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Cloning and expression of cultural filtrate proteins from novel and native strains of *Mycobacterium avium* subspecies *paratuberculosis* and their application in ELISA based sero-diagnosis of Johne's disease

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Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is endemic in livestock leading to low per animal productivity. MAP as survives pasteurization, poses a public health problem because of high exposure to animals and humans. There is an urgent need for newer diagnostic tests with high specificity and sensitivity as the current ones suffer from lower sensitivity and specificity. In present study, six *Mycobacterium avium* subspecies *paratuberculosis* (MAP)-specific culture filtrate proteins (CFPs) were produced and evaluated for sero-diagnosis of MAP infection in goat and cattle herds in India. Genes encoding for six MAP-CFPs were amplified and cloned into easy cloning vector pJET1.2/pTZ57R followed by sub-cloning into expression vector pET28a (+)/pET22b (+) containing C-terminal Histidine. Recombinant CFPs (r-CFPs) expressions were optimized in *Escherichia coli* (Rosetta cells) and purified using Ni-NTA affinity chromatography. In SDS-PAGE, MAP CFPs viz., 1693c, 2168c, ModD, 85C, Pep AN and Pep AC showed 22, 24, 55, 38, 20 and 25 kDa molecular masses, respectively. Identity of these r-CFPs was further confirmed by immuno-blotting. We developed six different ELISAs using the six individual r-CFPs and one additional ELISA *i.e.* cocktail ELISA (c-ELISA) was prepared using cocktail of all 6 r-CFPs. The performance of all seven ELISAs were further evaluated against whole cell protoplasmic based indigenous ELISA (i-ELISA). c-ELISA showed almost similar sensitivity as shown by i-ELISA. However, individual r-CFP based ELISA could not reach up to the sensitivity of cocktail of six r-CFPs. None of the r-CFP showed any false positive (as compare to i-ELISA) thereby specificity was 100%. Results of ELISA tests based on cocktail of r-CFPs, ModD and 85C were quite similar to i-ELISA from goat sera whereas in cattle serum c-ELISA was comparable with i-ELISA. Our study showed a comparable specificity of c-ELISA for the diagnosis of JD and it may have applicability in region where disease is endemic. Future validation of c-ELISA against gold standard or confirmatory tests would give a better insight on its diagnostic potential over i-ELISA.

Keywords: Cattle, Culture filtrate proteins (r-CFPs), c-ELISA & i-ELISA, Goats, Livestock

Johne's disease (JD) is chronic incurable granulomatous enteritis characterized by persistent or intermittent diarrhoea, progressive weight loss, debilitation and death[1]. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) that has wide host distributions, infecting livestock[2], wild animals[3] (rabbits[4], Blue-bull[5]) as well as primates[6] and human beings[7]. The disease is endemic in livestock leading to low per animal productivity[2]. Since MAP gets secreted in milk of affected domestic livestock and possibly survives during pasteurization as well, with the high exposure to animals and humans, it has emerged as a 'public health issue'[7]. Diagnostic tests may be helpful in identification and elimination of exposed animals in the early stage of infection[8]. JD control programs are hampered largely due to lower sensitivity and specificity of current diagnostic tests. Tests are based on 'antigen detection' (culture, ZN-staining, PCR, *in situ* hybridization and Immunofluorescence), 'antibody detection' (Cell mediated as well as humoral Immune response) and

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pathology based detection (gross and histopathology). Specificity and sensitivity of these tests varies and is often not consistent[9]. Therefore, newer diagnostic tests with high specificity and sensitivity need to be developed.

Culture filtrate proteins (CFPs)/secretary proteins are predictable to be immune-reactive or, immune-dominant due to their occurrence in the extracellular environment of cells where they are more possible to encounter the sensitized immune cells[10]. Cho *et al*.[11] checked infected serum by immune-blot analysis collected from MAP infected cattle, showed strong reactivity to CFPs in compare to antigens of intracellular origin. Using CFPs for sero-diagnostics of MAP, sensitivity of the tests has enhanced and similar results came using CFPs of other mycobacteria[12]. As compared to conventional ELISAs, the use of CFPs led to increase in sensitivity of the antibody detection by 20.0%[13]. Studies support the CFPs as better antigens for solid-phase ELISA assay performing better sensitivity[10,14]. Pradenas *et al*.[15] also reported that most of the CFPs having low molecular weight (>70 kDa) showed strong reactivity with serum from culture-positive animals. They have also observed a drastic alteration in immune reactivity of CFPs with MAP heavy shedders cattle and cattle having clinical cases of paratuberculosis. MAP infected small ruminants (goats and sheep) serum also displayed good reactivity with MAP CFPs examined by immune-blot assays[16]. However, selection of antigen remains a challenge, since no single CFP based antigen can recognize all the stages of infection especially sub-clinical infection. The results of earlier studies support the use of cocktail of CFPs and it could be a better candidate for sero-diagnosis of MAP infection.

In the present study, we cloned, expressed, and purified six MAP CFPs having potential sero-diagnostic value. Diagnostic potential of rCFPs were assessed by developing the different ELISAs (using individual CFP and cocktail of all 6 CFPs) and its comparison with whole cell protoplasmic antigen (derived from of native MAP strain - 'S-5 Indian Bison Type') based indigenous ELISA (i-ELISA).

Materials and Methods

Reference strain and sample collection

MAP reference strain 'S 5', 'Indian Bison Type' was a novel biotype obtained from Microbiology laboratory, Central Institute for Research on Goats (CIRG), Makhdoom, Mathura, UP, India. Culture was propagated as per standardized protocols[17]. Serum samples from 90 goats and 120 cattle were included for evaluation of different diagnostic ELISAs. Animals were from native livestock breeding farms of three states (Uttar Pradesh, Haryana and Madhya Pradesh) of India.

