

Original Research Article

Molecular Characterization of *Mycobacterium tuberculosis* complex in Clinical Isolates

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Abstract: Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis*, typically affects the lungs, but it can also affect other parts of the body. Diagnostics methods includes Serological, Radiological and Microbiological investigations. The current study was done for doing comparison between the specificity and sensitivity of ZN staining, culture and conventional PCR. 50 clinical isolates were collected and subjected for AFB smear preparation, Culture and Conventional -PCR (*IS6110*) for *mycobacterium tuberculosis* complex detection. 5 came positive for AFB smears, out of which 02 were pulmonary and 03 were extra pulmonary. 3 came positive for culture, out of which 01 was pulmonary and 02 were extra pulmonary. Conventional- PCR targeting *IS6110* gene was amplified at 123 base pairs with 340 base pairs as IC (internal control) was seen in 9 cases which include 2 pulmonary and 7 extra pulmonary. Thus, PCR was highly reliable for detection and conformation of *Mycobacterium tuberculosis bacilli* than AFB stain and Culture. This study showed that PCR can be considered as an alternative to ZN in combination with culture for the diagnosis of TB. Hence PCR is rapid and sensitive method for the early diagnosis of pulmonary and extra-pulmonary tuberculosis.

Keywords: Mycobacterium Tuberculosis complex, Conventional-PCR, Amplicons, Lowenstein-Jensen medium, Endometrial biopsy, Cerebrospinal Fluid

INTRODUCTION

Tuberculosis (TB) is believed to have been present in humans for thousands of years, as evidenced by the bones of ancient Egyptian mummies showing deformities consistent with the disease. Historically, pulmonary TB was known as the “Great White Plague” (causing about one in four deaths) of the 17th and 18th centuries in Europe, “phthisis” (a Greek term meaning to waste away), “scrofula” (swollen glands of the neck), and “consumption” (progressive wasting of the body) [1]. From 1700 to 1900, it is estimated that TB was responsible for one billion deaths and killed more people than any other disease [1,2]. Prior to the introduction of antibiotics in the 1950s, improved sanitation and living conditions significantly reduced the incidence of TB disease. Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis*, typically affects the lungs, but it can also

affect other parts of the body. The World Health Organization has noted that the global incidence of TB is increasing by 0.4% per annum [3,4]. *Mycobacterium tuberculosis* (MTB) belongs to the family Mycobacteriaceae and is the causative agent of most cases of tuberculosis (TB). First discovered in 1882 by Robert Koch, *M. tuberculosis* has an unusual, waxy coating on its cell surface (primarily mycolic acid), which makes the cells impervious to Gram staining. The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system, MTB infects the lungs. *M. tuberculosis* requires oxygen to grow. It does not retain any bacteriological stain due to high lipid content in its wall, and thus is neither Gram-positive nor Gram-negative; hence Ziehl-Neelsen staining or acid-fast staining, is used. While mycobacteria do not seem to fit the Gram-positive category from an empirical

standpoint (i.e., they do not retain the crystal violet stain), they are classified as acid-fast Gram-positive bacteria due to their lack of an outer cell membrane [4,5]. Diagnosis of tuberculosis is made thorough clinical history, including symptoms, and performing a physical examination [6]. Tests include special blood tests and a tuberculin skin test, which can detect if a person has been infected with the *Mycobacterium tuberculosis* bacterium or has had a vaccination for tuberculosis [7]. Lesions in the lungs that are due to tuberculosis may also be seen on a chest X-ray. These tests cannot detect if the infection has lead to active tuberculosis. Diagnosis of tuberculosis can be delayed or overlooked because there may be no symptoms. Molecular approaches targeting different markers is of utmost significance [8,9]. *IS6110* was first described by Thierry *et al.* *IS6110* gene - *IS6110* is an insertion sequence element found exclusively within the members of the *Mycobacterium tuberculosis* complex (MTBC), and because of this exclusivity, it has become an important diagnostic tool in the identification of MTBC species [10]. *IS6110* is 1,361 bp long and contains 28-bp, imperfect inverted repeats at its extremities with three mismatches and 3-bp direct repeats that probably result from repetition of the target sequence among the various mycobacterial species [11]. The restriction of *IS6110* to the MTBC is hypothesized to arise from the inability of these bacteria to exchange DNA [12]. The presence of *IS6110* indicates that lateral gene transfer has occurred among mycobacterial species, suggesting that the mycobacterial gene pool is larger than previously suspected [13,14]. Moreover, the element's presence in multiple copies and at differing locations in the genome, has provided an excellent method by which strains can be genotyped; because of these characteristics, *IS6110* has been used extensively for epidemiological studies different clinical samples [15,16]. *IS6110* is a novel *Mycobacterium* insertion element formed the basis of a reproducible genotyping technique. Bacteriological diagnosis of mycobacterial infections remains a problem and there is an urgent need for rapid and reliable diagnostic tests. Culturing of organisms has good sensitivity and specificity, but slow growth of the mycobacteria (4 to 6 weeks) results in delays in diagnosis. Identification of acid-fast bacilli stained smears provide rapid diagnosis, but this method identifies all the mycobacteria and requires that a relatively large number of bacteria is present in the sample. The current study was done on the *IS6110* as targets for the diagnosis of *Mycobacterium tuberculosis* complex in clinical specimens. This test is based on the principles of single-tube nested PCR method, which is a powerful and sensitive diagnostic tool for the

