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Molecular Diversity and Homology in Six CFPs Genes in the Novel Bio-Type, 'Indian Bison Type' of *Mycobacterium avium* subspecies *paratuberculosis* of Goat Origin *vs.* other Biotypes

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Abstract

Objective: Characterization of novel 'Indian Bison Type' bio-type of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) strain 'S5' (goat origin) was done through six MAP cultural filtrate proteins (CFPs) genes (*MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN and MAP Pep AC*) encoding for above six recombinant CFPs.

Material and Methods: Six immunogenic MAP CFPs genes (*1693c, 2168c, ModD, 85c, Pep AN* and *Pep AC*) were cloned and were confirmed by restriction digestions and sequencing. Evolutionary Analysis of the six genes was done using phylogenetic tree constructions and Sequence Identity plot.

Results and Discussion: Sequence analysis of six CFPs genes was studied extensively for their genetic composition, mutations and other variations in their open reading frames. Good percentage of homology has been found among the global biotypes/strains of the MAP along with the MAP strain 'S5' and a novel biotype (Indian Bison Type) of goat origin and other Indian isolates. Aligned sequences of the six genes have been submitted to NCBI. Genebank accessioned by following IDs (MG753462, MG753463, MG753464, MG753465, MG753466 and MG753467). However, certain insertions, deletions within the genes still required to be explored to identify the nature of the disease in Indian scenario with respect to genotypic influences in geographical entity. Mutations were observed in four genes of MAP 'S5' strain, *2168c, Mod D, Pep AN and Pep AC*. Phylogenetic tree analysis of *MAP 'S5'* genes showed *1693c* and *2168c* genes were taxonomically distant, on contrary *Mod D, Pep AN* were located taxonomically closer, 85C was present as out group of the first branch and *Pep AC* slightly away.

Conclusion: The study helped to understand molecular diversity and homology of CFPs genes of MAP 'S5' with other strains. Mutations may lead to modulations in the functions of the MAP metabolism thereby influencing the virulence and host response to the disease.

Keywords: *Mycobacterium avium* subspecies *Paratuberculosis*; Molecular diversity; Homology; CFPs; Indian bison type; Phylogenetic tree analysis; Sequence identity plot

Highlights

Characterization of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) 'S5' Indian Bison Type strain was done through six MAP cultural filtrate proteins (CFPs) genes (*MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN and MAP Pep AC*) which are responsible for encoding six recombinant CFPs.

This study will help to understand the molecular diversity and homology of MAP CFPs genes of MAP 'S5' with other available strains.

Mutations can lead to modulations in the functions of the MAP metabolism thereby influencing the virulence and the host response to the disease.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is not only highly pathogenic but also has widest host range infecting domestic livestock species, wild ruminants, other animals, non-human primates and human beings [1-4]. Potential involvement of MAP in human diseases has significant concerns for public health [5]. *MAP bacilli* has been recovered from intestinal biopsies from patients suffering from Crohn's disease, an incurable inflammatory enteritis that affects lower sections of small intestine and colon. Similarly other workers have also reported involvement of MAP in clinical symptoms and gross pathology of Crohn's disease (CD) in human beings and Johne's disease in animals [6-8]. In India, MAP has also been recovered from chronic patients of CD [9], suspected animal workers [10], human population from peri-urban areas [11], mass screening of human samples [1] and of patients suffering with advance clinical stage of CD [12].

In taxonomic classification, MAP has been placed under Order-Actinomycetes, Family- Mycobacteriaceae and Genus-*Mycobacterium*. Turenne et al. [13] reported 130 species and subspecies under single genus *Mycobacterium*. Generally, majority of the species and sub-species are non-pathogenic except some successful pathogens, which are closely related with soil bacteria like *Actinomyces* and *Streptomyces* [14,15]. *MAP* is a non-motile, acid-fast, aerobic rod shaped bacteria having complex and lipid-rich cell wall. Genetically, *MAP* is almost indistinguishable to *M. avium* subspecies *avium* (MAA) because they share 95.0% of their genes and exhibited more than 99.0% of homologies [16]. While sharing many genetic similarities with *MAA*, the phenotypic characteristics of *MAP* is different to *MAA*, with reports [17] observing *MAP* is less closely linked to mycobacteria from *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. Complete sequencing of genome of *MAP K10* 'Cattle Type' and *MAP 'S5'* Indian Bison Type [18] have led to many inroads into the MAP biology. Current information on the complete genomes of 'K10' and 'S5' strains indicated that size of *MAP* was 4.83 Mb and 4.79 Mb, respectively. *MAP* genome has 4350 protein-coding sequences including 3223 conserved proteins with known functions and 1088 conserved proteins with un-known functions and with 39 proteins with unique identity [19].

MAP 'S5' 'Indian Bison Type' biotype was isolated from a terminally sick goat of Jamunapari breed at ICAR-Central Institute for Research on Goats, Makhdoom, Mathura, UP, India. This strain has been characterized as recently evolved MAP biotype [20]. 'S5' strain of goat origin, is the source of antigens for 'Indigenous ELISA kit' and 'indigenous vaccine' for all 4 domestic livestock species [21,22]. Whole genome sequencing of *MAP 'S5'* strain was carried out to explore genetic organization and genes involved in physiology, pathogenicity and immunogenicity. This 'Whole Genome Shotgun' project has been deposited at DNA Data Bank of Japan, Mishima, Japan. (DDBJ)/ EMBL Nucleotide Sequence Database, Cambridge, UK. (EMBL)/ GenBank, NCBI, Bethesda, MD, USA. (Genbank) under accession number ANPD00000000. The version described in this paper is the first version, ANPD01000000 [18].