Targeted immunogenic CFPs

In this study, we targeted 6 CFPs encoding genes viz. *1693c*; *2168c*, *ModD*; *85C*; *Pep AN* and *Pep AC* for cloning, expression, purification and for developing ELISA tests. Immunoreactivity of these selected CFPs have been demonstrated in earlier studies[18] (Table 1).

Cloning of amplified gene segment of respective CFPs genes

PCR amplification of CFPs genes

Genes encoding MAP CFPs were amplified using MAP genes specific primers in PCR (Table 2). Cycling parameters were: Initial denaturation was done at 94°C, 5 min., followed by 37 cycles of denaturation at 94°C, 30 s, annealing at indicated temperatures (Table 2) for 30 s, extension at 72°C for 1.0 min, and final extension at 72°C for 5 min[17]. Amplified PCR products of MAP CFPs genes were purified from gel by agrose gel extraction DNA purification kit (GeneJet gel extraction kit, Thermo scientific, USA)[19].

Easy cloning

Purified PCR products were subjected to restriction digestion (Table 2), ligation into appropriate cloning vector, followed by transformation (Table 3) and plating on ampicilin-containing Luria Bertani (LB) agar plates[20]. Positive clones were confirmed by restriction digestion and sequence analysis. Products

Table 1 — Profile of secretome identified as potential antigens in native strain of MAP

Secretary protein	Putative function	Gene size	Approx. size of protein	Approx. size of proteins appeared with 6 histidine amino acids
MAP 1693c	Peptidyl-prolylcis–trans isomerase	474 bp	19 kDA	22 kDa
MAP 2168c	Hypothetical protein	453 bp	21 kDA	24 kDa
MAP ModD	Fibronectin attachment	998 bp	52 kDA	55 kDa
MAP 85C	Mycolyltransferase	999 bp	35 kDA	38 kDa
MAP Pep AN	Serine proteinase	423 bp	17 kDA	20 kDa
MAP Pep AC	Serine proteinase	540 bp	22 kDA	25 kDa

Table 2 — Culture filtrate proteins (CFP)s Primers used in the study

MAP Gene	Direction	Sequence	Restriction Enzyme	Product Length	Annealing temperature
MAP 1693c	Forward	5'-GAA TTC ATG GCC GAC TCC TGC CCG ACC GCC-3'	*EcoRI*	474 bp	64°C
	Reverse	5'-CTC GAG CTA GGT CGT GGC GCC GAG GAT C-3'	*XhoI*		
MAP 2168c	Forward	5'-GAA TTC ATG GCA GCG CCG ACC GGT CTG C-3'	*EcoRI*	453 bp	66°C
	Reverse	5'-CTC GAG TCA GTT CAT CGC GGT GGC CGC C-3'	*XhoI*		
MAP[#] Mod D	Forward	5'-CAT ATG GAT CCC GAG GTC CCG ACC-3'	*NdeI*	998 bp	54°C
	Reverse	5'-CTC GAG GGC CGA GAG GGT CTG CTG C-3'	*XhoI*		
MAP 85C	Forward	5'-GGA TCC ATG GCG GGT GGC TCC CCC GTC-3'	*BamHI*	999 bp	68°C
	Reverse	5'-AAG CTT TCA GGT GGC GGG CTG GGC C-3'	*HindIII*		
MAP PepAN	Forward	5'-GGA TCC ATG GCA CCG TCG GGC CTG GC-3'	*BamHI*	423 bp	58°C
	Reverse	5'-CTC GAG TCA GAC GAC CTT GCC GGC CAC C-3'	*XhoI*		
MAP PepAC	Forward	5'-GGA TCC ATG GCG CTC AAC CAG AGC GTC-3'	*BamHI*	540 bp	58°C
	Reverse	5'-CTC GAG TCA GGC CGG CGG CCC CTC C-3'	*XhoI*		

[[#]Cho *et al*., 2007[ref.18]]

Table 3 — Cloning and expression vector and kits, restriction enzyme used in this study

MAP CFPs Gene	Easy Cloning			Cloning in Expression Vector	
	Vector / kit	Antibiotics and supplements	Cells used in Transformation	Expression vector	Cells used in Transformation
MAP 1693c	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Ampicillin (100 µg/mL)	XL-1- Blue competent cells	pET28a(+) expression vector	XL-1- Blue competent cells
MAP 2168c	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Ampicillin (100 µg/mL)	XL-1- Blue competent cells	pET28a(+) expression vector	XL-1- Blue competent cells
MAP Mod D	pTZ57R/T Cloning vector, InsTAclone PCR Cloning Kit (#1214)	Ampicillin (100 µg/mL), X-gal (20 µg/mL) and IPTG (20µg/mL)	XL-10 Ultra-competent cells	pET22b(+) expression vector	XL-10 Ultra-competent cells
MAP 85C	pTZ57R/T Cloning vector, InsTA clone PCR Cloning Kit (#1214)	Ampicillin (100µg/mL), X-gal (20µg/mL) and IPTG (20µg/mL)	XL-10 Ultra-competent cells	pET28a (+) expression vector	XL-10 Ultra-competent cells
AP Pep AN	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Ampicillin (100µg/mL)	XL-1- Blue competent cells	pET28a(+) expression vector	XL-10 Ultra-competent cells
AP Pep AC	pJET1.2 / blunt vector, Clone JET PCR Cloning kit (#K1231)	Ampicillin (100µg / mL)	XL-1- Blue competent cells	pET28a (+) expression vector	XL-10 Ultra-competent cells

were named as pJET-MAP1693c, pJET-MAP2168c, pTZ57R/T-Mod D, pTZ57R/T-85C, pJET-MAP Pep AN and pJET-MAP Pep AC.

