



PCR-SSCP analysis in detecting point mutations targeting *rpoB*, *katG* and *inhA* genes for determining multi-drug resistance in *Mycobacterium bovis* and *Mycobacterium tuberculosis* strains

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ABSTRACT

The usefulness of polymerase chain reaction-single stranded confirmation polymorphism (PCR-SSCP) for determination of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* and *M. bovis* cultures from human and animal origin was investigated. Mycobacteria (81) in the study included, were 12 MDR-TB samples, 35 sputum samples, 3 lung and lymph node tissues from bovines and 11 *M. tuberculosis* and 18 *M. bovis* culture, *M. tuberculosis* H37Rv and *M. bovis* BCG strain. All the mycobacterial cultures were characterized on growth characteristics, biochemical test pattern, MTB complex specific IS6110 PCR and species specific 12.7 kb multiplex PCR. PCR-SSCP was used to determine resistance against rifampicin by targeting *rpoB* gene (305bp) and isoniazid by targeting *katG* (237bp) and *inhA* (261 bp). Rifampicin resistance was detected by PCR-SSCP in 1 out of 12 MDR-TB samples (8.3%), while isoniazid resistance was detected in 66.7% of MDR-TB samples using PCR-SSCP of *katG* and 75% of MDR-TB samples using *inhA* SSCP analysis.

Key words: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, PCR-SSCP

Mycobacterium bovis causes bovine and *Mycobacterium tuberculosis* human tuberculosis. The genetic similarities of these 2 species have made it difficult to differentially identify them in clinical samples. Emergence of multiple drug resistance (MDR) in tuberculosis impedes effective TB control across the globe. *M. bovis* appears to have a much wider host range than *M. tuberculosis* (Coleman and Cooke 2001) and the information about drug resistance in *M. bovis* has not yet been worked out as much as it is being reported in *M. tuberculosis*. The most common mechanisms of resistance to the primary anti-mycobacterial agents such as rifampin, isoniazid, and streptomycin in *M. tuberculosis* are mutations in the target genes, mostly point mutations, otherwise known as single nucleotide polymorphisms (SNPs). Single strand conformation polymorphism (SSCP) analysis involves amplification by PCR of a segment of the gene encoding for the specific drug target and comparison of PCR products of drug-sensitive and drug-resistant strains by SSCP in which mutations usually result in an altered pattern. This study was undertaken to explore the utility of PCR-SSCP in detecting point mutations targeting *rpoB*, *katG* and *inhA* gene for determining multi-drug resistance in *M. bovis* and *M. tuberculosis* strains.

MATERIALS AND METHODS

Mycobacteria and samples: Samples (81) in the study are: 12 MDR-TB cultures procured from the Tuberculosis Centre (NDTBC), New Delhi, 38 clinical samples included 35 sputum samples collected from the District Tuberculosis Hospital, Bareilly, 3 lung and lymphnode tissue samples from bovines, and 11 *M. tuberculosis* and 18 *M. bovis* culture, *M. tuberculosis* H37Rv and *M. bovis* BCG culture maintained at Mycobacteria Laboratory of the institute. Thirty-five sputum samples were decontaminated by the modified method of Petroff (1915). The decontaminated deposit was inoculated onto the surface of a Lowenstein-Jensen (LJ) slant with 1% glycerol and 0.5% sodium pyruvate; 25 out of 35 sputum samples were positive for acid-fast bacilli under smear microscopy and 18 samples cultured on LJ medium yielded growth. The lungs and lymphnodes processed as per Marks (1972) also yielded growth on LJ medium.

Genomic DNA was isolated directly from sputum samples. Briefly, sputum samples were mixed with 1.5–2 volumes of wash solution [6 M guanidinium hydrochloride, 50 mM Tris-Cl (pH 7.5), 25 mM EDTA, 0.5% sarcosyl, 0.1 M β-mercaptoethanol], incubated at room temperature for 20 min followed by addition of 15 ml sterile distilled water, centrifuged at 6000×g for 20 min at room temperature. The supernatant was discarded carefully and the sediment was washed with 2 ml of wash solution and re-centrifuged as

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described above. The pellet was washed thoroughly with 10 ml water and after centrifugation the pellet was re-suspended in 500 µl 0.05% Tween-80 and used for smear microscopy (20 %), culture (40 %) and the remaining (40%) of the sample was gently layered on 2 volumes of 50% (w/v) sucrose solution and centrifuged at 12,000 × g for 5 min. The supernatant was discarded and the pellet was washed with distilled water and then re-suspended in 50 µL TE buffer containing 0.05% Tween80 and incubated at 95°C for 10 min. The solution was cooled and centrifuged at 10,000 rpm for 5 min and the supernatant was stored at -20°C till further use (modified from Chakravorty and Tyagi 2005, Chakravorty *et al.* 2005, Victor *et al.* 1992, Thangaselvam *et al.* 2009).

