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Cloning and molecular characterization of fibronectin attachment protein gene (*ModD*) of *Mycobacterium avium* subspecies *paratuberculosis*

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ABSTRACT

Fibronectin attachment protein (FAP) also known as *ModD* gene of 987 bp size was identified in the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) genome. This gene was successfully amplified by PCR using specific primers and MAP genomic DNA template, in the presence of 10% DMSO and glycerol. The PCR product was cloned in pGEMT easy cloning vector and subsequently subcloned into pET-33b expression vector and sequenced. MAP *ModD* gene showed sequence identity ranging from 64 to 94% at nucleotide level and 47–87% at amino acid level, by WU-BLAST program with FAPs of other *Mycobacteria* species. MAP ModD gene showed 97% sequence homology with *M. avium* subsp *avium ModD* by Megalign tool of DNAStar programme. This strong identity was attributed to the more close genetic association of these two species. MAP *ModD* protein is mainly composed of beta sheets and is highly antigenic. Positive pET*ModD* recombinant clone was induced with IPTG for expression of the *ModD* protein. The MAP *ModD* protein was purified under native condition using nickel affinity column. The expressed recombinant *ModD* (52 kDa) was confirmed by Western blot with nickel-HRPO conjugate and rabbit hyperimmune serum raised against recombinant *ModD*.

Key words: Cloning, Fibronectin attachment protein, *ModD*, *Mycobacterium avium* subsp. *paratuberculosis*

Johne's disease (paratuberculosis) caused by *Mycobacterium avium* subsp. *paratuberculosis* is a chronic granulomatous infection of the gastrointestinal tract of dairy cattle (Kennedy *et al*. 2001). The presence of MAP in milk, resistance to pasteurization temperature, and association of this organism with Crohn's disease, suggests MAP as a public health significance organism (Herman-Taylor 2001). The exact mechanism involved in the interaction of MAP with the host intestinal mucosa is yet to be completely understood. Momotani *et al.* (1988) demonstrated that the mycobacteria were endocytosed by the M cells. Secott *et al.* (2001) demonstrated the importance of fibronectin (FN) in binding of the pathogen to the M cells. The ability of a microorganism to bind FN may potentially facilitate its colonization of the host through attachment to the extracellular matrix in areas of epithelial damage. Furthermore, because several host cell integrins have binding sites for FN, the ability of a microorganism to bind soluble FN establishes a bridge between the organism and the host cell cytoskeleton, a condition necessary for the internalization of the microbe by the cell. Fibronectin attachment proteins (FAPs) also called as *ModD*, comprise a family of FN-binding glycoproteins that are expressed by several species of mycobacteria (Zhao *et al*. 2000). This study was designed to clone and characterize FAP/*ModD* gene in Indian isolate (C-132) of MAP.

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MATERIALS AND METHODS

Bacterial culture: Liquid culture of *Mycobacterium avium* subsp. *paratuberculosis* strain C-132 was obtained from Goat Disease Laboratory, Division of Pathology, Indian Veterinary Research Institute (IVRI), Izatnagar. *Escherichia coli* DH5α, BL21 (DE3) and JM109 strains were obtained and revived from the repository of Division of Biological Standardization, IVRI.

Isolation of genomic DNA: Genomic DNA was extracted from *M. avium* subsp. *paratuberculosis* by Cetyl Trimethyl Ammonium Bromide (CTAB) lysis method (van Soolingen 1999).