Cloning in the expression vector

CFP genes were transferred from cloning vector to expression vector[20]. Briefly, expression vector (pET28a(+) pET22b(+)) was digested with appropriate restriction enzyme (Table 2), gel purified and dephosphorylated by alkaline phosphatase enzyme (Fermentas, USA). Gel purified and dephosphorylated vector and respective gene fragment (digested from cloned product) were ligated using T4 DNA Ligase (Fermentas, USA) and transformed into XL-1/XL-10 competent cells followed by plating on kanamycin (pET28a vector)/ampicillin (pET22b vector) containing LB agar plates. Positive clones were confirmed by colony PCR and sequencing using T7 universal primer. Products were named as pET28a-MAP1693c, pET28a-MAP2168c, pET22b-Mod D, pET22a-85c, pET28a-MAP Pep AN as well as pET28a-MAP Pep.

AC.**Expression of recombinant proteins**

To express recombinant CFP proteins, cloned constructs (pET28a-MAP1693c, pET28a-MAP2168c, pET22b-Mod D, pET22a-85c, pET28a-MAP Pep AN as well as pET28a-MAP Pep AC) vectors were further transformed into Rosetta competent cells, and a single colony for each CFP clone was picked up and propagated in 10 mL LB broth supplemented with antibiotics {kanamycin/ampicillin (50/100 µg per mL), respectively} at 37℃ in shaking mode (200 rpm) for 16 h. For expression, 500 mL LB broth supplemented with antibiotic was inoculated with 1% culture (overnight grown) and propagated for 5 h till absorbance reached between 0.5-0.6 at 595 nm. Then, isopropyl-β-D thiogalactopyranoside {IPTG} was added to a optimized concentration of 0.3 mM for induction of protein expression. Bacterial cells were incubated at indicated time and temperature (Table 4)

before being harvesting. Both soluble form (medium) and inclusion bodies (cell pellet) were assessed for optimum production of recombinant protein.

Recombinant MAP proteins that formed inclusion bodies (MAP 1693c and MAP 85C) were purified as follows: Pellet of Rosetta cells harbouring recombinant MAP CFPs were re-suspended by gentle stirring in 15 mL equilibration buffer (50 mM, Tris HCl, 200 mM NaCl, 5 mM DTT, 1.0 mM PMSF, pH 8.0). The 15 mL suspension was subjected to sonication (at optimized conditions). MAP CFPs purification was done using affinity chromatography method on a Nickel-nitrilotriacetic acid (Ni-NTA) gel matrix (Qiagen). A 50 mL conical tube containing 12 mL sonicated suspension equilibrated with 1.0 mL Ni-NTA resin in horizontal position for 8 h at 4°C. After incubation, the equilibrated suspension of MAP CFPs were centrifuged at 515 g, 10 min at 4°C. Supernatants (flow through) were removed and pellets were washed thrice with wash buffer (50 mM, Tris HCl, 200 mm NaCl, 5 mm DTT, 1.0 mm PMSF, pH 8.0 and 20 mM Imidazole) and supernatant was saved. All recombinant proteins was then eluted by increasing concentration of the imidazole to 250 mM (50 mM, Tris.HCl, 200 mM NaCl, 5 mM DTT, 1.0 mm PMSF, pH 8.0 and 250 mM). Fractions contained recombinant proteins was visualized by SDS-PAGE electrophoresis followed by Coomassie brilliant blue staining. For purification of soluble proteins (MAP 2168c; MAP Mod D; MAP Pep AN and MAP Pep AC), the medium was directly subjected to Ni-NTA affinity chromatography. The yield of proteins were in the range of 12 mg to 200 mg/L of bacterial culture (Table 4)

Confirmation, Immuno-reactivity and estimation of recombinant proteins

For confirmation, purified products were run in 12% SDS-PAGE and subjected to Coomassie blue staining to analyze the size of desired products. In addition to this, recombinant His-fusion proteins were analyzed by Western blot.

Confirmation of proteins and their size

Nitrocellulose membrane was incubated with 20 mL of peroxidase-conjugated anti-His_6 peroxidase (Cat. No. 11965085001, Roche Diagnostic GmBH, Germany) in 100 mU/mL for 1h at room temperature (25-30°C on shaker, followed by five times washing with Transfer buffer saline with Tween (TBST). Visualization of immune-reactive proteins bands were done using 2,3-diaminobenzidine (DAB) (Sigma-Aldrich).

Measure of the immunoreactivity

Nitrocellulose membrane was incubated with 20 mL of diluted sera (primary antibody) in 1:200 dilution and the membrane was kept at 4°C on rocker for over-night. Further, Nitrocellulose membrane was washed thrice using TBST buffer for 10 min. Reactivity was recorded by incubating nitro-cellulose membrane with per-oxidase-labelled rabbit based anti-species (Goat/Cattle) whole IgG antibody (Sigma Aldrich) in 1:2000 dilutions and incubated for 1.0 h at room temperature on rocker, followed by washing with TBST five times. Visualization of immunogenic proteins bands were completed using 2,3-diamino-benzidine (DAB) reactivity (Sigma-Aldrich).

Concentration of purified rCFPs

Concentration of purified rCFPs was estimated using Bradford protein estimation method at 595 nm using ELISA plate spectrophotometer[21] (Table 4).

Dot ELISA (d-ELISA)

Serum based d-ELISA was standardized for CFPs as per Singh et al., 2016[22]. Briefly, plastic d-ELISA strips (Cat No: CCN-12, MDI, India) were coated with either 0.5µg in 1.0 µL of standard antigen (MAP 'S 5' strain') used as 'positive control' or individual MAP recombinant protein used as 'test antigen'. Antigen-coated strips were blocked with 3% skimmed milk in 1XPBS (1h, 37°C). Test and positive control antigens were reacted with positive and negative control serum (1 h at 37°C). Rabbit anti-goat HRP conjugate was added (30 min at 37°C) and finally antigen antibody reaction was observed with absence of brown dot by adding substrate (DAB). Positive and negative controls were used to read the results of two antigens.