identification of *Mycobacterium Tuberculosis* complex. This assay is a two-step sequential assay. In the first step, the IS region of MTB complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123 bp amplification product. In this test, false positive reactions that may be caused by previous amplicon contamination are prevented by the use of uracil DNA glycosylase (UDG) and dUTP instead of dTTP.

MATERIALS AND METHODS

Specimen collection

A total of 50 Clinical specimens which includes Pulmonary such as Sputum, Pleural fluid, Bronchiolar alveolar lavage, bronchial secretions and extra pulmonary specimens such as Pus, Urine, Semen, Tissue, Endometrial biopsy, Cerebrospinal Fluid were considered for the study. Specimens were collected from patients attending Out Patient Department (OPDs) and In Patient Departments (IPDs) of different Departments of Shri Mahant Indresh Hospital, Dehradun, Uttarakhand, India. The current study was approved by institutional ethical clearance body and written consent was taken from patients. All the specimens were subjected for different parameters like Acid fast bacilli smear preparation by ZN staining method, Culture by using modified Petroffe method, conventional Polymerase Chain reaction (C-PCR). The Nucleic Acid (DNA) was extracted from the clinical specimen by Spin Column based Nucleic Acid Extraction method (Genetix) and the template was used for the C-PCR.

Comparision between AFB Staining, Solid Culture, and Conventional PCR:

A clinical samples were directly processed for AFB staining by ZN staining method, Culture by using modified Petroffe method and DNA isolation by Spin column method for conventional PCR.

Primer details and conditions for conventional PCR:

Forward primer (5'-CTCGTCCAGCGCCGCTTCGG-3') and reverse primer (3' CCTGCGAGCGTAGGCGTCCGG-5') were used for the amplification of *IS6110* gene of *Mycobacterium tuberculosis*. For the amplification of *IS6110* gene, cycling conditions as well as concentration and volume of the reagents were initially standardized and optimized. For standardizing cycling parameters i.e. denaturation, annealing, extension and final extension in terms of temperature (in degree centigrade) and

duration (in seconds) a PCR program was run. Each reaction (20µl) contained 1µl MgCl₂, 2 all deoxy nucleoside triphosphates, 0.2 µl of F and R primers, 5 µl Tris-HCl (pH 8.8), 11.4 µl nuclease free water and 0.2 µl of Taw DNA polymerase (MBI Ferments). The reaction mixture was subjected to an initial denaturation n at 94°C for 10 min and 35 cycles each of 30 sec at 94°C and annealing and extension at 68°C for 1min and 72°C for 1min, followed by a final extension at 72°C for 7.0 min. The products were analysed on a 1.6% arose gel and stained with ethidium bromide. The expected sizes of the amplicons for IS6110 gene of Mycobacterium tuberculosis is 123bp having internal control at 340bp (figure 2).