Amplification of ModD *gene by PCR and its characterization:* The region with high homology to *ModD* gene was searched in the MAP genome by BLAST analysis (Altschul *et al*. 1997) using *M. avium ModD* gene sequence. The forward and reverse primer sequences used for PCR amplification of gene were also aligned with this region to define the open reading frame (ORF) for *ModD* protein. Oligonucleotide primers were designed with restriction site at their 5' end to amplify the *ModD* gene of MAP, using GENE TOOL software. The sequences of the primers were forward (with *Nde*I at 5'end): 5'-CATATGGATC

CCGAGGTCCCGACC-3', reverse (with *Xho*I at 5'end): 5'-CTCGAGGGCCGAGAGGGTCTGCTGC-3'. PCR reaction was performed in a total volume of 25 µl. The reaction mixture contained 1.5 U of Pfu DNA polymerase, 2.5 µl of 10× PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20], 1.5 mM $MgCl_2$, 200 mM of each dNTP's, 10 pmol primers, 50 ng of template DNA. Dimethyl sulphoxide (DMSO) and glycerol was included in the reaction at 10% final concentration, to melt the high GC containing genomic DNA, which is characteristic to mycobacterial species. The amplification cycle was 30 cycles of denaturation at 95ºC for 1 min, primer annealing at 53ºC for 1 min and primer extension at 72ºC for 1 min. The amplified PCR product was characterized by digestion with *Hind*III restriction enzyme and the digested product was run on 1.0% agarose gel.

Cloning of ModD *gene:* The amplified *ModD* gene fragment was gel purified using gel extraction kit. This gel purified *ModD* gene fragment was ligated into a T/A cloning vector followed by transformation into competent *E. coli* DH5α cells. The recombinant pGEMT plasmids (pG*ModD*) were isolated using plasmid extraction kit following manufacture's instruction. Positive clones were confirmed by colony PCR and RE digestion using *NdeI* and *XhoI* restriction enzymes followed by analysis of digested product on 1.0% agarose gel. The insert DNA released from the recombinant pG*ModD* plasmid was purified and ligated into previously digested and purified pET-33b expression vector. The ligated vector-insert was transformed into DH5α cells and recombinant pETModD plasmid was isolated and confirmed by RE digestion and sequencing. The nucleotide sequence was subsequently submitted to GenBank database of NCBI. BLAST similarity search of MAPModD gene was conducted using WU-BLAST program and sequence information were analyzed using Megalign tool of DNASTAR software.

Expression of ModD *protein: E. coli* BL21 (DE3) cells were transformed with pETModD recombinant plasmids and grown at 37°C overnight, on LB agar plates containing kanamycin (50 µg/ml) and chloramphenicol (64 µg/ml). The transformed BL21 colonies were screened for the presence of *ModD* gene specific sequences by PCR. The positive individual colonies were grown at 30°C till the culture reached mid-log phase (OD 600nm - 0.4–0.5). The expression was induced at 30°C by using 1 mM isopropyl thiogalactoside (IPTG). Samples were collected at 0, 4, 5, 6, 7, 8 and 9 h post-induction (hpi) and were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as per standard procedures. Similarly, different concentrations of IPTG starting from 0.5–2 mM were also tried to find out the suitable concentration for maximum expression.

Purification of recombinant ModD *protein:* The recombinant *ModD* with 6× histidine residue at both the ends of protein was purified under native condition (applying different imidazole concentration) by using Ni-NTA agarose resin affinity chromatograph following manufacturer's protocol. The bacterial pellet was thawed on ice and 4 volume of lysis buffer (NaCl 300 mM, NaH_2PO_4 50 mM, immidazole 5 mM, pH-8.0) was added, and incubated for 60 min at room temperature. The cell lysate was centrifuged at 10,000×g for 30 min at room temperature to pellet the cell debris. The supernatant (cleared lysate) was added to pre-equilibrate Ni-NTA resin and mixed gently and incubated for 60 min at room temperature. The lysate-resin mixture was carefully loaded onto an empty column with the bottom outlet closed. After complete loading, the bottom outlet was opened and the flow-through was collected separately and the column was simultaneously packed. The column bed was then washed twice with equal volumes of wash buffer (NaCl 300 mM, NaH_2PO_4 50 mM, immidazole 10 mM, pH-8.0). The column wash was also collected separately and saved. The bound protein was eluted 4 times with 2 ml each of elution buffer (NaCl 300 mM, NaH_2PO_4 50 mM, immidazole 250 mM, pH-8.0) and the fractions were collected. The eluted fractions were analyzed for purification pattern of His-tagged expressed protein in SDS-PAGE as per standard procedures.

