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RESEARCH ARTICLE

Detection of *Mycobacterium tuberculosis* complex and rifampicin resistance using Xpert MTB/RIF Ultra assay in tissue samples from cattle and buffaloes

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ABSTRACT

Mycobacterium bovis belonging to *Mycobacterium tuberculosis* complex (MTBC) is the major causative agent of bovine tuberculosis in cattle and buffaloes. Inhalation and ingestion of bacilli remains the major route of transmission. Thus, to interrupt the transmission cycle, rapid and accurate detection of the infected animals is necessary. This study, evaluated the role of Xpert MTB/RIF Ultra assay in detecting bovine tuberculosis causing MTBC in postmortem tissue samples and its resistance to rifampicin. A total of 25 tissue samples were collected from cattle and buffaloes died of respiratory ailments and were used in the study. Conventional PCR was employed to confirm the presence of mycobacterial DNA. Xpert MTB/RIF Ultra assay detected the presence of MTBC in 5 out of 25 lung tissue samples, of which none of the samples positive displayed rifampicin resistance. Out of the 5 samples tested positive by Xpert MTB/RIF Ultra assay 2 samples gave positive *IS6100* PCR results. Thus, Xpert MTB/RIF Ultra assay could potentially serve as a valuable and easy to perform technique for diagnosing tuberculosis in postmortem tissue samples.

Keywords: MTBC, PCR, Tissue samples, Xpert MTB/RIF Ultra assay

INTRODUCTION

Tuberculosis (TB) continues to be a major contributor to illness and death, particularly in low and middle-income nations. According to the World Health Organization (WHO), approximately one-third of all TB cases are either undiagnosed or not reported[1]. Globally, India being endemic for tuberculosis, accounts for 26% of TB prevalence and 34% of tuberculosis deaths. The "End TB Strategy" by government of India (WHO & NTEP) will only be successful if all means of disease transmission like zoonosis and reverse zoonosis are properly addressed[2]

Mammalian tuberculosis is a persistent condition characterized by the formation of granulomas arising as a result of the infection with harmful bacteria within the *Mycobacterium tuberculosis* complex (MTBC). A significant contribution of disease in cattle comes from infection with *M. bovis.* The other members of MTBC includes *M. tuberculosis*, *M. caprae*, *M. africanum*, *M. microti*, *M. canettii* and *M. pinnipedii.* The disease reduces the productivity of livestock intended for both local consumption and export markets[3].

Although inhaling infected respiratory droplets is regarded as the primary means of transmitting MTBC in livestock, infection can also occur through other routes. These include ingesting contaminated milk, consuming meat taken from wild as well as domesticated animals which may not undergo veterinary examination, poses a potential risk of infection to humans[4].

Most prevalently used antibiotics in treatment of tuberculosis are rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), ethambutol (EMB) etc. Organisms belonging to *Mycobacterium tuberculosis* complex had developed resistance towards multiple antibiotics leading to multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium* in both humans and animals[5]. The rise of multidrug resistance in animals has garnered global concern, primarily because of the potential for pathogen transmission to humans[6].

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The Xpert MTB/RIF Ultra assay is a semi-quantitative, nested real-time PCR assay that has revolutionized the management of *Mycobacterium tuberculosis* in humans. It offers a faster and more accurate diagnosis of tuberculosis by simultaneously detecting *M. tuberculosis* and rifampicin resistance. According to the World Health Organization (2015), the implementation of

the Xpert MTB/RIF Ultra assay has led to significant improvements in human TB programs in more than 130 countries[7].

The assay focuses on multicopy sequences, specifically *IS6110* and *IS1081*, significantly enhancing DNA detection in samples with low bacterial load. *IS1081* and *IS6110* are unique to members of the *Mycobacterium tuberculosis* complex (MTBC), making them well-suited for samples collected from animals potentially infected with MTBC members. While the GeneXpert is primarily employed for detecting MTBC DNA in human samples, studies have demonstrated its accurate detection of *M. bovis* in wildlife specimens[8].

In this current study, we evaluated the application of Xpert MTB/RIF Ultra assay for detecting both *M. bovis* in animal lung tissue samples, collected from cattle and buffaloes died of respiratory ailments in comparison to results obtained from *IS6100* PCR.

MATERIALS AND METHODS

Study design and sample collection

The study was conducted in Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. All animal carcasses analysed in this study underwent examination for visible bTB lesions (Fig. 1). Lung tissue samples and lymph nodes from cattle and buffaloes died of respiratory ailments, with or without tubercle lesions were collected from Veterinary Pathology postmortem hall. All the samples collected were stored at -20°C till further processing.

All authors appreciate the ethical committee for permitting the use of animals and the IAEC/CPCSEA clearance may be accessed under reference number IAEC/2023/68/07.