RESULTS

A total of 50 specimens were collected for the proposed study. Different types of clinical specimens from the patients with the symptoms which includes; cough that lasts 3 weeks or longer, fatigue or weakness, chest pain, coughing up blood or sputum (phlegm from deep inside the lungs), loss of weight and appetite, chills, fever, sweating at night were collected from the various Departments of Shri Mahan Indirect Hospital, Patel Nagar, Dehradun which includes; TB & Chest, Medicine,

Surgery, Obstetrics and Gynaecology and Neurosurgery. Pulmonary specimens includes sputum, Bronchial alveolar lavage, bronchial secretion, pleural fluid, whereas extra-pulmonary specimens includes cerebrospinal fluid, ascetic fluid, biopsies, tissues , blood , urine , endometrial tissue and curetting and synovial fluid. Specimens includes; pleural fluid (n=02), ascetic fluid (n=02), CSF (n=13), tissue (n=01) pus (n=16) synovial fluid (n=02), EDTA blood(n=08), endometrial biopsy (n=01), Bronchial alveolar lavage (n=04), peritoneal fluid(n=01). Specimens were subjected further for decontamination by Modified Petofi method, AFB smear preparation and by conventional PCR for molecular characterization. Pulmonary and mucous containing specimens were decontaminated by N-Acetyl-L-Cysteine by Modified Petroffe method and subjected further for the different protocols. It was observed that out of 50 samples, 9 were found to be positive and 41 were found to be negative. Out of the 50 specimens processed, 3 (6%) came positive for modified Petroffe method culture (MPMC), 5 (10%) came positive for AFB smear and 9(18%) specimens turned positive for PCR with *IS6110* gene as target amplicon (Table 1).

Table-1: Comparison between the positivity rate of TB culture, AFB smear and PCR targeting *IS6110* gene.

Total no. of specimen	Positivity rate of TB culture	Positivity rate of AFB smear	Positivity rate of conventional PCR
50	6%	10%	18%

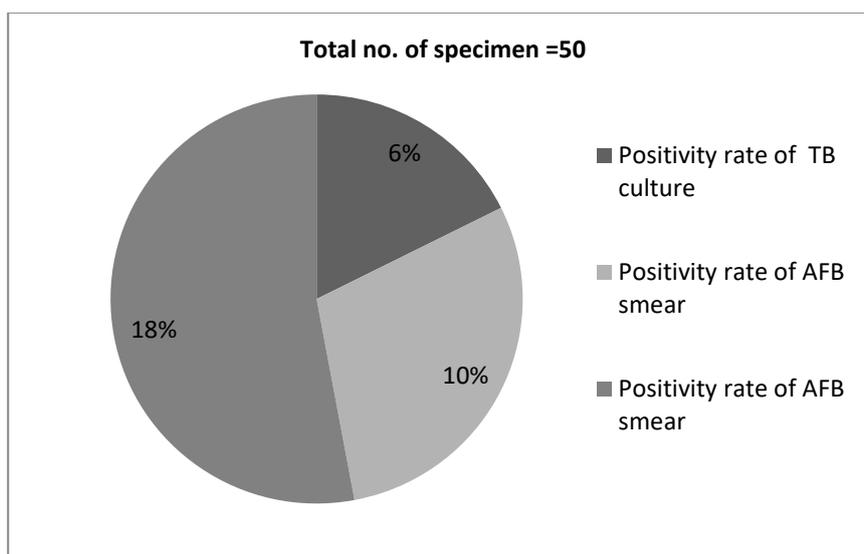


Fig-1 Bar graph between the positivity rate of TB culture, AFB smear and PCR targeting *IS6110* gene.

Table-2: Comparative results status for TB culture, AFB smear and PCR targeting *IS6110* gene.

S.no	Specimen type	Specimen positive for TB culture	Specimen positive for AFB smear	Specimen positive for <i>IS6110</i> gene
1	Pleural fluid (n=02)	No growth	Not seen AFB	Negative
2	Ascitic fluid(n=02)	No growth	Not seen AFB	Negative
3	Cerebro spinal fluid(n=13)	No growth	Not seen AFB	2
4	Tissue(n=01)	No growth	Not seen AFB	Negative
5	Pus(n=16)	2	3	4
6	Synonial fluid(n=02)	No growth	Not seen AFB	1
7	EDTA blood(n=08)	No growth	Not seen AFB	Negative
8	Endometrial biopsy(n=01)	No growth	Not seen AFB	Negative
9	Bronchial alveolar lavage(n=04)	1	2	2
10	peritoneal fluid(n=01)	No growth	Not seen AFB	Negative



Fig-2: Agarose gel electrophoresis of Nested PCR for *Mycobacterium tuberculosis* complex targeting *IS6110* gene.

