Sequence Analysis and Molecular Characterization of *mpb83* and *cfp2* Immunodominant Genes in Indian Field Strain of *Mycobacterium bovis*

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The *mpb83* and *cfp2* genes encoding MPB83 and CFP2 immunodominant proteins were amplified from *Mycobacterium bovis* field strain 3/86 by polymerase chain reaction. The recombinant plasmids were constructed *via* inserting the *mpb83* and *cfp2* genes into the pET vector and named pET28b-mpb83 and pET28b-cfp2, respectively. The nucleotide sequences of the insert PCR products showed an open reading frame of 600 and 360 base pairs for *mpb83* and *cfp2,* respectively. The sequenced genes and its deduced amino acid sequences were compared with the published sequences of reference strains. The sequences of the *mpb83* and *cfp2* genes share more than 99% similarity at both nucleotide and deduced amino acid level with published reference *M. tuberculosis* complex strains indicating their conserved nature.

Key words: *Mycobacterium bovis*, *mpb83* gene, *cfp2* gene, sequencing, homology.

Mycobacterium bovis, a member of *M. tuberculosis* complex is the principal cause of bovine tuberculosis (BTB). The infection in animal is generally chronic with tubercles in lungs, other visceral organs and associated lymph nodes. For BTB diagnosis, World Organization for Animal Health (previously OIE) recommend tuberculin skin test (TST) as a prescribed test for international trade[1]. In TST, the measurement of delayed-type hypersensitivity (DTH) reaction due to inflammatory reaction after the *in vivo* intradermal injection of tuberculin purified protein derivatives (PPD)[2]. But, a low specificity of PPD hinders BTB diagnosis that impedes designed national control programs. A number of immunologically dominant antigens have been explored from *M. bovis* in different studies that can be used for the specific diagnosis. Use of such defined antigens have improved sensitivity and specificity of BTB diagnostics. The immune dominant antigens, MPB83 and MTB12 (CFP2) are encoded by *Mb2898 (mpb83)* and *Mb2397c (cfp2)* genes, respectively (Uniprot). CFP2 is present in short term culture filtrate considered to be a strong inducer of pro-inflammatory cytokine[3]. MPB83 is a lipoprotein found attached to mycobacterial cell wall. MPB83 is expressed at high level by *M. bovis* than *M. tuberculosis*[4]. Beside this, it is a highly immunogenic and nucleic acid constructs expressing MPB83 antigen can induce both humoral[5] and T-cell immune responses[6]. So, in present study we reported cloning of *mpb83* and *cfp2* genes from Indian field strain of *M. bovis* 3/86 and their bioinformatics analysis.

MATERIALS AND METHODS

Mycobacterium bovis 3/86 strain used was obtained from Mycobacteria Laboratory,

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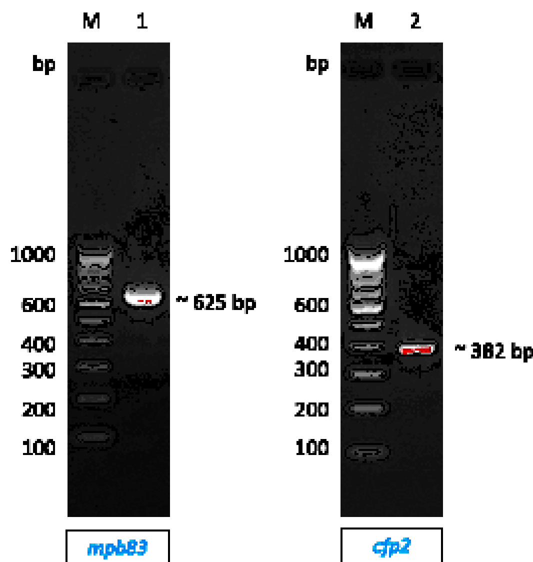
Division of Bacteriology and Mycology (IVRI, Izatnagar) and identified by biochemical and molecular methods[7, 8]. Primers for *mpb83* and *cfp2* genes were designed from the sequences obtained from available nucleotide database on the NCBI and GenBank using DNA STAR software. The *BamH*I recognition site was incorporated in the forward primer and *Hind*III site was incorporated in the reverse primer at 5' end of reverse primer along with tags in order to clone in pET28b (Table 1). Polymerase chain reaction (PCR) mixture (25µl) comprised of 0.5µl of 10mM dNTP mixture, 0.7µl of 25mM $MgCl_2$, 2.5ìl of 10×PCR assay buffer, 0.5ìl of each primers (10 pmol/ìl), 0.3ìl Pfu DNA polymerase (3U/ìl) and 1 ìl of genomic DNA. The cycling parameters were: initial denaturation at 94 °C for 10 min, followed by 30 three-step cycles, including denaturation at 94 °C for 1 min, annealing at a specific temperature for each gene (Table 1) for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The analysis of PCR products was performed by electrophoresis in ethidium bromide (0.5µg/ml) stained 1.2% (w/v) agarose gels and documented by gel documentation system.

The molecular cloning procedure was carried out by using the procedure of Sambrook et al[9]. Briefly, the purified PCR products were digested with *Bam*HI and *Hind*III (Thermo Scientific) followed by their purification and subsequently cloned into RE digested pET28b vector (Novagen). *E. coli* strain DH5á was used for the transformation and subsequent plasmid propagation. The recombinant plasmids were purified and confirmed for presence of desired insert by RE analysis. Insert DNA in recombinant plasmids were sequenced and sequences obtained were analyzed using DNA-STAR programme and sequence homology was checked with reported sequences using online basic local alignment search tool (BLAST). The multiple sequence alignments with neighbor joining method were performed using ClustalW method of MegAlign in DNA-STAR Version 4.0, Inc., USA.

