RAPID TOOLS- THE NEW ARMAMENTARIUM FOR DIAGNOSIS OF TUBERCULOSIS VS CLASSIC TOOLS

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ABSTRACT

BACKGROUND
Tuberculosis remains amongst deadliest communicable diseases. Number of diagnostic tests are available, but Nucleic acid amplification techniques, the new armamentarium for diagnosis are rapid and sensitive. The aim of this study is to compare such rapid diagnostic tests with conventional culture method and AFB smear examination.

MATERIALS AND METHODS
Total of 895 clinical samples (July 2015 to April 2017) were processed using smear microscopy, culture on LJ medium and real time PCR. Additionally, few samples were subjected for liquid culture (MGIT) and cartridge based nucleic acid amplification test (CBNAAT).

RESULTS
Out of 895 samples [including 72 pulmonary samples (8.05%) and 823 (91.95%) extrapulmonary] tested, smear microscopy was positive in 22 samples (2.45%), while culture was positive for 37 samples (4.13%). The TB PCR results were positive in 206 (23.00%) samples out of 895. MGIT (used in 75 samples) was positive in 21.33% samples (16 out of 75). CBNAAT was used on 50 PCR positive samples and was positive in 46 samples.

CONCLUSION
Conventional methods remain the gold standard for diagnosing pulmonary TB; however, poor performance of these on extrapulmonary samples and delayed diagnostic times demand for more rapid and sensitive nucleic acid amplification techniques. The major advantage of CBNAAT in simultaneously detecting Rifampicin resistance is especially beneficial in patients with MDR and HIV associated TB. Also, MGIT proved to be a valuable alternative to solid culture in terms of earlier detection. But for maximum recovery of Mycobacteria, both conventional and rapid methods should be incorporated.

KEYWORDS
Tuberculosis (TB), Ziehl-Neelsen (ZN), Lowenstein-Jensen (LJ) Medium, Polymerase Chain Reaction (PCR), Mycobacterium Growth Indicator Tube (MGIT), Cartridge Based Nucleic Acid Amplification Test (CBNAAT)/ Xpert MTB/RIF.

BACKGROUND

Tuberculosis (TB), one of the major air-borne infectious bacterial diseases that is caused by the Bacterium Mycobacterium tuberculosis (MTB) remains a major worldwide health problem. It ranks as the second leading cause of death from an infectious disease worldwide after the human immunodeficiency virus (HIV).[1] As per WHO Global TB Report 2016, out of the estimated global annual incidence of 10.4 million TB cases, India accounts for more than one-quarter of the World's TB cases and deaths.[2]

The situation is further exacerbated with the increasing incidence of drug resistant TB.

Hence, early diagnosis plays a vital role in control of TB.[3] There are a number of tests available for the diagnosis of mycobacterial infections; however, it remains a conundrum with no solution.

Tuberculosis can involve any organ system in the body. While pulmonary tuberculosis is the most common presentation, extra pulmonary tuberculosis (EPTB) is also an important clinical problem. The term EPTB has been used to describe isolated occurrence of tuberculosis at body sites other than the lung.[4]

Ziehl-Neelsen (ZN) smear microscopy is a rapid and cheap method to detect acid fast bacilli, but it has poor sensitivity.[5] Amongst the many different culture media devised for growing the tubercle bacillus, egg-based medium, in particular Lowenstein-Jensen (LJ) medium are among the best known for isolation of M. tuberculosis. Although, considered as the gold standard, but is slow and usually takes a long time to yield a final result and delays diagnosis by 6 - 8 weeks, and requires proper infrastructure and technical expertise.[1,4,5]

Major difficulty of these traditional bacteriological methods is seen especially with clinical samples that contain

small number of organisms or with the extrapulmonary samples (2). This can adversely affect the yield and hence cause diagnostic delays or misdiagnosis.[6]

Rapid diagnosis is vital for tuberculosis control and use of the most rapid methods available for isolation of Mycobacterium tuberculosis complex is therefore advocated.[7]

The Mycobacteria Growth Indicator Tube (BBL MGIT) is one such rapid liquid culture system, which has been found useful by a no. of studies conducted in a tertiary care setting in India.[8,9] It contains modified Middlebrook 7H9 broth base in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with ruthenium pentahydrate) in an atmosphere of 10% CO_2. This compound is sensitive to the presence of dissolved oxygen in the broth. As the microorganisms grow in the media, oxygen gets depleted, allowing the fluorescence to be detected automatically over time. When supplemented with MGIT Growth Supplement and PANTA, it provides an optimum medium for growth of a majority of mycobacterial species.[10]

