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Nontuberculous Mycobacteria diagnosis using conventional methods compared with INNOLiPA MYCOBACTERIA DNA probe assay

KEYWORDS: Nontuberculous mycobacteria, M. tuberculosis, biochemical test, molecular diagnostic method, Catalase test, presumptive diagnosis

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ABSTRACT *The utility of biochemical tests for the diagnosis of nontuberculous mycobacteria (NTM) was evaluated and the results were compared with the newer molecular diagnostic method using INNOLiPA MYCOBACTERIA v2 Amp Kit. A battery of biochemical tests was conducted for the identification and classification of the isolates to species level which included Niacin test, Nitrate Reduction test, Catalase activity (at 37°C and 68°C), Hydrolysis of Tween-80, Tellurite reduction test, test for production of Arylsulphatase, Pyrazinamidase and Urease activity. INNOLiPA MYCOBACTERIA v2 Amp Kit, a DNA probe based kit designed for the detection and identification of genus mycobacterium and 16 different mycobacterial species was used for the molecular diagnosis. On comparing the results of the ten samples studied, 8 results matched perfectly and the organisms were identified as M. scrofulaceum, M. fortuitum, M. gordonae, 2 strains as M. avium, 3 strains as M. chelonei. INNOLiPA MYCOBACTERIA v2 Amp Kit it is very costly. It cannot be used for routine identification as it a tedious process which requires expertise, time and also sophisticated equipments. So this method is not feasible in an ordinary lab and is not affordable for an ordinary patient. It was noted that activity of catalase at 68°C was one biochemical test that could be used to differentiate NTMs from M. tuberculosis and could be used as a presumptive diagnosis of NTM.*

INTRODUCTION

Mycobacterium tuberculosis is the most common pathogenic mycobacteria, but there are more than 20 species of Non Tuberculous Mycobacteria (NTM) that have also been associated with infection in humans [1]. With the emergence of HIV/AIDS, the incidence of infection due to NTMs has increased. According to published reports, the incidence of disease due to NTM in immunocompetent populations in various parts of the world varies between 0.9 to 2.0/1,00,000 [2-4], with *Mycobacterium avium* complex (MAC), *Mycobacterium kansasii*, being the NTM organisms most commonly associated with disease in several countries. In contrast, the incidence of *Mycobacterium Avium Complex* (MAC) bacteremia in patients with advanced HIV infection has been found to be as high as 43% [5-7]. The infections caused by *M. tuberculosis, M. bovis* (MTBC) and *M. leprae* have definite clinical entities while the disease caused by other mycobacteria have varied manifestations. Till date there is no definite method available for the identification of NTM. Acid fast staining, which has been the basic method for diagnosis, requires a relatively large number of bacteria to be present in the specimens and also the appearance of nontuberculous mycobacteria are indistinguishable from *M. tuberculosis.* Culture confirmation is the conventional method for the detection, but it takes 3 to 8 weeks and another 2 to 4 weeks for final identification by biochemical analysis. Although short-term culture methods are available they are too expensive requiring sophisticated instrumentation and needs expertise [8]. Other methods based on lipid profiling, such as high-performance liquid chromatography, thin-layer chromatography and gas chromatography, are cumbersome and expensive and are used in very few clinical laboratories [9-11]. Identification by use of molecular methods like amplification of targets sequences of *hsp65*, 16S rRNA gene, 16S - 23S rRNA internal transcribed spacer sequence (ITS) DNA and some transposable elements [12,13] are rapid but seldom in use owing to the problems concerning their complexity. Newer simpler technique like immunochromatographic test is used on direct culture positive specimens. Even though it can discriminate between MTBC and NTM, it cannot help in species level identification of NTMs [14]. There is a need for a simple, reproducible and specific method for the identification for nontuberculous mycobacteria causing pulmonary infections. The present study evaluated the utility of biochemical tests for the diagnosis of nontuberculous mycobacteria and comparison of results with the newer molecular diagnostic method using INNOLiPA MYCOBACTERIA v2 Amp Kit.

MATERIAL AND METHODS

Sputum samples were collected from patients attending the different TB treatment centers in Kerala. The data about the sample set and their drug susceptibility results has already published [15]. Sputum samples were processed by modified Petroff's method and were grown on Lowenstein-Jensen slope (L-J) and examined for colony morphology, growth rate and pigment production. Standard strains of H37Rv, H37Ra, BCG, *M. bovis, M. avium, M. kansasii, and M. fortuitum*were used as reference strains in the study. Based on the first set biochemical tests like the niacin test, nitrate reduction test and catalase activity at 37°C and 68°C, the 450 isolates used in this study were divided into two groups. The first group comprised of *M. tubercolosis* for which the above tests were positive except catalase activity at 68°C, while the second group included the NTMs. Accordingly, 30 strains that gave negative result for the first set of tests while positive for catalase activity at 68°C, was selected for the study. These strains were further characterized using second set of test which comprised of pigment production in dark and light, growth rate, and a battery of biochemical tests.

