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Improvement of the green fluorescent protein reporter system in *Leishmania* spp. for the *in vitro* and *in vivo* screening of antileishmanial drugs

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ABSTRACT

Development of new therapeutic approaches for leishmaniasis treatment requires new high throughput screening methodologies for the antileishmanial activity of the new compounds both *in vitro* and *in vivo*. Reporter genes as the GFP have become one of the most promissory and widely used tools for drug screening in several models, since it offers live imaging, high sensibility, specificity and flexibility; additionally, the use of GFP as a reporter gene in screening assays eliminates all the drawbacks presented in conventional assays and also those technical problems found using other reporter genes. The utility of the GFP as a reporter gene in drug screening assays with *Leishmania* parasites depends on the homogeneity and stability of the GFP transfected strains. Stable expression of the GFP in the Old World *Leishmania* species has been demonstrated using integration vectors; however, no reports exist yet about the success of this methodology in the New World species. Here we report the generation of New World *Leishmania* strains expressing the GFP protein from an integration vector, which replaces one copy of the 18S RNA in the chromosome with the GFP coding sequence by homologous recombination. We also prove that the expression of the integrated GFP is stable and homogeneous in the transfected parasites after months in culture without selective pressure or during its use in hamster infection assays. The fluorescent strains are useful for *in vitro*, *ex vivo* and *in vivo* drug screening assays since no considerable variations in virulence or infectivity where seen attributable to the genetic manipulation during both *in vitro* and *in vivo* infection experiments. The platform described here for drug testing assays based on the use of stable fluorescent *Leishmania* strains coupled to flow cytometry and fluorescent microscopy is more sensitive, more specific and faster than conventional assays used normally for the evaluation of compounds with potential antileishmanial activity.

1. Introduction

Leishmaniasis is a worldwide-expanded zoonotic disease caused by infection with protozoan parasites of the genus *Leishmania*, which has approximately twenty species capable of infecting humans. The clinical manifestation of the disease depends on the species that causing the infection. In the New World many species of the parasite have been associated with localized, disseminated, diffuse and atypical cutaneous and mucocutaneous leishmaniasis; most of those *Leishmania* species, as *L. braziliensis*, *L. panamensis*, *L. guyanensis* and *L. peruviana*, belong to the subgenus *Viannia* which is endemic of the tropical and subtropical regions of South America (WHO, 2010). *Leishmania* parasites have a digenetic life cycle shuttling between flagellate extracellular forms called promastigotes, which resides in the arthropod vector, to intracellular non-flagellate forms called amastigotes, which reside inside the mononuclear phagocytes of the mammalian host (Sacks et al., 1984, 1994).

Substantial differences between species of the genus *Leishmania* regarding the susceptibility to drugs and drug candidates have been demonstrated. Furthermore, strong differences in the susceptibility to drugs between Old and New World *Leishmania* species have been established (Croft et al., 2002, 2006). Current chemotherapy agents for leishmaniasis, which include pentavalent antimonials, miltefosine and amphotericin B are either toxic, expensive, or both, interfering with the effective treatment of people around the world. Identification of new potential drug compounds and molecular targets are the first steps toward new therapeutic approaches and rational drug design strategies (WHO, 2010).

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Testing a large number of drug candidates with the methodologies available to date is hard, because they are either time consuming or inappropriate for assessing the activity of the compounds against the intracellular forms of the parasite or to make *in vivo* assays; therefore is necessary to develop new techniques that decrease all of these drawbacks (Sereno et al., 2007).

Reporter genes are the most promissory approach on the field, since by using this technology the screening assays can be automatized and improved for high-throughput methods or *in vivo* assays. Recombinant parasites expressing diverse reporter genes have been generated as transient or stable transfectants. *L. amazonensis* strains expressing the B-galactosidase gene were generated, but its utility is restricted to the screening of drugs in the extracellular forms of the parasite, which is not the mammal-infecting form (Okuno et al., 2003). *L. major* and *L. amazonensis* strains expressing the B-lactamase gene in an episomal vector have also been generated, but despite their usefulness for drug screening in intracellular forms of the parasite, the utility is still limited because it is necessary to keep strong selective pressure over the parasite in culture to avoid the possibility of elimination of the episomal construct from the parasite. Besides, this methodology requires additional reagents to reveal the activity of the reporter gene, which makes it time consuming and laborious (Buckner and Wilson, 2005). The firefly luciferase gene has been introduced either as an episomal or an integrated transgene in *L. amazonensis*, *L. donovani*, *L. infantum*, and *L. major*. Its utility as a reporter gene was demonstrated in promastigotes and intracellular amastigotes; however, this methodology is still limited by the requirement of substrates for the detection of the luciferase activity, making this technique considerably expensive. Moreover, there are additional downstream procedures that difficult its use in high-throughput assays (Ashutosh et al., 2005; Lang et al., 2005; Roy et al., 2000; Sereno et al., 2001). Finally, the Green Fluorescent Protein (GFP) has been widely used for the generation of a large number of species for *in vitro* drug screening due to its flexibility and sensibility and the possibility of the automation of the screening process by using flow cytometry or fluorometry. Several *Leishmania* strains expressing the GFP have been generated using diverse transfection methodologies. *L. major*, *L. amazonensis*, *L. donovani* and *L. infantum* strains expressing the GFP reporter gene as an episomal transgene were generated in diverse studies, and showed their utility for *in vitro* drug screening assays with promastigotes (Kamau et al., 2001; Okuno et al., 2003; Singh and Dube, 2004). Further studies demonstrated that *L. donovani* carrying the episomal GFP were also useful for screening in intracellular amastigotes but the background noise still difficult the automatization of the technique (Dube et al., 2005). Similar results were obtained when Chan and coworkers developed a *L. amazonensis* strain harboring an episomal expression vector carrying the eGFP gene, and by using flow cytometry analysis it was possible to see that expression of the GFP was not homogeneous in the promastigote population (Chan et al., 2003). Despite promissory results, it was evident that the fluorescence in the transfected *Leishmania* populations was very heterogeneous because the number of copies of the GFP gene was not the same in all the parasites. Additionally, keeping the fluorescence requires that constant selective pressure over the parasites in culture is applied, which limits its use for *in vivo* assays or for extensive use in intracellular amastigotes.

