ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by Pharmascope Publications Journal Home Page: www.pharmascope.org/ijrps

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

Praveen Kumar V*[1], Sreenivasulu Reddy V[2], Suresh P[1], Vamsi Muni Krishna P[1]

[1]Ph.D Scholar, Bharath University, Agaram Road, Selaiyur, Chennai – 600073 Tamil Nadu, India
[2]Department of Microbiology, Sri Lakshmi Narayana Institute of Medical Sciences, Pondicherry-605502 India

Article History:

Received on: 02.07.2018
Revised on: 23.09.2018
Accepted on: 26.09.2018

Keywords:

Non-tuberculosis mycobacteria,
RGM,
SGM,
Inter Transcribed Spacer gene,
PCR – RFLP

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.

* Corresponding Author

Name: V.Praveen Kumar
Phone: +91-8142037662
Email: vpraveenkumar4@gmail.com

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i4.1654

Production and Hosted by

Pharmascope.org

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.leprae* 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 J 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 MACHEREY-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

B5 to the column and centrifuge for 30 s at 11,000 xg. Discard flow-through and place the column back into the Collection Tube. Centrifuge the column for 30 s at 11,000 x g. Elute highly pure DNA - Place the NucleoSpin® Microbial DNA Column into a 1.5 ml nuclease-free tube and add 100 µl Buffer BE onto the column. Incubate at room temperature for 1 min. Centrifuge 30 sec at 11,000 xg.

16S-23S ITS - PCR – based identification: An approximately 221-bp region of 16S-23S rRNA *ITS* gene was amplified by PCR using two specific primers. (*fig:1*) The primers of *ITS* gene were Sp 1(5'- ACC TCC TTT CTA AGG AGC ACC -3') Sp 2 (5'-GAT GCT CGC AAC CAC TAT CCA -3'). The PCR was performed with PCR reaction setup, consisting of 2µl of DNA, forward and reverse primers 0.5µl each, master mix 10µl and 7µl distilled water. The first PCR was performed by denaturing the samples 3min at 94°C, then 40 cycles including 94°C for 1min, 56°C for 1min, 72°C for 1 min and a final extension at 72°C for 10 min. Amplification of 221-bp product of the *ITS* gene was detected by 2% agarose gel electrophoresis.

Restriction fragment length polymorphism (RFLP): PCR amplified product of 221-bp of the *ITS* gene was aliquoted into two tubes both clinical and quality control strains were digested with the enzymes *BstEII* and *HaeIII* for 3 hours at 37°C. The digested products were visualized on 3% agarose gel electrophoresis and the RFLP patterns were analyzed according to fragment sizes. (Mi-Ae Jang *et al.,* 2014; Telenti A *et al.,* 1993; Shradha Subedi *et al.,* 2016; Chimara E, 2008)

RESULTS

During the study period, a total of 4295 clinical specimens were analyzed during this period. Of these 852 and 1138 were positive for AFB by ZN staining and culture method. Out of 1138 cultures, 96 (8.4%) were considered as Non-tuberculous mycobacteria. Of the 96 Non-tuberculous mycobacteria 59(61.4%) were from pulmonary sites and 37 (38.5%) were from extrapulmonary sites. The predominant age group in our study was 51 - 60 years (35.5%) followed by 61 – 70yrs (27.1%), 31 – 40yrs (15.2%), 41 – 50 (11.8%), <20 (5%) , 21-30 (3.3%) and 71 -80 (1.6%). Demographic characters of patients were mentioned in the *Table: 1*

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

Characteristic	Patient %
Sex	
Male: 37	37 (62.7%)
Female: 22	22 (37.2%)
Age	
<20	3 (5%)
21 – 30	2 (3.3%)
31 – 40	9 (15.2%)
41 – 50	7 (11.8%)
51 – 60	21 (35.5%)
61 – 70	16 (27.1%)
71 – 80	1 (1.6%)
Previous TB treatment	9 (15.2%)
HIV infection	1 (1.6%)
Other respiratory symptoms	24 (40.6%)
Type II Diabetes mellitus	7 (11.8%)
Characteristics of tuberculosis patients (n=59)	