Table 4 — Conditions optimized for expressed recombinant CFPs proteins and their concentrations

Proteins	Expression form	Temperature and Time	Protein concentration	Protein quantit/litre of culture
MAP 1693c	Inclusion bodies	30 h at 27°C	0.25 µg/µL in 2mL	50 mg
MAP 2168c	Soluble	30 h at 24°C	0.12 µg/µL in 2mL	24 mg
MAP Mod D	Soluble	16 h at 37°C	0.239 µg/µL in 4mL	92 mg
MAP 85C	Inclusion bodies	30 h at 27°C	0.06 µg/µL in 2mL	12 mg
MAP Pep AN	Soluble	16 h at 37°C	0.25 µg/µL in 4mL	100 mg
MAP Pep AC	Soluble	16 h at 37°C	1 µg/µL in 2mL	200 mg

Recombinant CFP-based ELISA

'Indigenous ELISA' (i-ELISA) using sPPA as developed in goats by Singh *et al*.[23] and in cattle by Sharma *et al*.[24] was employed. Either cocktail of 6 r-CFPs; MAP1693c; MAP 2168c; MAP ModD; MAP 85C; MAP Pep AN and MAP Pep AC were used at 6 µg of six r-CFPs (1.0 µg of each r-CFPs) to develop cocktail ELISA (c-ELISA); and 6 µg of each individual r-CFP to develop recombinant-ELISA (r-ELISA). Serum collected from MAP culture positive and culture negative animals were used as serum positive and serum negative controls, respectively. OD values of the samples were expressed as sample to positive (S/P) ratios[25]. Animals came positive and strong positive categories of the S/P ratios were considered positive for MAP infection. Sensitivity and specificity of c-ELISA and r-ELISAs were compared with i-ELISA.

S/P ratio and corresponding status of JD

S/P ratios revealed corresponding status of JD as between 0.00-0.09- Negative (N), 0.1-0.24- Suspected/Borderline (S), 0.25-0.39- Low Positive (LP), 0.4-0.99- Positive (P) and 1.0-10.0- Strong Positive (SP).

Sensitivity and Specificity

Sensitivity = (True Positive / True Positive + False Negative) × 100

Specificity = (True Negative / True Negative + False Positive) × 100

Statistical analysis

McNemar's test and kappa agreement was used to measure the statistical significance between new tests (c-ELISA and r-ELISAs) and i-ELISA (GraphPad software, USA)

Results

Cloning and expression of CFPs

Genomic DNA was isolated from 'S5' strain of novel 'Indian Bison Type' of MAP. Genes encoding 6 CFPs (MAP1693c; MAP2168c; MAP ModD; MAP 85C; MAP PepAN and MAP PepAC) were successfully amplified (Fig. 1) and cloned into pJET1.2/blunt or, PTZ57R/T cloning vector (Fig. 2) and then sub-cloned into expression vector viz., pET28a (+)/ pET22b(+). Recombinant clones of CFPs genes were confirmed by colony PCR, restriction digestion and nucleotide sequencing. Purified clones (plasmids) were transformed into competent cells (*E. coli* Rosetta) to evaluate expression of r-CFPs. Optimum r-CFPs production was observed at 0.3 mM IPTG concentration, 24-37°C temperature and 16-30 h of incubation (Table 4) and all expressed r-CFPs was purified using Ni-NTA resin and confirmed by SDS-PAGE (Fig. 3).

Comparison of sero-reactivity of six individual r-CFPs and their cocktail of six r-CFPs in Immunoblot and dot-ELISA system

SDS-PAGE analysis of purified r-CFPs revealed single thick band without detection of any other protein band suggesting optimum purity of recombinant products. Identity of r-CFPs was further assessed in immuno-blotting using anti-His antibody, where all the six r-CFPs showed their expected molecular mass (Figs. 4 & 5 A-C) except ModD, which seemed to be higher (55 kDa) than the expected size of 32 kDa. Immunoreactivity of these six r-CFPs was measured with positive serum of goats and cattle by immunoblot analysis. MAP 1693c, MAP 2168c and MAP Mod D, r-CFPs showed similar reactivity

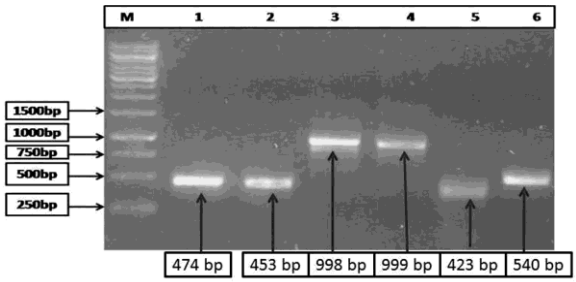

Fig. 1 — Amplification of Six MAP CFPs genes by PCR: M=1kb DNA ladder (#SM0313, Fermentas); lane 1= amplified product of MAP 1693c (474 bp); lane 2= amplified product of MAP 2168c (453 bp); lane 3= amplified product of MAP ModD (998 bp); lane 4= amplified product of MAP 85C (999 bp); lane 5= amplified product of MAP Pep AN (423 bp); lane 6= amplified product of MAP Pep AC (540 bp)

