Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 8
Open Access

Bridging Diagnostic Gaps In Tuberculosis: The Significance Of Is6110 And Mpb64 In Pulmonary And Extrapulmonary Tb, And The Role Of Nucleic Acid Amplification And Multidimensional Testing For Global Health And Wellbeing

Ashish Soni[1], Indu Sharma[1], Srishti Joshi Sharma[2], Nimisha Dandriyal[2], Kriti Novel[2], Narotam Sharma[2,4], Jagjeet Singh[2], Ankita Singh[2,3] and Palak Varma[2]

[1]NIMS Institute of Allied Medical Science and Technology, NIMS University, Jaipur, Rajasthan
[2]DNA Labs -A Centre for Applied Sciences, East Hope Town, Dehradun Uttarakhand, India
[3]Uttaranchal Institute of Technology, Uttaranchal University, Dehradun, Uttarakhand, India
[4]Department of Chemistry, Uttaranchal Institute of Technology, Uttaranchal University, Dehradun, Uttarakhand, India

Cite this paper as: Ashish Soni, Indu Sharma, Srishti Joshi Sharma, Nimisha Dandriyal, Kriti Novel, Narotam Sharma, Jagjeet Singh, Ankita Singh and Palak Varma (2024) Bridging Diagnostic Gaps In Tuberculosis: The Significance Of Is6110 And Mpb64 In Pulmonary And Extrapulmonary Tb, And The Role Of Nucleic Acid Amplification And Multidimensional Testing For Global Health And Wellbeing". *Frontiers in Health Informatics,* (8), 5598-5611

ABSTRACT

The causative agent of tuberculosis (TB) is Mycobacterium tuberculosis, one of the global health concerns with morbidity and mortality. TB presents in two forms; pulmonary tuberculosis (PTB) affects the lungs, and extrapulmonary tuberculosis (EPTB) affects organs other than the lungs. The study was carried out at DNA Labs, DLCAS, Dehradun, Uttarakhand, where 85 clinical materials, including broncho alveolar lavage (BAL), cerebrospinal fluid (CSF), pus, and biopsies were studied. ZN staining and PCR targeting IS6110 and mpb64 genes were performed as per standard protocol. Amplification of M. tuberculosis PCR assays included standard protocols. Fragment of DNA were analyzed using agarose gel electrophoresis. ZN staining detected AFB in 9.4% of 85 specimens. IS6110 was more frequently detected than MPB64 (27 IS6110 positive vs. 13 MPB64 positive by PCR). The performance of ZN was good for PTB but moderate for EPTB and low bacterial loads. PCR was more sensitive and specific and showed that the gene markers varied between specimen types. The EPTB and negative or mixed results of initial tests posed particular diagnostic challenges. These findings highlight the need for a multimodal diagnostic approach integrating ZN staining and higher-tier molecular methods, such as nested PCR. Patients showed gender- and age-based variations in tuberculosis detection, with specific symptoms including abscesses or meningitis having higher odds of positive results. With various diagnostic tests failing to detect all forms of malignant diseases, the new study underscores the importance of employing diverse diagnostic methods. Failure to apply these additional tests may lead to an incomplete diagnosis of tuberculosis.

Keywords - *Mycobacterium tuberculosis, pulmonary tuberculosis, extrapulmonary tuberculosis, IS6110, MPB64, cerebrospinal fluid*

INTRODUCTION

Mycobacterium tuberculosis is responsible for tuberculosis, a major global health concern. It has a significant rate of illness and death worldwide. There are two main types of TB: PTB, known as pulmonary tuberculosis,

and EPTB, referred to as extra-pulmonary TB. PTB impacts the lungs and is therefore the most common type; it accounts for the highest percentage of TB cases (Dorman, 2010; Golden & Vikram, 2005; Gupta et al., 2015; Hillemann et al., 2011; Sharma & Mohan, 2004). Typically, a diagnosis of PTB is conducted through sputum smear microscopy, culture, and NAATs (WHO, 2021). Nevertheless, PTB's dependence on sputum samples restricts its diagnostic capability, particularly for individuals unable to generate sputum or with negative sputum smears (Dorman, 2010). In contrast, EPTB indicates the infection of the pathogen in organs apart from the lungs: lymph nodes, pleura, bones, joints, the central nervous system, and the genitourinary tract (Sharma & Mohan, 2004). Diagnosing EPTB presents a bigger challenge due to the variety of affected sites and the paucibacillary nature of the disease, frequently leading to a reduced bacterial load in the clinical specimens (Golden & Vikram, 2005).

IS6110 is a commonly utilized target gene for identifying Mycobacterium tuberculosis because of its numerous copies in the M. tuberculosis complex genome, which is believed to improve diagnostic sensitivity (Golden & Vikram, 2005; Gupta et al., 2015; Lima et al., 2003; Rafi et al., 2007). Nonetheless, various research from different geographical areas has indicated that certain clinical isolates of M. tuberculosis may either possess a single copy or completely lack IS6110, which could result in possible false-negative outcomes (Dale et al., 2003). The fluctuation in IS6110 copy count can present difficulties in the precise identification of the pathogen, stressing the necessity for different or additional diagnostic targets.

MPB64 is a key secreted protein unique to the M. tuberculosis complex, with a molecular weight of about 23,000 Daltons. It was initially extracted from the culture fluid of M. bovis BCG Tokyo and designated MPB64. MPB64 has served as a target for primers in diagnostic assays aimed at identifying M. tuberculosis in clinical specimens, beyond just sputum, and has demonstrated high specificity for the M. tuberculosis complex. Due to their specificity, MPB64 primers present a promising alternative or addition to IS6110-based detection, rendering it an essential tool in the routine diagnosis of M. tuberculosis infection (Goletti, 2022; Lima et al., 2003; Rafi et al., 2007; Dale et al., 2003). This research seeks to evaluate the efficacy of these two primers in standard diagnostic procedures.

