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Comparative analysis of different identification techniques for detection of mycobacterial species in clinical samples

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ABSTRACT

Out of 906 cultures isolates, 263 (29.0%) were confirmed as NTM and 724 (71.0%) were identified as *Mycobacterium tuberculosis* complex. The diversity of NTM species was high and predominated by *Mycobacterium abscessus* (31.3%) followed by *M. fortuitum* (22%), *M. intracellulare* (13.6%), *M. chelonae* (9.1%), however, *M. abscessus* and *M. fortuitum* were the predominant species in both types of clinical isolates. Men (60.4%) and older patients aged greater than 55 years were the predominated risk group for NTM infection. Additionally, three HIV/AIDS cases (1.1%), as well as thirty five cases were found to be type two diabetes mellitus infected with non-tuberculous bacteria.

Keywords: NTM, Technique, BacT/Alert, Biochemical Test, Tuberculosis.

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INTRODUCTION

Non-tuberculous mycobacteria (NTM) infection associated with pulmonary and extra-pulmonary disease has been increasing globally. In many countries, NTM infections have become more common than *M. tuberculosis* (MTB). NTM is ubiquitous in nature and everyone is exposed with them; however only a little people can get a NTM infection. Despite an increase in incidence rate of NTM infection, its prevalence, species diversity, and antibiotic susceptibility pattern in India is largely unknown[1-4].

Occurrence of NTM has been reported in human respiratory and non respiratory diseases. A continuous global rise in NTM incidence is a big concern for physicians and microbiologists. Earlier in various developing countries, the higher occurrence of tuberculosis has surpassed the identification of NTM associated infections[5-8]. Most of these are present mainly in the form of saprophytes in the natural surrounding and have been identified as an etiological agent in human diseases. Now days this trend is changing with great pace. Higher reporting of NTM related pulmonary disease leads to international attention[6-10]. As of, 2014 estimated incidence of respiratory disease was 33–65 per 100,000 due to NTM[11-14].

However, culture of mycobacteria and detection of NTM are not regularly done in many regions of India. NTM infection prevalence in India still unclear. Therefore, in high burden resource areas; there is immediate requirement for the routine detection of NTM infected individuals. Since the recent standard of care does not include the characterization of bacteria ,in some positive NTM cases , is classified incorrectly as MTBC and receives conventional TB therapy, however some species of NTM can be resistant against treatment and many of them are unable to diagnose.

Restricted information on the occurrence of NTM and genotypic characterization among patients is available in India and infections of NTM are not well documented. In present study, we investigate; compare different identification techniques for detection of mycobacterium species in clinical samples[15-18].

MATERIALS AND METHODS

Study Design and Study Setting

This study designed as a prospective hospital based study in Northern India at department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, UP India.

Sample Sites

Pulmonary sites (Sputum, bronchoalveolar lavage) and extra pulmonary sites (Body fluids, pus and tissues, CSF, lymph node aspirates, pleural fluid, pus, bone marrow aspirates, peritoneal fluid, ascitic fluid, synovial fluid, and other body fluids) was collected from clinically assumed cases of tuberculosis.

Inclusion Criteria

- Clinically suspected of tuberculosis including pulmonary TB and extra-pulmonary TB was included
- Culture positives for mycobacteria was included
- HIV and other immune-compromised patients suspected of TB was included
- All age groups and sex supposed of having included
- NTM confirmed strains included

Exclusion Criteria

- MTBC excluded.
- Culture negative specimens excluded.
- Those patients not willing participates in this study were excluded.

Processing of Specimens

The processing of sample was divided in two parts: non sterile specimens (Sputum, bal fluid, wound swabs, pus, discharging fluids etc.), which includes bacterial normal commensal flora, were processed and decontaminated by standard N-acetyl-L-cysteine-NaOH (NALC-NaOH) process. It was centrifuged and sediment separated into two half, one half was utilized for AFB microscopy and another is used for culture. Sterile sites specimens (Synovial, ascetic fluid, pericardial fluid, CSF, pleural fluid, etc) were centrifuged and sediment divided into two half, one half was utilized for AFB microscopy and other is culture in BACT/ALERT 3D Culture.

