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Detection of NTM Species and Drug Susceptibility Testing by LPA Technique Using Genotype NTM-DR Kit

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A B S T R A C T

Non-tuberculous mycobacteria (NTM) are now widely recognised as significant pathogens, especially in areas where tuberculosis (TB) is endemic and smear-positive, CBNAAT-negative cases are frequently misinterpreted as TB. Effective management requires quick species identification and drug susceptibility testing. This study aims to find the NTM Species-level identification and drug susceptibility testing by LPA Technique using GenoType NTM-DR kit. At the Culture and Drug Susceptibility Testing Laboratory, KMCRI, Hubballi, a prospective study was carried out (June to December 2024). The MGIT 960 method was used to cultivate the clinical samples that were smear positive but CBNAAT negative. The GenoType NTM-DR assay was used to determine species and detect aminoglycoside and macrolide resistance. The majority of the 35 NTM isolates were from respiratory samples (74.2% sputum). The most prevalent species (45.7%) was Mycobacterium abscessus subsp. abscessus, which was followed by *Mycobacterium intracellulare* (5.7%), *M. chimaera* (2.8%), and *M. abscessus subsp. bolletii* (2.8%); the remaining 40% belonged to different NTM species. All the isolates were sensitive to aminoglycosides but around 70% isolates were resistance to Macrolide. Treatment outcomes illustrated that most patients had favourable outcomes, with a significant percentage of cases showing cure or treatment completion. This study found a high prevalence of the *M. abscessus* complex and considerable macrolide resistance among NTM isolates in northern Karnataka. CBNAAT-negative, smear-positive cases should be routinely evaluated for NTM testing. Effective molecular tests, such as LPA (GenoType NTM-DR Kits), must be included into regular diagnostic procedures to ensure early detection, appropriate treatment, and better clinical outcomes.

Introduction

Non-tuberculous mycobacteria (NTM) are also known as environmental mycobacteria, atypical mycobacteria, anonymous mycobacteria, mycobacteria other than *Mycobacterium tuberculosis* (Mtb) (MOTT), and its close relatives, *M. africanum, M. bovis, M. canetti, M. caprae, M. pinnipedii,* and *M. leprae*[1].These organisms

are prevalent in the environment and have been isolated from a variety of sources, including biofilms, wild animals, milk, food products, soil, dust, plants, natural and drinking water sources [2,3].

NTM are distinguished by a thin peptidoglycan layer that is enveloped in a thick outer lipid-rich coating. This coating allows NTM to adhere to uneven surfaces while additionally offering resistance to antibiotics and disinfectants, enabling NTM survival in low oxygen and carbon concentrations as well as other unfavourable situations. NTM are classified as either slowly growing mycobacteria (SGM; ≥7 days) or rapidly growing mycobacteria (RGM; <7 days) based on their growth characteristics from the culture. There is yet no proof of NTM latency. There are over 200 species and 13 subspecies in the taxonomy of the genus Mycobacterium[4].

In countries with a high tuberculosis (TB) incidence, diagnosing NTM diseases is challenging due to a lack of understanding among healthcare providers and limited availability to laboratory resources, such as mycobacterial culture and molecular tools for identification. In these resource-constrained settings, smear microscopy is heavily used to diagnose tuberculosis, and the diagnosis of NTM is commonly left out, and these patients are empirically treated as drug-sensitive and -resistant TB[4].

Numerous researches have indicated that within the past forty years, the prevalence rates of NTM have increased. While data from the UK indicate that the incidence of NTM-positive culture increased from 4/100,000 to 6.1/100,000 individuals between 2007 and 2012, data from the USA indicate that the present prevalence of NTM-positive culture ranges between 1.4 and 6.6/100,000 individuals[5]. According to a Canadian study, the prevalence of NTM-PD increased significantly from 29.3 cases/100,000 in 1998–2002 to 41.3 cases/100,000 people examined in 2006–2010[4].

