Iranian Biomedical Journal 8 (1): 13-18 (January 2004)

High Level Expression of Recombinant Ribosomal Protein (L7/L12) from *Brucella abortus* and Its Reaction with Infected Human Sera

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Received 6 July 2003; revised 12 August 2003; accepted 24 August 2003

ABSTRACT

Brucellosis, caused by *Brucella* spp., is an important zoonotic disease that causes abortion and infertility in cattle and undulant fever in humans. Various studies have examined cell-free native and recombinant proteins as candidate protective antigens in animal models. Among *Brucella* immunogenes, antigen based on ribosomal preparation has been widely investigated. In this study, the immunogenic ribosomal protein L7/L12 gene from *Brucella abortus,* S19, was amplified by PCR and sub-cloned to prokaryotic expression vector pET28a. *Escherichia coli* BL21 (DE3) pLysS was transformed with pET28a-L7/L12 and gene expression was induced by IPTG. The expressed protein was purified by affinity chromatography with Ni-NTA resin. The concentration of purified recombinant protein calculated to 8 mg/L of initial culture. The integrity of product was confirmed by Western-blot analysis using a standard rabbit anti *Brucella abortus* ribosomal protein L7/L12 antibody. Sera reactivity of five infected individual were further analyzed against the recombinant ribosomal L7/L12 protein. Data indicated that recombinant ribosomal L7/L12 protein from *Brucella abortus* was recognized by patient sera. *Iran. Biomed. J. 8 (1): 13-18, 2004*

Keywords: *B. abortus*, Brucellosis, L7/L12 gene, Ribosomal protein, Gene expression

INTRODUCTION

Brucellosis has been an emerging disease since the discovery of *Brucella melitensis* by Bruce in 1887. *Brucella* is a Gram-negative facultative intracellular bacterium with a great public health and economic importance throughout the world. The disease has a limited geographic distribution, but remains a major problem in the Mediterranean region, Western Asia, and parts of Africa and Latin America. The true incidence of human brucellosis is unknown. Reported incidence in endemic-disease areas varies widely, from <0.01 to >200 but in Iran, it is 132.4 per 100,000 population [1, 2]. Control of the disease depends on the ability to identify and remove contaminated animals and on the other hand on prophylactic measures such as vaccination [3]. At present, live attenuated strain 19 of *Brucella abortus* is used to immunize domestic animals, but this vaccine has several disadvantages: First, strain 19 can cause abortion when administered to pregnant cattle [4]; second, this strain could be pathogenic for human [5]; and finally, the induced antibodies in vaccinated animals may interfere with the diagnosis of natural infection [6]. Therefore, the development of more effective and safer vaccines is necessary for disease control in animals and in human [5]. The preferred strategy for many intracellular organisms, such as *B. abortus,* is to identify those antigens that are truly responsible for the induction of a protective cell mediated immunity response. These antigens, such as L7/L12 [3, 5], Cu-Zn superoxiddesmutase (SOD) [7], P39 [8], GroEL and GroES [9], YajC [10] and HtrA [11] could be candidate for research and development of subunit

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vaccines. A number of studies have focused on recombinant production or optimizing the vector and level of expression of these antigens [1, 3, 5]. In this regard, *Brucella abortus* ribosomal protein (MW ≅ 13.5 kDa) which correspond to L7/L12 gene has shown to be highly immunogenic for CD4 T cells and can protect different animal models against *Brucella* infection. Isolation, characterization and high level expression of *Brucella abortus* ribosomal L7/L12 gene in *E. coli* as a host was presented in this paper. We also showed that recombinant L7/L12 protein was recognized by infected human sera using Western blot analysis.

