JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 339-344

Cloning, Expression and Purification of *L7/L12* Ribosomal Gene of *Brucella melitensis* 16M

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(Received: 06 Augsut 2013; accepted: 20 October 2013)

Brucellosis is an important zoonotic disease transmitted to human and is also responsible for causing huge economic loss to livestock industry worldwide. Numerous candidate antigens of *Brucella* have been reported to induce protective effect against the organism, among which L7/L12 is an important one. In this study, the L7/L12 ribosomal gene of *Brucella melitensis* 16M was PCR amplified and cloned into prokaryotic expression vector pPROExHTb and the positive clones were confirmed by restriction enzyme digestion and sequencing. Recombinant clone was induced with 1mM of IPTG and the expressed protein was purified by Nickel affinity chromatography and quantified. The purified the recombinant L7/L12 protein was subjected to western blot analysis with hyperimmune serum, raised against it in rabbits and also with pooled clinical serum which revealed specific band of 16kDa confirming its antigenicity. This recombinant L7/L12 antigen can be used as protective antigen for control of brucellosis.

Keywords: *Brucella melitensis*, cloning, expression, L7/L12 gene

Brucella spp. are facultative intracellular Gram negative coccobacilli belonging to the alpha 2 subdivision of *Proteobacteria* responsible for causing brucellosis in animals and humans. Worldwide, brucellosis is responsible for causing huge economic loss to livestock industry and is also one of the important zoonotic diseases in humans. Transmission of brucellosis occurs by contact with infected animals or by consumption of their products while, person to person transmission is rare. Control of animal brucellosis has been reported to dramatically decrease the incidence of brucellosis in humans (Seleem *et al*., 2010). Vaccination is the only effective way to control brucellosis in endemic countries. Currently, available live vaccines against brucellosis like *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev1 are effective, but have certain disadvantages like ability to cause disease in humans, abortion in pregnant animals and interference in serological differentiation of infected and vaccinated animals (S19 and Rev1).

With recent development in molecular techniques, numerous approaches have been employed to develop more effective and safe vaccine against brucellosis. But, the major difficulty lies in the identification of immunodominant antigen of *Brucella* capable of inducing effective cell mediated immunity. Various cell surface and intracellular candidate antigens of *Brucella* have been assessed as protective antigens against *Brucella* with various degrees of success. Among them L7/L12 ribosomal protein was identified to be highly immunodominant with ability to induce T cell proliferation (CD4+) and cytokine production biased towards Th1 response (Brooks-Worreli and Splitter, 1992; Oliveira *et al*., 1994; Oliveira SC and

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Splitter, 1994; Oliveira *et al*., 2011). It also induces humoral immune response (Mallick *et al*., 2007b; Pakzad *et al*., 2009). The L7/L12 antigen was evaluated as recombinant protein vaccine or DNA vaccine or live vectored vaccine alone or in combination with other antigens in the presence of adjuvants by other researchers and was observed to induce significant degree of protection compared to live vaccines in mouse models (Oliveira and Splitter, 1996; Kurar and Splitter, 1997; Zhao *et al*., 2009). The aim of the present study was to clone, express and purify L7/L12 ribosomal gene of *B. melitensis* 16M and to characterize it by western blot analysis.

MATERIALS AND METHODS

Bacterial strains, vector

The *B. melitensis* 16M isolate available in the *Brucella* laboratory, Division of Veterinary Public Health (VPH), Indian Veterinary Research Institute (IVRI), Izatnagar, India, was grown on glucose dextrose agar for 48 h at 37°C under aerobic conditions. The prokaryotic expression vector pPROExHTb (Life Technologies, California) was used for recombinant protein production and the DH5α strains of *E. coli* were used as host strains. The *E. coli* cells were grown in Luria Bertani (LB) broth at 37°C with constant shaking at 150-200 rpm. The antibiotic ampicillin at the rate of 100 µg/ml was added to LB medium whenever required (Sambrook and Russell, 2001).

Isolation of genomic DNA

A loop-full culture of *B. melitensis* 16M strain was used for genomic DNA extraction using DNeasy Blood and Tissue Kit (Qiagen, USA) as per the manufacturer's recommendations. The quality of the DNA was analysed by 0.8% agarose gel electrophoresis in 0.5X TBE buffer and stored at -20°C until used.

