

Production of Recombinant Leishmania Tropica Parasites Expressing Two Proteins of Egfp and Luciferase Reporter

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J Babol Univ Med Sci; 19(8); Aug 2017; PP: 58-64

Received: Mar 11th 2017, Revised: Apr 4th 2017, Accepted: May 6th 2017.

ABSTRACT

BACKGROUND AND OBJECTIVE: *L. tropica* is the major cause of cutaneous and visceral leishmaniasis in Iran. Due to lack of an accurate, sensitive and noninvasive detection test in animal model is a major problem. Here, we designed *L. tropica* stably transfected with two reporter genes as fused form, enhanced green fluorescent protein (*egfp*) and Luciferase (*luc*), named as *egfp-luc* to use as a specific tools for detection and measurement of parasite load in live animals.

METHODS: In an experimental study, linearized cassette containing *egfp-luc* genes was stably integrated into Iranian strain of wild-type *L. tropica* genome, 18srRNA locus, by homologous recombination. Transfectants were screened by G418 resistance, confirmed by PCR and western blotting and the expression of EGFP signals were observed by fluorescent microscope and flow cytometry.

FINDINGS: Primary phenotype observations of parasite (by fluorescent microscopy and flow cytometry) were shown that recombinant *L. tropica*^{EGFP-LUC} was successfully produced EGFP protein into cytoplasm. Quantification of EGFP intensity was more than 90%. Furthermore, the results of PCR and western blotting verified the proper integration into genome and expression of both genes in promastigotes.

CONCLUSION: This is the first report to show the generation of recombinant *L. tropica*^{EGFP-LUC} expressing two reporter genes simultaneously.

KEY WORDS: *Leishmania tropica*, cutaneous leishmaniasis, Transfection, reporter genes, EGFP, luciferase, flow cytometry, fluorescent microscopy.

Please cite this article as follows:

Taheri T, Eskandar M, Rafati S. Production of Recombinant Leishmania Tropica Parasites Expressing Two Proteins of Egfp and Luciferase Reporter. J Babol Univ Med Sci. 2017;19(8):58-64.

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Introduction

Iran is one of the endemic areas of cutaneous leishmaniasis, which causes by both *Leishmania major* and *Leishmania tropica*. Skin leishmaniasis occurs in two different forms: rural cutaneous leishmaniasis (wet) caused by *Leishmania major* and urban cutaneous leishmaniasis (dry), which is caused by *Leishmania tropica*. Despite the similarities between the clinical signs of these two species of parasites, there are many differences between them that add to the treatment problems and the diagnosis of this disease (1). *Leishmania tropica* is transmitted from humans to humans and by sand blasts. *Leishmania tropica* can cause visceral leishmaniasis in addition to skin leishmaniasis (2). In addition, evidence of mucosal leishmaniasis from this parasite has also been reported in Iran (2,3). This parasite can be seen in two extracellular (amastigote) and intracellular (promastigote) forms throughout its life cycle.

No definite and effective remedy and vaccine has yet been found for leishmaniasis. One of the reasons is the lack of a quick and effective way to select and screen new and active drugs. In the old methods, the toxic effects of the compounds on the mastigotes were studied and evaluated in cell culture or in animal models after confirmation of contaminated macrophages and staining with Giemsa or microscopic preparation of smear (4).

Several methods are also used to measure the rate of infection of the lymph nodes in animal tissues or in infected macrophages in vitro. The infection in the foot of the mice is also estimated by limited dilution test or observation by microscopy after Giemsa staining (5). Molecular methods such as PCR and Real-time PCR are also commonly used diagnostic methods. Although the mentioned tests are standard methods, but they have some problems such as high cost, time consuming, sacrificing a large number of mice, and most importantly, these methods are not able to detect parasites and infection rates in the early stages of the disease in living tissue or when the amount of parasite in the tissue is low.

In the last decade, the use of reporter molecules whose activity in the cell is measurable has greatly expanded to observe the biological processes that occur in the cell. Meanwhile, visual reporter protein genes such as Luciferase (LUC) and Green Fluorescent Protein (EGFP) are the most appropriate and most used genes. Using the imaging of the mice during the course of the experiment, it is possible to examine them

repeatedly. Therefore, each animal can be studied as its own control during the experiment and during the experiment not only the animal is not eliminated, but the effect of the drug or vaccine on each animal is thoroughly investigated and will prevent a large number of mice being sacrificed.

