

Detection of *Mycobacterium tuberculosis* in Pulmonary and Extrapulmonary Samples Using Different Methods: Experience of a Tertiary Care Center in North India

ARTICLE INFO

Article Type Review Article

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How to cite this article

Zahoor D, Farhana A, Manzoor M, Kanth F, Nasir R. Detection of *Mycobacterium tuberculosis* in Pulmonary and Extrapulmonary Samples Using Different Methods: Experience of a Tertiary Care Center in North India. Infection Epidemiology and Microbiology. 2023;9(2):191-199.

Article History

Received: September 16, 2022

Accepted: June 15, 2023

Published: August 19, 2023

ABSTRACT

Backgrounds: Delay in the diagnosis of tuberculosis (TB) leads to poor response to treatment and the disease transmission to susceptible individuals. The Xpert MTB/RIF assay efficiently detects *Mycobacterium tuberculosis* (MTB). The present study aimed to compare acid-fast bacilli (AFB) microscopy, culture, and Xpert MTB/RIF assay in the diagnosis of pulmonary and extrapulmonary tuberculosis cases.

Materials & Methods: This retrospective study was conducted in the Department of Microbiology, Government Medical College, Srinagar, India over 18 months from February 2019 to July 2020. Samples were processed and evaluated using AFB microscopy, culture, and Xpert MTB/RIF assay.

Findings: Among the 1862 samples evaluated, 224 samples were found to be positive using AFB microscopy, culture, and Xpert MTB/RIF assay. The overall sensitivity and specificity of the Xpert MTB/RIF assay in diagnosing pulmonary TB cases was 98.23 and 97.69%, respectively. Among the smear-negative extrapulmonary samples, 52 (5.75%) and 86 (9.6%) samples were positive in culture and the Xpert MTB/RIF assay, respectively. The maximum recovery of MTB by Xpert MTB/RIF assay was from tissue biopsy specimens. Rifampicin resistance was observed in six samples.

Conclusion: Both culture and Xpert MTB/RIF methods were sensitive in detecting smear-positive samples. Although both techniques missed some smear-negative pulmonary and extrapulmonary TB cases, the Xpert MTB/RIF assay enhanced the detection rate of MTB compared to culture. The Xpert MTB/RIF assay enabled the accurate diagnosis of tuberculosis cases with a rapid turnaround time; therefore, it could assist clinicians to start timely therapeutic interventions for these patients.

Keywords: Pulmonary tuberculosis, Extrapulmonary tuberculosis, Diagnosis, Sensitivity and specificity.

CITATION LINKS

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Introduction

Tuberculosis (TB) is a pulmonary disease caused by the bacterium *Mycobacterium tuberculosis*. TB is one of the earliest diseases, whose causative agent was identified. Although effective vaccination and treatment have been developed for tuberculosis, it continues to be a major health problem around the globe. According to the global tuberculosis report published by the World Health Organization (WHO) in 2021, one-fourth of the world's population (nearly 2 billion individuals) is infected with the tuberculosis bacterium, among whom approximately 5–15% would develop an active disease at a certain point during their lifetime. India, among all countries in the world, accounts for the highest burden of tuberculosis cases (27% of all reported cases worldwide)^[1-3].

In tuberculosis, the main organ affected is the lung, although the number of extrapulmonary tuberculosis (EPTB) cases has increased over the past few years. Extrapulmonary TB cases account for more than 15% of all TB cases. Tuberculosis presents a dynamic spectrum of disease conditions, ranging from an asymptomatic disease to a life-threatening condition. Therefore, timely detection of tuberculosis is important to prevent disease progression, improve outcomes, and reduce disease transmission to susceptible uninfected populations. A large proportion of tuberculosis cases remain undiagnosed owing to the gradual growth of *M. tuberculosis*, the paucibacillary nature of the illness, and biosafety concerns that pose major challenges in the diagnosis of tuberculosis^[4].