Characterization of recombinant ModD: The specific immunoreactivity of the expressed recombinant ModD protein was checked by western blotting with Ni-NTA conjugate and rabbit antiserum against recombinant *ModD* protein. The purified recombinant protein was loaded and ran on SDS-PAGE using 10% gel. The resolved protein was electrophoretically transferred to a nitrocellulose membrane (NCM) at 0.8 $mAmp/cm^2$ constant current for 1 h using semi-dry blotting apparatus. After the transfer, the nitrocellulose paper was blocked overnight by 1% BSA. The membrane was incubated in a solution of primary antibody (rabbit *ModD* protein antiserum, 1:1,000 dilution) for 1 h at room temperature, washed with several changes of PBS-T and incubated with 1:15,000 dilution of secondary antibody conjugated to horse radish peroxidase for 1 h. The membrane was washed thoroughly with PBS-T and developed with diaminobenzidene (DAB) substrate solution. Further confirmation was done by western blot using Ni-NTA conjugates. This protocol uses standard western blotting procedures. Briefly, the western blot membrane carrying transferred *ModD* were washed twice for 10 min each time with TBS buffer. The membrane was incubated for 1 h in 3% BSA in TBS at room temperature. The membrane was washed thrice for 10 min with TBS-Tween buffer. After that, the membrane was incubated for 1 h at room temperature in TBS-Tween buffer containing a 1/1,000 dilution of Ni-NTA conjugate stock solution. After a brief washing, the membrane was stained with HRP staining solution until the signal was clearly visible. The reaction was stopped by rinsing the membrane twice in water. The membrane was dried and photographed for a future record.

RESULTS AND DISCUSSION

A 987 bp gene with homology/similarity to other FAP

genes was identified in the MAP genome, by Basic Local Alignment Search Tool (BLAST) analysis with *M. avium* and other mycobacterial FAPs. The open reading frame (ORF) of MAP *ModD* had a high G+C content (75.28%) which correlated to high GC content of *M. avium* and other mycobacterial FAPs (Holsti *et al*. 1998). MAP *ModD* gene could not be amplified without the use of a co-solvent namely dimethyl sulfoxide (DMSO) and glycerol which was later-on rectified by inclusion of DMSO and glycerol in the PCR reaction mixture, which facilitated denaturation/melting of the GC rich template DNA of MAP *ModD* gene that led to amplification of the gene. The beneficial roles of such cosolvents in reducing high levels of mispriming and in increasing efficiency of amplification of GC rich templates were reported (Newton and Graham 1994, Vardraj and Skinner 1994).

Genomic DNA from MAP was extracted and purified by CTAB method, used as template for PCR amplification of *ModD* gene yielded specific and non-specific product corresponding to the MAP *ModD* gene. *ModD* gene was amplified by PCR at an annealing temperature of 53°C with 1.5 mM $MgCl_2$ and 30 cycles of amplification, including DMSO and glycerol in the PCR reaction mixture at 10% level. No amplification was seen when DMSO and glycerol were used at less than 10% final concentration. Two close bands were found ~987 bp, subsequently these 2 bands were cut from the agarose gel and eluted and were checked for unique restriction enzyme digestion pattern of *ModD* gene using *Hind*III restriction enzyme. Eluted product from lower band gave 2 products of ~542 and ~445 bp size after digestion, which confirmed that this band was the specific product. Further, this eluted product used as template for PCR amplification. The size of the PCR product was as per the expectation; as primers designed were encompassing *ModD* (ORF-Signal Sequence) and RE sites for *Nde*I and *Xho*I. It was further confirmed by RE digestion with *Hind*III enzyme and as predicted by sequence analysis, 2 fragment of 542 and 445 bp were obtained upon agarose gel electrophoresis. The 987 bp PCR product representing MAP *ModD* gene was ligated into T/A cloning vector (pGEM-T Easy) and was transformed into competent *E. coli* DH5α, which yielded both blue and white colonies in LB agar plate containing ampicillin. Blue colonies usually carry wild-type plasmids containing active µ-galactosidase enzyme whereas white colonies carry recombinant plasmids that do not contain active µ-galactosidase because of insertion of PCR product into the T/A cloning vector and hence they do not form blue colonies in the presence of the chromogenic X-gal (Sambrook and Russell 2001). Recombinant pG*ModD* plasmids were confirmed by colony PCR and RE digestion using restriction enzymes (*Nde*I and *Xho*I) (Figs. 1, 2)

