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Bovine Tuberculosis

Conventional and molecular determination of drug resistance in *Mycobacterium tuberculosis* and *Mycobacterium bovis* isolates in cattle

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ABSTRACT

This study was aimed out to explore the presence of drug resistance among *M. tuberculosis* and *M. bovis* isolates (n = 51) of bovine origin by conventional broth microdilution method and molecular methods. By broth microdilution method, 16 isolates were found to be resistant to isoniazid, 08 isolates were resistant to pyrazinamide, 09 isolates were resistant to rifampicin and 07 isolates were found to be resistant for ethambutol. Two isolates showed resistance to rifampicin and pyrazinamide, one isolate showed resistance to pyrazinamide and ethambutol and 03 isolates showed resistance to isoniazid and ethambutol. None of isolates showed multi drug resistance (MDR). Other than the *M. bovis* strains, none of the other *M. tuberculosis* isolates showed any resistance to pyrazinamide. Molecular methods by multiplex PCR targeting *katG, pncA*, *rpoB* genes, multiplex allele specific PCR to detect mutation in *embB* codon 306 and sequencing showed point mutation in *katG* and *rpoB* gene. No mutation could be detected in the *embB* gene by multiplex allele specific PCR. The results indicates that further elaborate studies need to be carried out due to the presence of drug resistant *M. tuberculosis* in bovines which could be due to spill over from human in tuberculosis endemic areas making TB eradication programme more challenging.

1. Introduction

Bovine tuberculosis (BTB) is a chronic disease of animals caused mainly by *Mycobacterium bovis* causing direct and indirect economic losses in endemic regions. Although cattle are not maintenance hosts of *Mycobacterium tuberculosis* [1], several reports have demonstrated that cattle can get infected by the human bacillus in India [2–4]. Animal and human health is inextricably interwoven and both serve as reservoirs of zoonotic and reverse zoonotic diseases. The global scenario indicates that BTB is endemic in many countries and constitute a significant economic burden to the livestock industries. In spite of several control strategies for BTB, recent reports showed an increase in the prevalence of BTB in European Union countries [5]. In India, several authors have reported the occurrence of BTB in various states indicates, varying percentage of reactors from nil to 51.2% [6–9]. Though *M. tuberculosis* and *M. bovis* were isolated from TB like lesions in cattle, the ability of *M. tuberculosis* to transmit the same between animals or to human beings has not been proved. Effective BTB vaccines are also not currently available [10]. Attempts to treat with anti-tuberculosis drugs are providing controversial results [11]. In humans, global emergence of drug resistance to tuberculosis is a major concern and prevalence of multi-drug resistant tuberculosis (MDR-TB) as high as 26.8% has been reported. Controlled drug resistance clinical studies conducted at the National Institute for Research in Tuberculosis (NIRT) Chennai, (Tamil Nadu) showed that isoniazid, streptomycin, rifampicin resistant rate ranged from 10 to 16%, 8-3% and 1% respectively [12]. The increased incidence of *M. tuberculosis* in endemic regions like India could be a spill over infection from human being due to intertwining of human with animal habitats. Further there is also a possibility of drug resistant *M. tuberculosis* being contracted from the endemic environment by cattle [13,14]. In animals, drug resistance reports are very rare due to the fact that animals are not treated for tuberculosis. Hence, an attempt was made to identify the possibility of prevalence of drug resistant *Mycobacterium tuberculosis* complex in cattle, especially in tuberculosis endemic areas.

2. Materials and methods

2.1. Samples

All the isolates used in this study were obtained from the repositories available at Vaccine Research Centre – Bacterial Vaccines, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), India. The isolates were collected from slaughter house and farms

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located in and around Chennai, Tamil Nadu, India over a period from four years. The isolates were revived in Lowenstein Jensen (LJ) medium (Difco) with and without glycerol and maintained in Middlebrook 7H9 liquid medium (Difco). A total of fifty one isolates were revived, of which 43 isolates were identified as *M. tuberculosis* and 08 isolates were identified as *M. bovis* by 12.7 kb fragment PCR [15]. All the isolates (n = 51) were subjected for assessment of drug resistance by both conventional (Broth Microdilution Method-BMM) and molecular techniques.

