

## UTILITY OF ANTIGEN DETECTION TEST AND POLYMERASE CHAIN REACTION IN THE DIFFERENTIATION OF TUBERCULOUS AND NON-TUBERCULOUS MYCOBACTERIA

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### ABSTRACT

**Objectives:** Cultivation and identification of mycobacteria to species level remains difficult and time-consuming. Hence, easy and rapid diagnostic methods are necessary for the differentiation of *Mycobacterium tuberculosis* (MTB) from non-tuberculous mycobacteria (NTM). The present study aims to detect and differentiate MTB from NTM isolated from clinical samples by immunochromatographic test (ICT) and polymerase chain reaction (PCR).

**Methods:** Over a period of 1 year, clinical samples (n=496) received from suspected cases of TB, at the Department of Microbiology, Kasturba Medical College Hospital, Mangalore were cultured to isolate *Mycobacterium* spp. Identification of all the isolates was done by conventional biochemical technique, ICT, and PCR.

**Results:** Among the 496 samples processed, 49 (9.87%) were acid-fast bacilli smear positive and 59 (11.89%) samples showed the growth of *Mycobacterium* spp. Among these, 10 were rapid growers, 49 were slow-growing mycobacteria, out of which 30 were MTB as identified by conventional biochemical reaction. Out of 59 *Mycobacterium* isolates subjected to ICT for the detection of MPT 64 antigen, only 28 were identified as MTB. However, all the 30 isolates were correctly identified as MTB by PCR.

**Conclusion:** Hence, PCR is essential for rapid differentiation of non-tuberculous *Mycobacterium* from MTB. False negative results seen with immunochromatographic MPT 64 antigen assay could be due to mutations within the *mpt64* gene. Further studies are necessary to characterize these PCR-positive and immunochromatographic assay negative MTB isolates.

**Keywords:** *Mycobacterium tuberculosis*, Polymerase chain reaction, Culture, Antigen detection.

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### INTRODUCTION

Tuberculosis (TB) is one of the major public health problems worldwide and India accounts for the highest TB burden in the world, accounting for 2.2 million new TB cases each year [1]. Drug resistance in *Mycobacterium tuberculosis* (MTB) is a growing concern. As per the Global TB report 2014 data, there were 35,385 laboratory confirmed rifampicin resistant/MDR TB cases in India alone [2]. Hence, the important challenges before microbiologists are early and accurate diagnosis and differentiation of infection caused by MTB from that of atypical Mycobacteria. Rapid and accurate differentiation of MTB from non-tuberculous mycobacteria (NTM) will help in early initiation of appropriate treatment which in turn will stop the emergence and spread of multidrug-resistant TB and extensively drug-resistant TB, and also reduce morbidity and mortality.

Conventional methods for the diagnosis of TB such as microscopy, though rapid, lack sensitivity. Culture of mycobacteria and drug susceptibility testing by conventional method is time-consuming (approximately 8-10 weeks). Identification of mycobacterial growth to species level by conventional method delays the reports further by 2 more weeks [3]. Rapid and accurate differentiation of NTM from MTB is also necessary to avoid inappropriate treatment, thereby minimizes the emergence and spread of drug-resistant strains. Mycobacterial growth indicator tube (MGIT) is expensive and takes a minimum of 7-10 days for the growth to be indicated. Moreover, MGIT will detect the presence or absence of growth but cannot differentiate MTB from that of NTM [4]. Hence, an attempt is made in the present study to detect and differentiate MTB from that of NTM by rapid immunochromatographic antigen detection test and PCR.

### METHODS

#### Clinical samples included in the study

Over a period of 1 year (January to December 2014), 461 samples that consisted of bronchial alveolar lavage (62), cerebrospinal fluid (CSF) (73), urine (50), pus (52), and sputum (261), received at the Department of Microbiology, Kasturba Medical College Hospital, Mangalore, from adult patients who were more than 18 years of age, were included in the study by following random sampling method. Individuals who were <18 years of age and those adults who were on antibiotic and anti-TB treatment were excluded from the study. This study was approved by the Institutional Ethics Committee of Kasturba Medical College, Mangalore. All samples were transported at room temperature (25-30°C) and processed within 30 minutes of their receipt.

#### Microscopic examination of the samples

Smears were prepared directly from all samples and also from the concentrated deposits of the same samples and stained by Ziehl-Neelsen's staining to demonstrate acid-fast bacilli and interpreted as per the Revised National TB Control Programme guidelines [5].

