antimoniate; 100, 25, 6·25 and 1·56 $\mu g\ mL^{-1}$ miltefosine and pentamidine; 0·5, 0·125, 0·031 and 0·007 $\mu g\ mL^{-1}$ amphotericin B; and 13·3, 3·325, 0·831 and 0·21 $\mu g\ mL^{-1}$ C6. The infected cells were exposed 72 h to each compound at 37 °C in 5% CO_2; then the medium was removed and the cells were recovered with PBS–trypsin/EDTA. Finally, the cell suspension was transferred to a cytometry tube and analysed by flow cytometry as described previously. The infectivity of the strains was determined as the parasitic load. The results are expressed as EC_{50} calculated with the Probit model (Finney, 1978) as described below.

Data analysis

All parasitological tests [infective concentration 50 (IC_{50}) and EC_{50}] were performed by triplicate in two independent experiments. The infectivity of *L. tarentolae* strains (WT and EGFP) was determined according to infected cell percentages obtained from each dose of parasites. The results are expressed as IC_{50} calculated with the Probit model (Finney, 1978). On the other hand, the antileishmanial activity of the tested compounds over the *L. tarentolae* strains (WT and EGFP) is presented as the reduction in the percentage of infected cells and parasitic load for each concentration of the different compounds calculated according to the Equation: % infection = (% infected cells in the presence of the compound/% of infected cells without treatment) × 100. In turn, the percentage of reduction of the infection was calculated as: % of infection reduction = 100–% of infection. The values were subsequently used for the calculation of the EC_{50} using the Probit model (Finney, 1978). The degree of antileishmanial activity was established according to the CE_{50}: <25 $\mu g\ mL^{-1}$ = high activity, 25–70 $\mu g\ mL^{-1}$ = moderated activity, >70 $\mu g\ mL^{-1}$ = low activity. All these data are presented as average ± the S.D. For the statistical analysis of the differences between the WT and GFP strains we applied a Mann–Whitney test using the Graph Pad Prism 6 (San Diego CA, USA). *P*-values <0·05 were considered statistically significant.

RESULTS

Construction of the pIR3msc(−)-eGFP vector

The cloning and directionality of the eGFP coding sequence into the pIRmsc3(−) plasmid was verified by restriction mapping. A coding sequence of 700 bp was released from the pIRmsc3(−)-eGFP construct corresponding to the size of the eGFP gene (Pulido *et al.* 2012) (Fig. 1A). Sequencing of the purified plasmids confirmed the presence of the eGFP ORF in frame with the open reading frame (ORF) of the pIRmsc3(−) vector. Further digestion of the construct with the *SwaI* enzyme released a fragment of 6000 bp, which correspond to the integration cassette of the pIRmsc3(−)-eGFP (Fig. 1B).

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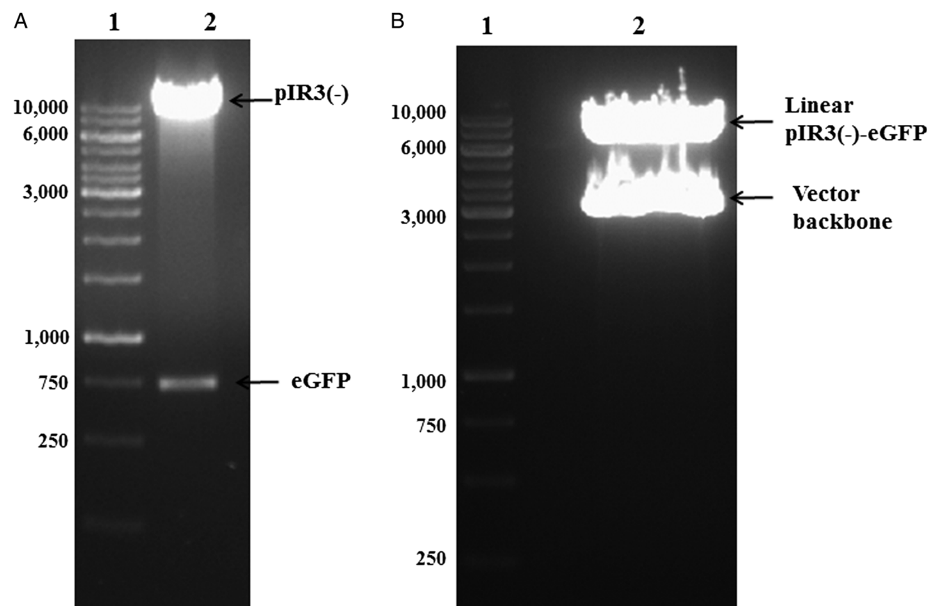

