International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.1, pp 72-79, 2014-2015

Cloning, Expression and Purification of Recombinant *Leishmania Tropica* P36 (Lack) Protein for Serodiagnosis and Vaccine Applications

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Abstract: Cutaneous Leishmaniasis (CL) is a group of diseases caused by a protozoan parasite of the genus *Leishmania* afflicting millions of people worldwide. Unfortunately, no ideal therapy for CL has been identified which emphasize the need for vaccine development. A suitable leishmaniasis vaccine candidate molecule must be immunogenic and have surface expression in amastigote, the infective stage for mammals. The location of the P36 (LACK) antigen on the parasite surface, its immunogenicity and its expression in promastigote and in amastigote of *Leishmania spp.* make it a potential candidate for CL serodiagnosis and vaccine. In the present work, we developed an expression system to produce recombinant *L. tropica*-P36. Using *L. tropica* genomic DNA as template, *P36* gene was amplified by PCR then cloned into the expression vector pRSET. The new plasmid construct (pRSET-P36) was efficiently able to express P36 protein in the cytoplasm of *E. coli*. P36 purification was achieved by one step metal affinity chromatography. The purity of the resulted protein was monitored by SDS-PAGE separation and coomassie blue staining, and its identity was confirmed by immunoblotting using specific anti-6×His tag antibody. Recombinant P36 was detected by sera from CL patients, suggesting its usefulness for CL serological diagnosis and perhaps as a vaccine for this endemic disease in Syria.

Keywords *L. tropica*; P36; pRSET; cloning; protein expression.

Introduction

Leishmaniasis is a neglected disease which affects 12 million people in nearly 90 countries presenting a worldwide public-health problem [1-3]. CL is caused by many species of *Leishmania*; *L. major*, *L. aethiopica* and *L. tropica*, in the Old World [4]. Nearly 90% of CL cases occurs in Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Sudan in addition to Syria [1].

Leishmaniasis is treatable; however, anti-leishmanial therapy is a confusing subject largely because of the complexities of the disease. Unfortunately, no ideal therapy for CL has been identified [5]. Drug resistance and toxicities associated with chemotherapy emphasize the need for a safe and effective vaccine [6]. Vaccine development for parasitic diseases is more difficult than for most bacteria and viruses due to the complexity of the pathogen and its interactions with the vertebrate host. Recently, much interest has been stimulated towards vaccination against leishmaniasis focused mainly on CL with fewer attempts against VL [7].

The recombinant DNA-derived *Leishmania* proteins can be used in diagnosis, therapy, and development of vaccines [8]. Several antigens have been identified and characterized that might be potential vaccine candidates. P36 is a 36-kDa protein, or alternatively named LACK antigen, expressed in promastigotes and amastigotes life stage of different *Leishmania* species [9-11] and P36 is one of the vaccine candidates for leishmaniasis [6, 12]. It has been clearly demonstrated that P36 is essential for the viability of the parasite and for the parasite establishment in the host. The function of P36 in *Leishmania* is not clear although the immunological response to this molecule has been well studied and used for experimental vaccine studies in the mouse model [6,13,14]. In addition, P36 has been used as a tool to investigate various immunity-related mechanisms, and tested in several immunization experiments, providing heterogeneous results [9,11, 15].

Because of CL spreading in Syria, which is mostly caused by *L. tropica*, and the prominent protective role of P36 protein as a vaccine candidate against leishmaniasis, we aimed to produce recombinant P36 protein to be used later as experimental vaccine against CL, as well to be used for the detection of leishmaniasis infection.

Materials and Methods

Bacterial Strains, Growth Conditions and Plasmid

E. coli strains TOP10 (Invitrogen) and BL21-Gold (DE3) (Novagen) were used in cloning and protein expression after transformation by electroporation with the plasmid pRSET (Invitrogen). For general maintenance and protein expression, *E. coli* were grown in Luria Broth (LB; 1 % Tryptone, 0.5 % yeast extract, 171 mM NaCl) (Bio Basic INC) with ampicillin antibiotic (Sigma; 100 g/ml) in orbit-rotating 37°C incubator.