MATERIALS AND METHODOLOGY

Site of Implementation of Work

The entirety of the experimentation was conducted at DNA Labs CRIS Centre for Research and Innovative studies (Parent organization) of DNA Labs- A Centre for Applied Sciences (DLCAS), situated in East Hope Town, Laxmipur, Dehradun, Uttarakhand.

Samples Collection and Analysis

A total of 85 clinical specimens were analyzed, comprising broncho alveolar lavage (BAL), endometrial curettings, pus, cerebrospinal fluid (CSF), ascitic fluid, pleural fluid, biopsies, tissue samples, blood, urine, and endometrial tissue, gathered from various hospitals and nursing homes. The samples were analyzed with acid-fast bacillus (AFB) smears and concurrently assessed using polymerase chain reaction (PCR), targeting both the mpb64 gene and the IS6110 gene via Nested PCR. The positivity rate for the Mycobacterium tuberculosis complex was determined through the identification of the IS6110 and mpb64 genes via PCR. A comparative analysis was performed on the PCR results of IS6110 and mpb64 for pulmonary and extrapulmonary samples.

Microscopy

Every clinical specimen, encompassing both pulmonary and extrapulmonary samples, underwent the Ziehl-Nielsen (ZN) staining method. Carbol fuchsin staining was utilized to dissolve the lipoidal substance in the

Mycobacterial cell wall, enabling it to infiltrate the cytoplasm when heat was applied, leading to red-colored cells. Acid-fast cells endured decolorization and maintained their red hue, whereas non-acid-fast cells faded in color. A methylene blue counterstain distinguished acid-fast cells from non-acid-fast ones.

Processing of Clinical Samples

Clinical samples, such as urine, tissue, broncho-alveolar lavage, pus, endometrial tissue, endometrial biopsy, and peritoneal biopsy, were handled by moving them into centrifuge tubes. Lysis buffer was introduced, and the samples were centrifuged to collect cells for later DNA extraction.

Extraction of DNA and Amplification of Specific Genes

(Zhao, 2019; Goletti, 2022; Parekh et al., 2006)

DNA was obtained through two distinct methods: the magnetic bead technique and the Norzen Biotech kit. In the magnetic bead technique, the procedure included incubation with proteinase K, then ethanol precipitation, column purification, and elution. The Norzen Biotech kit utilized a spin column extraction technique that depends on the attraction between nucleic acids and silica in the purification procedure. The polymerase chain reaction (PCR) was utilized to increase the specific DNA sequences of interest. Primers specifically created to target the repetitive sequences IS6110 and mpb64 were synthesized and arranged following standard protocols (Table I). The PCR master mix comprised PCR buffer, dNTPs, MgCl2, primers, Taq polymerase, and the DNA template. The PCR cycle included phases for denaturation, annealing, extension, and a final extension phase. The primers employed in the Nested PCR for IS6110 comprised the sequences 5'-CCTGCGAGCGTAGGCGTCGGT-3' (F) and 5'-CTCGTCCAGCGCCGCTTCGG-3' at 123bp, as well as the primer that amplified the 240 bp mpb64 repetitive sequence sourced from Sigma. The complementary strand arrangement is 5'-TCCGCTGCCAGTCGTCTTCC-3' (F) and 5'-CGGGTCCAGATGGCTTGC-3' (R) (Parekh et al., 2006; Tang et al., 2004)

Table I: First amplification master mix for nested PCR (µl)

Components of Master Mix	For 1 reaction(rxn)	For n reaction 1rxn*n no.
I Amplification Pre-mix	8.2	8.2(n)
Genei Hotstart Taq DNA Polymerase	0.33	0.33(n)
Uracil DNA glycosylase (UDG)	0.5	0.5(n)

A higher-level method called nested PCR utilizes two consecutive processes of amplification to improve the sensitivity and specificity of fundamental PCR. First, outer primers hybridize to a wider section of the DNA to form a first PCR product. As the first amplification can result in non-specific products, half of this initial PCR product is then used as a template for the second stage of amplification. Inner primers are designed to bind within the segment being amplified by the outer primers, overlapping a more specific and narrower region. The layered approach reduces the likelihood of amplifying unwanted sequences and enhances the test's general sensitivity.

Table II: First and Second amplification reaction profile for nested PCR (µl)

Temperature	Time	No. of cycles
22°C	10 min	1
94°C	5 min	
94°C	30sec	20X
68°C	1min	
72°C	1min	

72°C	7min	1
4°C	Storage	
Second amplification master mix (µl) for nested PCR		
Component/ Sample	**For 1rxn**	**1rxn*n no.**
II amplification pre-mix	14.7	14.(n)
Genei Hotstart Taq DNA Polymerase	0.33	0.33(n)
Second amplification reaction profile for nested PCR		
Temperature	**Time**	**No. of cycles**
94°C	5 min	1
94°C	30 sec	30X
68°C	30 sec	
72°C	30 sec	
72°C	7 min	1
4°C	Storage	

Preparation of II amplification master mix

First, it was mixed thoroughly after the II amplification pre-mix was thawed, where the number of samples that included a negative control and a positive control was counted. The necessary total volume was calculated for the master mix and then 14.7 µl was added to the amount for pipetting losses. After completing the first PCR, added 15 µl of the II amplification master mix to each tube. Used a fresh aerosol-resistant tip for each sample and carried out the second PCR using the following programme (Zhao, 2019; Goletti, 2022; Parekh et al., 2006).

Agarose gel electrophoresis

Amplified DNA products were analyzed by agarose gel electrophoresis. The agarose gel was prepared by dissolving agarose in an electrophoresis buffer, casting the gel, and then loading the DNA samples. Electrophoresis was conducted to separate the DNA fragments according to their size. Following separation, the gel was stained and the DNA fragments were visualized under UV light for subsequent analysis (Zhao, 2019; Goletti, 2022; Parekh et al., 2006).