The PCR is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The molecular based diagnosis by PCR technique is faster and sensitive. Nucleic acid amplification using the principle of polymerase chain reaction (PCR) has the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity.[11] The potential of PCR as a diagnostic test for tuberculosis has been investigated in a large number of studies.[12,13]

More recently, the WHO endorsed the GeneXpert (Xpert® MTB/RIF assay) for the diagnosis of TB.[14] The GeneXpert utilizes a DNA-PCR technique for simultaneous detection of Mycobacterium tuberculosis and Rifampicin resistance related mutations. It is the first fully automated bench top cartridge based nucleic acid amplification (CB-NAAT) assay for TB detection that includes all necessary steps of DNA-PCR. It gives results within 2 hours.[15]

A number of diagnostic tests are available, but Nucleic acid amplification techniques, the new armamentarium for diagnosis of Mycobacterium tuberculosis (MTB) in clinical specimens of pulmonary and extrapulmonary tuberculosis cases are rapid and sensitive. These techniques not only provide the advantage of rapidity of diagnosis, but also detect even low MTB genomic copies in various specimens. These not only help in early diagnosis and management of tuberculosis, but also curtail the transmission of the disease.[14]

In view of this, the present study was undertaken to determine the utility of such Rapid Diagnostic tools like MGIT, Real time PCR and CBNAAT with conventional modalities like ZN staining and LJ culture.

MATERIALS AND METHODS
This study was conducted from July 2015 to April 2017 in the Mycobacteriology and Molecular Lab. of a tertiary care hospital in northern India. A total of 895 clinical samples were tested for Tuberculosis (TB) using conventional microbiological techniques of diagnosis like Ziehl-Neelsen (ZN) staining and culture on LJ medium (according to the standard procedure) and with rapid techniques using real

time PCR assay (targeting 16S rRNA gene). Additionally, a few samples were also subjected for liquid culture (MGIT) and cartridge based nucleic acid amplification test (CBNAAT). The CBNAAT assay was done at Civil Hospital, Ludhiana.

Processing of the Samples
Clinical samples (both Pulmonary and Extrapulmonary) like Sputum, BAL, CSF, pleural fluid, ascitic fluid, pus, urine, lymph nodes, biopsies and other tissues from clinically suspected cases of TB were received at our laboratory in sterile containers. Two samples were received from each patient. One sample was processed for AFB smear and culture, while the second sample was processed for Real Time PCR.

For AFB smear and culture, the samples were first homogenized and concentrated using Petroff's method (4% NaOH solution). This mixture was homogenized by allowing it to stand at room temperature for 15 - 20 minutes. After this step, phosphate buffer was added and mixed well. The specimen was then centrifuged at 3000 rpm for 15 - 20 minutes.[16,17] After centrifugation, a portion of sediment was directly inoculated onto Lowenstein-Jensen medium slopes; the other portion was used for preparation of direct smear for Ziehl-Neelsen staining. Fluid specimens from sterile sites such as CSF, pleural fluid, etc. were centrifuged and a portion of sediment was directly inoculated in LJ medium without prior treatment (Petroff's method). The tissue biopsy specimens were minced and homogenized in a sterile homogenizer and a portion of the homogenate was directly inoculated onto Lowenstein-Jensen medium slopes and other portion was used for making smears for Ziehl-Neelsen staining. The Lowenstein-Jensen medium bottles were incubated at 37°C in the incubator. Culture readings were monitored weekly and discarded as negative in case of no growth at the end of 8 weeks.[18]

DNA extraction was performed on all the received samples by Genosen's DNA extraction mini kit (Genome Diagnostics Pvt. Ltd., New Delhi, India). TB DNA real time amplification was done by Genosen's MTb/ MOTT (rotor gene) Real Time PCR kit (Genome Diagnostics Pvt. Ltd., New Delhi, India) as per the manufacturer's instructions. PCR Assay Based on DNA Coding for 16S rRNA for detection and identification of mycobacteria in clinical samples was used.[19]

Few samples were processed by the Micro MGIT liquid culture system. A lyophilized vial of BBL MGIT PANTA (containing Polymyxin B, Azlocillin, Nalidixic acid, Trimethoprim and Amphotericin B) antibiotic mixture was reconstituted with 15 mL of BACTEC MGIT Growth Supplement (containing oleic acid albumin dextrose, catalase, Polyoxyethylene stearate) and 0.8 mL of this was added aseptically into the 7 mL MGIT tube prior to sample inoculation. After that 0.5 mL of the digested, decontaminated and concentrated specimen suspension was added to the MGIT tube and incubated at 37°C in the incubator for 42 days. Tubes are read daily starting from the second day of incubation in the BACTEC Micro MGIT Fluorescence Reader one by one for interpretation. Tubes showing reading above 14 mark upto 20 are considered as positive, whereas readings 1 - 13 are considered as negative. Positive tubes are stained for acid-fast bacilli for ruling out bacterial contamination.[20]