Plasmid Construction, Cloning and Subcloning

L. tropica genomic DNA was extracted. P36 gene was amplified by PCR with two specific primers P36-F (5'-TATATA**GGATCC**ATGAACTACGAGGGTCACCT-3') and P36-R (5'-TATATA**GAATTC**TTAC TCGGCGTCG GAGATG G-3'). Primers were designed according to P36 gene sequence of *L. major* (GeneBank accession number AF363975.1). These primers were designed to amplified the full length gene with the start and stop codons, and to add *Bam*HI restriction site at the 5' end and *Eco*RI at the other end of the PCR amplicon. PCR amplification program consisted of 2 min of denaturation step at 95 °C followed by 35 cycles of short denaturation step at 94 °C for 1 min, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. Amplified P36 fragment was purified using Invisorb Fragment CleanUp kit (Stratec molecular) then inserted into open plasmid pDrive cloning vector (Qiagen). The insert sequence was confirmed by sequencing. After that, the insert was subcloned into pRSET plasmid by digesting both, pDrive-P36 and pRSET with *Bam*HI and *Eco*RI restriction enzymes (Fermentas). Then, the insert and pRSET were ligated using T4 DNA ligase (Amersham). Freshly prepared electro-competent *E. coli* TOP10 cells were transformed with the new plasmid construct pRSET-P36 by electroporation. Colony PCR screening for positive P36 clones was performed using pRSET specific primers (T_7F/T_7R). Plasmid constructs were isolated from some positive clones by Plasmid Miniprep Kit (Qiagen) after being grown in LB/ampicillin medium. Successful cloning of these plasmid constructs was confirmed by digestion with restriction enzymes and by sequencing.

Expression and Purification of P36 Protein

Confirmed plasmid construct was used to transform by heat chock *E. coli* BL21-Gold (DE3) cells. Protein expression of P36 was performed in 250 ml shake flasks by growing the bacteria in LB medium till an optical density of 0.5 to 0.7 was reached and then expression was induced with 0.5 mM isopropyl β-D-thiogaldctoside (IPTG, Promega) for 16 h at 37 °C. After pelleting the cells, the pellet was resuspended in binding buffer (20 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, and 8 M urea), then lysed by sonication (Lab Sonic) on ice and the lysate was cleared by 20 min centrifugation at 8,000 rpm and 4 °C. Using fast protein liquid chromatography (FPLC) AKTA prime plus system (GE life science), recombinant P36 was purified from the cytoplasmic extract using a 5 ml column of Nickel charged Nitrilotriacetic acid (NTA) superflow Sepharose (Qiagen). After washing, the bound proteins were refolded on the column then eluted from the column with the elution buffer (20 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole and 8 M urea). The eluted fraction was dialyzed to eliminate the urea and refold the protein then concentrated on Vivaspin concentrators with a molecular mass cutoff of 10 kDa (Vivascience). The concentration of the purified protein was determined by Bradford method. The purity of P36 was evaluated in a coomassie-stained SDS-

PAGE. Protein samples were separated by SDS-PAGE using a Bio-Rad Mini-Protean Tetra Cell system following the manufacturer's instructions. Gels were prepared using stacking gel 5 % and running gel 12 %. After electrophoresis, the gel was stained in coomassie brilliant blue buffer (45 % methanol, 10 % acetic acid, 0.25 % coomassie R250) for 2 h and then washed several times in destaining buffer.

Immuno-Blotting of P36 Protein

For immuno-blotting, proteins in acrylamide gel were blotted onto 0.2 µm nitro-cellulose membranes (Whatman) using 1 × transfer buffer (800 ml dH2O containing 14.4 g glycine and 3 g tris, pH 8.3 and 200 ml methanol). After incubation in the blocking buffer (3 % BSA in tris buffered saline-tween-20, pH 7.5), blots were incubated once with 1/5000 dilution of rabbit anti-His antibody (Bethyl Laboratories), then incubated with 1/2000 dilution of goat anti-rabbit antibody conjugated to alkaline phosphatase (AP) (Bethyl Laboratories). Final revelation was done using NBT/BCIP (Nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate; Sigma) chromogen substrate in AP buffer (100 mM tris base, 100 mM NaCl and 5 mM $MgCl_2$, pH 9.5). Alternatively, blots were incubated with 1/1000 dilution of sera from CL patients, then incubated with 1/5000 dilution of goat anti-human antibody conjugated to AP (Thermo scientific) and then reveled as described previously.

Results

Cloning of P36 Gene Into Pdrive Cloning Vector

Full-length P36 gene was amplified from *L. tropica* genomic DNA using two specific primers. The amplified gene is about 1000 bp in length (Fig. 1A). Go*Taq* DNA polymerase, used in PCR reaction, adds an overhang A to the ends of the amplified sequence, which allows P36 gene insertion into linearized pDrive plasmid and the positive construct was used for the subsequent subcloning into pRSET expression vector (Fig. 1B). The plasmid pRSET is a T7 promoter-dependent system which produces cytoplasmic N-terminal His6-tagged proteins in *E. coli* cells. Tagging of recombinant proteins is indispensable for the subsequent steps of purification and detection.