Sample processing for Xpert ® MTB/RIF Ultra assay analysis

2-5 grams of tissue specimens was homogenized in a sterile mortar in a biosafety cabinet. Approximately 2 ml of sterile phosphate buffered saline was added into it. Approximately 0.7 ml of homogenised tissue without clumps was transferred into a conical screw-capped tube using a transfer pipette. Double the volume i.e 1.3ml of sample reagent was added to the tube using transfer pipette. The tube was vortexed for 10 seconds and incubated for 15 min at room temperature. 2ml of sample reagent treated specimen was added to Xpert® MTB/RIF Ultra cartridge and was loaded on to the instrument using a fresh transfer pipette to obtain results after 1 hr (Fig. 2).

Result interpretation of Xpert® MTB/RIF Ultra assay.

The results were interpreted by the GeneXpert DX System from measured fluorescent signals and embedded calculation algorithms and will be displayed in the laptop. Lowest Ct values represents a higher concentration of DNA template. The MTB result will be displayed as High, Medium, Low, Very low or Trace depending on the Ct value of the MTB target present in the sample.

- MTB DETECTED HIGH- MTB target is detected in the sample with a Ct range <16
- MTB DETECTED MEDIUM- MTB target is detected in the sample with a Ct range 16-22
- MTB DETECTED LOW- MTB target is detected in the sample with a Ct range 22-28
- MTB DETECTED VERY LOW- MTB target is detected in the sample with a Ct range >28
- MTB TRACE DETECTED- MTB target is detected in the sample with a Ct range One or both probes for multi-copy targets are positive with Ct <37 and no more than one *rpoB* probes have a Ct<40

RIF Resistance DETECTED, RIF Resistance NOT DETECTED, RIF Resistance INDETERMINATE will be displayed only in MTB DETECTED results.

- RIF Resistance DETECTED: a mutation in *rpoB* gene has been detected that falls within the valid Ct setting.
- RIF Resistance INDETERMINATE: the MTB concentration was very low and resistance could not be detected due to insufficient signal production.
- RIF Resistance NOT DETECTED: no mutation in *rpoB* gene is detected

DNA isolation and *IS6100* polymerase chain reaction

Sample DNA (n=25) was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturers' instructions along with appropriate adjustments and subsequently were accurately labelled and stored at -20°C until further analysis.

IS6100 PCR

The PCR technique utilized INS1 and INS1 primers designed to aim at the *IS6100* insertion sequences. These genetic markers are frequently utilized for identifying MTBC strains in samples, as described[9]. The amplified products were verified to be of the expected size by electrophoresis on a 1.5% (w/v) agarose gel (Thermo Scientific, USA) in 1X Tris Acetate EDTA buffer.

RESULTS

Results obtained from this study are shown in Table 1. A total of 25 tissue samples were subjected to molecular detection using Xpert MTB/RIF Ultra assay. 5 out of 25 samples were identified as MTC positive, of which 3 samples belonged to the category of "MTB detected very low (Fig. 3) and 2 samples under "MTB trace detected" (Fig. 4) category. Resistance to rifampicin was not

Table 1. Xpert MTB/RIF Ultra assay results from tissue samples in comparison to PCR results.

S.No.	Lab No.	Species	Age (yrs)	Smear Results	GeneXpert Ultra result	RIF results	IS6100 PCR
1.	TS1	Cattle	4.5Y	Negative	MTB detected very low	ND	Positive
2.	TS2	Buffalo	5Y	Negative	MTB trace detected	I	Negative
3.	TS5	Buffalo	7Y	Negative	MTB detected very low	ND	Positive
4.	TS8	Cattle	6Y	Negative	MTB detected very low	ND	Negative
5.	TS14	Cattle	5.5Y	Negative	MTB trace detected	I	Negative

detected in any of the collected samples that gave positive GeneXpert results. In samples under "MTB trace detected" category, RIF resistance detection is labelled "indeterminate" as it cannot be determined due to insufficient signal detection. Out of the 5 samples tested positive by Xpert MTB/RIF Ultra assay, only 2 samples were confirmed to be positive by *IS6110* PCR (Fig. 5). Out of 25 samples that were subjected to ZN staining, none of samples showed presence of acid-fast bacilli.



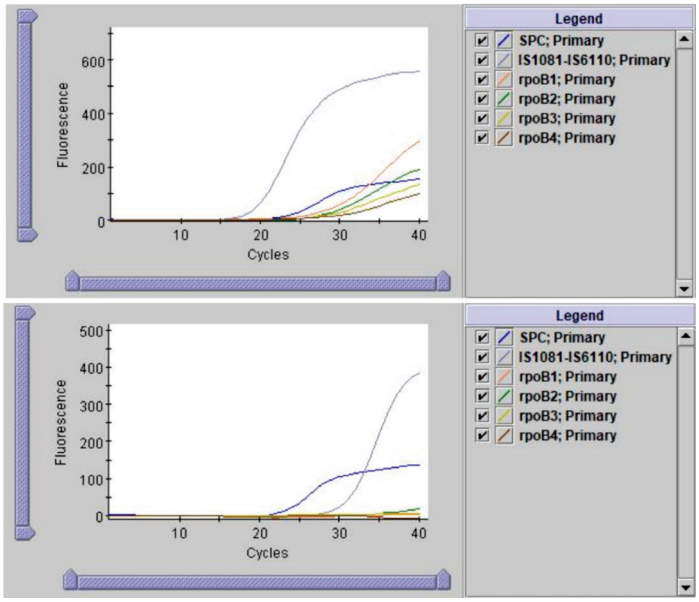

Fig. 1. Gross lesions showing tuberculous lesions in lung tissue; **Fig. 2.** Xpert® MTB/RIF Ultra machine along with its cartridge; **Fig. 3.** Amplification plot of Tissue samples using Xpert MTB/RIF Ultra assay in category, "MTB DETECTED VERY LOW", "RIF RESISTANCE NOT DETECTED"; **Fig. 4.** Amplification plot of Tissue samples using Xpert MTB/RIF Ultra assay in category, "MTB TRACE DETECTED", "RIF RESISTANCE INDETERMINATE".