Genomic DNA from mycobacterial culture was isolated by taking 2 loopful of the mycobacteria from the LJ slopes, suspended in 200 µL TE buffer [10mM Tris, 1mM EDTA pH 7.4] and incubated at 95°C for 10 min followed by centrifugation at 12,000 rpm for 5 min and supernatant was used as DNA template in subsequent PCR reaction. Lab maintained mycobacteria culture and mycobacterium culture from clinical samples were identified by MTB complex specific IS6110 region and species specific multiplex PCR targeting 12.7 kb region (Thangaselvam *et al.* 2009) (Table 1). DNA from 16 out of 25 acid-fast smear positive sputum and other mycobacteria showed 445 bp band in all samples indicating that they were *M. tuberculosis* complex and those yielded 389bp band in *M. tuberculosis* or 823 bp band as in *M. bovis*.

PCR-SSCP

PCR for *rpoB* (305bp), *katG* (237bp) and *inhA* were carried out separately in 25µl volume containing 2.5 µl 10× PCR buffer, 200 µM dNTP mix, 5pico mole forward and reverse primer each, 0.7mM MgCl₂, 1 unit Taq DNA polymerase and 80–100ng genomic DNA template. PCR

cyclic condition for amplification of *rpoB*, *katG* and *inhA* gene were as follows: initial denaturation at 94°C for 10 min followed by 30 cycle each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min (*rpoB*, *katG*), 55°C for 1 min (*inhA*), extension at 72°C for 1 min final extension at 72°C for 10 min and holding at 4°C. The PCR amplicon (5µl) were electrophoresed along with 100 bp molecular weight marker on a 1.5% agarose gel in Tris borate EDTA (TBE) buffer at 70V for 30 min.

Single strand conformation polymorphism (SSCP) analysis: SSCP analysis of the amplified genes (Table 2) were carried out by electrophoresis of 5 µl PCR amplicon product in 12% and 15% polyacrylamide gel at 150V at 4°C for 12 h followed by silver staining of gels for visualization of banding pattern (Bassam *et al.* 1991).

RESULTS AND DISCUSSION

Genes, *rpoB*, *katG* and *inhA* were amplified (Figs 1–3) in representative mycobacteria including 18 DNA isolated directly from sputum samples to explore the polymorphism (mutation) in these genes to detect multidrug resistance and subject SSCP analysis (Fig 1.1., 2.2., 3.3). DNA of all mycobacteria yielded similar patterns for *rpoB* genes by SSCP analysis. Only MDR-TB DNA (3893) gave a slightly different pattern. This may be either due to mutation is not principal reason for conferring resistance or mutation occurred in *rpoB* gene exterior to 305bp region. In *E. coli* substitutions of amino acids 146 and 687, which is located exterior to the residues encoded by the 305-bp region characterized in the *M. tuberculosis* gene, reportedly showed rifampin resistance (Ovchinnikov *et al.* 1983, Jin *et al.* 1988, Lisitsyn *et al.* 1984). Because of the presumed large size of *rpoB* in *M. tuberculosis* (Honore *et al.* 1993) no attempt was made to characterize other regions. Our finding was supported by numerous reports such as 3 out of 121 RIFr strain (Kapur *et al.* 1994), 7% of the *M. tuberculosis* isolates

Table 1. Primer sequence used in MTB complex specific IS6110 region and species specific multiplex PCR targeting 12.7kb to differentiate *M. tuberculosis* and *M. bovis*

Primer name	Sequence (5' to 3')	Primer length (bp)	Amplicon size (bp)
IS 6110 F	GACCACGACCGAAGAATCCGCTG	23	445
IS 6110 R	CGGACAGGCCGAGTTTGGTCATC	23	
12.7 kb F	CACCCCGATGATCTTCTGTT	20	
12.7 kb R1	GCCAGTTTGCATTGCTATT	19	823 in <i>M. bovis</i>
12.7 kb R2	GACCCGCTGATCAAAGGTAT	20	389 in <i>M. tuberculosis</i>

Table 2. Details of primers used for PCR-SSCP (Williams *et al.* 1994)