Another benefit of Luciferase is its high sensitivity than other genes (6). Previously, various types of leishmaniasis have been shown to express EGFP (7-11) proteins or red fluorescent protein mCherry (12) or luciferase (6,13-16) alone and have been used in numerous studies. Recently recombinant parasites *L. major* EGFP-LUC were produced and the amount of parasitic load and their pathogenicity were observed and measured with high precision in sensitive BALB/c mice in the minimal time (15 days after injection of the parasite) (15). The purpose of this study was to produce recombinant leishmania tropica (*L. tropica* EGFP-LUC), so that in future studies it can be used as an accurate and rapid tool for screening drugs and vaccines on infected BALB / c mice with this parasite. Considering the high sensitivity of the reporter genes, using the imaging method before sacrificing the rats, the estimated parasitic load in the tissue can be estimated.

Methods

Parasite culture: In this experimental study, the *Leishmania tropica* parasites of the wild straw of Iran (MOHM.IR.09.Khamesipour-Mashhad) or transfected with EGFP-LUC (*L. tropica*EGFP-LUC) in a complete M199 medium containing 10 % heat-inactivated fetal calf serum and supplements (40 mM HEPES, 2 mM L-glutamine, 0.5 mg/ml Hemin and 50 mg/ml gentamicin sulfate) were cultured (15,17).

Preparation and screening of the recombinant Leishmania Tropica: pLEXSY-EGFP-LUC-Neor plasmid was purified in a DH5 α bacterial host by plasmid DNA extraction kit (15,17). The 5'SSU-EGFP-LUC-Neor-3'SSU fragment was digested with the *Swa*I restriction enzyme and purified from the plasmid. Approximately 10^8 promastigotes of wild type *Leishmania tropica* in the growth logarithmic phase were solved in electroporation buffer (21 mM HEPES, 137 mM NaCl, 0.7 mM Na₂HPO₄ and 6 mM glucose, pH 7.5). 400 μ l of parasite suspension (containing approximately $10^7 \times 4$ parasites) was mixed with approximately 10 μ g of DNA (or without DNA as a negative control) and was poured inside the

2 ml cold bottle (Bio-Rad, USA) and was incubated for 10 minutes on ice. The parasites were then transfected with 500 microfarads using an electroporation device (Gene PulserEcell, USA) and two electrical pulses with a time interval of 20 seconds at 450 volts. Transfected parasites were transferred to 3 ml of complete M199 medium without antibiotics. For screening and selection of parasites recipient gene construct, parasites were transferred to plates containing 2% Noble agar and M1992x medium with or without G418 antibiotic after 24 hours, and were incubated until growing of clones at 26 °C (15,17).

Genotypic studies and confirmation of the similar recombination occurrence in transgenic or recombinant parasites: antibiotic-resistant parasite clones and their genomic DNA were extracted using a kit (GF-1, Vivantis). First, the presence of reporter genes in the genome was confirmed by using PCR and specific primers of egfp and luciferase genes (17). Then the homologous recombination in Lucas 18 rRNA was studied using specific F3001 primers (similar to the parasite genome sequence) and A1715 (similar to the plasmid sequence) (table 1).

Western Blot: Qualitative study of EGFP and LUC proteins was performed using Western Blot and monoclonal antibodies against GFP and LUC conjugated to HRP (Acris Antibodies GmbH). Deposition of suspension of recombinant parasites and wild type (as negative control) with 2X sample buffer (4.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol color Blu) was blended and boiled for 5 minutes and electrophoresed on SDS-PAGE % 12.5 gel. Separated protein bands were transferred to the nitrocellulose paper (Protean, Schleicher & Schuell) using a Bio-Rad blotting system. Nitrocellulose was coated with a buffer containing TBS buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween20) and 2.5% serum albumin protein and then it was adjoined for 2 hours with anti-GFP antibody (dilution 1: 5000) or LUC (dilution 1: 10000). After washing and removing free antibodies in

the environment, the paper was placed adjacent to the DAB substrate (3, 30-Diaminobenzidine) to allow the bands to appear and finally stopped by placing it in the water (15,17).

Investigation of EGFP expression by microscopic and flow cytometric observations: To measure the amount of EGFP fluorescence expressed in recombinant parasite promastigotes cytoplasm, parasite suspension was washed at a concentration of 10^6 para/ml in a PBS solution (8 ml Na₂HPO₄, 75.1 mM KH₂PO₄, 0.25 mM KCl, 137 mM NaCl, pH 7.2) and then examined in this solution using FOCS caliburBD (Becton Dickinson, Franklin Lakes, NJ) flow cytometry system (7,13,14). In addition, the expression of egfp gene was observed in terms of quality using a fluorescence microscope (Epi-fluorescence microscope, Nikon, E200) (17).

