

Identification and Characterization of a Gene Encoding 16kDa Protein from *Mycobacterium avium* Subspecies *paratuberculosis*

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In this study the effort was assumed to clone and express coding sequences of *M.a.paratuberculosis* to study their immune reactivity. Primers were designed for ORFs retrieved from MAP complete genome strain k10 (locus tag MAP 0862 and MAP 1087). The PCR amplified product of each gene fragment was cloned into *E. coli* expression vector pQE-30 and the resultant constructs were designated as pQE 441. The positive recombinant clones on induction with IPTG expressed the protein bands corresponding to 16kDa protein on SDS PAGE. The His-16protein was purified using single step Ni-NTA chromatography. The yield of the purified His-16protein was about 15 mg/L and from induced *E. coli* cultures harbouring plasmid pQE-441. Antigenicity of these proteins were evaluated by western blot using sera from a small number of cattle infected with MAP. The immunoproteomic analysis of culture filtrate (CF) and cellular extract (CE) of MAP revealed that serological tests may be improved by the use of MAP proteins derived from culture filtrates and not from cellular extracts. Development of sensitive serological tests for the rapid identification of infected animals at subclinical stage requires expression and characterization of proteins or secreted early from post infection MAP. Polyclonal antisera raised against purified His-16protein reacted with induced *E. coli* whole cell lysate harbouring pQE 441 and also with purified recombinant 16kDa protein on western blot. The recombinant His-16protein was recognized by rabbit hyperimmune sera of the MAP culture filtrates and also by serum from a goat with clinical paratuberculosis.

Keywords: DNA-Star, serological, *Mycobacterium avium*, MAP

Paratuberculosis (Johne's disease), chronic granulomatous enteritis of domestic ruminants¹ and wild animals^{2,3} caused by acid-fast, slow growing, fastidious *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is an enzootic disease on the B list of the Office des International Epizootes ('OIE') and causes huge economic losses to the dairy industry worldwide.

Detection of MAP, especially during the often-lengthy subclinical phase of the disease,

remains difficult due to intermittent shedding of small numbers of bacteria and a lack of effective diagnostic reagents⁴. The fecal culture test requires 12-16 weeks for cultivation and its sensitivity level is estimated to be approximately 38%⁵. So there is a need to improve the sensitivity or specificity of currently used diagnostic tests. Entry of microorganisms in Peyer's patches and infection of macrophages at this site allows presentation of antigens to immune cells and usually activates defense site allows presentation of antigens to immune cells usually activates defense mechanism. In paratuberculosis, as in other mycobacteria, Cell

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mediated immunity (CMI) is considered to be the principal mechanism for establishment or clearing of infection. Antibody is assigned are often seen in advanced clinical cases. In mycobacterial infections, CMI, which is highly initially, is associated with higher TH1 responses leading to IFN- γ production.

Development of sensitive serological tests for the rapid identification of infected animals at subclinical stage requires expression and characterization of early secreted proteins of MAP as important antigens for the diagnosis of paratuberculosis^{6,7}. Literature mining indicates the present work as the first of its kind involving epitopic region from MAP 0862 from the MAP genome strain k10 encoding immune reactive 16kDa protein.

Currently used diagnostic tests for Johne's disease need to be improved due to deficiencies in their sensitivity or specificity. Problems of specificity are due to the high degree of similarity that exists between MAP and environmental mycobacteria, especially the closely related MAA. It is not surprising therefore that cell mediated immune assays such as the intradermal (skin) test, which uses a complex, undefined MAP secreted protein preparation to stimulate an immune reaction in the host, are prone to false-positive results due to cross-reactivity with similar proteins present in other mycobacteria

MATERIALS AND METHODS

Materials and reagents

All the chemicals and biological used in the present study were of molecular biology grade. Agarose, Ammonium persulphate, Acryl amide, Bovine serum albumin, Bisacryl amide, β -mercapto- Life technology USA ethanol, Bromophenol blue, Calcium Chloride, Coomassie brilliant blue 250, Magnesium Chloride, Sodium Chloride, Potassium Acetate, Tween-20, Isopropyl thiogalactosepyranoside (IPTG), X-gal, lysozyme, Sodium dodecyl sulphate, Diaminobenzidine, TEMED, Glycine, Ethidium Bromide, Proteinase K, RNase, Triton X-100, PMSF, Trypan blue, Fetal calf serum, GMEM, Goat anti-rabbit IgG HRP conjugate, Rabbit anti-goat IgG HRP conjugate, Dialysis tube and Antibiotics (ampicillin and kanamycin) were obtained from, Sigma Chemicals,

