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Comparative evaluation of *pncA* gene, *IS*6110 and 12.7-Kb fragment based PCR assays for simultaneous detection of *Mycobacterium tuberculosis* complex (*M. tuberculosis* and *M. bovis*) in cultured strains and clinical specimens

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PCR based molecular techniques help in discrimination of two closely related *Mycobacterium tuberculosis* and *M. bovis*. Here, we analyzed 24 *M. bovis*, 39 *M. tuberculosis*, 21 fresh acid-fast positive sputum samples and standard mycobacterial strains with *pncA*, 12.7 Kb and *IS*6100 based PCR assays. DNA from cultures and sputum yielded a positive amplification of 185 bp with *M. tuberculosis* specific reverse primer *pncA*MT-2 but not with *M. bovis* specific reverse primer *pncA*MB-2 and all *M. bovis* strains showed a positive amplification of 185 bp with *M. bovis* specific reverse primer *pncA*MB-2 but not with *M. tuberculosis* specific reverse primer *pncA*MT-2. The 12.7 Kb fragment based PCR performed on DNA extracted from cultures of *M. tuberculosis* and sputum yielded product of 168 bp while *M. bovis* showed 262 bp products. *M. tuberculosis* complex specific *IS*6110 PCR assay performed on DNA extracted from *M. tuberculosis*, *M. bovis* cultures and sputum samples yielded *M. tuberculosis* complex specific 123-bp amplified products. The sequence analysis of representative PCR products of *IS*6110 and 12.7 Kb fragment showed 99-100% and 100% identity in amplicon products, respectively. To test reliability of primers, *M. tuberculosis* and *M. bovis* cultures were mixed and subjected to *IS*6110, *pncA* and 12.7 Kb PCR assay. *pncA* primers could not successfully and reliably discriminate the mixed culture, however, 12.7 Kb fragment primers successfully discriminated the mixed culture of *M. tuberculosis* and *M. bovis*.

Keywords: Tuberculosis (TB) diagnosis

Tuberculosis (TB) remains a major public health issue despite all the efforts of World Health Organization (WHO) and partners to improve its control[1]. The WHO statistics for TB in India for 2019 shows an estimated incidence 2.64 million cases. This is a rate of 193 per 100,000 population[2]. *Mycobacterium bovis*, precisely its zoonotic risk to humans represents a serious problem predominantly those who are living at animal-human interface[3]. The conventional tests are time consuming and these tests cannot identify closely related *M. tuberculosis* and *M. bovis*. In such a situation, discrimination between *M. tuberculosis* and *M. bovis* using PCR assays targeting allele specific genes plays a significant role in identifying *Mycobacterium* sp. These discriminating genes include *oxyR*[4], *pncA*[5], 500 bp[6] and RD9[7]. We require more information about these molecular techniques for improving the sensitivity and specificity. Development in this area has been very rapid and many PCR assays targeting different gene stretches of *M. tuberculosis* have been reported[8-11]. However, it would be difficult to comment on their relative merits, as limited comparative application data is available.

Therefore, in this study, we have made an attempt, first of its kind, for a comparative evaluation of three primers *pncA* gene, *IS*6110 and 12.7 Kb fragment using PCR assays for differential detection of *M. bovis* and *M. tuberculosis* in cultured strains and in clinical specimens and to determine the best nucleic acid target on the based on discriminatory power efficacy for field application.

Material and Methods

Mycobacterial strains

M. tuberculosis $H_{37}R_{V,}$ Strain C, DT, D4, PN and *M. bovis* AN5 Reference strains were included. A total of 68 mycobacterial strains maintained at Mycobacteria Laboratory, Division of Bacteriology and Mycology comprising of 44 *M. tuberculosis* (IVRI, Human Hospital and District Tuberculosis

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Table 1 — *Mycobacterium bovis* and *M. tuberculosis* cultures

