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Original Article

Non-tuberculous *Mycobacteria* in TB-suspected patients: a clinical, epidemiological and genetic study

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Abstract

Introduction: Non-tuberculous *Mycobacteria* (NTM) are opportunistic pathogens causing community and hospital infections, often misdiagnosed as *M. tuberculosis*; accurate, timely differentiation is essential due to differing treatment strategies.

Objectives: To genotype NTM from TB-suspected patients at a tertiary care hospital and correlate findings with clinical and epidemiological features.

Methodology: A prospective observational study (Aug 2018–Dec 2019) analysed 2,050 pulmonary and extrapulmonary specimens from TB-suspected patients. Specimens were processed conventionally, MTBC and NTM were differentiated via MPT64 antigen test, and 42 randomly selected NTM isolates were genotyped using line probe assay targeting the 23S rRNA gene.

Results: Of 2,050 specimens, 8.8% (180/2050) yielded mycobacterial growth. Non-tuberculous *Mycobacteria* were detected in 104 (5.1%) of the samples. Among these, 42.3% of patients were aged > 45 years, with a male predominance (62.5%). Nearly half of the cases (47.1%) presented clinically with pulmonary disease. Genotypic characterisation by line probe assay identified *M. abscessus* as the most frequent species, accounting for 35.6% of the isolates.

Conclusions: NTM is prevalent among TB-suspected patients, with pulmonary disease and rapid-growing *M. abscessus* predominating, emphasizing the need for accurate species identification to guide management and public health measures.

Key words: Non-tuberculous *Mycobacteria* (NTM); tuberculosis suspects; prevalence; 23S rRNA gene; line probe assay.

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Introduction

Non-tuberculous *Mycobacteria* (NTM) are ubiquitous environmental organisms responsible for both community-acquired and healthcare-associated infections. To date, more than 190 NTM species have been identified, many of which are pathogenic to humans [1]. Runyon classified NTMs into slow and rapid growers. Slow growers are further categorised as photochromogens, scotochromogen, and non-photochromogens [2]. Since NTM-related diseases are not notifiable in most countries, including India, their true prevalence remains largely unknown [3].

Globally, NTMs are increasingly recognised as human pathogens. This rise is attributed to a growing population of immunocompromised and vulnerable individuals, the increasing complexity of medical and surgical interventions, as well as increased awareness and diagnostic capabilities [4,5]. NTM can cause both pulmonary and extrapulmonary infections, with pulmonary disease accounting for the majority of cases (65-90%) [4].

Conventional phenotypic identifications require assessment of growth rate, pigment production, and a panel of biochemical tests, which are time-consuming and labour-intensive. When diagnosis is based on conventional methods, NTM infections may be misdiagnosed as M. Tuberculosis, resulting in inappropriate treatment and poor clinical outcomes [6]. To overcome these limitations, Advanced diagnostic techniques have been introduced [5]. Among them, molecular methods based on nucleic acid hybridisation, particularly the line probe assay (LPA), have shown high reliability for species-level identification of the most commonly isolated NTMs. Thus, this also reduces workload, turnaround time, and risk of misdiagnosis [7].

The present study aimed to perform genotypic characterisation of NTM isolated from suspected cases of tuberculosis attending a tertiary care referral hospital

and to correlate it with clinical and epidemiological features.

Methodology

Study Design and Setting

This prospective observational study was done in the Department of Microbiology at a tertiary care hospital, from August 2018 to December 2019, following institutional ethical approval (AIIMS/IEC/2018/651).

Study population and sample collections

A total of 2,050 non-repetitive specimens (pulmonary and extrapulmonary) were received from patients of all age groups clinically suspected of tuberculosis during the study period.

Sample processing

Specimens obtained from non-sterile body sites were decontaminated using the N-acetyl-l-cysteine and sodium hydroxide (NALC-NaOH) method before further processing. All samples were handled and interpreted according to the standard operating protocol of the Mycobacteriology laboratory.

Primary Objective

To perform genotypic characterisation of non-tuberculous *Mycobacteria* (NTM) isolated from clinically suspected tuberculosis cases attending a tertiary care referral hospital.

Secondary objective

To correlate the identified NTM species with clinical presentations and epidemiological features of the affected patients.