Secretary/culture filtrate proteins (CFPs) were considered highly immunogenic and immune-dominant due to their presence in the extracellular environment where they are more likely to encounter sensitized immune cells. This was confirmed in a study comparing CFPs from supernatants and antigens of intracellular origin [23]. Comparing the CFPs with cellular proteins showed greater sero-reactivity in animals infected with *MAP* [24]. Serum from infected cattle was used for immune-blot analysis, which showed that infected sera reacted more strongly to CFPs as compared to antigens of intracellular origin [25]. Sensitivity of sero-diagnostic tests improved with use of CFPs, and similarly for other mycobacterial pathogens, like *M. bovis* and *M. tuberculosis* [26-29]. Use of CFPs in ELISA increased sensitivity by 25.0% over 'commercial ELISAs' in low shedder animals [30]. Hence, CFPs are focus of research for DIVA based diagnostics and vaccine development. A range of new important CFPs have been identified using new technologies like 2D gel electrophoresis, chromatography, mass spectrometry and peptide mass fingerprint. Currently, semi-purified protoplasmic antigens (sPPA) are used for sero-diagnostics. More detailed categorization of antigen framework of *MAP* will be important to develop improved diagnostics. Cho et al. (2006) identified 14 CFPs of MAP JTC303 by immunoblot and mass spectrometry. Polyclonal rabbit antibodies (Rabbit anti-*M. paratuberculosis* JTC303) applied were directed against these 14 proteins where it reacted only with five CFPs (*MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep A* (Two subunit: *MAP Pep AN* and *MAP Pep AC*).

Present study aimed to characterize *MAP 'S5'* Indian Bison Type strain through these six *MAP* secretome genes (*MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN* and *MAP Pep AC*) which are responsible for encoding six recombinant secretory proteins of MAP.

Materials and Methods

Location of work

The study was conducted at Central Institute for Research on Goats, Makhdoom, and South Asian University, New Delhi, in India. Six MAP CFPs had encouraging sero-diagnostic values. Sequences of all six genes of novel 'Indian Bison Type' biotype of MAP (strain S5) of goat origin were amplified by PCR and cloning of all six genes was done in expression vectors pET28a (+)/pET22b (+). All Clones were amplified in *E. coli* cloning strain; XL1-Blue Competent Cells (Stratagene, Agilent) or, XL10-Gold Ultracompetent Cells (Stratagene, Agilent).

Cloning procedure and confirmation of orfs by sequencing

Six immunogenic MAP CFPs genes (*1693c, 2168c, ModD, 85c, Pep AN* and *Pep AC*) (Supplementary Figure 1) were cloned, since these genes express proteins which react with serum samples from animals infected with MAP (Table 1) [24].

S.NO	Name of CFPs	Putative Function	Gene Size
1	*MAP 1693c*	Peptidyl-prolylcis–trans isomerase	474 bp
2	*MAP 2168c*	Hypothetical protein	453bp
3	*MAP ModD*	Fibronectin attachment	998 bp
4	*MAP 85 C*	Mycolyltransferase	999 bp
5	*MAP Pep AN*	Serine proteinase	423 bp
6	*MAP Pep AC*	Serine proteinase	540 bp

Table 1: Short listing of potential antigens in Secretome of *MAP*.

CFPs genes were successfully amplified from *MAP 'S5'* genomic DNA using specific primers (Table 2) in thermal conditions with initial denaturation at 94C for 5 mins followed by 37 cycles of denaturation at 94°C for 30 sec, Annealing (64°C for *1693C*, 66°C for *2168C*, 54°C for *ModD*, 68°C for *85C* and 58°C for *PepAN* and *AC*) for 1 min and extension at 72°C for 5 min followed by final extension of 72°C for 5 min). Purification of PCR products was done using gel extraction purification kit (GeneJet plasmid miniprep kit, Cat. No. K0502, Thermo fisher scientific, USA). Easy cloning pJET1.2/blunt vector (CloneJET PCR Cloning kit, Cat. no.: #K1231, Thermo fisher Scientific), pTZ57R/T Cloning vector (InsTAclone PCR Cloning Kit, Cat. no.: #1214, Thermo fisher Scientific, USA) and expression vector, pET-28a (+) and pET-22b (+) were used. Both expression vectors contained a T7 promoter and C-terminal 6X His-tag coding sequence of multiple cloning regions.