PCR amplification

Primer specific for the L7/L12 gene of *B. melitensis*, forward primer RP-F (5'- 3'): GTG TAG GAT CCA TGG CTG ATC TCG CAA AG, and reverse primer RP-R (5'- 3'): GCC TAC TGC AGT TAC TTG AGT TCA ACC TTG GC, containing *BamHI* and *PstI* restriction site, respectively, was designed based on the nucleotide sequences available in Gene bank, and got synthesized commercially. PCR amplification of L7/L12 gene was carried out in 50 µl reaction volume containing 5µl of *pfu* Taq buffer (10X), 5µl of dNTP mixture (2mM each), 2 µl of each primers (10 pmol/µl), 1 µl *pfu* Taq DNA polymerase (2.5 U/µl), 5µl of genomic DNA and 30 µl of Nuclease free water. Amplification was performed using Mastercycler Gradient Thermocycler (Eppendorf, Germany) for 35 cycles, with denaturation at 94°C for 60s, annealing at 56°C for 45s and extension at 72°C for 60s followed by a final extension at 72°C for 5 min. Following amplification, the products were analysed by electrophoresis in 1.5% agarose gel and documented.

Cloning of *rL7/L12* gene in prokaryotic expression system

The PCR amplified L7/L12 gene was electrophorsed in 0.8% agarose gel and eluted using Qiagen gel extraction kit (Qiagen, USA) as per manufacturer's recommendations. The purity of the eluted product was checked by electrophoresis in 1.5% agarose gel. The prokaryotic expression vector pPROExHTb and purified insert (L7/L12) were subjected to double restriction enzyme (RE) digestion with *BamHI* and *PstI* (Fermentas, Lithuania) and purified by agarose gel electrophoresis and gel extraction. The digested vector and insert were ligated using T4 DNA ligase (Fermentas, Lithuania) at 22°C for 1h in thermal cycler (Eppendorf, Germany). The ligation product was transformed into *E. coli* DH5 α competent cells prepared by calcium chloride method (Sambrook and Russell, 2001), and plated on to LB agar plates containing 100 µg/ml ampicilin. The transformed clones were screened on LB agar plates after overnight incubation at 37°C and the positive clones were confirmed by RE analysis and sequencing.

Expression of recombinant L7/L12 protein

To standardize the expression conditions, the recombinant clone was grown in 10 ml LB broth supplemented with ampicillin at 37°C with constant shaking at 150-200 rpm and induced with different concentration of IPTG (0.5, 1 and 1.5 mM) after the concentration of cells has reached 0.6 OD. Following induction, 1 ml of broth was collected at hourly interval staring from 0 h up till 6 h and analysed by 15% SDS PAGE. The bulk induction of clone was carried out in 1 liter culture volume with optimized conditions, cells were pelleted by centrifugation and stored at 20°C until used.

Purification of recombinant protein

The expressed His tag-rL7/L12 fusion protein solubility was determined and purified using Ni-NTA column (Qiagen) according to protocol described by the manufacturer with slight modifications. Briefly, the induced clone pellet was thawed on ice for 15 min, resuspended in 4 volume of lysis buffer (NaH_2PO_4 20mM , NaCl 1M and Lysozyme 1 mg/ml) and incubated on ice for 30 min. PMSF (1mM) was added to it before sonication (Soniprep 150) for 6 burst of 10 s at 15 mA with 10 s cooling period between burst. The lysate was centrifuged at 12,000 rpm for 15 min at 4°C and supernatant was collected while the pellet was stored at -20°C. The soluble fraction of protein present in the supernatant was purified by Ni-NTA affinity chromatography and the flow through was also collected and stored at -20°C. The column was washed twice with wash buffer (NaH_2PO_4 20mM, Tris-Cl 0.1M; pH 7.4). The bound protein was eluted with stepwise increasing gradients of imidazole (10, 20, 40, 80, 150 and 200 mM) prepared in wash buffer and 4 fractions of 2 ml each were collected. The pellet, flow-through and different fractions of purified protein were analysed by SDS-PAGE (15 %). The protein concentration was quantified by Lowry's method (GeNeI, India) against standard curve of BSA and stored at -40°C until used.

