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# Genotypic identification of Non Tuberculous Mycobacteria from culture proven isolates- A 7 years retrospective study from a Referral laboratory, Chennai

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**ABSTRACT**

**Background:** Non-tuberculous Mycobacteria (NTM) are ubiquitous in the environment and gaining attention due to emerging outbreaks. Laboratory diagnosis of NTM is crucial for the proper management of the patients.

**Materials and Methods:** A total of 30 NTM isolates from 1416 clinical specimens were subjected to genome sequencing targeting hsp65 gene for the identification of NTM. Antibiogram for the isolates were put up for Amikacin (AMK), Ciprofloxacin (CFX), Moxifloxacin (MFX),Norfloxacin (NFX), Ofloxacin (OFX), Clarithromycin (CLR), Azithromycin (AZM), Imipenum (IMP), Tobramycin (TOB), Gatifloxacin (GAT) and Doxyclyclin (DOX) by disc diffusion method.

**Results:** Among 30 clinical isolates, majority of NTM were identified as Mycobacterium abscessus [11], followed by M. fortuitum [7], M. kansasii [2], M. massiliense [2] and one each of M. parascrofulaceum , M. conceptionense, M. africanum, M. senegalense. M. scrofulaceum, M. marinum, M. intracellulare, M. wolinskyi .The in vitro susceptibility of 30 NTM isolates showed sensitivity to AMK and TOB (29), AZM (27), CFX, MFX, IMP, CLR, GAT (26), OFX, DOX (25) and NFX (24).

**Conclusion:** Our study emphasized on rapid identification of NTM by Genome sequencing within 8-10 hours of isolation which was advantageous over the conventional method .The molecular technique explored in this study will aid the clinician for initiation of reliable, appropriate and timely treatment for the patients.

**Keywords**: DNA sequencing, Identification, Kirby Bauer disc diffusion, NTM, , PCR.

## INTRODUCTION

Non tuberculous Mycobacteria (NTM) are group of bacteria that do not belong to *Mycobacterium tuberculosis* (*M. tuberculosis*) complex. NTM are ubiquitous and are usually considered as saprophytes, and have been recovered from the environment, particularly in dust, watery soil and water distribution systems.[1] Recently 125 NTM species have been identified by molecular techniques especially using *16S rRNA* gene sequencing for new species cataloguing. [2] NTM is gaining attention now a days due to emerging outbreaks in human beings. The predisposing factors for disease from NTM are weakened immune system, lung disease, smoking, and alcohol abuse. In humans, NTM commonly affect the lungs, lymph nodes, skin, and soft tissue. Patients when presenting with NTM have respiratory distress with productive cough, abnormal chest x-ray and may also have fever, weight loss which often resembles tuberculosis, may require accurate laboratory diagnosis to differentiate *M. tuberculosis* and NTM.[3] Moreover, mycobacterial species differ in virulence and exhibit characteristic antimicrobial

patterns. Hence, rapid identification and antimicrobial pattern of NTM is highly crucial for the clinicians to start appropriate treatment for the patients. Thus, the present study was focused on the rapid identification of NTM by PCR based DNA sequencing targeting *hsp65 gene* from culture proven isolates received for mycobacteriological investigations and further determination of the drug resistance pattern of NTM by disc diffusion method.

## Materials and methods:

### Clinical isolates:

A total of 30 culture proven NTM isolates (13- biopsy/pus from various organs, 7- sputum, 6 - broncho alveolar lavage (BAL), 2 -corneal scraping and 1 each from sclera tissue and aqeous wash) from 1416 clinical specimens received for mycobacteriological investigations during the period of 2012-2019 were included in the study.

