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Molecular Confirmation of Conserved Nature of RD Region Encoded Genes from Indian Strain of *Mycobacterium bovis*

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***Mycobacterium bovis* 3/86 strain isolated from cattle was characterized based on RD region encoded*Mb3904, Mb3905* and *Mb2002c* gene sequences. PCR was performed to amplify Mb3904, Mb3905 and Mb2002c genes. Restriction enzymes digested amplified geneswere cloned in compatible pET vector and sequenced with vector specific primers. The sequenced genes and its deduced amino acid sequences were compared with the published sequences of reference strains. The sequences of the *Mb3904, Mb3905* and *Mb2002c* genes share 99.6 to 100% nucleotide homology and 99.5 to 100% deduced protein sequence homology for all studied genes with published reference mycobacterial strains indicating their conserved nature.**

Key words: *Mycobacterium bovis*, *Mb3904* gene, *Mb3905* gene, *Mb2002c* gene, sequencing

Mycobacterium tuberculosis complex (MTBC) contains genetically related acid fast Gram positive mycobacterium species namely *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canettii*. Among these, *M. bovis*is a causative agent of bovine tuberculosis which is a major infectious disease of cattle, but can be occasionally associated with other domestic and wild animals. The disease ischaracterized by progressive emaciation, a low grade fluctuating fever, weakness and in-appetence (OIE, 2012).Genome of *M. bovis* isa single circular chromosome with about 4.3 million base pairswith more than 99.95% nucleotide homology to that of *M. tuberculosis* (Garnier*et al*., 2003). Comparative genomics studies revealed some genomic regions that are present in *M. bovis*and *M. tuberculosis*,but absent in vaccine strain*M. bovis* BCG and several non-tuberculous mycobacteria (Polomino*et al*., 2007). These gene segments are called regions of differences (RD). A total 16 RD (RD1 to RD16) regions have been detected in *M. bovis*employing subtractive genomic hybridization and whole-genome DNA micro-arrays (Polomino*et al*., 2007). *M. bovis* RD regions genes encodes several immunogenic proteins out of which *Mb3904*, *Mb3905*, and *Mb2002c* genes encode culture filtrate protein 10 (CFP10), early secretory antigenic target 6 (ESAT6) and major protein of *M. bovis*64 (MPB64) proteins,respectively. The RD1 regionis deleted from all *M. bovis* BCG strains contains total eight ORFs, including ESAT6 gene and CFP10 (Brosch*et al*., 2000). MPB64,encoded in RD2 region, wasdeleted only in isolates of *M. bovis*BCG that were re-cultured after 1925 (Mahairas*et al*., 1996).ESAT6 and CFP10are potent T-cell antigens

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(Brusascaet al., 2001),while MPB64 protein is major immune dominant protein expressed by tuberculous bacteria (Elhayet al., 1998).Contemporary research has been taken up towards the specific diagnosis of bovine tuberculosisusing such RD region encoded proteins.These genes are known to be conserved in most of the MTBC isolates reported worldwide. In the present study, we reconfirmed the conserved nature of *Mb3904, Mb3905* and *Mb2002c* genes fromIndian strain of *M. bovis*.

MATERIALS AND METHODS

Mycobacteria strains, DNAisolationand species characterization

M. bovis AN5, *M. bovis* BCG and *Mycobacterium bovis* 3/86 strainswereobtained from Mycobacteria Laboratory(Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar). Originally, *M. bovis* 3/86 strain was isolated from tuberculosis positive cattle lymph node in 1986. Lowenstein-Jensen medium slant containing 0.5% sodium pyruvate was used for subculturing of *M. bovis* 3/86 strain. Two to three loopful of culture was used for DNA isolation and stored at -20R"C. *M. bovis* strain was identified as described previously by classical methods (Verma and Srivastava, 2001), as well as by PCR based on *IS*6110 and 12.7Kb multiplex PCR (Thangaselvamet al., 2009).

PCR Amplificationand cloning of PCR products

Primers for *Mb3905*, *Mb3904* and*Mb2002c* genes were designed from the sequences obtained from available nucleotide database on the NCBI and GenBankusing DNA STAR software. The *Bam*HI and *Hind*III RE sites were included in the primersfor directional cloning in the pET vector(Table 1). Polymerase chain reaction (PCR) mixture comprised of 200 µMdNTP mixture, 2mM $MgCl_2$, 2.5 µl 10 ×PCR buffer, 5 µMof each primers, 1.25 UPfu DNA polymerase (Fermentas, Lithuania),1 ¼l(50ng) of genomic DNA, and the volume was madeup to 25 µl with nuclease free water. The PCR amplificationconsisted of initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at a specific temperature for each gene (Table 1) for 1 min and extension at 72°C for 1 min, followed by a final extension at70°C for 5 min. The PCR amplified products were resolved on1% agarose gel in Tris-borate EDTA buffer (0.5×) and documented by gel documentation system (BioRad, USA). All three PCR-amplified products (*Mb3904, Mb3905* and *Mb2002c* genes) were digested with *BamH*Iand *Hind*IIIenzymes (Thermo Scientific, USA) and cloned directionally into compatible pET28b vector (Novagen, USA) as per the methods described in Sambrookeet al. 1989.Kanamycin resistant recombinant plasmids were isolated using GeneJET plasmid miniprep kit (Thermo Scientific, USA) following manufacturer's instructions and confirmed for presence of desired insert by RE analysis. All the plasmids were stored at -20ºC.

