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EVALUATION OF *CARE*TB REAL TIME PCR FOR RAPID DETECTION OF MYCOBACTERIUM TUBERCULOSIS IN PULMONARY SPECIMENS

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Abstract: *Rapid diagnosis of tuberculosis (TB) is one of the cornerstones for global TB control as it allows early epidemiological and therapeutic interventions. The slow growth of the tubercle bacillus is the greatest obstacle to rapid diagnosis of the disease. Newer molecular methods which are based on nucleic acid amplification (NAA) of different targets, aim to identify the M. tuberculosis complex in 6- 8 hours. In this study, we assessed the performance of careTB Assay, a Real Time based Polymerase Chain Reaction (PCR) for identification of IS6110 insertion sequence which is specific for Mycobacterium Tuberculosis Complex (MTC). Sputum specimens were collected from 100 patients with suspicion of pulmonary tuberculosis and tested for the presence of MTC using careTB kit. The results were compared against MTB culture. The sensitivity, specificity, positive predictive value, and negative predictive value of TB PCR were 92.98%, 88.37%, 91.38% and 90.48% respectively. PCR correctly identified MTC in four of the smear negative/ culture positive samples indicating high sensitivity of the assay. Four of the culture positive MOTT specimens were found to be negative by IS6110 PCR which is highly specific for MTC group of organisms. careTB test is simple, easy to perform and delivers the results in lowest possible turnaround time. Its sensitivity, specificity, and positive predictive value are satisfactory.*

Introduction

Tuberculosis (TB) remains a major global health problem and is the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). The "Global tuberculosis report 2013" released in Geneva states that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths(http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf). India has a heavy burden of tuberculosis accounting for 26% of all TB cases reported globally. Another issue faced by developing countries like India is emergence of drug resistant tuberculosis (TB), particularly multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) (Behera, 2013 and Michael, J.S. & John, T. J., 2012).

Timely diagnosis of tuberculosis and use of recommended therapy is essential to control the emergence and spread of MDR/ XDR strains. Although conventional culture and microscopy methods remain the cornerstone of diagnosis for Tuberculosis, these are either slow or their sensitivity is very low, especially with samples that contain small no. of organisms (Kulkarni *et al.*, 2012, Beige *et al.*, 1995). Furthermore, it requires sputum samples collected on consecutive days, making the procedure slow and making patient compliance with the diagnostic process difficult. Molecular assays based on PCR are proven to be quicker diagnostic method for rapid diagnosis of tuberculosis even in developing countries. Real Time PCR based assay is a single tube assay with no post PCR steps, thereby minimizing the chances of carryover contamination. The aim of the current study

was to evaluate the '*care* TB' real-time PCR (Qiagen China [Shenzhen] Co Ltd, Shenzhen, China) for direct detection of MTB from sputum specimens.

Materials and Methods

A prospective study was carried out on 100 patients who were investigated for pulmonary tuberculosis by smear and MTB culture at Metropolis Healthcare Ltd.

Modified Petroff's method, the most commonly used methods for *M. tuberculosis* culture was used for the decontamination and concentration of sputum samples. In brief, each sputum specimen was decontaminated with 4% *N*-acetyl-L-cysteine NaOH and suspended in phosphate-buffered saline (PBS; pH 6.8). From each suspended specimen, 0.5 ml was inoculated into Mycobacteria Growth Indicator Tube (BACTEC™ MGIT™ 960 System, BD, Sparks, MD, USA). The remaining portion was used for Real Time PCR. Smears were made from all the clinical specimens and stained by Ziehl Neelsen (ZN) staining method.

Culture:

Culture using MGIT 960 was performed according to the manufacturer's instructions. The tubes were incubated inside the device at 37°C and automatically monitored for 6 weeks or until an alarm signal indicated mycobacterial growth. ZN staining was performed on all the samples that signaled positive in the MGIT system to confirm presence of mycobacteria. An immunochromatographic assay, SD Bioline Ag MPT64 Rapid (®) was performed on all the positive samples to confirm MTB. This assay is earlier documented and shows excellent sensitivity (99%) and specificity (100%) (Fabre *et al.,* 2011).

Real Time PCR (careTB PCR Assay):

*care*TB PCR is a qualitative Real-Time PCR Test for identification of Mycobacterium tuberculosis complex (MTC) which uses *in vitro* nucleic acid amplification by Polymerase Chain Reaction (PCR) in combination with real-time detection of fluorescent probes for the detection and quantification of MTB DNA, Adoption of dUTP-UNG system in this kit prevents the possibility of carry-over contamination.

DNA was extracted from 900µl of liquefied sample (NaLC-NaOH treated) as per the manufacturers' instructions. The sample was centrifuged in 1.5ml eppendorf tubes at 13,000rpm for 10minutes. The upper liquid phase was discarded and the pellet was re-suspended in 1ml sterile physiological saline and centrifuged at the same speed after vortexing. The upper phase liquid was discarded and 30µl of TB DNA Extraction Solution was added to the pellet. The pellet was re-suspended by vortexing briefly, incubated at 37°C for 30 min, followed by incubation at 100°C for 10 min, and then centrifuged for 10 min at 13000 rpm. The supernatant was then used for real time PCR.