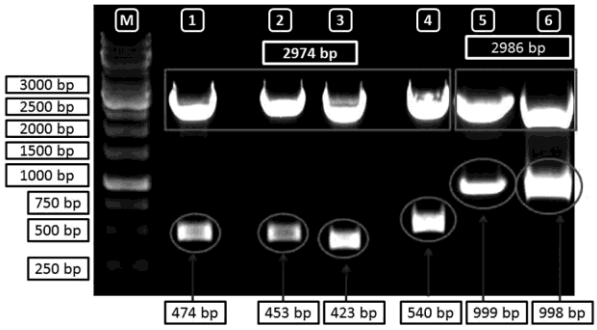

Fig. 2 — Easy cloning and confirmation in pJET 1.2 / pTZ57R/T vector: M=1kb DNA ladder (#SM0313, Fermentas); lane 1 =*Xho I* and *Eco RI* digested pJET-1693c; lane 2=*Xho I* and *Eco RI* digested pJET-2168c; lane 3 = *Bam HI* and *Xho I* digested pJET-Pep AN; lane-4 =*Bam HI* and *Xho I* digested pJET-Pep AC; lane 5= *Bam HI* and *Xho I* digested pTZ57R/T.85c; lane 6= *Nde I* and *Xho I* digested pTZ57R/T.Mod D

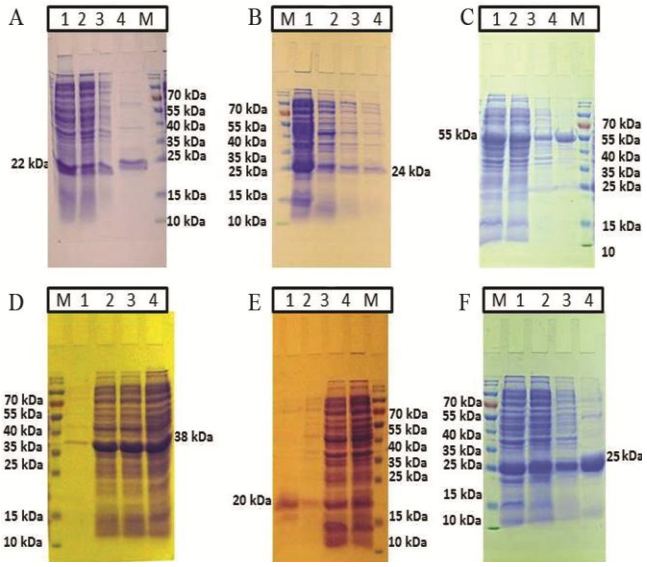

Fig. 3 — Purification of recombinant CFPs: (1) Purification of recombinant MAP 1693c protein expressed in Rosetta expression strain of *E.coli* (Left to right): lane 1. Crude sonicated extract, lane 2. Supernatant, lane 3. Wash buffer, lane 4. Eluted product, lane M: Marker (#SM0671); (2) Purification of recombinant MAP 2168c protein expressed in Rosetta expression strain of *E.coli* (Left to right): lane M: Marker (#SM0671), lane 1. Crude sonicated extract, lane 2. Supernatant, lane 3. Wash buffer, lane 4. Eluted product; (3) Purification of recombinant MAP Mod D protein expressed in Rosetta expression strain of *E.coli* (Left to right): lane 1. Crude sonicated extract, lane 2. Supernatant, lane 3. Wash buffer, lane 4. Eluted product, lane M: Marker (#SM0671); (4) Purification of recombinant MAP 85C protein expressed in Rosetta expression strain of *E.coli* (Left to right): lane M: Marker (#SM0671), lane 1. Crude sonicated extract, lane 2. Supernatant, lane 3. Wash buffer, lane 4. Eluted product; (5) Purification of recombinant MAP Pep AN protein expressed in Rosetta expression strain of *E.coli* (Left to right): lane 1. Crude sonicated extract, lane 2. Supernatant, lane 3. Wash buffer, lane 4. Eluted product, lane M: Marker (#SM0671); (6) Purification of recombinant MAP 85C protein expressed in Rosetta expression strain of *E.coli* (Left to right): lane M: Marker (#SM0671), lane 1. Crude sonicated extract, lane 2. Supernatant, lane 3. Wash buffer, lane 4. Eluted product.

with goats and cattle serum. Whereas, Pep AN and Pep AC showed higher reactivity with goat serum and 85c showed more reactivity in serum of cattle as compared to goats (Fig. 5 B & C). All individual recombinant CFPs showing good antigenicity by individual antigen based dot-ELISA (Fig. 6).

Comparison of sero-reactivity of six individual r-CFPs and their cocktail of six r-CFPs with sPPA in ELISA system

OD values of six individual r-ELISA, cocktail ELISA and i-ELISA were expressed as S/P ratio and positivity was determined by comparing S/P ratio of positive and negative controls[25] (Table 5). c-ELISA showed almost similar sensitivity as shown by i-ELISA (Table 6). However, individual r-CFPs based ELISA could not reach up to the sensitivity of cocktail of six r-CFPs in c-ELISA (Table 6). Sero-reactivity of individual r-CFPs to positive goat serum was in the order of ModD > 85C > 2168c > PepAC > PepAN > 1693c (Fig. 7) while in

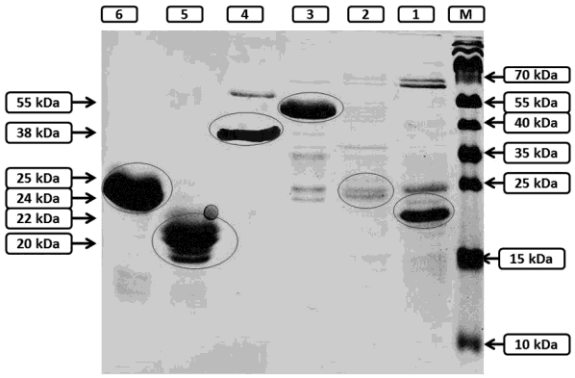

Fig. 4 — Purified expressed recombinant CFPs (Right to left): lane M= Pre-stained page ladder (#SM0671), lane 1= MAP 1693c purified protein, lane 2= MAP 2168c purified protein, lane 3= MAP Mod D purified protein, lane 4= MAP 85C purified protein, lane 5= MAP Pep AN purified protein, lane 6= MAP Pep AC purified protein.

