

Expression of a Gene Encoding 30.7 kDa Protein of *Mycobacterium avium subsp. paratuberculosis* in *E.coli*

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Johne's disease or paratuberculosis is a chronic infectious enteric disease of ruminants caused by the intracellular pathogen. The control of the Johne's disease is hampered by lack of specific diagnostic tests. A unique ORF 843 bp encoding 30.7 kDa hypothetical protein of *Mycobacterium avium subsp. paratuberculosis* (MAP) was generated using PCR. The gene was cloned in frame into *E. coli* expression plasmid pQE 30. The recombinant plasmid designated as pQE 30.7 was transformed into *E. coli* M15 and induced with IPTG revealed the high level expression of 30.7 kDa protein which was confirmed by immunoblotting. Recombinant 30.7 kDa protein was then purified by Ni-NTA agarose chromatography. The polyclonal antiserum raised against purified recombinant 30.7 protein reacted with induced *E. coli* whole cell lysate as well as recombinant purified proteins on western blot. The 30.7 kDa expressed in the present study will prove useful as reagent in diagnostic test.

Keywords: 30.7 kDa, *Mycobacterium avium subsp. paratuberculosis*, accession no. EF 210209.

Johne's disease (JD) or paratuberculosis is a chronic, progressive, and incurable intestinal disease caused by *Mycobacterium avium subsp. paratuberculosis* (Map) in domestic and wild ruminants¹. The disease causes severe economic losses to agriculture industry due to reduced milk production and premature culling (Ott *et al.*, 1999). Infected animals with clinical signs shed the organism in feces (Clarke *et al.*, 1997, Stehman *et al.*, 1996) and milk (Taylor *et al.*, 1981), resulting in greater risk for other animals as well as human exposure (Gangadhararao *et al.*, 2013)

The progression of Map infection to different stages mainly depends on the immune status of individual animals. Any single diagnostic test is not full proof to detect Map infection at every stage. The control of JD was severely hampered due to lack of early diagnostics and

vaccines; there are many commercially available tests for JD each with their own advantages and limitations. Most tests perform well at the herd level. But for the identification of individually infected animal, a combination of complementary tests, and repeated sampling can increase the diagnostic sensitivity for efficient control programs.

Diagnosis of the Map is a challenge due to the chronic nature and prevalence of stages of disease. Currently practiced serological testing for JD in animals includes agar gel immunodiffusion (Sherman *et al.*, 1984) and commercial ELISA tests are generally preferred. The intradermal Johnin test and interferon gamma assays (IFN- γ) (Wood *et al.*, 1991, Rothel *et al.*, 1992) are CMI based tests and the results are not reliable because of using the crude antigens which showed variability in potency and cross reactivities. The bacterial isolation from fecal and tissue samples is considered as a gold standard for definitive diagnosis, though consuming.

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Since the sequencing and analysis of the entire Map genome it has become possible to identify several unique ORF's. These unique ORF's on expression in heterologous *E. coli* system can be used to generate proteins for specific detection of Map in infected animals. In the present study the heterologous expression, purification and characterization of 843 bp unique ORF encoding 30.7 kDa protein of *M. a. paratuberculosis* has been studied.

MATERIALS AND METHODS

Bacterial Strains and Plasmid

M. a. paratuberculosis strain 316F was obtained from Central Diengenees Kundig Tieh Institute, Lelystad, The Netherlands, and maintained at Gene Expression Laboratory, Veterinary Biotechnology Division, IVRI, Izatnagar, India. The mycobacteria were grown and maintained at 37°C on Middlebrook 7H10 agar (Difco laboratories, Detroit, USA) enriched with 0.1% glycerol (v/v) and 10% oleic acid dextrose catalase (Difco laboratories and supplemented with 20 mg/L mycobactin J (Allied Monitor, Fayette, USA) was also included. *E. coli* strain (M15, pREP4) supplied by Qiagen (Valencia, USA) was grown at 37°C in Luria Bertani broth containing kanamycin (25 µg/mL), as the strain carries kanamycin resistant plasmid. The expression vector plasmid pQE30 was purchased from Qiagen. The plasmid contains a T5 promoter and a 6 X His-tag coding sequence at 5' to the multiple cloning region. The plasmid also contains an ampicillin resistance marker.

