Isolation of non-tuberculosis mycobacterial strains from suspected pulmonary tuberculosis patients by 16S-23S rRNA inter transcribed spacer PCR – RFLP

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ABSTRACT
Pulmonary infections due to Non-Tuberculosis Mycobacteria (NTM) has emerged as an increasingly prevalent clinical entity in the past two to three decades. The prevalence of tuberculosis has reduced in developed countries, but infections due to NTM are on rising, whereas in developing countries like India, tuberculosis is still a significant health problem. This study was undertaken to investigate the overall burden and diversity of NTM among clinical pulmonary isolates from a south Indian population. This study was conducted from March 2017 to December 2017, to identify species of Non-tuberculous mycobacteria from specimens suspected with pulmonary tuberculosis. We applied conventional biochemical test like growth on the LJ medium containing PNBA (para-nitrobenzoic acid) for differentiation of NTM and M. tuberculosis and PCR-RFLP of the 16S-23S rRNA ITS gene for species identification. A 221 bp fragment of ITS gene was amplified and the amplification product was digested by two restriction enzymes, BstEII and HaeIII. Digested products were analyzed using polyacrylamide gel electrophoresis. Out of 1138 positive cultures 96 were identified as Non-tuberculous mycobacteria. Of the 96 Non-tuberculous mycobacteria 59 were from pulmonary sites. Among the 59 NTM, 74.5% (44/59) were rapidly growing mycobacteria (RGM) and 15 (25.4%) were slow growing mycobacteria (SGM). The most predominant NTM species among RGM was Mycobacterium abscessus followed by Mycobacterium simiae, Mycobacterium fortuitum, Mycobacterium chelonae, Mycobacterium gordonae. Regular documentation of NTM isolated from clinical specimens and reporting their antibiogram is essential to be aware of the clinical spectrum of disease associated and preferred treatment option.

INTRODUCTION
The genus Mycobacteria comprises more than 170 officially recognized species. Non-tuberculosis mycobacteria (NTM) also termed as mycobacteria other than tuberculosis (MOTT), environmental mycobacteria and atypical mycobacteria are ubiquitous and widely distributed in water, soil and animals. (L. Garcia-Agudo et al.; Tarcisio Fedrizzi et al., 2017) Non-tuberculosis mycobacteria include all Mycobacterium species that doesn’t cause tuberculosis or leprosy, thus excluding the species of M.tuberculosis complex, M.leprae and
M. lepromatosis. Although *M. tuberculosis*, *M. bovis* and *M. lepra* are established pathogens, Non-tuberculosis mycobacteria (NTM) causing pulmonary infections mimics tuberculosis, extrapulmonary infections affecting lymph nodes, skin and soft tissues, multifocal disseminated infections. (Ali Nour – Neamatollahie et al., 2007; Jesudason MV et al., 2005).

The infections caused by these organisms are reported to have been increased in the past few years in both immunocompromised and immunocompetent. Most of the NTM are only partially susceptible or notably resistant to regularly used anti-tubercular drugs. Different species have different susceptibility to antibiotics, determination of Mycobacteria to species level is crucial and provides useful data epidemiologically and facilitates successful outcomes inpatient treatment (Ali Nour – Neamatollahie et al., 2007; Jesudason MV et al., 2005; Sharma B et al., 2010; Sarika Jain et al., 2014; Wenjuan Nie et al., 2015).

Pulmonary disease is the most common manifestation comprising 90% of Non-tuberculous mycobacterial infections. Pulmonary infections due to Non-tuberculosis mycobacteria are often associated with various conditions such as COPD, cystic fibrosis of lung, bronchiectasis, emphysema, cases underwent treatment for pulmonary tuberculosis and lung cancer. (Park et al., 2014; Katoch V.M. et al., 2004; Wallace R Jr et al., 1990)

The prevalence of Non-tuberculous mycobacterial infections is not known in India, as NTM infection is not a reportable condition due to the lack of awareness among clinicians coupled with lack of laboratory capacity to diagnose these infections. Conventional identification of mycobacteria through cultural and biochemical tests are time-consuming and cumbersome to perform leading to a significant delay in diagnosis (Park et al., 2014). Various molecular assays like PCR and other nucleic acid amplification techniques are extensively used to overcome the limitations in isolation. Among the several techniques, PCR – RFLP is preferred, as it is economical and straightforward assay and it can be used for various genes like 16S-23Sr RNA internal transcribed spacer (ITS), 16S ribosomal DNA (16S rRNA), heat shock protein 65 (hsp 65 or hsp 60), dnaJ, groES, and DNA-directed RNA polymerase beta chain rpoB genes (Ali Nour – Neamatollahie et al., 2007)