2.2. Determination of drug resistance by broth micro dilution method (BMM)

All the isolates (n = 51) maintained in LJ slants and the test organism were inoculated from LJ slant to Middlebrook 7H9 broth enriched with ADC (Albumin Dextrose Catalase) with and without glycerol and incubated for five days at 37 °C with mild shaking. Culture suspension was prepared by vortexing 2 mL of culture for 2 min and allowed to stand for the coarse particles to settle for 30 s. The supernatant was taken in clean 5 mL glass test tubes and cultures were diluted with sterile distilled water to reach a turbidity that matched with the turbidity of 0.5 Mac Farland standard. The first line antimycobacterial drugs viz., isoniazid, pyrazinamide and ethambutol (Himedia, India) were dissolved in sterile distilled water and rifampicin (Himedia, India) in methanol as 1000 μg/mL stock solution [16].

BMM was carried out in 96 well 'V' bottom microtitre plate (Nunc) for each isolate in duplicates. All the wells were dispensed with 100 μl of Middlebrook 7H9 medium. Serial two fold dilution was done for each drug with the final concentration ranging between 1000 μg/L to 0.048 μg/L. Five microlitre of culture suspension (0.5 Mc Farland standards) suspension was added to all the 12 wells. Positive and negative controls were also included in each microtitre plate. Similarly drug resistance was also carried out for the reference bacterial strain used as positive controls was *M. tuberculosis* H37Rv and *M. bovis* BCG. The plates were sealed and put in sterilized autoclave bags and incubated at 37 °C. Macroscopic examination of bacterial growth (turbidity/button formation) was examined once on 2, 14, 20 and 28 days after incubation to determine the resistance pattern. The critical concentration was compared as described by Gumbo [17].

2.3. Multiplex PCR for drug resistance genes

DNA was isolated from the isolates using Quiagen DNeasy kit. Single tube multiplex PCR was performed with all isolates (n = 51) with specific primers with two component, representing both mycobacterial identification (*hsp*65) and MTBC (IS*6110* for MTBC and RD9 for) detection, targets genes (*katG* - isoniazid, *rpoB*- rifampicin *pncA*-pyrazinamide) responsible for drug resistance was amplified for DNA sequencing to detect mutations associated with resistance to first line drugs. The *hsp*65, IS*6110,* RD9 genes were included for additional confirmation of *M. tuberculosis*. Single tube multiplex PCR was carried out as described by Pere-Osorioet et al. [18] and primer sequence are mentioned in Table 1. The thermal cycling conditions are initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 50 s, extension at for 60 s and final extension at 72 °C for 5 min. PCR amplification was carried out in 25 μl reaction mixtures containing 2 μl of DNA template, 20pM of 6 μl of primer mix (RD9, *hsp65*, *katG, pncA*, *rpoB*, IS*6110*), 12.5 μl of 2× red dye PCR master mix (Amplicon) and 4.5 μl of nuclease free water. The final PCR amplified product was separated by 1.5% agarose gel electrophoresis and gel documented.

2.4. Sequencing of the PCR amplicons

Representative purified PCR amplicons (n = 15) of *katG*, *pncA*, *rpoB* and *embB306* amplicons were subjected to sequencing using capillary gel forming ABI Prism 3700 DNA sequencer and analyser. The representative sequences were selected based on different localities, expecting the chance of identifying variation among sequences. The sequencing was carried out by cycle sequencing using big dye terminator ready reaction kit with fluorescent dye-termination incorporation method. The sequences were analysed for any nucleotide change with the referral sequence available with the Genbank.

Table 1
Nucleotide sequences of primers used in the multiplex PCR assay for Drug Resistance Genes and Multiplex Allele-Specific PCR.