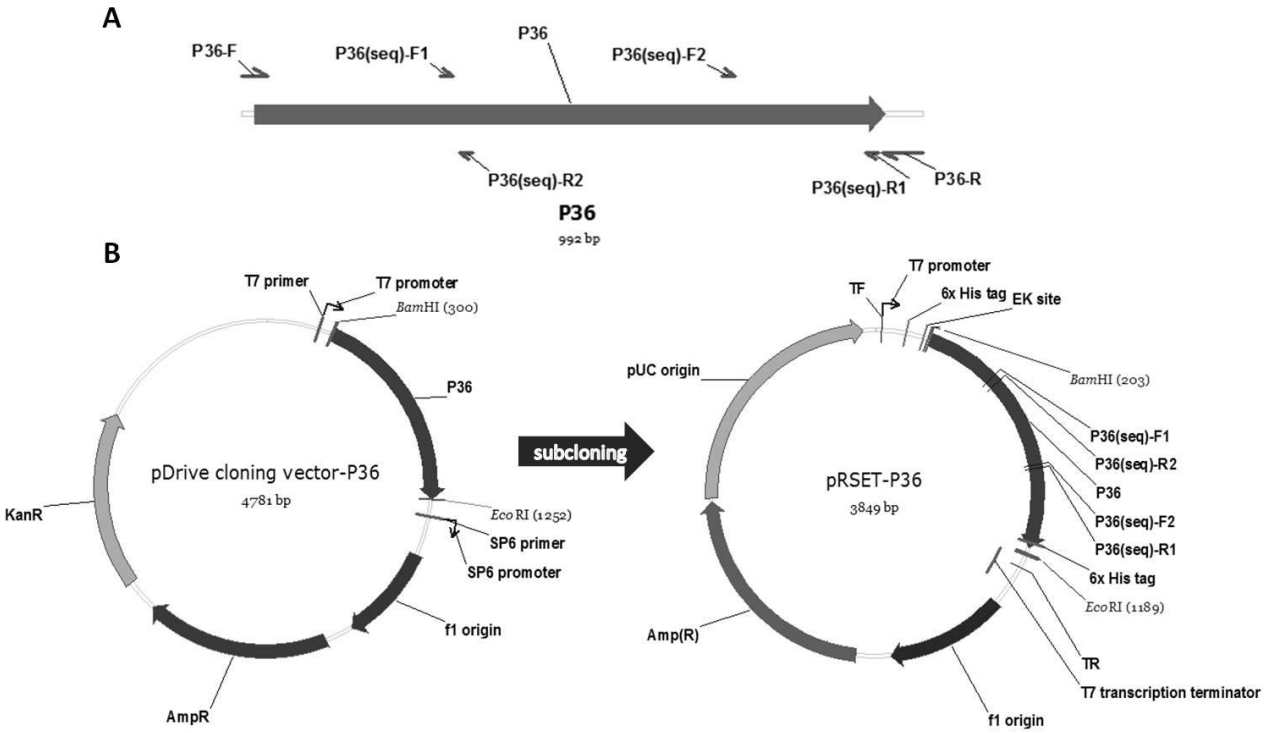

Fig. 1. Scheme of P36 gene, pDrive plasmid used in cloning and pRSET plasmid used in protein expression.

(A) P36 gene and the positions of different primers used in the cloning and sequencing. (B) Maps of the resulted plasmid constructs (pDrive-P36) for cloning and sequencing and (pRSET–P36) for expression where the position of the inserted P36 gene is indicated. The most important elements of the plasmids are indicated. These include T7 promoter, His6 tag downstream the two restriction sites (*Bam*HI and *Eco*RI) used for P36 gene ligation. Positions of the primers used for PCR positive colonies screening and for sequencing are shown.

PCR amplified P36 gene was successfully cloned in pDrive plasmid as confirmed by colony PCR using a combination between a vector-specific primer and an insert-specific primer. Such reaction confirms the insert orientation, since cloning using the A-overhang left by *Taq* DNA polymerase and T-tailed vector is not a technique that will retain orientation. After cloning, P36 was sequenced (Data not shown).

