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RESEARCH ARTICLE
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EMERGING BIOMARKERS FOR THE DIFFERENTIAL DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS AND NONTUBERCULOUS MYCOBACTERIA

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Abstract
Tuberculosis (TB), primarily caused by Mycobacterium tuberculosis (MTB), remains a major global health threat, particularly in developing countries. However, nontuberculous mycobacteria (NTM) are increasingly implicated in pulmonary infections, often mimicking TB clinically and radiologically. Misdiagnosis could cause unsuitable treatment plans and higher morbidity. This research investigates emerging molecular biomarkers that distinguish between MTB and NTM infections, highlighting the clinical significance of accurate identification. N. tuberculosis (NTM) was found in 33 clinical samples using real-time PCR that targeted IS6110 for M. tuberculosis (MTB) and MPT64. Results revealed that 51.5% were MTB-positive, 27.3% NTM-positive, and 21.2% negative for both. The findings underscore the importance of incorporating molecular diagnostics in TB-endemic settings to improve therapeutic outcomes and guide public health strategies.

Key Words: Interferon Gamma (IFN-γ), Bacillus Calmette-Guérin (BCG), Cell-Mediated Immunity (CMI), Complement Receptors (CR), Fc Receptors (FcR), Surfactant Protein A (SP-A) Receptors, Scavenger Receptor Class A, Toll-like Receptors (TLR), and Active Tuberculosis (ATB).

Introduction:

Tuberculosis (TB), caused by Mycobacterium tuberculosis (MTB), remains a major global health, social, and economic burden. Despite extensive research, key questions about its pathogenesis and immune response persist (Delogu et al., 2013). 10.8 million new cases and 1.25 million fatalities from

tuberculosis were reported in 2023. Five to ten percent of people worldwide will develop active TB, while one-third are latently infected. Global efforts have saved 79 million lives since 2000. The Mycobacterium tuberculosis complex (MTBC), including species like M. bovis, M. africanum, and others, shares over 99.5% genetic similarity. M. bovis, primarily a cattle pathogen, can also infect humans (Ahmad, 2011).

Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mtb), has afflicted humans for over 4,000 years, with high mortality in the 17th–18th centuries, earning names like "consumption" and "white plague" (Bussi et al., 2019; Daniel et al., 1994). Robert Koch identified Mtb in 1882, a milestone now marked as World TB Day. Sanatorium care preceded the antibiotic era, which began with streptomycin (1943), followed by isoniazid and rifampicin—key drugs in TB therapy (Sakamoto, 2012). The BCG vaccine, introduced in the 20th century, provides partial protection, especially in children (Abebe et al., 2007).

Nontuberculous mycobacteria (NTM), excluding M. tuberculosis and M. leprae, were first isolated in the late 1800s but recognized as pathogens in the 1950s. Mycobacterium avium complex (MAC), Mycobacterium kansasii, and Mycobacterium abscessus are common species (Miller Jr., 1994). NTM infections have risen, especially in immunocompromised individuals and those with lung conditions (Koh & W.J., 2017). CF patients and older women are frequently affected, even though soil and water are significant reservoirs (Johnson et al., 2014; Olivier et al., 2003). Accurate species identification is crucial due to diverse drug resistance patterns (Marras et al., 2002).

Mycobacterium tuberculosis (MTB) spreads via inhalation of aerosolized droplets from individuals with active pulmonary TB (Getahun et al., 2015). Droplet sizes (<0.1 to >7.0 μm) influence respiratory deposition and transmission (Bussi et al., 2015). Latent TB infection (LTBI) is widespread and primarily transmitted by untreated cases. Progression to active TB depends on host immunity, bacterial load, and environmental factors (Cadena et al., 2016). Effective LTBI management requires treating the infection and addressing risk factors like drug use, alcohol abuse, and smoking through behavioral interventions (Getahun et al., 2015). Comorbidities, especially HIV, significantly increase the risk of progression to active TB, with HIV-positive individuals facing a 10% annual risk due to weakened immunity (Sia et al., 2019).