Primer name	Sequence (5' to 3')	Primer length (bp)	Amplicon size (bp)
<i>rpoB</i> F	CAGACGTTGATCAACATCCG	20	305bp
<i>rpoB</i> R	TACGGCGTTTCGATGAAC	18	
<i>katG</i> F	GCCCGAGCAACACCC	15	237bp
<i>katG</i> R	ATGTCCCGCGTCAGG	15	
<i>inhA</i> F	CGGGTGACCGAGGCGATCG	19	261bp
<i>inhA</i> R	GACCGTCATCCAGTTGTAG	19	

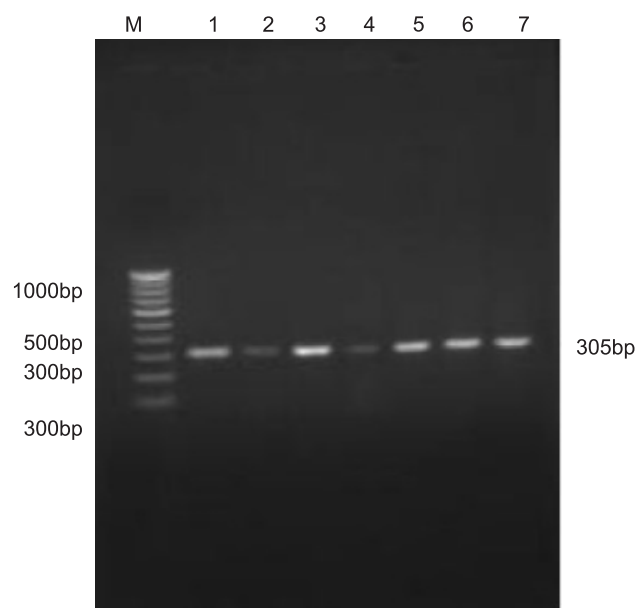


Fig. 1. Amplification of 305 bp region of *rpo B* gene in *Mycobacterium* spp. M, 100 bp ladder; Lane 1 (*M. tuberculosis* H37RV); Lane 2 (*M. bovis* BCG); Lane 3,4 (MDR *M. tuberculosis*); Lane 5, 6 (*M. tuberculosis*); Lane 7 (*M. bovis*).

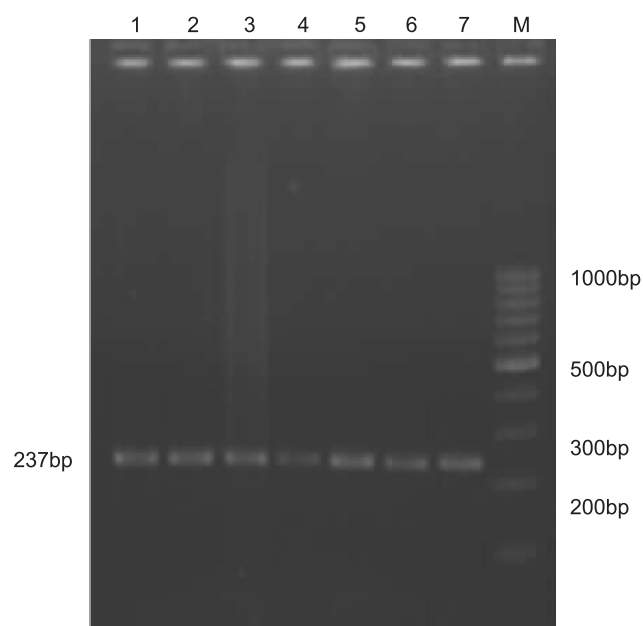


Fig. 2. Amplification of 237 bp region of *katG* gene in *Mycobacterium* spp. M, 100 bp ladder; Lane 1 (*M. tuberculosis* H37RV); Lane 2, (*M. bovis* BCG); Lane 3 & 4 (MDR *M. tuberculosis*); Lane 5 & 6 (*M. tuberculosis*); Lane 7 (*M. bovis*).

