Utility of smear microscopy and GeneXpert for the detection of *Mycobacterium tuberculosis* in clinical samples

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Abstract

Introduction Rapid identification of *Mycobacterium tuberculosis* (MTB), its resistance to rifampicin and differentiation of MTB from nontuberculous mycobacteria (NTM) is necessary in the management of mycobacterial diseases. Culture, the "gold standard" for the detection of MTB, is time consuming. In spite of its rapidity and low cost, smear microscopy has poor sensitivity for the detection of acid-fast bacilli (AFB). A real-time PCR based rapid diagnostic method like GeneXpert MTB/RIF assay can simultaneously detect *M. tuberculosis* and rifampicin (RIF) resistance. Hence, we aim to compare the performance of GeneXpert MTB/RIF assay with smear microscopy and culture.

Methods In this descriptive cross-sectional study, we compared the performance of GeneXpert in pulmonary (N=127) and extrapulmonary (N=48) clinical specimens with other diagnostic methods like culture, Auramine O (AO), and Ziehl Neelsen (ZN) staining. Rifampicin resistance was detected only by GeneXpert. Demographic data and clinical history of the subjects were collected from the patient's hospital records.

Results AO and ZN staining when compared with mycobacterial growth indicator (MGIT) culture showed the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of 68.6, 95.7, 80, 92.4, 90.3% and 65.7, 95.7, 79.3, 91.8, 89.7%, respectively. The sensitivity, specificity, PPV, NPV and accuracy of GeneXpert was 88.6, 93.6, 77.5, 97.0 and 92.6%, respectively.

Conclusions GeneXpert is the best available rapid diagnostic method as it can detect MTB and rifampicin resistance gene simultaneously. Accuracy and negative predictive value of GeneXpert was found to be better than AFB staining. Thus, a negative GeneXpert test can rule out TB. Further, a negative GeneXpert and a positive smear microscopy results indicate the presence of NTM. However, GeneXpert is expensive and needs sophisticated instrument when compared to smear microscopy.

Keywords Tuberculosis, GeneXpert, molecular diagnostic method, non-tuberculous mycobacteria, *Mycobacterium tuberculosis*, culture, staining.

Introduction

In India, tuberculosis (TB) is a community health problem. According to WHO Tuberculosis Report (2017), 24% of the global TB burden is seen in India.¹ Commonly used diagnostic tests like smear microscopy and conventional culture have reduced sensitivity and long turnaround time, respectively.^{2,4} Delay in

diagnosis increases morbidity, mortality, and spread of disease in the community.

Xpert MTB/RIF, a programmed, cartridge-based real-time PCR, detects *M. tuberculosis* (MTB) and rifampicin resistance using molecular beacons within two hours.⁵ WHO strongly recommends Xpert assay for the diagnosis of TB and multidrug-resistant (MDR) TB in patients

Received: 24 October 2019; revised: 23 January 2020 and 04 March 2020; accepted: 03 April 2020.

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Article downloaded from www.germs.ro

Published June 2020

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ISSN 2248 - 2997

ISSN - L = 2248 - 2997

with HIV infection (WHO 2013).⁶ Most of the studies for the optimization and validation of the GeneXpert test are in patients with HIV infection from African and other countries and a few from India.^{7,9}

Acid fast bacilli (AFB) smear is the least expensive and widely used diagnostic tool for pulmonary tuberculosis. However, it has low sensitivity and needs a concentration of 10000 colony forming units/mL to be seen as positive under a microscope. Hence a sample with low bacterial count results in a negative report.¹⁰ Accurate and timely diagnosis of TB will reduce the transmission of the disease and unnecessary antibiotic use.¹¹ Therefore the present study is undertaken to compare the performance of GeneXpert and smear microscopy with mycobacterial growth indicator (MGIT) culture to choose the best available test for the diagnosis of TB.

Methods

Study design and duration

This is a descriptive cross-sectional study, performed for a period of six months (June to December 2017), at the Department of Microbiology, Kasturba Medical College (KMC), Mangalore, India. This study was approved by the Institutional Ethics Committee, KMC, Mangalore, Manipal University, Ref No: IEC KMC MLR 02-16/34.

Sampling method

Samples (n=175) from suspected TB patients (age >18 years) received at the Department of Microbiology, KMC hospitals, Mangalore, India for routine GeneXpert MTB/RIF assay and AFB culture were included in the study by convenient sampling method. All sputum samples of patients who were not having signs and symptoms of TB and samples from patients below 18 years of age were excluded from the study.