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-tuberculosis 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

Table 1: Diversity of NTM species from pulmonary clinical isolates

Specimens	*M.abscessus*	*M.simiae*	*M.fortuitum*	*M.chelonae*	*M.gordonae*
Sputum	11	4	8	2	-
Bronchial washings	13	7	4	1	1
Bronchoalveolar Lavage	5	3	-	-	-
Total	29 (49.1%)	14(23.7%)	12(20.3%)	3(5%)	1(1.6%)

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 predominated 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 Umarao 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. chelonae* 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.chelonae 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.chelonae 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

REFERENCES

AK Maurya, S Kant, VL Nag, Rash Kushwaha, TN Dhole. Detection of the 123bp fragment of the insertion element IS6110 Mycobacterium tuberculosis for diagnosis of extrapulmonary tuberculosis. Indian Journal of Medical Microbiology, (2012) 30(2): 182-6.

Ali Nour – Neamatollahie, Nayereh Ebrahimzadeh, Seyed Davar Siadat, Farzam Vaziri, Mona Eslami, Abbas Akhavan Sepahi, Sharareh Khanipour, Morteza Masoumi, Fatemeh Sakhaee, Morteza Ghazanfari Jajin, Ahmadreza Bahrmand, Abolfazl Fateh. Distribution of non-tuberculosis patients by heat shock protein 65 PCR – RFLP. Saudi Journal of Biological Sciences (2007) 24, 1380-1386.

Chakrabarti, M. Sharma, M. Dubey, Isolation rates of different mycobacterial species from Chandigarh (North India), Indian J. Med. Res. 91 (1990) 111–114.

Das, V. Sharma, L. Bhau, et al., Characterization of mycobacterial strains from clinical specimens, Indian J. Pathol. Microbiol. 25 (1982) 19–27.

HY Kim, SY Lee, BJ Kim and YH Kook. Allele-specific duplex polymerase chain reaction to differentiate Mycobacterium abscessus

subspecies and to detect highly clarithromycin-resistant isolates. Indian Journal of Medical Microbiology, (2016) 34 (3): 369-374.

Jason E.Stout, Won –Jung Koh, wing Wai Yew. Up to date on pulmonary disease due to non-tuberculous mycobacteria. International Journal of Infectious Diseases.45 (2016) 123-124.

Jesudason MV, Gladstone P.Non tuberculous mycobacteria isolated from clinical specimens at a tertiary care hospital in South India. Indian J Med Microbiol. 2005 Jul;23(3):172-5.

Jyoti Umrao, Dharamveer Singh, Amreen Zia, Swati Saxena, Surendra Sarsaiya, Shushma Singh, Jahanarah Khatoon, Tapan N. Dhole. Prevalence and species spectrum of both pulmonary and extrapulmonary nontuberculous mycobacteria isolates at a tertiary care centre. International Journal of Mycobacteriology 5 (2016) 288 – 293.

K. Karak, S. Bhattacharyya, S. Majumdar, et al., Pulmonary infection caused by mycobacteria other than M. tuberculosis in and around Calcutta, Indian J. Pathol. Microbiol. 39 (1996) 131–134.

Katoch V.M. Infections due to Non-Tuberculous Mycobacteria (NTM). *Indian J Med Res* 2004; 120: 290-304.

Koneman EW, Allen SD, Janda WM, Schrekenberger PC, Winn WC JR. Colour atlas and textbook of diagnostic microbiology. 5th ed JB Lippincott Company; 1997. P.877-996.

L.Garcia-Agudo, P.Garcia-Martos. Clinical significance and antimicrobial susceptibility of rapidly growing mycobacteria.

