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Cloning, Expression and Purification of Recombinant A_2 Protein from *Leishmania infantum* for Diagnosis of Visceral Leishmaniasis in Iran

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Abstract

Visceral leishmaniasis (VL) is a fatal disease caused by *Leishmania infantum* in the Mediterranean basin and Iran. Different methods are used for diagnosis of VL. The aims of this study were expression and purification of recombinant A2 (rA2) protein of *L.infantum* and its application in the diagnosis of VL. The serological diagnosis of VL was applied using rA2 protein. In this study, A2 gene of *L.infantum* sequence was ordered for the synthesis, cloned in *E.coli* strain TOP10F' and proliferated in pET22-b vector. The expression and purification of rA2 proteins applied in host *via* BL21 and Ni-NTA respectively. The A2 gene sequences were synthesized and the construct transformed to pET22-b vector. A

520bp fragment was identified in digested pEASY-A2 plasmid. The gene was successfully cloned in to pET22-b standard expression vector and transformed in *E.coli* BL21. Expression of rA2 was confirmed by SDS-PAGE and a 27KD protein was detected. The antigenicity of A2 protein was assessed using both pooled dog sera and C9 anti-A2 monoclonal Ab. This study recommends rA2-ELISA as alternative assay to detect VL. More evaluation should be made to develop a cheap and reliable serologic test for detection of *L.infantum* among infected hosts.

Keywords: A2 protein, *Lieshmania infantum*, Kala-azar, rA2, visceral leishmanisis, Iran

Introduction

Visceral leishmaniasis (VL) or Kala-azar is a disease that transmitted by female sand flies and caused by *leishmania* species. Humans, domestic and wild animals could be infected by VL and found throughout parts of the old and new worlds [1]. VL is characterized by the presence of; fever, hepatosplenomegaly, swollen lymph nodes and weight loss, depending on the *Leishmania* species and the host's immune response against the parasite [2]. Different methods are used for the diagnosis of VL based on aspirates or biopsy specimens of visceral tissues (spleen, liver, bone marrow), which are subjected to microscopic examination and culture, and serological methods such as the indirect fluorescent antibody (IFA), the indirect hemagglutination assay (IHA), the direct agglutination test (DAT) and enzyme-linked immunosorbent assay (ELISA) [3]. Several antigens (Ag) are used for serological detection of antibody but most common is crud soluble Ag (CSA). Nevertheless, the use of total soluble antigens is limited due to problems of reproducibility and manufacturing [4]. Recombinant proteins such as rK39 [5], rK26 [6], rKE16 [7] are used for detecting of antibody (Ab) against VL and evaluated in attempt to replace native Ags in serological tests. A2 is as a family of proteins that expressed only in amastigote forms and display a variable number of repeats of a unit of 10 amino acids [8,9] have shown that A2 protein, an amastigote stage specific, was reactive in ELISA with 60% and 82% of sera of patients with kala-azar from Indian and Sudan, respectively. Due to attractive of A2 protein as an Ag for detecting Ab in sera samples, the aim of the present study was molecular cloning, expression and purification of rA2 of *L. infantum.*

Materials and Methods

Gene Synthesis

Under the A2 gene sequences available in Gene Bank Databases with Accession number NO: AY255808, a part of the A2 gene sequences was selected which contain immune dominant sequences and less number of repetitive sequences. Therefore, we predict a 6 histidin in the C terminal, which was synthesized by Sinaclon Company, Iran **(Figure 1)**.

CCCTTCCATGGGGTCCCTGCAGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAG
TCAAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAA
AGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTA
ACGTTCTTGTACGACGGTATTGAAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAA
CGATATTATTGAGGCTCACCGCGAACAGATTGGAGGTGAACCGCACAAAGCGGCAGTGGATGTTGGCC
CGCTGAGCGTAGATGTGGGTCCGCTGTCCGTTGGTCCTCAGTCCGTGGGTCCGCTGTCCGTAGGCCCGC
AAAGCGTAGGTCCACTGTCCGTCGATGTCGGCCCTCTGAGCGTAGGCCCGCAGAGCCATCACCACCAT
CATCACTAAGGATCCGAATTCGAGCTCAAGCTTGCGGCCGCCTCGAG

Figure1. The A2 gene sequences

The A2 Gene (pEASY–A2) was transformed to Top10 *E. coli* competent cell by thermal shock (30 min on ice, 90 Second thermal bath at 42°C) (Figure 2).

