

Cloning of *Leishmania Major* P4 Gene

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Abstract

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Objective: *Leishmania major* P4 gene is normally expressed during amastigote form of the parasite and can be good candidate for producing an effective vaccine. In this study we cloned this gene in suitable vector (pQE-30) for further vaccine preparation studies.

Materials and Methods: *Leishmania* promastigotes were grown in N.N.N. medium and culture in RPMI 1640 cell culture medium. Total genomic DNA was extracted by centrifugation of promastigotes. The pellet was suspended in lysis buffer and followed by boiling method. PCR was carried out using P4 gene specific primers. PCR product was detected by agarose gel electrophoresis and cloned into Bluescript plasmid via T/A cloning method. Reaction was transformed into XL1- Blue competent cell and recombinant plasmid screened using agar plate contained X-gal and IPTG. The product was extracted, digested by restriction enzyme and electrophoresed on agarose gel.

Results: Plasmid was extracted and cloned gene was released by restriction enzyme and subcloned into pQE-30 expression vector.

Conclusion: This construct is ready for protein expression in *in-vitro*.

Keywords: P4 Gene, Cloning, *Leishmania Major*

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Introduction

Leishmania is an obligate intracellular protozoan parasite with two forms in its life cycle: One as a flagellated promastigote within the phlebotomus vector digestive tract and another one as an amastigote within the host mammalian macrophages (1).

Different species of *leishmania* cause a wide spectrum of diseases, ranging from self-limited cutaneous to the more severe diffuse cutaneous and visceral leishmaniasis. Because of clinical treatment failures, significant adverse effects of drugs and drug resistancy, preparation a suitable vaccine is paid more attention (2).

Antigens specific for the amastigote stage of the parasite have been the main interest in the construction of a leishmanial vaccine, as such developmentally regulated molecules may be biologically important for the intracellular survival of the parasite. Since the amastigote stages causes the human diseases, the identification and producing of it's antigens in laboratory is important for

synthesis of probable vaccine (3-5).

The previous experiments reported the production of 8 monoclonal antibodies (mAb) specific for the amastigote stage of a strain of *Leishmania* (*L.*) *pifanoi* (p1 through p8). The mAb p2, P4 and p8 also reacted with *L.amazonensis* amastigote (4) and P4 precipitated a band at 34KDa (4, 5). The amastigote specific antigens recognized by these mAbs. Each of these antigens represents a potential vaccine candidate or a target for parasite control (5). Later on, the *L.pifanoi* gene encoding the P4 member protein was cloned and sequenced by Sujata K. in the year 2000 (3). The presence of six tandemly arrayed copies of the P4 gene in *L.pifanoi* is indicated and homologues of the P4 gene are found in all other species of examined *leishmania*. This antigen localized in the endoplasmic reticulum of the amastigote possesses single strand nuclease activity and it is thought that the role of P4 antigen is in RNA stability

(gene expression) or DNA repair (3). The P4 has been purified from *L. pifanoi* axenic amastigotes and used to induce significant protection (3, 5). If *Corynebacterium parvum* used as an adjuvant, three intraperitoneal injections of 5 mg of P4 antigen would provide partial to complete protection of BALB/c mice challenged with 10 (5) to 10 (7) *L. pifanoi* promastigotes. These immunized mice developed significantly smaller or no lesions and exhibited a 39 to 1.6x10 (5) fold reduction of lesion parasite burden after 15 to 20 weeks of infection (6). Taking into consideration, P4 is as one of the amastigote's antigen that will be suitable for vaccine development (3-6).

The aim of this study was cloning the P4 gene as a leishmania vaccine candidate.

Materials and Methods

Parasite and DNA extraction

Leishmania major (*L. major* strain MRHO/ IR/75/ ER) promastigotes were grown in Novy-Mac Neal- Nicolle (N.N.N: HIMEDIA, India) medium at 22°C and cultured in RPMI1640 cell culture medium (Gibco BRL) with Fetal Calf Serum 10% (FCS: Sigma-Aldrich), respectively (1). Promastigotes were collected by centrifugation at 8000 rpm for 5min (min) and lysed by the lysis buffer (0.33 M glucose, 10 mM Tris, 5mM MgCl₂, 2% Triton-x100) and subjected to DNA extraction by boiling method as described by Brown TA., 1999 (7).