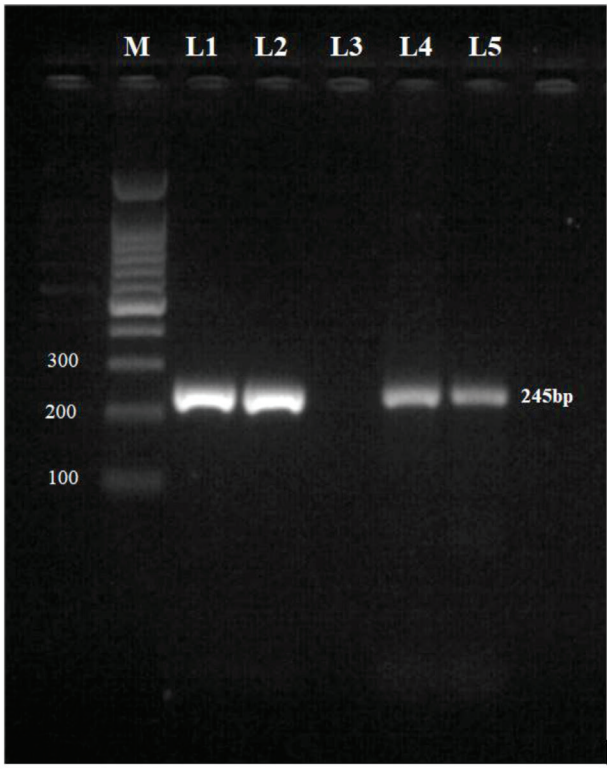

Fig. 5. Agarose gel electrophoresis of amplified IS6100 gene of MTC at 245 bp using INS1/INS2 primers for tissue samples. Lane M: 100 bp ladder. Lane 1,2: Positive control of M. bovis, Lane 3: Negative control, Lane 4,5: Positive samples for IS6100 PCR

DISCUSSION

The objective of this pilot study was to assess the feasibility of detecting MTBC DNA in *M. bovis*-infected cattle and buffaloes using post-mortem tissue samples with the rapid Xpert MTB/RIF Ultra assay. The original Xpert MTB/RIF assay was among the most commonly utilized tests for tuberculosis and drug resistance in humans. However, since its endorsement by the World Health Organization (WHO) in 2010, the manufacturers have consistently improved its diagnostic capabilities. Unintentionally,

these enhancements have also transformed it into a potential diagnostic tool for detecting *M. bovis* in animals[10].

Research conducted using human sputum samples has proved that GeneXpert results offer an indication of the bacterial load in a specimen. There is a direct relationship between the semi-quantitative GeneXpert results and the detection of acid-fast bacilli through smear microscopy. GeneXpert results indicating "MTB trace detected" or "MTB not detected" have been linked with the absence of acid-fast bacilli, while results indicating "MTB detected high" and "medium" typically correspond to positive results in smear microscopy [11]. In the current study, none of the samples gave positive smear results may be because GeneXpert results of all 5 positive samples belonged to "MTB detected very low" (n=3) and "MTB trace detected" (n=2) category.

Out of 5 samples giving positive GeneXpert results, surprisingly only 2 were found to be positive by PCR. *IS6110* PCR did not identify 3 of the positive samples. In a previous study[12], utilizing GeneXpert to identify MTBC DNA with tissue samples, similar results were observed and the reason for this phenomenon remains unidentified.

While mycobacterial culture of post-mortem tissues and the identification of bacterial isolates continue to be regarded as the gold standard method for confirming *M. bovis* infection in animals, this approach is constrained by challenges in obtaining tissue samples without contamination and the suboptimal sensitivity of culture techniques[13]. In many previous studies, the utilization of GeneXpert with tissue samples gathered from cattle buffaloes and wildlife to identify MTBC DNA was evaluated[14-17]. This method was determined to be a secure, straightforward, and efficient approach to screen animals for the existence of *M. bovis*.

Limitations of this study includes a limited sample size and the occurrence of multiple freeze-thaw cycles on samples before testing. Another limitation of the study is the inability of Xpert MTB/RIF Ultra assay to conclusively determine whether the detection of MTBC DNA was from the animals or environmental contamination. In conclusion, the Xpert MTB/RIF Ultra assay emerges as a rapid diagnostic test capable of detecting MTBC DNA in tissue samples obtained from cattle and buffaloes.

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