Demographic data like age, sex, history of lung diseases, blood glucose level, and HIV sero-status were collected from the laboratory information system. Clinical data like degree and duration of fever, chest pain, dyspnea, hemoptysis, weight loss, duration of cough, and

the extrapulmonary sites of tuberculosis was collected from patient hospital records.

Test procedures

All pulmonary and extrapulmonary samples were subjected to Auramine O (AO) fluorescent staining, Ziehl Neelsen (ZN) staining, GeneXpert MTB/RIF (Cepheid, Sunnyvale, US) assay and MGIT culture. Non-sterile clinical samples were pre-treated according to the conventional N-acetyl-L-cysteine-NaOH digestion-decontamination procedure. Specimens from sterile sites were centrifuged and used directly.¹² All stains and chemicals used in the study were procured from Hi Media Laboratories Ltd. Mumbai, India unless otherwise specified.

AFB smear and staining

Four smears were made from the sample; two direct and two smears after concentration and decontamination. Smears were stained by ZN and AO staining to demonstrate AFB and interpreted as per the Revised National Tuberculosis Control Programme (RNTCP) guidelines.^{13,14}

GeneXpert MTB/RIF assay

Samples were utilized for Xpert MTB/RIF as per the manufacturer's instructions. Briefly, samples were treated with NaOH and isopropanol at 1:2 ratios. After 15 min time interval, the mixture was injected into the Xpert MTB/RIF cartridge which was subsequently loaded to the GeneXpert instrument.⁶ In samples detected as positive for *M. tuberculosis* by the device, presence or absence of rifampicin resistance gene was also noted.

AFB culture

Clinical samples from the non-sterile site were treated with N-acetyl-L-cysteine-NaOH and sterile specimens were used directly for culture after concentration.¹² The concentrated and decontaminated sample was emulsified in 1 mL of sterile phosphate buffered saline (pH 6.8) and 500 µL of the concentrate was inoculated into MGIT 960 tubes.¹⁵ All inoculated tubes were incubated in the MGIT 960 instrument (Becton

Dickson and Company, Andover, MA, USA) until they were detected as positive by the machine. All negative tubes were incubated for at least eight weeks and then discarded. Isolates were identified as MTB by their rate of growth, and immunochromatographic test kit (SD MPT64TB Ag kit). Any acid-fast bacterial growth that was not detected as MTB by the immunochromatographic method was considered as NTM.

Genus *Mycobacterium* and MTB specific PCR targeting the mycobacterial *hsp65* gene was used to confirm the discrepant results of GeneXpert and culture. Duplex PCR was performed on extracted DNA from culture and clinical samples as described by Singh et al.¹⁶ The following primer pairs were used: MTB: F5'TGCTCGAGAAGGTCATCGGA3' and R5'-TCACCTGACCACCGGTGAGA3', NTM: F5'GAGGTGGCCAAGAAGACGGAC-3' and R5'-AGCAGCGGCTTGCCCTCTGA3'. Amplicon size of 195 and 515 bp indicated the presence of MTB and NTM, respectively.

Data analysis

Descriptive data is presented as frequencies and percentages. Sensitivity, specificity, negative predictive value (NPV) and positive predictive values (PPV) were calculated for GeneXpert and smear microscopy using culture as the gold standard, and expressed as percentages.

Disease prevalence was taken into consideration (MTB culture positive cases 20%) for the calculation of PPV, NPV and accuracy. The diagnostic test evaluation calculator MEDCALC easy-to-use statistical software https://www.medcalc.org/calc/diagnostic_test.php was used for statistical analysis.

Results

Demographic and clinical data

A total of 175 samples were included in our study. Samples comprised of sputum (n=33), bronchial alveolar lavage (BAL n=86), pleural fluid (n=8), cerebrospinal fluid (CSF n=18), pus (n=7), gastric lavage (n=13) and lymph node aspirate (n=10). Among the study subjects, 70.8% (n=124) were males, and 29.1% (n=51) females. The age of the patients ranged from 18

to 89 years with a median of 55 and interquartile range (Q3-Q1) of 25.5 years.

Diabetic patients represented 24% (n=42) of the study population, comprised of 33 men and nine women. Among the study population, 4% (n=7) were seropositive for HIV antibodies: four males and three females. History of TB was documented in 13.7% (n=24), whereas 15.4% (n=27) had a history of lung disease other than TB, like chronic obstructive pulmonary disease (COPD), pneumonia, pneumoconiosis, bronchiectasis, etc. They were positive for TB by GeneXpert and or MGIT culture. However, 120 (68.5%) patients had no history of TB or any other lung disease.