Regardless of the evidence supporting the robustness of the GFP as a reporter gene in *Leishmania* and its utility for drug screening assays, very few works have directed their efforts to the generation of *Leishmania Viannia* strains expressing the GFP. In our lab we developed a *L. panamensis* strain expressing the GFP as an episomal transgene (Varela et al., 2009). Those parasites were useful for drug screening either in promastigotes and intracellular amastigotes, as evaluated by flow cytometry. However, the fluorescence in those parasites was very heterogeneous, as seen in other experiments using episomal vectors, because of the variation in the number of copies of the transgene. Additionally, it was observed that the level of fluorescence and the number of fluorescent cells in the population depend on the grade of selective pressure with tunicamycin. These disadvantages limit the use of these parasites for intracellular drug screening because of the background generated for the low-fluorescent cells present in the intracellular amastigote population, since tunicamycin cannot be added to the infected macrophages. It is also expected that those parasites lose their fluorescence once used for experimental infection in murine models during *in vivo* drug screening assays.

For all the reasons discussed above many efforts have been focused on the development of new methodologies that allow the production of stable recombinant strains expressing exogenous genes. Singh et al. published in 2009 the development of a *L. donovani* strain which constitutively expressed the GFP gene through integration into the 18S rRNA locus by homologous recombination. In this work they proved that expression was stable and homogeneous in the parasite population even in the intracellular stages (Singh et al., 2009). Recently Bolhassani and coworkers developed *L. major*, *L. infantum* and *L. tarentolae* strains expressing the GFP as an integrated transgene. They used an integration vector (pLEXSY) designed initially for transgenic expression in *L. tarentolae* system (LEXSY) by integration of the construct into the *ssu* locus. The pLEXSY-GFP shown to be very efficient for the generation of different strains of GFP-*Leishmania* strains, the fluorescence was homogeneous and persists for long periods of time. Those GFP parasites kept their fluorescence in the intracellular stages, and more surprisingly, it was possible to detect the fluorescence of the parasite in the footpath lesion of experimentally infected mice (Bolhassani et al., 2011). Other approaches for stable expression of reporter genes in *Leishmania* are based on transfection with integration vectors as the pIR1SAT, patented by Dr. Stephen Beverley (2001), which allowed the integration of the GFP gene into the *ssu* locus by homologous recombination showing high expression rates of the reporter gene as demonstrated by flow cytometry. Variations to the pIR1SAT vector have been generated, such as the pIRmcs3(−), created by Clos and coworkers. That vector has the same backbone of the pIRSAT1 plasmid, but a different multicloning site (MCS) was inserted in the unique Bgl II site (Hoyer et al., 2004). Resultant pIRmcs3− plasmid allows the cloning of the gene of interest flanked by leishmanial regulatory regions, in the middle of the 18S rRNA sequence. pIR vectors represent a very powerful tool for stable modification of *Leishmania* genome through integration of the transgenes into the *ssu* locus by homologous recombination. As a result, the inserted gene is transcribed under the control of the rRNA promoter yielding high transcription rates and, since the maturation of the transcripts is made by the autologous mechanisms of *Leishmania*, pIRmcs3− allows the production of functional exogenous proteins inside the parasites.

Here we report the generation of different *Leishmania* strains expressing the GFP reporter gene as a stable-integrated transgene using the pIRmcs3(−) system. The expression of the transgene is homogeneous in the population and keeps stable over long periods of time in absence of selective pressure. These GFP-*Leishmania* strains have been improved for drug screening assays in intracellular amastigotes without any considerable background noise. We also used the fluorescent *L. panamensis* parasites for experimental infections in murine models, and found that the parasites maintained their fluoresce during all of the infection period and do not lose their infectivity or virulence since they are recovered by aspiration from the lesion several weeks after infection and still can be used for a new *in vitro* infection experiments.