TBSURE: An Improved Multiplex Real-Time PCR for TB Detection and Differentiation from Nontuberculous Mycobacterial Diseases in Adult Indian Population

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Abstract

Background: Tuberculosis (TB), an infectious disease caused by bacterium Mycobacterium tuberculosis, has crossed the borders of developing nations and has now emerged as disease of global emergency due to an alarming increase in cases of co-infection with human immunodeficiency virus (HIV). Conventional diagnostics used for TB diagnosis like microscopy and culture although reliable are time consuming. Rapid diagnosis of the disease is required for early commencement of treatment of TB patients. The diagnosis of extra pulmonary tuberculosis (EPTB) is more troublesome due to limited accessibility of infection sites and lack of accurate differentiation from other granulomatous diseases. Currently, WHO advises use of Xpert MTB/RIF assay for EPTB diagnosis, but this assay cannot be used in resource constrained settings prevalent in most of parts of India.

Aim: We aimed to develop a multiplex real-time PCR assay which not only detects Mycobacterium tuberculosis complex (MTBC) but also differentiates them from nontuberculous mycobacteria (NTM).

Methods: For development and validation of TBSURE, we included 3, 709 EPTB samples from adult Indian patients suspected for TB. TBSURE is a real time PCR diagnostic which uses three Taqman probes based on IS6110, MPB64 and 16S rRNA genes. The sensitivity and specificity of TBSURE was compared with the gold-standard culture test and XpertMTB/RIF assay for TB detection.

Results and Conclusion: TBSURE gave a sensitivity of 92% and specificity of 91% as compared to Xpert MTB/RIF assay, when samples from TB suspected individuals were subjected to both the tests. As compared to AFB culture, TBSURE gave a sensitivity of 93.1% and specificity of 96.5%. Thus, TBSURE is an in-house efficient and affordable diagnostic test for EPTB diagnosis.

Keywords: Tuberculosis; Multiplex real-time PCR; MTBC; NTM; Xpert MTB/RIF assay

Introduction

During the last decade, resurgence of TB has been seen in both developed and developing nations; especially due to increase in cases of co-infection with human immune deficiency virus (HIV). India contributes to approximately one quarter of global TB burden. In 2015, 2.2 million new cases of active TB were detected in India, out of global incidence of 9.6 million [1].

Rapid and reliable diagnosis of disease is required for early start of lengthy treatment for TB. Acid fast bacterial (AFB) culture followed by Ziehl-Neelsen staining of Mycobacteria is time consuming and often not able to differentiate between tuberculous and nontuberculous mycobacteria. The EPTB is even more difficult to detect by AFB culture and smear methods due to low bacillary load. The paucibacillary nature of EPTB, lack of uniform distribution of mycobacteria at various sites in the body makes the early diagnosis of this disease even more cumbersome [2,3].

The diagnosis of smear positive pulmonary tuberculosis (PTB) has been established but that of EPTB, smear negative PTB and HIV-TB co-infection is still posing many challenges to scientists throughout the world. The routinely used smear microscopy for diagnosis of EPTB fails to distinguish between MTBC and NTM. EPTB samples have been diagnosed by a variety of tests like smear microscopy, Interferon gamma release assay (IGRA), histopathology, tuberculin skin test, culture identification, each having its own limitation. These tests also fail to distinguish between MTBC infection and other granulomatous diseases like sarcoidosis, leprosy, systemic lupus erythematosus and NTM [4,5].

Molecular methods like polymerase chain reaction (PCR) are increasingly replacing old methods for EPTB diagnosis due to their precision and lack of need for BSL3 laboratory. Real-time PCR (qPCR) is widely used for laboratory diagnoses of infectious diseases caused by
viruses, bacteria, parasites or fungi [6]. qPCR has further advantages, including high sensitivity, reproducibility, accuracy, reduced false positives due to reduced contamination and does not require running of agarose gel after every reaction, as in conventional PCR reactions.

The insertion sequence IS6110 has emerged as an attractive target for molecular detection of MTBC in a large number of diagnostic methods as it is the most plentiful IS element in the genome of the MTBC bacteria [7-10]. A meta-analysis and meta-regression study established that use of IS6110 probes in PCR, significantly increased the reliability of PCR based diagnostics for MTBC [11]. The presence in multiple copies in mycobacterial genome made this sequence a standard for RFLP analysis to distinguish between isolates of MTBC.