GeneXpert testing was performed according to the manufacturer's instructions.[21] The Xpert MTB/ RIF assay

can be used directly on CSF specimens and homogenized extrapulmonary specimens (from biopsies of lymph nodes or other tissues). Pleural fluid is considered a suboptimal specimen; however, pleural biopsy provides the preferred specimen. Specimens such as stool, urine or blood are not recommended for the testing given the lack of data on the utility of Xpert MTB/ RIF on these specimens.[21] The sample is mixed with the reagent that is provided with the assay at a ratio of 2: 1, manually agitated and kept for 10 mins at room temperature, then shaken again and kept for 5 mins; 2 mL of the inactivated material was transferred to the test cartridge and inserted into the test platform. All processing from this point on is fully automated. Results from the Xpert MTB/ RIF assay indicate whether or not MTBC was detected in the sample. If MTBC was detected, the results also state whether resistance to RIF was detected or not detected. (Detected: Mycobacteria have a high probability of resistance to RIF; Not detected: Mycobacteria are probably susceptible to RIF).[22]

The study was initiated after due approval by Institutional Ethical Committee and samples collected after informed written consent.

RESULTS

Out of 895 samples [including 72 pulmonary samples (8.05%) and 823 (91.95%) extrapulmonary] tested, smear microscopy was positive in 22 samples (2.45%) which included 6 sputum, 4 BAL, 6 Pleural Fluid, 2 tissues, 3 Ascitic fluid and 1 Pus sample, while culture was positive for 37 samples (4.13%) which included 8 sputum, 3 Pus, 6 BAL, 8 Pleural Fluid, 4 tissues, 4 Ascitic fluid and 2 CSF samples. The TB PCR results were positive in 206 (23.00%) samples out of 895.

- MGIT (used in 75 samples) was positive in 21.33% samples (16 out of 75).
- CBNAAT was used on 50 PCR positive samples and was positive in 46 samples.
- The sample distribution is shown in Figure 1.
- The mean time to detection for MTB was 28.81 days (7 - 48 days) by LJ media, 9.66 days (2 - 39 days) by MGIT, less than 1 day by PCR following extraction and less than 2 hours by CBNAAT.

| Type of Specimen	Smear Positive (%)	Culture Positive (%)	PCR Positive (%)	MGIT Positive (%)	CBNAAT Positive (%)
Sputum	33.33%(06/18)	44.44% (08/18)	50.00% (09/18)	66.66% (04/06)	-
BAL	7.40% (04/54)	11.11% (06/54)	33.33% (18/54)	14.28% (03/21)	100% (1/1)
CSF	-	0.64% (02/310)	13.22% (41/310)	0% (0/5)	91.66% (22/24)
Pleural Fluid	4.13% (06/145)	5.51% (08/145)	26.8% (39/145)	21.42% (06/28)	85.71% (06/07)
Tissue	1.48% (02/135)	2.96% (04/135)	25.9% (35/135)	25.0% (01/4)	85.71% (06/07)
Urine	-	2.50% (02/80)	15.0% (12/80)	-	-
PUS	1.29% (01/77)	3.89% (03/77)	31.16% (24/77)	16.66% (01/6)	100% (06/06)
Ascitic Fluid	3.94% (03/76)	5.26% (04/76)	36.84% (28/76)	20.0% (01/5)	100% (05/05)
Total	**2.45% (22/895)**	**4.13% (37/895)**	**23.00% (206/895)**	**21.33% 16/75**	**92.00%(46/50)**
Table 1. Type of Specimens and Positivity Rate					
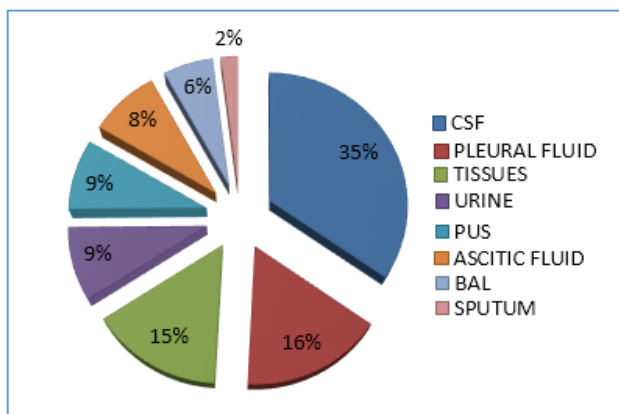
Figure 1. Distribution of Various Clinical Samples

