# Biochemical Tests And Molecular Methods In Ntm Species Identification

**Ashish Pal[1*], Dr. Madhurendra Singh Rajput[2]**

[1*]Research Scholar, Malwanchal University, Indore (M.P)
[2]Associate Professor), Malwanchal University, Indore (M.P)

***Corresponding Author:** Ashish Pal
*Research Scholar, Malwanchal University, Indore (M.P)

**ABSTRACT**
In general, NTM has aided in focused treatment and illness management by fast separation and species-level distinction utilising genetic methods. The purpose of this research is to learn how common NTM species are in TB patients (both pulmonary and extrapulmonary) in a tertiary care hospital in Pondicherry, to compare several phenotypic and genotypic approaches to NTM identification, and to find out what patterns their DSTs show.

**Keywords:** Diseases, infections, Treatment, pulmonary, management.

## INTRODUCTION

Although nontuberculous mycobacterium (NTM) has been studied for a century, both microbiologists and clinicians are deeply concerned about the current trend of NTM prevalence. When it comes to chronic lung disorders, NTM are a major killer and source of illness progression. There are about 125 known species of NTM, and at least 42 of those species are associated with illnesses affecting NTM. Guidelines for the diagnosis of NTM diseases, including clinical symptoms, radiographic findings, and microbiological criteria, have been prepared by the American Thoracic Society and the Infectious Diseases Society of America. Treatment for NTM and Mycobacterium tuberculosis complex (MTBC) infections is usually distinct, even when their clinical presentations are similar or identical. Infections of the skin and soft tissues, as well as lymphatic, disseminated, and nosocomial infections after surgery, were caused by several species of NTM. These infections might be mild, moderate, or severe, and could occur in outbreaks or sporadic outbreaks. Most cases of NTM-related disseminated illness are associated with HIV/AIDS and other severe immunosuppressant diseases.

Though they are laborious, time-consuming, and need complex safety measures, conventional biochemical techniques may identify mycobacterial species. The line probe test has been suggested by the World Health Organisation (WHO) as a means to cut the time it takes to identify, culture, and detect drug resistance down to as little as two days. Mycobacteria may be detected and identified simultaneously using DNA strip technology, also known as line probe tests. This method relies on the reverse hybridization of PCR products to their corresponding probes. Developed by Hain Life science of Nehren, Germany, the GenoType Mycobacterium common mycobacteria/additional species (CM/AS) test is a brand-new commercial kit for differentiating and identifying NTM species from cultures. The process begins by amplifying a section of the 23S rRNA gene in DNA, and then it moves on to reverse hybridization using oligonucleotide probes that have been immobilized on membrane strips.

Both kits are highly accurate and dependable for mycobacterial species identification; the CM kit identifies 15 different species of Mycobacterium, including the M. tuberculosis complex, and the AS kit aims to differentiate 16 additional, less common NTM species. The detection rate for lung illnesses and the prevalence of NTM in India ranged from 0.7% to 34%. All pulmonary/extrapulmonary illnesses and clinical practitioners in India and throughout the world should be concerned about the increasing prevalence of NTM infection. Recent and comprehensive data on the incidence of NTM infection in cases of extrapulmonary tuberculosis (EPTB) in India's tertiary care institutions is lacking. The purpose of this research was to determine the frequency of NTM at tertiary care centres in the Northern area of India that treat patients with extrapulmonary TB.

## LITERATURE REVIEW

Background According to Gardini (2021), the most common way to get pulmonary nontuberculous mycobacterial (pNTM) infection is to breathe in bioaerosols. The current recommendations for pNTM management place more emphasis on diagnosis and therapy than prevention, which is why physicians seldom prescribe behavioural limitations to vulnerable groups. The purpose of this analysis is to determine whether or not suggestions for risk- reducing behaviours should be standard fare in pNTM prevention. Approach The biomedical database PubMed was used. Using keyword combinations such as "nontuberculous mycobacteria," "water," "soil," and "exposure," we narrowed our search to publications published between 2000 and 2020. A comprehensive screening was conducted on the titles and abstracts of the chosen papers. The study only included articles that were published in English, French, German, or Italian and had complete texts available via the digital library of the University of Brescia (Italy).