Based on the clinical signs and symptoms, three patients were treated with first-line anti-tuberculosis drugs, although their samples tested negative for AFB by all the methods used. They showed improvement during treatment. After thorough investigations, they were clinically diagnosed to have TB vasculitis and meningoencephalitis, TB of the spine and sarcoidosis. The majority of the signs and symptoms shown by the patients are depicted in Figure 1.

Evaluation of different diagnostic methods

Results of various diagnostic tests for the detection of AFB in pulmonary and extrapulmonary specimens are illustrated in Table 1. Among the 40 samples positive by GeneXpert, five (12.5%) cases were rifampicin resistant. The performance of different staining methods and GeneXpert in comparison to MGIT culture is presented in Table 2, along with their sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) values. The performance of different staining methods in comparison with GeneXpert is presented in Table 3.

Discussion

This study assesses the diagnostic usefulness of smear microscopy and GeneXpert to detect MTB in both pulmonary and extrapulmonary samples (Table 1). The results obtained are compared with the MGIT culture, which is taken as the gold standard.

Figure 1. Signs and symptoms seen in the study subjects

Table 1. Comparison of the results of different diagnostic methods for pulmonary and extrapulmonary samples

Sample type	Auramine O staining		Ziehl Neelsen staining		MGIT culture		Gene Xpert	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
Pulmonary sample (n=127)	23 (18.1)	104 (81.9)	21 (16.5)	106 (83.5)	26 (20.5)	101 (79.5)	29 (22.8)	98 (77.2)
Extrapulmonary samples (n=48)	7 (14.5)	41 (85.5)	8 (16.6)	40 (83.4)	9 (18.8)	39 (81.2)	11 (22.9)	37 (77.1)
Total (n=175)	30 (17.1)	145 (82.9)	29 (16.6)	146 (83.4)	35 (20)	140 (80)	40 (22.9)	135 (77.1)

The staining methods AO and ZN showed the sensitivity, specificity, PPV, NPV and accuracy of 68.6, 95.7, 80, 92.4, 90.3% and 65.7, 95.7, 79.3, 91.8, 89.7%, respectively. However, AO is less time consuming than ZN staining. The same has been reported by earlier workers also.¹⁷ A study by Agarwal et al. showed the sensitivity and specificity of ZN staining to be 22.2, and 78.5%.¹⁸ In our study AO staining had a higher sensitivity (65.7%), and both had similar specificity (95.7%). This is because the earlier study had used the direct samples for staining, and we have used two smears each of direct and NALC-NaOH treated samples.

MGIT culture showed a negative result for six (3.4%) sputum samples (five patients on

treatment and one not treated), but these samples were positive by AO staining (Table 2). It could be the dead bacilli which took up the stain, and one sample could have been a false positive as this sample was negative for *Mycobacterium* by PCR. BAL samples (n=12) were positive by GeneXpert and negative by AO staining (Table 3). AFB smear examination requires 10000 CFU/mL to give a positive result.¹⁰ Our BAL samples also could have had few bacilli making the smear negative. Moreover, these GeneXpert positive BAL samples were positive by PCR and had grown MTB by MGIT culture, showing that the GeneXpert result was truly positive.

In the present study, five samples were negative by GeneXpert and positive by ZN

Table 2. Comparison of results of staining techniques and GeneXpert with MGIT culture

Tests	Results	MGIT culture		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
		Positive n (%)	Negative n (%)					
Auramine O staining	Positive, n (%)	24 (80)	6 (20)	68.6	95.7	80	92.4	90.3
	Negative, n (%)	11 (7.6)	134 (92.4)					
Ziehl Neelsen staining	Positive, n (%)	23 (79.3)	6 (20.7)	65.7	95.7	79.3	91.8	89.7
	Negative, n (%)	12 (8.2)	134 (91.8)					
GeneXpert	Positive, n (%)	31 (77.5)	9 (22.5)	88.6	93.6	77.5	97.0	92.6
	Negative, n (%)	4 (3.0)	131 (97.0)					

