Comparison of Polymerase Chain Reaction With Fluorescent Microscopy for Detecting Pulmonary Tuberculosis in Smear-Negative Sputum Samples in Quetta, Pakistan

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Abstract

Background: One of the common clinical manifestations of extra-pulmonary tuberculosis is tuberculous meningitis (TBM). New methods, which are simple and effective, are necessary for early diagnosis of TBM.

Objective: This study aimed to assess the use of fluorescence microscopy and polymerase chain reaction (PCR) techniques targeting pncA and IS6110. PCR is a fast and reliable technique for diagnosing tuberculosis (TB), particularly in paucibacillary specimens such as smear-negative sputum for diagnosing patients with Mycobacterium tuberculosis.

Materials and Methods: The pncA and IS6110 multiplex-PCR methods were applied to analyze sputum using a specific pair of primers designed to amplify the insertion sequence, pncA, and IS6110 in the M. tuberculosis genome. A total of 200 sputum samples were collected from patients with TB. Out of 200 patients, 54.0% (n = 108) and 46.0% (n = 92) were males and females at Fatima Jinnah General and Chest Hospital, Quetta, respectively.

Results: M. tuberculosis was detected as 15 (16.3%) and 18 (16.7%) for pncA and IS6110 using PCR and fluorescence microscopy, respectively. M. tuberculosis DNA detected by PCR and smear-negative fluorescence microscopy was 16.50% (n = 33) and 14.70% (n = 18), respectively. In other words, PCR assay detected the increased prevalence of M. tuberculosis. Risk factors revealed high exposure to receiving TB infection which was associated with urban areas (OR = 1.07, 95% CI: 0.34–3.35), TB in family (OR = 1.22, 95% CI: 0.38–3.88), over crowdedness (OR = 2.93, 95% CI: 0.37–23.7), malnutrition (OR: 1.39; 95% CI: 0.52–3.74), and rural areas (OR: 1.54, 95% CI: 0.72-3.31).

Conclusion: A wide range of molecular assays are now being developed and reviewed for detecting TB. We conclude that the use of pncA and IS6110 PCR assays is beneficial in the quick diagnosis of TB meningitis.
microscopy was limited due to the high cost of mercury vapor light sources, the need for frequent maintenance, and the requirement for a dark room. Further, rapid nucleic acid amplification techniques such as polymerase chain reaction (PCR) were found to be more sensitive and specific as they strive to detect specific sequences of DNA from the organism under examination. Various facilities, notably IS\textsubscript{6110}, have tested the M. sequences of TB-specific DNA.\textsuperscript{6}

The factors contributing to PCR reliability include DNA amplification utilizing primers specific to different target sequences in the mycobacterial genome, proper DNA insulation, and PCR methods. In clinical samples, the PCR sensitivity and specificity for \textit{M. tuberculosis} range from 50–90% in certain laboratories to 60–100% in others.\textsuperscript{7} Because of the genome of TB, it is an appealing target for PCR amplification since it could improve assay sensitivity.\textsuperscript{8} The efficacy of the IS\textsubscript{6110} sequence for TB diagnosis has been investigated in several studies.\textsuperscript{6,9,10} In this study, we discuss our experience using the IS\textsubscript{6110} and \textit{pnc}A-based PCR techniques to detect \textit{M. tuberculosis} DNA directly from smear-negative sputum samples.

### Materials and Methods

#### Study Area and Population

Quetta is the sixth largest city of Pakistan with a population of 2.2 million. It is the provincial capital of Balochistan. People belong to different races and languages. The study was conducted at two phases where fluorescent microscopy, sputum specimen collection, and its decontamination were performed at the Provincial Reference Laboratory of Fatima Jinnah General and Chest Hospital, and PCR-based molecular characterization was conducted at the center for Advanced Studies in Vaccinology and Biotechnology (ASVAB), University of Balochistan, Quetta.