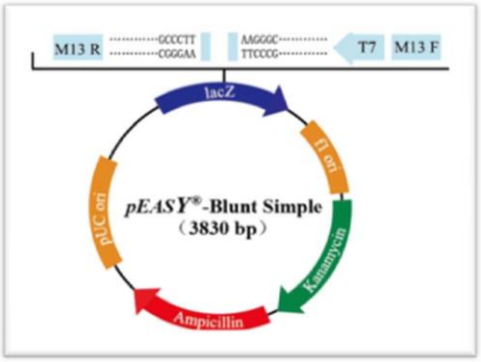

Figure2. pEASY-truncA2-520bp-Forward

The competent cells were prepared from the TOP10 strain of *Escherichia coli* using the calcium chloride method. One hundred µl of *E. coli* was cultured with 5 ml of new Luria-Bertani (LB Broth) and incubated at 37˚C for 14 hours. The next day, 200µl of these cells were cultured in 10 ml of LB broth and incubated at 37˚C for three hours. After incubation, the culture was centrifuged at 10000 rpm at 4˚C for 10 minutes. The supernatant was discharged and 900µl of cold calcium chloride (100 mM) was added to the pellet and mixed gently before incubation on ice for 30 minutes. The suspension was centrifuged at 10000 rpm at 4˚C for 10 minutes. The supernatant was discharged and 600 µl of cold calcium chloride added to the pellet and mixed gently before being incubated on ice pieces for one hour. Following this, after centrifugation and adding 100 µl, 10µl of the A2 Gene (pEASY-A2) was added to this suspension and incubated on ice pieces for 30 minutes. Then, the suspension for heat shock was incubated at 42˚C for 90 seconds and immediately transferred onto ice. Subsequently 500µl of LB broth, with Kanamycin and Ampicillin antibiotic, was added and incubated at 37˚C for one hour. These cells cultured on plates of Luria-Bertani (LB) agar medium containing; 100mg/ mL of Ampicillin and 100mg/mL Kanamycin. The plates were

incubated at 37˚C for 16 hours. Recombinant plasmids (pEASY-A2) were extracted from the LB broth medium according to the protocol for the plasmid extraction kit. The recombinant plasmid (pEASY-A2) and expression vector (pET22) were digested by EcoRI and NcoI enzymes. The double digestion reactions were prepared in the same way for each in 150µl volumes containing 90µl of the plasmid extraction product, 39µl of water, 15 µl of tango buffer (Fermentas®, Germany), 3 µl of EcoRI and 3 µl of NcoI enzymes. These reactions were incubated at 37°C for 16 hours. The products of digestion were analyzed by electrophoresis on 1% agarose gel. The bands belonging to the A2 fragment and digested expression plasmids (Pet22) were purified from the agarose gel by a gel purification kit (Roche, Germany). Pet22 fragments were purified from the gel.

Expression of Recombinant Protein

In order to express recombinant protein (pET-A2), *E-coli* BL21 cells were used as host cell. The expression of A2 was induced at OD 600 of 0.6 by adding of 1mM of Isopropyl-β-D-Thiogalactopyranoside (IPTG) and incubation at 37°C. The induced bacteria were harvested by centrifugation at 6000 *g* for 15 min; BL21 cells were cultured in LB Broth medium for 4 hours with IPTG (0.1 M). The A2 expression was analyzed by 12% SDS-Polyacrylamid Gel Electrophoresis (SDS-PAGE). The pellet of 1 ml of induced bacteria was incubated in 200 µl of the lysis buffer (50 mM Tris-Cl, 500 mM NaCl, pH= 7.8, 1mg/ml lysozyme) at room temperature (RT) for 1 hr. The cell were lysed by sonication 30", 60 pals and centrifuged at 16000 g for 20 min at 4°C to separate the insoluble proteins from the soluble ones. Finally, the supernatant and pellet were analyzed by SDS-PAGE [2].

