Cloning and Constructing a Plasmid Encoding *Leishmania* Eukaryotic Initiation Factor Gene of *Leishmania* major Fused with Green Fluorescent Protein Gene as a Vaccine Candidate

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ABSTRACT

Objective: *Leishmaniasis is usually treated with chemotherapy; however, toxicity, resistance and high-cost limit use of the chemical drugs.* Leishmania *eukaryotic initiation factor (LeIF) protein acts the same as interleukin (IL)-12 and reduces the secretion of IL-4 in lymph node cells of mice infected with* Leishmania *major. The aim of this study was cloning of the gene encoding LeIF antigen into eukaryotic expression plasmid pEGFP-N1.*

Methods: *DNA was extracted from Iranian strain of the L major (MRHO/IR/75/ER) promastigotes. The full-length sequence of LeIF was amplified with Pfu DNA polymerase using a specific primer. The amplified LeIF was cloned into a pJET1.2/blunt vector. Then this fragment was digested with HindIII and EcoRI and was subcloned into the pEGFP-N1 vector. Confirmation of the cloning was done by colony polymerase chain reaction (PCR).*

Results: Leishmania *eukaryotic initiation factor gene was successfully cloned and subcloned into pJET1.2 and pEGFP-N1 plasmids, respectively. The results of colony PCR, restriction analysis and sequencing confirmed them.*

Conclusion: *We cloned LeIF gene which could be expressed in eukaryotic cells* in vivo *and could be used as a vaccine candidate against leishmaniasis in future studies.*

Keywords: Cloning, DNA vaccine, *Leishmania* eukaryotic initiation factor, *Leishmania* major

Clonación y construcción de un plásmido que codifica el gen del factor iniciador eucariótico de *Leishmania* de *Leishmania* major fundido con el gen de la proteína verde fluorescente como vacuna candidata

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RESUMEN

Objetivos: *La leishmaniasis generalmente se trata con quimioterapia. Sin embargo, la toxicidad, la resistencia, y el alto costo limitan el uso de los medicamentos químicos. La proteína del factor iniciador eucariótico de Leishmania (LeIF) actúa igual que la interleucina (IL)-12 y reduce la secreción de IL-4 en células de ganglios linfáticos de ratones infectados con Leishmania major. El objetivo de este estudio fue la clonación del gen que codifica el antígeno LeIF transformádolo en el plásmido de expresión eucariótica pEGFP-N1.*

Métodos*: Se extrajo ADN de una cepa iraní de los promastigotes (MRHO/IR/75/ER) de L major. La secuencia de LeIFen toda su longitud fue amplificada con la Pfu DNA polimerasa utilizando un primer específico. El LeIF amplificado fue clonado y convertido en el vector de pJET1.2/blunt. Luego este fragmento fue digerido con HindIII y EcoRI, subclonado para convertirlo en el vector pEGFP-N1. La confirmación de la clonación fue realizada mediante reacción en cadena de la polimerasa (RCP) de colonias.*

Resultado: *El gen del factor iniciador eucariótico de Leishmania fue exitosamente clonado y subclonado obteniéndose plásmidos pJET1.2 y pEGFP-N1, respectivamente. Estos fueron confir-*

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mados por los resultados de RCP de colonias, el análisis de restricción, y la secuenciación. ***Conclusión:*** *Clonamos el gen LeIF que podría expresarse en las células eucariotas in vivo y podría ser utilizado como vacuna candidata contra la leishmaniasis en futuros estudios.*

Palabras claves: clonación, vacuna ADN, factor iniciador eucariótico de *Leishmania*, *Leishmania* major

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INTRODUCTION

Leishmania is an obligate intracellular protozoa causing leishmaniasis. The disease is an important public health problem that has infected 12 million people in 88 countries. Also, two million new cases and 60 000 deaths have been reported due to leishmaniasis per year. The disease rate has risen with HIV in Europe (1, 2). Leishmaniasis is usually treated by chemotherapy (3). The first-line drugs that are used to treat leishmaniasis include glucantime and Pentostam. There are other drugs such as pentamidine and amphotericin B that may also be administered (4, 5). However, the emergence of resistance to the traditional antileishmanial compounds greatly reduces their efficiency (6). In addition, treatment with alternative drugs and new formulations of old compounds are not suitable because of high toxic effects, resistance issues, excessive prices and long-term administration (7, 8).

