



Laboratory and field evaluation of polymerase chain reaction assays for diagnosis of zoonotic tuberculosis in bovine post mortem samples

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ABSTRACT

The diagnosis of zoonotic tuberculosis revolves around detection of pathogen by gold standard culture method. In the live animals, tuberculin testing and interferon gamma assays are the options with their own limitation of specificity and cost effectiveness respectively. Clinical examination along with the affordable methods can lead to a definitive diagnosis which could help in decision making for not only the individual animal but also for the whole farm. During post-mortem examination, the post-mortem lesions with visible tubercle and acid fast staining give a fair idea of tuberculosis infected carcass for tuberculosis. The molecular testing of tissue samples with proven mycobacterial genus, *Mycobacterium tuberculosis* complex (which includes *M. bovis* also) and *M. bovis* species-specific primers can add to the diagnostic strength for bovine tuberculosis. In this regard, we evaluated sequential PCRs for mycobacterial genus specific primer (*hsp65*), *M. tuberculosis* complex specific primer (IS6110 & IS1081) and *M. bovis* specific primer (RD4 and 500 bp) in spiked and field tissue samples of cattle. The research was carried out in 2019-20 in Division of Animal Health for framing out a diagnostic confirmation protocol for TB infected cattle on tissue samples. All the PCR assays were almost equally efficient in detection of *M. bovis* in spiked samples, if supported by an efficient DNA extraction method which is the foremost limiting factor. It was concluded that a combination of minimum of two assays can give a definitive result along with the support of the clinical history or post mortem outcome of the animals.

Keywords: Bovine, *Mycobacterium bovis*, Polymerase chain reaction, Tuberculosis

Bovine tuberculosis (bTB) is a global animal health problem and a neglected zoonotic disease in middle and low level income countries (Chauhan *et al.* 2019). bTB is basically caused by *Mycobacterium bovis* which falls under *Mycobacterium tuberculosis* bacillus complex (MTBC). Many developed countries have eradicated or have a negligible level of bTB but it is widespread in developed and developing countries of Asia, Africa and Latin America. *M. bovis* causes 5% global and 10% of the total human TB cases in developing countries (Olea-Popelka *et al.* 2017). In India, till 1916, TB in animals was very rare because indigenous cattle are considered somewhat resistant. Moreover, animals were kept in open rearing system, which made them less susceptible. A systematic study using random effects and standardized mean revealed a prevalence of 474, 385, 218, and 326 by culture, microscopy, PCR and spoligotyping respectively per

1,000 slaughtered cattle in India (Ramanujam *et al.* 2020). A meta-analysis study by Srinivasan *et al.* (2018), a pooled prevalence of 7.3% (21.8 million cattle) was estimated for bTB in India. A study using random effects meta-regression model by Refaya *et al.* (2020) states that approximately 21.8 million are infected in India which transcends the overall population of dairy herds in the United States of America.

The diagnosis of tuberculosis infection in humans and animals caused by *M. bovis* is often difficult because the diagnostic approaches rely mainly on conventional methods like clinical features and acid-fast bacilli microscopy. Screening of herds is done by single, double, and comparative tuberculin tests using PPD for *M. bovis* but has limitations for sensitivity and specificity because of several cross reactive proteins being present in other Mycobacteria (Kumar *et al.* 2021). Therefore, there is a need for molecular biological assays like PCR for early and accurate detection of organism, speciation of Mycobacteria and determination of disease burden caused by Mycobacteria. Many simplex PCR assays have been claimed to detect Mycobacteria up to species level of which the commonest used genes to detect Mycobacteria at genus

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level is *hsp65*, *M. tuberculosis* complex level (IS6110 & IS1081), and species level (RD9 for *M. tuberculosis*- *M. bovis*) (RD4, 500 bp fragment for *M. bovis*) and many others. There is discrepancy between sensitivity of detection found with direct testing of tissues and purified mycobacterial cultures due to limited mycobacterial DNA from tissue homogenate (Taylor *et al.* 2007). In the present study, we described the performance of PCR to detect *M. bovis* in tissue homogenate using genus-specific (*hsp65*), complex-specific (IS6110 & IS1081) and species-specific (RD4 & 500 bp fragment) gene targets.