Target gene	Sequence (5′-3′)	Product size	References
katG	F-GAG CCCGATGAGGTCTATTG R-GTCCTTGGCGGTGTATTGC	498 bp	[16]
pncA	F- GACGTATGCGGGCGTTGA R-CCATCAGGAGCTGCAAACCA	569 bp	
rpoB	F- CGAGGTGCCGGTGGAAAC R- GTC GTCGTGCTCCAGGAAGG	721 bp	
RD9	F-GTGTAGGTCAGCCCCATCC R-GTAAGCGCGTGGTGTGGA	369 bp	
*hsp*65	F-ACCAACGATGGTGTGTCCAT R- CTTGTCGAACCGCATACCCT	441bp	
IS*6110*	F-GGATCCTGCGAGCGTAGGCGTCGG R-CCTGTCCGGGACCACCCGCGGCAA	200 bp	
Multiplex Allele-Specific PCR			
Emb1F	GGGCGGGGCTCAATTGCC	324 bp	[17]
Emb2R	GCGCATCCACAGACTGGCGTC		
Emb 306A(F)	GACGACGGCTACATCCTGGGCA	160 & 210	
Emb 306B(R)	GGTCGGCGACTCGGGCC	bp	

2.5. Multiplex allele-specific PCR (MAS-PCR)

Multiplex allele-specific (MAS)-PCR was carried out as described by Mokrousov et al. [19] and primer sequences are mentioned in Table 1. MAS-PCR uses the two outer primers (Emb1F and Emb2R) and two inner primers (Emb306A and Emb306B). A strain with the *embB306* wild type allele would produce two allele-specific bands of 160 bp and 210 bp, a strain with *embB306* mutated in the first base would produce 210 bp fragment only and a strain with *embB306* mutated in the third base would produce only 160 bp fragment only. In addition a 324 bp fragment is invariably amplified by the outer primers. In case of wild strain three band profile implying no mutation. The cycling conditions include initial denaturation at 95 °C for 4 min followed by 6 cycles of 94 °C for 1 min, 75 °C for 1 min and 72 °C for 20 s; followed by 6 cycles of 94 °C for 1 min, 74 °C for 40 s, and 72 °C for 20 s; followed by 20 cycles of 94 °C for 1 min, 73 °C for 30 s and 72 °C for 20 s; with a final elongation at 72 °C for 2 min. PCR amplification was carried out in 25 μl reaction mixtures containing 2 μl of DNA template, primers (5 pmol of Emb2R and Emb306A primers, 50 pmol of Emb1F and Emb306B) and 12.5 μl of 2× red dye PCR master mix (Amplicon) and 0.5 μl of nuclease free water. The final PCR amplified product was separated by 1.5% agarose gel electrophoresis and gel documented.

3. Results

3.1. Determination of drug resistance by broth micro dilution method (BMM)

All the isolates (n = 51) along with reference strain *M. tuberculosis* H37Rv and *M. bovis* BCG were screened for drug resistance with the first line drugs *viz.*, rifampicin, ethambutol, pyrazinamide and isoniazid. Turbidity or button formation in dilution above the critical concentration or breakpoint was considered as resistant for the drug [Fig. 1]. The critical concentration for all the isolates [Table .2] and results of drug resistance are summarized in Table .3.

