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# Identification And Molecular Characterization Of Non-Tuberculous Mycobacteria In Tuberculosis Suspected Patients.

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**A B S T R A C T**

Introduction: The study aims to identify and characterize nontuberculous mycobacteria at the molecular level in patients suspected of having tuberculosis, as standardized identification criteria are recommended by the American Thoracic Society for quicker clinical and laboratory procedures.
Method: 772 samples were collected from 386 patients (2 each) in selected districts of Jammu and Kashmir. In addition to phenotypic, molecular methods were also used to detect the number and species of Non tuberculosis mycobacteria. Records of patients were collected for clinical information, such as symptoms and radiological findings. Results: Out of 772 samples, 180 (23.31%) were positive for acid-fast bacteria, with 164 (91.11%) and 16 (8.89%) identified as M. tuberculosis complex and NTM strains. Mycobacterium abscessus and M. intracellular isolates were the most frequent. Common symptoms included cough, fever, shortness of breath, weight loss, sputum production, appetite loss, night sweating, and thrombocytosis. Conclusion: A study of 386 patients revealed that most were over 40 years old, with a higher rate of non-tuberculous mycobacteria (NTM) infections in males. Common symptoms included cough, fever, shortness of breath, weight loss, and sputum production. Positive cultures showed 91.11% MTB complexes and 8.89% NTM growth. The study identified 5 different mycobacterial species with 100% concordance.

## INTRODUCTION

Tuberculosis, an ancient disease, has been present in humans since prehistory. It may have first appeared around 150 million years ago[1]. Despite the first humans leaving Africa 1.7 million years ago[2], they likely brought TB with them. Written accounts date back as long as 2300 years in China and 3300 years in India[3,4].
Mycobacterium tuberculosis was once the only Mycobacterium infection in humans, causing significant social impact. Other mycobacterium species, known as anonymous or atypical mycobacteria, mycobacteria other than tuberculosis (MOTT), and non-tuberculous mycobacteria (NTM), are more common and have thicker, lipid-rich cell walls. Mycobacteria, resistant to hydrophilic nutrients, heavy metals, antibiotics, and disinfectants, are found in various environments. Non-tuberculous mycobacteria (NTM) spread disease at varying rates, and host factors are now considered more significant in the pathophysiology of NTM infections, despite environmental variables being suspected. Host-organism interaction can occur due to environmental exposure or host components[5,6]. Soil and water sources have high concentrations of NTM, which contributes to biofilm growth and antibiotic resistance. NTM's hydrophobicity allows preferential aerosolization from water, and many species can withstand high temperatures and low pH[7,8].

There is limited evidence that NTM is spread from human to human, unlike leprosy and tuberculosis. Since NTM infections cannot widely spread, they are not reported; yet, there are few surveillance data available. It has been demonstrated that over 140 distinct mycobacteria species are hazardous to humans. Among NTM species that cause sickness in humans, Mycobacterium avium complex (MAC) and Mycobacterium kansasii are the most common[9,10].

From the early 20th century, reports of non-tuberculous mycobacteria from clinical specimens were infrequent. However, in the 1950s, a new concept emerged, focusing on mycobacterial infection and the relationship between infection with organisms other than the tubercle bacillus and mild tuberculin responses. Two "novel" mycobacterial infections were described, establishing mycobacteria other than tunberculous bacilli as serious human illnesses. NTM infections are not reportable in India, making it difficult to determine prevalence due to clinical, radiographic, and microbiological criteria. Factors such as immune-suppressive medications, immune deficiency disorders, and chronic structural lung illnesses contribute to the increase in NTM infections[11,12].

NTM infection can manifest in various areas, including the lung, skin, soft tissues, lymphadenitis, empyema, eye, central nervous system, and genitourinary infections. Initially believed to be untransferable, genetic evidence suggests human-to-human transmission[13]. Treatment typically involves antibiotics, with surgery for non-responsive patients, posing a high risk of complications[14].

Non-tuberculous mycobacterial pulmonary disease (NTM-PD) is a common comorbidity in patients with underlying respiratory diseases like bronchiectasis, cystic fibrosis, and chronic obstructive pulmonary disease[15]. Common NTM species causing NTM-PD include Mycobacterium avium complex, Mycobacterium kansasii, Mycobacterium xenopi, Mycobacterium abscessus, and Mycobacterium malmoense[16]. The discussion of pulmonary NTM disease in relation to tuberculosis is motivated by two factors: NTM-related lung illness shares symptoms with TB, and NTM isolates may become resistant to first-line anti-TB drugs[17].