RESULTS

M. tuberculosis which reflects red or pink color and is in the shape of rods as shown in Figure I was the case. This phenomenon is caused by the cell walls of the mycolic acid-containing bacteria which retain the dye fuchsin of the carbol despite the decolorization step with the acid-alcohol. The existence of bacilli that are stained red under a microscope is evidence for the microbe called M. tuberculosis. This technique is especially helpful in the case of the pulmonary tuberculosis type of the disease that is not only very fast but also easy and affordable. Nevertheless, the ZN test has some limitations for the identification of extra pulmonary tuberculosis and mild bacterial loads, so the combination with other diagnostic tests is also required.

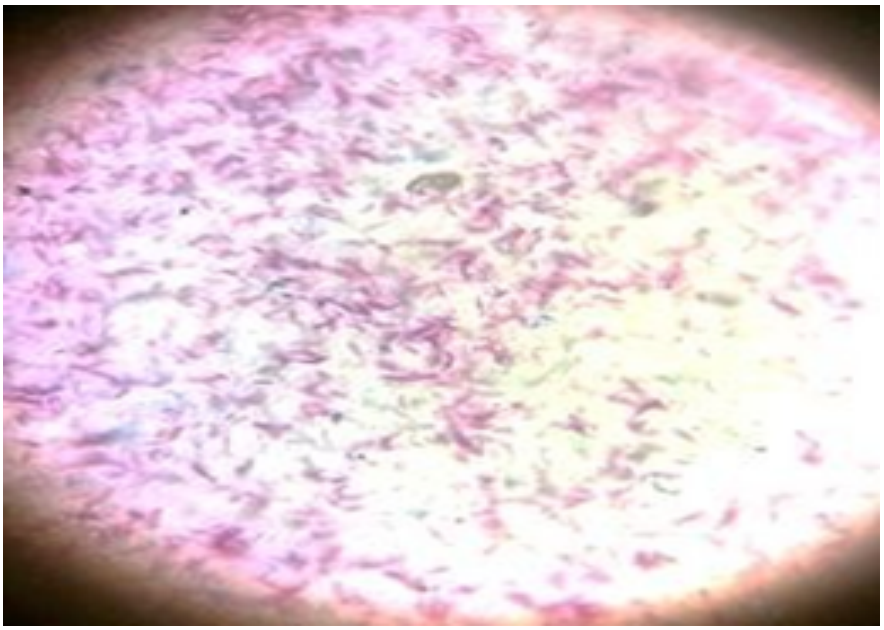

Figure I: Mycobacterium tuberculosis appearing as pink rods under microscope

TB Classification

Among the 85 clinical specimens received (urine, blood, CSF, pus, pleural fluid, tissue, BAL, menstrual/endometrial samples) from patients suspected of having TB, significant diagnostic information was obtained. AFB staining, IS6110 gene detection and mpb64 gene test were performed to confirm the TB infection. Most of the cases were negative for all three diagnostic markers, indicating a low detection rate using conventional methods. In contrast, a subset of infected patients were positive for IS6110 and mpb64 genes and thus Mycobacterium tuberculosis infection with pus and tissue samples.

Notably, IS6110 and/or mpb64 were detected in the patients who had TB meningitis, septic arthritis, and chronic abscesses, and some actually turned an occasional positive AFB stain. The sporadic positive cases in BAL and CSF samples again emphasized molecular diagnostic tests to diagnose extrapulmonary tuberculosis. The need for multidimensional tests was once again demonstrated by a few cases showing positive IS6110 gene expression in the absence of mpb64 positivity. Based on the findings, nucleic acid amplification tests enhance detection of TB, particularly during cases of non-pulmonary pathology, while AFB staining alone may miss several cases. The current research emphasizes the role of molecular diagnostics in improving TB detection in all kinds of specimens.

Out of 85 specimens, 8 tested positive for Acid-Fast Bacilli (AFB), indicating the presence of *Mycobacterium tuberculosis* in these samples. The remaining 77 specimens did not show AFB, which constitutes approximately 90.6% of the total samples. These samples did not have detectable *Mycobacterium tuberculosis* by the AFB stain (Table III). Among the specimens, 27 were positive for the IS6110 gene, a genetic marker for *Mycobacterium tuberculosis* suggesting the presence of the bacterium in these cases.

Table III: Overall results interpretation of AFB staining and molecular characterization of IS6110 and mpb64 genes

Total no. of specimen	Total no. of AFB positive	Total no. of AFB negatives	Total no. of *IS6110* positive gene	Total no. of *IS6110* negative gene	Total no. of *mpb64* positive Gene	Total no. of *mpb64* negative gene
85	08	77	27	58	13	72

Conversely, 58 samples were negative for the IS6110 gene, these samples did not show the presence of this specific genetic marker. 13 specimens tested positive for the MPB64 gene, which is another specific marker for *Mycobacterium tuberculosis* (Fig. I). 72 samples were negative for the MPB64 gene. This indicates that these samples did not contain the MPB64 gene. The overall detection of *Mycobacterium tuberculosis* using the IS6110 gene marker was higher than the MPB64 gene marker. A significant number of specimens were negative for both AFB and the specific gene markers, reflecting the challenge in detecting TB in certain cases.