However, the number of isolates with zero copy of IS6110 (zero band in RFLP analysis) are now not only restricted to South-East Asia, but a lot of such cases have been reported in different parts of USA as well, leading to false-negative results affecting the sensitivity of the assay [12,13]. Therefore, use of other probes in addition to IS6110, appears to be a method of choice, to increase specificity of the test. Singh et al. recently developed a multiplex PCR test which uses IS6110, in combination with two other unrelated genes like namely, Hsp65 and DNAJ. This test proved to be better and rapid than AFB culture and smear microscopy, but it had a sensitivity ranging from 73.33% to 84.61%, and the specificity of 80% [14]. For diagnosis of different types of EPTB, IS6110 has been used by different groups to give a specificity of around 100%, but none of these tests gave a good sensitivity [15-18]. There are two reports by Sharma et al. one on TB meningitis and other on osteoarticular TB that uses multiplex PCR using IS6110, MPB64, in conjunction with a 38KDa protein respectively [19,20].

In order to improve sensitivity of current diagnostic procedure for TB, in TBSURE, we have introduced a second probe, MPB64 (a secretory protein) from Mycobacterium tuberculosis H37Rv, which has a role in virulence of the bacterium [21,22]. Both the probes were labeled with FAM-TAMRA to increase sensitivity. MPB64 helps in selection of those MTBC strains in which IS6110 is absent. MPB64 has been used previously also to detect Mycobacterium tuberculosis in pleural and meningeal tuberculosis cases, where it gave a sensitivity of 70% and specificity of 88% [23]. We have also used another probe for 16S rRNA for differentiation of mycobacteria from NTM. Here in this study, we have used TBSURE for 3,709 EPTB samples including Pleural fluids (664), body fluids (402), pus (754), CSF (291), endometrium (1152), ascites fluid (1), urine (228) and miscellaneous samples (217). We have created three different groups of patients and tested them by TBSURE and culture method; TBSURE and Xpert MTB/RIF assay and with all the three methods. Additionally, we have also done Sanger sequencing on 109 patient samples to detect rifampin resistance in MTBC positive samples, so as to compare its sensitivity with gold standard Haintest and Xpert MTB/RIF assay.

### Materials and Methods

#### Ethics statement

All the experiments on virulent Mycobacterial cultures were done in DBT sponsored BSL3 Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi. All the experiments on human samples were done in compliance with the guidelines of Good Society for Ethical Research, Shahdra, and New Delhi, India.

**Sample size**

A total of 3,709 EPTB samples of different origins were collected from adult patients of Indian origin for TB detection and differentiation from NTM. Out of these samples, 3002 samples were tested by TBSURE and AFB culture, 583 samples were tested with TBSURE and Xpert MTB/RIF assay and 122 samples were tested by all three methods. Rifampin resistance was tested in 109 patient samples by Sanger sequencing, Xpert MTB/RIF assay and Hain’s assay. Table 1 shows the types of EPTB samples collected at various collection centres of Dr. LalPathLabs Ltd. and different methods used for their diagnosis.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>TBSURE vs. XpertMTB/RIF</th>
<th>TBSURE vs. Culture</th>
<th>TBSURE vs. Xpert MTB/RIF vs. Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleural fluid</td>
<td>256</td>
<td>392</td>
<td>16</td>
</tr>
<tr>
<td>Body Fluids</td>
<td>114</td>
<td>288</td>
<td>-</td>
</tr>
<tr>
<td>Pus</td>
<td>284</td>
<td>436</td>
<td>34</td>
</tr>
<tr>
<td>Cerebrospinal Fluid (CSF)</td>
<td>74</td>
<td>216</td>
<td>1</td>
</tr>
<tr>
<td>Endometrium</td>
<td>-</td>
<td>1152</td>
<td>-</td>
</tr>
<tr>
<td>Ascites Fluid</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Urine</td>
<td>-</td>
<td>227</td>
<td>1</td>
</tr>
<tr>
<td>Misc.</td>
<td>85</td>
<td>132</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>813</td>
<td>2843</td>
<td>53</td>
</tr>
</tbody>
</table>

**Table 1:** Types and numbers of extra-pulmonary tuberculosis samples diagnosed through various tests. Body Fluid types include samples of various origins like Peritoneal, Pericardial, Synovial and Gastric juice. Miscellaneous samples include lymph node, fine needle aspirates, ET secretion and trans-bronchial needle aspirates.