Subcloning of P36 Gene Into Prset Plasmid

P36 was digested with *Bam*HI and *Eco*RI restriction enzymes, resulting in a sticky ended fragment ready for ligation in linearized pRSET plasmid with the same two restriction enzymes (Fig. 2C). Ligated products were used to transform *E. coli* TOP10 cells by electric shock and positive colonies on the selective plates were screened by PCR using pRSET-specific forward primer (T_7F) and P36-spicefic sequencing reverse primer ($P36_{seq}$-R2). This approach enabled distinguishing between two types of colonies; empty pRSET-containing colonies and pRSET-P36-containing colonies which gave a fragment of 506 bp due to the presence of the insert gene within (Fig. 2D).

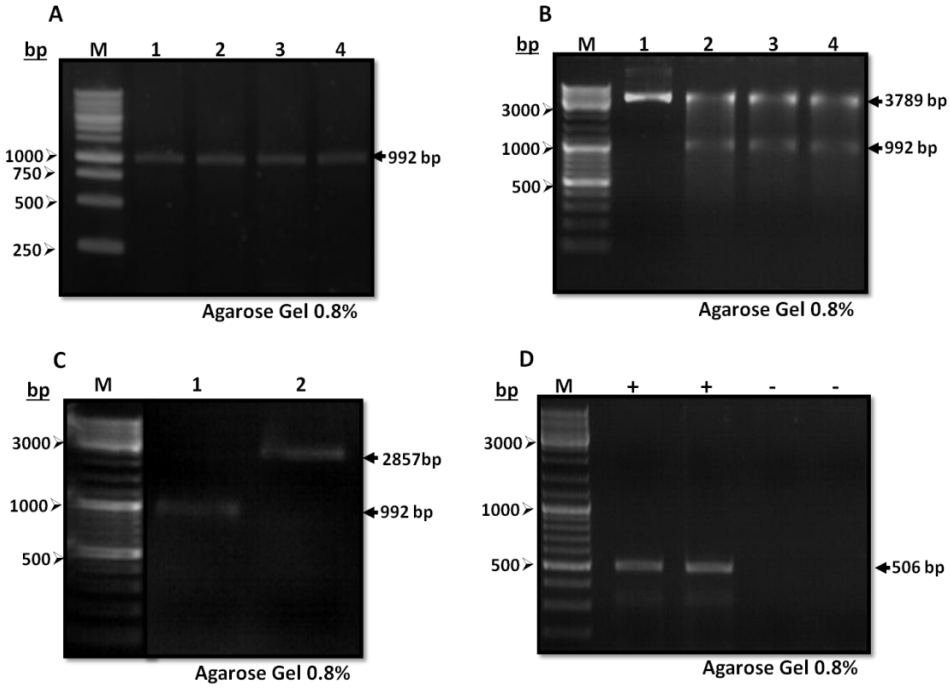

Fig. 2. Amplification, cloning and subcloning of P36 gene.

(A) PCR reaction was performed on genomic DNA extracted from *L. tropica,* using P36 specific primers (lanes 1-4). **(B)** Digestion of pDrive cloning vector-P36 after cloning and sequencing for subcloning; lane 1: the purified construct (pDrive-P36) without digestion as a control; lane 2: the same construct digested using *Bam*HI; lane 3: the construct digested using *Eco*RI; lane 4: the construct digested using both *Bam*HI and *Eco*RI restriction enzymes. (C) Shows the purity of digested P36 gene (lane 1) and pRSET plasmid (lane 2) using both *Bam*HI and *Eco*RI restriction enzymes for ligation reaction. (D) Results of colony PCR screening performed on 4 randomly selected clones after *E. coli* TOP10 transformation with the products of the ligation reaction.

Expression and Purification of P36 Protein In E. Coli

Production of P36 as recombinant protein was obtained after transformation of *E. coli* BL21-Gold (DE3) cells with the confirmed pRSET-P36 plasmid construct. Cells were grown in LB medium supplemented with antibiotic and protein expression was then induced by IPTG. Purification of P36 from cytoplasmic extract was done on immobilized-metal affinity chromatography, after treatment with urea to dissolve the formed inclusion bodies, using Nickel-charged NTA column installed on AKTA prime system. The UV-detector, supplemented with this system, enabled real-time monitoring of the different steps of P36 purification (Fig. 3A and 3B). The protein expression and purification procedures of recombinant P36 were followed by SDS-PAGE and immunoblotting for purity assessment (Fig. 3C and 4). A remarkable expression of P36 could be observed after IPTG induction and incubation for 16 h at 37 °C. Although, expressed protein was totally purified from bacteria cytoplasmic extract by column purification which yielded 90 % pure P36. The yield of purified recombinant protein estimably reached ~500 mg/L of bacteria culture.