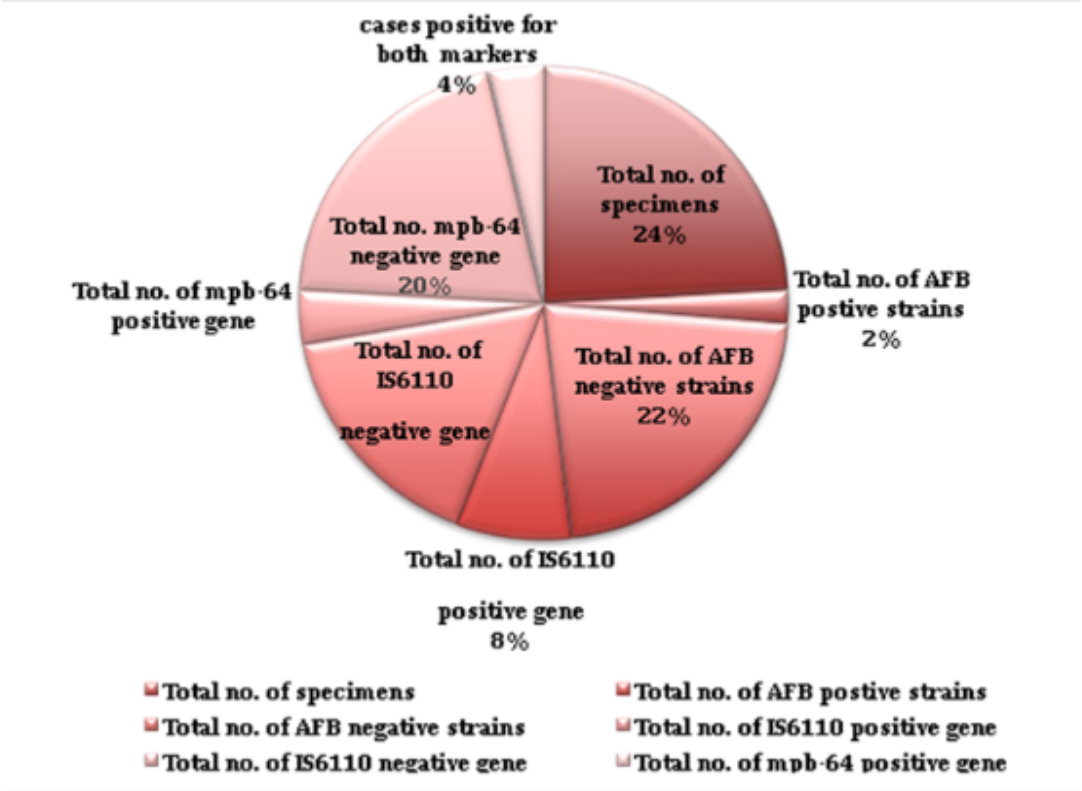

Figure II: Pie charts depicting the results interpretation for *mpb64* gene, *IS6110* gene and AFB

The evidence shows that in this sample set, the prevalence and the detectability of IS6110 might be much higher or more accessible to be discovered than it is in case of MPB64. It seems that the quite big number of AFB negative results along with the info on gene markers live up to the importance of the usage of various diagnostic methods to make sure that the accurate one is detected, particularly in cases when the initial tests don't give a clear answer.