S.No	MAP Gene name	Oligos Name	Direction	Sequence	Restriction Enzyme	Product Length	Reference
1	*MAP 1693c*	MAP 1693c.28a.FP	Forward	5'- GAA TTC ATG GCC GAC TCC TGC CCG ACC GCC -3'	EcoRI	474 bp	In-house designed
		MAP 1693c.28a.RP	Reverse	5'- CTC GAG CTA GGT CGT GGC GCC GAG GAT C -3'	XhoI		
2	*MAP 2168c*	MAP 2168c.28a.FP	Forward	5'- GAA TTC ATG GCA GCG CCG ACC GGT CTG C -3'	EcoRI	453 bp	In-house designed
		MAP 2168c.28a.RP	Reverse	5'- CTC GAG TCA GTT CAT CGC GGT GGC CGC C -3'	XhoI		
3	*MAP Mod D*	MAP Mod D.22b.FP	Forward	5'- CAT ATG GAT CCC GAG GTC CCG ACC -3'	NdeI	998 bp	Cho et al., 2007
		MAP Mod D.22b.RP	Reverse	5'- CTC GAG GGC CGA GAG GGT CTG CTG C-3'	XhoI		
4	*MAP 85 C*	MAP 85c.28a.FP	Forward	5'- GGA TCC ATG GCG GGT GGC TCC CCC GTC -3'	BamHI	999 bp	In-house designed
		MAP 85c.28a.RP	Reverse	5'- AAG CTT TCA GGT GGC GGG CTG GGC C -3'	HindIII		
5	*MAP Pep AN*	MAP Pep AN.28a.FP	Forward	5'- GGA TCC ATG GCA CCG TCG GGC CTG GC -3'	BamHI	423 bp	In-house designed
		MAP Pep AN.28a.RP	Reverse	5'- CTC GAG TCA GAC GAC CTT GCC GGC CAC C -3'	XhoI		
6	*MAP Pep AC*	MAP Pep AC.28a.FP	Forward	5'- GGA TCC ATG GCG CTC AAC CAG AGC GTC -3'	BamHI	540 bp	In-house designed
		MAP Pep AC.28a.RP	Reverse	5'- CTC GAG TCA GGC CGG CGG CCC CTC C -3'	XhoI		

Table 2: MAP CFPs Primers designed from novel native 'Indian Bison Type' strain (S5) of MAP goat origin.

Sn	MAP CFPs Gene	Easy Cloning			Restriction Enzyme	Cloning in Expression Vectors	
		Vector/ Kit	Antibiotics and Supplements	Cells used in Transformation		Expression Vectors	Cells used in Transformation
1	*MAP 1693c*	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Tetracyline (20 µg/ml)	XL-1-Blue competent cells	EcoRI XhoI	pET28a(+) expression vector	XL-1-Blue competent cells
2	*MAP 2168c*	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Tetracyline (20 µg/ml)	XL-1-Blue competent cells	EcoRI XhoI	pET28a(+) expression vector	XL-1-Blue competent cells
3	*MAP Mod D*	pTZ57R/T Cloning vector, InsTAclone PCR Cloning Kit (#1214)	Tetracycline (20 µg/ml), X-gal (20 µg/ml) and IPTG (20 µg/ml)	XL-10 Ultracompetent cells	NdeI XhoI	pET22b(+) expression vector	XL-10 Ultracompetent cells
4	*MAP 85c*	pTZ57R/T Cloning vector, InsTAclone PCR Cloning Kit (#1214)	Tetracycline (20 µg/ml), X-gal (20 µg/ml) and IPTG (20 µg/ml)	XL-10 Ultracompetent cells	BamHI HindIII	pET28a(+) expression vector	XL-10 Ultracompetent cells
5	*MAP Pep AN*	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Tetracyline (20 µg/ml)	XL-1-Blue competent cells	BamHI XhoI	pET28a(+) expression vector	XL-10 Ultracompetent cells
6	*MAP Pep AC*	pJET1.2/blunt vector, CloneJET PCR Cloning kit (#K1231)	Tetracyline (20 µg/ml)	XL-1-Blue competent cells	BamHI XhoI	pET28a(+) expression vector	XL-10 Ultracompetent cells

Table 3: Details of Cloning and expression vector and kits, restriction enzyme used in this study.

Purified PCR products were ligated to pJET1.2/blunt vector or, pTZ57R/T cloning vector as per manufacturer's protocols. Recombinant clones were transformed into competent *E.coli* XL-1/XL-10. After sequencing, the inserted ORFs were cloned into sites for directional ligation into the pET-28a (+) or, pET-22b (+) vector after digestion with specific restriction enzymes. Ligated products were transformed into XL-1/XL-10 competent cells followed by plating on kanamycin (pET28a vector)/ampicillin (pET22b vector) containing LB agar and incubated at 37°C for 12-18 h. We observed kanamycin/ampicillin resistant colonies of transformed cells containing respective

ligated vector compared to control empty vector. Six to ten colonies were selected from respective transformed plates and cloned products were confirmed by colony PCR. Colony PCR confirmed transformed colonies of respective transformants were cultured in LB media containing respective antibiotics followed by mini preparation of plasmid DNA. Plasmid DNA of corresponding clones were confirmed by digestion with respective restriction enzymes (Table 3) indicating successful cloning of the gene of interest.