DISCUSSION

In this study, we compared the conventional methods with rapid methods for diagnosing TB. Current global TB control efforts are based on diagnosis of cases followed by adequate treatment. Rapid diagnosis of TB is absolutely necessary in a developing country like India where early detection of TB cases is the key to successful treatment and reduction of disease transmission. In areas with high prevalence of TB and HIV, better and more efficient diagnostic tests are required.[2]

Sample distribution in our study varied greatly as maximum samples were of CSF followed by pleural fluid and tissues, whereas the pulmonary samples were less. So our study had predominantly extrapulmonary samples. In a study by Muhammad et al, pulmonary samples predominated as compared to extrapulmonary samples and there was 35.7% smear positive by ZN staining, 52.0% by culture and 62.2% by PCR.[23] The high smear and culture positivity could be attributed to the significantly more number of sputum samples as compared to our study. Siddiqui et al observed in their study that the maximum number of samples received were of CSF (37%).[24] This is similar to the study done by us, wherein CSF samples predominates 507 (40.17%). The next 2 commonest samples received in study by Siddiqui et al were ascitic fluid (29%) and pleural fluid (24%) respectively. In another study from Iran, the most frequent type of samples received among suspected extrapulmonary was CSF (33.84%) followed by pleural fluid (27.69%).[25]

Major drawback of direct smear examination using Ziehl-Neelsen (ZN) staining for the diagnosis of tuberculosis (TB) as employed in most low-income countries is cheap and easy to use, but has very low sensitivity and is especially problematic with paucibacillary specimens. Mycobacterial culture using LJ medium, although considered as the gold standard has drawbacks in being slow and usually takes 4 - 6 weeks' time to yield a positive result. It also requires proper infrastructure and technical expertise.[26]

In our study mean time to detection varied among both the culture techniques, results by MGIT liquid culture medium come earlier as compared to LJ medium. Similar

findings has also been stated in another study by Sebastian et al and various other studies.[27,28]

Although, MGIT was also carried out in our centre for TB diagnosis and is considered as a rapid method, but due to its use in very less number of samples in our study the results cannot be accurately compared with PCR and other conventional modalities.

PCR techniques though very rapid as compared to conventional methods are costly. It has the potential to be a cost-effective alternative, provided the diagnosis can be determined with one specimen examination. If diagnosis can be established faster, and the diagnostic process becomes less cumbersome for the patient PCR may reduce delay both in diagnosis and in the start of treatment.[29] PCR has a good potential and can be a useful adjunct to diagnose clinical tuberculosis, particularly in smear negative paucibacillary cases. The primary limitation of PCR arises from the absence of a suitable gold standard to assess its efficiency. When culture is used as a gold standard in comparison studies, samples containing non-viable Mycobacteria may lead to a false positive PCR, thereby misleading clinicians. However, studies with larger numbers need to be taken up in order to validate these results.[30]

In a study from Delhi, the sensitivity of PCR for extrapulmonary tuberculosis (suspected tubercular lymphadenitis) was highest (82%) among all the investigations in comparison with the combined sensitivity of AFB Smear and Culture (36% and 23% respectively).[31] In this study, the physician integrated the results of all procedures before pronouncing a diagnosis, the PCR results were compared with the gold standard which was the result of all tests combined. In this study, author also mentioned that the use of more sensitive microbiological techniques such as liquid culture system would result in higher culture positivity. In another study on lymph node aspirate from 23 patients in whom the cytological diagnosis was consistent with tuberculosis, a PCR positivity of 83% was reported based on the amplification of IS 6II0 insertion sequence, which is present at a copy number of between 1 and 24 in M. tuberculosis.[32] The authors concluded that the diagnosis of granulomatous lymphadenitis is consistent with TB can be given, even though AFB smears are negative. Likewise, a study on fine needle aspirate from 31 patients with tubercular lymphadenitis reported a PCR sensitivity of 61% in comparison with AFB smear and culture positivity of 10% and 19% respectively.[33] In a study by Negi et al, PCR test was also shown to be reasonably sensitive (75.9%) in diagnosis of extrapulmonary TB.[34] Less positivity rate in our study could be due to less representative samples in our study.