Purification of A2 proteins by Ni-NTA affinity chromatography

Immobilized Metal Affinity Chromatography (IMAC) is one of the most popular techniques for the purification of recombinant proteins, which use the metal binding property of certain amino acids, in particular histidine are known as a 6X His tag. Nickel is bound to an agarose bead by chelating using nitroloacetic acid (NTA) beads [10, 11]. For purification, we used the plastic column and a 50 ml falcon tube. No pump was necessary; simply allowed gravity to draw the buffer and sample through the column. The 200 ml culture were collected and centrifuged and then re-suspended in 10 ml of binding buffer (50 mM Tris-Cl, 5 mM imidazole, 500 mM NaCl). The purification process performed at three pH values of binding buffer: 7.0, 7.5, and 8.0. The cell suspension was sonicated for 7 min. The cell lysates were centrifuge at 12000 g for 30 min. After equilibration the column with binding buffer, the supernatant was applied to the column at flow rate of 1 ml/min. Then the column was washed step-wised with 5 column volumes of binding buffer. In the next step the column was washed step-wised with 25, 20, 15 and 10 ml of binding buffer containing 20, 40, 60 and 80 mM of imidazole.

Then the absorbed protein was eluted with binding buffer containing 400 mM imidazole. Finally, the purified protein was analyzed with SDS-PAGE [12].

SDS-PAGE and Western blotting

SDS–PAGE was performed under denaturing and reducing conditions with a 12% acrylamide–3% bis acrylamide gel, as described previously by Laemmli [13]. The purified recombinant protein was lysed in ice-chilled SDS-PAGE sample buffer [45 *m*M Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercapto-ethanol and 0.05% (w/v), bromophenol blue and urea 8 M], and the lysate was boiled for 4 min. Each lysate was then centrifuged at 16,000 g for 10 min [14].The pre-stained protein ladder was used, which contains ten proteins ranging in size from 11- 180 kDa (Cinnagen Co, Iran). The gels were stained with Coomassie blue G250 and then investigated by western blotting after transfer to polyvinylidene difluoride (PVDF) membrane in transfer buffer (25*m*M Tris, 192 *m*M glycine). The membrane was probed with pooled dog sera, briefly; each membrane sheet was quenched overnight, at 4°C, in TBS–Tween [25 mM Tris-HCl, 137 mM NaCl, 0.05% (v/v) Tween-20] containing 3% (w/v) skimmed milk. The membrane was then washed once in TBS–Tween before being incubated, in a 1:100 dilution of the pooled dog sera, and 1/50 dilution of C9 monoclonal antibody which was kindly provided by Dr. G. Matlashewski (McGill University, Canada) for 2 hour at room temperature (20–23°C). After three more washes in TBS–Tween, the membrane was incubated in a 1:20000 dilution of peroxidase–anti-dog-IgG conjugate (Sigma, USA) for dog sample and a 1:4000 dilution of peroxidase–anti-mouse-IgG conjugate for C9 monoclonal antibody before 3, 3′-diaminobenzidine tetrahydrocholoride (DAB) substrate (Sigma) as the chromogenic substrate was added . Color development was stopped by washing the membrane in distilled water. The resultant bands were scanned so that their optical densities (OD) could be recorded and compared in the Quantity One H Software Package (Bio-Rad Laboratories, Hercules, and CA). Finally, the weight of dark bands was determined [6, 7, 15].

Results

The A2 gene sequences were synthesized and the construct transformed to pET22-b as an expression vector. The size of the synthesized A2 gene of *L. infantum* in our study was similar to earlier predictions. A 520 bp fragment was identified after agarose gel electrophoresis of digested pEASY-A2 plasmid. PEASY-A2 was digested and the gene was successfully cloned in to pET22-b standard expression vector and transformed in *E. coli* BL21. The expression plasmid was digested by EcoRI and NcoI restriction enzymes that shown in **Figure 3**.

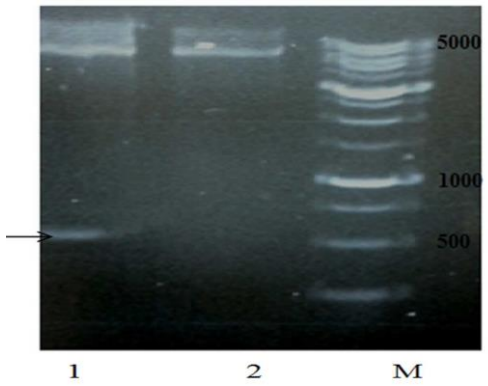

Figure 3. Recombinant Plasmid (pET22-A2)
Digested by EcoRI and NcoI Enzymes and Electrophoresis on 1% Agarose Gel; 1: recombinant plasmid uncut, 2: recombinant plasmid cut, M: DNA Ladder: base per (bp)