#### Collection of the Sputum Specimens

A total of 200 sputum samples were collected in screw-capped sterile pre-labeled containers. The participants of this study consisted of all individuals who exhibited the classical symptoms of the disease such as a history of prolonged chronic cough more than two weeks, fever, and chest pain. All samples were recorded, along with the bio data of the patients.

#### Sample Processing

Using the standard N-acetyl-L-cysteine-sodium hydroxide process, the collected sputum specimens were liquefied and decontaminated.\textsuperscript{11} These samples were then concentrated through a centrifuge at the Provincial TB Reference Laboratory with Biosafety Level 3. For the preparation of acid-fast bacilli (AFB) smears, the rest of the resuspended sediments (500 µL) were purified by heat killing. Then, the purified specimens were brought to ASVAB and placed at 20 °C for molecular characterization.

#### Fluorescent Microscopy

AFB smears were prepared by applying the auramine-O staining technique,\textsuperscript{11} and all smeared slides were then examined under it using a 40X objective fluorescent microscope (Figure 1).

#### Amplification of Mycobacterium DNA Using IS\textsubscript{6110}

The cetyl trimethyl ammonium bromide method was selected for performing PCR standardized by Ausubel et al.\textsuperscript{12} Using two sets of oligonucleotide primers (Macrogen), PCR was performed through the extracted DNA as TB1 (F) CCTGCGAGCGTAGGCGTCGG, and TB2 (R) CTCGTCCAGGGCGCTTCGG for IS\textsubscript{6110} and \textit{pnc}ATB-1.2 (F) ATG CGGGCGTTGATCATCGTC, \textit{pnc}AMT-2 (R) CGGTGTGCCGGAGAAGCGG for \textit{pnc}A. The multiplex PCR was done by an amplifying185-bp fragment of the \textit{pnc}A gene for \textit{M. tuberculosis} and a 123-bp segment of IS\textsubscript{6110} explicit for the \textit{M. tuberculosis} complex (Figure 2). In the process of multiplex PCR, a 25 µL reaction mixture was prepared which consisted of 1X Taq buffer, 2.5 mM MgCl\textsubscript{2}, 50 µM of each dNTP, 1.25 units of Taq DNA polymerase, 10 pmol of each of both oligonucleotide primers IS\textsubscript{6110} and \textit{pnc}A (forward and reverse), and 4 µL of template DNA. Amplification was performed using an automated thermal cycler (applied biosystems, 2720). The mixture was first denatured at 96°C for 5 minutes, followed by 35 PCR cycles with template denaturation for 45 seconds at 94°C, annealed for 45 seconds at 57°C, then extended for 45 seconds at 72°C, and the final extension lasted 7 minutes at 72°C. A positive control containing \textit{M. tuberculosis} DNA and a negative control with no DNA were also included in each PCR run. The PCR amplification products were run...
by agarose gel electrophoresis (2% W/V) for one hour at 100 Volts followed by ethidium bromide (0.5 mg/mL) staining. The electrophoresed amplicons were visualized and documented using an ultraviolet transilluminator (Wealtec Dolphin-View, the USA). The 50-bp DNA marker (Vivantis, Malaysia) was used as a reference to estimate the DNA bands.

Statistical Analysis of Data
Statistical package for the social sciences software version 20 was used to analyze the data. Pearson chi-square test was used to explore the association between TB and risk factors, and logistic regression was performed to assess the strength of association between risk factors and the disease. Odds ratios (OR) with 95% confidence interval (CI) were obtained for the risk variables. P values of ≤0.05 were considered significant.

Results
Among the 200 samples of sputum, 108 (53.3%) were females and 92 (46.7%) were males. Fluorescent microscopy was used to detect the existence of *M. tuberculosis* in the sputum samples. The findings revealed that fluorescent microscopy showed 15 (7.5%) sputum specimens positive, and PCR indicated 33 (16.5%) on both *pncA* and *IS6110* primers (Figure 3). PCR detected all 15 smear-positive specimens. Furthermore, all those samples which were negative on fluorescent microscopy 18 (9%) were shown to be positive on PCR. In all 33 (16.5%) positive samples, 15 (16.30%) were females and 18 (16.60%) were males. The sensitivities of *IS6110*, *pncA*-PCR, and auramine-O fluorescent microscopy were 100%, 100%, and 45%, respectively; however, the specificities of all three were 100% as shown in Tables 1 and 2.

