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Molecular cloning and expression of MPT63 gene of *Mycobacterium tuberculosis*

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ABSTRACT

The present investigation was undertaken to amplify, clone and express MPT63 gene of *Mycobacterium tuberculosis* to discern this secretary protein as a potent diagnostic and immunogenic antigen. Primer specific for MPT63 gene with restriction enzyme sites, viz *BamH*I and *Hind*III were designed. MPT63 gene was amplified using DNA from *M. tuberculosis* 198/94 (IVRI) strain, with designed primers by polymerase chain reaction (PCR). The 412 bp amplicon was purified and first cloned into pGEM-T vector. Positive clone was confirmed by colony PCR and restriction enzyme digestion. The pGEM-T released insert was ligated with *BamH*I and *Hind*III digested pET32b expression vector using T4 DNA ligase and transformed into *Escherchia coli*. Recombinant clones were selected by colony PCR and restriction enzyme digestion and induced with 1mM final concentration of Isopropyl-ß- D-thiogalactopyranosidase (IPTG) for expression of the recombinant MPT63 protein. The expressed protein of 33 kDa was obtained during 2 h post induction. Western blot with Ni- NTA anti-histidine HRP conjugate and hyperimmune serum raised in rabbits confirmed the presence and purity of recombinant MPT63 protein by immuno reactivity at the unique 33 kDa region. Further confirmation of recombinant protein was done by dot blot and indirect ELISA using rabbit hyperimmune serum. The recombinant MPT63 protein was evaluated as a skin test antigen to produce delayed type hypersensitivity reaction in guinea pigs. Recombinant MPT63 protein produced skin erythema of 3 to 4 mm diameter in guinea pigs sensitized with killed *M. tuberculosis* and *M. bovis* culture. From the present study, it may be concluded that understanding of the immunogenic components of an infectious agent is essential through molecular characterization, in regard to the serological diagnosis, and the development of strategies for efficient immune protection and eradication of the disease.

Key words: DTH, MPT63 gene, *Mycobacterium tuberculosis*, pGEM–T cloning vector, Polymerase chain reaction, PPD, SDS PAGE, Western blot

Bovine tuberculosis is of public health concern affecting livestock, humans and ecosystem in low-income developing countries (Michel *et al*. 2010). The primary methods used for detection of TB in humans and ruminants include measurement of a delayed-type hypersensitivity (DTH) to purified protein derivative (PPD) and an indirect *in vitro* assay that measures the concentration of gamma interferon (IFN-γ) produced in response to stimulation with PPD (Monaghan *et al.* 1994, Wood *et al*. 1992, Wood *et al*. 2001). But these tests lack sensitivity and specificity because of a cross-reactive immune response to T and B-cell epitopes conserved on orthologous molecules present in nonpathogenic mycobacteria and *M. avium* subsp. *Paratuberculosis* (De la *et al*. 2006, Pollock *et al.* 2005).

Mycobacterium secretes several proteins into the extracellular environment and utilizes it to interact with their surroundings, which facilitate survival of the bacterium within its host. These proteins have been identified experimentally (Wiker *et al*. 1991, Young *et al*. 1991) and computationally (Gomez *et al*. 2000). The secretary proteins of *M. tuberculosis* are an important source of antigens that induce protective immunity and immune responses having diagnostic value (Andersen *et al*. 1994, Cooper *et al*. 1995). So, they seem to be the possible candidates for the development of complex-based test systems and vaccines. The conserved MPT63 protein is found in species of the *M. tuberculosis* complex (MTC), including virulent *M. tuberculosis*, *M. africanum*, *M. bovis*, and attenuated *M. bovis* BCG (Nagai *et al*. 1991). Like other secreted proteins of *Mycobacterium* (Horwitz *et al*. 1995), MPT63 is a target for the immune response in the infected host and can be the important for TB pathogenesis. T-cell epitopes mapping shows that MPT63 is an immunodominant protein (Lee and Horwitz. 1999) and is cell envelope associated (Braunstein *et al*. 2000). Being uniquely specific to the *M. tuberculosis* complex, MPT63 is a plausible candidate for *M. tuberculosis* complex-specific diagnostics. The present study describes cloning and expression of MPT63 gene of *M. tuberculosis* in a prokaryotic expression (pET32b) vector and evaluation of diagnostic potential of the expressed

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recombinant MPT63 protein as a skin test antigen.

