

# Molecular Cloning of *Leishmania major* gp63 Gene in BALB/c Mouse CT26 Cell Line

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**Background:** Leishmaniasis is now accounted as a worldwide disease, which is caused by different species of *Leishmania* parasite. This parasitic disease is now endemic in many areas including 16 developed and 72 developing countries. An estimated 12 million cases of leishmaniasis exist worldwide and a further 367 million are at the risk of acquiring the disease. A high rate of *Leishmania* and HIV co-infection has now been reported from 30 countries around the world. Th1 immune responses have the main role in eliciting immunity to *Leishmania* parasite. Many attempts have been made and different strategies have been approached to develop a potent vaccine against this parasite.

**Objectives:** The main objective of this study was to clone and detect the gene encoding *Leishmania major* gp63 in mouse CT26 cell line.

**Materials and Methods:** In this observational study, the *Leishmania major* gp63 gene segment was amplified using specific primers and then sub-cloned into pcDNA3 expression vector. The new construct was transfected into mouse CT26 cell line.

**Results:** The results clearly showed that pcDNA3 *L. major* gp63 was successfully constructed and transfected into CT26 mouse cell line and these cells have a high potency in accepting and expressing *Leishmania* genes.

**Conclusions:** A full length of *L. major* gp63 gene was cloned in to mouse CT26 cell line, which can be used in future vaccine studies for *Leishmania*.

**Keywords:** *Leishmania major*; gp63; Cloning; BALB/c

## 1. Background

Leishmaniasis as a parasitic disease is now considered as a neglected disease by WHO in many countries around the world. However, the high incidence rate of the disease occurs in tropical and temperate regions where the *Leishmania* vector lives [1]. According to WHO, the number of leishmaniasis cases has been increasing during last two decades where more sensitivity with a high rate of *Leishmania* and AIDS co-infection cases is reported in patients suffering from AIDS in many areas [2-5]. Chemotherapy against *Leishmania* infestation, because of the toxicity and drug resistance, is not totally worthwhile [6, 7]. It has been shown that Th1 immune responses are crucial in immunity to *Leishmania* parasite [8] and because of the capability of single antigenic proteins in inducing Th1 immune responses, these proteins are now in main focus of vaccine candidates against the pathogen [9, 10].

Vaccination is postulated as the most appropriate opportunity for the prevention and safe treatment of all forms of the disease so developing a safe, effective and affordable anti-leishmanial vaccine is accounted as a promising approach for *Leishmania* researchers [11].

Several studies have been carried out to prove the immunogenicity of killed *Leishmania* parasite using different adjuvants such as Bacillus Calmette Guerin (BCG) [12] but no significant protection was observed following the in-

jection of the vaccine. However, the mixture was safe and induced *Leishmania* Skin Test (LST) conversion with weak but measurable IFN- $\gamma$  production. Using live *Leishmania* as a potent vaccine (leishmanization) has been considered as another strategy in the prevention of leishmaniasis [13]. Although, the live vaccine is of low cost and highly immunogenic, it still lacks standardization and quality control.

Genetically modified *Leishmania* is carried out either by mutagenesis and or gene targeting methods in which either a foreign gene is introduced into the parasite genome or by knocking out virulence genes [14]. Recently, recombinant proteins of *Leishmania* species have been used to produce the immunity against different species of *Leishmania*. Different proteins have been tested to be used as a vaccine including recombinant acylated surface protein B1 (HASP B1) and promastigote surface antigen-2 (PSA-2), which are immunogenic and can develop variable levels of immunity in different animal models.

*Leishmania* gp63 or leishmanolysin, an immunogenic *Leishmania* protein, is shown to be present in all *Leishmania* species and accounted as a good vaccine candidate in *Leishmania* studies [15, 16]. Injection of gp63 in saline, Complete Freud's Adjuvant (CFA), Bacillus Calmette Guerin (BCG) and *Corynebacterium parvum* induced significant protection in mice. So, cloning *Leishmania* gp63 into a

BALB/ mouse cell line would be a feasible strategy in developing a potent cell line expressing an immunogenic Leishmania protein to be used for Leishmania vaccines.

## 2. Objectives

The main objective of this study was to clone and detect the gene encoding *L. major gp63* in mouse CT26 cell line.

## 3. Materials and Methods

In this observational study, N-methylurethane-induced BALB/c murine colon carcinoma (CT-26) cells were gifted by Prof Ian Hart (St Thomas hospital) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) + 10% Fetal Calf Serum (FCS).

### 3.1. Preparation of *L. major* cDNA

CMV promoter pNUT *L. major gp63* DNA was kindly gifted by Prof Mack Master (Department of Medical Genetics, University of British Columbia, Vancouver, Canada). The construct was first sequenced by MWG-Biotech using the primers ATCACGAAGTCGGTGTG, GCACATCACCGAGGGCTT and AGAGCGAGGACGCCATCC and checked for mismatches against the sequence of the gene (code number Y00647) in the gene bank.