Acid-fast bacilli (AFB) smear microscopy, culture, and molecular tests are common microbiological methods used to diagnose tuberculosis. AFB microscopy could be performed in peripheral laboratories with minimal biosafety requirements, although

its use is limited due to its lower sensitivity with a detection limit of approximately 5000–10000 bacilli per mL of specimen. Culture remains the gold standard for the laboratory confirmation of tuberculosis. In contrast to AFB microscopy, only 10–100 viable bacilli per mL are required for a positive culture. The culture of a specimen for *M. tuberculosis* on a solid medium requires approximately 8 weeks prior to being reported as negative. On the contrary, liquid culture has a shorter turnaround time (approximately 12 days), although it is associated with higher contamination rates. Another tuberculosis diagnosis method is molecular diagnosis, which involves the detection of genes specific to *M. tuberculosis*. The use of the Xpert MTB/RIF assay for the detection of *M. tuberculosis* in pulmonary and extrapulmonary specimens has been endorsed by the World Health Organization. The assay is fully automated and performed based on real time polymerase chain reaction (RT-PCR) using single-use cartridges that simultaneously detect *M. tuberculosis* bacilli as well as rifampicin resistance directly in clinical samples. Rifampicin inhibits the bacterial RNA polymerase enzyme encoded by the *rpoB* gene; mutations in this gene are associated with resistance to this drug. Rifampicin resistance serves as a surrogate marker for multidrug-resistant tuberculosis (MDR-TB). The assay involves the addition of sample diluents to chemically inactivate the specimen. The process is simple with minimum hands on time, and results are available within 2 hours^[4-8].

Objectives: Considering the availability of numerous modalities for the detection of *M. tuberculosis*, the current study was conducted to evaluate the performance of AFB smear microscopy, culture, and Xpert MTB/RIF assay in diagnosing pulmonary and extrapulmonary TB cases. The aim of this study was to evaluate the potential

of these available diagnostic strategies in enhancing tuberculosis diagnosis.

Materials and Methods

Setting: The present study was designed as a retro-prospective cross-sectional study carried out in the Department of Microbiology, Government Medical College, Srinagar, J&K, India over 18 months from February 2019 to July 2020. Clinical specimens, including both pulmonary (sputum, bronchoalveolar lavage fluid, and gastric lavage) and extrapulmonary (pleural fluid, tissue biopsy, pus, cerebrospinal fluid, ascitic fluid, pericardial fluid, etc.) samples, which were taken for routine mycobacterial testing, were included in the present study. Two samples from each patient were preferred: one for Xpert MTB/RIF assay and the other one for AFB smear and culture. Samples from patients who were already under anti-tubercular treatment and follow-up samples were excluded from the study. Ethical clearance for the study was obtained from the ethical clearance committee of the institute (99/ETH/GMC/ICMR).

Sample processing: Decontamination of non-sterile specimens was done using the conventional N-acetyl-L-cysteine-sodium hydroxide (NaOH) method. Sterile body fluids were concentrated by centrifugation. Tissue samples were excised into small pieces using a sterile blade and then ground using a tissue grinder until a homogenous suspension was obtained.

AFB staining: After sample decontamination, smears were prepared and subjected to Ziehl-Neelsen (ZN) staining. The sample was considered AFB-positive if at least one of the slides showed acid-fast bacilli.

AFB culture: Solid medium, automated liquid medium (BacT Alert; Biomerieux, India), or both were used for culture, depending on the availability of each medium. Solid cultures on Lowenstein Jensen (LJ) media (HiMedia,

India) were incubated for 8 weeks prior to being reported as negative. The cultures were examined regularly for contamination with rapidly growing bacteria and morphologies inconsistent with the *M. tuberculosis* complex (MTBC). *M. tuberculosis* identification was performed based on colony morphology, ZN staining, and biochemical tests on the growth of bacteria on LJ medium. Cultures on BacT Alert were reported negative after incubation for 6 weeks. When the instrument flagged positive, the presence of *M. tuberculosis* was confirmed based on flaked clumpy growth, the presence of AFB bacilli, and immunochromatographic detection of the MPT64 antigen. The culture was considered positive if the solid or liquid culture or both presented a positive result.