Table-3: Number of positive cases

Total number of case for tuberculosis screening	Specimen positive for TB culture	Specimen positive for AFB smear	Specimen positive for <i>IS6110</i> gene
50	3	5	9

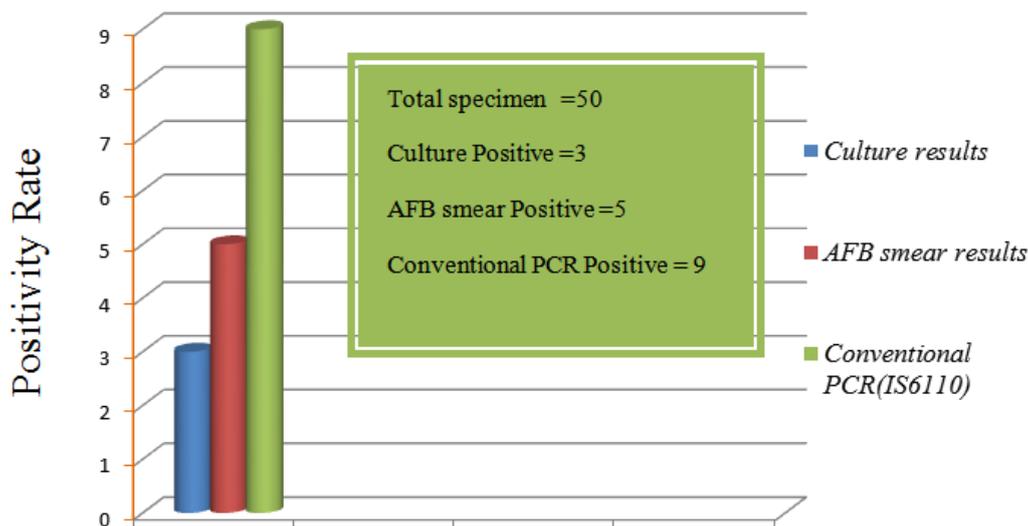


Fig-3: Bar diagram depicting comparative results status for TB culture, AFB smear and Conventional-PCR targeting IS6110 gene.

DISCUSSION AND CONCLUSION

Mycobacterium tuberculosis is an aerobic bacterium; Gram-positive that divides every 16 to 20 hours, an extremely slow compared with other bacteria. It is a major socio-economic burden in India, afflicting 14 million people, mostly in the reproductive age group (20-60 years). In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children. People living with HIV accounted for 1.2 million (11%) of all new TB cases. Tuberculosis is a disease with worldwide significance. This disease is persistent problem in the developing world and the biggest cause of mortality due to a single pathogen. Effective treatment requires the rapid detection of *Mycobacterium tuberculosis*. Bacteriological diagnosis of Mycobacterial infections remain a problem and there is an urgent need for a rapid and reliable diagnostic test. The culture method is sensitive and specific but the slow growth of Mycobacteria results in delay in diagnosis. Identification of acid fast bacilli stained smears provide a rapid diagnosis, but this method identifies all strains of Mycobacteria and requires that a relatively large number of bacteria should be present in the sample. During the past 30 years molecular techniques have been under development, however these have had a rapid and tremendous progress in recent year [16, 17]. Among molecular techniques, PCR and its different variations are highlighted as the most commonly used in laboratories and research institutes

[18]. Thus, these have contributed to identification and characterization of several organisms and understanding of physiopathology of diverse diseases in human, animal and plant. Also these have provided clues for future research directions in specific topics with impact in public health such as genetics and biochemistry of antimicrobial resistance [19]. In the present study we came to conclude and analyzed a total of 50 pulmonary and extra-pulmonary samples processed for PCR, culture and AFB staining. In few samples, ZN smear examination and PCR results were positive but culture was negative, this could be due to the presence of nonviable mycobacteria in the sample. While PCR was 100% sensitive and culture was 85% sensitive and specific, culture required up to 4-6 weeks of incubation and additional time to perform biochemical testing to identify the isolated micro-organism. Acid-fast bacilli stain had a specificity of about 80% and did not differentiate among Mycobacterial species. In contrast, the results from PCR were available within 10h and did not require additional testing to attain a final result. PCR was highly reliable for detection and conformation of *Mycobacterium tuberculosis bacilli* than AFB stain and Culture. This study showed that PCR can be considered as an alternative to ZN in combination with culture for the diagnosis of TB. Hence PCR is rapid and sensitive method for the early diagnosis of pulmonary and extra-pulmonary tuberculosis.

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Conflict of Interest: None

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