PCR Reaction

A pair of primer was designed based on P4 gene (GenBank accession number AF057351) sequence: forward, 5'-GGATCCTCATGCCTGCGCCTGTCAGTC-3' and reverse, 5'-CTCAGTGCAAGTGGGATCCCAG-3' with BamHI restriction enzyme site at 5' end of forward and reverse primers. Each PCR reaction contained 1µg DNA, 20 p mol each of forward and reverse primers, 4 mM MgCl₂, 0.4 mM dNTPs, 1x PCR buffer, 1.25 unit of Taq DNA polymerase (CinnaGen, Iran) and dH₂O up to 30µL. PCR amplification was carried out within 94°C for 10min, followed by 35 cycles of denaturation at 94°C for 40sec, annealing at 62.5°C for 60sec and extension at 72°C for 60sec and after the PCR cycling 72°C for 5min by Corbett DNA thermal cycler (2, 3, 8).

Electrophoresis

PCR product was submitted to electrophoresis using 1.5% agarose gel (Agarose: Molecular grade, TAE 1X buffer) (TAE: Tris-base, Acetic acid, EDTA) and stained by ethidium bromide 10mg/ml. The DNA band was visualized under an ultraviolet light (UV transilluminator) (9).

Gene cloning

The PCR product was electrophoresed on 1% Low Melting point (LMP) agarose gel and the DNA band was sliced under long wave UV, recovered by the previously

described method (10). The recovered DNA was cloned in pBluescript cloning vector via T/A cloning method. Briefly, EcoRV blunt digested pBluescript was 3' tailed using dTTP by terminal deoxy nucleotidyl transferase (11). The ligation reaction was transformed in *E. coli* XLI-blue strain competent cells (12) and dispensed on LB (Luria-Bertani: LB, Agar) agar plate containing 50mg/ml ampicillin. The colonies were screened by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal: 20 mM) and Isopropyl thio-β-D-galactoside (IPTG: 200mg/ml). The white colonies containing recombinant plasmid were selected (13) and extracted (14), then digested by BamHI restriction enzyme. The released DNA band was purified and subcloned in BamHI digested pQE-30 expression vector. The reaction was transformed and bacterial colonies containing recombinant plasmids were screened, followed by mass cultured on LB medium. The recombinant plasmids were confirmed by Pmacl restriction enzyme which had a restriction site only on P4 gene and no restriction site on intact plasmid. Cloned gene orientation was confirmed by PCR using forward plasmid universal primer and P4 specific reverse primer.

Results

P4 detection

After culture the leishmania, it subjected to DNA extraction and PCR amplification. The 965bp PCR product as P4 gene is shown in figure 1 (Lane 2) next to the Marker (Lane 1).

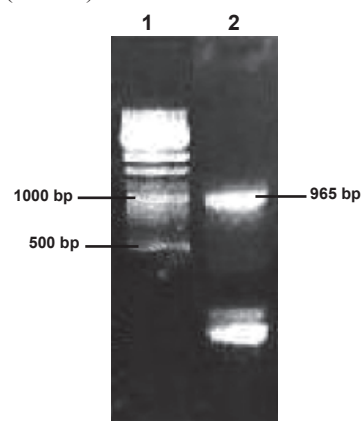


Fig 1: 1.5% agarose gel electrophoresis of PCR product
Lane 1: 100 bp DNA Ladder Marker (Fermentase), Lane2: 965 bp PCR product as P4 gene (using P4 specific forward and reverse primer)

Confirmation of recombinant plasmid

The PCR product was cloned in T-vector, then subcloned in pQE-30 expression vector and transformed in bacterial competent cell. The extracted recombinant plasmid, shown in figure 2 (Lane 4), confirmed by Pmacl restriction enzyme which has a restriction site on P4 gene and converted the super coil band of recombinant plasmid into the linear band (Lane 3) next to the

extracted intact plasmid (Lane 1) having no restriction site for Pmacl enzyme. Therefore, there is no conversion after digested by the Pmacl enzyme (Lane 2).

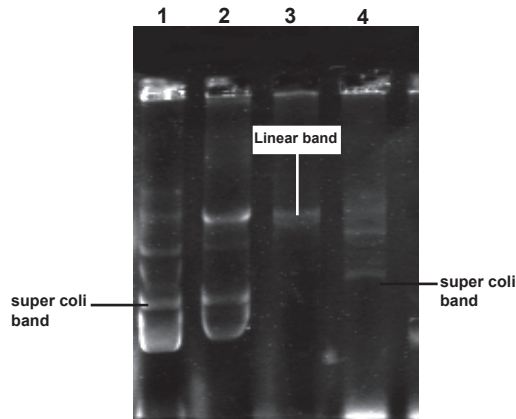


Fig 2: Confirmation of recombinant plasmid: 0.8% agarose gel electrophoresis of plasmids, Lanes 1 and 2: No recombinant plasmid (not digested by Pmacl), Lane 3: Pmacl.digested recombinant plasmid, Lane 4: No digested recombinant plasmid (Pmacl=0.5 μ l, Plasmid: 20 μ l, Reaction volume: 40 μ l, incubated at 37 $^{\circ}$ c for 2 hours)

Orientation detection of cloned gene

The 1050bp PCR product confirmed the favorable orientation of the cloned gene in the expression vector which is shown in figure 3 (Lane 1) next to pQE-30-4 as a recombinant plasmid (Lane 2) and Marker (Lane 3).