MATERIALS AND METHODS

Bacterial culture: *M. tuberculosis* strain 198/94 used in the present study was obtained from Mycobacteria Laboratory, Division of Bacteriology and Mycology, Indian Veterinary Research Institute (IVRI), Izatnagar.

Genomic DNA isolation: M. tuberculosis culture was grown in Lowenstein-Jensen (LJ) medium slants containing glycerol. The inoculated slants were incubated at 37°C for 4 to 6 weeks. Genomic DNA of the *M. tuberculosis* culture was extracted by standard cetyl trimethyl ammonium bromide (CTAB) lysis method (Van Soolingen *et al.* 1998).

PCR amplification of MPT63 gene: The oligonucleotide primers were designed to have restriction enzyme sites *BamH*I in the forward primer and *Hind*III in the reverse primer (FP 5´-CAG CAG GAT CCC GCC TAT CCC ATC ACC GGA -3´; RP 5´- GCC CAA GCT TCG GCT CCC AAA TCA GCA G - 3´). The primers were designed in such a way to express protein in pET32b expression vector and procured commercially. The amplification of MPT63 gene (412 bp) including the restriction sites was carried out in a standard 25 ml reaction with an initial denaturation of DNA strands at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and strand elongation at 72°C for 1 min. Thereafter 1 cycle of final extension of the strands was given at 72°C for 10 min. The PCR amplification was confirmed by visualization of product in 1% agarose gel stained with ethidium bromide following electrophoresis.

Molecular cloning of MPT63 gene: The amplified MPT63 gene of *M. tuberculosis* strain 198/94 was purified using PCR purification kit following manufacture's protocol. Thereafter competent *Escherichia coli* DH5α cells were prepared following standard calcium chloride treatment method. Ligation for cloning of MPT63 (amplified from *M. tuberculosis* strain 198/94) into pGEM-T easy cloning vector and transformation of DH5α cells was carried out following standard protocol. The positive clones were identified by ampicillin resistance blue white colony screening method. Further confirmation was done by colony PCR of the recombinant plasmid DNA isolated from the white colonies as well as restriction enzyme digestion with *BamH*I and *Hind*III following standard protocol (Sambrook *et al*. 2001). The restriction digestion reaction was carried out at 37°C for 3 h. A stab culture of recombinant clone containing desired MPT63 gene was custom DNA sequenced commercially. The sequence information received was analyzed using DNASTAR and Gene Tool software.

Prokaryotic expression of MPT63 gene: In order to clone the gene for expression, the positive recombinant plasmid of MPT63 gene and pET32b vector was digested with *BamH*I and *Hind*III restriction enzymes simultaneously in a 50µl reaction to facilitate directional cloning. The reaction was carried out at 37°C for 3 h. Both the recombinant plasmid and vector were purified by gel extraction kit following manufacture's protocol. Directional cloning of recombinant plasmid into expression vector was carried out by performing a ligation reaction at 4°C overnight. The transformation of *E. coli* BL21 (PLys) cells, grown in LB broth containing chloramphenicol (34 mg/ml) was carried out with the recombinant plasmid. Confirmation of the clones was done by colony PCR and restriction enzyme digestion with *BamH*I and *Hind*III. Ten positive colonies harbouring the desired MPT63 gene were selected for induction. The colonies were inoculated in 5ml broth containing ampicillin (100 mg/ml) and chloramphenicol (34 mg/ml) and grown overnight at 37°C with constant shaking at 120 rpm. Fifty microlitre of overnight growth were again inoculated in 5 ml LB broth containing appropriate antibiotic and further incubated at 37°C with constant shaking until mid log phase (approximately 3 h). One milliliter of the culture was collected from each tube and kept as an uninduced control. To the rest of the culture, IPTG was added at a final concentration of 1mM and kept at 37°C with constant shaking at 120 rpm. One milliliter of the induced culture was collected at 1, 2, 4, 6, 8 and 24 h post induction. All the cultures collected were pelleted by centrifugation at 12,000 rpm and kept at - 20°C till further use. The pellets of recombinant *E. coli* cells collected at different hourly intervals of induction as well as, the uninduced cultures were subjected to electrophoresis by SDS-PAGE (12% gel) at 8–10 V/cm^2 until the tracking dye reached the bottom of the gel. The gel was stained using coomassie brilliant blue R-250.