Fig. 1. (A) Verification of pIR3(−)-eGFP construct with *NdeI* y *BglII*. 1% agarose gel electrophoresis. eGFP was amplified by PCR and the PCR product and the pIRmsc3(−) were digested with *BglII* and *NdeI* enzymes (NEB); the digested products were ligated in presence of T4 ligase (NEB) and finally transformed in DH5α cells. Positive clones containing the pIR3msc(−)-eGFP construct were verified for PCR and *BglII-NdeI* digestion. Line 1: molecular weight marker; lines 2 and 6: digested pIR3(−)-eGFP construct; lines 3, 4 and 5: negative control. (B) Liberation of pIR3 (−)-eGFP construct using *SwaI*. 1% agarose gel electrophoresis. Line 1: molecular weight marker; line 2: negative control; lines 3 and 4: pIR3(−) eGFP construct.

Table 1. Sensitivity of *Leishmania tarentolae*-WT and *L. tarentolae*-EGFPT to compounds with antileishmanial activity

Compound	Lt-WT Microscopy (optical)	Lt-GFP Microscopy (fluorescence)	Lt-GFP Flow cytometry
Amphotericin B	0·06 ± 0·01	0·03 ± 0·005	0·08 ± 0·01
Mitelfosine	14·52 ± 3·32	10·32 ± 1·87	11·42 ± 0·31
Meglumine antimoniate	14·01 ± 2·75	18·19 ± 3·35	53·52 ± 4·11
Pentamidine	25·33 ± 3·63	15·75 ± 1·78	0·75 ± 0·08
C6	1·28 ± 0·28	0·89 ± 0·17	1·61 ± 0·23

Data represent the mean value of EC_{50} (in μg mL) ± S.D. of two independent experiments by triplicate.

Bioinformatic analysis of the integration and regulation sequences of the pIRmsc3(−)-eGFP.

Previous reports have shown that the vector pLEXY (Jena Biosciences) can be successfully integrated into the rRNA locus of *L. tarentolae* by homologous recombination. pLEXY is an integration vector containing the homologous sequences of 18S ssu rRNA of *L. tarentolae* allowing successful recombination of the integration elements into the desired locus (Breitling *et al.* 2002). In the present work, we used the vector pIRmsc3(−) created by Clos *et al.* (Hoyer *et al.* 2004) by introducing an alternative multiple cloning site (MCS) into the pIRSAT1 vector (Beverley, 2000). Both pIRmsc3(−) and pIRSAT1 are non-commercial integration vectors widely used in academic research. pIR vectors contain 2 integration arms homologous to *L. major* 18S ssu rRNA and it has been shown that it is suitable for its use in several *Leishmania* strains (Pulido *et al.* 2012). Sequence alignment of the 18S ssu sequences of *L. major* (accession number GQ332361) and *L. tarentolae* (accession number M84225) sowed 99·6% identity and 99·6% similarity, in the same way alignment of the recombination arms of the pIRmsc3(−)-eGFP vector and the pLEXY vector recombination arms with the 18S ssu-rRNA locus of *L. tarentolae* showed that 99·4% identity and similarity for the 5′ recombination arm and 100% identity and similarity for the 3′ recombination arm (Table 1). Regardless the genealogical distance between both *Leishmania* strains it is clear that the 18S RNA locus is highly conserved in the same fashion as it has been described before between the *Leishmania* and *Viannia* subgenus (Pulido *et al.* 2012); therefore, targeting these locus in *L. tarentolae* with the pIRmsc3(−) vector should not affect significantly the occurrence of the