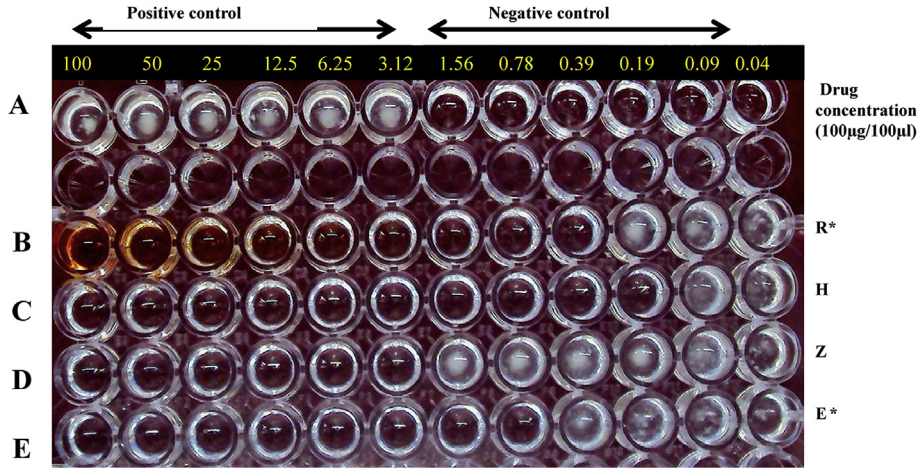

Fig. 1. Microdilution MIC plate for a susceptible strain of *M. tuberculosis*. The wells contained; **Row A:** 1- 6 wells (positive control), 7–12 wells (negative control). **Rows B, C, D, and E** - *M. tuberculosis* isolate with two fold dilutions of rifampicin (100 µg/100 µl), pyrazinamide(100 µg/100 µl), isoniazid (100 µg/100 µl), ethambutol (100 µg/100 µl). Critical concentration are; R* – Rifampicin (MIC – 0.10 µg/100 µl) - RESISTANT. Z - Pyrazinamide (MIC - 10 µg/100 µl) - SENSITIVE. H – Isoniazid (MIC – 0.10 µg/100 µl) - SENSITIVE. E* – Ethambutol (MIC – 0.5µg/100 µl) - RESISTANT.

3.2. Multiplex PCR for drug resistance and sequencing

DNA from the isolates were used as a template for multiplex PCR targeting *hsp*65, IS*6110*, RD9, *katG*, *rpoB*, *pncA* gene produce an amplicon sizes of 441 bp, 200 bp, 369 bp, 498 bp, 721 bp and 569 bp respectively. This multiplex PCR further confirms MTBC by IS*6110, M. tuberculosis* by RD9, *hsp*65 for mycobacteria species and also followed by amplified genes responsible for drug resistance as shown in Fig. 2. The PCR amplicons for *katG*, *rpoB*, *pncA* gene ie. 498 bp, 721 bp and 569 bp respectively were purified and subjected for sequencing for the determination of mutation in drug resistance genes. The representative PCR amplicons (n = 15) (five samples for each target gene of which *M. tuberculosis*- 03 samples, *M. bovis* – 02 samples) were selected randomly based on resistance by BMM for *katG*, *rpoB* and *embB* genes were purified and sequenced. *M. tuberculosis* (n = 3) and *M. bovis* (n = 2) showed point mutation in *katG* in the codon 315 (AGC to ACC), *M. tuberculosis* (n = 1) and *M. bovis* (n = 1) showed point mutation in *rpoB* gene in the codon 531 (TCG to TTG). None of *M. tuberculosis* isolates showed any mutation in *pncA* and *embB* codon 306. *M. bovis* are naturally resistant to pyrazinamide and none of the isolates showed any mutation in *embB* codon 306.

3.3. Multiplex allele specific PCR (MAS-PCR) for ethambutol

All the isolates were screened with multiplex allele specific PCR to detect mutation in *embB* 306. All the isolates exhibited a similar band profile at 324 bp, 210 bp and 160 bp indicative of wild type strains.

4. Discussion

Bovine tuberculosis continued to be a major problem in countries where test and slaughter principles are not affordable and these programs are partially effective due to the fact that wild life act as reservoir host and it maintains the infection [20]. While several investigators from western countries have stressed the possible zoonotic importance of bovine tuberculosis, very limited data on this aspect are available from Asian countries including India where the disease is endemic [21]. Recent reports indicate increased incidence of *M. tuberculosis* in bovines contrary to *M. bovis* and all the *M. tuberculosis* isolates subjected to spoligotyping grouped to Manu 1 strain which is frequently encountered in human beings [13,22,23]. Hence there is a possibility of reverse zoonosis and since cattle are not generally treated for tuberculosis, identifying drug resistant *M. tuberculosis* isolates can possibly be a spill-over from human beings.

4.1. Minimum inhibitory concentration by broth microdilution method (BMM)

Every *M. tuberculosis* population is heterogeneous and contains a subpopulation of drug-resistant bacilli. There is a dearth of information on and drug susceptibility of MTBC circulating in cattle since treatment for tuberculosis is not economically feasible. Though proportion method [24] is considered as a gold standard method, due to the technical complexity broth dilution method as described by Liete et al. [16] was carried out which could read the results in 14–20 days. Yamane et al. [25] examined 1217 clinical *M. tuberculosis* isolates by using Middlebrook broth microdilution method that had 98–99% concordance with proportion method.