Immunosuppressive conditions like malnutrition, aging, diabetes, renal failure, and certain therapies increase the risk of TB reactivation (Ahmad, 2011). Genetic factors, including MSMD, highlight the role of the IFN-γ/IL-12 axis in TB immunity (Boom et al., 2012). Antibodies against antigens like ESAT-6 are being studied to differentiate LTBI from active TB (Abebe et al., 2007). NTM pulmonary disease commonly affects individuals with structural lung issues (e.g., bronchiectasis, COPD, prior TB, CF) or immunocompromised states like HIV, organ transplants, or cancer therapies (Holland & S.M., 2001; Phillips et al., 2001). Environmental exposure to water, soil, and aerosols increases risk, with elderly women often developing nodular bronchiectasis and middle-aged men cavitary disease (Marras et al., 2002).

Mycobacterium tuberculosis (MTB) produces tuberculosis through a cycle comprised of infection, latency, and reactivation. It spreads via aerosols and survives in alveolar macrophages by evading destruction (Sasindran et al., 2011). Granulomas produced by the immune system to enclose the infection cause latent tuberculosis. Reactivation occurs under immunosuppression, causing tissue damage and active disease (Sasindran et al., 2011). Culture (slow, gold standard) and AFB smear (fast, low sensitivity) are used for TB diagnosis. TST lacks specificity due to BCG/NTM cross-reactivity. IGRAs using ESAT-6 and CFP-10 provide higher specificity for LTBI (Abebe et al., 2007). Tuberculosis affects all age groups, with the highest burden observed by the WHO from Southeast Asia, Africa, and the Western Pacific Regions. The lungs are primarily affected, although systemic spread is possible. This study aims to investigate the microorganisms behind TB through biopsy-based molecular analysis.

Materials and methodology:

Site of implementation of work:
DNA Labs-CRIS (Centre for Research and Innovative Studies), of DNA Labs—A Centre for Applied Sciences, located in East Hope Town, Laxmipur, Dehradun, Uttarakhand, was the site of all the experiments.

Materials:
Suspected samples, BSL lab, micropipettes, motor pistol, microcentrifuge, tubes, vortexer, centrifuge, liquefaction buffer, nucleus-free water, lysis buffer, proteinase K, binding reagent (ethanol), wash buffer (1 & 2), ethidium bromide (EtBr), loading dye bromophenol blue, hypochlorite solution, parafilm, elution buffer, hypochlorite solution, analytical-grade agarose powder, 1X Tris-Acetate-EDTA (TAE) buffer, electrophoresis casting tray, casting dams, electrophoresis tank and power supply, UV transilluminator, sample set (Samples 1–10), master mix tubes, reconstitution buffer, positive control kit, and no template control (NTC).

Biopsy and DNA Extraction:
Biopsy samples were collected under sterile conditions and stored at -20°C. Samples were defrosted, homogenized, and treated with nuclease-free water and liquefaction buffer. After centrifuging the homogenate, we delicately pipetted a portion of the supernatant to proceed with the DNA extraction. The careful handling at this stage sets the stage for discovering the genetic blueprint hidden within!

DNA Extraction Using the Silica Column Method:
DNA was isolated using a protocol involving lysis buffer, proteinase K, the sample, and ethanol. The lysate was bound to a silica column, washed twice with Wash Buffers 1 and 2, and eluted using an elution buffer. DNA was stored for downstream molecular testing.

Gel Electrophoresis:
A 1% agarose gel was used to validate DNA integrity. The intercalating dye was ethidium bromide. The samples were processed in a TAE buffer after combining loading dye and bromophenol blue. Visualization was done using a UV transilluminator.