#### Sampling

10-100 ml of urine sample, body fluids, ascites fluid, pus and pleural fluid and 2 ml CSF samples were collected in sterile disposable containers. For biopsies, fresh tissue or bone tissue was kept in normal saline. All the samples were transported to the National Reference Laboratory, Dr. LalPathlabs Ltd. at 4°C. Paraffin embedded tissue block was cut in 8-10 µm sections and kept at room temperature. For bone tissue samples of size 250 mm³, decalcification was done 3-4 times, by adding 10 ml of 0.5 M EDTA, pH 8.0, after washing and centrifugation the samples were proceeded for DNA isolation, by using guanidine thiocyanate method.

#### Target population

The samples of different origins were collected from adult patients of Indian origin clinically suspected for EPTB.

#### Study setting

This study compared TB diagnostic tools and estimated levels of misdiagnosis in a resource limited setting. Furthermore, we estimated the diagnostic utility of three-different techniques in TB diagnostics viz GeneXpert, Culture and Hain assay.
Case definition

**Tuberculosis suspect:** Any person who presents with symptoms or signs suggestive of EPTB. The most common symptom of EPTB is pain and swelling of the involved area which may be accompanied by other constitutional symptoms like loss of appetite, weight loss, fever, night sweats, and fatigue.

**Case of tuberculosis:** A definite case of EPTB (defined below) or one in which a health worker (clinician or other medical practitioner) has diagnosed EPTB and has decided to treat the patient with a full course of TB treatment.

**Definite case of tuberculosis:** A patient with Mycobacterium tuberculosis complex identified from a clinical specimen, either by culture or by a newer method such as GeneXpert.

Inclusion criteria

Patients clinically suspected of tuberculosis presenting symptoms or signs suggestive of EPTB. Most commonly patients present enlarged lymph nodes in case of TB lymphadenitis; headache, neck stiffness, altered mental status, and cranial nerve abnormalities in case of meningeval TB; abdominal swelling, pain, fever, change in bowel habits and haem-positive stool in case of abdominal TB and dysuria, haematuria, urinary frequency, pelvic pain, menstrual disorders or infertility in women in case of genitourinary TB.

Exclusion Criteria

Samples received without any clinical history or without correct requisition and consent form. Samples were also excluded if the volume of the sample is inadequate or the sample is not transported in sterile and refrigerated conditions. Blood samples were also excluded from the study.

Pre-treatment of extra- pulmonary tuberculosis samples

For all the samples except bone tissue samples, the samples were mixed thoroughly for 15 sec on a vortex mixer and centrifuged at 10,000 rpm for 1 min. The supernatant was discarded and the pellet was incubated with decontamination liquid without N-acetyl L-cysteine (NALC) for 10 min. The sample was centrifuged again, the supernatant was discarded and 1 ml of 10 mM Tris-HCl, pH 8.0 was added to each vial. It was again centrifuged at 20°C and the supernatant was aspirated. 200 µl of proteinase K solution was added after treatment was given overnight and then proceeded with DNA isolation and quantification.

DNA isolation and quantification

Genomic DNA was isolated from the pre-treated samples using Guanidine thiocyanate method. 1 ml of lysis buffer 1 containing 480 g/l guanidine thiocyanate, 1% Triton X 100 in Tris EDTA buffer was added to each reaction vial. The Celite suspension was thoroughly mixed on a vortex mixer and 50 µl of the suspension was pipetted to each reaction vial. The reaction vials were mixed on a vortex mixer and shaken for 10 min and were then centrifuged at 10,000 rpm for 1 min. The supernatant thus obtained was discarded and to the remaining pellet, 1 ml of lysis buffer 2 containing 480 g/l guanidine thiocyanate in Tris buffer (pH 6.4) was added and mixed thoroughly. The pellet was washed with 70% ethanol, dried and suspended in 10 mM Tris-HCl. The vials were incubated for 10 min in a water bath at 56°C. The contents of the vials were mixed on a vortex to resuspend the Celite and centrifuged at 10,000 rpm for 2 min. The supernatant thus obtained was used for PCR. Figure 1 shows pictorial representation of methods used in TBSURE.