Strain/Isolate	Source
(A) *M. bovis* strains	
AN5	Standard
481/85	Bovine Lymph node
2/86	Bovine Lymph node
3/86	Bovine Lymph node
4/86	Bovine Lung
3/87	Bovine Lung
1/87	Bovine Lung
30/88	Bovine Lymph node
24/88	Bovine Lymph node
39/89	Bovine Lymph node
57/90	Bovine Lung and Lymph node
356/90	Deer Lung
89/91	Buffalo Lung
83/91	Buffalo Lung
93/91	Buffalo Lung
85/91	Buffalo Lung
90/91	Buffalo Lung
259/95	Bovine Lung
227/95	Deer Lung
324/96	Bovine Lung
372/98	Bovine Lung
391/98	Bovine Lung
417/99	Bovine Lung
CML	Camel Lung / Lymph node
(B) *M. tuberculosis* strains	
$H_{37}R_v$	Standard
DT	- do -
D4	- do -
PN	- do -
10/86	Human Sputum
12/87	Human Sputum
29/88	Human Sputum
30/88	Human Sputum
35/89	Human Sputum
86/89	Human Sputum
45/90	Human Sputum
191/91	Human Sputum
94/91	Human Sputum
87/91	Human Sputum
128/92	Human Sputum
162/93	Human Sputum
380/93	Human Sputum
178/94	Human Sputum
203/94	Human Sputum
174/94	Human Sputum
197/94	Human Sputum
193/94	Human Sputum
186/94	Human Sputum
320/94	Human Sputum
198/94	Human Sputum
175/94	Human Sputum
356/96	Human Sputum
320/98	Human Sputum
321/96	Human Sputum
199/97	Human Sputum
417/96	Human Sputum
373/98	Human Sputum
439/01	Human Sputum
458/04	Human Sputum
491/05	Human Sputum
1/86	Bovine Lymphnode
5/87	Bovine Lung and Lymphnode
25/88	Bovine Lung
37/89	Calf Lymphnode
92/91	Calf Lymphnode
91/91	Guinea Pig Spleen / Lung / Kidney
125/92	Swine Lung
82/97	Buffalo Lung

Hospital, Bareilly) and 24 *M. bovis* (from IVRI Dairy Farm, Post-Mortem Room and NRC on Camel, Bikaner) were used (Table 1). Mycobacteria were confirmed biochemically by niacin test, nitrate reduction test, pyrazinamidase test, tellurite reduction test and growth on thiophen-2-carboxylic acid hydrazide biochemical tests[12].

Clinical specimens

Fresh sputum samples from 48 clinically suspected tuberculosis individuals were collected from the Tuberculosis Hospital, Bareilly with the due permission of the District Tuberculosis Officer. Sputum smears were stained for acid fast bacteria (AFB) by the Ziehl-Neelsen method, and the specimen was processed for DNA isolation. Each smear was graded as per the standard protocol into four scales *viz*., +3, +2, +1 and scanty.

Extraction of genomic DNA from mycobacterial cultures

Mycobacterial colonies were suspended into 200 µL 1X TE followed by incubation at 95°C for 45 min[13]. After centrifugation of the suspension at 10000 ×g for 10 min, the supernatant containing DNA was harvested and stored at −20°C until further use.

DNA isolation from sputum samples

An equal volume of 4% NaOH was added to the sputum sample, shaken for 15 min and then centrifuged at 3000 ×g for 15 min. The supernatant was poured off into a disinfectant and the pellet was washed with 15-20 mL of sterile distilled water. Isolation of DNA was done using GeneJet Genomic DNA Purification Kit (Thermo Scientific, USA) with slight modifications. The eluted DNA was stored at –20°C until use. The quality and integrity of DNA samples were analyzed by agarose gel electrophoresis performed in 1% horizontal slab gels containing ethidium bromide. The concentration of DNA extracted from

mycobacterial strains were measured by a NanoDrop spectrophotometer (Thermo Scientific, USA).

PCR assays

PCR assay based on *IS*6110[14], allele-specific *pncA* gene based PCR[4] and PCR based on 12.7 Kb fragment[15] were performed (Table 2). Briefly, the PCR was performed in thin walled 0.2 mL PCR tubes containing approximately 100 ng of bacterial genomic DNA, 5 µL 10X PCR buffer, 2 mM $MgCl_2$, 1.0 µL of 10 mM dNTPs, 10 µM of the respective primer sets (6110F and 6110R for *IS*6110 based *M. tuberculosis* complex detection; *pncA*TB-1.2F and *pncA*MT-2R for *pncA* based *M. tuberculosis* detection; *pncA*TB-1.2F and *pncA*MB-2R for *pncA* based *M. bovis* detection; CSB1, CSB2 and CSB3 for 12.7 Kb fragment based *M. tuberculosis* complex detection), 1U of Taq DNA Polymerase (Thermo Scientific, USA) and the volume was made up to 50 µL with nuclease free water. The PCR amplification was performed in a thermocycler and consisted of initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 1.0 min, annealing at 56°C and extension at 70°C for 1.0 min each, followed by a final extension at 70°C for 10 min. A non-template control was run in all the PCR experiments to rule out the possibility of contamination in the samples. The PCR amplified products were resolved on 2% agarose gel in Tris acetate EDTA (TAE) buffer (1X). The agarose gel was stained with ethidium bromide and documented under UV light in a gel documentation system.