Hypothesis

Non-tuberculous *Mycobacteria* (NTM) are frequently misidentified as *Mycobacterium tuberculosis* in routine diagnostic settings. Genotypic characterisation will reveal distinct species distributions that may be associated with specific clinical and epidemiological features.

Research Question

What are the genotypic profiles of NTMs isolated from clinically suspected tuberculosis cases, and how do these profiles relate to the clinical presentations and epidemiological characteristics of the patients?

Ziehl-Neelsen acid-fast staining

Ziehl-Neelsen acid-fast staining was performed on both processed clinical specimens and culture-positive broths using the ZN Acid Fast Stains Kit (Himedia Laboratories, Mumbai, India).

Culture for Mycobacteria

Mycobacterial culture was performed in BACT/ALERT (Biomérieux, France) bottles supplemented with the PANTA antimicrobial mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) according to *the* manufacturer's instructions. Liquid culture material from positive-signalled bottles was used for ZN staining, and the MPT 64 rapid antigen detection test, and 2-3 ml aliquots were stored at -80°C for subsequent line probe assay.

The SD Bioline TB Ag MPT64 Rapid kit (Standard Diagnostics, Inc., South Korea) was used to differentiate MTBC from NTM in positive culture broths with acid-fast bacilli. A 100µL aliquot of culture material was applied to the test device, and results were read after 15 minutes: a control band alone indicated NTM, while both a control and test bands indicated MTBC (Figure 1A).

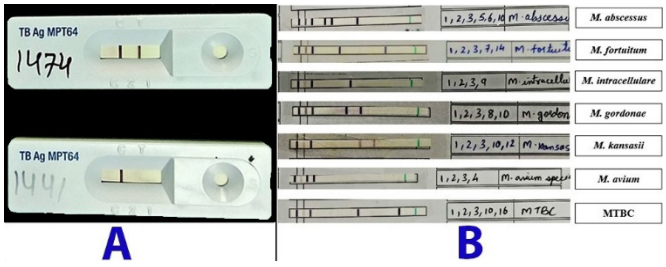
Inclusion and exclusion criteria for Genotyping

All consecutive, non-repetitive NTM isolates, confirmed by the MPT 64 Rapid Antigen Detection Test, from pulmonary and extrapulmonary samples of clinically suspected tuberculosis patients during the study period were included. Of 2,050 specimens processed, NTM were isolated in 104 cases. Due to limited reagent availability, 42 isolates were randomly selected by a blind lottery for genotyping using a line probe assay.

Data Collection

After written informed consent, clinico-epidemiological data were obtained from NTM-positive patients using a structured questionnaire, and patients were followed up.

Figure 1. MPT64 immunochromatographic test results: positive (top) and negative (bottom) (1A); nitrocellulose strips showing species-specific band patterns (1B).



Line Probe Assay

The Genotype® Mycobacterium CM and AS tests (Hain Lifescience, Germany), based on DNA-STRIP® reverse hybridisation targeting species-specific sequences of the 23S rRNA gene, were used to speciate 42 NTM isolates, following the manufacturer's instructions.

DNA was extracted using the Genolyse® VER1.0 kit (Hain Lifescience, Nehren, Germany). Briefly, 500 µL of liquid culture was processed according to the manufacturer's protocol, and 5 µL of the extracted DNA was used for PCR.

Multiplex PCR was performed using biotinylated primers with hotstar Taq® DNA polymerase (Hain Lifescience, Nehren, Germany) in a thermocycler (Applied Biosystems, Thermo Fisher Scientific, USA) following the manufacturer's protocol: initial denaturation at 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds and 58 °C for 2 minutes, 20 cycles of 95 °C for 25 seconds, 53 °C for 40 seconds, 70 °C for 40 seconds, and a final extension at 70 °C for 8 minutes.

Reverse hybridisation was performed using the Genotype® Mycobacterium CM/AS assay on the automated GT-Blot 48 system (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. Hybridisation, washing, conjugation, and substrate reactions were carried out automatically, and developed strips were analysed on evaluation sheets.

Evaluation of band patterns on nitrocellulose strips was performed using the kit template to identify NTM species (Figure 1B).