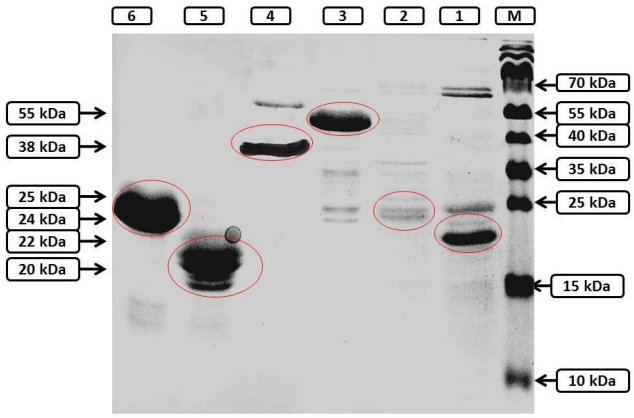

Figure 1: Purified expressed recombinant CFPs (Right to left): Lane M=Pre-stained page ladder (#SM0671), Lane 1=*MAP 1693c* purified protein, Lane 2=*MAP 2168c* purified protein, Lane 3=*MAP Mod D* purified protein, lane 4=*MAP 85c* purified protein, lane 5=*MAP Pep AN* purified protein, Lane 6=*MAP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

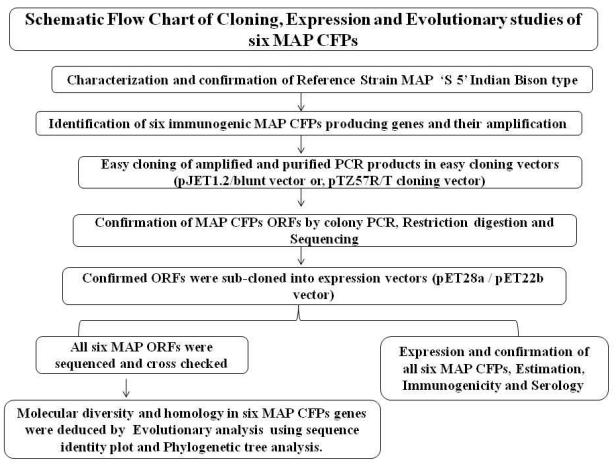

Figure 2: A schematic flow chart of the whole process (Cloning, expression and evolutionary analysis using constructions of Sequence identity Plot and Phylogenetic tree analysis).

In order to cross-check the cloned gene of interest, plasmid DNA of each clone were sequenced using T7 universal primer to obtain the in-frame gene of insert into the expression vector (pET28a/ pET22b). All the six purified cloned plasmids were transformed into *E. coli* Rosetta cells to evaluate expression of r-CFPs. Optimum r-CFPs production was observed at 0.3 mM IPTG concentration, 24°C-37°C temperature and 16-30 h of incubation and all expressed r-CFPs was purified using Ni-NTA resin and confirmed by SDS-PAGE (Figure 1). The whole process of cloning and expression of CFPs was presented in a schematic flow chart (Figure 2).

Evolutionary analyses

Phylogenetic tree construction of six MAP secretary/CFPs genes including (*MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN and MAP Pep AC*) of *MAP 'S5'* Indian Bison Type strain was done using MEGA6 software [31]. Evolutionary history was inferred using the Minimum Evolution method [32,33].The percentage of replicate trees in which the associated taxa were clustered together by bootstrapping analysis (500 replicates). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [34] and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [35]. The Neighbor-joining algorithm [36] was used to generate the initial tree. Submission of sequence data of all genes sequences generated during the current study for MAP S5 CIRG Bison type (*MAP 1693c, MAP 2168c, MAP Mod D, MAP Pep AN and MAP Pep AC)* were aligned and submitted to the Genbank™ with the accession numbers MG753462, MG753463, MG753464, MG753465, MG753466 and MG753467, respectively (Supplementary Figures 8-13).

The whole process of cloning, expression and evolutionary analysis (Sequence Identity Plot and Phylogenetic tree analysis) of all six *MAP* CFP gene sequences were portrayed in a schematic flow chart (Figure 2).

Results

All six MAP CFPs were expressed and purified and their molecular weight (kDa) were verified by SDS-PAGE (Figure 1). Six MAP CFP genes were sequenced and analyzed using Sequence Identity plot and Phylogenetic tree analysis.

A sequence identity plot was constructed for MAP 1693c secretome for Indian bison type strain of CIRG (Indian Bison CIRG or, Indian Bison/CIRG), *MAP K10* (MAP K10 AE016958.1), *MAP/TANUVAS* (MAP/TANUVAS/TN/India/2008 CPO 15495.1), *MAP 4* (MAP 4 CP005928.1 positions 169), *MAP E93* (MAP E93_CP010114.1 Position 1), *MAP FDAARGOS_305* (MAP FDAARGOS_305_CP022095.1 Po), *MAP JII-1961* (MAP JII-1961) and *MAP E1* (MAP E1_CP010113.1 Positions 16) standard reference stain. There were five nucleotide (nt) deletions (CCACC) found in *MAP K10, MAP/TANUVAS, MAP JII-1961* at nucleotide position 100 to 104. Similarly, at nt154 to nt156 position, there was three nucleotide (AGA) deletion in *MAP K10, MAP/TANUVAS* and *MAP JII-1961* while the deletions were absent in Indian Bison CIRG, *MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* strain. Similar deletions were also noticed at nt420-421, nt447-448, nt470-471 for *MAP K10, MAP/TANUVAS* and *MAP JII* while Indian Bison CIRG strain featured no such deletions. Similar 4 nucleotide insertion sequence was observed in nt382-386 for *MAP K10, MAP/TANUVAS, MAP JII* which is not observed in other compared sequences.