Sequence analysis of PCR products

Representative PCR products amplified by *IS*6110 assay, *pncA* and 12.7 Kb PCR assay from selective *M. bovis* and *M. tuberculosis* isolates were purified using GeneJet PCR purification kit (Thermo Scientific, USA) as per the manufacturer's protocol. The purified PCR products were got sequenced by gene-specific forward primer using the Big dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, CA, USA) following the manufacturer's instructions on an automated DNA sequencer (Applied Biosystems 3730xl DNA Analyzer, Applied Biosystems, CA, USA) at Europhins Banglore (India). The sequence chromatogram was annotated with BioEdit Sequence Alignment Editor Software vs 7.0.5 (Isis Therapeutics, Carlsbad, CA, USA). The annotated sequences were identified based on being the closest match to the sequences submitted in the NCBI database on BLAST analysis.

Table 2 — Details of primers

Primer	Sequence (5' to 3')	Length (bp)
1. *IS*6110 PCR[14]		
6110 F	CTCGTCCAGCGCCGCTTCGGG	21
6110 R	CCTGCGAGCGTAGGCGTCGG	20
2. *pncA* gene based PCR[4]		
*pncA*TB-1.2 F	ATGCGGGCGTTGATCATCGTC	21
*pncA*MT-2 R	CGGTGTGCCGGAGAAGCGG	19
*pncA*MB-2 R	CGGTGTGCCGGAGAAGCCG	19
3. 12.7 Kb fragment based PCR[15]		
CSB1	TTCCGAATCCCTTGTGA	17
CSB2	GGAGAGCGCCGTTGTA	16
CSB3	AGTCGCCGTGGCTTCTCTTTTA	22

Evaluation of primers in DNA of *M. bovis* and *M. tuberculosis* mixed culture

DNA extracted from *M. bovis* and *M. tuberculosis* mixed in equal concentration were analyzed to check the reliability of the *pncA* gene and 12.7 Kb fragment-based PCR assays. DNA from Randomly selected *M. bovis* strains (481/85, 2/86, 3/86, 4/86, 3/87 and 1/87) were mixed in equal concentration with DNA from *M. tuberculosis* (10/86, 12/87, 29/88, 30/88, 35/89 and 86/89), respectively. On these DNA mixtures, the *pncA* gene based PCR with all three specific primers for the *pncA* gene (*pncA*TB1.2, *pncA*MT-2 and *pncA*MB-2) added in a single tube. Also, 12.7 Kb PCR assay[16] with their respective three primers was were performed on the same mixed DNA samples. To confirm the target, PCR amplification, 5 µL of the PCR products from each tube were electrophoresed on 1.2% agarose gel. Amplified products were visualized as compact bands of expected size under UV light and documented by a gel documentation system.

Results

Cultivation of Mycobacteria

A total of 62 mycobacteria isolates were maintained in Mycobacteria Laboratory which comprises of 39 field isolates of *M. tuberculosis* and 23 field isolates of *M. bovis*. Six reference mycobacterial strains with 62 isolated mycobacteria cultured on LJ medium showed a characteristic growth pattern within 6 weeks of incubation at 37°C. All the mycobacterial cultures were found pure by Ziehl-Neelsen staining and showed positive results in respective biochemical tests.

Clinical specimens

Smears prepared directly from 48 sputum samples were examined. Of them, 21 were found positive for AFB and briefly after microscopic examination for AFB, the smears were graded as +3 (11 sputum samples), +2 (3 sputum samples), +1 (4 sputum samples) and scanty (2 sputum samples).

PCR assay based on IS6110

All strains *M. bovis* and *M. tuberculosis* showed specific amplification product of a 123-bp (Fig. 1A). *IS*6110 PCR yielded a specific amplification of 123 bp with 19 sputum sample DNA, while two were found negative in this assay.

Allele specific *pncA* gene based PCR

DNA obtained from different mycobacterial strains and directly from sputum samples were subjected to differential amplification by *pncA* gene PCR assay. All the cultured *M. tuberculosis* strains yielded a positive amplification of 185 bp with common *pncA*TB-1.2 forward primer and *M. tuberculosis* specific reverse primer *pncA*MT-2 but not with *M. bovis* specific reverse primer *pncA*MB-2. *M. bovis* strains showed a positive amplification of 185 bp with common *pncA*TB-1.2 forward primer and *M. bovis* specific reverse primer *pncA*MB-2 but not with *M. tuberculosis* specific reverse primer *pncA*MT-2 (Fig. 1B). Out of 21 DNA samples isolated directly from sputum samples, 18 showed specific amplification of 185 bp with common *pncA*TB-1.2 forward primer and *M. tuberculosis* specific reverse primer *pncA*MT-2. None of the DNA showed any amplification with *M. bovis* specific reverse primer *pncA*MB-2.

PCR based on 12.7 Kb fragment

A 12.7 Kb fragment based PCR assay showed 168 bp band for *M. tuberculosis* while and 262 bp band for *M. bovis*. The same PCR assay performed on DNA isolated from sputum samples, generated amplification of 168 bp for 18 sputum samples. None of the samples yielded a 262 bp amplification product for *M. bovis* indicating that all were diagnosed for *M. tuberculosis* (Fig. 1 C1 and C2).