Biochemical Characterisation of NTM strains

A battery of biochemical tests were conducted for the identification and classification of the strains to species level which included Niacin reduction test, Catalase activity at 68°C, Hydrolysis of Tween-80, Tellurite reduction test,

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test for the production arylsulphatase, pyrazinamidase and urease activity etc as described elsewhere [16].

Molecular Techniques

a. Isolation of genomic DNA

Mycobacterial strains were grown on Lowenstein-Jensen slants and used for DNA isolation using the standard protocol [17]. Briefly the colonies were scraped from the slant and taken in a screw-capped tube containing 500µl of normal saline, centrifuged at 10,000 rpm for 5 minutes in order to remove the medium. The pellet was resuspended in 500µl of homogenization buffer (300 mM Tris HCl, pH 8.0, 100 mM NaCl, 5nM EDTA, pH 8). Glass beads of 1 mm diameter were added and the cells were disrupted using a Mini bead beater at 2500 rpm for 1 minute. DNA was extracted from the above suspension twice with phenol: chloroform and once with chloroform. DNA was then precipitated with 2.5 volume absolute alcohol and 1/10th volume of 3M sodium acetate, pH5.2. The DNA resuspended in TE (10mM Tris HCl,pH 8.0, 1mM EDTA pH8.0) was treated with ribonuclease A (20mg/ml) at 42°C for 30 minutes followed by extraction with chloroform-isoamyl alcohol (24:1) and precipitated with 2.5 volume absolute alcohol and 1/10th 3M sodium acetate pH 5.2. The concentration of the DNA was quantitated with UV spectrophotometer at 260 and 280nm. The DNA was aliquoted and stored at –20°C.

b. PCR analysis using primers specific for M. tuberculosis

To confirm whether the samples in the study were NTM and not *M. tuberculosis* and to correlate with the result of biochemical tests, a PCR analysis which amplified sequence specific for *M. tuberculosis* was used in the study [18].

c. INNO-LiPA MYCOBACTERIA based v2 kit for the Identification of NTMs

Those samples which gave negative result for the *M. tuberculosis* specific PCRs were further used for the species identification studies for which INNOLiPA MYCOBACTERIA v2 kit from Innogenetics, Belgium was used. It's a DNA probe test designed for the detection and identification of genus mycobacterium and 16 different mycobacterial species [19]. Speciation using INNO-LiPA was done as per the manufacturer's protocol. Briefly [20], the method involved the amplification of the 16S-23S rRNA spacer sequence from the genomic DNA of the Mycobacterial isolates (using biotinylated primers). Biotinylated DNA material (16S-23S rRNA spacer region) was hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane based strips. After hybridization, streptavidin labeled with alkaline phosphatase was added and bound to any Biotinylated hybrid previously formed. Incubation with 5-bromo, 4-chloro, 3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) chromogen resulted in a purple/brown precipitate. The strips were read using the template provided in the kit.

RESULTS

Four hundred and fifty mycobacterial strains were used in this study. Based on the first set of biochemical tests mycobacterial isolates were grouped into two groups i.e. Group I MTBCs (420/450) and Group II the remaining 30 as NTMs. Second set biochemical tests were conducted for all the 30 strains, which gave positive result for catalase activity at 68°C. Of the 30 isolates studied, only 10 were identified to the species level using biochemical tests. Among these 10 strains, 6 were rapid growers, of which three isolates were *M. fortuitum* and three were *M. chelonei*. Among the remaining four isolates, two were identified as *M. avium* and one each was identified as *M. scrofulaceum* and *M. gordonae*. The remaining twenty isolates were difficult to identify as they answered the biochemical reactions not in a classical way even though they were reported as NTM. There were lots of variability among the biochemical test results leading to misidentification.