In the present study, monodrug resistance for isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) were found to be 21.56%, 13.72%, 11.76% and 5.88% respectively. Some of the isolates exhibited dual resistance for E and Z (1.96%), Z and R (3.92%), H and E (5.88%). None of the isolates exhibited multidrug resistance. Similar resistance patterns were reported by Paramasivam et al. [26] in his survey in North Arcot of Tamilnadu which revealed primary drug monoresistance for isoniazid (23.4%), rifampicin (2.8%) and ethambutol (4.6%). Multiple drug resistance for H and E was 1.4%. In the present study, monoresistance detected in cattle isolates may be due to primary resistance or transmitted drug resistance due to reverse zoonosis because animals do not undergo treatment for tuberculosis. Critical concentration of anti TB drugs is close to MIC for wild type susceptible strains and it is prone to yield poor reproducible results [27]. Hence the drug resistance noticed in cattle isolates could be of primary resistance since cattle are generally not treated for tuberculosis or it could have acquired from human by reverse zoonosis. Among the *M. bovis* isolates, 2 isolate showed resistance to rifampicin and 1 isolate showed resistance to ethambutol and no resistance was noticed for isoniazid. The possible reason of *M. bovis* showing drug resistance may be likely that some human cases diagnosed as tuberculosis could be overlooked cases of bovine tuberculosis since culture based diagnostic systems cannot differentiate between the two pathogens at species level. Bapat et al. [28] detected *M. bovis* from 12.6% human cases in Central India having direct or indirect contact with animals. Unrestricted movement of cattle prevalent in our country owing to economic compulsions and religious taboos synergised with high population leads to a potential situation for zoonosis and reverse zoonosis.

4.2. Multiplex PCR for drug resistance

Anti-tuberculosis drugs are a two-edged sword. While they destroy pathogenic *M. tuberculosis*, they also lead to drug resistance due to non - adherence and pharmacokinetic variability among individuals.

Table 2
Minimum inhibitory concentration by Broth microdilution method (BMM) for all isolates (n = 51).