Area of Analysis
According to the area, wise distribution data were analyzed in rural (66.0%) and urban (34.0%) areas similarly. The existence of TB evidence expressively affected the lifestyle of patients ($\chi^2 = 6.21$, df = 1, $P = 0.264$). A progressive infection ratio was observed, and people's survival rate was 18.90% in a rural area, while individuals who inhabited in an urban area (13.23%) were twofold at the risk for TB (OR: 1.54, 95% CI: 0.72-3.3; Table 3).

Ethnicity
All-out share (53.50%) with ethnicity group individuals were related to Pathans, Baloch contributed to 19.0%, and other ethnicity groups (i.e., Punjabi, Hazara, Urdu-speaking, and the like) had 27.0% of TB infection. Among ethnic groups, 18.40% of Pathan (16/107) were infected mainly through *M. tuberculosis* as detected by smear and

![Figure 2](image-url) Detection of *Mycobacterium tuberculosis* Through Multiplex PCR Using *IS6110* and *pncA* From Sputum Samples. Note. PCR: Polymerase chain reaction. PC: Positive control; NC: Negative control. Lanes 1, 3, 6, 7, 8, 9, 10, 12, 13, 14, and 15: Positive for *M. tuberculosis* complex species and *M. tuberculosis*. Lanes 2, 4, 5, 11, and 16: No amplification: Negative samples.

![Figure 3](image-url) Incidence of Positive TB Cases Noticed Through Fluorescent Microscopy and PCR Using *IS6110* and *pncA* Specific Genes. Note. TB: Tuberculosis; PCR: Polymerase chain reaction.

| Result for Specimen | FM Smear | IS6110-PCR | pncA-PCR |
|---------------------|----------|------------|----------|
| Positive TB cases   | 15       | 18         | 33       |
| Negative TB cases   | 0        | 167        | 0        |
| Sensitivity (%)     | 45.45%   | 100%       | 100%     |
| Specificity (%)     | 100%     | 100%       | 100%     |

Note. FM: Fluorescent Microscopy; PCR: Polymerase chain reaction; TB: Tuberculosis; OR: Odds ratio; CI: Confidential interval.
PCR techniques, while the frequency of TB in Baloch turned out to be 14.5% (7/102) as presented in Table 3. As regards the Baloch group, Pathan presented the risk for emerging TB 1.4 times higher (95% CI: 0.8-2.5) than the Baloch group. This risk factor was statistically not found to be related to TB existence (P > 0.05).

**Discussion**

The diagnosis of TB is a serious health problem and a challenge, especially in developing countries such as Pakistan. Early diagnosis of TB mainly by paucibacillary specimens such as smear-negative specimens is a great challenge. In Pakistan, radiography and smear microscopy are mainly used for the diagnosis of TB. There is no suitable diagnostic tool in most hospitals in Baluchistan. Poor prognosis and treatment pose significant challenges to the fight against TB. Microscopy is a simple, low-cost, and speedy diagnostic tool. Microscopy, on the other hand, lacks sensitivity and specificity. It can only detect 60% to 70% of AFB in culture-positive samples and cannot distinguish between living and dead TB pathogens. Although the cultural approach is the gold standard, it is time-consuming. Early identification of TB is critical for interrupting the disease transmission chain. Most clinicians focus on smear-positive patients because they are deemed highly infectious. According to Campos et al, smear negative was quite common among TB patients. Smear negative instances are on the rise and play a role in the spread of TB. As a result, the current study was designed to use PCR for detecting smear-negative patients. In this study, we compared fluorescence microscopy with a PCR for the early and rapid detection of TB. Out of 200 cases, 15 (7.5%) were smeared positive, while 33 (16.5%) were PCR positive, indicating that PCR was more sensitive than fluorescence microscopy. All 15 smear-positive samples were tested positive for TB bacilli by PCR. PCR was used to detect 18 (9.6%) smear-negative samples. For amplification of the \textit{pnc}A gene and \textit{IS6110}, multiplex PCR was used with oligonucleotides. This molecular technique enables the differentiation of \textit{M. tuberculosis} from other \textit{M. tuberculosis} complex species.