### 3.2. PCR Amplification

PCR was performed using a DNA thermal cycler (Thermo Hybaid, USA) for amplification of the whole gene. Primers were supplied by Sigma Genosys (UK). For amplification by PCR, 1 µL of cDNA was mixed with 5 µL of 10 × PCR buffer, 0.8 µL each of 10 mmol dNTP, 20 pmol each of primer solutions, 1.25 unit of thermostable Taq polymerase (Bioline), 1.5 mmol MgCl<sub>2</sub> (Bioline) and then water was added to a total volume of 50 µL. The PCR program was set up as an initial melting step at 95°C for 10 minutes followed by 35 cycles including 1 minute at 95°C for denaturation, 45 seconds at 58°C for annealing and 2 minutes or 45 seconds (only for screening) at 72°C for extension and a final extension step at 72°C for 10 minutes. PCR products were visualized using a 1.5% (w/v) agarose gel containing 1 µg/mL of ethidium bromide (BDH Laboratories, UK).

### 3.3. Ligation of *L. major gp63* Gene and pcDNA3 Vector

The *L. major gp63* gene produced by PCR using the primers containing HindIII and EcoRI restriction sites and the pcDNA3 vector were digested with relevant restriction enzymes and run onto the gel agarose. The gene and the vector were then cut and extracted from the gel by DNA extraction kit (Gene Flow) with accordance to the manufacturer's protocol and ligated together using ligation enzyme (promega). The ligation was set up by adding 0.5 µL ligation enzyme, 1 µL buffer and 6.5 µL water to 2 µL of each DNA (adjusted at 10 µL). The ligated DNA was incubated at 4°C overnight. The pcDNA3 vector containing *L.*

*major gp63* gene was reproduced by transforming *E. coli* and extracting the DNA from the bacteria.

### 3.4. Antibiotic Sensitivity Assay

In presence of a serial concentration of Geneticin (G418) (Sigma) from 50 to 900 µg/mL, 1×10<sup>6</sup>/well CT26 tumour cells were cultured in 24-well plates; wells for each concentration of the antibiotic were put in duplicate. The cells were incubated at 37°C with 0.5 CO<sub>2</sub> for 10 days. The lower concentration (500 µg/mL) of the antibiotic in which all the CT26 tumour cell died in 7-10 days was chosen for transfection.

### 3.5. Transfection of CT26 Tumour Cells With *L. major gp63*

Lipofectamine 2000 (Invitrogen) was used to transfect CT26 tumour cells with pcDNA3 *L. major gp63*. Briefly, the culture of CT26 tumour cells at 1×10<sup>6</sup> per well in 24-well plates with 90% confluence was chosen. pcDNA3 *L. major gp63* and lipofectamine 2000 were diluted in serum free DMEM media at 0.8 µg/50 µL and 2 µg/50 µL respectively and incubated for 5 minutes at room temperature. The diluted DNA and lipofectamine 2000 were mixed together and incubated again at room temperature for 20 - 30 minutes. After removing the supernatant, the DNA-lipofectamine was added to the CT26 cell culture and the culture was incubated at 37°C with 0.5% CO<sub>2</sub> for 4 - 6 hours. One milliliter per well DMEM media containing 10% FCS was then added to the culture. Sixteen until 24 hours later, the media was replaced with a fresh media complemented with 10% FCS and 500 µg/mL G418.

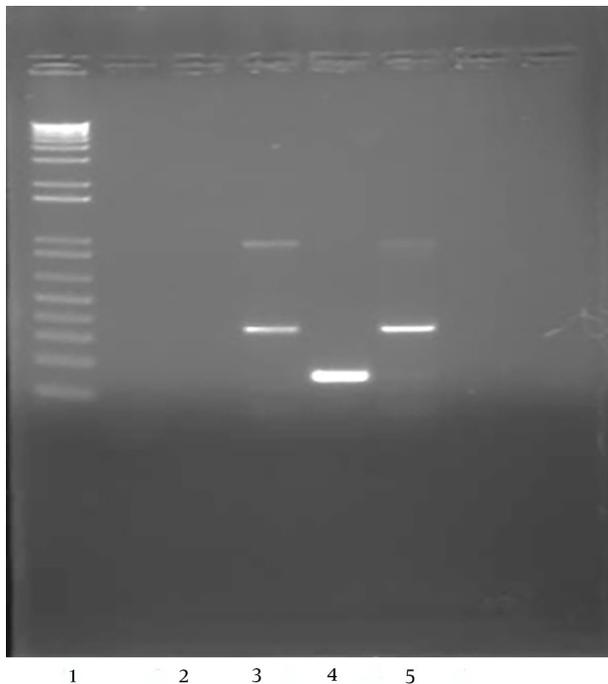
### 3.6. Screening the Expression of *L. major gp63* Gene by RT-PCR