Sequence analysis

Sequences obtained with rrepresentative *IS*6110 assay, *pncA* and 12.7 Kb PCR products were analyzed using the DNA-STAR programme and sequence homology was checked with reported sequences using an online basic local alignment search tool (BLAST). BLAST analysis of sequences confirms that it belongs to the *M. tuberculosis* complex. Similarly, amplicon products from 12.7 Kb PCR from *M. bovis* and *M. tuberculosis* confirmed respective species.

PCR in mixed *M. bovis* and *M. tuberculosis* DNA samples

The reliability of the *IS*6110, *pncA* gene and 12.7 Kb fragment based PCR assays were checked in the present study. A mixed DNA template randomly selected from *M. bovis* (481/85, 2/86, 3/86, 4/86, 3/87 and 1/87) were mixed in equal concentration with DNA from *M. tuberculosis* (10/86, 12/87, 29/88, 30/88, 35/89 and 86/89) for PCR assays. The *pncA* gene based PCR with all three specific primers for the *pncA* gene (*pncA*TB1.2, *pncA*MT-2 and *pncA*MB-2) was added in a single tube and PCR was performed. Similarly, single tube PCR for 12.7 Kb PCR with three primers (CSB1, CSB2 and CSB3) was performed and analyzed the banding pattern on agarose gel.

All the mixed *M. bovis* and *M. tuberculosis* DNA showed amplification product of a 123-bp (Fig. 2A). The *pncA* gene-based PCR with all three specific primers (*pncA*TB1.2, *pncA*MT-2 and *pncA*MB-2) gave a single amplification product of 185 bp (Fig. 2B). In contrast to this, 12.7 Kb PCR with three primers (CSB1, CSB2 and CSB3) gave two amplification products of 168-bp and 262-bp (Fig. 2C).

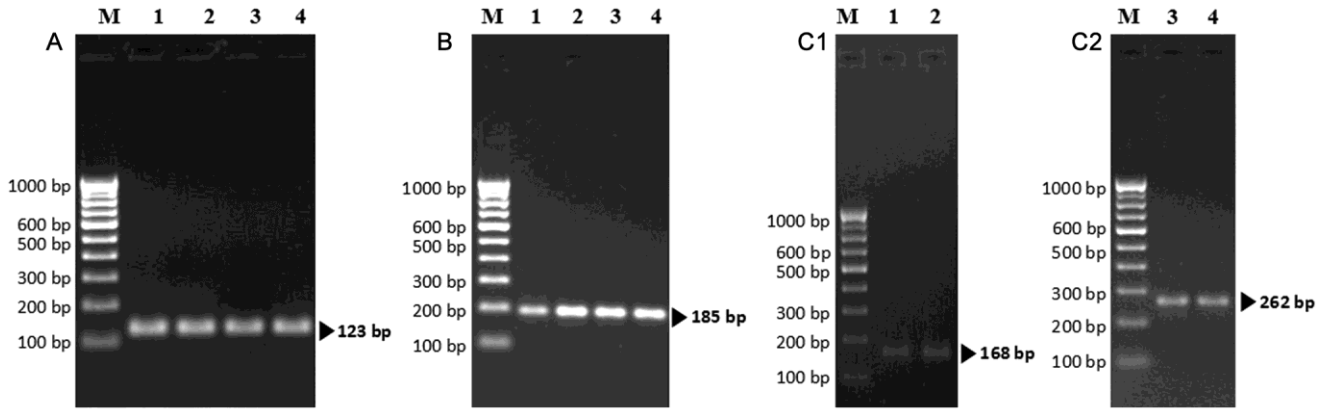

Fig. 1 — (A) IS6110 PCR on *M. bovis* and *M. tuberculosis* DNA with 123 bp amplification; (B) pncA PCR on *M. bovis* and *M. tuberculosis* DNA with 185 bp amplification; and C1 & C2, 12.7kb PCR on *M. tuberculosis* DNA with 168 bp and 262 bp amplification, respectively [M, 100 bp ladder; 1, *M. tuberculosis* 25/88; 2, *M. tuberculosis* 91/91; 3, *M. bovis* 30/88; and 4, *M. bovis* 39/89]