Oligonucleotide primers

To amplify a 17 kDa protein of Map, the primers: Map17F (sense) 5' 5' TACGAGCTCATG CCG TCG CTG AAG CTC AC -3' 29 mer and Map17R (antisense) 5' CGG AACCTT TCA GTCGATCCGGTCG -3' -30 mer, respectively, containing *SacI/HindIII* restriction endonuclease sites were designed on the basis of sequence information of Map str. k10, complete genome Gene Bank Accession No. AE016958 tag Map 3437c region 3816702-3817544.

Preparation of DNA

The genomic DNA from *M. a. paratuberculosis* was isolated from the grown culture by the method of Portillo et al. (Portillo et

al., 1991). Plasmid DNA extraction from *E. coli* was carried out by the alkaline lysis method (Sambrook et al., 2001).

PCR Amplification and Cloning

The amplification reaction was performed in a 25 µL reaction volume containing 100 ng of *M. a. paratuberculosis* DNA; 2.5 µL of *Taq* DNA polymerase buffer 10 mM/L Tris.HCl (pH 9.0), 50 mM/L KCl, 1.5mM/L MgCl₂ and 0.01% (w/v) gelatin; 200 µM of each dNTP; 0.5 µM each primers; 1 units of *Taq* DNA polymerase. The final volume was made up with sterile distilled water. The reaction was carried out in a PTC-100 reactor (MJ Research Inc., Waltham, USA) for 30 cycles each cycle consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The amplified product was analyzed by submarine gel electrophoresis (Genei, Bangalore, India) on 1% agarose gel. The amplified gene product was purified from agarose gel using a QIAEXII gel extraction kit (Qiagen). For the purpose of cloning, about 500 ng of the PCR product was ligated into the plasmid pQE30 expression vector. The resulting plasmid p30.7 was transformed into competent *E. coli* M15 cells. The recombinant clones were selected on LB agar containing ampicillin (75 µg/mL) and kanamycin (25 µg/mL).

Transformants were further screened by restriction enzyme analysis of the plasmids with *SacI* and *HindIII* (Sambrook et al., 2001). pUC18 *Sau* 3AI/*TaqI* digest were used as standard molecular weight marker for agarose gel electrophoresis (Genei).

Expression and Purification of the Recombinant 30.7kDa His-Fusion Protein

E. coli M 15 cells harbouring the plasmid pQ30.7 were grown in LB medium containing (75 µg/mL) and kanamycin (25 µg/mL) and induced with 1.0mM IPTG for 4–6 hours. The purification of recombinant 30.7 kDa His fusion protein under denaturing conditions was carried out by single step affinity chromatography using Ni-NTA (nickel-nitrilotriacetate) agarose (Qiagen) and renatured as described previously (Basagoudanavar et al., 2004). The protein concentration was determined spectrophotometrically (Lowry et al., 1951). The protein solution was sterilized by filtration, and aliquots were stored at 70°C, until used.

Two New Zealand White rabbits (8–10

weeks old) obtained from Laboratory Animals Resource section, IVRI, Izatnagar were used to raise antibody against the recombinant 30.7 kDa His-fusion protein. Rabbits were immunized subcutaneously with 150 μ g of immunogen with incomplete Freund's adjuvant (IFA) (Genei, Bangalore, India), and boosters of 100 μ g of immunogens with IFA were given intramuscularly after 3 weeks and again 2 weeks later. Animals were bled ten days after the second booster and sera samples separated and stored at -20°C. All the sera used in this study were preabsorbed with *E. coli* antigens following the procedure of Harlow and Lane (Laemmli *et al.*, 1970). In brief, recombinant 30.7 kDa His-fusion protein was initially electrophoresed and transferred to nitrocellulose membrane. The specific protein band was excised from the blot and incubated with polyclonal rabbit serum diluted 1 : 3 in buffer I (0.1M NaH₂PO₄, 0.01M Tris.Cl, pH 7.5) at 37°C, overnight. Following washing the membrane in buffer I, the monospecific antibodies were eluted in buffer II (0.1M NaH₂PO₄, 0.01M Tris.Cl, pH 6.3). These were utilized in western blotting experiments.