The sensitivity of smear-microscopy and PCR was 45.45% and 100%, respectively. Alli et al reported that the diagnosis of TB by PCR is more authentic than microscopy. Our study findings are in the line with a previous study conducted by Zakham et al who reported...
that the results of the IS6110 PCR in-house showed high sensitivity and specificity (92.4%, 98.0%, respectively). The higher positivity of PCR is attributed to the fact that fewer than ten mycobacteria could be detected and to the repetitive nature of the target sequence IS6110 amplified by PCR. The comparative result of two techniques used for TB diagnosis indicated a statistically significant difference ($\chi^2 = 82.064, \text{df} = 1, P < 0.001$).

In a routine test, the PCR technique specificity is critical. IS6110 is a frequent marker used to amplify the DNA of mycobacteria and is found in $M. tuberculosis$ complex species. It is also a useful marker for distinguishing $M. tuberculosis$ complex species from other non-TB mycobacteria. The $pncA$ gene, which contains a single nucleotide polymorphism (cytosine at position 169), is only found in $M. tuberculosis$ and is a likely genetic marker for distinguishing $M. tuberculosis$ from other $M. tuberculosis$ complex species. The current investigation found no relationship between gender and an increased risk of TB infection ($P > 0.05$). However, males were found to have more positive TB cases than females (18 versus 15 cases). Several other studies were reported by Ndungu et al. in Kenya and Fleming et al. in Russia.

As reported by WHO, Males in Russia have a greater rate of TB infection than females. Sixteen-year-old males had a greater TB incidence than females. The causes for the increased occurrence and prevalence among boys are unknown, requiring further investigation. Age is regarded as a significant risk factor for TB. When compared to patients under the age of 20, patients aged 41–60 years had a 1.7-fold (95% CI: 0.25–2.45) higher likelihood of developing the condition (20.89% vs. 13.0%). This could be due to weakening and damaged immune systems as people get older. Age, on the other hand, was not shown to be substantially connected to TB infection ($P > 0.05$). Our findings are consistent with prior research that has shown that the risk of contracting TB increases dramatically with age. As ethnicity groups were a focus of complex species from $M. tuberculosis$, the Pashtoon ethnic group accounted for 19.0% and 27.0%, respectively. Another study by Shafee et al. found that Pashtoon ethnic group accounted for 19.0% and 27.0% compared to Baloch and all other ethnic groups combined who had a greater TB incidence than females. The causes for the increased occurrence and prevalence among boys are unknown, requiring further investigation.

Conclusion
In recent decades, advances in molecular TB diagnostics have resulted in TB tests that are more accurate and faster than traditional microbiological testing; in addition, future technologies promise to keep this trend going. The results of this study strongly suggested the use of the multiplex PCR targeting IS6110 and $pncA$ as a highly potential tool for the rapid diagnosis and diagnosis in the routine analysis due to its increased sensitivity and specificity.

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Authors’ Contribution
MR, AS, and AI contributed to the study concept and design. MR, FSB, MN, and SKA collected samples and prepared them for the experiment. MR, AI, and MG analyzed and interpreted the data. MR and AI drafted the manuscript.

Conflict of Interest Disclosures
The authors have declared that no competing interests exist.

Ethical Approval
All investigations were performed following the center for ASVAB at the University of Balochistan, Quetta. The local ethics committee approved the study protocol and the informed consent form was obtained from all subjects.

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