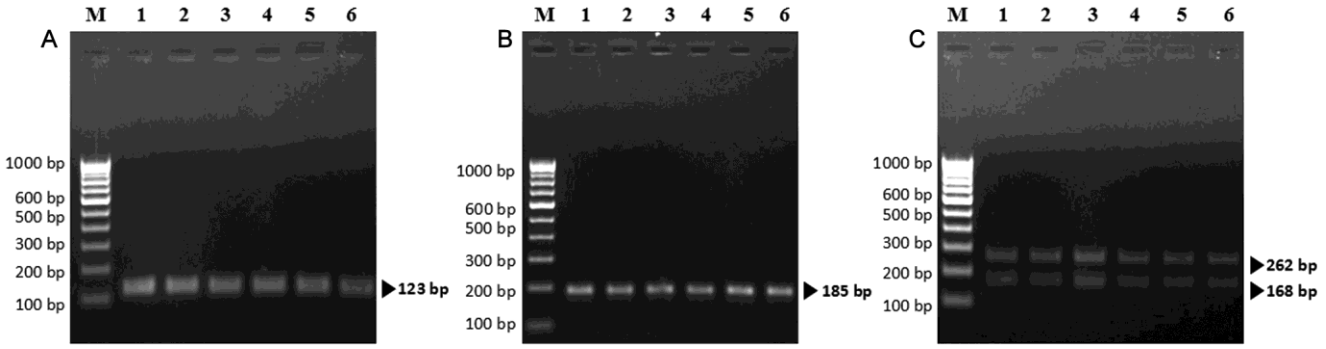

Fig. 2 —Reliability of (A) IS6110 PCR; (B) pncA PCR; and (C) 12.7kb PCR on mixed *M. bovis* and *M. tubersulosis* DNA. [1, *M. bovis* 481/85 + *M. tuberculosis* 10/86; 2, *M. bovis* 2/86 + *M. tuberculosis* 12/87; 3, *M. bovis* 3/86 + *M. tuberculosis* 29/88; 4, *M. bovis* 4/86 + *M. tuberculosis* 30/88; 5, *M. bovis* 3/87 + *M. tuberculosis* 35/89; and 6, *M. bovis* 1/87 + *M. tuberculosis* 86/89]

Discussion

The traditional detection of *M. bovis* from *M. tuberculosis* based on culture and biochemical tests is tedious and slow. Biochemical typing is not 100% reliable because of intermediate strains, such as niacin and T2CH variant *M. tuberculosis*[16,17] and PZA variant *M. bovis*[18]. Thus, the ability of discern and tracking individual *Mycobacterium* strain is of critical importance. Most isolates of the MTB complex contain six copies of the insertion sequence *IS*1081[19], and hence make it a better target when testing for *M. tuberculosis* isolates, which have few copies or even lack *IS*6110[20]. *M. bovis*, which usually contains a single *IS*6110 copy in the direct repeat (DR) region of the genome[21]. However, variation in *IS*6110 copy number has been associated with *M. bovis* isolates of different geographical origin[22]. The sequence analysis of the *M. tuberculosis* and *M. bovis* genomes revealed that *M. bovis* lacks a 12.7 kb fragment which is present in the genome of *M. tuberculosis*[23]. The analysis of the 12.7 kb fragment showed that it represented a deletion in *M. bovis* rather than an insertion in *M. tuberculosis*. This deletion removed most of the mce-3 operon, one of the four closely related operons involved in cell entry. Therefore, this deletion might contribute to differences in virulence or host range in the two species. Interestingly, all the *M. tuberculosis* isolates studied showed 12.7 kb fragment, while all the *M. bovis* strains lack this fragment[23]. Similarly, a PCR assay based on oligonucleotide primers from a 500 bp genomic fragment well conserved in *M. bovis* and the *pncA* gene (based on *M. tuberculosis* specific nucleotide polymorphism, a cytosine residue at position 169), specific for *M. tuberculosis* was developed and standardized in our laboratory[24,25]. With this background, we chose three primers *viz*. *IS*6110 because of its popular use and the presence of 0 to 25 copies in *M. tuberculosis* complex, the differential ability of the *pncA* gene and 12.7 Kb fragment-based oligonucleotide primers for their use on several DNA isolated from *M. tuberculosis* and *M. bovis* (isolated from divergent animals species, cattle, camel, deer) and DNA extracted from direct fresh sputum smear positive samples. The quality control of our PCR assay was done using standard *M tuberculosis* and *M. bovis* AN5 cultures.

To test the comparative evaluation of differential primers *pncA* and 12.7 Kb, *M. tuberculosis* and *M. bovis* cultures were mixed and subjected to *IS*6110, *pncA* and 12.7 Kb primers. The PCR showed that the *pncA* primers could not reliably discriminate the mixed culture: however, 12.7 Kb fragment-based primers efficiently discriminated the mixed culture of *M. tuberculosis* and *M. bovis*. Moore & Curry[26] compared the diagnosis of tuberculosis or other mycobacterial infections by a PCR (Amplicor *M. tuberculosis* test) and culture techniques. The foremost question would be to determine whether it infected a patient who is smear positive with *M. tuberculosis?* Since the sensitivity of the PCR for smear-positive specimens as reported was 99% and has been reported by others to be 94-95%, PCR can be used to rapidly determine if an infection detected by a positive smear result is caused by *M. tuberculosis*. It is important to determine, if a new smear-positive result from a patient because of therapy, has previously turned smear negative is caused by *M. tuberculosis* or another organism. Confirming that the positive smear results from continued *M. tuberculosis* infection is an early indication of possible treatment failure, and appropriate measures can be taken[26].