### DNA extraction and PCR targeting *hsp 65* gene:

DNA extraction was performed by using Qiagen DNA extraction mini kit (Hilden, Germany) according to the manufacturer's instructions. For the PCR amplification a 50µl reaction was set with 5µl of 10XPCR buffer (100mM Tris-HCl[pH 8.3], 500mM KCl, 0.1% gelatin, 15mM MgCl2), 8µl of 200µM of each dNTPs, 1µl of 1 picomole of each primer [4][Forward primer Tb 11: 5' ACCAACGATGGTGTGTCCAT 3', Reverse primer Tb12: 5' CTTGTCGAACCGCATACCCT 3'], 0.75µl of 2 units of *Taq* polymerase, 5µl Sterile Glycerol and 5µl of extracted DNA was used as the template and the final volume was made up to 50µl with sterile Milli Q water. *M. tuberculosis* H37Rv ATCC DNA was used as a positive control for the experiment. The PCR thermal profile consists of 40cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. Amplification of the 439-bp product of the hsp65 gene was detected by 2% agarose gel electrophoresis incorporated with 0.5 µg/ml ethidium bromide for visualization by using UV transilluminator.

### DNA sequencing of the amplified PCR product of 439 bp:

DNA Sequencing of amplified products were carried out using an ABI prism 3110 automated DNA sequencer (Applied biosystems, USA). Cycle sequencing of the amplified products were performed in a 10-µl reaction volume, containing 1.5 µl of RR mix, 2.5 µl of sequencing buffer, 1 µl of forward primer/Reverse primer , 4 µl MilliQ water, and 1 µl of PCR amplified product. Amplification was carried out in the Perkin- Elmer thermo cycler using 25 cycles at 96°C for 10 s, at 50°C for 5 s, and at 60°C for 4 min, with initial denaturation at 96°C for 1 min. The cycle-sequenced products were then purified and sequenced using ABI Prism 3130 AVANT (Applied Biosystems, USA) genetic analyzer, which works based on the principle of Sanger's dideoxy termination method.

### Determination of drug resistance for NTM by disc diffusion method:

The isolated NTM were put up for amikacin (AMK), ciprofloxacin (CFX), moxifloxacin (MFX),norfloxacin (NFX), ofloxacin (OFX), clarithromycin (CLR), azithromycin (AZM), imipenum (IMP), tobramycin (TOB), gatifloxacin (GAT) obtained from Himedia Laboratories for determination of sensitivity pattern of the isolated NTM by Kirby Bauer disc diffusion method as per standard CLSI guidelines.

## Results:

### PCR based DNA sequencing targeting *hsp 65* gene:

All 30 NTM isolates were positive for PCR targeting *hsp 65* gene. DNA sequencing of the amplified products revealed 12 different species of NTM from 30 clinical isolates (**table 1**).



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**Table1: Details of NTM isolates identified by PCR based DNA sequencing targeting *hsp 65* gene**

| S.no | NTM isolates (N) | Details of Clinical specimens N |
|---|---|---|
| 1 | *M. abscessus* (11) | Biopsy/pus from various organs 4<br>BAL 4<br>Corneal scraping 2<br>Sputum 1 |
| *2* | *M. fortuitum* (7) | Biopsy/pus from various organs 5<br>Sputum 2 |
| *3* | *M. kansasii* (2) | sclera tissue 1<br>Aqeous wash 1 |
| 4 | *M. massiliense* (2) | Pus from unknowm region 1<br>BAL 1 |
| 5 | *M. parascrofulaceum* (1) | BAL |
| 6 | *M. conceptionense* (1) | Sputum |
| 7 | *M. africanum* (1) | Sputum |
| 8 | *M. senegalense* (1) | Pus from cervical vertebrae |
| 9 | *M. scrofulaceum* (1) | Sputum |
| 10 | *M. marinum* (1) | Pus from unknowm region |
| 11 | *M. intracellulare* (1) | Sputum |
| 12 | *M. wolinskyi* (1) | Pus from cervical vertebrae |

**Drug susceptibility testing by Kirby bauer disc diffusion test for 30 NTM isolates:**

The *in vitro* susceptibility of 30 NTM isolates showed sensitivity to AMK and TOB (29), AZM (27), CFX, MFX, IMP, CLR, GAT (26), OFX (25) and NFX (24) by Kirby bauer disc diffusion method (**table 2**).