There are different vaccine candidates against leishmaniasis such as heat shock proteins (HSP), *Leishmania* eukaryotic initiation factor (LeIF), cysteine proteases, L major stress-inducible 1 (LmSTI1) *etc* (9). *Leishmania* eukaryotic initiation factor is a prototype of the DEAD-box protein family that induces the production of interleukin (IL)-12, interferon gamma (IFN-ɤ), IL-10 and tumour necrosis factor-alpha (TNF-α) by macrophages and dendritic cells (10). Both N-terminal and C-terminal parts of LeIF protein induce their production (11). Green fluorescent protein (GFP) is derived from different organisms such as the jellyfish *Aequorea victoria* (12) and is used as a reporter of gene expression (13). The protein intrinsically has fluorescent characteristics, while other fluorescent proteins need a cofactor. Also, GFP does not interfere with normal cell activities and does not have high toxicity (14). Therefore, it has been used for gene expression measurement (14). There are no efficient vaccines for leishmaniasis, therefore, development and production of an effective vaccine is necessary. The aim of this study was construction of a plasmid encoding LeIF protein into eukaryotic expression vector pEGFP-N1 using it as a DNA vaccine candidate in the next studies.

METHODS

Culture of parasites

Leishmania major promastigotes (MRHO/IR/75/ER, Iranian strain) were cultivated at 26 °C in RPMI-1640 (Sigma) plus L-glutamine (20 mM), supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/mL) and streptomycin (100 µg/mL; Sigma).

Amplification of LeIF gene by polymerase chain reaction

Genomic DNA was extracted from the promastigote stage according to the procedure of Genomic DNA Extraction Kit (Bioneer, Korea). We used a pair of oligonucleotide based on the LeIF gene sequence obtained from GenBank [accession no. XM_003721551] (15). The full-length encoding sequence of LeIF was amplified (about 1212 bp) with PFU polymers to produce a blunt ended fragment by specific primers. Upstream primer contained a HindIII site (underlined) and downstream primer contained an EcoRI site (underlined) and without a stop codon (upstream: 5'- CAATTA AAGCTT ATG GCG CAG AAT GAT AAG ATC GCC- 3' and downstream: 5'- CAT GGA ATT CCG CTT ACT CGC CAA GGT AGG CAG -3'). Polymerase chain reaction (PCR) amplification was performed using the following programme: initial denaturation at 95 °C for five minutes, denaturation at 95 °C for 1 minute, annealing at 48–54 °C for one minute, extension at 72 °C for one minute, 35 cycles and final extension at 72 °C for 10 minutes.

Cloning of LeIF gene into pJET1.2/blunt vector and transformation into E coli

The PCR product was recovered from agarose gel by Vivantis kit and was ligated into pJET1.2/blunt cloning vector (Fermentas) at 22 °C for 60 minutes; the ligation reaction product was transformed into *Escherichia coli* bacteria strain TOP10 by calcium chloride method. The transformed bacteria were cultured on Luria-Bertani agar media containing 100 µg/mL ampicillin and were incubated overnight at 37 °C. Cloning confirmation was done by two methods: colony PCR and sequencing, and then colonies containing recombinant plasmids were selected for subsequent works.

Subcloning of LeIF gene into eukaryotic expression plasmid pEGFP-N1

Recombinant plasmid was digested with EcoRI and HindIII enzymes (Fermentas) and the LeIF gene was recovered from agarose gel and was subcloned into the same restriction sites of the eukaryotic expression vector pEGFP-N1. The recombinant plasmid containing LeIF and enhanced GFP genes was purified by GF-1 Plasmid DNA Extraction Kit (Vivantis, Malaysia). Comparison of the recombinant pEGFP-LeIF and non-recombinant pEGFP-N1 plasmids, colony PCR and enzymatic analysis were confirmed subcloning.