A repetitive 123 bp sequence of *M. tuberculosis* DNA was amplified by PCR, from sputum samples,

for the diagnosis of pulmonary tuberculosis in 78 sputum specimens. *M. tuberculosis* was detected by PCR in all smear-and culture-positive and smear-negative, culture positive cases were found positive by PCR to *M. tuberculosis*. PCR detected 4 of 9 cases clinically suspected of tuberculosis smear and culture negative were detected by PCR. According to Tanil and co-workers[27] DNA amplification by PCR is a sensitive and specific method for the diagnosing tuberculosis in routine clinical samples. The routine use of PCR assay for the direct detection of *M. tuberculosis* in expectorated sputum specimens was assessed[20]. A pair of primer (20 mer) was designed to amplify the 38 kd protein of *M. tuberculosis*. They first assayed 31 *M. tuberculosis* strains and 15 atypical mycobacterium species and subsequently applied the assay on 519 sputum specimens from 85 patients of a chest hospital in Hong Kong. They concluded that PCR was a useful technique for the diagnosing untreated and recently treated cases of pulmonary tuberculosis20. Our study involved using of *IS*6110, *pncA* and 12.7 Kb fragment primers by PCR for differentiation of *M. tuberculosis* and *M. bovis* cultures. Out of 21 sputum samples, 19 were positive for *IS*6110 PCR and 18 positive to *pncA* and 12.7 Kb fragment. The possible explanation in case of negative PCR results may be inhibitors in the sputum or inadvertent error in DNA extraction due to paucity of tubercle bacillus in the sample. PCR, detected *M. tuberculosis* in clinical material such as pleural fluid, bronchial washings, and biopsies, and these results were compared with those obtained by classical bacterial culture[28]. Of 34 *M. tuberculosis* strains, 5 did not carry the amplifiable 158 bp fragment, usually as a single copy in the chromosome. By this technique about 103 *M. tuberculosis* bacteria were detectable in sputum10. Chayada *et al*.[29] have demonstrated novel targeting of the *lepB* gene using PCR with confronts two-pair primers for simultaneous detection of *M. tuberculosis* complex and *M. bovis*. One of the reasons for the low impact of animal tuberculosis particularly bovine tuberculosis on human health may be that whenever isolates from human patients with tuberculosis are typed, they are rarely identified as *M. bovis*[30]. Therefore, our major contention of the study was to assess the capability of primers as a discriminatory power between *M. tuberculosis* and *M. bovis* both in cultures and on direct DNA of sputum samples.

Though various PCR methods are available today, majority of them lack reliability and sensitivity. The bottlenecks are the potential inhibitors that undermine a sensitive PCR reaction and the marker's discrepancy. The laboratories that use PCR tests have produced their own tests with various primers, probes, and extraction, amplification, and detection techniques. This has led to wide variations in reported assay performances. In India, we have used PCR assays based on gene targets like RD97, pncA5, 500 bp6, *IS*61109, 12.7 Kb fragment15, 27 bp difference in the C-terminal part of the hupB gene[31]. The results of a study in bovine tuberculosis in swine suggested that the PCR using *IS6110* or the combined application of bacteriological culture and PCR, may serve as an accurate diagnostic tool to confirm bovine tuberculosis in swine samples in Argentina[32]. Various PCR based methods have been developed and customized to identify specific MTBC organisms, based on detecting mycobacterial DNA, from either cultured isolates or directly from ante or post-mortem samples[33]. However, we have not come across publication reporting the use of three primers simultaneously on the same sample and in particular on samples comprising mycobacterial cultures from diverse sources. Representative PCR products of the *IS*6110 and the 12.7 Kb fragment-based subjected to sequence analysis showed 99-100% identity with most of *IS*6110 sequences of *M. tuberculosis* complex. Similarly, there was 100% identity of *M. bovis* and 99-100% *M. tuberculosis* for 12.7 Kb fragment. Over all, the comparative evaluation of primers under study revealed that 12.7 Kb primer was superior as compared to *pncA*. 12.7 Kb fragment based primers had more discriminatory power to differentiate the mixed culture of *M. tuberculosis* and *M. bovis* and it can be performed in a single tube reaction.

Identifying mycobacteria to species level is crucial since it provides a great deal of useful information on epidemiology and facilitates successful treatment of patients[34]. It is difficult to directly detect *M. bovis* which time-consuming due to slow growth of bacteria and the paucity of tubercle bacilli in antemortem samples. They opined that development of rapid PCR assays may improve direct detection of MTBC in the future and it may be standardized enabling its use in the field setting[35]. Two emerging PCR based molecular tools for wildlife samples reported include VetMAX™ *M. tuberculosis* complex PCR kit (Thermo Fischer Scientific, Waltham, MA, USA) and cartridge-based GeneXpert® (Cepheid, Sunnyvale, CA, USA) technology[33].