MATERIALS AND METHODS

The experiment was carried out in 2019-20 in Division of Animal Health, ICAR-Research Complex for North East Hill Region, Meghalaya. A preliminary experiment was done for the whole process starting from stock preparation, meat (beef) inoculums and PCR simulation by using laboratory available non-pathogenic *E. coli* (ATCC 25922) culture and then with a characterized avirulent standard ATCC culture of *Mycobacterium phlei* (MTCC1724).

Experiment on *M. bovis*: The optimized experiment was carried out with the *M. bovis* AN5 strain cultured in our laboratory. The culture was checked for its purity through its growth and colony study in solid media of Lowenstein-Jensen pyruvate (LJ-P) media (Hi-media, India). Later, the isolate was reconfirmed with *hsp65*, IS6110, IS1081, RD4 and 500 bp fragment PCR and finally sub-cultured in LJ-P media in order to use fresh culture for the experiment by taking the culture growth in log phase of the bacteria.

Titre of stock *Mycobacterial suspension*: The *Mycobacterial* colony were taken from solid media by disposable loop in biosafety cabinet class 3 and suspended in 200 µl sterilized PBS. The colonies were further washed in PBS once and then centrifuged at 1000 rcf for 1 min and the bacterial pellet was re-suspended in sterile PBS and adjusted to the optical density of McFarland Standard-4 (1.2×10^9). Serial dilutions (10^9 - 10^1 cfu/ml) of this *M. bovis* stock organism were made in a total volume of 9 ml of sterile PBS.

Preparation of inoculums in tissue: The lung tissue (cattle) which was to be used for spiking study was checked for its *Mycobacterial* contamination by inoculating it in duplicate LJ (P) after digestion-decontamination following procedure of Petroff *et al.* (1915) and incubated at 37°C for 2 months. Simultaneously, the tissues were subjected to PCR detection based on *hsp65*, IS6110, IS1081, RD4 and 500 bp fragment, standardized in our laboratory. The tissues which were confirmed negative by culture and PCR were used for the experiment. Tissue (24 g) was triturated in mortar and pestle with 75 ml of PBS and 9 ml each of these inoculums was added to eight sterile centrifuge tubes. To each of this centrifuge tubes, 1 ml of the serially diluted (10^9 - 10^1 cfu/ml) mycobacterial stock solution prepared in PBS was inoculated to make the final concentration of inoculums as 10^8 to 10^1 cfu/ml of tissue homogenate. Altogether, two sets of spiked *M. bovis* inoculums with

varying concentrations is obtained; one with the PBS diluted culture (10^9 - 10^1 cfu/ml of PBS) and the other being the spiked tissue (10^8 - 10^1 cfu/ml of homogenate), both of which were used for DNA extraction and PCR. The spiking experiment (PBS diluted and spiked tissue) was repeated thrice to assess the replicability and robustness of the experiment.

DNA extraction: Qiagen Bacterial Genomic DNA isolation kit (Qiagen, Germany), Blood DNA extraction kit (GCC Biotech, India) and boiling-snap-chill method was used for DNA extraction. The DNA was extracted from both of the sets (the one diluted in PBS and another set of spiked one) for all the dilutions of *M. bovis*, by above mentioned methods. From each batch, we could elute effectively around 30 µl from Qiagen kit, 60 µl from GCC kit and took 100 µl suspension for boiling-snap-chill method. The quantity and quality of DNA extracted from Qiagen and GCC was measured by nanodrop and 0.8% agarose gel electrophoresis.