RESULTS

DNA was extracted from *Leishmania* promastigotes and assessed on 1% agarose gel. The LeIF gene was amplified by

specific primers and PCR product showed a single band about 1212 bp. The PCR product was cloned into pJET1.2 blunt plasmid and the only bacteria containing inserted plasmids with LeIF gene grown on Luria-Bertani agar with 100 µg/mL ampicillin. Sequencing and colony PCR (Fig. 1) confirmed cloning. Sequencing results indicated 100% identity with *Leishmania major* strain Friedlin in GenBank (accession no. XM_003721551) and was submitted to GenBank with the accession number of KF752601. Colony PCR results demonstrated that the LeIF gene had been properly subcloned into eukaryotic expression vector pEGFP-N1. Also, the analysis of enzymatic restriction displayed that recombinant plasmid was digested by EcoRI and HindIII enzymes and was isolated in the LeIF gene (Fig. 2).

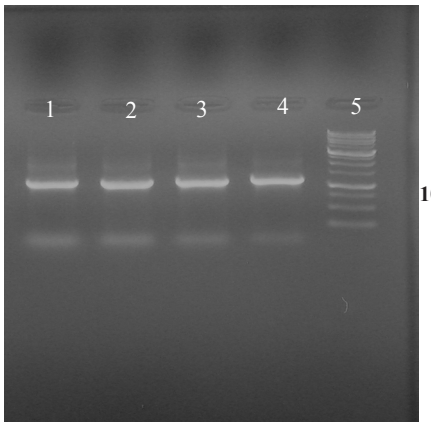

Fig. 1: Electrophoresis of colony polymerase chain reaction of pJET-LeIF plasmid on 1% (w/v) agarose gel. Lanes 1–4: LeIF gene (about 1212 bp), Lane 5: 1Kb ladder

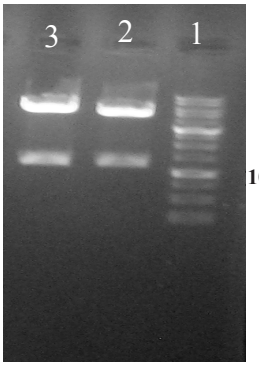

Fig. 2: Electrophoresis of LeIF-pEGFP-N1 plasmid on 1% (w/v) agarose gel. Lane 1: 1 Kb ladder, Lanes 2 and 3: pEGFP-N1-LeIF plasmid was digested with HindIII and EcoRI.

DISCUSSION

DNA vaccines are bacterial plasmids encoding antigen whose release into living cells leads to expression of recombinant protein (16). These plasmids were used for protective and therapeutic targets (17). The vaccines are able to induce both humoral and cellular immune response against the encoded antigen. Because plasmid DNA contains CpG ODN sequences, it can also activate the innate immune response which results in up-regulation of co-stimulatory molecules, production of IL-12, and inflammatory cytokines and oxidant radicals by macrophages (18).

Proteins containing GFP can be directly detected in a living cell during real time using time lapse fluorescence microscopy. And it does not require fixing and staining cells with specific antibodies to the protein that may be fixed artefacts (12). The LeIF stimulates production of proinflammatory cytokines such as IFN-ɤ by IL-12/IL-18-dependent mechanisms but it down-regulates production of IL-4 (10, 15). Furthermore, LeIF induces generation of IL-2 and TNF-α and reduces production of IL-10 in peripheral blood mononuclear cells (10).

In this study, the LeIF gene was cloned into pEGFP-N1 plasmid which contains cytomegalovirus promoter and is used as a powerful system for expression of recombinant proteins in eukaryotic cells. The cloned gene does not have a stop codon and will be expressed as a fusion protein that is located in the upstream of enhanced GFP gene. Enhanced green fluorescent protein fragment has a kozak sequence which enhances protein expression level; also, using GFP gene, the expression of this protein can be detected. According to the previous studies, LeIF antigen acts as a Th1-type natural adjuvant and stimulates Th1 response by IL-12 in the persons infected with *Leishmania* (9). *Leishmania eukaryotic* initiation factor, in combination with other proteins or separately, is used as an antigen to construct a recombinant protein or a DNA vaccine. For example, LeIF is a part of a recombinant polyprotein vaccine, namely Leish-111f that protects against several species of *Leishmania* (19). Finally, we made a recombinant plasmid encoding fusion of LeIF and GFP genes, which we can use for future studies, such as for a DNA vaccine.

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