MATERIALS AND METHODS

Strains, plasmids, media and sera. *B. abortus* strain *S19* (a gift from the Pasteur Institute of Iran) was grown on trypticase soy agar and Brucella broth for 48 hours. The media were supplemented with serum-dextrose and incubated with 5% CO_2 at 37˚C. Bluescript plus (pSK+, Stratagene) vector together with *E. coli* strain DH5α (Stratagene) (*f⁻ gyr A96 Nalr, recA1 relA1 Thi-1 hsdR17 r⁻k m+k*) were used for initial cloning, sequencing and maintenance of different DNA fragments. For recombinant protein production, a prokaryotic expression vector pET28a (Novagene) was used. This vector can express a fusion protein with a six-histidine tag, a thrombin recognition site and a T7 tag at the N-terminus. These additional amino acids increase the size of the expressed protein about 5 kDa. The recombinant pET28a (pET28a-L7/L12) is transformed into *E. coli*, BL21 (DE3) pLysS (*f⁻ ompt hsdB, rB⁻ mB⁻, dcm gal, DE3, pLYsS cmr*) as host strain. The required antibiotics were added to LB media according to the reference recommendation [12]. We received standard rabbit anti recombinant L7/L12 sera from Philippe Langella (Unite de Recherches Laitieres et de Genetique Appliquee, INRA, Domaine de Vilvert, 78352 Jouy en Josas cedex, France) and 5 acute phase patients sera from Dr. Darabi (Immunology Department, the Pasteur Institute of Iran, Tehran). All chemicals were obtained from Merck (Germany) and all of the enzymes from Fermentas (Lithuania) or Cinagen (Iran) Companies.

Extraction of chromosomal DNA. Chromosomal DNA was prepared according to standard CTAB/NaCl method [12]. Briefly, after resuspended the pellet of bacterial culture in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8), the bacteria were lysed by SDS and proteinase K. The chromosomal DNA was extracted by CTAB/NaCl solution (10% CTAB and 0.7 M NaCl). The cell debris and proteins were removed by two times phenol/chloroform/isoamylalcohol (25:24:1) mixture. DNA was precipitated by isopropanol and washed in ethanol (70%), dried, and then resuspend in TE buffer. Quality and quantity of the purified genomic DNA were assayed by 0.8% agarose gel electrophoresis in 1× TBE buffer and spectrophotometrically (260/280 nm), respectively.

Primers design. Primers were designed according to the published sequence for ribosomal protein of *B. abortus* (accession number: L19101). The forward primer, L28F, (5'GGA AAT GGATTC ATG GCT GAT CTC GCA AA) starts from the beginning of the gene and contains *Bam*HI site. Reverse primer, L28R, (5'CCA CTCGAG CTT GAG TTC AAC CTT GGC CA) contain recognition site for *Xho*I and no stop codon. The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure.

Gene amplification of L7/L12. PCR was performed in a 50 µl total volume containing 500 ng of template DNA, 1 µM of each primer, 2.5 mM Mg^{2+}, 200 µM (each) deoxynucleoside triphosphates, 1× PCR buffer and 2.5 unit of *pfu* DNA polymerase (Fermentas). The following conditions were used for amplification: hot start at 94˚C for five minutes, followed by thirty cycles of denaturation at 94˚C for one minute, annealing at 63˚C for one minute and extension at 72˚C for one minute. The program followed by a final extension at 72˚C for five minutes. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1× TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified from the agarose gel by high pure PCR product purification kit (Roche Diagnostic) according to the manufacturer recommendation. The purity of eluted PCR product was checked by electrophoresis in 1% agarose gel in 1 × TBE buffer. For further analyses and before sequencing, the PCR product was digested with *Hind*III and *Eco*RI.

Cloning of L7/L12 gene in bacterial expression vector. The PCR product was digested with *Bam*HI and *Xho*I and ligated to pSK+ and pET28a, which were digested by the same restriction enzymes, using T4 DNA ligase at 16˚C over night. *E. coli* DH5α and *E. coli* BL21 (DE3) plysS competent

cells were prepared by calcium chloride method and were used for transformation of pSK-L7/L12 and pET28a-L7/L12 plasmids, respectively. The transformed bacteria were selected by screening the colonies on media containing antibiotic. The suspected colony was further analyzed by restriction enzyme digestion and PCR.

Expression and purification of recombinant L7/L12. *E. coli* BL21 (DE3) pLysS was transformed with pET28a-L7/L12 and grown in LB broth supplemented with Kanamycin (100 μg/ml) at 37˚C with agitation. In order to optimize the expression condition, different concentrations of IPTG (0.5, 0.7, 0.9 and 1 mM) at different bacterial growth rates (OD 0.6, 0.8, and 1) were tested for two and four hours and analyzed on 18% SDS-PAGE. The expressed protein was purified using Ni-NTA column according to manufacture instructions (Qia-gene). The purified protein was dialyzed twice against PBS (pH 7.2) at 4˚C over night. Quantity of the purified recombinant L7/L12 protein was analyzed by Bradford methods and subsequently it's quality was assayed by SDS-PAGE 18% (2.5 μg /well). In order to analyze the cross-reaction between fused segment (near 5 kDa) of L7/L12 protein with patient sera, an *E. coli* BL21 (DE3) plysS containing pET28 a vector was induced by IPTG.