Table 3. Comparison of results of staining techniques with GeneXpert

Tests	Results	Gene Xpert		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
		Positive n (%)	Negative n (%)					
Auramine O staining	Positive, n (%)	28 (93.3)	2 (6.7)	70	98.5	92.2	92.9	92.8
	Negative, n (%)	12 (8.3)	133 (91.7)					
Ziehl Neelsen staining	Positive, n (%)	24 (82.8)	5 (17.2)	60	96.3	80.2	90.6	89
	Negative, n (%)	16 (11)	130 (89.0)					

staining, of which two were AO staining positive and had grown NTM (Table 3). Thus, culture and staining will be helpful when NTM infections are suspected, as GeneXpert can detect only *M. tuberculosis*. However, three GeneXpert negative and ZN staining positive samples did not show growth in culture and were negative by PCR for MTB. These three patients were on anti-tuberculosis drugs based on clinical suspicion of TB. Hence it could be the dead bacilli or atypical mycobacteria, which showed ZN staining positive. Similar results were reported by Chao et al.¹⁹

MGIT 320 was used for AFB culture as it detects growth and drug resistance faster than culture using Lowenstein Jensen (LJ) medium.²⁰ In our study, the sensitivity, specificity, PPV, NPV and accuracy rate of GeneXpert was 88.6,

93.6, 77.5, 97.0 and 92.6%, respectively (Table 2). Our results are consistent with earlier studies.^{21,22} A meta-analysis study consisting of 9557 participants from 27 studies revealed the sensitivity and specificity of GeneXpert assay on respiratory specimens to be 89 and 99% respectively.²³ A survey on extrapulmonary samples by Habous et al. reported the sensitivity specificity, PPV and NPV of GeneXpert to be 82.7%, 100%, 100%, and 92.8%, respectively.²⁴ Slightly lower sensitivity and specificity were seen in the present study; this could be due to the small sample size that included even a few treated cases.

GeneXpert was positive for nine samples, (five were BAL, two were sputum, and one was pleural fluid) that were negative by MGIT culture (Table 2). However, these nine samples were confirmed

as positive for MTB by PCR. All these nine patients were on treatment, which could be the reason for negative MGIT culture result. In our study, 15.3% (n=27) patients had a history of lung disease (Figure 1), and they were positive for TB by GeneXpert and or MGIT culture. First-line anti-tuberculosis drugs were prescribed for three patients even though their samples tested negative by all the methods. They improved during treatment. These findings emphasize the need for correlation of laboratory test result with the clinical presentation of the patients. If the clinical findings are strongly suggestive of tuberculosis, patients should be started on anti-tuberculosis drugs irrespective of the laboratory results (WHO 2010).²⁵

Multidrug-resistant tuberculosis (MDR-TB) is an emerging problem and is estimated to be 3.9% in new TB cases and 21% in treated cases.^{26, 27} In our study, 12.5 % (5/40) of the samples were rifampicin (RIF) resistant as detected by GeneXpert, which included three treated cases and two new cases. A study from Punjab, India, reports RIF resistance to be 9.9% by GeneXpert.²⁷ This shows RIF resistance varies from one geographic area to the other. Moreover, their sample size was larger when compared to ours.

Our study has a few limitations, such as the distribution of pulmonary and extrapulmonary samples, which was not uniform. Further, the sensitivity and specificity of GeneXpert assay in detecting rifampicin resistance could not be determined, as the phenotypic method was not available for confirmation.

Conclusions

GeneXpert is found to be a rapid test for the diagnosis of TB and rifampicin resistance when compared to smear microscopy and culture. A smear-positive and GeneXpert negative result gives a high suspicion of NTM infection. Thus, smear microscopy done in conjunction with GeneXpert will have immense diagnostic value in differentiating MTB, RIF resistant TB and NTM infections in resource poor country where TB culture facilities are not available.

Authors' contributions statement: DA performed the experiments, interpreted the results, analyzed the data and drafted the manuscript. DB designed the study, supervised the experiments, critically revised the manuscript. All the authors approved the final version of the manuscript to be published, and agree to be accountable for all aspects of the work.

Conflicts of interest: All authors – none to declare.

Funding: The study has received financial support from Indian Council of Medical Research New Delhi, India (Reference ID: 2016-00558).

Acknowledgements: DA thanks the Indian council of Medical Research, New Delhi for providing short term student research grant for two months to carry out the study. Authors thank the staff of the Microbiology department and Dean KMC Mangalore for their support.

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Please cite this article as:

Arora D, Dhanashree B. Utility of smear microscopy and GeneXpert for the detection of *Mycobacterium tuberculosis* in clinical samples. *GERMS* 2020;10(2):81-87. doi: 10.18683/germs.2020.1188