RT-PCR Master Mix and Setup:
Samples were pooled and processed in sterile MCTs. A sample mix, a positive control, and a no-template control were all contained in master mix tubes. Reagents included reconstitution buffer, nuclease-free water, and lyophilized master mix. Vials were loaded onto the rotor disk of the PCR machine.

PCR Instrument Setup:
PCR parameters were configured using Rotor-Gene software. Initial denaturation lasted 3 minutes at 95°C. After that, there were cycles of annealing at 55°C for 25 seconds and denaturation at 95°C for 15 seconds. The red, green, and yellow channels were selected for detecting fluorescence.

Quality Control Measures:
All reactions included positive and negative controls. Work areas were kept under sterile conditions, and pipetting was carried out with calibrated instruments.

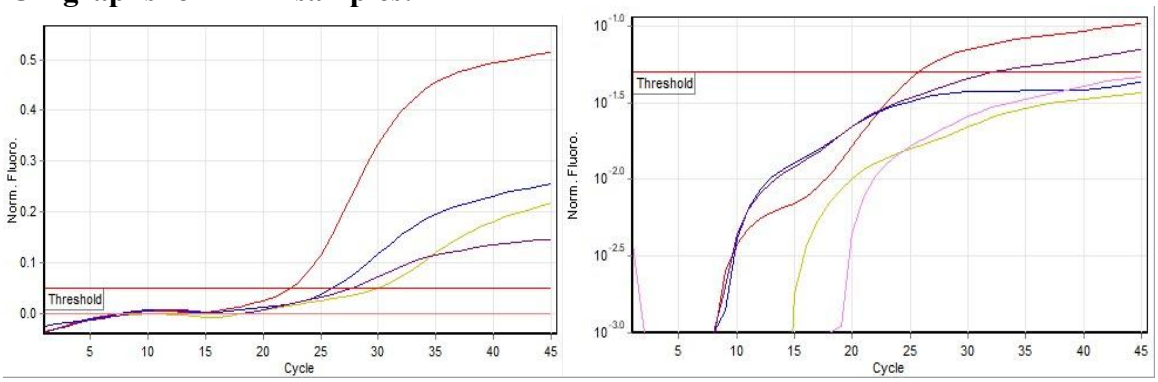
Result:

MTB-NTM RESULTS

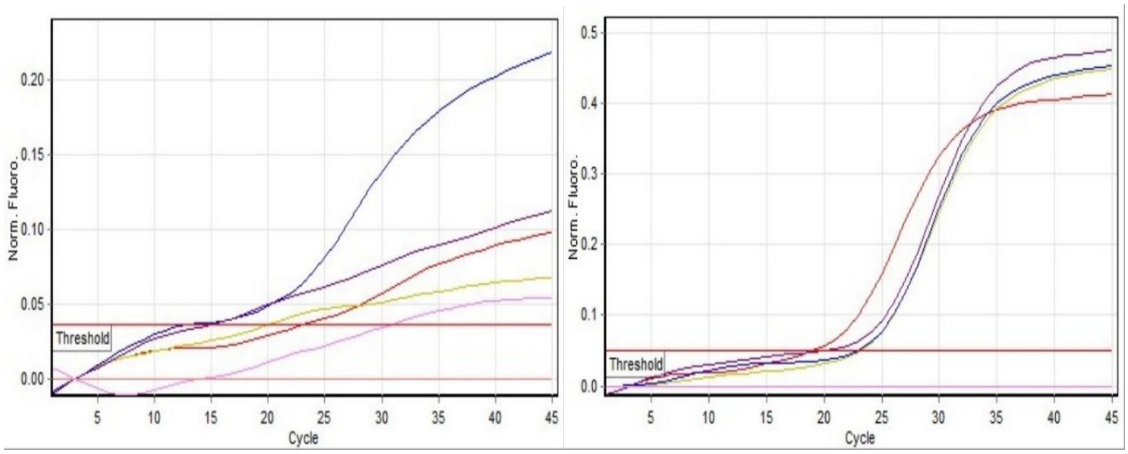
Table 1: List of Patients showing positive or negative MTB-NTM