The sequence map for *MAP 2168c* secretome gene showed multiple mutations including nucleotide deletions of 'TGGCG', 'CCGCC'

observed between nt36-40, nt71-75 respectively in MAP bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* while the same was absent in *MAP K10, MAP/TANUVAS, MAP JII.* Similarly, more such deletions viz., 'AATT', 'AATT', 'GGCGG' and 'CGCCA' were also observed at nt126-129, nt360-363, nt414-418, nt449-453 positions, respectively with the same pattern.

While the picture for *MAP Mod D* secretome gene showed seven major deletions in MAP Indian bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305, MAP E1* at nt355-357, nt369-372, nt416-421, nt445-452, nt542-544, nt608-613, nt634-636, respectively. The deletions were absent in *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS* and *MAP JII* strain for the *MAP MOD D* secretome gene.

In MAP 85c, four sequence (CGCC) deletion was observed at position nt70-73 in *MAP K10, MAP/TANUVAS* and *MAP JII* strain while Indian Bison CIRG strain does not showed the deletion. Similarly, four sequence (GGCG) deletions was observed at nt123-126 in MAP bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* while the same was absent in *MAP K10, MAP/TANUVAS, MAP JII.* Interestingly 5 nucleotide sequences (nt259-263) present in *MAP K10, MAP/TANUVAS* and *MAP JII* was not observed in Indian bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* strain. Similar 4 nucleotide insertion sequence was observed in nt345-350 for *MAP K10, MAP/TANUVAS, MAP JII* which is not observed in other compared sequences.

According to the sequence plot of *MAP Pep AN* secretome gene comparison with the standard reference *MAP* strains, there was one major deletion (GGTGCCGC) observed at nt25-32 position in MAP K10, MAP Indian Bison *CIRG, MAP TANUVAS* and *MAP JII* strains while *MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* strain does not show this deletion. On contrast the sequence 'GCGGCACC' observed at nt405-412 position in *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS* and *MAP JII* strain was found it deleted in *MAP 4, MAP E93, MAP FDAARGOS* and *MAP E1* strain.

Three major deletions and two major insertions were observed for the secretome gene *MAP Pep AC.* Major deletion observed included nt17-20, nt328-331, nt425-427 positions for *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS* and *MAP JII* strain, whereas the other compared strains did not show such deletions. There are 2 major insertions observed at nt405-412 and nt437-439 positions for *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS, MAP JII* which is not observed in other compared sequences.

Phylogenetic tree analysis

Phylogenetic tree analysis was conducted using minimum evolution method completed by using neighbor joining algorithm for secretome of *MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN, MAP Pep AC* (Supplementary Figures 2-7). For *1693c*, there are two major branches with the *MAP 'S5'* Indian bison CIRG strain present as an out group of the first branch grouped along with other taxa such as *MAP FDAARGOS_305, MAP E1, MAP E93* and *MAP 4*, while *MAP K10, MAP/TANUVAS* and *MAP JII* were grouped in a separate branch (Figure 3). Similar picture was also observed for phylogenetic tree of secretome gene *2168c* where, *MAP 'S5'* Indian bison CIRG strain is present as an outgroup of the first branch associated with other strains such as *MAP 4, MAP E-93, MAP E1* and *MAP FDAARGOS_305* grouped together in same branch but diverged into separate subclades (Figure 3). Interestingly for *MAP Mod D* secretome gene, Indian bison *CIRG* type was present together with *MAP K10, MAP/TANUVAS, MAP JII,* while *MAP E93, MAP E1, MAP 4* and *MAP_FDAARGOS_305* strain are present in a different branch of the tree with *MAP E93* as an out group of that branch (Figure 3). For secretome *MAP 85C*, there are two different branches with *CIRG* strain present as an out group of the first branch while *MAP K10, MAP/TANUVAS, MAP JII* are present as a separate branch (Figure 3). *MAP Pep AN* showed as similar picture along the lines of *Mod D* with *CIRG* strain group together in the first branch along with *MAP K10, MAP JII, MAP/TANUVAS* while the second branch featured the other compared strain (*MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1*) (Figure 3). The phylogenetic tree for *MAP Pep AC* showed an extra branch among all the secretome genes analyzed with two outgroups. With the first group been MAP Indian bison *CIRG* strain and second *MAP/TANUVAS.* The first branch has two clades with the first clade comprised *MAP FDAARGOS_305, MAP E1, MAP E93* and *MAP 4* and the second clade with *MAP K10* and *MAP JII* (Figure 4).

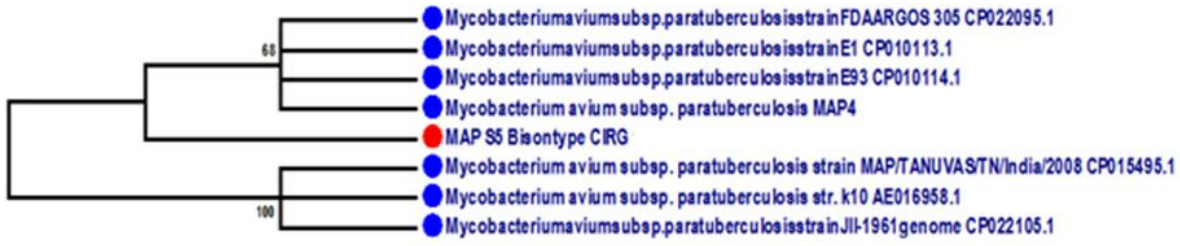

Figure 3: Evolutionary relationships of taxa for CFP genes viz., *MAP1693c, MAP2168c, MAP 85C, MAP ModD* and *MAP PepAN* of various *MAP* species including the *MAP* Indian Bison *CIRG* strain.