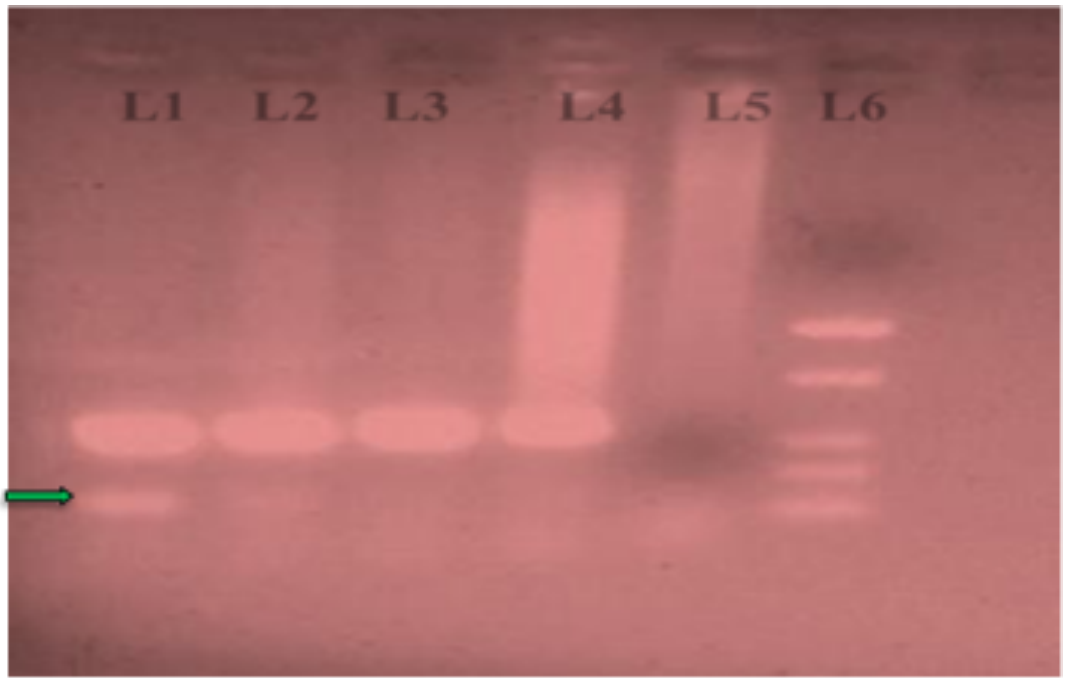

Figure III: L6 guided by 100bp ladder and L1 with 124bp of IS6110

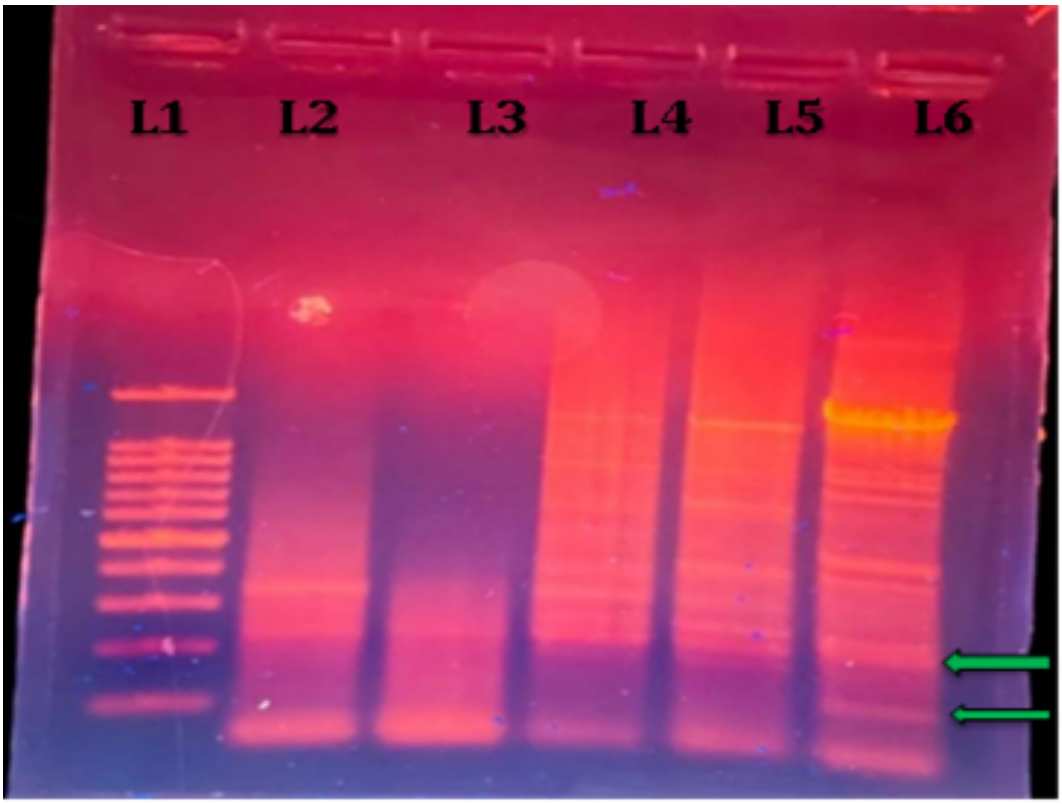

Figure IV: L4,L5 and L6 show 240bp band of *Mpb64* and with 124bp of IS6110 Guided by 100bp DNA ladder

The findings expose the discoveries in TB diagnosis, which occurs mostly in the cases of extra-pulmonary TB, and give attention that underpin a number of issues like to the improvement of CD4 diagnostics right through amplification approaches such as nested multiplex PCR.

DISCUSSION

Age Group Insights:

In Children and Adolescents (Under 18 Years) Eye infections in two young patients, a 6-year-old girl (case 33) with pus and a 4-year-old with a thigh abscess (case 60), were among the affected who tested positive for tuberculosis markers. This implies that tuberculosis might be invading the patients of the tender age group, and emphatically, with acute symptoms like scrofula or the development of deep septal infections. In equally frequent cases of this age group, for example, pus or CSF is obtained from a 4-month-old male hippo (case 39) and a 16-year old to the lip at the base of his tongue (case 37) where the he had hypoglossal cyst, however, the fungi could not be found in the samples are better understood and in turn, the pathogen is mostly not the TB one. In fact, the aforementioned symptoms that were observed might be characteristic of other disorders. In Adults (18 to 65 Years) among the titillations were ones with the positive candidatures of pus or CSF samples, for instance, a perfect example can be the case of the same pus from the right hip joint in a 12-year-old (case 25), that was the case of chronic breast sinuses in a 36-year-old (case 26), and CSF from the 30-year-old with appendicitis (case 40). The pathogen's presence in such cases points to the prolonged state of active disease and hints at its abounding capacity for embarking on other parts of the body. Not only this observation of adults with symptoms like secondary infertility or fever (e.g., cases 1-24) was determinant of the tuberculosis negative status in the patient group, it was also evidence to the possibly other health disorders. In Elderly (Over 65 Years) the levels of tuberculosis in afore-mentioned cases were like the following ones, namely: pus in a 65-year-old male (case 48) and pleural abscess in a 65-year-old female (case 56). The tuberculosis in these instances confirms that older adults are still vulnerable to the disease. On the other hand, events of malaise found to be unrelated to tuberculosis came to the elderly such as adults with blood samples (case 80) and/or cerebrospinal fluids (case 54) that are negative which goes to show that the elderly can alternatively have other health complaints apart from tuberculosis.

Gender Insights

Positive responses on the part of the males are the one which showed in the cases such as discharge of pus from the right hip joint (case 25), chronic breast sinus (case 26), and CSF from a 15-year-old male with meningo encephalitis (case 51). This means that TB can assume different forms in males and it is, therefore, often times a harder diagnosis than in females. The male belong to the negative cases such as those with usually symptoms as blunt chest trauma (case 4) and fever (case 22), were no found with any tuberculosis markers, and hence, it seems like the symptoms were a result of other health problems. Sufficed cases of tuberculosis in females include pus samples (cases 33, 49) and pleural abscesses (case 56), meaning that tuberculosis can severely affect females, especially those showing symptoms such as abscesses or those who have chronic conditions. Moreover, the females with the disease were absolutely not diagnosed with specific symptoms as for example the secondary infertility (cases 6, 15) and the menstrual abnormalities (case 30). Notably not all negative results of females are those of tuberculosis indicating symptoms such as secondary infertility (cases 6, 15) and the menstrual abnormalities (case 30) being the case. To sum up, these findings would allow us to believe that tuberculosis is not necessarily the ailment causing some of the symptoms, in females, who appear to have tuberculosis. From the tuberculosis test, females showed variations in the predicted health outcomes only some of them had the

versions that lead to the disease. Concomitantly, BTC was shown to be an illness.