Regarding the geographical distribution of NTM, a study discovered that after examining NTM data from 30 nations across six continents, the species distribution among NTM isolates from lung specimens varies by continent and country within these continents. Thus, changes in species distribution may influence the occurrence and signs of pulmonary NTM disease in different geographical areas. Other researchers observed changes in NTM isolation and dispersion. For example, a comprehensive review and meta-analysis in mainland China revealed that *M. abscessus* was the most commonly isolated species between 2000 and 2014, while *M. intracellulare* was more prevalent between 2015 and 2019. The geographic diversity of different species demonstrated that environmental and economic variables influence NTM distribution. Overall, this epidemiological data can shed light on the differences in clinical significance and treatment outcomes of NTM diseases.[6]

Surveillance of NTM for monitoring prevalent species and their medication resistance profiles would be critical for improving infection management and treatment. In many of the countries, reporting NTM infection to health authorities is voluntary. However, retrospective cohort or laboratory-based investigations have lately been reported in Germany[7], China [8,9], the United States of America (USA) [10], and other countries. In Italy, there is no significant data on the incidence and drug susceptibility of NTM infections[6].

NTM infections can be both localised and widespread, with the most common organ affected. Patients with disseminated NTM infections (involvement of two or more non-contiguous body organs) typically have a generalised immunological deficiency, such as HIV/AIDS. Additionally, 2-8 percent of these patients may have concurrent pulmonary involvement. Identifying the underlying immunological deficiency is essential for early diagnosis, therapy, and prevention. Patients with NTM illness and primary immunodeficiencies often present in childhood or adulthood, but acquired immunodeficiencies can present at any age[4].

Even an experienced physician may find it difficult to diagnose NTM infection because its clinical signs are identical to those of tuberculosis. Four clinical forms of NTM infections are classified: (i) persistent Parkinson's disease (PD); (ii) lymphadenopathy; (iii) skin and soft tissues; and, in rare cases, bones and joints; and (iv) generalised illness.[4]

Nontuberculous mycobacteria (NTM) comprise roughly 200 species, some of which can cause disease in humans by infecting pulmonary and extrapulmonary organs. The lungs are the most affected, however NTM species are phenotypically diverse and cause a wide range of clinical symptoms in other organs. Mycobacterium avium complex (MAC), *M. xenopi, M. kansasii,* and *M.*

abscessus are the most common causes of pulmonary illness. The *M. avium complex* can also cause widespread infections, whereas *M. fortuitum, M. chelonae*, and *M. marinum* are mostly responsible for skin and soft tissue infections resulting from surgery or unintentional wounds.[6]

The NTM are intrinsically resistant to various medications, such as anti-TB treatments, and must be treated with antibiotic combinations determined by susceptibility testing. This presents significant problems for new medication discovery and treatment of pulmonary and extrapulmonary infections developed by these species. Drug resistance in NTM might be inherent (natural) or acquired. Several intrinsic resistance mechanisms evolved over time, including decreased cell envelope permeability, increased efflux systems, and other mechanisms (such as drug degradation and target changes), as well as NTM presence in biofilms and granulomas, which effectively reduced drug uptake. Rather, acquired resistance describes situations where a resistant strain arises from a previously drug-susceptible population, frequently as a result of extended antibiotic therapy.

If the target protein is encoded by a single gene copy, acquired resistance is especially severe, increasing the likelihood of acquiring mutations following single-drug treatments. In November 2018, the Clinical and Laboratory Standards Institute (CLSI) amended and expanded its antimycobacterial susceptibility testing (AST) guidelines, which were established in 2011. Both guidelines included recommendations for NTM AST using population distribution, comparative breakpoints, clinical evidence, and the expertise of a panel of mycobacteriology specialists[6].

NTM laboratory diagnosis is often time-consuming because it is based on culture. Culture is often carried out utilising a continuously tracked broth culture instrument system, which lowers incubation time. Microscopy with acid-fast stains can be used to detect mycobacteria; however, its sensitivity is average and depends on bacterial load and the microscopist's expertise. Furthermore, it lacks specificity, as other bacteria can stain acid-fast. Importantly, it cannot consistently distinguish between NTM and MTB, nor can it predict viability. earlier, NTM were identified using a series of metabolic reactions, growth rate, and colony colouration, but this was time-consuming and inconsistent.[11]