Conclusion

Results of the three primers based 12.7 Kb fragment PCR assays in this study have shown the best discriminatory primer which may be a tool for identifying two closely related species of mycobacteria e.g. *M. tuberculosis* and *M. bovis*. A study with a large sample size at multi location sites would be a way forward to translate the findings of this research work into practice.

Conflicts of interest

Authors declare no competing interests.

References

1 World Health Organization. Global tuberculosis report, 2020.

2 India TB Report 2021 https://tbcindia.gov.in/

3 Michel AL, Maller B & van Helden PD, *Mycobacterium bovis* at the animal-human interface: A problem, or not? *Vet Microbiol*, 140 (2011) 371.

4 De los Monteros LEE, Galan JC, Gutierrez M, Samper S, Marin JFG, Martin C, Dominguez L, de Rafael L, Baquero F, Gomez M & Blazquez J, Allele-specific PCR method based on *pncA* and *oxyR* sequences for distinguishing *Mycobacterium bovis* from *Mycobacterium tuberculosis*: Intraspecific *Mycobacterium bovis pncA* sequence polymorphism. *J Clin Microbiol*, 36 (1998) 239.

5 Shah DH, Verma R, Bakshi CS & Singh RK, A multiplex-PCR for differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *FEMS Microbiol Lett*, 214 (2002) 39.

6 Rodriguez JG, Mejia GA, Del Portillo P, Patarroyo ME & Murillo LA, Species-specific identification of *Mycobacterium bovis* by PCR. *Microbiology*, 141 (1995) 2131.

7 Das SC & Verma R, Occurrence of RD9 region and 500bp fragment among clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *Microbiol Immunol*, 51 (2007) 231.

8 Baevis KG, Litchy MB, Jungkind DL & Giger O, Evaluation of amplicor PCR direct detection of *Mycobacterium tuberculosis* from sputum specimens. *J Clin Microbiol*, 33 (1995) 2582.

9 Eisenach KD, Sifford MD, Cave MD, Bates JH & Crawford JT, Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. *Am Rev Resp Dis*, 144 (1991) 1160.

10 Hermans PW, Schuitema AR, Van Soolingen D, Verstynen CP, Bik EM, Thole JE, Kolk AH & van Embden JD, Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. *J Clin Microbiol*, 28 (1990) 1204.

11 Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K & Bodmer T, Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet*, 341 (1993) 647.

12 Kubica GP & Wayne LG (Editors), *The Mycobacteria: A Source Book*, Part A and Part B (Microbiology Series Volume 15). (Marcel Dekker, New York, USA) , 1984, 537-545.

13 Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, Gicquel B, Tibayrenc M, Locht C & Supply P, High-resolution minisatellite based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. *Proc Natl Acad Sci USA*, 98 (2001) 1901.

14 Eisenach KD, Cave MD, Bates JH & Crawford JT, Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis*, 161 (1990) 977.

15 Bakshi CS, Shah DH, Verma R, Singh RK & Malik M, Rapid differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* based on a 12.7-Kb fragment by a single tube multiplex-PCR. *Vet Microbiol*, 109 (2005) 111.

16 Verma R, Sharma AK, Vanamaya PR, Khanna PN, Siddique IH & Gupta BR, Prevalence of tuberculosis in an organized farm and its public health implications. VII Annual Conference of IAVMI held at Veterinary College, Hisar (21-23 Dec., 1987).

17 Grange JM, Yates MD & de Kantor I, Guidelines for specimen within the *Mycobacterium tuberculosis* complex. 2nded World Health Organization Unpub. Document WHO/EMC/ZOO/ 96.4. (Eds. Thoen CO & Steele JH;Geneva), 1996.

18 Niemann S, Harmsen D, Rüsch-Gerdes S & Richter E, Differentiation of clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis. *J Clin Microbiol*, 38 (2000) 3231.

19 Collins DM & Stephens DM, Identification of an insertion sequence, IS1081, in *Mycobacterium bovis*. *FEMS Microbiol Lett*, 67 (1991) 11.

20 Yuen KY, Chan KS, Chan CM, Ho BS, Dai LK, Chau PY & Ng MH, Use of PCR in routine diagnosis of treated and untreated pulmonary tuberculosis. *J Clin Pathol*, 46 (1993) 318.

21 Hermans PWM, van Soolingen D, Bik EM, de Hass PEW, Dale JW & van Embden JDA, The insertion element *IS*987 from *M. bovis* BCG is located in a hot spot integration region for insertion elements in *M. tuberculosis* complex strains. *Infect Immun*, 59 (1991) 2695.