Purification of recombinant MPT63 protein: Purification of recombinant MPT63 protein was done under native condition. The induced cells were grown in 50 ml culture. The cells were pelleted and resuspended in 2 ml lysis buffer containing 5mM imidazol (pH 8). Lysozyme was added to it and kept in ice for 30 min. The cell lysate was sonicated and centrifuged at 10,000 rpm for 30 min at 4°C to pellet the cell debris. The supernatant containing solubilised inclusion bodies was added into 2 ml of 50% Ni-NTA agarose slurry and mixed gently at 50– 200 rpm for 1 h at 20°C. The lysate-resin mixture was then carefully loaded onto an empty 5 ml polypropylene column. The flow-through of the column was collected in a separate tube and the column was simultaneously packed. The column was subsequently washed 10 times with wash buffer containing 10 mM imidazol (pH 8). The bound histidine rich recombinant protein was eluted with 4 ml of elution buffer containing 250 mM imidazol (pH 8). The purity of the protein was checked by electrophoresis on SDS-PAGE using 12% gel. The protein was aliquoted in 0.5 ml volume and stored at –20°C. Following purification, the concentration of recombinant protein was assayed by Nanodrop.

Western blot analysis of the recombinant MPT63 protein: The specific immunoreactivity of the expressed recombinant MPT63 protein was checked by western blotting with Ni-NTA conjugate. The purified recombinant protein was loaded and ran on SDS-PAGE using 12% gel.

The resolved protein was electrophoretically transferred to a PVDF membrane using a blotting apparatus (BIORAD) at a constant current of 1–2mA/cm^2 for 1h. The membrane carrying transferred MPT63 protein was washed twice for 10 min each time with PBS-Tween 20 (0.05%); the membrane was incubated for 1 h in 3% blocking solution in PBS-T at room temperature followed by washing. After that, the membrane was incubated for 1 h at 37°C in PBS-T 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. Further confirmation of expressed recombinant MPT63 protein was made by hyperimmune serum raised in rabbits. Following electro transfer of the protein, the membrane was soaked in 5% blocking buffer overnight at 4°C and washed thoroughly with PBS-T. Primary antibody (polyclonal MPT63 antibody raised in rabbit) was added at a concentration of 1 in 50 and incubated for 1 h at 37°C and washed. Secondary antibody (anti rabbit IgG–HRPO conjugate) was added at a concentration of 1 in 1,000 and incubated at 37°C for 1 h followed by stringent washing in PBS-T. The membrane was immersed in substrate solution (DAB 10 mg/50 ml) containing fresh hydrogen peroxide.

Dot blot analysis of the expressed recombinant MPT63 protein: A single drop of recombinant MPT63 protein was coated on nitrocellulose membrane and allowed to dry at 37°C for 1 h. The membrane was then washed with PBS-T and soaked with 5% blocking buffer for 1 h at 37°C followed by washing. To each drop of antigen, 1 drop of anti-MPT63 rabbit hyper immune serum was added in a dilution of 1:25, 1:50, 1:100 and 1:200. One drop of negative serum was added to antigen and one drop of hyperimmune serum was added to *E. coli* cell lysate. The membrane was incubated at 37°C for 1 h with continuous shaking and then washed. After washing, the nitro cellulose membrane was incubated with goat anti-rabbit IgG-HRPO conjugate, diluted to 1:5000 dilutions in PBS. Following incubation for 1 h at 37°C, the membrane was washed with PBST and subsequently developed with DAB solution as substrate.