RESULTS AND DISCUSSION

After biochemical and molecular confirmation of *M. bovis* (3/86), *mpb83* and *cfp2* genes under study were successfully amplified by PCR. On agarose gel electrophoresis specific amplification product of 625 bp for *mpb83* and 382 bp for *cfp2* were generated (Figure 1). Amplicon were purified, digested and cloned as described above. Positive colonies of *E. coli* strain DH5á transformed with pET28b plasmids were grown in fresh medium containing kanamycin (100 µg/ml). The isolated recombinant plasmids (named pET28b-mpb83 and pET28b-cfp2) were confirmed by *BamHI/HindIII* digestion which released specific inserts. The complete nucleotide sequences of the insert in pET28b were determined, and one open reading frame encoding a protein composed of encoding 200 and 120 amino acids were identified for pET28b-mpb83 and pET28b-cfp2, encoding MPB83 and CFP2 proteins, with predicted molecular weight of 22 kDa and 13.2 kDa, respectively. Resulting sequences were submitted to GenBank nucleotide sequence databases under accession no. KJ614485 and KJ614488.

BLAST analysis of *mpb83* and *cfp2* gene sequences confirms that it belongs to MTBC. The nucleotide sequence and predicted amino acid sequence were aligned and compared with available standard strain sequences of MTBC members, namely, *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. canettii* using ClustalW method



M: 100 bp DNA ladder, 1: Amplified *mpb83* full length gene, 2: *cfp2* gene

Fig. 1. PCR Amplification of *mpb53, mpb63, mpb83* and *cfp2* from *M. bovis* 3/86

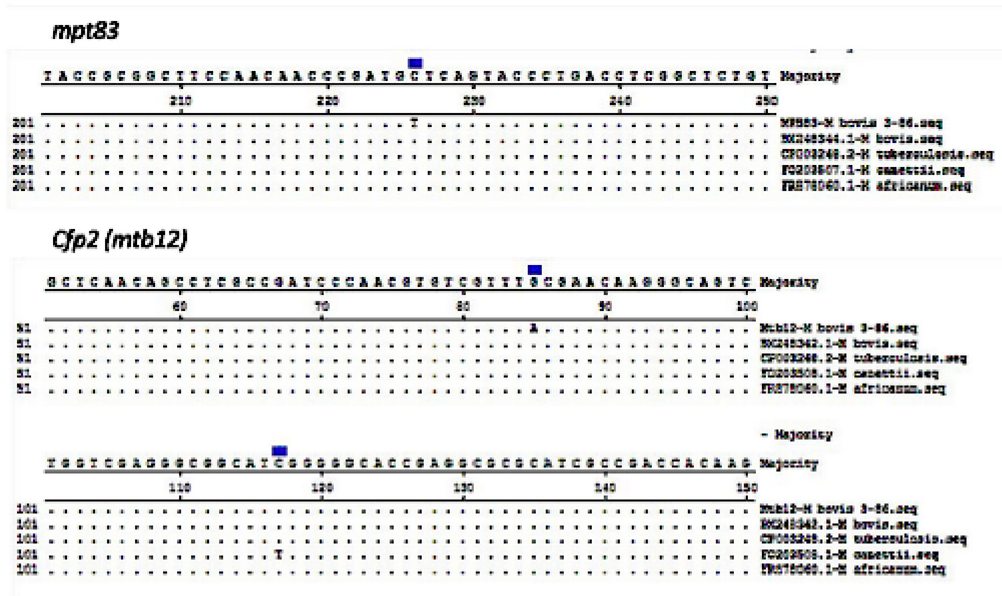


Fig. 2. Nucleotide alignment reports of *Mb3905*, *Mb3904* and *Mb2002c* genes

Table 1. Forward and reverse primer details with their annealing temperatures

Gene	Primer sequence (5' — 3')	Annealing temp
1. mpb83	F *gaa ttc* GGA TCC ctt ctt agc ggg ttg ttc g	54ºC
	R *ctc gag* AAG CTT ctg tgc cgg ggg cat cag	
2. *cfp2*	F *cag ca*G GAT CCc gac ccg gca tcc gc	56ºC
	R *gcc c*AA GCT Tgt tcc ctg cgg cct g	

of MegAlign module in DNA-STAR. The *mpb83* gene revealed 99.8 to 100% nucleotide homology and 99.5 to 100% inferred protein sequence homology for all studied genes. One nucleotide substitution in DNA sequence of *mpt83* gene of *M. bovis* 3/86 at position 226 (from nucleotide C to T) resulted in point mutation at 76 codon position from amino acid L to F (Figure 2) with 99.5% inferred amino acid sequence homology. The nucleotide sequence of 360 bp *cfp2* gene shows 99.7% homology with *M. tuberculosis, M. bovis* and *M. africanum* while 99.4% with *M. canettii*. One nucleotide substitution was found at 85 nucleotide position from G to A resulting in an amino acid change from A to T at position 29 found in *M. bovis* strain 3/86 (Figure 2). Results of our study indicated that nucleotide sequences of *mpb83* and *cfp2* gene and deduced amino acid sequences are highly conserved in different members of MTBC species. DNA sequence knowledge has become an essential pre-requisite in basic biological research, and in several applied fields microbiology. Further studies in our laboratory are aimed towards expressing these immune dominant proteins and evaluation of their use in development of BTB diagnostics.

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