Unfortunately, it can be challenging to diagnose NTM infections, and patients commonly receive a delayed or inaccurate diagnosis, which can have detrimental long-term effects. The purpose of this research was to identify and characterize non-tuberculous mycobacteria at the molecular level in people who may have tuberculosis.

## MATERIAL AND METHODS

A study analyzed 772 clinical samples of 386 patients suspected of having tuberculosis between June 2021 and August 2024 in Jammu and Kashmir. The majority were male. Standardization from ATS/IDSA and American Thoracic Society was used to identify NTM isolates[18]. Patients with at least two positive cultures were included.

### NTM identification using phenotypic and genotypic testing

The study involved decontaminating sputum specimens with N-acetyl-L-cysteine, staining them with Ziehl-Neelsen, and preparing them for Acid Fast Bacilli (AFB) smear microscopy. Cultured in Lowenstein-Jensen medium[19], they were incubated for eight weeks[20]. To differentiate between MTB and NTM[21], SD Bioline TB Ag was used to expose AFB-positive growth to the MPT64 antigen. PCR and biochemical testing were used to identify the species, all based on Centers for Disease Control (CDC) procedures for the isolation of NTM strains.

### Immunochromatographic assay (ICA):

The SD Bio-Line MPT64 TB test was used for rapid immunochromatographic assay (ICA) to differentiate between MTB and NTM21. 200 µL of extraction buffer was used to emulsify three or four colonies of mycobacterial strains culture, adding 100 µL to each well, and visually assessing the results using color development after 15 minutes of incubation.

### Molecular methods of identification

#### Extraction of DNA by boiling

A colony of bacteria was aseptically extracted and subsequently placed in an eppendorf tube containing 100 µl of sterile water. The sample was subjected to vortexing for a duration of 10 to 15 seconds. Following this, the tube was heated to 99°C for five minutes utilizing a Biometra T 3000 thermal cycler. After centrifugation at 12,000 rpm for 10 minutes, the supernatant was carefully removed and stored at 4°C. To determine the concentration of DNA, a NanoDrop™ spectrophotometer (Thermo Scientific) was employed to evaluate the concentration and purity of the final DNA extract obtained from the bacterial culture. The NanoDrop was calibrated according to the manufacturer's instructions. Once the system was operational, the sensor was cleaned with 1 µl of sterile water. After performing a second blanking, 1 µl of the DNA sample was applied to the sensor, and the results were

recorded.

PCR Preparation for all NTM isolates:
Several targets were amplified from the mycobacterial DNA using PCR. All reactions shared the same basic reaction mix composition, with the following little adjustments needed for different targets. Every amplification was done in a 50µl final reaction volume. The master mix concentration for each target is displayed in Table 1.

Table 1. Throughout this investigation, BiolineTM products (core kit) were utilized.

| Table 1: Master Mix volumes for target | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target | 10X Buffer | 25mM MgCl2 | 100mM dNTP | Primer mix F/R | Taq Polymerase | DNA Template | H2O | Total |
| Hsp65 | 5 µl | 3 µl | 1 µl | 1 µl | 0.5 µl | 3 µl | 35.5 µl | 50 µl |

Primer Sets for identification of NTMs

| Amplifying region | 5’ 3’ |
|---|---|
| Hsp65 (TB11/12) | TB11-ACCAACGATGGTGTGTCCAT |
| | TB12-CTTGTCGAACCGCATACCCT |

Molecular Diagnosis of NTM
NTM strains were analyzed using restriction enzyme techniques and polymerase chain reaction for species identification. The hsp65 protein is universally present in all mycobacterial species, with both common and unique epitopes. Standard primers including Tb11 (5′ – CAACGATG GTGTGTCCCAT) and Tb12 (5′ – CTTGTCGAACCGCAT ACCCT) were used for amplification, and distinctiveness enabled differentiation into specific species or subspecies. Agarose gel electrophoresis separated digestion products, revealed patterns associated with individual species.

Restriction enzyme analysis
The 10 µl of PCR product was digested with BstE II and HaeIII enzymes, respectively, in a solution containing 0.5 U of enzyme, 2.5 U of restriction buffer, and 11.5 µl of water. The mixture was incubated at 60°C for 60 minutes, and at 37°C for the same process. The digested products were loaded on 3% agarose gel electrophoresis at 100 V for 2 hour. To interpret the restriction analysis profiles obtained by different species, 50 bp ladder DNA size marker was used. The fragments were detected with ethidium bromide staining and UV transilluminator.