Quality Control

M. Tuberculosis H37Rv and molecular-grade water served as positive and negative controls, respectively.

Statistical Analysis

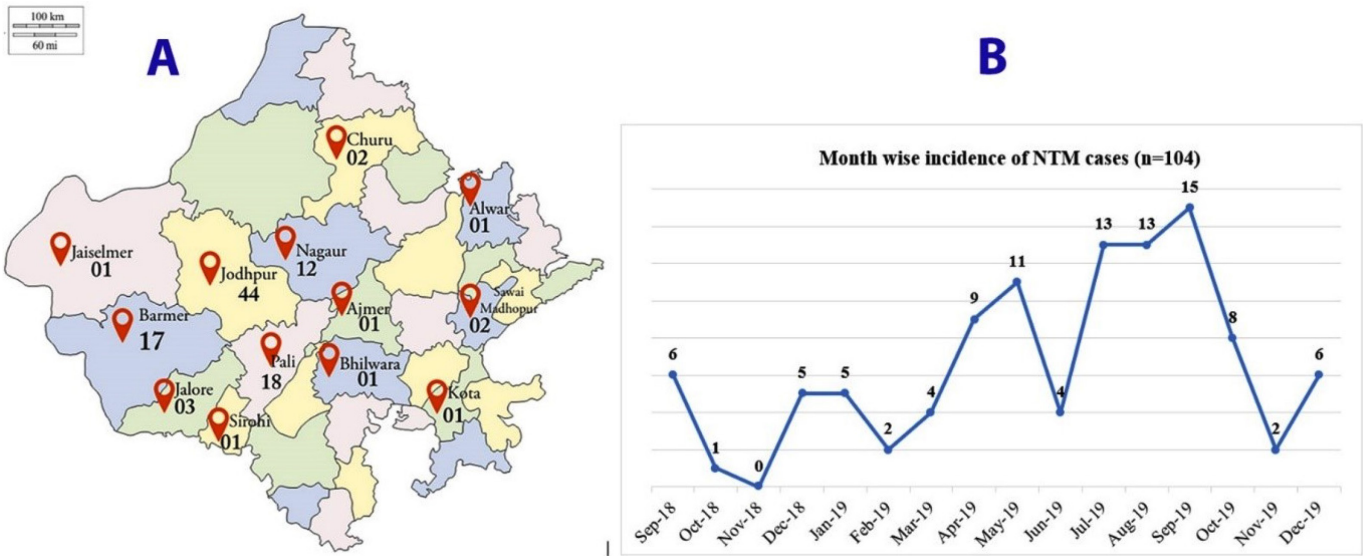
Categorical variables (e.g., age, gender, clinical features, comorbidities, sample type, and species) were expressed as frequencies and percentages, while continuous variables were summarised as median with range. Statistical analysis was performed using SPSS, with significance set at $p < 0.05$.

Results

Of 2,050 specimens, 8.8% (180/2050) yielded mycobacterial growth, confirmed by BACT/ALERT and ZN microscopy. Based on the MPT64 rapid antigen test, the prevalence of NTM and MTBC was 5.1% (104/2050) and 3.7% (76/2050), respectively. Among culture-positive specimens (n=180), NTM accounted for 57.8% (104/180) and MTBC for 42.2% (76/180). Only 32.7% (34/104) of NTM isolates were AFB positive on direct smear microscopy.

Among 104 NTM-positive patients, the median age was 33.5 years, with 42.3% aged > 45 years. Males predominated (62.5%). Most patients were from Jodhpur (44, 42.3%), followed by Pali (18, 17.3%), Barmer (17, 16.3%), and Nagaur (12, 11.5%) (Figure 2A); cases peaked in September (15, 14.4%) (Figure 2B). Based on the clinical spectrum, pulmonary disease was present in 47.1% of cases. However, based on specimen type, the majority of NTM were isolated from extrapulmonary specimens (67.3%), with FNA (16.3%) and pleural fluid and biopsy (12.5% each) being the

Figure 2. Geographic distribution (2A); and month-wise incidence of NTM cases (2B).



commonest specimens. Comorbidities were present in 25% of patients. Median ESR was 31 mm/hr, elevated in 73%, 35.6% had a history of smoking, and 16.4% had prior tuberculosis (Table 1).