Real Time PCR

For detection of MTBC and NTMs, multiplex real-time PCR was set up in 96 well plate. Each reaction mix was of 15 µl containing 12.5 µl Premix Ex Taq (Takara Biosciences, USA), 0.5 µl ROX and 2 µl Primer and probe mix. To this reaction mix, 10 µl DNA sample was added. Each run contained a direct positive PCR control (from genomic DNA of Mycobacterium tuberculosis H37Rv), sensitivity control, no template control (NTC) and negative sample preparation control. For direct positive PCR control, the genomic DNA was suitably diluted after taking the OD260, to yield 40 fg/µl, 10 µl of which (400 fg) was used to put up positive control. This control monitored the run for correct reconstitution and amplification of master mix:1:10 dilution of above was added to the master mix to serve as the sensitivity control to monitor sensitivity of the run. Each DNA sample was used for TBSURE as well as internal amplification control (IAC) real time PCR reactions separately. Vector and primers for IAC ‘lambda phage’ were obtained from TIB Mol Biol, Germany. 50,000 copies of lambda phage DNA was spiked in each sample and negative water control. DNA was isolated and amplified with lambda phage primers. This control was used to monitor the DNA isolation procedure and presence of inhibitors in the sample. For IAC reaction, each reaction vial contained 12.5 µl SYBR® Premix Ex Taq”, 0.5 µl SYBR® ROX (50X), 2 µl primer mix to give a total volume of 15 µl, to which 10 µl DNA sample was added. The cycling parameters used for TBSURE are 95°C for 15 sec, 60°C for 30 sec and 60°C acquiring at FAM and JOE. The cycling parameters for IAC PCR are 95°C for 15 sec, 60°C for 30 sec and 60°C acquiring at SYBR, followed by melting curve analysis. For melting...
curve analysis, each cycle started from 60 °C and the temperature was increased by 0.5°C till 95°C.

**Specificity of the test**

The specificity of all the primers and probes was checked by amplification of DNA from all the five members of MTBC namely, M. tuberculosis, M. bovis, M. microti, M. africanum and M. canetti. The genus specific probe was used to check the specificity. DNA from non-mycobacterial genera like M. avium, M. fortuitum, M. xenopie and M. bovis was used for amplification with test primers and probes as well as with the genus specific probes.

**Limit of detection (LOD) and reproducibility**

Genomic DNA was extracted from Mycobacterium tuberculosis H37Rv culture and OD260 was measured and the 10-fold dilution of DNA was made and so as to give 2,00,000 to 2 genome equivalents per 10 µl as one bacterium has approximately 10 fg of DNA. The dilutions were tested in triplicate, and the LOD was determined as the dilution at which all replicates were positive.

**Xpert MTB/RIF assay**

The Xpert MTB/RIF assay (Cepheid Inc., Sunnyvale, CA, USA) was performed according to the manufacturer's instructions. This assay is a cartridge based, fully automated, rapid and real-time based nucleic acid amplification test which is used for the detection of MTBC and rifampin resistance in an MTB infection. The device amplifies a region of the rpoB gene which is specific for MTB. It also determines rifampin resistance by using molecular beacons for mutations within the rifampin resistance determining region [24].

**Culture of Mycobacterium tuberculosis from EPTB samples**

After pre-treatment of samples with N-acetyl-L-cysteine and sodium hydroxide method and centrifugation step, the specimens containing bacterial load was resuspended in phosphate buffer, pH 6.8 and this was used to inoculate culture media in the MGIT™ (mycobacterial growth indicator) 960 tubes and incubated at 37°C. The results were recorded as positive or negative in numerical growth units, and this was used to inoculate culture media in the MGIT™ culture.

**Sanger sequencing for rifampin resistance determination**

DNA was extracted from decontaminated samples as described earlier. Rifampin resistance determining region of rpoB gene was PCR amplified using specific primers. Each reaction was of 25 µl containing 0.8 picomoles of primers, 1X SYBR mix, 0.5 µl ROX, template DNA and nuclease free water. An annealing temperature of 65°C was used increased by 0.5˚C till 95˚C. A clinical sample which shows rifampin resistance in an MTB infection.

**Results**

**TBSURE Real time PCR results interpretation**

The results of a completed test were considered reliable if the test samples and the control samples met the following criteria. The sensitivity and direct positive PCR should give amplification curve for both NTM (JOE) and MTBC (FAM). The Direct PCR negative control should not give any amplification. The negative sample preparation water controls should show amplification for only IAC and not for NTM/MTBC. The IAC should give expression in all the samples except for the ones having inhibitors. The expression of IAC is checked by the melt curve analysis wherein the melting temperature should be 85.7°C +/- 2.5°C. A clinical sample which shows amplification for MTBC (FAM) having a Ct value below the sensitivity control (Ct value: 37.3) is considered positive for MTBC infection and a clinical sample which shows amplification curve for NTM(Ct value below 37.3) and not for MTBC is considered positive for NTM infection. All the MTBC/NTM negative samples and negative sample preparation controls should have positive expression of IAC.