In many studies, problems with false positive PCR results have been reported. Specificity of PCR results varies between laboratories due to procedural differences, differences in cross-contamination rates and the choice of primers. The drawbacks of PCR are its high cost, specific requirement of infrastructure, equipment and expertise. The conventional in-house NAATs require well-trained technical staff and sophisticated equipment. Also, for these PCR, there are no validation studies done in large sample size. As the conventional NAATs have various steps from DNA isolation to amplification, there are also chances of cross-contamination from environmental factors or carry-over contamination

from other samples.[35] In our study, all the efforts were taken to overcome these limitations by proper preventive laboratory techniques and use of sterilized methods.

At present, India is experiencing an epidemic of TB. Considering the number of cases diagnosed with TB in India, there is an urgent need to use multiple/ newer diagnostic modalities for rapid detection of MTB to control the transmission of TB. Molecular methods such as PCR and CBNAAT are underutilized, and it is becoming imperative to have these diagnostic tests available at as many health centres as possible. The clinical utility of detecting MTB by Molecular Nucleic Acid Amplification Methods is its reduction in the time to detection and its accuracy in detecting the pathogen in AFB smear-negative paucibacillary specimens. These tests should be incorporated as a suitable public health tool in a country like India, as the test is rapid and early diagnosis of TB is crucial for prompt treatment and for the control of disease transmission.

Xpert MTB/RIF, an automated cartridge-based molecular technique detects mycobacterium tuberculosis and rifampicin resistance within two hours has been endorsed by WHO for rapid diagnosis of TB. It is a simple bench top point of care diagnostic assay that can be performed with minimal training.[15] There have been various studies from India with a relatively large sample size that have evaluated the efficacy and performance of CBNAAT/ Xpert MTB/ RIF in patients with pulmonary samples; however, very few studies have evaluated its performance on extrapulmonary samples. In a study by Sharma et al,[36] it was observed that the Xpert assay detected 71% of the "confirmed TB" cases where culture and response to anti-TB treatment were positive. It also identified 68% of "possible TB" cases where culture biochemical and histopathology reports were negative and only the response to anti-TB treatment was positive. Of the cases where all parameters were negative, Xpert MTB/ RIF detected 0.8% of these cases as positive. High specificity of the assay in all the specimens explains the low false positivity achieved by this diagnostic tool, which can thus be a useful rule-in test for EPTB diagnosis. In another study by Vadwai et al,[37] Xpert MTB/ RIF demonstrated a sensitivity of 83% and specificity of 73% for 533 EPTB patients.

While Xpert MTB/ RIF maybe the foremost choice amongst all molecular diagnostic tests, it has its own limitations. Resistance to RIF is taken as a surrogate marker for MDR-TB, but certain strains may exhibit only mono-resistance to RIF that may not warrant full line MDR therapy, thus leading to over-estimation of the MDR-TB cases. Other drawbacks of Xpert MTB/ RIF are requirement of stable electrical power supply, temperature control and annual calibration of instrument. Regardless of all these limitations, addition of Xpert MTB/ RIF assay to the present set of diagnostic modalities for TB on account of its unambiguous, rapid results, and high sensitivity and specificity will facilitate early diagnosis.[38]

The primary limitation in our study was the differences in sample size to compare various diagnostic modalities. Since the sample size was too small, and therefore studies with larger numbers need to be taken up in order to validate these results.

In conclusion, this study discusses methods which are able to detect M. tuberculosis rapidly and directly in clinical samples and might become a valuable, cost-effective and

alternative tool for quick diagnosis of tuberculosis. However, further work is needed for improving sensitivity, specificity and reproducibility of this test and to make it more user friendly and cost effective.

CONCLUSION

Conventional methods of smear microscopy and culture remain the gold standard for diagnosing pulmonary TB; however, poor performance of these conventional methods on extrapulmonary specimens and delayed diagnostic times demands for more sensitive and specific nucleic acid amplification techniques using polymerase chain reaction. PCR is a very rapid and accurate diagnostic tool for early detection of TB, particularly for EPTB. In a country like India with such high burden of TB and limited resources for diagnosing TB PCR is found to be very valuable for rapid identification and early diagnosis, hence necessary for earlier isolation, treatment, improved patient outcome and more effective public health interventions. The major advantage of CBNAAT in simultaneously detecting Rifampicin resistance is especially beneficial in patients with MDR and HIV associated TB and should be studied further. Also, MGIT proved to be a valuable alternative to solid culture in terms of earlier detection in settings where PCR is not available. But for maximum recovery of Mycobacteria, both conventional and rapid methods should be incorporated.

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