PCR assays: The extracted DNA was used for running five sets of simplex PCR viz. (i) *Mycobacterial* genus specific *hsp65* as per Telenti *et al.* (1993), (ii) *M. tuberculosis* complex specific IS6110 (Eisenach *et al.* 1990) and IS1081 (Collins and Stephens 1991, Wards *et al.* 1995), (iii) flanking primer for RD4 specific to *M. bovis* (Sales *et al.* 2014) and 500 bp fragment synonym to RvD1-Rv2031c sequence (Rodriguez *et al.* 1995). The primers used in the study were selected based of their robustness, specificity, sensitivity as reported by the authors and there subsequent researcher evaluations. The genus-specific and complex-specific primer could detect all the species related to bovine tuberculosis viz. *M. bovis*, *M. orygis*, *M. caprae*. Although the targets genes for species differentiation of *M. bovis* has been reported but co-amplification of other species such as *M. orygis* and *M. caprae* need to be ascertained when the disease-causing entity is of primary interest.

The *hsp65*, IS6110, IS1081, 500 bp fragment and RD4 PCR assay was standardized for a 20 µl reaction with 2 µl of template DNA isolated from serially diluted *M. bovis* in PBS and in subsequent PCR with 4 µl and 6 µl template DNA. Then, the same PCR assays were repeated on *M. bovis* spiked tissue samples for final experiment in triplicate using GGC blood genomic DNA kit. With DNA isolated of Qiagen bacterial DNA kit from the spiked samples, we performed all the above mentioned five PCR assays on two template DNA volume i.e., 2 µl and 6 µl owing to shortage of template DNA from one single elute. The snap-chill isolated template DNA was run for all the dilution but with only one template volume of 6 µl due to unsatisfactory observations in preliminary experiments for other lower volume (2 and 4 µl).

Detection limit of PCR: Analytical sensitivity of *M. bovis* was done by serially diluting pure DNA of concentration 95 ng/µl upto 0.095 fg/µl. All the five PCR assays (*hsp65*, IS6110, IS1081, 500 bp fragment, RD4) were run on all the dilutions and their results were recorded.

Field evaluation: A total of 30 lung tissue obtained

from slaughter animals were evaluated by all the five PCR assays (*hsp65*, IS6110, IS1081, 500bp and RD4) using the DNA isolation protocol which performed best in the spiking experiment and all these PCR assays were also repeated thrice.

RESULTS AND DISCUSSION

DNA quantity and quality: The DNA quantity isolated by GCC blood DNA kit in PBS and spiked tissues for all three times of experiment was in the range of 4 to 9.4 ng/μl and 1.2 to 23 ng/μl respectively. Similarly, with Qiagen bacterial kit, it varied from 1.4 to 7.6 ng/μl and 1 to 8.6 ng/μl respectively. The yields were lower as compared to other general bacteria due to the thick mycolic acid of *Mycobacterium* which hinders the DNA isolation. It is also claimed that recovery of DNA from intact cultured *M. bovis* cells added to tissue homogenate could be as low as 22% of the expected yield (Taylor *et al.* 2007). The Qiagen bacterial kits yields were even lower but the use of Qiagen Tissue Blood and DNA extraction kit would have given better results. In this study, different extraction protocols were used for comparison and possible cost reduction. Additionally, lung tissue was the tissue of choice for the spiking experiment as most of the clinical presentation of pulmonary tuberculosis is seen in the lungs. Technical issues during manual trituration and centrifugation with this type of elastic tissue could arise which can be minimized by use high speed electric homogenizer.

***M. bovis* experiment:** The results of spiking experiment for *M. bovis* in diluted PBS and spiked tissue homogenate showed positive amplification for all the genes (*hsp65*, IS6110, IS1081, 500 bp fragment and RD4) with the DNA extracted by using GCC Blood genomic DNA kit in all dilutions and volume of template used. Using the DNA isolated by Qiagen Bacterial kit, the five PCR assays could also detect *M. bovis* missing few replicates and dilutions irrespective of primer used. PCR assays on DNA from snap-chill method could detect occasionally only for PBS diluted samples and for spiked tissues very few higher concentration were detected.