Xpert MTB/RIF assay. To perform this assay, the manufacturer's instructions were followed. The decontaminated sample and the sample reagent (containing NaOH and isopropyl alcohol) were added in a ratio of 1:2 to kill the mycobacteria and liquefy the samples. SR (sample reagent) buffer was added directly to sterile body fluids at a ratio of 2:1 by volume. However, in the case of cerebrospinal fluid (CSF) samples, if the volume was <1 mL, SR buffer was added to obtain a volume of 2 mL. The sample-SR mixture was incubated for 20 min with intermittent shaking. Subsequently, 2 mL of the sample-SR mixture was transferred to the cartridge, which was then loaded into the Xpert MTB/RIF assay instrument for automated operation of all subsequent steps. Results were interpreted automatically as negative, positive, or invalid. The Xpert MTB/RIF assay detects mutations in the core region of the *rpoB* gene that confers rifampicin resistance. When some errors or invalid/no results were reported at the end of the operation, the whole process was repeated if sufficient materials were available. Regarding the samples that showed negative

results in culture but positive results in the Xpert MTB/RIF assay, the results were interpreted as positive considering the high specificity of the Xpert MTB/RIF assay and the paucibacillary nature of the illness.

Findings

A total of 1862 samples were received in the TB lab during the 18-month study period. All patients were HIV-negative. Among all included patients, 638 (34.26%) cases were suspected of pulmonary tuberculosis, and 1224 (65.74%) cases were suspected of extrapulmonary tuberculosis (Fig. 1). Among the evaluated samples, 224 (12.03%) samples were positive for *M. tuberculosis* in culture or the Xpert MTB/RIF assay or both. The total recovery rate was 12%. Among all positive samples, 139 (62%) cases were pulmonary samples, while 85 (38%) cases

were extrapulmonary samples. The number of different clinical samples received in the laboratory and the positivity percentage of each type of sample are provided in Fig. 2. The most frequent site of involvement was the lymph node, followed by pus and aspirates from different parts of the body.

AFB positivity: The AFB positivity rate in the present study was 4.03%. Among all positive samples, 60 (9.4%) cases were pulmonary samples, while 15 (1.23%) cases were extrapulmonary samples.

Pulmonary samples: Culture: Out of the 638 pulmonary samples received, 60 (9.4%) samples were smear-positive, while 578 (90.6%) samples were smear-negative. Among the 60 smear-positive samples, 55 samples were used for culture, of which 51 (92.72%) samples were positive for *M. tuberculosis*, while two (3.64%) samples