Immunoblot analysis. For Western blot analyses, 0.5 μg of purified recombinant L7/L12 protein was used per well. As a negative control, the bacterial lysate from induced *E. coli* BL21 (DE3) pLysS contain pET28a vector was analyzed by Western blot. The gel was blotted on to Polyvinylidine difluoride (PVDF Membrane, Roche Diagnostic) membrane using transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine and %20 methanol at 90 volts for 1.5 hours at 4˚C. The blotted membrane was blocked with 2.5% (w/v) BSA in TBST buffer (0.5M NaCl, 0.02 M Tris pH 8.5, 0.05% Tween 20) for 1 hour at room temperature. Membranes were incubated for 2 hours at room temperature with rabbit and patients sera, diluted 1:100 and 1:50, respectively. Normal sera from rabbit and human were used as controls. After reactions with the primary antibody, the blots were washed three times with TBST and incubated with peroxidase conjugated goat anti-rabbit IgG and anti-human Ig (G, A, M) at a 1:2500 dilution in TBST. The blots were then washed three times with TBST and reactions were developed by diamino Banzidine (DAB) Solution (Roche Diagnostic).

RESULTS

DNA preparation and amplification. The chromosomal DNA of *B. abortus* was prepared and the concentration was adjusted to 500 μg/ml, which was used as template for amplification of the gene encoded L7/L12. The amplified fragment had the expected size of 375 bp comparing to100 bp DNA ladder (Fermentas) (Fig. 1).

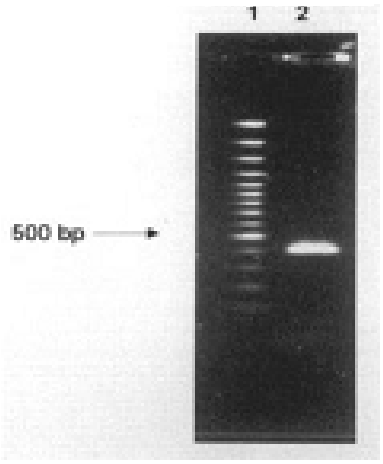

Fig. 1. L7/L12 gene amplification by PCR. Lane 1, Molecular weight marker (100 bp ladder); Lane 2, amplified L7/L12 with 2.5 mM Mg^{++}.

PCR product analysis and cloning. The eluted PCR product was analyzed by digestion with *Hind*III and *Eco*RI, the resulting fragments were compared with 100 bp ladder (Fig. 2). The restriction enzyme analysis showed expected sizes with *Hind*III (69 and 305 bp) and *Eco*RI (169 and 206 bp). The recombinant plasmid (pSK-L7/L12) was sequenced by standard T7/T3 primers and dideoxy chain termination method. The sequencing result was confirmed by comparing with the databases and using basic local alignment search tool (BLAST) Software (data not shown).

Expression and purification of recombinant L7/L12. Expression of PET28a-L7/L12 in *E. coli* BL21 (DE3) pLysS was induced and the expressed protein was purified by Ni-NTA column (Fig. 3). SDS-PAGE analyses showed the expected molecular mass of near 19 kDa fusion recombinant protein. The results showed that the best conditions for recombinant L7/L12expression can be achieved when 1 mM of IPTG and $OD_{600nm} = 0.6$ for four hours was used. The concentration of recombinant protein was assayed and calculated to 8 mg purified protein per liter of the initial culture.