Although the clinical relevance and treatment outcomes of nontuberculous mycobacteria (NTM) infections vary depending on the causative organism and resistance profile, the incidence of NTM infections among individuals is continuously rising. Drug susceptibility testing (DST) and the differential and precise identification of NTM species are therefore highly desirable in the medical field. Precision identification of *Mycobacterium avium complex* (MAC) species and Mycobacterium abscessus subspecies in clinical specimens is becoming increasingly necessary due to differences in treatment outcomes and epidemiological consequences. However, only specialised laboratories are now able to identify these species. Moreover, the gold standard approach for NTM DST is broth microdilution (BMD), which is time-consuming, particularly for slow-growing mycobacteria.[12]

The major clinically encountered NTM, such as MAC species (*M. avium, M. intracellulare, and M. chimaera*), *M. chelonae*, and subspecies of *M. abscessus*, such as *M. abscessus subspecies abscessus, M. abscessus subspecies massiliense*, and *M. abscessus subspecies bolletii*, can be identified at the species or subspecies level using the GenoType NTM-DR (NTM-DR; Hain Lifescience, Nehren, Germany). Antibiotic resistance to aminoglycosides and macrolides can also be detected using the NTM-DR assay. In particular, mutations at locations 2058/2059 in the rrl gene and polymorphisms (T28 or C28) at position 28 in the erm(41) gene are indicative of macrolide resistance.

Similarly, locations 1406 to 1408 of the rrs gene are used to identify aminoglycoside resistance. The NTM-DR assay's mutation probes have been developed to hybridise to alleles with certain mutations: one mutation in the rrs gene (A1408G) and four mutations in the rrl gene (A2058C (MUT1 probe), A2058G (MUT2), A2059C (MUT3), and A2059G (MUT4) [12].

Treatment for NTM disease can be difficult because it necessitates a lengthy course of combination therapy with aminoglycosides and macrolides, sometimes in addition to surgical resection. Many patients experience relapses or re-infection even after receiving effective treatment, especially when they have macrolide resistance.

To evaluate the results of patients with lung disease brought on by macrolide-resistant M. avium complex (MAC), Park *et al.,* performed a meta-analysis. Over the

course of a year, they discovered a moderate 21% sputum culture conversion rate and a 10% all-cause death rate. It is essential to look into new therapy options because macrolide resistance increases the chance of treatment failure.[11]

Therefore this study aims to find the NTM Species-level identification and drug susceptibility testing by LPA Technique using GenoType NTM-DR kit.

Materials and Methods

A prospective study was conducted at Culture and Drug Susceptibility Testing Laboratory on 35 isolates from June-2024 to December-2024.

Samples were handled in a class II biosafety cabinet in a biosafety level 3 (BSL) lab. The samples were digested and decontaminated with N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH). All the 35 samples were Smear Positive & CBNAAT negative. The MGIT 960 method was used to cultivate the samples. After the culture flags positive, it was then subjected to LPA using the GenoType NTM-DR kit.

GenoType NTM-DR testing

Geno Lyse v (2.0) (Hain Lifescience, Nehren, Germany) kit was used to extract DNA in accordance with the manufacturer's protocol. Following DNA extraction as previously mentioned, the NTM-DR assay was used for amplification and hybridisation in accordance with the manufacturer's suggested procedure. In short, 5µl of extracted DNA was combined with 35 µl of AM-A (containing buffer, nucleotides, and Taq polymerase) and 10 µl of AM-B (containing salts, particular primers, and dye). Under the following setting, PCR amplification was performed: Ten cycles of 30 seconds at 95°C and 120 seconds at 65°C make up the first round of amplification after one cycle at 95°C for 15 minutes. Twenty cycles of 25 seconds at 95°C, 40 seconds at 50°C, and 40 seconds at 70°C comprised the second round of amplification, followed by an 8-minute final extension at 70°C.

In accordance with the manufacturer's instructions, reverse hybridisation and detection were performed in a shaking water bath (TwinCubator; Hain).

The manufacturer's chart was used to interpret the developed strips, which were fixed to an evaluation sheet. In a blinded manner, three different observers independently interpreted the findings[12].

Results and Discussions

Study population and sample characteristics

A prospective study was conducted at Culture and Drug Susceptibility Testing (DST) Laboratory, KMCRI, Hubballi for the period one year from June-2024 to December-2024. A total of 35 samples processed for non-tuberculous mycobacteria (NTM) test.