For the expression of *Mod*D gene in prokaryotic vector, plasmid DNA of recombinant clone having *Mod*D gene (pG*Mod*D) and prokaryotic expression vector pET-33b were isolated, digested with *Nde*I and *Xho*I enzyme, ligated together and transformed into competent BL21 (DE3) cell.

Fig. 1. Confirmation of recombinant pGModD clones by colony PCR. Lane M, 100 bp DNA ladder; lane 1, amplified product from clone 1; lane 2, amplified product from clone 2; lane 3, no amplification from clone 3; lane 4, amplified product from clone 4; lane 5, amplified product from clone 5; lane 6, no amplification from clone 6; lane 7, amplified product from clone 7.

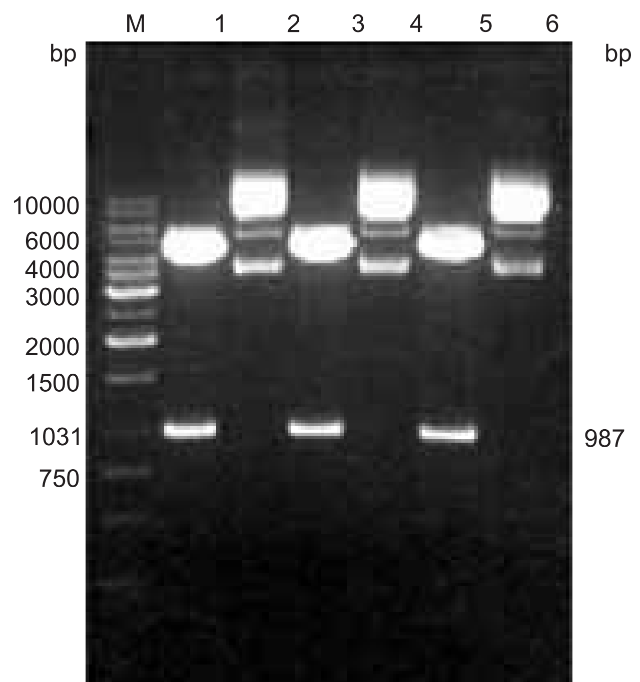
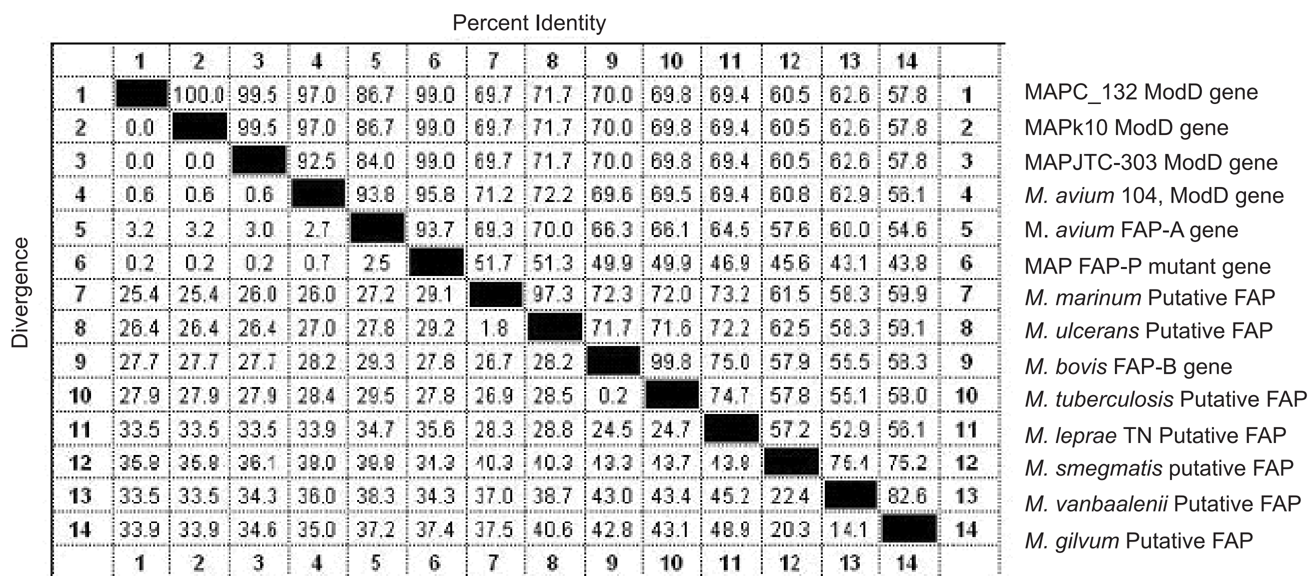