Further, all the PCR assays are working efficiently in laboratory setting without showing any non-specific band. The GCC isolated DNA showed better results followed by Qiagen bacterial kit and the snap-chill method proved to be the least reliable. Very few numbers of studies on direct tissues have been done with literature getting complex in this aspect for comparison because researchers have tested different types of samples, with different parameters and criteria. Examination of literature revealed that initial processing of mycobacterial samples and extraction of mycobacterial DNA plays a key role in detection of *M. bovis* by PCR.

Altogether, all the PCR assay (*hsp65*, IS6110, IS1081, 500bp & RD4) stands to be efficient in detecting the *M. bovis* from direct sample if that is supported by an efficient DNA extraction method. This work did not assess the sensitivity and specificity of the PCR but as per

literature search, considering PCR setting, for detection of *M. bovis*, IS1081 having 5 copies is well detected than single or multiple copy of IS6110 in *M. bovis* among MTB complex specific primers (Zanini *et al.* 2005). IS1081 is also known to detect *M. bovis* in milk samples (Zanini *et al.* 1998) and 100% sensitive in detection of *M. bovis* in tissue confirmed for tuberculosis (Ward *et al.* 1995). IS1081 is also claimed to be 100% specific in detection of *M. bovis* in bovine semen (Ahmed *et al.* 1999). IS6110 is routinely used for human *M. tuberculosis* diagnosis and is also reported to have 100% (Zanini *et al.* 2001) and 71.4% (Liebana *et al.* 1995) sensitivity in detection of *M. bovis* from direct tissue samples. The 500 bp fragment is widely used for detection of *M. bovis* and can be detected in spiked milk (Rodriguez *et al.* 1995, 1999) and lymph node (Cardoso *et al.* 2009) but it also had recorded most of the errors in discriminating *M. bovis* and *M. tuberculosis* (Metaxa-Mariatou *et al.* 2004, Shah *et al.* 2004). Sechi *et al.* (2000) recorded that 500 bp PCR failed to detect 4/30 *M. bovis* culture. RD4 flanking primer (Sales *et al.* 2014) is claimed to be best for discriminating *M. bovis* and *M. tuberculosis*. A study have used multiplex PCR for the selected region targeting 500 bp fragment with IS6110 and found to be efficient in direct tissue detection of *M. bovis* (Figueiredo *et al.* 2009, Carvalho *et al.* 2015).

Detection limit of PCR assays: The pure *M. bovis* culture DNA with a concentration of 95 ng/μl was serially diluted up to 0.095 fg/μl and used for amplification of the genes included in our study. All the five PCR assays were able to amplify *M. bovis* from 95 ng/μl to 95 pg/μl and failed to detect further lower concentrations of DNA (Fig. 1). The analytical sensitivity for all the five PCR assays showed

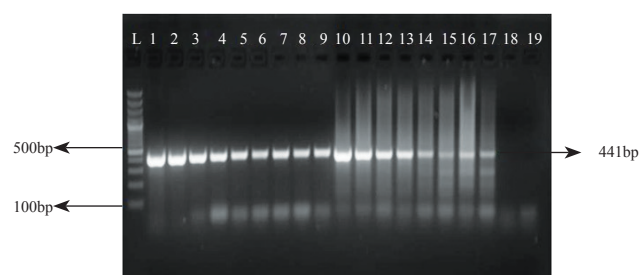


Fig. 1. Detection limit of *hsp65* PCR on *M. bovis* using DNA isolated by GCC kit. Lane L, Ladder (Gene Ruler 100 bp plus DNA ladder); Lane 1, Positive Control (*M. bovis* AN5); Lane (2-9), Serial dilutions of standard *M. bovis* in PBS (10^9 - 10^2); Lane (10-17), *M. bovis* in Spiked tissues (10^8 - 10^1); Lane 18, Negative control (*E. coli* ATCC 25922); Lane 19, NTC.

similar trend proving their same efficiency.