Of 104 NTM isolates, 42 were randomly selected for genotyping and identified to species level (Table 2). Among extrapulmonary cases, *M. abscessus* (28.6%) was most common, followed by *M. fortuitum* (25%) and *M. intracellulare* (10.8%). Pulmonary cases were also dominated by *M. abscessus* (50%), followed by *M. intracellulare* (21.6%). Overall, *M. abscessus* (35.6%) and *M. fortuitum* (19%) were the most frequently identified species across all specimens. None of the 42 speciated isolates was misidentified as *M. tuberculosis*.

Discussion

The prevalence of non-tuberculous *Mycobacteria* (NTM) has increased significantly in recent decades, influenced by factors such as genetic evolution of the organism, climate changes, host immunodeficiency, longer life expectancy, improved diagnostics, and greater clinical awareness [8]. In this study, NTM prevalence among suspected TB patients was 5.1%, compared to 3.7% for M. tuberculosis complex (MTBC). Reported NTM prevalence ranges from 0.6% to 8.2% [3–5,9–14]. Reflecting differences in geography, climate, population density, and host factors [15].

Table 1. Clinico-epidemiological characteristics of NTM patients.

Variable	NTM detected by MPT-64 (n = 104)	Genotyped NTM (n = 42)
Age (years)		
Mean ± SD	39.2 ± 22.3	40.7 ± 23.3
Median (Range)	33.5 (0.33-85)	33.0 (0.33–76)
Sex		
Male	65 (62.5%)	24 (57.1%)
Female	39 (37.5%)	18 (42.9 %)
Specimen category		
Pulmonary	34 (32.7%)	14 (33.3%)
Extra-pulmonary	70 (67.3%)	28 (66.7%)
Clinical presentation		
Pulmonary disease	49 (47.1%)	22 (52.4%)
Lymphadenitis	18 (17.3%)	4 (9.5%)
Disseminated disease	15 (14.4%)	6 (14.3%)
Osteoarticular disease	6 (5.8%)	2 (4.8%)
CNS infection	8 (7.7%)	4 (9.5%)
SSTI	8 (7.7%)	4 (9.5%)
Co-morbidities* present	26 (25%)	13 (31%)
Raised ESR#	76 (73%)	28 (66.7%)
History of smoking	37 (35.6%)	13 (31%)
HIV status		
Negative	80 (77%)	31 (73.8%)
Positive	2 (1.9%)	1 (2.4%)
Unknown	22 (21.1%)	10 (23.8%)
Previous history of TB	17 (16.4%)	7 (16.7%)

*Comorbidities included diabetes mellitus, malignancy, chronic kidney disease, hypertension, cardiac disorders, and systemic lupus erythematosus. #The normal value of ESR was considered 0-15 mm/hr in males and 0-20 mm/hr in females.

The median patient's age was 34.5 years, with older individuals (> 45years) representing 42.3% of cases, consistent with studies showing increased NTM incidence in older populations due to weaker immunity and higher comorbidity [9,10,16–18]. Males predominated (62.5%), likely reflecting higher rates of smoking, previous TB, respiratory complaints, and surgeries [19,20]. Seasonal trends are rarely reported; however, while an Australian study observed winter peaks [21], we found slightly increased isolation during the summer, likely due to the region's hot-arid climate and sandy environment.

Clinically, pulmonary disease was observed in 47.1% of patients. Microbiologically, most NTM isolates came from extrapulmonary specimens (67.3%), largely pleural fluid and biopsy tissues. These findings highlight the varied clinical spectrum of NTM infections, with a substantial burden of extrapulmonary disease. The high proportion of isolates from pleural fluid and tissue biopsies underscores the importance of considering NTM in the differential diagnosis of chronic pleural and soft tissue infections. Early recognition and accurate species-level identification are essential, as clinical presentation alone may not reliably distinguish NTM disease from tuberculosis or other bacterial infections [15,22].

Worldwide, NTM is more frequently reported from pulmonary samples due to active respiratory specimen testing, possibly underestimating extrapulmonary NTM cases. This diagnostic bias has been described in several epidemiological studies, where the higher clinical suspicion and systematic testing for pulmonary tuberculosis facilitate concurrent detection of pulmonary NTM, whereas extrapulmonary samples are less consistently investigated for *Mycobacteria* [23,24]. Notably, eight isolates were obtained from CSF, all from patients with CNS symptoms, highlighting the importance of clinical correlation in invasive

Table 2. Distribution of NTM species and their association with pulmonary and extra-pulmonary specimens.