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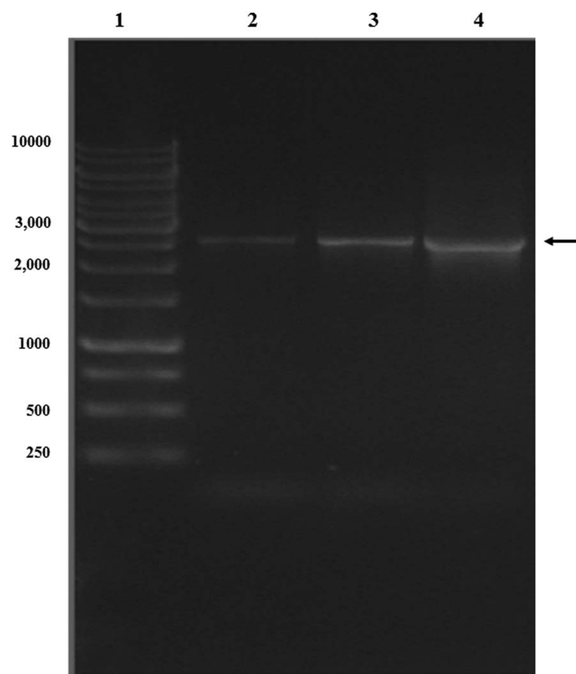

Fig. 2. Verification of insertion of pIR3(−)-eGFP construct in the 18S RNA. 1% agarose gel electrophoresis. Integration cassette was confirmed by conventional PCR and further cloned into the pTZ vector. The amplified product is observed at different annealing temperatures. Line 1: molecular weight marker; line 2: 60°C; line 3: 59.6°C; line 4: 58.7°C. The arrow shows the expected amplicon.

homologous recombination events and the subsequent integration and overexpression of the integrated genes according to the works published by others (Papadopoulou and Dumas, 1997). On the other hand, comparison of the regulatory sequences included in the pIRmsc3(−) vector with its homologous regions in the *L. tarentolae* parasite showed a rather large difference. The IR-DST showed the highest conservation between *L. major* and *L. tarentolae* (68·4% identity and 73·2% similarity); meanwhile the IR-LPG1 showed the lowest conservation between the species with <40% identity and 40·78 similarity. The IR-CYS2 showed a low extend of conservation as well, with <60% identity and similarity, respectively (Table 1).

The pIRmsc3(−)-eGFP was successfully integrated into the 18S ssu-rRNA locus of L. tarentolae

We performed conventional PCR experiments using gDNA of the transfected *L. tarentolae* strain as template. The amplified fragments of the regions flanking the integration cassette of the pIRmsc3 (−)-eGFP correspond to the expected sizes as reported (Pulido *et al*. 2012): 2·7 and 3·2 kb of the 5′ and 3′ regions of the integration cassette, respectively (Fig. 2). Further sequencing of the pTZ-cloned PCR products confirmed the presence of the eGFP gene as well as the recombination arms

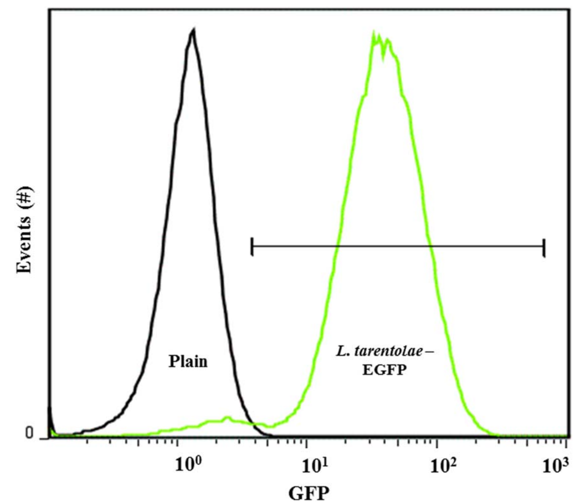

Fig. 3. Flow cytometry analysis of GFP expressing parasites. Promastigotes of *L. tarentolae*-GFP at logarithmic phase of growth were analyzed according to fluorescence intensities. The figure shows two populations clearly distinguishable: black histogram representing non-transfected parasites and green histogram representing transfected parasites. The shift shows the relative increase in the average fluorescence for *L. tarentolae*-EGFP compared to plain parasites. Over 98% of transfected parasites demonstrate high and homogeneous expression of GFP.

of *L. major* replacing at least one of the native 18S ssu-rRNA copies of the 18S ssu rRNA of *L. tarentolae* in the targeted locus.