#### Culture of samples and identification of isolates

Sterile samples such as CSF were concentrated by centrifugation and the deposit were inoculated onto the Lowenstein-Jensen (LJ) medium. Samples from non-sterile site such as bronchoalveolar lavage, and pus, sputum were digested/decontaminated by Petroff's method and inoculated onto LJ medium. Cultures were incubated at 37°C. Cultures were first examined after 72 hrs and later at weekly interval for 8 weeks or until growth occurs. The growth on LJ medium was observed for pigmentation; colony morphology and time taken for growth were

noted. Smears were prepared from suspected colonies and identified by standard biochemical reactions such as thermostable catalase test, nitrate reduction test, niacin production, and urease test [6].

#### MPT64 antigen detection by immunochromatographic test (ICT)

The SD BIOLINE TB Ag MPT64 RAPID kit was used according to the manufacturer's instruction manual. Three or four colonies of mycobacterial growth from LJ medium were suspended in 200 µL of the extraction buffer and 100 µL of the suspension was added to the sample well. Incubated for 15 minutes at room temperature. Chromatographic diffusion of a specific MPT64 antigen of MTB complex reacts with anti-MPT64 monoclonal antibodies. Mouse monoclonal anti-MPT64 antibodies were immobilized on a nitrocellulose membrane as the capture material. Another antibody, which recognizes a different epitope of MPT64, has been conjugated with colloidal gold particles, which was used for antigen capture and detection in a sandwich-type assay. The presence of a control band (C) alone indicates a negative result, whereas the presence of two pink color bands one at C and other at T indicates a positive result. Culture of *Mycobacterium* H37 RV strain grown on LJ medium was employed as the positive reference control, which gave two pink color bands.

#### Differentiation of MTB from NTM by polymerase chain reaction (PCR) [7]

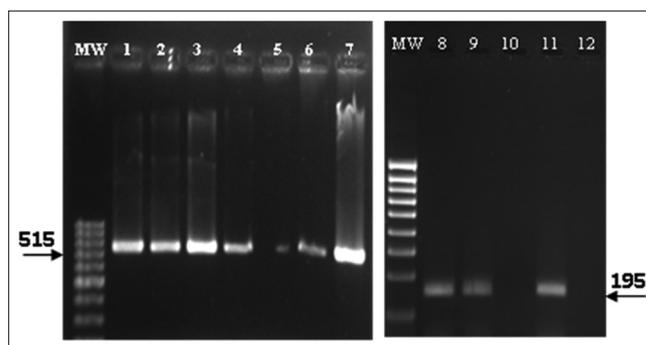
PCR targeting the mycobacterial *hsp65* gene was performed on the DNA extracted from colonies of mycobacterial isolates and *Mycobacterium* H37 RV strain using the primers F5'-TGCTCGAGAAGGTCATCGGA-3' and R5'-TCACCTGACCACCGGTGAGA-3' for MTB complex and F5'-GAGGTGGCCAAGAAGACGGAC-3' and R5'-AGCAGCGGCTTGCCCTCTGA-3' for NTM. PCR cycle included initial denaturation at 95°C for 5 minutes, 30 cycles (30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C), and a final elongation at 72°C for 5 minutes. Amplified product was detected using 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transilluminator. Amplicon size of 195 and 515 bp will indicate the presence of MTB and NTM, respectively.

#### RESULTS

Among 59 culture positive samples, 10 (16.94%) were rapid growers and 49 (83.05%) were slow growers. All rapid growers were biochemically identified as NTM. Among the 49 slow growers, 30 (50.84%) isolates were MTB as identified by conventional biochemical test. All the biochemically confirmed mycobacterial isolates were subjected to ICT for the detection of TB Ag MPT64 and only 28 (93.33%) gave the test and control bands and rest of the isolates (n=31) showed only control bands. Hence, only 28 isolates were identified as MTB by ICT. All 59 mycobacterial isolates were subjected to PCR and 30 isolates gave a 195 bp band specific for MTB *hsp65* gene and 29 isolates produced 515 bp bands that corresponded to NTM *hsp65* gene as shown in Fig. 1. Two MTB isolates tested negative by ICT were positive by PCR and conventional biochemical reactions. Various clinical samples tested and performance of different tests used in the study are shown in Tables 1 and 2.