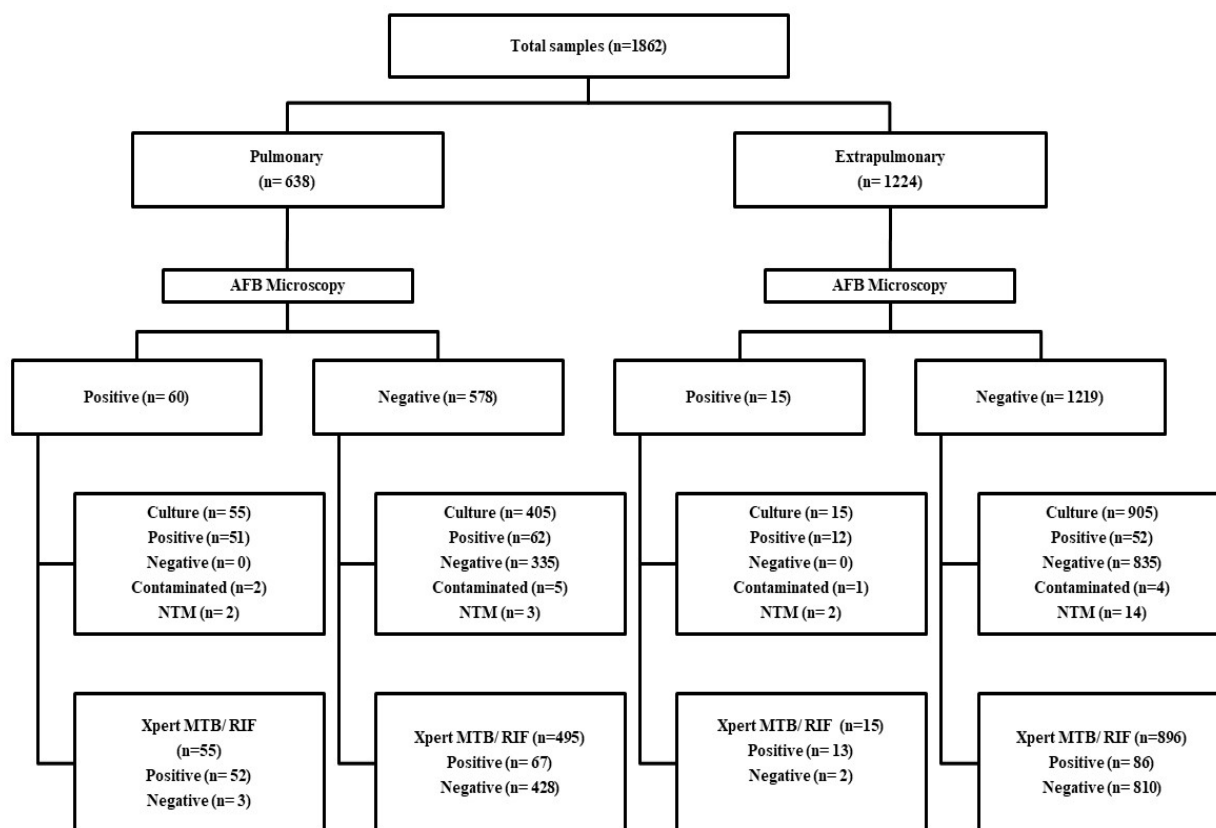


Figure 1) Culture and Xpert MTB/ RIF assay results in pulmonary and extrapulmonary samples processed in the mycobacteriology laboratory

Table 1) Sensitivity, specificity, PPV, NPV, and diagnostic accuracy of the Xpert MTB/RIF assay when using pulmonary samples

	Sensitivity %	Specificity %	PPV %	NPV %	Diagnostic Accuracy %
Total	98.23 (93.78–99.51)	97.69 (95.52–98.83)	93.28 (87.29–96.55)	99.41 (97.89–98.81)	97.83 (96.04–98.81)
Smear positive pulmonary	100 (93–100)	100 (92.06–100)	98.08 (89.88–99.66)	100 (92,100)	98.08 (89.88–99.66)
Smear negative pulmonary	96.77 (88.98, 99.11)	97.96 (95.85, 99.01)	89.55 (79.97, 94.85)	99.41 (97.87, 99.84)	97.78 (95.83, 98.83)

presented contaminated growth, and the remaining two (3.64%) samples exhibited the growth of non-tuberculous mycobacteria (NTM). Among the 578 smear-negative samples, 405 samples were used for culture, of which 62 (15.31%) samples were positive for *M. tuberculosis*, while three (0.74%) samples were NTM-positive, and five (1.23%) samples exhibited contaminated growth. The remaining 335 samples were negative (Fig. 1).

Xpert MTB/RIF assay: A total of 55 AFB-positive samples were subjected to the Xpert MTB/RIF assay. Among these, 52 (94.54%) samples were positive for *M. tuberculosis*, while three (5.46%) samples were negative. Two AFB-positive but Xpert-negative samples were subsequently detected as NTM-positive in culture. Among the 495 smear-negative pulmonary samples subjected to Xpert MTB/RIF assay, 67 samples (13.53%) were positive for *M. tuberculosis* (Fig. 1).