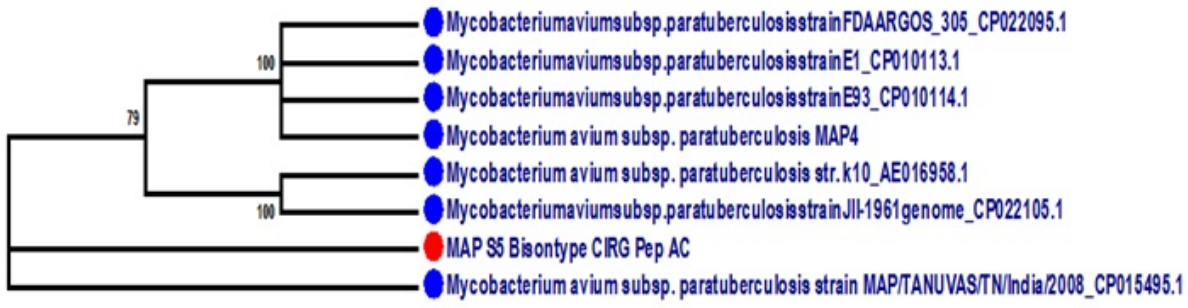

Figure 4: Evolutionary relationships of taxa for *MAP Pep AC* gene of various *MAP* species including the *MAP* Indian Bison *CIRG* strains.

Discussion

Nucleotide composition in the ORF of *MAP 1693c* secretome showed variations between the strains compared including Indian bison *CIRG, MAP K10, MAP/TANUVAS, MAP 4, MAP E93, MAP FDAARGOS_305, MAP JII-1961* and *MAP E1* standard reference stain. Nucleotide (nt) deletions (CCACC) were found in *MAP K10, MAP/TANUVAS, MAP JII-1961* at nucleotide position 100 to 104 and another deletion (AGA) between positions nt154 to156 in *MAP K10, MAP/TANUVAS* and *MAP JII-1961* while no deletion in Indian Bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* strain. To the best of our knowledge, there were no known previous studies available to compare and further analyze these mutations and its effect on the function of *MAP1693c* secretome. *MAP 1693c* is a Peptidyl-prolyl cis-trans isomerase (PpiA) which catalyses the interconversion of cis-trans peptide bonds and can accelerate protein folding, with implications in cell surface recognition. The increased role of PpiA in the pathogenic process, and its potential to alter cell surface recognition, makes cue that this protein may be involved in stealth, evading the immune response [37]. *MAP 1693c* secreted protein has a molecular weight of 18.3 kDa and conserved in MAP having unknown localization [38]. The function of *MAP1693c* was analyzed using the mutant strain WAg915 (*M. avium* subsp. *paratuberculosis* ΔppiA), defective in the peptidyl-prolyl cis-trans-isomerase, showed a mild attenuated phenotype relative to the wild-type strain and resulted in limited tissue colonization following challenge with parental *M. avium* subsp. *paratuberculosis* strain [39]. Later, a study reported that *MAP 1693c* protein specific antisera with their cognate native MAP protein in CF by immune-blotting and ELISA. *MAP1693c* showed higher specificity (>88%), and is more significant (P<0.05) in infected than uninfected bovine sera samples by immune-blotting. As per data generated during his study, he suggested that *MAP1693c* contain epitope capable of sensitive detection, and antigen-specific rat antisera data imply that these epitopes may be pathogen-specific. Leroy et al. [40] found *MAP1693c* were both specific and antigenic. Since there is no concrete information or research findings unequivocally describing the function of *MAP1693c* secretome is available, the function needs to be explored based on the structural similarity it carries with the other known proteins or by full-proof gene knock out studies on the lines of O'Garra et al. [39].

ORF of *MAP 2168c* secretome showed variations in nucleotide composition compared including Indian bison *CIRG, MAP K10, MAP/TANUVAS, MAP 4, MAP E93, MAP FDAARGOS_305, MAP JII-1961* and *MAP E1* standard reference stain. The sequence of *MAP 2168c* secretome gene showed multiple mutations including nucleotide deletions of 'TGGCG', 'CCGCC', 'AATT', 'AATT', 'GGCGG' and 'CGCCA' at nucleotide position 36-40, 71-75, 361-363, 414-419 and 449-453, respectively in *MAP* bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305, MAP JII* and *MAP E1* while absent in *MAP K10, MAP/TANUVAS, MAP JII*. There were no known previous studies available to compare and further analyze these mutations and its effect on the function of *MAP 2168c* secretome. *MAP 2168c* is a hypothetical protein of 15.9 kDa MW having unknown functions and localization. Cho et al. [24] used Rabbit anti-CF antibodies produced significantly higher ELISA A450 values (p<0.05) with 5 (1693c, 2168c, ModD, 85C, PepA-C) of the 14 recombinant proteins than was seen with the negative control protein (bovine fibronectin). The recombinant protein MAP2168c showed the highest specificity for *M. paratuberculosis* yielding high ELISA A450 values with rabbit anti-*M. paratuberculosis* CF and low ELISA A450 values with rabbit anti-*M. avium* CF. Again, the function of this secretome needs to studied to understand its function and role in virulence or pathogenicity or immunogenicity. To the best of our knowledge, there is no literature available to compare the possible effects of the reported mutations during the study.