USA. Potassium chloride, Disodium hydrogen orthophosphate, Potassium dihydrogen orthophosphate, Hydrogen peroxide, Certrimide, Agar, CTAB, Glucose and Glycerol, Sodium nitrite and Orthophosphoric acid, Sodium carbonate, Sodium bicarbonate, Isopropanol, Phenol, Chloroform, Ethylene diamine tetra-acetic acid, Boric acid, Glacial acetic acid, Isoamyl alcohol, Sodium hydroxide, Sodium acetate, Sodium dihydrogen phosphate, Magnesium sulphate were purchased from Qualigens & Merck, India respectively. QIAGEN, Germany supplied QIAEX II Gel Extraction Kit while Difco, USA supplied Luria-Bertani Media, SOB Media, and Middlebrook 7H10 agar and OADC salt. Restriction endo-nucleases and T4 DNA ligase were procured from New England Biolabs, UK and MBI Fermentas, Germany respectively. All other reagents were acquired from commercial sources.

Oligonucleotide primers

A set of specific oligonucleotide primers 862 F and 862 R from (locus tag MAP 0862 region 884392-884892), were synthesized by integrated DNA technologies, inc. Coralville, IA USA, based on sequence information of MAP strain k10 complete genome GeneBank Accession No: AE016958. Linkers with restriction endonuclease sites *Bam*HI at 5' end and *Pst*I at 3' end were included in the forward and reverse primers respectively.

MAP PPG 5 Forward 5'-GTA GGATCC ATG CTC GGC TAC GTT CTC-3' 27mer

MAP PPG 6 Reverse 5'-TAT CTG CAG TCA AAA CGT GGG GAC CG-3' 26mer

Mycobacterial strain and antigen

Bacterial strain of *M. a. paratuberculosis* (MAP) strain 316F were obtained from Biological Products Division of IVRI, Izzatnagar and later maintained at Gene Expression lab, Division of Animal Biotechnology, IVRI, Izzatnagar. Antigen against MAP culture filtrate and clinical sera from the goat affected with paratuberculosis were available in the lab.

Plasmid and host strains

Prokaryotic expression vector pQE-30 was purchased from QIAGEN, Germany

Laboratory animals

New Zealand white rabbits were obtained from Laboratory Animal Resource Section, IVRI, Izzatnagar. Standard prescribed guidelines for care

and use of laboratory animals were followed during the experimentation with these animals.

METHODS

Culture and growth of *M. a. paratuberculosis* and *E. coli*

MAP organisms were grown on Middlebrook 7H10 agar enriched with 0.1% glycerol v/v and 10% oleic acid dextrose catalase (OADC) with additional supplementation of Mycobactin J (2mg/l) while *E. coli* cells were grown in Luria Bertani (LB) medium with shaking at 180 rpm. *E. coli* M15 cells containing pQE-30 vector were grown in presence of kanamycin @ 50 mg/ml and ampicillin @ 50 mg/ml. Both organisms were grown at 37°C.

Isolation of genomic DNA from *M. a. paratuberculosis*

The genomic DNA from MAP was isolated by the method of Portillo *et al.* (1991) with slight modifications. The bacterial colonies scrapped from two months old Middlebrook 7H10 agar slants were washed thrice with 1X TE and resuspended in 500 µl of 1X TE. Lysozyme was added to the final concentration of 5 mg/ml. After incubation at 37°C for two hrs, SDS and proteinase K were added (@1% and 250 µg/ml respectively) and incubated further at 65°C for 30 min. To this, added 80 µl of 5M NaCl followed by addition of 64 µl of CTAB/NaCl solution and vortexed. The suspension was again incubated at 65°C for 30 min. DNA was extracted once with phenol, once with phenol: chloroform (1:1) and finally with chloroform: isoamyl alcohol (24:1). The aqueous phase containing DNA was pelleted by centrifugation and washed with 80% ethanol, dried and re-dissolved in 200 µl of 1x TE. And ethanol precipitation. Contaminating RNA was removed from DNA by incubating with 100 µg/ml RNase. The treatment was given for one hour at 37°C, followed by phenol: chloroform extraction and ethanol precipitation. The DNA was quantitatively estimated using the following formula, by recording the absorbance at 260nm wavelength: Concentration of dsDNA (µg/ml) = A₂₆₀ X 50 X dilution factor

Agarose gel electrophoresis

The DNA preparations were analyzed on 1% agarose gel and visualized under UV

transillumination and documented by photography in Uvitech Gel Documentation System (Austria).