The strains harbouring the pIRmsc3(−)-eGFP integration element successfully express the EGFP gene product

Flow cytometry analysis of the transfected strains showed that the expression of the EGFP is highly homogeneous in the parasite population after selection with the antibiotic NTC (Fig. 3). The analysis was done several weeks after the selection pressure was withdrawn from the culture, which evidences the high degree of stability of the expression of EGFP in the transfected strain. Fluorescence microscopy confirmed the flow cytometry findings. The promastigotes of the transfected strain showed a normal morphology and natural behaviour (mobility), the fluorescence is spread all over the cytoplasm of the parasites and contrast with light microscopy images showed that the population is homogeneous with no detection of non-fluorescent cells (Fig. 4).

The L. tarentolae-*EGFP strain behaves* in vitro *as* L. tarentolae-*WT strain*

Genetic manipulation of the parasites may lead to changes in the behaviour of the transfected strains with respect to the WT strains. We therefore performed a series of experiments intending to

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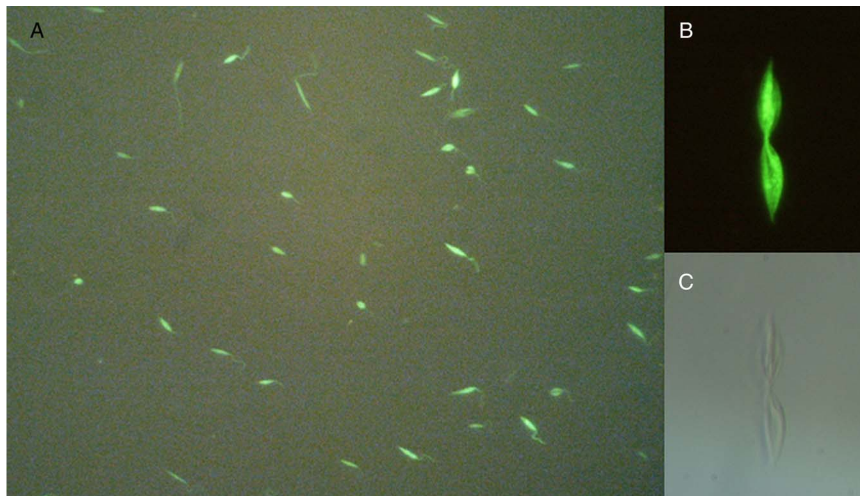

Fig. 4. Detection of GFP expression in transgenic parasites by epifluorescence microscopy. Fluorescent microscopic images show expression of GFP in transfected *L. tarentolae* after glinting of fluorescence during logarithmic phase of growth (5-days old promastigotes. Images were captured under a 40x lens (A); 100x green excitation filter (B) and 100x oil immersion lens (C).

determine if *L. tarentolae*-EGFP strain preserves the same biological properties of the WT strains *in vitro*. The growing curve of the transfected strain showed that the genetic manipulation does not modify the growing kinetics of the parasites compared with the WT strain (Fig. 5). Furthermore, we prove that the transfected parasites conserve the capacity to infect U937 cell lines in the same fashion as the WT strain as evidenced by fluorescence microscopy experiments and standard Giemsa stain assays (Fig. 6). Analysis of the IC_{50} showed that both strains present closely IC_{50} values ($23{\cdot}0 \pm 2{\cdot}4$ for the WT and $19{\cdot}5 \pm 2{\cdot}0$ for the transfected strain) ($P > 0{\cdot}05$).

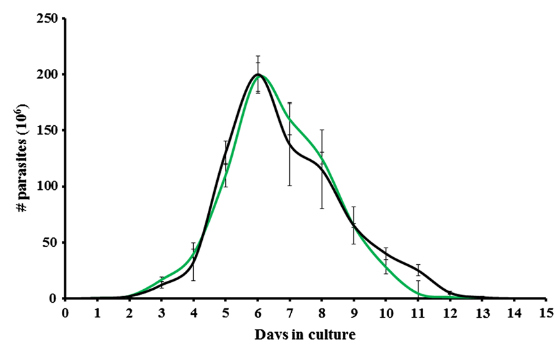

Fig. 5. *In vitro* time proliferation curve of promastigotes *L. tarentolae*. Parasites were cultured at 26°C in Schneider's insect medium supplemented with 10% of fetal bovine serum and antibiotics. Promastigotes were harvested at every day during 15 growth days and counted on a microscope. Figure shows the growth of *L. tarentolae*-WT (black line) and *L. tarentolae*-EGFP (green line). Data represent the mean value of parasite counts ± standard deviation of two independent experiments by triplicate.