Mi-Ae Jang, Won-Jung Koh, Hee Jae Huh, Su-Young Kim, Kyeongman Jeon, Chang-Seok Ki, Nam Yong Lee. Distribution of Nontuberculous Mycobacteria by Multigene Sequence-Based Typing and Clinical Significance of Isolated Strains. Journal of Clinical Microbiology.

Park, C.K.Kwon, Y.S., 2014. Respiratory review of 2014: tuberculosis and non-tuberculous mycobacterial pulmonary disease. Tuberc. Respire Dis (Seoul) 77, 161-166.

Prabha Desikan, Karuna Tiwari, Nikita Panwalkar, Saima Khaliq, Manju Chourey, Reeta Varathe, Shaina Beg Mirza, Arun Sharma, Sridhar Anand, and Manoj Pandey. Public health relevance of non-tuberculous mycobacteria among AFB positive sputa. Germs. 2017 Mar; 7(1): 10–18.

Sarika Jain, Manimuthu M Sankar, Navneet Sharma, Sarman Singh, and T D Chugh. High prevalence of non-tuberculous mycobacterial disease among non-HIV infected individuals in a TB endemic country – experience from a tertiary centre in Delhi, India Pathog Glob Health. 2014 Mar; 108(2): 118–122.

Sharma B, Pal N, Malhotra B, Vyas L. Evaluation of a Rapid Differentiation Test for Mycobacterium Tuberculosis from other Mycobacteria by Selective Inhibition with p-nitrobenzoic Acid using MGIT 960. J Lab Physicians. 2010 Jul;2(2):89-92. doi: 10.4103/0974-2727.72157.

Shraddha Subedi, Fanrong Kong, Peter Jelfs, Timothy J. Gray, Meng Xiao, Vitali Sintchenko, Sharon C-A Chen. 16S-23S Internal Transcribed Spacer Region PCR and Sequencer-Based Capillary Gel Electrophoresis have Potential as an Alternative to High-Performance Liquid Chromatography for Identification of Slowly Growing Nontuberculous Mycobacteria. Journal pone.0164138 October 17, 2016.

Singh AK, Maurya AK, Umrao J, Kant S, Kushwaha RA, Nag VL, Dhole TN. Role of GenoType Mycobacterium Common Mycobacteria/Additional Species Assay for Rapid Differentiation Between Mycobacterium tuberculosis Complex and Different Species of Non-Tuberculous Mycobacteria.J Lab Physicians. 2013 Jul;5(2):83-9. doi: 10.4103/0974-2727.119847.

Tarcisio Fedrizzi, Conor J. Meehan, Antonella Grottola, Elisabetta Giacobazzi, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, De Sanctis V, Rumpianesi F, Pecorari M, Jousson O, Tortoli E, Segata N. Genomic characterization of Nontuberculous Mycobacteria. Sci Rep. 2017 Mar 27;7:45258. doi: 10.1038/srep 45258.

Telenti A, Marchesi F, Balz Marianne. Rapid Identification of Mycobacteria to the Species Level by Polymerase Chain Reaction and Restriction Enzyme Analysis. J Clin Microbiol. 1993;31:175–178.

V.P. Myneedu, A.K. Verma, M. Bhalla, J. Arora, S. Reza, G.C. Sah and D. Behera. The occurrence of Non-Tuberculous Mycobacterium In Clinical Samples - A Potential Pathogen. Indian Journal of Tuberculosis. 2013; 60: 71 – 76.

Wallace R J Jr, O'Brien R, Glassroth J, Raleigh J, Dutta A. Diagnosis and treatment of disease caused by Non-Tuberculous Mycobacteria. *Am Rev Respir Dis* 1990; 142:940-53.

Wenjuan Nie, Hongfei Duan, Hairong Huang, Yu Lu, and Naihui Chu. Species Identification and Clarithromycin Susceptibility Testing of 278 Clinical Nontuberculosis Mycobacteria Isolates. Biomed Res Int. 2015; 2015: 506598.