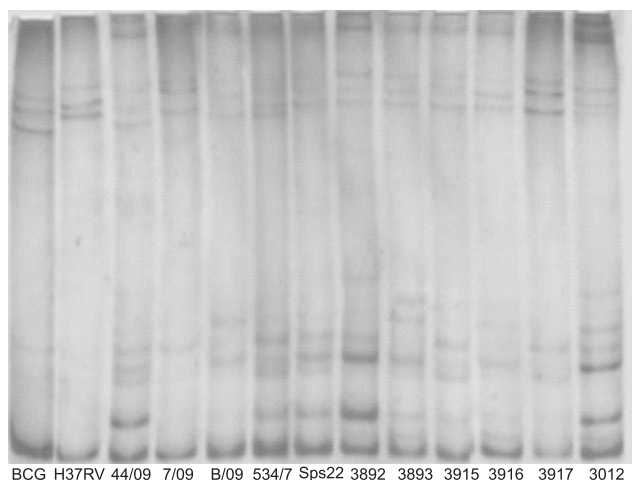


Fig. 1.1. SSCP profile of *rpo B* gene; Lane 1 to 4, *M. bovis*; Lane 5 to 8, MDR-*M. tuberculosis*; Lane 9 to 12, *M. tuberculosis*; Lane 13, *M. Tuberculosis* H37RV.

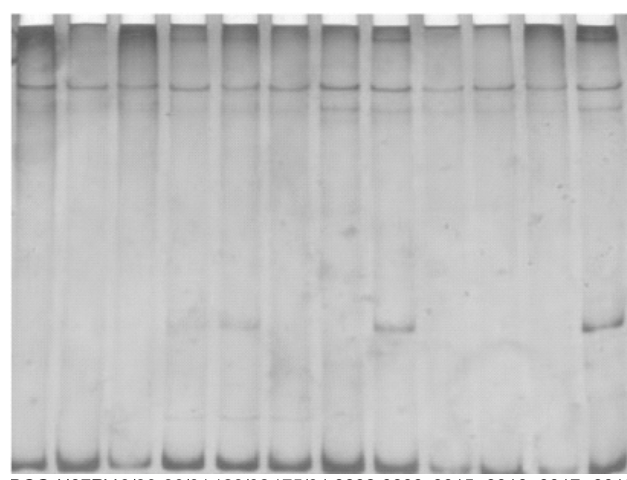


Fig. 2.1. SSCP profile of *katG* gene. Lane 1, *M. bovis* BCG; Lane 2, *M. tuberculosis* H37RV; Lane 3 & 4, *M. bovis*; Lane 5 & 6, *M. tuberculosis*; Lane 7 to 12, MDR-*M. tuberculosis*.

with 100% resistance to rifampin (Williams *et al.* 1994), 2 out of 66 (3.3%) Rif^r (Telenti *et al.* 1993) showed lack of mutation in *rpoB* targeted region, confirmed by DNA sequencing. For analyzing role of point mutation outside this 305bp region in evolving drug resistance TB organism, a 200bp fragment exterior to this genomic region was sequenced but no mutations were observed in that region in any strain. It clearly underlines that there may be another mechanism to acquire resistance in addition to point mutation.

In the present study, SSCP analysis of *katG* fragment (a 237bp product) showed 2 patterns among the *M. tuberculosis* organisms differentiating INH^r from INH^s.

Some of MDR *M. tuberculosis* strains (3915, 3916, 3917, 3139) showed a pattern similar to *M. tuberculosis* H37Rv indicating that mutation elsewhere may have been responsible for the resistance. Another finding was slightly different in the electrophoretic mobility between *M. tuberculosis* and *M. bovis* that may be indicative of a possible nucleotide polymorphism between these 2 species, but which has no relevance with antibiotic resistance. None of the DNA samples tested had a complete deletion of *katG* deletion as reported by Zhang *et al.* (1992). Isoniazid resistance is associated with alterations in the catalase peroxidase gene (*katG*) or the *inhA* gene, which encodes an enzyme involved in mycolic acid bio-synthesis (Zhang