Fig. 2. Confirmation of recombinant pGModD clones by RE digestion. lane M, 100 bp DNA ladder; lane 1, undigested pGModD clone-1; lane 2, digested pGModD clone-1; lane 3, undigested pGModD clone–2; lane 4, digested pGModD clone-2; lane 5, undigested pGModD clone-4; lane 6, digested pGModD clone-4.

The recombinant clones (pET*ModD*) were all white irrespective of presence or absence of insert DNA because these vectors did not carry the gene for β-galactosidase enzyme. Subsequently, nucleotide sequencing of MAP *ModD* gene in pET*ModD* recombinant plasmid was carried out to understand the primary structure of the protein and to further dissect the molecule into structural, functional domains and for comparison of its sequence for similarity to other proteins of this family. Sequence information of the gene also confirmed the proper orientation of insert in the expression vector. Selected clone, bearing the specific insert, was commercially sequenced by di-deoxy chain termination method and subsequently submitted in NCBI database (accession number KF021287). MAP *ModD* gene sequence found similar to sequence of standard strain of MAP (K10), and in contrast to finding of Cho *et al*. (2007), which showed 18 bp deletion. MAP *ModD* gene sequence showed highest homology (97%) to *M. avium* subsp. *avium ModD* gene (Fig. 3). This strong nucleotide identity between *M. avium* and *M. paratuberculosis ModD* is due to the high degree of genetic relatedness of these 2 species, which are grouped within the *M. avium* complex (MAC), even though they differ between each other with respect to some phenotypic properties (Bannantine *et al*. 2003). Phylogenetic mapping analysis of MAP *ModD* gene with other FAP revealed that the *ModD*/FAP of *M. avium* was closely related. It was slightly distant related to *M. bovis* and *M. tuberculosis* whereas other mycobacteria were distantly related (Fig. 4).