Symptom-Based Insights

Abscesses and pus samples with a greater number of active tuberculosis indicators (cases 25, 33, 60) showed an increased rate of TB positivity. This indicates that pus samples, particularly from abscesses, are decisive in the diagnosis of active TB conditions. Also, some pus samples (cases 34, 35) were positive, which suggests that there might be a detection variability depending on the type or phase of infection. The CSF cases with reactions such as meningitis and hydrocephalus (cases 39, 40, 51) with diagnosis markers positive indicate that the TB can involve the central nervous system and exact investigation is required for the correct diagnosis. Several CSF samples from patients reporting symptoms such as fever or headache (cases 74, 75) showed negative results, which means that the symptoms might be from other neurological or infectious causes. On the other hand, in cases with symptoms such as fever (case 22) or trauma (case 4) but the markers of tuberculosis were negative the latter might not be related to TB but need other investigations.

This study underlines that individuals of all ages and both sexes may have tuberculosis, but the presence of particular symptoms and the kind of specimen play a role in the detection of the disease. In some cases, when dealing with unusual presentations or negative findings, the best result, usually, is to look further into the matter.

The importance of multidimensional diagnosis

The old methods took a lot of time; looking at tissues under a microscope and growing bacteria were not as good at finding EPTB. New ways to test DNA PCR, have made it easier to spot TB in both PTB and EPTB. These tests are very accurate and quick helping doctors find the TB germ in patient samples fast (Hillemann et al., 2011; Sharma & Mohan, 2004; WHO, 2021). Different test samples and patient cases show many things about TB and other health issues. In quite a few cases, like those from pee, blood, fluid around the lungs, and brain fluid (cases 1-24), all checks for the TB germ came back negative. This means they didn't find any TB germs or their DNA parts (IS6110 gene and mpb64 gene). This tells us these samples don't have active TB. It's worth noting that even patients with specific problems like trouble having babies, chest injuries from accidents, or high temperature didn't show any signs of TB.

A handful of samples showed positive results for TB markers in pus specimens. Key examples include pus from the right hip joint (case 25) long-term breast sinus (case 26), and pus from the thigh abscess (case 60). These samples tested positive for ZN staining, IS6110 gene, and mpb64 gene pointing to an active TB infection. Some CSF samples such as those from a patient with meningitis and hydrocephalus (cases 39 40), and TB meningitis (cases 74, 75), tested positive for the IS6110 gene hinting at an ongoing TB infection or related issues like hydrocephalus. Some samples showed mixed results. For example, case 33 involved a pus sample from a 6-year-old girl with positive ZN staining, IS6110, and mpb64 genes suggesting a strong TB presence. However, cases like 34 and 35 showed positive results for the IS6110 gene but negative for mpb64 suggesting possible differences in the TB strain or detection methods. Many samples were negative despite clinical signs suggesting TB. Cases with symptoms like fever and suspected meningitis (cases 37 83) showed negative results for TB markers pointing to other possible causes for the symptoms. In Breast and Chest Tissue like Case 50, which had a chest wall sinus, the test showed IS6110 but not mpb64. This suggests a TB infection maybe in an unusual form. Case 56, with a pleural abscess, tested positive for both IS6110 and mpb64 genes. A few older patients with CSF samples (cases 54, 55, 76) had negative or mixed results. This might point to a long-term infection or

other age-related changes in how the disease shows up.

To sum up, the findings show why it's key to use many ways to diagnose TB. This is true when symptoms are there but first tests come back negative. Positive results in pus and CSF samples give a clearer sign of TB. Negative or mixed results mean we need to look deeper or try other ways to diagnose.

According to research, Mycobacterium tuberculosis has between 1 to 10 iterations of IS6110, which serves as a target for the PCR-based diagnostics (Narayan et al., 2002). This emphasizes the need of utilizing the right primers, for example, those that are aimed at repetitive regions or those that amplify shorter regions of DNA that are difficult to annex. In the drought-prone areas of South India, where there could be M. tuberculosis strains with little or no IS6110 components, the work of Narayan et al., (2002) showed that having only IS6110 based PCR tests could lead to misdiagnosis through the 'no' method. This leads towards the development and implementation of other approaches like most universal genomic primers that are associated with diverse strains of M. tuberculosis, for example the MPB64 protein. This aspect is critical in the regions which have high incidence of the disease because timely and precise diagnoses are necessary for effective TB management and prevention.

The analysis shows that adding the MPB64 system to the IS6110-based testing method would help reduce false negatives in Indian strain samples that do not contain IS6110 copies. The IS6110 system produced PCR positivity rates between 83% and 87% according to earlier studies. Researchers examined how various PCR assays perform in identifying Mycobacterium DNA within granulomatous lymphadenopathy cases which frequently indicate tuberculosis (TB). The research presents important findings as it pinpoints both limitations and potential benefits of applying molecular methods especially PCR-based tests to detect TB in clinical samples where standard diagnostic approaches like acid-fast bacillus staining or culture testing show reduced sensitivity (Totsch et al., 1996). The diagnostic precision for TB in regions with variable IS6110 copy numbers could improve when two PCR systems work together.

The research results show that initial diagnostic tests alone are insufficient for fully understanding tuberculosis infection. The detection of positive results in particular specimens like pus and CSF serves as a reliable marker for active tuberculosis. Negative or mixed test results in different cases that display clinical TB symptoms show the importance of using multiple diagnostic techniques. Accurate diagnosis requires this method for cases that remain difficult to diagnose when traditional tests fail to show conclusive results or when patients continue to display symptoms after initial negative results.