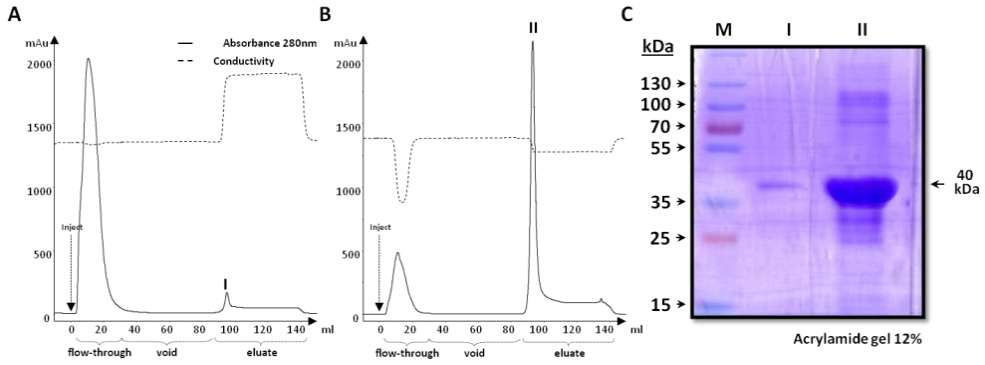

Fig. 3. The expression and purification of P36 protein.

(A) and **(B)** diagrams of purification procedure using Ni+-NTA column installed on FPLC AKTA prime system. Continuous line represents the absorbance of the eluate and the dashed line represents the conductivity of the eluate. Different purification steps are shown below and peaks of the flow-through sample and of purified P36 are indicated. In diagram A, extraction and purification were accomplished using PBS, while in diagram B extraction and purification were accomplished under denaturing conditions using Urea. **(C)** SDS-PAGE (acrylamide 12 %) of the purified protein samples, with PBS (lane I) or with PBS+Urea (lane II).

In immunoblotting, purified P36 protein was detected by anti-His6-AP conjugated antibody (Fig. 4B) or by diluted sera from CL patients (Fig. 4C) in which the recombinant green florescent protein (GFP) was used as a negative control with positive and negative sera.

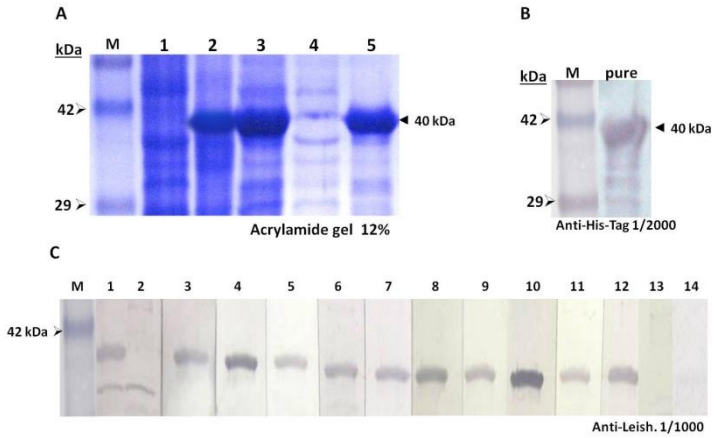

Fig. 4. SDS-PAGE analysis and immunoblotting of P36 protein.

Protein migration in SDS-PAGE (acrylamide 12 %) of protein samples obtained after different steps of purification. Total cytoplasmic extract after 16 h of IPTG induction (lane 3; while lanes 1 and 2 show before and after induction respectively), and purified P36 protein (lane 5) in comparison with flow-through (lane 4). Detection of migrated proteins was done either by coomassie blue staining **(A)** or by immunoblotting using rabbit anti-His antibody (1/2000) **(B)** or using positive serum from CL patients (1/1000) **(C)** (lanes 1, and 3-12 different positive serums; lanes 2, and 13 GFP protein as a control with positive and negative sera respectively; and lane 14 purified P36 with negative serum). The location of P36 in the gel is indicated and defined as 40 kDa compared to the protein molecular weight ladder in the first lane (M).

Discussion

In recent years, great efforts were performed searching for an effective vaccine against Leishmaniasis in particular Cutaneous Leishmaniasis. Although a great deal of knowledge has been gained from studies on the immunobiology of leishmaniasis, there is still no universally acceptable, safe, and effective vaccine against the disease [16]. As a second generation vaccines against Leishmaniasis several antigens have been identified and characterized that might be potential vaccine candidates [6]. According to previous studies, P36 (LACK) protein is one of these candidates [7, 12, 17].