Different technique for identification of NTM using biochemical test

Biochemical test commonly was done for identification of the commonly encountered NTM species. The following test was carried out arylsulfatase, iron uptake, nitrate reductase, catalase, urease, Tween80, tolerance to 5% NaCl, and utilization of the carbohydrates mannitol, inositol, and citrate and Mac-Conkey agar absence of crystal violet as described.

SD- Bioline Assay

A 100 µl supernatant of sample from liquid culture was put into the sample well. From solid culture used few colonies mix into buffer and the suspension was added into the well. After 15 minutes, interpretation of the test was done.

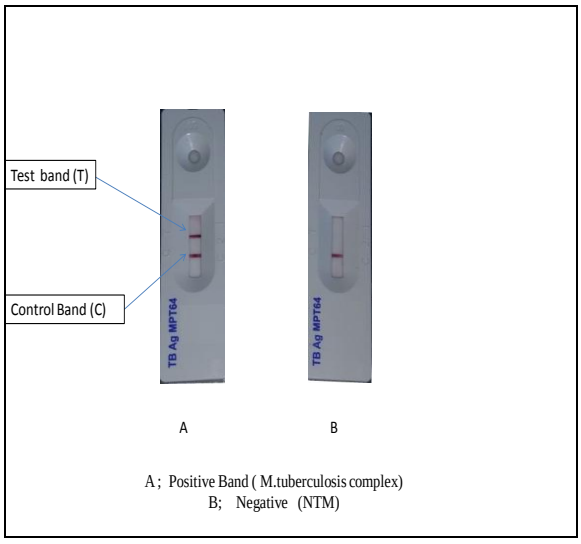

Figure 1: differentiation of NTM and MTBC by bio-line SD AG MPT64 antigen test

Band of Pink color in the 'C' region of control confirmed as a test is valid. The pink band in the 'T' region test line and 'C' region was control line reported as positive for MPT64 antigen test. Only pink band in the 'C' region and band not present in the 'T' region were regarded as negative for MPT 64 antigen. There have no bands in 'C' region was considered as invalid test. Both test line and control line is present is called as MTBC and in control line is present interpretate as MOTT (Figure 1).

Genotype® Mycobacterium CM/AS Assay

The detection of NTM and differentiation (RGM and SGM) species was completed by the GenoType® Mycobacterium CM/AS assay of BacT/ALERT® MP positive culture isolates of mycobacteria.

Dna Extraction

Extraction of DNA was subjected for 15 minutes using sonication method, followed by heating for 15 min to 100^0C. Positive culture broth was centrifuged at 13, 400 g for 2 minutes and the supernatant was further used for the PCR.

PCR

PCR was carried out using specific primers set designed to amplify a species specific 23S rRNA gene sequence of mycobacterium species. Amplification mixture (45µl) was made in DNA free area, containing 5 µl extracted DNA (20-100 ng DNA) in reaction mixture included 35 µl PNM, 5 µl 10X polymerase incubation buffer, $MgCl_2$ solution 2 µl 25 mM, HotStar Taq 0.2 µl (QIAGEN, Hilden, Germany) and 3 water µl (biology grade water) and after that used 0.5 µl extracted DNA. Finally vortex the tubes and subjected to PCR. Amplification was completed in a thermal cycler (Gene Amp PCR System 9700 Thermal Cycler), which involved 01 cycles is denaturation for 15 at 95°C minutes, then annealing of primers for 30 seconds at 95°C, 2 minutes at 58°C for 10 cycles, then at 95°C for 25 seconds of 20 cycles, 53°C for 40 seconds, and 70°C for 40 seconds, and final extension of primer at 70°C, 8 minute for 01 cycle. And then amplified PCR results were stored at +8 to -20°C till hybridization was not performed in machine of hybridization (Profiblot; Tekan, Maennedorf, Switzerland).