Molecular characterization of the 30 samples under study was conducted to confirm whether these were true NTM strains, for which a multiplex PCR analysis which amplified sequence specific for *M. tuberculosis* was used in the study. It was found that some of the samples gave positive result for the PCR for the presence of *M. tuberculosis* specific sequence and on repeating the biochemical the variability of the results still remained from typical *M. tuberculosis* to NTM. DNA of the 10 biochemically identified NTM isolates which gave negative results for the amplification tests were used for the species level identification using INNOLiPA MYCOBACTERIA v2 Amp Kit. Of the 10 isolates whose species level identification was conducted, 2 samples were identified as *M. gordonae,* 4 samples as *M. chelonae Complex (group III & M. abscessus),* 3 samples as *M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense* and remaining one as *M. fotuitum-M. peregrinum Complex*. On comparing the results of Biochemical tests and INNOLiPA MYCOBACTERIA v2 Amp Kit most of the results matched one to one and was identified as *M. scrofulaceum* and *M. gordonae, M. fortuitum.M. chelonei* (3 strains), *M. avium* (2 strains) using both methods (Table-2). There was disparity among the two results with strains 8 and 9 being reported as *M. fortuitum* using biochemical test but as *M. gordonae* and *M. chelonae* using INNO-LiPA MYCOBACTERIA v2 Method.

DISCUSSION

Results obtained from the study confirm the presence of NTM among the local isolates. Several NTM species has been reported to cause disease in the patients. The samples were collected from the patients who were categorized to be having tuberculosis and this study shows that the patients were actually not infected with *M. tuberculosis*, but with some other species of mycobacteria. Under current diagnostic and treatment strategy, when a patient is suspected to have TB, he/she is subjected to sputum microscopy examination and / or chest X-Ray. If the sputum smear gives positive results, they are considered as tuberculosis patients and they are provided with the DOTs treatment irrespective of whether they are infected with *M. tuberculosis* or NTM and in most cases the drug sensitivity pattern of the bacteria are also not determined. DOTs is very useful and effective in treating tuberculosis if the organism is not MDR, but not at all effective in infections by NTM. Many of the pulmonary diseases caused by NTM are not identified but rather treated with conventional anti-TB treatment which eventually fails because majority of NTM are resistant to conventional TB treatment [21,22]. The treatment regimen is entirely different and hence there is an immediate need for the isolation and proper identification of NTM strains causing infections.

Molecular characterization using multiplex PCR which amplified sequences specific for *M. tuberculosis* gave positive result for some of the NTM samples and on repeating the biochemical tests, the variability of the results still remained from typical *M. tuberculosis* to NTM. The reason for such disparity may be due to multiple infections in the same patient with more than one type mycobacterial strain [23, 24].

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On comparing the results of Biochemical tests and INNOLiPA MYCOBACTERIA v2 Amp Kit most of the results matched. At the same time there was disparity among the results. This disparity may be due to the fact that the reference table for biochemical tests are for general guidance and variations in a few biochemical properties are common for several mycobacterial strains so it is difficult for clear cut identification [16].

Even though species identification can be done using INNOLiPA MYCOBACTERIA v2 Amp Kit it is very costly. It cannot be used for routine identification as it a tedious process which requires expertise, time and also sophisticated equipments. So this method is not feasible in an ordinary lab and is not affordable for an ordinary patient. From all the results obtained, it was striking to notice that one biochemical test can differentiate mycobacterial strains into *M. tuberculosis* and NTM is Catalase production at 68°C. All the NTMs gave positive result for stable catalase production at 68°C, which can be used for the presumptive diagnosis of NTMs.

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Table-I Banding pattern and interpretation of the INNO-LiPA MYCOBACTERIA v2 Method.

RGTB No.	Banding Position	Interpretation
30	MAIS	M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense
126	MGO	M. gordonae
182	MAIS	M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense
192	MFO	M. fotuitum-M. peregrinum Complex.
193	MCH1 & MCH 2	M. chelonae Complex (group III & M. abscessus)
199	MCH1 & MCH 2	M. chelonae Complex (group III & M. abscessus)
222	MCH1 & MCH 2	M. chelonae Complex (group III & M. abscessus)
230	MGO	M. gordonae
350	MCH1 & MCH 2	M. chelonae Complex (group III & M. abscessus)
572	MAIS	M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense

Table-II Comparison between the results obtained from INNO-LiPA MYCOBACTERIA v2 Method and Biochemical Tests

RGTB No.	INNO-LiPA MYCOBACTERIA v2 Method	Biochemical test
30	M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense	M. avium
126	M. gordonae	M. gordonae
182	M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense	M. avium
192	M. fotuitum-M. peregrinum Complex.	M. fortuitum
193	M. chelonae Complex (group III & M. abscessus)	M. chelonae
199	M. chelonae Complex (group III & M. abscessus)	M. chelonae
222	M. chelonae Complex (group III & M. abscessus)	M. chelonae
230	M. gordonae	M. fortuitum
350	M. chelonae Complex (group III & M. abscessus)	M. fortuitum
572	M. avium, M. intracellulare, M. scrofulacium, MAC, M. malmoense	M. scrofulacium

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