Percival, (2013) Mycobacteria are bacilli that look like rods; they are Gram-positive, slim, non- motile, and non-spore forming. The cell shape might vary from a coccoid to a long, thin rod. The opportunistic pathogen mycobacterium causes a wide variety of illnesses, including AIDS, skin lesions including post- injection abscesses, swimming pool granulomas and Buruli ulcers, lesions that resemble TB, and lymphadenitis after infection. Soil and sea water aren't the only places you may find the Mycobacterium avium complex (MAC). There is an abundance of mycobacterial species in man-made environments, including water distribution systems. In people, MAC may lead to three distinct illnesses: pulmonary disease, cervical lymphadenitis, and disseminated MAC. Aerosol inhalation and gastrointestinal tract ingestion are two routes of exposure. There are those who think the latter is more to blame for the spread of the disease. The ability of mycobacteria to create biofilms has been discovered. Regardless, the exact processes by which they construct biofilms are still a mystery. Studies on Mycobacterium MAC have revealed that it is 700–3000 times more chlorine resistant and 100–50 times more resistant to chlorine dioxide and ozone than Escherichia coli. In this chapter, we will cover all the bases when it comes to Mycobacterium, from its basic microbiology and natural history to its metabolic and physiology, clinical features, pathogenicity and virulence, treatment, environmental survival, water survival, epidemiology, evidence of biofilm growth, detection methods, antimicrobial control, and risk assessment.

Mycobacterial infections, according to Hosseiniporgham (2022), may be caused by either fast-growing or slow-growing mycobacteria and can be fatal. Every year, millions of lives are lost due to certain mycobacteria, such Mycobacterium tuberculosis. The capacity of mycobacterial pathogens to acquire resistance to standard antibiotics and the difficulties encountered in diagnosing these germs impact the control of mycobacterial illnesses. The intracellular nature of mycobacterial pathogens, in conjunction with the lipid- enriched composition of their cell wall, makes detection of mycobacterial infections very challenging. In addition, new research has shown that over 20% of M. tuberculosis (Mtb) infections are MDR, meaning they are resistant to several drugs. So, the purpose of this study was to provide an overview of several mycobacteriophages that have been studied, with a focus on those that infect the three problematic mycobacteria in question, M. pneumonia, M.L. family Avium. multi-organ failure (MAP), and M. abscessus, demonstrating their medicinal theranostic potential.

Michael Babarola. (2015) This presents the Mycobacterium tuberculosis SWOT (Strengths, Weaknesses, Opportunities, and Threats) analysis, as well as the initial proposal to implement this novel concept in tuberculosis research. The goal is to provide a comprehensive framework, targeted information, and methods to weaken the tubercle bacilli's strength and ultimately overcome the disease. The methods and materials used in this study included a systematic review that aimed to identify M's advantages, disadvantages, new prospects, and risks. tb, by searching this topic with relevant keywords and themes in a number of articles. The lungs are particularly susceptible to TB because of their anatomy and the fact that inhaled aerosols from inhaled air are essential for latent infection. Dangers to M. T. tuberculosis includes: creating and using a sensitive combination of microbiological tests as the standard for HIV- infected patients; conducting TB tests annually; reintroducing sanatoria to selectively isolate TB patients; prioritizing genomic drug targets; maintaining global TB funds; developing a powerful vaccine; imaging deep-seated infections using Infecton; and developing and using a computer tomography and positron beam tomography live imaging system to characterize active TB in lesions.

According to Delogu (2013), tuberculosis (TB) is far from gone; in fact, it has been on the rise again in nations that were once believed to have eradicated the illness. It is widely believed that new diagnostics, a vaccine, and medications are desperately needed to rein in the worldwide pandemic. However, a big roadblock to developing these control tools is our current lack of knowledge about the pathogenesis mechanism of Mycobacterium tuberculosis (Mtb). Recent years have seen a paradigm shift away from the old dichotomy between latent and active disease and towards a dynamic equilibrium between the host and the bacilli, encompassing a continuous spectrum of conditions referred to as the TB spectrum. This review will summarise the latest advances in our understanding of Mycobacterium tuberculosis biology and the pathogenesis of Mycobacterium tuberculosis infection, with a focus on latent infection. We will also talk about the consequences for how we diagnose and manage diseases in certain populations.