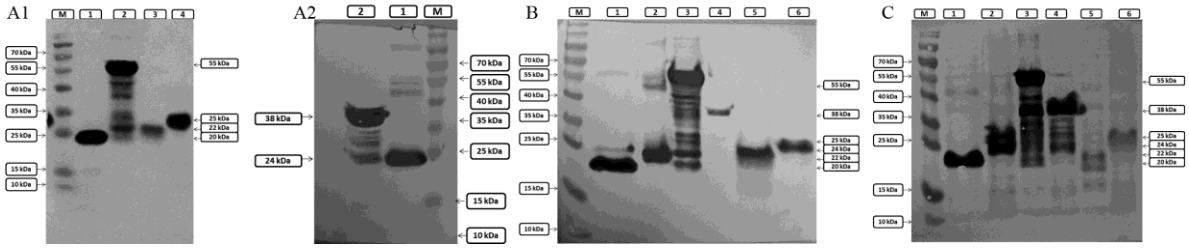

Fig. 5 — (A) The four expressed and purified proteins were subjected to immunoblot detection using anti-6 His antibody. (from left to right): lane M- Pre-stained ladder (#SM0671), lane 1- MAP 1693c, lane 2- MAP Mod D, lane 3- MAP Pep AN and lane 4- MAP Pep AC; (A1) The two expressed and purified proteins were subjected to immunoblot detection using anti-6 His antibody. (from right to left): lane M- Pre-stained ladder (#SM0671), lane 1- MAP 2168c, lane 2- MAP 85C; and (B & C) Imuunoreactivity of secretory proteins with goat & cattle positive serum by Western Blot analysis (from left to right): lane M- Pre-stained ladder (#SM0671), lane 1- MAP 1693c, lane 2- MAP 2168c, lane 3- MAP Mod D, lane 4- MAP 85c, lane 5-MAP Pep AN and lane 6- MAP Pep AC

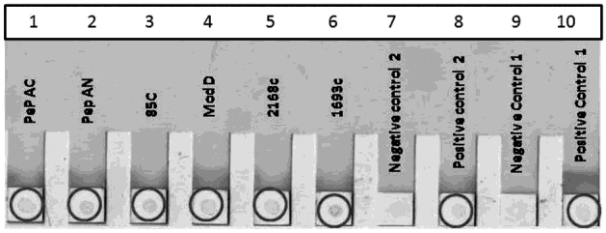

Fig. 6 — Dot-ELISA test for MAP recombinant CFPs showing brown dot for positive samples for MAP infection. (Left to Right): lane 1. MAP Pep AC, lane 2. MAP Pep AN, lane 3. MAP 85C, lane 4. MAP Mod D, lane 5. MAP 2168c, lane 6. MAP 1693c, lane 7. Negative control 2, lane 8. Positive control 2, lane 9. Negative control 1, and lane 10. For lane 1 to 6, 8 and 10 positive sera was used of MAP infected goats, and for Lane 7 and 9 negative sera was used from healthy goats. For lane 7 to 10 sPPA was used as an antigen.

Table 5 — Screening of goats and cattle serum using sPPA based i-ELISA and six recombinant CFPs based six individual r-ELISA and a c-ELISA

Comparison	Goats – 90		Cattle – 120	
	Positive	Negative	Positive	Negative
sPPA- i-ELISA	58	32	86	34
c-ELISA	57	33	83	37
1693c-ELISA	24	66	77	43
2168c-ELISA	40	50	78	42
Mod D-ELISA	56	34	80	40
85C-ELISA	54	36	79	41
Pep AN-ELISA	34	56	75	45
Pep AC-ELISA	37	53	76	46

Table 6 — Sensitivity and specificity of recombinant CFPs-based ELISA in detecting anti-MAP antibodies in goat sera

Comparison	Animal species	+ve	–ve	False +ve	False –ve	Sensitivity and specificity (%)
c-ELISA	Goat	57	32	0	1	98.0 & 100.0
	Cattle	83	34	0	3	96.5 & 100.0
1693c	Goat	24	32	0	34	41.4 & 100.0
	Cattle	77	34	0	9	89.5 & 100.0
2168c	Goat	40	32	0	18	69.0 & 100.0
	Cattle	78	34	0	8	90.7 & 100.0
Mod D	Goat	56	32	0	2	96.6 & 100.0
	Cattle	80	34	0	6	93.0 & 100.0
85C	Goat	54	32	0	4	93.1 & 100.0
	Cattle	79	34	0	7	91.9 & 100.0
Pep AN	Goat	34	32	0	24	58.6 & 100.0
	Cattle	75	34	0	11	87.2 & 100.0
Pep AC	Goat	37	32	0	21	63.8 & 100.0
	Cattle	76	34	0	10	88.4 & 100.0

cattle it was ModD > 85C > 2168c > 1693c > PepAC > PepAN (Fig. 8).

