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Cloning and Expression of 16kDa Protein of Johne's Disease as Diagnostic Antigen

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The present work was undertaken 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. Polyclonalantis era raised against purified His-16protein reacted with induced *E. coli* whole cell lysate harbourin gpQE 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.

Key words: *Mycobacterium avium*, Paratuberculosis, Kilodalton, Isopropyl β-D-thogalactosidase.

Paratuberculosis (Johne'sdisease), chronic granulomatous enteritis of domestic ruminants[1] and wild animals[2,3] caused by acid-fast, slow growing, fastidious *Mycobacterium avi Freum* 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 reagents[4] .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. 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 immunoreactive 16kDa protein.

MATERIALS AND METHODS

Materials and reagents

All the chemicals and biological used in the present study were of molecular biology grade.

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Agarose, Ammonium persulphate, Acryl amide,Bovine serum albumin, Bisacryl amide, b-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, Rabbi t 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 ace tic 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 Rfrom (locus tag MAP 0862 region884392-884892), were synthesized by integrated DNA technologies, inc.Coralville, IA USA, basedon sequence information of MAP strain k10 complete genome GeneBankAccession No:AE016958 Linkers with restriction endo-nuclease sites *BamH*1 at 5' end and *Pst*1 at 3' end were included in the forward and reverse primers respectively.

MAP PPG 5 Forward 5'-GTA GGA TCC 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 LaboratoryAnimal 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 Middle brook 7H10 agar slants were washed thrice with 1X TE and re-suspended in 500 ml 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 65ºC 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 65ºC 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 37ºC, 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 denatuaration 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 441bpin 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:

a. pQE-30 : 1 µl (50ng)
b.441 bp PCR product: 1 µl (50ng)
c. 2X ligation master mix : 5 µl
d. 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 ºC on ice for 1hr. Heat shock was given to this mixture for 2min at 45ºC and then was rapidly chilled on ice for 10 min. Then 600 µl of SOC medium was added and incubated at 37ºC 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 37ºC overnight and later stored at 4ºC.

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 harbouringpQE (OD600 of 0.6 was induced with 1mM IPTG to express the recombinant 16protein and grown again for 4 hr at 37oC in a shaker incubator at 180 rpm. The *E. coli* cells were pelleted by centrifugation at 6,000 rpm for 10 min.& frozen at – 20ºC overnight followed by thawing at room

temperature for 15min. These pellets were re-suspended 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 (20-25oC) and about 600µl of the cleared lysate supernatant containing the 6X His-tagged protein was loaded onto a pre-equilibrated (with 600 µl 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 600µl of wash buffer (buffer C - 8M urea, 0.1M NaH2PO4, 1M TrisCl - pH 6.3) and eluted twice with 200 µl of elution buffer (buffer E - 8M urea, 0.1M NaH_2PO_4, 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 anddistained 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 4ºC against PBS (pH 7.4) in dialysis tubing with 8 kDa molecular weight cut-off, and stored in aliquots at –20ºC until used.

Hyperimmunization for raising antisera

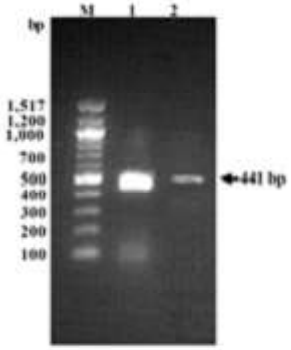
New Zealand white 8-10 weeks old rabbits were immunized to raise antibody against the purified His-16by inoculating with 150 µg of the immunogen in 0.5ml PBS (pH 7.4) along with equal volumes of incomplete Freund's adjuvant (IFA) subcutaneously. After three weeks, subsequent boosters of 100 µgimmunogens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

Western blotting

Samples were electrophoresed as before and the protein bands were transferred to nitrocellulose membrane by semi dried blotting according to Bjerrum and Schafter- Nielsen (1986) utilizing transblot buffer (Tris Glycine, pH 8.2 with 20% methanol). Non specific sites were blocked with 5% skim milk powder in PBS-T (phosphate buffer saline pH 7.4 containing 0.05% Tween 20) for 2hr at 37°C. The membrane was washed three times with PBS-T and the blot was incubated for 2hr at 37°C with polyclonal hyperimmune sera raised in rabbit against His-16and His-16 proteins (1:500 in PBS-T). After washing the blot with PBS-T, they were further incubated for 1hr with HRP labeled goat anti-rabbit IgG (diluted to 1:15000 in PBS-T). After washing, the membrane was dipped in substrate solution (0.1% diaminobenzidine and 0.03% hydrogen peroxide in 50mM Tris, pH 7.6). After the development of sufficient colour, the reaction was stopped by washing the blot in PBS (pH 7.4).

Dot blot assay

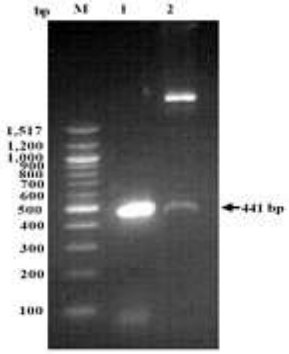
20 µl of His-16proteins were spotted on a nitrocellulose membrane as a small dot, dried at room temperature and processed to confirm the



Agrose (1.2%) gel electrophoresis showing PCR amplified 441 bp gene fragment of *M. a. paratuberculosis*

Lane M : DNA molecular weight marker 100bp ladder
Lane 1 : PCR amplified 441 bp gene fragment
Lane 2 : Gel eluted and purified PCR amplified 441 bp gene fragment

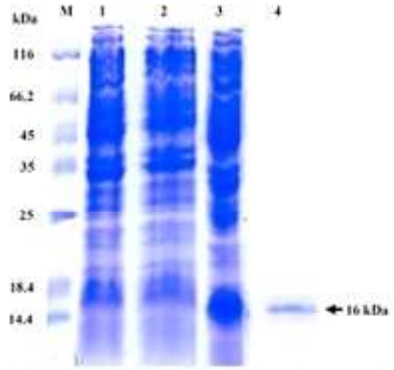
Fig. 1.