Detection of the recombinant MPT63 protein by antiserum raised in rabbits by indirect ELISA: The purified MPT63 protein was diluted serially (1:25, 1:50, 1:100, 1:200) in PBS and incubated overnight at 4°C followed by washing with PBS-T. The wells were soaked with 5% blocking buffer and incubated at 37°C for 1 h. After washing, 2 antigen-coated wells for each dilution were incubated with 100 ml of anti-MPT63 rabbit hyper immune serum (1:50 dilution) and 2 wells were incubated with 100 ml of normal rabbit serum (1:50 dilution). Plates were incubated for 1 h at 37°C with continuous shaking. After washing, wells were incubated with 100 ml of anti-rabbit IgG-HRPO conjugate, diluted to 1:8,000 dilutions in PBS. Following incubation for 1 h at 37°C, the wells were washed 5 times using PBS-T; 100 ml of OPD substrate solution were added to each well and allowed for development of colour. The reaction was stopped by addition of 100 ml 1M sulphuric acid and the optical density was measured at 492 nm.

Guinea pig sensitization and skin tests: Apparently healthy, adult female guinea pigs (200–300 g) were housed under conventional conditions in the animal shed and provided green fodder, concentrate and water *ad lib.* Killed *M. bovis*, *M. tuberculosis* and *M. avium* bacilli were suspended in autoclaved liquid paraffin and made into an emulsion with Freund's incomplete adjuvant. 0.5 ml of this suspension was inoculated intramuscularly into the guinea pigs. In the first group, 8 guinea pigs were sensitized by autoclaved *M. tuberculosis*; in the second group, 8 guinea pigs were sensitized by autoclaved *M. bovis* and in third group, 8 guinea pigs were sensitized by autoclaved *M. avium.* The tuberculin test was carried out after 21 days post sensitization. For skin testing, guinea pigs were shaved on the back and injected intradermally with different concentrations of the purified recombinant MPT63 protein (1µg, 2µg, 3µg, 4µg and 5µg) and checked for erythema after 24 h. After observing visible erythema when given at 4µg concentration of recombinant protein each guinea pig was injected with 0.1ml recombinant protein in PBS along with 0.1ml PPD and 0.1ml PBS as control. The diameter of both axis of skin erythema were independently measured and recorded at 24 h after antigen injection.

RESULTS AND DISCUSSION

A significant number of mycobacterium proteins have been proposed to be secreted or exported during growth (Gomez *et al*. 2000). Nicod (2007) showed that these secreted proteins are potent inducers of cell mediated immune responses in host which is crucial in the control of *M. tuberculosis* infection. MPT63 is a novel secretary antigen found only in species of the *M. tuberculosis* complex

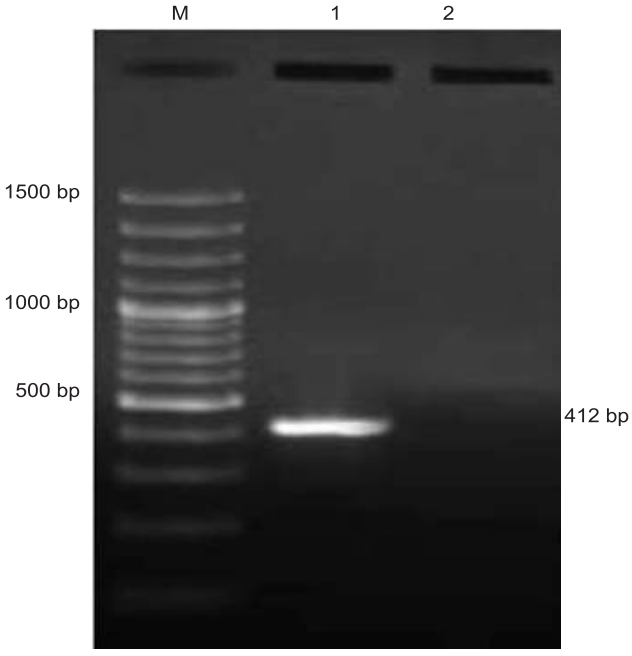

Fig. 1. PCR amplification of gene MPT 63. Lane M, 1 kbp DNA ladder; lane 1, 412 bp DNA band; lane 2, distilled water control.