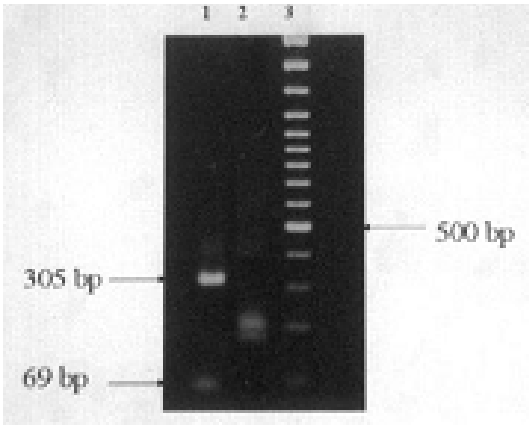

Fig. 2. Restriction enzyme analyses of L7/L12 gene. Lane 1, digestion of L7/L12 gene by *Hind*III (305 and 69 bp fragments); Lane 2, digestion of L7/L12 gene by *Eco*RI (206 and 169 bp fragments); Lane 3, Molecular weight marker (100 bp ladder).

Immunoblotting analysis in rabbit and infected human. To determine the antigenicity of recombinant L7/L12 in patients infected with *B. abortus*, the recombinant L7/L12 was assayed by Western-blotting. The standard anti recombinant L7/L12 rabbit serum and five patients sera were used. Figure 4 illustrates the specific interaction between standard anti L7/L12 rabbit antibody and purified recombinant L7/L12 protein. A normal serum from rabbit used as a negative control.

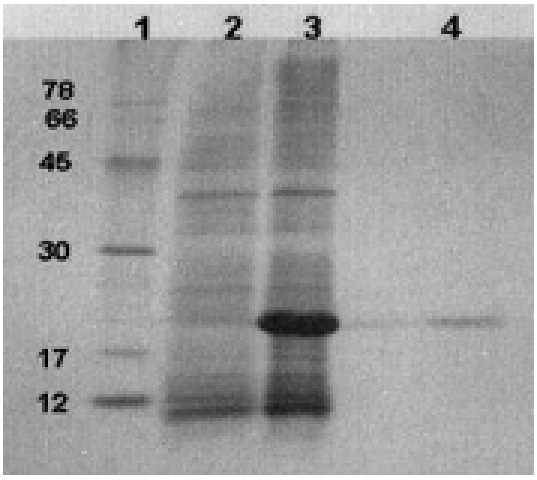

Fig. 3. Expression of recombinant L7/L12 protein and its purification. Lane 1, protein marker; Lane 2, pET28a-L7/L12 before induction; Lane 3, pET28a-L7/L12 after induction; Lane 4, elution of recombinant L7/L12 protein through Ni-NTA column.

Antigenicity of the expressed product was confirmed by Western blot analysis using patient sera. The specific antibody response from five patient sera were observed, Figure 5 (lanes 3-7). Serum sample from normal individual was also tested as negative control and no anti-L7/L12 antibodies were detected, Figure 5 (lane 8). There was no reaction between the expressed pET28a in *E. coli* BL21 (DE3) pLysS and patient serum (data not shown).

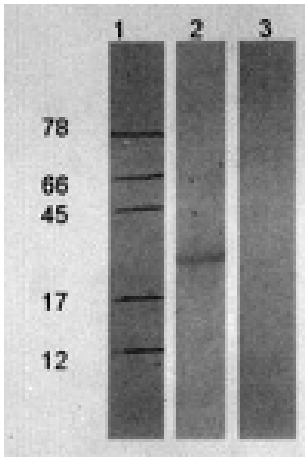

Fig. 4. Western blot analyses against recombinant L7/L12 protein by rabbit sera. Lane 1, Protein Marker; Lane 2, Western blotting by standard anti L7/L12 rabbit serum; Lane 3, Western blotting by normal rabbit serum (Negative Control).

DISCUSSION

B. abortus strain S19 is one of the most commonly used attenuated live vaccines against bovine brucellosis, which induces high level of protection in cattle [13]. The presence of smooth lipopolysaccharide in this vaccine may interfere with the discrimination between infected and vaccinated individuals and impair the test and slaughter strategy [14]. Moreover, this strain can cause abortion when administered to pregnant cattle [4] and is still fully virulent for human [15]. In order to avoid this drawback, alternative vaccination approaches are needed. Among these, subcellular vaccines, able to induce protective Th1 cell mediated immune response are being developed. Numerous protein antigens derived from *B. abortus* have been used in laboratory animals to study the humoral and cellular responses to experimental Brucellosis. Among these recombinant antigens L7/L12 [3, 5], P39 [8] and peptides comprising certain epitops of Cu-Zn SOD