Extrapulmonary samples:

Culture: Out of the 1224 extrapulmonary samples received, 15 (1.23%) samples were smear-positive, while 1219 (98.77%) samples were smear-negative. Among the 15 smear-positive samples, 12 (80%) samples were positive for *M. tuberculosis* in culture, one (6.67%) sample exhibited contaminated growth, and the remaining

two (13.33%) samples exhibited the growth of non-tuberculous mycobacteria. Among the 1219 smear-negative samples, 905 samples were used for culture, of which 52 (5.75%) samples were positive for *M. tuberculosis*, while 14 (1.55%) samples were NTM-positive, and four (92.7%) samples exhibited contaminated growth (Fig. 1). **Xpert MTB/RIF assay:** A total of 13 (86.67%) AFB-positive samples were detected to be positive in the Xpert MTB/RIF assay, while two (6.6%) samples were negative. Two (6.67%) AFB-positive but Xpert-negative samples were subsequently detected to be NTM-positive in culture. Among the 896 smear-negative samples subjected to the Xpert MTB/RIF assay, 86 (9.6%) samples were positive for *M. tuberculosis*, while 810 (90.4%) samples were negative (Fig. 1).

Determination of rifampicin resistance:

A total of six (2.75%) samples were detected to be resistant to rifampicin, while indeterminate results were obtained for four (1.8%) samples. Among the rifampicin-resistant isolates, one sample was pulmonary, while the remaining five samples were extrapulmonary.

Comparison of AFB microscopy, culture, and Xpert MTB/RIF assay: Among the 55 AFB-positive pulmonary samples, 51 samples were positive in culture, and 52

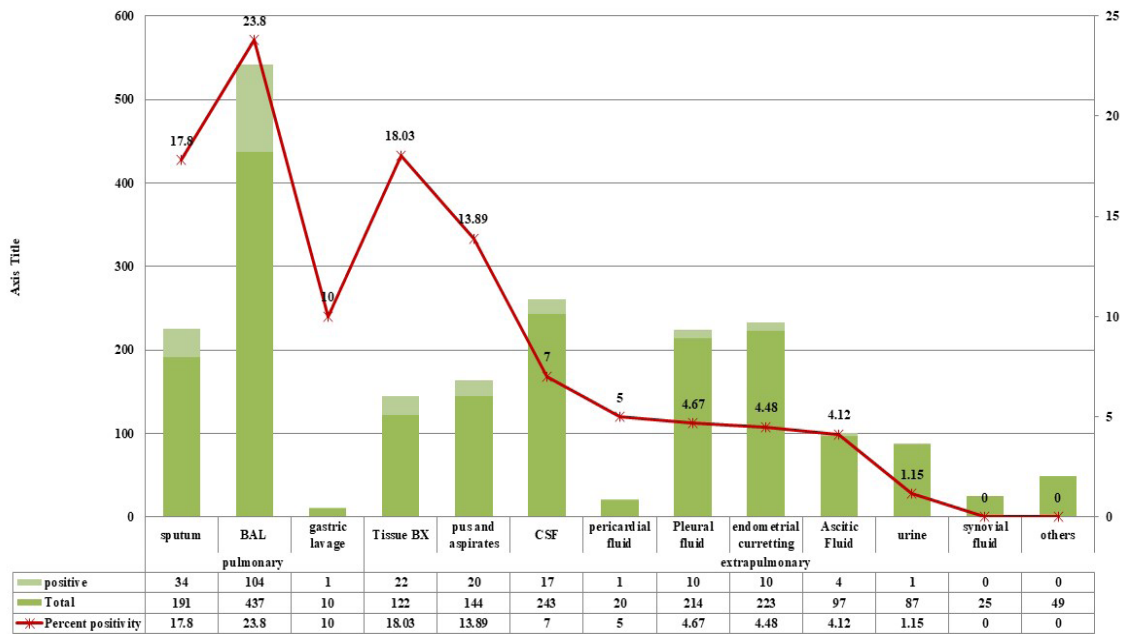


Figure 2) Diagrammatic representation of specimens processed and their positivity percentage