The majority of the samples were respiratory samples, predominantly sputum (26, 74.2%) & 6(17.1%) were Bal fluid samples. Patients belonged to a wide age range, with ages spanning from young adults to the elderly. Both sexes were represented, with a slight predominance of female patients.

Most patients were residents of districts from northern Karnataka, including Belgaum, Dharwad, and Dakshina Kannada.

Of the 35 individuals included in the study, most of the patients were HIV non-reactive (34/35; 97.1%), and only one patient (1/35; 2.8%) was HIV reactive. Regarding comorbidities, three individuals (3/35; 8.6%) had diabetes mellitus. The remaining patients either had other comorbidities like alcoholism (2/35; 5.7%) or no concomitant conditions (28/35; 80%).

Species distribution of NTM isolates

Species-level identification demonstrated a predominance of Mycobacterium avium complex (MAC) organisms.

Treatment outcome

Treatment outcomes were documented using WHO-defined TB programmatic outcome categories**,** adapted for NTM reporting:

- **Cured**: 13 patients
- **Treatment completed**: 5 patients
- **On treatment** at the time of analysis: 6 patients
- **Treatment failure**: 1 patient
- **Died**: 5 patients

Additionally, three patients were lost to follow-up, and two cases were subsequently identified as wrongly diagnosed, leading to treatment discontinuation.

Non-tuberculous mycobacteria (NTM) are becoming more extensively recognised as potent human pathogens, especially in nations with high rates of tuberculosis (TB), where diagnostic overlap with *Mycobacterium tuberculosis* complex (MTBC) frequently results in underdiagnosis or misdiagnosis[15,18,19]. The current prospective investigation, which was carried out over a six-month period at the Culture and Drug Susceptibility Testing Laboratory, KMCRI, Hubballi, offers important insights into the species distribution, drug resistance trends, and treatment outcomes of NTM isolates from northern Karnataka.

All the samples investigated the study were smear-positive and CBNAAT-negative, underscoring a crucial diagnostic difficulty in standard TB programs. Smear positivity is often mistaken for tuberculosis in high-TB burden countries like India, which results in the empirical start of anti-TB treatment. Our results highlight the necessity of mycobacterial culture and species-level identification in such situations, supporting earlier findings that a minority of smear-positive, CBNAAT-negative cases may actually reflect NTM infections. [18,19,26]

The prevalence of NTM varies globally based on patient factors, including age, gender, and region.[11]. Over 90% of the samples were respiratory, especially sputum, which is correlated with NTM's known tendency for pulmonary involvement. This is noteworthy as NTM cultivated from non-sterile areas frequently indicates colonisation or contamination[26].The broad age range and modest female predominance seen in this study are consistent with existing literature indicating higher susceptibility among the elderly and females, probably due to structural lung disease, hormonal issues, or immunological abnormalities.[18,20,29]

HIV infection was shown to be rare among patients with non-tuberculous mycobacterial (NTM) disease in the current study; only one HIV-reactive case was observed. According to this study, NTM infections are not only seen in HIV-infected populations but are also increasingly being found in immunocompetent people in our settings. The result is consistent with previous data from India and around the world, which show an increasing prevalence of NTM disease among non-HIV patients, most likely due to enhanced diagnostic capabilities and clinical awareness[13].

In this study, diabetes mellitus was found to be a more common comorbidity than HIV. People who have chronic hyperglycemia are more susceptible to mycobacterial infections, such as NTM, because it is known to affect both innate and adaptive immune responses. A proportion of patients have diabetes, which emphasises the illness's significance as a risk factor for NTM disease, especially in nations like India where the rate of the illness is regularly rising[14]. Additionally, all diabetic individuals in this investigation were HIV-negative, underscoring a concept that non-HIV immunosuppressive diseases, such as diabetes, may play an important role in NTM aetiology. These results highlight the significance of routinely screening patients suspected of NTM infection for metabolic comorbidities, since diabetes may affect clinical outcomes, treatment responsiveness, and the severity of the disease.