Agrose(1.2%) gel electrophoresis of the 441 bp cloned gene fragment encoding 16kDa protein of *M. a. paratuberculosis* in expression vector pQE-30

Lane M : DNA molecular weight Marker 100bp ladder
Lane 1 : PCR amplified 441bp gene fragment
Lane 2 : Release of 441 bp gene fragment after digestion with *BamHI* and

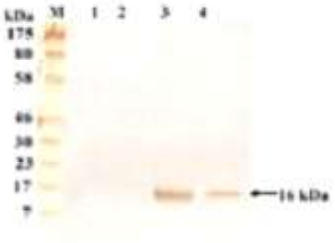
Fig. 2.



Coomassie Brilliant Blue stained 12% SDS-PAGE gel, showing expressed His-16 protein

Lane M : Prestained protein molecular weight marker
Lane 1 : Whole cell extract of *E. coli* M15
Lane 2 : Whole cell extract of *E. coli* M15 harbouring His-16-kDa protein (IPTG uninduced)
Lane 3 : Whole cell extract of *E. coli* M15 harbouring His-16kDa protein (IPTG induced for 4 hr)
Lane 4 : Purified recombinant His16 protein

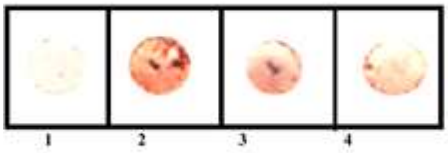
Fig. 3.



Western blot assay of the 16kDa protein expressed in *E.coli* probed with anti His-16 protein

Lane M : ColorPlus Prestained Protein marker (7-175 kDa)
Lane 1 : Whole cell extract of *E. coli* M15
Lane 2 : Whole cell extract of *E. coli* M15 harbouring 16kDa protein (IPTG uninduced)
Lane 3 : Whole cell extract of *E. coli* M15 harbouring 16kDa protein (IPTG induced for 4 hr)
Lane 4 : Purified recombinant His-16 protein

Fig. 4.



Dot blot assay showing sero reactivity of purified His- 16 protein

1 : Uimmunised rabbit sera (control)
2 : Probed with rabbit antisera against His- 16 protein
3 : Probed with rabbit antisera against MAP culture filtrate antigen
4 : Probed with Clinical sera obtained from goat affected by paratuberculosis

Fig. 5.

reactivity of the proteins with the hyperimmune sera raised against the recombinant 16kDa protein, MAP culture filtrate and also from goat with clinical paratuberculosis as per the procedure described for western blotting.

Sequence analysis

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

RESULTS

PCR amplification of 441bp*M. a. paratuberculosis*

PCR amplification of 441 bp was carried out using MAP 862F and R specific primers, atannealing temperature 58°C in 34 cycles amplified a product of 441 bp size band (Fig. 1, lane 1).

Screening and confirmation of recombinant pQE 441

Isolated Plamid DNA from randomly selected 15 colonies (from pQE 441) was double digested with *BamH*I/*Pst*I restriction enzymes released a fragment of 441 bp(Fig. 2, lane 2).

Expression of recombination 16kDa proteins in *E.coli*

IPTG induced *E.coli*cells harbouring the recombinant plasmids pQ E441 12% SDS-PAGE resolved a predominant band of 16kDa corresponding to the recombinant 16kDa (His 16) protein **(Fig. 3, lane 3, 4).**

Purification of the His-16 protein

The purified protein analyzed by12% SDS-PAGE, showing the monomeric band of 16kDa (Fig. 5, lane 4) with a concentration of 15 mg/L at flask level.

Immunogenicity of the His-16 protein

The pooled polyclonal sera raised in rabbits against purified His-16protein, reacted with 4 hour IPTG induced culture of *E.coli* M15 harbouringpQE 441as well as recombinant 16kDa protein on western blot. (Fig. 4, Lane 4)

Dot ELISA

The His-16was also recognized by the polyclonal serum again MAP culture filtrates and also by the serum from a goat naturally infected with MAP (Fig. 5, Lane 4).

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 ORFsof 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

Johne'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-?) 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 Bannatineet. 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 development[13]. Therefore, in the present work keeping in view the importance of identifying MAP specific antigens through the cloning and hetrologous 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 *BamH*I and *Pst*I were incorporated into the oligonucleotide

primers to facilitate directional cloning. The resulting plasmid pQE 441 containing an open reading frame encoding successively 6X histidinepolypeptide, 16kDa. The recombinant pQE 441 clones were confirmed by release of the insert by double digestion with *BamH*I and *Pst*I 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-16proteins strongly reacted with the *E. coli* expressed recombinant 16kDa and 16 kDa proteins on immunoblot. This has not only confirmed the heterologous expression but also suggested that the recombinant proteins retained their antigenicity.Reactivity of the His-16proteins 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 antigen city 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 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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