Sequencing and analysis

The sequencing of recombinant plasmids containing all three genes (*Mb3904, Mb3905* and *Mb2002c*) was carried out separately by vector specific primers using ABI 3500 sequencer (Applied Biosystems Inc., CA, USA)following the manufacturer'sinstructions at GCCBiotech (India) Pvt. Ltd.Kolkata, India.Sequences obtained for genes wereanalyzed 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

M. bovis strain 3/86 was subjected to various biochemical tests like niacin test, nitrate reduction test and growth on T_2CH medium. The culture wasnegative to niacin andnitrate reduction and did not grow in T_2Ch medium due to its susceptibility for this medium. In *IS*6110 PCR assay, *M. bovis*strain 3/86showed a *M. tuberculosis* complex specific 445 bp band. In 12.7 kb PCR assay, *M. bovis*strain 3/86showed an 823 bp band specific for *M. bovis*(Figure 1).

All the genes under studywere successfully amplified by PCR. On agarose gel electrophoresis specific amplification product corresponding to each of the gene including RE sites (310 bp for *Mb3905*, 322 bp for *Mb3904* and 709 bp for *Mb2002c*)were generated (Figure 2).No amplification was observed with *M. bovis* BCG DNA template for any of the gene studied. All the

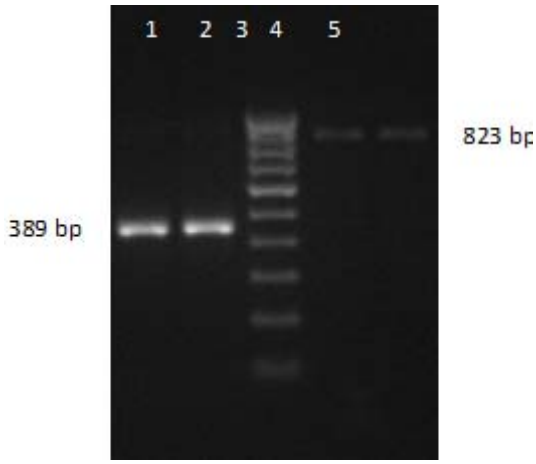
Table 1. Forward and reverse primer details and annealing temperatures

Gene	Primer sequence (5' — 3')	Annealing temp
1. Mb3905	F- gaattcggatcc cat gacagagcagcagtg gBamHIR- ctc gag aagctttgcgaa cat ccc agtgac gHindIII	54°C
2. Mb3904	F- cag cag gat cccatggca gag atgaagaccBamHIR- gcccaagcttgaagcccatttgcgaggHindIII	54°C
3. Mb2002c	F- gaattcggatcccgtgcg cat caa gat ctt cBamHIR- ctc gag aagcttggc cag cat cgagtc gat cHindIII	52°C

Table 2. Nucleotide and protein sequence homology in studied genes of *M. bovis*3/86 strain

Gene	Nucleotide length	Total amino acids	Nucleotide sequence homology (%)	Protein sequence homology (%)
1. Mb3904	285 bp	95	99.6	100
2. Mb3905	300 bp	100	100 to 99.7	100
3. Mb2002c	684 bp	228	100 to 99.9	100 to 99.6