Field evaluation: Evaluation of the PCR assay on bovine lung tissue was performed with the standardized assays targeting all the five genes/region using GCC Blood genomic DNA isolation kit on 30 such samples. Only one sample was positive by all the five set of PCR assays (Fig. 2, *hsp65* PCR as a representative) and this sample was collected from a bovine lung showing gross tubercle nodule which was positive by microscopy and culture growth also.

There are many previous claim for efficient detection of

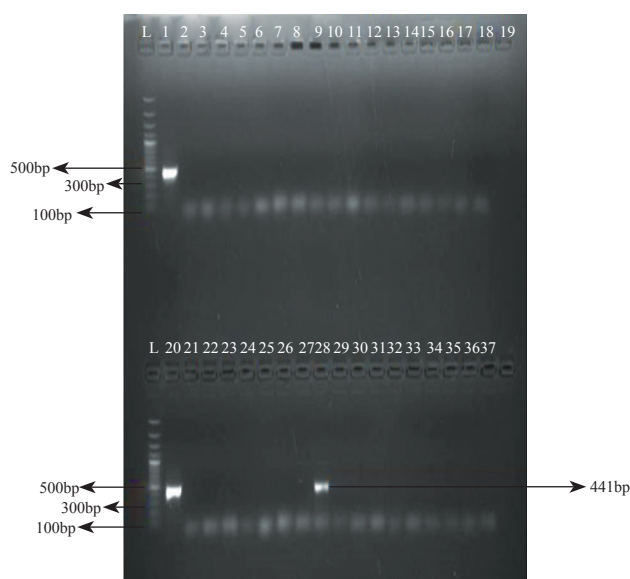


Fig. 2. Screening of lung tissues by hsp65 PCR. Lane L, Ladder (Gene Ruler 100 bp plus DNA ladder); Lane 1, 20, Positive control (*M. bovis* AN5); Lane (2-16), Tissue samples L1-L15; Lane (21-35), Tissues Samples L16-L30; Lane 17, 36, Negative control (*E. coli* ATCC 25922); Lane 18, 37, NTC.

M. bovis from culture as well as from direct tissue samples with their own pros and cons. Out of the all claimed, we have used the well-known proven PCR primers and some of them are being used for the last two decades for *M. bovis* detection. Still we compared these proven selected primers so as to know which one could be even more efficient in present time as during the course of time, the efficiency of DNA extraction and PCR efficiency with improved polymerase had increased tremendously. In the present study, *hsp65* (genus specific), IS6110, IS1081 (*M. tuberculosis* complex) and 500 bp, RD4 (*M. bovis* species specific) all stood to be good for detection of bovine tuberculosis agents in tissue samples using the mentioned primer sets in the study. These PCR assays supported by any efficient DNA extraction protocol will help in detection for the agents of bovine tuberculosis directly from the tissues.

The present study showed the confidence in diagnosis of zoonotic tuberculosis which emphasis a combined approach of clinical, conventional and molecular confirmation. Here, we were able to conclude that for molecular approach, a combination PCR of genus specific (*hsp65*), complex (IS6110/IS1081) and species specific (500bp/RD4) PCR on tissue samples would be a good approach to confirm the diagnosis, if supported by an robust DNA extraction method.

Future prospects and applicability of the study: The combination PCR of genus, complex and species specific PCR on direct tissue samples would certainly help in the accurate diagnosis of zoonotic tuberculosis. This would help to screen the positive tissue which will give the accurate status of infection in farms through backtracking and then these suspected farms can be vigorously screened

for infected animals through clinical, conventional and molecular approach. This approach will certainly help in removing the positive animals and will reduce the zoonotic TB burden in the farms and in a one health approach will reduce the burden of zoonotic TB in public also.

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