Results

Preparation of recombinant leishmania tropica parasites and clone screening: The gene of EGFP-LUC reporter proteins was stably transfected in the wild type Leishmania tropica genome, and clones containing genes receiving resistance gene were selected in the presence of G418 antibiotic.

Genotypic studies and confirmation of the similar recombination occurrence in transgenic or recombinant parasites: Using various primers (table 1), the presence of egfp (EGFP2, EGFP1) and luc (LUC2 and LUC1) genes in the genome, as well as the precise location of these two genes into the genome in the locus 18srRNA F3001, A1715) was confirmed. In Figure 1, the binding position of primers has been determined. Figures 2a and 2b show PCR results for the presence of egfp genes (fragment of approximately 750 bp) and luciferase (fragment of approximately 1650 bp), and Figure 2c shows the correct recombination in the recombinant parasite genome (fragment of approximately 1000 bp).

Table 1. List of primers used in this study

Gene or position name	Primer name	Primer sequence	Product length (bp)
egfp	EGFP1	5'-ATGATATCAAGATCTATGGTGAGCAAGGGC-3'	~750 bp
	EGFP2	5'-GCTCTAGATTAGGTACCCCTGTACAGCTCGTC-3'	
luciferase	LUC1	5'-GCTAAGCTTATGGAAGACGCCAAAAACATAAAG-3'	~1650 bp
	LUC2	5'-ATTCTAGATTACACGGCGATCTTTCCGGCAC-3'	
Similar recombination	F3001	5'-GATCTGGTTGATTCTGCCAGTAG-3'	~1000 bp
	A1715	5'-TATTCGTTGTCAGATGGCGCAC-3'	



Figure 1. Gene's position and primer binding site. Plasmid gray line, black line genome and the arrows show the primer binding site.

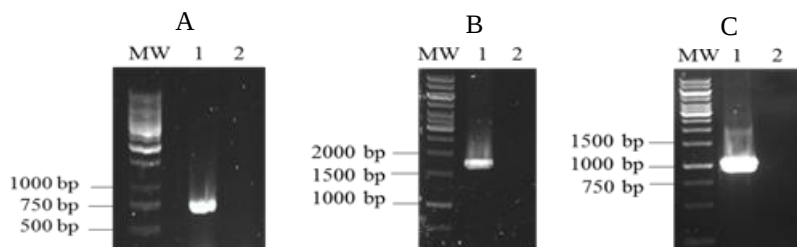


Figure 2. PCR results to confirm the presence of egfp (a), luc (b) genes and confirmation of recombination occurrence in the recombinant parasite genome (c). MW: Molecular Weight Index, Column 1: Recombinant Parasite and Column 2: Wild type parasite

Investigating the expression of reporter proteins using western blot: expression of reporter conjugated proteins with the presence of a band of about 89 kDa in recombinant parasites using western blot and anti-EGFP antibodies (Fig 3, column 2) and anti-luciferase (Fig 3, Column 3) were verified. Given that the weight of EGFP and luciferase proteins are 27 and 62 kDa respectively, a band of about 89 kDa represents the expression of two reporter genes as conjugated. Columns 1 and 4 show the wild type parasite that was used as a negative control.

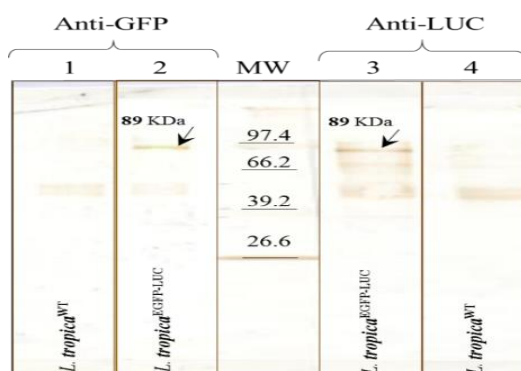


Figure 3. Results of reporter gene expression in recombinant parasites using western blot and anti-EGFP and luciferase antibodies. Columns 2 and 3: Recombinant parasites, columns 1 and 4: wild type parasites and MW: Molecular Weight Index

Investigation of EGFP expression using fluorescence microscopy and flow cytometry: phenotype of *L. tropica*^{EGFP-LUC} parasites was investigated for expression of EGFP protein using fluorescence microscope. As shown in Fig. 4a, the

EGFP reporter protein was expressed in the parasite cytoplasm so that the whole parasite body can be seen in green fluorescent. To investigate quantitatively and estimate the amount of EGFP fluorescence, several clones of transfected parasites were studied using flow cytometry. The percentage of fluorescent EGFP in different clones was 91 to 98%. Figure 4b shows the flow cytometric results of one of the recombinant parasite clones