PCR reaction mixture

Reagent/Solution	**Volume (µl) for single reaction**	**Final Concentrations**
PNM	35 µl	
PCR water	5 µl	10X
$MgCl_2$ solution,	2 µl	25 mM
HotStar Taq	0.2 µl	
biology grade water	3 µl	
DNA	0.5 µl	

Reverse Hybridization

Further after the amplification this was followed by hybridization (Profiblot; Tekan, Maennedorf, Switzerland). **;** in which denaturation solution (DEN, blue) 20 µl of was distributed in corner of every well, and then 20 µl of product amplified PCR was dispensed and incubated at room temperature for 5 minutes. Then pre-heated 1 ml of hybridization buffer (HYB, Green) was dispensed, after that shake gently until a homogenous color was appeared. Then strip was placed in a systematic way to make sure whole flooding of solution above on the strips. After that the tray was put in TwinCubator and was incubated at 45^0C for 30 minutes, then total aspiration of hybridization buffer.

Washing requires 1 ml of stringent wash solution to every strip and incubated at 45^{0}C for 15 minutes in TwinCubator. Further washing of strips was done with 1 ml of rinse solution on TwinCubator for 1 minute. After that each strips were diluted 1 ml conjugate solution and incubated for 30 minutes on TwinCubator. Again Strips were washed with 1 ml of rinse solution for 1 minute, followed by addition of 1 ml of diluted substrate to each strips and incubated in absence of light without shaking for 3-20 minutes. To stop this reaction rinsing was carried out twice with distilled water. At the end, strips were taken out and dried between two layers of blotting paper. Evaluation and interpretation of results were done based on the presence and absence of different bands and compared with reference band as provided in the kit given as;

The developed strips were pasted in the selected fields by line up the universal control (UC) and conjugate control (CC) and genus control (GC) bands with the particular lines on the evaluation page.

The Conjugate control (CC); this band must develop to confirm the effectiveness of the conjugate binding and the substrate reaction.

The universal control (UC) band detect all known *Mycobacterium* species and constituent of the faction of Gram positive bacteria with high G+C content. If the bands conjugate control, the universal control band and other bands are positive but pattern that cannot be allocate to exact *Mycobacterium* species. Merely those bands whose strength is about as strong as or stronger than that of the universal control are to be considered.

The genus control (G-C) band confirms the organism is a constituent of the *Mycobacterium* genus, the intensity of the band varies depending on the *Mycobacterium* species.

Results interpretation was complete according to the absence and presence of bands compared with reference provided in the kit. The organisms were recognized based on the position and number of bands formed on the strip (Figure 2).

Data Analysis

The clinical and demographic data were analyzed by using SPSS version 19.0 (IBM, NY, USA) software package. Genotype CM/AS assay results were interpreted based on manufacturers protocol.

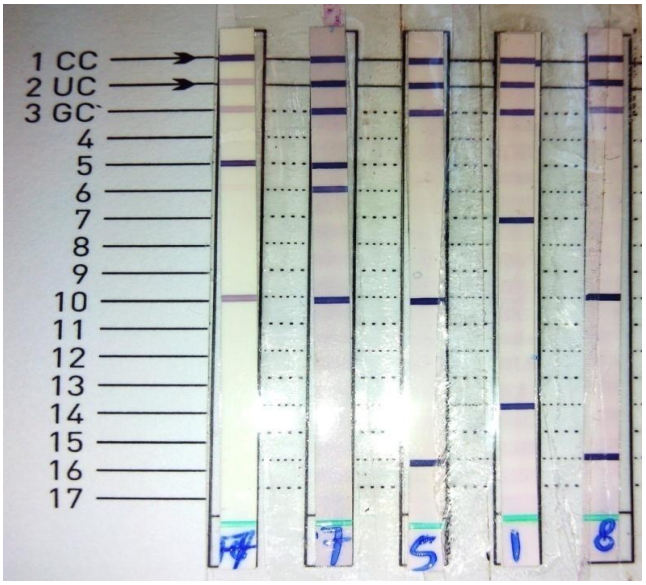

Figure 2: Discrimination of different species of mycobacteria strains no 17, 7, and 1 was identified as NTM and 5, 8 AS MTBC using Genotype® CM/AS assay.