Expression of recombinant MAP *ModD* was carried out using pET33-b expression vector. This expression system is one of the most powerful systems for expression in *E. coli*, which utilizes bacteriophage T7 RNA polymerase, a processive enzyme for transcription having highest



Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1		100.0	99.5	97.0	86.7	99.0	69.7	71.7	70.0	69.8	69.4	60.5	62.6	57.8	1
2	0.0		99.5	97.0	86.7	99.0	69.7	71.7	70.0	69.8	69.4	60.5	62.6	57.8	2
3	0.0	0.0		92.5	84.0	99.0	69.7	71.7	70.0	69.8	69.4	60.5	62.6	57.8	3
4	0.6	0.6	0.6		93.8	95.8	71.2	72.2	69.6	69.5	69.4	60.8	62.9	56.1	4
5	3.2	3.2	3.0	2.7		93.7	69.3	70.0	66.3	66.1	64.5	57.6	60.0	54.6	5
6	0.2	0.2	0.2	0.7	2.5		51.7	51.3	49.9	49.9	46.9	45.6	43.1	43.8	6
7	25.4	25.4	26.0	26.0	27.2	29.1		97.3	72.3	72.0	73.2	61.5	58.3	59.9	7
8	26.4	26.4	26.4	27.0	27.8	29.2	1.8		71.7	71.6	72.2	62.5	58.3	59.1	8
9	27.7	27.7	27.7	28.2	29.3	27.8	26.7	28.2		99.8	75.0	57.9	55.5	58.3	9
10	27.9	27.9	27.9	28.4	29.5	27.8	26.9	28.5	0.2		74.7	57.8	55.1	58.0	10
11	33.5	33.5	33.5	33.9	34.7	35.6	28.3	28.8	24.5	24.7		57.2	52.9	56.1	11
12	35.9	35.9	36.1	39.0	39.8	31.3	40.3	40.3	43.3	43.7	43.9		76.4	75.2	12
13	33.5	33.5	34.3	36.0	38.3	34.3	37.0	38.7	43.0	43.4	45.2	22.4		82.6	13
14	33.9	33.9	34.6	35.0	37.2	37.4	37.5	40.6	42.8	43.1	48.9	20.3	14.1		14
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	

Fig. 3. Sequence distance between MAP C-132 *ModD* (FAP) with other FAPs.

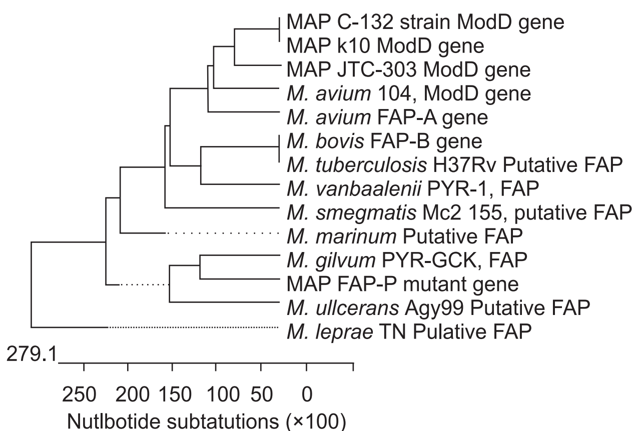
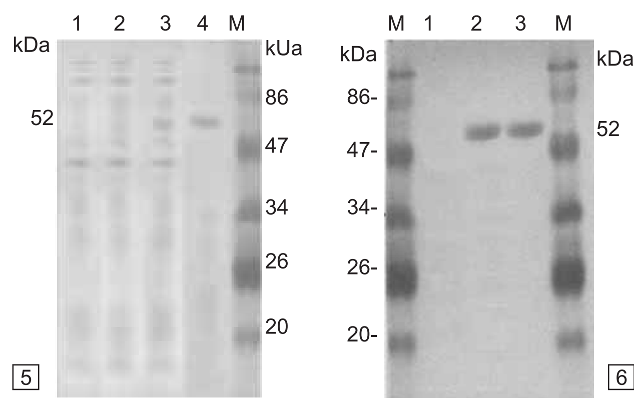


Fig. 4. Phylogenetic map.