Raising of antiserum and western blot analysis

The hyper immune serum against the recombinant L7/L12 protein was raised in three apparently healthy adult New Zealand White rabbits procured from Laboratory Animal Resources (LAR), IVRI. The rL7/L12 protein (200 µg) was emulsified with Freund's incomplete adjuvant (IFA) and injected subcutaneously at multiple sites. A booster dose of the antigen with IFA was given on day 14 and the blood was collected 3 weeks post immunization. The serum was separated and stored at -20°C. Production of antigen specific antibody was analysed by western blot analysis (Towbin *et al*., 1979). Briefly, the purified rL7/L12 protein was subjected to SDS-PAGE along with pre-stained marker and electro-blotted onto nitrocellulose membrane (NCP) using transfer buffer (Tris 0.1M, Glycine 0.192M and 5% methanol) by tank transfer method (GeNeI, India) at 90 volts for 1 h. The unbound site on NCP was blocked with 5% skim milk powder in Tris buffered saline and tween 20 (TBST; Tris 50 mM, NaCl 150mM and 0.2 % Tween 20) for 2 h at 37°C. Membranes were incubated at 37°C for 1 h with 1:500 dilution of hyper-immune serum raised against recombinant proteins in rabbits. After incubation with primary antibody, the blot was washed thrice with TBST and incubated with 1:2000 dilution of HRP conjugated goat anti-rabbit IgG for 1 h at 37°C. The blots were finally washed thrice with TBST and developed with diamino-benzidine (Sigma, USA) (6 mg/10 ml of 50 mM Tris buffer; pH 7.6) in the presence of 4 µl of H_2O_2 (30%). Similarly, western blot analysis was also carried out with pooled human clinical serum (1:500) and rabbit anti-human IgG HRP conjugate (GeNeI, India) at 1:2000 dilution.

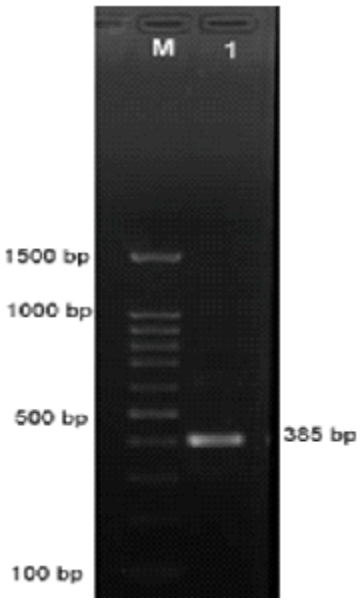
RESULTS

PCR amplification and Cloning of L7/l12 gene

Amplification of L7/L12 ribosomal protein gene of *B. melitensis* 16M yielded approximately 385 bp amplicon as observed by agarose gel electrophoresis (Figure 1). This amplicon was cloned successfully in pPROExHTb and RE analysis of the plasmid extracted from clones using *BamHI* and *PstI* revealed the presence of correct insert by release of around 385 bp insert (Figure 2). The sequencing of plasmid pPROExHTb-L7/L12 using M13-pUC rev (-49) primer revealed the correct sequence and orientation of the insert in the construct. The sequence was found to be similar to that of published sequences by nucleotide blast analysis and was submitted to GenBank (Accession number: KF362131).

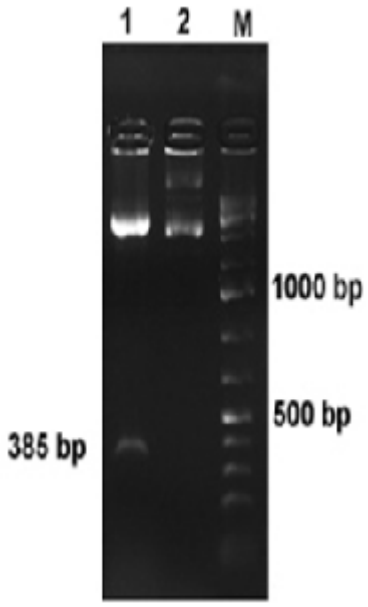
Expression and purification of recombinant protein

Optimization studies for induction indicated that induction with 1 mM of IPTG for 5 h at 37°C with agitation was optimum for expression of rL7/L12 protein. The recombinant L7/L12 protein was found to be expressed as soluble protein and was purified under native condition by Nickel affinity chromatography. Maximum elution of recombinant protein occurred in elution buffer containing imidazole between 40 to 150 mM, with highest elution occurring at 80 mM concentration of imidazole with a yield of 1.2 mg/ml of elute. The SDS-PAGE analysis revealed the expected recombinant fusion protein of around 16 kDa (Figure 3).



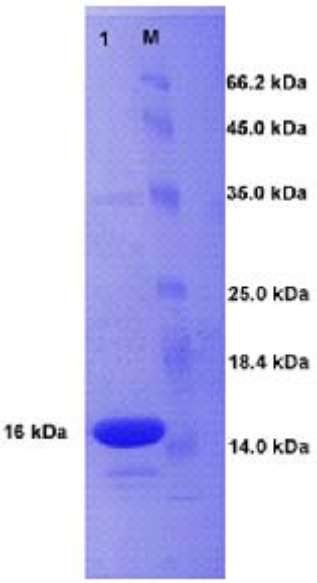
Lane M: 100 bp DNA ladder.
Lane 1: L7/L12 amplicon (approximately 385 bp)

Fig. 1. Agarose gel electrophoresis of PCR amplified L7/L12 gene of *B. melitensis* 16M