**MATERIALS AND METHODS**

The present study was conducted to investigate the prevalence of NTM strains from clinical specimens of all suspected pulmonary tuberculosis patients received between March 2017 to December 2017. Ethical approval is granted by the institutional Ethics committee. Since NTM are ubiquitous and is a possible laboratory contaminant, the isolation of these organisms from specimens should meet specific criteria to confirm their etiological significance such as a). Repeatedly isolating the same isolate from the patient b). Supportive, positive clinical and radiological evidence and c). Histopathological condition (Jesudason MV et al., 2005).

**Clinical specimen collection:** Three early morning sputum specimens, bronchoalveolar lavage, gastric aspirate, pleural fluid and bronchial wash specimens were collected from patients with radiological and clinical findings suggestive of tuberculosis. The specimens were processed with the standard protocol (Singh AK et al., 2013).

**Smear examination, Culture and differentiation of *M. tuberculosis* and NTM:** The clinical specimens should be processed on the same day for microscopy, performing the ZN staining of direct and concentrated smears (N-acetyl-L-cystine-NaOH concentration method) (LYFECTOL, Tulip Diagnostics). The concentrated samples were simultaneously inoculated on LJ medium, for each isolates two tubes of Lowenstein-Jensen medium (Hi-Media) were used, one containing everyday media and the other LJ medium containing Para nitrobenzoic acid) and incubated at 37°C temperature under aerobic conditions. AFB cultures were also performed simultaneously in BACT/ALERT culture media as well.

**DNA extraction:** Extraction of DNA was done by using NucleoSpin® Microbial DNA from MACHERY-NAGEL GmbH & Co. KG. 0.5ml of culture from BACT/ALERT culture media was taken using sterile disposable syringe cultures were centrifuged at 10,000 rpm to form a pellet. Add 100 µl of Elution Buffer BE to the pellet and resuspend the cells. Transfer the suspension of cells into the NucleoSpin® Bead Tube Type B (provided). Add 40 µl Buffer MG. Then, add 10 µl Liquid Proteinase K and close the tube. Agitate the NucleoSpin® Bead Tube on a swing mill or vortexer device. Centrifuge NucleoSpin® Bead Tube for 30 seconds at 11,000 xg to clean the lid. Add 600 µl Buffer MG and vortex for 3 seconds and Centrifuge the tube for 30 sec at 11,000 xg. Transfer the supernatant (~500–600 µl) into the NucleoSpin® Microbial DNA Column, placed in a 2 ml Collection Tube (provided). Centrifuge for 30 s at 11,000 xg. Discard collection tube with flow-through. Put column into a fresh collection Tube (2 ml, provided). First wash: Add 500 µl Buffer BW. Centrifuge for 30 s at 11,000 xg. Discard flow-through and place the column back into the Collection Tube. Second wash: Add 500 µl Buffer...
The majority of NTM were isolated from pulmonary specimens, including bronchial wash 26 (42.3%), sputum 25 (38.9%), bronchoalveolar lavage 8 (13.5%). Of these 74.5% (44/59) were rapidly growing mycobacteria (RGM) and 15 (25.4%) were slow growing mycobacteria (SGM). The distribution of NTM species is mentioned in the Table: 2. The most predominant NTM species among RGM was *Mycobacterium abscessus* 29(49.1%) followed by *Mycobacterium simiae* 14(23.7%), *Mycobacterium fortuitum* 12(20.3%), *Mycobacterium chelonae* 3(5%), *Mycobacterium gordonae* 1(1.6%) Table 2.