22 Liébana E, Aranaz A, Domínguez L, Mateos A, Gonzalez-Llamazares O, Rodríguez-Ferri EF, Domíngo M, Vidal D & Cousins D, The insertion element IS*6110* is a useful tool for DNA fingerprinting of *Mycobacterium bovis* isolates from cattle and goats in Spain. *Vet Microbiol*, 54 (1997) 223.

23 Zumarraga MJ, Marti C, Samper S, Alito A, Latini O, Bigi F, RoxoE, Cicuta ME, Errico F, Ramos MC, Cataldi A, Van Soolingen D & Romano MI, Usefulness of spoligotyping in molecular epidemiology of *Mycobacterium bovis* related infections in South America. *J Clin Microbiol*, 37 (1999) 296.

24 Shah DH, Evaluation of polymerase chain reaction (PCR) assay for species-specific detection of *Mycobacterium bovis* and *Mycobacterium tuberculosis* based on a 12.7 Kb fragment by a single tube multiplex PCR. *Vet Microbiol*, 109 (2002) 111.

25 Bannalikar AS & Verma R, Detection of *Mycobacterium avium* and *Mycobacterium tuberculosis* from human sputum cultures by PCR-RFLP analysis of hsp65 gene & pncA PCR. *Indian J Med Res*, 123 (2006) 165.

26 Moore DF & Curry JI, Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by Amplicor PCR. *J Clin Microbiol*, 33 (1995) 2686.

27 Tanil KI, Engin Y, Erff O, Sesin K, Murat H, Sachdeva M & Chambers HF, Detection of *Mycobacterium tuberculosis* in sputum samples by polymerase chain reaction using a simplified procedure. *J Clin Microbiol*, 31 (1993) 1435.

28 Van M, Cleff KN, Githui W, Nganga K, Odhiambo J & Klaster P, Cost effectiveness of PCR versus Ziehl-Neelsen smear microscopy for diagnosis of tuberculosis in Kenya. *Int J Lung Dis*, 9 (2005) 877.

29 Chayada ST, Sorasak I, Usanee A, Ponrut P, Bordin B, Santhasiri O, Sirikwan S, Surachet A, Boonchai C, Naowarat K, Watchara K & Khajornsak T, Novel targeting of the lepB gene using PCR with confronting two-pair primers for simultaneous detection of *Mycobacterium tuberculosis* complex and *Mycobacterium bovis*. *J Med Microbiol*, 65 (2016) 36.

30 Amanfu W, The situation of tuberculosis and tuberculosis control in animals of economic interest. *Tuberculosis*, 86 (2006) 330.

31 Soto ME, Del Carmen Ávila-Casado M, Huesca-Gómez C, Alarcon AV, Castrejon V, Soto V, Hernandez S, Espinola-Zavaleta N, Vallejo M, Reyes PA & Gamboa R, Detection of *IS6110* and *HupB gene* sequences of *Mycobacterium tuberculosis and bovis*in the aortic tissue of patients with Takayasu's arteritis. *BMC Infect Dis*, 12 (2012) 194.

32 Barandiaran S, Aguirreburualde MSP, Maerfil MJ, Vivot MM, Aznar N, Zumaeeaga M & Perez, Bayesian assessment of the accuracy of a PCR based rapid diagnostic test for bovine tuberculosis in swine. *Front Vet Sci*, 4 (2019) 204.

33 Goosen WJ, Kerr TJ, Kleynhans L, Buss PE, Cooper DV, Warren RM, van Helden PD, Schroder B, Parsons SDC & Miller MA, The VetMAX^{TM} *M tuberculosis* complex PCR kit detects MTBC DNA in antemortem and postmortem samples from white rhinoceros (*Ceratotherium simum*), African elephants (*Loxodonta africana*) and African buffaloes (*Syncerus caffer*). *BMC Vet Res*, (2020) doi: 10.1186/s12917-020-02438-9.

34 Jagielski T, van Ingen J, Rastogi N, Dziadek J, Mazur PK & Bielecki J, Current methods in the molecular typing of *Mycobacterium tuberculosis* and other mycobacteria. *Biomed Res Int*, (2014) 645802. doi: 10.1155/2014/645802.

35 Bernitz N, Kerr TJ, Goosen WJ, Chileshe J, Higgitt RL, Roos EO, Meiring C, Gumbo R, de Waal C, Clarke C, Smith K, Goldswain D, Sylvester TT, Kleynhans L, Dippenaar A, Buss PE, Cooper DV, Lyashchenko KP, Warren RM, van Helden PD, Parsons SDC & Miller MA, Review of Diagnostic Tests for Detection of *Mycobacterium bovis* Infection in South African Wildlife. *Front Vet Sci*, 2021 doi.org/10.3389/fvets.2021.588697.