SDS-PAGE and Western Blotting

SDS-PAGE of the expressed recombinant 30.7 kDa His-fusion protein was carried out on a vertical slab mini apparatus (Atto, Tokyo, Japan) using polyacrylamide gels run under denaturing conditions as described by Laemmli in (Towbin *et al.*, 1979). The 12% separating and 4% stacking polyacrylamide gel contained 0.1% SDS. About 25 μ g of each sample was boiled in equal volume of 2X sample loading buffer prior to loading. Electrophoresis was carried with Tris-glycine electrode buffer (1X), pH 8.3 at 90V for 2 hours; the gels were stained overnight with Coomassie brilliant blue G 250 (Sigma, Milwaukee, USA) (Bjerrum *et al.*, 1986). The coloured protein marker range from 17 to 175 kDa (New England Biolab, USA) was used as size markers. Samples electrophoresed on 12% SDS-PAGE were transferred to nitrocellulose membranes (0.45 μ M) using semidry electroblotting (Atto, Tokyo, Japan) at 0.8mA/cm², following the method of Bjerrum and Schaffer-Nielsen (Kyte *et al.*, 1982). The blots were blocked with 2% skimmed milk powder in PBS-T buffer (PBS containing 0.1% Tween-20) for 2 hours at room temperature. After washing with PBS-T buffer three times, the membranes were

incubated for 2 hours at 37°C with antisera against 30.7kDa His-fusion protein (1: 10000 in PBS) raised in rabbit. Following further washing, the blots were incubated with a 1: 1000 dilution of HRP-labelled goat anti-rabbit IgG (Sigma) for 1 hour. After washing, the blot was dipped in substrate solution (0.02% diaminobenzidine suspended in PBS containing 0.03% hydrogen peroxide in PBS pH 7.4) for a minute, till brown colour developed.

Nucleotide Sequencing and Deduced Amino Acid Analysis

The nucleotide sequence determined of the 843 bp gene encoding 30.7 protein of Map strain 316F has been deposited in nucleotide database. The deduced amino acid sequence of gene encoding 30.7 kDa protein from Map was analyzed for hydrophobic domains according to Kyte and Doolittle algorithm (Li *et al.*, 2005) using Lasergene software (DNASTAR, Madison, USA). The homology prediction was also done with the help of public server UNIPROT and PSI-BLAST.

RESULTS

PCR Amplification and Construction of Recombinant pQ30.7

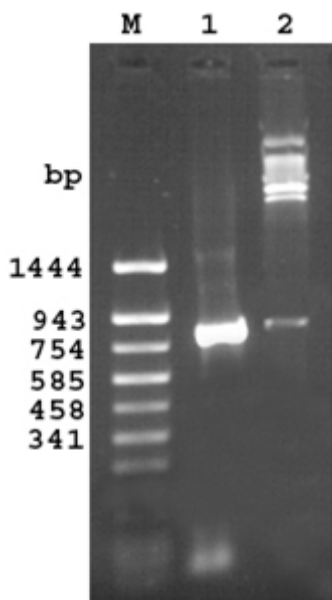
A PCR product of 858 bp (843bp gene and 15 bp linker) was obtained on amplification at 55°C. Restriction digestion of the recombinant plasmid pQ30.7 with SacI and HindIII released an identical size fragment as seen on a 1% agarose gel (Figure 1).

Expression of Recombinant 30.7kDa Protein in *E. coli*

When *E. coli* M15 cells harbouring the recombinant plasmid pQ30.7 were induced for 6 hours with 1 mM/L IPTG and analysed by 12% SDS-PAGE and Coomassie staining, a predominant band corresponding to that predicted for 30.7 kDa protein was detected in total cell extract of the *E. coli* (Figure 2, lane 2). The recombinant protein formed a major (more than 30%) portion of the total *E. coli* extract. No such protein band was observed with *E. coli* M15 cells or in uninduced *E. coli* M15 cells harbouring recombinant plasmid pQ30.7 (Figure 2, Lanes 3 and 4).