Mycobacterium abscessus complex, specifically M. abscessus subsp. abscessus (45.7%), was the most common species identified through the GenoType NTM-DR kit. This observation is clinically notable because M. abscessus is recognised for its aggressive clinical course, inherent multidrug resistance, and poor treatment outcomes [17,23,24]. Similar patterns have been documented from a number of Asian nations, such as China and India, where rapidly multiplying mycobacteria are becoming the most prevalent respiratory pathogens [17,19, 29].

According to worldwide epidemiological trends, a significant percentage of isolates were Mycobacterium avium complex (MAC), which includes M. intracellulare and M. chimaera. MAC organisms are often linked to immunocompromised conditions and chronic lung disease, and they are among the most prevalent causes of NTM pulmonary disease globally. Despite the fact that M. chimaera has only been identified in one isolate, is vital to discuss due to its rising clinical relevance and connections to invasive infections.[28]

The presence of quite a few of "other NTM species" emphasises NTM's diverse ecology and the limitations of conventional diagnostic techniques, which do not allow for thorough species identification.[18, 27]

An important attribute of this study is utilisation of a molecular line probe assay to assess macrolide and aminoglycoside resistance. A substantial proportion of the reported NTM species (70%) showed macrolide resistance while remaining susceptible to aminoglycosides.

Fig.1 Interpretation chart of LPA GenoType NTM DR

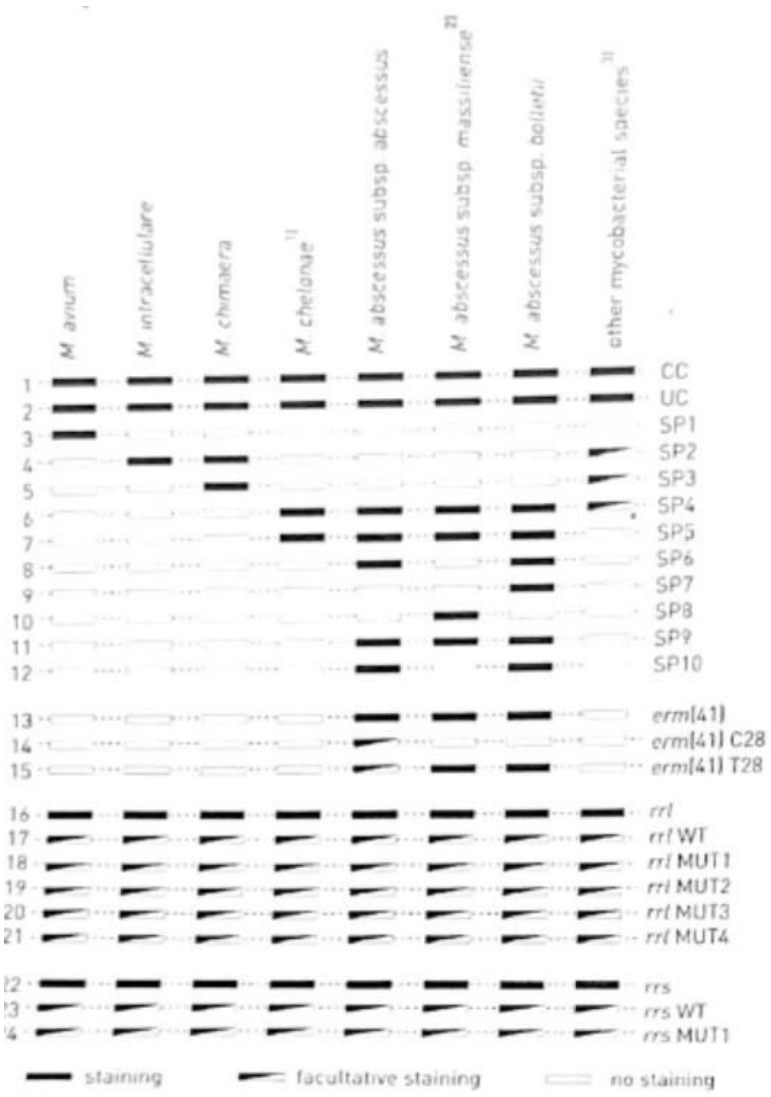


Table.1 Species distribution of NTM isolates

NTM Species distribution	Number of isolates	Sensitive to both macrolides & aminoglycosides	Resistance to macrolides & Sensitive to aminoglycosides
Mycobacterium abscessus complex subsp. Abscessus	16(45.7%)	5	11
Mycobacterium intracellulare	2(5.7%)	1	1
Mycobacterium chimaera	1(2.8%)	0	1
Mycobacterium abscessus Subsp.bolletii	1(2.8%)	0	1
Other Species	15(42.8%)	0	0

MAC species together constituted the largest proportion of NTM isolates, consistent with WHO-recognized epidemiological patterns.