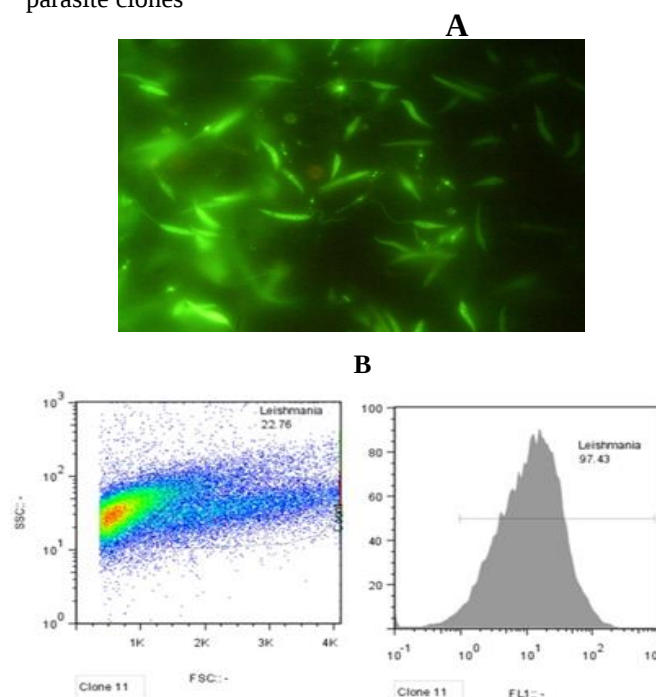


Figure 4. A. The image of the *L. tropica*^{EGFP-LUC} parasite using a fluorescence microscope (1000 × magnification). B) Results of EGFP fluorescence intensity in *L. tropica*^{EGFP-LUC} parasite using flow cytometry

Discussion

In this study, *Leishmania tropica* parasite expressing both EGFP and luciferase proteins was produced. The fluorescence fluctuation analysis showed more than 90% fluorescence in recombinant parasites. On the other hand, the results of western blotting using both anti-LUC and EGFP antibodies indicate that two proteins are successfully coupled together. Previously, egfp or luciferase genes have been used separately in the *Leishmania* parasite. But this is the first report that two egfp and luc-linked genes have been transfected into *Leishmania tropica*. In the case of pathogenicity of this type of *Leishmania* parasite in the host mammal and the existence of an appropriate animal model, there is not enough knowledge (18), and this increases the importance of studying this parasite.

Also, the use of tools with high specificity and sensitivity that can detect the minimum amount of parasite in the infected tissue in the shortest possible time and not being invasive for animal and have the least possible damage or victimization of the mouse seems necessary in studies and research. In previous studies, it has been shown that by using recombinant *leishmania major* parasites expressing egfp and luciferase reporter genes, alone or in association with each other, the presence of parasitic infections and the progression of disease in the site of injection of parasite or in the lymph nodes can be traced and measured by using imaging techniques before scarifying mouse (15, 7). There are very few reports on the expression of reporter genes in *Leishmania tropica*. In 2013 Parbhu-Patel et al., produced *Leishmania Tropica* containing egfp gene and used only in vitro

tests (19). In 2012, Talmi-Frank et al. injected *leishmania tropica* transfected with the LUC gene into the rat ear, but only one day after the infection the parasitic load was observed in the infected position using the bioluminescence imaging (18).

One of the benefits of the reporter protein is their neutrality, and one of the most controversial aspects of using these proteins is the effect of their expression on the immune system of the mouse. There are many studies in this field and there are conflicting reports. Some investigations refer to their ineffectiveness, and the results of some studies also suggest that EGFP proteins affect the immune system (21, 20, 5, 4). Sadeghi et al. showed that there is no difference between the wild type *L. major* with *L. major* EGFP-LUC recombinant class in vitro (22).

In a similar study, Seif et al. indicated that although the recombinant parasites in BALB / c mice exhibit more inflammation than wild type parasites in the infected position, there is no significant difference in parasitic load and immune response between them (23). Based on the results of this study, recombinant *leishmania tropica* parasites expressing both the EGFP and LUC reporter protein were simultaneously successfully produced.

Acknowledgments

Hereby, we would like to thank the cooperation of Ms. Motamedi in the department of virology (collaborative work on flow cytometry) and Mr. Shahram Gholam Ali Zad in the department of immunotherapy and research on *Leishmaniasis* vaccine.

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