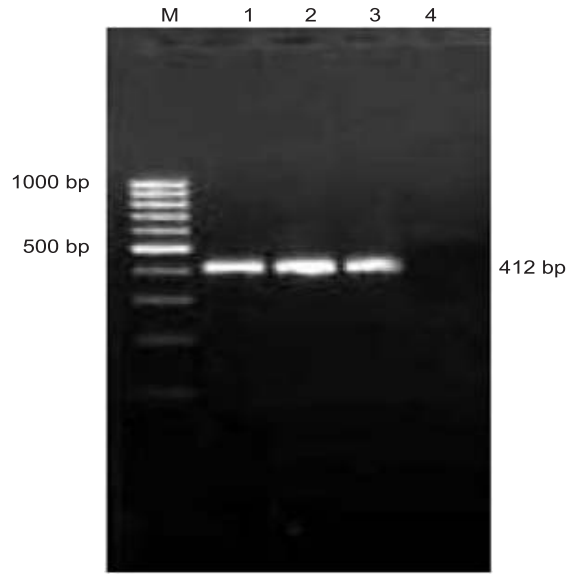

Fig. 2. Colony PCR of pGEMT/MPT 63 clone. Lane M, 100 bp DNA ladder; lane 1–3, 412 bp product of positive clone; lane 4, distilled water control.

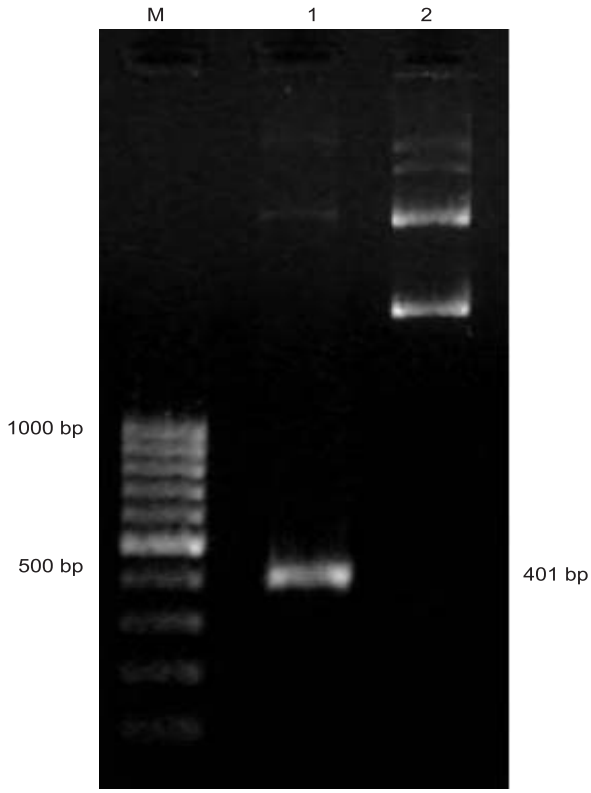

Fig. 3. Release of insert of 401 bp from pGEMT/MPT 63 clone. Lane M, 100 bp DNA ladder; lane 1, 401 bp insert released by BamHI/HindIII digestion; lane 2, plasmid DNA of positive clone.

and polyclonal antibodies against MPT63 do not cross-react with proteins of a common environmental mycobacterial species, *M. avium* (Manca *et al.* 1997).

Amplification and molecular cloning of MPT63 gene: The MPT63 gene from *M. tuberculosis* 198/94 strain was amplified using the specific forward and reverse primers. The amplicon was resolved as a single band of 412 bp size (Fig. 1) which was further purified for ligation in pGEM-T Easy cloning vector. The selection of positive clones containing the insert was done by colony PCR with specific primers and restriction enzyme digestion of recombinant plasmids with *BamH*I and *Hind*III for the release of insert. The results of colony PCR and restriction enzyme digestion were checked by agarose gel electrophoresis (Figs 2, 3). The positive clones were selected and sequenced for nucleotides. The nucleotide sequence revealed 100% homology with all the members of MTB complex and three nucleotide differences with *Mycobacterium canettii* (Fig. 4). The amino acid sequence of *M. tuberculosis* 198/94 strain showed 100% homology with other mycobacteria of MTB complex. The antigenic index showed that the MPT63 protein has 7 strong antigenic peptides. The sequence information was submitted to Gene Bank under the accession number KF155517.