## RESEARCH METHODOLOGY

The Mahatma Gandhi Memorial Medical College ' institutional ethics committee in Indore has given its stamp of approval (No. IEC/C- P/48/2014).

Patient selection: This research included patients whose diagnosis of NTM illness was validated by the American Thoracic Society. The patient's full demographic information, as well as their clinical and radiological records, were gathered throughout the enrollment process.

### Biochemical reactions:

### Controls:

The research used standardized identification procedures that were closely monitored. Positive and negative controls were acquired from JALMA, Agra.

As controls, we used the standard ATCC strains of Mycobacterium TB complex H37Rv (ATCC 27294), Mycobacterium fortuitum (ATCC 6841), and Mycobacterium chelonae (ATCC 19539).

### Molecular methods of identification:

### DNA extraction:

A product from MACHEREY-NAGEL GmbH & Co. KG called "NucleoSpin® Microbial DNA" was used for DNA extraction.

The BacT/ALERT MP vial was aspirated with a sterile disposable syringe to remove 0.5 mL of culture.
The cultures were centrifuged at 10,000 rpm until they formed a pellet.
Before resuspending the cells, mix the pellet with 0.1 millilitres of elution buffer (BE).
Now, the supplied "NucleoSpin® Bead Tube Type B" is used to transmit this suspension.
Now, add 40 microliters of Buffer MG. 10 µl of Liquid Proteinase K is added thereafter, and the tube is then sealed. Use a vortex mixer to agitate the NucleoSpin® Bead Tube.
To clean the cover, spin the NucleoSpin® Bead Tube in a centrifuge for 30 seconds at 11,000 rpm.
Then, after waiting three seconds, add 0.6 millilitres of "Buffer MG" and mix well. Set the centrifuge to 11,000 rpm and spin for 30 seconds.
The NucleoSpin® receives about 500-600 µl of the supernatant. A two millilitre collection tube is used to hold the microbiological DNA column (supplied).
After 30 seconds of centrifugation at 11,000 rpm, discard the collecting tube and the flow-through.
Use the included collection tube to transfer the column to a fresh 2 ml tube.
This is the first wash: Pour 500 µl of buffer at room temperature. Reintroduce the column to the Collection Tube after centrifuging it for 30 seconds at 11,000 xg; remove the flow-through.
To do the second wash, pour 0.5 millilitres of Buffer B5 into the column and spin it at 11,000 revolutions per minute for 30 seconds. After that, the flow- through is thrown away while the column is placed back into the Collection Tube.
The column should be centrifuged at 11,000 rpm for 30 seconds.
To extract the DNA that is very pure, place the NucleoSpin® Microbial DNA Column into a 1.5 ml tube that does not contain nucleases, and then pour 100 µl of Buffer BE over the column.
At room temperature, incubate for one minute. Place in a centrifuge and spin at 11,000 rpm for about 30 seconds.