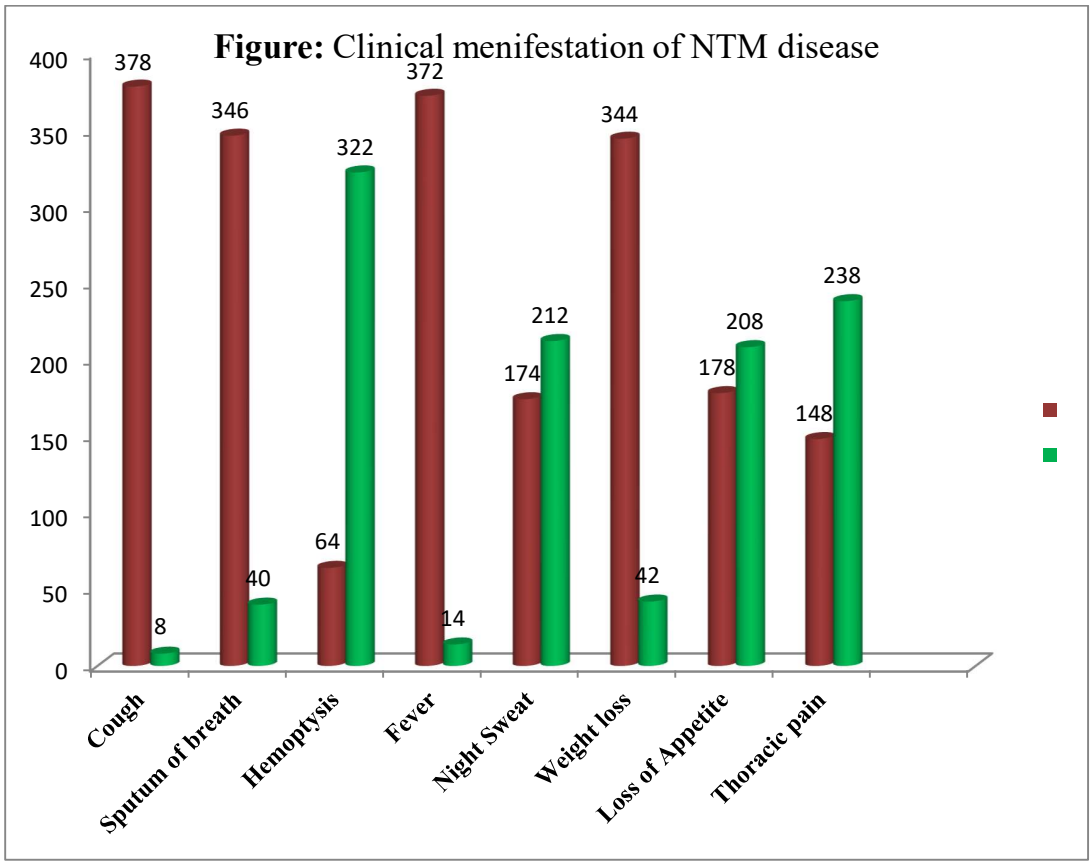
| Table 3: Age group wise distribution of suspected cases of pulmonary tuberculosis | | |
|---|---|---|
| Age groups | No. of cases | Percentage |
| 19-29 | 178 | 46.11 |
| 30-40 | 116 | 30.05 |
| >40 | 92 | 23.84 |
| Total | 386 | 100 |

**RESULTS**

The study collected demographic data from 386 patients, categorized into three age groups based on ten-year intervals. The highest age group was those over 40 years, with 178 cases. The 30-40year age group had 116 cases, and the 19-29year age group had 92 cases.
The study revealed a higher prevalence of NTM infections among males, with 204 males and 182 females identified out of 386 patients.

| Table 2: Gender wise distribution of suspected cases of pulmonary tuberculosis | | |
|---|---|---|
| Gender | No. of cases | Percentage |
| Male | 204 | 52.85 |
| Female | 182 | 47.15 |
| Total | 386 | 100 |

Mycobacterium infection symptoms include cough, fever, shortness of breath, weight loss, sputum production, appetite loss, night sweat, Thoracic pain, and hemoptysis.