Polymerase chain reaction

The PCR was carried out in 25 µl reaction volume using 1 µl of genomic DNA (10 ng) as template, 2.5 µl of PCR buffer, 1 µl of MgCl₂ (1.5 mM), 1 µl (25 µM) of each primers, 1 µl of dNTP mix (200 µM of each dNTP) and 1 U of Taq DNA polymerase. The volume was made up to 25 µl by adding DNase free water. The thermal cycling steps were carried out in PTC-200 thermo-cycler MJ Research Inc, USA with initial denaturation at 94°C for 5 min followed by 30 cycles with denaturation at 94°C for 1 min, annealing of 1 min at 58°C for 441bp followed by extension at 72°C for 30 Seconds and final extension at 72°C for 1 min. Size of the amplified product was confirmed by using DNA molecular weight marker in a 1.2% agarose gel and quantified by spectrophotometric analysis. The DNA fragment of 441 bp was eluted from agarose gel using QIAEXII gel extraction kit.

Cloning of 441bp in prokaryotic (*E. coli*) expression vector pQE-30

Ligation

Plasmid pQE 30 was isolated from *E. coli* cells by small scale alkaline lysis method followed by the ligation of eluted PCR product of 441 bp as follows:

- pQE-30 : 1 µl (50ng)
- 441 bp PCR product: 1 µl (50ng)
- 2X ligation master mix : 5 µl
- nuclease free water: 3 µl

The 10 µl ligation mixture was kept at 16°C overnight and the resulting plasmid was designated as pQE 441 which was stored at -20°C.

Transformation and Screening of recombinant pQE 441

10 µl ligation mixture was diluted to 200 µl TCM (1X), to which, 200 µl of the competent cells were added with gentle mixing and left at 0 °C on ice for 1hr. Heat shock was given to this mixture for 2min at 45°C and then was rapidly chilled on ice for 10 min. Then 600 µl of SOC medium was added and incubated at 37°C for 1hr with shaking. The transformed cells were spread on LB agar plate containing ampicillin (100 µg/ ml) and kanamycin (25 µg/ ml). Plates were incubated at 37°C overnight and later stored at 4°C.

Screening of recombinant pQE441

About twenty randomly selected colonies

were picked up containing pQE 441 clones and grown on LB broth containing Ampicillin and Kanamycin and incubated at 37°C overnight in a shaker incubator at 180 rpm. Plasmid DNA was extracted & positive clones were identified by Colony PCR and restriction endonuclease analysis.

Expression and purification of the recombinant 16kDa (His 16) Protein

Fresh *E. coli* culture harbouring pQE (OD₆₀₀ of 0.6 was induced with 1mM IPTG to express the recombinant 16protein and grown

again for 4 hr at 37°C in a shaker incubator at 180 rpm. The *E. coli* cells were pelleted by centrifugation at 6,000 rpm for 10 min. & frozen at –20°C overnight followed by thawing at room temperature for 15min. These pellets were resuspended in 1/25th volume of lysis buffer (buffer B-8M urea, 0.1M NaH₂PO₄, 1M TrisCl- pH 8.0) and incubated with agitation for 1hr at RT. Removed the cell debris by centrifugation at 10,000 x g for 20-30min at RT (20-25°C) and about 600µl of the cleared lysate supernatant containing the 6X His-