Nucleotide composition in ORF of *MAP ModD* secretome exhibited variations between Indian bison *CIRG, MAP K10, MAP/TANUVAS, MAP 4, MAP E93, MAP FDAARGOS_305, MAP JII-1961* and *MAP E1* standard reference stain. The sequence in *MAP ModD* ORF showed seven major deletions in *MAP* Indian bison *CIRG, MAP 4, MAP E93, MAP FDAARGOS_305, MAP E1* at 355-357, 369-372, 416-421, 445-452, 542-544, 608-613, 634-636 nucleotide positions, respectively. The deletions were absent in *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS* and *MAP JII* strain for the *MAP ModD* secretome gene. Mycobacterial ModD is an alanine and proline-rich secretory protein, with an expected size of 45-47-kDa MW possessing immunogenic glycoprotein belonging to fibronectin attachment protein (FAP) family present in several species [41-43] and is conserved in several mycobacterial species including *M. tuberculosis*, *M. bovis*, *M. vaccae*, *M. avium*, and *M. leprae* [44]. The ModD homologue was investigated as a dominant secreted antigen of *M. tuberculosis* and *M. Bovis* and mycobacteria containing phagosomes [45]. Zhao et al. [46] reported that fibronectin binding peptides blocked the *in vitro* binding of fibronectin with *M. avium*. This indicated that *M. avium* binds with fibronectin with FAPS present on its surface. Middleton et al. [47] demonstrated that addition of recombinant FAP to human respiratory tract organ cultures inhibited the binding of *M. avium* with extracellular matrix (ECM). They further

demonstrated that FAP knock-mutants of *M. smegmatis* failed to bind with ECM which was restored when a plasmid carrying multicopy FAP gene was transformed into mutant. Secott et al. [48] demonstrated that the fibronectin binding was pH dependent and could be enhanced by acid treatment. They found that MAP contained coding sequences for FAP homologue that bound fibronectin in a FAP dependent manner and bound with M cells. FAPs are important for internalization and invasion of epithelial cells by MAP, and deletion of this gene leads to enhanced aggregation in *M. smegmatis* [49].

Structural proteomics of ModD has also been evaluated by previous works, which shows MAP ModD proteins were mainly composed of beta sheets. Functional antigenic analysis showed strong antigenicity in ModD due to residues 1-250 possessing high antigenic index when analyzed using protean tool of DNAstar. It has been reported that the fibronectin attachment protein of MAP induces CD^{4+} and CD^{8+} T cell activation through TLR-mediated DC activation [50,51]. Presence of ModD in association with MHC class II molecules in late endosomal/lysomal compartments suggests that it may be processed and presented in association with MHC class II molecules. The major deletions that were observed in the coding region of the ModD suggests potential alteration in the invasive capability of the MAP. This also cues a possible increase or decrease in the virulence of the strains and genotypes reported from India. As in this case, the mutations obtained for *MAP S5* Indian Bison type needs further experiments to connect them with virulence capabilities.

Nucleotide composition of ORF of *MAP 85c* exhibited variations between Indian bison *CIRG, MAP K10, MAP/TANUVAS, MAP 4, MAP E93, MAP FDAARGOS_305, MAP JII-1961* and *MAP E1* standard reference stain, there was four sequence deletion at nt70-73 nucleotide position in *MAP K10, MAP/TANUVAS* and *MAP JII* strain while this deletion was absent in Indian Bison CIRG. Similarly five nucleotide sequence deletion at nt259-263 observed at in *MAP K10, MAP/TANUVAS* and *MAP JII* strain which was not deleted in Indian Bison CIRG. While, 4 nucleotide insertion sequence at nt345-350 featured in *MAP K10, MAP/TANUVAS, MAP JII* was not observed in Indian bison CIRG. The Ag85 complex is a 30-32 kDa family of three proteins (Ag85A, Ag85B) which have mycolyl transferase activity [52] and play a major role in bacterial cell wall synthesis [53], each of which possesses an enzymatic mycolyl-transferase activity required for the biogenesis of cord factor (trehalose-dimycolate) [54,55]. In previous works [56], the role of 85C in the transfer of mycolates have been assessed by inactivation of the gene coding for antigen 85C by transposon mutagenesis. The resulting mutant was shown to transfer 40% fewer mycolates to the cell wall with no change in the types of mycolates esterifying arabinogalactan or in the composition of non-covalently linked mycolates. As a consequence, the diffusion of the hydrophobic chenodeoxycholate and the hydrophilic glycerol, but not that of isoniazid, was found to be much faster through the cell envelope of the mutant than that of the parent strain. Taken together, these data demonstrate that: (i) antigen 85C is involved directly or indirectly in the transfer of mycolates onto the cell wall of the whole bacterium; (ii) the enzyme is not specific for a given type of mycolate; and (iii) the cell wall-linked mycolate layer may represent a barrier for the diffusion of small hydrophobic and hydrophilic molecules. This confirms that these enzymes play a critical role in physiology and pathogenesis of *M. tuberculosis* [57].