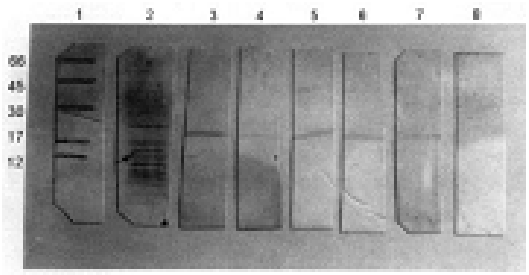

Fig. 5. Western blot analyses against recombinant L7/L12 protein by infected and normal human sera. Lane 1, Protein marker; Lane 2, *Brucella abortus* lysate react with serum from patient No; 1; Lanes 3-7, Western blotting by patients sera; Lane 8, human normal sera (Negative control).

[7] were induced some level of protection in a mouse model of infection. But, there was not any report about the antigenicity or immunogenicity of these proteins in humans. The *B. abortus* L7/L12 ribosomal protein has been identified as an immunodominant antigen in animals [16, 17]. Ribosomal proteins L7 and L12 are two proteins encoded by the same gene (*rplL*) and differ from each other only by an acetylic post translation modification that occurs at the L12 N terminus converting it to L7. L7/L12 consists of an N terminal domain that anchors the L10 ribosomal protein and a C terminal domain which is important in binding of elongation factor G and Tu. Therefore, the L7/L12 ribosomal protein is essential in bacterial ribosome for proper function in protein synthesis [14]. Due to the importance of high level production of recombinant protein in immunological studies, after identification of nucleotide sequence of *B.abortus* L7/L12 ribosomal protein [18], several groups cloned this gene in different expression vectors and recombinant protein was produced [18-20]. The maximum amount of expression reported has been approximately 6 mg of pure recombinant protein under the control of T5 promoter (pQE-30 QIA-GEN) system per liter of initial bacterial culture. In this study, we have sub-cloned and expressed *B. abortus* gene that encodes L7/L12 ribosomal protein under the control of T7 promoter and optimized condition. The expression level was increased to 8 mg (30% increase) of the purified protein per liter of initial bacterial culture [3]. In agreement with other findings [21], our results also indicate that the highly regulated expression vector with powerful T7 promoter (pET28a,), in conjunction with suitable host cell [*E. coli*, BL21 (DE3) pLysS], could influence the rate of expression. The expressed protein in pET system contains several extra amino acids (6xHis tag and T7 tag) linked to the C or N terminal extension of protein. These additional amino acids increase the size of expressed protein approximately 5 kDa, as shown in Figure 3. These extra amino acids may interfere with the subsequent immunological analyses. In order to investigate the effect of additional amino acids, pET28a vector in *E. coli* [BL21 (DE3) pLysS] was also induced by IPTG and further analyzed by Western blotting with rabbit and human sera. The results, in the agreement with other researcher [19], showed that there was no interfere related to fused amino acids.

During the infection by intracellular bacteria such as *Brucella* and *Mycobacteria*, some of the bacterial structural components, such as the L7/L12 ribosomal protein, were involved in the immune response developed in the host. However, the highly conserved sequences of the ribosomal L7/L12 protein among bacteria and even eukaryotes raised questions about the nature of the antigenic motif that was recognized by immune response of the infected host [3]. Here, by using the Western blot analysis, we showed that the *B.abortus* purified recombinant L7/L12 doesn't has any cross reactivity with normal human sera which was vaccinated against pathogenic bacteria such as *Mycobacterium tuberculosis*. Other groups demonstrated that recombinant *B. abortus* L7/L12 ribosomal protein acts as an immunogenes and induces partial immunity in animal models [5, 7], but there are no reports concerning the antigenicity of the recombinant L7/L12 in human. Our data showed that recombinant L7/L12 protein could be detected as an antigen by sera from acute phase of brucellosis in infected human. Additionally, other studies demonstrated that this protein can elicit cellular immunity in animals [3, 5, 22], but in human models it needs more investigation. We suggest that L7/L12 in combination with other molecular subunits of *B. abortus* would provide superior protection to *B. abortus* infection. As observed in malaria and leprosy models, solid protective immunity requires immunization with several proteins rather than a single moiety [23, 24].

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