The study involved collecting 772 sputum samples using a modified Pertroff's method. After decontamination, the samples were inoculated onto Lowenstein-Jensen medium slants and incubated at 37°C for up to four weeks. 124 samples tested positive for ZN staining, and 180 samples contained positive mycobacteria. The MPT 64 antigen-based immunological test was used to analyze the presence of MTB complexes or non-tuberculous mycobacteria (NTM) growth. Biochemical tests, including niacin, nitrate, and heat-resistant catalase, confirmed the positive growth findings. The results showed that 16.65% of the samples were NTM growth, while 95.34% were identified as M. tuberculosis complexes.

| Table 5: Identification of Mycobacterium species by various methods | |
|---|---|
| Test method | Test Positive |
| Direct ZN staining | 124/772 (62 out of 386 patients) |
| Culture on LJ media | 180/772 (90 out of 386 patients) |
| MPT64 antigen | 162/180 (81 out of 90 patients) |
| MTB Complex | 164/180 (82 out of 90 patients) |
| NTM | 16/180 (8 out of 90 patients) |

**Figure :** Direct staining, Culture result and MPT 64 Ag

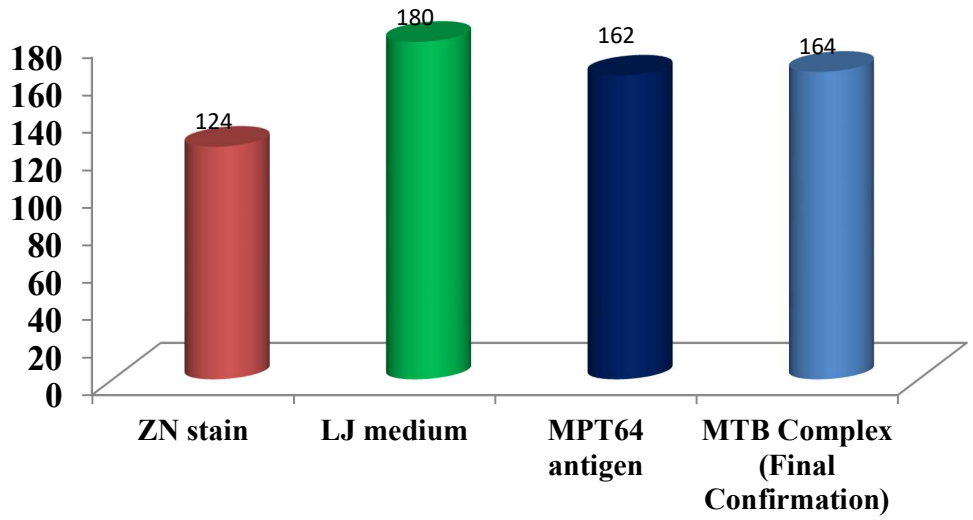
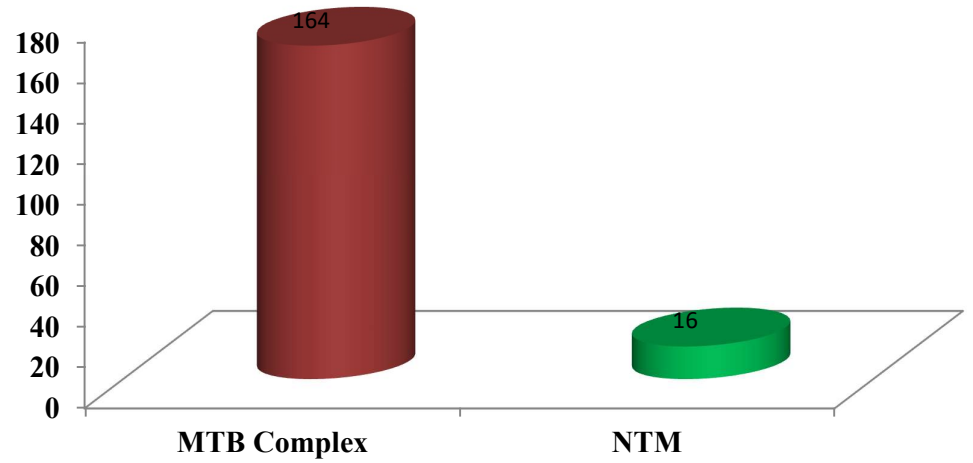



**Figure:** Detection of NTM and MTB complex




Lung fibrosis, observed in 88.86% of cases, is the most prevalent clinical manifestation, followed by cervical lymphadenopathy at 68.65%, nodularity at 61.40%, and unilateral lung involvement at 60.88%.