Tuberculosis (TB) is hard to diagnose given that it has variable symptoms and limitations of traditional methods of diagnosis. Because more than one organ is affected by TB and has variable clinical symptoms, multi-faceted methods of approach to diagnose must be used for specific identification and cure of tuberculosis. Multi-faceted methods of approach to diagnose include several methods of diagnosis to improve sensitivity, specificity, and general correctness in diagnoses. Traditional methods of diagnoses like culture and histopathologic examination have inherent limitations. Histopathologic testing involves much technique and much time, whereas cultures take several weeks to provide results and may not identify infections of low bacterial loads, especially in extra-pulmonary tuberculosis (EPTB) (Boehme, 2010). Cultures are equally ineffective in their capacity to identify EPTB in low bacterial loads or in other than sputum specimen cases (Gonzalez, 2013). Polymerase chain reaction (PCR)-based molecular methods of diagnoses have transformed tuberculosis by allowing sensitive, rapid, and specific Mycobacterium tuberculosis DNA detection. Polymerase chain reaction for genes like IS6110 and mpb64 has shown excellent diagnostic specificity in significantly augmenting pulmonary and extra-

pulmonary TB identification rates (Pai, 2014; Lange, 2016). For example, IS6110 has found extensive utility as a target for tuberculosis detection given that it occurs in several copies in genomes of numerous M. tuberculosis strains, thus having higher sensitivity (Raman, 2018). However, PCR alone may not distinguish actively infected from latent infections or distinguish non-tuberculosis from tuberculosis mycobacteria, thus underscoring complementary methods of approach to diagnose (Zhao, 2019).

Employing a multi-dimensional approach to diagnostics results in more comprehensive evaluation. Using PCR in combination with traditional methods like Ziehl-Nielsen (ZN) staining and culture maximizes yield of diagnoses. ZN staining is a quick but not so sensitive method of detecting acid-fast bacilli that is beneficial in resource-poor settings (Miller, 2015). Culturing is still gold standard for TB diagnosis but is normally followed by molecular testing to make results faster and more specific. Clinical experience normally illustrates the necessity of multi-dimensional diagnostics. In some research articles, cases of EPTB have remained unnoticed by initial AFB staining or culture but have been established by molecular diagnostics (Kumar, 2017; Bertagnolio, 2020; Rao, 2021; Goletti, 2022). As in our research CSF and pus samples with mixed or negative results for traditional testing but molecular marker-positive results illustrate the necessity of multi-modal integration to avoid false negative results and provide proper diagnosis.

CONCLUSION

The integration of numerous diagnostic approaches that merge molecular testing with traditional approaches ensures more solid and comprehensive tuberculosis diagnosis. Through this approach, drawbacks of solo testing in situations of symptoms but negative initial results are attended to. Advances in future diagnostic technology and more concentration on multidimensional testing must take place in order to improve TB diagnosis in complicated or atypical situations.

In conclusion, integration of several diagnostic methods where molecular diagnostic is employed in combination with traditional methods is required for more reliable and detailed diagnoses of tuberculosis (TB). Employing more than one method has the benefit of compensating for inherent limitations of some methods of diagnostics in cases where clinical presentation is compatible with TB but initial testing results turn to be negative. The benefit of molecular methods such as PCR is that they have potential to diagnose Mycobacterium tuberculosis even in low bacterial loads or where non-tuberculosis mycobacteria complicate for condition of diagnosis. Through supplementation of traditional methods such as Ziehl-Nielsen (ZN) staining and culture, PCR has higher sensitivity and turn-around that is needed for having on-time and precise diagnoses.

Traditional methods such as ZN staining, though quick and affordable, have the drawback of not selecting cases of TB in extra-pulmonary tuberculosis (EPTB), where loads of bacteria may not be high or where acid-fast bacteria may get masked by granulomatous inflammation. As much as culture is gold in TB diagnosis, it is cumbersome to carry out and so much time to give results, hence not ideal in cases where results have to be quick. Molecular diagnostics far surpass conventional methods in yield by providing quick and sensitive results even in cases where conventional methods would not provide results. Experience of receiving mixed or negative results in CSF and pus by conventional methods depicts how molecular methods have an edge in selecting cases of TB in atypical or complicated cases. Not only was PCR beneficial in providing us with confirmation but in avoiding false negative results that would have led to delayed initiation of treatment with resultant severe clinical implications.

Future advancements in technology for diagnostics need to advance TB diagnosis in resource-poor settings as well as in complicated cases where atypical presentation of EPTB or atypical TB has to be excluded or included.

Ongoing focus on multidimensional diagnostic strategy that integrates PCR, conventional smear microscopy, and culture shall play crucial role in augmenting diagnostic performance, minimizing false negative results, and allowing initiation of proper treatment in good time. As challenges of TB in global settings evolve more so in high burden settings, adoption of integrated diagnostic strategy that maximizes benefits of more than one method is the way to go. Eventually, it shall have integral contribution towards reduction of patient suffering through improvement in patient outcomes, allowing more effective disease surveillance, and in global control of TB.

ACKNOWLEDGEMENT

The experimental setup was carried out at DNA Labs-A Centre for Applied Sciences, East Hope Town, Dehradun, Uttarakhand, India with facility of BSL 2 with practices of BSL 3. Some of the samples were also received from Gautam Buddha Chikitsa Mahavidyalaya, Dehradun, Uttarakhand, India.

Corresponding Author

Dr. Narotam Sharma, Scientist and Laboratory Director, DNA Labs-A Centre for Applied Sciences, East Hope Town, Dehradun and Assistant Professor, Department of Chemistry UIT, Uttaranchal University (Research Faculty) , Dehradun, Uttarakhand, sharmanarotam5@gmail.com, +91 9557650069

Funding Source

Self-funded project

Ethical Statement

All experiments and procedures were performed following the ethical guidelines of DNA Labs-A Centre for Applied Sciences, East Hope Town, Dehradun, Uttarakhand, India , and were approved by the laboratory ethical committee for medical research (Laboratory Consent Letter ref. no.: DLCA/2023/LAB-2/CL/203).