Evaluation of the sensibility of L. tarentolae*-EGFP strain to standard antileishmanial compounds*

Evaluation of the EC_{50} for both strains by either light or fluorescence microscopy of the transfected parasites upon exposure to different antileishmanial agents showed that the sensitivity of the EGFP expressing strain is not significantly modified with respect to the WT strain ($P < 0{\cdot}05$) (Table 2). Nevertheless, it was noticed that the analysis by flow cytometry showed statistically significant variations between both the strains, especially for the MA and pentamidine; these variations were not detected using microscopic analysis. Additionally, in the microscopic analysis we detected significant variations in the sensitivity profile to amphotericin B and pentamidine. We cannot determine whether the differences are attributed to experimental errors during the microscopic analysis, or whether those can be attributed to the influence of the genetic manipulation of the parasite; however, considering the heterogeneity of the results between the different tested compounds we interpreted that the slight variations can be mostly attributed to the experimental methods used during the analysis. It has been shown in several reports that manual procedures, as the microscopy, are prone to statistical errors since they rely mostly in the expertise of the personal performing the manual count (Sereno *et al*. 2007). In addition, flow cytometry analysis has been reported as an efficient method for the determination of antileishmanial activities in intracellular amastigotes.

DISCUSSION

A previous study showed that *L. tarentolae* is a suitable model for evaluating antileishmanial activity *in vitro* reducing the risk of infection in laboratory workers handling these parasites in culture (Taylor *et al*. 2010). However, evaluating the effect of compounds on intracellular parasites requires visualization

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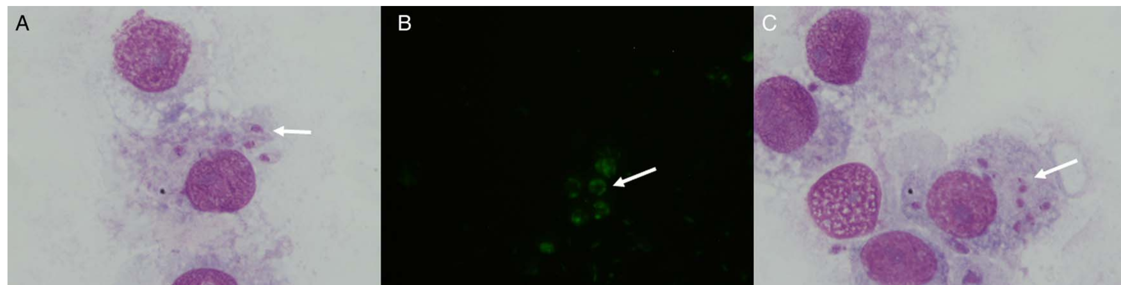

Fig. 6. Human macrophage infectivity by *L. tarentolae*. Cells were infected with stationary growth phase promastigotes (5-days old) at 20:1 parasite:cell ratio. Intracellular amastigotes were clearly visible 24 h following infection with *L. tarentolae*-WT or *L. tarentolae*-EGFP. (A) U-937 cells infected with *L. tarentolae*-WT Giemsa stained; (B) U-937 cells infected with *L. tarentolae*-EGFP observed at epifluorescence microscopy; and (C) U937 cells infected with *L. tarentolae*-EGFP Giemsa stained. All images were captured under a 100x oil immersion lens.