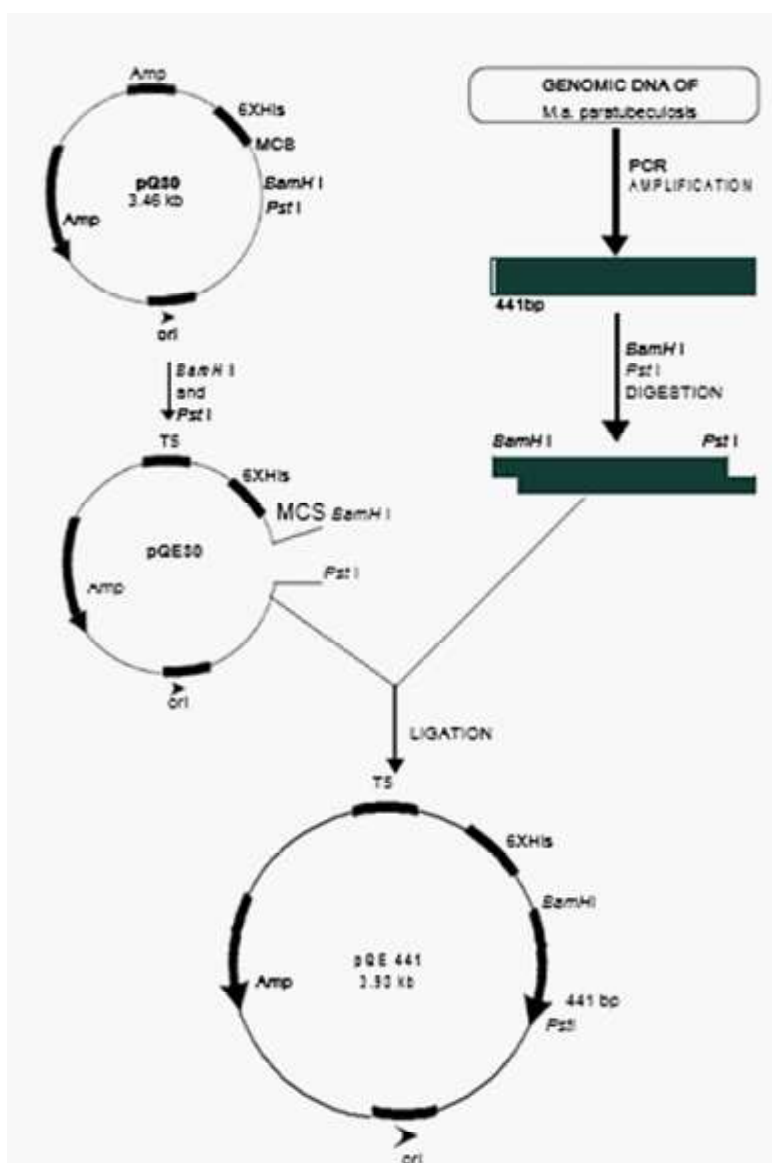


Fig.1. Cloning of PCR amplified 441 ORF into Expression vector pQE-30

tagged protein was loaded onto a pre-equilibrated (with 600 μ l of buffer B) Ni-NTA spin column and centrifuged for 2min at 700 x g. The column Ni-resin bound 6X His-tagged proteins was washed twice with 600 μ l of wash buffer (buffer C - 8M urea, 0.1M NaH₂PO₄, 1M TrisCl - pH 6.3) and eluted twice with 200 μ l of elution buffer (buffer E - 8M urea, 0.1M NaH₂PO₄, 1M TrisCl - pH 4.5).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE)

SDS-PAGE was carried out on a vertical slab mini apparatus (Atto, Japan). After electrophoresis the gel was stained with Coomassie

brilliant blue G250 and destained in solution containing methanol and acetic acid. The fraction containing the purified recombinant 16kDa protein was resolved and approximate molecular weight of the protein was determined by comparison with protein molecular weight marker in Uvitec gel documentation system using Uvipro software.

Dialysis of the recombinant 16kDa

The selected fractions of each protein were pooled and extensively dialyzed at 4°C against PBS (pH 7.4) in dialysis tubing with 8 kDa molecular weight cut-off, and stored in aliquots at -20°C until used.