<table>
<thead>
<tr>
<th>FAM</th>
<th>JOE</th>
<th>IAC(SYBR)</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-ve NTM/MTBC</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+ve NTM, -ve MTBC</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ve MTBC, -ve NTM</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>Inhibitors present, repeat sample</td>
</tr>
</tbody>
</table>

**Table 2:** TBSURE real time PCR results interpretation tab
Table 2 shows the result interpretation for signals obtained in the three channels and Figure 2 shows representative graphs for all the four combinations of signals obtained in the three channels.

Figure 2: Real-time amplification curves for MTBC (red) and NTM (green) using 16S rRNA, IS6110 and MPB64 gene in FAM and JOE channel. Amplification curves for MTBC (A), amplification curves for NTM (B), amplification curves for negative sample with no MTBC/NTM (C) and amplification curves if inhibitor of PCR is present in sample (D).

Limit of detection (LOD)

The limit of detection was calculated by the method described in Materials and Methods section. The LOD value for the test varies between 6-20 bacteria per test. This test was done for six different bacterial cultures and six different NTM cultures, so as to check reproducibility also.

Sensitivity and specificity

On comparison with GeneXpert, the sensitivity of TBSURE came out to be 92% and specificity came out to be 91%. On comparison with AFB culture, the sensitivity and specificity of the TBSURE came out to be 93.1% and 96.5% respectively. Tables 3-5 show percentage concordance of results when same sample was tested by TBSURE and AFB culture, TBSURE and GeneXpert and by all the three tests respectively. PPV and NPV calculated for different groups of samples tested by TBSURE and Culture was 0.58 and 0.98 and when calculated for TBSURE and GeneXpert was 0.89 and 0.93 respectively.

Table 3: Variants obtained by EPTB samples tested by TBSURE and culture.

<table>
<thead>
<tr>
<th>Variant</th>
<th>No. of Samples</th>
<th>% Share</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBSURE +ve, Xpert MTB/RIF +ve</td>
<td>242</td>
<td>41.3</td>
</tr>
<tr>
<td>TBSURE +ve, Xpert MTB/RIF --ve</td>
<td>30</td>
<td>5.2</td>
</tr>
<tr>
<td>TBSURE --ve, Xpert MTB/RIF +ve</td>
<td>22</td>
<td>3.7</td>
</tr>
<tr>
<td>TBSURE NTM, Xpert MTB/RIF --ve</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>TBSURE +ve, Xpert MTB/RIF Inhibition</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>TBSURE --ve, Xpert MTB/RIF Inhibition</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>TBSURE --ve, Xpert MTB/RIF --ve</td>
<td>285</td>
<td>49</td>
</tr>
<tr>
<td>Total</td>
<td>585</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4: Variants obtained by EPTB samples tested by TBSURE and Xpert MTB/RIF assay.

<table>
<thead>
<tr>
<th>Variant</th>
<th>No. of Samples</th>
<th>% Share</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBSURE --ve, Xpert MTB/RIF +ve, Culture --ve</td>
<td>47</td>
<td>38.5</td>
</tr>
<tr>
<td>TBSURE +ve, Xpert MTB/RIF +ve, Culture +ve</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>TBSURE +ve, Xpert MTB/RIF +ve, Culture --ve</td>
<td>10</td>
<td>8.2</td>
</tr>
<tr>
<td>TBSURE --ve, Xpert MTB/RIF +ve, Culture +ve</td>
<td>6</td>
<td>4.9</td>
</tr>
<tr>
<td>TBSURE +ve, Xpert MTB/RIF +ve, Culture --ve</td>
<td>20</td>
<td>16.4</td>
</tr>
<tr>
<td>TBSURE --ve, Xpert MTB/RIF --ve, Culture +ve</td>
<td>6</td>
<td>4.9</td>
</tr>
<tr>
<td>TBSURE +ve, Culture +ve, Xpert MTB/RIF --ve</td>
<td>5</td>
<td>4.1</td>
</tr>
<tr>
<td>TBSURE --ve, Culture --ve, Xpert MTB/RIF --ve</td>
<td>5</td>
<td>4.1</td>
</tr>
<tr>
<td>Total</td>
<td>122</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 5: Variants obtained by EPTB samples tested by TBSURE, Xpert MTB/RIF assay and culture.