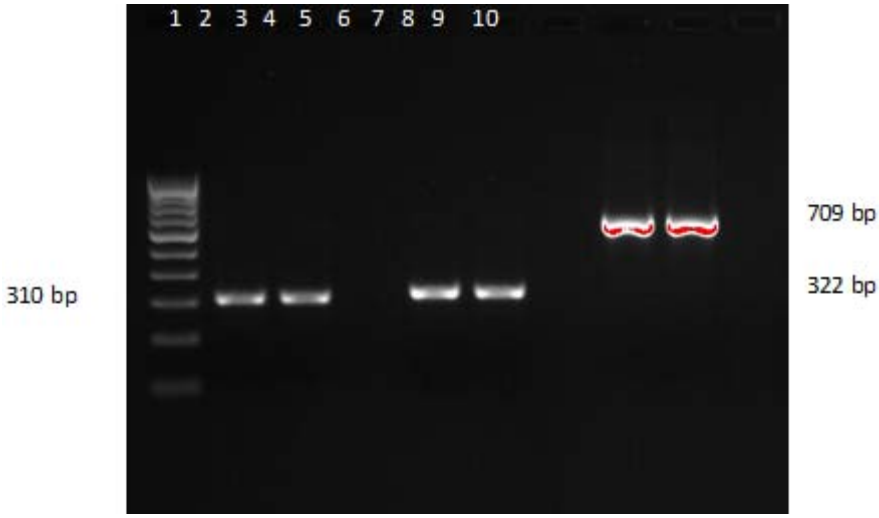
amplified products were purified, REdigested, and cloned as described above.Recombinant plasmids (pET28b-Mb3905, pET28b- Mb3904, pET28b-Mb2002c) isolated from the Kanamycin (100µg/ml) resistant colonies were confirmed by *Bam*HI/*Hind*III digestion which released specific inserts. Positive plasmids were sequenced and resulting sequence were submitted to NCBI GeneBankunder accession no. KJ614483, KJ206083 and KJ614484. BLAST analysis of *Mb3904*, *Mb3905* and *Mb2002c* gene sequences confirmed that all were 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. bovis* BCG, *M. africanum* and *M. canettii* using ClustalW method of MegAlign module in DNA-STAR. It revealed 99.6 to 100% nucleotide homology and 99.5 to 100% inferred protein sequence homology for all studied genes.



*IS*6110 PCR: 1.*M. bovis* 3/86, 2.*M. bovis*BCG, 3. 100 bp Molecular Marker
12.7Kb PCR: 4.*M. bovis* 3/86, 5.*M. bovis*BCG

Fig. 1. *IS*6110 and 12.7Kb PCR

The nucleotide sequence of *Mb3904* gene shows 99.6% homology with MTBC which indicates close relationship. Only one nucleotide substitution in DNA sequence of*Mb3904* gene of *M. bovis* 3/86 at position 147 (from nucleotide G to A) resulted in silent mutation (amino acid E to E)(Figure 3) with 100% inferred amino acid sequence homology. The nucleotide sequence of *Mb3905* and *Mb2002c* gene shows 100% homology with all members of MTBC except with *M. canettii*, where A was replaced with G in both cases at 183 and 551 nucleotide position, respectively (Figure 3). The results indicated that nucleotide sequences of*Mb3904*, *Mb3905* and *Mb2002c*genes and deduced amino acid sequences are highly conserved across the different members of MTBC species. Further studies in our laboratory are aimed towards expressing these immune dominant proteins and evaluation of their uses in BTB diagnostics.



1.100 bp Molecular Marker
*Mb3905*PCR: 2.*M. bovis* 3/86, 3. *M. bovis* AN5, 4. *M. bovis* BCG
Mb3904 PCR: 5.*M. bovis* 3/86, 6. *M. bovis* AN5, 7.*M. bovis* BCG
Mb2002c PCR: 8.*M. bovis* 3/86, 9. *M. bovis* AN5, 10.*M. bovis* BCG

Fig. 2. PCR amplification of *Mb3905, Mb3904*and *Mb2002c* genes

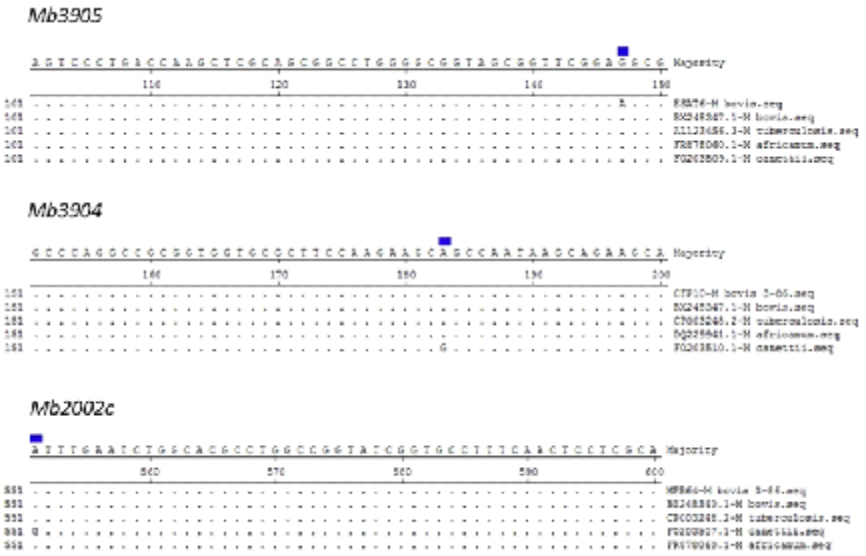


Fig. 3. Nucleotide alignment reports of *Mb3905, Mb3904* and *Mb2002c* genes

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