expression levels and tightest control over basal expression. Isopropyl thiogalactoside (IPTG) was used for induction of protein expression since the T7 RNA polymerase gene in the expression vector is under the control of IPTG-inducible lacUV5 promoter. Accordingly different concentrations of IPTG (0.5–2.0 mM) were attempted for expression of MAP *ModD* gene 4 h post-induction. Though there was no induction at 0.5 mM IPTG concentration, highest amount of induction was observed at 1, 1.5 and 2 mM level. One noteworthy observation in this study was MAP *ModD* gene got expressed when the bacterial cultures were incubated at 30°C before and after induction but there was no significant expression when incubated at 37°C. The reason could be attributed to the fact that the bacteriophage T7 promoter expression system in pET vector expresses very high levels of foreign gene/cDNA and thus the bacterial



Figs. 5–6 **5.** Western blot analysis of recombinant *ModD* protein using rabbit hyper-immune serum. Lane M, pre-stained protein molecular weight marker; lane 1, bacterial cells (BL-21) lysate; lane 2, uninduced recombinant bacterial cells (BL-21) lysate; lane 3, induced recombinant bacterial cells (BL-21) lysate; lane 4, purified *ModD* protein. **6.** Western blot analysis of recombinant *ModD* protein using Ni-NTA HRP conjugate. Lane M, pre-stained protein molecular weight marker; lane 1, negative control; lane 2, Ni-NTA agarose purified protein (batch no.1); lane 3, Ni-NTA agarose purified protein (batch no. 2).

synthetic and processing apparatus could easily become overloaded. Under such circumstances, if the conditions like subnormal temperature (30°C) were provided during incubation of cultures, will result in slow induction or growth of cells which in turn leads to higher levels of foreign protein expression (Sambrook and Russell 2001).

The apparent molecular mass of the expressed MAP *ModD* gene was ~52 kDa, which was greater than the predicted size of MAP *ModD* gene product (~32 kDa) including the fusion protein (~4 kDa). However, the authenticity of the MAP *ModD* gene/DNA that was ligated and subsequently expressed was checked and confirmed by nucleotide sequencing. The unexpectedly high apparent size could be attributed to the presence of high amount of proline (22.49% by frequency), alanine (19.76% by frequency) and glycine (10.03% by frequency) residue in the protein, which was arrived from the deduced amino acid sequence using the MAP *ModD* gene by DNAStar programme. Similar observations were reported for the recombinant proteins of mycobacterial FAPs, which was attributed to high proline, alanine and glycine content of the expressed protein (Cho *et al*. 2007, Secott *et al*. 2001).

Recombinant *ModD* protein is soluble in nature; therefore purification of protein was carried out under native condition using different concentration of imidazole and eluted using 250 and 500 mM imidazole. Imidazole (250 mM) was optimum and thus used subsequently. There were no contaminating proteins in the preparation and concentration of purified recombinant *ModD* protein was 2 mg/l. Recombinant *ModD* protein carrying an exposed histidine tract bind to resin charged with divalent nickel ions, and thus contaminating proteins were removed by appropriate washings and the protein of interest was eluted by a soluble competing chelator like imidazole. These findings were found similar to previous studies (Cho *et al*. 2007, Secott *et al*. 2001). The advantage of studying the cloned gene products obtained from recombinant *E. coli* is that the protein is free from other mycobacterial proteins as well as mycobacterial cell wall component. This purified protein was used to raise polyclonal antiserum in rabbits which was specific for a single mycobacterial antigen. Immunoblot of recombinant *ModD* protein showed strong immunoreactivity with rabbit anti-*ModD* sera (Fig. 5). Protein expression was also confirmed using nickel-HRPO that showed strong reacting band in induced culture in the region of 52 kDa, because of affinity binding of nickel-HRPO to hexa-histidine tag in the recombinant protein (Fig. 6). The recombinant *ModD* protein can be useful in production of polyclonal serum against *ModD* which allowed purification of natural *ModD*, as natural *ModD* provided significantly higher diagnostic sensitivity than recombinant *ModD*. Further, this recombinant *ModD* protein modulates dendritic cells so, understanding the mechanism of modulation may lead to development of effective MAP vaccine.

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