**Biochemical reactions in identification of NTM species:**

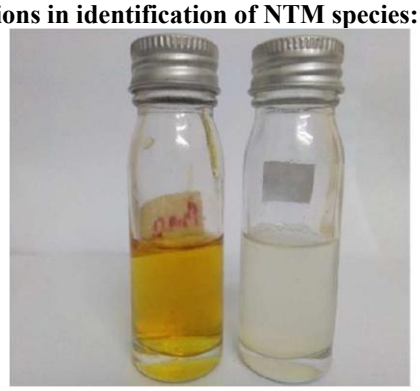

**Fig:1: Niacin accumulation test Fig: 2: Nitrate reduction test**

**Fig:3: Thermo stable Catalase Test Fig:4: Catalase test**

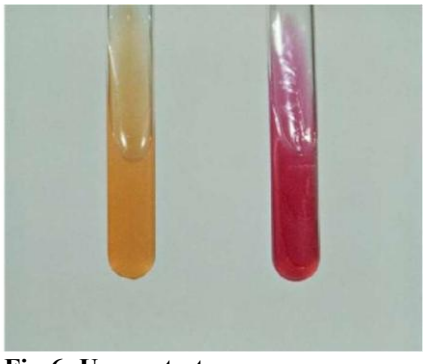

**Fig:5: Iron uptake test Fig:6: Urease test**

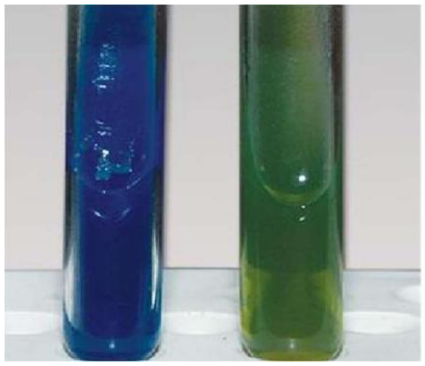

**Fig:7: Citrate utilization test**

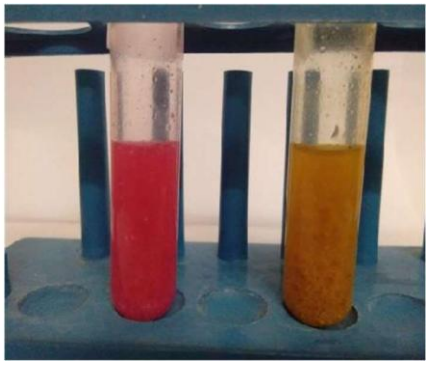

**Fig:8: Tween 80 hydrolysis test**

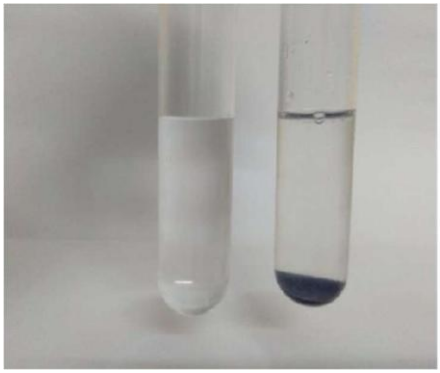

**Fig: 9: Tellurite reduction test**

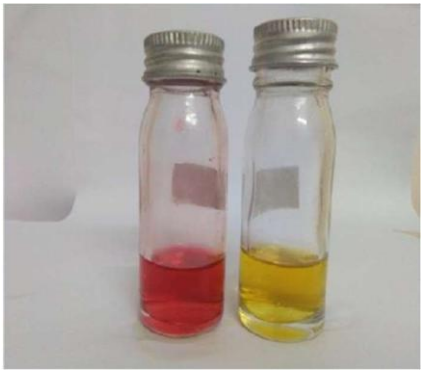

**Fig:10: Aryl sulphatase test**

**Table:1. NTM Species identified by biochemical reaction:**

| Ni a | Ar y 5D | Ar y 2W | Ni t | G. Rat e 25 | G. Rat e 37 | SQ CT | Pi g M | Tel. Re d | T wee | P N B | M A C | Iro n | P Z A 4d | P Z A7d | T C H | Ur E a | NTM Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | P | P | N | P | P | >45 M M | N | <7 | P | P | P | P | P | P | P | P | ***M.abscessu s*** |
| N | N | N | N | N | P | >45 M M | P | >7 | N | P | N | N | N | N | P | P | ***M.simiae*** |
| N | P | P | P | P | P | >45 M M | N | <7 | P | P | P | P | P | P | P | P | ***M.fortuitu m*** |
| N | P | P | N | P | P | >45 M M | N | <7 | P | P | P | P | P | P | P | P | ***M.chelonae*** |
| N | | | | | | | | >7 | | | | | | | | | ***M.gordonae*** |

**Table: 2. Distribution of species by biochemical reactions:**

| S. No | NTM species | NTM (n=450) | |
|---|---|---|---|
| | | Total number | Percentage |
| 1 | *M.abscessus* | 253 | 56.22% |
| 2 | *M.simiae* | 86 | 19.11% |
| 3 | *M.fortuitum* | 58 | 12.88% |
| 4 | *M.chelonae* | 49 | 10.88% |
| 5 | *M.gordonae* | 4 | 0.88% |
| | | **450** | |

Second positive smears, together with clinical and radiological symptoms, were necessary for the commencement of anti-NTM medication. After one month of treatment with Anti-NTM, 111/242 (91.32% of the samples) remained positive, whereas 21/242 (8.67%) cultures were determined to be negative. Patients who tested positive and those who tested negative were both closely monitored for treatment results for a full 18 months. At the end of the first month of therapy, all twenty-one cultures tested negative. This trend persisted throughout the trial.