**Figure:** X-ray findings of lung with suspected TB infections

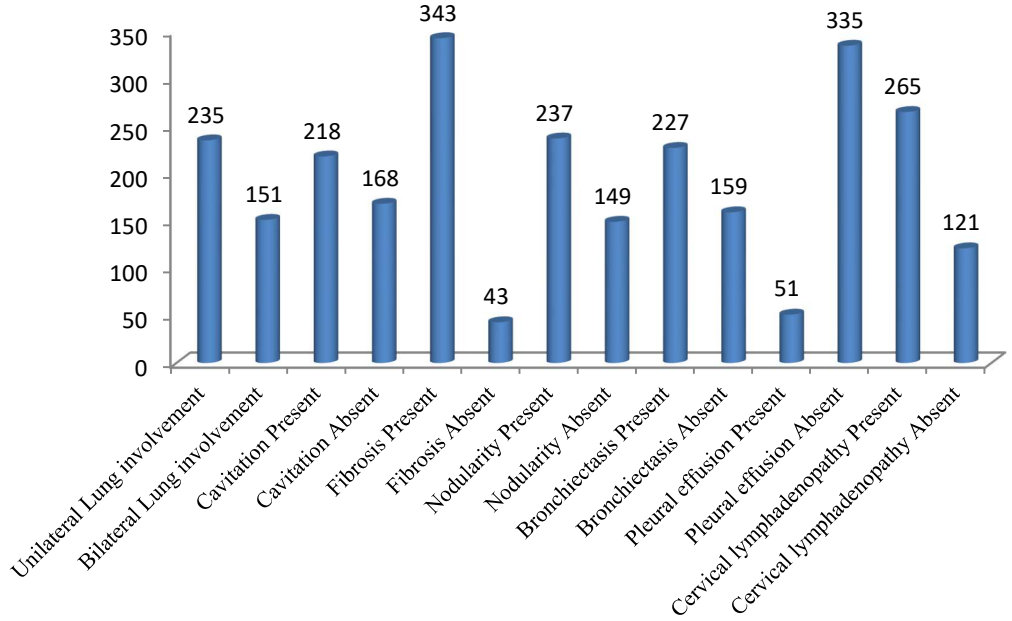


Figure: DNA amplification band

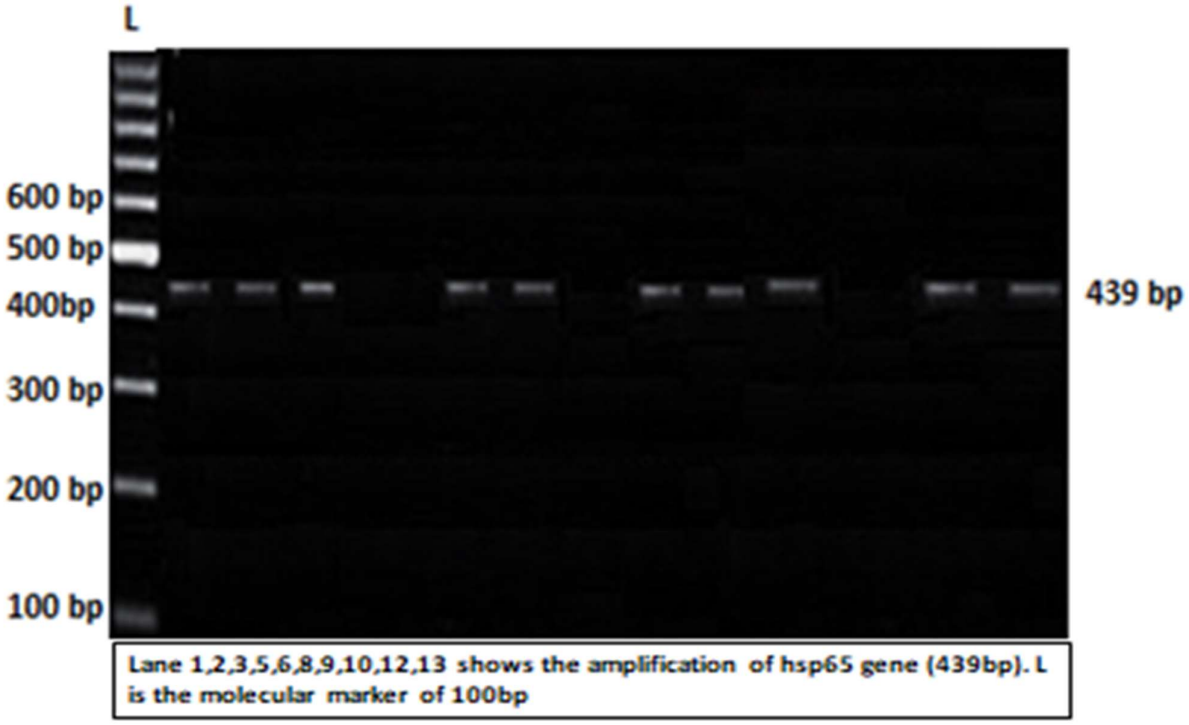
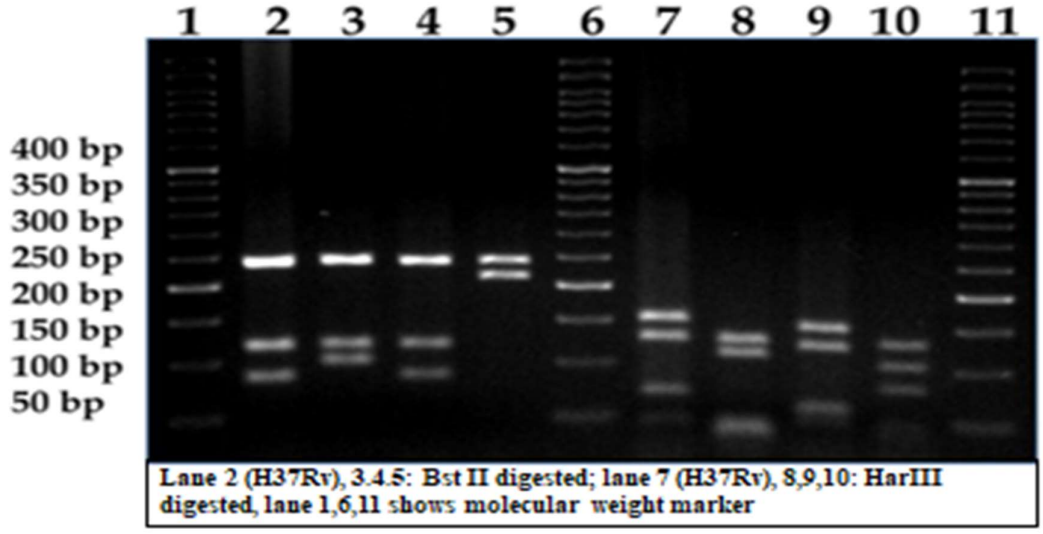
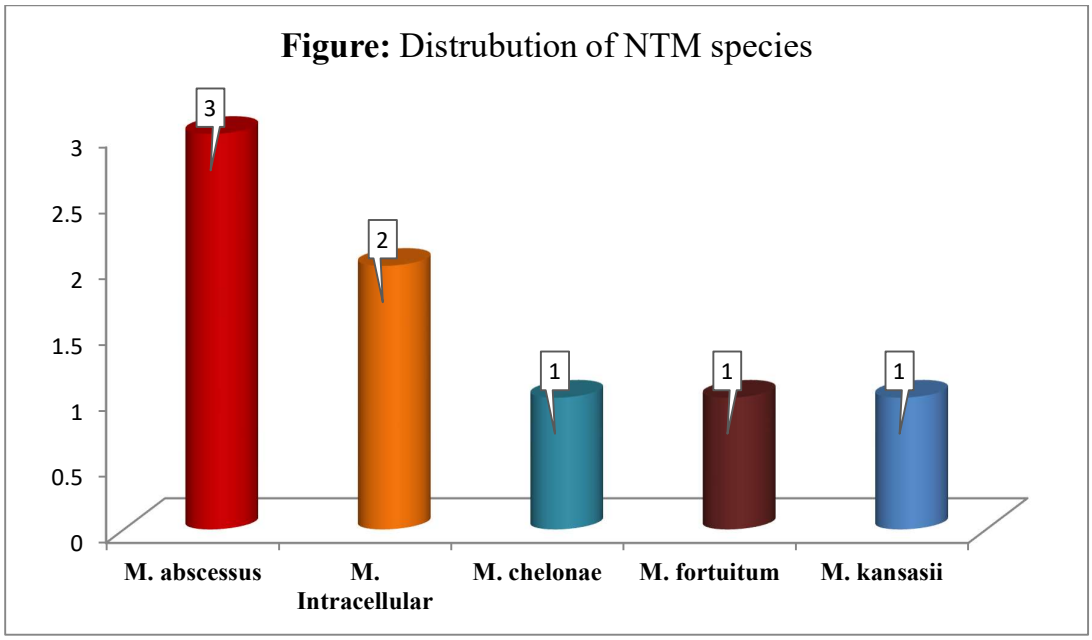



Figure: Restricted enzyme analysis




The PCR-REA method used to identify mycobacteria, revealed five distinct species. The study identified all eight strains of Mycobacteria at the species level when digested with BstE11 and Hae111 restriction enzymes. The most frequently encountered species was Mycobacterium abscessus. The PCR-REA test showed a strong correlation with biochemical identification, achieving a 100% concordance rate. The identified species were M. chelonae (12.5%), M. abscessus (37.5%), M. intracellular (25%), M. fortuitum (12.5%), and M. kansasii (12.5%). 50% of samples contained isolates classified as rapidly growing mycobacteria, specifically M. abscessus and M. chelonae.