#### DISCUSSION

TB is a public health problem in our country. Detection of TB cases rapidly using a cost-effective method is the need of the hour. Many of the well-equipped microbiology laboratories in the medical colleges of the country still use conventional biochemical reactions for the identification and differentiation of MTB from NTM. This process will delay the initiation of treatment and will lead to the spread of the disease in the community. Hence, many researchers have developed rapid, cost-effective methods such as enzyme-linked immunosorbent assay, PCR and antigen detection test [8]. One such rapid method is the ICT. In this rapid test, MPT64 antigen is detected. Earlier studies have shown that MPT 64 and culture filtrate protein - 2 antigens are found only in MTB complex and are TB-specific candidate antigens [9].



**Fig. 1: Agarose gel picture showing the amplification product of polymerase chain reaction (PCR) performed on mycobacterial culture isolates for the detection of *hsp65* gene: PCR products of 515 and 195 bp correspond to *hsp65* gene of non-tuberculous mycobacteria (NTM) and MTB, respectively. Lanes: Mw: Molecular weight marker; 1, 2, 3, 4, 6: NTM isolates; 5 and 12: Negative control; 7: Positive control (*Mycobacterium fortuitum*); 8, 9: *M. tuberculosis* complex isolates; 10: Negative culture; 11: Positive control (*Mycobacterium* H37 RV strain)**

Earlier few studies from India and abroad have shown that immunochromatographic TB Ag MPT64 rapid test to be 100% specific and sensitive [10-13]. However, in the present study, we have found that TB Ag MPT64 rapid test could not identify two isolates of MTB which was otherwise identified as MTB by biochemical test as well as by PCR. Hence, negative results of ICT rapid card tests must be interpreted with caution and such negative results need confirmation by PCR or other alternate tests. In the present study, due to false negative results of ICT, the sensitivity of the test is found to be 93.33%, specificity 100%, positive predictive value to be 100%, and negative predictive value to be 93.5%. However, an earlier Indian study has also found the sensitivity of the test to be 99% due to one single false negative result. Our culture isolates were only 59, whereas earlier study, they have used 102 mycobacterial isolates from extrapulmonary samples [14]. Hence, further studies are necessary to know whether mycobacterial isolates from different clinical samples behave differently. Another possible explanation for the false negative results could be due to mutations within the *mpt64* gene of these strains, which may have led to the production of an incomplete protein as reported by earlier workers [15,16]. Hence, DNA sequencing of isolates which tested negative by ICT and positive by PCR are necessary to substantiate the negative findings.

Moreover, ICT test being rapid will markedly reduce the turnaround time in MTB culture and identification. Hence, in spite of small proportion of false negative results, this can be an alternative rapid and cost-effective identification method for the differentiation of MTB from NTM. In the study, PCR was found to be 100% specific and sensitive when compared with the conventional technique and ICT. PCR though costly is essential for rapid differentiation of NTM from MTC, especially those suspected isolates which are negative by ICT. Thus, PCR can be used as confirmatory test for those isolates which give doubtful result as conventional biochemical tests are time-consuming. In spite of various rapid detection of MTB, control of drug-resistant TB can be brought about only by change in knowledge, attitude, and practice by pharmacist [17].

#### CONCLUSION

Detection and differentiation of MTC from NTM by ICT test and/or multiplex PCR will considerably reduce the turnaround time in the laboratory diagnosis of TB, which contributes in a significant way to early treatment and TB control program.

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Table 1: AFB smear and culture results of the different clinical samples

Types of samples (n)	AFB smear (%)		Culture (%)	
	Positive	Negative	Positive	Negative
BAL (62)	5 (08.06)	57 (91.93)	13 (20.96)	49 (79.03)
CSF (73)	1 (01.36)	72 (98.63)	1 (01.36)	72 (98.63)
Urine (50)	4 (08.0)	46 (92.0)	5 (10.0)	45 (90.0)
Pus (52)	2 (03.84)	50 (96.15)	5 (09.61)	47 (90.38)
Sputum (261)	37 (14.17)	224 (85.82)	35 (13.40)	226 (86.59)
Total (496)	49 (9.87)	447 (90.12)	59 (11.89)	437 (88.10)

BAL: Bronchial alveolar lavage, AFB: Acid-fast bacilli, CSF: Cerebro spinal fluid

Table 2: Results of culture, PCR, and TB Ag MPT64 rapid test

Method	MTB complex		Non-TB mycobacteria		Total
	No positive	No negative	No positive	No negative	
Conventional method	30 (100%)	0	29	0	59
PCR	30 (100%)	0	29	0	59
TB Ag MPT64 rapid	28 (93.3%)	02	0	29	59

TB: Tuberculosis, PCR: Polymerase chain reaction, MTB: *Mycobacterium tuberculosis*

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