samples were positive in the Xpert MTB/RIF assay. Two of the samples detected as negative in the Xpert MTB/RIF assay were subsequently revealed to be NTM-positive in culture. The third sample that was detected as negative in the Xpert MTB/RIF assay was later found to be contaminated and therefore eliminated. Similarly, among the 405 smear-negative pulmonary samples, 62 (out of 405) samples were detected as positive in culture, while 67 (out of 405) samples were detected as positive in the Xpert MTB/RIF assay. The sensitivity, specificity, PPV (positive predicative value), NPV (negative predicative value), and diagnostic accuracy of the Xpert MTB/RIF assay in comparison to the gold standard culture method are presented in Table 1.

Among the 15 AFB-positive extrapulmonary samples, 12 samples were culture-positive, and 13 samples were positive in the Xpert MTB/RIF assay. Two of the samples that were detected as negative in the Xpert MTB/RIF assay were subsequently revealed to

be NTM-positive in culture. Among the 896 AFB-negative extrapulmonary samples, 52 samples were detected as positive in culture, while 86 out of 896 samples were positive in the Xpert MTB/RIF assay.

Discussion

The present study highlighted the challenges encountered in the diagnosis of tuberculosis, which is essential to improve disease outcomes and terminate the chain of transmission. Diagnostic delays lead to delays in treatment and an increased risk of transmission of *M. tuberculosis* infection to healthy individuals.

The national tuberculosis elimination program recommends the use of AFB microscopy for the diagnosis of pulmonary tuberculosis as this method is cost-effective, requires less technical expertise, and offers rapid diagnosis and better specificity, all of which are beneficial in countries with a high burden of TB disease. The major drawback of the smear microscopy method is its low

sensitivity. Smear-negative pulmonary and extrapulmonary samples represent a huge challenge in tuberculosis diagnosis due to their paucibacillary nature. In the present study, the smear positivity rate for pulmonary tuberculosis was 9.4%, while this value for extrapulmonary tuberculosis was 1.23%. The paucibacillary nature of extrapulmonary tuberculosis has made smear microscopy an extremely insensitive and unreliable evaluation method in the diagnosis of this disease [9-10].

In the present study, both culture and Xpert MTB/RIF methods performed equally well in detecting *M. tuberculosis* in smear-positive samples. A total of four specimens that were smear-positive (two pulmonary and two extrapulmonary samples) exhibited the growth of non-tuberculous mycobacteria and were detected as negative in the Xpert MTB/RIF assay. It was observed that when using both Xpert MTB/RIF and culture methods for the diagnosis of this disease, patients who were falsely positive on smear microscopy were unlikely to be misdiagnosed. This issue has also been reported in several previous studies [9, 11].

In the case of smear-negative pulmonary tuberculosis samples, the Xpert MTB/RIF assay was able to detect five more TB cases that were culture-negative, resulting in a 7.46% increase in the detection rate in the present study. The sensitivity and specificity of this assay for smear-negative pulmonary cases (96 and 97%, respectively) were lower than for smear-positive cases (100 and 100%, respectively). Oliveira et al. (2019) reported a 7.8% increase in the sensitivity of the Xpert MTB/RIF assay compared to culture [12]. Horne et al. (2019) reported a pooled sensitivity of 67% (62-70%) for this assay, and Lombardi et al. (2017) reported an improved detection rate of 36.5% by Xpert MTB/RIF assay in smear-negative pulmonary TB samples [6, 11].