The proteins are encoded by three paralogous genes (fbpA, fbpB and fbpC) located in distinct regions of the bacterial genome [58]. The genes encoding the three Ag85 components from MAP have been sequenced, and at the protein level, a 99% sequence identity with *M. avium* was found, with a single amino acid residue difference for each protein, isoleucine/threonine in position 284 of the mature Ag85C protein in MAP and *M. avium*, respectively. Comparisons have also been made between the mature protein sequences of *MAP Ag85* and *M. bovis* Ag85. For the 85C protein (MAP 3531c) 87% of amino acid sequences are identical between MAP and *M. bovis* [54].

Ag85C (294 aa) were synthesized as 20-mer peptides overlapping by 10 residues, with the exception of two 18-mer peptides (aa 31 to 50 and 41 to 60, residues 33 and 34 lacking in Ag85C) and the carboxy-terminal 14-mer peptide (aa 281 to 294). MAP FbpC2 (Ag85C2) is important for the covalent attachment of mycolic acids to arabinogalactan [59]. The recent crystal structure of FbpC2 has revealed a catalytic triad, formed by Ser124, Glu228 and His260, a hydrophobic pocket and tunnel corresponding to a probable TMM binding site, and a trehalose binding site [60,61]. A number of conserved residues among FbpC2 highlighted potential site (s) for interaction with human fibronectin was found [62].

Response against synthetic peptides from Ag85C were much lower. As the Ag85C homologues from *M. avium* subspecies *paratuberculosis* and *M. Tuberculosis* share the highest percentage of identical amino acids, this suggests that low responsiveness to the Ag85C component is probably caused by low expression levels, rather than by low immunogenicity. Low response levels to the Ag85C component have also been described in tuberculosis [63]. MAP 85C induce a strong Th1 response and confer protection against MAP infection [64,65].

The current study could map some of the differences in the coding regions of the Indian Bison type of MAP. Despite this, there might also be variations between global strains and genotypes of MAP, which needs to be explored. The valid point that could leverage this exercise is to study the variations in the MAP85C secretome protein synthesized by various genotypes vis-à-vis their antigenicity and potential marker for their differentiation. Besides, the cord factor associated with wide range of MAP genotypes can be analyzed for applicability in marker assisted DIVA assays.

The nucleotide composition of ORF of *MAP Pep AN* secretome gene having variations between Indian bison *CIRG, MAP K10, MAP/TANUVAS, MAP 4, MAP E93, MAP FDAARGOS_305, MAP JII-1961* and *MAP E1* standard reference stain. There was one major deletion (GGTGCCGC) observed at 25-32 nucleotide position in MAP Indian Bison *CIRG, MAP K10, MAP TANUVAS* and *MAP JII* strains while *MAP 4, MAP E93, MAP FDAARGOS_305* and *MAP E1* strain does not show this deletion. On contrast the eight nucleotide sequence 'GCGGCACC' insertion was observed at nt405-412 position in MAP Indian bison *CIRG, MAP K10, MAP/ TANUVAS* and *MAP JII* strain but deleted in *MAP 4, MAP E93, MAP FDAARGOS* and *MAP E1* strain.

Three major deletions and two major insertions were observed for the secretome gene *MAP Pep AC*. Major deletion observed included nt17-20, 328-331, 425-427 positions for *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS* and *MAP JII* strain, whereas the other compared strains did not show such deletions. Major insertions observed included nt405-412 and nt437-439 positions for *MAP K10, MAP* Indian bison *CIRG, MAP/TANUVAS, MAP JII* which is absent in other compared sequences. But the current mutations need to be validated with multiple sequencing of MAP strains from the same geographical region encountering clinical Johne's disease. Moreover, the functional analogy of these insertions and deletions in the coding

region would unravel the MAP genotypes evolution under the Indian perspective.

MAP PepA is a serine protease and has a putative signal sequence at the N-terminus. Native PepA was significantly more antigenic in infected sheep, goats, and deer [66], and PepA is conserved among *Mycobacterium* spp. for example a virulent H37Ra strain, virulent Erdman strain, H37Rv strain, and clinical isolate CSU93 with 100% identity [42]. The antigenicity and diagnostic sensitivity of PepA and ModD was evaluated in patients with Crohn's disease (CD). It was found that, CD patient sera were found to contain antibodies to ModD and PepA. Interestingly, these two proteins are also highly reactive with sera from cattle with *paratuberculosis* and are known to be secreted or exported proteins of *M. tuberculosis* [67]. Native ModD and PepA had a significantly higher diagnostic sensitivity than the recombinant forms of these proteins.

Conclusion

Diagnosis of MAP in larger population requires robust techniques and antigen candidates for eradication studies. DIVA strategy is inevitable in such cases. Secretomes or CFPs produced by MAP could form an excellent candidate for detection of infected animals among the vaccinated herd. The sequence analysis of 6 secretome genes were studied extensively for their genetic composition, mutations and other variations in their open reading frames. Good percentage of homology has been found among the global strains of the MAP along with the Indian Bison type and other Indian isolates. However, certains insertions, deletions within the gene needs to be explored to identify the nature of the disease its dynamics in Indian scenario. These mutations can lead to modulations in the functions of the MAP metabolism thereby influencing the virulence and the host response to the disease.

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Conflict of Interest

The authors declare that they have no conflict of Interest.

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