The stable transfected CT26 tumour cells were screened for the presence of the *L. major gp63* gene by RT-PCR. Total RNA was isolated from the cells using RNA STAT-60 (AMS Biotechnology, UK) following manufacturer's instructions. Briefly, CT26 *L. major gp63* were cultured in T25 tissue culture flasks. After removing the media, 1 mL of RNA-STAT60 was added to the cell culture followed by incubating at room temperature for 5 minutes, adding 0.2 mL of chloroform, vigorously shaking for 60 seconds and leaving at room temperature for 3 minutes. The homogenate was then centrifuged at 14,000 rpm for 10 minutes and the aqueous phase was separated. Total of 0.5 mL of isopropanol was added the aqueous phase of samples followed by 8 minute incubation at room temperature and 15 minute centrifugation at 14,000 rpm. After removing the supernatant, the RNA pellet was washed with 75% ethanol, dried and resuspended in molecular grade water.

### 3.7. RNA was Then Reverse Transcribed into cDNA as Follow

Two microgram of RNA and 0.5 µg of oligo (dT15) primer



**Figure 5.** *L. major* gp63 RT-PCR:

1: standard DNA, 2: PCR control, 3, 5: mouse GAPDH, 4: *L. major* gp63 cDNA.

#### 4.3. Transfection of CT26 Tumour Cells With *L. major* gp63

pcDNA3 *L. major* gp63 was applied onto CT26 tumour cells for transfection of the cells using lipofectamine 2000 (see materials and methods). The presence of the gene was examined with detection of its mRNA using RT-PCR. The following mouse GAPDH primers 5'-ACTCCACTCACGGCAAATTC-3' and 5'-CCTTCCA CAATGCCAAAGTT-3' were used to check the efficacy of the mRNA extracted from the CT26 transfected cells and specific *L. major* gp63 primers used to check the presence of the gene (Figure 5).

### 5. Discussion

The results of our study clearly showed that *Leishmania* gp63 successfully transfected into CT26 mouse cell line and these cells have a high potency in accepting *Leishmania* genes and expressing the parasitic proteins.

Developing specific resistance after infection approves the feasibility of vaccine approaches against *Leishmania* [17]. Different attempts have been made to generate *Leishmania* vaccines using recombinant immunogenic *Leishmania* proteins expressed in microbial vectors but none has yet been introduced as a vaccine for *Leishmania* [18, 19]. However, the potency of mammalian cells in expression of *Leishmania* immunogenic genes is not yet fully cleared.

Cloning and characterization of antigenic *Leishma-*

nia genes is now postulated as a promising approach in *Leishmania* vaccination. So far, different studies have been conducted to clone, express or manipulated *Leishmania* genes. Cloning and characterization of a gene encoding class I nuclease in *L. infantum* has already been carried out by Farajnia et al. [20]. Also, there are other reports on cloning and expression of different *Leishmania* genes such as HSP70 and glucose-regulated protein 94 (GRP94) gene from *L. infantum* [21, 22], surface protein encoding genes from *L. major* [23, 24] and *L. donovani* avastin gene [25]. Some other researchers tried to find similarity between genes cloned in other protozoa and the *Leishmania* ones in order to develop immunity against *Leishmania* [26]. There are some attempts for establishing cell lines specific for *Leishmania*, which were promising [17]. One of the characterised immunogenic proteins in all *Leishmania* species is *Leishmania* gp63 or leishmanolysin [16]. It has been shown that vaccination based on gp63 DNA or recombinant gp63 protein is potential to induce T cell stimulation against *Leishmania* in human producing high levels of INF- from effector T cells [27, 28]. Our previous studies also proved the immunogenicity of peptides predicted for either class I or II derived from this protein [29, 30]. We also showed that expression of *Leishmania* gp63 protein in host's cells by either injection or using the gene gun can elicit immunity to *Leishmania* [31]. Similar results have been demonstrated for LACK antigen where in presence of recombinant IL-12 evokes CD4+ T cell inducing protection in mice against *L. major* infection [32]. Therefore, cloning *Leishmania* gp63 into a BALB/mouse cell line would be a feasible strategy in developing a potent cell line expressing an immunogenic *Leishmania* protein to be used for *Leishmania* vaccines.

In present study, it was tried to clone a gene derived from protozoan cells and express it in mammalian cells in order to develop a potent mammalian cell line expressing an immunogenic *Leishmania* protein, which can be used for *Leishmania* vaccines. To clone the gene, *L. major* gp63 DNA was first cut from a week construct (pNUT vector) and sub-cloned into pcDNA3 vector using specific primers.

In conclusion, a full length of *L. major* gp63 gene was cloned into the mouse CT26 cell line and the stable expression of the gene was evaluated. The results confirmed that *Leishmania* genes can be successfully expressed in mammalian cell lines, which could be used as a potential vaccine against *Leishmania*.

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