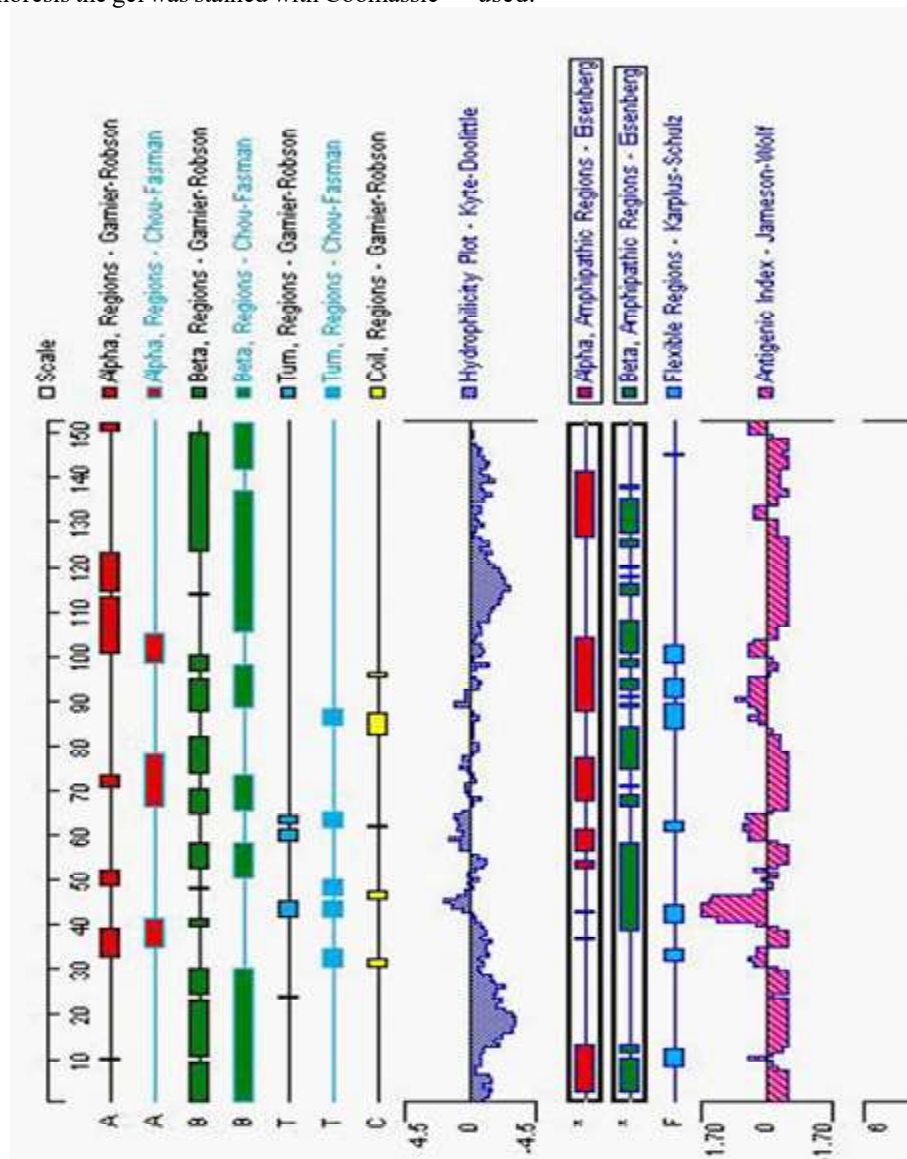


Fig. 2. DNA sequence and deduced amino acid sequence analysis

Hyperimmunization for raising antisera

New Zealand white 8-10 weeks old rabbits were immunized to raise antibody against the purified His-16 by inoculating with 150 µg of the immunogen in 0.5ml PBS (pH 7.4) along with equal volumes of incomplete Freund's adjuvant (IFA) subcutaneously. After three weeks, subsequent boosters of 100 µg immunogens were given intramuscularly at weekly intervals. The rabbits were bled a week after third booster and sera were separated and stored at -20 °C in aliquots.

Sequence analysis

DNA sequencing of the plasmid pQE 441 was carried out commercially.

RESULTS

Expression of Recombination 16kDa Proteins in *E.coli*

PCR amplified 441 bp gene fragments each was each digested with Bam HI /Pst restriction enzymes and inserted in-frame

The nucleotide and protein sequence analysis of plasmid pQE 441 containing 441 bp gene fragments of MAP 316 F strain revealed about 100% homology with retrieved ORFs of the MAP k10 strain by laser gene software DNA star. The predicted primary protein composed of 146 amino acids with a mature protein of 16kDa respectively. Analysis of the deduced amino acids sequence of 16 kDa protein according to Kyte and Doolittle (1982) algorithm showed presence of three and two highly hydrophobic region

DNA sequence and deduced amino acid sequence analysis

The nucleotide and protein sequence analysis of plasmid PQE 441 containing 441 bp gene fragments of MAP 316F strain revealed about 100% homology with retrieved ORFs of the MAP k10 strain by laser gene software DNA star. The predicted primary protein composed of 166 amino acids with a mature protein of 16kDa. Analysis of the deduced amino acids sequence of MAP16kDa according to Kyte and Doolittle (1982) algorithm showed presence of three hydrophobic regions.