NTM species (N; %)	Pulmonary n (%)	Extra pulmonary n (%)
M. abscessus (15; 35.6)	7 (50.0)	8 (28.6)
M. fortuitum (8; 19)	1 (7.1)	7 (25.0)
M. gordonae (2; 4.8)	1 (7.1)	1 (3.6)
M. interjectum (1; 2.4)	1 (7.1)	0
M. intracellulare (6; 14.2)	3 (21.6)	3 (10.8)
M. malmoense (2; 4.8)	1 (7.1)	1 (3.6)
M. avium (2; 4.8)	0	2 (7.1)
M. chelonae (2; 4.8)	0	2 (7.1)
M. kansasii (2; 4.8)	0	2 (7.1)
M. peregrinum (2; 4.8)	0	2 (7.1)
Total	**14**	**28**

N = total number of isolates of each NTM species; n = number of isolates within pulmonary or extra-pulmonary specimens; percentages are calculated column-wise.

specimens. NTM involvement of the CNS, although uncommon, has been documented particularly in immunocompromised individuals and is associated with significant morbidity. The recovery of NTM from sterile sites such as CSF strongly supports true infection rather than colonization or contamination. These observations emphasize the importance of correlating microbiological results with clinical presentation and radiological findings, especially in invasive specimens, to ensure timely diagnosis and appropriate management [22,25].

Smoking 35.6% (37/104), prior TB 16.4% (17/104), and other comorbidities were important risk factors, consistent with previous studies [18,26,27]. Elevated ESR was observed in 73% (76/104) of patients (median 31 mm/hr), supporting its role in predicting active NTM disease and prognosis [28]. Only 1.9% of patients were HIV-positive, indicating that NTM infections are rising in immunocompetent individuals [3,9,19].

Of the 42 isolates randomly selected for genotyping, a total of 10 NTM species were identified. *M. abscessus* (35.6%) was the most common, followed by *M. fortuitum* (19%) and *M. intracellulare* (14.2%), similar to findings by Umrao *et al.* [9] and other reports [4,13,16,27,29]. While slow-growing *Mycobacteria* such as *M. avium*, *M. kansasii*, and *M. intracellulare* are often predominant in pulmonary specimens [10,15,30]. In our study, *M. abscessus* was the most frequent pulmonary isolate, likely reflecting local environmental exposures [10,29]. Among extrapulmonary specimens, *M. abscessus* (28.6%) was most common, while other studies report *M. intracellulare*, *M. avium*, and *M. fortuitum* as prevalent [9,13,27,31]. The predominance of rapidly growing *Mycobacteria*, particularly *M. abscessus* and *M. fortuitum*, is notable, as these species are increasingly recognized as significant pathogens in both pulmonary and extrapulmonary disease. Their intrinsic resistance to multiple first-line anti-tubercular agents underscores the importance of accurate species-level identification to guide appropriate antimicrobial therapy. Furthermore, the absence of misidentification as *M. tuberculosis* highlights the reliability of the genotyping method used and reinforces its utility in differentiating NTM from tuberculosis in high-burden settings [22,25,32].

There are a few limitations of our study. First, this was a single-center study; the results may not fully represent the epidemiological pattern of NTM infections in other geographic regions. Second, not all NTM isolates (only 42 out of 104) underwent molecular characterization, potentially introducing selection bias despite random sampling. In addition, detailed clinical data, including immune status, treatment outcomes, and radiological correlations, were limited, restricting comprehensive clinico microbiological analysis.

Finally, antimicrobial susceptibility testing was not systematically performed for all isolates, preventing assessment of resistance patterns and their correlation with specific species. Future multicenter studies with larger sample sizes and integrated clinical, radiological, and microbiological data would provide a more robust understanding of NTM epidemiology and disease burden.

Conclusions

This study highlights a notable prevalence of NTM among patients clinically suspected of tuberculosis, with a predominance of pulmonary disease and frequent isolation of rapid growing species such as *M. abscessus*. These findings underscore the importance of accurate species-level identification for guiding appropriate patient management and form public health strategies aimed at improving diagnostic and therapeutic approaches.

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Conflict of interest

No conflict of interest is declared.

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