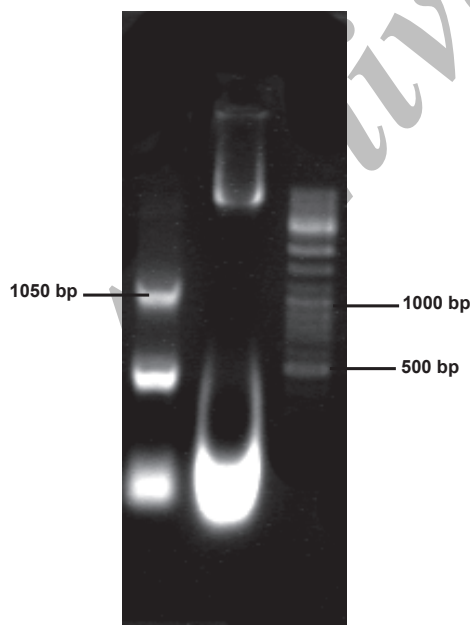


Fig 3: Orientation detection of cloned gene in expression vector: 1.5% agarose gel electrophoresis of PCR product Lane 1: 1050bp PCR product as P4 gene (using forward plasmid universal primer and P4 specific reverse primer) Lane 2: recombinant pQE-30(dilution: 1/10), Lane 3: 100 bp DNA Ladder Marker

Discussion

Leishmania is an obligate intracellular parasite. The flagellated promastigote stages are in phlebotomus insect vector and amastigote stages in mammalian host (1). Because of clinical treatment failure, significant adverse effects of drugs and drug resistancy, producing a suitable preventing vaccine is paid attention more (2, 3). P4 gene product which is localized in endoplasmic reticulum of amastigote is a good candidate for vaccine development (3).

Some studies demonstrated that P4 is amastigote specific antigen and purified protein may be useful in taxonomical, epidemiological and immunological studies (2-5).

Sujata et al reported that purified antigen P4 provided partial to complete protection in BALB/c mice against infection with *L.amazonensis*. Also, it was observed to elicit a preferential Th1- like response in patients with American cutaneous leishmaniasis (3). To explore the potential of a DNA-based vaccine, Campbell et al worked with *L.amazonensis* gene encoding P4 nuclease and murine interleukin-12(IL-12) and *L.amazonensis* HSP70 (as a chaperon). The susceptible BALB/c mice were immunized with the DNA encoding P4 alone, P4/IL-12, or P4/HSP70 prior to challenge with *L.amazonensis* and the result indicated that P4/IL-12 genes could provide potent immune protection against challenge with *L.amazonensis*, which were not sufficient in protection against *L.major* challenge. On the other hand, while the P4/HSP70 vaccine produced delayed lesion development in *L.amazonensis* infected mice, it led to a self-healing phenotype in *L.major*-infected host. Thus, it seems that P4 and HSP70 can be a suitable in DNA- based vaccines for protection of New and Old World Leishmaniasis (2).

With regard to the presented results, we cloned the P4 gene of Iranian strain of *L.major* as a vaccine which may protect against Iranian strain of *L.major*. This strain, apparently, could be the most beneficial for Iranian population. Our finding in comparison to the survey by Farajnia et al which cloned the Class 1 nuclease from amastigote stage of *L.major* (LmaC1N) revealed that LmaC1N had a high similarity (87%) to the *L.pifanoi* P4 protein (15, 16) and it was a *L.major* homologue to the P4 nuclease (15). Also after evaluating the recombinant LmaC1N as a potential human vaccine candidate, it showed an immunogenic in human during *L.major* infection and eliciting immunological response relevant to immunoprophylaxis of leishmaniasis (15).

To access the P4 gene, in this study the DNA of promastigote was extracted because there was no intron in the DNA of leishmania (17). Knowing that DNA of amastigote and promastigote stages was homogeneous, and P4 gene was expressed in amastigote stages, we preferred to use the DNA of promastigote. It seemed that, these procedures were more rapid, simple and free of complication than utilizing the lesion obtaining of

laboratory animals (16). Meanwhile, the preparation of specific culture media for amastigote stages (2) were terribly time consuming to obtain the genes which expressed in amastigote stages.

Conclusion

The *Leishmania major* P4 gene successfully was cloned in pQE-30 expression vector and was ready for in vitro expression of recombinant protein.

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