DISCUSSIONS

John's disease with a potential of causing economic losses worldwide could not be

eradicated successfully due to the lack of simple and specific diagnostic tests for the early detection prior to the appearance of disease signs' such as fecal shedding of MAP in the environment^{4,8}. Early diagnosis is important to identify and remove potential fecal shedders of MAP to prevent the spread of John's disease, and requires the development of sensitive and specific diagnostic tests. Indeed fecal culture the current gold standard tests, which can detect both clinical and subclinical stages is time consuming, requires more than 15 weeks and also labour intensive. Poor specificity of the intradermal skin test and interferon gamma (IFN-γ) release assay, agarose gel immuno diffusion (AGID), complement fixation (CF) and ELISA use a complex ill defined mixtures of proteins derived from either, whole cell or fractionated extracts of MAP^{9,10,11}. To date a number of antigens have also been identified but the data about the characterization of proteins secreted early post infections from MAP are still lacking. Recently Bannantine et al.¹² constructed the partial protein array and examined the antibody profiles of subclinically MAP infected animals and study has focused on the detecting antigens eliciting early antibody response. Since, proteins produced by the MAP are in very low concentration, purification becomes highly cumbersome and uneconomical. Therefore, for high level expression of proteins, *E. coli* has been widely used for the expression of large number of genes at level sufficient for structural and biochemical analysis and even for product development¹³.

The recombinant proteins will further be useful in the development of diagnostic reagents for subclinical detection paratuberculosis in animals. While further studies using sera from experimentally as well as naturally infected animals are needed to lay down the solid foundation towards the development of feasible ELISA based assay for subclinical detection of MAP infected animals. Although current serological tests are useful in detecting animals with clinical paratuberculosis, the application of this procedure in identifying animals in early stages of infection or in subclinical stages has proven to be of limited value. Also the sensitivity of the commercial ELISA might at least in part be due to heterologous nature of the antigen they are apparently based on derived from *M. avium* subsp

Therefore, in the present work keeping in view the importance of identifying MAP specific antigens through the cloning and heterologous expression of 441 BP coding sequences found in the MAP genome encoding 39.7kDa proteins was chosen for expression and immuno-reactivity. Based on the sequence information of two genes encoding 16kDa from MAP strain k10, complete genome Gene Bank Accession No. AE016958 (tag MAP 0862 and MAP 1087 coding for hypothetical proteins) and also the information about multiple cloning sites of the expression vector pQE-30, restriction sites *Bam*HI and *Pst*II were incorporated into the oligonucleotide primers to facilitate directional cloning. The resulting plasmid pQE 441 containing an open reading frame encoding successively 6X histidine polypeptide, 16kDa. The recombinant pQE 441 clones were confirmed by release of the insert by double digestion with *Bam*HI and *Pst*II restriction enzymes and on induction with IPTG for 4 hours appearance of 16kDa protein band. Expression of the recombinant proteins was induced by IPTG which bind to the lac repressor protein, inactivating it leading to transcription of sequences downstream of the promoter. Using pQE-30 vector, Goswami *et al.*¹⁴ and Basagoudanavar *et al.*¹⁵ have got maximum expression of the recombinant protein after 4-6 hrs post induction. Therefore, it was decided to purify the recombinant proteins 4 hours post induction from *E. coli* culture. The presence of 6X histidine tag of 840 Dalton at the N-terminal of the recombinant proteins facilitates single step affinity purification, which is poorly immunogenic and may not interfere with the protein immunogenicity its functional structure; hence the tag was not removed by protease cleavage. The polyclonal antisera raised in rabbit against the recombinant His-16 proteins strongly reacted with the *E. coli* expressed recombinant 16kDa and 16 kDa proteins on immunoblot.

To date a number of antigens have also been identified but the data about the characterization of proteins secreted early post infections from MAP are still lacking. The partial protein array and examined the antibody profiles of subclinically MAP infected animals and study has focused on the detecting antigens eliciting early antibody response. For functional studies and diagnostic purpose large amount of pure

protein is required. Since, proteins produced by the MAP are in very low concentration, purification becomes highly cumbersome and uneconomical. Expression of the recombinant proteins was induced by IPTG which bind to the lac repressor protein, inactivating it leading to transcription of sequences downstream of the promoter.

This has not only confirmed the heterologous expression but also suggested that the recombinant proteins retained their antigenicity. Reactivity of the His-16 proteins with polyclonal sera against culture filtrate of MAP on Dot ELISA revealed the secretory nature of the recombinant proteins. Further reaction of these recombinant proteins with clinical sera from par tuberculosis affected goat suggested that these proteins have retained their antigenicity even after purification. The result of these studies suggested that these recombinant proteins will further be useful in the development of diagnostic reagents for subclinical detection of par tuberculosis in animals. While further studies using sera from experimentally as well as naturally infected animals are needed to lay down the solid foundation towards the development of feasible ELISA based assay for subclinical detection of MAP infected animals.

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