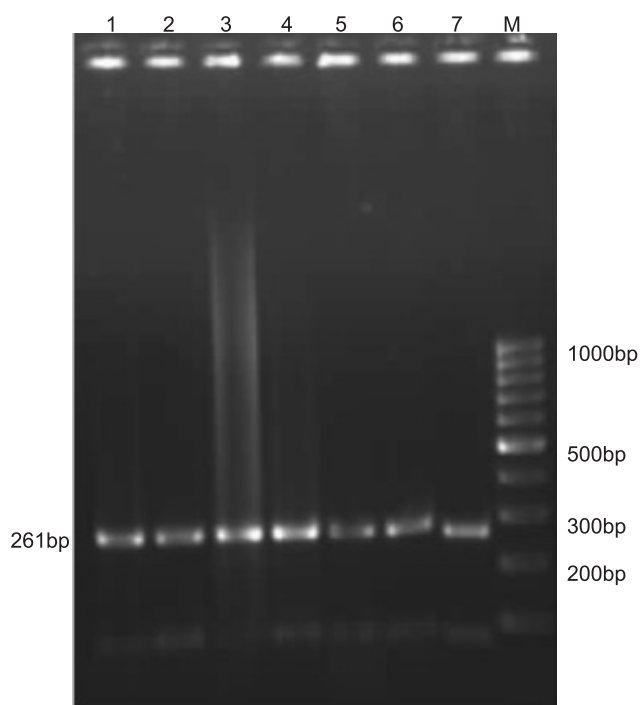


Fig. 3.1. SSCP profile of *inhA* gene Lane 1, 6, MDR-*M. tuberculosis*; Lane 7, 8, *M. tuberculosis*; Lane 9 to 11, *M. bovis*; Lane 12, *M. tuberculosis* H37RV; Lane 13, *M. bovis* BCG.

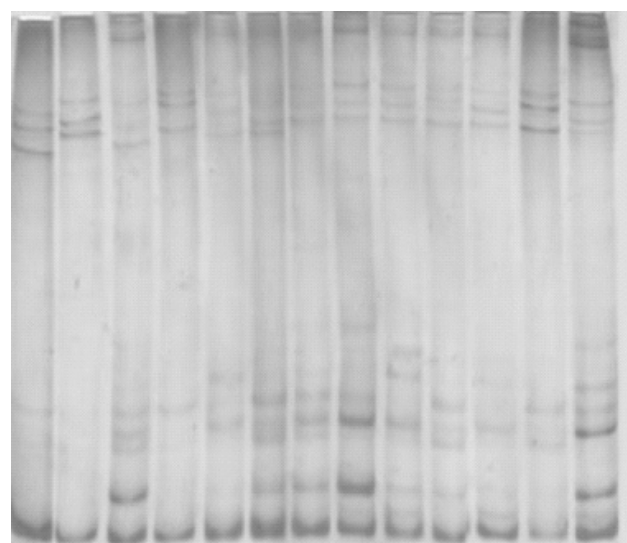


Fig. 3. Amplification of 261 bp region of *inhA* B gene in *Mycobacterium* spp. M, 100 bp ladder, Lane 1 (*M. tuberculosis* H37RV). Lane 2 (*M. bovis* BCG), Lane 3 & 4, (MDR *M. tuberculosis*), Lane 5 & 6 (*M. tuberculosis*), Lane 7 (*M. bovis*)

et al. 1992, Banerjee *et al.* 1994). The *inhA* gene (261 bp) was analysed by SSCCP. Two distinct patterns could be identified distinguishing antibiotic resistant and susceptible organisms. Sechi *et al.* (2000) studied molecular basis of isoniazid resistance in *M. bovis* strains isolated in Italy. To detect mutations involving isoniazid resistance of *M. bovis* they analysed *katG*, *oxyR-aphC* and *inhA* gene regions by sequencing. They failed to detect mutations for *oxyR-aphC*, but found polymorphisms in the *katG* and *inhA* gene

regions. They found only 1 mutation in the *inhA* region however they found 3 mutations for the *katG* region, which may explain our finding of different banding patterns even among the MDR-TB samples.

Similar to *katG* gene, some MDR organisms showed common SSCP pattern resembling with SSCP pattern of drug susceptible mycobacterial isolates. 2 out of 3 MDR isolates showed same SSCP pattern with susceptible mycobacterial isolates that further strengthened hypothesis that resistance in MDR-TB may be due to either mutation in different genes or different regions of same gene or mutations in different genes caused some physiological or metabolic changes that allowed mycobacteria organism in presence of commonly used first line of anti tuberculosis drug such as rifampin and isoniazid. In this study, 3 band SSCP pattern (*katG*) found in many isolates for this gene region may reflect the presence of 2 possible conformations for 1 of the DNA strands. This may be due to multiple mutations in targeted region as reported 3 mutations in *katG* region and only 1 mutation in the *inhA* region (Sechi *et al.* 2001). A high concordance between SSCP and DNA sequencing for isoniazid resistance in Brazilian *M. tuberculosis* isolates was reported but 25% false results were interpreted on the basis of SSCP pattern (Silva *et al.* 2003). On the basis of our limited study in particular *M. bovis*, SSCP analysis seemed efficacious method for determining drug resistance, however repeatability of the technique is less and resistance may be due to mutation in exterior to genomic region targeted, complex drug resistance mechanism, it should always be complemented by DNA sequencing and drug susceptibility testing to confirm drug resistance and understanding the mechanism of drug resistance.

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