**Table 2: Phenotypic drug susceptibility pattern exhibited by 30 NTM isolates by Kirby Bauer disc diffusion method**

| S.no | Number of the NTM isolates | Antibiogram pattern | |
|---|---|---|---|
| | | Sensitive (no of isolates) | Resistant (no of isolates) |
| 1 | *M. abscessus* (11) | AMK, TOB (10) | AMK, TOB (1) |
| | | AZM, CLR (9) | AZM,CLR (2) |
| | | CFX, MFX, NFX, | CFX, MFX, NFX, |



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| | | | |
|---|---|---|---|
| | | GAT, IMP (8) | GAT, IMP (3) |
| | | OFX (7) | OFX (4) |
| *2* | *M. fortuitum* (7) | AMK, OFX, CFX, IMP, MOX, (7) | AZM, NFX, TOB, CLR, GAT (1) |
| | | AZM, NFX, TOB, CLR, GAT (6) | |
| *3* | *M. massiliense* (2) | AMK, AZM, TOB, CLR (2) | IMP,CFX,OFX, NFX,GAT,MFX (1) |
| | | IMP,CFX,OFX, NFX,GAT,MFX (1) | |
| 4 | *M. marinum* (1) | AMK, AZM, CPX, MOX, OFX, TOB,IMP,CLR | NFX |
| 5 | *M. kansasii* (2) | AMK, AZM, CFX, MFX, NFX, OFX, TOB, IMP, CLR, GAT | Nil |
| 6 | *M. parascrofulaceum* (1) | | |
| 7 | *M. conceptionense* (1) | | |
| 8 | *M. africanum* (1) | | |
| 9 | *M. senegalense* (1) | | |
| 10 | *M. scrofulaceum* (1) | | |
| 11 | *M. intracellulare* (1) | | |
| 12 | *M. wolinskyi* (1) | | |

**Discussion:**

Rapid diagnosis of NTM is an important factor for providing appropriate treatment for the patients and to avoid inaccurate diagnosis which may lead to therapeutic approaches consistent with *M. tuberculosis* infection that are highly inappropriate because of the development of drug resistance. Conventional method of diagnosis of NTM is time consuming and fails to identify upto species level of mycobacteria. Thus, the PCR based DNA sequencing method targeting either *16s rRNA* or *hsp65* gene would rapidly identify the NTM both from clinical specimens and isolates. In our earlier study, we reported a case of *M. massiliense* in a corneal ulcer by PCR-based DNA sequencing targeting the *hsp 65* gene and confirmed the identification by PCR-based RFLP targeting the hsp65 gene. [5] In a recent South Korean study by Kim et al, the authors evaluated the sequences of NTM for *16S rRNA*, *hsp65*, and *rpoB* genes collected from GenBank, checked manually and constructed a database for the genes by multilocus sequence analysis (MLSA) using 109 clinical isolates. They discovered that, the rates of species- level identification of NTM were 71.3%, 86.79%, and 81.55% with *16S rRNA*, *hsp65*, and *rpoB* genes respectively and finally identified 97.25% of the NTM isolates to the species level using MLSA.[6] Dastranj et al from Iran studied PCR based DNA sequencing of *16S rRNA* and *rpoB* genes on 37 NTM sputum isolates and identified 12



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different species of NTM and the majority were *Mycobacterium simiae* (22%), *M. fortuitum* (19%), and *M. abscessus* (13%).[7] As a view from Indian scenario, Pooja Sharma et al, performed NTM identification by PCR- restriction analysis (PRA) and gene sequencing targeting *hsp 65* gene. From their study, *M. intracellulare* was the commonest species isolated, followed by *M. gordonae*, *M. abscessus and M. avium* .[8]

In the present study, 30 NTM were identified by PCR based DNA technique targeting hsp65 gene and the antibiotic drug resistance pattern exhibited by NTM were analysed using Kirby Bauer disc diffusion method. From our study, *M. abscessus* (37%) was the predominant NTM identified, followed by *M. fortuitum* (23%), *M. massiliense* (7%), *M. kansasii* (7%). Regarding the sensitivity pattern, 97% of the NTM isolates were sensitive to AMK and TOB and high resistance (20%) was observed with NFX. The main limitation of this study was the sample size which had low statistical value. To conclude, our study emphasized on rapid identification of NTM by Genome sequencing within 8-10 hours of isolation which was advantageous over the conventional method .The molecular technique explored in this study will aid the clinician for initiation of reliable, appropriate and timely treatment for the patients.

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