Expression of recombinant MPT63 protein: The *BamH*I and *Hind*III digested pET32b expression vector and recombinant MPT63 plasmid were ligated overnight at 4°C. The transformed BL21 (PLys) cells were grown in presence of antibiotic ampicillin and chloramphenicol. Following an overnight incubation, colony PCR (Fig.5) as well as restriction enzyme analysis (Fig. 6) confirmed the presence

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	
1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.2	1
2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	99.2	2
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	99.2	3
4	0.0	0.0	0.0		100.0	100.0	100.0	100.0	99.2	4
5	0.0	0.0	0.0	0.0		100.0	100.0	100.0	99.2	5
6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	99.2	6
7	0.0	0.0	0.0	0.0	0.0	0.0		100.0	99.2	7
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0		99.2	8
9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8		9
	1	2	3	4	5	6	7	8	9	

1 *M. africanum*
2 *M. bovis BCG* str. Tokyo
3 *M. tuberculosis* 198–94
4 *M. tuberculosis* CTRI-2
5 *M. tuberculosis* H37Rv
6 *M. tuberculosis* Ut205
7 *M. bovis BCG* str. Mexico
8 *M. bovis BCG* str. Pasteur
9 *Mycobacterium canettii* CIPT

Fig. 4. Nucleotide sequence alignment of MPT63 gene with other *Mycobacteria* of MTB.

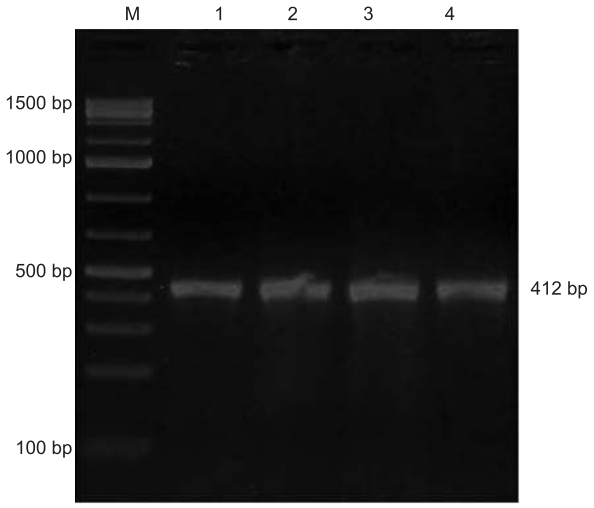

Fig. 5. Colony PCR of pET32b/MPT 63 of clones; lane M: 1 kbp DNA ladder; lane 1–4: 412 bp product of positive clones.

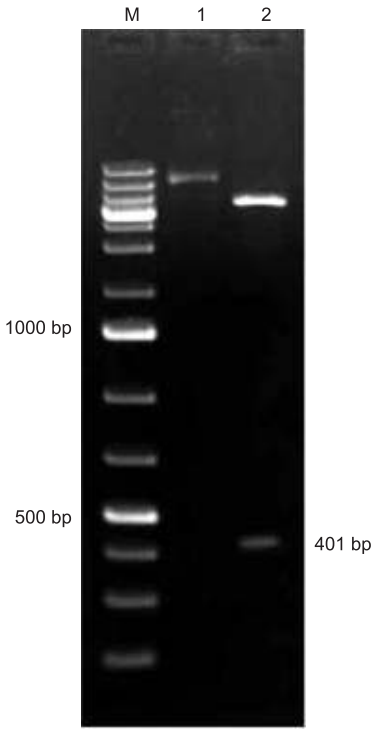

Fig. 6. Release of insert 401 bp pET32b expression vector; lane M: 1 kbp DNA ladder; lane 1, plasmid DNA of positive clone; lane 2, 401 bp insert released from pET 32b MPT63 plasmid.