Conflict Of Interest

The authors declare no conflict of interest

REFERENCES

Bertagnolio, S., (2020). The use of molecular diagnostics for the detection of extrapulmonary tuberculosis. *Journal of Tuberculosis Research, 8*, 185–195. https://doi.org/10.4236/jtr.2020.84017

Boehme, C. C. (2010). Performance of nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis in adults in resource-limited settings: A systematic review and meta-analysis. *Lancet Infectious Diseases, 10*, 240–250. https://doi.org/10.1016/S1473-3099(10)70067-3

Dale, J. W., Al-Ghusein, H., and Al-Hashmi, S. (2003). Evolutionary relationship among strains of *Mycobacterium tuberculosis* with few copies of IS6110. *Journal of Bacteriology, 185*, 2555-2562. https://doi.org/10.1128/JB.185.8.2555-2562.2003.

Dorman, S. E. (2010). New diagnostic tests for tuberculosis: bench, bedside, and beyond. *Clinical Infectious Diseases, 50*(Suppl 3), S173–S177. https://doi.org/10.1086/651488

Golden, M. P., and Vikram, H. R. (2005). Extrapulmonary tuberculosis: an overview. *American Family Physician, 72*, 1761–1768. PMID: 16300038

Goletti, D.,(2022). Current and future diagnostic approaches for tuberculosis: An overview of advances and challenges. *Expert Review of Molecular Diagnostics, 22*, 115–130. https://doi.org/10.1080/14737159.2022.2040468

Gonzalez, J. (2013). Diagnostic challenges in extrapulmonary tuberculosis. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases, 1*, 15–21. https://doi.org/10.1016/j.jctube.2013.08.001

Gupta, R. K., Lucas, S. B., Fielding, K. L., and Lawn, S. D. (2015). Prevalence of tuberculosis in post-mortem studies of HIV-infected adults and children in resource-limited settings: A systematic review and meta-analysis. *AIDS (London, England), 29*, 1987–2002. https://doi.org/10.1097/QAD.0000000000000802

Hillemann, D., Rüsch-Gerdes, S., Boehme, C., and Richter, E. (2011). Rapid molecular detection of extrapulmonary tuberculosis by the automated GeneXpert MTB/RIF system. *Journal of Clinical Microbiology, 49*, 1202–1205. https://doi.org/10.1128/JCM.02268-10

Kumar, P., (2017). Challenges in the diagnosis of extrapulmonary tuberculosis: Case series and review. *Clinical Infectious Diseases, 64*, 1522–1531. https://doi.org/10.1093/cid/cix165

Lange, C.,(2016). The role of molecular diagnostics in the diagnosis and management of tuberculosis. *International Journal of Tuberculosis and Lung Disease, 20*, 1330–1340. https://doi.org/10.5588/ijtld.16.0135

Lima, D. M., Colares, J. K., and Fonseca, B. A. (2003). Combined use of the polymerase chain reaction and detection of adenosine deaminase activity on pleural fluid improves the rate of diagnosis of pleural tuberculosis. *Chest, 124*(3), 909-914. https://doi.org/10.1378/chest.124.3.909

Miller, M. A, (2015). Ziehl-Neelsen staining: Diagnostic utility and interpretation. *Clinical Microbiology Reviews, 28*, 218–232. https://doi.org/10.1128/CMR.00055-14

Narayan, S., Das, S., Garg, R (2002). Molecular epidemiology of tuberculosis in a rural area of high prevalence in South India: Implications for disease control and prevention. *Journal of Clinical Microbiology, 40*, 4785-4788. https://doi.org/10.1128/JCM.40.12.4785-4788.2002.

Pai, M., (2014). T cell–based assays for the diagnosis of latent tuberculosis infection: A systematic review and meta-analysis. *European Respiratory Journal, 43*, 24–35. https://doi.org/10.1183/09031936.00095213

Parekh, K. M., Inamdar, V., Jog, A., and Kar, A. (2006). A comparative study of the diagnosis of pulmonary tuberculosis using conventional tools and polymerase chain reaction. *Indian Journal of Tuberculosis, 53*, 69-76.

Rafi, W., Venkataswamy, M. M., and Ravi, V. (2007). Rapid diagnosis of tuberculous meningitis: A comparative evaluation of in-house PCR assays involving three mycobacterial DNA sequences, IS6110, MPB-64, and 65 kDa antigen. *Journal of Neurological Sciences, 252*, 163-168. https://doi.org/10.1016/j.jns.2006.10.027

Raman, P (2018). Evaluation of IS6110 PCR for the diagnosis of pulmonary tuberculosis. *Journal of Clinical Microbiology, 56*, e00890-18. https://doi.org/10.1128/JCM.00890-18

Rao, S.,(2021). Evaluating the diagnostic efficacy of molecular and conventional methods for tuberculosis in clinical practice. *Journal of Clinical Pathology, 74*, 386–390. https://doi.org/10.1136/jclinpath-2020-206823

Sharma, S. K., and Mohan, A. (2004). Extrapulmonary tuberculosis. *Indian Journal of Medical Research, 120*, 316–353. https://doi.org/10.1080/17476348.2021.1927718

Tang, Y. W., Meng, S., Li, H., Stratton, C. W., Koyamatsu, T., and Zheng, X. (2004). PCR enhances acid-fast bacillus stain-based rapid detection of *Mycobacterium tuberculosis* . *Journal of Clinical Microbiology, 42*(4), 1849-1850. https://doi.org/10.1128/JCM.42.4.1849-1850.2004.

Totsch, M., Böcker, W., Brommek, (1996). Diagnostic value of different PCR assays for the detection of *Mycobacterium* DNA in granulomatous lymphadenopathy. *Journal of Pathology, 178*, 121-126. https://doi.org/10.1002/(SICI)1096-9896(199602)178:2

World Health Organization. (2021). *Global tuberculosis report 2021*. World Health Organization. https://www.who.int/publications/i/item/9789240037021

Zhao, Y, (2019). Molecular detection of non-tuberculous mycobacteria using PCR and sequencing methods. *Diagnostic Microbiology and Infectious Disease, 95*, 137–142. https://doi.org/10.1016/j.diagmicrobio.2019.03.003