Discussion

For any diagnostic test to be validated for diagnosis of any disease, the most important parameters are repeatability, robustness and reproducibility as elaborated in ISO 17025 and Principles and methods of validation of diagnostic assays [28]. In 1998, the STOP TB initiative was started with aim to eliminate TB from the world, in order to accomplish this aim the rapid and accurate diagnosis of TB became the most important prerequisite. Under this initiative, a core group called the New Diagnostics working group was established [29]. Foundation for Innovative Diagnosis (FIND), is a non-profit organization working for developing new diagnostics for poverty related diseases like TB, malaria etc. Xpert MTB/RIF assay done on the machine GeneXpert was developed by FIND in collaboration with Professor David Alland at the University of Medicine and Dentistry of New Jersey (UMDNJ) and Cepheid Inc., Sunnyvale, CA, USA. This is a cartridge based diagnostic assay that gives sensitivity of 81.3% and specificity of 99.8% for EPTB samples of different anatomic origins [30]. This system and its cartridge is available at concessional rates for many developing countries, but still the cost remains excessively high (Table 6).
In this study, we have developed an improved, indigenous, affordable and sensitive molecular diagnostic test for TB, called 'TBSURE' which uses 16S rRNA, IS6110 and MPB64 gene targets in a multiplex real-time PCR format. This is the first in-house reliable, rapid method that distinguishes between MTBC and NTM. NTM infections are becoming increasingly prevalent globally, due to immunodeficiency, hospital exposures and cosmetic procedures [31]. The precise differentiation of MTBC from NTM is essential as the antibiotic susceptibilities of different species of NTMs are very different from that of MTBC. For treatment of TB, it is important to identify the correct antibiotics for early commencement of treatment [32]. It is important to study NTMs because immune response to NTM infection may influence responsiveness to currently used BCG vaccination, especially in paediatic cases [33]. Multiplex PCR using IS6110/hsp65 and duplex PCR targeting regions different between rpoB gene of MTBC and NTM has been used by different groups for MTBC/NTM differentiation between 476 and 78 samples respectively [31,34]. Here in this study, we have employed far more sensitive multiplex real time PCR, TBSURE on a large no. of EPTB samples i.e. 3709 samples and got highly promising results.

The advantage of using multiplex real-time PCR is that it can also be used on samples which are inadequately stored as it requires just the DNA of the bacteria, while this is not the case with phenotypic tests. Moreover, microbiological diagnoses like culture and phenotypic tests often take more than three weeks' time, and the diagnosis still fails to distinguish between MTBC and NTM and does not give reliable results for drug susceptibility. TBSURE gives a reliable result in not more than 2 h after DNA extraction. In addition to this, we have also used sequencing to find out rifampin resistance in RRDR region with a sensitivity of 93.1% and specificity of 96.5% in wide variety of EPTB samples, when compared to culture. Figure 3 summarises the validation of TBSURE on 3,709 clinical samples.

### Conclusion

WHO issued a policy update on use of XpertMTB/RIF assay for TB detection in adults and children which estimated pooled specificities and sensitivities of different studies across the globe [35]. The sensitivity of the assay in CSF samples, lymph nodes and gastric fluid samples came out to be 79.5%, 83.7% and 83.8%, respectively, while all these gave a good specificity of greater than 98% [35]. The TBSURE test gives a comparable sensitivity (93.1%) and a decent specificity (96.5%), making it an affordable alternative. A single reaction by our in-house PCR along with rifampin resistance test is cheaper than XpertMTB/RIF assay. Ultimately TBSURE test can be transferred to a solid platform to develop a unique single test diagnostics system that distinguishes between MTBC and NTM, which can provide clinician with first hand reliable data that can be used to start correct treatment of infected individual and at the same time provide unambiguous EPTB cases for epidemiological data of the country.

### Acknowledgements

This work is supported by Department of Science and Technology (DST), Govt. of India. AB is recipient of UGC-BSR Senior Research Fellowship. RG would like to acknowledge DST- INSPIRE Faculty Award scheme.

### Compliance with Ethical Standards

All the experiments on virulent mycobacterial cultures were done in DBT sponsored BSL3 Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi. All the experiments on human samples were done in compliance with the guidelines of Good Society for Ethical Research, Shahdra, New Delhi, India (approval no.: GSER/2015/ND/AP/045). Prior consent was taken from all the patients for using their samples for research purposes.
Funding

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Conflict of Interest

The authors declare that they have no conflict of interest. All authors have seen and approved the submission. This manuscript or any related work has not been communicated or is being considered elsewhere.

References

25. BACTECTM MGIT™ 960 System.