In the current work, a high-level expression system of His6-tagged *L. tropica* P36 was established in *E. coli*. Firstly, *P36* gene was amplified, cloned and sequenced. The genomic DNA was used as a template in PCR reaction, since *P36* gene does not contain introns (*L. major P36*; GeneBank accession number AF363975.1). Cloning was performed using pDrive cloning vector which provides superior performance through the direct ligation of the blunt-ended PCR products, allowing sequence analysis using standard sequencing primers. Following sequence confirmation, DNA insert was subcloned into pRSET plasmid using specific restriction enzymes. The pRSET vector is a pUC-derived expression vector designed for high-level protein expression and purification from cloned genes in *E. coli*. The presence of the T7 promoter provides high level expression of the down-stream cloned gene [18, 19]. In addition, DNA inserts are positioned downstream and in frame with a sequence that encodes an N-terminal polyhistidine tag that functions as a metal binding domain in the translated protein [20]. High level of recombinant P36 protein expression was obtained in *E. coli* BL21-Gold (DE3). However, the product precipitated in cytoplasmic inclusion bodies. Protein aggregation is a major bottleneck during the bacterial production of recombinant proteins. In general, the induction of gene expression at sub-optimal growth temperatures improves the solubility of aggregation-prone polypeptides and minimizes inclusion bodies formation [21]. Choosing appropriate conditions for cell growth and induction can influence the formation of these insoluble structures [22], especially, temperature which affects directly proteins stability and confirmation [23]. Increased temperature has been found to stimulate aggregation in several cases [24]. Low temperature affects the quality of the recombinant protein, especially within the insoluble cell fraction. The fraction of aggregated protein (i.e. IB) was largely decreased at low temperatures (below 37 ^{o}C), and the conformational quality was improved [21]. Traditionally, very high concentrations of denaturing buffers are usually used to solubilize proteins in the inclusion bodies. Urea is one of classic denaturants used for inclusion bodies dissolving and refolding aggregated proteins [25-27]. On the other hand, polyhistidine-tagged fusion proteins that form IBs can be extracted with urea and purified directly by nickel-chelate affinity chromatography. Removal of contaminating proteins and refolding by exchange to non-denaturing buffer conditions can then be performed directly on the purification column just before the elution step [28, 29]. To overcome IBs formation in future, a new system which combines improved expression, solubility screening and purification efficiency can be used. The system is based on newly constructed vector, which generates a protein with an N-terminal His tagged green fluorescent protein (GFP) fusion to allow rapid quantitation of soluble protein [30]. Previous studies reported the use of GFP as an excellent expression tag for fusion proteins, which can improve their expression while preserving their function and native-like structure. Fusion protein method allows the purification and the detection of a protein of interest even when no specific antibody is available [30, 31].

Previous published works described production of recombinant P36 protein from other different *Leishmania spp.*, for application as an experimental vaccine with or without adjuvant against VL or CL caused by different *Leishmania spp.* such as *L. amazonensis* [9], *L. mexicana* [13], *L. major* and *L. infantum* [12]. Another study determined the ability of P36 DNA to induce protection after challenging with *L. major*

promastigotes. The induced protection was similar to that achieved by P36 protein and rIL-12, but superior to P36 protein without rIL-12 [10].

The recombinant P36 protein presents a new applicable approach for CL serological diagnosis since this protein gave a positive result in immunoblotting using many different serums from CL patients. As well, recombinant P36 may be able to detect CL disease which is caused by different *Leishmania* species since P36 is a conserved antigen between different *Leishmania* species. But this point still needs more investigations. Additionally, recombinant P36 presents a tool for developing one of CL candidates vaccine derived from *L. tropica*, and more steps can be achieved for production and testing of P36 as a recombinant protein and/or DNA vaccine.

In summary, a new approach was developed for *L. tropica* P36 production as a recombinant protein. Recombinant P36 may be useful as a diagnostic tool since good positive results were obtained with western blot using CL patient's serum. In addition, recombinant P36 may be useful in the development of an efficient vaccine against Old World cutaneous leishmaniasis.

Acknowledgments

The authors would like to thank Damascus University for the continuous support throughout this work; Atomic Energy Commission of Syria Department of Molecular Biology and Biotechnology for help with DNA sequencing and protein purification; and the supervisors of this work for critically reading the manuscript.

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