Sensitivity and specificity of recombinant CFPs based ELISA in detecting anti-MAP antibodies in comparison to i-ELISA

Sensitivity of recombinant CFPs based ELISA tests was compared with i-ELISA. None of the recombinant CFPs reacted to produce false positive results thereby considered as 100% specific as

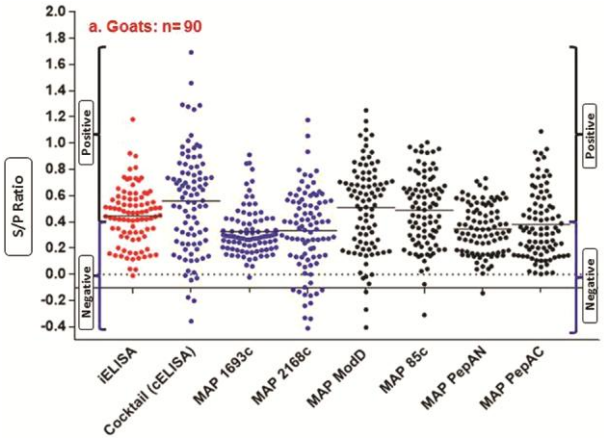

Fig. 7 — Preliminary results using the expressed proteins as solid phase antigens for screening of sera from goats. [Representative results for six individual recombinant proteins based ELISA with traditional i-ELISA and c-ELISA (Cocktail of all six recombinant secretory proteins)]

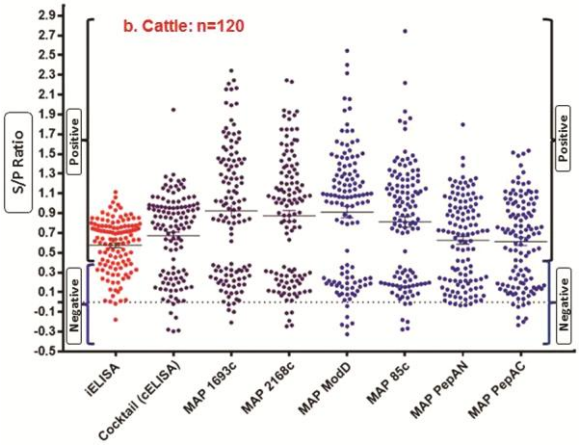

Fig. 8 — Preliminary results using the expressed proteins as solid phase antigens for screening of sera from cattle. [Representative results for six individual recombinant proteins based ELISA with traditional i-ELISA and c-ELISA (Cocktail of all six recombinant secretory proteins)]

compared to i-ELISA. However, sensitivity varied from 41.4 to 98% in serum of goats and 87.2 to 96.5% in bovine serum samples. None of them could provide up to 100% sensitivity even with the use of the cocktail of six CFPs (Table 6).

The correlation between two different ELISA tests was done to detect anti-MAP antibodies, sensitivity and specificity, Mc-Nemar calculation as well as kappa agreement. Results of ELISA tests based on cocktail of r-CFPs, Mod D and 85C were quite similar to i-ELISA in detecting anti-MAP antibodies in goat sera whereas in cattle serum c-ELISA was comparable with i-ELISA (Table 7).

Table 7 — Statistical evaluation between r-CFP/(s) based ELISA and i-ELISA in detection of MAP infection in goats and cattle

Recombinant CFP/(s) used in ELISA	Relative agreement with i-ELISA					
	Goat			Cattle		
	Kappa	95% CI	P value	Kappa	95% CI	P value
c-ELISA	0.976±0.024	0.929-1.000	1.000	0.940±0.034	0.873-1.000	0.2482
1693c	0.334±0.067	0.202-0.466	<0.0001	0.829±0.054	0.723-0.935	0.0077
2168c	0.612±0.075	0.465- 0.760	<0.0001	0.847±0.052	0.745-0.948	0.0133
Mod D	0.952±0.033	0.887-1.000	0.4795	0.883±0.046	0.793-0.974	0.0412
85C	0.906±0.046	0.816-0.996	0.1336	0.865±0.049	0.768-0.961	0.0233
Pep AN	0.502±0.076	0.354-0.650	<0.0001	0.794±0.058	0.681-0.908	0.0026
Pep AC	0.556±0.076	0.407-0.705	<0.0001	0.812±0.056	0.702-0.921	0.0044

[Kappa value (0.0-0.20, poor; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial and 0.81-100, perfect); P value showing 0.05 or less is considered as significant variation between the two ELISAs; CI: confidence interval]

Discussion

Specific and early diagnosis of MAP infection is a major challenge for the control of MAP infection in livestock. Increased production of interferon gamma in the view of MAP infection is the earliest and first immune response (cell mediated), and is slowly replaced by response by humoral immunity (antibody based)[26]. However, few studies established that some elevated level of antibodies is recognized considerably earlier and hence serological ELISA could also be used as early marker for MAP diagnosis in livestock[27]. Selection of the particular early secretary protein (antigen) is challenging, since no single MAP CFPs can detect all the MAP infected animals, especially those in sub-clinical stages of disease[28,29]. However, in view of enhanced sero-reactivity to MAP infected serum, secretary CFPs are the focus of research for the development of DIVA based diagnostics and vaccines[30,31].

In the present study, six MAP CFPs known to sero-react with anti-MAP serum were successfully cloned and expressed in bacterial expression system (pET-28a/pET-22b). Yield (12 mg to 200 mg/L of culture) of the purified recombinant proteins was sufficient enough to establish ELISA test. No significant amount of undesired protein band was observed in the purified products suggesting their purity. However, solubility of purified proteins was variable, some of them were soluble proteins (MAP 2168c, MAP ModD, MAP Pep AN and MAP Pep AC) whereas other formed inclusion bodies (MAP 1693c and MAP 85C). Expressed products were detected in Western blot using both anti-His antibody and anti-MAP serum suggesting their in-frame fusion as well as intact immunoreactivity.

Predicted molecular mass of maximum of the recombinant purified CFPs, based on their amino acid composition, matched with observations accomplished by SDS–PAGE assay and immune-blot analysis. Only exception was observed with MAP ModD protein that moved slowly in SDS–PAGE than predicted speed. This unusual movement pattern was probably due to its high proline composition in its peptide sequence, and due to this reason we observed the higher molecular weight band on SDS-PAGE than expected size.