S.No	Isolate ID	Isoniazid (H) (μg/100 μl)		Pyrazinamide(Z) (μg/100 μl)		Rifampicin (R) (μg/100 μl)		Ethambutol (E) (μg/100 μl)	
		Critical concentration (0.10μg/100 μl)		Critical concentration (10μg/100 μl)		Critical concentration (0.10μg/100 μl)		Critical concentration (0.5 μg/100 μl)	
		R	S	R	S	R	S	R	S
Mtb 1	62	–	0.195	–	0.048	–	0.195	**100**	–
Mtb 2	59	–	0.048	–	0.048	**0.25**	–	–	0.048
Mtb 3	15	–	0.048	–	0.0975	–	0.048	–	0.078
Mtb 4	56	–	0.048	–	0.048	–	0.048	–	0.048
Mtb 5	63	–	0.048	–	0.195	–	0.048	**3.125**	–
Mtb 6	21	–	0.09	–	0.048	**0.78**	–	–	0.39
Mtb 7	16	–	0.048	–	0.048	–	0.195	–	0.048
Mtb 8	44	**100**	–	–	0.048	–	0.048	**100**	–
Mtb 9	9	–	0.048	–	0.048	–	0.048	–	0.048
Mb10	20	–	0.048	**25**	–	–	0.048	–	0.39
Mb11	42	–	0.19	**12.5**	0.048	–	0.195	–	0.09
Mtb 12	46	-	0.19	-	0.048	-	0.195	-	0.048
Mtb 13	11	**100**	–	-	0.048	-	0.195	-	0.19
Mtb 14	14	-	0.19	-	0.048	-	0.048	-	0.048
Mtb 15	36	**100**	–	-	0.048	-	0.048	-	0.048
Mtb 16	3	-	0.19	-	0.048	-	0.048	-	0.09
Mtb 17	12	-	0.097	-	0.048	-	0.048	-	0.048
Mtb 18	48	-	0.19	-	0.048	-	0.048	-	0.048
Mtb 19	2	-	0.19	-	0.048	-	0.048	-	0.048
Mtb 20	38	**25**	–	-	0.048	-	0.048	-	0.048
Mtb 21	17	**50**	–	-	0.195	-	0.048	–	0.048
Mtb 22	7	-	0.048	-	0.048	**0.78**	–	–	0.048
Mtb 23	39	-	0.048	-	0.048	**0.78**	–	–	0.048
Mtb 24	13	-	0.048	-	0.048	-	0.048	–	0.048
Mtb 25	23	-	0.048	-	0.048	**3.125**	–	–	0.048
Mtb 26	10	-	0.19	-	0.048	-	0.195	**12.5**	–
Mtb 27	65	-	0.048	-	0.048	-	0.048	–	0.048
Mtb 28	29	-	0.048	-	0.048	-	0.048	–	0.048
Mtb 29	5	**0.78**	–	-	0.048	-	0.048	–	0.19
Mtb 30	64	-	0.048	-	0.048	-	0.195	–	0.048
Mb31	mvc 23	-	0.048	**12.5**	-	-	0.048	–	0.048
Mtb 32	49	–	0.048	–	0.048	-	0.195	–	0.19
Mb33	6	–	0.19	**50**	–	**0.78**	–	**50**	–
Mtb 34	33	–	0.048	–	0.195	-	0.195	–	0.048
Mb35	40	–	0.195	**50**	–	**0.78**	–	–	0.048
Mtb 36	61	–	0.048	–	0.048	**0.78**	–	–	0.048
Mtb 37	35	–	0.19	–	0.048	-	0.048	–	0.09
Mtb 38	43	–	0.19	–	0.048	-	0.048	–	0.048
Mb39	32	–	0.19	**12.5**	–	-	0.048	–	0.048
Mtb 40	1	**100**	–	–	0.048	-	0.048	**50**	–
Mtb 41	8	–	0.19	–	0.048	-	0.048	–	0.048
Mtb 42	46	**1.56**	–	–	0.048	-	0.19	–	0.048
Mb43	41	–	0.048	**25**	–	-	0.09	–	0.048
Mtb 44	45	**100**	–	–	0.048	-	0.19	–	0.19
Mtb 45	34	**3.125**	–	–	0.048	-	0.048	**12.5**	–
Mtb 46	18	**25**	–	–	0.048	-	0.048	–	0.19
Mtb 47	50	**25**	–	–	0.048	-	0.048	–	0.048
Mtb 48	19	**100**	–	–	0.048	-	0.19	–	0.048
Mtb 49	31	**100**	–	-	0.048	-	0.19	–	0.048
Mtb 50	4	-	0.048	-	0.048	**0.78**	-	-	0.048
Mb51	24	-	0.048	**12.5**	0.048	–	0.048	-	0.048

R-Resistance, S-sensitive.
Mtb - *Mycobacterium tuberculosis* isolates.
Mb - *Mycobacterium bovis* isolates.

Table 3
Results of drug resistance to first line antimycobacterial drugs.

Isolates resistant to *anti*-TB drugs in species level (n = 51)	No. of isolates resistant to Isoniazid (H)	No. of isolates resistant to Rifampicin (R)	No. of isolates resistant to Pyrazinamide (Z)	No. of isolates resistant to Ethambutol (E)
M. tuberculosis (n = 43)	14	7	–	6
M. bovis (n = 8)	2	2	8	1
% of drug resistance	**31.37 (16)**	**17.6 (9)**	**15.6 (8)**	**13.72 (7)**
% of mono drug resistance	**21.56 (11)**	**13.72 (7)**	**11.76 (6)**	**5.88 (3)**
% of dual drug resistance	**H and R- Nil**		**Z and E -1.96 (1)**	
		R and Z -3.92 (2)		
	H and E-5.88 (3)			