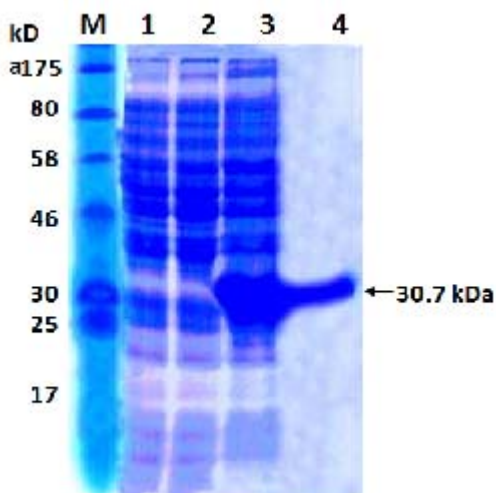
Purification of the Recombinant 30.7kDa Protein

The His-tagged purified protein, when analyzed on SDS-PAGE, showed a monomeric band of about 30.7 kDa size (Figure 2, Lane 1). Purification



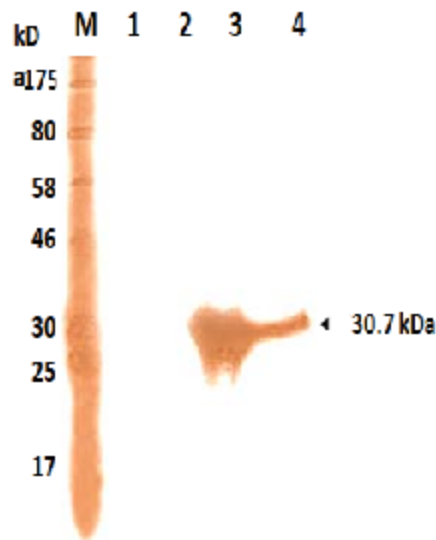
Lane M: DNA molecular weight marker pUC18 Sau 3AI/TaqI digest
 Lane 1: PCR amplified fragment encoding 30.7 kDa protein
 Lane 2: Released insert of 843bp after SacI and Hind III digestion

Fig. 1. Agarose gel (1.2%) of the cloned fragment encoding 30.7 kDa protein of *M. a. paratuberculosis*



Lane M: Protein Marker
 Lane 1: Cell extract of *E. coli* M15
 Lane 2: Cell extract of *E. coli* M15 harbouring pQ30.7 clone un-induced
 Lane 3: Cell extract of *E. coli* M15 harbouring pQ30.7 clone induced
 Lane 4: Purified 30.7 kDa protein

Fig. 2. SDS PAGE analysis of the recombinant 17kDa protein



Lane M: Protein Marker
 Lane 1: Cell extract of *E. coli* M15
 Lane 2: Cell extract of *E. coli* M15 harbouring pQ17 clone un-induced
 Lane 3: Cell extract of *E. coli* M15 harbouring pQ17 clone induced
 Lane 4: Purified 17 kDa protein

Fig. 3. Western Blot analysis of the recombinant 17kDa protein

of the recombinant protein was nearly 80%, as visualized on SDS-PAGE. The yield of the pure recombinant protein was about 10–15 mg/L of culture at shake flask level.

Immunoreactivity of the Recombinant Protein

The polyclonal antiserum raised against purified 30.7 kDa protein in rabbits could bind to the IPTG that induced whole cell extract expressing recombinant as well as purified 30.7 kDa protein on western blot (Figure 3, Lanes 2 and 1). However no such band was visible in uninduced total cell extract of M15 harbouring pQ30.7 plasmid (Figure 3, Lane 3)

Deduced Amino Acid Analysis

The nucleotide sequence of the pQ 30.7 plasmid having 843 bp gene of Map has been deposited in Gene bank database under accession no. EF 210209. The Blast search of the sequence revealed that no other mycobacterial sequences have homology. The predicted 281 amino acids of the 843 bp gene fragment had a mature are protein of 30.7kDa. Analysis of the deduced 281 amino

acids sequence indicated that the protein contained four major hydrophobic regions (amino acids 18–25, 119–126, 217–224 and 264–271)

DISCUSSION

JD caused by *M.a. paratuberculosis*, with its significant impact on livestock economy worldwide, has become a disease of concern. Currently, all antigen based diagnostic tests for JD use a complex mixtures of antigens, many of which are highly conserved among mycobacterial species. Diagnostic antigens appear to show promise, but none has been proved useful as a routine diagnostic tool.