It is rationale to use mixture (cocktail) of MAP-specific immunogenic CFPs that might upsurge the sensitivity and specificity of JD sero-diagnosis. In the development of six individual r-ELISA and cocktail ELISA, we assessed these six recombinant secretory protein antigens (MAP 1693c; MAP 2168c; MAP ModD; MAP 85C; MAP Pep AN and MAP Pep AC) with i-ELISA. c-ELISA showed almost similar sensitivity as shown by i-ELISA (Table 6). Whereas, individual r-CFPs based ELISA could not reach up to the sensitivity of cocktail of six r-CFPs in c-ELISA (Table 6). Results showed that single antigen may not be sufficiently sensitive during entire course of infection and therefore future experiments with cocktails of MAP-specific recombinant protein antigens might improve the test sensitivity and enable detection of infected animals at different stages of JD. Among the six r-CFPs, ModD and 85C revealed high sensitivity of 96.6 and 93.1% in goats and 93.0 and 91.9% in bovine sera, respectively, while, 1693c, Pep AN and Pep AC showed lowest sensitivity of 41.4, 58.6, 63.8% in goats and 89.5, 87.2 and 88.4% in cattle, respectively.

Several other studies supports the sero-diagnostic value of CFPs. Dheenadhayalan *et al*.[32] has reported the immunogenicity of five recombinant antigens (rAgs), MAP 2411; ClpP (MAP 2281c); Ppa (MAP 0435c), MAP 0593c and GreA (MAP 1027c). These antigens were tested with serum from MAP infected (41) and non-infected (41) control sheep where these

antigens, MAP0593c and ClpP, reacted with 58.5% and 46.3% positive serum and 12.1 and 4.9% of the negative serum, respectively. Another study also tested rAgs of Ag85A, Ag85B, Ag85C as well as SOD in ELISA with serum from 60 MAP shedders and 22 non shedders cows[33]. Ag85 complex and SOD were showing high reactivity with serum of MAP infected cows and very low reactivity with non-infected. Conserved proteins of Ag85 complex were observed in all other mycobacterial species[34]. At protein level, there were three Ag85 components were presented in MAP and *M. avium* subspecies *avium* (MAA) sharing 99% sequence identity[35]. Bannantine *et al*.[36] have identified 21 potential coding sequence of MAP by comparative genomic approach. All the 21 genes were successfully cloned and five of the 21 genes were only expressed and getting purified in its denaturing conditions and examined by immune blotting with serum of MAP immunized rabbits and mice found immunogenic. Western blot analysis with serum of 9 healthy and 10 MAP infected cattle showed the same five antigens recognized in context of disease. Leroy *et al*.[37] have identified 25 antigen candidates for diagnosis of MAP infection. They tested diagnostic potential of 5 antigens from 25 antigen candidates in an ELISA assay with small panel of serum collected from fields and their mixture (all three antigens) were contested in performance with other assays available commercially, getting a diagnostic specificity of 97.9% and sensitivity of 94.7%. Mon *et al*.[28] expressed 54 proteins by recombinant technology and spotted on nitrocellulose membrane and gave exposure with serum of 25 MAP infected, 10 healthy and 8 *M. bovis* infected animals. Out of 54, only 7 MAP antigens (MAP 2513, 1693, 2020, 0038, 1272, 0209c and Map 0210c) reacted with serum of MAP infected animals. Developed ELISA using Cocktail of these antigens reacted with 18 serums of the 25 MAP infected animals and did not showed any cross-reactivity with healthy animals and showed low reactivity with serum from diseased cattle from bovine tuberculosis. Gupta *et al*.[16] reported that most of the CFPs governs low molecular mass (>70 kDa) could be used as potential targets for development of new diagnostics for paratuberculosis carrying high specificity and improved sensitivity in comparison to crude protoplasmic antigens (PPA) prepared by sonication of whole cell. Hatamifar *et al*.[38] have developed a secretary proteins based ELISA and evaluated it's sensitivity and specificity as 70 and 100%, respectively, which can detect the infection in its early stage. In another study, a recombinant MAP 1637c (r20.8) protein was used in development of an ELISA for the diagnosis of bovine paratuberculosis, reported high specificity and sensitivity when compared with the PPD skin test[39]. Facciuolo *et al*.[40] have identified four MAP specific proteins were uniquely reacted with serum from MAP infected cows. They demonstrated the specificity of these proteins by calf and uninfected cow sera containing no detectable antibodies to these antigens. So, above reports given by other authors also suggest the recombinant CFPs incorporated single multiplex (cocktail) ELISA to be valuable for detection of the disease at all stages of infection in animals[16].

Conclusion

Six genes encoding MAP CFPs were cloned, expressed, purified and evaluated in ELISA to detect anti-MAP antibodies in infected animals. While sensitivity of individual CFP-based ELISA was low, the ELISA developed by cocktail of six recombinant CFPs antigen had high sensitivity and it was comparable to whole cell protoplasmic i-ELISA. Our study shows c-ELISA has comparable specificity than i-ELISA and may have better specificity to evaluate further against gold standard test and provide a better understanding on its diagnostic potential over i-ELISA for the diagnosis of JD and it's applicability in region where infection is widespread.

Ethical approval

Central Institute for Research on Goats, Makhdoom, Mathura ethical committee chaired by Member Secretary, Institutional Animal Ethics committee (IAEC) and The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi has approved works were performed under Indian Council of Medical Research Project [grant number 5/8/5/28/TF/2013/ECD-I], ICMR, New Delhi, India under reference number IAEC/CIRG/16-17 dated 12.05.2016 and confirmed that this project do not have any ethical issue. Serum samples were collected/ received only for laboratory analysis. We have avoided unnecessary pain and suffering of the animals. Samples were not collected from endangered or protected species.

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Conflict of interest

Authors declare no conflict of Interest.

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