**Molecular diagnosis of NTM:**
**a. PCR – Restriction Endonuclease Analysis:**

**Table:3. HaeIII and Bst II restriction patterens of hsp65gene and results of mycobacterial strains isolated by PRA assay.**

| Restriction fragment size | | Species | Number | Percentage |
|---|---|---|---|---|
| HaeIII | BSTII | | | |
| 160 bps,60 bps | 245 bp,220 bp | *M.abscessus* | 253 | 56.22% |
| 160, 140 | 245, 220 | *Msimiae* | 86 | 19.11% |
| 155/135 | 245,120/80 | *M.fortuitum* | 58 | 12.88% |
| 210 | 325/140 | *M.chelonae* | 49 | 10.88% |
| 235,115 | 125, 80 | *M.gordonae* | 4 | 0.88% |

We used BstEII and HaeIII enzymes to selectively digest a 439 bp fragment of the hsp 65 gene. The resulting patterns of digested fragments were then analysed using 2% polyacrylamide gel electrophoresis (PAGE). The 452 isolates were categorised into 450 NTM species and 2 MTB complex isolates; however, we did not include them in our analysis. Figure shows the patterns of digested hsp65 PCR products.

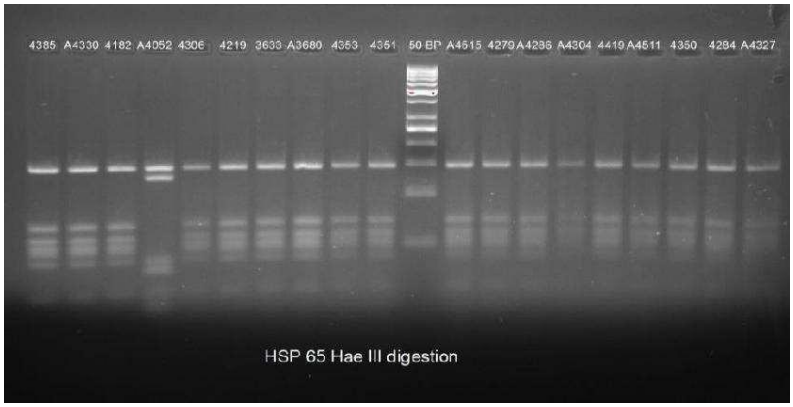


**Fig:11: hsp 65gene restriction digestion with Hae III enzyme.**

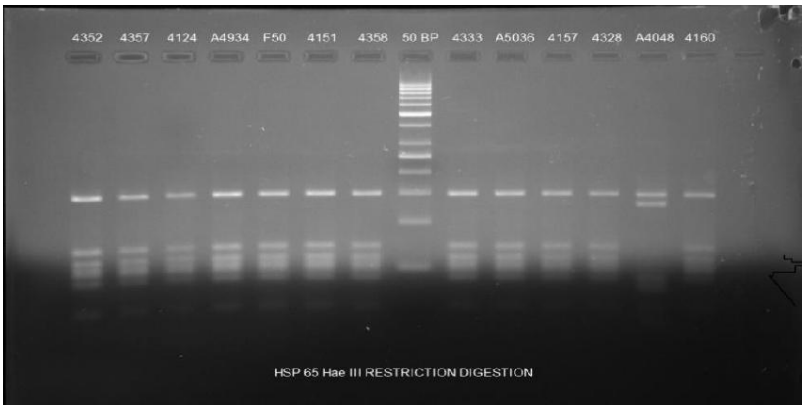


**Fig:12: hsp 65gene restriction digestion with Hae III enzyme.**


**Fig:13: hsp 65gene restriction digestion with Bst II enzyme**


**Fig:14: Restriction digestion of ITS gene with HaeIII enzyme.**

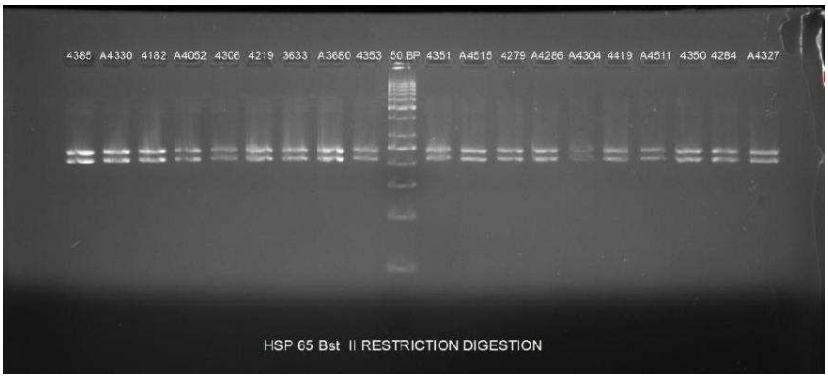


**Fig:15: Restriction digestion of HSP 65 gene with Bst II**

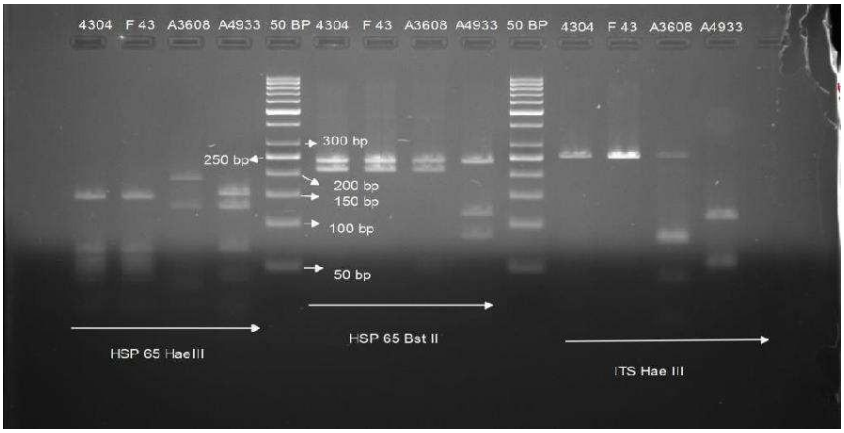