Sample	MTB Positive/Negative	NTM Positive/ Negative
Sample 1	Positive	Negative
Sample 2	Positive	Negative
Sample 3	Positive	Negative
Sample 4	Positive	Negative
Sample 5	Positive	Negative
Sample 6	Positive	Negative
Sample 7	Negative	Negative
Sample 8	Positive	Negative
Sample 9	Positive	Negative
Sample 10	Positive	Negative
Sample 11	Positive	Negative
Sample 12	Positive	Negative
Sample 13	Positive	Negative
Sample 14	Positive	Negative
Sample 15	Negative	Negative
Sample 16	Negative	Positive
Sample 17	Negative	Positive
Sample 18	Negative	Negative
Sample 19	Negative	Positive
Sample 20	Negative	Negative
Sample 21	Positive	Negative
Sample 22	Negative	Positive
Sample 23	Negative	Positive
Sample 24	Negative	Positive
Sample 25	Negative	Positive
Sample 26	Negative	Positive
Sample 27	Negative	Negative
Sample 28	Negative	Negative
Sample 29	Positive	Negative
Sample 30	Positive	Negative
Sample 31	Negative	Negative
Sample 32	Negative	Negative
Sample 33	Positive	Negative

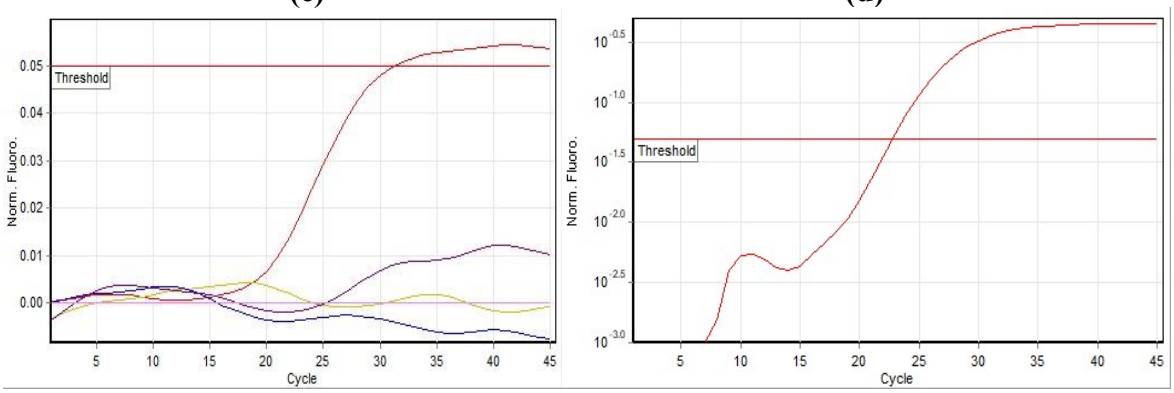
RT-PCR graphs for MTB samples:



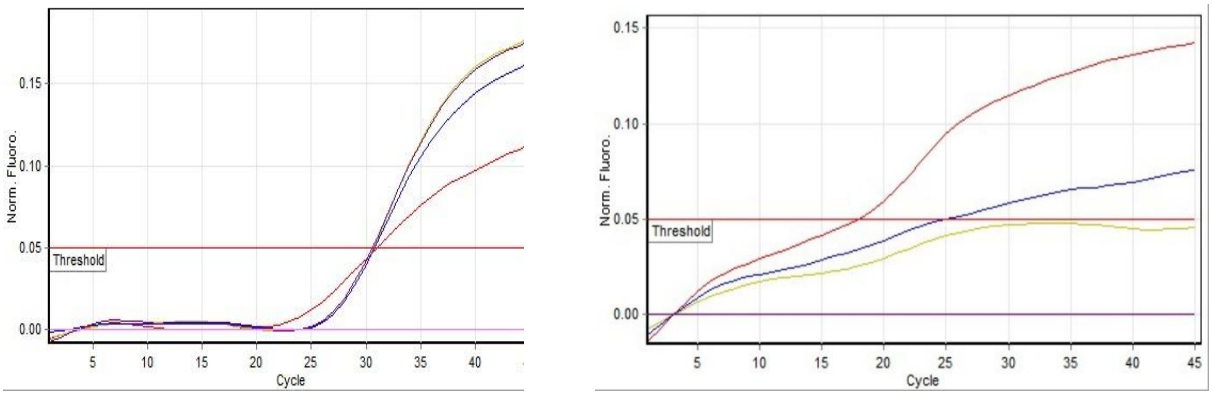
(a) **(b)**



(c) (d)

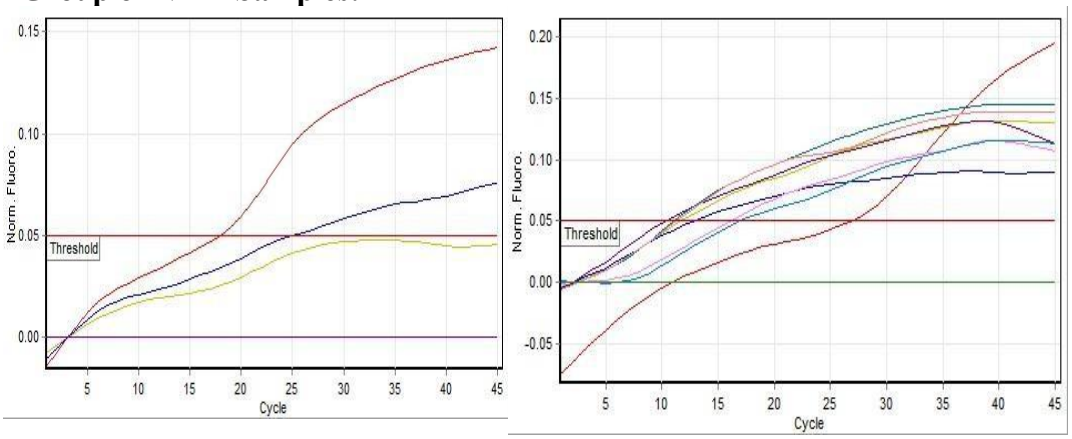


(e) (f)

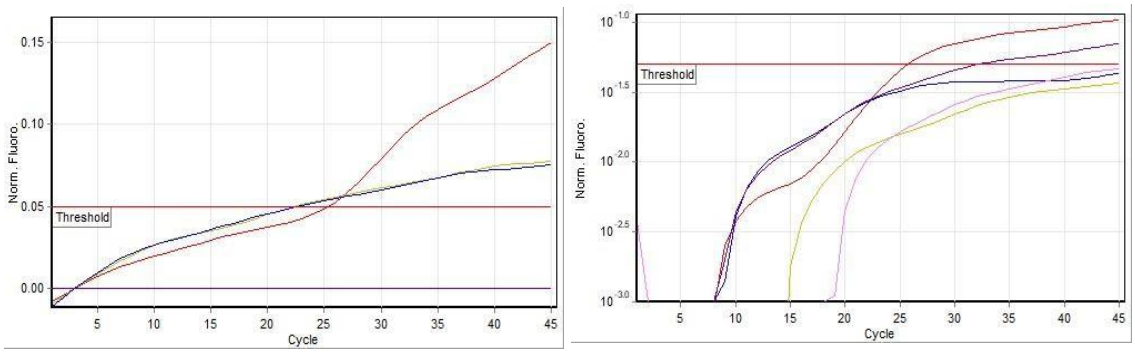


(g) (h)

RT-PCR Group of NTM Samples:



(i) (j)