Fig.2 Interpretation of *Mycobacterium chimaera*

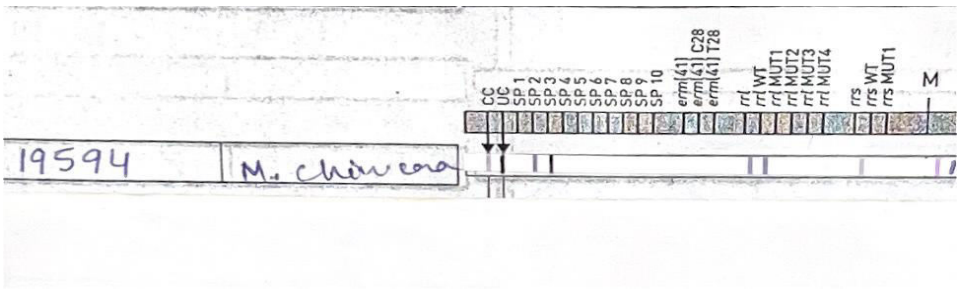


Fig.3 Interpretation of *Mycobacterium intracellulare*

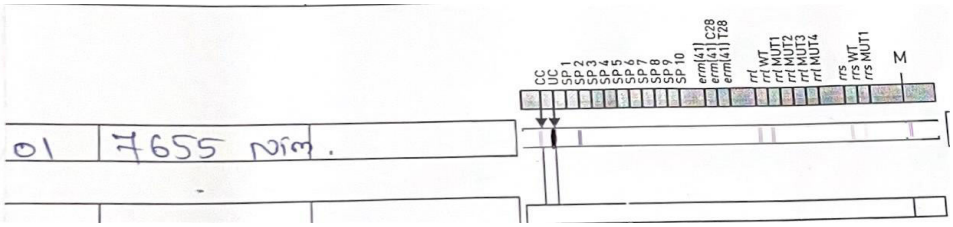
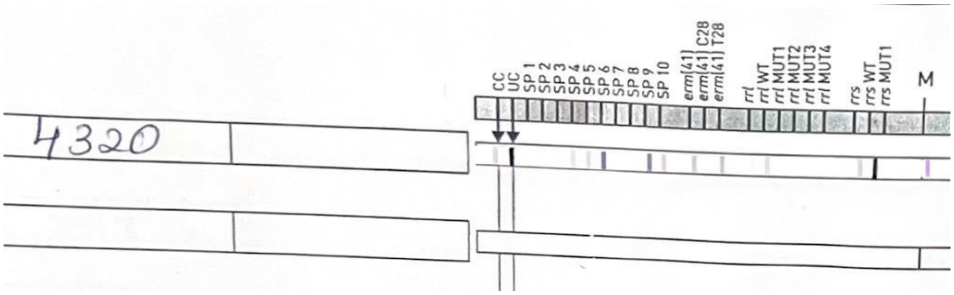
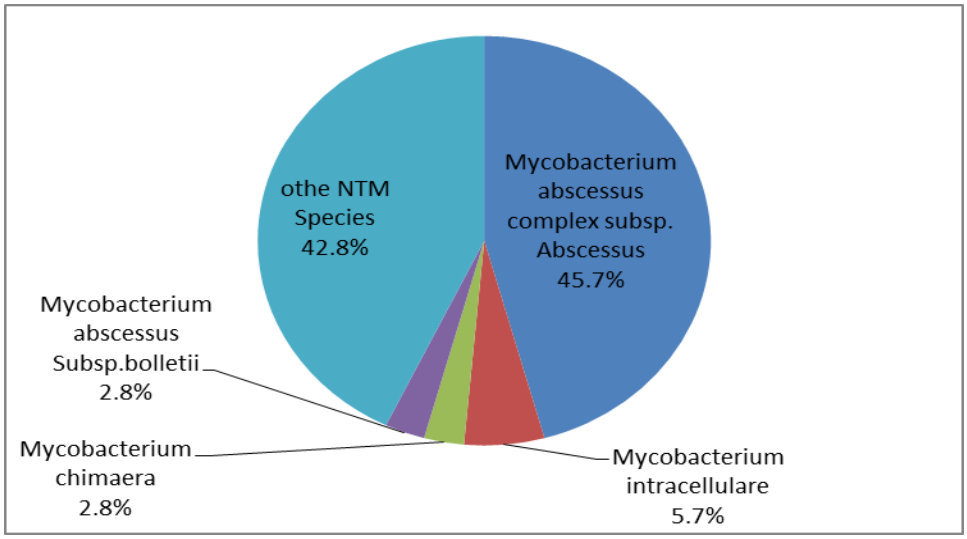