of MPT63 gene specific product. The recombinant clone showed high level of expression under T7 promoter of the vector when induced with 1mM concentration of IPTG. Highest amount of induction was observed at 1mM concentration of IPTG as it was also reported by other workers (Kulshrestha *et al.* 2005, Wang *et al*. 2005, Wu *et al*. 2010). The protein was expressed as a 33 kDa fusion protein as visualized by SDS-PAGE. The protein kinetic study showed that expression of recombinant protein started

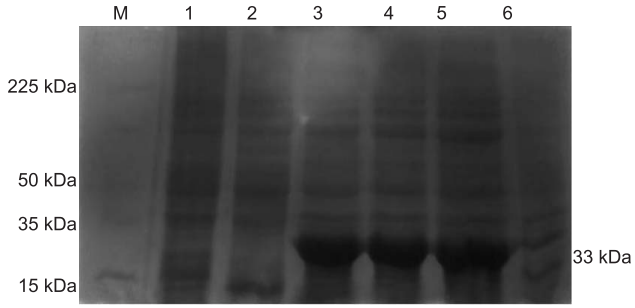

Fig. 7. SDS-PAGE showing expression of MPT 63 gene at different intervals after IPTG induction. Lane M, protein molecular weight marker; lane 1, uninduced sample collected at 4 h; lane 2, sample collected at 1 h post induction; lane 3, sample collected at 2h post induction; lane 4, sample collected at 4 h post induction; lane 5, sample collected at 6 h post induction; lane 6, sample collected over night post induction.

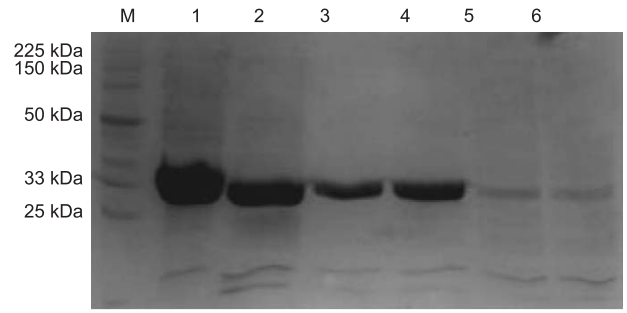

Fig. 8. SDS- PAGE analysis showing elution pattern of purified His-tagged recombinant. MPT 63 protein in native state. Lane M, protein molecular weight marker; lane 1, first elute; lane 2, second elute; lane 3, third elute; lane 4, fourth elute; lane 5, fifth elute; lane 6, sixth elute.

after second hour of induction and subsequently reached maximum at 6 h (Fig. 7). In some cases mycobacterial recombinant protein expressed at 4 h onward (Wu *et al*. 2010). Purification of the fusion protein was done by Ni–NTA chromatography and purity was checked by SDS-PAGE analysis (Fig. 8).

Western blot analysis of expressed recombinant MPT63 protein: The specific reactivity of the recombinant MPT63 protein was done by western blotting. Ni-NTA antihistidine HRP conjugate and anti MPT63 rabbit hyper immune serum when used for specific reactivity to the recombinant His tagged protein, confirmed the presence and purity of histidine tagged protein with immunoreactivity at the unique 33 kDa region specific for MPT63 protein on the nitro cellulose membrane (Fig. 9). Hence, immunoblot of recombinant MPT63 protein showed strong immuno-reactivity with rabbit anti MPT63 sera which was in agreement with other reports (Webb *et al*. 1998, Johnson *et al*. 2001, Farshadzadeh *et al*. 2010).

Dot blot analysis of the expressed recombinant MPT63 protein: On dot blot analysis, rabbit anti MPT63 sera reacted strongly with recombinant MPT63 protein. But the preimmune sera did not react with the recombinant protein

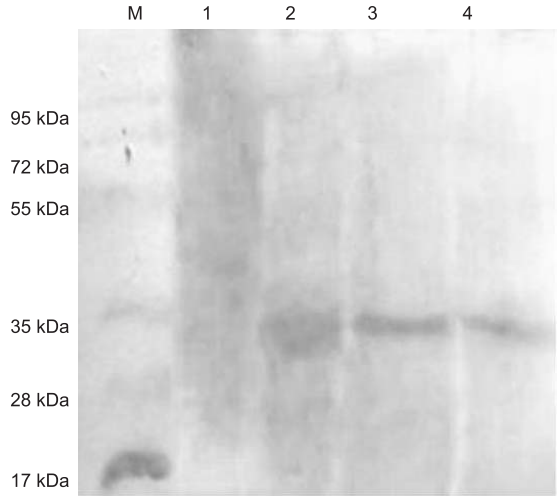

Fig. 9. Western blot analysis of recombinant MPT 63 protein. Lane M, protein molecular weight marker; lane 1, uninduced cell lysate; lane 2, crude cell lysate from induced bacterial culture; lane 3, 4, purified MPT 63 protein.