SDS-PAGE analysis of induced bacteria showed expression of 27 KDa, which was absent in uninduced bacteria. The recombinant A2 protein (rA2) was confirmed by SDS-PAGE and a 27 KD protein was detected. The antigenicity of A2 protein was surveyed in western blotting using both pooled dog sera and C9, an anti-A2 monoclonal antibody **(Figure 4).**

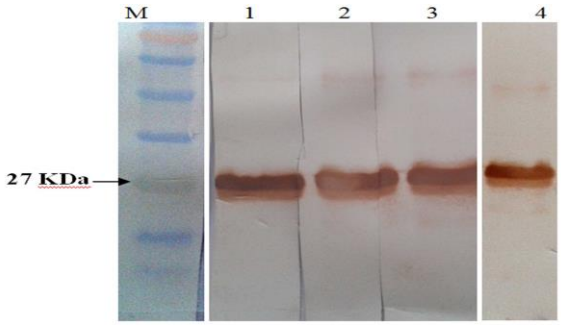

Figure 4. Western blot analysis of purified rA2 protein among different dilution of anti A2 C9 monoclonal Antibody:
1- 1/70, 2-1/50, 1/40.4- primary antibody pooled dog serum 1/100. M: protein ladder 11-160 kDa

We were purified the soluble fraction of rA2 by using IMAC and observed variation in the purity of rA2 at different pH values of binding buffer. We experiment three pH 7, 7.5 and 8 as explained in materials and methods and SDS of protein sample showed that the purity is the best in pH=8. The purification was considerably improved by increasing pH from 7.0 to 8.0 **(Figure 5).**

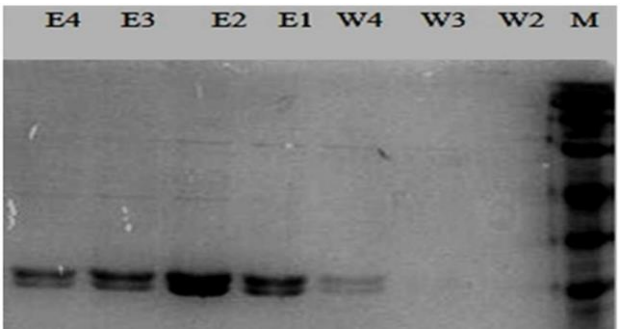

Figure 5. Purification of A2 protein.
The purification that was considerably improved by increasing pH from 7.0 to 8.0. FT(Flow Through),W (Wash 1,2,3,4) ,E (Elution 1,2,3,4).

Discussion

Many methods are used for diagnosis of VL, however detection of antibody is important. Some antigens are used for serological assays, but the A2 protein is an attractive and powerful immunogen due to its virulence factor [16, 9]. A2 has been investigated as antigen for diagnosis of leishmaniasis in humans and dogs by detecting of anti-A2 antibodies using various methods including western-blotting, ELISA and immune-precipitation [3, 17, 18]. In this study, the A2 gene sequences were synthesized and the construct transformed to a vector. A fragment was identified in digested plasmid. The gene was successfully cloned into expression vector and transformed in *E. coli*. The plasmid was digested by enzymes and expression of rA2 was confirmed by SDS-PAGE and its antigenicity was assessed. In this process, the expression of A2 protein is investigated and the rA2 protein was purified in single step by IMAC at the optimized pH and the highly pure protein was obtained. The full length of A2 was difficult to express and according to the epitop mapping of A2 protein, the repeated sequences were recognized. Some of these repeated sequences were synthesized as epitops for B and T cells[19]. In some study, PCR product was cloned in T/A cloning vector and ligation was performed in pET16b/ORF with BamHI[15].but we used pET22 digested with EcoRI/NCOI and BL21 as an expression host. The soluble rA2 was purified at pH=8, because the *pI* of histidin residue is 6 and is recommended perform the purification in pH 7-8 to make sure that histidin is binding to the Ni^{+} ions and has negative charge and we have high purity at pH=8.0. Carlvahlo *et al* [20] used two preparations of rA2 antigen by fusing A2 protein into the glutathione transferase (GST-A2). The A2 recombinant protein contained a tag of six residues of the amino acid histidine (A2-His). They have detected cross reactivity of sera with GST, but not with a six histidin tag in canine sera. This study recommends rA2-ELISA as alternative assay to detect VL. More evaluation should be made to develop a cheap and reliable serologic test for detection of *L. infantum* among infected hosts.

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Conflict of interest.
The authors declare that there is no conflict of interest.

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