**DISCUSSION**

NTM's status as a major human pathogen has been cemented by a noteworthy increase in both individual cases and outbreaks over the past ten years. Previously, mycobacteria were distinguished at the species level through biochemical tests, a process that usually takes three to six weeks [22,23], this study investigates the use of various molecular techniques to quickly distinguish NTM isolates from two hospitals in southern Ireland at the species and sub-species levels Previously, isolates of mycobacteria (NTMs) were identified as separate species in hospital labs. NTMs, found in various environments, can cause various clinical symptoms. Diagnosing NTM pulmonary illness is challenging due to their similarity to tuberculosis. More precise diagnostic methods are needed, as smear microscopy cannot differentiate between MTBC and NTM infections. Special techniques are needed for treating MTBC and NTM effectively. However, because finding the organism does not necessarily indicate a clinical illness, it is challenging to determine the frequency and prevalence of NTM lung infections with accuracy. In the current study, 8 NTMs (2.07%)were found among 386 Pulmonary TB suspected patients (8 out of 90 culture positive patients, 8.88%). In a study conducted in Delhi, Jain et al. found that 13 NTMs were found in 237 lung samples, or 5.48% of the total. Nine of the thirteen NTMs in total were taken from extra pulmonary samples, whereas the other four were taken from pulmonary samples [24]. This investigation's findings aligned with the current study's findings. NTM levels in soil and water samples from Sewagram, Wardha, and those in patient clinical specimens were found to be related in 2007 by R. Narang et al. [25]. Twenty of the 26 NTM isolates that were found were isolated from soil, while six were isolated from water samples. Mycobacterium avium was the only species found in both environmental and clinical samples out of seven distinct NTM species. The majority of the individuals who took part in our research were men. Several studies conducted in India and other nations have shown that men were more susceptible to environmental NTM isolates due to their exposure in the workplace. In a study by M.V. Jesudason et al, [26], it was found that 64 cases (56%) involved males, while 51 cases (44%) involved females. K. G De Mello [27] from Brazil and Alejandro Hernandez-Solís from Mexico [28] represented 62.1%.

In this research, 9 isolates did not show positive results in the SD Bioline Ag MPT64 test, but 8 isolates tested negative using CBNAAT, indicating the presence of NTM. Based on Arora et al's findings, the SD Bioline Ag MPT-64 test may yield false negative outcomes due to a point mutation in the MPT64 gene. Therefore, for definitive confirmation of the presence of NTM, further molecular testing is necessary [29], which in our study was carried out using CBNAAT. Our research results indicate that the MPT 64 TB Ag rapid Immunochromatographic test has a sensitivity of 88.89%. The research conducted by S. Shenai [30], E. Streit [31], B. Varghese [32], and D. P. O'Brien [33] highlighted that NTM had a greater impact on the respiratory system compared to extra pulmonary infections.

Based on our investigation, Mycobacterium abscessus 3/8 (37.5%) appears to be the most prevalent NTM type,