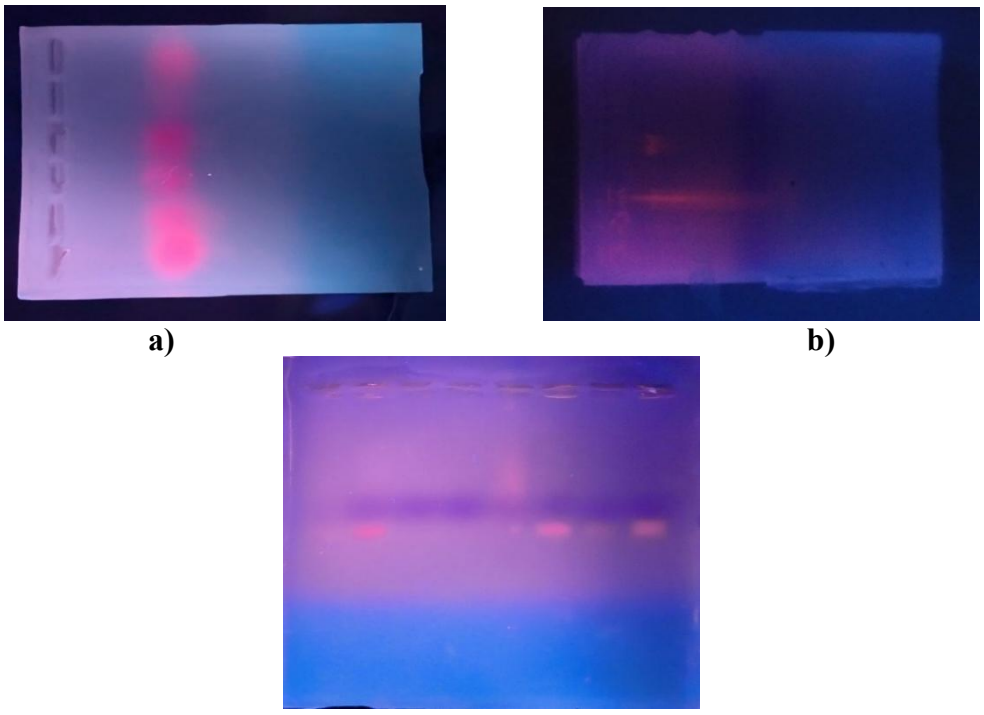


(k) **(l)**

RT-PCR graphs result for MTB and NTM samples: Fig.(a): Baseline RT-PCR setup or control curve. Fig.(b): Amplification curve showing positive MTB detection with a clear Ct value. Fig.(c): Melt curve indicating specific amplification with a single peak. Fig.(d): Strong amplification suggesting high MTB load. Fig.(e): Weak or no amplification; likely MTB-negative sample. Fig. (f): Standard curve showing assay efficiency and linearity. Fig.(g): Negative control with no amplification, confirming no contamination. Fig.(h): Confirmatory amplification of a second MTB target or replicate. Fig.(i): Positive MTB amplification with clear Ct. Fig.(j): Specific melt curve for sample (i). Fig.(k): Standard curve for quantification. Fig.(l): Negative control with no amplification.

Expression/Amplification of reference gene:

The amplification efficiency of the IS6110(MTB), MPT64(NTM) genes was evaluated using real-time PCR. These genes were selected due to their widespread application as internal controls in human gene expression studies and their reported stable expression in MTB-NTM. Agarose gel electrophoresis was used to assess the existence and clarity of particular bands. The ensuing gel pictures, which show discrete bands at the anticipated product size across a variety of sample types, validate that the IS6110 and MPT64 gene was amplified successfully. These visual findings provide qualitative confirmation of IS6110 gene expression under the investigated circumstances and corroborate the molecular data.