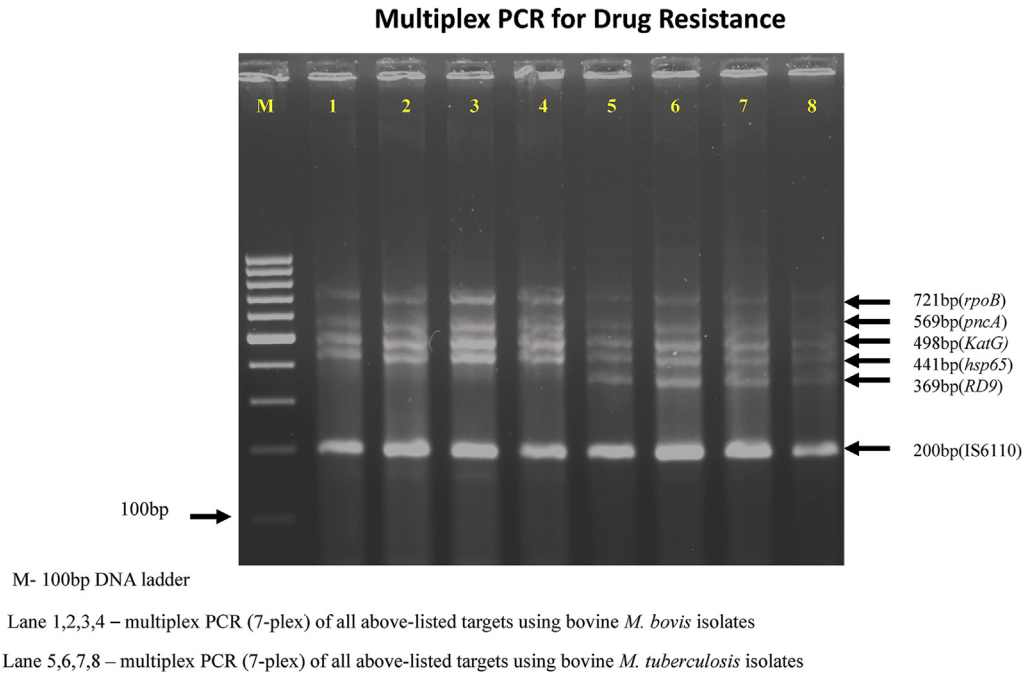

Fig. 2. Multiplex PCR for Drug Resistance. M- 100bp DNA ladder. Lane 1,2,3,4 – multiplex PCR (7-plex) of all above-listed targets using bovine *M. bovis* isolates. Lane 5,6,7,8 – multiplex PCR (7-plex) of all above-listed targets using bovine *M. tuberculosis* isolates.

Resistance to first line *anti*-TB drugs has been linked to mutations in at least 10 genes, *katG, inhA, ahpC, kasA* and *ndh* for isoniazid resistance, *rpoB* for rifampicin resistance, *embB* codon 306 for ethambutol resistance, *pncA* for pyrazinamide resistance. The use of molecular methods for the identification of mutations in resistance-causing genes may offer a means to rapidly screen *M. tuberculosis* isolates for antibiotic resistance. PCR amplification followed by DNA sequencing is the most widely used technique to identify mutations associated with drug resistance in TB [29]. A multiplex PCR method, Mycobacterial Identification and Drug Resistance Screening (MID-DRS) assay, which allowed identification of members of MTBC and the simultaneous amplification of targets for sequencing-based drug resistance screening of rifampicin, isoniazid, and pyrazinamide resistant tuberculosis. High percentage of isolates lacking mutations suggests that phenotyping methods remain an important complement to genotyping methods for drug susceptibility testing and the genotype analysis determined 15 of the 52 (28.8%) strains which had phenotype resistant to rifampicin had no mutation on both *rpoB* codon 526 and *rpoB* codon 531 [30]. There were also 29 of the 46 (63%) strains phenotype resistant to isoniazid which had no mutation on *katG* codon 315 and revealed 19% difference between phenotype and genotype properties of all isolates detected by multiplex PCR of *rpoB*526, *rpoB*531, and *katG*315.

M. bovis strains isolated (68%) from cattle in Sardinia were found to be resistant to rifampicin and isoniazid. The most frequent mutation, encountered in 6 of 10 strains (60%), was in the *rpoB* gene; it occurred, at codon position 521 and resulted in leucine changed to proline. Resistance to isoniazid was found by phenotypic method but no sequence variation was detected [20]. Silaigwana et al., [31] showed that 10/11 samples were resistant to both isoniazid and rifampicin *i.e.*, multi-drug resistant (MDR). The most and least frequent *rpoB* mutations detected in rifampicin resistant samples were H526Y (9/10) and D516V (2/10) respectively. None of the isoniazid resistant samples harboured mutations in the *katG* gene. However, all of them harboured the T8A mutation in the *inhA* gene.