followed by Mycobacterium Intracellular 2/8 (25%). These results align with and deviate from various studies conducted in India. A study by Prabha Desikan et al. [34] in Bhopal reported M. abscessus as the most frequently identified species at 53.8%, while research by Paramasivan et al. [35] in Chennai, South India, identified NTM in pulmonary samples of patients within the BCG clinical trial area, with M.avium/intracellularae being the most commonly isolated species at 22.6%. A study by B Nasr-Esfahani and colleagues [36] involved the amplification of a 441 base pair segment of the hsp65 gene of NTM. This was followed by restriction digestion using BstEII and HaeIII endonucleases, and subsequent analysis of the restriction fragment patterns, leading to the identification of 19 (86.4%) NTM isolates. Three species showed profiles that differed from the known profiles of RFLP, making it impossible to ascertain. Turenne and Tschetter's research [37] demonstrated that hsp 65 PRA was the most effective method for identifying certain NTM strains, such as M. gastri and M. kansasii, which could not be detected using alternative techniques. Wong and Yip's study [38] revealed that the hsp65 gene PCR-RFLP can identify approximately 74.5% of NTM isolates. Telenti and Marchesi [39] utilized PCR-RFLP to categorize ten non-tuberculous mycobacteria (NTM) isolates to the species level. They amplified a 439 bp segment and subjected it to digestion using BstEII and HaeIII restriction enzymes. Our research revealed that M. abscessus accounted for 37.5% of NTM, M. intracellulare for 25%, M. chelonae for 12.5%, M. fortuitum for 12.5%, and M. kansasii for 12.5%. We observed that M. abscessus was the most frequently identified NTM in respiratory samples. V.P.Myneedu et al [40] documented a high prevalence of M. simiae isolates in patients with pulmonary and extrapulmonary diseases in New Delhi. In pulmonary samples, M. fortuitum was detected in 12.88% and ranked as the third most common NTM, while M. chelonae was present in 10.88% and ranked fourth. MV Jesudasan from Vellore identified M. chelonae as the most prevalent isolate at 8.1%, followed by M. fortuitum, accounting for 67% of NTM isolated from pulmonary samples. In the research conducted by Chakrabarthi et al., B Goswami et al., and Das et al. [41, 42], M. fortuitum was found to be the most common isolate. Analysis of molecular sequences from conserved genes like 16S rRNA, hsp65, rpoB, and the 16S-23S internal transcribed spacer region has resulted in faster diagnoses. To validate RFLP findings, sequence analysis was performed targeting the amplified hsp65 gene product of NTM strains. Eight strains were confirmed as NTM species: Mycobacterium abscessus (3), M. Intracellular (2), M. chelonae (1), M. fortuitum (1), and M. kansasii (1). After analyzing 436 suspected isolates for NTM, Delhi-based Sarika Jain [42] and colleagues found 68 NTM isolates of 16 different species, 17 Mycobacterium tuberculosis complex isolates, and 3 each of Rhodococcus equi, Tsukamurella pulmonis, Paenibacillus spp., and Nocardia carnea isolates. Like Grace and colleagues from Zambia, their study's results demonstrate that all potentially dangerous strains can be found using DNA sequence analysis of the ITS region, with M.intracellularae being the most prevalent isolate (27.8%). In contrast, InesJoao et al. partially sequenced the hsp65 and 16S rRNA genes of 54 clinical isolates and 22 reference strains. 16S rRNA sequencing alone is insufficient to reliably identify NTM species. To identify NTM at the species level, it is recommended to combine 16S rRNA gene and hsp65 gene sequence analysis with various databases. Compared to 16S rRNA, hsp65 performed better when a single gene was employed [43].

## CONCLUSION

A study involving 386 patients found that the majority were over 40 years old, with a higher rate of non-tuberculous mycobacteria (NTM) infections observed in males. Common symptoms of mycobacterium infection included cough, fever, shortness of breath, weight loss, sputum production, loss of appetite, night sweats, thoracic pain, and hemoptysis. Sputum samples were collected and processed for culture, with negative cultures confirmed by smear examination. Positive cultures were further evaluated for mycobacterial growth using immunochromatography and biochemical tests. The results showed that 91.11% of positive cultures were MTB complexes, while 8.89% were identified as NTM growth. It was found molecular methods are accurate to differentiate species comparing with phenotypic methods and also biochemical tests are time consuming. A molecular diagnosis of NTMs identified M. abscessus as the most common species at 37.5%. All eight mycobacterial strains were analyzed for species identification using Polymerase chain reaction – restriction enzyme analysis, revealing that all mycobacteria possess a hsp65 protein with unique and common epitopes. The PCR-REA method successfully identified 05 different types of mycobacterial species, with 100% concordance in species identification.

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Ethics statement

This study was approved by the Institutional Ethics Committee, Santosh Medical College Ghaziabad, UP, India (Letter No: SU/2019/1531[5])

AUTHOR CONTRIBUTIONS:
Nadeem Gul Dar and Geeta Gupta conceived the idea of the manuscript. Nadeem Gul Dar wrote the first draft of the manuscript. Nadeem Gul Dar, Nazia Khanum, Sachin Kishore commented and edited subsequent versions of the manuscript. All co-authors participated in data collection and analysis. All authors have reviewed and approved the final version of the manuscript submitted to the journal.

Conflict of interest:
The authors declare that there is no conflict of interest.

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