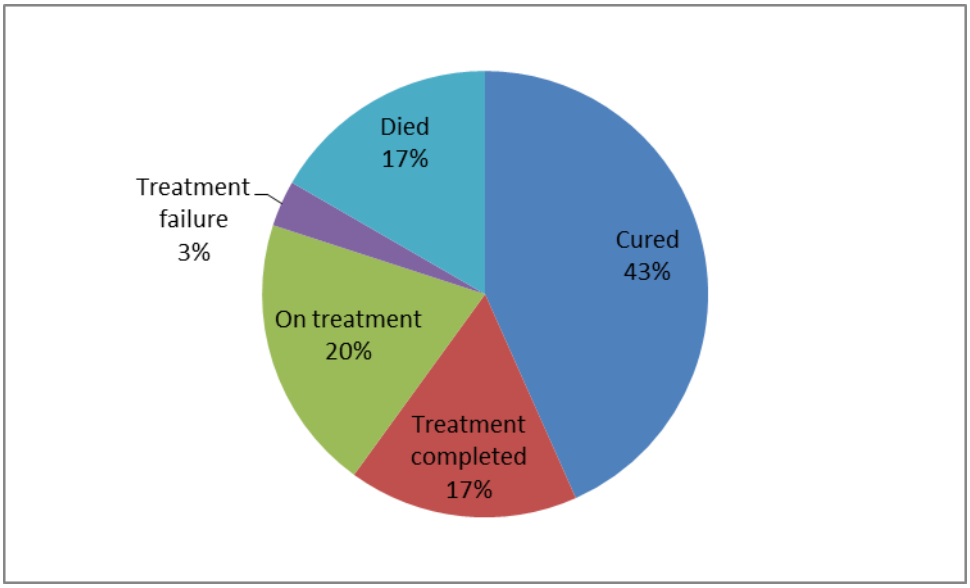
Fig.4 Interpretation of Mycobacterium abscessus complex subsp. Abscessus



Graph.1 Representing NTM Species distributions



Graph.2 Representing the treatment outcomes



This pattern is especially worrying because macrolides are the cornerstone of NTM treatment regimens, particularly for MAC and M. abscessus infections.[15, 21, 22] The erm (41) gene or mutations in the rrl gene often mediate macrolide resistance in M. abscessus, resulting in induced or acquired resistance[21,23,24]. The significant resistance rate found in this study reflects prior macrolide exposure, extended empirical therapy, or delayed diagnosis, all of which are prevalent among TB-endemic areas. Although retaining aminoglycoside susceptibility provides some therapeutic alternatives, their usage is limited because to their toxicity and need for continuous parenteral administration.[22]

These results demonstrate strong reason for the routine integration of drug susceptibility testing into NTM diagnostic workflows in order to offer individualised therapy and avoid inefficient regimens.[15, 25]

Analysis of treatment outcomes illustrated that most patients had favourable outcomes, with a significant percentage of cases showing cure or treatment completion. This indicates that even with NTM infection, which is frequently thought to be difficult to treat, promptly diagnosis, adequate antimicrobialtreatment, and routine follow-up can result into favourable outcomes. Although, the reported mortality and treatment failure are few in numbers underscore the inherent difficulties of NTM infections, such as delayed diagnosis, antibiotic resistance, comorbidities, and longer treatment duration. The diagnostic complexity of NTM in TB-endemic settings, where clinical and radiological overlap with tuberculosis might result in improper treatment initiation and termination, is further reflected by loss to follow-up and misdiagnosis.