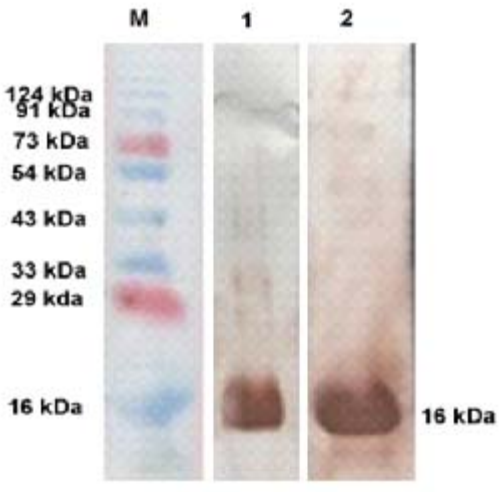
Lane 1: Restriction enzyme digested plasmid showing release of the insert (approximately 385 bp).
Lane 2: Uncut recombinant plasmid
Lane M: 100 bp plus DNA ladder

Fig. 2. Restriction enzyme analysis of the recombinant plasmid in agarose gel



Lane 1: Purified His-tagged fusion protein L7/L12.
Lane M: Protein molecular weight marker

Fig. 3. SDS-PAGE analysis of the purified recombinant L7/L12 protein



Lane M: Pre-stained protein molecular weight marker
Lane 1: Recombinant L7/L12 fusion protein with hyperimmune serum. Lane 2: Recombinant L7/L12 fusion protein with human clinical serum.

Fig. 4. Western blot analysis using hyperimmune serum raised in rabbits and human clinical serum

Immunoblot analysis

The antigenicity of the purified recombinant L7/L12 protein was confirmed by production of antigen specific antibodies as illustrated by specific interaction between purified antigen and hyperimmune sera raised in rabbit and also with clinical serum derived from human patients (Figure 4).

DISCUSSION

Control of brucellosis through vaccination was reported to the most economic way of controlling the disease in highly endemic countries. However, the currently available live vaccines against brucellosis are considered to be far from ideal (Oliveira *et al*., 2011). The search for an alternative vaccine which is effective, safe and able to provide complete protection still continues. Several approaches have been employed in the development of an alternative vaccine against brucellosis by researchers worldwide, like use of rough vaccine strains, mutants, recombinant protein vaccines, DNA and RNA vaccines, live vectored vaccines, over-expression of protein antigens (Oliveira *et al*., 2011). The use of a subcellular vaccine in the presence of an adjuvant has been considered to be a much safer approach. Among the various antigens analysed, L7/L12 protein has been identified to be highly immunodominant, capable of inducing T lymphocyte (CD4+) proliferation and cytokine production (Brooks-Worreli and Splitter, 1992; Oliveira *et al*., 1994; Oliveira SC and Splitter, 1994), besides being a major component in the antigenicity of brucellin INRA, responsible for inducing DTH response (Bacharach *et al*., 1994). It has been shown to induce significant degree of protection in mice when used as recombinant protein vaccine (Oliveira and Splitter, 1996) or as DNA vaccine (Kurar and Splitter, 1997). Later, in an attempt to improve its immunogenicity Mallick *et al*. (2007a, b) employed a liposomised form of antigen and found induction of a strong Th1 immune response with better systemic clearance of the challenge strain *B. abortus* 544.

In this study, cloning, expression, purification and western blot analysis of *B. melitensis* 16M L7/L12 ribosomal gene with an aim to use it as a recombinant vaccine with some other protein antigen of *Brucella*. The L7/L12 ribosomal protein gene of *B. melitensis* 16M was amplified, ligated with pPROExHTb prokaryote expression vector and transformed into *E. coli* DH5 α cells. Single positive clone was induced and purified under native condition by affinity chromatography using Ni-NTA column. The rL7/L12 protein was found to be antigenic based on its ability to induce antibody production in immunized rabbit and reacted specifically with the antigen in western blot analysis. It was also recognized by the serum derived from human brucellosis patients, indicating that this antigen is highly immunodominant able to induce antibody response in infected individual and can be used as a potential vaccine candidate antigen as has been reported earlier too (Abtahi *et al*., 2004).

In conclusion, we successfully cloned, expressed and purified rL7/L12 protein of *B. melitensis* 16M, in order to be used in future study as a vaccine candidate in mouse model in the presence of suitable adjuvant.

ACKNOWLEDGEMENTS

The Authors are thankful to the Head, Division of Veterinary Public Health and Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out this work. The fellowship received by Dr. S. Rajagunalan in the form of ICAR-SRF is also duly acknowledged.

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