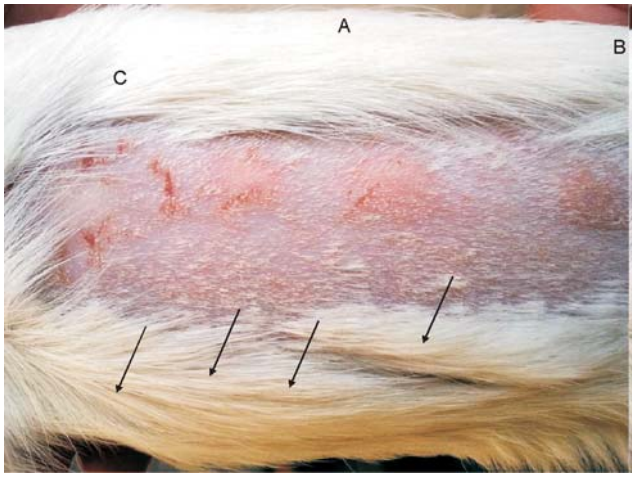

Fig. 10. Tuberculin test to measure delayed type of hypersensitivity (DTH) reaction. A, Injection with PPD; B, injection with recombinant MPT 63 protein; C, injection with *M. avium* protein.

and anti MPT63 serum did not react with *E. coli* cell lysate which confirmed the specificity of anti MPT63 serum.

Indirect ELISA: The purified MPT63 protein was diluted in the ratio of 1:25, 1:50, 1:100 and 1:200 respectively and used as coating antigen with fixed dilution (1:50) of rabbit hyperimmune serum and preimmune serum. Two to four times differences found in the OD value of preimmune and hyperimmune rabbit serum with different dilutions of coating antigen, although maximum P/N value was found with 1:50 dilution of recombinant antigen. These observations of dot blot and indirect-ELISA confirmed the specificity of anti MPT63 serum and immuno reactivity of recombinant protein.

DTH reactivity to recombinant MPT63 in guinea pigs: Different doses of recombinant MPT63 protein were tested for their ability to produce DTH responses in guinea pigs sensitized with killed *M. tuberculosis, M. bovis, and M. avium.* Recombinant protein with a concentration of 4 µg was found to produce visible skin erythema than lower concentration of recombinant protein.The three groups were injected intradermally with PBS as the negative control, PPD as positive control and recombinant MPT63 as test antigen. All animals sensitized with killed *M. tuberculosis, M.bovis* and *M.avium* had positive reactions to PPD by an erythema response of greater than 5 mm in diameter after 24 h. The recombinant MPT63 protein induced skin erythema of 3–4 mm in animals sensitized with killed *M. tuberculosis* and *M.bovis* but no skin reaction found in animals sensitized with killed *M.avium* (Fig. 10). Similar studies were conducted by other workers to measure the DTH response in sensitized guinea pig with different doses of recombinant CFP–10 (Pollock *et al*. 2003, Wu *et al.* 2010). Skin erythema of 5–9 mm size when induced by recombinant CFP 10 protein also reported by Wu *et al*. (2010).

The results suggested that recombinant MPT63 protein obtained in the present work alone is a weak skin antigen as compared with the PPD. Hence, we opined that, MPT63 protein may be used along with other immunogenic antigens for the development of a better tuberculin than PPD. The specificity of MPT63 for *M. tuberculosis* complex and lack of cross reactivity between MPT63 and *M. avium* proteins strongly encourage further evaluation of recombinant MPT63 in terms of specific tuberculin test antigen.

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