The study of the complete genome of the *M. a. paratuberculosis* has accelerated the understanding the biology of this significant animal pathogen and also provide information about several ORFs specific only to pathogen (Leroy *et al.*, 2009). Analysis of the *M. a. paratuberculosis* genome has revealed a total of 87 predicted coding sequences that are not present in available sequence databases, suggesting that these genes are unique and specific to *M. a. paratuberculosis* (Skeiky *et al.*, 2000).

The next critical step will be learning functional roles of all the protein encoded by the ORFs. An achievement of this objective depends on the rapid expression and purification of proteins on a large scale. Therefore, the present study was envisaged to address the critical importance of identifying species specific antigens through the cloning and heterologous expression of unique predicted coding sequence found in the *M. a. paratuberculosis* genome.

An unique sequence of 843 bp from the Map genome with no homology with other mycobacteria and efficient expression system, based on the pQE30 vector, to produce 30.7kDa protein of Map in *E.coli*, so as to facilitate further characterization of the protein. Based on the sequence information of the gene encoding 30.7 kDa protein of Map str. k10, complete genome Gene Bank Accession No. AE016958 (tag Map 1636c region 1790543-1791016 coding for hypothetical protein) and also the information on the multiple cloning site of the expression vector pQE30 vector, restriction sites for *SacI/HindIII* were incorporated into the primers to facilitate directional cloning. In

the design of the forward primer, consideration was given to inserting the amplified gene of 30.7kDa protein in frame into the pQE30 vector under the T5 promoter. The resulting plasmid pQ30.7 contained an open reading frame encoding successively 6X.His polypeptide and the 30.7kDa protein. The recombinant pQ30.7 clones were confirmed by release of the insert by double digestion with *SacI/HindIII* enzymes and on induction with IPTG appearance of the 17kDa band consistent with the predicted size of 30.7kDa protein. A similar strategy of cloning in pQE series expression vector system was applied to generate high levels of 35 kDa protein of Map (Basagoudanavar *et al.*, 2004) and a 26kDa protein of *Brucella abortus* (Sisk *et al.*, 1994). Expression of recombinant proteins is induced by, IPTG which binds to the *lac* repressor protein, inactivating it leading to transcription of sequences downstream of the promoter Expression of the gene facilitated the production of large amounts of the recombinant protein for immunological studies. The presence of 6X-histidine tag is of 840 Da in size at N-terminal of the recombinant protein facilitated single step affinity purification. It rarely interferes with protein immunogenicity, protein functional structure, hence the tag was not removed by protease cleavage (Sisk *et al.*, 1994).

Authenticity of the purified recombinant protein was confirmed by reaction of the protein with the antisera raised against the protein. The antisera reacted strongly against purified 30.7 kDa as well as recombinant 30.7 kDa expressed in *E. coli* lysate in western blot showing their specificity and antigenicity.

The analysis of the deduced amino acid sequence according to the Kyte and Doolittle algorithm (Kumar *et al.*, 2008), showed the presence of four highly hydrophobic regions, which could be the membrane segment of the protein. The homology study and deduced amino acid sequence are unique to Map and have the potential to be developed as diagnostic reagent.

The characterization of *M. a. paratuberculosis* specific ORF was one of the major challenge that has been addressed in this work. The high level expression of recombinant 30.7 kDa provides opportunity for its further characterization. The results of this study suggested that a specific protein 30.7kDa could be

useful for the development of diagnostic reagent. Extensive screening with sera of clinical and subclinically affected cases of paratuberculosis are needed to ascertain the diagnostic potential with regard to specificity and sensitivity with 30.7kDa as antigen.

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