**Fig.16: RFLP of 4 samples with Hae III and Bst II HSP 65 gene and Hae III ITS gene**

Five distinct NTM species were found using PCR-RFLP analysis of the hsp65 gene. Of these, 253 (56.22%) belonged to the M.abscessus family, 86 (19.11%) to the M.simiae, 58 (12.88%) to the M.fortuitum, 49 (10.88%) to the M.chelonae, and 4 to the M.gordonae.

Our 450 M. chelonae isolates all thrived at different temperatures (25C, 37C, and 42C), showed signs of growth on MacConkey agar, and were positive for urease and reduced nitrates to nitrites; nevertheless, they were negative for iron absorption and Tween 80 hydrolysis. S.Jain et al., from Delhi, reported similar biochemical test results with an M.chelonae isolate in a case of catheter-related bacteremia; Rajini et al., from Kolar, Karnataka, identified an NTM isolate from a post-operative infection and confirmed its identity as M.chelonae based on growth at different temperatures, urease test, nitrate test, tween 80 hydrolysis, and the Thiophene -2-carboxylic acid hydrazide (TCH) test.

## CONCLUSION

An rise in immunocompromised states and the development of more precise diagnostic tools may be contributing factors to the rising incidence of NTM infections. Lung, lymph gland, bone, skin, and wound infections are among the many human illnesses caused by NTM. It is critical for microbiologists and doctors to be aware of NTM and to diagnose it correctly. The primary goal of NTM should be to distinguish between diseases and colonisation. The alarmingly high frequency of these isolates in otherwise healthy people highlights the need to educate physicians and microbiologists

about NTM illnesses so that patients may be diagnosed early and undergo targeted treatments to lessen the severity of NTM infections.

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