The use of smear microscopy without confirmation culture or molecular identification may result in incorrect treatment. The GenoType NTM-DR assay proven to be a fast and accurate method of species identification and resistance detection, confirming its usefulness in conventional laboratory settings.[15,16,26] Strengthened laboratory capacity, increased clinician awareness, and the establishment of national guidelines for NTM diagnosis and treatment are critically needed due to the growing burden of NTM disease, particularly in TB-endemic areas[15,18,19]

This prospective study offers significant epidemiological and clinical insights into non-tuberculous mycobacterial infections in northern Karnataka. Our findings indicate a prevalence of the Mycobacterium abscessus complex, a high incidence of macrolide resistance, and inferior treatment outcomes, highlighting the complexities of NTM illness management. NTM infection should be extensively investigated in a crucial group of smear-positive, CBNAAT-negative individuals. Drug susceptibility testing and species-level identification are vital for providing an appropriate treatment and to

improve patient outcomes. Integrating NTM diagnostics into conventional TB laboratory procedures, as well as increasing clinician awareness and surveillance, is vital to managing India's growing public health burden posed by NTM infections.

Author's Contribution

Nivedita R D: Collected the samples, performed the preliminary and main tests, Conceptualization, Methodology, Writing - Original Draft Preparation, Visualization, Project Administration, and Funding Acquisition. Dr Nirmala A: Software, Validation, Formal Analysis, Writing - Review & Editing, Constructed the agreements and errors, Supervision.

List of Abbreviations

- **AIDS** – Acquired Immunodeficiency Syndrome
- **AM-A** – Amplification Mix A
- **AM-B** – Amplification Mix B
- **AST** – Antimycobacterial Susceptibility Testing
- **BAL** – Bronchoalveolar Lavage
- **BMD** – Broth Microdilution
- **BSL** – Biosafety Level
- **CBNAAT** – Cartridge-Based Nucleic Acid Amplification Test
- **CLSI** – Clinical and Laboratory Standards Institute
- **DST** – Drug Susceptibility Testing
- **ERM** – Erythromycin Ribosomal Methylase
- **FDA** – Food and Drug Administration
- **HIV** – Human Immunodeficiency Virus
- **IDSA** – Infectious Diseases Society of America
- **KMCRI** – Karnataka Medical College and Research Institute
- **LPA** – Line Probe Assay
- **MAC** – Mycobacterium avium Complex
- **MGIT** – Mycobacteria Growth Indicator Tube
- **MDR-TB** – Multidrug-Resistant Tuberculosis
- **MOTT** – Mycobacteria Other Than Tuberculosis
- **MTB** – *Mycobacterium tuberculosis*
- **MTBC** – *Mycobacterium tuberculosis* Complex
- **NALC–NaOH** – N-acetyl-L-cysteine–Sodium Hydroxide
- **NTM** – Non-tuberculous Mycobacteria
- **NTM-PD** – Non-tuberculous Mycobacterial Pulmonary Disease
- **PCR** – Polymerase Chain Reaction
- **PD** – Pulmonary Disease
- **RGM** – Rapidly Growing Mycobacteria
- **SGM** – Slowly Growing Mycobacteria
- **TB** – Tuberculosis
- **USA** – United States of America
- **WHO** – World Health Organization
- **XDR-TB** – Extensively Drug-Resistant Tuberculosis

Declarations

No Conflict of Interest: "The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper."

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Ethical committee approval: The authors of this manuscript declare that this scientific work complies with reporting quality, formatting and reproducibility guidelines set forth by the EQUATOR Network.

Plagiarism Check: We also certify that we have not plagiarized the contents in this submission and have done a Plagiarism Check.

Informed Consent: Informed consent was obtained for experimentation and that it conforms to the standards currently applied in the country of origin.

Clinical trial registry: The authors have not registered this study with the Clinical Trial Registry as it is not applicable.

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