Table 2. Identity and similarity between the recombination and regulatory sequences of the pIR3 (−) vector and the homologous sequences in *Leishmania tarentolae*

Gene	Identity (%)	Similarity (%)
L. major 18S ssu RNA	99·6	99·6
L. major DTS-IR	68·40	73·20
L. pifanoi CYS2-I	52·85	58·81
L. donovani LPG IR	<40%	40·78

Analysis was done using the BLAST tool of the TriTrypDB database (http://tritrypdb.org/tritrypdb) Needle EMBOSS alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle).

and quantification of the viable parasites under optical microscopy in samples stained with Giemsa, a method that is time-consuming and requires personnel highly trained to differentiate between living and dead parasites. To optimize the methods for assessing antileishmanial activity using automated methods, several *Leishmania* strains expressing different reporter genes have been generated and they have demonstrated their usefulness in automated methods such as colorimetry, luminometry and fluorometry (Sereno *et al*. 2007).

Among all these different types of reporter genes, the GFP gene has shown high utility and versatility, allowing the automation of the evaluation processes of natural and synthetic antileishmanial compounds by flow cytometry or fluorometry. Over the last decade, several *Leishmania* species expressing the GFP gene have been generated using different systems or expression vectors. In the specific case for *L. tarentolae*, a fluorescent strain was constructed with the pLEXSY system (Jena Bioscience). Although it is commercially available, the cost is very high and the access to this system is limited in countries where commercial representatives do not exist. Over the past 5 years we have been working in the optimization of methodologies for *in vitro* mass screening of antileishmanial of compounds. Using the pIRmsc3(−) system (Hoyer *et al*. 2004) several fluorescent strains of pathogenic *Leishmania* species were constructed: *L. panamensis, L. braziliensis, L. guyanensis, L. Mexicana, L. amazonensis* and *L. infantum* (Pulido *et al*. 2012). Here, a non-commercial genetic modification approach was used for the generation of a fluorescent strain of *L. tarentolae*, which can be used for the initial screening of antileishmanial drugs without complex biosafety systems.

The expression system pIRmsc3(−)-eGFP was successfully integrated in the genome of *L. tarentolae* resulting in the generation of a new fluorescent *L. taretolae* strain. The integration of the construct into the 18S ribosomal subunit allowed stable fluorescence levels in the parasite population over time with more than 90% of fluorescent population after several passages (10–15) in culture thus, facilitating the *in vitro* assays. EGFP transgene insertion using the pIRmsc3(−) system did not affect neither the morphology of the parasite or its growth in culture nor its infectivity and sensitivity to many of the known antileishmanial drugs. Analysis showed that the IC_{50} of the *L. tarentolae*-EGFP and *L. tarentolae*-WT were very similar ($19{\cdot}5 \pm 2{\cdot}0$ and $23 \pm 2{\cdot}4$ for the EGFP and WT strains, respectively) ($P > 0{\cdot}05$). These results demonstrate that genetic manipulation of the *L. tarentolae* strain did not induce greater alterations in the biological properties of the parasite *in vitro*.

The high expression of the EGFP was evidenced by the presence of homogeneous fluorescence in almost 98–99% of the parasite population with a notorious stability through *in vitro* subcultures without requiring a constant selective pressure, which reduces the cost of production and maintenance in culture and promoting thus the massive use of these parasites in the search of candidate molecules for antileishmanial drugs.

Additionally, we probed that the homologous recombination of the pIR3(−)-eGFP into the 18S ssu-rRNA locus of *L. tarentolae* is as efficient as it was for other *Leishmania* species as shown in

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previous works (Hoyer *et al*. 2004; Pulido *et al*. 2012). An even more interesting observation is that *L. tarentolae* can recognize and process mRNA transcripts containing regulatory sequences from the genealogically distant *Leishmania* species *L. major, L. pifanoi* and *L. donovani* despite the notorious differences presented between the regulatory regions of the mentioned species and *L. tarentorale*. These findings account to two interesting insights: (i) mRNA maturation and translation regulation mechanisms allows high acceptance and plasticity between *Leishmania* species, even with those genealogically distant, a concept already explored in other studies (Beverley, 2000); (ii) the mRNA maturation mechanisms of *L. tarentolae*, although genetically divergent, is effective in the recognition and maturation of mRNAs containing maturation signals from other species of *Leishmania*.