**Table 2: Demographic characteristics of the 59 non-tuberculosis cases**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male: 37</td>
<td>37 (62.7%)</td>
</tr>
<tr>
<td>Female: 22</td>
<td>22 (37.2%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>21 – 30</td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>31 – 40</td>
<td>9 (15.2%)</td>
</tr>
<tr>
<td>41 – 50</td>
<td>7 (11.8%)</td>
</tr>
<tr>
<td>51 – 60</td>
<td>21 (35.5%)</td>
</tr>
<tr>
<td>61 – 70</td>
<td>16 (27.1%)</td>
</tr>
<tr>
<td>71 – 80</td>
<td>1 (1.6%)</td>
</tr>
<tr>
<td>Previous TB treatment</td>
<td>9 (15.2%)</td>
</tr>
<tr>
<td>HIV infection</td>
<td>1 (1.6%)</td>
</tr>
<tr>
<td>Other respiratory symptoms</td>
<td>24 (40.6%)</td>
</tr>
<tr>
<td>Type II Diabetes mellitus</td>
<td>7 (11.8%)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Infections with Non-tuberculous mycobacteria (NTM) are increasingly reported worldwide and emerging as a significant cause of chronic pulmonary infection, posing some challenges for both clinicians and researchers. While some studies worldwide have described an increasing prevalence of NTM pulmonary disease over time, population-based data are relatively sparse and subject to ascertainment bias. Non-tuberculous mycobacteria are emerging worldwide as a significant cause of pulmonary infection, posing some challenges for both clinicians and researchers. Several studies, worldwide have described an increasing prevalence of NTM pulmonary disease over time. The treatment of NTM infections associated with respiratory infection caused by *M. abscessus* is difficult in
comparison to other species (Jason E.Stout et al.2016; AK Maurya et al. 2012) In the present study RGM constituted of 74.5% (44/55), of all non-tuberculous mycobacterial isolates M. abscessus being the most prevalent isolate in our study.

Many studies from India have reported a variation in the incidence of Non-tuberculous mycobacteria infections. A study by Chakrabarti et al. from Chandigarh reported 7.4% incidence of NTM from various clinical specimens. Similarly, Das B.K. et al. reported 8.3% of NTM incidence in their study and Mycobacterium fortuitum was the predominant isolate in their studies. Our study was conducted in a tertiary care hospital in South India and we found similar findings, we reported 8.4% NTM in pulmonary specimens, whereas M. abscessus being the predominant species in our study. A study from Kolkata by Karak et al., documented a high incidence of NTM 17.4%, from sputum specimens (B. Das et al., 1982; A. Chakrabarti et al., 1990; K. Karak et al., 1996)

Among the Rapidly Growing Mycobacteria, Mycobacterium abscessus species occupy the highest position to cause respiratory and soft tissue infections and disseminated disease in both immunosuppressed and immunocompetent patients (HY Kim et al., 2016). Mycobacterium abscessus being the most prevalent isolate in our study accounted for (29) 49.1%, similar findings were seen with Jyoti Umrao et al. from Lucknow and Prabha Desikan from Bhopal reported M.abscessus as the most frequently reported species (Jyoti Umrao et al., 2016; Prabha Desikan et al., 2017)

In our study, M.simiae was the second most predominant NTM in pulmonary samples. The high frequency of M.simiae isolate was reported by V.P.Myneedu et al. from New Delhi in patients with pulmonary and extrapulmonary diseases (V.P. Myneedu et al., 2017). In the present study, M. fortuitum 12(20.3%) was the second most prevalent and M. chelonea 3 (5%) was the third most prevalent NTM isolated in pulmonary samples. Whereas M. fortuitum being the most prevalent isolate in the study of Chakrabarti et al. and Das et al. MV Jesudasan from Vellore reported M.chelonea as the most prevalent isolate followed by M.fortuitum, both accounted for 67% of NTM isolated from respiratory specimens in contrast in our study M.fortuitum and M.chelonea strains accounted for 25.4%. (Jesudason MV et al. 2005).

Diabetes and HIV infection are known to suppress the cellular arm of immunity and hence predispose the patient to NTM infection. Both are considered as important risk factors for NTM infection. In the current study, the HIV positivity rate was found to be lower (1.6%). In our study, M. abscessus was the most prevalent NTM species found in both pulmonary and extrapulmonary infections, this suggests M. abscessus could be the highly prevalent strain in this region

CONCLUSION

Even though an increase in the incidence rate of NTM infection, its prevalence, species diversity, and circulation pattern in India is mostly unknown. With new and improved tuberculosis diagnostic tools, pulmonary infections due to non-tuberculous mycobacterium (NTM) are increasingly recognised worldwide. This study evaluated a novel method of identification of NTM by amplification of the mycobacterial ITS gene followed by RFLP. The clinical spectrum of NTM can be assessed by regular documenting and reporting the isolates in clinical settings along with their antibiogram for better treatment outcome.

Acknowledgement

We want to acknowledge Dr.E.Prabhakar Reddy, Professor, Dept. of Biochemistry, SLIMS, Pondicherry and Dr. A.Neelima Sudharshan, Asst. Professor, Dept. of Microbiology, NIMS, Secunderabad, for their support in research work and collaboration.

Conflicts of Interest

NIL

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