RESULTS

Differentiation of MTBC and NTM

Primary screening of culture positive isolates i.e., (906 cases) were done by colony morphology using different biochemical tests such as catalase test, nitrate reduction and niacin production, which identified 651 (71.9%) MTBC and 255 (28.1%) NTM. Further, another technique Rapid BIO-LINE SD Ag MPT64 TB was carried out on total positive isolates of mycobacteria irrespective of their day of culture positivity. Out of Nine Hundred Six culture positive isolates, 641 isolates confirms the subsistence of MPT 64 antigen appearance and thus shows presence of MTBC while remaining 265 isolates were identified as NTM (Table). A confirmatory (gold standard) test was carried out using GenoType® Mycobacterium CM/AS assay which confirmed 643 (70.9%) isolates, showed presence of distinct band at position 10 and 16 which reflected a positive test for MTBC while other band patterns confirmed 263 (29.1%) different species of NTM (Table 1).

Table 1: Distribution of mycobacterial isolates according to the identification test performed.

Biochemical test		MPT 64 Ag test		Genotypic CM/AS Assay	
Positive	Negative	Positive	Negative	MTBC	NTM
651(71.8%)	255 (28.1%)	641(70.7%)	265(29.2%)	643(70.9%)	263(29%)

Specificity and sensitivity of different techniques

Among different diagnostic tests applied for specificity, sensitivity, negative (NPV) and positive predictive value (PPV), GenoType® Mycobacterium CM/AS assay which is gold standard method shows highest specificity and sensitivity as compared to MPT64 Ag test and other biochemical test respectively (Table 2). From this study, it can be concluded that molecular test are more sensitive and specific for the identification of NTM and MTBC in given samples.

Table 2: sensitivity and specificity of MPT64 AG test and biochemical test in comparison to genotype® mycobacterium CM/AS assay for discrimination between NTM and MTBC

Parameters	MPT64 Ag test	Biochemical test
Sensitivity	99.68%	98.78%
Specificity	99.24%	96.95%
PPV	99.68%	98.78%
NPV	99.24%	96.95%

DISCUSSION AND CONCLUSION

Smear microscopy for AFB is rapid, on the other hand does not distinguish between NTM and MTBC. Phenotypic techniques such as staining of a growth and a group of various biochemical methods are not able to differentiate more than 100 species of Mycobacteria, as they overlap in their biochemical properties. Two isolates were correctly detected as MTBC by LPA were falsely diagnosed as NTM by using MPT 64 test. In earlier studies , it is reported that MPT64 BIO- LINE SD Ag kit sensitivity of 90- 100% for differentiation of NTM and MTBC species. In this study we have identified that the 99.4% sensitivity for MTBC isolates by BIO- LINE SD MPT64 Ag. Many other studies that compared the GenoType® Mycobacterium CM/AS technique with various genotypic and phenotypic tests and reported outstanding outcomes .The benefit of using this assay is quick and correct detection of a wide range of Mycobacterial species the same as evaluate with molecular techniques and phenotypic, which fails to distinguish various species of NTM accurately. The additional uses of this are very less time as compared with conventional tests and molecular methods.

In this research we have found higher prevalence of NTM from both site clinical specimens. Rapid growing species *M.abscessus* and *M.fortuitum* are most recurrently isolated and slow growers *M.intermedium and M.malmoense* are least isolated in our study. The risk factors that were found to be related with infections of NTM gender, age groups respiratory symptoms presence and HIV infection. Molecular methods are an indispensible tool as it diminishes the possibility of an inadequate treatment of infection related to NTM. This may gradually replace conventional techniques for the identification and discrimination of NTM. The higher occurrence of NTM and its species diversity suggests the urgent need and exact characterization including antibiotic susceptibility connected with NTM required for appropriate management and treatment of patients.

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