4.3. Multiplex allele specific PCR for ethambutol

In the present study, by conventional method 13.72% of isolates showed resistance to ethambutol and none of the isolates showed resistance by Multiplex allele specific PCR (MAS-PCR). In *M. tuberculosis*, mutations in *emb CAB* operon, in particular *embB*, are responsible for ethambutol resistance. About 35% of ethambutol resistant isolates did not show mutations in *embB* suggesting that there may be other mechanisms for ethambutol resistance. The absence of mutation in resistant isolates by drug susceptibility testing may be due to the involvement of other codon position at the same gene or other genes. This MAS-PCR can detect only the known mutation and mutation can vary depend upon the geographical condition [32].

Due to the polygenic nature of ethambutol resistance, individual nonsynonymous mutations in *embB* had a marginal effect on ethambutol resistance. Ethambutol drug resistance tests frequently provide discordant results and previous studies report that a small percentage of ethambutol resistant isolates do not show mutation in the *embB* gene [33].

4.4. Sequencing for determination of mutation in drug resistant target genes

The evolving of drug resistance among *M. tuberculosis* isolates is a serious concern in tuberculosis endemic countries like India. This Mycobacterium evades susceptibility to the first line of anti mycobacterial drugs by various mechanisms, including mutations in genes that code for drug target proteins.

The *katG* gene encodes mycobacterial catalase peroxidase which is the only enzyme in *M. tuberculosis* capable of activating the pro-drug isoniazid to active form. In our present study, 06 isolates showed point mutation only at the codon 315 with serine to threonine substitution (AGC to ACC), which was in concordance with Abdelaal et al. [34] who reported about 93.3% of point mutation in the *katG* and 7.7% wild type *katG*. In contradiction, Silaigwana et al. [31] did not find any resistance in mutation in the *katG*, but they found mutation in the *inhA* from MTBC isolates in cattle. This could be due to the mutation which could vary depending upon the prevalence of diseases from different geographical area.

Rifampicin is lipophilic derivative, binds to β subunit of RNA polymerase and inhibits the RNA transcription. In our present study, one sample from *M. tuberculosis* and one from *M. bovis* showed a point

mutation at the codon 531 with serine to leucine substitution which is in concordance with Perez-Osorio et al. [18] with about 59% mutation in *rpoB* in the codon 531.

In the present study, sequencing of *M. tuberculosis* isolates did not show mutation to pyrazinamide where as *M. bovis* are naturally resistant to pyrazinamide. Mutation of the *pncA* gene was suggested as the major mechanism of pyrazinamides resistance but resistant strains containing the wild-type gene have also been described, suggesting additional resistance mechanisms exist besides a lack of pyrazinamidase activity [35].

The representative amplicon sequencing for ethambutol resistance did not show any mutation in *embB306* codon. Similar finding was reported by Bahrami et al. [32] who found 70.8% of the resistant isolates did not show any mutation in the *embB* codon 306. The resistance could be due the possible involvement of other codon position at the same gene or other genes.

5. Conclusion

India is considered as the hotspot for tuberculosis and drug resistance among humans has been reported over the decades. Though *M. bovis* is considered as the principal etiological agent for bovine tuberculosis, recent reports indicate *M. tuberculosis* as a predominant etiological agent in tuberculosis endemic areas. The possibility of reverse zoonosis can't be altogether denied because in endemic regions, drug resistant *M. tuberculosis* from humans may end up as spill - over infection to cattle due to the close human animal interaction. Further work is being carried out to substantiate the reverse zoonosis in cattle. This study demonstrates prevalence of drug resistant strains among bovine isolates in India by conventional and molecular techniques. None of the *M. tuberculosis* isolates were found resistant to pyrazinamide except the 08 naturally resistant *M. bovis* strains included in the study. The exact magnitude of the problem can be known only by well conducted studies. The reason for selective pressure to cause mutation in genes responsible for antibiotic resistance need to be further investigated. Further detailed studies are needed to assess the drug resistance pattern among animals since human and animal coexistence may result in potentially harmful interactions. This study emphasizes that control and eradication programs of tuberculosis should involve both human and bovine tuberculosis monitoring.

Conflicts of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.12.005.

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