The *care*TB assay targets MTC specific IS6110 gene and uses TaqMan hydrolysis probe labelled with an FAM (6-carboxyfluorescein) fluorophore at the 5'-end and a TAMRA (tetramethylrhodamine) quencher molecule at the 3'-end. PCR was performed in a final volume of 20 µl containing 17.8 µl master mix, 0.2 µl Taq DNA polymerase, 0.03 µl uracil-N-glycosylase (UNG) enzyme and 2 µl template DNA. The thermalcycling conditions were 1 cycle at 37°C for 5 min, 94°C for 1 min, and followed by 40 cycles of 95°C for 5 s, 60°C for 30 s. The assay was performed in 0.1 ml tubes using *Rotor-Gene Q* instrument (QIAGEN, Germany). Samples with no CT value were considered as MTC negative. And the ones with CT value ≤37.0 were reported as MTC positive. The samples with CT value between 37.0 and 40.0 (37.0<CT<40.0) were re-tested and were regarded as MTC negative if re-test result shows no CT value. Otherwise, it was MTB positive.

In our study, MTB culture was considered as the gold standard method for lab diagnosis of pulmonary tuberculosis and AFB smear and *careTB* assay were evaluated with respect to culture.

Results

Of all the 100 clinical specimens tested, 53 were MTB positives, 4 were Mycobacteria other than tuberculosis (MOTT) and 47 were culture negatives. Out

of the 4 MOTT samples, 3 of them were smear positives and 1 was smear negative. There were about 6 specimens that were culture positive and smear negative. The overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of AFB smear were found to be 87.72%, 93.02%, 94.34% and 85.11% respectively (Table 1).

Table 1:
Comparison of Culture with AFB Smear (N=100)

	Culture Positive	Culture Negative
Smear Positive	50 (3 MOTT)	3
Smear Negative	7 (1 MOTT)	40
Sensitivity – 87.72%, Specificity – 93.02%, PPV – 94.34%, NPV – 85.11%		

The MTB culture results were then compared with *careTB* PCR. Out of 100 sputum samples, 54 were PCR positive and 46 were found to be negative. *CareTB* PCR demonstrated better sensitivity of 92.98% (Table 2) in comparison to smear (sensitivity - 87.72%).

Table 2:
Comparison of Culture with *careTB* (N=100)

	Culture Positive	Culture Negative
careTB PCR Positive	53 (4 MOTT)	5
careTB PCR Negative	4	38
Sensitivity – 94.44%, Specificity – 93.48%, PPV – 94.44%, NPV – 93.48%		

Out of the 7 PCR+/Smear- samples (Table 3), four of the smear negative samples were found to be culture positive. This indicates good sensitivity of PCR and culture in comparison to smear. There were 5 samples that were PCR positive/culture negative (Table 2), of which 2 were smear positive and three were smear negative. Two samples showed false negative results by real time PCR since they were positive by both, smear and culture.

Table 3:
Comparison of AFB Smear with *care*TB (N=100)

	AFB Smear Positive	AFB Smear Negative
careTB PCR Positive	47	7
careTB PCR Negative	6	40
Sensitivity – 88.67%, Specificity – 85.11%, PPV – 87.04%, NPV – 86.96%		

Discussion

Rapid and accurate diagnosis of tuberculosis is required for treatment and prevention of tuberculosis. Molecular based methods have emerged as a rapid and reliable diagnostic tool for identification of MTB infection for many years and a wide range of commercial assays are now available for clinical use (Miller *et al.,* 2011, Moure et al., 2012, Lee *et al.,* 2011, Jeong *et al.,* 2011). Many of the PCR based assays described in the literature are based on amplification of IS6110, an insertion element that is believed to be restricted to members of the M. tuberculosis complex. The presence of multiple copies of this element in the majority of M. tuberculosis strains undoubtedly enhances the sensitivity of PCR (Kulkarni *et al.*, 2012, Caws *et al.,* 2000, Narayanan et al., 2001, Chakravorty et al., 2005), though occasional certain M. tuberculosis strains in India lack this gene (Nagdev et al., 2008, Das et al., 1995).

We evaluated the performance of *care*TB real time PCR kit for the detection of MTB in sputum specimens. We have compared the clinical performance of the *care*TB PCR Assay with ZN smear test, which is routinely performed in our lab, using 100 sputum specimens. The *care*TB PCR Assay demonstrated 92.98% sensitivity and

88.37% specificity in comparison to MTB culture. *care*TB assay was earlier evaluated by Chen et al. (Chen et al., 2012). They had compared Real Time PCR assay with Transcription Mediated Amplification (TMA) based Amplified MTD® Test (AMTD) and had reported overall sensitivities and specificities of 91.6% and 100% respectively for real-time PCR, and 95.2% and 97.9% for AMTD.

Three specimens (2 culture+/smear- and 1 culture-/smear-) were not picked by *careTB* assay, probably due to absence of IS6110 element in these isolates. careTB assay amplifies IS6110 region of MTB and certain isolates are known to lack this element. Four smear-/culture+ specimens were successfully detected by *care*TB PCR Assay as true positive, indicating that the PCR assay is more sensitive than smear. On the other hand, three smear positive MOTT specimens were correctly called as MTB Complex negative by the PCR assay, showing that the PCR assay demonstrated overall better specificity than smear.

In conclusion, *care*TB Real Time PCR is rapid and reliable assay with good sensitivity and specificity for rapid detection of MTB in clinical specimens for use in a diagnostic laboratory. However, PCR should be followed by MTB culture to increase overall sensitivity of diagnosis.

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