In the case of smear-negative extrapulmonary TB samples, the culture method and the Xpert MTB/RIF assay detected 52 and 86 samples as positive, respectively. The increase in the detection rate using the Xpert MTB/RIF assay was 39.43%. Vadwai et al. (2011) analyzed both smear-positive and smear-negative extrapulmonary samples and reported the sensitivity of the Xpert MTB/RIF assay as 81 and 64%, respectively, compared to the composite reference standard. Habous et al. (2019) reported a sensitivity of 100 and 93.2% for smear-positive and smear-negative EPTB cases [3,9]. In their study, Lombardi et al. (2017) demonstrated an improved detection rate of 63.4% using the Xpert MTB/RIF assay [6]. Among the Xpert-positive samples, 34 samples were found to be culture-negative, while six culture-positive samples were found to be negative in the Xpert MTB/RIF assay. Therefore, the culture method is insensitive in the diagnosis of EPTB, as reported in several previous studies. In addition to the low sensitivity of culture, the diagnosis of EPTB is also difficult because of its paucibacillary nature and the use of invasive techniques for sample collection. Therefore, based on this study findings, culture should not be considered as the gold standard for the diagnosis of extrapulmonary tuberculosis. In the absence of culture, the use of a composite reference standard based on microbiological evaluations, clinical characteristics, and additional tests is recommended to increase the chances of detection [13-15].

The findings of the present study are consistent with those reported in other studies in terms of the variation observed in the sensitivity of the Xpert assay for different types of extrapulmonary specimens. The maximum recovery of MTB by Xpert MTB/RIF assay was from tissue biopsy specimens, including the lymph node biopsies, followed by pus and aspirates. Moderate sensitivity

was observed for CSF, while the lowest sensitivity was observed for various body fluids such as pleural fluid and ascitic fluid [10,16]. The lower sensitivity of Xpert MTB/RIF for body fluids is attributed to the low bacterial load. Many authors have recommended the use of tissue biopsies rather than body fluids for the diagnosis of peritoneal and pleural tuberculosis [16].

Rifampicin resistance was observed in six samples, of which five samples were extrapulmonary. Indeterminate results were obtained for six cases. The pooled sensitivity and specificity of the Xpert MTB/RIF assay for the detection of rifampicin resistance were 96 and 98%, respectively [11].

The Xpert MTB/RIF assay was proven to be a valuable tool for the detection of *M. tuberculosis* and rifampicin resistance. This assay offers benefits such as a rapid turnaround time of approximately 2 hrs, a hands-on time of less than 5 min per specimen, and minimal biosafety requirements [17]. However, even with the adoption of this assay, there is still a need for conventional TB culture in the case of smear-negative pulmonary tuberculosis and EPTB. Non-tuberculous mycobacteria are increasingly isolated from clinical specimens and are often indistinguishable from tuberculosis infection during diagnosis. Therefore, accurate diagnosis of NTM is important because NTM are resistant to many drugs used to treat tuberculosis. In the present study, none of the NTM-positive samples in culture were detected as positive in the Xpert MTB/RIF assay. This finding is consistent with those of previous studies as well [17-20].

According to the findings of the present study, it was inferred that both culture and the Xpert MTB/RIF assay could detect cases with high bacterial loads, while they may miss cases with low bacterial loads. The Xpert MTB/RIF assay was revealed to be an

efficient tool for the rapid detection of *M. tuberculosis* as well as rifampicin resistance and therefore should be used in the routine workup of patients with suspected tuberculosis. It is important to maintain a high level of suspicion towards patients with smear-negative pulmonary tuberculosis and EPTB.

Limitation of the study: Since the present study was a retrospective study, a composite reference standard was not included to evaluate the performance of the Xpert MTB/RIF assay in the diagnosis of extrapulmonary tuberculosis.

Acknowledgments

The authors would like to thank the technical staff for carrying out this work.

Ethical permissions: The institute's ethical review committee approved the study [99/ETH/GMC/ICMR].

Authors' contributions: Study concept and design: DZ and AF, drafting the manuscript and data analysis: DZ and MM, critical review of the manuscript: RN and FK.

Conflicts of interests: None declared by authors.

Funding/Supports: None declared by authors.

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