One of the goals of the present study was the generation of a non-commercial *L. tarentolae* strain suitable for the evaluation *in vitro* of new antileishmanial compounds in semiautomatic platforms in laboratories with no access to biosafety level II setups. Therefore, the sensitivity of *L. tarentolae*-EGFP strain to the current standard antileishmanial drugs was tested in order to rule out if the genetic manipulation may induce changes in the sensitivity profile of *L. tarentolae* to the antileishmanial compounds. The sensitivity of *L. tarentolae*-EGFP to antileishmanial drugs was not affected as a result of genetic manipulation, since EC_{50} values for the *L. tarentolae*-WT strain and the transgenic strain, assessed by optical and fluorescence microscopy were similar. Likewise, the results obtained with the *L. tarentolae*-WT were similar to those obtained previously by Taylor *et al*. (2010). These results confirm that the transfected strain behaves as a suitable experimental model and can replace the use of the WT strain. Although differences between the EC_{50} obtained by flow cytometry and fluorescence microscopy were observed, especially for MA and pentamidine but not miltefosine, amphotericin B and C6; these results may be due to differences in the quantification method but cannot be attributable to effects of the genetic manipulation.

Leishmania tarentolae may present a series of biological properties that mask the reality of the infection by human-pathogenic species. Nevertheless *L. tarentolae* has been increasingly calling attention of the scientific community for the study of virulence factors and the knowledge of the mechanisms of infectivity by human-pathogenic species. On the other hand, genome sequencing of the *L. tarentolae* strain (Raymond *et al*. 2012) showed that despite the lack of most of the genes required for the mammalian infection and the underrepresentation of other genes involved in intracellular persistence and infectivity, *L. tarentolae* shares >90% of gen content with human-pathogenic species. The genome derived data showed the presence of important virulence factors as GP63 in *L. tarentolae* although the data contrast with previous publications about the existence of LPG or LPG-like factors in the parasite (Azizi *et al*. 2009). Nevertheless, this finding yet leaves room for exploring novel drug targets in *L. tarentolae* that may apply as well in pathogenic species. In addition, genomic data and other genetic studies support the hypothesis that *Sauroleishmania* species derived from human-pathogenic species and that, the differences in the virulence and pathogenic mechanisms between them, are the result of adaptive processes to the vectors and hosts (Azizi *et al*. 2009; Raymond *et al*. 2012;). Therefore, *in vitro* studies of novel compounds over *L. tarentolae* can indeed supply valid preliminary information about the applicability and validity of novel antileishmanial drugs.

The lack of infectivity of *L. tarentolae* to mammals supports the poor applicability of *L. tarentolae*-EGFP for *in vivo* studies. However, in a previous work, we demonstrated that *L. tarentolae*-WT strain is able to infect *in vitro* human derived cell lines as well as hamster cells where the infection persisted for at least 3 weeks (Taylor, *et al*. 2010); we also probed that axenic and intracellular amastigotes of *L. tarentolae* WT exhibit comparable susceptibility with the conventional antileishmanial compounds; therefore, we sustained that this intracellular model is applicable to *in vitro* screening of future antileishmanial drugs. Now we probed that genetic modification using the pIRmsc3(−) system does not interfere with the infectivity, virulence and susceptibility to antileishmanial compounds of *L. tarentolae* parasites *in vitro*.

Thus, our results support the applicability of fluorescent *L. tartentolae* for *in vitro* studies during initial stages of drug development, which involves *in vitro* studies in the extracellular or the intracellular forms of the parasites. These studies may include library screenings, high-throughput screening methods and molecule improvement processes, which can be performed in laboratory environments where protocols of biosafety level 2 or higher are not implemented.

Finally, the data presented here support the application of genetic modification approaches in *L. tarentolae* without interfering with the main biological properties of the parasite. This observation combined with the stability and homogeneity of the EGFP in the transfected parasites probes that the pIRmsc3(−) vector is as well a suitable tool for the implementation of functional studies of genes in *L. tarentolae* parasites. The methodology presented here can be easily implemented in any laboratory with any biosafety level thus favouring either the development of biochemical studies in *Leishmania* or the process of discovering new leishmanicidal activities using optimized methods such as flow cytometry and fluorometry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with the development of this work.

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ETHICAL ASPECTS

The development of this work involved exclusively *in vitro* assays. Waste disposal was performed according to the standard procedures established in the special plan for management of recyclable, biological and chemical waste at the University of Antioquia.

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