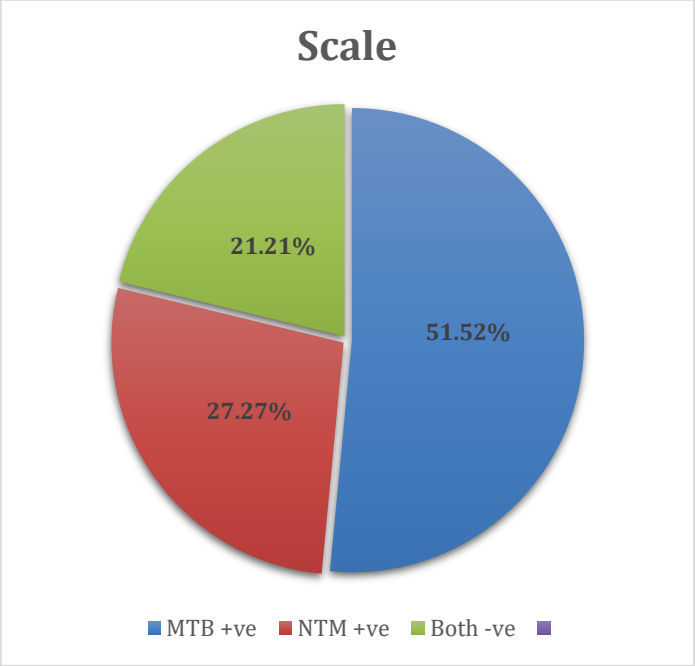


a) **b)**

c)

Agarose gel electrophoresis image showing PCR amplification of the IS6110 and MTP64 evaluated as a reference gene for internal control in MTB-NTM detection. a), b) are the gel results of MTB patients, and c) are the results of NTM patients.

Analysis of MTB-NTM Patients:



Distribution of MTB-NTM infections of positive and negative results from clinical samples

In this study, 33 clinical samples suspected of tuberculosis were analyzed using molecular diagnostic methods, including real-time PCR (RT-PCR) targeting IS6110 for Mycobacterium tuberculosis (MTB) and MPT64 for nontuberculous mycobacteria (NTM). Of the total samples, 17 were found to be MTB-positive, 9 were NTM-positive, and 7 tested negative for both, suggesting potential low bacterial load, alternate infections, or procedural limitations. Gel electrophoresis confirmed the presence and integrity of DNA in most samples, indicating successful DNA extraction. The RT-PCR results provided clear amplification curves with appropriate cycle threshold (Ct) values, while internal controls demonstrated no evidence of contamination or reaction inhibition. Quantitative analysis confirmed reliable detection, with the positive control consistently yielding expected Ct values. The differentiation between MTB and NTM was critical in identifying cases that may have otherwise been misdiagnosed, reinforcing the value of molecular tools in clinical diagnostics. These findings highlight the necessity of incorporating specific genetic markers for precise identification, as well as the increasing clinical relevance of NTMs in patients with compromised immunity or underlying pulmonary conditions.

Discussion

This study confirms the diagnostic value of IS6110 and MPT64 in distinguishing MTB from NTM. Traditional AFB staining lacks the sensitivity and specificity provided by molecular methods. NTM infections are particularly prevalent among immunocompromised patients and require different treatment regimens than MTB. Thus, misdiagnosis can lead to therapeutic failure, extended illness, and unnecessary exposure to anti-TB drugs. The integration of RT-PCR into routine diagnostic workflows enhances accuracy and informs better clinical decisions. The 21.2% negative results suggest the potential for co-infections or alternative diagnoses, warranting further investigation.

Conclusion

Accurate diagnosis of mycobacterial infections is essential for effective treatment and public health management. This study highlights the utility of molecular diagnostics, particularly RT-PCR targeting

IS6110 and MPT64, in differentiating MTB and NTM. Implementing such tools in TB-endemic regions can significantly improve patient outcomes and reduce the burden of misdiagnosis.

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Conflict of Interest
Authors of this paper declare no conflict of interest

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